Studies on Disease Prevention and Control, Decontamination and Sterilization, Microbial Adaptive Responses and Survival, Alternative Therapies, and Sustainability

Submitted by

Neil J. Rowan (M.Sc. Ph.D.)

[Volume 2]

September 2024

A Thesis Submitted in Fulfilment of the Requirements for the Higher Degree of Doctor of Science (D.Sc) in the Faculty of Science

Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:  Date: 27th September, 2024

Abstract

This thesis describes my independent research studies starting in 1996 on five related areas that have advanced disease prevention and control including sustainable technologies to meet significant societal challenges. There are ever increasing demands for specialist foods and sophisticated devices to meet complexities of modern society including serving vulnerable groups. Whilst there is an expanding volume of published literature on developing food production, and to a much lesser degree medical devices, there is a need to understand why traditional and emerging decontamination and sterilization modalities work and what conditions or circumstances operating at the interface between microbial destruction and maintaining a desired product functionality could support microbial survivors and potentially foodborne or iatrogenic-mediated infection. There is also a dearth in knowledge surrounding the real-time detection of viable fastidious pathogenic microorganisms (such as complex parasites or drug-resistant fungi) post selection of appropriate technologies to safely treat foods and to decontaminate complex reusable medical devices. There is also a dearth of published information on appropriate cellular and molecular indicators to inform critical mechanistic information underpinning testing, verification and validation of new decontamination technologies. Elucidating holistically, the key parameters governing reliable and effective decontamination, provides evidence-based data to inform next-generation products from design thinking to automation in order to meet emerging societal needs.

The **first section** provides critical new insights and knowledge on conditions promoting the potential survival of microbial pathogens in sensitive foods such as reconstituted foods destined for vulnerable populations. It describes preparation and storage-abuse conditions promoting adaptive microbial survival and toxin production leading. It describes occurrence of such abuse conditions in hospital-prepared feeds in a HIV ward along with implemented of my recommended solutions that informed new guidelines of practice and helped to mitigate against future food-borne illnesses. This section characterizes processing conditions promoting the occurrence of atypical pathogens in sensitive foods, such as thermal-stresses leading to atypical cellular appearance and virulence factor expression *Listeria monocytogenes* that also enabled survival in human polymorphonuclear leukocytes.

The **second section** elucidates the first reporting on reliable and repeatable operational conditions underpinning non-thermal processing technologies (pulsed UV light, pulsed-plasma gas-discharge, pulsed electric fields), which also encompasses key mechanistic knowledge on critical cellular and molecular determinants governing irreversible microbial cell death. Commensurate studies report on development of alternative biomarkers to monitor and evaluate real-time disinfection performance including first report of a combined cell culture-qPCR assay for complex entero-parasites. New methodologies for toxicological end-point determinations in processing technologies are described.

First reporting on microbial kinetic inactivation and modelling for non-thermal technologies. Studies also elucidate appropriate treatment dose for effective killing of biological indicators including development of vaporized hydrogen peroxide as a new thermal sterilization modality for medtech.

The third section elucidates and develops non-thermal decontamination and sterilization technologies at commercial scale for established and new applications including for medical devices, food/feed, and for pollination industry (such as for decontaminating heat-sensitive pollen of complex pathogens fed to bees). Depending on the application, these studies include technologies encompassing x-ray, electron-beam, gamma-irradiation, pulsed UV and the co-development of real-8578/-+parametric release for treated products. Understanding the holistic interplay of all applied and inimical stresses governing effective microbial lethality *defines* critical knowledge including desirable end-to-end sterility assurance conditions ranging from elucidation to verification and validation of technological applications that meets safety. This section describes first classification system for effective cleaning of complex features in reusable medical devices and revisits efficacy of Spaulding's classification for device sterilisation and patient safety using this combinational new cleaning method. A holistic subject-matter knowledge of decontamination helps society meet unforeseen threats such as my elucidation and the first published recommendation of appropriate sterilization technologies and conditions for the safe reuse of PPE arising from critical supplying chain shortages during COVID-19 pandemic along with for sustainable waste management. Studies advance shellfish depuration and decontamination for recalcitrant fastidious norovirus pathogens attached to bivalve tissue.

The **fourth** section elucidates and develops novel alternative therapies and approaches for combatting antimicrobial-drug resistant (AMR) pathogens including bacteria and fungi, such as for lung delivery and for animal feed applications. Studies also address diagnostics for AMR pathogens that are at crisis point for society linked to decontamination. Studies also address elucidation of alternative antimicrobial and biofilm-disrupting bioactives used synergistically and in combination, yet tolerant of and suitable for medical device production-processes for smart coating applications.

The **last section** describes development of sustainable innovation including use of appropriate disinfection technologies. This includes first studies on development of an integrated multi-trophic aquaculture system in the peatlands aligned with zero-waste, zero pollution and climate action principles. It addresses digital transformation including new 'in-field' real-time monitoring and combined use of bioinformatics and next-generation sequencing to advance sustainable food innovation. It develops and applies new models including life cycle assessment and ecological tools.

Acknowledgements

Life is a journey that we are told is taking one step after another, that is lonely road. When we have someone to walk with us and support us along the way, that is living. I dedicate this thesis to my parents Ruth and Breffni, to my wife Michelle, to my daughter Chloe and to my sons Kevin and Liam for their endless love, support and encouragement! This thesis has been a psychological journey that captured precious time that was not shared enough with them. I apologise to them for when I have been distant and not focusing on their needs. I discussed this higher DSc submission with my father Breffni sadly before he passed way in 2014; it's a real relief (catharsis) that have I eventually managed to address it.

I was the first of many generations to have attended University. I feel this background has positively influenced my steadfast commitment to pursuit of research excellence and has helped with my engagement with students who attend university from all walks of life. I am grateful to the University of Galway for awarding me a professional soccer scholarship during my undergraduate years as a microbiology student. I am grateful to the late Professor John E Smith offering me an AFRC educational scholarship to pursue postgraduate studies at Strathclyde University. Thank you to Prof John G. Anderson and Prof Scott MacGregor for their friendship and encouragement at Strathclyde University as I forged an independent research pathway. I was pleased that my grading of 5* during the UK Research Assessment Exercise of 2023 reflected my commitment to this pursuit of academic excellence and to growing research and innovation at the University of Strathclyde.

To my friends at Dublin Institute of Technology, thank you for keeping me sane given that I commuted ca. 6 hours every day for 3 years to lecture there; thus, affording our children the opportunity of attend school locally in Athlone. Thank you to the School of Medicine at the University of Galway for appointing me as an Adjunct Professor. Also, to the University of KwaZulu Natal, and the University of Malaysia for Honorary Professorship appointments in Biotechnology. To the University of Stanford for recognising my contributions to these fields of study as reflected in my appearance on 2023 list of top 2% scientists globally. To the Technological University of Shannon (TUS), for bestowing the inaugural 'Outstanding Contribution to Research' award earlier this year. I have spent the past 20 years working at TUS here in Ireland (formerly Athlone Institute of Technology (AIT)) that was predominantly teaching-led. I thank TUS for my appointment as the first Director of the Bioscience Research Institute since 2012 and for its trust in me to inform process changes to grow research scale and capacity that contributed to it becoming a new university in October 2020.

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Section Four – Complementary and Alternative Therapies and Approaches'

This explored in part a collaboration with Prof John Laffey in Clinical Anaesthesia and Intensive Care Medicine at the University College Galway. Studies describe the first isolation, purification and extraction of sterile beta glucan from shiitake mushroom that was used to combat antibiotic resistant *Klebsiella pneumoniae* using a lung model. An understanding of appropriate novel bioactives that could prime immune system to combat antimicrobial resistant bacteria (AMR) that could potentially survive novel device processing would be deemed important from a preventive disease perspective. Studies based on isolation of beta-glucans from other medicinal fungi also revealed positive immunological markers for lung health application. There is a growing volume of literature on this beta-glucan topic where our independent studies revealed shortcomings with bioactive extraction, purification and dose internationally that has stalled production of a safe product for clinical use. Studies using shiitake mushroom-derived beta-glucans provided some insights for therapies to combat complex lung health conditions and had relevance for SARS-COV-2 virus. The use of medical fungi and extracts as immune-stimulants did not produce undesirable toxicological end-points when studied in fisheries including zebra-fish. Bio-based products (such as Nisin, zince oxide, silver nitrate and chitosan) were studied using a new mild-temperature extrusion process for inhibiting or inactivating AMR bacterial pathogens and biofilm development (with and without non-processing) such as for medical device coating research and applications. This can inform future use of appropriate Green bioactive alternatives and linked smart polymer research that is described in this thesis. The role of alternative biocides for addressing complex pathogens such as emergence of drug-resistant fungi is also described.

Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Purified β-glucans from the Shiitake mushroom ameliorates antibiotic-resistant *Klebsiella pneumoniae*-induced pulmonary sepsis

C.H. Masterson[1], E.J. Murphy[2], H. Gonzalez[1], I. Major[3], S.D. McCarthy[1], D. O'Toole[1], J.G. Laffey[1,4] and N.J. Rowan[5]

1 Lung Biology Group, National University of Ireland, Galway, Ireland
2 Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland
3 Materials Research Institute, Athlone Institute of Technology, Athlone, Ireland
4 Anesthesia and Intensive Care Medicine, University Hospital Galway, Galway, Ireland
5 Centre for Disinfection, Sterilisation and Biosecurity, Athlone Institute of Technology, Athlone, Ireland

Significance and Impact of the Study: Bacterial infection remains the main cause of acute respiratory distress syndrome and the need for an effective therapeutic intervention is evident. This study has demonstrated for the first time that purified β-glucans from the Shiitake mushroom *Lentinus edodes* can be used to attenuate the injury resulting from antibiotic-resistant *Klebsiella pneumoniae* pulmonary infection. Intravenous administration of β-glucan shows potential for treating sepsis-induced lung injury as it effectively reduces bacterial load, inflammatory white cell influx, protein leakage to the lungs and improves lung physiological parameters. Use of lentinan shows promise as potential therapeutic intervention to combat bacterial pulmonary sepsis.

Keywords
beta glucans, *Klebsiella pneumoniae*, lung injury, medicinal mushrooms, sepsis.

Correspondence
Claire Masterson, Lung Biology Group, Biosciences Research Building, National University of Ireland, Galway, Ireland.
E-mail: Claire.mast@gmail.com

2020/0583: received 1 April 2020, revised 26 June 2020 and accepted 16 July 2020

doi:10.1111/lam.13358

Abstract

Bacterial infection remains the main cause of acute respiratory distress syndrome and is a leading cause of death and disability in critically ill patients. Here we report on the use of purified β-glucan (lentinan) extracts from *Lentinus edodes* (Shiitake) mushroom that can reduce infection by a multidrug-resistant clinical isolate of *Klebsiella pneumoniae* in a rodent pneumonia model, likely through immunomodulation. Adult male Sprague–Dawley rats were subjected to intra-tracheal administration of *K. pneumoniae* to induce pulmonary sepsis and randomized to three groups; vehicle control (Vehicle, $n = 12$), commercial lentinan (CL, $n = 8$) or in-house extracted lentinan (IHL, $n = 8$) were administered intravenously 1 h postinfection. Physiological parameters and blood gas analysis were measured, bacterial counts from bronchoalveolar-lavage (BAL) were determined, along with differential staining of white cells and measurement of protein concentration in BAL 48 h after pneumonia induction. Use of IHL extract significantly decreased BAL CFU counts. Both CL and IHL extractions reduced protein concentration in BAL. Use of IHL resulted in an improvement in physiological parameters compared to controls and CL. In conclusion, administration of lentinan to treat sepsis-induced lung injury appears safe and effective and may exert its effects in an immunomodulatory manner.

Introduction

Acute respiratory distress syndrome (ARDS) is a leading cause of death and disability in critically ill patients (Rubenfeld *et al.* 2005) resulting from a loss of respiratory system compliance and hypoxia due to alveolar–capillary barrier disruption (Ferguson *et al.* 2005) which can lead to multiple organ failure and death (Ware and Matthay 2000). Although ARDS can arise from many causes, sepsis-associated ARDS is the most common type (Bellani *et al.*

2016) with approximately 40% of patients with severe sepsis, and 23% of mechanically ventilated patients developing ARDS (Bellani *et al.* 2016, McNicholas *et al.* 2018). The inflammatory response associated with the presence of a microbial infection involves the recruitment of inflammatory white cells and an over-production of inflammatory cytokines leading to alveolar–capillary barrier damage and pulmonary oedema instigating further infiltration, inflammation and widespread damage (Grommes and Soehnlein 2011). Interventions to combat the progression of ARDS to date have been mostly unsuccessful (Laffey and Kavanagh 2018; Lewis *et al.* 2019). Thus far the most promising therapy for the treatment of ARDS has been the implementation of supportive therapies such as fluid management, ventilatory strategies and prone positioning to improve oxygenation (Kon *et al.* 2015). The collective failure of pharmacologic therapies shows a clear need for the introduction of novel treatments for sepsis and associated ARDS. Targeting the immune response as a method of controlling the inflammatory response would serve as an attractive option in combating the initial infection and detrimental outcome of an overzealous inflammatory milieu in sepsis-induced ARDS.

β-glucans are polysaccharides naturally found in the cell walls of plants, bacteria, fungi, yeast and algae (Sullivan *et al.* 2006). They are comprised of chains of D-glucose rings connected commonly through a 1–3 glycosidic bond. Depending on source, β-glucans vary in chain length and level of side branching. Mushroom β-glucans, including lentinan extracts from *Lentinus edodes* (Shiitake), consist of a 1,3 linked back bone with 1,6 linked side branches (Wan-Mohtar *et al.* 2016). The molecular structure of different β-glucans confers different properties including solubility and receptor binding which is of interest in relation to leukocyte activation. For example, β-glucans with poor solubility confer direct leukocyte activation due to clustering of the receptor binding site (Sahasrabudhe *et al.* 2016), whereas modified soluble forms of β-glucan enhanced leukocyte activity without complete activation (Poutsiaka *et al.* 1993). Dectin-1 is a β-glucan-specific receptor (Herre *et al.* 2004) found predominantly on macrophages and neutrophils as well as dendritic cells and T-Lymphocytes (Brown *et al.* 2003; Taylor *et al.* 2002) and functions as a receptor specific for β-glucans with 1,3 and/or 1,6 linkages (Willment *et al.* 2003). It has been known for quite some time that β-glucans are the effective components in fungal products, and mushroom extracts have been used as medicine for thousands of years (Chang 2002; Sullivan *et al.* 2006).

Recently, there has been an increasing interest in the use of β-glucans from natural sources for treating infection in animals and humans (Carballo *et al.* 2019), including use of Shiitake mushrooms (Zhang *et al.* 2019). Carballo *et al.* (2019) exploited use of β-glucans from yeast in feed to modulate immune responses to control *Vibrio* genus-related infection in farmed fish. McCarty *et al.* recently described the potential role of β-glucan as a natural nutraceutical for boosting type 1 interferon response to RNA viruses such as influenza and coronavirus (Mccarty and Dinicolantonio 2020). Sepsis, influenza and coronavirus cause an inflammatory storm in the lungs and it is this inflammatory storm that leads to respiratory distress, organ failure and death (Shi *et al.* 2020). There is emerging evidence to suggest that certain nutraceuticals, such as β-glucans, may help reduce inflammation in the lungs with RNA viruses and other problematic bacterial pathogens (Vetvicka and Vetvickova 2015). β-glucans may also help boost type 1 interferon response to these pathogens, which is the body's primary means of help to create antimicrobial antibodies to fight off these infections (Mccarty and Dinicolantonio 2020). Here we aimed to investigate the potential for our in-house purified lentinan β-glucan preparation (compared to commercially produced lentinan β-glucan) in combating antibiotic-resistant *Klebsiella pneumoniae* pulmonary infection.

Results and discussion

The characteristics of β-glucan compounds varies depending on preparation

Immunomodulatory polysaccharides, such as β-glucans, are nontoxic and do not result in side effects commonly seen with the use of bacterial or synthetic compounds (Rice *et al.* 2004) making them an attractive adjunct therapeutic strategy in critically ill or high-risk patients. Megazyme analysis of commercial lentinan (CL) and IHL samples revealed differences in both β and α-glucan % w/w content. CL was shown to have a significantly higher α glucan content at $16 \pm 1{\cdot}5\%$ compared with only $2 \pm 0{\cdot}5\%$ for IHL extract. However, IHL had a significantly higher β-glucan content at $76 \pm 3{\cdot}5\%$ compared with $48 \pm 3{\cdot}0$ for CL samples (Fig. S1). Further studies by our group using the same samples revealed differences between specimens including variances in particle size and particle dispersity and elemental content, but also that each sample contained the same β-glucan compound (Murphy *et al.* 2020). This corroborates findings of Zhang *et al.* where similar medicinal fungi may exhibit significant differences in the molecular mass range profile and chain conformation of the beta-glucan samples. A study conducted by Vetvicka and Vetvickova (2018) suggested that the use of a highly purified yeast-derived β-glucan was superior to crudely isolated, less active β-glucans from various sources. In rodent models of septic shock

and as we have shown here, the IHL which contains a purer composition of β-glucan out-performs the commercially sourced CL. β-glucans at concentrations used in this study (20 mg kg^{-1}) were based on the maximum dose of CL that could be administered due to the viscosity of the solution, and did not affect viability or proliferation of *K. pneumoniae* bacteria (data not shown). This suggests an indirect effect of the compounds as described previously (Vetvicka and Vetvickova 2015) whereby β-glucans act upon the immune system to instigate clearance of infection and are not directly antibacterial themselves. Recent linked *in vitro* studies using same β-glucan extracts revealed that both IHL and CL demonstrated low to minimal toxicity in pulmonary cell lines. We observed that IHL and CL had both inflammatory and anti-inflammatory properties, with IHL showing a greater immune-modulating profile compared to CL supporting the immunomodulation potential of this novel β-glucan extract (Murphy *et al.* 2020).

B-glucan can effectively attenuate bacterial counts and white cell infiltrates to the lungs during pneumonia

Whilst direct antibacterial properties of CL and IHL could not be demonstrated, here we have shown that the numbers of viable bacteria present in the BAL fluid of treated animals was significantly reduced. The administration of 20 mg kg^{-1} β-glucan was significantly effective at lowering BAL WCC compared to vehicle (Fig. 1, panel b, $P < 0{\cdot}05$), however, only the IHL was shown to significantly reduce the numbers of viable *K. pneumoniae* in the BAL fluid (Fig. 1, panel a, $P < 0{\cdot}05$). Differential counts of the total white cells in the BAL fluid revealed that both CL and IHL could reduce the numbers of inflammatory PMNs infiltrating to the lung (Fig. 1, panel c, $P < 0{\cdot}01$) but only IHL increased the numbers of monocytes/macrophages in the BAL fluid (Fig. 1, panel d, $P < 0{\cdot}05$). It has been shown in several studies that β-glucans exert a potent effect on cells of the innate immune system which would explain the reduction in bacterial numbers and the differences in innate immune cell populations as shown here. B-glucans are not produced by humans or animals and are therefore classed as 'non-self' upon introduction to the body activating innate and adaptive immune responses (Brown and Gordon 2005). B-glucans can bind to and activate circulating monocytes increasing their cytotoxic activity, phagocytic activity, reactive oxygen species and nitric oxide production and cytokine secretion (Chan *et al.* 2009). In mouse models of influenza yeast β-glucan has been shown to increase dendritic cell activation conferring protection from infection (Camilli *et al.* 2018, Vetvicka and Vetvickova). In humans, administration of β-glucan has been previously shown to increase blood monocyte proliferation and maturation of dendritic cells (Chan *et al.* 2007), whereas we have demonstrated an increase in monocyte numbers in the BAL fluid of

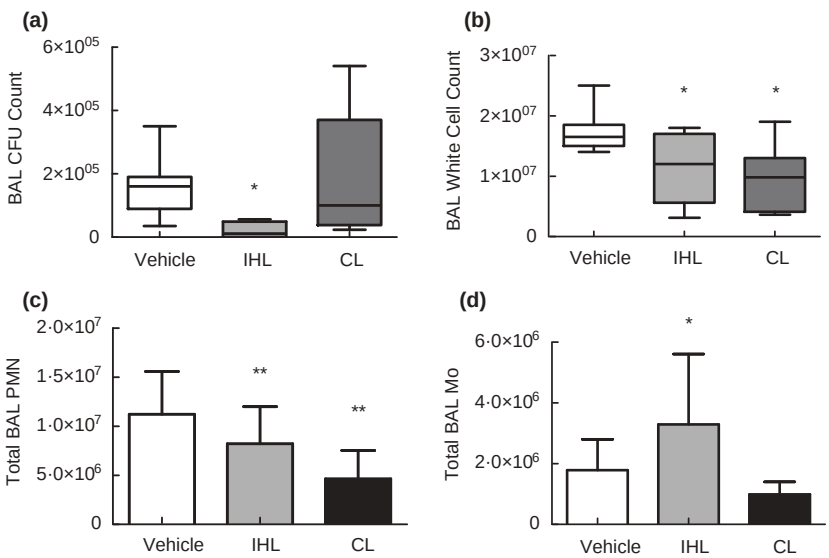

Figure 1 The effects of IHL and CL on the bacterial counts and white cell infiltrates to the lungs during pneumonia. Rodent models of *Klebsiella pneumoniae* infection were treated intravenously with 20 mg kg^{-1} of IHL or CL β-glucan. Bacterial and white cell counts were performed on the BAL fluid (panels a & b). Differential staining of the BAL white cells allowed numeration of the PMN (panel c) and Mo fractions (panel d). Data are expressed as mean ± SD (n = 6–10/group). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ (one-way ANOVA with Newman–Keuls multiple comparison test) with respect to Vehicle group. IHL = In-house lentinan; CL = Commercial lentinan; CFU = colony forming unit; BAL = bronchoalveolar lavage; PMN = Polymorphonuclear neutrophils; Mo = Monocytes.

treated animals whilst also demonstrating a decrease in the inflammatory PMN fraction.

B-glucans improve lung function and levels of circulating metabolites

Administration of IHL significantly improved arterial oxygenation ($P < 0{\cdot}05$) during pneumonia compared to vehicle controls. CL also improved arterial oxygen levels, albeit not significantly (Fig. 2, panel a). Static lung compliance measurements revealed that lung structural indices were also improved. Blood gas analyses of circulating metabolites revealed a significant decrease in plasma lactate levels after treatment with IHL β-glucans (Fig. 2, panel b, $P < 0{\cdot}05$). The administration of IHL β-glucan also returned blood glucose levels back to normal compared to vehicle and sham (Fig. 2, panel c).

While both CL and IHL increased compliance towards normal values, only IHL reached significance (Fig. 3, panel a, $P < 0{\cdot}05$). The wet:dry ratio, an indicator of lung leak and tissue oedema, was positively decreased by IHL compared to vehicle control (Fig. 3, panel b, $P < 0{\cdot}001$) and arterial–alveolar gradient values trended towards a decrease in the IHL-treated group (Fig. 3, panel c).

B-glucans enhance the inflammatory response to infection

Cytokine measurements in the BAL of animal models revealed that the treatment of *K. pneumonia* using β-glucans seemed to enhance the inflammatory response to infection by significantly decreasing the levels of anti-inflammatory IL-10 ($P < 0{\cdot}01$) and modestly increasing levels of TNF-α ($P = 0{\cdot}108$) compared to vehicle control (Fig. 4, panels a & b). Measurement of BAL protein levels indicated that treatment with β-glucan significantly decreased the levels of protein representing a decrease in alveolar–capillary leakage (Fig. 4, panel c, $P < 0{\cdot}05$) and supports the finding that IHL could improve lung structural indices as shown by measurements in static lung compliance and wet:dry ratio (Fig. 3, panels a,b). A study has demonstrated that the prophylactic administration of β-glucan can confer a protective effect, lowering TNF-α production and enhancing endotoxin clearance (Vereschagin *et al.* 1998). Here however, we have demonstrated a very modest increase in inflammatory TNF-α accompanied by a significant decrease in anti-inflammatory IL-10. This may be explained by the prophylactic administration used in previous studies which may have resulted in a state of 'trained immunity' (Quintin *et al.* 2012). In contrast, β-glucan in the present study was administered 1h postbacterial inoculation.

There is strong potential to produce commercially scalable production of β-glucans from Shiitake mushroom mycelium using controlled bioreactor studies for global research and innovation (Wan-Mohtar *et al.* 2016). With more research such as the present study and others (Vetvicka and Vetvickova 2018) showing that the production process can significantly impact the activity and efficacy of the extracted β-glucan it is imperative that it is given due attention. Here we have shown that the isolation of a pure β-glucan product from the Shiitake mushroom is effective in attenuating the injury parameters associated with bacterial pneumonia and propose the use of naturally derived immunomodulators to be a novel strategy in combating ARDS.

Materials and methods

Klebsiella pneumoniae-Induced Lung Injury

All work was approved by the Animal Care Research Ethics Committee of the National University of Ireland, Galway and conducted under license from the Health

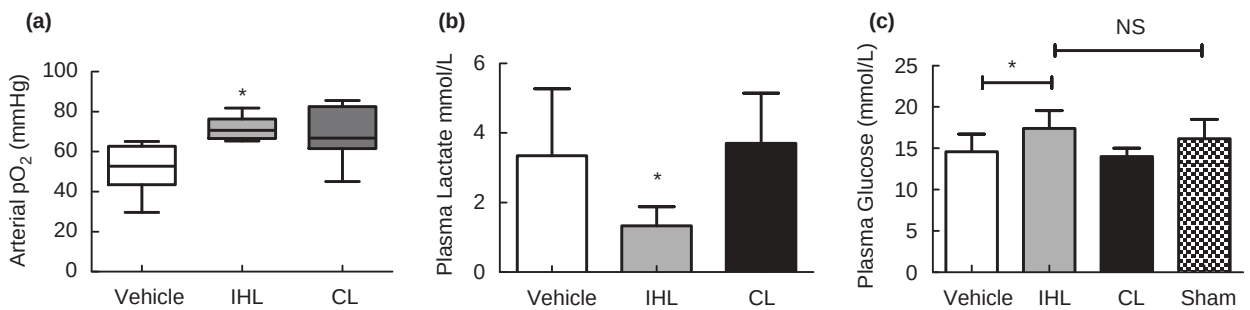

Figure 2 The effects of IHL and CL on blood gas indices during pneumonia. Rodent models of *Klebsiella pneumoniae* infection were treated intravenously with 20 mg kg^{-1} of IHL or CL β-glucan. Samples of arterial blood were analysed using a blood gas analyser during mechanical ventilation. Arterial oxygenation (panel a), and plasma lactate and glucose readings were recorded (panels b & c). Data are expressed as mean ± SD (n = 6–10/group). *$P < 0{\cdot}05$, NS = not significant (one-way ANOVA with Newman–Keuls multiple comparison test) with respect to Vehicle group and/or Sham group. IHL = In-house lentinan; CL = Commercial lentinan; pO_2 = partial pressure of oxygen.

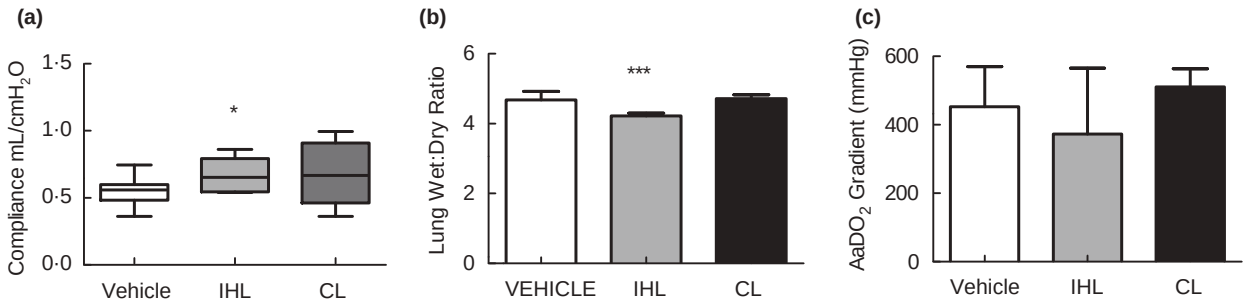

Figure 3 The effects of IHL and CL on physiological parameters during pneumonia. Rodent models of *Klebsiella pneumoniae* infection were treated intravenously with 20 mg kg^{-1} of IHL or CL β-glucan. Static compliance was measured under anaesthesia (panel a), and samples of lung tissue were dried postmortem for analysis of wet : dry ratios (panel b). Samples of arterial blood were analysed using a blood gas analyser during mechanical ventilation at FiO_2 1·0 and $AaDO_2$ was calculated (panel c). Data are expressed as mean ± SD (n = 6–10/group). $^*P < 0{\cdot}05$, $^{***}P < 0{\cdot}001$, (one-way ANOVA with Newman–Keuls multiple comparison test) with respect to Vehicle group. IHL = In-house lentinan; CL = Commercial lentinan; $AaDO_2$ = arterial alveolar oxygen gradient.

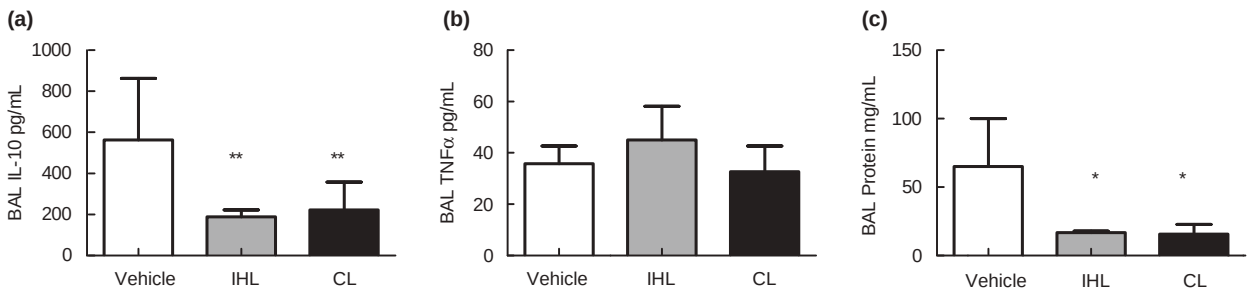

Figure 4 The effects of IHL and CL on cytokine profiles during pneumonia. Rodent models of *Klebsiella pneumoniae* infection were treated intravenously with 20 mg kg^{-1} of IHL or CL β-glucan. Postmortem BAL samples were analysed for anti-inflammatory IL-10 (panel a) and inflammatory TNF-α (panel b) and protein concentration was assessed using a protein assay of the BAL fluid (panel c). Data are expressed as mean ± SD (n = 6–10/group). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, (one-way ANOVA with Newman–Keuls multiple comparison test) with respect to Vehicle group. IHL = In-house lentinan; CL = commercial lentinan; BAL = bronchoalveolar lavage; IL-10 = interleukin 10; TNF-α = tumor necrosis factor alpha.

Products Regulatory Authority, Ireland (Licence number AE19125/P053, Number of animals approved on licence —246; Animals enrolled in this series—28). Specific-pathogen-free adult male Sprague–Dawley (CD) rats (Charles River Laboratories, Kent, UK) weighing between 300 and 450 g were used in all experiments. These animals were chosen based on the successful establishment of bacterial pneumonia using intratracheal *Escherichia coli* by our research group which was used in several studies (O'croinin *et al.* 2005; Chonghaile *et al.* 2008; Devaney *et al.* 2015; Masterson *et al.* 2018). The use of rats allows for adequate sample sizes of arterial blood, bronchoalveolar lavage (BAL) fluid and tissues for analysis.

In all groups, animals were anaesthetised using vaporized isoflurane (Iso-Vet, Chanelle, Co. Galway, Ireland). The animals were orotracheally intubated under direct vision using a guide wire and a 14G catheter (BD Insyte®; BD Biosciences, Berkshire, UK). A bolus of 1×10^9 CFU of *K. pneumoniae* in a 300-μl PBS suspension was instilled followed by a bolus of air. The animals were allowed to recover from anaesthesia as previously described (Curley *et al.* 2017; Devaney *et al.* 2015; Devaney *et al.* 2013; O'Croinin *et al.* 2005; O'Croinin *et al.* 2008) before proceeding to treatment. Piloting results for the *K. pneumoniae* pneumonia model are included in the supplementary data (Fig. S2).

Clinical isolate

Klebsiella pneumoniae clinical isolate was provided by Tullamore General Hospital (Health Service Executive, Co. Offaly, Ireland) as a stab culture. Using a Clinical and Laboratory Standards Institute (CLSI)-compliant Vitek 2 automated analyser to obtain antibiotic sensitivities, this organism was determined to be resistant to gentamicin and aztreonam and therefore categorized as a multidrug-resistant extended-spectrum beta-lactamase as per European Antimicrobial Resistance Surveillance Network guidelines 2017 (Giske *et al.* 2017). Of note, the patient from whom the isolate was derived was successfully

treated using meropenem following the failure of broad-spectrum antibiotics. The sample was isolated from blood but originated from the urine via a urinary tract infection complication.

Klebsiella pneumoniae cultures were propagated using tryptone soya broth (Fisher Scientific Ireland) and colonies were identified using UTI Brilliance Clarity Agar (LIP Fannin, Galway, Ireland). Colony forming units were quantified using a combination of serial dilution cultures and optical density readings at 600 nm. Cultures were prepared for animal models of pneumonia at 1×10^9 CFU per 300 μl in sterile PBS (Sigma Aldrich, Wicklow, Ireland).

Beta glucan isolation and preparation

Commercial lentinan was sourced from Carbosynth (FL33321, Berkshire, UK) and resuspended in sterile H_2O (Sigma Aldrich). In-house produced lentinan (IHL) was extracted from the fruiting bodies of *L. edodes* which were purchased from Fancy Fungi Ltd (Co. Wexford, Ireland). Lentinan extracts were analysed for (1,3)-(1,6)-beta-glucan content using the Megazyme yeast and mushroom kit (K-YBGL; Megazyme Ltd Co. Wicklow, Ireland). Assays were carried out according to the manufacturer's instructions. Briefly, samples were milled, and placed in 12 mol l^{-1} H_2SO_4 at −4°C for 2 h to solubilize the glucans. The samples were then hydrolyzed in 2 mol l^{-1} H_2SO_4 at 100°C for 2 h. After incubation any remaining glucan fragments were quantitatively hydrolyzed to glucose using a mixture of *exo*-1,3-β-glucanase and β-glucosidase which gives a measurement of total glucan. The alpha glucan and sucrose content of the sample is determined by hydrolyzing specifically to D-glucose and D-fructose. Glucose was measured with amyloglucosidase and invertase using Glucose Determination Reagent. β-glucan was determined by the difference in both measurements.

In-house vs commercial lentinan beta glucans in bacterial pneumoniae

One hour following intra-tracheal instillation of *K. pneumoniae* bacteria, animals were randomized to receive: (i) vehicle (PBS, 300 μl, $n = 12$); (ii) 20 mg kg^{-1} IHL β-glucan ($n = 8$); (iii) 20mg kg^{-1} CL β-glucan ($n = 8$) and the degree of injury assessed at 48 h postpneumonia induction.

In vivo assessment

Animals were anaesthetized with subcutaneous ketamine 75 mg kg^{-1} (Ketalar; Pfizer, Cork, Ireland) and medetomidine 0·5 mg kg^{-1} (Domitor; Vétoquinol, Dublin, Ireland), intravenous and intra-arterial access was secured, and a tracheostomy tube was inserted. Anaesthesia was maintained with 2 mg kg^{-1} Alfaxan® (Jurox PLC, UK) and paralysis with 0·5 mg kg^{-1} atracurium besylate (Tracrium; GlaxoSmithKline, Dublin, Ireland), and mechanical ventilation commenced using 7 ml kg^{-1} tidal volume. Arterial blood pressure, airway pressure, lung static compliance and arterial blood gas analyses were performed as previously described (Curley *et al.* 2012; Devaney *et al.* 2015).

Ex vivo analyses

Following exsanguination under anaesthesia, bronchoalveolar lavage (BAL) was performed, and BAL fluid differential leukocyte counts and lung bacterial colony counts were completed. BAL concentrations of TNF-α and IL-10 cytokines were determined using ELISA (R&D Systems, Abingdon, UK) and total BAL protein was measured (Micro BCA; Pierce, Rockford, IL). All *ex vivo* analyses were performed by blinded investigators.

Statistical analysis

A sample size of 8–12 animals per group was determined for a three-group design based on previously published experimental data from these models by our group (Chonghaile *et al.* 2008; O'Croinin *et al.* 2005; Devaney *et al.* 2015). Data are reported as means (±SD) or as medians (interquartile range). Data were analysed using Sigma Stat (SYSTAT® software, Richmond, CA). The distribution of all data was tested for normality using Kolmogorov–Smirnov tests. Data were analysed by Student's unpaired *t*-tests or one-way ANOVA as appropriate, with post hoc testing using Student–Newman–Keuls between group comparisons as appropriate. Underlying model assumptions were deemed appropriate based on suitable residual plots. A two-tailed P value of $<0{\cdot}05$ was considered significant.

Acknowledgements

This work was supported by the Irish Health Research Board (HRB-2015-POR-644).

Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. β-Glucan content was analysed using the Megazyme analysis kit and α (white bars) and β-glucan (grey bars) content expressed as % (w/w) of total glucan in each sample (adapted from Murphy *et al.* 2020).

Figure S2. *Klebsiella pneumoniae* models were established using a dose escalation of viable bacteria (2×10^7–1×10^9 CFU per animal) given intratracheally.

Science of the Total Environment 732 (2020) 139330

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

β-Glucan extracts from the same edible shiitake mushroom *Lentinus edodes* produce differential in-vitro immunomodulatory and pulmonary cytoprotective effects — Implications for coronavirus disease (COVID-19) immunotherapies

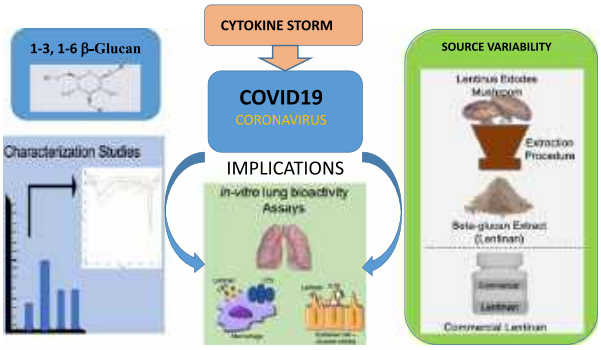
Emma J. Murphy [a,*], Claire Masterson [b], Emanuele Rezoagli [b,c,d], Daniel O'Toole [b], Ian Major [e], Gary D. Stack [a], Mark Lynch [a], John G. Laffey [b,c], Neil J. Rowan [a,f]

[a] Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland
[b] Lung Biology Group, Regenerative Medicine Institute at CURAM Centre for Medical Devices, National University of Ireland Galway, Galway, Ireland
[c] Anaesthesia and Intensive Care Medicine, University Hospital Galway, Galway, Ireland
[d] Department of Medicine and Surgery, University of Milano-Bicocca, Monza (MB), Italy
[e] Materials Research Institute, Athlone Institute of Technology, Athlone, Ireland
[f] Centre for Disinfection, Sterilization and Biosecurity, Athlone Institute of Technology, Athlone, Ireland

HIGHLIGHTS

- β-Glucans from shiitake mushroom reduces IL-1β, IL-6 in in vitro lung injury model.
- β-Glucans from same source can differ in immunomodulatory and pulmonary cytoprotective effects.
- β-Glucans can reduce oxidative stress and activate macrophages.
- β-Glucans may ameliorate cytokine storm that causes ARDS as seen with COVID-19.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 April 2020
Received in revised form 7 May 2020
Accepted 8 May 2020
Available online 11 May 2020

Editor: Damia Barcelo

Keywords:
β-Glucans
Medicinal mushroom
COVID-19
Immunomodulation

ABSTRACT

Coronavirus pneumonia is accompanied by rapid virus replication, where a large number of inflammatory cell infiltration and cytokine storm may lead to acute lung injury, acute respiratory distress syndrome (ARDS) and death. The uncontrolled release of pro-inflammatory cytokines, including interleukin (IL)-1β and IL-6, is associated with ARDS. This constituted the first study to report on the variability in physicochemical properties of β-glucans extracts from the same edible mushroom *Lentinus edodes* on the reduction of these pro-inflammatory cytokines and oxidative stress. Specifically, the impact on the immunomodulatory and cytoprotective properties of our novel in 'house' (IH-Lentinan, IHL) and a commercial (Carbosynth-Lentinan, CL) Lentinan extract were investigated using in vitro models of lung injury and macrophage phagocytosis. CL comprised higher amounts of α-glucans and correspondingly less β-glucans. The two lentinan extracts demonstrated varying immunomodulatory activities. Both Lentinan extracts reduced cytokine-induced NF-κB activation in human alveolar epithelial A549 cells, with the IHL extract proving more effective at lower doses. In contrast, in activated THP-1 derived

* Corresponding author at: Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Ireland.
E-mail address: emurphy@ait.ie (E.J. Murphy).

https://doi.org/10.1016/j.scitotenv.2020.139330
0048-9697/

Cytokine storm
Disease mitigation

macrophages, the CL extract more effectively attenuated pro-inflammatory cytokine production (TNF-α, IL-8, IL-2, IL-6, IL-22) as well as TGF-β and IL-10. The CL extract attenuated oxidative stress-induced early apoptosis, while the IHL extract attenuated late apoptosis. Our findings demonstrate significant physicochemical differences between Lentinan extracts, which produce differential in vitro immunomodulatory and pulmonary cytoprotective effects that may also have positive relevance to candidate COVID-19 therapeutics targeting cytokine storm.

1. Introduction

The pandemic outbreak of coronavirus disease 2019 (COVID-19) is rapidly spreading globally (Zhang et al., 2020; Rowan and Laffey, 2020). Reports from China showed that about 20% of COVID-19 patients developed severe disease, resulting in a fatality of 4% (Zhang et al., 2020). A large part of COVID-19 patients in China experienced severe complications including acute respiratory distress syndrome (ARDS) requiring admission to intensive care unit (ICU) (Zhang et al., 2020). ARDS is a devastating condition of severe respiratory failure with 40% mortality and for which novel therapies are urgently needed (Rezoagli et al., 2017). It is characterized by widespread inflammation of the lungs, where extrapulmonary infections are a key aetiology of ARDS onset (Laffey and Matthay, 2017). The inflammatory insult results in lung parenchyma injury and activation of the immune system with an up-regulation of pro-inflammatory cytokines (Laffey and Matthay, 2017). The current international standard of intervention includes ventilatory management and organ support (Bellani et al., 2016). The innate immune system plays a pivotal role in the pathophysiology of ARDS (Chousterman et al., 2017).

COVID-19 causes an inflammatory or cytokine storm (CS) in the lungs with the excessive and uncontrolled release of pro-inflammatory cytokines, including interleukin (IL)-1β and IL-6 (Conti et al., 2020). The binding of COVID-19 to the Toll-Like Receptor (TLR) causes the release of pro-IL-1β which is cleaved by caspase-1, followed by the production of IL-1β that is a mediator of lung inflammation, fever and fibrosis. Researchers have confirmed level of inflammatory factors in patients with COVID-19 including elevation of IL-6 in non-survival groups (Huang et al., 2020), as compared with that of the survivals. Therefore, how to block the CS and when to initiate anti-inflammation therapy is critical for reducing the death rate of COVID-19 (Channappanavar and Perlman, 2017; Chousterman et al., 2017). Suppression of pro-inflammatory IL-1 family members and IL-6 has been shown to have a therapeutic effect in many inflammatory diseases, including viral infections (Zhang et al., 2020).

There is an increasing interest in the medicinal use of mushrooms nutraceuticals that have been previously reported to exhibit wide-ranging activities including anti-inflammatory, anti-tumor and immune-modulating capabilities (Pelizon et al., 2005; Zheng et al., 2005; Akramiene et al., 2007; Kumar, 2015). β-Glucans are one of the main active components derived from mushrooms (Smith et al., 2002; Zhu et al., 2015). These are glucose polymers that are linked together through 1,3 linear β-glycosidic chains. Complexity and variation in the compound derive from side branching structures (Stone, 2009). β-glucans isolated from fungi commonly possess side branching at the 1,4 or 1,6 position (Kaur et al., 2020). Variance will also occur with chain length and many these variances are species-specific and dictate biological activity (Sullivan et al., 2006; Chaichian et al., 2020). Lentinans are a specific class of β-glucans extracted from the edible mushroom *Lentinus edodes*, and are composed of a β-(1–3)-glucose backbone with two (1–6)-β-glucose branches of each five glucose units (Sullivan et al., 2006; Kaur et al., 2020). There has been an increasing interest in their use for treating disease in animals and humans (Carballo et al., 2019; McCarty and DiNicolantonio, 2020). McCarty and DiNicolantonio (2020) recently described the potential role of β-glucan as a natural nutraceutical for boosting type 1 interferon response to RNA viruses such as influenza and coronavirus.

Putative use of β-glucans in mitigating lung infections correlates with findings from our recent in vivo studies to address ARDS (Masterson et al., 2019). However, there were significant challenges in identifying a reliable and repeatable source of β-glucans suitable for lung delivery as findings from screening of over 20 natural and commercial products screened revealed that they were unsuitable for lung delivery due to microbial contamination or exhibiting very low levels of β-glucan. We recently reported that purified β-glucans (Lentinan) from the Shiitake mushroom *Lentinus edodes*, obtained using our in-house novel extraction method can be used to reduce populations of clinical isolate *Klebsiella pneumoniae* harbouring multiple antibiotic resistances in an in vivo lung infection model (Masterson et al., 2019). We reported that administration of Lentinan shows potential for treating sepsis-induced lung injury as it effectively reduces bacterial load in arterial blood and BAL, reduces white cell count protein inflammation to the lungs, and improves lung physiological parameters. Evidence also showed that in-house Lentinan extracted supported vital pO2 along with promoting lung cellular repair.

This constitutes the first study to compare a commercially sourced Lentinan extract from the edible mushroom *Lentinus edodes* (referred to as Carbosynth-Lentinan) to that of an in-house hot-water extract (IHL) of the same mushroom in order to evaluate immunomodulatory properties. These were characterized using an in vitro lung injury model with a focus on profiling components associated with cytokine storm. It is hypothesized that β-glucans derived from exotic mushrooms have the potential to alleviate the immune cascade in pathological conditions, such as ARDS that is experienced by COVID-19 patients.

2. Materials and methods

2.1. Materials

Commercial Lentinan (CL) was sourced from Carbosynth (FL33321, Compton, Berkshire, UK). Fruiting bodies of *Lentinus edodes* were purchased from Fancy Fungi (Ringaheen, Co. Wexford Ireland). IHL was extracted from the fruiting bodies using a novel process. A549 cells (used at passage 90) and THP-1 cells (used at passage 10) were obtained from the American Type Culture Collection, (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/mL) and streptomycin (100 μg/mL) solution (Sigma-Aldrich) at 37 °C in 95% air/5% CO_2 environment. For differentiation into macrophages, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) (Peprotech EC, London, UK) at a concentration of 100 ng/mL for 48 h.

2.2. Physicochemical characterization of β-glucan samples

2.2.1. Megazyme analysis

Extracts were analyzed for (1,3)-(1,6)-β-glucan content using the Megazyme yeast and mushroom kit (K-YBGL) (Megazyme Ltd., Bray, Co. Wicklow, Ireland). Assays were carried out according to manufacturer's instructions. Briefly, samples were milled, and placed in 12 M

H_2SO_4 at −4 °C for 2 h to solubilize the glucans. The samples were then hydrolyzed in 2 M H_2SO_4 at 100 °C for 2 h. After incubation, any remaining glucan fragments were quantitatively hydrolyzed to glucose using a mixture of *exo*-1,3-β-glucanase and β-glucosidase which gives a measurement of total glucan. The alpha glucan and sucrose content of the sample is determined by hydrolyzing specifically to D-glucose and D-fructose. Glucose was measured with amyloglucosidase and invertase using a glucose oxidase peroxidase GOPOD reagent. β-Glucan was determined by the difference in both measurements.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR).

ATR-FTIR was carried out on a Perkin Elmer Spectrum fitted with a universal ATR sampling accessory. All data were recorded at ambient temperature, in the spectral range of 4000–650 cm^{-1}, utilizing a 16 scan per sample cycle and a fixed universal compression force of 80 N. Subsequent analysis was carried out using Spectrum software.

2.2.2. Scanning electron microscopy (SEM)

SEM was performed on a Mira SEM (Tescan Oxford Instruments, UK) using a range of magnifications to evaluate the surface morphology of the extracts using the function of secondary electrons. Samples were placed on an aluminum stub and were gold-coated using Baltec SCD 005 sputter coater (BAL-TEC GmbH, Chemnitz Germany) for 110 s at 0.1 mbar vacuum before observation.

2.2.3. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were obtained using an Agilent Technologies Ultra High Field (UHF) 800 MHz NMR system. Spectra were analyzed using ACD NMR software. Samples were prepared in deuterated water (D_2O).

2.3. Immunomodulatory properties of β-glucan samples from Lentinus edodes

2.3.1. Cell viability assays

Cells were seeded at a density of 4×10^5 cells/well in a 96 well plate. After 24 h, cells were treated with varying concentrations of both CL and IHL. Cells were incubated with samples for a further 24 h. For MTT assay, media was aspirated and cells were treated with 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in RPMI for 3.5 h at 37 °C in a humidified incubator with 5% CO_2. MTT and media were aspirated and formazan product was solubilized through the addition of 100 μL of DMSO per well. Solubilized product was quantitatively measured at 540 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, USA). Results were expressed as percentage viability with respect to vehicle control.

2.3.2. Enzyme linked immunosorbent assay (ELISA)

Human Duoset sandwich ELISA kit (RnD Systems MN, USA) was used to measure cytokine levels in the medium after β-glucan exposure. All ELISAs were performed according to manufacturer's instructions. Results were expressed either in pg/mL or in ng/mL.

2.3.3. Cell injury

Pulmonary alveolar type II A549 cells were seeded at a density of 4×10^5 cells/well in 96 well plates. After 24 h cells were injured with 1 ng/mL of IL-1β (Peprotech, Rocky Hill, NJ) in RPMI supplemented with 1% penicillin/streptomycin. THP-1 cells were seeded at a density of 4×10^5 cells/well in 96 well plates and 24 h later injured with 100 ng/mL LPS (Sigma) in RPMI supplemented with 1% penicillin/streptomycin. Cells were then treated with β-glucan samples for a further 24 h.

2.3.4. Luciferase assays

A549 cells stably transfected with NF-κB-luc reporter gene were obtained from the National University of Ireland Galway. These A549 cells were seeded at a density of 4×10^5 cells/well in 96 well plates. After 24 h, IL-1β and β-glucan samples were added to each well for 3.5 h at 37 °C in a humidified incubator with 5% CO_2. 100 μL of luciferase substrate (SolarGlow Molecutools, Dublin, Ireland) which includes lysis buffer, was added to each well. After brief agitation on an orbital shaker, luminescence was assessed in a Microplate Reader (BioTek, USA). Results were expressed as fold induction with respect to vehicle treated controls.

2.3.5. Phagocytosis assay

The Vybrant™ phagocytosis assay kit (Invitrogen, Carlsbad, CA) was used to measure the phagocytosis capacity of THP-1 cells after exposure to β-glucan samples. Samples were analyzed according to manufacturer's instructions. Phagocytic index was calculated and results were graphed as percentage phagocytic index with respect to vehicle treated controls.

2.3.6. Hydrogen peroxide (H_2O_2) injury assay

To determine the effect of H_2O_2 on cell viability, cell apoptosis and necrosis were assessed using propidium iodide (PI) and Annexin-VFITC conjugated antibodies (Miltenyl Biotech). Cells were initially treated with a dose range of H_2O_2 from 0.001 mM to 20 mM. As 10 mM of H_2O_2, reduced cell viability by 50%, it was selected as the injuring concentration for further experiments. Cells were simultaneously treated with β-glucan samples. After 24 h of incubation, cells were complexed with Annexin-VFITC conjugated antibody at 1:1000 dilution for a further 10 min in the dark. Cells were washed in PBS three times and resuspended in flow buffer (Miltenyl Biotech). Cells were then automatically incubated with PI using the MACS Quant analyzer. Cells from each sample were then analyzed by MACSQUANT Analyzer 10 (Miltenyl Biotech). Data was analyzed using Flowlogic software(Miltenyl Biotech) with a guide to interpretation of findings represented in Table 1.

2.4. Statistical data analysis

Continuous data were described as mean ± standard deviation (SD). Differences in glucan content between CL and IHL were tested using unpaired Student's *t*-test. Differences of κb induction in A459 cells over increasing doses of CL and IHL were explored using a two-way analysis of variance. The viability of A549 cells was tested using increasing doses of β-glucans (i.e. IHL or CL) using a one-way analysis of variance. Post-hoc multiple comparison of increasing doses of CL and IHL versus 0 mg/mL was performed by controlling the false discovery rate using the two-stage step-up method of Benjamini, Krieger and Yekuteli. Differences in cytokine levels and phagocytosis were tested between different groups (i.e. LPS, CL and IHL) or between different doses (i.e. 1, 5 and 10 mg/mL) of β-glucans using a one-way analysis of variance. Post-hoc multiple comparison versus PBS was performed by controlling the false discovery rate using the two-stage step-up methods of Benjamini, Krieger and Yekuteli. Statistical significance was reached with a *p*-value < .05 (2-tailed). Statistical analyses were performed using GraphPad Prism 7a (GraphPad Software, San Diego, CA, USA) and Microsoft Excel for Mac 2017, Version 15.32 (Microsoft Corporation, Redmond, WA).

3. Results & discussion

3.1. Physicochemical characterization of β-glucan samples

Megazyme analysis of Carbosynth-lentinan (CL) and In-house Lentinan (IHL) samples are displayed in Fig. **1**. Data shows both β and alpha glucan % w/w content. CL was shown to have a significantly higher alpha glucan content. IHL was shown to have a significantly higher β-glucan content and were purer and cleaner in composition. Scanning electron microscopy paired with **energy dispersive X-ray analysis was carried out to determine particle size and elemental content, with results displayed in Figs. 2 and 3 respectively. Fig. 2, a**

Table 1
Interpretation of H_2O_2 assay derived from using flow-cytometry data.

Quadrant location:	Location representation:	Annexin V:	Propidium iodide:	Cell condition:
Lower left		Negative	Negative	Living cells
Lower right		Positive	Negative	Early apoptotic
Upper right		Negative	Positive	Late apoptotic cells
Upper left		Positive	Positive	Necrotic cells

dimensional representation of particle size, shows that CL has a uniform particle dispersity. Conversely, IHL shows a more heterogeneous particle mix. Fig. 3 shows that IHL contains fewer elements compared to CL, as sulfur, silicon and chlorine were not present in IHL. These results, paired with Megazyme results, reaffirm that IHL is a cleaner preparation with fewer constituents.

FT-IR spectra of the CL and IHL are shown in Fig. **4**. The IR analysis reveals a strong absorption peak in the fingerprint region at approximately 1015 cm^{-1} in both the commercial and in-house lentinan samples. The presence of equivalent absorption peaks, in the absorption range characteristic of polysaccharides, strongly suggests that both samples contain the same β-glucan compound. **Preliminary NMR analysis of the commercial and in-house Lentinan suggests significant differences between the products. The ^{1}H spectra of the compounds are shown in Fig. 5.**

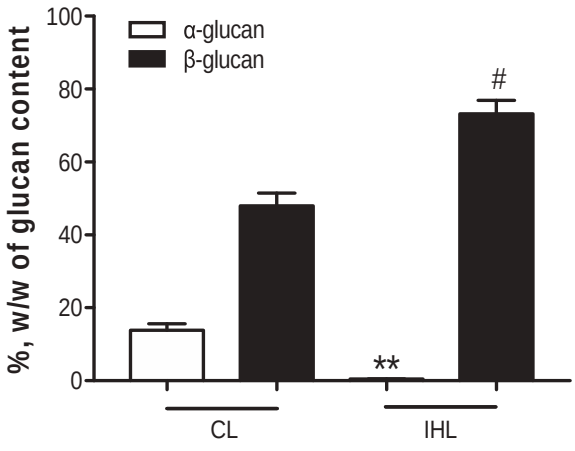


Fig. 1. Comparison of glucan content in commercial and in-house samples using Megazyme analysis. $^{**}p < .01$ versus alpha glucan C; $\#p < .05$ versus β-glucan CL.

Previous researchers have reported that variability in β-glucan chain length, branching and composition can vary based principally on on source (Kaur et al., 2020). Extraction procedures will have an effect on the structure and purity of the β-glucan product, which may have an effect of its bioactivity. Thus, there has been an obstacle in understanding biological activities and this has hindered potential therapeutic development (Zhang et al., 2011). Scanning electron microscopy paired with **energy dispersive X-ray analysis showed that the IHL had a diverse heterogenous particle composition in comparison to CL, which had a more uniform composition. IHL was also found to have less chemical elements (sulfur, silicon and chlorine) compared to CL. These results suggest that IHL is a purer β-glucan product, compared to the commercially available sample. FTIR characterization of the samples shows that both samples contain the same β-glucan compound (Fig. 4). NMR analysis contradicted this and showed that there is a significant difference between both samples (refer to supplementary information). Bak and colleagues** carried out a study to measure the glucan contents in the fruiting bodies of *L. edodes* mushroom from various cultivars which was found to be variable based on cultivar **(**Bak et al., 2014). Gil-Ramirez and co-authors found a variance between mushroom samples based on growth conditions, degree of fruiting body maturing body as well as a difference between fresh and fruiting bodies (Gil-Ramirez et al., 2011). Studies have also shown variance in β-glucan content dependent on the country of origin (Bak et al., 2014). *L. edodes* from Japan (49.5% β-glucan w/w) having a higher content than that of mushrooms purchased in Iran (38% β-glucan w/w).

Immunomodulatory properties of β-glucan samples from Shiitake mushroom *L. edodes*.

To investigate the anti-inflammatory effects of the β-glucan samples, transformed human airway epithelial cells (A549) were used. Initial experiments showed that a dose of 10 ng/mL of IL-1β gave an IL-8 release response, indicative of NF-κB activation. To further examine

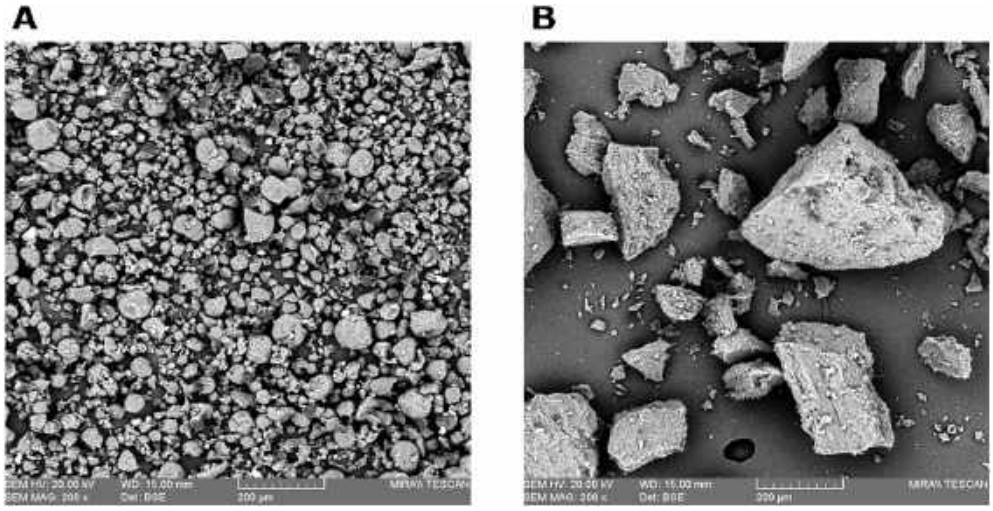


Fig. 2. Dimensional representation of commercial (A) and in-house (B) samples analyzed by scanning electron microscopy.

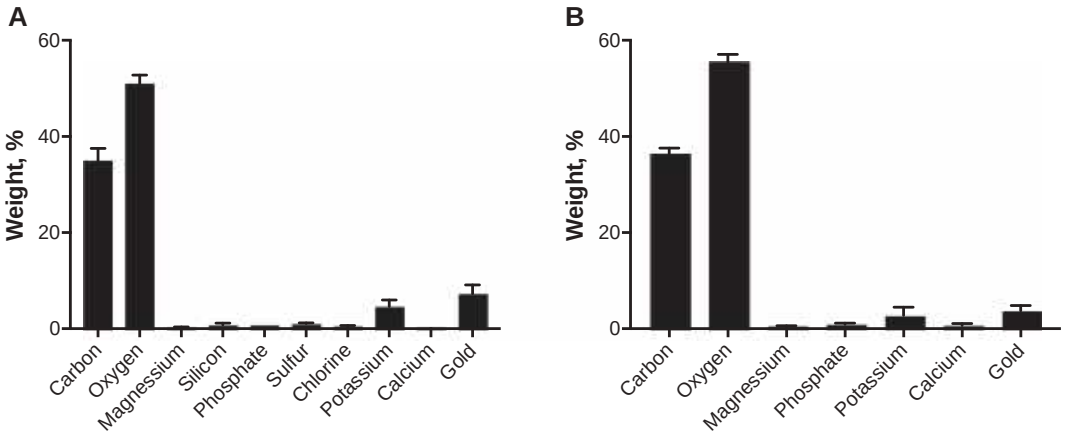


Fig. 3. Quantitative analysis of elemental contents in commercial (A) and in-house (B) samples determined by energy dispersive X-ray analyzer.

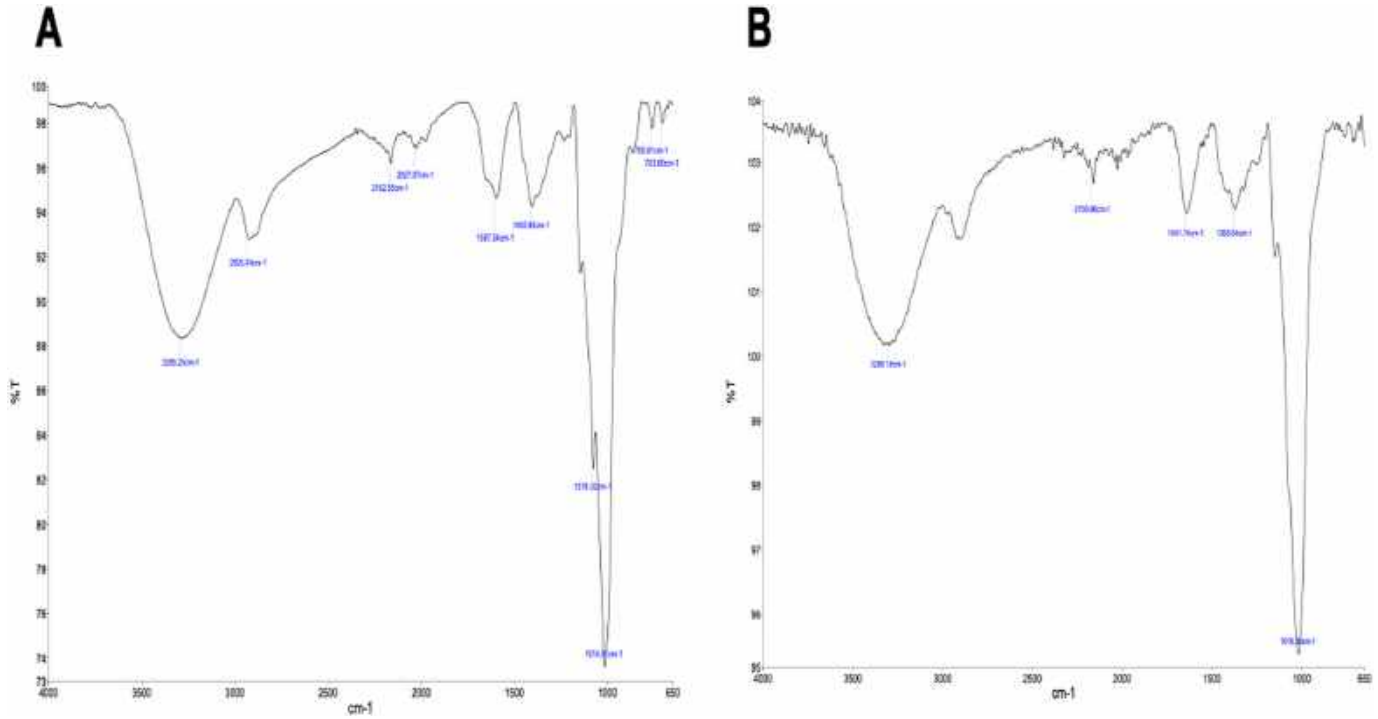


Fig. 4. FTIR spectra of the commercial (A) and in-house lentinan (B).

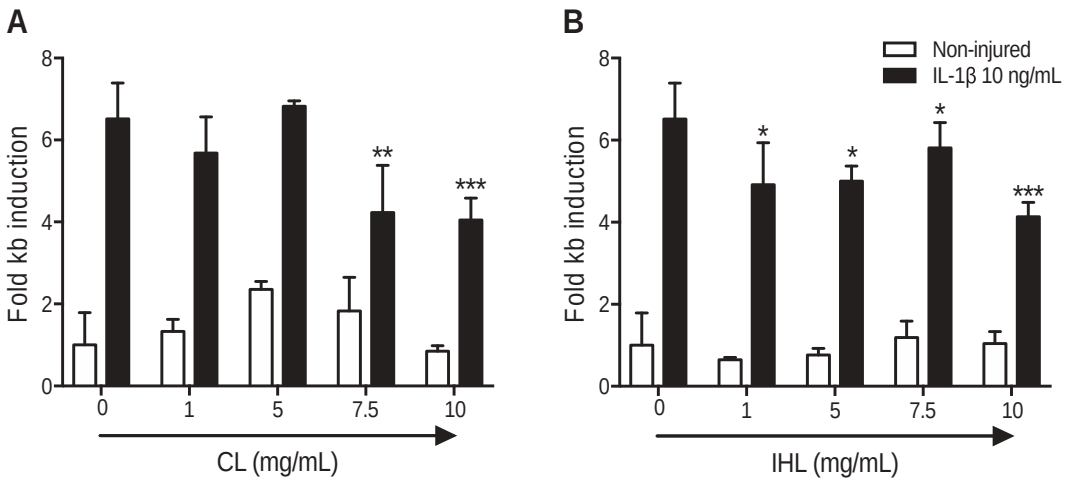


Fig. 5. A549 cells transfected with NF-KB reporter gene were stimulated with IL-1β for 1 h before administration of β-glucan samples. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ versus 0 mg/mL.

the effects of the β-glucan samples on the NF-κB pathway, A549 cells stably transfected with the NF-κB luciferase reporter gene were used. IL-1β stimulation caused a 6-fold increase in luciferase expression. Both β-glucan samples reduced this expression, with IHL inhibiting this increase at a dose of 1 mg/mL (Fig. 5B). CL was able to reduce IL-1β induced NF-κB pathway activation at the higher concentrations (Fig. 5A). This IL-1β pulmonary model finding is directly relevant to COVID-19 as IL-1β is a prominent part of the 'cytokine storm' response (Zhang et al., 2020). Hypercytokinaemia is considered a prominent mechanism of injury in COVID-19 patients. Furthermore, there are reports of the IL-1 Ra blocker Anakinra being effective for COVID-19, where Anakinra is being tested as a potential therapy for clinical trials. There are currently no treatments directed at halting the cytokine storm and acute lung injury to stop the progression from manageable hypoxia to frank respiratory failure and ARDS in patients with COVID-19 infection. Preventing progression from early acute hypoxia and cytokine release syndrome to frank hypoxic respiratory failure and ARDS could have a huge impact on the foreseeable overflow of the ICU units (https://clinicaltrials.gov/ct2/show/NCT04330638). The aforementioned reported that in ventilated patients, preventing the onset of ARDS, or shortening ICU stay could also be crucial in this regard. Furthermore, the clinical status after 15 days treatment was evaluated to measure the effectiveness of tocilizumab, tocilizumab and anakinra, siltuximab, siltuximab and anakinra and anakinra on restoring lung homeostasis, using single IV injection (siltuximab or tocilizumab) combined or not with daily subcutaneous injections of anakinra until 28 days or hospital discharge, whichever is first.

IHL was able to achieve these effects at the lower concentrations analyzed. MTT assays were preforming to ensure that cell viability was not contributing to the anti-inflammatory effects observed. Results show that both extracts did not elicit any cytotoxic effects at all concentrations, despite a trend toward a decrease in viability that was seen at the highest dose tested (10 mg/mL), but was only evident for the CL group ($p = .096$) (Fig. 6). No statistical differences were observed for multiple comparisons of singe *p*-values for MTT findings, which is attributed to the broad data variability represented by large standard deviation observed (Fig. 6). Post-hoc multiple comparisons of increasing doses of CL and IHL versus test control (0 mg/mL) were used to ensure that MTT findings were not over-stated in the assessment of preclinical data to reduce risk of false discovery. Statistical analysis data of multiple comparisons for single *p*-values for MTT findings are also provided in supporting information.

The capability of the β-glucan samples to induce or inhibit phagocytosis was also investigated. In the absence of injury, both CL (Fig. 7A+C) and IHL (Fig. 7B+C) appeared to reduce phagocytic index. After injury, both samples reduced phagocytic index (Fig. 7).

Macrophages and monocytes recognize β-glucans by various receptors present on their membrane (Vaclav et al., 2013). This recognition will result in the secretion of cytokines (Netea et al., 2008). Therefore, the immunomodulatory activity of the β-glucan samples was analyzed, in relation to their ability to influence the secretion of cytokines from macrophages. Results demonstrated that the samples induced/suppressed cytokine release at different concentrations. For example, IHL induced the secretion of IL-6 — conversely CL suppressed it. Lentinan analyzed by previous researchers was found to increase the release of TNF-α and IL-6 (Morales et al., 2019). This immunomodulatory effect was also analyzed by their ability to influence the phagocytic activity of macrophages. Both samples suppressed phagocytosis after LPS insult, but CL exclusively suppressed phagocytosis in the absence of injury. Dectin-1 receptor is assumed to be one of the main receptors responsible for the recognition of β-glucans. Activation of dectin-1 with 1,3–1,6 β-glucans can trigger cytokine release and phagocytosis (Brown and Gordon, 2001, 2005; Willment et al., 2003; Herre et al., 2004).

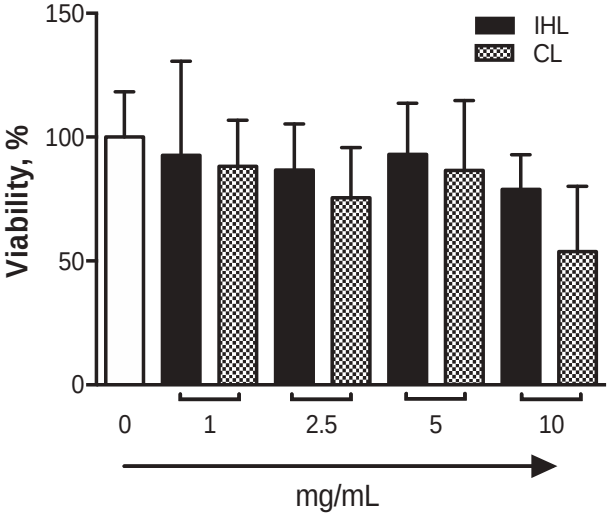


Fig. 6. MTT analysis of A549 cells treated with β-glucan samples to ensure anti-inflammatory properties' are not related to a reduction in toxicity.

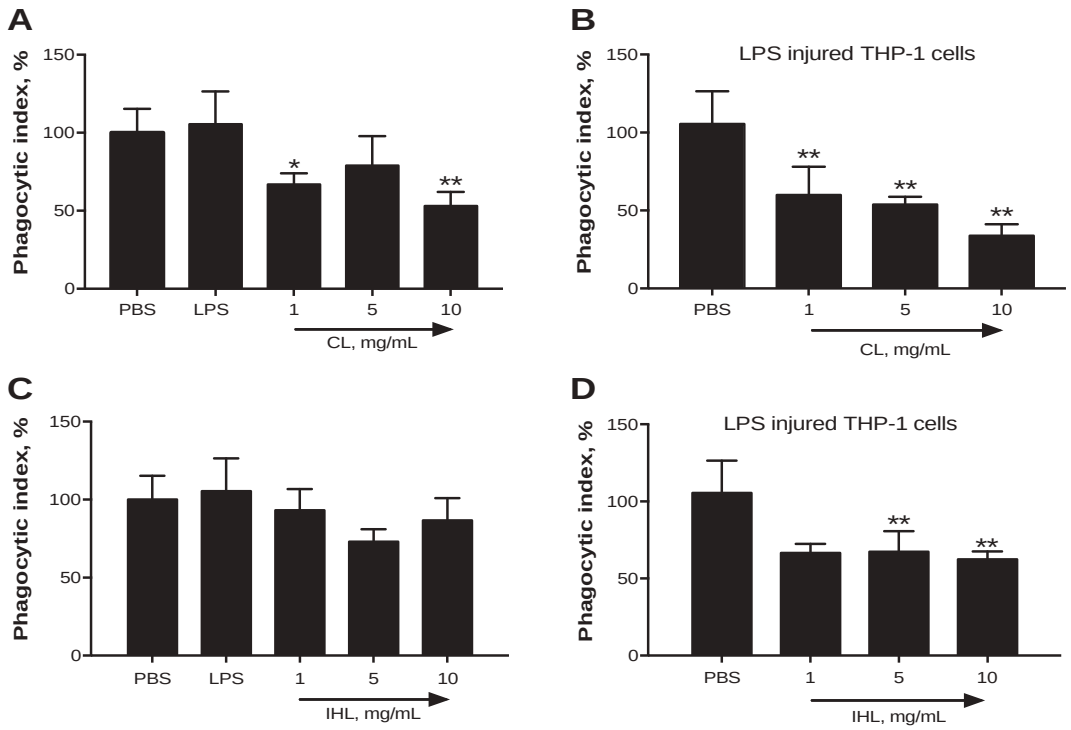


Fig. 7. The potential of β-glucan samples to alleviate phagocytic index of THP-1 cells was determined using Vybrant Phagocytosis assay kit $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ versus PBS.

To determine the potential alleviating effects of the β-glucans on oxidative stress injury, THP-1 cells were treated with 10 mM of H_2O_2 to induce oxidative stress subsequent to β-glucan treatment. In terms of viability (Panel a), all doses of β-glucans increased viability after injury, CL however significantly increased viability at all doses administered (1, 2.5 and 5 mg/mL). CL significantly reduced early apoptosis after injury at all doses tested (Fig. 8B). IHL appeared to reduce both early and late apoptosis (Fig. 8A+B), however this was not significant. IHL at 1 mg/mL and 5 mg/mL, or CL at 1 mg/mL and 2.5 mg/mL appear to increase necrosis although not significantly (Fig. 8C).

Reactive oxygen species (ROS), which are generated as intermediates in metabolic pathways, are prevalent in pathological conditions. Oxidative stress can occur at a pulmonary level (Rahman and MacNee, 2000). Cellular-derived ROS are produced enzymatically by inflammatory and epithelial cells (Marwick et al., 2007). It has been suggested that oxidants play a contributing role to cell injury as well as leakage of fluid into the lung interstitial space (Liu, 2008). **The potential of β-glucan to alleviate oxidative stress is relatively unknown.** β-Glucans were used to investigate the ability to alleviate oxidative stress in H_2O_2-treated THP-1 cells where apoptosis and necrosis were measured using flow cytometry. Propidium iodide (PI) in conjunction with Annexin V was used to determine if THP-1 cells were viable, apoptotic or necrotic. Cell status was assessed based on differences in plasma membrane integrity and permeability (Vermes et al., 2000; Farrell et al., 2011). The results from this experiment were variable and effects were source dependent. Both CL and IHL sources at both conditions tested had the ability to increase viability after injury. CL significantly reduced early apoptosis, and IHL showed a trend to reduce early apoptosis although not significantly. Our results are in line with the findings reported by Zi and colleagues who observed that Lentinan had the ability to alleviate oxidative stress (Zi et al., 2019). Reduction in oxidative stress by use of Lentinan is relevant to COVID-19 intervention. The renin-angiotensin (RAS) signaling pathway, oxidative stress and cell death, cytokines storm and endothelial dysfunction are four major pathways involved in the pathogenesis of COVID-19 (Kouhpayeh et al., 2020). Therapeutic candidates that inhibit RAS and quench oxidative stress would be relevant for COVID-19.

An important assay to determine glucan effect is measurement of cytokine production. In this study, we investigated the effect β-glucan samples had on pro and anti-inflammatory cytokine expression. Inflammatory chemokines and cytokines measured included IL-8, IL-2 and TNF-α. Pleiotropic cytokines included TGF-β1 and IL-6. Anti-inflammatory cytokines included IL-10 and IL-22 (Fig. 9). LPS (100 ng/mL) was also tested as it is a known inducer of inflammatory cytokines in immune cells. IHL (1 mg/mL) significantly induced the secretion of inflammatory mediators IL-8 and TNF-α. CL significantly induced the secretion of IL-8. IHL significantly increased the secretion of IL-10 and significantly decreased the secretion IL-22, as did CL. Other cytokines measured included IL-6 and TGF-β1. CL reduced the secretion of IL-6 and TGF-β1. Conversely, IHL significantly increased the secretion of IL-6 and CL decreased secretion. No differences in TGF-β1 concentration were observed when IHL was administered, however CL significantly reduced secretion.

In conclusion, findings of this in-vitro investigation showed that β-glucan from *Lentinus edodes* demonstrated potential for the treatment of lung injury. When compared to a commercial source of the same mushroom, the in-house Lentinan extract contained higher levels of β-glucan and lower levels of α-glucan. Both Lentinan products reduced inflammation in a lung epithelial model, and IHL achieved this effect at lower doses. Physicochemical characterization studies found important differences in the composition of the Lentinan extracts, as determined by SEM, ATR-FTIR, and NMR, with the CL exhibiting higher amounts of alpha-glucans and correspondingly less β-glucans. The two Lentinan extracts demonstrated varying immunomodulatory activities. Both Lentinan extracts reduced cytokine induced NF-κB activation in human alveolar epithelial A549 cells, with the IHL extract proving more effective at lower doses. In contrast, in activated THP-1 derived macrophages, the CL extract more effectively attenuated pro-inflammatory cytokine production (TNF-α, IL-8, IL-2, IL-6, IL-22). The CL extract attenuated oxidative stress-induced early apoptosis, while

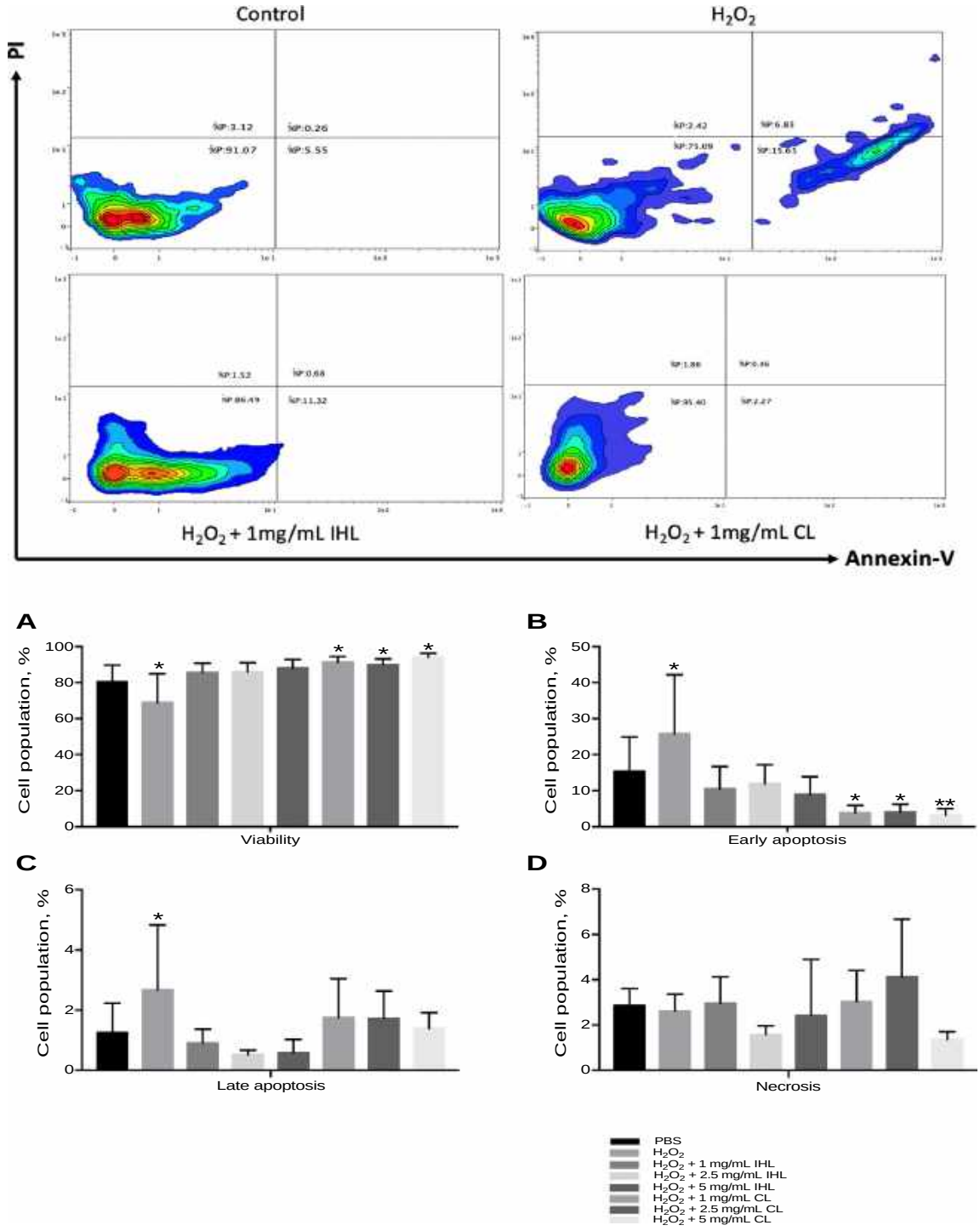


Fig. 8. Flow cytometric analysis of apoptosis (early and late) and necrosis of THP-1 cells after oxidative stress induction and subsequent β-glucan treatment $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ versus 0 mg/mL.

the IHL extract attenuated late apoptosis. The implications of these findings infers that defining a reliable and repeatable source of β-glucan, where processes can be tailored to control chain lengths may potentially reduce key cytokines involved in cytokine storm experienced in severe cases of COVID-19. In the future, β-glucans may be delivered as a tailored cocktail matched against critical time-points in the form of future nutraceutical-based intervention for tackling COVID-19. Such β-glucans immunomodulatory cocktails may also have adjacent applications for addressing ARDS that is an important pathophysiological event seen with sepsis. These purified β-glucan combinational cocktails may be produced on a large commercial scale using bioreactors for global deployment (Tafuek et al., 2020).

To maintain functional bioactivity and to increase β-glucan yield, less harmful extraction processes are required that includes cessation of enzyme and harsh chemical usage as adopted using this IHL approach. Findings from this timely study highlight the putative potential

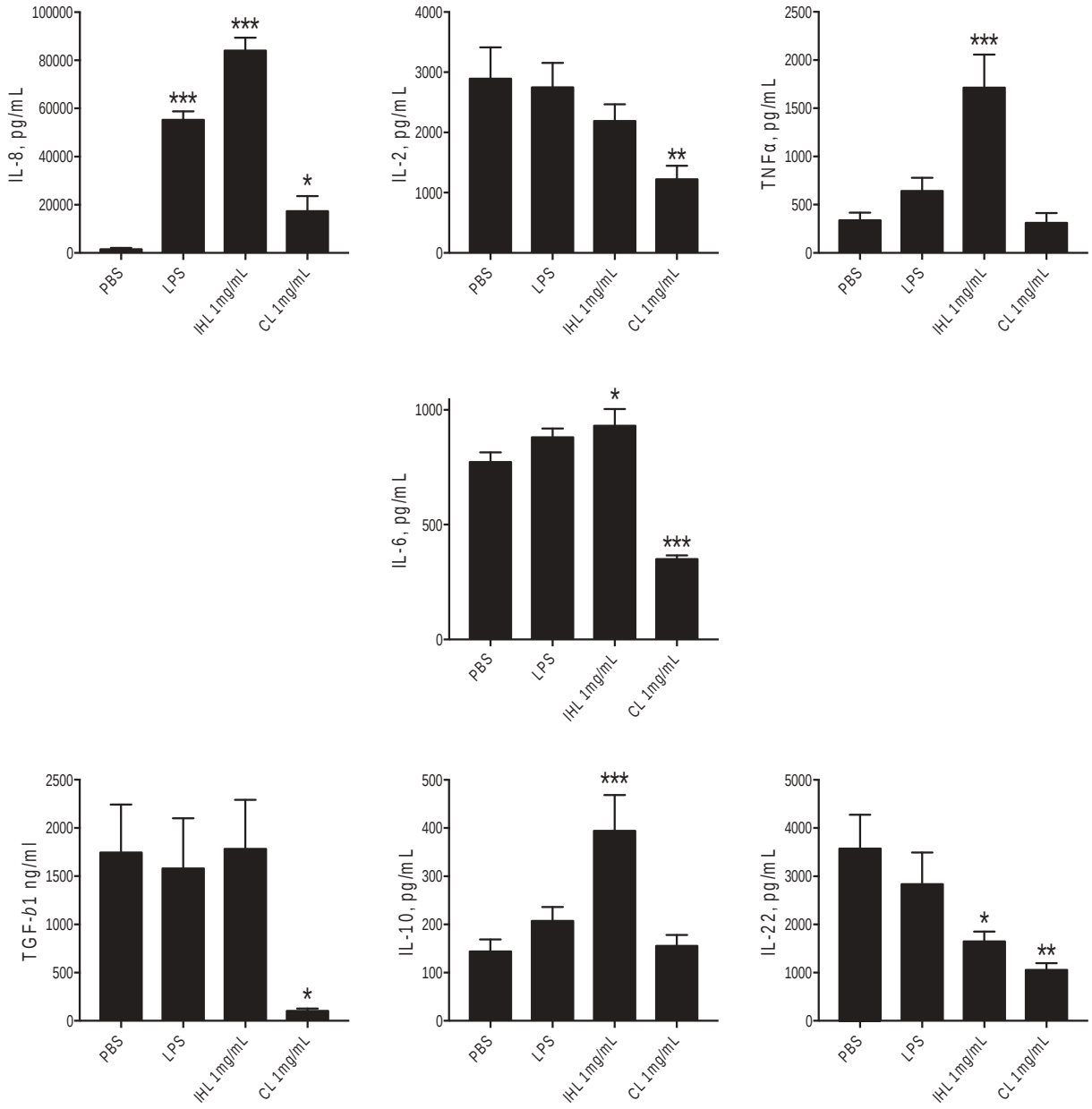


Fig. 9. The effects of β-glucan samples on cytokine expression of THP-1 cells preactivated with PMA. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ versus PBS.

for use β-glucan extractions from the edible mushroom Future research is also required to study putative relationship (if any) of variation in the extraction methods producing different β-glucan preparations and cytokine storm associated with COVID-19. *L. edodes* may also have future potential by way of influencing immunotherapies for addressing COVID-19 that rely on reducing cytokine storm. Further clinical studies are merited to refine β-glucan as a countermeasure for tackling cytokine storm that causes ARDS, as evident with COVID-19.

Funding

The authors acknowledge funding support from the Health Research Board Project [grant number HRB-HRA-POR-2014-664], Republic of Ireland, and Athlone Institute of Technology Doctoral Scholarship [grant number AIT0614], Republic of Ireland.

Contributions to publication

All author contributed equally to this research paper.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to all the researchers whom we cited in this study for their significant and valuable research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139330.

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Science of the Total Environment 809 (2022) 152177

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Immunomodulatory activity of β-glucan polysaccharides isolated from different species of mushroom – A potential treatment for inflammatory lung conditions

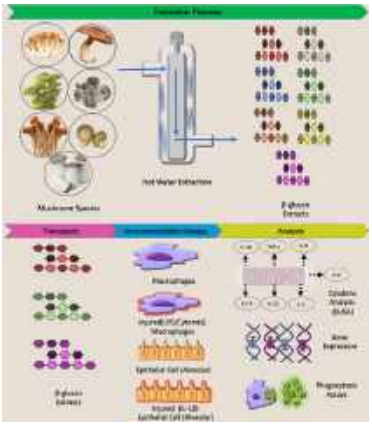
Emma J. Murphy [a,b,1], Emanuele Rezoagli [c,d,e,*,1], Robert Pogue [a,f], Bianca Simonassi-Paiva [a], Ismin Izwani Zainol Abidin [g], Gustavo Waltzer Fehrenbach [a], Emer O'Neil [a], Ian Major [g], John G. Laffey [c,d], Neil Rowan [a]

[a] Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland
[b] Department of Graduate Studies, Limerick Institute of Technology, Limerick, Ireland
[c] Lung Biology Group, Regenerative Medicine Institute at CURAM Centre for Medical Devices, School of Medicine, National University of Ireland Galway, Galway, Ireland
[d] Anaesthesia and Intensive Care Medicine, University Hospital Galway, Galway, Ireland
[e] Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy
[f] Post-Graduate Program in Genomic Sciences and Biotechnology, Catholic University of Brasilia, Brazil
[g] Materials Research Institute, Athlone Institute of Technology, Athlone, Ireland

HIGHLIGHTS

- There is biological variance in glucan content between different mushroom species.
- β-glucans extracts reduce inflammatory biomarkers in macrophages after LPS and Cytomix injury.
- β-glucans extracts reduce inflammatory cytokine secretion in in-vitro lung cells after injury with inflammatory biomarkers.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 August 2021
Received in revised form 29 November 2021
Accepted 30 November 2021
Available online 4 December 2021

Editor: Lotfi Aleya

Keywords:
β-Glucans

ABSTRACT

Acute respiratory distress syndrome (ARDS) is the most common form of acute severe hypoxemic respiratory failure in the critically ill with a hospital mortality of 40%. Alveolar inflammation is one of the hallmarks for this disease. β-Glucans are polysaccharides isolated from a variety of natural sources including mushrooms, with documented immune modulating properties. To investigate the immunomodulatory activity of β-glucans and their potential as a treatment for ARDS, we isolated and measured glucan-rich polysaccharides from seven species of mushrooms. We used three models of in-vitro injury in THP-1 macrophages, Peripheral blood mononuclear cells (CD14+) (PMBCs) isolated from healthy volunteers and lung epithelial cell lines. We observed variance between β-glucan content in extracts isolated from seven mushroom species. The extracts with the highest β-glucan content found was *Lentinus edodes* which contained 70% w/w and *Hypsizygus tessellatus* which contained 80% w/w with low levels of α-glucan. The

* Corresponding author at: Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy.
E-mail address: emanuele.rezoagli@unimib.it (E. Rezoagli).
[1] E.J. Murphy and E. Rezoagli equally contributed.

http://dx.doi.org/10.1016/j.scitotenv.2021.152177

THP-1 macrophages
Lung injury
ARDS
Medicinal mushrooms
One-health

extracts had the ability to induce secretion of up to 4000 pg/mL of the inflammatory cytokine IL-6, and up to 5000 pg/mL and 500 pg/mL of the anti-inflammatory cytokines IL-22 and IL-10, respectively, at a concentration of 1 mg/mL in THP-1 macrophages. In the presence of cytokine injury, IL-8 was reduced from 15,000 pg/mL to as low as 10,000 pg/mL in THP-1 macrophages. After insult with LPS, phagocytosis dropped from 70–90% to as low 10% in CD14+ PBMCs. After LPS insult CCL8 relative gene expression was reduced, and IL-10 relative gene expression increased from 50 to 250-fold in THP-1 macrophages. In lung epithelial cells, both A549 and BEAS-2B after IL-1β insult, IL-8 levels dropped from 10,000 pg/mL to as low as 6000 pg/mL. TNF-α levels dropped 10-fold from 100 pg/mL to just below 10 pg/mL. These results demonstrate the therapeutic potential of β-glucans in inflammatory lung conditions. Findings also advance bio-based research that connects green innovation with One Health applications for the betterment of society.

1. Introduction

Acute respiratory distress syndrome (ARDS), is the most common form of acute severe hypoxemic respiratory failure in the critically ill (Rezoagli et al., 2017). The syndrome is defined by: acute onset of hypoxemia (PaO2:FiO2 ratio <300) and bilateral pulmonary opacities not explained by cardiac failure or fluid overload (Bellani et al., 2016). ARDS is a diffuse inflammatory reaction and can be characterised by an explosive acute inflammatory response in lung parenchyma (Crimi and Slutsky, 2004), impairing the principal function of gas exchange, which can lead to hypoxaemia. Treatment is mainly focused on clinical management as there remains no effective direct pharmacological therapy for this condition (Rezoagli et al., 2019). There is an urgent need for treatment as mortality and morbidity are unacceptably high at 40% (Horie et al., 2020). Furthermore, infection by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has led to further incidences of COVID-19-related ARDS, which is associated with 70% of fatal cases (Rezoagli et al., 2021; Li et al., 2020; Zhou et al., 2020). In both ARDS and in COVID-19-induced ARDS there is a marked increase in serum levels of inflammatory cytokines and chemokines, which is a major contributor to disease severity and ultimately death (G. Chen et al., 2020; X. Chen et al., 2020; Huang et al., 2020; Mehta et al., 2020; Qin et al., 2020). The pathophysiology of ARDS is associated with numerous target immune cells including macrophages. The lung microenvironment during injury determines the functional phenotype of macrophages, which can promote either wound healing or inflammation (Nanchal and Truwit, 2018). Thus, in developing potential therapeutics it is important to understand the potential effects of these therapeutics on lung tissue and on macrophages. An ideal treatment for this condition would aim at reducing the effects of the proinflammatory cascade and would seek to maximize the anti-inflammatory immunomodulatory response (Zambelli et al., 2021).

β-Glucans are defined as complex polysaccharides that are found in an abundance of sources including fungi, yeast, grain, bacteria, and algae (Murphy et al., 2021). β-Glucans can be classified structurally as either 1,3 1,4-linked or 1,3 1,6-linked, which is dependent on their source (Cui et al., 2011; Pogue et al., 2021; Murphy et al., 2020). These molecules are widely marketed as biologically active molecules (bioactives) (Wang et al., 2017a). There are over 200 clinical trials registered for their use for a range of applications. There are also licenced drugs containing β-glucans on the market since 1980 in Japan, for the treatment of cancer (Novak and Vetvicka, 2008; Takeshita et al., 1991; Yang et al., 2019). β-Glucans as pharmaceutical agents have also been authorised in several countries, including the United States of America, Canada, Finland, Sweden, China and Korea (van Steenwijk et al., 2021). The diverse functional effects of these molecules include alteration of lipid and glucose metabolism, cholesterol reduction, obesity regulation and reduction of cardiovascular and diabetic risk, modulating the gut microbiome, altering lipid and glucose metabolism and beneficial effects on gastrointestinal conditions such as irritable bowel syndrome (Drozdowski et al., 2010; Maki et al., 2003; McRorie and McKeown, 2017; Sima et al., 2018; Tiwari and Cummins, 2011). β-Glucans, specifically from non-cereal sources, are widely documented for their immunomodulatory properties, with the ability to stimulate the immune response and initiate inflammatory properties, and to promote resistance to infections (Ooi and Liu, 2012). (Bohn and BeMiller, 1995). Mushroom-derived β-glucans are the most potent immune modulators (Borchers et al., 1999; Ooi and Liu, 2012; Lorenzen and Anke, 1998; Ooi and Liu, 1999; Tzianabos, 2000; Wasser and Weis, 1999). Moreover, they have demonstrated therapeutic effects in alleviating infective respiratory conditions (Fuller et al., 2012; Jesenak et al., 2013; Yamauchi et al., 2008). They have also been documented to reduce pro-inflammatory cytokines, increase anti-inflammatory cytokines, increased formation of antioxidants as well reduction of inflammatory cells in preclinical lung injury models (Bedirli et al., 2007; Jedinak et al., 2011; Johnson et al., 2009; Kofuji et al., 2012; Soltys and Quinn, 1999; Yamada et al., 2007). These beneficial effects can also be seen in clinical trials. When patients were administered β-glucans for the prevention of nosocomial pneumonia and sepsis, the treatment group compared to the control group had lower incidences of pneumonia as well as a lower mortality rate (De Felippe et al., 1993).

We have previously investigated the effects of a commercial β-glucan and an in-house extract of β-glucans from the mushroom *Lentinus edodes* (Masterson et al., 2020; Murphy et al., 2020a, 2020b, 2020c). Specifically, Murphy et al. (2019) showed that β-glucans from the same mushroom, one isolated by hot water extraction and one sourced commercially had different effects, namely reduction in inflammatory cytokines, reduction in phagocytic activity of macrophages after LPS insult and reduction of inflammatory response in in-vitro lung cells. Thus, to continue this work and understand the potential immunomodulatory properties of other mushroom β-glucans as a potential treatment for inflammatory lung conditions like ARDS we decided to replicate the assays and include additional test parameters. In the current study, we first extracted and measured β-glucans from seven species of mushroom to determine BRM variance among species by applying them to a monocytic cell line and an in-vitro lung injury model. Second, we isolated CD14+ monocytes from healthy volunteers and exposed the cells to the extracts, then measured phagocytic activity. Third, we simulated an injurious environment on two types of alveolar cell lines using IL-1β and measured cytokine expression. Finally, we extended this assay to a monocytic cell line, which was inflamed with different insults (LPS and cytomix). It has recently emerged that macrophages are reduced and equally as inflamed as lung cells during COVID-19 infection. Therefore, after injury we measured cytokine release, gene expression, and phagocytosis of these cells to determine immune-modulatory potential in an inflammatory micro-environment.

2. Materials & methods

Commercial Lentinan (CLE) was sourced from Carbosynth (FL33321, Compton, Berkshire, UK). Fruiting bodies of mushrooms were kindly gifted by Garryhinch Wood Exotics Ltd. Garryhinch, Portarlington, Co Offaly, Ireland. The fruiting body of *Agaricus blazeii* was kindly gifted by Professor Leo van Griensven, Wageningen University, The Netherlands. Other species of mushroom included; *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), Pleurotus eryngii (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

2.1. β-Glucan extraction

To extract β-glucans from the fruiting bodies of the mushrooms, the method used previously by Murphy et al. (2019) was employed. Briefly –

the fruiting bodies were washed and dried. The samples were blended into a fine powder. Roughly, 100 g of dried blended biomass was placed in 1 Litre of water and autoclaved. After autoclaving the polysaccharides were precipitated from supernatant using 100% Ethanol. Precipitates were dried and solubilised in PBS for analysis.

2.2. β-Glucan quantification

Extracts were analysed for 1-3 1-6 β-glucan content using the Megazyme yeast and mushroom kit (K-YBGL) (Megazyme Ltd., Bray, Co. Wicklow, Ireland). Assays were carried out according to manufacturer's instructions. After milling, samples were placed in 12 M H_2SO_4 at −4 °C for 2 h to solubilize the β-glucans. Samples were then hydrolysed in 2 M H_2SO_4 at 100 °C for a further 2 h. Any remaining β-glucan fragments were quantitatively hydrolysed to glucose using a mixture of exo-1,3-β-glucanase and β-glucosidase which gives a measurement of total β-glucan content after substrate addition. The α-glucan content of the sample was determined by hydrolysing specifically to D-glucose and D-fructose. Glucose was measured with amyloglucosidase and invertase using a glucose oxidase peroxidase GOPOD reagent. β-Glucan content was determined by the difference between the two measurements.

2.3. Blood donor cell collection

Blood sample collection was approved by the Athlone Institute of Technology Ethics Committee. Blood samples were obtained from healthy volunteers for isolation of immune cells. A total of 15 mL was collected from each donor. Individual cells were isolated from 5 mL aliquots of collected blood. Samples were magnetically labelled with whole blood microbeads (Miltenyi Biotec, Germany) to isolate cells based on specific surface molecules according to the manufacturer's instructions, using the autoMACS separator (Miltenyi Biotec).

2.4. Cell culture

A549 cells (used at passage 90), BEAS-2B cells (used at passage 10), and THP-1 monocyte cells (used at passage 20), were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/mL) and streptomycin (100 μg/mL) solution (Sigma-Aldrich), at 37 °C a 5% CO_2 environment. For differentiation into macrophages, THP-1 monocyte cells were treated with phorbol 12-myristate 13-acetate (PMA) for differentiation into THP-1 macrophages. (Peprotech EC, London, UK), at a concentration of 100 ng/mL, for 48 h.

2.5. CD14+ PBMCs

CD14+ cells were positively isolated based on the surface molecule CD14, which is primarily found on monocytes (Shin et al., 2019). Isolated cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal calf serum (Sigma-Aldrich), 1% penicillin G (100 U/mL)/streptomycin (100 μg/mL) solution (Sigma-Aldrich) and 50 ng/mL of macrophage colony stimulating growth factor (MCSGF) (RnD Systems MN, USA) at 37 °C in a 5% CO_2 environment.

2.6. Cell injury and β-glucan treatment

All cell types were treated with 1 mg/mL of β-glucan in PBS based on other published work (Jung et al., 2007; Murphy et al., 2020a, 2020b, 2020c; Sari et al., 2020; Sivinski et al., 2020). For injury assays THP-1 PMA differentiated macrophage cells were seeded at a density of 4×10^5 cells/well in 96-well plates, and 24 h later were injured with two different types of insult: either LPS (100 ng/mL) (Sigma) or cytomix (TNF-α, IFN-ϒ, IL-1β), at 25 ng/mL (Immunotools), in RPMI supplemented with 1% penicillin/streptomycin. After 24 h cells were washed three times in PBS and treated with 1 mg/mL of extracts for a further 24 h before analysis. Pulmonary alveolar type II A549 cells were seeded at a density of 4×10^5 cells/well in 96 well plates. After 24 h cells were injured with 1 ng/mL of IL-1β (Peprotech, Rocky Hill, NJ) in RPMI supplemented with 1% penicillin/streptomycin.

2.7. Enzyme linked immunosorbent assay (ELISA)

A human Duoset sandwich ELISA kit (RnD Systems MN, USA) was used to measure cytokine levels in the medium after β-glucan exposure. All ELISA assays were performed according to the manufacturer's instructions. Results were expressed either in pg/mL or in ng/mL.

2.8. Phagocytosis assays

To determine Phagocytic activity, THP-1 macrophages (PMA differentiated) and CD14+ cells were seeded into 96-well plates at 4 × 105 cells/well. After 24 h, cells were either injured or treated with PBS. After a further 2 h, cells were treated with β-glucan extracts. After a further 24 h cells were washed with PBS and incubated with Alexa Fluor 488-conjugated E.coli (K-12 strain) Bioparticles (E13231; Life Technologies) for 2 h, after which cells were washed three times with PBS to remove residual particles before resuspension in FACS flow buffer and measured for fluorescent particles by flow cytometry (Miltenyi Biotec, Germany).

2.9. RNA extraction

For RNA extraction from THP-1 macrophage cells, Media was removed, the cells were washed 3× with PBS, and RNA was extracted using the Purelink RNA Mini kit (Thermo-Fisher), according to the manufacturer's instructions. RNA was analysed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA) to determine RNA concentrations and A260/A280 ratios.

2.10. cDNA synthesis and real-time PCR

cDNA was prepared for the replicate samples using the SensiFAST cDNA Synthesis kit (Bioline), according to the manufacturer's instructions. RNA input for all samples was normalized to the sample, so that 325.5 ng of total RNA were used in each reaction. Real-time quantitative PCR was performed using pre-designed TaqMan Gene Expression Assays for the respective genes, together with the TaqMan Gene Expression Master Mix (Thermo-Fisher). The transcripts examined were: TLR2, IL-10, CCL8, CLEC-7a and MCSGF. Reactions were carried out on the LightCycler 96 equipment (Roche), using the GAPDH transcript as endogenous control. Relative gene expression was calculated using the 2ˆ-ddCq method.

2.11. Statistical analysis

Continuous data were expressed as mean and standard error of the mean (SEM). Differences of continuous variables between species of mushrooms and PBS and injury (i.e. LPS or Cytomix or IL-1 β) were assessed by one-way analysis of variance for independent measures. Post-hoc comparisons were investigated by controlling the False Discovery Rate using the two-stage step-up method of Benjamini, Krieger and Yekutieli test. Statistical significance was considered with a p-value < 0.05 (two-sided). Statistical analyses were performed using STATA/MP 16.0 for Windows (StataCorp LLC, College Station, TX 77845, USA) and GraphPad Prism 8 for Windows (Version 8.0.2, FraphPad Software, Inc.).

3. Results

3.1. β-Glucans quantification

β-glucans were extracted from seven species of mushrooms as previously described (Murphy et al., 2020a, 2020b, 2020c). After extraction

and isolation, the Megazyme 1,3 1,6 kit was used to determine the concentration of α- and β-glucans.

There was variance between species as can be seen in the relative concentrations of β-glucan and α-glucan displayed in Fig. 1. P.E appeared to have the purest concentration of β-glucan compared to other extracts. A.B and P.O appeared to have high levels of contaminating α-glucan present. H.T yielded the highest concentration of β-glucan with little contaminating α-glucan. H.T yielded the highest concentration of β-glucan with little contaminating α-glucan.

3.2. Effect of β-glucans on macrophages

3.2.1. The direct effect of β-glucans on THP-1 macrophages

To understand the effects of β-glucan extracts on macrophages, THP-1 macrophages were treated with each of the extracts and cytokine secretion levels were measured by an ELISA. Results show (Fig. 2) that each extract had a different effect on the cytokine release profile from THP-1 macrophages. Extracts had the potential to increase secretion of both inflammatory cytokines (IL-6, IL-8, TNF-α) and anti-inflammatory cytokines (IL-10 and IL-22). CLE is a commercial source of β-glucan, and its extraction method is unknown; all other extracts were processed as described in the methods Section 2.1. Although the extracts have different effects on the cytokine secretion profile, the pattern was generally similar except for CLE. CLE induced lower secretion levels of IL-6 (Panel A), IL-22 (Panel C), and IL-10 (Panel E), compared to the other extracts and lower levels of IL-2 (Panel F) compared to the PBS control. The remaining extracts increased IL-6, TNF-α (Panel B) and IL-10 secretion and maintained IL-22 and IL-2 compared to PBS control. CLE and some of the extracts (L.E, C.L.E and P. C) appeared to increase the secretion of the chemokine IL-8 compared to control (Panel D).

3.2.2. Effect of β-glucans on phagocytosis by THP-1 and CD14+ PBMC macrophages

To compare the effects of β-glucan extracts on a macrophage cell line (THP-1) and on fresh PBMCs (CD14+), cells were treated with extracts, and after 24 h phagocytic activity was measured and displayed in Fig. 3. Results varied: Panel A shows the effects on THP-1 macrophages; LPS significantly increased phagocytosis relative to untreated cells. Four extracts (P. M, P.C, P. E and H.T) were able to significantly reduce phagocytosis, with PM showing by far the greatest reduction. CD14+ PBMCs (Fig. 3, Panel B) showed varying responses to the extracts in terms of phagocytosis, as expected due to donor variability. The extract A.B significantly reduced the phagocytic activity, although the overall percentage phagocytosis was low in these cells. The extracts showed a tendency toward reduction in PBMCs, however, the results do not show significance, potentially due to donor variability.

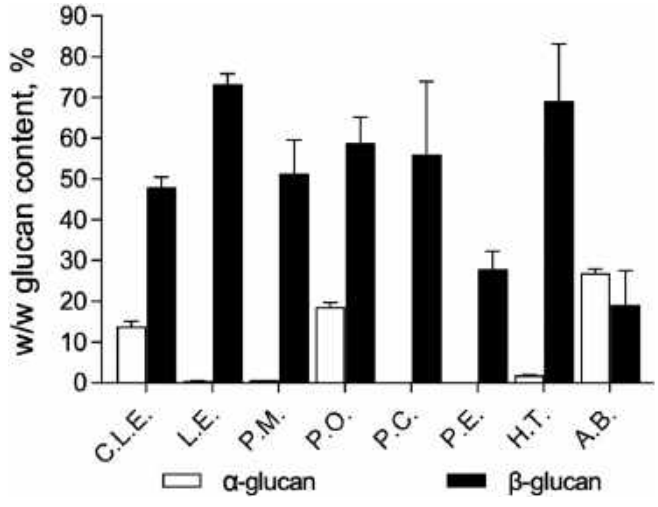


Fig. 1. The percentage w/w α-glucan and β-glucan content in mushroom extracts using Megazyme. Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

To gain a further understanding of the mechanism, expression levels of four genes were measured. Two genes were related to cytokine/chemokine response: IL-10 (Fig. 4; Panel B), and CCL8 (IL-8; Fig. 4, Panel D). Two genes corresponded to cell surface ligands associated with β-glucan recognition: toll-like receptor 2 TLR-2 (Fig. 4, Panel A), and dectin-1 (CLEC7a, Fig. 4, Panel C).

Expression of TLR2 in THP-1 macrophages showed no significant difference in induction between LPS and the β-glucans. However, compared to PBS control, P.O induced expression of TLR2. All the extracts significantly inhibited the relative gene expression levels of CLEC7a compared to both PBS control and LPS in THP-1 macrophages. IL-10 expression showed no significant increase in gene expression compared to controls except for with P.M. The extracts did not significantly induce the expression of CCL8 compared to PBS controls and induction was significantly lower compared to LPS.

To determine the effect of β-glucans on macrophages after injury, THP-1 macrophages were injured with Cytomix (IL-1β, TNF-α & IFN-γ), and then treated with β-glucan extracts (Fig. 5). ELISA assay results show the β-glucan extracts from L.E, P.E, H.T and A.B significantly increased the secretion of IL-6 after insult (Panel A). P.O and P.C did not have the same induction profile as when directly treated with injury (Fig. 2 Panel A), which induced ~2000 pg/mL secretory levels of IL-6. After insult with cytomix P. O and P.C induced ~1000 pg/mL secretory levels of IL-6. IL-8 secretion was increased after cytomix treatment alone (Fig. 5, Panel B). However, the strongest inducers of IL-8 secretion with direct treatment were L.E, C.L.E and P.C (Fig. 2 Panel D), all of which significantly reduced the secretion of this inflammatory chemokine after injury except for LE which was not significant. There was no significant effect of β-glucan treatments on TNF-α secretion after cytomix insult (Fig. 5, Panel C).

Both THP-1 macrophages and PBMCs were analysed for phagocytic activity (Fig. 6) after injury with LPS. The THP-1 macrophages after injury (Fig. 6, Panel A) had a very similar response to those with β-glucan extracts alone (Fig. 3 Panel A) compared with treatment after injury. When CD14+ cells were treated with β-glucan extracts, only A.B significantly reduced phagocytosis (Fig. 3 Panel B). However, when administered after LPS, all β-glucan extracts reduced percentage phagocytosis (Fig. 6, panel B). Panel C displays the phagocytosis percentage of THP-1 macrophages after cytomix insult; C.L.E, P.M, P.O, P.C and P.E reduced the phagocytic activity after insult and treatment.

Inflammatory gene expression and anti-inflammatory gene expression markers were analysed after both types of injury, as displayed in Fig. 7. The inflammatory marker CCL8 was measured after LPS injury (Panel A) and cytomix (Panel B). In the presence of LPS, CCL8 relative gene expression was significantly reduced after treatment with all extracts. In the presence of cytomix insult, L.E, P.O, P.C and H.T all increased the relative gene expression of CCL8. The anti-inflammatory marker IL-10 was measured after LPS injury (Panel C) and cytomix (Panel D). After LPS insult L.E, H.T and A.B all significantly increased the expression of IL-10 gene compared to injury alone. All the extracts increased the expression of IL-10 compared to injury alone.

3.3. Effect of β-glucans in an in-vitro lung injury model

In previous work carried out by this group, we established that β-glucan extracts (L.E) have the potential to reduce inflammation in alveolar A549 cell lines (Murphy et al., 2019). To further expand on this work, the same assays were repeated with another alveolar cell line BEAS-2B, using six new extracts.

3.3.1. A549 cells

To determine the direct effects of the β-glucan extracts on lung cells, extracts were incubated with A549 cells for 24 h and supernatant was

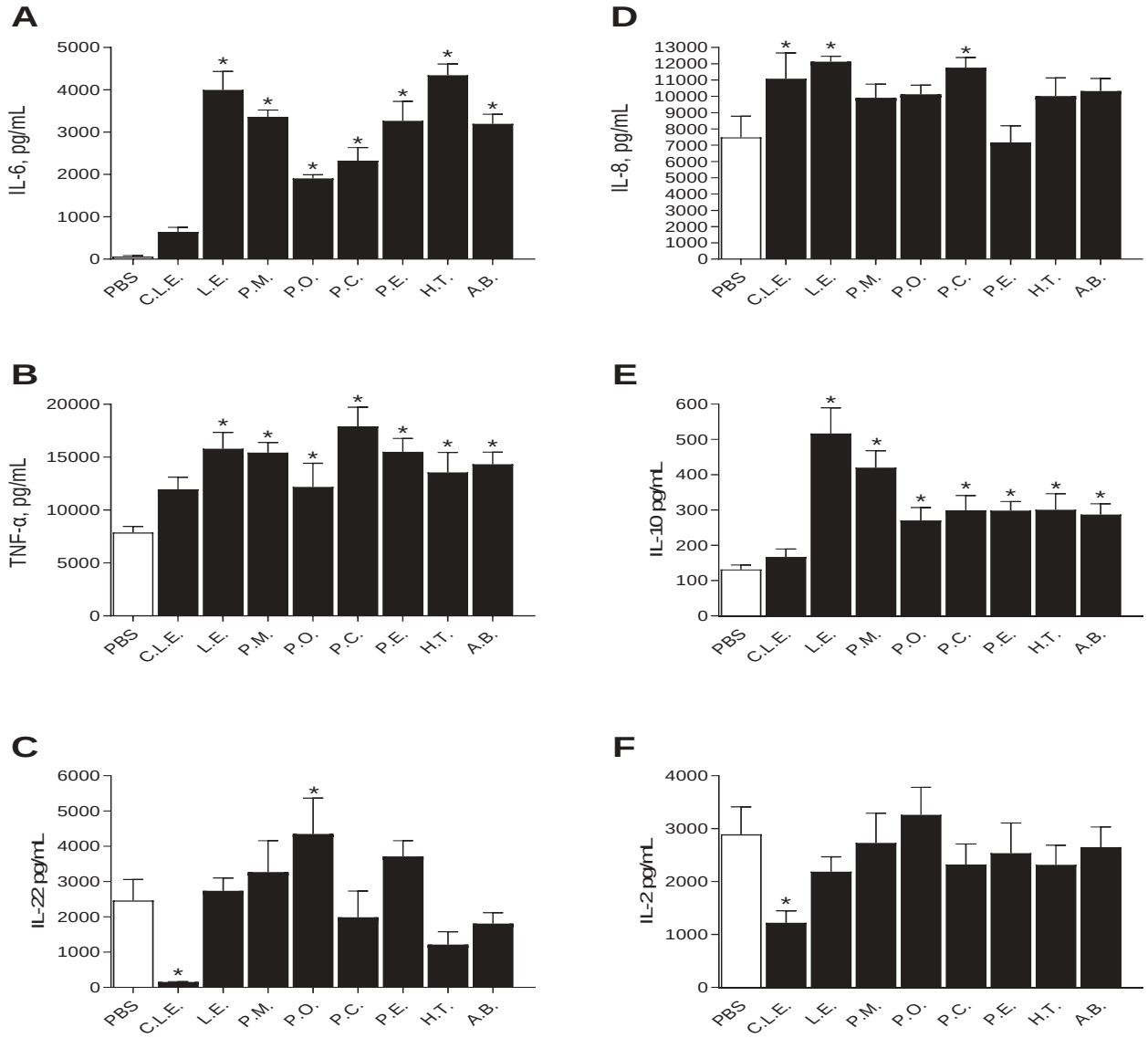


Fig. 2. The effect of the β-glucan extracts on cytokine expression in THP-1 macrophages (PMA differentiated) measured using ELISA. Panel A; IL-6, Panel B; TNF-α, Panel C; IL-22, Panel D; IL-8, Panel E; IL-10 Panel F; IL-2. $p < 0.05$ versus PBS. Cells were treated with 1 mg/mL of extracts for 24 h before cytokine analysis. Phosphate buffer saline (PBS), Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

measured for cytokines. Results are displayed in Fig. 8. Panel A shows the release of IL-6 after treatment; P.M, P.A, P.E, H.T and A.B all significantly induce the secretion of IL-6 compared to PBS control. Panel B shows the secretion of IL-8. All the extracts induced the secretion of IL-8 from A549 cells with respect to PBS control except for P.E. When A549 cells were treated with the extracts, TNF-α secretion was increased with L.E, P.O, P.E, H.T and A.B with respect to PBS control (Panel C).

A549 cells were then treated with IL-1β to induce a cytokine injury. Subsequently the cells were treated with the β-glucans extracts and the cytokine analysis was repeated, as displayed in Fig. 8. Panel D shows that C.LE., P.M and P.A slightly reduced IL-6 secretion (though not significantly), after insult except for L.E which increases secretion with respect to injury alone. Panel E shows that after insult L.E, C.L.E, P.O, P.E and A.B reduce the section of IL-8, which is an opposite response to when cells are treated in the absence of injury (Fig. 8; Panel B). The extracts P.E, H.T and A.B significantly reduced TNF-α secretion after IL-1β insult (Panel F) which is again an opposite response to when the cells are treated alone with extracts (Panel C).

3.3.2. BEAS-2B

To understand if the β-glucan extracts would have a similar effect in another lung epithelial cell line, BEAS-2B cells were treated with the β-glucans extracts. The supernatant was then measured for cytokine secretion. Results are displayed in Fig. 9. All extracts induced the secretion of IL-6 (Panel A), and IL-8 (Panel B) with respect to PBS control. Panel C shows the secretion of TNF-α after treatment, C.L.E increased secretion but the other extracts had no effect.

Like the A549 cells, BEAS-2B cells were then treated with IL-1β to induce a cytokine injury; cells were then treated with the β-glucans extracts and the cytokine analysis was repeated, as displayed in Fig. 9. There was no effect on IL-6 secretion (Panel D). Panel E shows that after insult C.L.E, P.A, P.E, H.T and A.B reduced the section of IL-8 which is an opposite response to when cells are treated in the absence of injury (Fig. 9; Panel B). The extracts P.E, H.T and A.B significantly reduced TNF-α secretion after IL-1β insult (Fig. 9; Panel F) however these levels were the same in the absence of injury (Fig. 9; Panel C) suggesting expression levels are maintained in the presence of injury.

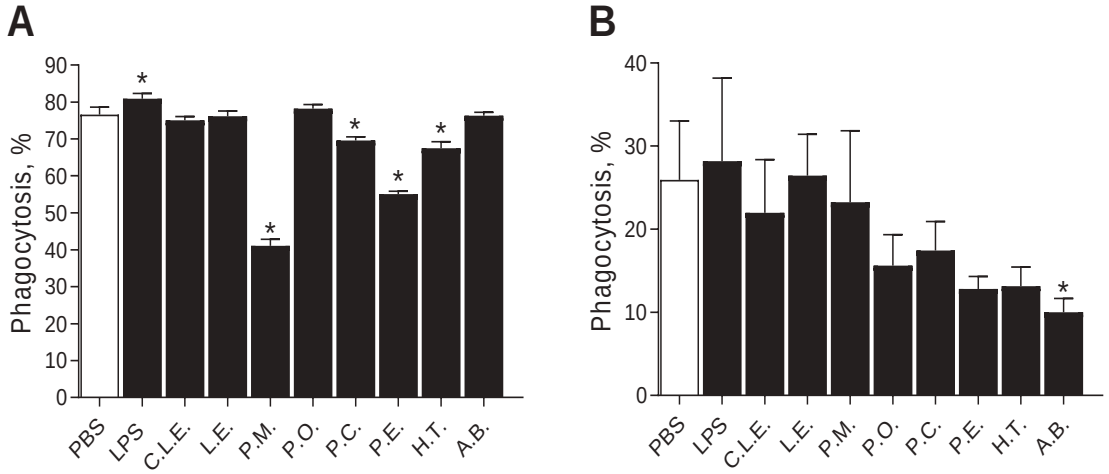


Fig. 3. The effects of the β-glucan extracts on percentage phagocytosis measured using flow cytometry analysis of uptake of Alexa Fluor 488-conjugated E.coli (K-12 strain) Bioparticles. THP-1 macrophages were treated with 1 mg/mL of extracts for 24 h before phagocytic analysis. Panel A; THP-1 macrophages; Panel B; CD14+ primary macrophages. Phosphate buffer saline (PBS), Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

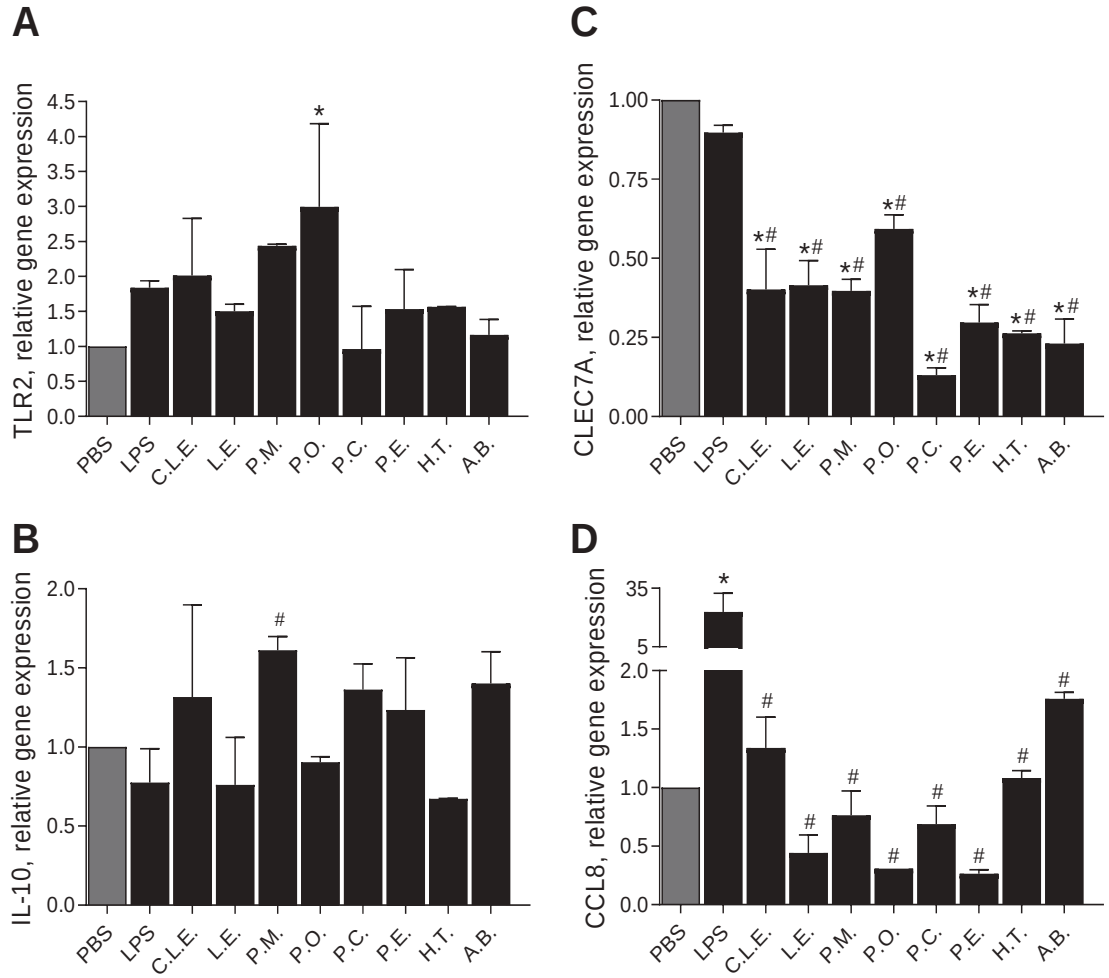


Fig. 4. The effect of the β-glucan extracts on gene expression levels of THP-1 macrophages (PMA differentiated), relative to PBS-treated cells (expression level = 1.0). Panel A; TLR2, Panel B; IL-10, Panel C; CLEC7a, Panel D; CCL8. Differences in relative gene expression *$p < 0.05$ versus PBS; #$p < 0.05$ versus LPS. Cells were treated with 1 mg/mL of extracts for 24 h before analysis of gene expression levels. Phosphate buffer saline (PBS), Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

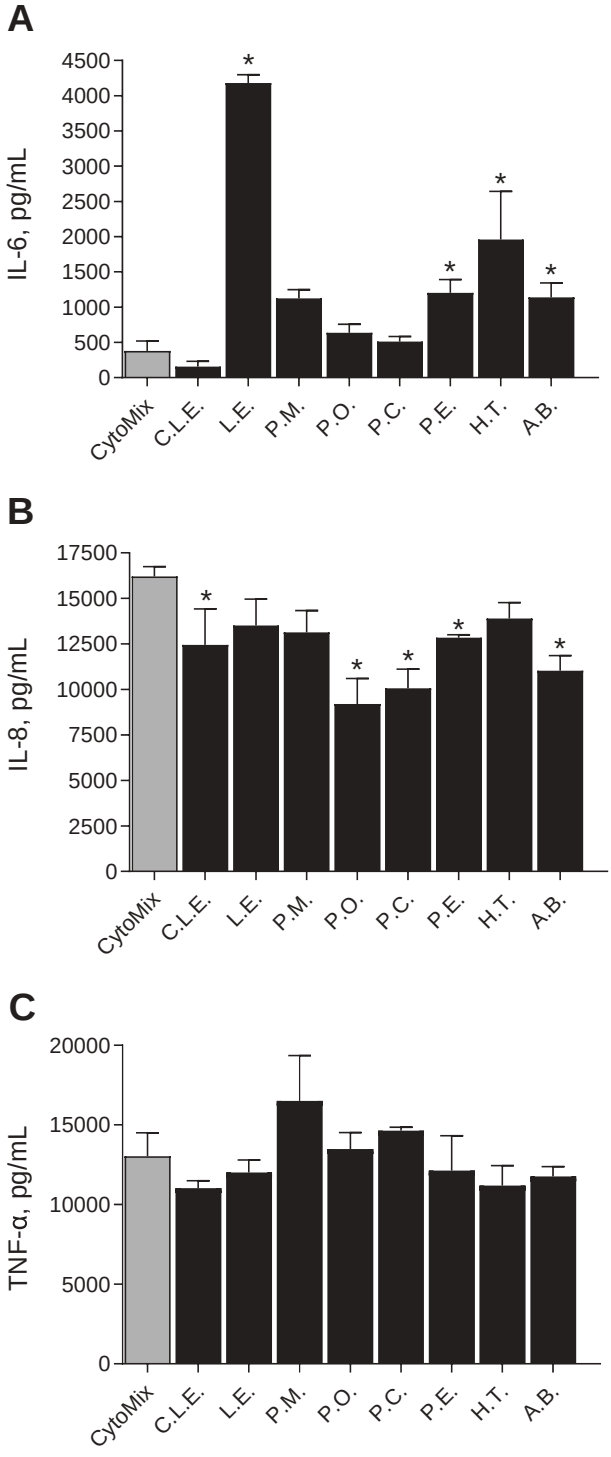


Fig. 5. The effects of the β-glucan extracts on THP-1 macrophages (PMA differentiated) after cytokine insult measured using ELISA. Panel A; IL-6, Panel B; IL-8, Panel C; TNF-α. *$p < 0.05$ versus cytomix. Cells were treated with cytomix (TNF-α, IFN-ϒ, IL-1β), at 25 ng/mL for 24 h, after which they were washed with PBS and treated with extracts (1 mg/mL) for 24 h before cytokine analysis. Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

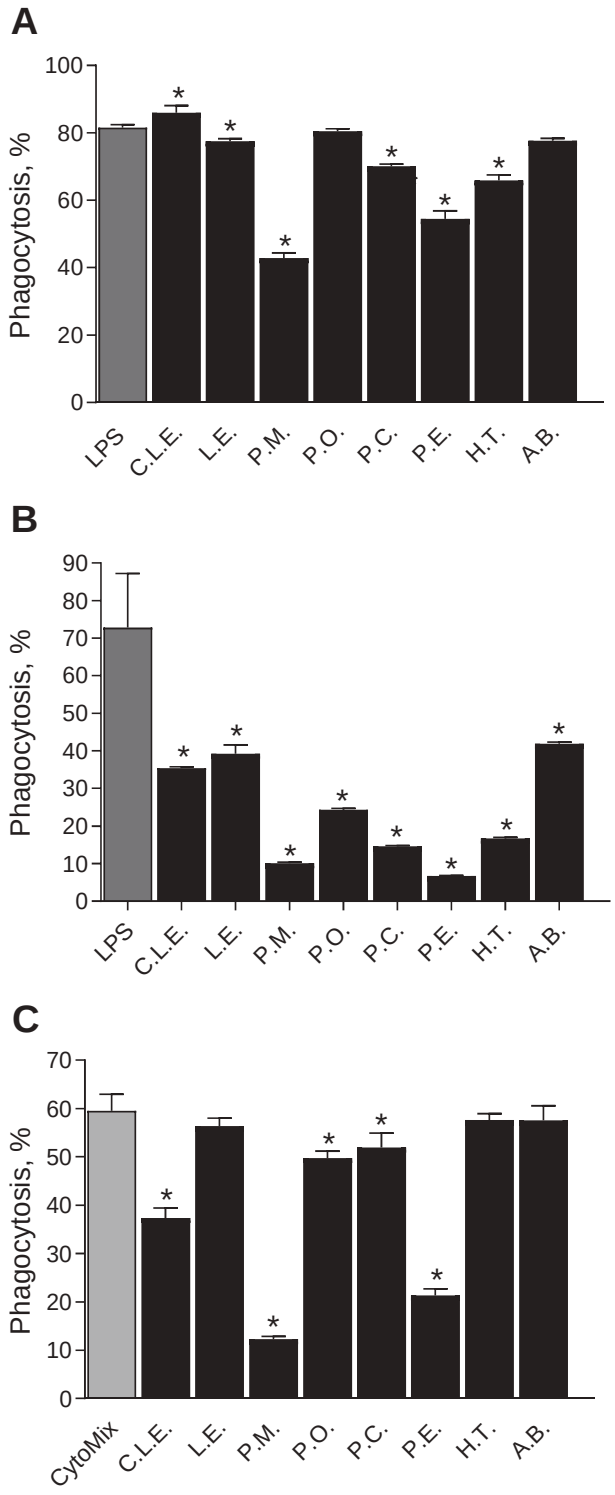


Fig. 6. The effects of the β-glucan extracts on THP-1 macrophages (PMA differentiated) after cytokine insult or LPS insult. Percentage phagocytosis measured using flow cytometry analysis of uptake of Alexa Fluor 488-conjugated *E. coli* (K-12 strain) Bioparticles. Panel A; THP-1 macrophages (PMA differentiated) treated with LPS, Panel B; CD14+ PBMCs treated with LPS. Panel C; THP-1 macrophages (PMA differentiated) treated with cytomix. *$p < 0.05$ versus Cytomix or LPS. Cells were treated with cytomix (TNF-α, IFN- ϒ, IL-1β), at 25 ng/mL or LPS for 24 h after which they were washed with PBS and treated with extracts (1 mg/mL) for 24 h before phagocytic activity analysis. Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazeii* (A.B).

4. Discussion

ARDS-associated lung injury develops a state which is marked by an increase in serum levels of inflammatory chemokines and cytokines; this is a

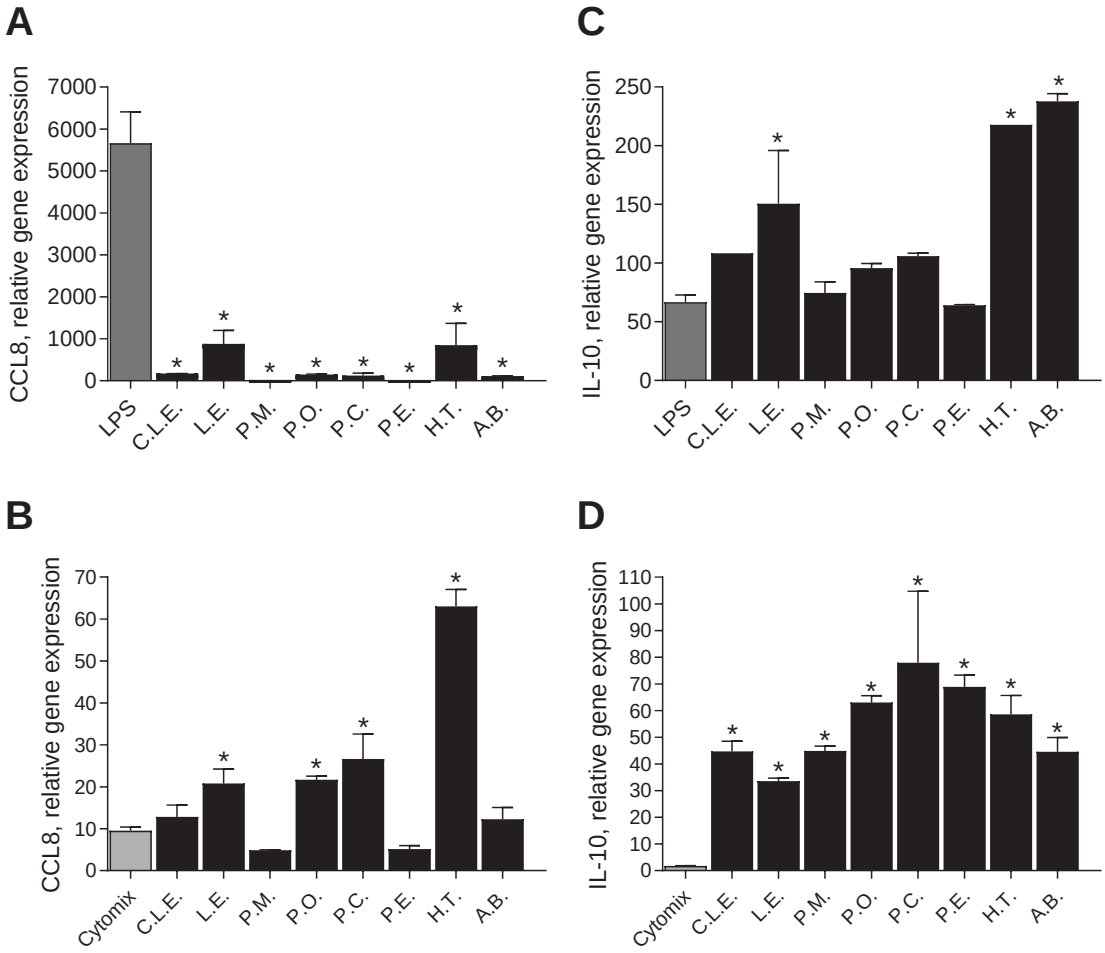


Fig. 7. The effects of the β-glucan extracts on THP-1 macrophages (PMA differentiated) after LPS (Panels A, C) or Cytomix (Panels B, D) injury relative to PBS-treated cells (expression level = 1.0). Panels A and B show IL-8 expression after β-glucan treatments, relative to insult; Panels C and D show IL-10 expression after β-glucan treatments, relative to insult. *$p < 0.05$ versus LPS /Cytomix. THP-1 macrophages (PMA differentiated) were treated with cytomix (TNF-α, IFN- Y, IL-1β), at 25 ng/mL or LPS for 24 h after which they were washed with PBS and treated with extracts (1 mg/mL) for 24 h before gene expression analysis. Commercial Lentinan (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazei* (A.B).

major contributor to disease severity and ultimately death (G. Chen et al., 2020; X. Chen et al., 2020; Huang et al., 2020; Mehta et al., 2020; Qin et al., 2020). There is currently a vast literature relating to the immunomodulatory effects of β-glucan (Rao et al., 2020), and there is a huge level of enthusiasm regarding their therapeutic potential. Thus with this in mind, there are three aims to this work. Firstly, to understand if β-glucans extracted in the same way from different species of mushroom contained the same levels of β-glucan content and if these extracts had the same effect on a key player in cellular immunity and response – macrophages. The second aim was to determine if the samples could elicit a response or prime immune cells, and whether there would be a difference in priming effects. Our final aim was to determine whether there may be the potential for the extracts to be used in hyperinflammatory conditions such as ARDS. To understand this, two types of in-vitro models were used – injured macrophages and lung injury models. This approach will potentially facilitate a greater understanding into the biological variance of these compounds and realising their therapeutic potential.

It is recognised in the literature that β-glucans from different sources can exert different biological effects, and that different extraction methods may help to optimize performance. Our experiments were performed using the same extraction method on all seven mushroom species. As such, we can compare the immunomodulatory effects of the β-glucan extracts by the same extraction procedure. Further studies may examine the effects of altering extraction parameters on β-glucan activity. Furthermore, future structural analyses would allow us to deepen the structure-function relationship. Further studies may examine the effects of altering extraction parameters on β-glucan activity. Furthermore, future structural analyses would allow us to deepen the structure-function relationship. The diverse mechanisms of action of β-glucans is unknown. There are differences in the effects of β-glucans that can be observed between similar preparations from the same species or source. The cellular pathways that are activated after recognition are also not fully understood. β-Glucans appear to be recognised as pathogen associated molecular patterns (PAMPs) and modulate immune function via this pathway (Brown and Gordon, 2005; Borchers et al., 1999). However, the exact mechanism by which β-glucans suppress inflammatory cytokines and induce anti-inflammatory cytokines are complex, and incompletely understood. With this in mind, previous work by this group, Murphy et al., 2019 investigated the differential effects of two β-glucan extracts in an in-vitro lung injury model and in an in-vivo model of pulmonary sepsis (Masterson et al., 2020). Once, determined fungal β-glucans had immune-modulatory effects in lung injury pre-clinical models, the next advancement is to highlight other potential fungal derived β-glucans with immune-modulatory activity. Once identified, future studies will investigate the structure-activity relationship to gain an understanding of how these molecules elicit their effects and the pathways associated with these effects.

The Key findings of this work include - There is a variance in the levels of β-glucan between mushroom species extracted in the same way. This study found that *Lentinus edodes* and *Hypsizygus tessellatus* had the highest levels of β-glucan content when measured using the Megazyme assay.

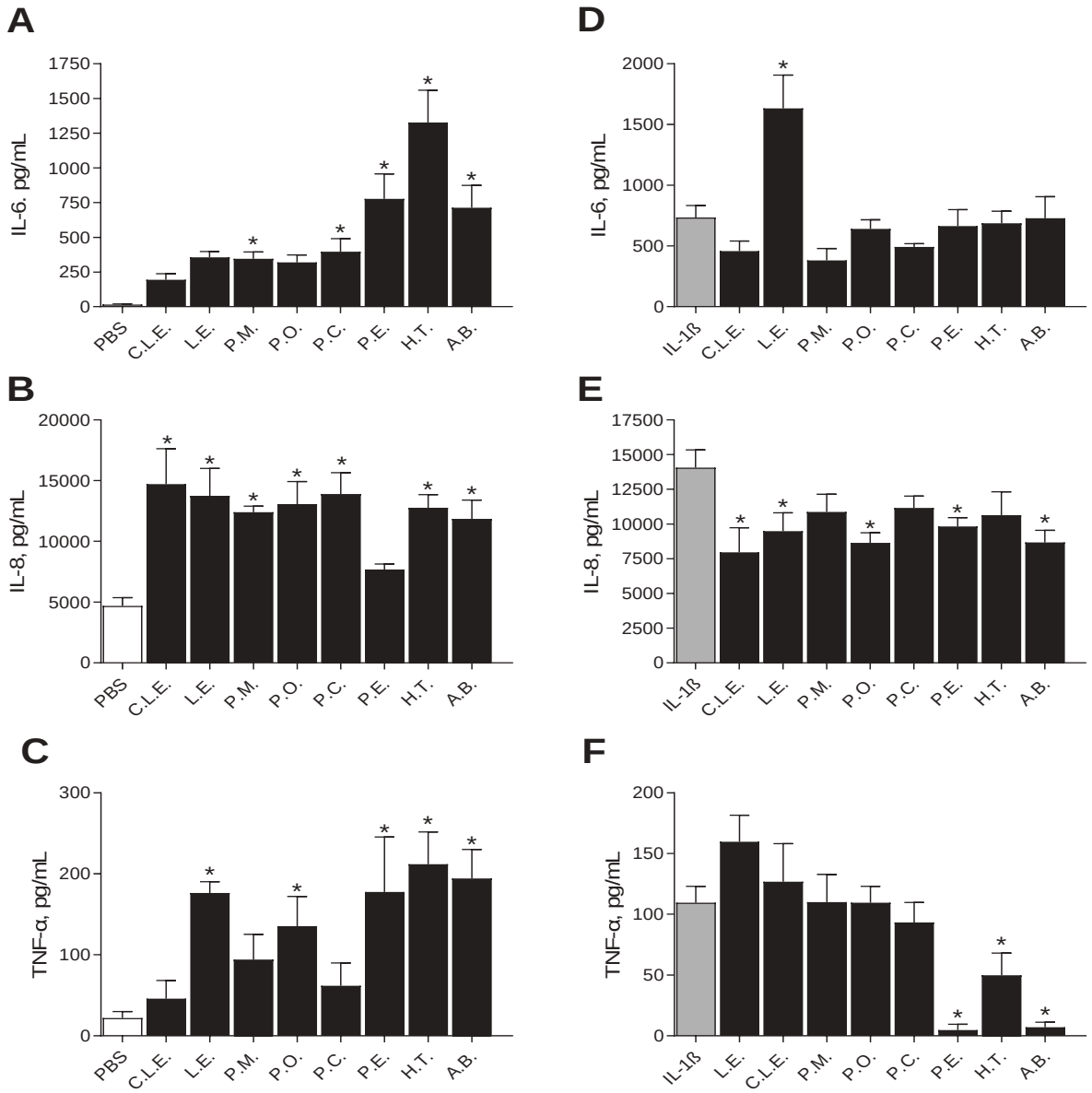


Fig. 8. The effect of the β-glucan extracts on cytokine expression in A549 cells measured using ELISA. Panel A; IL-6, Panel B; IL-8, Panel C; TNF-α. The effect of the β-glucan extracts on cytokine expression in BEAS-2B cells after IL-1β insult, measured using ELISA. Panel D; IL-6, Panel E; IL-8, Panel F; TNF-α. $p < 0.05$ versus PBS or IL-1β. Uninjured cells were treated with extracts (1 mg/mL) for 24 h before cytokine analysis. Cells were treated with IL-1β at 1 ng/mL for 24 h after which they were washed with PBS and treated with extracts (1 mg/mL) for 24 h before cytokine analysis. Phosphate buffer saline (PBS), *Commercial Lentinan* (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazei* (A.B).

Most extracts had the ability to induce both pro and anti-inflammatory cytokines individually at a concentration of 1 mg/mL in THP-1 macrophages. In the presence of a paracrine insult of a cocktail of cytokines; IL-8 was reduced in THP-1 macrophages. Also observed was a reduction in phagocytosis in THP-1 macrophages and CD14+ macrophages in the presence and absence of injury. After LPS insult, CCL8 relative gene expression was reduced, and IL-10 gene expression was increased in THP-1 macrophages. In lung epithelial cells, the extracts had the ability to reduce two cytokines (IL-8 and TNF-α) which are heavily correlated to pathogenesis of inflammation in the presence of IL-1β.

4.1. β-Glucans quantification in mushroom species

Hot water extracts were prepared from seven species of mushroom, and β-glucan content was determined. Results show that although extracts were isolated by the same method, each species yielded different levels of α- and β-glucans. Although there is some evidence to suggest that α-glucans can have immune-modulating properties (Masuda et al., 2017; Okamoto et al., 2007). There is substantially more evidence to suggest that the β-glucan molecule is the immune-stimulating compound found in mushrooms. These results highlight the variability between β-glucan contents in the different mushroom species. Two other studies using the same analysis procedure found variance among mushroom species (McCleary and Draga, 2016; Sari et al., 2017). Other studies have found that α- and starch glucans are usually of low abundance in cultivated mushrooms (Bak et al., 2014; Sari et al., 2017; Synytsya et al., 2008).

4.2. Effects of β-glucans on macrophages

Macrophages have the potential to intensify inflammation or exhibit regulatory repair activity during injury (Wynn and Barron, 2010).

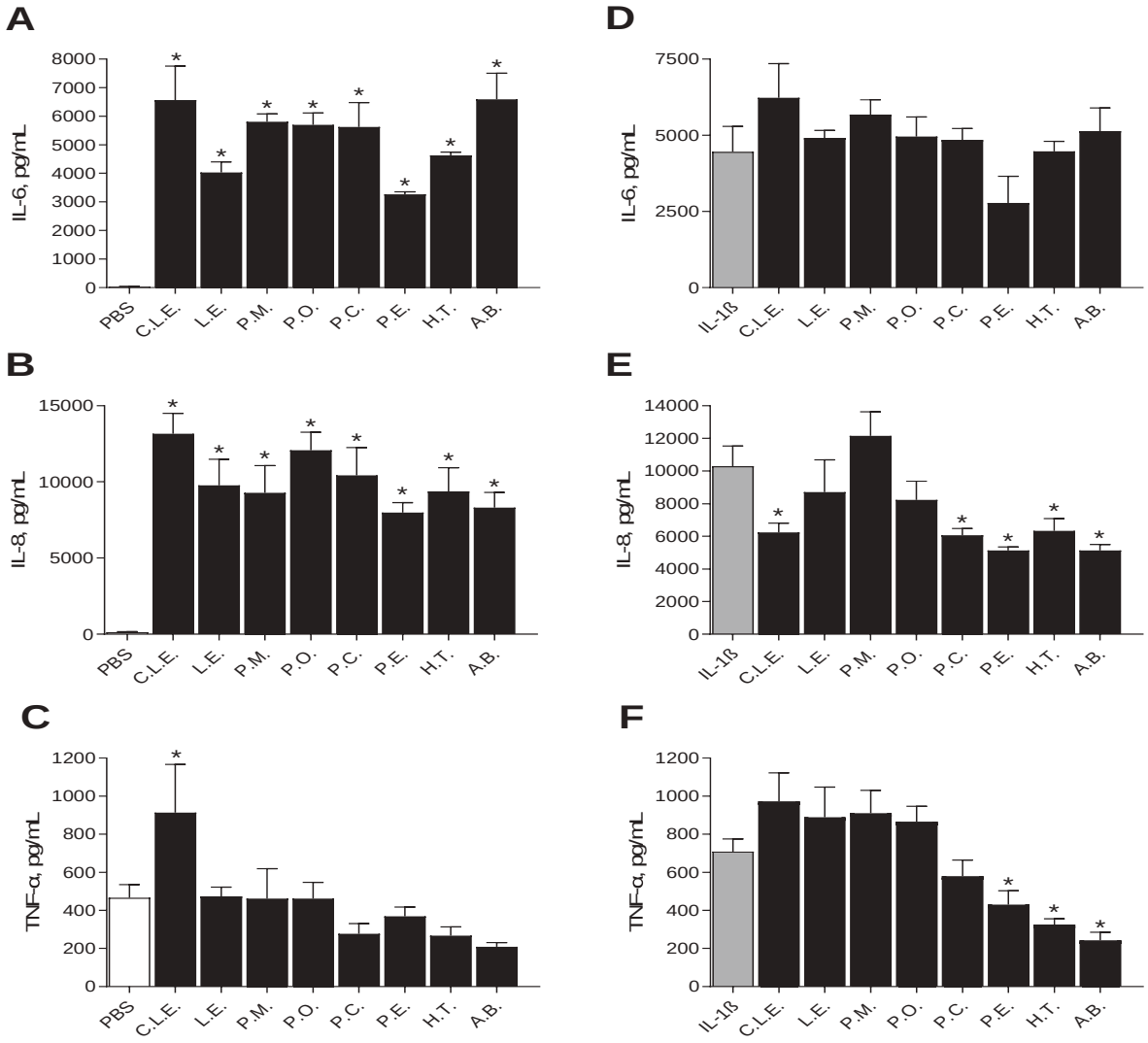


Fig. 9. The effect of the β-glucan extracts on cytokine expression in BEAS-2B cells measured using ELISA. Panel A; IL-6, Panel B; IL-8, Panel C; TNF-α. The effect of the β-glucan extracts on cytokine expression in BEAS-2B cells after IL-1β insult, measured using ELISA. Panel D; IL-6, Panel E; IL-8, Panel F; TNF-α. $p < 0.05$ versus PBS or IL-1β. Uninjured cells were treated with extracts (1 mg/mL) for 24 h before cytokine analysis. Cells were treated with IL-1β at 1 ng/mL for 24 h after which they were washed with PBS and treated with extracts (1 mg/mL) for 24 h before cytokine analysis. Phosphate buffer saline (PBS), *Commercial Lentinan* (C.L.E.), *Lentinus edodes* (L.E), *Pholiota microspora* (P.M), *Pleurotus ostreatus* (P.O), *Pleurotus citrinopileatus* (P.C), *Pleurotus eryngii* (P.E), *Hypsizygus tessellatus* (H.T) and *Agaricus blazei* (A.B).

As well as variance in content there is also evidential variance in response, which is most evident in Fig. 2 Panel A, measurement of IL-6. P.M and P.O have similar levels of β-glucan content (Fig. 1), yet P.M induced THP-1 macrophages to produce nearly double the amount of IL-6 in comparison to P.O, according to the ELISA assay. This could be correlated to the higher levels of α-glucan, in the P.O sample or to structural variances between β-glucans from different species. However, the high amount of α-glucan present in A.B sample does not hinder its activity in stimulating IL-6 secretion.

Variance can also be seen in phagocytic activity (Fig. 3, Panel A), where some samples (P.M, P.C and H.T) reduced phagocytosis in THP-1 macrophages. A.B reduced phagocytic activity in the donor PBMCs. Other extracts had no effect on phagocytic index. The THP-1 macrophages were differentiated using PMA, and the PBMCs were differentiated using MCSGF. Thus, as they should have a high phagocytic potential in this assay, it is interesting that some of the samples appeared to reduce this. This ability is potentially useful especially in conditions where macrophages are hypersensitive, and phagocytosis is uncontrolled.

Previous research has also shown that varied sources and structures lead to a varied biological response (Bohn and BeMiller, 1995; Bose et al., 2014; Demleitner et al., 1992; Driscoll et al., 2009; Goodridge et al., 2009; Volman et al., 2008; Wang et al., 2017b). As such, our results are in agreement with the literature in that β-glucan from different mushroom sources can induce varied responses. Further investigation into these correlations may identify optimized β-glucan sources for treatment of different pathological conditions.

Dectin-1 is a type II membrane receptor, which is documented as one of the principal receptors for β-glucans (Baert et al., 2015). TLR 2, 4 and 6 co-bind to dectin-1 after β-glucan recognition (Guo et al., 2015), modulating and contributing to cell responses including the release of pro and anti-inflammatory cytokines and phagocytic activity (Kanjan et al., 2017). The results of the present study showed a low- to absent expression for the gene dectin-1 receptor (CLEC7a). This could be for two reasons; a limitation of this study was that the samples were taken at 24 h when the gene could be (temporarily) switched off. Secondly, dectin-1 does not recognise all β-glucans equally; studies have shown that dectin-1 reacts differently based on structural determinants such as side-branching and size of the molecule (Adams et al., 2008). No gene expression could also be correlated to inhibition of CLEC7a, which could be correlated to a negative feedback effect. This result warrants a further timeline study to understand this mechanism.

Nonetheless these results demonstrate that the β-glucan samples are recognised by macrophages of a cell line lineage and from fresh PBMCs. This recognition can induce the secretion of both pro- and anti-inflammatory cytokines, reduce phagocytic activity, and alter gene expression levels reducing pro-inflammatory chemokines and increasing the secretion of the anti-inflammatory marker IL-10. Extracts increased secretion of both inflammatory cytokines (IL-6, IL-8, TNF-α) and anti-inflammatory cytokines (IL- 10 and IL-22). M1 macrophage polarization is associated with the secretion of pro-inflammatory cytokines: IL-1β, IL-6, and TNF-α (Bouhlel et al., 2007). M2 macrophage polarization is associated with the secretion of anti-inflammatory cytokines IL-10 (Arora et al., 2018; Wang et al., 2014). As the β-glucan extracts induce the secretion of both, it is possible that they stimulate the cells into a mixed population of M1/M2 macrophages. The commercial sample C.L.E had a different effect on the cells. L.E and C.L.E are isolated from the same mushroom species, again showing the great variances between β-glucan samples which can be dependent on cultivation, seasonal variation as well as extraction procedure. Taken together these results show the potential of β-glucans from mushrooms to behave as biological response modifiers.

To understand the immunomodulatory effects of β-glucan in an inflammatory M1 phenotype-inducing environment two types of insult were used. Firstly, LPS which stimulates macrophages toward an M1 phenotype (Zheng et al., 2013) and secondly a cocktail of cytokines (cytomix) was used to stimulate an inflammatory environment (Farley et al., 2009). After insult β-glucan samples were added to determine if the effects of the insult could be tempered.

After treatment with cytomix, some of the β-glucan extracts increased the secretion of IL-6. However, after insult, some of the extracts (P.O and P.C) induced less secretion of IL-6, compared with β-glucan alone, thus suggesting that the immune response is reduced in the presence of an injuring agent (cytomix) (Fig. 5 Panel a). One interesting finding in this study is the reduction of phagocytosis of PBMCs after LPS insult. All β-glucan extracts reduced the phagocytic index in the presence of LPS to just under half of the activity of positive controls. This result demonstrates the potential of β-glucans to modulate macrophage activity as these cells are from healthy volunteers. There is donor variation in these samples which is to be expected; future studies would investigate this effect in larger groups of healthy volunteers. The β-glucans also reduced phagocytosis after cytomix insult.

Impressively, the β-glucan samples reduced IL-8 gene expression levels after LPS injury and increased the gene expression levels of IL-10. This demonstrates an intracellular shift from an inflammatory phenotype to an anti-inflammatory phenotype in the presence of LPS. Although IL-8 was not reduced in the presence of cytomix, IL-10 was increased, again demonstrating a shift to a more anti-inflammatory response.

During SARS-CoV-2 macrophages communicate with target cells through chemokines and phagocytic signaling (Qi et al., 2020). Macrophages respond to initial infection as a result of the inflammatory cytokines secreted by type II alveolar cells which include IL-1β, IL-6 and TNF-α (Denney and Ho, 2018). When aiming to reduce the response of macrophages in inflammatory conditions, it is also important to target the alveolar cells at the centre of the injury.

4.3. Effect of β-glucans in an in-vitro lung injury model

Cytokines and chemokines have an important role in immunity as well as in immune pathology as a dysregulated response has the potential to cause extensive tissue and organ damage, especially in the lungs (Pedersen and Ho, 2020). As SARS-Cov-2 infection is associated with the production of inflammatory cytokines we investigated the effects β-glucans would have in an inflammatory environment by measuring cytokine production after IL-1β insult on two types of alveolar cell lines; A549 and BEAS-2b. When A549 (Fig. 8) and BEAS-2B cells (Fig. 9) were treated with the β-glucan extracts, all inflammatory cytokines were elevated. However, in the presence of inflammatory insults, some of the inflammatory cytokines were reduced significantly. Figs. 8 and 9 Panel E and F, shows that the extracts had the ability to reduce IL-8 and TNF-α in both A549 cells and BEAS-2B. In reducing the cytokine expression and inflammation of lung tissue the inflammatory process can be avoided and blood gas transfer potentially unaffected or minimally affected. After injury, when the invading pathogen is eliminated, large numbers of inflammatory monocytes and macrophages can be recruited to the distal alveolar space because of chemokine gradients, this can also exceed the total number of resident macrophages (Davies et al., 2013; Galli et al., 2011).

As epithelial cells are the main source of anti-viral responses in the first 24–48 h window after infection, this is an important result. Important signals are transmitted to innate immune cells which are translated to adaptive immune responses (Geller and Yan, 2020). By firstly priming these cells with bioactives such as β-glucans to respond to infection, innate cells are recruited, and a memory is created for prevention of a secondary infection. More importantly, if the cells are primed, the hyper inflammatory reaction might not occur as cells are modulated by the β-glucans.

As the infection is more lung-centred than multi-organ-centred (McGonagle et al., 2020).

In-vitro lung epithelial cells represent a good model to determine potential targets. This study has shown that β-glucan extracts from mushrooms can reduce inflammatory responses in models of in-vitro lung injury.

5. Conclusions

There is a growing awareness of therapies directed to modulate the immune response in many pathological contexts. Medicinal mushrooms, which contain the complex β-glucans sugars have been used to treat an array of conditions for centuries including inflammatory conditions. Previously, we have demonstrated that β-glucans from the same mushroom isolated by different methods have differential immune-modulation abilities in an in-vitro model as well as in an in-vivo preclinical model. Following on from this, the current work has demonstrated the potential of β-glucans as immunomodulators with dual functions, firstly as immune priming agents that may bolster the capacity of the body to maintain homeostasis in the face of infectious and other challenges, and secondly to temper the immune response following infection, thus helping to avoid the serious sequelae associated with immune hyper-inflammatory response in immune and epithelial cells in inflammatory lung conditions such as ARDS. Future work will investigate relationship between the structure of β-glucans and mechanistic effects at cell and molecular levels. The main findings of this research also strongly align with emergence of green innovation for OneHealth applications (Rowan and Galanakis, 2020).

CRediT authorship contribution statement

Emma Murphy: conceptualization, methodology, data curation, visualization, formal analysis, writing original draft, review and editing. **Emanuele Rezoagli:** conceptualization, methodology, data curation, visualization, formal analysis, writing original draft, review and editing. **Robert Pogue:** methodology, data curation, formal analysis, review and editing. **Bianca Simonassi-Paiva:** methodology, formal analysis, review and editing. **Ismin Izwani Zainol Abidin:** methodology, formal analysis, review and editing. **Gustavo Waltzer Fehrenbach:** methodology, formal analysis, review and editing. **Emer O'Neil:** methodology. **John Laffey:** supervision, funding acquisition, conceptualization, review and editing. **Ian Major:** methodology, supervision, review and editing. **Neil Rowan:** funding acquisition, project administration, conceptualization, supervision, methodology, review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Entry

β-Glucans

Emma J. Murphy [1,2], Emanuele Rezoagli [3,4,5], Ian Major [2], Neil Rowan [1] and John G. Laffey [3,4,*]

1 Bioscience Research Institute, Athlone Institute of Technology, N37 HD68 Athlone, Ireland; emurphy@ait.ie (E.J.M.); nrowan@ait.ie (N.R.)
2 Materials Research Institute, Athlone Institute of Technology, N37 HD68 Athlone, Ireland; imajor@ait.ie
3 Lung Biology Group, Regenerative Medicine Institute at CURAM Centre for Medical Devices, School of Medicine, National University of Ireland Galway, H91 TK33 Galway, Ireland; emanuele.rezoagli@unimib.it
4 Anaesthesia and Intensive Care Medicine, University Hospital Galway, H91 TK33 Galway, Ireland
5 Department of Medicine and Surgery, University of Milan-Bicocca, 20900 Monza, Italy
* Correspondence: john.laffey@nuigalway.ie; Tel.: +353-91-49349

Definition: Definitionβ-glucans are complex polysaccharides that are found in several plants and foods, including mushrooms. β-glucans display an array of potentially therapeutic properties.

Keywords: β-glucan; clinical trials; biomedicine; immunomodulation; metabolism

1. Introduction

β-glucans/Beta-glucans are a large class of complex polysaccharides that can be found in an abundance of sources. Depending on origin, β-glucans can be classified as cereal or non-cereal derived. Cereal sources of β-glucans include oat and barley and non-cereal sources can include mushroom, algae, bacteria and seaweed [1]. β-glucans are biologically active compounds that have been widely reported to improve health [2]. Specific to this group of polysaccharides is a 1,3 beta-glycosidic linked backbone; separate to this, the polysaccharide can take many forms, dictated by origin.

There is a growing interest in foods that have the potential to lower the risk or incidences of chronic diseases or promote lifespan as well as have anti-aging properties. This has led to an increase in awareness of the effect of diet on health [3,4].

In 1979, Stephen DeFelice devised the term nutraceutical, which may be isolated nutrients, dietary supplements, genetically engineered foods and herbal products [5]. Nutraceuticals are defined as a food or food component that provides medical or health benefits, including prevention and/or treatment of disease [5]. Similarly, bioactive compounds are defined as "essential and non-essential compounds that occur in nature, are part of the food chain and are shown to have an effect on human health" [6].

Bioactive substances in food provide health benefits beyond the nutritional benefits of the product [7]. β-glucans are reported to be both a bioactive and a nutraceutical. Their therapeutic effects can also be largely classified into two categories, metabolic/GI effects or immune-modulatory effects, which is largely based on structure, determined by source [1,8].

Metabolic effects are usually observed with cereal derived β-glucans. Effects include modulation of the gut microbiome, cholesterol reduction and decreased cardiovascular and diabetic risk. Non-cereal β-glucans are associated with immune-modulatory effects, anti-tumor effects, wound healing and alleviation of immune-related conditions, as demonstrated in Figure 1 [1]. β-glucans are also administered as an animal and fish feed additive to increase [9,10]. These molecules also have applications in the food industry for thickening and for gelation purposes [11].

In this encyclopedia entry on β-glucans, we start from their initial discovery, then examine β-glucan sources, characterize their complex and diverse structures, and examine the implications of their structural variations on their activity profile. The therapeutic

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Citation: Murphy, E.J.; Rezoagli, E.; Major, I.; Rowan, N.; Laffey, J.G. β-Glucans. *Encyclopedia* **2021**, *1*, 831–847. https://doi.org/10.3390/encyclopedia1030064

Academic Editor: Fabrizio Araniti

Received: 9 June 2021
Accepted: 11 August 2021
Published: 19 August 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

potential in different disease conditions is then discussed, and the barriers to fully realizing this potential is dissected in some detail. Finally, we examine other uses of β-glucan in animal health and their application in the food industry.

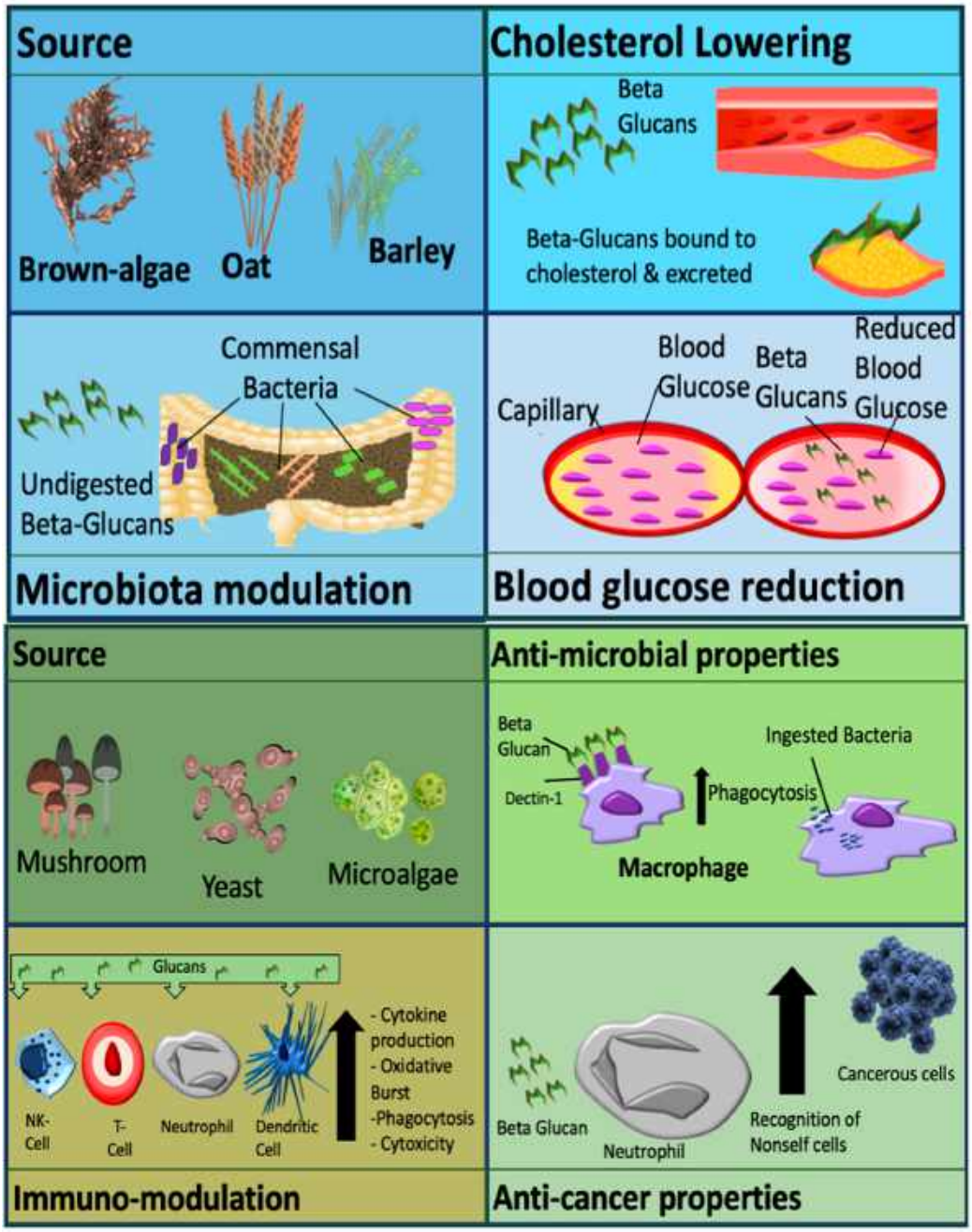

Figure 1. Mechanisms and activity of β-glucan which are dependent on source. β-glucan can be classified as cereal derived (upper panel), or non-cereal derived (lower panel). Picture originally published in [1]. Modified with permission.

2. History of β-Glucans

As β-glucans are a structural component of mushrooms, their use as an unknown therapeutic in medicinal mushrooms dates back hundreds of years [12]. β-glucans, as an active compound, followed two different pathways of discovery. In Western medicine, they were initially discovered by Pilliner et al., in 1954 [13]. β-glucans were discovered unintentionally through the discovery of Properdin in the compliment system. Zymosan was the stimulatory agent used in these experiments. It is a crude mixture of yeast cell

wall particles, which is now established to contain high levels of β-glucans. In 1961, Riggi and Dilugio identified the active compound in zymosan to be a polysaccharide. They concluded that the polysaccharide required a 1,3 beta type linkage for activity [13].

Independently, concurrently in Japan, β-glucans were being investigated for antitumor properties, with the first study in 1969. Chikara et al. isolated β-glucans from the mushroom *Lentinus edodes* and demonstrated it to have an ability to inhibit sarcoma in mice [14]. β-glucans were previously used as a traditional medicine for cancer therapy [15–17]. To date, β-glucans isolated from *Lentinus edodes* named Lentinan and Polysaccharide K are two licensed drugs in Japan [18].

Developments

In the wake of these pioneering studies, β-glucan research has gained considerable attention and advancements, with these bioactives being registered in clinical trials for an array of conditions including inflammatory conditions, cardiometabolic diseases, obesity and cancer, and more recently, as a nutritional supplement for the treatment of COVID-19 in conjunction with vaccination (Efficacy and Tolerability of ABBC1 in Volunteers Receiving the Influenza or Covid-19 Vaccine—Full Text View—ClinicalTrials.gov) [19]. The US Food and Drug Administration has also recognized the benefits of consuming β-glucans, recommending a 3 g/day intake of oat bran, a source of β-glucan and registered as a cholesterol-reducing food [20].

3. Sources and Structure of β-Glucans

As mentioned, β-glucans can be classified by source: cereal or non-cereal. This classification is largely based on structure, as β-glucans from cereal have a different structure than those originating from non-cereal sources. Secondly, β-glucans from cereal sources can differ; for example, β-glucans from barley can be structurally different to those of oat. The same diversity can occur between β-glucans from non-cereal sources. All β-glucans are homo-polysaccharides and essentially composed of glucose units linked together and thus have a characteristic 1,3 linked backbone [21,22], which is fundamental to activity [23]. The structural difference occurs at branching off this backbone, which is dictated by source. β-glucans can be unbranched or branched [24]. Branching can usually occur at either the 1,4 or 1,6 position [24]. These molecular and structural characteristics will determine functional activity as β-glucans have a defined structure–activity relationship [25].

Cereal or grain derived β-glucans usually have 1,3 1,4 glycosidic linkages without any 1,6 bonds or branching [26,27]. Non-cereal sources usually have 1,6 linked branches off the main side chain. Figure 2 demonstrates the structural differences between β-glucans. Other glucans have no branching, such as Curdlan, a glucan isolated from *Agrobacterium* that contains no side branching, just a beta-D glucan backbone [28]. There are, however, exceptions—*Sorghum arundinaceum*, an ancient cereal grain, was found to contain β-glucans with alpha 1,4 linked D-glucopyranose residues with 1,3, 1,6 branching points [29]. Moreover, different species of Sorghum have different structures; *Sorghum bicolor* contains 1,3 with 1,4 linkages [30].

β-glucans from the same species source may have variances in structure. Variances can include chain length, degrees of branching, polymerization and 3D conformational structure. Three-dimensional conformational structure can be random coil, single helix or triple helix [2]. Furthermore, other factors that can influence the structure include growth conditions, extraction procedure and analysis [20,31].

It has been shown that β-glucans specifically of mushroom origin have anti-tumor properties. Lentinan is a β-glucan isolated from mushroom which has a β-helix conformation [32,33]. If the helix is destroyed or removed, the anti-tumor activity decreases. Other studies demonstrated that molecular weight and chain conformation are all dependent on anti-tumor activity [34]. The ability of β-glucans to interact and modulate immunity is correlated to polymer length, degree of branching and tertiary structure [23,35]. Larger β-glucans have been shown to activate leucocytes directly. Activation initiates phagocytosis,

antimicrobial activities and production of cytokines. Medium to smaller-sized β-glucans induce NF-κB and mediate inflammation [36].

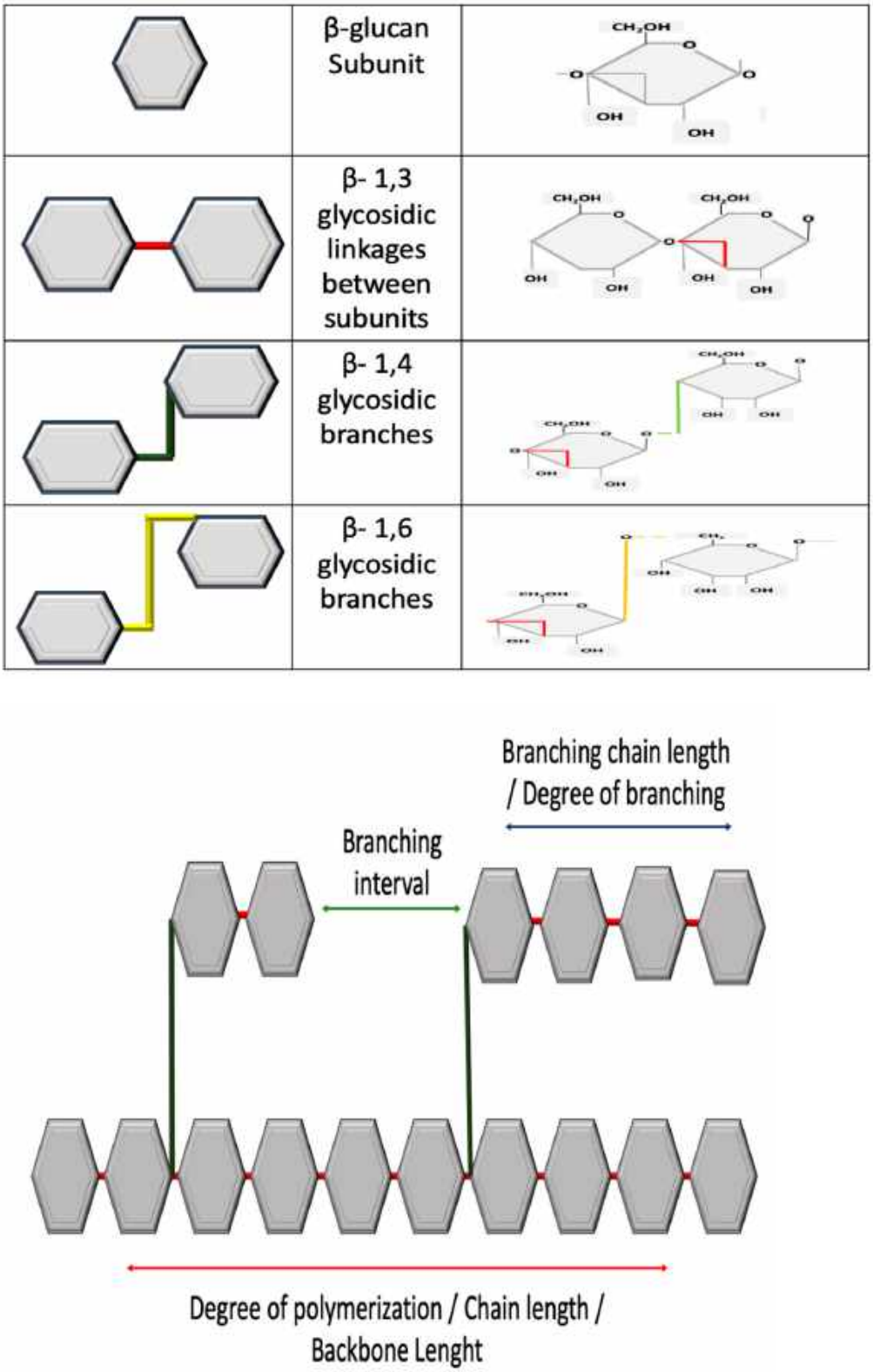

Figure 2. All β-glucans have a 1,3 linked backbone. The variances in structure occur with glycosidic side branches. Variances can also occur in chain length or degrees of polymerization, branching interval and branch length. Source and extraction technique will influence final structure. Various analytical methods can be used for the characterization of β-glucans, as outlined in Table 1.

β-glucans can also be isolated from macro and microalgae sources. Microalgae are unicellular photosynthetic organisms, whereas macroalgae are larger multicellular organisms. Laminarian is a water-soluble β-glucan extracted from the cell wall storage of *Eisenia bicyclis*, a brown macroalgae. Similarly, to other non-cereal sources, the structure consists of a 1,3 linked backbone with 1,6 side branching. The glycosidic bonds have different ratios depending on the habit or season of extraction [37,38].

Laminarian possesses anti-inflammatory, anti-apoptotic, anti-tumor, antioxidant and anticoagulant properties. More recently, anti-cancer effects via enhanced apoptotic cellular death and angiogenic potential have been reported [37]. This polysaccharide is also being exploited as a carrier for gene delivery [39]. Topical laminarian-based creams improve wound healing via collagen deposition and promotion of skin regeneration [40]. Oral supplementation of laminarian has also been shown to suppress the progressive development of precancerous lesions in mice [38].

Soluble β-glucan from macro algae has been applied in the aquaculture industry to fish in the early stages of development as well as cultures of crustaceans as a bath treatment to enhance immunity in early life [41]. Species of brown algae (*Phaeophyceae*) and red algae (*Gracilariaceae*) contain a 1,3 1,6 linked β-glucan with immunomodulatory properties—via activation of spleen lymphocytes [41].

The microalga *Euglena gracilis* is a well reported source of β-glucans. The microalga is of the division Euglenoophyceae and family Euglenales [42,43]. β-glucans isolated from this source are called paramylon, which are large, linear, unbranched 1,3 β-glucans with the potential to support immune function [44]. A clinical trial that investigated the effects of supplementation with *E. gracilis* that contained a 50% β-1,3-glucan on immune function reduced the severity of upper respiratory tract infections (URTI), decreasing sick days and symptoms compared to placebo [43].

Extraction, Purification and Characterisation Methods

There are four types of extraction procedures used for isolation of β-glucans: alkaline extraction, acidic extraction, enzymatic extraction and water extraction. More advanced methods can include ultrasound-assisted extraction, microwave-assisted extraction and superheated water extraction.

Cereal derived β-glucans are generally located in the aleurone (proteins stored as granules), in the sub-aleurone or in the cell wall of endospores, which are all located in oat, barley, wheat and rice [45]. Extractions from cereal sources can be difficult and therefore, β-glucans from these sources are commercially more expensive [46]. There is no standard method for the extraction of β-glucans. Most researchers modify commonly published methods. The method selected is usually based on solubility of the β-glucan in hot water or solvents and is followed by precipitation using 2-propanol or ethanol [47]. Other published methods include accelerated solvent extraction (ASE) [48], sodium hydroxide as initial extraction solvent [47]. Table 1 lists the various extraction methods for β-glucans as well as analytical methods for identification.

For β-glucans from non-cereal sources, hot water extraction is the most common method [49]. Samples are usually milled, extracted with water and then precipitated with solvents such as ethanol [50]. Other methods have used ethanol precipitation and chromatography methods using Sephadex or Di-Ethyl-Amino-Ethyl (DEAE) [47,51].

Simple extraction techniques will isolate the β-glucans to some extent. Usually, there are other contaminating materials present, e.g., proteins. Enzymatic techniques can be used for the removal of proteins. The isolated β-glucans, by simple extraction techniques, are usually fit for purpose. Purification and extensive extraction techniques can be expensive and tedious. However, different extraction and purification techniques will yield different products and cause inconsistences between results. A way to control this is to fully characterize β-glucans after extraction, as it is important that β-glucans retain their structural characteristics after extraction [52]. Methods applied for characterization include gel permeation chromatography (GPC) [53] for molecular weight estimation, Fourier-transform

infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR) for structural characterization [54,55]. For the detection of β-glucans in products, enzymatic reactions are also available [56].

Table 1. Extraction and characterization methods for β-glucan.

Source	Extraction Method	Analytical Method	Ref
Hull-less Barley Bran	Ultrasonic extraction, Hot water extraction, Microwave extraction, Microwave assisted ultrasonic extraction	Megazyme commercial quantification kit, Structure determined using FTIR, Molecular weights determined using gel permeation chromatography (GPC)	[57]
Hull-less Barley	Alkali extraction and ethanol precipitation.	High pressure size exclusion chromatography (HPSEC), Methylation, Gas Chromatography Mass Spectroscopy (GC-MS)	[21]
Barley	Ethanol Extraction and enzyme treatment with amylase.	Megazyme commercial quantification kit, Molecular weight was determined using size-exclusion ultra-high-performance liquid chromatography	[58]
Barley Bran	Enzyme extraction using α-amylase, protease, glucoamylase, pullunlanase and xylanase	Megazyme commercial quantification kit, HPSEC, Rheology, SEM	[59]
Barley	Pressurized aqueous ethanol	Megazyme commercial quantification kit, GPC	[60]
Barley	Ethanol and water extraction	N/A	[61]
Oat and Barley	Multistage approach; Solvent (acetone) and enzyme extraction α-amylase and hot water extraction	Megazyme commercial quantification kit, Asymmetric flow field-flow fractionation (AF4) coupled to multiangle light scattering (MALS), differential refractive index (dRI) and fluorescence (FL) detection, High performance anion exchange chromatography	[62]
Oat	Multistage enzymatic and solvent extraction—enzymes α-amylase, amyloglucosidase, and papain	Megazyme commercial quantification kit	[63]
Oat	Subcritical-water extraction	Megazyme commercial quantification kit HPLC	[64]
Oat	Enzymatic extraction- α-amylase	Megazyme commercial quantification kit	[65]
Corn pericarp	Anion exchange chromatography and affinity chromatography	HPLC, NMR, Methylation	[66]
Castanea mollissima	Water extraction followed by ethanol extraction purified using anion exchange chromatography	Phenol- sulfuric acid method, High performance gel permeation chromatography (HPGPC) HPLC, FTIR, Methylation analysis	[67]
Pueraria lobata	Ethanol extraction followed by cold water extraction followed by further ethanol extraction. Fractions of extracts collected using DEAE-Sepharose chromatography column	Carbohydrate content determined by phenol-sulfuric acid method, HPGPC, Congo red method used to determine triple helix structure.	[68]
Ziziphus jujuba Mill	3 Phase extraction, Aqueous alkaline extraction; acidic precipitation of proteins at their isoelectric point, precipitation of glucans with absolute ethanol	Megazyme commercial kit, FTIR, Surface structural differences determined using scanning electron microscope.	[69]

Table 1. *Cont.*

Source	Extraction Method	Analytical Method	Ref
Tuber melanosporum	Extracted pressurized liquids. Water and ethanol used as extraction solvents.	Analyzed by NMR and Gas chromatography mass spectroscopy GC_MS	[70]
Phaseolus vulgaris	Sonication of cell wall residue	Gel filtration chromatography, Methylation analysis, NMR	[71]
Punica granatum	Alkaline treatment, isoelectric precipitation, alcohol precipitation	FTIR	[72]
Euglena cantabrica	Pressurized liquid extraction (PLE) using different temperature 40–180 °C using green solvents (ethanol-water) mixtures.	Extracts analyzed by high-pressure size-exclusion chromatography coupled to an evaporative light-scattering detector.	[73]
Laminaria hyperborea	Hydrothermal assisted extraction—acid extraction, temperature and pressure treatment, filtration and freeze drying.	Concentration calculated using Megazyme commercial kit.	[74]
Saccharomyces cerevisiae	Hot water Extraction & Enzymatic Treatment; enzymes-protease and lipase	Concentration calculated using Megazyme commercial kit.	[75]
Saccharomyces cerevisiae	n/a	Congo red assay—colorimetric	[76]
Saccharomyces cerevisiae	Yeast was cultured in yeast extract-peptone-glucose (YBG) broth to produce ß-glucans. Cells were sonicated. Alkaline-acid extraction used as extraction method.	n/a	[77]
Saccharomyces cerevisiae	Acid-base extraction method	FTIR analysis of structure, HPLC	[78]
Saccharomyces cerevisiae	Cell exposure to hot water (autoclaving), thermally induced autolysis, homogenization in a bead mill, sonication	FTIR, Megazyme commercial kit	[79]
Saccharomyces cerevisiae	Alkaline and acidic extraction	FTIR, NMR	[80]
Pleurotus eryngii	Water extraction at different temperatures and pressures	High pressure size exclusion chromatography (HPSEC), Gel permeation chromatography (GPC)	[81]
Cantharellus tubaeformis	Pressurized hot water extraction (80–240 °C)	Megazyme commercial kit	[82]
Pleurotus sajor caju	Hot aqueous extraction	NMR spectroscopy, HPSEC, Methylation analysis	[83]
Agaricus bisporus, Lentinula edodes and Pleurotus ostreatus	Pressurized water extraction (PWE)	Megazyme commercial kit	[84]
Mushroom by-products	Mechanical agitation and ultrasound assistance in ethanol/water solutions	Megazyme commercial kit	[85]
Ganoderma lucidum	Hot water extraction, Soxhlet extraction, ultrasound assisted extraction.	Phenol Sulfuric acid assay, HPGPC, Content and ration of branching determined by enzymatic-HPAEC-PAD detection, FTIR, SEM	[86]
Pholiota nameko	Defatting process with cold water, Hot aqueous extraction Enzyme treatment with amylase	NMR, Methylation	[87]

Table 1. *Cont.*

Source	Extraction Method	Analytical Method	Ref
Pleurotus ostreatus	Methanol extraction. Hot water extraction; Acid hydrolysis	Megazyme commercial kit, GPC, Hydrophilic interaction chromatography	[88]
Lentinus edodes-	Orthogonal alkaline extraction with sodium hydroxide—further purification ethanol precipitation and anion exchange chromatography	^{1}H-NMR High-performance gel permeation chromatography–refractive index–multi-angle laser light scattering (HPGPC-RI-MALLS) Methylation analysis	[89]

4. Activity

4.1. Absorption

Although other routes of administration of β-glucans have been explored, oral administration is the most common, as β-glucans are a food-derived compound. When orally administered, studies have shown that small β-glucan concentrations in other tissues are low. Studies have also demonstrated that β-glucans reach the small intestine intact. The primary reaction occurs between the molecule and the gut epithelia [90]. Peyer's patches are groupings of lymphoid follicles in the ileum region of the small intestine. It is in this location that immune stimulatory components are taken up to lymphatic circulation. In the Peyer's patches, there are circulating antigen presenting cells (APCs) [91]. When β-glucans reach the Peyer's patches, dendritic cells or microfold (M cells) recognize β-glucans and present them to other immune cells located here [92,93].

Pathogen-associated molecular patterns (PAMPs) are essentially the signature markings of pathogens. It is how they are recognized by immune counterparts. PAMPs that contain a sugar component such LPS from Gram-negative bacteria are classified as sugar complexed PAMPs (SCPs). SCPs are functional and structural components of the pathogen [94]. Receptors recognize these unfamiliar microbial structures and respond. β-glucans are a structural component of fungi and therefore are recognized as a PAMP. This is beneficial as β-glucans initiate an immune response without introducing an infectious agent such as a fungus or bacteria. As PAMPs, β-glucans can stimulate immune cells in isolated form—i.e., they are not required to be attached to the infectious agent—they have been developed as adjuvants and immunotherapeutics [95].

There is huge diversification of carbohydrate structure and branching, creating a range of complex monomers and polymers, and there are specific receptors to recognize these conformations which elicit the necessary response [96,97]. The PAMP recognition ability of the immune cells requires receptors, namely, pathogen recognition receptors (PRRs) [98].

4.2. Cellular Activation

It is well established that cellular biological activities—namely, immune modulatory activity—are dependent on recognition and binding of receptors. C-type lectin receptors (CLRs) are the main pathogen recognition receptors involved in fungal recognition [99]. Initial or broad-spectrum recognition of β-glucans is based on the 1,3 backbones usually by a receptor called Dectin-1 or the β-glucan receptor [92]. It is reported that β-glucans activate intestinal epithelial cells to secrete pro-inflammatory cytokines in a Dectin-1 and Syk-dependent manner [100].

Dectin-1, a CLR-type receptor, is present on macrophages, dendritic cells, neutrophils and on pathways where pathogens invade [101–103]. After activation, Dectin-1 will trigger the NF-κB pathway, which will induce cytokine synthesis by Syk-dependent pathways. Pathways can include NF-κB inducting kinase (SYK-NIK) pathways or Syk independent pathways, including the Ras associated factor-1 (RAF-1) pathway [104].

Complement receptor 3 (CR3) is mainly expressed on NK cells, dendritic cells, macrophages and neutrophils [105]. This specific receptor usually responds to iC3B opsonized cells and pathogens [106].

CR3 is involved in the recognition of endogenous ligands, but also acts as an opsonic receptor for the complement component and as a non-opsonic receptor for β-glucan. The anti-tumorigenic properties of β-glucans are correlated to recognition of this receptor and deficiency is correlated to a loss in activity [107].

Other receptors that recognize β-glucan include lactosylceramide receptor, scavenger receptors and Toll like receptors (TLR), namely TLR2 [108,109]. TLR 2,4 and 6 co-bind to dectin-1 after glucan recognition [110]. Lactosylceramide receptors are found in plasma membrane of many different cells [95]. Binding and recognition of these receptors induces ingestion, respiratory burst, microbial killing, inflammatory processes and the release of chemical mediators that activate and recruit other cells [111].

Dectin-1 can interact with β-glucans over a wide range of affinities; although 1,6 branching does not influence binding, branched β-glucans appear to have a stronger affinity in comparison to linear β-glucans [112]. Other influences over recognition include chain conformation and polymerization. Extraction procedure can have a huge impact on β-glucan conformation [113]. Thus, the isolation and purification procedures must be carefully selected, as it will ultimately influence the activity of β-glucans.

5. Therapeutic Potential and Challenges

5.1. Therapeutic Potential

In May 2006, the US Food and Drug Administration acknowledged that β-glucans reduce the risk of coronary heart disease. Health claims relating to β-glucans specifically for maintenance of blood cholesterol were approved in Europe in 2009 [114].

From a metabolic viewpoint, β-glucans have the ability to lower cholesterol and improve glycemic control [115–117]. Viscous β-glucans have been shown to modulate host bile acid metabolism [117]. In lowering cholesterol and triglycerides, the risk of cardiovascular diseases is also reduced [118]. Yeast β-glucans serve as prebiotics, improving gastrointestinal function by enhancing intestinal microbiota [1,119].

Immune modulatory properties include stimulation of the immune response and initiation of inflammatory processes as well as improving resistance to infections [120]. Non cereal β-glucans alleviate allergic conditions [121,122]. β-glucans have also been shown to enhance bacterial clearance from blood and reduce mortality in a rat model of intra-abdominal sepsis [123].

Immuno-oncology is the study of treatments that utilize the immune system to target cancer [124]. β-glucans have shown promise in this area as they stimulate immune cells to target cancerous cells. β-glucans have been demonstrated to induce macrophages and NK cells to attack and destroy cancerous cells [125]. Mushroom β-glucans promoted T-cell immunomodulation and neutrophil infiltration into tumors in mice, which lead to tumor growth inhibition [126]. Oral administration of β-glucans has similar effects to other routes of administration when used as an anti-tumor immunotherapy [107].

Promising results in clinical trials using β-glucans as an adjuvant in conjunction with monoclonal antibodies for cell mediated tumor killing has established them as a potential intervention in the field of immune-oncology [127].

β-glucans have also gained attention as vaccine adjuvants. When combined with vaccines, they have the ability to enhance sensitivity by stimulating immune cells [128]. Stimulants that are used for immune modulatory functions are referred to as immune adjuvants [129]. As β-glucans are recognized by immune counterparts as PAMPs, they are widely investigated for their potential as immune adjuvants [130,131]. They have therefore been administered as adjuvants in vaccines or counterparts in vaccine delivery systems [132,133].

5.2. Therapeutic Challenges

The therapeutic promise of β-glucans for an array of clinical conditions is considerable [127]. The translation to clinical application is not as successful for several reasons. Firstly, like all naturally derived products, β-glucans can represent a complex mixture of

ingredients which can all contribute to or prevent activity. Extraction methods will greatly affect the final compound. Intense extraction procedures can alter the natural configuration of the β-glucan compound. Softer extraction procedures can leave other contaminating residues present, which can dilute, contaminate or even contribute to activity [27].

Secondly, there is huge diversity among β-glucan structures. Diversity occurs between β-glucans from different species, but there are also variances in extracted samples from the same species. These differences include degree of branching, monosaccharide composition, linkage ratio and linkage type [134]. The molecular weight of the β-glucan greatly influences activity, which can be dictated by growth conditions and the extraction procedure. For example, β-glucans isolated from barley in one experiment was shown to have a molecular weight of 3×10^4 g/mol and in a separate experiment found to have a molecular weight of 270×10^4 g/mol [31]. The distribution of polysaccharides from aleurone to endosperm in cereal sources can also vary depending on origin. In one study, aleurone was found to contain 26% w/w β-glucan in comparison to endosperm, which contained 70%. A study investigating the individual cytolytic composition of different barley varieties found that cytolytic malt modifications have a lower β-glucan content. This work confirms that breeding progress reduces β-glucan content in barley [135]. These variances can cause huge inconsistencies in reported therapeutic effects. These two issues can potentially be resolved by producing standardized methods for the extraction of β-glucans categorized by source, as the location of β-glucans is different in each. At present, there is no standardized extraction method.

Furthermore, very few reports state the way in which β-glucans are extracted and the final concentration yield. A potential solution to this is that all β-glucans should be fully characterized in terms of structure, molecular weight and degree of purity. Therefore, activity can be compared, receptor pathways defined and mechanisms of action elucidated.

Finally, disease targets are also very broad. In categorizing and defining structure and disease targets, clinical effects can be better understood. Optimal routes of administration and general applications such as length of treatment and dose also vary in both preclinical and clinical trials, leading to conflicting findings [136]. These too need to be defined for comparison to overcome inconsistences, as there is huge enthusiasm for the clinical use of β-glucans.

5.3. Animal Health

β-glucans administered as adjuvants in vaccines have also shown promise in animal models. In vivo models have shown upregulated macrophage and dendritic cell recruitment and maturation. Antigen-specific CD8+ T-cell responses were also increased. Delivery by various routes of administration showed similar activity [137,138].

There are constant influxes of studies demonstrating the immune modulating properties effects of β-glucans. There is also a ban on the use of antibiotics administered to farmed animals for growth and immune promoting purposes. The use of β-glucans as an alternative is increasing [139]. In fisheries, it is tedious and costly to vaccinate fish to improve immunity and immune strength against pathogens; one of the major benefits of food component immune modulators is that they can be administered as food and still achieve the same effects. Studies have shown that feeding β-glucans to healthy fish has increased non-specific and specific immunity levels and increased protection against bacterial infections [140].

Algae β-glucans have demonstrated improved immunity in dogs [141]. In mice, the bioactives had significant effects as a prophylactic treatment to reduce anthrax infection and inhibition of cancer cells through stimulation of cytokines [142,143]. Sheep oral supplementation to ewes had positive effects on reproductive performance, growth rate and body composition [144]. Lambs, when administered β-glucans, had a significant increase in phagocytic and respiratory burst activities [145,146]. In pigs, there was an improvement in health status [139] and when administered to piglets, there was a reduction in the susceptibility of newborn piglets to enterotoxigenic bacterial infection [147]. When

chicks are administered dietary supplementation with yeast derived β-glucans, Salmonella colonization in the cecum was reduced [148]; this effect was also observed in turkeys [149]. With these studies and many more, β-glucans are proving to be an attractive safe alternative to antibiotic administration.

5.4. Applications of β-Glucans in the Food Industry

β-glucans are added to food products for a variety of reasons, most often as texture enhancers [150]. β-glucans originating from barley have been added as a thickener to liquid products as well as a source of dietary fiber [21]. β-glucan have also been demonstrated to increase water absorption [151], be a fat substitute [152] and act as a stabilizer in the formation of foam and emulsion. [153]. When used as a fat substitute in meat, it improved texture and retained moisture [152]. When added to food, β-glucans can enhance texture and replace fats but also have beneficial therapeutic effects against metabolic conditions. β-glucans are not digestible by enzymes in the human gastrointestinal tract, and therefore, when ingested in food, they behave as soluble dietary fibers [154]. Highly viscous β-glucans have the ability to lower cholesterol and improve glycemic control as well as satiety control [117]. There is also evidence to suggest that when eaten, β-glucans interact and positively influence the gut microbiome [155].

The circular economy repurposes residues or by-products into raw materials for other processes, with the aim of reducing waste. Brewers spent grain (BSG) is an abundant by-product from the brewing industry. It contains many bioactives and is a source of 1,3 1,4 β-glucans. Previously, BSG was mainly used in animal nutrition. However, β-glucans can be extracted from BSG and used for human nutrition or included in food with beneficial effects, including reduction in the postprandial glycemic response by up to 50% [156].

6. Conclusions and Perspectives

β-glucans are a diverse class of complex polysaccharides that can be found in an abundance of sources and are classified by source as cereal or non-cereal, based on differences in their branching patterns. These structural differences, caused in part by their source, and the extraction and purification methods used, confer different effect profiles on members of the β-glucans family. There is a significant body of literature attesting to their therapeutic potential, which vary from anti-tumor and immunomodulatory effects described predominantly with mushroom derived β-glucans, to metabolic effects such as improved glycemic control and cholesterol-lowering effects with cereal β-glucans, to probiotic properties.

While over 200 registered clinical trials of β-glucans clearly attest to the interest in these compounds, significant barriers exist to realizing their therapeutic effects. A key issue is the fact that the structure versus function profile remains incompletely understood. Addressing these barriers will require optimization of isolation and purification procedures, careful characterization of the relationship between specific variations in β-glucan structure and their effect profile. This approach would facilitate a greater understanding β-glucan, enabling identification of the most promising compounds for clinical testing, and should realize the promise of this intriguing class of compounds.

Author Contributions: Conceptualization, J.G.L. and E.J.M.; original draft preparation, E.J.M., E.R. and J.G.L.; writing—review and editing, E.J.M., E.R., I.M., N.R. and J.G.L.; visualization, E.J.M.; supervision, J.G.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Entry Link on the Encyclopedia Platform: Https://encyclopedia.pub/14401.

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foods

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Review

Food Ingredients and Active Compounds against the Coronavirus Disease (COVID-19) Pandemic: A Comprehensive Review

Charis M. Galanakis [1,2,3,*], Turki M.S. Aldawoud [2], Myrto Rizou [1], Neil J. Rowan [4,5] and Salam A. Ibrahim [6,*]

1 Research & Innovation Department, Galanakis Laboratories, 73131 Chania, Greece; myrtorizou@chemlab.gr
2 College of Science, King Saud University, Riyadh 12372, Saudi Arabia; tdawoud@ksu.edu.sa
3 Food Waste Recovery Group, ISEKI Food Association, 1190 Vienna, Austria
4 Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, N37 F6D7 Athlone, Ireland; nrowan@ait.ie
5 Empower Eco Sustainability Hub, Lough Boora, R35 DA50 Co. Offaly, Ireland
6 Food and Nutritional Sciences Program, North Carolina Agricultural and Technical State University, Greensboro, NC 27401, USA
* Correspondence: cgalanakis@chemlab.gr (C.M.G.); ibrah001@ncat.edu (S.A.I.)

Received: 30 September 2020; Accepted: 16 November 2020; Published: 20 November 2020

Abstract: As media reports have noted, the COVID-19 pandemic has accelerated market mainstreaming of immune-boosting food bioactives, supplements, and nutraceuticals. However, most studies reporting on the potential of bioactives against COVID-19 transmission have been uploaded as preprints with little opportunity to revise content for benefit and impact. The current review discusses current best evidence and information underpinning the role of food ingredients and bioactive compounds in supporting immune functions in humans and animals, specifically in the prevention and treatment of COVID-19 disease. Up to now, some evidence from randomized population and clinical trials has suggested that vitamin D levels may be linked to COVID-19 transmission and severity. Numerous theoretical studies have pointed to polyphenols and particularly flavonoids as potential inhibitors of SARS-CoV-2 infection. There is also inconclusive evidence to support the future use of β-glucan to address COVID-19 due in part to variability in immune response arising from heterogeneity in polysaccharide branch and chain length for different sources and the absence of a standardized extraction method. To confirm the promising outcomes and hypotheses for bioactive compounds, more randomized and controlled clinical studies are needed. The results of such studies would have a profound effect on the prospects of food supplements and nutraceuticals as potential prophylaxis against COVID-19 and serve to help consumers to protect themselves during the post-lockdown recovery era.

Keywords: SARS-CoV-2; COVID-19; vitamin D; immune system; functional foods; nutraceuticals

1. Introduction

Over the past 20 years, much scientific focus has been trained on the identification of active ingredients in food products (e.g., vitamins) that promote human health. The terms "functional foods" and "superfoods" have become very popular concerning foods and food products that claim health benefits. To follow this trend, numerous food ingredients and active compounds have been investigated as health-promoting agents with possible antimicrobial functions, anti-inflammatory activities, and potential antiviral actions [1]. Except for the vitamins, the compounds under investigation include bioactive peptides, polysaccharides, bioactive lipids, and natural polyphenols [2]. Nutraceuticals' demand increases despite the false efficacy claims, the concerns about quality, the lack of clinical

evidence, and the self-medication implications for severe illness [3]. There is still uncertainty about the "nutraceutical" term, as these products have been reported as neither nutritious nor pharmaceutical. In many countries, there is no legal definition of "functional food" and "nutraceuticals" terms that are used interchangeably. The term "nutraceutical" (originally coined by Stephen DeFelice in 1989) is broadly used to include various naturally occurring products, such as fortified foods, functional foods, and food supplements. However, while the definition of a "food supplement" is clear, the definition of a "nutraceutical" (syncretic neologism of the words nutrient and pharmaceutical) fits in the grey area between food, food supplements, and pharmaceuticals [4]. In addition to their essential nutritional value, nutraceuticals are intended to promote health benefits, where these products typically comprise ingredients that are referred to as generally recognized as safe (GRAS) internationally.

As the world entered the COVID-19 pandemic, the market of bioactive ingredients supporting the immune system was among the four aspects of food systems (including also food safety, food security, and sustainability) directly affected by this crisis [5,6]. In particular, consumers around the world stocked up on vitamin C and other botanical ingredients, whereas panic buying and shortages ensued [7,8]. Subsequently, the efforts of researchers and the scientific community to investigate bioactive compounds that could support the immune system, protect against viruses of the lower respiratory tract, and restrict the transmission of SARS-CoV-2 (the novel coronavirus causing COVID-19 disease) accelerated, resulting in some recent reviews summarizing the potential of natural compounds against SARS-Cov-2 and coronaviruses in general [9–11].

Since there is still relatively limited knowledge about SARS-CoV-2, the urgent need for fast-track prevention and treatment strategies has led researchers to publish articles in "torrents" [12]. The disadvantage of this expeditious approach to glean new knowledge is that the associated surge in recently published studies has as yet to be subjected to any review for benefit or impact. It is envisaged that incremental innovations sustaining agri-food and new products will be influenced by pressing need for new health interventions to combat COVID-19, along with responding to a surge in interest in accelerating green deal innovations and climate change [13].

Therefore, this current timely study provides a comprehensive review on the role of food and plant ingredients and active compounds against COVID-19 disease, as claimed in the torrents of early and fast-released studies. In addition, it commensurately explores the important contribution of food supplements and nutraceuticals in supporting a healthy society including considering "long COVID-19" for survivors [14]. For example, peripheral inflammation caused by COVID-19 may have long-term consequences in those that recover, leading to chronic medical conditions such as dementia and neurodegenerative disease, likely through neuroinflammatory mechanisms that can be compounded by an unhealthy diet [14]. Thus, there is a pressing need for wider access to healthy foods, and people should be made aware that healthy eating habits may reduce susceptibility to and long-term complications from COVID-19.

2. The Role of Food Ingredients and Active Compounds in Supporting the Human Immune System

As it is well known from mechanistic and clinical data, vitamins and folate, polysaccharides and dietary fiber, lipids, peptides, and natural polyphenols are important for the body's immune system against viruses [1,15,16]. Table 1 presents a summary of the health benefits and possible action mode against SARS-CoV-2 virus of food ingredients and bioactive compounds. These bioactive compounds can be found in fruits and vegetables, the consumption of which is highly recommended [17,18]. For example, citrus, kiwi, and vegetables such as broccoli contain high amounts of vitamin C (ascorbic acid), which is necessary for the repair of body tissues and immune's function [19]. Likewise, vitamin C can restrict infection of the lower respiratory tract under certain conditions [20] and prevent the common cold [21]. Vegetables such as sweet potato, spinach, and carrots contain high amounts of vitamin A (comprised of oil-soluble compounds such as β-carotene, retinol, and retinoic acid). Vitamin A is known to support immune function and protect against infections when administered

at pharmacological or high dosage (10 nM or higher) due to its regulatory roles in humoral immune processes and cellular immune responses [22].

Bioactive peptides are composed of several of amino acids arranged in different configurations, and their molecular weights are <6000 kDa. Currently, more than 1500 different bioactive peptides have been reported. Although the correlation between structure and functional properties is not well established, many bioactive peptides share some structural features that include a peptide residue length between 2–20 amino acids, and the presence of hydrophobic amino acids in addition to proline, lysine, or arginine groups [23]. Bioactives are classified by their action mode (e.g., antimicrobial, antioxidant, immunomodulatory, anti-inflammatory, antithrombotic) and their binding capacity to micronutrients such as minerals. Dairy products are the primary sources of peptides, e.g., < 3 and 3–10 kDa peptide fractions obtained from fermented milks with specific *Lactobacillus plantarum* strains [24]. However, peptides can be obtained from other animal sources such as bovine blood, gelatin, meat, egg, and fish. Some plant sources of bioactive peptides are soy peanut, sorghum, pumpkins, mushroom, and rice. Peptides can also be released during the fermentation process of cheese, yogurt, and other fermented products. Peptides can be produced during cheese ripening, whereas lactic acid bacteria in general can produce a wide range of them during fermentation. For instance, some anti-inflammatory activities of peptide fractions can be obtained from fermented milk with specific *Lactobacillus plantarum* strains [24]. The probiotic strain of *Lactobacillus gasseri* SBT2055 (LG2055) has also been shown to reduce virus respiratory infection by suppressing the replication of the virus. Results demonstrated that following the respiratory syncytial infection, LG2055 enhanced the expression of IFN-β and IFN-γ at the gene level in the mice's lungs. Another study demonstrated that several lactic acid bacteria-induced small protein molecules known as immune interferon (IFN-γ or IFN-β) in the mice's lungs, contributing to clearance of respiratory virus [23]. Finally, another study has shown that L. paracasei strains cultured in the presence of artichokes' phenolic extracts (4 mg/mL) have anti-inflammatory effects on dendritic cells, suggesting potential synergistic effects between different bioactive compounds [25].

Polysaccharides are complex carbohydrate polymers that are naturally found in various food sources including seaweeds and cereal grains and are also produced by microorganisms such as lactic acid bacteria. Polysaccharides have many bioactivities such as antioxidant, immunomodulatory, antidiabetic, anticancer, as well as liver and renal protective functions. A large number of studies have been conducted on the isolation and structural identification of polysaccharides from edible and medicinal mushrooms, and a total of more than forty polysaccharides were reported. In addition, these studies have shown that polysaccharides from mushrooms have antivirus activity against a wide range of viruses, including hepatitis B, influenza, enterovirus, herpes simplex virus, porcine circovirus, rotavirus, and others. For instance, *Coriolus versicolor*'s polysaccharide peptide has been demonstrated to possess immunomodulatory properties with the ability to triggers a Toll-like receptor 4 showing insignificant toxicity [26]. One explanation for this antiviral activity could be attributed to the restriction of adsorption and penetration of the virus. The polysaccharides from mushrooms exhibited potential anti-HIV activity by downregulating replication of the virus and upregulating certain antiviral chemokines (Stromal Cell Derived Factor-1 alpha, SDF-1α, and Macrophage Inflammatory Protein, MIP-1α/β). These chemokines block the coreceptors of HIV-1 in THP1 cells from leukemia patients and blood mononuclear cells [26].

Table 1. Health benefits and possible action mode against SARS-CoV-2 virus of food ingredients and bioactive compounds.

Compounds	Type of Study	Health Benefits	Mode of Action against SARS-CoV-2
Bioactive peptides (e.g., <3 and 3–10 kDa peptide fractions obtained from fermented milks with specific *Lactobacillus plantarum* strains)	In vitro	Control hormone release, anti-inflammatory, anti-hemolytic, anti-mutagenic, antioxidant and antimicrobial activities [23–25]	Disruption of viral spike protein [26]
Polysaccharides	In vitro In vivo	Antiviral activity stimulate ROS [1], reduce risk factors for chronic diseases, improve metabolism and digestibility [27]	Reduction in inflammatory responses Prevention of ARDS [2] [28–32]
Vitamins (A, C, E, and D)	In vitro Clinical	Support immune function Protect against infections Prevent common cold [19–22]	Restriction of ACE2 [3] activity Promotion of innate immunity [33–38]
Medicinal Herbs	Clinical In vitro	Prevention of influenza viruses [33,34,39,40]	Improve COVID-19 patients recovery [41,42]
Bioactive lipids (fatty acids, phytosterols, carotenoids)	In vivo	Enhance immune response Anti-inflammatory activities Reduce risk of cardiovascular diseases [43]	Inhibition of ACE [4] and restriction of virus ability to enter the cells [44]
Natural polyphenols (flavonoids, phenolic acids, stilbenes, lignans)	In vitro In silico	Anti-inflammatory, antimicrobial and antioxidant activities, antiviral capacity, prevent digestion issues, reduce the risk of chronic diseases [45–53]	Inhibition of viral replication Disruption of viral spike protein Inhibition of SARS-CoV-2 protease [26,54–69]

[1] ROS: reactive oxygen species; [2] ARDS: acute respiratory distress syndrome; [3] ACE2: angiotensin-converting enzyme 2; [4] ACE: angiotensin-converting enzyme.

Bioactive lipids comprise several endogenous molecules affecting a wide array of biological processes. Typical examples of lipids include omega-3 fatty acids and their metabolic products, carotenoids, phytosterols, and fat-soluble vitamins, phenolic lipids, and acylglycerol derivatives. Numerous studies have found an association between the consumption of certain bioactive lipids and the prevention, delay, or treatment of chronic and acute diseases such as cardiovascular disease, cancer, osteoporosis, and immune disorders. One possible protective mechanism for the bioactive lipids against virus infection is the prevention of inflammation. Bioactive lipids such as oleic acid have exhibited antiviral protection by inducing leakage and even lysis of cell membranes including the virus lipid membrane. It is also known that these bioactive lipids show phagocytic capacity of macrophages and can facilitate the removal of any damage by viral infection [27].

Polyphenolic compounds from plant foods, extracts, and food processing by-products have well known antioxidant activities [28–32]. Polyphenolic compounds can be subdivided into flavonoids, phenolic acid, polyphenolic amides, resveratrol, and other polyphenols. These compounds are antioxidants and also have antimicrobial and antiviral activities. Several recent studies explored the in vitro antiviral capacity of different polyphenols [33–35]. In addition, Vázquez-Calvo et al. [36] conducted a study related to the effect of polyphenols (e.g., epigallocatechin, epigallocatechin gallate, epicatechin, catechin, cyanidin, and delphinidin) on Dengue, Zika, and Nile viruses. These enveloped plus-strand RNA viruses are transmitted by mosquitoes, posing a serious threat to health. The results of this study showed that the aforementioned polyphenols influenced the attachment and entry step of the RNA into the host cells were dose-dependent in a 1–10 μM range and thereby reduced the infectivity of the viruses.

3. Food Ingredients and Active Compounds against COVID-19 Disease

The oral supplementation or intravenous administration with food bioactives and nutraceuticals has been proposed as an alternative approach against COVID-19 disease based mainly on their anti-inflammatory properties, but also to reflect their ability to inhibit viruses' (e.g., SARS-CoV, MERS-CoV and SARS-CoV-2) activity by disrupting their protein envelopes [37]. For instance, these compounds can enhance the response of type 1 interferon to RNA viruses such as influenza and coronavirus [38]. The possible mode of action against SARS-CoV-2 of several bioactive compounds and food ingredients is presented in Table 1.

Plant secondary metabolites (e.g., β-carboline, quinoline alkaloids like cinchonine, skimmianin, dictamine, and quinine, as well as isoquinoline alkaloids such as emetine, berberine, and sanguinarine) can act as DNA intercalators (similar to chloroquine's proposed in vitro mechanisms of action against SARS-CoV-2) inhibiting the replication of the virus [39]. Another anti-replication mechanism is through binding of angiotensin-converting enzyme 2 (ACE-2). This enzyme is a type I transmembrane metallocarboxypeptidase that mainly expressed in the renal tubular epithelium, and the vascular endothelial cells, but also in the lung, and the kidney [40–42]. Different studies have referred to ACE-2 as a cellular entry for SARS-CoV-2 in the body together with other factors [43,44]. In particular, the spike proteins of the virus surface binds on ACE-2 and diffuses the virus into the target cells [45]. Subsequently, compounds that exhibit a binding affinity for the core amino acid of ACE-2 have been proposed as potential preventive or therapeutic agents (these compounds mainly attack free viral particles and to a lesser extent viruses that have already entered host cells), since they are able to interfere with or avoid the host–viral interaction [39,46]. For example, omega-3 fatty acids and their metabolites inhibit angiotensin-converting enzymes (ACE, which is a precursor of ACE-2) and thus suppress ACE2-expression, reducing the availability of receptors to SARS-CoV-2 and subsequently restricting its ability to enter the target cell [37,47].

Using simulations of binding free energy and molecular dynamics, stilbene-based compounds such as resveratrol has been shown to be potential anti-COVID-19 candidates that can theoretically disrupt the virus's spike protein [48]. Relevant action mechanisms have also been proposed for other bioactive compounds such as folic acid. Folic acid is a water-soluble form of vitamin B

that can inhibit furin activity that facilitates the cleavage between ACE-2 and spike proteins of SARS-CoV-2 [49]. Those proteins have also been studied as possible targets of bioactive peptides in mitigation of SARS-CoV-2 pathology. In particular, bioactive peptides with unique amino acid sequences can mitigate such targets including, type II transmembrane serine proteases (TMPRSS2) inhibition, furin cleavage, and the renin–angiotensin–aldosterone system (RAAS) members. Based on current evidence and structure-function analysis, multiple bioactive peptides present potency to neutralize the virus [50].

Adem et al. [51] conducted a molecular docking study to identify the ability of 80 flavonoid compounds to bind 3-chymotrypsin-like protease (3CLpro), which is known to be an important enzyme for the replication of SARS-CoV. Other polyphenols and flavonoids such as protocatechuic acid, punicalagin, theaflavin gallate, kaempferol, theaflavin digallate [52], pedunculagin, tercatain, punicalin, [53], epigallocatechin gallate [54], riboflavin, daidzein, genistein, phycocyanobilin, cyanidin [55] hispidin, lepidine E [56], and hesperidin [57] have been proposed as potential inhibitors of the main SARS-CoV-2 protease in similar studies. Besides, hesperidin and other flavonoids have been referred to possess better binding poses than common drugs against COVID-19, e.g., nelfinavir, chloroquine, and hydroxychloroquine sulfate [51,58]. Another phenolic compound, quercetin, is known to display a broad range of antiviral properties that can interfere at multiple steps of pathogen virulence (virus entry, replication, and protein assembly). These therapeutic effects can be augmented by the co-administration of vitamin C. Furthermore, quercetin has recently been identified as an inhibitor of SARS-CoV-2 3CLpro [59], and also, it has been shown to act synergistically with vitamin D and have anti-viral action against SARS-CoV-2 with the proposed dosage for both substances being up to 500 mg taken two times a day for prophylaxis and mild cases [60]. Considering the different flavonoids, a thorough bioinformatic analysis of small molecules interacting with ACE-2 using the SUMMIT supercomputer indicated the structural analogue eriodictyol (5,7,30,40-tetrahydroxyflavanone) of luteolin as the most potential inhibitor of SARS-CoV-2 [61]. Finally, both quercetin and other flavonoids from different plants (e.g., litchi seeds) have shown similar activity when applied in traditional Chinese medicine [62]. In general, supplementation with dietary flavonoids up to 1–2 g/day is considered to be safe, but higher cumulative dosages should be avoided, as they can negatively affect the metabolism of the liver. Flavonoids can be obtained from different plant sources, but it is peanut shells that may affect persons allergic to peanuts, or fava beans, consumption of which could cause hemolytic anemia to Mediterranean extraction persons who lack the enzyme glucose-6-phosphate dehydrogenase (G6PD). Such patients should also not be administered the antimalarial drugs chloroquine and hydroxychloroquine, which have been advocated based on anecdotal reports for the treatment of COVID-19 [63,64].

Nevertheless, although these theoretical approaches and hypotheses about the potential role of plant secondary metabolites against COVID-19 seem to be reasonable, most of them have not been justified yet either with in vitro studies or with clinical trials. In one of the first relevant studies, Murphy et al. [65] used an in vitro lung injury model to study the activity of β-glucans from the *Shittake* mushroom. The authors using 1, 5–10 mg/mL of β-glucans reported that they reduce inflammatory responses associated with acute respiratory distress syndrome (ARDS) in vitro that includes reduced cytokine production, oxidative stress, necrosis, and apoptosis, suggesting potential for amelioration of SARS-COV-2 through a role in preventing cytokine storm. The latest occurs when white blood cells accumulate by infections such as SARS-CoV-2, releasing inflammatory cytokines. Differential in vitro immunomodulatory and pulmonary cytoprotective effects were attributed in part to variance in β-glucan structure that will have cross-cutting relevance to COVID-19 interventions. ARDS was evident in severe complications experienced by COVID-19 patients in China admitted to intensive care units [66], where the specific involvement in reducing cytokines IL-1β and IL-6 production is a strategy for COVID-19 intervention [67]. Although not studied yet, β-glucan from other sources could result in similar responses, e.g., β-glucan derived from algae (in a diet of 108 mg/kg) has been shown to enhance immune responses of weaned pigs experimentally infected with a pathogenic E.

coli. In particular, the supplementation of β-glucan also reduced white blood cells, neutrophils, serum tumor necrosis factor (TNF)-α, cortisol, and haptoglobin and down-regulated ($p < 0.05$) the mRNA expression of several immune genes (IL1B, IL6, and TNFA) in ileal mucosa of E. coli challenged pigs, compared with the control diet [68].

On the other hand, Hetland et al. [69] recently highlighted the potential for medicinal mushrooms, such as *Basidiomyceta Agaricus blazei Murill*, *Hericium erinaceus*, and *Grifola frondosa,* for prophylactic and therapeutic potential against severe lung inflammation that often follows COVID-19 infection. An AbM-based mushroom extract (Andosan™), also containing *Hericium erinaceus* and *Grifola frondosa*, has been shown to significantly reduce bacteremia and increase survival in mice with pneumococcal sepsis and to improve symptoms and quality of life of patients with inflammatory bowel disease via an anti-inflammatory effect. Hence, such mushroom extracts could have prophylactic or therapeutic effect against the pneumonic superinfection and severe lung inflammation that often complicates COVID-19 infection.

Besides, several authors highlighted the potential benefits of boosting immune response and wellbeing of individuals undergoing self-quarantine for prevention and treatment of COVID-19; such as, the consumption of foods rich in vitamins A and D, zinc, and selenium that are found in mushroom, oily fish (salmon, sardines), egg yolk, milk, cheese, whole grain and wheat bran cereals, and dark green leafy vegetables [70–72].

Novel therapies are urgently required to address ARDS that is associated with severe respiratory failure with approximately 40% mortality [73]. Up-regulation of pro-inflammatory cytokines associated with lung parenchyma injury is an important causation element of ARDS [74,75]. β-Glucans from Shiitake also reduced populations of a multiple-antibiotic resistant isolate *Klebsiella pneumoniae* in an in vivo lung infection model [76,77]. Findings showed that β-glucan improved lung physiological parameters, reduced white cell count protein inflammation to the lung, reduced bacterial levels in the bronchoalveolar lavage and arterial blood parameter, and supported vital partial pressure of oxygen (pO2) along with promoting lung cellular repair. The same research group recently reported on a novel means of delivering nebulized bioactives in aerosols for effective lung delivery that has potential implications for use of smart nutraceuticals in future treatment and recovery of COVID-19 patients [78]. The aforementioned also supports the findings of Bediril et al. [79], whereas in an experimental sepsis model, β-glucan attenuated inflammatory cytokine release (tumor necrosis factor-α, interleukin-1β and interleukin-β) and prevented acute lung injury. While there is emergence of smart infection models, such as for lung [65,76,77] or gut delivery [80], there is a pressing need to pursue controlled clinical studies on use of β-glucan to prove efficacy for human health.

Moreover, Luo et al. [81] reported on the use of bilberry (*Vaccinium myrtillus* Linnaeus), which investigated the anti-inflammatory effects of bilberry extract (containing 42% anthocyanin) on alleviating liver injury and croton oil-induced ear edema using a rodent infection model. In particular, bilberry extract inhibited effectively liver inflammation and croton oil-induced ear edema caused by *P. acnes*. The administration of bilberry extract suppressed the protein levels of nuclear factor-kB, tumor necrosis factor-α, and inducible nitric oxide synthase, and the increase in liver mRNA levels of interleukin-6, tumor necrosis factor-α (TNF-α), interleukin-1β, and inducible nitric oxide synthase. Bilberry extract treatment also reduced liver malondialdehyde and nitric oxide contents. These results suggest anti-inflammatory potential for future use in natural products and healthy food, such as for COVID-19 needs. Garden blue blueberry (*Vaccinium ashei Reade*) anthocyanin extracts were also recently reported for their capacity and underlying mechanisms in protecting from lipopolysaccharide (LPS)-stimulated inflammation in vitro [82]. Enzyme-linked immunosorbent assay (ELISA) studies indicated that blueberry extract restricted importantly the production of interleukin-1β, prostaglandin E2, interferon-γ, and nitric oxide. Further analysis with real-time PCR showed that in LPS-stimulated RAW 264.7 cells, the mRNA expression levels of cyclooxygenase 2, tumor necrosis factor-α, monocyte chemoattractant protein-1, interleukin-6, and interleukin-1β were suppressed. However, detailed

mechanistic information underpinning the use of anthocyanin in disease mitigation remains to be elucidated.

Other reviews and studies discuss the role of vitamins (particularly A, E, and D) against COVID-19 transmission. For example, isotretinoin, which is a derivative of vitamin A, was recently suggested to restrict the activity of angiotensin-converting enzyme 2 (ACE2) [83]. In contrast, it is known that the decrease in levels of vitamin D and E in cattle could increase the infection possibilities by bovine coronavirus [84]. Besides, the increased risk of acute respiratory distress and lung injury syndrome is attributed to vitamin D deficiency. In particular, it is known that vitamin D promotes innate immunity processes, e.g., it suppresses adaptive immunity by decreasing the maturation of dendritic cells, diminishing the ability of dendritic cells to present antigen to CD4 cells, suppressing the proliferation of CD4 cells, and their differentiation into Th1 and Th17, and via promotion of Th2 and Treg [85]. Taking into account that vitamin D possesses immunomodulatory and anti-inflammatory properties as well as antiviral effects indicated by numerous studies with well-established data [86], the respective supplementation of patients may support their immune system against COVID-19 [87]. Ilie et al. [88] confirmed this using randomized population studies. More specifically, the mean levels of vitamin D for 20 European countries and morbidity and mortality caused by COVID-19 were acquired. Negative correlations between mean levels of vitamin D (average 56 mmol/L ± 10.61) in each country and the number of COVID-19 cases/1 M population (mean 295.95 ± 298.7, and mortality/1 M population (mean 5.96 ± 15.13) were observed. Vitamin D levels are severely low in the aging population especially in Spain, Italy, and Switzerland. In another effort, Daneshkhah et al. [89] combined data from two clinical studies to suggest that vitamin D may reduce COVID-19 severity by suppressing the cytokine storm in patients. A possible link between high C-reactive protein (CRP) and vitamin D deficiency and calculated an odds ratio of 1.8 among the elderly (age greater than or equal to 60 years) in low-income families and an odds ratio of 1.9 among the elderly (age greater than or equal to 60 years) in high-income families. COVID-19 patient-level data show a notable odds ratio of 3.4 for high CRP in severe COVID-19 patients. Lau et al. [90] conducted a retrospective observational study, reviewing the medical records of COVID-19 and suggesting that vitamin D insufficiency may play a role in the progress of COVID-19 disease. However, in order to validate this kind of hypotheses, more randomized controlled studies are needed, since the latest investigation did not adequately address risk-stratify subjects.

Another prevention strategy against the transmission of SARS-CoV-2 may be the consumption of herbs. Herbal medicines (e.g., traditional Chinese or Unani) are rich in dietary antioxidants such as polyphenols and vitamins and have shown some promising results for the treatment of relevant diseases. For example, *Astragulus membranaceus* extracts have shown in vitro anti-influenza virus activity [91,92]. Moreover, the consumption of ginseng root has been proposed for the prevention of influenza [93]. Hui et al. [94] revised the outcomes of population, cohort, and clinical studies that used Chinese herbal formulas for the prevention of SARS and H1N1 influenza transmission in older people and high-risk populations, suggesting the potential protective role of such formulas against COVID-19 disease. The most frequently used herbs in these studies included *Fructus forsythia* (Lianqiao), *Radix glycyrrhizae* (Gancao), *Radix saposhnikoviae* (Fangfeng), *Radix astragali* (Huangqi), *Lonicerae Japonicae Flos* (Jinyinhua), and *Rhizoma Atractylodis Macrocephalae* (Baizhu). Luo et al. [95] conducted an empirical study based on the treatment of 54 COVID-19 patients from Wuhan (China), noting that traditional Chinese medicine improved patients' recovery. Ang et al. [96] analyzed 28 (26 Chinese and two Korean government-issued) guidelines and numerous herbal formulas, identifying different patterns for mild, moderate, severe, and recovery stages of COVID-19 disease. Glycyrrhizae Radix et Rhizoma was the most used herb in the Chinese guidelines. Silveira et al. [97] studied the possible positive effects of 39 herbal medicines on COVID-19 patients and concluded that five of them (*Althaea officinalis, Commiphora molmol, Glycyrrhiza glabra, Hedera helix* and *Sambucus nigra*) can be used as immune system boosters in early and mild cases, whereas the benefits/risks assessment of 12 (*Allium sativum*, *Andrographis paniculata*, *Echinacea angustifolia, Echinacea purpurea, Eucalyptus globules* essential oil, *Justicia pectoralis, Magnolia officinalis*, *Mikania glomerata*, *Pelargonium sidoides*, *Pimpinella anisum*, *Salix* sp, *Zingiber*

officinale) others were promising. Similarly, ibuprofen showed promising results, although there was not enough evidence to endorse the use of paracetamol and/or codeine. In any case, it is important to confirm the potential protective role of the above formulas with prospective and rigorous clinical studies [94,96].

4. The Prospects of Food Supplements and Nutraceuticals in the Era of the COVID-19 Pandemic

The pandemic has disrupted citizen purchase behavior in a way that is referred to as the "ripple effect"; an upstream propagation of the disruptions to all actors involved in the supply chains. Subsequently, a considerable effort is required by authorities to support the primary sector in terms of economy planification, digitalization, and products' eco-labeling [98]. To this line, forthcoming technologies for the agri-food sector will be influenced by the growing demand to produce safer and nutritious foods to meet growing populations that reflects dynamic changes in eating habits such as personalized nutrition, alternative protein sources, and attitudes towards climate change and digitization [99]. The interplay of food insecurity, malnutrition, and obesity on dietary behaviors amid the COVID-19 pandemic indicate opportunities for stakeholders to address social and structural determinants of healthy eating as a treatment strategy to improve the health span of individuals [100].

Thereby, the COVID-19 pandemic has widely affected the food sector with consumers increasingly seeking sustainable, organic, and functional foods [101,102]. Immunity was among consumers' highest priorities, but the current pandemic has forced them to re-evaluate their eating patterns and lifestyles. According to a survey of 23,000 individual consumers by FMCG Gurus [103], 72% of European shoppers are eager to change their eating behavior by turning to healthier choices following the era of COVID-19 pandemic. Consequently, products fortified with bioactive ingredients and nutraceuticals are more popular than ever [104]. These products may be developed from food processing by-products [30,105], plants, yeast, seaweeds, algae [106], fungi, or mushrooms that reduce inflammatory responses that are typically associated with cytokine storm in severe COVID-19 patients. At the same time, there is a significant interest from existing businesses and various start-ups in animal protein alternatives, bio-based fibers, and other bio-based products (e.g., biofuels to bioplastics) that fall in the concept of the circular bioeconomy. In order to be socially accepted, the latest needs to rely on residual bio-based feedstock and waste, hence reducing its dependency on crops that compete with food markets [99].

Following this trend, several companies are releasing relevant products such as chocolate balls containing β-glucan (recovered from mushrooms) that are targeting improving children's immunity during the post-lockdown period [107]. Subsequently, the food industry claims more recognition of immune-boosting ingredients [108], as well as highlighting the emerging need for more systematic research and collaboration with academic and governmental institutions in this field [109,110]. Indeed, despite many studies suggesting the protective role of food ingredients, the legal framework for relevant health and nutritional claims is strict, especially in Europe [111]. In this regard, organizations such as the Food and Drug Administration (FDA) and the Global Organization for Eicosapentaenoic Acid and Docosahexaenoic Acid (GOED) have released warnings and informative letters for the related food industries to avoid product claims of general immunity. It is feared that such premature or untested claims could lead to further confusion with regard to food and nutrition-related prevention or treatment of COVID-19 disease [112]. Chinese authorities have gone so far as to call out the names of products officially (e.g., probiotic pulp, vegetables, and tea) and respective companies that have unproven health claims against COVID-19 [112].

COVID-19 will present both challenges and opportunities for the development of new innovative nutraceuticals and business models that may lead to techno-socioeconomic disruption in the food ecosystem throughout the supply chain and marketplace [9]. This disruption will lead to innovative products that converge disciplines such as innovation in manufacturing, services, business processes, and Internet and communication technologies (ICT). Virtual accelerator hubs for connecting micro with small and medium enterprises (SMEs) that exploit advances in ICT through immersive technologies will become more popular for informing disruptive innovation in situ and for remote end-user

applications. This tendency will enable hurdling restrictions that may come with networking and training innovators or employees in meeting rooms that may persist as a barrier to innovate for post-COVID-19 disruptors [99]. Priority is to achieve international consensus on datasets to harmonize methods for reliable and repeatable processing and to inform clinical trials [10] that can be facilitated by promoting open access to findings. COVID-19 pandemic has provided an almost instantaneous void or dearth in critical information to inform consumer market preferences, beliefs, perceptions, attitudes, and barriers towards change that will meet this particular need during and post this pandemic [113]. There is a pressing need to exploit existing or to create new multi-agency enterprise hubs related to academia that will support and accelerate innovators and businesses (such as the ones in agri-food), and full-span commercialization of products and services using the nine-stage technology readiness assessment. This fact should include off-site pilot-data generation where there is an increasing trajectory towards sustainable innovation, the green agenda, and digitization [98]. Therefore, establishing convincing and compelling evidenced-based information as to the real benefits to human health from existing and new nutraceuticals will be important moving forward. Besides, there is a trend towards online purchases of nutraceuticals as well as greater focus on security and adulteration [114].

5. Conclusions

The promising outcomes of the above studies along with the well-documented role of food bioactive ingredients in supporting the immune system and the supplementation of consumers' diets with vitamins, tannins, polyphenols, flavonoids, bioactive lipids, and herbs are driving current market growth trends in the food and nutraceutical sector. Moreover, these trends will most likely continue to drive the market in the post-lockdown era [4]. However, as of 10 November 2020, at the time of this writing, there has not been adequate published evidence correlating the consumption of food bioactives with direct prevention or recovery from COVID-19 disease. Some evidence (including randomized population and clinical trials) does exist regarding the role of vitamin D against COVID-19 disease. In addition, numerous theoretical studies have suggested polyphenolic compounds (mostly flavonoids) as potential inhibitors of SARS-CoV-2 transmission. The potential for using β-glucan to address COVID-19 has also been suggested by taking into account the variability in immune response arising from heterogeneity in polysaccharide branch and chain lengths of different sources. Researchers have recommended drinking plenty of water, along with consuming foods rich in minerals such as magnesium and zinc and vitamins C, D, and E, in addition to a better life style that can boost immunity to help fight infection [115]. Because these supplements can support or improve the function of the immune system, the market prospects for nutraceuticals and functional foods within the post-lockdown period remain high, as a result of the increased interest of health-conscious consumers. Businesses will thus be seeking to fill the demand for new knowledge surrounding consumer preferences, needs, and attitudes toward nutraceuticals and functional foods in order to address the challenges and opportunities created by COVID-19 disease. The aforementioned changes that are coming will also tend to inform or even accelerate innovation across the food industry ecosystem including advances in ICT and manufacturing.

Author Contributions: Conceptualization: C.M.G., T.M.S.A., N.J.R., and S.A.I.; writing—original draft: C.M.G., T.M.S.A., N.J.R., and S.A.I.; writing—review and editing: C.M.G., T.M.S.A., M.R., N.J.R., and S.A.I. All authors have read and agreed to the published version of the manuscript.

Funding: Author (S.A.I.) would also like to acknowledge the support of the Agricultural Research Station at North Carolina Agricultural and Technical State University (Greensboro, NC 27411, USA). This research was funded, in part, by Grants (project Number NC.X337-5-21-170-1 and NC.X341-5-21-170-1) from the National Institute of Food and Agriculture (NIFA). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIFA.

Conflicts of Interest: The authors declare that they have no competing or conflict of interests.

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International Journal of
Molecular Sciences

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Use of Zebrafish Embryo Assay to Evaluate Toxicity and Safety of Bioreactor-Grown Exopolysaccharides and Endopolysaccharides from European *Ganoderma applanatum* Mycelium for Future Aquaculture Applications

Wan Abd Al Qadr Imad Wan-Mohtar [1,2,3], Zul Ilham [2,4], Adi Ainurzaman Jamaludin [4] and Neil Rowan [3,5,*]

1 Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur 50603, Malaysia; qadyr@um.edu.my
2 Bioresources and Bioprocessing Research Group, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur 50603, Malaysia; ilham@um.edu.my
3 Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, N37 WO89 Athlone, Westmeath, Ireland
4 Environmental Science and Management Program, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur 50603, Malaysia; adiainurzaman@um.edu.my
5 Empower Eco Innovation Hub, Lough Boora, Co., R35 DA50 Tullamore, Offaly, Ireland
* Correspondence: nrowan@ait.ie; Tel.: +353-(0)90-64671838

Abstract: Natural mycelial exopolysaccharide (EPS) and endopolysaccharide (ENS) extracted from bioreactor-cultivated European *Ganoderma applanatum* mushrooms are of potential high commercial value for both food and adjacent biopharmaceutical industries. In order to evaluate their potential toxicity for aquaculture application, both EPS (0.01–10 mg/mL) and ENS (0.01–10 mg/mL) extracts were tested for Zebrafish Embryo Toxicity (ZFET); early development effects on Zebrafish Embryos (ZE) were also analyzed between 24 and 120 h post-fertilization (HPF). Both EPS and ENS are considered non-toxic with LC_{50} of 1.41 mg/mL and 0.87 mg/mL respectively. Both EPS and ENS did not delay hatching and teratogenic defect towards ZE with <1.0 mg/mL, respectively. No significant changes in the ZE heart rate were detected following treatment with the two compounds tested (EPS: 0.01–10 mg/mL: 176.44 ± 0.77 beats/min and ENS: 0.01–10 mg/mL: 148.44 ± 17.75 beats/min) compared to normal ZE (120–180 beats/min). These initial findings support future pre-clinical trials in adult fish models with view to safely using EPS and ENS as potential feed supplements for supplements for development of the aquaculture industry.

Keywords: *Ganoderma applanatum*; exopolysaccharide; endopolysaccharide; zebrafish; toxicology

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Citation: Wan-Mohtar, W.A.A.Q.I.; Ilham, Z.; Jamaludin, A.A.; Rowan, N. Use of Zebrafish Embryo Assay to Evaluate Toxicity and Safety of Bioreactor-Grown Exopolysaccharides and Endopolysaccharides from European *Ganoderma applanatum* Mycelium for Future Aquaculture Applications. *Int. J. Mol. Sci.* **2021**, *22*, 1675. https://doi.org/10.3390/ijms22041675

Academic Editor: Toshio Ohshima
Received: 21 January 2021
Accepted: 4 February 2021
Published: 7 February 2021

1. Introduction

Over the past two decades, medicinal mushroom exopolysaccharides (EPS) and endopolysaccharide (ENS) have been increasingly exploited for human benefits including clinical trials [1], neurological protection [2], potent in vitro biological activities [3], chicken patty [4], anti-oral cancer [5], and breast cancer models [6]; however, there is still limited development as yet on their commercial use by the aquaculture industry. Development and exploitation of medicinal mushroom products has significant potential to improve farmed fish health, where appropriate innovation in fish feed and formulation potentially offers safe, affordable and future intensive sustainability needs [7–9]. Recent evidence suggests the use of bioreactor-grown medicinal mushroom *Ganoderma* sp. extracts in aquaculture-like areas including fish-feed [10], fish toxicity [11], and have passed potential clinical application [12]. These potential applications are largely attributed to its bioactive EPS-ENS components with immuno-stimulatory properties, which can be scaled efficiently and repeatedly for commercial use by deploying a specifically-tailored mushroom bioreactor system [13,14].

Currently, there is an innovation gap in terms of specific use and development of fungal biotechnology for meeting emerging needs in the aquaculture industry globally. However, there are some examples of specific mushroom usage including Shiitake fruiting body for fingerlings of Carps [15] and Rainbow trout feed [16]; thus, use of Ganoderma-derived EPS-ENS that comprise beta-glucans warrants further investigation and testing. Mushroom scientists have previously reported that *G. applanatum* EPS-ENS demonstrated promise as novel disease mitigation therapeutic [17–19], suggesting potential for use as disease prevention counter-measures for adjacent aquaculture industry in the form of prebiotics [20].

The creative idea of using extracts from naturally-occurring algae to address key challenges and opportunities in fish health has been exemplified by Marino et al., [21] who studied use of algal extracts as potential alternatives to that of antibiotics for farming sea bass and gilthead sea bream. Marino et al., [21] highlighted increased interest in the use of natural-alternatives materials to that of frontline antibiotics and biocides, particularly where there is a trend towards producing farmed high-value fish with organic status. Cascio et al., [22] reported on the potential "game-changer" use of a microalgae (Spirulina) targeting beneficial effects in the gastrointestinal tract of Zebrafish; this has implications for potentially stimulating the breeding of animals. Such studies show the importance of Zebrafish (*Danio rerio*) assay due to the ability of Zebrafish stomach system to recognize small extracts such as mRNA and immune-priming genes. The use of beta-glucans-derived from medicinal mushrooms, yeast, algae, seaweed and so forth is of increasing interest as these bio-based therapeutics have shown potential for immune-priming of fish for disease mitigation and for added food security. However, the vast majority of studies to date have focused on extraction of bioactives from raw materials, but not from bioreactor grown systems that are important for larger pilot scale and commercial deployment. It is therefore necessary to test and verify the toxicity status of EPS-ENS polysaccharide extract prior to pilot and commercial use; such as, the use of a zebrafish larvae model to evaluate these bioactive substances as part of a fish feed formulation study [23,24]. For example, use of antibacterial polysaccharides from *Undaria pinnatifida* (macroalgae) was reported by Rizzo et al., [25] for the treatment of prominent aquaculture disease *Vibrio harveyi* causes death of marine fish [26] due to vasculitis, gastro-enteritis and eye lesions.

Our study used the ZFET strategy as it was previously reported to be a fast, affordable and sensitive approach [11]; it also offers greater flexibility in terms of using multiple sample insertions to evaluate toxicological effects due to early stages of embryonic development [11] and for continuity in terms of relevance to the gastrointestinal anatomy in small intestine mammals [22]. This study reports on the use of ZFET assay for testing European *G. applanatum* extracts using seven different EPS-ENS concentrations that specifically addresses LC_{50}, embryonic hatching delays, teratogenic defect, and heart rate response with clear microscopic images.

2. Results

2.1. Effects of EPS and ENS on the Survival Rate of Zebrafish Embryos

In the ZFET assay, the survival rate of the embryos of the zebrafish in the range of 0 to 120 h old at the cell culture concentration of 0.01 to 10 mg/mL was analyzed. Based on standard, the normal hatching for zebrafish embryos is median of 48 to 72 h of post-fertilization (HPF), thus the survival rate of the embryos (prior hatch) and larva (post hatch) treated with EPS-ENS extract was determined for maximum of five days (120 h). In Figure 1, we observe that 100% of untreated embryos survived for up to 120 HPF. However, among pretreating cells with EPS-ENS doses of 0.01 to 1 mg/mL, the cell survival rate was 88% at 72 HPF. The survival rate dropped from 88% at 72 HPF to 0% for all cells pretreated at the highest EPS-ENS concentrations (5–10 mg/mL). No embryos at >72 HPF managed to survive and developed at EPS concentration >5 mg/mL (Figure 1a). In the meantime, Figure 1b, on low concentrations (0.5–1 mg/mL) of ENS extract, an average drop in survival rate was relatively small (85%), while at higher concentrations (5–10 mg/mL), a lower

survival rate (<30%) was observed at just 4 days of age (96 HPF). No embryo survived after 120 HPF for ENS at concentrations >5 mg/mL. Collectively, it shows that EPS-ENS delay hatching optimum concentrations of less than <1 mg/mL.

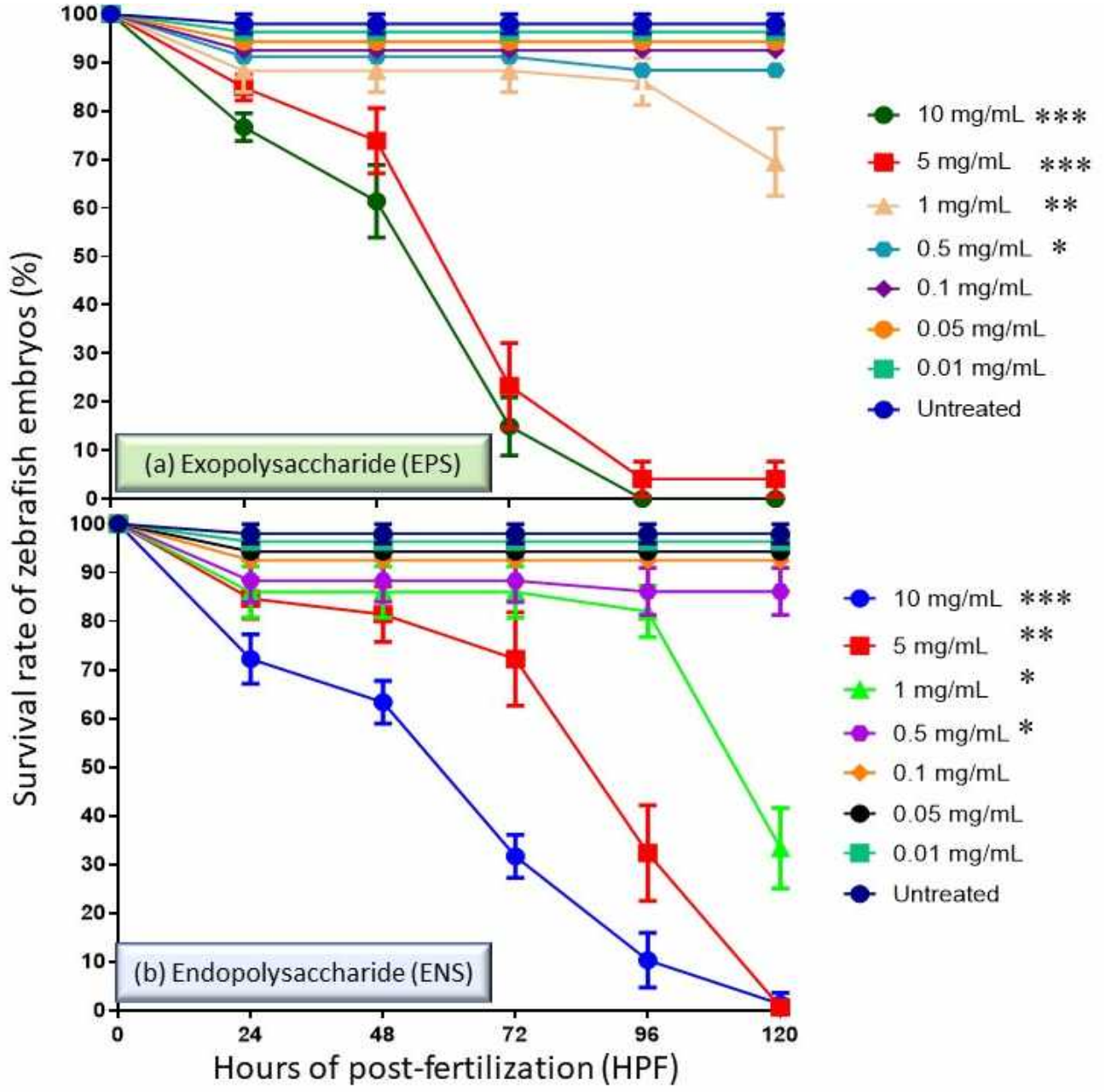

Figure 1. The performance of *Ganoderma applanatum* (**a**) EPS and (**b**) ENS extract at concentrations of 0.01–10 mg/mL on the survival rate of zebrafish embryos at 0–120 h. No embryos survived for both samples at concentration tested > 5.0 mg/mL after 96 h post fertilization (HPF). Symbols: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.2. Performance of EPS-ENS Doses on the Zebrafish Embryos Mortality

Overall, extracts of European *G. applanatum* EPS-ENS have lethal effects that vary with dose and time. In Figure 2, at low concentrations, EPS (<3 mg/mL) and ENS (<1 mg/mL) were both able to prevent 90% of zebrafish embryos from dying. However, at high concentrations, 4 mg/mL EPS and 2 mg/mL ENS did not perform better (with low survival rate) throughout the embryo development stage, showing dead embryos post 72 HPF. Consequently, the LC_{50} value of zebrafish embryos exposed to EPS extract tested was

1.41 mg/mL, whereas the LC_{50} value for ENS extract tested was much lower than this at 0.87 mg/mL.

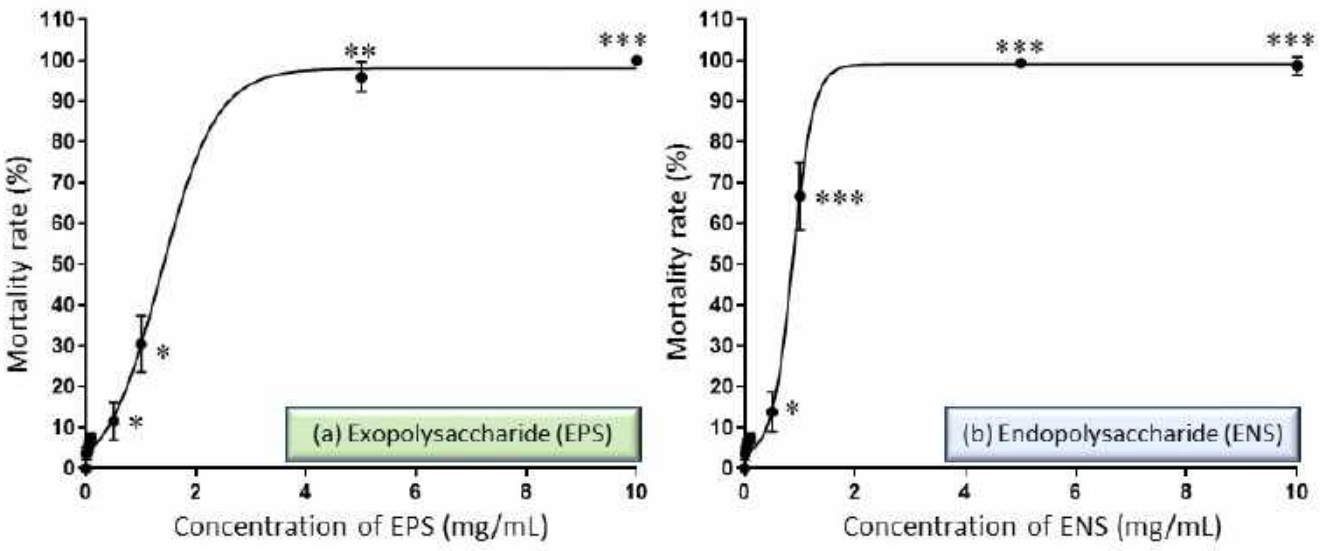

Figure 2. Effect of *Ganoderma applanatum* (**a**) EPS extract at concentrations of 0.01–10 mg/mL and (**b**) ENS at concentrations of 0.01–10 mg/mL on zebrafish embryos mortality rate after 120 HPF. The LC_{50} value for EPS extract was 1.41 mg/mL while LC_{50} value for EPS extract was 0.87 mg/mL. Symbols: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.3. Performance of EPS and ENS Doses on the Zebrafish Embryos Hatching Capability

Subsequently, different EPS-ENS concentrations of extract may affect the hatchability percentages. Figure 3 shows a trend of decreasing hatching rates with increased concentrations of the extract (>1 mg/mL) once treated into zebrafish embryos. It also depicts the hatching rate during (a) EPS treatments at 0.01–10 mg/mL and (b) ENS treatments at 0.01 to 10 mg/mL during 0 to 120 HPF period. For EPS, there was no visible changes in terms of hatching capability at concentrations less than 1 mg/mL, but the rate dropped to 35% at 48 HPF once exposed to 5 mg/mL. Zebrafish did not hatch or survive when treated with a concentration of 10 mg/mL EPS, indicating that high amounts of EPS causes strong suppression of zebrafish embryo at 24 HPF. On the other hand, less than 81% of the embryos hatched on 48 h treatment with ENS at concentrations >0.5 mg/mL; although, zebrafish larvae treated with ENS concentrations at 5 to 10 mg/mL showed the lower hatching rate (<65%) due to high mortality rate after 72 HPF.

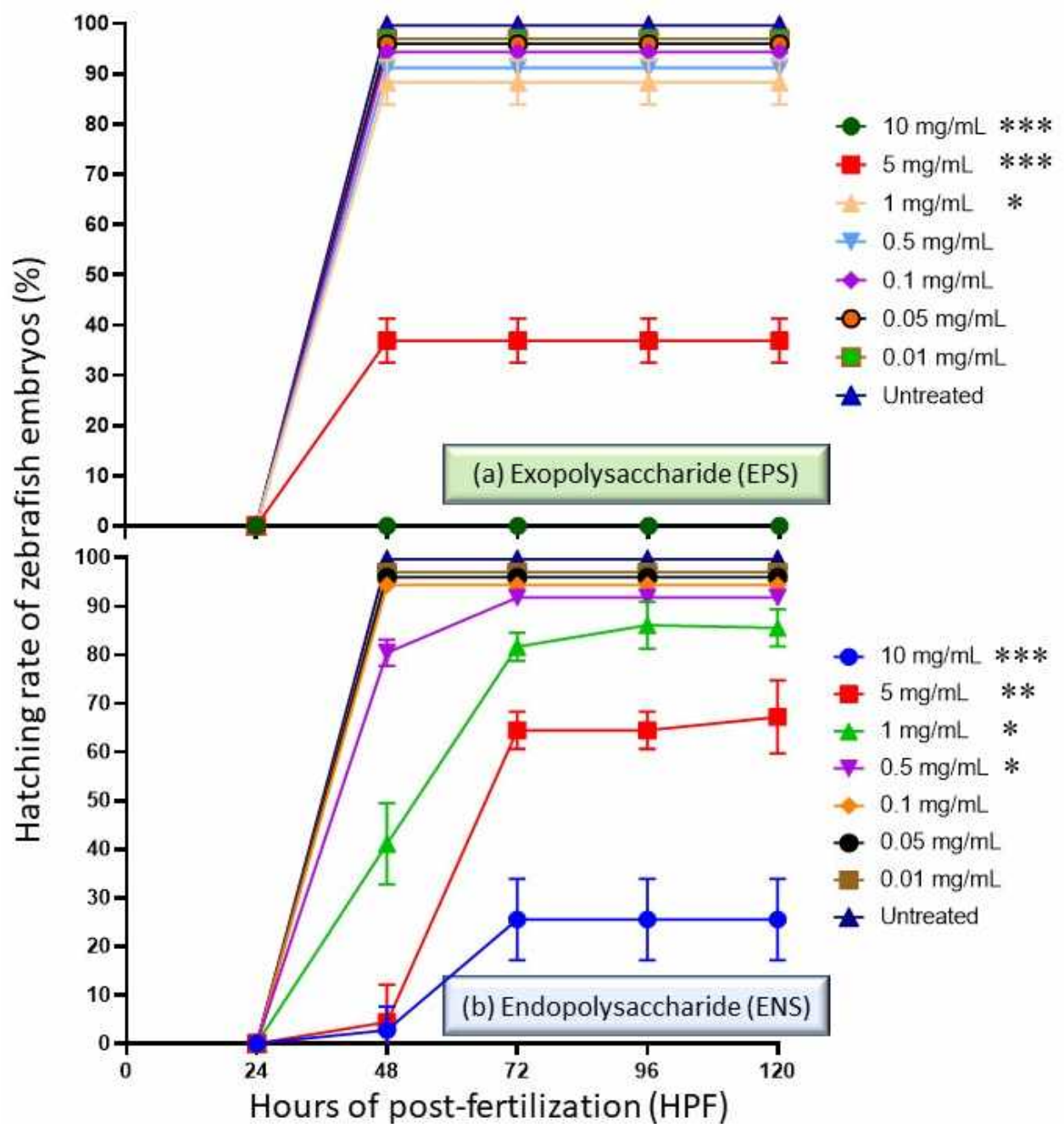

Figure 3. Hatching rate of zebrafish embryos at 0 to 120 h of post exposure with *Ganoderma applanatum* EPS and ENS extract at concentrations of 0.01–10 mg/mL. (**a**) For EPS, low hatching rate (<40%) was observed at concentration 5.0 mg/mL due to high mortality rate. Meanwhile, (**b**) for ENS, low hatching rate (<30%) was observed at concentrations 10 mg/mL due to high mortality rate. High hatching rate was observed at concentrations >1.0 mg/mL (>80%). Symbols: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2.4. The Effects of EPS and ENS on the Heart Rate of Zebrafish Embryos

In the development of many model organisms, zebrafish heart represents the core of functional organ [27]. Based on Figure 4, both extracts of EPS and ENS at low concentrations <1 mg/mL, showed no significant difference towards the heart rate of untreated zebrafish embryo at 96 HPF. The heart rate at day 4 (96 HPF) for EPS (Figure 4a) treatment was healthily recorded at 176.44 ± 0.77 beats min^{-1} at concentrations <1 mg/mL compared to normal average zebrafish embryonic heart rate (120–180 beats min^{-1}). Meanwhile, ENS (Figure 4b) treatment also gave normal heart rate (148.44 ± 17.75 beats min^{-1}) at

concentrations <1 mg/mL. Since EPS extract at 5 and 10 mg/mL killed the zebrafish at 96 HPF, the embryonic heart rate at these concentrations were omitted. However, the embryo was barely alive when treated with 5 mg/mL of ENS (90 beats min^{-1}) and unrecorded at 10 mg/mL due to death. Both EPS and ENS treatments verified the previous report in which the normal heart rate of zebrafish embryo nearly mimics human condition at 120 to 180 beats min^{-1} [28].

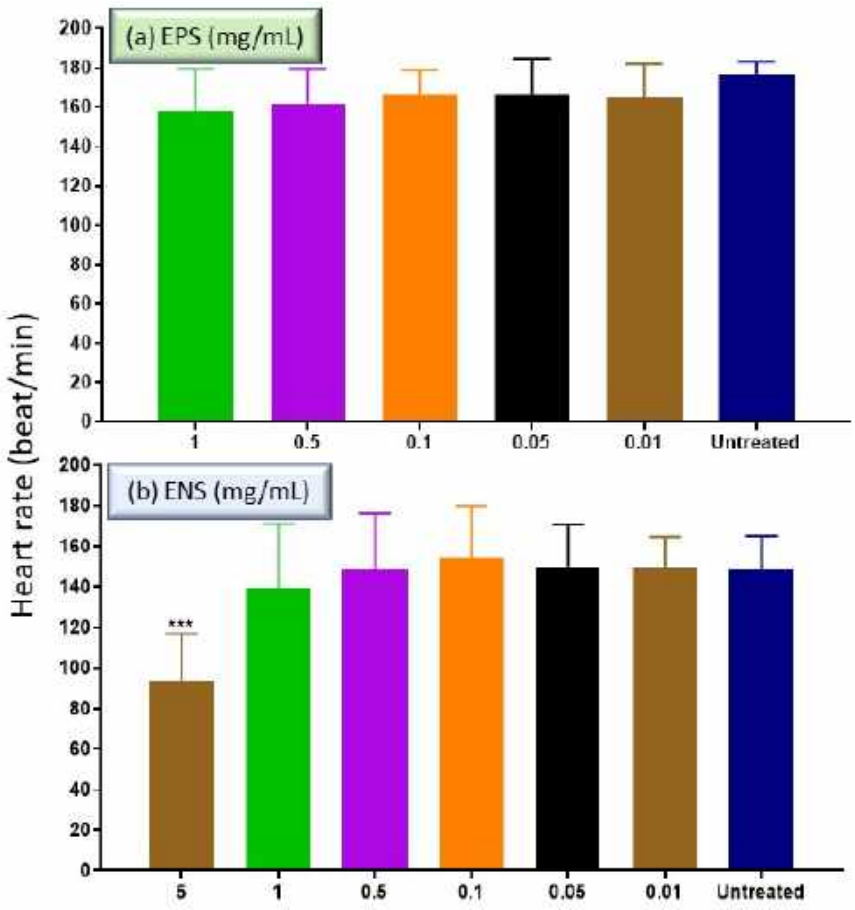

Figure 4. Effect of *Ganoderma applanatum* EPS and ENS extract at concentrations of 0.01–1.0 mg/mL on heart rate of zebrafish embryos at 96 HPF. For (**a**) EPS, no data at concentrations >5.0 mg/mL due to embryo death. Meanwhile, (**b**) for ENS, no data at concentrations 10.0 mg/mL recorded due to embryo death. *** $p < 0.05$ significantly different from the untreated group (zebrafish embryos in medium only).

2.5. Performance of EPS and ENS Doses on the Morphology of Larvae and Zebrafish Embryos Development

The performance of EPS and ENS doses were observed from 0 to 120 HPF period particularly on larvae and zebrafish embryos morphological defects. Figure 5 depicts no visible teratogenic effect of either EPS or ENS at concentrations <1 mg/mL on the embryogenesis stages at 120 h after exposing the embryos to them. Based on these data, it clearly suggested that either EPS or ENS has affected larvae and zebrafish embryos development before and after hatching.

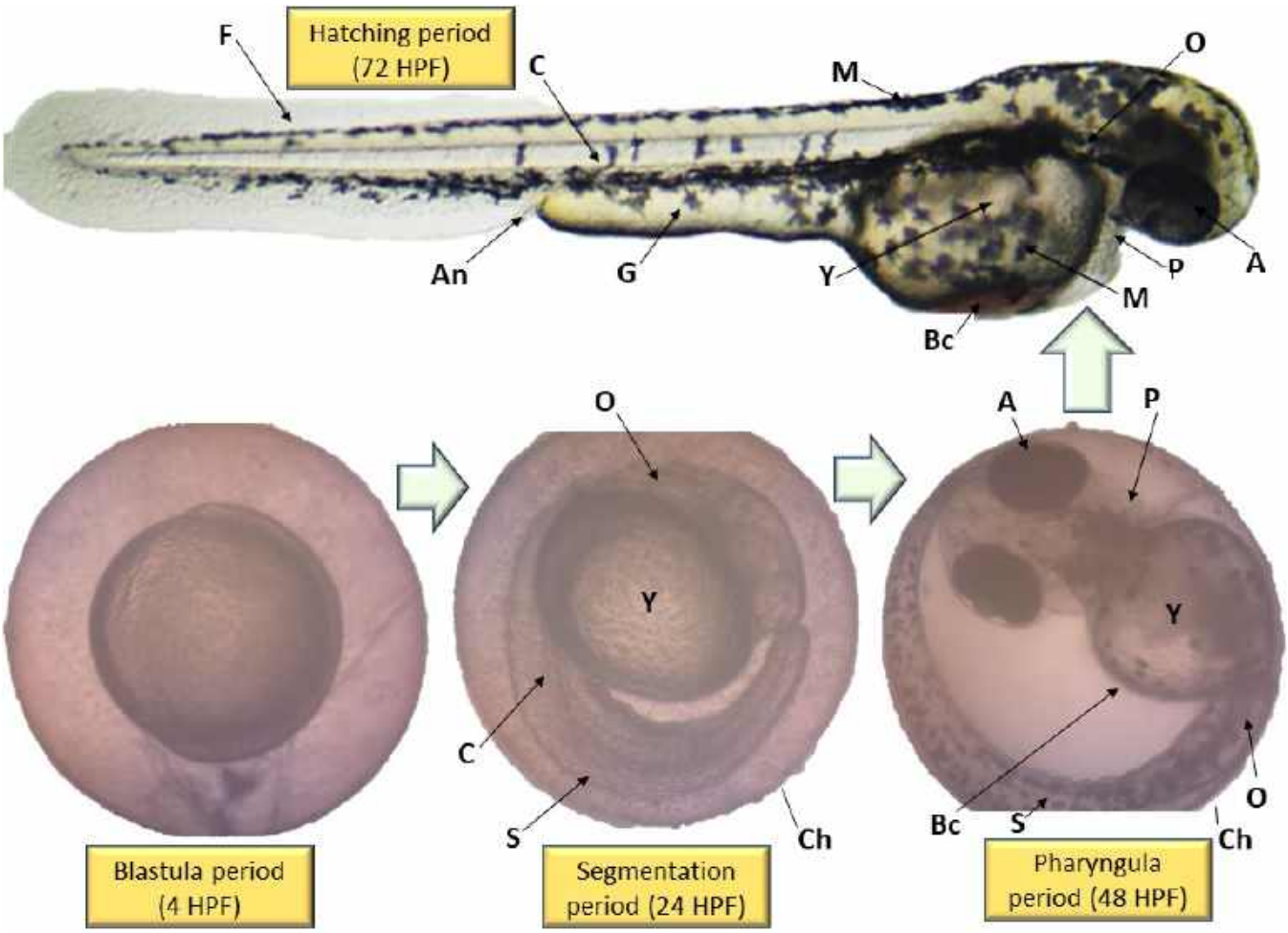

Figure 5. Effect of EPS-ENS extracts (0.01–1 mg/mL) of European *Ganoderma applanatum* showing normal zebrafish embryogenesis at different HPF development. The inverted microscope was used to produce the images and edited using paint 3D [11]. There were 4 periods depicted: Blastula (4 HPF), Segmentation (24 HPF), Pharyngula (48 HPF), and Hatching (72 HPF). Y—yolk sac; S—somites; P—pericardium; O—ear bud; M—melanophores; G—gut; F—fin; Ch—chorion; C—chorda; Bc—blood cells; An—anus; A—eye anlage. Scale bar = 0.5 mm.

As both zebrafish embryo and larvae development were unaffected when treated with 1 mg/mL EPS and 1 mg/mL ENS, various abnormalities were observed as the concentration significantly spiked to 5 mg/mL EPS (Figure 6) and 1.31 mg/mL ENS (Figure 7). One of the most prone abnormalities was observed for ENS showing the period of coagulated embryos from 24 HPF (segmentation) to 48 HPF (pharyngula), and loss of yolk sac, resulting in unhatched embryos even after 120 HPF. Meanwhile, ENS-treated zebrafish managed to hatch at 72 HPF, however with tail malformation and broken blood cells after 120 HPF. Most of normal features were also absent such as fin, gut, and melanophores.

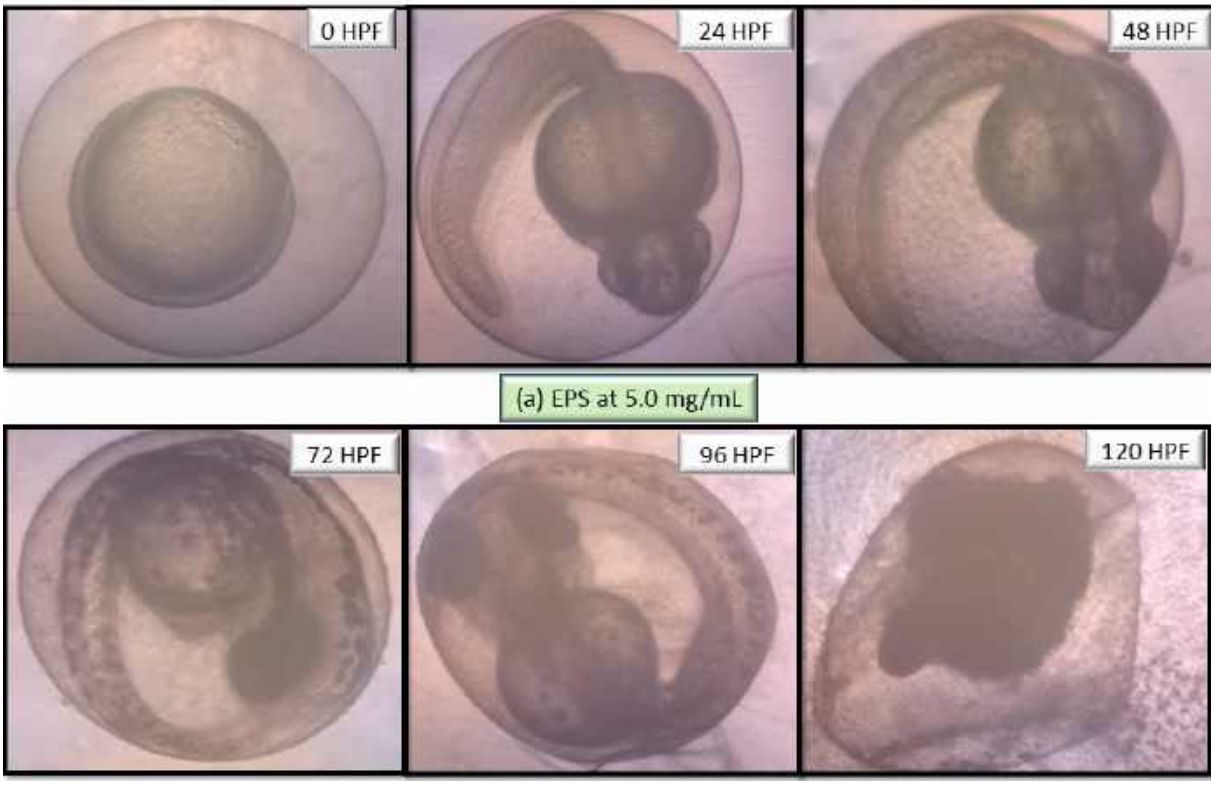

Figure 6. Images of zebrafish embryo and larvae development at 0 HPF, 24 HPF, 48 HPF, 72 HPF, 96 HPF and 120 HPF after treated with European *Ganoderma applanatum* at high EPS concentration of 5.0 mg/mL. Images were captured using inverted microscope at 100× (0 and 24 HPF) and 40× magnification (48–20 HPF).

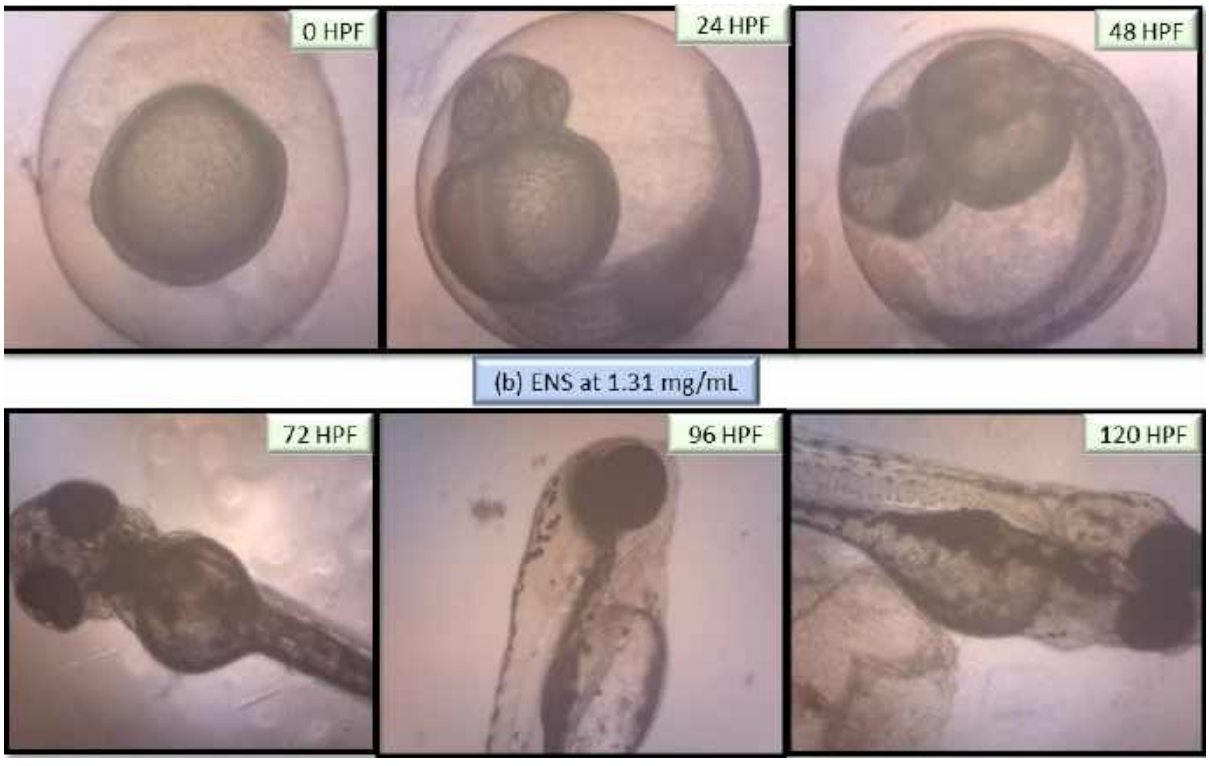

Figure 7. Images of zebrafish embryo and larvae development at 0 HPF, 24 HPF, 48 HPF, 72 HPF, 96 HPF and 120 HPF after treated with European *Ganoderma applanatum* at high ENS concentration of 1.31 mg/mL. Images were captured using inverted microscope at 100× (0 and 24 HPF) and 40× magnification (48–20 HPF).

3. Discussion

In this study, both EPS and ENS extracted from bioreactor-grown European *G. applanatum* were analyzed for acute toxic effects in treated zebrafish embryo. EPS-ENS originates from edible mushroom species and has potential to be used as high-value product in the form of dietary fish feed supplement [29]. Recently, use of EPS-ENS (or beta-glucan) extracts has increased in interest for various applications, such as for preventing biofilms [30] and for fish-feed [10]. Beta-glucans derived from mushrooms and other sources, have also been reported as potential new therapeutic to help fight Coronavirus disease (COVID-19) in the form of potential immunotherapies [31]; to alleviate use of frontline antibiotics for combatting resilient bacteria [32]; as an alternative to the use of silver nanoparticles [33]; or for other potential applications such as cosmetics [34], immune priming or boosting [12], and prevention of cell death [2]. However, study on the toxicity of these specific compounds are lacking, including use of Zebrafish testing, which would help inform product development and applications.

Using this ZFET technique, healthy zebrafish embryos, or larvae, have been used as surrogate animal model, which offers several advantages such as efficient, fast, reliable and comparable to human model [23,35]. This suits the "Zebrafish 3.0" toxicity model as the strategy share many physiological and cellular characteristics with higher vertebrates. In addition, the current ZFET assay uses 96-well plate that have a direct contact with EPS-ENS, mimicking the close contact with the demersal characteristic of zebrafish embryo. Secondly, extra-uterine and transparency can be examined, representing clear phenotypic embryonic development changes. In this study, teratogenic and embryotoxic effects evaluations are essential for EPS-ENS to determine the concentration that is safe for consumption. With booming global health industry and products from plants and fungi, claimed to have strong pharmacological responses, most of them are still dubious on the toxicological profiles.

In this study, fertilized zebrafish embryos were exposed to concentrations of *G. applanatum* extract, EPS (0.01–10 mg/mL) and ENS (0.01–10 mg/mL) that were shown to be non-toxic using this ZFET technique. Overall, EPS at concentrations <1 mg/mL and ENS at <1 mg/mL did not cause delay hatching towards the embryo and with 88% survival rate at 24 to 120 HPF. Additionally, there were no significant changes in both EPS and ENS at concentration <1 mg/mL on the embryo heart rate compared to the normal ones. Moreover, teratogenic effect and zebrafish embryo abnormalities can only be observed at concentrations >5 mg/mL and >1 mg/mL in EPS and ENS, respectively. The test showed that EPS has a higher LC_{50} value of 1.41 mg/mL, meaning that it is better than ENS with lower LC_{50} value (0.87 mg/mL). Even though both EPS and ENS extracts originated from *G. applanatum* mycelium, they may differ in terms of compound composition which originated from fruiting body and different mycelial extraction procedures [36–38]. Former research verified that similar EPS from the sister *G. lucidum* mycelium exhibit a broad range of bioactivities, including immunostimulant and antitumorigenic effects [39], which are higher than those of the fruiting bodies [34]. Meanwhile, ENS has given a lower LC_{50} value than EPS due to its different mycelial extraction methodology; EPS is directly extracted from the surface of fungal mycelium while ENS has undergone series of physico-chemical extractions from the internal part of dried fungal mycelium [5].

Morphological deformities, including tail malformation, could restrict the embryo's ability to break the chorion (Ch in Figure 6) and hatch out post 5 days. Likewise, the absence of heartbeat and coagulated embryo is considered as lethal effects. The work of Dulay et al. [40] has confirmed that tail malformation in zebrafish embryo does occur when exposed to 10 mg/mL of *Ganoderma* sp. fruiting body extract. In addition, it depicted that the safety threshold concentration is <10 mg/mL for any Ganoderma sp. fruiting body extract [40], which is in-line with the current data described for *G. applanatum* EPS and ENS possessing less toxic responses.

Some medicinal mushrooms have also been studied on the effects of toxicity towards zebrafish embryos in comparison to *Ganoderma* species. Exposure of termite-mound mushroom *Termitomyces clypeatus* extract at concentrations of >0.1% reduces the zebrafish

embryos hatchability and <50% extract gave visible teratogenic effects post 48 HPF [41,42]. On the other hand, exposure of grey oyster mushroom *Pleurotus ostreatus* ethanol extract killed the zebrafish embryos using 2.5 and 5% concentrations post 12 HPF, while obstructed growth and tail malformation can be observe at 1% dose [43].

Recent studies have reported that *G. applanatum* polysaccharides are heavily used as food supplements for human species [19,44,45] to improve growth and immunity but none was tested as aquatic feed. The closest comparison were only by its popular sister *G. lucidum* at acceptable concentrations ranging from 1.0 to 1.5 g/kg on giant freshwater prawn (*Macrobrachium rosenbergii*) [46] and grass carp (*Ctenopharyngodon Idella*) [47], which enhance innate immune response and development. Therefore, to gain safety concentration for both aquaculture feed and human drug, both EPS and ENS extracts from *G. applanatum* toxicity reports are essential. Henceforth, the current novel ZFET data may provide useful information for assessing the potential health risks of the EPS-ENS consortia. Still, further tests are merited to evaluate the LC_{50} value of EPS-ENS extract for informing large-scale human trials and for larger animals (e.g., pig, rabbit, and adult trout), before this innovation can be deployed for commercial use.

This constitutes the first toxicology study that addresses bioreactor-cultured European *G. applanatum* (temperature climate mushroom) extracts where findings are compared to that of extracts derived from its sister *G. lucidum* (Table 1). Three studies have previously reported on the evaluation of EPS from *G. lucidum* using ZFET assay (2648 µg/mL) [11], normal prostate cell line (500 µg/mL) [48], and normal human lung cell (1000 µg/mL) [49]. The findings from this present study reports on testing and evaluation of both non-toxic mycelial EPS (1410 µg/mL) and ENS (870 µg/mL) of stirred-tank bioreactor grown *G. applanatum* samples using the zebrafish 3.0 toxicity model. Moreover, this study provided evidence to support the safe use of European *G. applanatum* bioactive polysaccharides via Zebrafish model.

Table 1. Comparison with published non-toxicity assessment of exopolysaccharide (EPS) and endopolysaccharide (ENS) from the mushroom *Ganoderma* sp.

Source	Toxicological Model	Non-Toxic Concentrations (µg/mL)		References
		Exopolysaccharide (EPS)	Endopolysaccharide (ENS)	
G. applanatum BGS6Ap	In vivo—Zebrafish embryos and larvae	1410	870	Current study
G. lucidum QRS 5120	In vivo—Zebrafish embryos and larvae	2648	NA	[11]
G. lucidum BCCM 31549	In vitro—normal human prostate cell line (PN2TA)	500	NA	[48]
G. lucidum	In vitro—normal human lung cell (WRL68)	1000	NA	[49]

4. Materials and Methods

4.1. European Ganoderma Applanatum Bioreactor Fermentation

European *G. applanatum* strain BGS6Ap was isolated by Prof. Dr. Anita Klaus from the Republic of Serbia in the wild region of Mount Kosmaj at temperate environment (18 °C–24 °C), coordinate of 44°27′57″ N, 20°33′52″ E, and maintained at 4 °C on Malt Extract Agar (MEA) slants prior to fermentation setup. The bioreactor fermentation of *G. applanatum* consists of double-seed culture stages. The seed culture medium condition was previously optimized for Ganoderma sp. liquid batch fermentation at fixed metrics (g/L): Yeast extract 1, Glucose 30, KH_2PO_4 0.5, $MgSO_4$ 0.5, and NH_4Cl_4 4 [50]. The fungus was cultivated for ten days at pre-optimized condition of 25 °C, pH 6, 10 g/L of glucose and 150 rpm in a 5-L stirred-tank (STR- Labfors, Infors H-T, Basel, Switzerland) bioreactor [51] to produce fungal biomass.

4.2. G. applanatum EPS-ENS Preparations

The fungal biomass from the harvested *G. applanatum* culture was subjected to Buchner funnel filtration, followed by triple washing with distilled water. To obtain EPS, the filtrate was precipitated by the addition of four volumes of 99% (*v*/*v*) cold-ethanol-shock treatment and left overnight at 4 °C. After 24 h, crude EPS was formed naturally by agglomeration, and centrifuged at 10,000 rpm for 20 min. Meanwhile, to obtain ENS, the initial washed-fungal biomass was dried in a dryer until it powdered. The dried biomass was mixed with 1 g:20 mL distilled water, then subjected to heat-extraction (24 °C for 30 min) [52]. The resulting crude ENS was precipitated using the similar procedure of crude EPS. Finally, both EPS and ENS crude extracts were slow-dried to constant weight prior to toxicity studies. Stock powder of dried EPS-ENS was diluted in embryo medium (Danio-SprintM solution) producing working solutions (10 mg/mL). The mixture was diluted in 2-fold serial dilutions obtaining six concentrations ranging from 0.01–5 mg/mL in a 96-well microplate (200 µL). Embryos as in embryo medium were assigned as standard control (untreated).

4.3. Zebrafish Maintenance and Breeding

The use of zebrafish was approved under the by the Institutional Animal Care and Use Committee (IACUC), Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Briefly, zebrafish of both sexes were placed into a breeding tank on the day the breeding tank was prepared. Following the first day, embryos were collected, washed, and then incubated in Danio-SprintM solution. Dead and coagulated embryos were discarded in an attempt to find healthy embryos [11].

4.4. Zebrafish Embryo Toxicity (ZFET) Assay

The standard of Organization for Economic Cooperation and Development (OECD) standard was used in the ZFET assay [53]. Initially, one zebrafish embryo per well system at 0 HPF were subjected to diluted EPS-ENS samples (200 µL) in 96-well microplates (Corning® 96 Well Clear Polystyrene Microplate, Corning, NY, USA) at seven different concentrations ranging from 0.01–10 mg/mL. The EPS-ENS and untreated samples were tested with a total of 24 replicate of embryos per exposure group slight above minimum standard [54,55]. Successful treated embryos were incubated at room temperature (25–28 °C) for 5 days. The cumulative mortality and developmental malformations of embryos and larvae were observed and determined every 24 h from 0–120 HPF as previously suggested by Parenti et al., [56]. The results indicate that mortality, hatching rate, heart rate, morphological malformation or teratogenic defects were observed using THUNDER Imager 3D Live Cell & 3D Cell Culture & 3D Assay, Leica Microsystems GmbH, Wetzlar, Germany. Heartbeat (1 min) and lethal endpoints (no heartbeat and clear coagulation) were assessed as according to Taufek et al., [11]. Anomalous development includes yolk sac oedema, curved body, non-hatched, bent tail and pericardial oedema. The principle of toxicity (LC_{50}) values for EPS-ENS derivative >1 mg/mL are considered relatively harmless, 0.1–1 mg/mL are considered practically non-toxic, 0.01–0.1 mg/mL are considered slightly toxic, 0.001–0.01 mg/mL are considered moderately toxic, 0.0001–0.001 mg/mL are considered highly toxic and <0.0001 mg/mL are considered super toxic. Video S1: EPS-ENS Untreated vs. 0.01, 0.5 and 1 mg/mL treatments was supplied as supplementary data.

4.5. Statistical Analysis

ZFET response graphs and the lethal concentration at 50% (LC_{50}) of treated samples toward zebrafish embryos were produced by using GraphPad Prism version 9.0 (GraphPad Software, Inc., 2365 Northside Dr. Suite 560, San Diego, CA, USA). Heart rate response was presented as mean ± standard error of mean (S.E.M) from three different animals. One-way analysis of variance (ANOVA) was used to carry out the significant differences with a post hoc test via Dunnett's Multiple Comparison. The significant difference was

considered at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between the means of treated group as compared to embryos in embryo medium.

5. Conclusions

In conclusion, this represents the first study to report on the use of zebrafish embryo toxicity (ZFET) assay on bioreactor-grown European medicinal mushroom *G. applanatum* EPS and ENS extracts. EPS (LC_{50}: 1.41 mg/mL) was harmless while ENS (LC_{50}: 0.87 mg/mL) are practically non-toxic. The ZFET assay offers a fast, affordable, robust, and efficient early development approach to evaluating extracts from medicinal fungi for future use in aquaculture [57]. Findings will inform and guide future "foodomics" research spanning molecular toxicology and linked proteomics and metablomics as it relates to targeting specific transcripts for biosensor development that will support end-to-end monitoring of value chain, including using of machine learning for mitigating food waste and improving "circularlity" for the food sector. Specifically, this study provides new knowledge that will inform efficacy for future immune-priming and disease mitigation innovation for global food security.

Supplementary Materials: The following are available online at https://www.mdpi.com/1422-0067/22/4/1675/s1, Video S1: EPS-ENS Untreated vs. 0.01, 0.5 and 1 mg/mL treatments.

Author Contributions: Conceptualization, W.A.A.Q.I.W.-M. and N.R.; methodology, W.A.A.Q.I.W.-M. and N.R.; software, W.A.A.Q.I.W.-M. and N.R.; validation, Z.I. and A.A.J.; formal analysis, W.A.A.Q.I.W.-M.; investigation, W.A.A.Q.I.W.-M.; resources, N.R.; data curation, W.A.A.Q.I.W.-M. and N.R.; writing—original draft preparation, W.A.A.Q.I.W.-M. and N.R.; writing—review and editing, Z.I. and A.A.J.; visualization, W.A.A.Q.I.W.-M.; supervision, W.A.A.Q.I.W.-M.; project administration, W.A.A.Q.I.W.-M. and N.R.; funding acquisition, W.A.A.Q.I.W.-M. and N.R. All authors have read and agreed to the published version of the manuscript.

Funding: We want to thank Universiti Malaya under the Research University Grant- Faculty Program GPF084A-2020 and MOHE- Top 100 (IIRG)-IISS: IIRG003A-2020IISS awarded to Wan-Mohtar. The work was also funded by H2020 MSCA RISE ICHTHYS project [number 872217/19] awarded to Neil Rowan.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Organization for Economic Cooperation and Development (OECD) No. 236; Fish Embryo Acute Toxicity (FET) Test (OECD, 2013) under compliance of IACUC UPM.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Acknowledgments: We would like to thank Anita Klaus for European *G. applanatum* strain BGS6Ap isolated by from the Republic of Serbia in the wild region of Mount Kosmaj. Sincere gratitude to Danio Assay Team, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor for technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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Future Foods 3 (2021) 100018

Contents lists available at ScienceDirect

Future Foods

journal homepage: www.elsevier.com/locate/fufo

Investigations on the use of exopolysaccharide derived from mycelial extract of *Ganoderma lucidum* as functional feed ingredient for aquaculture-farmed red hybrid Tilapia (*Oreochromis* sp.)

Wan Abd Al Qadr Imad Wan-Mohtar[a,c], Norhidayah Mohd Taufek[b], Jaganath Puvanen Thiran[b], Janathu Ferdaus Pajaru Rahman[b], Grema Yerima[b], Kumeera Subramaniam[b], Neil Rowan[c,d,*]

[a] *Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia*
[b] *AquaNutri Biotech Research Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia*
[c] *Bioscience Research Institute, Athlone Institute of Technology, Ireland*
[d] *Empower Eco Sustainablity HUB, Lough Boora, Co. Offaly, Ireland*

ARTICLE INFO

Keywords:
Ganoderma lucidum
Exopolysaccharides
Red hybrid Tilapia
Growth performance
Sustainable aquaculture
Functional Food

ABSTRACT

There is growing interest in medicinal fungi for promoting immunomodulation benefits in humans and animals. In this study, exopolysaccharide (EPS) from *Ganoderma lucidum* was used in feed (EFF) on production of farmed-red hybrid Tilapia performance for sustainable aquaculture. The feeding trial was conducted for a period of 42 days. Tilapia weight gain (WG) was significantly the highest (18.75 ± 0.03 g/fish) using 3 g/kg of EFF compared to control (0 g/kg EFF; 12.4 g/fish). Fish fed with 3 g/kg of EFF were shown to be healthiest as attested by measured organosomatic indices that achieved an hepatosomatic index (HSI) of 2.22% and condition factor (CF) of 1.46% compared to control (1.97% HSI; 1.60 CF). Antioxidant activity of Glutathione S-transferase (GST) exhibited significantly lower activities in 1 and 2 g/kg of EFF while increasing catalase (CAT) activity was detected in higher EFF (2-3 g/kg of EPS). Muscle compositions were not affected by the EFF feeding regimes. In contrast, EFF feeding produced significantly higher haemoglobin (7.43 g/dl), haematocrit (37.5%), red blood cell (2.69 10^6/mm^3), and white blood cell (176.7 10^6/mm^3) compared to control group. In summary, EFF was beneficial in promoting growth, stimulating antioxidant enzymes, and health status of red hybrid Tilapia, which highlights the potential for future use in intensive sustainable aquaculture practices.

1. Introduction

Aquaculture is a rapidly growing food sector that has been characterised by considerable investment in many parts of the world including Malaysia (O'Neill et al., 2019; Tahar et al., 2018a; Tahar et al., 2018b; Ruiz-Salmón et al., 2020). It has been identified as a sustainable solution for addressing problems of depleting natural wild fish stock globally (O'Neill et al., 2020). Rowan and Galanaksi (2020) have also described the important role of developing intensive aquaculture practices for transitioning beyond the global economic recession, which has been caused by ongoing COVID-19 pandemic. Over the past four decades, a significant increase in fish supply has been achieved through the intensity of aquaculture production that has led to a stressful environment for the fish cultured in captivity (FAO, 2018; Naughton et al., 2020). Operational flux, such as the imposition of stressful environmental growth conditions in aquaculture, may lead to elevated susceptibility to diseases where administration of immunomodulatory therapeutics in feed has been reported previously to alleviate onset or severity including positive impact on fish gut microbiome (Carballo et al., 2019).

The utilisation of antibiotics in managing diseases is a concern in the aquaculture industry due to the increased risk of developing antimicrobial resistance in animal production (Naughton et al., 2020). A growing interest in organic farming has warranted the use of natural alternatives, such as medicinal plants and fungi as a feed supplement, as a way to reduce the adverse side effect of chemical residue from antibiotics (Baba et al., 2015; Wan-Mohtar, 2021). These prebiotic and probiotic ingredients have been reported to improve immunity as well as a growth promoter in fish and shrimp without compromising their health and also sustainable for the environment (Mohan et al., 2019a; Rufchaei et al., 2019). Probiotics are important feed supplements in aquaculture due to their capacity to enhance health and to prevent disease (Carballo et al., 2019). Carballo and co-workers (2019) recently reported that such ingredients are typically non-digestable molecules such as yeast-derived beta-glucans, which selectively modulate the intestinal microbiome by

* Corresponding author at: Bioscience Research Institute, Athlone Institute of Technology, Ireland.
E-mail address: nrowan@ait.ie (N. Rowan).

https://doi.org/10.1016/j.fufo.2021.100018
Received 13 October 2020; Received in revised form 2 February 2021; Accepted 4 February 2021

promoting indigenous microbial populations and commensurately prevention of unwanted pathogen proliferation. In spite of demonstrating the potential benefits of using prebiotics to improve animal health, their use in aquaculture remains limited and their mode of mechanistic action is far from understood (Dimitrogou et al., 2011; Akhter et al., 2015; Morrison et al., 2016; Ruiz-Salmón et al., 2020; Ruiz-Salmón et al., 2021)

Use of medicinal mushrooms has been previously reported to provide health benefits in both animals and humans through immunomodulatory function (Smith et al., 2002; Rowan et al., 2003; Sullivan et al., 2006; Masterson et al., 2020; Murphy et al. 2020a; Murphy et al., 2020b; Galanakis et al., 2020). *Ganoderma lucidum* is one such medicinal mushroom known for its valuable properties to treat many diseases (Cao et al., 2018). Regarded as a 'marvellous herb', *G. lucidum* is widely used in many countries, particularly in East Asia due to its therapeutic effects (Kozarski et al., 2019; Wan-Mohtar et al., 2020). It contains functional metabolites that possess antifungal, antibacterial and anti-inflammatory properties (Rowan et al., 2003; Cao et al., 2018; Wan-Mohtar et al., 2017). The bioactive metabolites, particularly polysaccharide extracted from *G. lucidum,* have demonstrated immunomodulatory and anticancer properties (Meng et al., 2014). However, the low yield of fungal polysaccharides extracted from fruiting bodies and mycelium of *G. lucidum* is appreciated as a technological limitation: consequently, it has prompted advance research in the area of fungal exopolysaccharide (EPS) through submerged liquid fermentation (Supramani et al., 2019b). Technically, the production of EPS is viewed to be less-harsh during the extraction process, hence reduce product degradation and resulted in cost-effectiveness (Yuan et al., 2012).

Tilapia production is projected to increase from 58% in 2016 to 62% in 2030 (FAO, 2018) In Malaysia, red hybrid tilapia (*Oreochromis* sp.) is considered as the top commercial species besides catfish (DOF, 2018). However, there are many constraints in developing sustainable, yet intensive culture of tilapia. One of the top challenges is the disease threat that could results in economic loss. Several bacteria disease such as Streptococcosis, Columnaris and Haemorrhagic septicaemia are amongst common infections of tilapia culture in Malaysia. Viral infection such as Tilapia Lake Virus (TiLV) is currently a significant threat to global tilapia production and major outbreak has also been reported in Malaysia. The interaction of pathogenic bacteria and TiLV was reported to be synergistic, which resulted in strengthening of both pathogens and increasing severity of the disease (Amal et al., 2018; Elumalai et al., 2019; Jansen et al., 2019). According to USDA (2019) there is no vaccine available for the treatment of TiLV and appropriate alternative mitigation and biosecurity measures are needed to address this challenge. Hence, to reduce the challenges in the stressful event during intensive tilapia culture, introducing feed additive containing immunostimulant properties would be an advantage to boost their immunity. Therefore, this constitutes the first study to examine the potential of EPS fish-feed (EFF) extracted from the mycelium of *G. lucidum* on growth, antioxidant responses, body composition and haematological indices of Malaysian red hybrid tilapia.

2. Materials and method

2.1. Production of fungal exopolysaccharides from the mycelium of Ganoderma lucidum

Fungal biomass was pre-grown in a 5-L stirred-tank bioreactor system (STR, Fermetec, Malaysia) under controlled submerged fermentation strictly according to Usuldin et al. (2019) using *Ganoderma lucidum* strain QRS 5120 (Supramani et al., 2019a) obtained from Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Universiti Malaya. The fermented *G. lucidum* mycelial biomass was subjected to cold 95% (v/v) ethanolic extraction at 1:4 ratio and left for 48 hours at 4 °C for macromolecules precipitation. The method of extraction was conducted according to Hassan et al. (2019) whereas the centrifuged (9000 rpm, 15 min) macromolecule precipitates was freeze-dried to produce crude EPS.

Table 1
Formulation and nutritional composition of exopolysaccharide fish feed (EFF) originated from the mycelium of *Ganoderma lucidum.*

Ingredients (g/kg)	Control [5]	1 g/kg	2 g/kg	3 g/kg
Fish meal (g)	300.0	300.0	300.0	300.0
Corn meal (g)	193.9	193.5	193.1	192.7
Rice bran meal (g)	199.3	198.9	198.6	198.2
Soybean meal (g)	236.8	236.6	236.3	236.1
Exopolysaccharide (g)	0.0	1.0	2.0	3.0
Lysine (g)	10.0	10.0	10.0	10.0
Methionine (g)	5.0	5.0	5.0	5.0
Vitamin premix(g) [1]	1.0	1.0	1.0	1.0
Mineral premix(g) [2]	3.0	3.0	3.0	3.0
Dicalcium phosphate (g)	10.0	10.0	10.0	10.0
Fish Oil (ml)	40.0	40.0	40.0	40.0
Total (g)	1000	1000	1000	1000
Nutritional composition				
Protein (%)	37.86	36.30	37.02	35.19
Lipid (%)	6.29	6.29	4.84	6.67
Fiber (%)	1.63	1.70	1.92	1.76
Dry matter (%)	85.65	85.69	85.73	85.77
Ash (%)	12.00	12.86	12.40	12.45
Carbohydrate (%) [3]	42.22	42.85	43.82	43.93
Gross energy (kJ/g) [4]	18.98	18.72	18.49	18.80

[1] The vitamin premix supplied the following per 100g diet: vitamin A, 500IU; vitamin D_3, 100IU; vitamin E, 0.75 mg; vitamin K, 0.02 mg; vitamin B_1, 1.0 mg; vitamin B_2, 0.5 mg; vitamin B_3, 0.3 mg; vitamin B_6, 0.02 mg; folic acid, 0.1 mg; biotin, 0.235 mg; pantothenic acid, 1.0 mg; inositol, 2.5 mg.

[2] The mineral premix supplied the following per kg diet: selenium, 0.2 mg; iron, 8.0 mg; manganase, 1.0 mg; zinc, 8.0 mg; copper, 0.15 mg; potassium chloride, 0.4 mg; magnesium oxide, 0.6 mg; sodium bicarbonate, 1.5 mg; iodine, 1.0 mg; cobalt, 0.25 mg. [3]Carbohydrate = 100 – (% protein+ % lipid + % fiber + % ash).

[4] Gross energy was calculated as 23.9, 39.8, and 17.6 kJ/g for protein, lipid, and NFE, respectively.

[5] Feed: Control = 0.0 g/kg of EPS,

2.2. Preparation of experimental diets

Four experimental EFF were formulated to be isoenergetic composition (~19g/kJ) containing 0 (control), supplementation of 1(1E), 2(2E) and 3(3E) g EPS per kg diet (Table 1). The supplements (in dried powder form) were uniformly mixed with dry ingredients and pelleted with extruder machine (KCM, Y132M-A). The pelleted feed was then oven-dried at 60^0C and later stored in 4^0C until further use.

2.3. Experimental animal and setup

Red hybrid Tilapia fingerlings were purchased from a local fish farm in Sungai Buloh, Malaysia and acclimated for two weeks prior to the feeding trial. Fish were hand-fed with commercial diet, *ad libitum*, twice per day during the acclimatisation period. One hundred twenty fish (16.19 ± 0.24 g) were stocked into each of the eight tanks (100 L) with a density of 15 fish per treatment in duplicate cultures as justified by Wan-Mohtar et al., (2020). Feeding was performed twice daily at a 3% body weight ratio throughout the 42-days of feeding trial and feeding intake was recorded.

Each tank was equipped with filtration and constant aeration with dechlorinated water throughout the study. Water temperature were maintained at 28–29°C, pH at 6.0–6.8, DO above 4.0, ammonia <0.80 mg/L, and nitrate <1.9 mg/L. Approximately 25-30% of water was changed every two days, and uneaten feed and faeces were collected every day by siphoning at the bottom of the tanks to maintain water quality.

All procedures of fish rearing and handling were performed according to the Universiti Malaya Animal Care and Use Policy (UM ACUP)

(IACUC-002), which was established according to the Malaysian Animal Act 1953 (Act 647).

2.4. Growth performance and organosomatic indices

After termination of the feeding trial at day 42, fish were starved for 24 hours before weighing. All the fish were anaesthetised with clove oil and weigh individually to determine the growth performance including body weight gain (BWG), specific growth rate (SGR), feed conversion ratio (FCR), survival rate (SR) and feed intake (FI). Organosomatic indices including visceromatic index (VSI), hepatosomatic index (HSI) and condition factor (CF) were also measured post-feeding trial.

$$BWG\ (g/fish) = final\ weight\ (g) - initial\ weight\ (g)$$

$$FI\ (g/fish) = total\ feed\ for\ the\ feeding\ period\ (g)/number\ of\ fish\ alive$$

$$SGR\ (\%) = \left[(Ln\ final\ weight) - (Ln\ initial\ weight)/days\ of\ trial\right] \times 100$$

$$FCR = feed\ intake\ (g)/weight\ gain\ (g)$$

$$SR\ (\%) = final\ number\ of\ fish\ alive/initial\ number\ of\ fish\ alive \times 100$$

$$CF = [body\ weight\ (g)/\ (body\ length\ (cm)] \times 100$$

$$VSI\ (\%) = \left[viscera\ weight\ (g)/\ whole\ body\ weight\ (g)\right] \times 100$$

$$HSI\ (\%) = \left[wet\ weight\ of\ liver\ (g)/\ final\ weight\ of\ fish\ (g)\right] \times 100$$

2.5. Sample collection

2.5.1. Liver

Five fishes were randomly selected in each tank and sacrificed for its liver sample collection. Data for the body weight and liver weight were recorded individually according to each sacrificed fish. A total of 1.0 g of each liver was homogenized in 10ml buffer containing 25mM sodium phosphate buffer (pH 7.4), 0.1 mM protease inhibitor, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 0.1 phenylthiourea (PTU). The sample was then homogenized using a laboratory homogeniser at 150 rpm for 2 minutes until a distributed uniform liquid was achieved. Later, the homogenates were centrifuged at 100,000 rpm (Beckman Coulter Optima L-100K Ultracentrifuge), at 4°C for 30 minutes and the supernatants were separated and later stored at -80°C for further analysis.

2.5.2. Blood and serum

Blood samples were collected randomly from five fishes in each tank through the caudal puncture. Approximately 1 ml of the blood sample was collected by using a 1-ml syringe with a needle size of 22G 1 $\frac{1}{2}$ inch from each fish.. For each individual fish, 0.5 ml of the blood was transferred to a heparinised vacutainer tube for haematological analysis (complete blood counts) and the remaining 0.5 ml was transferred into a non-heparinised serum vacutainer tube and later centrifuged at 5000 x g at 4°C for 10 min, according to the method described by Zhang et al. (2019). The five blood samples from each replicate were pooled together. The isolated serum was then stored at -20°C for further analysis.

2.6. Analytical methods

2.6.1. Proximate composition

Proximate composition of the feed ingredients (Table 2), experimental diets (Table 1) and fish muscle were determined according to the Standard Method of AOAC (2005). Crude protein was analysed by using the semi-auto Kjeldahl system (N × 6.25) (Kjeltec semi auto-analyzer). Crude lipid was determined by Soxhlet extraction method using petroleum ether as the solvent. Moisture was measured by drying the sample in an oven at 105 °C overnight and later continue in a muffle furnace for ash content at 600 °C for 6 h. The crude fibre was analyzed after acid and alkali digestion. Carbohydrate or NFE data were obtained through calculation [100 - (% crude protein + % crude lipid + % crude fiber + % crude ash)] and gross energy was determined by using the physiological values (crude protein x 23.9) + (crude lipid x 39.8) + (carbohydrate x 17.6) according to Schulz et al. (2005).

2.6.2. Antioxidant enzyme assay

Glutathione S-transferase (GST) was analysed spectrophotometrically by measuring the activity towards 1-Chloro-2,4-dinitrobenzene (CDNB) as a substrate at 340 nm as described by the method from Habig et al. (1974). Catalase activity (CAT) was measured according to the decline in H_2O_2 at 240 nm according to Claiborne (1985). Both antioxidant enzyme activities were expressed in nmol/min/mL.

2.6.3. Haematology and serum protein analysis

Complete blood counts (CBC) which include red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), haematocrit (HCT) and RBC indices namely: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were measured using automatic haematology analyser (Sysmex XN, Germany). Serum protein analysis was measured by using Advia 2400 Chemistry System (Siemens Healthineers, Germany)

2.7. Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to compare the means between individual treatments using SPSS software version 23 (SPSS Inc., Chicago IL. USA). Data were expressed as mean ± standard deviation (SD) values and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Growth performance, feed efficiency and organosomatic indices

At the end of the 6-week trial, the effect of different EFF on growth performance and feed utilisation in red hybrid Tilapia is shown in Table 2. Precise observation of fish morphological measured bi-weekly was characterized in Figure 1. The groups treated with 2E (2 g/kg EFF) and 3E (3 g/kg EFF) had significantly higher WG and SGR compared with those fed with control although no significant difference was observed in 1E (1 g/kg EFF) group. In addition, FCR, FI and SR did not affect all the experimental diets ($P > 0.05$) throughout the feeding trial. Based on the organosomatic indices results, HSI and VSI were significantly affected by the supplementation of EFF. Groups with either high EFF (2 and 3 g/kg) and control shows significantly higher HSI values compared to 1E (1 g/kg EFF). In contrast, significantly lower VSI value was recorded in 3E (3 g/kg EFF) fed fish compared to other diets ($P < 0.05$). However, the supplementation of EFF did not influence the CF of red hybrid Tilapia during the experimental period.

3.2. Oxidative stress of experimental fish

The GST activity in liver of red hybrid Tilapia was found to be significantly higher ($P < 0.05$) in 3 g/kg of EFF (38.17 nmol/min/ml)

Table 2
Growth performance of the red hybrid Tilapia treated with EPS fish feed (EFF) originated from the mycelium of *Ganoderma lucidum.*

Performance [1]	Experimental EPS feed					
Control [5]	1 g/kg	2 g/kg	3 g/kg	F	*P*-value	
Initial Weight (g/fish)	17.45 ± 1.48	16.20 ± 0.14	15.40 ± 1.41	15.65 ± 1.91	0.85	0.54
Final weight (g/fish)	28.36 ± 0.22^{a}	32.75 ± 0.35^{ab}	33.75 ± 2.21^{ab}	34.40 ±1.88^{b}	3.58	0.13
BWG(g/fish) [4]	11.87 ± 0.64^{a}	16.55 ± 0.21^{ab}	18.37 ± 0.80^{b}	18.75 ± 0.03^{b}	12.00	0.02
SGR (%/day)	1.16 ± 0.22^{a}	1.60 ± 0.11^{ab}	1.88 ± 0.06^{b}	1.89 ± 0.16^{b}	7.10	0.05
FCR	2.00 ± 0.05	1.72 ± 0.23	1.32 ± 0.06	1.31 ± 0.14	3.19	0.14
FI (g/fish)	23.78 ± 0.60	26.25 ± 0.98	24.24 ± 2.13	24.61 ± 2.60	1.25	0.12
SR (%)	100 ± 0.00	93.33 ± 6.70	100 ± 0.00	96.67 ± 3.33	3.27	0.25
Organosomatic indices [2]						
HSI (%)	1.97 ± 0.14^{b}	1.42 ± 0.51^{a}	2.27 ± 0.40^{c}	2.22 ± 0.45^{c}	5.67	0.06
VSI (%)	11.02 ± 0.99^{b}	9.75 ± 1.06^{ab}	10.71 ± 1.18^{ab}	9.42 ± 0.87^{a}	3.23	0.04
CF (%)	1.60 ± 0.19	1.51 ± 0.08	1.48 ± 0.04	1.46 ± 0.05	1.80	0.18

[3]Mean values in the same row with different superscripts are significantly different (P < 0.05)
[1] The results represent mean± SD of 15 fishes per tank (*14 fishes for control) on growth performance
[2] The results represent mean± SD of 3 fishes per tank on organosomatic indices
[4] BWG: Body Weight gain;, SGR: Specific growth rate, FCR: Feed conversion ratio, SR: Survival rate, FI: feed intake, HSI: Hepatosomatic index, VSI: Viscerosomatic index, CF: Condition factor
[5] Feed: Control = 0.0 g/kg of EPS

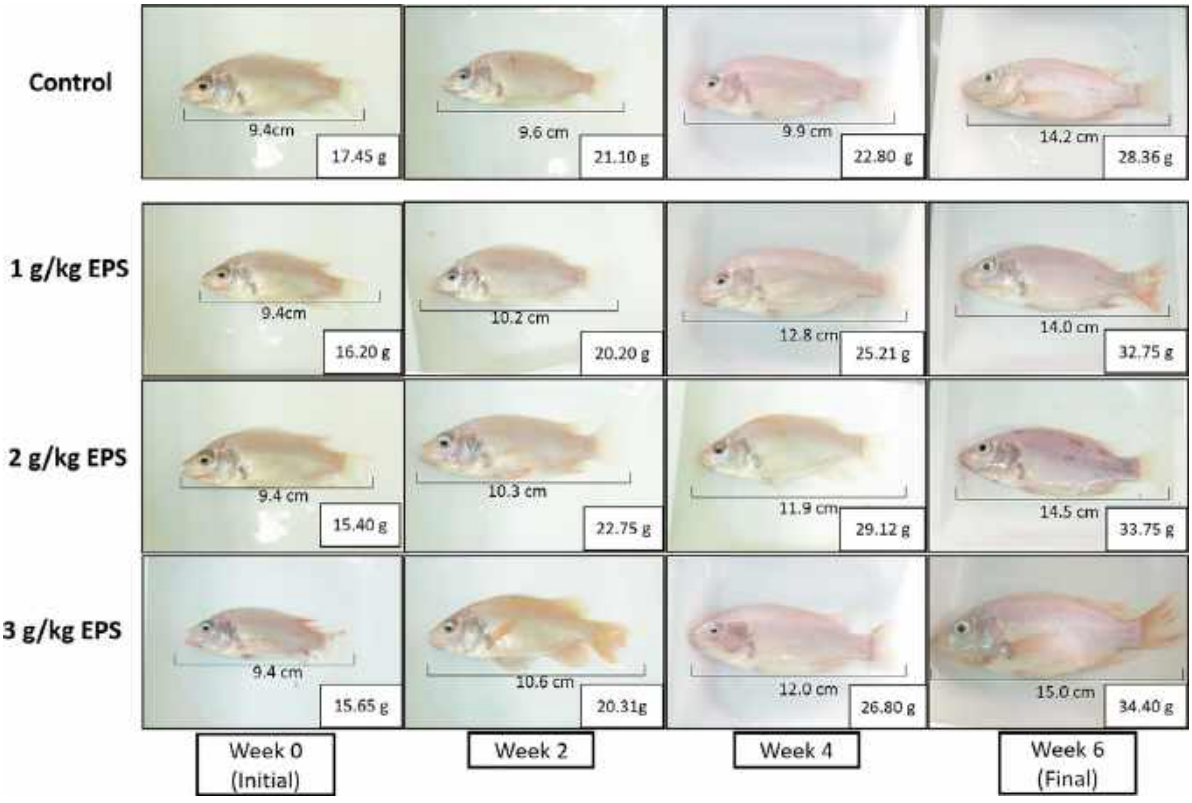

Fig. 1. Bi-weekly weight gain of red hybrid Tilapia (*Oreochromis* sp.) treated with different concentration of EPS fish feed (EFF) originated from the mycelium of *Ganoderma lucidum* in 6-week feeding trial.

(Table 3). On the other hand, no significant difference was detected in the activity of dietary 1E (1 g/kg EFF) and 2E (2 g/kg EFF) compared to control ($P > 0.05$). Similar trend was observed in 3E (3 g/kg EFF) with significantly higher CAT activity (230.51 nmol/min/ml) compared to other diets ($P < 0.05$).

3.3. Fish muscle nutritional composition

The fish muscle composition treated with EFF after 42-day of feeding trial is presented in Table 4. The data revealed that only fibre was significantly affected by EFF treatments. The fibre content of fish treated with 2 g/kg EFF is comparatively higher ($P < 0.05$) compared to other diets while control was recorded as the lowest group. Other nutrient components including crude protein, crude lipid, dry matter, ash and gross energy did not have a significant effect amongst the treatment ($P > 0.05$). In contrast, 1E (1 g/kg EFF) diet had a significantly lower level ($P < 0.05$) of carbohydrate compared to other EFF. Control EFF did not differ significantly ($P > 0.05$) compared to 2E (2 g/kg EFF) and 3E (3 g/kg EFF) in terms of carbohydrate muscle content.

3.4. Haematological indices

Haemoglobin (Hb) and RBC of red hybrid Tilapia were affected by experimental EFF and elevated significantly ($P < 0.05$) in a linear fashion

Table 3
Antioxidant responses of red hybrid Tilapia (*Oreochromis* sp.) treated with different concentration of EPS fish feed (EFF) originated from the mycelium of *Ganoderma lucidum* after 42 days.

Antioxidant enzymes (nmol/min/mg protein) [4]	Experimental EPS feed					
Control [3]	1 g/kg	2 g/kg	3 g/kg	F	*P*-value	
Glutathione-S-transferase	20.77 ± 0.99^{a}	11.18 ± 1.14^{a}	16.91 ± 1.03^{a}	38.17 ± 6.85^{b}	21.52	0.006
Catalase	117.81 ± 7.24^{a}	176.73 ± 7.99^{b}	225.90 ± 2.17^{c}	230.51 ± 13.04^{c}	75.99	0.001

[1]The results represent mean± SD of 5 fishes per tank
[2]Mean values in the same row with different superscripts are significantly different ($P < 0.05$)
[3] Feed: Control = 0.0 g/kg of EPS
[4] Specific activity of enzyme measured in nmol/min/mg protein.

Table 4
Fish muscle composition of red hybrid Tilapia (*Oreochromis* sp.) treated with different concentration of EPS fish feed (EFF) originated from the mycelium of *Ganoderma lucidum* after 42 days.

Components (%)	Experimental fish feed [3,4]					
Control [5]	1 g/kg	2 g/kg	3 g/kg	F	*P*-value	
Crude protein	81.87 ± 1.73	88.70 ± 2.01	83.80 ± 3.13	83.21 ± 1.47	3.75	0.12
Crude lipid	1.50 ± 0.09	1.68 ± 0.11	1.37 ± 0.25	1.45 ± 0.33	0.72	0.59
Crude fiber	0.03 ± 0.00^{a}	0.20 ± 0.00^{bc}	0.22 ± 0.08^{c}	0.06 ± 0.06^{ab}	7.18	0.04
Dry matter (wet weight basis)	24.20 ± 0.14	24.40 ± 0.11	25.40 ± 0.20	25.70 ± 0.12	0.42	0.53
Ash	6.60 ± 0.41	6.93 ± 0.63	5.90 ± 0.89	6.55 ± 1.06	0.79	0.56
Carbohydrate [1]	10.00 ± 1.32	2.50 ± 2.38	8.70 ± 3.66	8.73 ± 2.89	3.14	0.15
Gross energy (kJ/g) [2]	21.93 ± 0.22	22.52 ± 0.26	22.11 ± 0.20	22.00 ± 0.03	3.73	0.12

[1] Carbohydrate % = 100 – (crude protein % + crude lipid % + ash % + crude fiber %).
[2] Gross energy was calculated as 23.9 kJ/g, 39.8 kJ/g, and 17.6 kJ/g for protein, lipid, and carbohydrate, respectively
[3] The result represents the mean ± SD of 3 fishes per tank
[4] Mean values in the same row with different superscripts are significantly different ($P < 0.05$).
[5] Feed: Control = 0.0 g/kg of EPS

Table 5
Haematological parameters of red hybrid Tilapia (*Oreochromis* sp.) treated with different concentration of EPS fish feed (EFF) originated from the mycelium of *Ganoderma lucidum* after 42 days.

Parameters[3]	Experimental diets[1,2]					
Control[4]	1 g/kg	2 g/kg	3 g/kg	F	*P*-value	
HGB (g/dl)	5.75 ± 0.70^{a}	6.98 ± 0.10^{b}	7.08 ± 0.51^{b}	7.43 ± 0.22^{b}	10.18	0.001
HCT (%)	30.00 ± 3.46^{a}	36.00 ± 1.41^{b}	39.00 ± 2.31^{b}	37.50 ± 0.58^{b}	12.66	0.001
RBC (10^{6}/mm^{3})	2.04 ± 0.29^{a}	2.47 ± 0.14^{b}	2.57 ± 0.24^{b}	2.69 ± 0.02^{b}	7.92	0.004
WBC (10^{3}/mm^{3})	133.88 ± 27.84	155.20 ± 6.64	161.63 ± 36.34	176.65 ± 21.99	1.92	0.18
MCV (fl)	148.00 ± 3.46^{bc}	145.75 ± 4.35^{b}	152.50 ± 4.04^{c}	139.50 ± 1.29^{a}	9.57	0.002
MCH (pg)	28.20 ± 0.68	28.28 ± 0.94	27.83 ± 4.52	27.65 ± 0.62	0.07	0.98
MCHC (g/L)	190.75 ± 2.22	193.75 ± 4.35	182.25 ± 24.84	198.50 ± 2.89	1.15	0.37
Serum total protein (g/dl)	3.20 ± 0.12	2.75 ± 0.40	3.23 ± 0.10	3.03 ± 0.26	3.01	0.07

[1] The result represents mean ± SD of 5 fishes per tank
[2] Mean values in the same row with different superscripts are significantly different ($p< 0.05$).
[3] HGB = Haemoglobin; HCT = hematocrit; RBC = red blood cell; WBC = white blood cell; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration.
[4] Feed: Control = 0.0 g/kg of EPS

as the supplementation level EFF increased from 0 (control) to 3 g/kg (Table 5). Similarly, a significant higher level of HCT was observed in experimental diets compared to control. White blood cell (WBC) count, MCH and MCHC values of fish did not vary significantly ($P > 0.05$) among different treatments, albeit discernible improvement was seen in WBC as the EFF increased. Nevertheless, the MCV was significantly lower in high EFF (3E, 3 g/kg) than other diets ($P < 0.05$). For biochemical serum total protein, no significant differences were observed between all treatments.

4. Discussion

The emergence of bacterial antibiotic-resistance due to their excessive use has stimulated research and development to elucidate alternative eco-friendly compounds from fungal products. In addition to been considered inexpensive, safe and biodegradable, use of fungal polysaccharides (such as β-glucans) are unlikely to influence bacterial resistance to front-line antibiotics that is reached a crisis-point, according to the World Health Organisation (Reverter et al., 2014; WHO, 2019). One of the most promising medicinal mushroom, *G. lucidum,* has demonstrated potential for human and animal use by way of improved immune function: however, these bioactives have not been developed or tested as potentially essential aquaculture-based protein for farmed fish. Moreover, recent adjacent findings have shown that exopolysacharides, extracted from the same *Ganoderma lucidum,* is considered as non-toxic, as demonstrated by use zebrafish embryo toxicity (ZFET) assay: thus, inferring it's safe use in aquaculture feed (Taufek et al., 2020).

Aligned with previous findings, the results of this preliminary study revealed that administration of EPS from the mycelium of *G. lucidum* improved growth performance and feed efficiency, antioxidant activities and haematological indices in red hybrid Tilapia. To the best of our knowledge, this constitutes the first study to examine the performance

of EPS from the fermented mycelium of *G. lucidum* as a feed supplement for red hybrid Tilapia. Previous published studies were limited to using mature fruiting bodies of *G. lucidum* for animal feed trials including fish (*Ctenopharyngodon Idella*) (Chithra et al., 2016), prawn (Mohan et al., 2016) and poultry (Bederska-Łojewska et al., 2017; Khan et al., 2019). Previous study by Chitra et al, (2016) indicated that administration of 1.0g/kg of *Ganoderma lucidum* polysaccharides regulate better performance of grass carp. Hence in the current study, the concentration of EPS was observed at 1, 2 and 3 g/kg diet. This study demonstrated that the supplementation of 2 to 3 g/kg EPS in red hybrid Tilapia diet provide better growth when observed in terms of WG, SGR and organosomatic indices of HSI. Moreover, improved FCR values were also observed in these supplemented diets when compared to control, which demonstrates the ability of EPS to promote fish growth.

Antioxidant activity has been regarded as an essential measure to detect fish well-being. Glutathione S-transferase (GST) plays a critical role in protecting cells in terms of toxicity of xenobiotics electrophiles and from a foreign contaminants perspective (Dasari et al., 2018). In aquatic animals, significant alteration of GST activities will reflect the metabolic disturbance and cell damage in specific organs (Mohan et al., 2015; Raji et al., 2018). The increasing level of GST in any material implies that it may contain a compound that could trigger the biotransformation of xenobiotics. Our findings suggest that incorporation of EPS did not differ significantly in between control, 1E and 2E although 1E diet exhibited numerically lower activities than other treatments.

As the first line of defence mechanism, catalase (CAT) is correlated with an increasing concentration of H_2O_2 (Wilhelm Filho et al., 2005). In contrast to GST, high CAT activity was observed in EPS supplemented groups when compared to control, whereby group fed with 3E shows significantly higher activity than the others. This might corresponds to the previous study by Wu et al. (2016) which reported the presence of ß-1,3/1,6 glucan polysaccharides in cell wall of *G.lucidum*. The CAT activity trend is in accordance to the growth performance analysis where high SGR and WG, as well as numerically low FCR value, was observed in the group fed with 3E, which might be attributed to high metabolic rate (Ahmed et al., 2017; Taufek et al., 2016). Ahmed et al. (2017) investigated the effect of hot water extract (HWE) of the waste mushroom-stalk (*Pleurotus sp.*) on CAT activity of tilapia. They found a significant increase in CAT activity in 0.5% and 1% of HWE when compared to control, which is contributed by the presence of EPS. Oxidative stress response findings reported in this current study will inform new innovation in terms of fish supplement. Consequently, there are pressing opportunities that include potential immediate issues in fish farming from farm to fork, that focues on reduced environmental stress conditions.

Fish fillet is the edible part of the fish that is expected to contain high nutritional value. Consequently, the nutritional composition of fish fillet was addressed in this study with a particular role on investigating the influence of EPS supplementation on key determinants. Specifically, high protein content was observed in all EPS-supplemented fish. This contrasts to previous studies that focused on red tilapia body composition where fish were fed a diet supplemented by 10% with mushroom stalk meal (*Pleurotus sajor-caju*) (Abdul Rahman Jabir et al., 2012). Other researchers have also reported that the lipid content of fish fed the experimental diets (1.37 – 1.68 %) were higher than Nile tilapia that were fed supplemented diets containing red algae (*Gracilaria arcuata*) (Younis et al., 2018).

Haematological parameters are essential tools in understanding the physiological status of experimental fish. Overall, the HGB, HCT, and RBC had significant effects on all EPS group as compared to control, whereby the highest level was detected in 3E group. It was observed that increasing RBC is correlated with the increasing trend of HGB and HCT due to their synergistic linkage (Audu et al., 2014). Also, EPS-treated groups produced higher WBC despite no significant differences were found amongst treatments, which corresponds to the trend of RBC as immunity indicator. A similar trend was observed in the study made by Chitsaz et al. (2018) during supplementation of a medicinal mushrooms extract *(Lentinula edodes,)* in great sturgeon juvenile (*Huso huso*). Increasing in RBC and WBC values were also reported by Mohan et al. (2019b) in shrimp fed with exopolysaccharides. Red blood cell indices are mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) which used to diagnose the level of anaemia (Şahan, Duman, 2010). The maturation of red blood cells determined the value of MCV and MCH. The unstable trend was observed in MCV of fish in all treatments suggesting the presence of immature red blood cells which have a larger cell volume and lower haemoglobin (Carvalho, Fernandes, 2006).

The findings of this present study correlate well with the trend towards the use of prebiotics and probiotics in feed supplements for aquaculture production (Akhter et al., 2015; Carballo et al., 2019; Dimitroglou et al., 2011; Galankis et al., 2020). This also reflects a trend towards the use of natural processes for disease mitigation in aquaculture and adjacent food production processes for intensive sustainability (Rowan, 2019). Use of probiotics and prebiotics are normally non-digestable molecules that selectively modulate the intestinal micobiome by promoting indigenous microbial populations and by preventing pathogen proliferation (Carballo et al., 2019). Recent findings have demonstrated that certain carbohydrates (exopoly and oligosaccharides) as promising prebiotics for animal and human health including beta-glucans that are fermented in the gut resulting in enhanced production of short chain fatty acids (SCFA) such as formate, acetate, proponate and butyrate (Carballo et al., 2019; Mahdhi et al., 2020; Morrison, Preston, 2016). These secondary metabolites also modulate production and release of cytokines, chemokines and leukocyte recruitment for health benefits (Morrison, Preston, 2016). Use of beta-glucans derived from medicinal fungi, as described in this present study, shows potential as an immune-priming feed supplement for aquaculture production. However, future studies focusing on interaction of beta-glucans derived from medicinal fungi with gut microbiota in fish is merited.

Conclusion

EPS fish-feed formulation, based on the mycelium of *Ganoderma lucidum,* exerted potent benefits for red hybrid Tilapia growth performance and improved antioxidant activity of GST and CAT enzymes as well as haematological indices of RBC, HCT and HGB. Nutritional muscle composition was minorly affected by EFF supplementation. Findings from this preliminary study highlight the potential benefits of using EFF in fish feed supplementation to enhance the health and immunity of Tilapia and other farmed fish along with adjacent scale-up studies for commercial aquaculture deployment. This timely study has significant implications for the smart intensification of aquaculture through innovative steps in feed formulation, which will provide vital future food for meeting the pressing dietary needs of our growing global populations. Developing sustaining and disruptive technologies in the the food sector will also enable pivoting of society beyond recession created by ongoing COVID-19 (Rowan and Galanakis, 2020) through provision of safe and nutritious supply chain that includes health benefits (Galanakis et al., 2021). Transnational modelling of key socio-economic drivers informing sustainable development of marine industry, enabled by life cycle assessment of products and services (Ruiz-Salmón et al., 2020), will inform policy that includes Europe's Green New Deal (Rowan and Galanakis, 2020). This transdisciplinary study has significant implications for the smart sustainable intensification of aquaculture through innovative steps in feed formulation, which will provide future safe nutritious food to meet pressing needs for our growing global populations.

Declaration of Competing Interest

The authors declare that they have no competing conflict of interest and the authors alone are responsible for the content and writing of the paper.

Acknowledgement

We want to thank the Ministry of Higher Education Malaysia on RU Geran-Fakulti Program Universiti Malaya (GPF084A-2020) to Dr Wan-Mohtar and for the Fundamental Research Grant Scheme (FRGS FP066-2018) awarded to Dr Norhidayah. The work was also sponsored in part by collaborative H2020 MSCA RISE ICHTHYS project [number 872217/19] Prof Neil Rowan.

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In vivo toxicity of bioreactor-grown biomass and exopolysaccharides from Malaysian tiger milk mushroom mycelium for potential future health applications

Siti Rokhiyah Ahmad Usuldin[1,2], Wan Abd Al Qadr Imad Wan-Mohtar[2,3,6,8], Zul Ilham[3,4], Adi Ainurzaman Jamaludin[4], Nur Raihan Abdullah[5] & Neil Rowan[6,7,8]✉

Natural mycelial biomass (MB) and exopolysaccharides (EPS) of Malaysian tiger milk mushroom *Lignosus rhinocerus* are considered high-end components due to their high commercial potential value in drug discovery. This study aims to evaluate the toxicity of the mushroom extracts' generated in a bioreactor using the zebrafish embryo toxicity (ZFET) model assay as a new therapy for treating asthma. Both MB and EPS extracts, at concentrations 0.16–10 mg/mL, were tested for ZFET and early development effects on Zebrafish Embryos (ZE) during 24–120 h post-fertilisation (HPF). Findings revealed that MB was deemed safe with an LC_{50} of 0.77 mg/mL; the EPS were non-toxic (LC_{50} of 0.41 mg/mL). Neither MB nor EPS delayed hatching nor teratogenic defects in the treated ZE at a 2.5 mg/mL dose. There were no significant changes in the ZE heart rate after treatments with MB (130 beats/min) and EPS (140 beats/min), compared to that of normal ZE (120–180 beats/min). Mixing both natural compounds MB and EPS did not affect toxicity using ZFET testing; thus, intimating their safe future use as therapeutic interventions. This represents the first study to have used the ZFET assay on MB and EPS extracts of *L. rhinocerus* for future health applications.

Mushrooms are filamentous fungi that have been used worldwide since prehistoric times[1,2], and medically since at least 3000 BCE[3,4]. Mushrooms are heterotrophic organisms and have become increasingly important in research and industry[5]; food, medicines, cosmetics, detergents, and biofuels are examples of high-value products manufactured from fungi[6,7]. Furthermore, mushroom-derived extracts are becoming increasingly popular due to their potential usage in a wide range of vital health applications[8,9]. A focused interest in biorefining these products, such as via Green New Deal innovations from food waste streams, reflects a stronger emphasis on expanding 'circularity' and bioeconomy[10–14]. Mushrooms are classified in the kingdom of Fungi and have many active constituents, including, but possibly not limited to, polysaccharides, polysaccharide peptides, proteins, terpenoids, and nucleotides[15]. The most studied and used medicinally active ingredient in mushrooms is β-glucan. Previous research has revealed that β-glucans have broad metabolic and gastro-intestinal effects, including modulating the gut microbiome, altering lipid and glucose metabolism, and reducing cholesterol; thus, leading to the use of β-glucan as potential therapy for treating metabolic syndrome, obesity and diet regulation, gastrointestinal conditions, and to reduce the risk of cardiovascular and diabetes[16,17].

The bioactive extracts derived from mushrooms can modulate the immune response affecting hematopoietic stem cells, lymphocytes, macrophages, T cells, dendritic cells, and natural killer cells. Murphy et al.[18] reviewed

[1]Agro-Biotechnology Institute, Malaysia (ABI), National Institutes of Biotechnology Malaysia (NIMB), c/o HQ MARDI, 43400 Serdang, Selangor, Malaysia. [2]Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia. [3]Bioresources and Bioprocessing Research Group, Institute of Biological Sciences, Faculty of Sciences, Universiti Malaya, 50603 Kuala Lumpur, Malaysia. [4]Environmental Science and Management Program, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia. [5]Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia. [6]Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland. [7]Empower Eco Innovation Hub, Boora, Co. Offaly, Ireland. [8]These authors contributed equally: Wan Abd Al Qadr Imad Wan-Mohtar and Neil Rowan. ✉email: nrowan@ait.ie

over 200 patents that highlighted the therapeutic potential of β-glucans; this is evidenced by the fact that two glucans were licensed in Japan as immune-adjuvant therapy for treating cancer. Moreover, the pronounced immune-modulatory effects of β-glucans[18,19] promoted their usage as adjuvant agents for treating cancers, immune-mediated conditions, rhinitis, respiratory infections, and to enhance wound healing[16,20]. However, further clinical testing and translation of β-glucans face significant challenges due to differences in source and extraction procedures[16]. We recently identified the active ingredients of *Lignosus rhinocerus* using 2D NMR analyses and reported on the antioxidative potential of (1,3)-β-D-glucan as an essential constituent[21]. However, many other compounds extracted from medicinal mushrooms have yet to be named, which are often referred to by gel chromatography fraction[15]; thus, highlighting the need to conduct and report on their safety.

The tiger milk mushroom, *Lignosus rhinocerus*, belongs to the Basidiomycota section of the Polyporaceae family and is classified as a filamentous fungus[22,23]. *L. rhinocerus* was grown in submerged-liquid fermentation (SLF) using a laboratory-scale stirred-tank bioreactor to achieve bulk cultivation and commensurate production of polysaccharides[21]. When compared to solid-state fermentation (SSF), SLF has several advantages, including limited space requirements, ease of scale-up, reliable and reproducible processing, ease of monitoring, and versatility[24]. Artificially cultivated *L. rhinocerus* is also an excellent replacement in developing therapeutic items. For example, exopolysaccharides (EPS) isolated from mushroom mycelial biomass (MB) have pharmacological properties as immunomodulatory, anti-inflammatory, antibacterial, antiviral, and antioxidant activities[25]. Chen et al.[26] discovered that *L. rhinocerotis* mycelium grown in SLF does not cause mutagenicity or genotoxicity. The US Food and Drug Administration (FDA) standards, on the other hand, demand substantial proof of no hazard for commercial usage[26].

Asthma affects 300 million individuals worldwide and is caused by a complex combination of inherited and environmental variables[23]. Allergic asthma is a long-term condition characterised by wheezing, shortness of breath, chest tightness, and coughing. In Malaysia, indigenous peoples have long used *L. rhinocerus* to treat asthma, while the majority of today's asthma medications are made up of steroids and other anti-inflammatory drugs[23]. Recent studies have reported on the efficacy of using EPS from medicinal mushrooms to ameliorate pro- and anti-inflammatory responses using ex vivo and in vivo infection models with therapeutic potential[16,18,27]. Aqueous extracts of *L. rhinocerotis* were reported to help reduce asthma-related variables in an asthma model[28]. In addition, a previous toxicity study indicated that feeding 1000 mg/kg of *L. rhinocerus* extract to rats had no detrimental consequences, hence it was considered safe[29]. As a result, more effective asthma treatment is required using *L. rhinocerus* as a helpful adjuvant or alternative to currently available asthma medications.

Zebrafish embryos have been extensively studied and documented as a reliable and popular model for developmental biology, toxicity, and, more recently, drug discovery[30]. Zebrafish may be readily bred, reared, and maintained in the laboratory[31]. Zebrafish embryos develop quickly, where they are fully developed five days after conception. Light microscopy can straightforwardly examine morphological structures and internal organs, such as the brain, eyes, heart, liver, and kidney due to the embryo's transparency. Dyes can be used to measure organ-specific and overall developmental toxicity visually or quantitatively. Due to its small size, a single Zebrafish embryo can be maintained in low fluid volumes for the first six days of development, including microtiter plates. The permeability of zebrafish embryos is prominent; for example, small chemicals added to fish water permeate the embryos, simplifying drug administration and assay processing[32]. Chemical screening can be completed after a few days due to the embryo's rapid development. The zebrafish is therefore a unique vertebrate model for high-throughput chemical screening, beneficial for pre-clinical drug discovery and toxicity assessment[33,34].

A recent publication evaluating the toxicity of biomass-EPS comparable medicinal mushroom mycelial extracts revealed that the zebrafish embryo toxicity (ZFET) assay could be deployed as a safety screening approach before pre-clinical testing according to national and international standards[35]. Compared to human cell lines, research on the ZFET model is quick, resilient, efficient, and cost-effective for early development investigations; it also represents relevant genetic structure and equivalent critical organs and tissues[36,37]. Thus, this study aims to determine the toxicity of mushroom extracts using ZFET before they are developed and potentially commercialised as a new therapeutic intervention. To the best of our knowledge, there have been no toxicity studies using the ZFET model describing the use of MB and EPS of *L. rhinocerus* generated in the bioreactor.

As a result, this constitutes the second study to determine the toxicity levels of extracted MB and EPS from *L. rhinocerus* using a ZFET model to ensure product safety throughout the pre-commercialisation phase. Notably, the present rare *L. rhinocerus* strain ABI (Agro-Biotechnology Institute Malaysia) was successfully isolated and identified from a tropical forest near Lata Iskandar, Pahang, Malaysia[21]; however, limited information has been published on its therapeutic potential. This study reports on the use of ZFET assay on bioreactor-grown Malaysian medicinal mushroom *L. rhinocerus* MB and EPS extracts. This study explicitly addresses $LC_{50,}$ embryonic hatching delays, teratogenic defect, and heart rate response with clear microscopic images. Furthermore, these findings also support the possibility of future pre-clinical trials involving the safe use of MB and EPS for prospective health applications in respiratory diseases.

Results

Zebrafish embryo survival rate after MB and EPS exposure. The survival rate of zebrafish embryos following MB and EPS exposure was studied between 0 and 120 h at MB and EPS extract concentrations of 0.16–10 mg/mL. The study period included larvae as the zebrafish embryos hatch typically 48 to 72 h post-fertilisation (HPF). The survival rate of untreated embryos, between 0 and 120 HPF, was 100% (Fig. 1a). At 48 HPF, the survival percentage for embryos treated with MB fell to 85% and 60% at > 5 mg/mL and 10 mg/mL, respectively. At 72 HPF, the survival rate declined to 80%, 65%, and 10% at < 2.5 mg/mL, 5 mg/mL, and > 10 mg/mL, respectively. At concentrations > 1.25 mg/mL, the survival rate at 96 HPF was 20%, and after 120 HPF; it was observed that no embryos survived at concentrations > 1.25 mg/mL (Fig. 1a). The survival rate of embryos

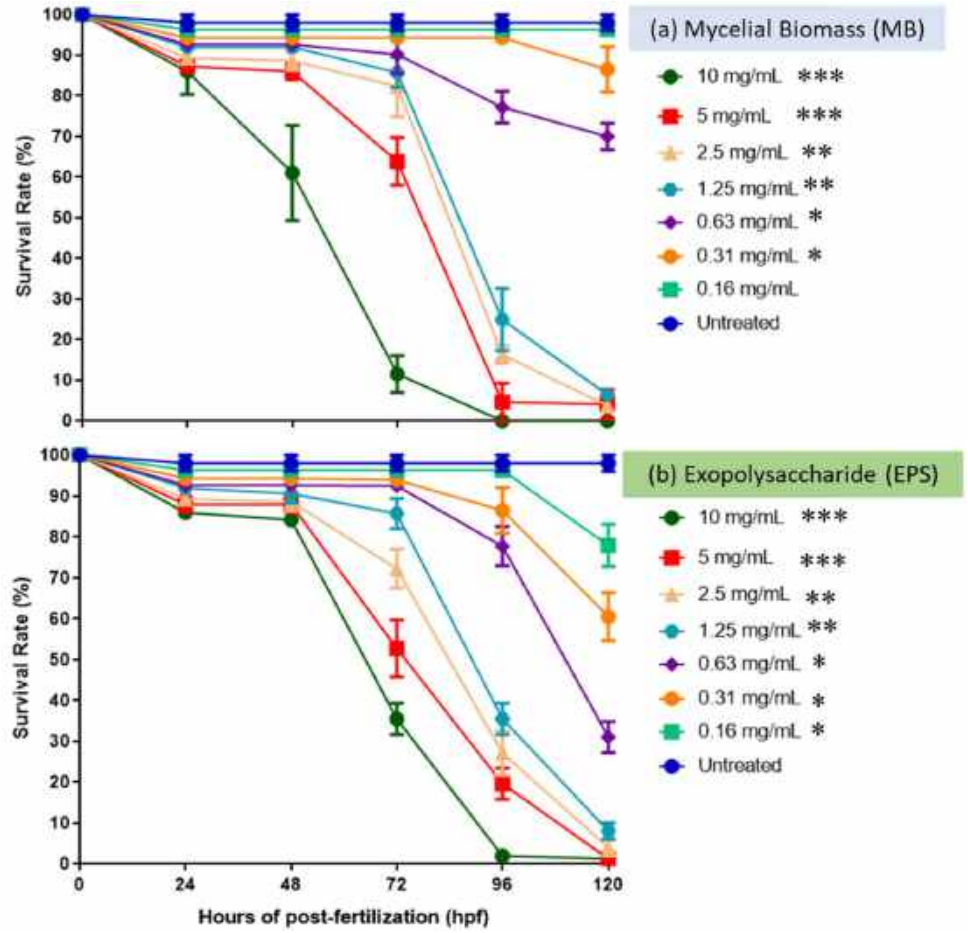


Figure 1. The performance of Tiger milk mushroom, *Lignosus rhinocerus* strain ABI (**a**) MB and (**b**) EPS extract at concentrations of 0.16–10 mg/mL on the survival rate of zebrafish embryos at 0–120 h. Symbols: $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$. No embryos survived for both samples at concentration tested > 5.0 mg/mL after 96 h-post-fertilization (HPF).

(prior to hatching) and larvae (post-hatching) treated with EPS (0.110 mg/mL) during the five days is shown in Fig. 1b. Between 0 and 120 h of HPF, untreated embryos (control) exhibited a 100% survival rate. After 72 h of HPF exposure, the survival rate declined to 90%, 85%, and 50% at a concentration of 0.63 mg/mL, 1.25 mg/mL, and 5 mg/mL, respectively. At 96 HPF, the survival rate declined to 75% at concentrations < 0.63 mg/mL and 30% at concentrations > 1.25 mg/mL. At 120 HPF, survival rates at concentrations 0.63 mg/mL declined to 30%, while survival rates at concentrations > 1.25 mg/mL were 0%, with no surviving embryos. Overall, the results suggest that MB and EPS extracts delay hatching at doses < 1.25 mg/mL.

Zebrafish embryos mortality after MB and EPS exposure. Overall, MB and EPS extracts had dose- and time-dependent fatal effects. Figure 2 shows a high survival rate (90%) of zebrafish embryos at concentrations of MB and EPS extracts < 1.25 mg/mL. Both MB and EPS extracts had a low survival rate at high concentrations (> 1.25 mg/mL), and none survived after 96 HPF. As a result, the fatal concentration for 50% (LC_{50} value) of zebrafish embryos exposed to MB was 0.77 mg/mL, while the LC_{50} value of the EPS extract was 0.41 mg/mL.

Zebrafish embryos hatching after MB and EPS exposure. Based on the embryo observations, increasing the mushroom extract concentrations can decrease the percentage hatchability. Figure 3a illustrates the hatching rate of zebrafish embryos treated with MB and EPS (both at 0.1610 mg/mL) at 0–120 HPF. No significant changes in the hatching rate were found when the zebrafish embryos were treated with MB extract at a 0.63 mg/mL concentration. However, at 48 HPF, the rate declined to 80% at concentrations > 1.25 mg/mL. At 72 HPF, the hatching rate was lowered to 65% at 5 mg/mL. Further reduction was observed (25% hatching rate) when treated with 10 mg/mL MB, implying a high death rate after 72 HPF. The hatching rate of EPS did not alter significantly after the treatment with 0.63 mg/mL MB. Less than 85% of the embryos hatched were observed after a 48-h treatment with EPS at > 1.25 mg/mL. However, due to a significant mortality rate at 72 HPF, zebrafish larvae treated with EPS at 10 mg/mL doses had the lowest hatching rate (30%).

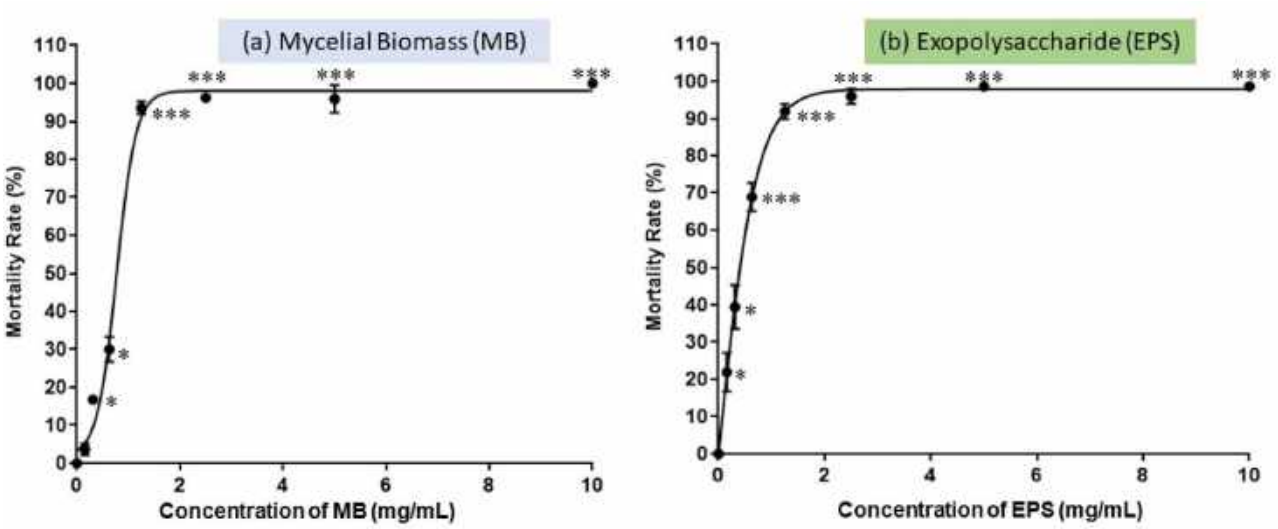


Figure 2. Effect of Tiger milk mushroom, *Lignosus rhinocerus* strain ABI (**a**) MB extract at concentrations of 0.16–10 mg/mL and (**b**) EPS at concentrations of 0.01–10 mg/mL on zebrafish embryos mortality rate after 120 HPF. Symbols: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. The LC_{50} value for MB extract was 0.77 mg/mL while LC_{50} value for EPS extract was 0.41 mg/mL.

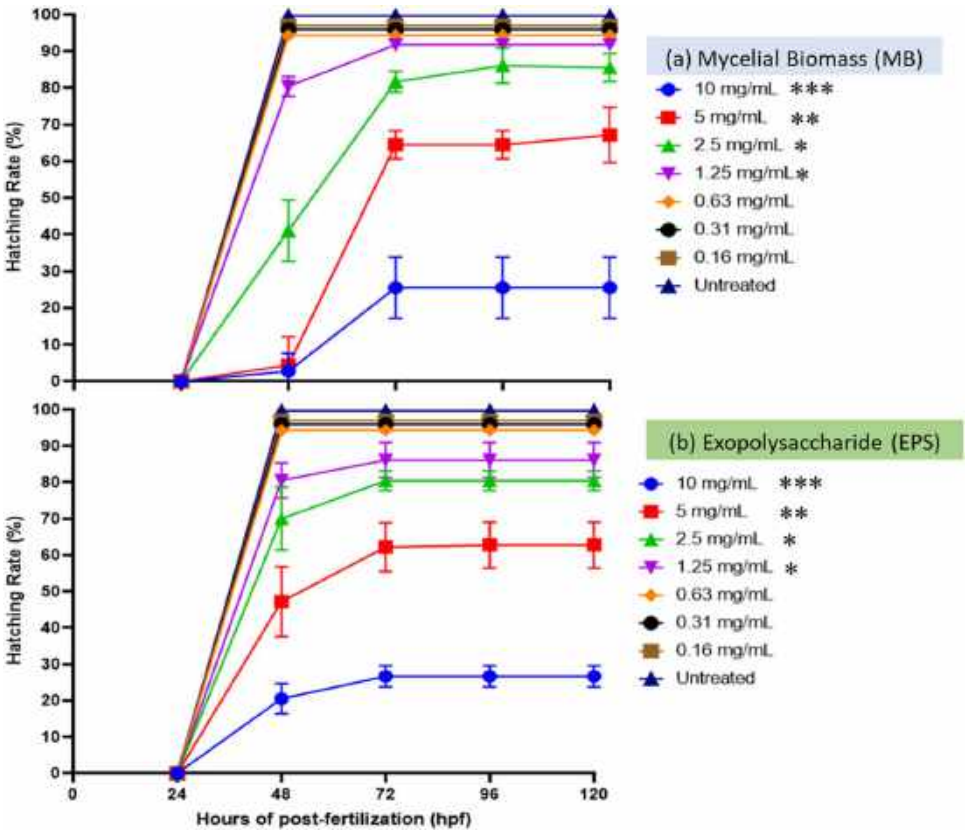


Figure 3. Hatching rate of zebrafish embryos at 0 to 120 HPF with Tiger milk mushroom *Lignosus rhinocerus* strain ABI, with MB and EPS extract at concentrations of 0.16–10 mg/mL. Symbols: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. (**a**) For MB, a low hatching rate (< 25%) was observed at concentration 10.0 mg/mL due to a high mortality rate. Meanwhile, (**b**) for EPS, a low hatching rate (< 30%) was observed at concentrations of 10 mg/mL due to a high mortality rate. High hatching rate was observed at concentrations > 1.25 mg/mL (> 80%).

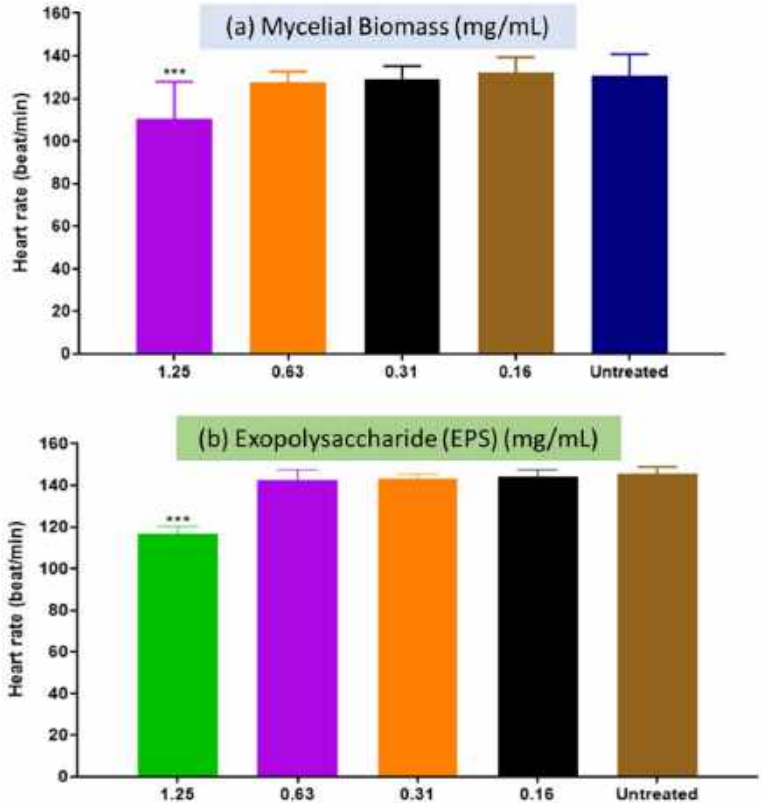


Figure 4. Effect of Tiger milk mushroom *Lignosus rhinocerus* strain ABI, with MB and EPS extract at concentrations of 0.16–10 mg/mL on the heart rate of zebrafish embryos at 96 HPF. ***$p < 0.05$ significantly different from the untreated group (zebrafish embryos in medium only). *$P < 0.05$ significantly different from the untreated group (zebrafish embryos in medium only). For (**a**) MB, no data at concentrations > 2.5 mg/mL due to embryo death. Meanwhile, (**b**) for EPS, no data at concentrations > 2.5 mg/mL was recorded due to embryo death.

Zebrafish embryos heart rate after MB and EPS exposure. During the development of many model species, including zebrafish, the heart is the major functioning organ[38]. Previous research has shown that the average heart rate of zebrafish embryos is 120–180 bpm, which is much closer to that of humans[39]. As shown in Fig. 4, the heart rates of zebrafish larvae at 96 HPF (4 days) for both the MB (Fig. 4a) and EPS (Fig. 4b) treatments were 130 and 140 bpm, respectively. Both extracts exhibited no significant difference in the heart rate of zebrafish larvae at 96 HPF at lower concentrations (relative to higher doses in Fig. 3), ranging between 0.161.25 mg/mL for MB and 0.161.25 mg/mL for EPS. The heart rate of zebrafish larvae at these concentrations was not determined because both MB and EPS extracts at 2.5, 5, and 10 mg/mL demonstrated very little to no survival at 96 HPF.

Morphology of the larvae and zebrafish embryos after MB and EPS exposure. Potential morphological abnormalities in embryos and larvae were measured from 0 to 120 HPF. There was no apparent teratogenic effect on embryos and larvae after 120 h of exposure to MB and EPS at 0.63 mg/mL and 1.25 mg/mL, respectively (Fig. 5). These findings infer that MB and EPS have no teratogenic effects on zebrafish embryo development prior to- and post-hatching. The unaffected development of zebrafish embryos and larvae after exposure to 0.63 mg/mL MB and 1.25 mg/mL EPS are shown in Fig. 6 and Fig. 7; however, numerous defects were observed when the concentration of MB and EPS increased to 10 mg/mL (Fig. 8 and Fig. 9). Coagulated embryos observed between 24 HPF (segmentation) and 48 HPF (pharyngula), along with the loss of yolk sac preventing hatching, were the most common abnormalities reported using MB treatments. Moreover, EPS-treated zebrafish hatched at 72 HPF, where tail deformity and damaged blood cells were observed after 120 HPF, with various defects included missing fins, guts, and melanophores.

Discussion

Lignosus rhinocerus is well-known for its therapeutic values, particularly as potential treatment of respiratory diseases. Previous reports have highlighted that the sclerotia, mycelium, and exopolysaccharides of *L. rhinocerus* contain similar bioactive compounds to β-glucans[21]. Nowadays, β-glucans have gained appeal for several emerging applications, including biopolymers[40] and biomedicines[41]. Notable potential therapeutic properties recently uncovered of mushroom-derived β-glucans include: (a) new or complementary immunotherapies

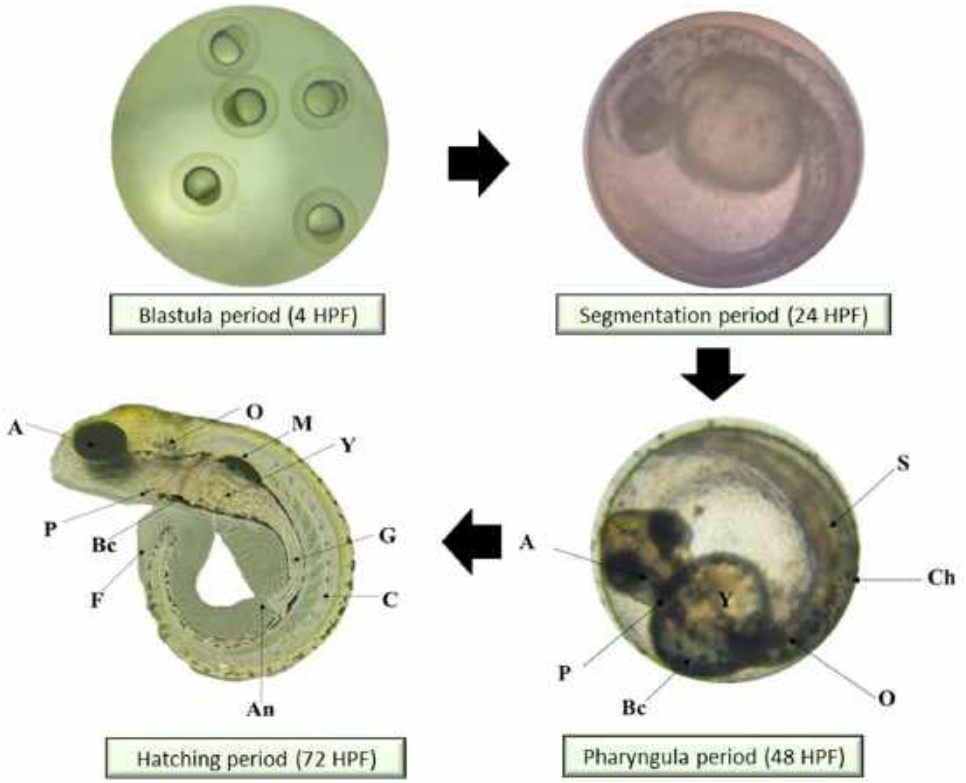


Figure 5. Effect of MB-EPS extracts (0.16–10 mg/mL) of Tiger Milk mushroom *Lignosus rhinocerus* strain ABI showing normal zebrafish embryogenesis at different HPF development. There were four periods depicted as according to Taufek et al.[16]: (**a**) Blastula (4 HPF), (**b**) Segmentation (24 HPF), (**c**) Pharyngula (48 HPF), and (**d**) Hatching (72 HPF). A—eye anlage; An—anus; Bc—blood cells; C—chorda; Ch—chorion; F—fin; G—gut; M—melanophores; O—ear bud; P—pericardium; S—somites; Y—yolk sac. Scale bar = 0.5 mm. The inverted microscope procedure was used to produce the images.

against Coronavirus disease (SARS-CoV-2)[18]; (b) new therapeutic agent for mitigating diseases associated with gastrointestinal mucosal damage, such as peptic ulcers and inflammatory bowel disease[42]; (c) anticancer drugs for lung and breast cancer[43]; and (d) asthmatic treatment[28,44]. However, there is a substantial gap in knowledge surrounding the toxicity (if any) of these mushroom-derived bioactive compounds, particularly on MB-EPS extracts. Therefore, the use of Zebrafish trials could aid product development and implementation. Hence, this work investigated and reported on the acute toxicity of zebrafish embryos post exposure to MB and EPS derived from a rare Malaysian-origin Tiger Milk mushroom *L. rhinocerus* grown in a bioreactor.

The ZFET approach was used to expose fertilised zebrafish embryos to quantities of *L. rhinocerus* extract, MB (0.16–10 mg/mL), and EPS (0.16–10 mg/mL) shown to be non-toxic. Overall, both MB and EPS at 2.5 mg/mL concentrations did not delay embryo hatching and had a > 80% survival rate between 24 and 120 HPF. In addition, there were no significant differences in the embryo heart rate between the MB and EPS concentrations of 1.25 mg/mL. At MB and EPS doses of > 0.63 mg/mL and > 1.25 mg/mL, respectively, teratogenic effects were observed with evident zebrafish embryo defects. The test revealed that MB has a larger LC_{50} value of 0.77 mg/mL than EPS, with a lower LC_{50} value of 0.41 mg/mL. Although both MB and EPS extracts were obtained from *L. rhinocerus* mycelium, the compound composition may differ owing to the fruiting body and mycelial extraction procedures[45,46]. *L. rhinocerus* mycelium and culture broth demonstrated similar or increased bioactivities, including antioxidant capacities, compared to the use of fruiting bodies[45]. Moreover, EPS exhibited a lower LC_{50} value than MB did due to its different mycelial extraction methodology. This is possibly related to MB being directly obtained from dried fungal mycelium, whereas EPS is derived from post series of physicochemical extractions using active fungal mycelia[21,47]. The embryo's ability to burst through the chorion (Fig. 8) and hatch after five days may be limited by morphological defects such as tail deformity. A coagulated embryo and the absence of a heartbeat are both considered deadly.

Certain medicinal mushrooms have also been tested for their toxicity on zebrafish embryos in comparison to *Lignosus* species. Recent research on *Ganoderma lucidum* exposure found that MB did not affect ZE hatching at concentrations ranging from 250 to 5000 g/mL and EPS at 3000 g/mL. Notably, neither MB nor EPS were teratogenic at concentrations < 3000 g/mL[35]. Neither EPS or endopolysaccharide (ENS) concentrations of 1 mg/mL in *G. applanatum* cause embryo hatching delays. They were shown to have an 88% survival rate when tested from 24 to 120 HPF[48]. Consequently, this new ZFET data could be helpful in the identification of potential health risks associated with the MB-EPS consortia. However, more testing is merited to identify the LC_{50} value of MB-EPS extract for large-scale human trials and larger animals before this innovation may be used commercially

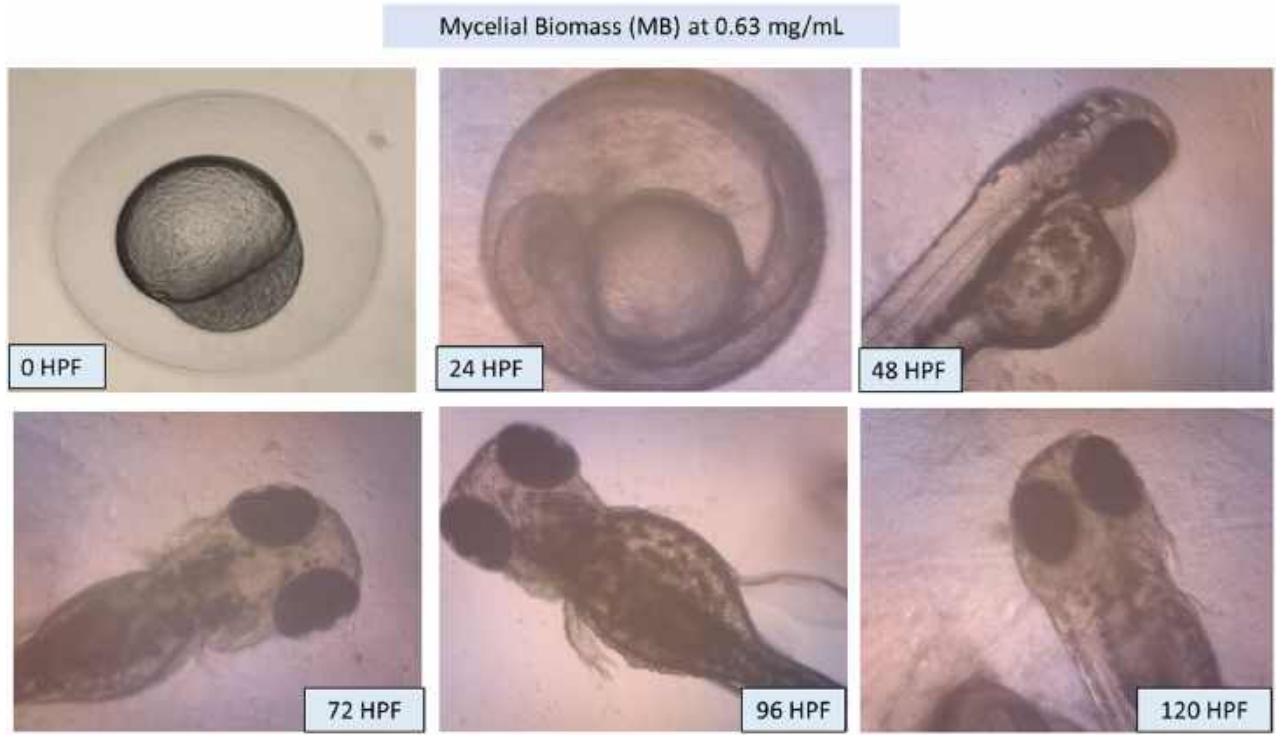


Figure 6. Illustrations of zebrafish embryo and larvae development after treated with Tiger Milk mushroom *Lignosus rhinocerus* strain ABI at EPS concentration of 0.63 mg/mL. Descriptions were captured using an inverted microscope at 100X (0 and 24 HPF) and 40X magnification (48–20 HPF).

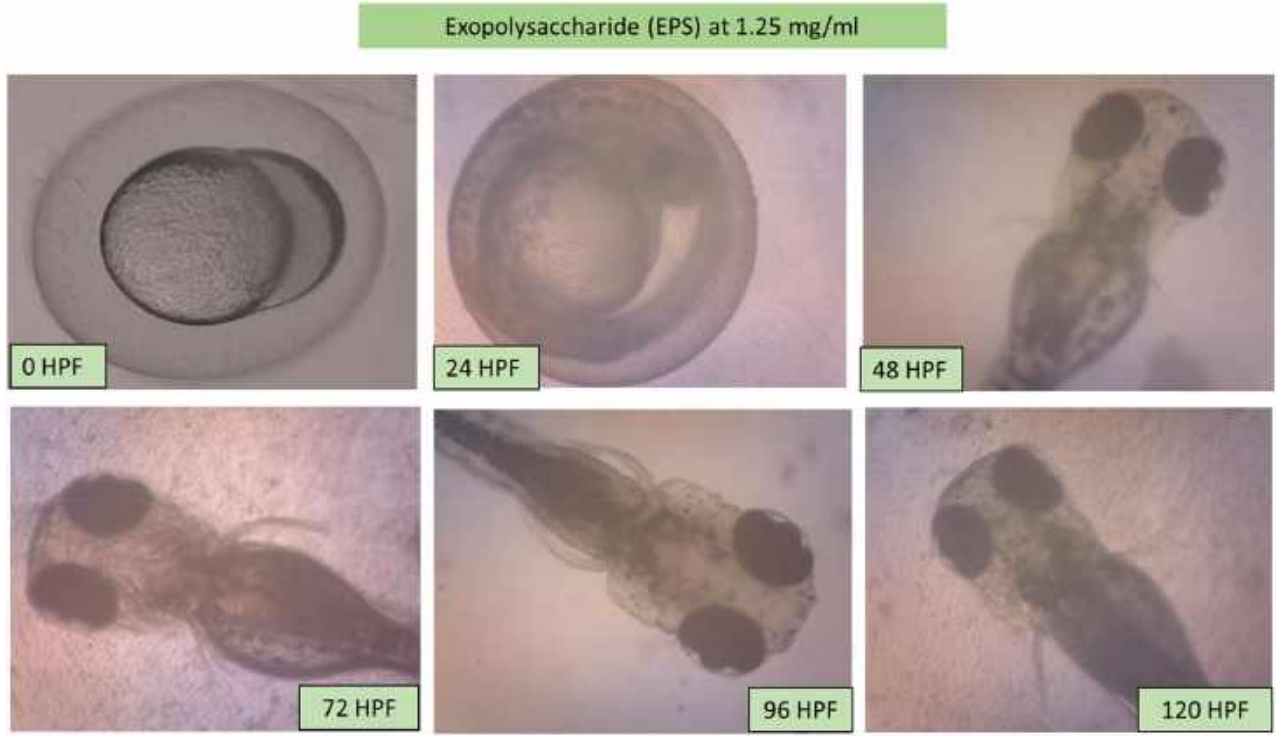


Figure 7. Illustrations of zebrafish embryo and larvae development after treated with Tiger Milk mushroom *Lignosus rhinocerus* strain ABI at EPS concentration of 1.25 mg/mL. Descriptions were captured using an inverted microscope at 100X (0 and 24 HPF) and 40X magnification (48–20 HPF).

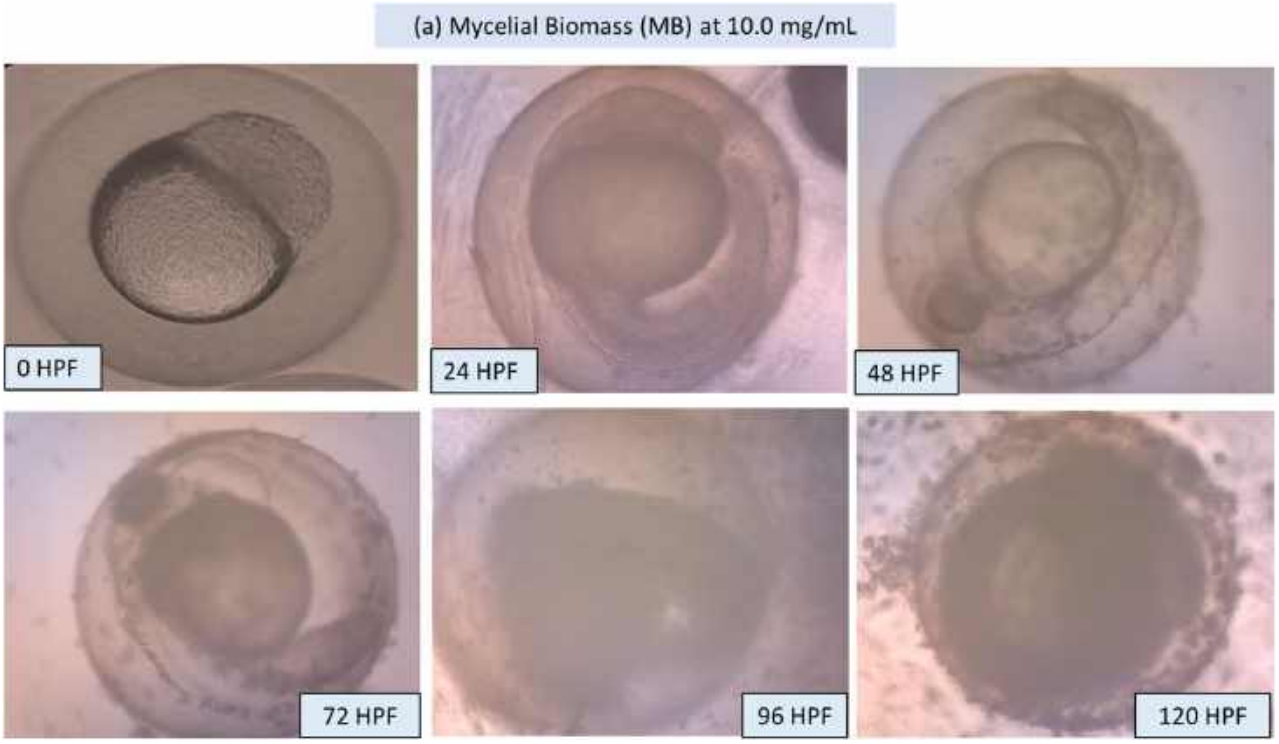


Figure 8. Illustrations of zebrafish embryo and larvae development after treated with Tiger Milk mushroom *Lignosus rhinocerus* strain ABI at high EPS concentration of 10.0 mg/mL. Descriptions were captured using inverted microscope at 100X (0 and 24 HPF) and 40X magnification (48–20 HPF).

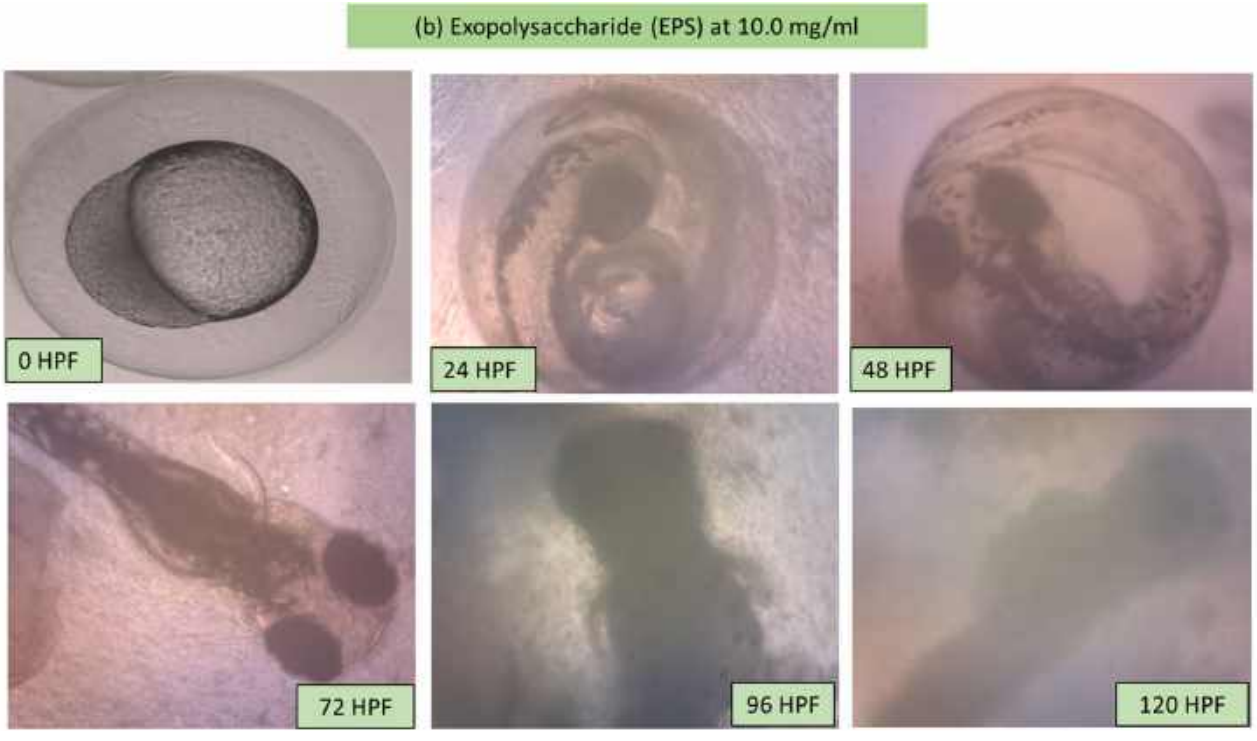


Figure 9. Illustrations of zebrafish embryo and larvae development after treated with Tiger Milk *Lignosus rhinocerus* strain ABI at high EPS concentration of 10.0 mg/mL. Descriptions were captured using an inverted microscope at 100X (0 and 24 HPF) and 40X magnification (48–20 HPF).

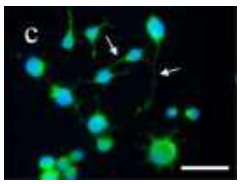
Fungal source	Toxicity model	Image	Non-toxic concentrations (mg/mL)		References
			Mycelial Biomass (MB)	Exopolysaccharide (EPS)	
L. rhinocerus	In vivo—Zebrafish embryos and larvae		0.77	0.41	*Current study*
L. rhinocerotis	In vitro—Cervical cancer cells (Ca Ski, HPV-16)	NA	25	–	[50]
L. rhinocerotis	In vitro—Differentiating mouse neuroblastoma (N2a) cells		1.75–5.93	–	[51]
L. rhinocerotis	In vitro-MTT assay	NA	0.2	–	[45]
L. rhinocerotis	In vivo—Developmental toxicity in pregnant Sprague–Dawley (SD) rats	NA	3.4	–	[52]

Table 1. Similarity with literature for non-toxicity evaluation of mycelium biomass (MB) and exopolysaccharide (EPS) from the rare Tiger milk mushroom *Lignosus* sp. [NA: Not Available].

(e.g. pigs, rabbits, and adult trout). A similar biosafety approach using zebrafish was used to test EPS from the wild-Serbian mushroom *G. applanatum*[49], which exhibited a higher yet safe LC_{50} value (1.41 mg/mL) than that of the current wild-Malaysian *L. rhinocerus* study (0.41 mg/mL). The MB of *L. rhinocerus* demonstrated a harmless biosafety status of bioreactor cultivated *L. rhinocerus* mycelia and EPS products; thus, supporting further pre-commercialisation trials. Usuldin et al.[21] found that MB production (~ 6 g/L: 30 g dry form) from a 5-L bioreactor culture supports high EPS yield, which can be produced in large quantities. When compared to dried polysaccharides, powdered MB is more applicable in the pharmaceutical industry. The latter is notable as 300 mg of dry tuber biomass from the Malaysian *L. rhinocerus* has been reported to potentially improve respiratory health in both in vivo and in vitro models[34].

This study therefore constitutes the first toxicity investigation of *L. rhinocerus* grown in a bioreactor, with the results compared with that of extracts from other *Lignosus* species. Table 1 shows details of four studies assessing the effect of *L. rhinocerus* MB on cervical cancer cells (24 mg/mL)[50], neurite bearing cells (1.75–5.93 mg/mL)[51], MTT assay for normal human cells (200 µg/mL)[45], and developmental toxicity in pregnant Sprague–Dawley (SD) rats (3.4 mg/mL)[52]. Notwithstanding this, there is no published research on the toxicity of EPS. The study results are significant where the Zebrafish 3.0 toxicity model was used to evaluate and assess what was to be non-toxic mycelial biomass (0.77 mg/mL) and EPS (0.41 mg/mL) in *L. rhinocerus* bioreactor samples. This Zebrafish model offered evidence that the use of Malaysian bioactive mycelial biomass and polysaccharides *L. rhinocerus* may be safe as a new therapeutic intervention.

Furthermore, the findings from this research highlight the increasing trend towards the intensive yet sustainable exploitation of bio-based resources from food and marine ecosystems, from the emergence of the bioeconomy[11]. These bio-inspired materials may be refined and scaled up for commercial use through advances in biotechnology, as described here[49]. Notably, this emerging area will be future-proofed through accelerating digitalisation, where metadata outputs will potentially inform food for therapeutics, cosmetics, personal care products, and smart packaging, along with offering putative interventions to help mitigate the Covid-19 disease[10,51,53].

Conclusion

In conclusion, this is the first study on the use of ZFET assay on bioreactor-grown Malaysian medicinal tiger milk mushroom *L. rhinocerus* MB and EPS extracts. MB (LC_{50}: 0.77 mg/mL) was harmless, whereas EPS (LC_{50}: 0.41 mg/mL) are practically non-toxic. The ZFET assay offers a fast, affordable, robust, and efficient early development approach to evaluating extracts from medicinal fungi for future use as asthmatic medication. Specifically, this study provides evidence of the potential of *L. rhinocerus* as an alternative or adjuvant to the current drugs used for the management of respiratory diseases. Additionally, for the early medication development process, zebrafish can be utilised to quickly discover potentially dangerous chemicals and prioritise compounds for additional pre-clinical and clinical testing. The adaptation of conventional instruments in conjunction with new nanotechnology discoveries will help to further increase the use of zebrafish for drug screening.

Methods

Tiger milk mushroom. Wild Malaysian tiger milk mushroom, *L. rhinocerus* strain ABI was isolated from Lata Iskandar, Pahang, Malaysia, from the tropical rainforest (23 °C to 28 °C; 4.1949° N, 101.1923° E)[21]. The sclerotium was cultured on a potato dextrose agar (PDA) plate (Sigma-Aldrich, Dorset, UK) and incubated at 30 °C under dark conditions. The strain was stored and maintained on PDA slants at 4 °C[54].

Culture conditions. The fungal inoculum was prepared according to Wan Mohtar et al.[55] blueprints fungal production plan, including two seed culture stages. The mycelium was cultivated for ten days under dark conditions at an initial pH of 5, 150 rpm, and 30 °C with slight adjustments for the first seed culture. Four mycelial agar squares (1 cm x 1 cm each) were cut from a ten-day-old plate culture and inoculated in a 250 mL Erlenmeyer flask using sterile scalpels (100 mL of medium). The first seed culture was then homogenised for 10 sec with a sterile Waring hand mixer to produce more hyphal tips with uniform mycelium diameters. The homogenised mycelial culture was transferred to a 500 mL shake flask (200 mL medium) as the inoculum for the second seed culture and incubated for 11 d under dark circumstances on an orbital shaker at initial pH 5, 150 rpm, and 30 °C. Unless otherwise stated, the liquid culture medium of seed cultures contained glucose (3% (w/ v), yeast extract (0.1% (w/ v), peptone (0.2% (w/ v), potassium dihydrogen phosphate (KH_2PO_4) (0.046% (w/ v), dipotassium hydrogen phosphate (K_2HPO_4) (0.1% (w/ v), and magnesium sulphate heptahydrate (MgSO4.7H).

High-scale bioreactor fermentation. A stirred-tank (STR) bioreactor was used with a total volume of 5 L (3.5-L working volume) (Sartorius Stedim, Biostat B-plus, Germany). Blueprint of Usuldin et al.[21] was followed; 10% (v/v) of the seed culture was used to inoculate the STR using parameters as follows: temperature (30 °C); pH 5.0; dissolved oxygen (DO) (20–40%); air flow rate 3 L/min; agitation speed (200 rpm). The mycelium was cultured in the bioreactor for 11 d and the resulting mycelial pellets were isolated. The media formulation for the bioreactor used was the same as that for the shake flask, unless otherwise stated.

Mycelial biomass and exopolysaccharide production. The bioreactor's harvested mycelial biomass (MB) was filtered three times with distilled water using a vacuum Buchner funnel filter. The filtered MB was dried at 35 °C in a food dehydrator until it reached a consistent weight[47]. The filtrate was precipitated by adding 95% (v/ v) ethanol at a ratio of 1:4 to the filtrate and left overnight at 4 °C to obtain the EPS. After that, the sample was centrifuged for 15 min at 10,000 rpm. The supernatant was discarded, and the pellet was dried at 35 °C in a food dehydrator until it reached a constant weight.

Sample preparation for the toxicity test. Dried MB and EPS were prepared at room temperature for toxicity testing. A 10 mg/mL of stock solution was prepared by dissolving dried MB and EPS in embryo media (Danio-SprintM media), which was then diluted two-fold and further in a 96-well microplate (200 µL/well) using serial dilutions to obtain seven different concentrations in the 0.16–10 mg/mL range. For a standard control, zebrafish embryos in embryo media solution were used as an untreated control sample (0 mg/mL).

Upkeep and breeding of zebrafish system. A couple of adult zebrafish were placed in a breeding tank the day before the breeding occurred to set up the system. The following day, embryos were cleansed and incubated in the embryo medium (Danio-SprintM media) for two hours. Only healthy fertilised embryos were selected for the ZFET testing; meanwhile, the dead and coagulated embryos were discarded[35].

Zebrafish embryo toxicity (ZFET) test. Firstly, at 0 HPF, zebrafish embryos were exposed to samples (200 µL) in 96-well microplates (embryo/well) at seven different concentrations ranging from 0.16 to 10 mg/mL. The experiments were designed with an exposure group, both treated and untreated, containing 12 embryos each. The successfully treated embryos were cultured at ambient temperature (25 °C to 28 °C) for five days. The cumulative mortality and development abnormalities of zebrafish embryos and larvae were observed and examined for every 24 HPF from 0 to 120 HPF. Data of the survival rate, hatching rate, heart rate, morphological malformations, and teratogenic defects were captured and recorded using an inverted microscope coupled with a digital camera. The heartbeats were counted using a stopwatch (three embryos/min). Lethal endpoints were defined based on coagulation and the nonappearance of a heartbeat. Developmental defects such as pericardial oedema, yolk sac oedema, non-hatched, twisted body, and twisted tail were observed and recorded. The LC_{50} values were considered based on the principle of toxicity, in which > 1 mg/mL are considered relatively harmless, 0.1–1 mg/mL non-toxic, 0.01–0.1 mg/mL slightly toxic, 0.001–0.01 mg/mL moderately toxic, 0.0001–0.001 mg/mL highly toxic, and > 0.0001 mg/mL are super toxic.

Ethics declaration. The breeding of Zebrafish (Danio rerio F. Hamilton, 1822) broodstocks and the in vivo methodology was approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM), Malaysia and a licensed Danio Assay Laboratories Sdn. Bhd. (1,075,617-T), Director, Animal Biochemistry & Biotechnology Laboratory (ABBTech), Department of Biochemistry, Faculty of Biotechnology & Biomolecular Sciences, UPM, Selangor. The research was carried out in accordance with the Organization for Economic Cooperation and Development (OECD) No. 236: Fish Embryo Acute Toxicity (FET) Test (OECD, 2013)[56], under compliance of IACUC UPM using triplicates of all samples and ARRIVE guidelines.

Statistical evaluation. All of the graphs and figures were produced using GraphPad Prism v.8.0. (GraphPad Soft-ware, Inc.). The lethal concentration at 50% (LC_{50}) of treated samples toward zebrafish embryos was

evaluated using the same methods. The heart rates of three different animals were presented as a mean standard error of mean (SEM). A one-way analysis of variance (ANOVA) was used to determine significant differences, followed by a Dunnett's Multiple Comparison post-hoc test. differences between the means of the treated group and embryos in embryo media were set at p 0.001***, p 0.01**, p 0.05*.

Received: 3 August 2021; Accepted: 12 November 2021
Published online: 29 November 2021

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Acknowledgements

We want to thank Universiti Malaya under the GPF084A-2020 and IIRG003A-2020IISS awarded to Dr Wan-Mohtar, ST007-2021 (Dr Ilham) and Agro-Biotechnology Institute (ABI), Malaysia, under the Bio-Analytical Industry Development Programme (BIDP) awarded to Ms Siti Usuldin. The work was also funded by H2020 MSCA RISE project (number 872217/19) and Interreg Atlantic Area Neptunus Project (EAPA_576/2018) awarded to Prof Neil Rowan.

Author contributions

Conceptualization, A.U.; methodology, W.M.; software, W.M.; validation, A.J; formal analysis, N.R.; investigation, Z.I.; resources, N.R.; data curation, N.R.; writing—original draft preparation, A.U.; writing—review and editing, A.J.; visualization, R.A.; supervision, Z.I.; project administration, W.M.; funding acquisition and writing—review and editing, N.R. All authors reviewed the manuscript. A.U: Ahmad Usuldin; R.A: Raihan Abdullah; Z.I: Zul Ilham; A.J: Ainurzaman Jamaludin; W.M.: Wan-Mohtar; N.R: Neil Rowan.

Funding
Universiti Malaysia Scholarship, GPF084A-2020, GPF084A-2020, and ST007-2021. Horizon 2020 Framework Programme, 872217/10, Just Transition, MRTT 112. Interreg Atlantic Area Neptunus Project (EAPA_576/2018).

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to N.R.

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Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Science of the Total Environment 800 (2021) 149545

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Development of a low-temperature extrusion process for production of GRAS bioactive-polymer loaded compounds for targeting antimicrobial-resistant (AMR) bacteria

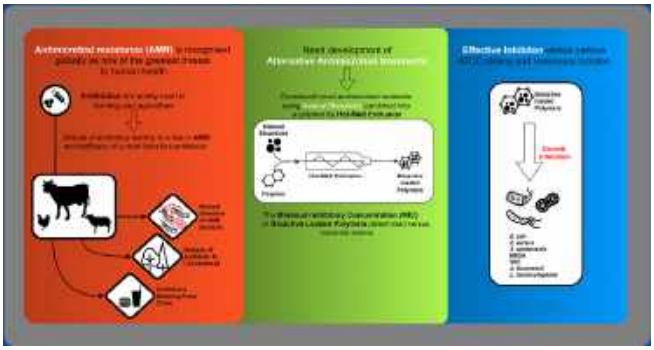
Kevin Masterson [a,*], Elaine Meade [d], Mary Garvey [d], Mark Lynch [a], Ian Major [b], Neil J. Rowan [a,c]

[a] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[b] Materials Research Institute, Athlone Institute of Technology, Ireland
[c] Centre for Disinfection, Sterilization and Biosecurity, Athlone Institute of Technology, Ireland
[d] Department of Life Science, Sligo Institute of Technology, Ash Lane, Sligo, Ireland

HIGHLIGHTS

- New copolymer process for delivery of heat-sensitive antimicrobial bioactives
- Efficacy against a range of veterinary antimicrobial resistant bacterial isolates
- Process appropriate for delivery of next-generation bioactives
- A new 'One-health' approach for potentially addressing priority AMR challenges

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 4 June 2021
Received in revised form 5 August 2021
Accepted 5 August 2021
Available online 10 August 2021

Editor: Damia Barcelo

Keywords:
Disease mitigation
Novel materials
GRAS
Thermally-sensitive
Antimicrobial-resistance
Polymer processing

ABSTRACT

Antimicrobial resistance (AMR) is recognised globally as one of the greatest threats to human and animal health; thus, discovery of alternative antibacterial agents to address AMR is a priority challenge. This study constitutes the first report of a low-melting temperature, polymer- extrusion process for the smart delivery of thermally-sensitive antimicrobial bioactives, including generally-regarded-as-safe (GRAS) bioactives derived from various sources. Bioactives were assessed before and after extrusion by determining their respective minimum inhibitory concentrations (MIC). WHO-priority AMR-bacterial isolates causing zoonotic infections were evaluated along with use of standard ATCC strains. Findings revealed that this copolymer method was capable of delivering thermally-sensitive bioactives with varying degrees of growth inhibition against the AMR-bacterial strains. The extrusion process was found to increase the effect of nisin against MRSA (4-fold increase) and *L. monocytogenes* (6.4-fold increase), silver nitrate ($AgNO_3$) against *E. coli* (3.6-fold increase) and *S. epidermidis* (1.25-fold increase), and chitosan against *S. aureus* (1.25-fold). Findings show the potential applicability of this polymer extrusion process for developing future bioactive-loaded polymer compounds; thus, highlighting the potential of converging bio-based industry with novel materials for enabling 'One-Health' solutions.

1. Introduction

Antimicrobial resistance (AMR) is recognised globally as one of the greatest threats to human health (Interagency Coordination Group on

* Corresponding author.
E-mail address: k.masterson@research.ait.ie (K. Masterson).

https://doi.org/10.1016/j.scitotenv.2021.149545

Antimicrobial Resistance, 2019). The World Health Organisation (WHO) has called for immediate action to counteract what has become an "antibiotic resistance crisis" (World Health Organistaion, 2020). The emergence and spread of AMR is directly related to misuse and overuse of antibiotics across various sectors (Talebi Bezmin Abadi et al., 2019). WHO have developed the antibiotic classification framework, Access, Watch, Reserve (AWaRe), for aiding antibiotic stewardship (WHO report on Surveillance of Antibiotic Consumption, 2018). Access antibiotics are classified as having the lowest potential for resistance, Watch antibiotics are those that are prone to AMR, while Reserve antibiotics are classed for use as "last resort" only. It was reported that between 2000 and 2015, global consumption of Watch antibiotics increased by 90·9% per-capita, while consumption of Access antibiotics increased by 26·2% (Klein et al., 2021). On any given day, about one in every three hospital patients in Ireland are being treated with antibiotics (Plachouras et al., 2018). This is further complicated by antibiotic use in the agricultural sector, which has also been identified as a major contributor to AMR on a global scale (Economou and Gousia, 2015; Manyi-Loh et al., 2018).

In collaboration with other major World Organisations, the WHO has designed an approach, known as "One-Health", which aims to combine resources across multiple sectors particularly converging public health and animal health (FAO et al., 2008). The concept of One-Health is not new; however, in recent times it has become increasingly important such as in the area of AMR (Evans and Leighton, 2014). Studies of plasmid-encoded colistin resistance (mcr-1), initially found in pigs in China, have shown its presence in humans and animals worldwide; thus highlighting the one health link between humans, animals and the environment in the context of AMR (Caniaux et al., 2017). Additionally, there are various types of antimicrobial resistant organisms (ARO), some of which are resistant to "last resort" antibiotics (Tacconelli et al., 2018). As such, there is a pressing need to develop new or alternative antimicrobial compounds to combat the global threat of AMR for human and animal intervention (Aminov, 2010; Interagency Coordination Group on Antimicrobial Resistance, 2019; Ventola, 2015).

There are a number of potential alternatives currently under investigation, such as phages and various organic and inorganic compounds; however, such alternatives hold limitations in treating bacterial infections (Fenton et al., 2013; Gill et al., 2006; Kwiatek et al., 2012; O'Flaherty et al., 2005; Wilson and González, 2003; Wittebole et al., 2014). Bacteriophages have a narrow spectrum of effect and are generally tailored per bacterial species, leading to increased costs and higher probability of inefficacy versus infections with more complex aetiology (Breyne et al., 2017; Gomes et al., 2016; Porter et al., 2016). Biofilms also limit the efficacy of therapeutics by reducing or preventing interaction with their targets (Marques et al., 2017). For example, certain biomedical devices are complex in design with narrow lumen diameters and are prone to unwanted biofilm formations as are challenging to clean (Bhattacharya et al., 2015; Wagner et al., 2011). However, alternative interventions include using polymer-based materials in order to prevent a build-up of bacterial cells in such devices; thus, stopping initial attachment and preventing or disrupting the formation of biofilm (Costerton, 2005; Konai et al., 2018). Development of alternative bio-based therapeutics and smart materials also has implications for efficacy of reprocessing and sterilization (Chen et al., 2019).

Polymer-based therapeutic delivery solutions are plausible through the use of smart manufacturing approaches, such as hot-melt extrusion (HME), which necessitates the capacity of the active compound to withstand thermal processing to maintain bioactivity (Simões et al., 2019). However, traditional antibiotics have low thermal stability, excluding them as possible candidates for HME applications (Wylie et al., 2021). There is growing interest in exploiting bio-based ingredients as potential antimicrobial therapies; however, there is a gap in knowledge as to effective non-thermal processing and delivery approaches (Rowan and Galanakis, 2020; Rowan and Casey, 2021). HME has seen growing use in the area of pharmaceuticals as it allows the production of biologically active polymers with various formulations, dosage forms, and can enhance the physical properties of the bioactive component (such as increased water solubility, improved stability and shelf-life) (Patil et al., 2016).

This constitutes the first study to report on use of low-temperature polymer extrusion process using four bioactive compounds for one health applications. Silver nitrate ($AgNO_3$), Zinc Oxide (ZnO), nisin and chitosan were assessed similarly to that of antibiotics, by determining their minimum inhibitory concentration (MIC) against three standard, ATCC bacteria strains. These bioactives were assessed for their processing potential by incorporating them into a polymer by HME and then re-assessing their antibacterial capabilities. By assessing their potential as antibiotic alternatives, further studies can be conducted to determine suitable areas they can be utilised, replacing traditional antibiotics; thus, potentially lowering their overall use and mitigating against occurrence of AMR development as a consequence of antibiotic misuse.

$AgNO_3$ is the most commonly used and documented silver salt derivative, and has raised great interest recently for its revival as an antimicrobial (Atiyeh et al., 2007; Balazs et al., 2004; Gao et al., 2018; Prabhu and Poulose, 2012; Russell and Hugo, 1994). While previous studies have shown that $AgNO_3$ incorporation into hydrogels and liquid-based polymers can increase it's antimicrobial efficacy (dos Santos et al., 2012; Wu et al., 2018), this present study represents the first to incorporate the compound into a solid-form polymer by HME and assess its antimicrobial abilities.

ZnO is a commonly-used ingredient in cosmetics and other various areas, categorised generally recognised as safe (GRAS) by the FDA; it has recently elicited interest due to its antimicrobial abilities, particularly when used as a nano-particle (Beyth et al., 2015; Padmavathy and Vijayaraghavan, 2008; Pasquet et al., 2014; U.S. Food and Drug Administration, 2019). ZnO and ZnO nanoparticles (ZnO-NPs) have shown numerous successes in their ability as antimicrobials against bacteria and fungi. A number of studies in the food packaging area have shown ZnO-NPs suitability for polymer incorporation, demonstrating their resilience to processing, holding their antimicrobial abilities while in polymer form (Espitia et al., 2012; Silvestre et al., 2016).

Nisin, a bacteriocin, has been studied for use in food packaging to reduce spoilage. While recent studies have focused primarily on its use as a general antibacterial agent, previous works have shown its active properties within polymer preparations (Kawada-Matsuo et al., 2019; Lewies et al., 2018; Tong et al., 2014). Chitosan has been also studied in food packaging, drug delivery and as a synergistic carrier of antimicrobials (Kim et al., 2017; Kravanja et al., 2019). Both nisin and chitosan are recognised GRAS compounds, where they were previously investigated for use in active food packaging due to their known antimicrobial abilities and established non-toxicity in mammalian studies (Asli et al., 2017; Bastarrachea et al., 2015; Cardozo et al., 2014; Moon et al., 2007; J. Wu et al., 2007; Yang et al., 2014).

While the antimicrobial efficacy of these bioactives have been previously reported, this constitutes the first study to report on use alone and combined by way of incorporation into a solid polymer by HME for antimicrobial use. While their efficacy toward bacteria is important, the ability of these bioactives to be utilised in polymer forms potential creates opportunities for their adaptability across a number of important sectors such as biomedical devices (implants, catheters etc.) and in veterinary products. The HME process was chosen as it allows for complex formulations of the active ingredients, and incorporation into various polymer carriers allowing for refinement of the final product's physical characteristics. In addition, this supports and potentially enables incorporation of smart bioactive products that have been biorefined from difference sources for antimicrobial use; thus, providing a process for current markets and practises. The HME process will also enable combinations of individual bioactives that will support broad-spectrum antimicrobial properties. Specifically, this study assesses the efficacy of

these bioactive-polymer combinations against wild-type and AMR bacteria, including pathogenic veterinary strains.

2. Materials & methods

2.1. Isolation and characterization of veterinary zoonotic strains

Bacterial isolates including *Escherichia coli*, Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant Enterococci (VRE), *Listeria monocytogenes* and *Acinetobacter baumannii* were obtained from diagnostic testing of canine, equine and farm animals manifesting with conditions such as bacteraemia, renal infection, open wound infections, and mastitis. Collected samples of infection or disease in the form of urine, blood material, milk and swabs were provided by registered veterinary personnel in sterile containers (Cruinn Diagnostics, Dublin, Ireland). Liquid samples were immediately inoculated onto nutrient agar and incubated at 37 °C for 24 h. Swabs were inoculated in nutrient broth and incubated at 37 °C for up to 24 h under rotary conditions (125 rpm) before streaking onto nutrient agar plates.

Individual colonies were re-streaked for isolation and pure isolated colonies inoculated into nutrient broth for further biochemical characterization. Colonies were identified based on their morphological characteristics, biochemical profile, and growth on selective agars, specifically CHROMagar™ Acinetobacter (CHROMagar™, Paris, France), Harlequin™ *E. coli*/Coliform Medium, Harlequin™ Listeria Chromogenic Agar, Baird Parker agar (LabM, Cruinn Diagnostics Ltd., Dublin, Ireland) and BBL™ Enterococcosel™ Agar (Becton, Dickinson and Company, Dublin, Ireland). Identity was confirmed via polymerase chain reaction (PCR). Specifically, a single colony of each bacterial test isolate was subcultured in nutrient broth and incubated overnight at 37 °C. Genomic DNA was directly extracted using the GenElute™ Bacterial Genomic kit (Sigma Aldrich, Dublin, Ireland) according to the manufacturer's instructions. The bacterial primers ITS_8F (5′- AGAGTTTGATCCTGGCTCAG -3′) and ITS_U1492R (5′- GGTTACCTTGTTACGACTT -3′) (Sigma Aldrich, Dublin, Ireland) were used for amplification of 16s rRNA gene. PCR was performed in a total reaction volume of 20 μL, containing 17 μL red Taq 1.1× master mix (VWR, Dublin, Ireland) 1 μL ITS_8F, 1 μL ITS_U1492R and 1 μL of pure genomic DNA eluate. DNA amplification was performed in a thermo cycler (VWR, Dublin, Ireland) using the recommended parameters. Following DNA amplification, the PCR products were examined by electrophoresis on a 1% w/v agarose gel run at 120 V for 50 min. Successful reactions were sent to Source Bioscience (Waterford, Ireland) for clean-up and gene sequencing of products. Strains were stored long term in 20% glycerol at −20 °C and short term in nutrient broth at 5 °C. Identity of strains was confirmed via Gram stain and selective agars prior to each experimental set up.

2.2. Antibiotic resistance profile of veterinary isolates

Antibiotic resistance profiles were established using CHROMagar™ agars selective for Extended Spectrum Beta-Lactamase (ESBL), vancomycin-resistant enterococci (VRE) and Methicillin-resistant *Staphylococcus aureus* (MRSA) (CHROMagar™, Paris, France) and a range of antibiotic susceptibility disks (ThermoFisher Scientific, Ireland) as per European Committee for Antibiotic Susceptibility Testing (EUCAST) guidelines (EUCAST, 2020). Specifically, colonies of an overnight bacterial culture suspended in sterile saline at a density of 0.5 McFarland (ca. 1×10^8 cfu/mL) were overlaid on to Mueller-Hinton agar (MHA) (4 mm) in 90 mm circular petri plate as per the EUCAST disk diffusion method (EUCAST, 2020). An antibiotic inoculated disk was placed in the centre of the plate and incubated inverted for 18 h at 37 °C. Antibiotics used during profiling include Streptomycin, Vancomycin, Chloramphenicol, Erythromycin, Ampicillin, Amoxicillin/Clavulanic acid, Cefpodoxime, Cefotaxime, Aztreonam, Doripenem, Meropenem, Ciprofloxacin, Levofloxacin, Colistin, Doxycycline.

Zones of inhibition were measured and used to determine the bacterial species resistance profile. The absence of a zone of inhibition denotes complete resistance (R) of the species against the tested antibiotic. Susceptible species were graded as being completely susceptible (S), or as having intermediate susceptibility (I), based on the ability of the test drug to produce a zone diameter according to EUCAST zone diameter guidelines. MRSA and VRE, which are listed as high importance, were assessed for resistance to vancomycin and quinolones amongst other therapeutics. Isolates that tested positive on CHROMagar™ ESBL and displayed resistance to the extended-spectrum cephalosporin group of antibiotics were selected for phenotype confirmation of ESBL production. ESBL detection and characterization are recommended for public health and infection control purposes (EUCAST, 2020). This was carried out by placing cefpodoxime (10 μg) and cefpodoxime/clavulanate (10 μg/1 μg) discs on an inoculated MHA plate, 30 mm apart. Plates were then incubated overnight at 37 °C. A zone diameter of ≥5 mm is considered positive for ESBL production.

2.3. Hot-melt extrusion (HME) co-polymer process

The bioactive compounds, nisin (2.5% w/w, SKU: N5764, CAS: 1414-45-5), Chitosan (low molecular weight, SKU: 448869, CAS: 9012-76-4), Zinc Oxide (ZnO, nanopowder: <100 nm particle size, SKU: 544906, CAS: 1314-13-2) and Silver Nitrate ($AgNO_3$, SKU: S8157, CAS: 7761-88-8) were purchased from Sigma Aldrich (Sigma-Aldrich Ireland Limited, Wicklow, Ireland), and incorporated into a polymer by hot-melt extrusion (HME) process using a PRISM Twin Screw Extruder-16-TC (Twin bore diameter: 16 mm, screw diameter: 15.6 mm, channel depth: 3.3 mm, barrel length: 384 mm). Polymers were processed using an upper barrel temperatures 140 °C and screw speed of 100 RPM. The HME process was selected to match material stability of bioactives such that it enabled processing without affecting material functionality or activity post-processing. Poly-vinyl-pyrrolidone/vinyl acetate (PVPVA) was purchased from BASF (BASF SE Headquarters, Ludwigshafen, Germany) and chosen as the copolymer carrier due to its low melting temperature, which would ensure minimal thermal damage to the test bioactives. PVPVA is a hydrophilic polymer matrix that dissolves readily in water; thus, forming bioactive polymer solution when loaded with drugs. Processed PVPVA also has a very solid yet brittle composition allowing it to be ground to a fine powder, allowing for easier solution preparation.

2.4. Bioactive solution preparation

Chitosan was dissolved in 1% (v/v) acetic acid and then adjusted to pH 5.5 with 0.4 M sodium hydroxide (NaOH). ZnO was suspended in dH_2O. Nisin was dissolved in a solution of 400 mM sodium chloride (NaCl), pH 3.25. These solutions were then sterilised by autoclaving. Nisin concentrations are reported in terms of active nisin content, where 1 g of commercial nisin powder contains 25 mg of active nisin. $AgNO_3$ was put to a solution of 28% (v/v) Poly (ethylene glycol), average molecular weight 400 (PEG-400) and 26% (w/v) d-sorbitol This solution was then filter sterilised by use of a 0.2 μm syringe filter tip. The bioactive polymers were ground into a fine powder using a mortar and pestle and prepared as per their respective bioactive, apart from $AgNO_3$-ploymer, which was dissolved in dH_2O and sterilised by autoclaving.

2.5. Antibacterial assay

The minimum inhibitory concentration (MIC) was determined for each standard bacterial and veterinary isolate strain by use of the broth microdilution method, adapted from previous literature (Wiegand et al., 2008). The standard strains, *Escherichia coli* (ATCC 25922, NCTC 12241) and *Staphylococcus aureus* (ATCC 29213, NCTC 12973) were purchased from Public Health England (Culture

Collections, Public Health England, Salisbury, UK). *Staphylococcus epidermidis* (ATCC 35984) was purchased from ATCC (LGC Standards, Middlesex, UK). Briefly, microdilution assays were carried out in untreated 96-well plates using Mueller-Hinton broth (MHB). Bacterial strains were subcultured twice from frozen stocks before use. Working stock dilutions of the bioactives and bioactive polymers were prepared in MHB, aliquoted into the first column of a 96-well plate and serial diluted along the plate. Treatment vehicles (T_v) were included to assess any effects that the solutions may have (excluding the bioactive itself). Two columns were left untreated, with one of these left uninoculated to act as a sterility and positive control (SC) and the other as a growth and negative control (GC). Colonies of the test bacteria were taken from an overnight streak plate used to prepare a 0.5 MacFarland bacterial suspension (1×10^8 cfu/mL). This suspension was adjusted and aliquoted into the wells of the plate (excluding the SC) giving a final in-well bacterial concentration of 5×10^5 cfu/mL. Plates were incubated in a shaker incubator (120 rpm) for 18 h at 37 °C. Wells were observed for growth as determined by visible turbidity and absorbance readings (625 nm). The MIC was determined as the lowest concentration of a treatment to prevent cell growth (i.e. broth turbidity).

2.6. Statistical analysis

Experiments were carried out in replicates of three or four. Significance between MIC values of the bioactives before and after polymer processing was determined by use of either a paired *t*-test (one-tailed), or a two-way ANOVA model with Bonferroni post-hoc test. A P value <0.05 was considered significant.

3. Results

3.1. Resistance profile of veterinary isolates

Resistance profiles were established in accordance with the WHO priority pathogen list for veterinary isolates MRSA, VRE, *L. monocytogenes*, with critically important *E. coli*, and *A. baumannii* also being assessed for resistance to 3rd generation cephalosporins and carbapenems, amongst other drug classes (Table 1). *A. baumannii* and *E. coli* both exhibited resistance to vancomycin and ampicillin, with ESBL activity, which was confirmed, with the bacteria exhibiting zones of 15 mm and 20 mm vs Cefpodoxime discs, and 23 mm and 28 mm vs Cefpodoxime + clavulanic acid discs, respectively. *E. coli* also demonstrated resistance to amoxicillin/clavulanic acid. *E. coli* and *A. baumannii* displayed intermediate susceptibility to an array of antibiotics including streptomycin (10 mm, 15 mm), erythromycin (9 mm, 9 mm), and chloramphenicol (21 mm, 9 mm) respectively.

MRSA give clear indication of resistance to streptomycin and 3rd generation cephalosporins, with intermediate susceptibility to vancomycin (16 mm), tetracycline (11 mm), and erythromycin (22 mm). The high priority pathogen VRE demonstrates clear resistance to streptomycin, cephalosporins and quinolones, with low susceptibility to the macrolide erythromycin (7 mm) and full susceptibility to tetracyclines (doxycycline, 30 mm). *L. monocytogenes* displays resistance to ampicillin, erythromycin, and cephalosporins.

Table 2
Antibiotic Minimum Inhibitory Concentrations for Veterinary Isolate strains. Table shows the minimum inhibitory concentration (MIC) of various traditional antibiotics versus veterinary bacterial isolates.

		Bacterial species			
		A. baumannii	*E. coli (93)*	*MRSA*	*L. monocytogenes*
Antibiotic (μg/mL)	Streptomycin	8	4	R	16
	Vancomycin	R	256	1	0.5
	Erythromycin	32	R	1	16
	Azithromycin	4	8	8	32
	Amoxicillin	256	128	256	4
	Ceftazidime (3rd)	64	16	32	R
	Cefotaxime (3rd)	128	16	32	R
	Ceftriaxone (3rd)	64	4	32	R
	Cefepime (4th)	64	2	32	16
	Aztreonam	256	8	–	–
	Meropenem	0.5	0.125	32	16
	Doxycycline	16	64	128	8
	Tetracycline	128	128	128	16
	Ciprofloxacin	0.125	0.25	0.25	2
	Levofloxacin	0.25	0.25	0.25	2

3.2. Minimum inhibitory concentration of antibiotics

The MIC of an array of antibiotics was determined against each veterinary isolate of MRSA, VRE, *E. coli* and *A. baumannii* (Table 2).

3.3. Minimum inhibitory concentration of bioactives

The MIC values of the bioactive compounds were determined for each ATCC bacterial strain and veterinary isolate. Results of the MIC assays gave positive indication to the bacterial inhibitory properties of $AgNO_3$, chitosan, ZnO and nisin versus the three ATCC bacterial strains, *E. coli*, *S. aureus* and *S. epidermidis*. The $AgNO_3$ preparation exhibited

Table 1
Resistance profile of veterinary isolates to a range of antibiotics as determined by zones of inhibition with reference to EUCAST cut off points.

			Zone diameter (mm) of bacterial species				
Drug class	Antibiotic	Conc. (μg/disc)	*A. buamannii*	*E. coli (93)*	MRSA[a]	VRE[a]	*L. monocytogenes*
Aminoglycoside	Streptomycin	10	15	10	R	R	11
Glycopeptide	Vancomycin	30	R	R	16	R(12)	13
Chloramphenicol	Chloramphenicol	30	9	21[24]	22(18)[24]	20	27
Macrolide	Erythromycin	15	9	9	15(18)[26]	7	15(25)
Penicillin	Ampicillin	10	R	R	R	15	10(16)
Penicillin-like	Ampicillin/clav	20:10	25	14(19)	11	24	25
Cephalosporins	Cefpodoxime	10	10	20(21)	R	15	R
	Cefotaxime	5	16	16(17)	R	R	R
Monobactam	Aztreonam	30	32	25(21)	–	–	–
Carbapenems	Doripenem	10	29	22[9]	16	25	38
	Meropenem	10	25(15)	23(16)	30	12	30(26)
Quinolones	Ciprofloxacin	5	30(21)	36(22)	28(24)	R(15)	10
	Levofloxacin	5	32(20)	31[33](19)	29(22)	R(15)	26
Polymyxin	Colistin	10	13	12[9]	–	–	–
Tetracycline	Doxycycline	30	10	12	11	30	40

R donates complete resistance to antibiotic.
( ) EUCAST 2020 cut-off zone diameter (mm) for antibiotic resistance for certain species and antibiotic. Zones below this are deemed resistant.
[ ] EUCAST 2019 cut-off zone diameter (mm) for antibiotic resistance for certain species and antibiotic. Zones below this are deemed resistant.
[a] WHO high priority pathogens.

Table 3
Bioactive Minimum Inhibitory Concentrations versus *ATCC Bacterial Strains*. Table shows the mean minimum inhibitory concentration (MIC) of silver nitrate ($AgNO_3$), chitosan, zinc oxide (ZnO) and nisin against the ATCC bacterial species, *E. coli*, *S. aureus* and *S. epidermidis*, as determined by use of broth microdilution assays. Bioactives were assessed before and after extrusion with the co-polymer PVPVA via hot-melt extrusion (HME). Significance changes of MIC were determined by use of a 2-way ANOVA and shown in terms of a P value. N = 4.

		Bacterial species		
		E. coli (ATCC 25922)	*S. aureus* (ATCC 25913)	*S. epidermidis* (ATCC 35984)
Bioactive MIC (μg/mL)	$AgNO_3$	31.25	42.97***	19.53
	$AgNO_3$ -PVPVA	8.789	109.4***	15.63
	Chitosan	156.3	390.6	156.3
	Chitosan-PVPVA	175.8	312.5	208.3
	ZnO	203.125*	156.25	97.6625
	ZnO-PVPVA	562.5*	312.5	140.625
	Nisin	No MIC[a]	6.833**	4.885
	Nisin-PVPVA	No MIC[a]	19.53**	8.3

[a] Up to 0.125 mg/mL tested.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

effective growth inhibitory effects, as determined by the absence of broth turbidity, against *all tested bacterial strains* at concentrations between 15 and 62.5 μg/mL (Tables 3 & 4). The $AgNO_3$ T_v, consisting of PEG-400 and d-sorbitol, exhibited no effects upon bacterial growth.

Chitosan showed efficacy versus all test strains at concentrations between 78 and 625 μg/mL except for VRE which required up to 1250 μg/mL (Tables 3 & 4). The Chitosan T_v only held noticeable effect at the higher concentrations of 0.125–0.25% acetic acid (AcOH), which is equivalent to a chitosan concentration of 1250–2500 μg/mL. The AcOH concentration in the MIC range would be between 0.0137 and 0.0235% AcOH. This indicates that the T_v has no effect on the acquired MIC values as they are much too low.

The ZnO suspension held varying degrees of efficacy, with a mean concentration range of 78.125–312.5 μg/mL versus ATCC while the MRSA vet isolate required up to 7500 μg/mL and VRE vet isolate showed no inhibition, even with testing up to 30 mg/mL (Tables 3 & 4). The ZnO suspension was effective in inhibiting growth of the three ATCC strains, being least effective against *E. coli* and most effective against *S. epidermidis*. This can be accounted for by the bacteria's intrinsic resistance and pathological strengths. Gram-negative bacterium, such as *E. coli*, have multiple, thin layers of membrane combined with an inner peptidoglycan cell wall-layer, which present formidable barrier for therapeutics. Gram-positive bacteria, such as *S. aureus* and *S. epidermidis*, comprise of a single, outer cell membrane under a thick peptidoglycan layer that has actually been shown to enable therapeutics by aiding their absorption into the cell. Furthermore, *S. epidermidis* is a well-known opportunistic, biofilm forming bacteria recognised as an etiological agent in complex device-mediated infection in healthcare and in veterinary practice. It generally exhibits low resistance to antimicrobials while in planktonic forms, requiring biofilm formation to become more resistant, thus justifying its lower MIC compared to *E. coli* and even *S. aureus*. The resistance of VRE to ZnO may be accounted for by its alternative peptidoglycan synthesis, which alters the bonding potential of its outer peptidoglycan layer, preventing ZnO from interacting and penetrating the bacteria cell (Ahmed and Baptiste, 2018).

Nisin was effective in inhibiting the growth of the ATCC strains of *S. aureus* and *S. epidermidis*, with MIC values between 3.9 and 7.81 μg/mL. Effective inhibition was also seen against the veterinary isolates MRSA, VRE and L. *monocytogenes*, with MICs ranging between 12.5 and 15.6 μg/mL. Nisin held no inhibitory effect against *E. coli* (both ATCC and veterinary isolates) or *A. baumannii*, even with concentrations up to 125 μg/mL. This finding is appreciated given that nisin cannot carry out its mechanisms of growth inhibition versus Gram negative bacteria due to outer cell membrane preventing this bioactive from interacting with its target, the intramembrane molecule lipid II.

3.4. Minimum inhibitory concentration of bioactive loaded polymer compounds

The bioactives were extruded into a polymer and assessed using the broth microdilution assay to determine their MIC versus both ATCC and veterinary isolates. Samples of the stock polymer, PVPVA, were also included at concentrations up to 60 mg/mL to assess their effects upon the bacteria. The MIC values of the bioactives before and after polymer incorporation versus the ATCC bacterial strains are presented together for comparison (Tables 3 & 4).

$AgNO_3$ held notable inhibitory effects against tested strains, before and after polymer processing (Tables 3 & 4). MIC values against *E. coli* and *S. epidermidis* obtained after extrusion into the PVPVA polymer were lower than those obtained from $AgNO_3$ pre-hot melt extrusion, noting an increase in its efficacy. $AgNO_3$ proved the most consistently effective of the four compounds, displaying consistently low MIC values against each test bacterial strain while also exhibiting lowered MIC values following polymer incorporation. This showing that not only does $AgNO_3$ still hold function following the HME process but appears to have its mechanism of actions enhanced. Additional observations have shown the bioactive to have greater stability when incorporated into the polymer, denoted by a lack of colouration after long-term air exposure, an occurrence normally seen with unmodified $AgNO_3$.

Chitosan was quite effective against tested bacterial strains, with very little interference seen from its T_v. Following HME, chitosan exhibited no notable change in efficacy (Tables 3 & 4). Although the MICs versus *S. aureus*, *L. monocytogenes* and *E. coli (isolate)* were lowered 1.25-fold (390.6 → 312.5 μg/mL), 1.5-fold (234.38 → 156.25 μg/mL) and1.5-fold (234.38 → 156.25 μg/mL) respectively, showing an increase in efficacy, these were not considered significant ($P > 0.05$).

Table 4
Mean Minimum Inhibitory Concentrations versus *Veterinary Isolates*. Table shows the minimum inhibitory concentration (MIC) of silver nitrate ($AgNO_3$), nisin, zinc oxide (ZnO) and chitosan, before and after incorporation with the polymer PVPVA against the veterinary isolates, *MRSA*, *VRE*, *L. monocytogenes*, *E. coli* and *A. baumannii*. Significant changes in MIC were determined by use of either a paired *t*-test (nisin) or a two-way ANOVA with Bonferroni post-test, and shown in terms of a P value. N = 3.

		Veterinary Isolate				
		MRSA	VRE	*L. monocytogenes*	*E. coli (isolate)*	*A. baumannii*
Bioactive (μg/mL)	$AgNO_3$	20.83	20.83	15.63	15.63**	13.02
	$AgNO_3$-PVPVA	31.25	31.25	15.63	31.25**	13.02
	Nisin	15.6**	15.6**	12.5**	No MIC	No MIC
	Nisin-PVPVA	3.9**	1.95**	1.95**	No MIC	No MIC
	Chitosan	208.33	1250	234.38	234.38	260.42
	Chitosan-PVPVA	260.42	1666.67	156.25	156.25	416.67
	ZnO	4583.33**	No MIC[a]	937.5	390.63	364.58
	ZnO-PVPVA	25000**	No MIC[a]	937.5	1562.5	208.17

[a] Up to 30 mg/mL tested.
** $P < 0.01$.

ZnO appeared to have its antimicrobial abilities greatly hindered by the polymer processing, as all MIC values were observed to increased. *E. coli* and *S. aureus* MIC values saw a 2.8-fold (203.13 → 562.5 μg/mL) and a 2-fold (156.25 → 312.5 μg/mL) increase, respectively (Table 3). MRSA and *E. coli (isolate)* MICs were seen to increase 5.5-fold ($P < 0.01$) and 4-fold respectively (Table 4). The MIC against *S. epidermidis* demonstrated the lowest effect from the polymer process, with a 1.4-fold increase (97.66 → 140.63 μg/mL). While it is unlikely that the heat from the polymer process caused this loss of potency (as ZnO has a melting temperature of 1975 °C), it is possible that the shearing effect of the twin-screw extruder may have denatured it. Although it is probable that the polymer itself bound too strongly to the ZnO molecules; thus preventing it from freely interacting with bacterial cells.

Incorporation of nisin into a PVPVA polymer through HME exhibited a significant decrease in efficiency, as denoted by an increase in MIC values against the ATCC strains *S. aureus* (6.83 → 19.53 μg/mL) and *S. epidermidis* (4.89 → 8.3 μg/mL), which represents a 2.9-fold ($P < 0.01$) and 1.7-fold increase in MIC values respectively (Table 3). However, the opposite was observed in assays against the veterinary isolates, which resulted in nisin-PVPVA achieving lower MIC values versus MRSA (15.6 → 3.9 μg/mL), VRE (15.6 → 1.95 μg/mL) and *L. monocytogenes* (12.5 → 1.95 μg/mL); thus, representing 4-fold, 8-fold and 6-fold decreases, respectively (Table 4). This increase in potency is noteworthy as was found to be statistically significant ($P < 0.01$). Another noted characteristic of the nisin-PVPVA polymer was its increased shelf life in solution. Many of the additional components of the commercial nisin powder are intentionally left within its composition in-order to increase the compounds shelf life. However once in solution, it loses potency after 7–10 days. The nisin-PVPVA were observed to hold same level of potency for up to 30 days following solution preparation, which could be accredited to the polymer supporting the nisin's polycyclic structure stability.

Overall, the four bioactives exhibited satisfactory bacterial growth inhibition against *E. coli*, *S. aureus* and *S. epidermidis*. In terms of effective treatment dose, $AgNO_3$ and nisin held the greatest efficiency to lowest concentrations. Chitosan had equally consistent results across bacterial strains and, along with $AgNO_3$, was the least negatively affected following HME with PVPVA. All bioactives have demonstrated their suitability for HME as they have retained sufficient activity post-processing, with enhanced solubility and potency lifetime.

4. Discussion

There has been a pressing need for alternative therapies in the treatment of bacterial infections and diseases. With the current antibiotic resistance pandemic, stringent restrictions and management are being implemented to hinder its impact nationally and globally. The use of antibiotics in medicine has taken a hesitant approach in recent years due to their misuse being observed in various sectors worldwide, including agriculture, which having a huge influence on the increasing levels of AMR bacteria. The monitoring and documenting of AMR is a vital phase in controlling its emergence and instigating future actions. Simply differentiating which drugs bacteria susceptible to, and which they are resistant to, is essential to clinicians, where susceptibility results guide treatment options. The emergence of *S. aureus* strains displaying intermediate susceptibility (VISA) or full resistance (VRSA) to vancomycin, is currently a significant threat to public health and safety where they are associated with hard-to-treat nosocomial infections globally. The evidence of varying levels of resistance in these veterinary isolates contributes to the association between the veterinary use of antibiotics and emergence/proliferation of antibiotic resistance. With the increasing demand on livestock production, there will undoubtedly be an increase in the veterinary use of antibiotics with proliferation of AMR and environmental pollution with resistant genes, which can impact upon the human health sector. In conjunction with monitoring resistance profiles, research on the sensitivity of treated bacteria to antibiotic alternatives is important for monitoring and controlling the threat of AMR.

Here, four bioactive compounds, silver nitrate ($AgNO_3$), nisin, chitosan and zinc oxide (ZnO), were presented and assessed for their antibacterial abilities under conditions equal to that of antibiotic testing. Studies have been conducted to evaluate and compare $AgNO_3$ and silver nanoparticles (AgNPs), with reported $AgNO_3$ MIC values ranging between 31 and 140 μg/mL against *E. coli* and 31–80 μg/mL against *S. aureus*, which closely resemble MIC values determined the in the present study (Lima et al., 2019; Salman, 2017). Another study investigated the antibacterial and cytotoxic properties of $AgNO_3$, reporting MIC values of 6 μg/mL versus *E. coli* and *S. aureus*, which are slightly lower than those described in the present study (31.25 and 42.97 μg/mL respectively) (Mulley et al., 2014). The present study has found that $AgNO_3$ not only holds antibacterial ability following HME, but exhibited an increased efficacy against ATCC *E. coli* and *S. epidermidis* strains.

While nisin has been extensively studied for its antibacterial abilities, there appears to be large variation between studies in terms of reported MIC values (Kawada-Matsuo et al., 2019; Lewies et al., 2018; Tong et al., 2014). Kawada-Matsuo et al. (2019) reported on a similar solution prepartion method where determined MIC values (3.2–6.4 μg/mL) against *S. aureus*, were similar to what were observed in this present study (6.8 μg/mL). Tong et al. (2014) reported MIC values of 1000 μg/mL against *Enterococcus* species, which is much greater than those reported here (15.6 μg/mL versus VRE). The variation may be a result of the preparations used in its testing, where the current study utilises a set pH and salt content for preparing the nisin solution; where some literature reports using a dilute acid in-order to dissolve the nisin after which the solution is sterilised by autoclaving or filtration. Other studies have reported the need of a specific salt content in-order to stabilise nisin in solution, without which would increase its susceptibility to the damaging effects of the autoclaving process (Rollema et al., 1995; Yamazaki et al., 2000). Results from this study show nisin to inhibit bacterial species in a similar capacity as before in relation to *S. aureus*, or, in some cases, to even higher efficacy in terms of *Enterococcus* species tested.

The majority of studies that focus on use of nisin are concerned with its incorporation into food packaging materials, either as an active component or as a coating on processed polymer films. There are numerous polymer materials examined as a carrier for nisin, such as polylactic acid (PLA), nitrocellulose (NC), methylcellulose (MC), polyethylene oxide (PEO), and even natural polymers such as chitosan (Cutter et al., 2001; Cha et al., 2003; Jin and Zhang, 2008; Jin et al., 2009; Imran et al., 2010; Imran et al., 2014; Han et al., 2017). Many of these studies have reported increased antimicrobial activity following nisin incorporation. However, many of these methods involve numerous and time-consuming steps for the preparation of the nisin/polymer, requiring mixing of the components in a liquid form and then allowing them to dry. The incorporation of nisin into a polymer by extrusion has also been documented, although is reported that the heat and shear forces of the process have negative effects upon the antimicrobial abilities of nisin (Gharsallaoui et al., 2016). The present study represents the first known to extrude nisin using the PVPVA co-polymer. Results show successful incorporation of the nisin into the polymer, retaining activity after HME and even showing increased efficacy against the veterinary isolate strains, MRSA, VRE, *L. monocytogenes*. Additionally, several advantages over previous studies can be noted in that the preparation was combined using dry stocks, the product was processed in a much shorter time-span with a prolonged shelf.

The reported antimicrobial abilities of chitosan can vary greatly, with studies by Aliasghari et al. (2016) and Zaghloul (2015) showing MIC values much higher than those presented here (ranging between 625 and 1250 μg/mL). While a separate study by Shanmugam et al. (2016) reported lower MIC values of 100 μg/mL versus *E. coli* and *S. aureus*. The lower MIC reported by Shanmugam et al. (2016) is quite significant in comparison to the values presented here (156.3–390.6 μg/mL), however it should be noted that the study extracted and produced its own form of chitosan, whereas the present study utilised

commercial chitosan, similarly to the two other mentioned studies. Modifications of chitosan to increase its antimicrobial potential have also shown success by reporting lowered MIC values (Hassan et al., 2018); however, these were not superior to the present study (Hassan et al., 2018). Chitosan itself being a polymer, commonly used in HME and other processes, owes for its resilience. Notably, the HME process alone without bioactives, as reported in this study, had no significant effect upon antibacterial effect. This present study also reported that ZnO can be effectively incorporated into a polymer for antimicrobial action. Pantani et al. (2013) noted that ZnO extrusion with the polymer polylactic acid (PLA) hindered diffusion of ZnO within the polymer, which would interfere with its antimicrobial abilities and may explain the reduced antimicrobial efficacy exhibited during this study.

5. Conclusion

The present study has demonstrated the potential application of four GRAS bioactives, namely $AgNO_3$, chitosan, ZnO and nisin, as alternative antibiotic compounds. The bioactives were able to inhibit bacterial growth of various standard ATCC strains and antibiotic resistant veterinary strains under similar conditions to that used in traditional antibiotic testing. Furthermore, the bioactives gave a clear indication to their suitability for polymer processing and incorporation, opening the way for their application as potential alternatives in many areas that include antibiotic use. In addition, the bioactive-polymer incorporation supports their use where traditional antibiotics were not previously appropriate (due to over/under-exposure, leading to AMR development) such as general disinfectants, antimicrobial-infused wound treatments, antimicrobial-embedded polymer devices and biofilm-based infection treatments. This constitutes the first study to report on the novel use of GRAS bioactives that have been embedded in a polymer carrier with view to use as potential, alterative antimicrobial therapeutic for challenging zoonotic and human infections. Future studies merited include determining biofilm disruptive capabilities of these bioactive-polymer combinations along with commensurate biocompatibility. The relationship between use of nonthermal reprocessing and sterilization modalities, such as electron beam and vaporized hydrogen peroxide, and maintenance of bioactive-polymer functionaty post treatment are also merited (McEvoy and Rowan, 2019; McEvoy et al., 2021). Also, studes revealing molecular and cellular mechanisms underpinning the effectiveness of this novel GRAS bioactive-polymer combintion on targeted AMR bacteria will help advance this platform technology (Farrell et al., 2011). Initial studies surrounding use of this novel co-polymer delivery process shows promise for use of heat-sensitive bioactives, such as for bio-based bioactives mined from food and from the bioeconomy.

Funding acknowledgement

The authors acknowledge funding support from Athlone Institute of Technology Presidential Doctorate project (AIT-2017-008) for this work.

CRediT authorship contribution statement

Kevin Masterson: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Elaine Meade:** Data curation, Formal analysis, Methodology, Writing – original draft. **Mary Garvey:** Formal analysis, Investigation, Methodology, Writing – original draft. **Mark Lynch:** Formal analysis, Methodology, Supervision, Writing – original draft. **Ian Major:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Neil J. Rowan:** Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

There is no conflict of interest for authors.

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Synergy Assessment of Four Antimicrobial Bioactive Compounds for the Combinational Treatment of Bacterial Pathogens

Kevin Masterson [1,*], Ian Major [2], Mark Lynch [1] and Neil Rowan [1]

[1] Bioscience Research Institute, Technological University of the Shannon, N37 HD68 Athlone, Ireland; marklynch@tus.ie (M.L.); nrowan@tus.ie (N.R.)
[2] PRISM Research Institute, Technological University of the Shannon, N37 HD68 Athlone, Ireland; imajor@tus.ie
* Correspondence: k.masterson@research.ait.ie

Abstract: Antimicrobial resistance (AMR) has become a topic of great concern in recent years, with much effort being committed to developing alternative treatments for resistant bacterial pathogens. Drug combinational therapies have been a major area of research for several years, with modern iterations using combining well-established antibiotics and other antimicrobials with the aim of discovering complementary mechanisms. Previously, we characterised four GRAS antimicrobials that can withstand thermal polymer extrusion processes for novel medical device-based and therapeutic applications. In the present study, four antimicrobial bioactive—silver nitrate, nisin, chitosan and zinc oxide—were assessed for their potential combined use as an alternative synergistic treatment for AMR bacteria via a broth microdilution assay based on a checkerboard format. The bioactives were tested in arrangements of two-, three- and four-drug combinations, and their interactions were determined and expressed in terms of a synergy score. Results have revealed interesting interactions based on treatments against recognised test bacterial strains that cause human and animal infections, namely *E. coli*, *S. aureus* and *S. epidermidis*. Silver nitrate was seen to greatly enhance the efficacy of its paired treatment. Combinations with nisin, which is a lantibiotic, exhibited the most interesting results, as nisin has no effect against Gram-negative bacteria when used alone; however, it demonstrated antimicrobial effects when combined with silver nitrate or chitosan. This study constitutes the first study to both report on practical three- and four-drug combinational assays and utilise these methods for the assessment of established and emerging antimicrobials. The novel methods and results presented in this study show the potential to explore previously unknown drug combination compatibility measures in an ease-of-use- and high-throughput-based format, which can greatly help future research that aims to identify appropriate alternative treatments for AMR, including the screening of potential new bioactives biorefined from various sources.

Keywords: antimicrobial resistance; bioactives; synergy analysis; drug combinations; two-drug combination; three-drug combination; four-drug combination; checkerboard assay; broth microdilution

Citation: Masterson, K.; Major, I.; Lynch, M.; Rowan, N. Synergy Assessment of Four Antimicrobial Bioactive Compounds for the Combinational Treatment of Bacterial Pathogens. *Biomedicines* **2023**, *11*, 2216. https://doi.org/10.3390/biomedicines11082216

Academic Editor: Maria A. Bonifacio

Received: 29 June 2023
Revised: 22 July 2023
Accepted: 31 July 2023
Published: 7 August 2023

1. Introduction

Antimicrobial resistance (AMR) has become a topic of academic interest, as it has reached a crisis point that has driven scientists to consider novel appropriate solutions to overcome it [1–3]. An ever-increasing number of antibiotic resistant bacterial species have emerged that pose serious threats to modern medicine, causing a loss in efficacy of critical front-line therapeutics [4]. Antibiotics remain our primary means of eliminating pathological bacterial infections, and while there has been a recent resurgence in the development of novel antibiotic compounds, additional ways of tackling AMR bacteria are urgently needed [5,6]. Research that aims to discover alternative antimicrobials has

been a major topic of interest, as it particularly hopes to circumvent the emerging resistance to mainstay antibiotics [7–9]. Additionally, the co-development of methods that can assess the efficacy of appropriate combinations of already established antimicrobial compounds is important to reduce reliance on a single treatment [10–12]. While a vast number of antimicrobial compounds are in use today, many have specific modes of action and, thus, have a narrow effective spectrum in terms of the bacterial species that they can target [13]. This issue can reduce their suitability in medical settings, which ideally require a more broad-spectrum treatment, given the frequent occurrence of co-infections [14]. Furthermore, selecting a narrow-effect spectrum that relies on a singular mechanism of microbial inactivation or inhibition can also make it easier for exposed bacteria to develop unwanted resistance. Thus, the use of two or more treatments in combination to treat bacterial infections represents a highly promising avenue of research. Checkerboard assays are well-documented methods used to assess the effects of different treatments when used in combination, whereby serially diluted concentrations of treatments are combined across a 96-well microtiter plate [15–18]. The resulting effects of combination therapy can be described as synergistic, additive, or antagonistic [16,19,20]. Synergy describes a total effect greater than the sum of the individual effects. An additive effect shows that the combined drugs exhibit a total effect equal to the sum of the individual effects, being no lesser or greater. An antagonistic effect describes combinations in which the total effect is lessened compared to the sum of the individual effects [21]. Combination therapies that result in an overall synergistic effect can allow a much greater impact to result from treatments that would normally hold less or, perhaps no, effect when used alone, such as in the case of AMR bacteria. While co-treatment therapies have been widely used in the treatment of diseases such as cancer, there is a rising interest in the synergistic abilities of previously established antimicrobial compounds [10,12,16].

In a previous study reported by these authors, the individual antibacterial capabilities of four GRAS bioactive compounds—silver nitrate ($AgNO_3$), nisin, chitosan and zinc oxide (ZnO)—were assessed against a number of type-strain bacterial species, as well as AMR wild-type strains [22]. These GRAS bioactives were unusual in the sense that they withstood temperatures used to extrude and process polymers used in the manufacturing of medical devices; thus, these bioactives offer interesting options for new therapeutic research. In the present study, these four bioactives will be assessed regarding their antimicrobial capabilities in combination with one another, using arrangements of two-, three- and four-drug combinations. For this initial combinational study, three standard type strains will be used, namely *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. *E. coli* and *S. aureus* were chosen as they represent Gram-negative and Gram-positive bacteria, respectively. *S. epidermidis* was included as it represents opportunistic Gram-positive bacterial pathogens and was observed to hold atypical behaviour against these four compounds in the previous study relative to *S. aureus*. The antimicrobial abilities of the two-drug combinations will be determined via use of a standard broth microdilution protocol in a checkerboard assay format, through which growth will be measured using turbidity absorbance readings. Three- and four-drug combinations will be assessed via use of novel versions of the checkerboard assay developed in the present study. The readings will be used to calculate the % growth of each treatment relative to the 100% growth control. While the checkerboard assay is a method commonly utilised to assess combination effects, there are various methods and programs developed for analysis of results [15–18]. The end results identified in the present study will analysed via use of the recently developed "synergy" python package, which can analyse large amounts of combinations and report their synergy scores [23].

2. Materials and Methods

2.1. Bioactive Solution Preparation

Silver nitrate ($AgNO_3$) (SKU: S8157, CAS: 7761-88-8), nisin, 2.5% (SKU: N5764, CAS: 1414-45-5), chitosan of low molecular weight (SKU: 448869, CAS: 9012-76-4), zinc oxide

(ZnO) and nanopowder of <100 nm in particle size (SKU: 544906, CAS: 1314-13-2) were purchased from Sigma-Aldrich/Merck (Merck Life Science Limited, Arklow, Co. Wicklow, Ireland). Chitosan was dissolved in 1% (v/v) acetic acid and adjusted to pH 5.5 using 0.4 M sodium hydroxide (NaOH). ZnO was suspended in dH_2O. Nisin was dissolved in a solution of 400 mM sodium chloride (NaCl), which had a pH of 3.25. These solutions were then sterilised through autoclaving. Nisin concentrations were reported in terms of active nisin content, with 1 g of commercial nisin powder containing 25 mg of active nisin. $AgNO_3$ was placed into a solution of 28% (v/v) Poly (ethylene glycol), which had an average molecular weight of 400 (PEG-400) and 26% (w/v) d-sorbitol [24]. This solution was then filter sterilised through use of a 0.2-micrometer syringe filter tip.

2.2. Bacterial Cell Culture

The standard strains, namely *E. coli* (ATCC 25922, NCTC 12241) and *S. aureus* (ATCC 29213, NCTC 12973), were purchased from Public Health England (Culture Collections, Public Health England, Salisbury, UK). *S. epidermidis* (ATCC 35984) was purchased from ATCC (LGC Standards, Middlesex, UK). Cultures were prepared via overnight incubation using tryptone soy agar (TSA). Colonies were then suspended in Mueller–Hinton broth (MHB) to 0.5 MacFarland absorbance for use as inoculum [25,26].

2.3. Two-Drug Combinational Broth Microdilution Assay

All steps were conducted under aseptic conditions or in closed systems. The antimicrobial properties of each bioactive solution in combinations of two were assessed in terms of their growth inhibitory capabilities, as determined via use of the broth microdilution method adapted from a previously published protocol [26]. Broth microdilution assays were carried out in flat bottom 96-well plates (untreated) against three chosen bacterial strains, namely *E. coli*, *S. aureus* and *S. epidermidis*. Before use, microplate lids were treated using a hydrophilic coating (20% (v/v) of isopropyl alcohol (IPA), 0.5% (v/v) of Triton-X100) [27]. Bacterial inoculums were prepared to give a final in-well concentration of 5×10^5 cfu/mL, as determined via absorbance readings. Two-drug combination assays were prepared in an 8 × 8 checkerboard layout, allowing a total of 64 combinations. Dilutions of drug A and B were prepared in Mueller–Hinton broth (MHB) at a concentration four times higher (4×) than the highest desired final in-well concentration. Serial dilutions (1:2) of drug A and drug B were prepared in separate 96-well plates and combined in the final test plate (1:2 dilution) (See Figures S1 and S2). Each well was then inoculated with the prepared bacterial inoculum (1:2 dilution). Absorbance of the plate was measured using a BioTek® Synergy HT microplate reader and Gen5 Microplate Reader Software (Version 2.01.14) (BioTek® Instruments GmbH, Bad Friedrichshall, Germany). The plate was read using an endpoint absorbance read at 625 nM, and results were recorded as time-point 0 (t = 0) before incubation. This process allowed measurement of any turbidity caused by treatments and was be used as a blank. The plate was placed in a container to help prevent loss of well volume due to evaporation. The container was placed on a rotary incubator at 120 RPM, 37 °C for 18 h. Following incubation, plate absorbance was read (variable shake, and the 1-min endpoint absorbance was read at 625 nm). Results were recorded as timepoint 18 (t = 18). The absorbance values were used to calculate the % inhibition for each treatment well.

2.4. Three-Drug Combinational Broth Microdilution Assay

Three-drug combination assays were carried out as per the two-drug combination assay, albeit using a 6 × 6 checkerboard layout. Six such checkerboards were prepared by combining drug A and drug B, as per a two-drug combination assay, and different concentrations of drug C was added to each individual checkerboard. This setup allowed 6 concentrations of drug A, drug B and drug C to be assessed in combination (6 × 6 × 6), with a total of 216 combinations (See Figure S3 for example layout). The experiment was split across three 96-well plates, allowing two 6 × 6 checkerboards per plate. A separate

broth microdilution assay of single treatments was also carried out as a control to ensure that the treatments and bacteria tested performed in a nominal manner. Incubations and absorbance readings were carried out as per two-drug combinational assay.

2.5. Four-Drug Combinational Broth Microdilution Assay

Four-drug combination assays were carried out in a 4 × 4 checkerboard layout, which built on the three-drug layout design. The layout was designed in such a way that four 4 × 4 checkerboards (CBs) were set up within four 96-well plates. Each CB combined drug A with drug B. Each of these four CBs then had a different concentration of drug C added to it. To all CBs within each plate, a difference concentration of drug D will be added. The resulting system will yield a 4 × 4 × 4 × 4 combination (totalling in 256 combinations) (see Figure S4 for example layout).

A separate broth microdilution assay was also carried out using drugs A, B, C and D in tandem with the four-drug combination assay, which was used as a control to ensure that the individual treatments and bacteria being tested performed in a nominal manner. Plate and inoculum preparation, incubations and absorbance readings were all carried out as per the two-drug combinational assay.

2.6. Analysis of Results for the Determination of Synergy/Antagonism

Results from drug combination assays were analysed to determine drug interactions in terms of synergy or antagonism via the "synergy" python package [28]. Input data for synergy were prepared in an excel document using the concentration of each drug (μg/mL) and the % growth. Input data contained an individual column for the concentration of each drug ("drug1.conc", "drug2.conc", "drug3.conc" or "drug4.conc"). The Bliss model was chosen due to its simplicity and ability to analyse four-drug combinations. The reported response was expressed in terms of % growth. The response was input under the column "effect" and expressed as a decimal fraction of 1 (i.e., 100% growth = 1.0, 50% = 0.5, 0% = 0.0). Data were then exported as a .csv file. The synergy package was opened and run using PyCharm (version 2020.2) (JetBrains s.r.o, Prague, Czech Republic), which is a python-integrated development environment (IDE). Following the synergy documentation, input data were imported and analysed using the Bliss model. Results were expressed in terms of a synergy score, with a positive score indicating synergy, a score of 0 representing no effect and negative scores representing antagonism.

3. Results

Due to the number of combinations analysed during this study, only the three highest-scoring interactions of each combination and their average values will be reported and discussed. Synergy scores represent the magnitude of the combination interactions, where a positive score indicates synergy, scores close to 0 indicate an additive effect, a score of 0 represents no effect and negative scores represent antagonism. 2D heat-maps of all 64 combinations of each two-drug combination against each test bacterial species are presented in Figures 1–6 showing each combination's synergy score based on a colour scale, which is shown in the legend. Bar graphs have been prepared and presented in Figures 7–9 for each two-drug, three-drug and four-drug combination respectively, showing the three highest-scoring drug combinations (Combo 1–3 on the *x*-axis) at the drug concentrations (μg/mL) shown on the left *y*-axis, with the calculated Bliss synergy score shown on the right *y*-axis (see Tables S1–S5 for the graphed data).

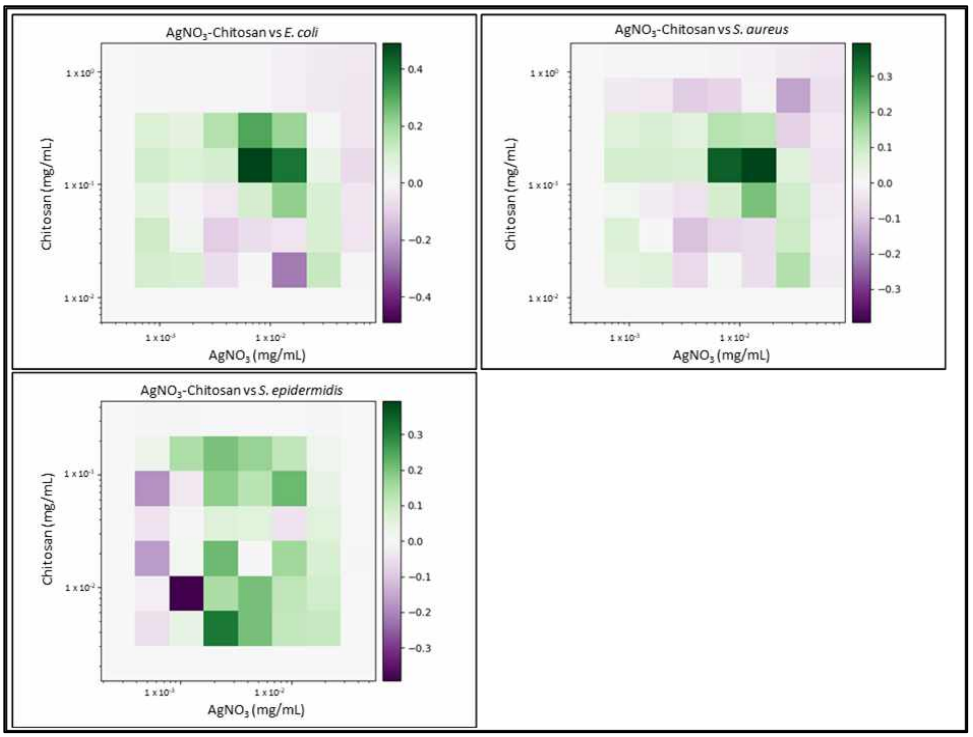


Figure 1. $AgNO_3$–chitosan synergy heat map: Graphs show heat map of synergy between silver nitrate ($AgNO_3$) and Chitosan in inhibiting *E. coli, S. aureus* and *S. epidermidis* growth as determined via broth microdilution and absorbance readings. Inhibition results were analysed using the synergy python package using the Bliss synergy model. The synergy python package produced the heatmap graphs of each combination result, giving visual presentations of combinations of synergy (green) or antagonism (purple). $n = 3$.

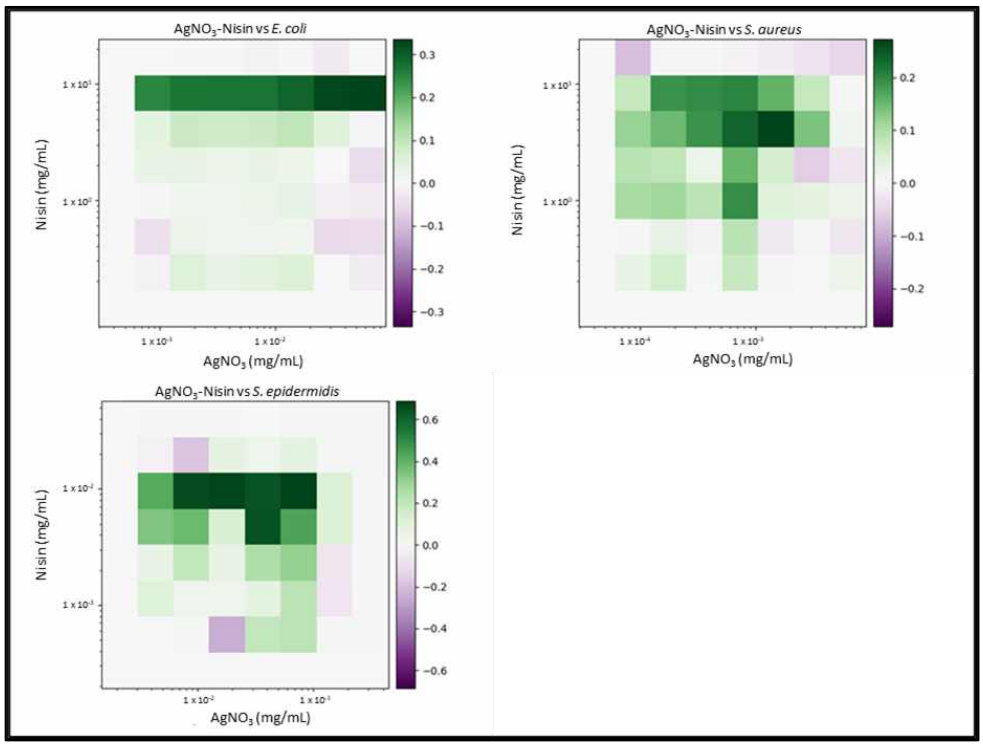


Figure 2. $AgNO_3$–nisin synergy heat map: Graphs show heat map of synergy between silver nitrate ($AgNO_3$) and Nisin in inhibiting *E. coli*, *S. aureus* and *S. epidermidis* growth as determined via broth

microdilution and absorbance readings. Inhibition results were analysed with the synergy python package using the Bliss synergy model. The synergy python package produced the heatmap graphs of each combination result, giving visual presentations of combinations of high (green) and low (purple) synergy. $n = 3$.

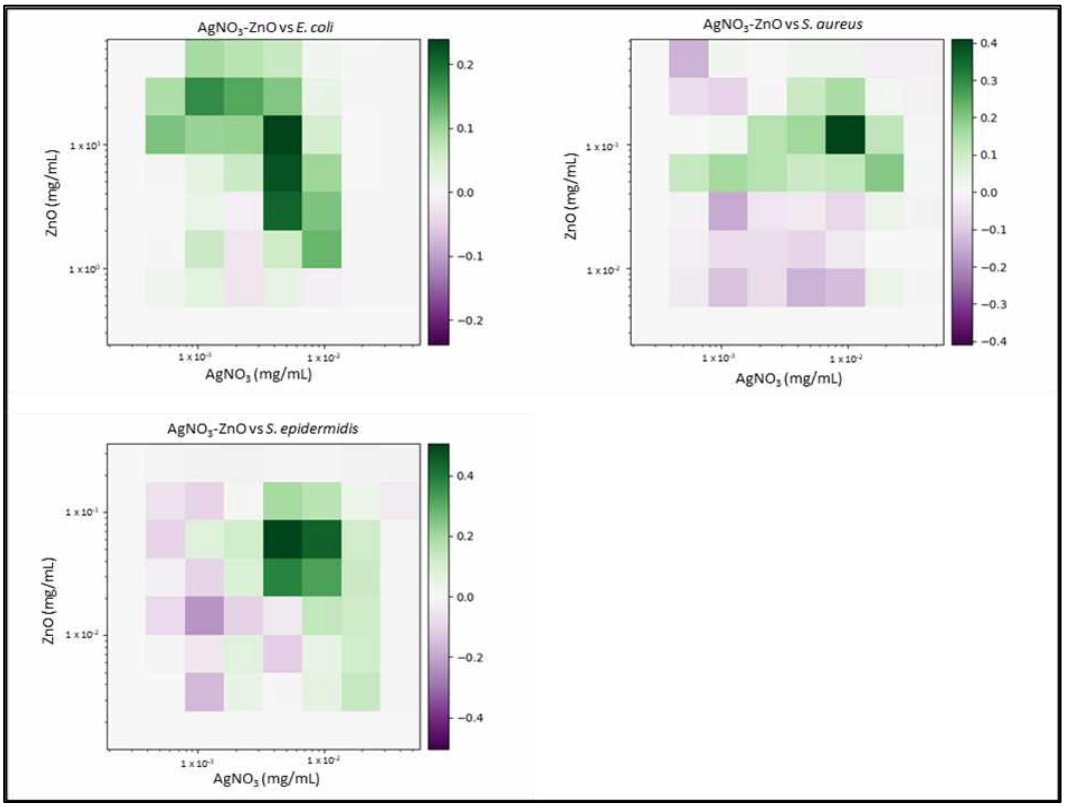


Figure 3. $AgNO_3$–ZnO synergy heat map: Graphs show heat map of synergy between silver nitrate ($AgNO_3$) and zinc oxide (ZnO) in inhibiting *E. coli*, *S. aureus* and *S. epidermidis* growth, as determined via broth microdilution and absorbance readings. Inhibition results were analysed via the synergy python package using the Bliss synergy model. The synergy python package produced the heatmap graphs of each combination result, giving visual presentations of combinations of high (green) and low (purple) synergy. $n = 3$.

3.1. Two-Drug Combinations

- $AgNO_3$–Chitosan

$AgNO_3$ and Chitosan reported good synergistic interactions against each bacterial strain. The combination reported the highest average synergy scores against *E. coli* (average 0.4) and *S. aureus* (average 0.32). While the average concentration of chitosan was similar to that of the MIC versus *E. coli*, $AgNO_3$ was reported to be present in lower concentrations. The most effective combination versus *S. aureus* reported concentrations that were 1/2 the MIC, with inhibition being approximately 69%. Results versus *S. epidermidis* reported good overall synergy, as much lower concentrations of each treatment exhibited more effective inhibition, and the second reported combination exhibited 99% inhibition, with 1/3 of the MIC of $AgNO_3$ and less than 1/2 of the MIC of chitosan being used.

- $AgNO_3$–Nisin

$AgNO_3$ and Nisin demonstrated a number of highly synergistic combinations (an average 0.32 versus *E. coli* and an average 0.24 versus *S. aureus*), as well as reporting the highest two-drug score from this study (average 0.68 versus *S. epidermidis*). While the highest-scoring combinations versus *E. coli* did not report inhibition exceeding 70%, there was moderate synergy observed compared to $AgNO_3$ used alone at the same concentrations.

The third highest-scoring combination versus *S. aureus* reported 99% inhibition, using less than 1/4 MIC of $AgNO_3$ and 1/10 MIC of nisin. The three highest-scoring combinations versus *S. epidermidis* indicated that a concentration of 10 μg/mL $AgNO_3$ was most effective in enabling nisin, which was reported to be present in relatively low concentrations, while still having a notable effect upon bacterial growth.

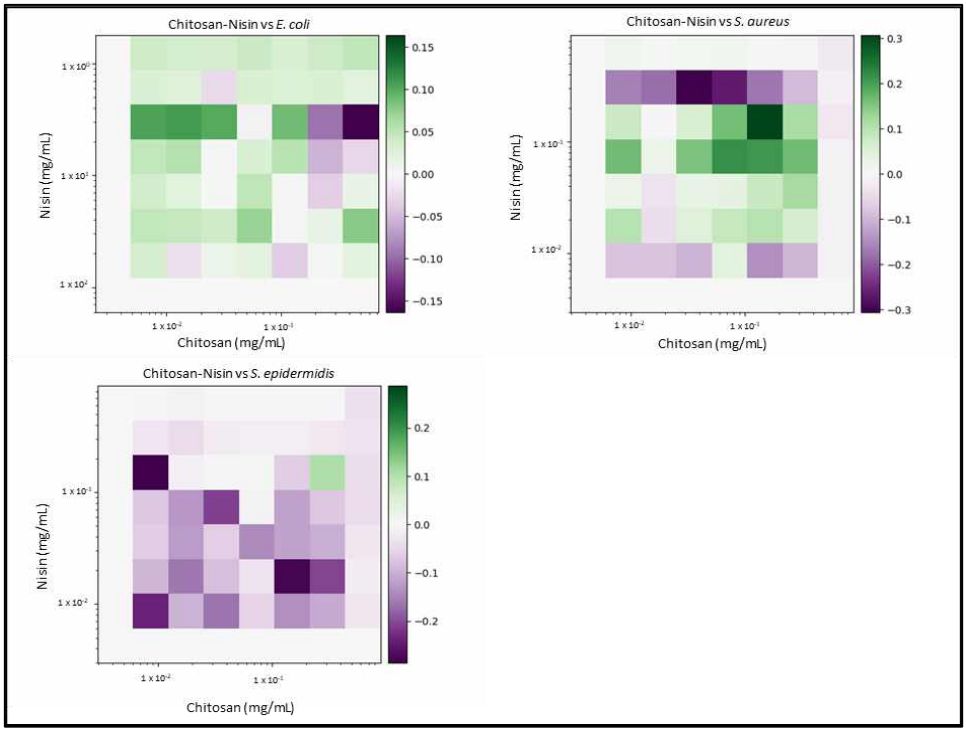


Figure 4. Chitosan–nisin synergy heat map: Graphs show heat map of synergy between Chitosan and Nisin in inhibiting *E. coli*, *S. aureus* and *S. epidermidis* growth, as determined via broth microdilution and absorbance readings. Inhibition results were analysed via the synergy python package using the Bliss synergy model. The synergy python package produced the heatmap graphs of each combination result, giving visual presentations of combinations of high (green) and low (purple) synergy. $n = 3$.

- $AgNO_3$–ZnO

$AgNO_3$ and ZnO reported moderate synergy against *E. coli* (average 0.22) and *S. aureus* (average 0.26), as well as relatively high synergy versus *S. epidermidis* (average 0.44). The highest reported *E. coli* combination exhibited 98.5% growth inhibition at an $AgNO_3$ concentration 1/4MIC and a ZnO concentration 1/2.5MIC, demonstrating a noticeable increase in the efficacy in both treatments. *S. aureus* results reported that lower concentrations of both $AgNO_3$ and ZnO exhibited greater effect when combined. One reported combination exhibited 95.5% growth inhibition using 1/1.8MIC $AgNO_3$ and 1/2.5MIC ZnO. $AgNO_3$ and ZnO demonstrated the second highest-scoring average of all two-drug combinations (average 0.44) versus *S. epidermidis*. Reported combinations exhibited effective growth inhibition at much lower concentrations, even reaching 95.4% growth inhibition with 1/1.6MIC $AgNO_3$ and 1/3.33MIC ZnO.

- Nisin–Chitosan

Nisin and chitosan reported mixed results in combination. The highest-scoring combinations were identified versus *S. aureus* (average 0.24); however, the highest inhibition of these combinations reached only 50%, with no major reductions being seen in the concentrations of nisin or chitosan. Results versus *E. coli* show that greater concentrations of chitosan were needed to enable nisin; however, these concentrations exceeded the MIC of chitosan, making the combination ineffective. Results versus *S. epidermidis* demonstrated

no major interactions, being close a synergy score of 0 in all combinations. Only one combination reported effective synergy, which exhibited 87.3% inhibition with a score of 0.11; however, the concentration of nisin used in this combination exceeded that of its MIC when tested alone.

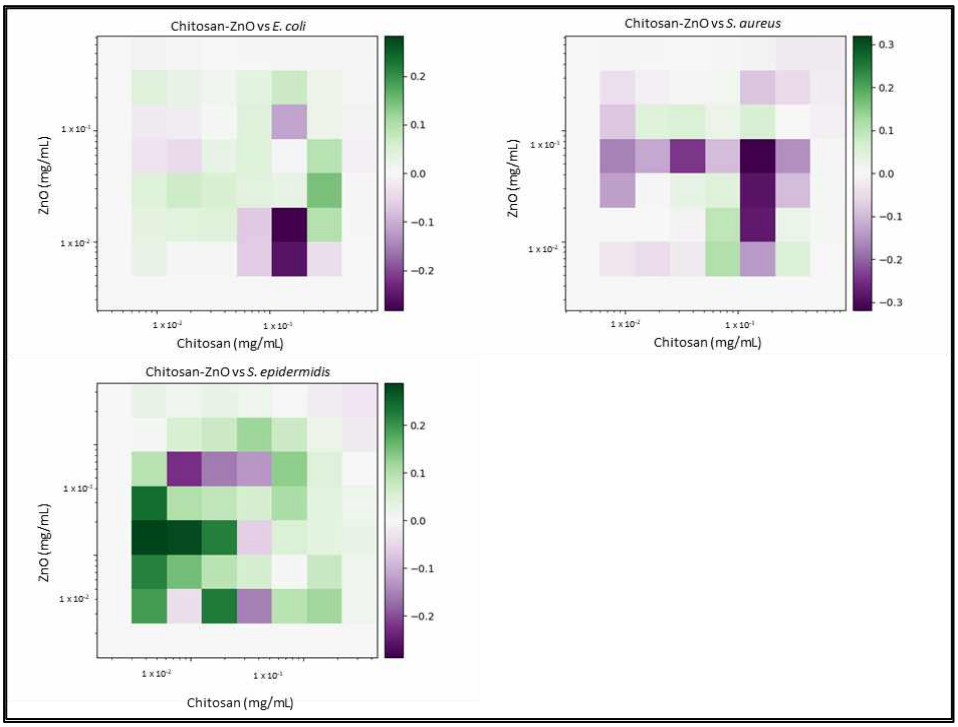


Figure 5. Chitosan–ZnO synergy heat map: Graphs show heat map of synergy between Chitosan and zinc oxide (ZnO) in inhibiting *E. coli*, *S. aureus* and *S. epidermidis* growth, as determined via broth microdilution and absorbance readings. Inhibition results were analysed via the synergy python package using the Bliss synergy model. The synergy python package produced the heatmap graphs of each combination result, giving visual presentations of combinations of high (green) and low (purple) synergy. $n = 3$.

- Chitosan–ZnO

Chitosan and ZnO reported very few synergistic interactions versus *E. coli* (average 0.11), *S. aureus* (average 0.09) and *S. epidermidis* (average 0.27). Analysis of interactions versus *E. coli* show that a high concentration of chitosan was required for synergy to be identified; however, the amount of chitosan was 2 × MIC, and the synergy score was relatively low. While synergy was seen versus *S. aureus* at quite low concentrations of the two highest-scoring combinations, the exhibited growth inhibition was not noteworthy (3.1%, 2.6%, respectively). Combinations versus *S. epidermidis* reported moderate synergy at quite low concentrations of each combination; however, the inhibition did not exceed 31%.

- Nisin–ZnO

Nisin and ZnO reported low synergy versus *E. coli* (average 0.08), *S. aureus* (average 0.06) and *S. epidermidis* (average 0.14). The highest-scoring combination versus *E. coli* (0.09) did not yield noteworthy inhibition, while the next highest-scoring combinations reported concentrations of ZnO that exceed the MIC in order to enable nisin. Highest-scoring combinations versus *S. aureus* reported low concentrations of each treatment; however, they had no noteworthy growth inhibitory effect (3.7–14%). Combinations versus *S. epidermidis* exhibited moderate inhibitory effects; however, the concentrations of ZnO exceeded that of the average MIC, and concentrations of nisin were not much lower that the previously reported MIC average.

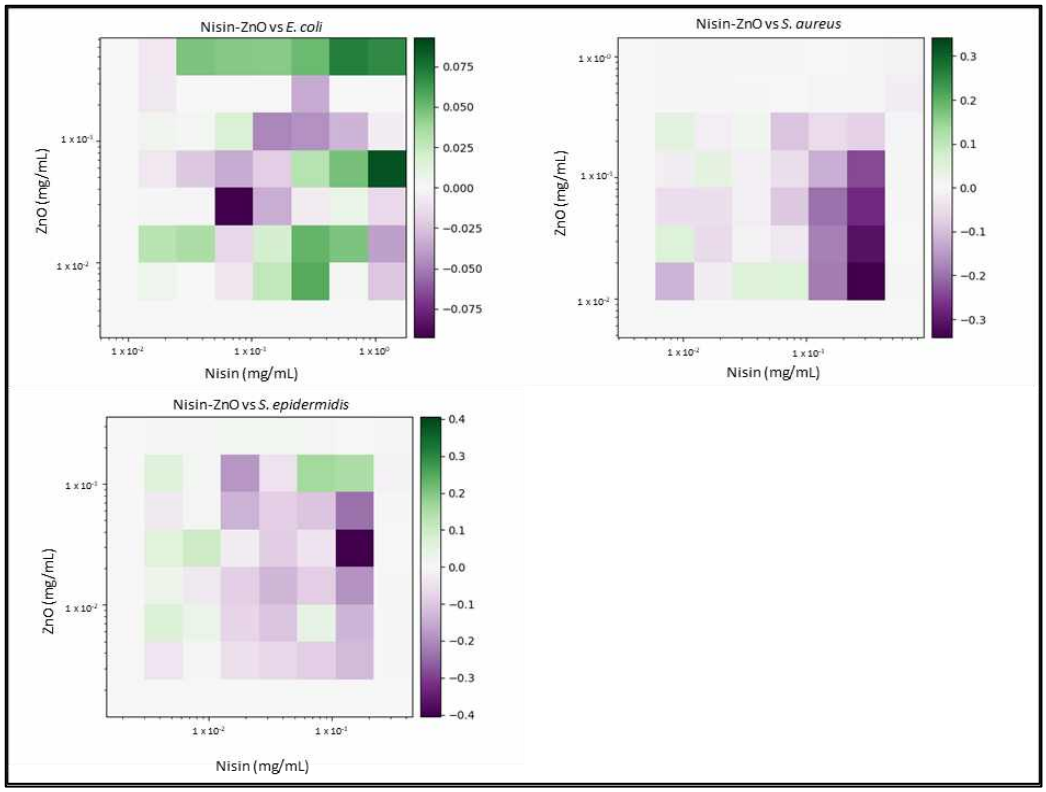


Figure 6. Nisin–ZnO synergy heat map: Graphs that show heat map of synergy between nisin and zinc oxide (ZnO) regarding the inhibition of *E. coli*, *S. aureus* and *S. epidermidis* growth, as determined via broth microdilution and absorbance readings. Inhibition results were analysed via the synergy python package using the Bliss synergy model. The synergy python package produced the heatmap graphs of each combination results, giving visual presentations of combinations of high (green) and low (purple) synergy. $n = 3$.

3.2. Three-Drug Combinations

- Chitosan–$AgNO_3$–Nisin

Chitosan, $AgNO_3$ and nisin reported moderate-to-high synergy in growth inhibition versus *E. coli* (average 0.38), *S. aureus* (average 0.56) and *S. epidermidis* (average 0.43). While the higher scoring combinations versus *E. coli* included high concentrations of chitosan (80–160 μg/mL), reported concentrations of $AgNO_3$ were low (2–4 μg/mL) and had 99% inhibition. Concentrations of nisin were rather high, relative to those of the other test species (3.91–7.81 μg/mL). The highest-scoring combination versus *S. aureus* reported relatively low concentrations of each compound (78.13 μg/mL chitosan, 8 μg/mL $AgNO_3$ and 10.63 μg/mL nisin) and expressed 99% inhibition. The second highest-scoring combination showed similar concentrations and levels of inhibition; however, when used twice, the amount of $AgNO_3$ (16 μg/mL) was still less than the previously reported MIC. The third highest-scoring combination reported lower chitosan (39.06 μg/mL), though it did not fully inhibit *S. aureus* growth (71.45%). These combinations versus *S. aureus* reported the second highest average score of all three-drug test combinations. Combinations versus *S. epidermidis* reported near full inhibition (92–96%), as well as good synergy and low concentrations of chitosan (39.06–78.13 μg/mL) and nisin (0.63–1.25 μg/mL); however, concentrations of $AgNO_3$ were near to the MIC (8–16 μg/mL).

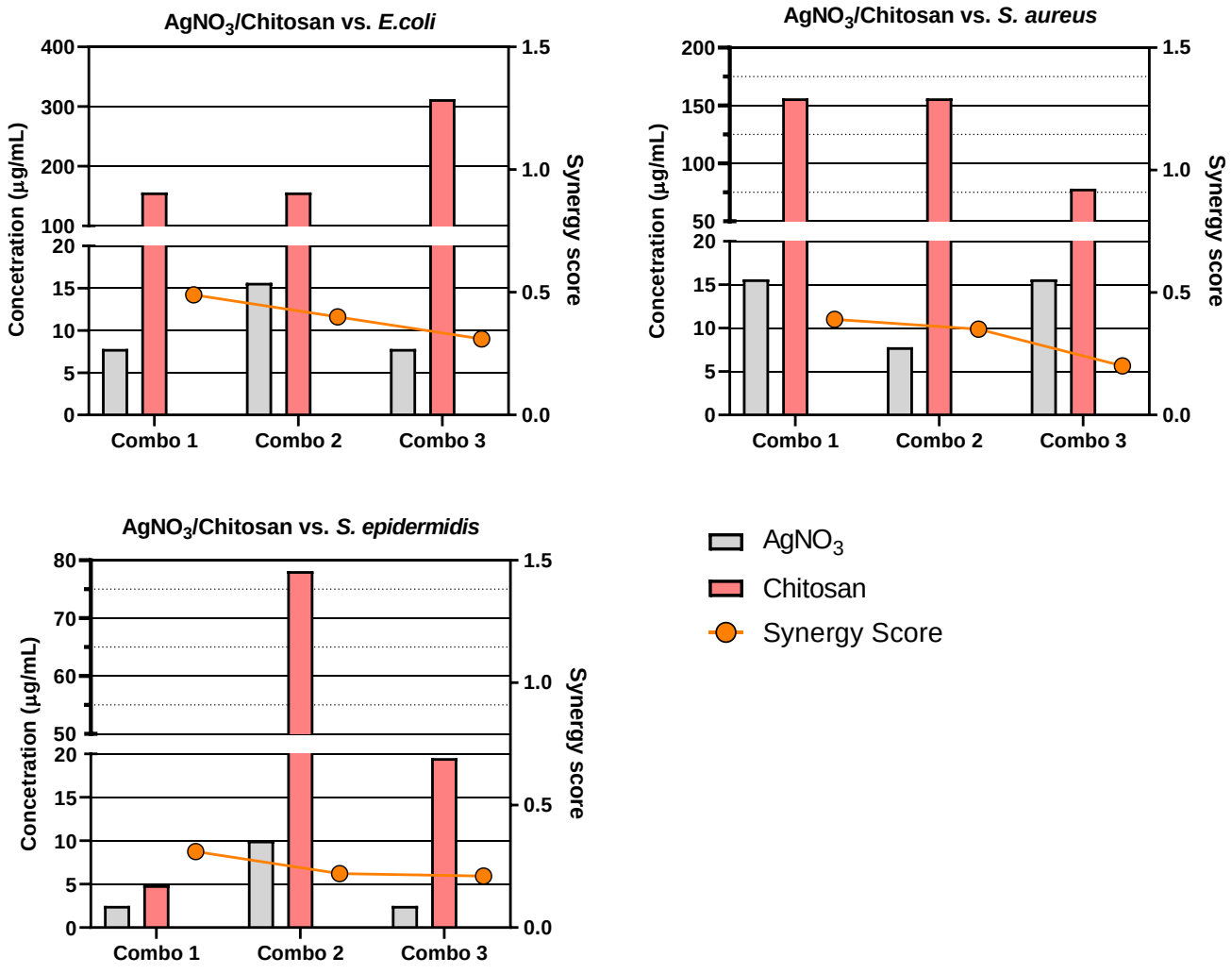
- Chitosan–$AgNO_3$–ZnO

Chitosan, $AgNO_3$ and ZnO held moderately low synergy versus *E. coli* (average 0.14), *S. aureus* (average 0.28) and *S. epidermidis* (average 0.35), with combinations versus *E. coli* having the lowest scores of all three-drug combinations. Scores for *E. coli* were quite low,

and each of the reported combinations exhibited full inhibition (97.15–100%) at low concentrations of chitosan (40–80 μg/mL), $AgNO_3$ (0.5–4 μg/mL) and ZnO (20 μg/mL). The highest scores versus *S. aureus* reported consistently low concentrations of ZnO (62.5 μg/mL), as well as low concentrations of chitosan (39.06–78.13 μg/mL) and $AgNO_3$ (8–16 μg/mL), while growth inhibition was high (97.55–98.32%). Combinations versus *S. epidermidis* reported good growth inhibition (87.95–98.4%) at relatively low concentrations of chitosan (20–40 μg/mL), $AgNO_3$ (2–4 μg/mL) and ZnO (10 μg/mL).

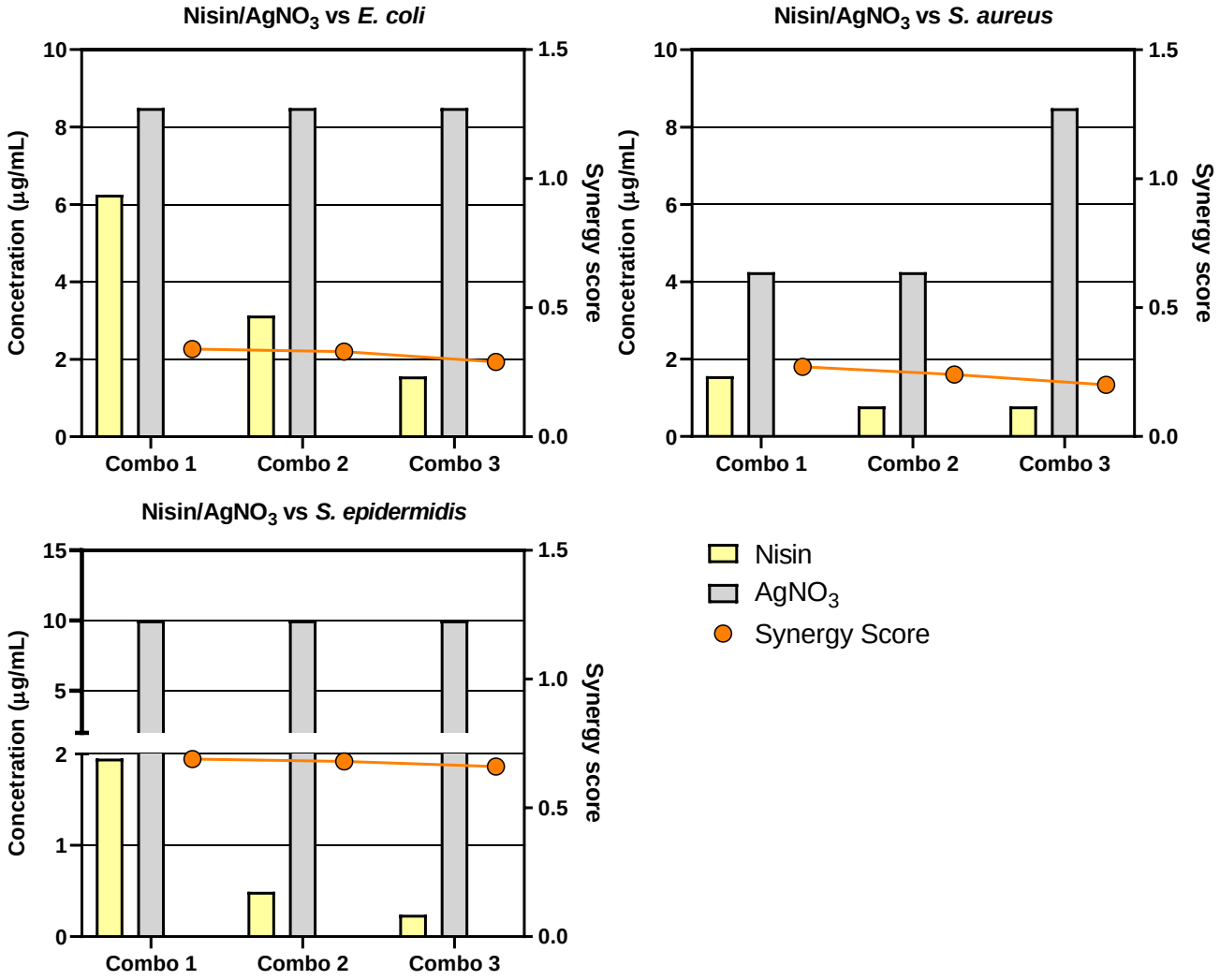
- Nisin–$AgNO_3$–ZnO

Nisin, $AgNO_3$ and ZnO reported strong synergy versus *E. coli* (average 0.36), *S. aureus* (average 0.38) and *S. epidermidis* (average 0.53). Results versus *E. coli* show that very high concentrations of nisin (31.25 μg/mL) yielded high synergy at low concentrations of $AgNO_3$ (0.5–1 μg/mL) and ZnO (31.25–62.5 μg/mL); however, growth inhibition did not exceed 77%. Combinations versus *S. aureus* reported moderate synergy at low concentrations of nisin (0.63–1.25 μg/mL) and moderate concentrations of $AgNO_3$ (4–16 μg/mL) and ZnO (39.06 μg/mL). Combinations versus *S. epidermidis* reported the third highest average score synergy at low concentrations of nisin (1.25–2.5 μg/mL), $AgNO_3$ (2–4 μg/mL) and ZnO (5 μg/mL); however, these combinations exhibited low-to-moderate growth inhibition (33.6–72.3%).



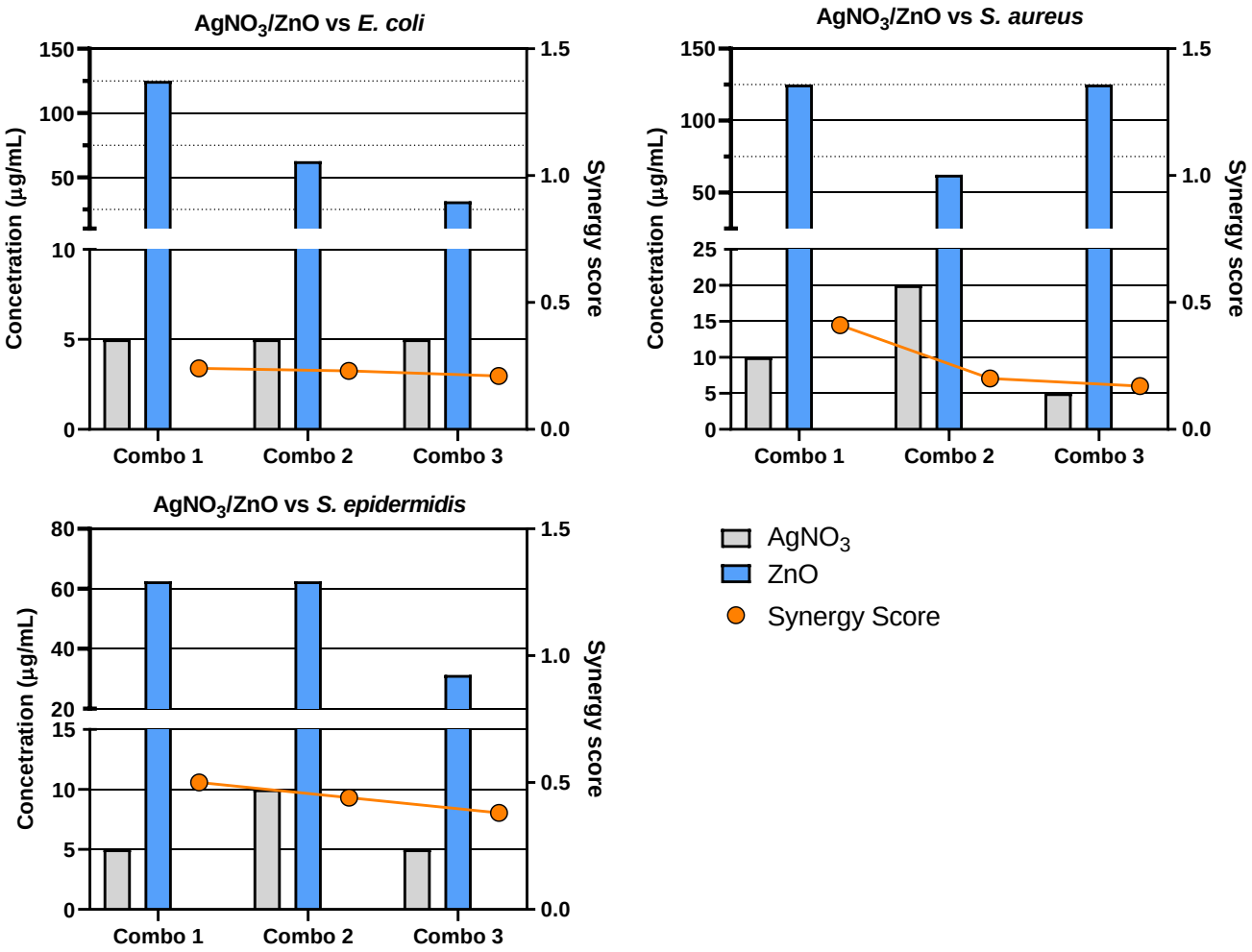
(**A**)

Figure 7. *Cont.*



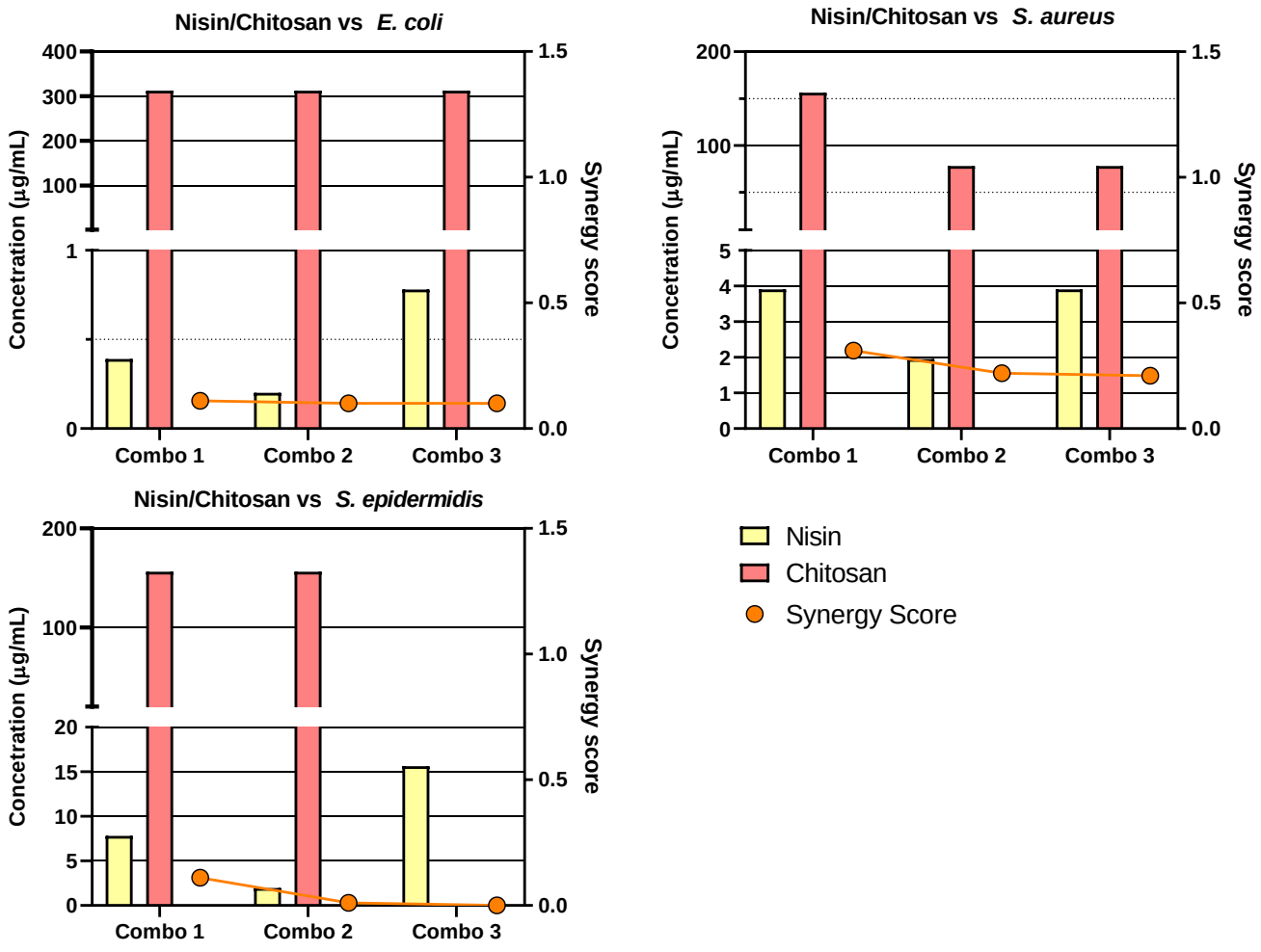
(**B**)

Figure 7. *Cont.*



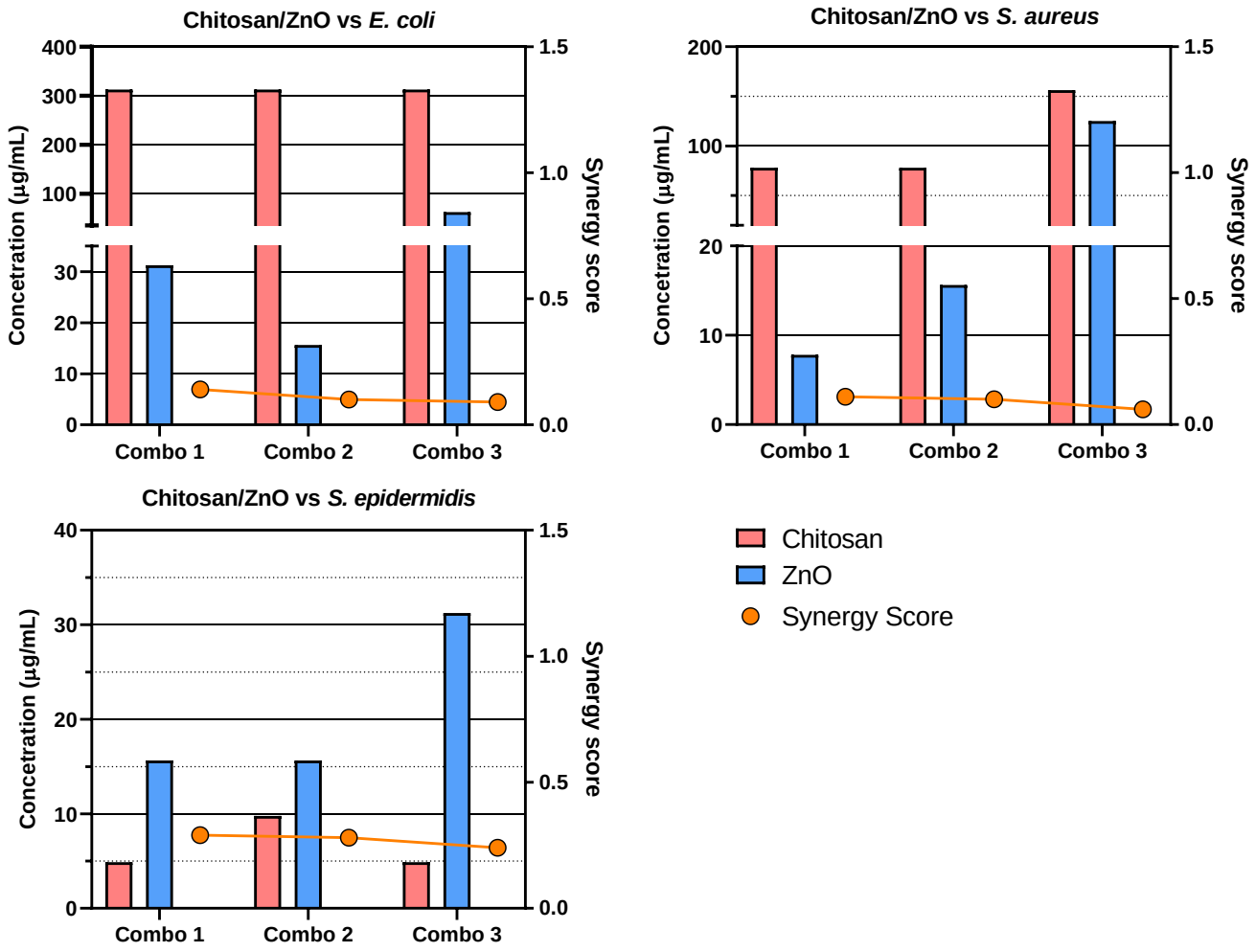
(**C**)

Figure 7. *Cont.*



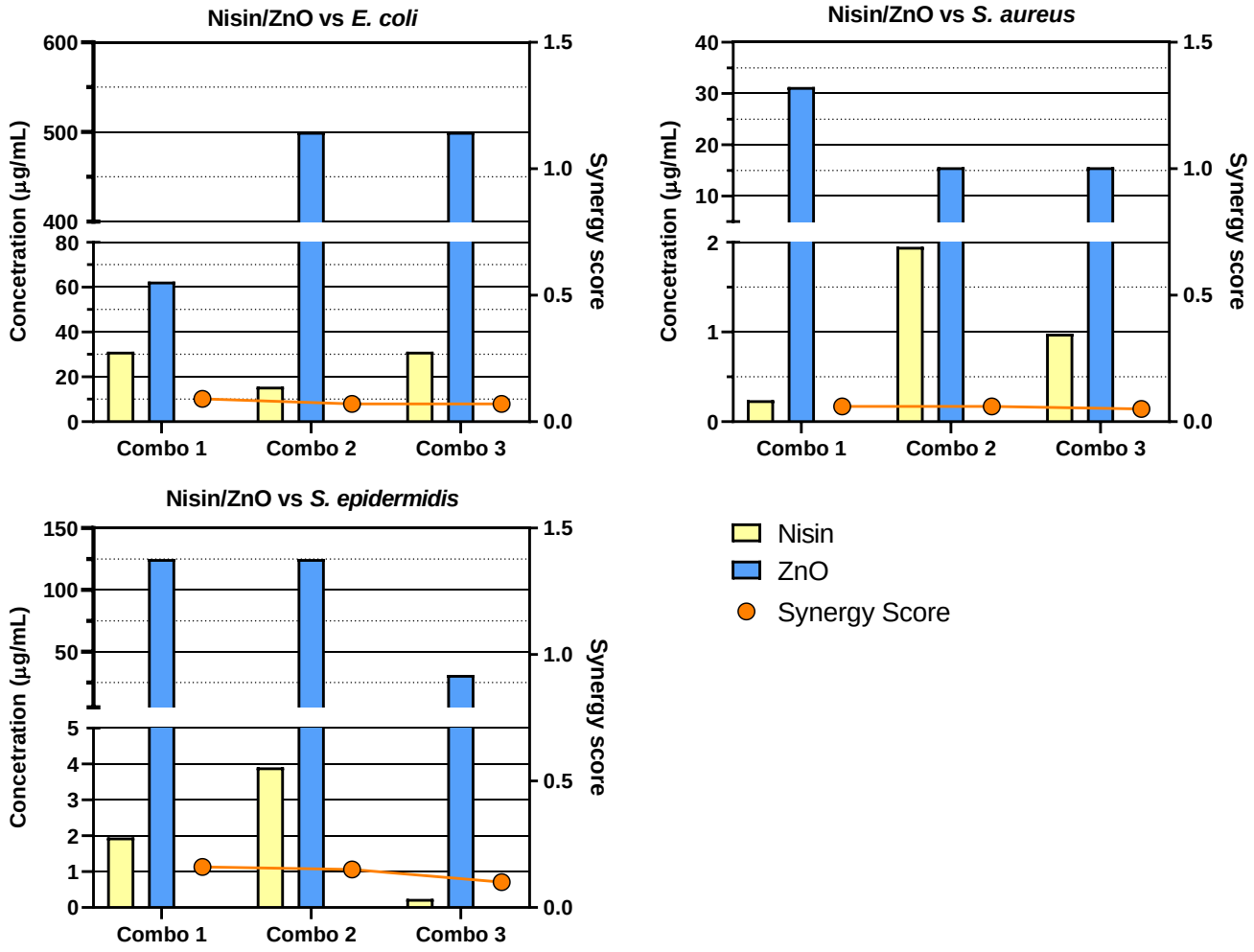
(**D**)

Figure 7. *Cont.*



(**E**)

Figure 7. *Cont.*



(**F**)

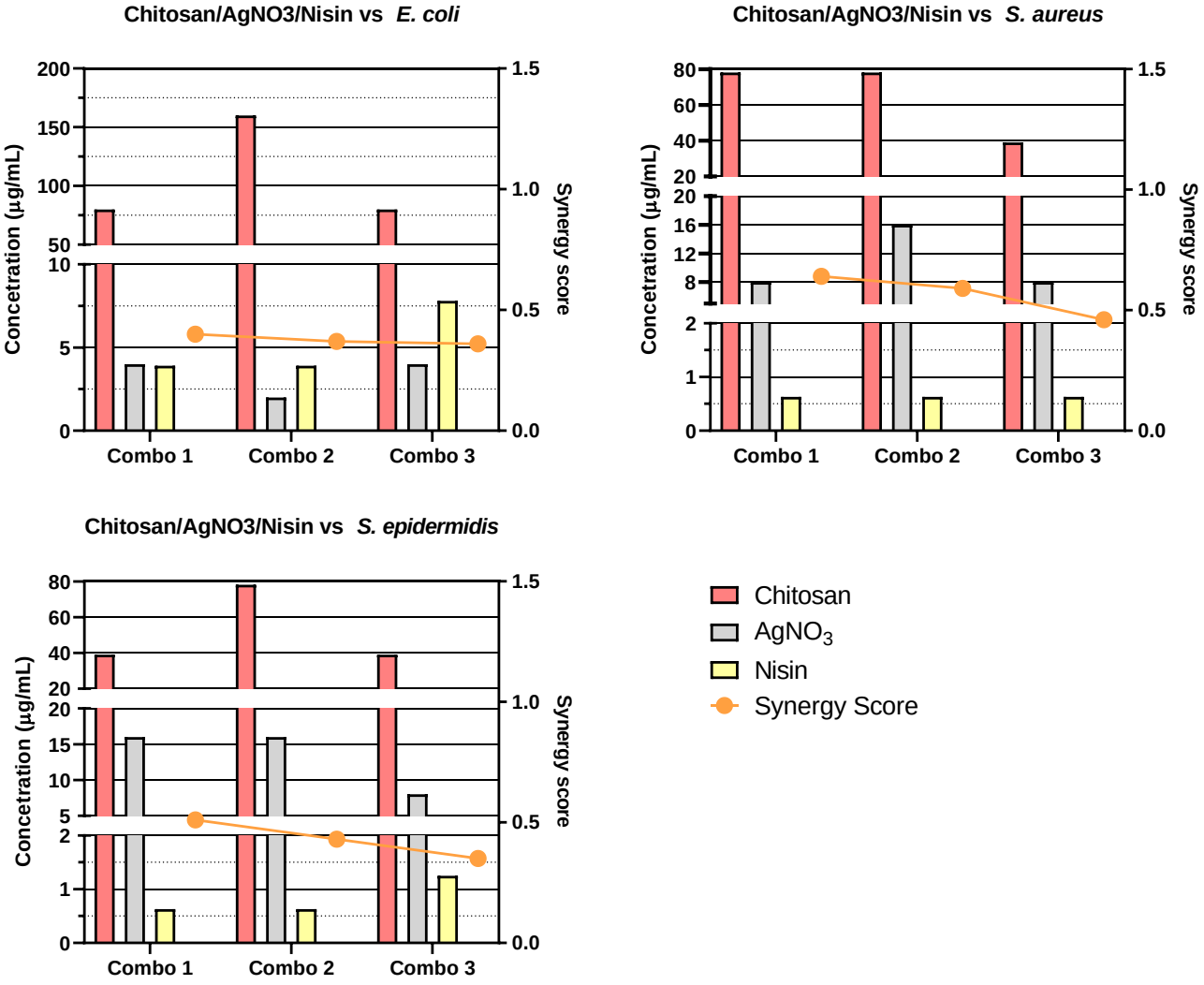
Figure 7. Synergy score results of the top three two-drug combinations: Bar graphs that present the concentrations of each drug and their respective synergy scores from the top three two-drug combinations (**A**–**F**) against *E. coli*, *S. aureus* and *S. epidermidis*. (**A**) $AgNO_3$/Chitosan, (**B**) Nisin/$AgNO_3$, (**C**) $AgNO_3$/ZnO, (**D**) Nisin/Chitosan, (**E**) Chitosan/ZnO and (**F**) Nisin/ZnO. Bars show drug concentrations, as indicated on the left *y*-axis, and the line/symbols show each combination's (combo) synergy score, as indicated on the right *y*-axis.

- Nisin–Chitosan–ZnO

Nisin, chitosan and ZnO reported low inhibition synergy versus *E. coli* (average 0.23) and *S. epidermidis* (average 0.21); however, combinations versus *S. aureus* reported the highest synergy score across all three-drug combinations (average 0.83). Concentrations of the reported combinations versus *E. coli* indicated poor synergy between treatments, as high concentrations of chitosan (9.77–312.5 µg/mL) and ZnO (31.25–125 µg/mL) were utilised. Combinations that used low concentrations of each drug exhibited very low inhibition (26.6%).

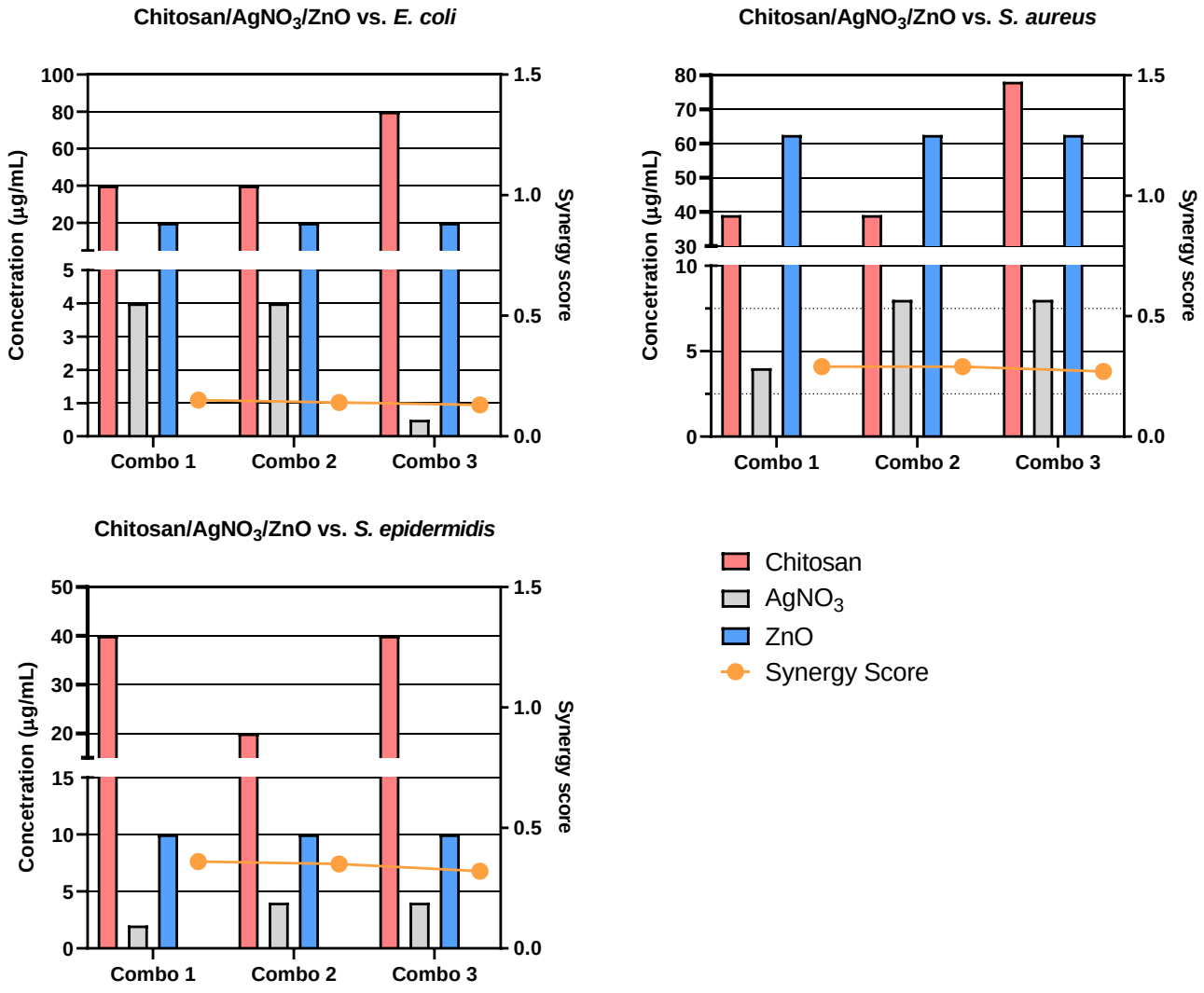
The highest-scoring combination (0.93) versus *S. aureus* reported low concentrations of nisin (3.91 µg/mL), chitosan (39.06 µg/mL) and ZnO (62.5 µg/mL) with high inhibition (98.7%). The second highest-scoring combination (0.84) also reported low concentrations of nisin (0.977 µg/mL), chitosan (156.25 µg/mL) and ZnO (62.5 µg/mL) with high inhibition (99.4%). While the third highest-scoring combination reported low concentrations of nisin (0.977 µg/mL), chitosan (78.13 µg/mL) and ZnO (62.5 µg/mL), the reported growth

inhibition was moderate (71.5%). Moreover, while results versus *S. epidermidis* reported low concentrations of nisin (0.98–1.95 µg/mL), chitosan (39.06 µg/mL) and ZnO (31.25 µg/mL), along with high growth inhibition (99%), there was little synergy observed, as denoted by the two highest scores. The third highest-scoring combination reported a very high concentration of chitosan (625 µg/mL).



(**A**)

Figure 8. *Cont.*



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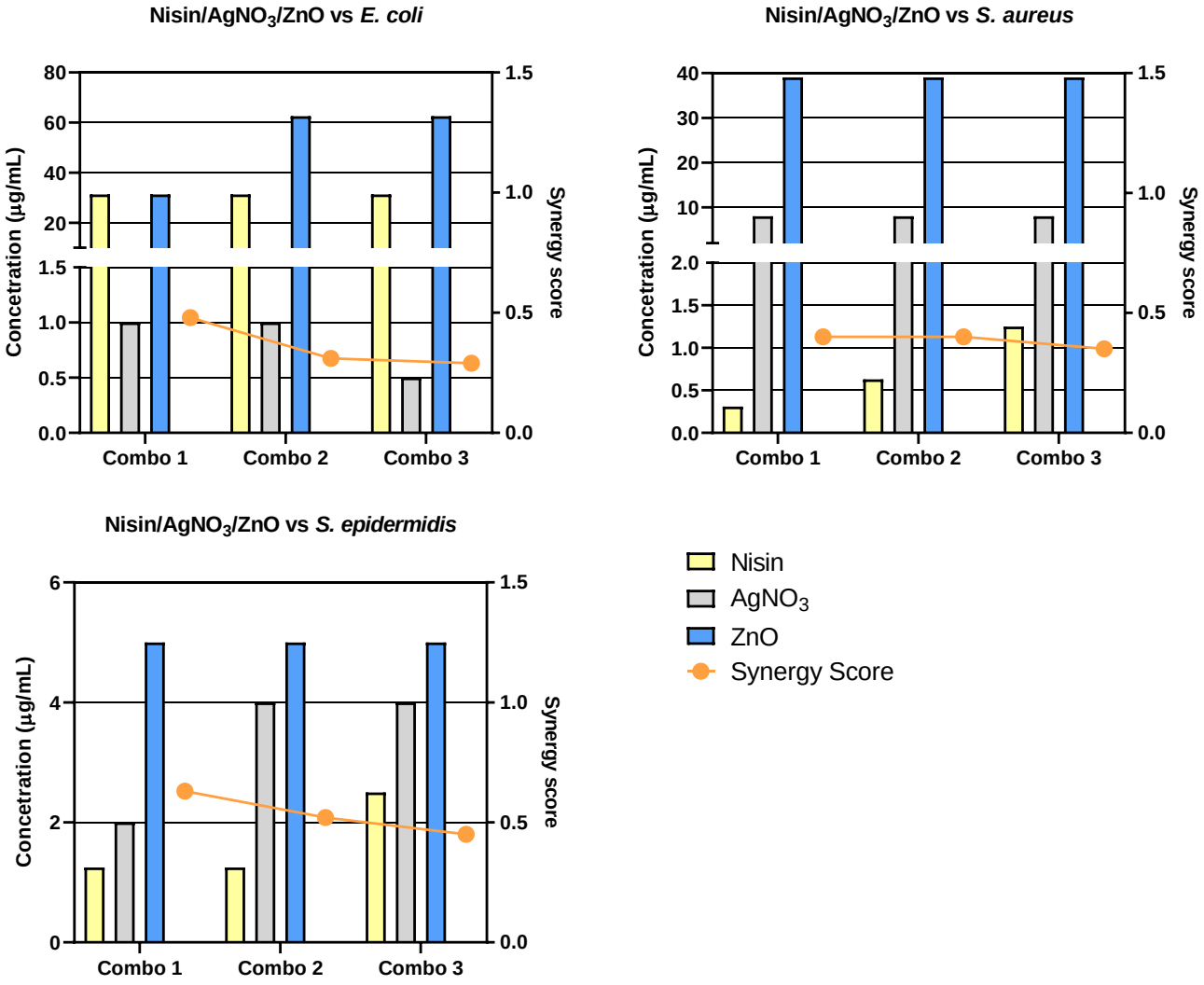
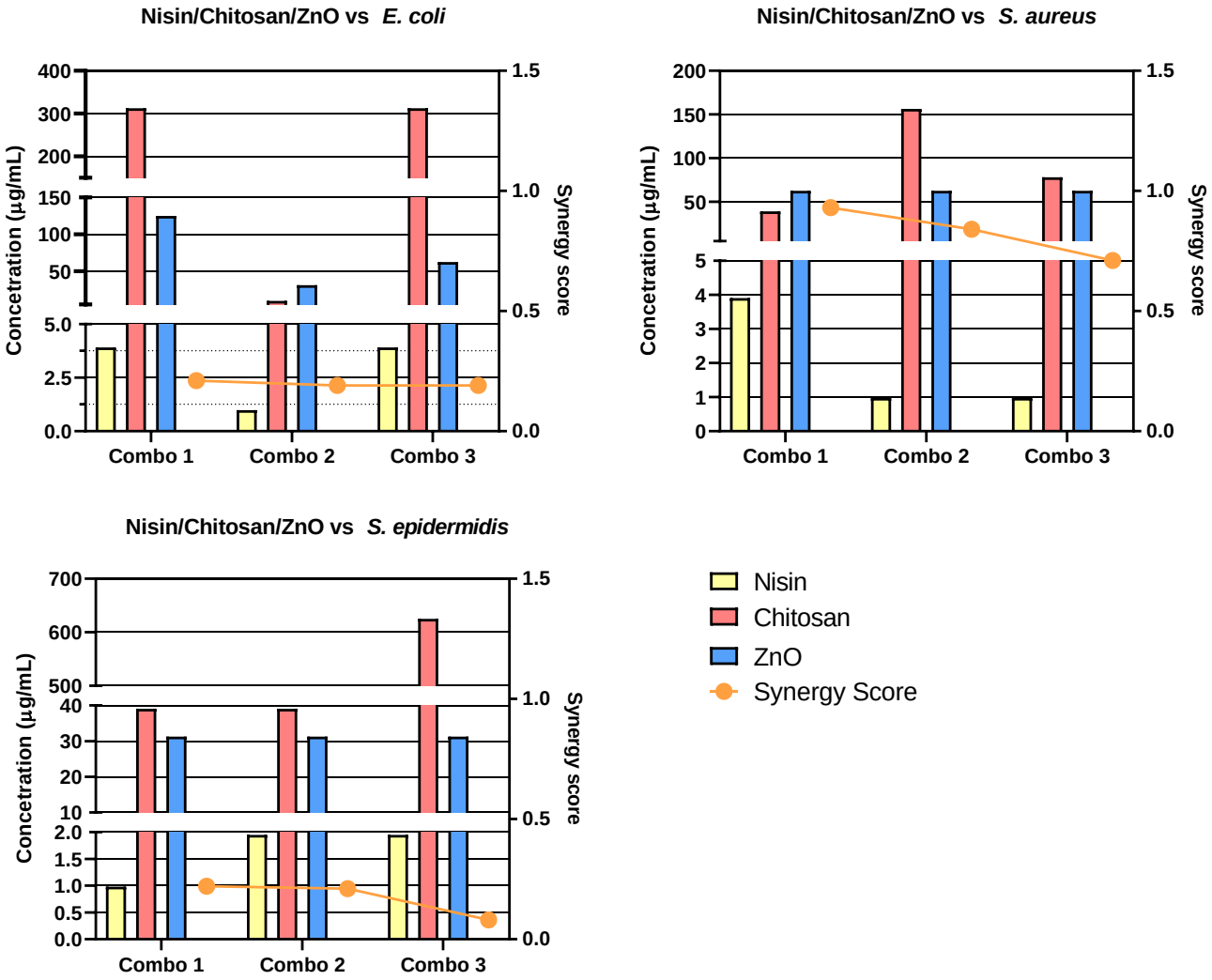


(**C**)

Figure 8. *Cont.*



(**D**)

Figure 8. Synergy score results of top three three-drug combinations: Bar graphs presenting the concentrations of each drug and their respective synergy scores from the top three three-drug combinations (**A**–**D**) against *E. coli*, *S. aureus* and *S. epidermidis*. (**A**) Chitosan/$AgNO_3$/Nisin, (**B**) Chitosan/$AgNO_3$/ZnO, (**C**) Nisin/$AgNO_3$/ZnO and (**D**) Nisin/Chitosan/ZnO. Bars show drug concentrations as indicated on the left *y*-axis and the line/symbols show each combination (combo) synergy score as indicated on the right *y*-axis.

3.3. Four-Drug Combinations

- $AgNO_3$–Nisin–Chitosan–ZnO

Chitosan, nisin, $AgNO_3$ and ZnO exhibited moderately high synergy in combination versus *E. coli* (average 0.36) and very high synergy versus *S. aureus* (average 0.91) and *S. epidermidis* (average 1.11). While the compound's average synergy score versus *E. coli* is lower than that versus the other two test species, results indicate the positive contributions of each treatment at low concentrations of chitosan (80 µg/mL), nisin (1.95–31.25 µg/mL), $AgNO_3$ (8 µg/mL) and ZnO (10–40 µg/mL), which showed effective inhibition (69–98.9%). The highest-scoring combination, which exhibited 98.9% inhibition, reported a very high concentration of nisin (31.25 µg/mL), indicating that nisin had a strong influence within the combination.

Reported synergy scores versus *S. aureus* are quite high, with the top scoring combinations exhibiting effective inhibition (97.5–99.5%) at low concentrations of chitosan (19.53–78.13 µg/mL), nisin (0.39–1.56 µg/mL) and $AgNO_3$ (4 µg/mL) and moderate concentrations of ZnO (62.50 µg/mL).

Combinations versus *S. epidermidis* reported the highest synergy scores identified within the present study at low concentrations of chitosan (20–80 µg/mL), nisin (1.25 µg/mL), $AgNO_3$ (8 µg/mL) and ZnO (10–40 µg/mL). While the top combination reported a very high synergy score (1.3), the reported inhibition greatly deviated (stdev 69.18), having an average value of 24.96%. The second highest (1.13) and third highest (0.9) scoring combinations exhibited stable inhibition (98.7–99.1%) at similarly low concentrations of each treatment.

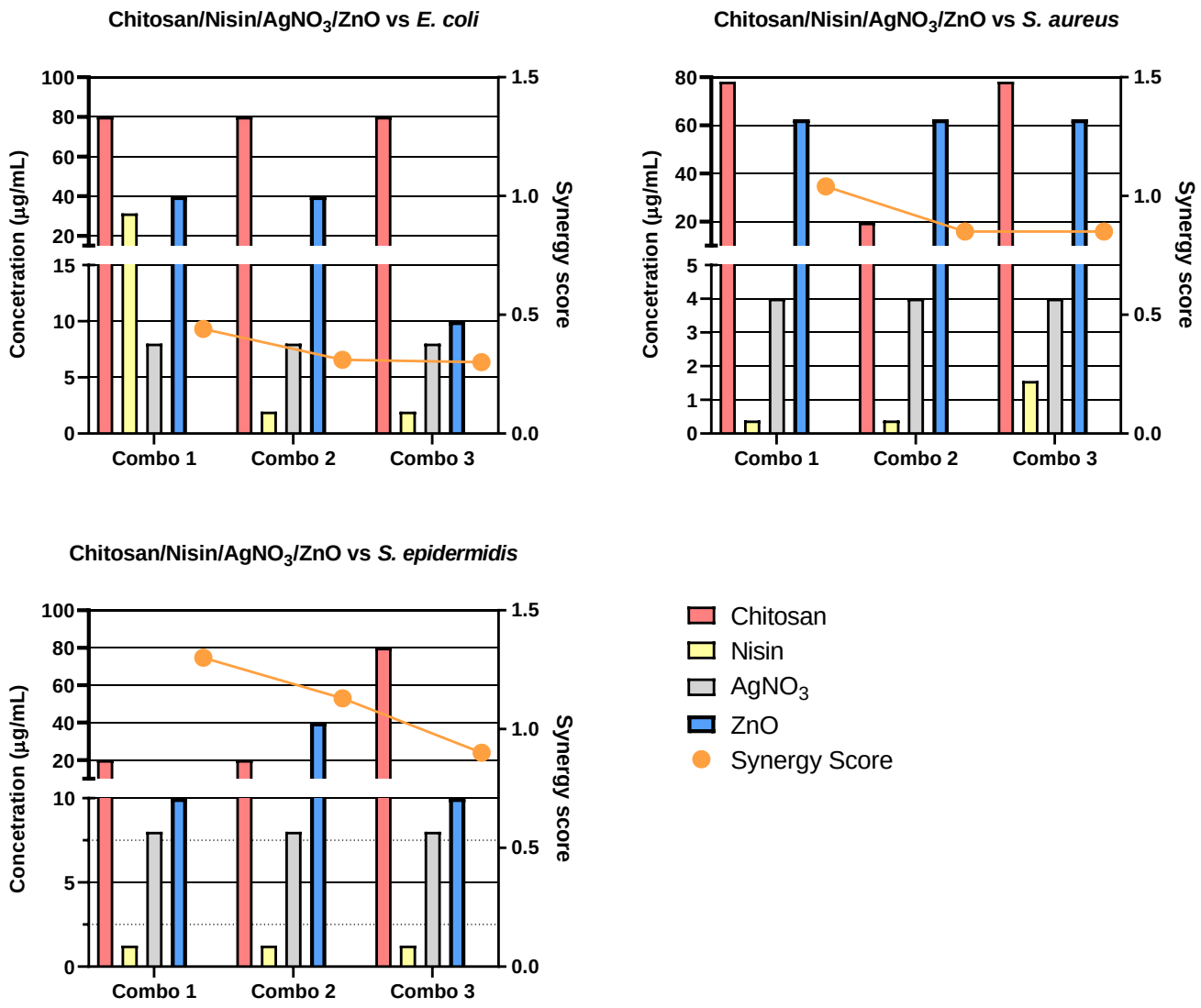


Figure 9. Synergy score results of the top three four-drug combinations: Bar graphs that present the concentrations of each drug and their respective synergy scores from the top three four-drug combinations (Chitosan/Nisin/$AgNO_3$/ZnO) against *E. coli*, *S. aureus* and *S. epidermidis*. Bars show drug concentrations, as indicated on the left *y*-axis, and the line/symbols show each combination (combo) synergy score, as indicated on the right *y*-axis.

4. Discussion

Antimicrobial synergy holds great promise as a solution for use in meeting the AMR crisis for several reasons. While a bacterial species may hold or even develop resistance to a single therapeutic agent, co-treatment with an alternative compound that exhibits

alternative modes of antimicrobial action could help to alleviate this issue. Additionally, certain groups of bacteria hold intrinsic metabolic or physical characteristics that can prevent certain classes of antimicrobials from exhibiting their effect. Co-treatment using a compound that can disrupt these characteristics would allow the primary treatment to carry out its effect unimpeded. Gram-negative bacteria are an example of one such group, as they have an additional outer membrane that can act to prevent compounds from reaching their target ligands. Following this example, nisin is a poly-cyclic lantibiotic that targets the inner-membrane-bound lipid II molecule. Due to the presence of an outer membrane, nisin is prevented from reaching its target, thus rendering it ineffective [29,30]. However, in theory, it would be possible to enable nisin by combining it via treatment with an additional compound that targets the outer membrane. By removing the outer membrane or compromising its integrity, nisin could freely interact with its lipid-II target. While this interaction can be clearly deemed to be synergistic, it is not enough on its own to observe a positive end result from the combination. While it would stand to reason that combining two or more already well-known and effective treatments would produce a greater gross effect than that of each individual treatment, previous studies of drug combinations have shown this predicted outcome to be incorrect, as have the results presented in this study [16,20,31]. To determine the synergistic abilities of two or more compounds, it is necessary to assess an array of various concentrations in different combinations. It is not important to determine the highest effect of combined treatments; the concept is to instead determine combinations that express a higher effect than that of the individual drugs at an identical concentration. The aim is to more easily discern the ratio of each drug required to enable another's mechanism of action, thus giving the most efficient synergy.

4.1. Inhibition and Synergy

Previously, $AgNO_3$ was shown to be the most effective bacterial growth inhibitor of the tested bacterial species [22]. Nisin was shown to have very efficient inhibitory effects on test Gram-positive bacterial species, while having no effect on Gram-positive bacteria. Both compounds differ majorly in their modes of action, with $AgNO_3$ permeating bacterial membranes through reactive silver ions (Ag^{2+}), while nisin has specific binding affinity to the lipid-II molecules bound in the inner bacterial membrane. Nisin's inability to affect Gram-negative bacteria is based on its inability to breach its outer membrane and interact with the lipid-II ligand. By combining both $AgNO_3$ and nisin, it was hypothesised that the reactive Ag^{2+} ions of $AgNO_3$ breach the Gram-negative outer bacterial membrane, allowing nisin to reach its target ligand [32–35]. Similar hypotheses were devised regarding ZnO and nisin, as ZnO had efficacy against Gram-negative and Gram-positive bacteria, as well as a similar mode of action wherein it destabilises membranes through release of Zn^{2+} ions and reactive oxygen species (ROS) [36–39]. Chitosan also had a noteworthy effect on all test strains, though it also had an alternate mechanism via which it targeted the bacterial cell wall [40,41]. The varying mechanisms had great significance for combinational studies and allowed us to observe whether effects unlock one other treatments' drawbacks ($AgNO_3$–nisin, ZnO–nisin), stack upon against them ($AgNO_3$–ZnO) or complement them (chitosan–nisin, chitosan–$AgNO_3$, chitosan–ZnO).

In this study, the combinatory compatibility of four chosen bioactives was successfully established, as were the magnitude of their interactions with one another. The checkerboard assay was utilised to screen the inhibitory effect of bioactive combinations against each test bacterial strain. The checkerboard assay was a well-established method used to screen drug combinations in various areas of clinical research [12,15–18]. Through the use of the synergy python package and the Bliss model, the synergy score of each test combination was successfully determined. The results of this study presented interesting interactions between the bioactives, many of which were predictable, though others were unanticipated.

4.2. Two-Drug Combination Synergy

The results of two-drug combination studies carried out against the Gram-negative bacteria *E. coli* have yielded varying results. Nisin, which is a lantibiotic that targets the inner-membrane-bound lipid II molecule, is hindered by the outer membrane found in Gram-negative bacteria, which prevents nisin from carrying out its mechanism of action. It was hypothesised that combining nisin with a compound capable of penetrating the outer membrane, such as $AgNO_3$ or ZnO, would enable nisin, with the resulting interaction being marked as synergetic. Combinations of nisin–$AgNO_3$ exhibited moderate-to-high synergy (average 0.32), showing a consistent concentration of $AgNO_3$ (8.49 μg/mL) to be the most accommodating compound for varying concentrations of nisin. While the inhibition ranged between 64 and 68% for this combination, it shows that nisin was able to have an effect upon a previously unaffected target. In contrast, ZnO was not found to enable nisin; rather, it appeared that nisin was antagonizing ZnO, as the concentrations of ZnO in the most synergistic combinations were higher than that of its previously determined MIC. Combinations of nisin–chitosan also exhibited undesired results, with higher concentrations of chitosan being utilised to observe an inhibitory effect. While such results are unfavourable, they still present a promising observation, showing that nisin had an effect on Gram-negative bacteria. Combinations of $AgNO_3$–chitosan exhibited a strong synergistic interaction, with effects being evident at lower concentrations of $AgNO_3$, which would indicate chitosan's ability to enable it. Chitosan has also shown to enable ZnO, which also exhibited lower concentrations; however, these combinations scored quite low, which reflects the fact that the concentration of chitosan was quite high.

Two-drug combinations used to inhibit *S. aureus* and *S. epidermidis* growth presented some moderate-to-strong synergistic combinations; however, there was a pattern of ZnO not effectively combining with nisin or chitosan. $AgNO_3$ demonstrates itself to be the most effect bioactive, enabling all other bioactives with which it is combined, reporting lower concentrations with higher inhibition responses. The highest overall scoring two-drug combination involved nisin–$AgNO_3$ versus *S. epidermidis*. Chitosan also demonstrated notable synergy with most bioactives, though it only effectively combined with ZnO against *S. epidermidis*, as much lower concentrations of both gave a greater response; however, the inhibition response was weak.

4.3. Three-Drug Combinations against Gram-Negative Bacteria

Increasing the combination number can further alter the effect exhibited by treatments, as is evident from three-drug combinations. Combinations that included nisin were shown to demonstrate high synergy versus *E. coli* with near full inhibition. Following the two-drug analysis, it was predictable that chitosan–$AgNO_3$–nisin would effectively synergise, presenting the highest-scoring combination versus *E. coli*. Furthermore, the relatively high concentrations of nisin in this combination showed that it had an active effect on *E. coli*, as it can be presumed to be heavily involved in enabling resistance (i.e., a concentration close to 0 would indicate little-to-no input). A more unpredictable result was seen regarding combinations that involved ZnO, as two-drug combinations demonstrated ZnO to be a poor component in combination, though three-drug combinations showed opposing results. Nisin–ZnO was the lowest scoring combination versus *E. coli*; however, with the inclusion of $AgNO_3$ or chitosan, these combinations were the second and third highest-scoring three-drug combinations against *E. coli*, respectively. Most interestingly, the nisin–$AgNO_3$–ZnO combination highlights how little $AgNO_3$ was reported in the higher scoring combinations, while relatively high concentrations of nisin were reported. This result again indicates nisin's active role in the combination, whereas $AgNO_3$ is at too low a concentration to have an inhibitory effect. This result could also demonstrate the ability of $AgNO_3$ to enable the mechanism of nisin. While chitosan–$AgNO_3$–ZnO reported low scoring combinations, the results seemed to be promising, with low concentrations of all three bioactives and nearly full inhibition reported. This result, once again, does not follow the patterns observed in two-drug combinations of the same bioactives.

4.4. Three-Drug Combinations against Gram-Positive Bacteria

Three-drug combinations versus *S. aureus* and *S. epidermidis* offered interesting points of comparison. The chitosan–$AgNO_3$–nisin combination scored highly versus both bacterial strains; however, concentrations of $AgNO_3$ were quite high at low concentrations of nisin. Scores from combinations that involved ZnO also proved to be quite unpredictable versus Gram-positive bacteria. Combinations of nisin–ZnO and chitosan–ZnO against *S. aureus* scored quite poorly; however, nisin–chitosan–ZnO reported the highest score of all three-drug combinations. In contrast, this combination had the second lowest score against *S. epidermidis*. The individual concentrations were quite low, and the reported synergy scores were also quite low. An interesting observation of this combination is that it was also predictable based on the two-drug combinations of nisin–chitosan, nisin–ZnO and chitosan–ZnO, which produced synergy scores that very closely averaged that of the nisin–chitosan–ZnO synergy score. Concentrations versus *S. aureus* indicate that ZnO enabled the effects of chitosan and $AgNO_3$; however, concentrations versus *S. epidermidis* did not indicate that any single bioactive enabled another bioactive, highlighting the even distribution of activity between the three bioactives.

Nisin–$AgNO_3$–ZnO demonstrated strong synergy versus both Gram-positive bacteria. While the reported synergy was particularly high against *S. epidermidis*, the reported inhibitory effects were quite low. Two-drug reports show that nisin–ZnO combinations interacted very poorly, which implies that the influence of $AgNO_3$ caused the three-drug combinations to more favourably interact, which was also predictable given the synergy scores of nisin–$AgNO_3$ and $AgNO_3$–ZnO.

4.5. Four-Drug Combinations

While a four-drug combination gave valuable insights into interactions between all four treatments at once, the 4×4 sized checkerboard had some disadvantages relative to larger 6×6 or 8×8 checkerboards, primarily the fact that it could not accommodate enough combinations to generate a full model of the possible combinational interactions. However, if determined via a two- or three-drug assay, key concentrations can be selected and utilised within the four-drug assay for further investigation. As such, the present four-drug assay layout should be used as a follow-on study, rather than as an initial combinational study, due to such limitations. Likewise, with the chosen three-drug assay layout, the four-drug assay layout allowed reduced experimental size and a faster setup.

Four-drug combinations reported a marked increase in the efficacy of all four bioactives relative to their individual capabilities against each bacterial strain. The combination of chitosan–nisin–$AgNO_3$–ZnO against *E. coli* exhibited some predictable results, with synergy scores comparable to scores derived from two- and three-drug combinations. While concentrations of each bioactive in the highest-scoring combinations were lower than their individual MICs, concentrations of chitosan and $AgNO_3$ were still quite moderate. Furthermore, only the highest-scoring combination reported complete inhibition while using a high concentration of nisin, which was expected due to nisin's inability to target Gram-negative species.

The reported four-drug synergy scores against *S. aureus* and *S. epidermidis* were very high relative to other scores determined during this study. Concentrations versus *S. aureus* were notably lower than their individual MICs, thus having strong inhibitory effects. While *S. epidermidis* reported the highest synergy score of this study, its highest-scoring combination reported low inhibition. Compared to the other two reported combinations, a slight increase in either chitosan or ZnO was sufficient to push the effects toward complete inhibition, albeit remaining well below their individual MICs. From analysis of the most effective combinations against each individual bacterial strain, the most effective concentrations of each drug in combination were determined, which gave a four-drug combination that could cause complete inhibition against all tested bacterial strains, while the amount of each drug used was limited (See Table 1).

Table 1. Most effective concentrations of the four bioactive compounds in combination against *E. coli*, *S. aureus* and *S. epidermidis*. These concentrations were established by evaluating the highest-scoring combinations against each bacterial species and determining the lowest concentrations of each compound that would cause complete inhibition of all three bacterial species.

Bioactive	Most Effective Concentration (μg/mL)
Chitosan	80
Nisin	2
$AgNO_3$	8
ZnO	60

5. Conclusions

Combinational antimicrobial bioactive studies hold great potential in many areas of clinical, pharmaceutical and medical research. The discovery of cross-treatment synergism could potentially unlock many new avenues of therapy for various pathogens and conditions. While drug synergy is a key target of combinational studies, drug antagonism is also a well-documented occurrence in pharmaceuticals, and while there are several models under development for its prediction, in many cases, it is difficult to determine which treatments may interact negatively without performing pre-clinical or clinical studies. Though it is important to find compatible combinations of drugs, another key goal is finding combinations in which the individual drugs are more effective within a combination than they are when acting alone. Determining synergy scores is an efficient method of screening many combinations of treatments and deducing the most effective option. It is evident from results presented here that treatment interactions cannot be accurately predicted and can differ greatly between bacterial strains. Furthermore, increasing the combination number has also been shown to have an unpredictable effect, as two-drug combinations cannot predict the effects of three-drug combinations of the same components, and likewise, two- and three-drug combinations cannot predict the effects of four-drug combinations. Current findings show that models previously used to predict drug combinations cannot be wholly trusted, as there are aberrant results presented in this study that contradict predictive models. While such results may not be considered advantageous, they provide knowledge critical to the development of combinational treatments. Within the scope of investigating new or previously unsuitable compounds as alternative antimicrobial treatments, the possibility of using as little of each compound possible while still holding an antimicrobial effect holds great potential. Identification of antimicrobial compounds based on complementary modes of action also provides an additional avenue for the discovery of novel treatments that combat AMR bacteria, wherein the activity of one treatment may enable the activity of another treatment that is naturally ineffective against the bacteria, as was the case of nisin against Gram-negative species in the present study.

There is also a pressing need to use effective screening methods to evaluate alternative bioactives that are biorefined via various environmental and food waste streams to help address the shortage in appropriate antimicrobials and the development of AMR [42,43]. Interestingly, there is increased interest in exploring new sources of antimicrobials, such as marine, peatland and food waste streams, that may present stressful environments that favor the production of unique antimicrobial bioactives [44]. This simple mass-throughput screening-based approach to evaluating combinational bioactives will also help address the surge in resistance to anti-fungal drugs among problematical fungi that cause significant human and animal infections [45,46]. There is also a proportionate interest in progressing interdisciplinary research through Quadruple Helix Hub frameworks (combining academia, industry, society and regulators), which use shared access to specialist equipment and subject-matter experts across disciplines to overcome these challenges [47].

The four chosen bioactives, namely $AgNO_3$, ZnO, nisin and chitosan, which have previously been characterised in terms of their individual antimicrobial abilities, have now been characterised for their combinational interactions in two-, three-, and four-drug

arrangements. Using this data, we accurately determined the most effective concentrations of each compound required for most effective microbial growth inhibition, limiting the amount of each compound needed to inhibit bacterial growth while having a broader spectrum of effect. Using this data, it is clear that the use of these compounds in combination produces a much more effective inhibitor of microbial growth, as the concentration of each individual bioactive is much lower than that of their MIC alone. As the current study relied on combinations of serial dilutions, more extensive analyses must be carried out regarding the most synergistic combinations, with further testing used to determine the most ideal concentration combinations and verify their efficacy. Additionally, the methods and analysis procedures presented have been shown to produce detailed and high-throughput assessments of drug combinations, and as such, they should be carried forward to evaluate additional treatments and combinations. The methodology could also be adjusted accordingly to allow studies of pharmaceuticals in other fields of research.

Supplementary Materials: The following supporting information can be downloaded via the following link: https://www.mdpi.com/article/10.3390/biomedicines11082216/s1, Figure S1. Drug combination setup, Figure S2. Two-drug combination assay final layout, Figure S3. Three-drug combination assay final layout, Figure S4. Drug combination assay final layout; Table S1. Three highest Bliss scoring two-drug combinations against *E. coli*, Table S2. Three highest Bliss scoring two-drug combinations against *S. aureus*. Table S3. Three highest Bliss scores of each two-drug combination versus *S. epidermidis*. Table S4. Three highest Bliss synergy scores of each three-drug combination versus *E. coli*, *S. aureus* and *S. epidermidis*. Table S5. Three highest Bliss synergy scores and the average of each four-drug combination versus *E. coli*, *S. aureus* and *S. epidermidis.*

Author Contributions: Conceptualisation, K.M., I.M. and N.R.; methodology, K.M.; investigation, K.M.; writing—original draft preparation, K.M.; writing—review and editing, all authors; supervision, N.R., I.M. and M.L.; funding acquisition, N.R. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge funding support provided by DAFM-2022-PSS125 and Athlone Institute of Technology (grant No. AIT-2017-008) for this work.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Efficacy of frontline chemical biocides and disinfection approaches for inactivating SARS-CoV-2 variants of concern that cause coronavirus disease with the emergence of opportunities for green eco-solutions

Neil J. Rowan[1,2,a], Elaine Meade[3] and Mary Garvey[3]

Abstract

The emergence of severe acute respiratory disease (SARS-CoV-2) variants that cause coronavirus disease is of global concern. Severe acute respiratory disease variants of concern (VOC) exhibiting greater transmissibility, and potentially increased risk of hospitalization, severity and mortality, are attributed to molecular mutations in outer viral surface spike proteins. Thus, there is a reliance on using appropriate counter-disease measures, including non-pharmaceutical interventions and vaccination. The best evidence suggests that the use of frontline biocides effectively inactivate coronavirus similarly, including VOC, such as 202012/01, 501Y.V2 and P.1 that have rapidly replaced the wild-type variant in the United Kingdom, South Africa and Brazil, respectively. However, this review highlights that efficacy of VOC-disinfection will depend on the type of biocide and the parameters governing the activity. VOC are likely to be similar in size to the wild-type strain, thus implying that existing guidelines for use and re-use of face masks post disinfection remain relevant. Monitoring to avoid injudicious use of biocides during the coronavirus disease era is required as prolonged and excessive biocide usage may negatively impact our receiving environments; thus, highlighting the potential for alternative more environmental-friendly sustainable biocide solutions. Traditional biocides may promote cross-antimicrobial resistance to antibiotics in problematical bacteria. The existing filtration efficacy of face masks is likely to perform similarly for VOC due to similar viral size; however, advances in face mask manufacturing by way incorporating new anti-viral materials will potentially enhance their design and functionality for existing and potential future pandemics.

Addresses

[1] Centre for Disinfection and Sterilisation, Athlone Institute of Technology, Dublin Road, Athlone, Ireland

[2] Department of Nursing and Healthcare, Athlone Institute of Technology, Dublin Road, Athlone, Ireland

[3] Department of Life Science, Institute of Technology, Sligo, Ash Lane, Sligo, Ireland

Corresponding author: Rowan, Neil J (nrowan@ait.ie)

[a] Given his role as Guest Editor Neil J. Rowan had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Damia Barceló Culleres.

Current Opinion in Environmental Science & Health 2021, **23:**100290

This review comes from a themed issue on **Environmental Toxicology 2021: Disruptive Green Deal Innovations**

Edited by **Neil J. Rowan** and **Robert Pogue**

For a complete overview see the Issue and the Editorial

https://doi.org/10.1016/j.coesh.2021.100290

Keywords

COVID-19, Disinfection, PPE, Biocides, SAR-Cov-2 variants, Healthcare, Environment.

Introduction — coronavirus and its implications for maintaining healthcare provision

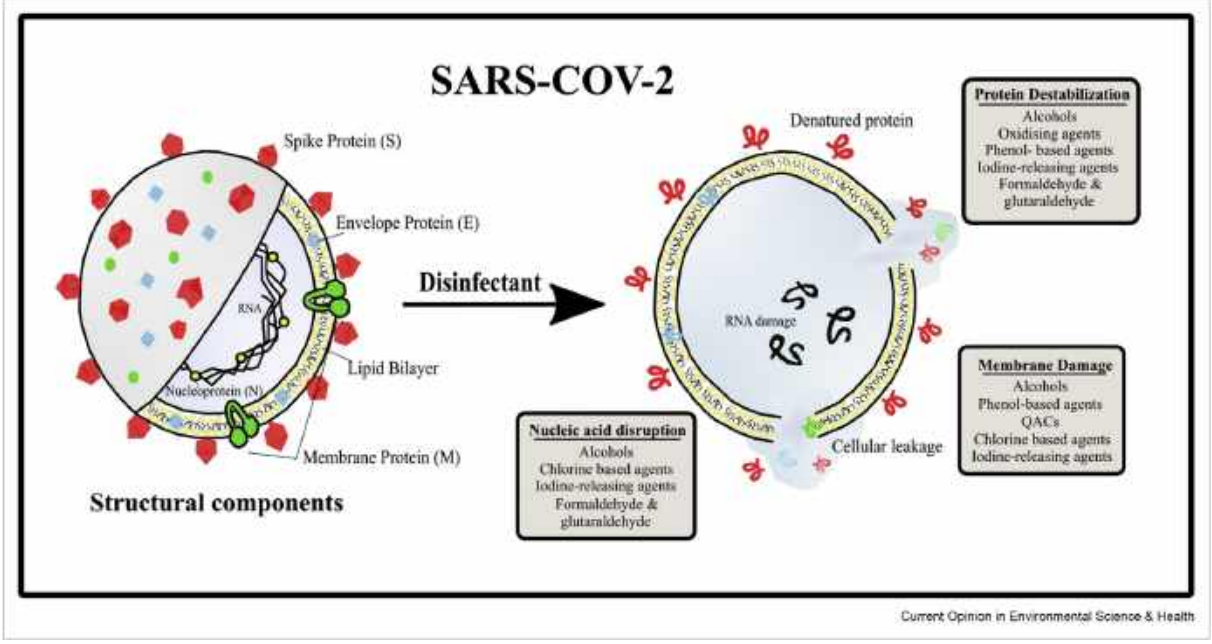
The coronavirus disease (COVID-19) pandemic, caused by the severe acute respiratory coronavirus 2 (SARS-CoV-2), has imposed tremendous challenges on healthcare systems globally [1—3]. At the time of writing (30th March, 2021), there have been 127,628,928 cases of COVID-19 worldwide, including 2,791,055 deaths [4]. COVID-19 elicits a broad infection spectrum ranging from very mild, non-respiratory symptoms to severe acute respiratory illness, sepsis with organ dysfunction and death; however, some infected people can be asymptomatic [1]. Evidence highlighting the contributions of super-spreaders of infectious airborne viral particles, including the more transmissible SARS-CoV-2 variants of concern (VOC), has also contributed to the occurrence of third and fourth waves of COVID-19 infections [5—7]. Addressing the ongoing COVID-19 pandemic has created unprecedented logistical challenges to maintain critical supplies of single-use personal and protective equipment (PPE) [8,9], where reuse and

disinfection occurred under emergency use authorization. At the outset of COVID-19, there was a void in knowledge to effectively counter this disease; however, there is an increasing understanding of the potential role of different strategies to address COVID-19, including adopting non-pharmaceutical interventions (NPIs, such as correct wearing of face masks, hand hygiene, use PPE, maintaining social distancing, detection testing, contact tracing), along with delivering new vaccines [8,9]. Data generated from predictive mathematical modelling of multiple-contributing factors influencing the occurrence of COVID-19, and commensurate efficacy of disease counter-measures, is increasing, such information is translated to calculating risk probability to monitor and manage the basic reproduction number R_0 in the following [9]. It is challenging to appreciate the actual efficacy of specific COVID-19 disease interventions in real-time given the swiftly moving pace of this pandemic. This current opinion focuses on understanding the efficacy of frontline biocides and disinfection approaches against SARS-CoV-2 variants of concern.

Coronaviruses and implications for meeting personal and protective equipment supply chain shortage and disinfection reprocessing

SARS-CoV-2 is a large positive-stranded RNA virus with an outer lipid envelope containing glycoprotein spikes (Figure 1) [10]. In general, enveloped viruses, such as coronaviruses, are more sensitive to environmental deleterious stresses, such as chemical biocides, than similarly-treated naked viruses, due to the presence of a lipid membrane [9,11,12]. Coronaviruses range from 60 to 140 nm in size, which is below the 300 nm pore diameter used in multiple layers of material used to make face masks. However, the use of multiple layers in single-use plastic face mask reduces the probable risk of penetration and transmission by acting as a barrier to respiratory droplets [13,14], which is likely to apply similarly to mitigating against VOC transmission. The effectiveness of single-use plastic filtering-face piece respirators face masks varies based on type and certification that is defined across three levels of protection depending upon leakage of particles into the interior of the mask that are *22%* (FFP1, such as medical and procedural masks), and 8% (FFP2, such as N95-type respirators), and *2%* for non-disposable FFP3-type respirators [2]. Use of non-thermal biocidal and disinfection approaches, such as vaporized hydrogen peroxide (30–35% VH2O2) and moist heat (60–65 °C for 30 min), and ultraviolet light at 254 nm (or fluence at 2000 mJ/cm^2), has been applied for reprocessing FFP1 and FFP2 type respirators, such as under emergency use authorization [2,25–27]. Non-thermal disinfection approaches of FFPs have been selected to enable retention of filtration performance, material compatibility, comfort fit, and pressure drop. In addition, there has been increased usage of alternative, cost-effective, home-made, cloth or fabric face coverings by the general public, where particle penetration efficacy was improved by using more than one layer of cotton-polypropylene and by introducing pleats when compared to testing using a fitted

Figure 1



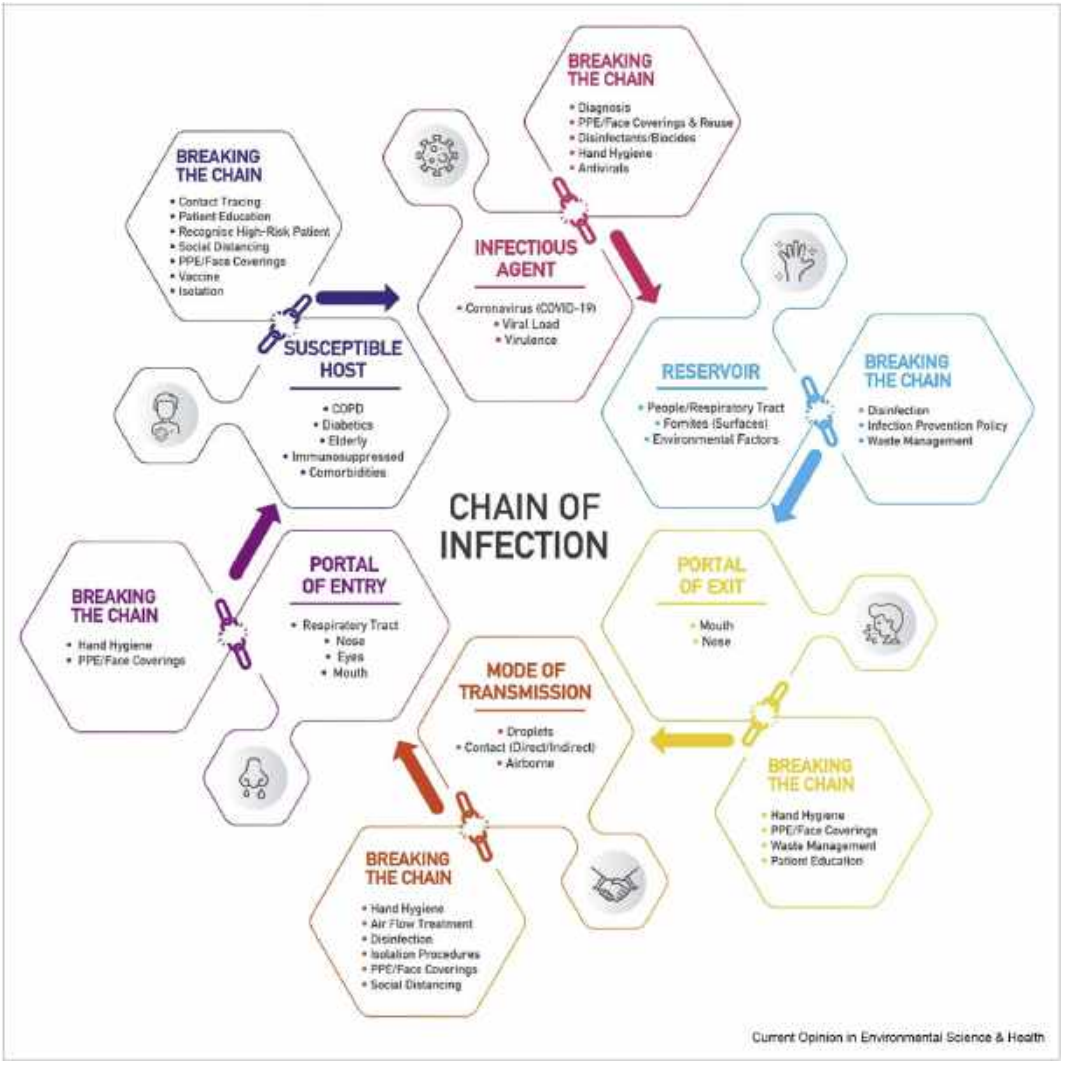
Structural components of SARS-COV-2 (left) and effective biocidal agents known to deactivate the virus (right).

N95 FFP2-type control [15–17]. Combining similar mild heat conditions, along with the use of detergent, for reuse of face coverings is theoretically plausible for coronavirus-disinfection; however, there remains a lack of information on disinfection and efficacy for informing the frequency of reuse that maintains filtration functionality [2,9]. Post COVID-19, it is likely that the changes in medical practice will drive sustaining or increasing high demand for PPE.

Understanding coronaviruses and the role biocides in breaking cycle of COVID-19 infection

Coronaviruses are typically inactivated on different surfaces within 4–5 days at ambient room temperatures on different surfaces, such as tissue, wood, glass, plastic, stainless steel, surgical masks, and paper, that can be influenced by humidity, viral load, presence of organic matter [9,18]; for example, colder conditions, such as refrigeration (4 °C), may extend SARS-COV-2 viability on surfaces beyond 14 days [19,20]. It took 14 days at 20 °C to reduce SARS-CoV-2 on nitrile gloves by 5 log orders using simulated typical infectious body fluids from infective patients; however, viral persistence was evident up to 21 days on plastic face shields, N100 FFPs, and polyethylene overalls [21]. These observations imply that the colder conditions associated with winter may support longer survival of SARS-CoV-2 on contact surfaces and when suspended in aerosols. There is a pressing need to understand the role of different interventions in breaking the cycle of SARS-CoV-2 infection (Figure 2) to protect frontline healthcare workers and patients [9]. This includes generating data over a longitudinal period to evaluate and harmonize deployment of these interventions (singly or combined) to prevent or reduce the risk of COVID-19 with a focus on

Figure 2



Use of chemical biocides and other disinfectants to break cycle of COVID-19 disease.

infections agent (SARS-CoV-2 and co-infections), reservoir, portal of exit, mode of transmission, portal of entry, and susceptible host (Figure 2). The efficacy of such data will be informed by mathematical modelling and randomized control studies [9]. The use of disinfectants or chemical biocides and disinfection approaches will feature strongly as key disease counter-measures in breaking the cycle of infection (Figure 2). Factors that influence the efficacy and performance of different types of biocides are varied and complex [22,23]; however, the parameters governing selection and performance of different biocide types are generally well-established, where the degree of application depends upon categories of risk to patients aligned with a commensurate level of treatment required to be achieved on contact surfaces and in the environment (Table 1). 'COVID-19 fatigue' of citizens is likely to play a contributing role in meeting compliance with deploying disease counter-measures to effectively manage new cases to protect frontline healthcare workers [24].

SARS-CoV-2 VOC

The World Health Organization monitors public health events associated with SARS-CoV-2 VOC [3]. Key features for these VOC are presented in Table 2. VOC 202012/01, 501Y.V2, and P.1 have commonly demonstrated an increase in transmissibility compared to wild-type (non-VOC) variants and have demonstrated a propensity for rapidly replacing other circulating SARS-CoV-2 strains. Variants 202012/01, 501Y.V2, and P.1 rapidly replaced the wild-type variant in the United Kingdom, South Africa, and Brazil, respectively. However, it is highly likely that more transmissible and pathogenic VOCs will emerge during this pandemic as the virus has ample opportunity to mutate given the high numbers of infected hosts globally. However, in terms of existing VOC, the WHO deploys 'a logistic model of competitive growth that highlighted additive increases in the effective reproduction number (Rt) relative to the wild-type variant that was estimated at 41% (95% CI: 41−42%) for 202012/01, 36% (95% CI: 32−40%) for 501Y.V2, and 11% (95% CI: 7−16%) for P.1' [3]. The transmissibility of P.1 is such that it is rapidly replacing the wild-type variant at a local level. Recent studies have shown VOC 202012/01 may be associated with an increased risk of hospitalization, severity, and mortality. There is a growing body of evidence on vaccine-induced neutralizing antibody activity against VOCs (Table 2). The findings support that neutralizing activity is largely sustained against this variant. However, these findings highlight the importance of using combinational approaches, including the use of biocides for surface disinfection, as important to limit transmission of VOCs. Key mutations affect viral non-structural proteins that are unlikely to affect the efficacy of frontline biocides described in Tables 3 and 4. There is an increasing interest in the future proof design of face masks by also incorporating potentially new antiviral materials with the provision for more environmentally friendly non-metal nanomaterials [66].

Indication of biocide efficacy against coronavirus

Biocides encompass chemicals with antiseptic, disinfectant, and/or preservative activity (Table 3). Biocides are used for a broad range of purposes, 'usually with inanimate objectives (hard surface disinfectants), externally on the skin (antiseptics and topical antimicrobials), to prevent or limit microbial infection for pre-operative skin infection or incorporated (preservatives) into pharmaceutical, cosmetic, or other types of products to prevent microbial contamination' [28]. Desirable properties of biocides include virucidal within the time that can allow for it to be in contact with materials to be treated; effectiveness not diminished under conditions of disinfection; does not damage material treated, has a suitable spectrum of activity; low toxicity and resistance to it has not emerged; inexpensive. Biocidal efficacy is influenced by several, and sometimes, inter-related factors — notably concentration, period of contact, pH, temperature, presence of organic or other interfering or enhancing materials or compounds, nature, numbers (dose), location (planktonic, biofilm), and condition of microorganisms (recalcitrant endospores vs sensitive enveloped viruses) (Table 3). For example, concentration exponent (η) is particularly important as it measures the effect of concentration of dilution based on the activity of the biocide [22,23]. Biocides with high η-values (such as alcohols, phenols) rapidly lose efficacy when diluted, whereas those with low η-values (such as QACs, chlorhexidine, orthopthalaldehyde) retain considerable activity on dilution. This difference is highly relevant when considering both lethal disinfection activity and potential implications on receiving environment, where the potentially deleterious impact of biocide residues must be considered [28]. In addition, many frontline biocides have optimal pH activity, such as hypochlorite and phenolics are most effective at acid pH, whereas glutaraldehyde and cationic biocides (e.g. QACS) are most potent at alkaline pH. Several researchers have reported that biocide activity can be influenced by interaction with organic matter (e.g. dirt, blood, serum, vomit, the presence of biofilm), and non-ionic surfaces, and adsorption on containers and other contact surfaces (Table 3).

Coronaviruses are incapable of supporting independent life; thus, biocide disinfection is determined by using *in vitro* bioassays, where reduction of cytopathic effects in tissue culture monolayers are observed that is attributed to a reduction in viral infectivity compared with untreated controls. Surviving viral fractions are typically expressed through Log_{10} reductions enumerated either by determining the 50% titration reduction endpoint for infectivity (known as tissue culture infectious dose 50%,

Table 1

Factors governing anti-viral efficacy of biocides.

Factors[a]	Comments	Relevance and implication for usage in practice
Factors characteristic of biocide		
Concentration	Understand the effect of dilution upon activity — concentration must be 'cidal' to viruses	Appropriate staff training
Contact time	Length of exposure can often enhance biocidal efficacy	Appropriate staff training
Organic load	Diminish the activity of biocide and protect other contaminating bacteria of concern	Understand physicochemical factors governing biocidal action
Formulation	Influences inactivation performance against coronaviruses and intended surface or application for treatment	Understand nation of active agent and impact on intended contact material
Temperature	Increased activity against viruses can be achieved with higher temperatures and relevant for some devices (e.g. endoscopes)	Appropriate staff training
pH	Affects biocide (stability and ionization) and affects growth of co-infective microorganisms	Less relevant for healthcare environment
Biological and environmental factors		
Presence of biofilm	Provides protective menstrua or environment that can be found on equipment and in certain surfaces	Combine physical cleaning along with chemical action
Viral load	The greater the population number of viruses present the more difficult it can be to disinfect	Biocides often used in excess at high level concentration — SARS-CoV appear sensitive to low and moderate levels
Categories of risk as defined for patients and treatment of surfaces, equipment, environment		
High risk		Sterilization such as use of VH2O2
Intermediate risk		Disinfection
Low risk		Cleaning and drying usually sufficient; disinfection
Requirements of chemical biocides or disinfectants		
Spectrum of activity		'Cidal' as opposed to 'static' activity as latter is not appropriate
Efficacy		Rapid action, particularly on surfaces
Incompatibility		Should not be neutralized or diminished easily
Toxicity		Should be minimal
Damages to surfaces, or materials		Corrosiveness should be minimal, especially at dilution of use. Should not damage contact surface to be disinfected
Costs		Should be affordable, particularly to ensure supply chain

[a] Factors listed in order of importance – adapted from the study by Michie, West, and Harvey [24].

Table 2

Synopsis of key information on SARS-CoV-2 variants of concern, as reported by World Health Organization on 23rd March, 2021.

Emerging information[a]	Variant of concern (VOC)		
Next strain clade	20I/501Y.V1	20H/501Y.V2	20J/501Y.V3
PANGO lineage	B.1.1.7	B.1.351	B.1.1.28.1 (alias P.10
Alternate name	VOC 20201/01	VOC 202012/02	
First detected	United Kingdom	South Africa	Brazil
First appearance	20 September, 2020	Early August, 2020	December 2020
Key spike mutations	H69/V70 deletion; Y144 deletion; N501Y, A5700, P681H	L242/A243/L244 deletion; K417N E484K, N501Y	N417T, E484K; N501Y
Key mutation in common		5106/G107/F108 deletion in Non-Structural Protein 6 (NSP6)	
Transmissibility	Increased (36−75%), increased secondary attack rate (10−13%)	Increased (1.50 (95% CI: 1.20−2.13) times more transmissible than previously circulating variant	Increased, more transmissible than previous circulating variants
Severity	Possible increased risk of hospitalization, severity and mortality	Possible increased risk in hospital mortality by 20%	Under investigation, limited impact
Neutralization capacity	Slight reduction, but overall neutralising titres still remained above levels expected to confer protection	Decreased, suggesting potential increased risk of infection.	Decreased reinfections reported
Potential Impacts on vaccines	• No significant impact on post-vaccine neutralization by Moderna, Pfizer-BioNTech, Oxford-AstraZeneca, Novavax • No significant change in prevention of disease by Oxford-AstraZeneca, Novavax, and Pfizer-BioNTech • Evidence for prevention of infection evidence limited — reduced effect reported for Oxford-AstraZeneca	• Post-vaccine neutralization reductions range from minimal to moderate for Moderna and Pfizer; however, there is also some evidence of more substantial reductions • Substantial reductions found for Oxford-AstraZeneca products • There is no evidence to inform vaccine impact on asymptomatic infection by 501Y.V2	• Limited to modest reduction in post-vaccine neutralization by Oxford-Astrazeneca, Moderna, and Pfizer-BioNTech vaccines. • Preliminary suggestion of loss of neutralization following vaccination with Sinovac
Countries reporting new cases (newly reported in last week)	125 (7)	73 (11)	41 (3)

[a] Adapted from WHO [ ]; note, consult this reference report for more detailed information on emerging information on key VOC.

Table 3

General properties, strengths and limitations of frontline chemical biocides against coronaviruses.

Biocide type and active igredient	Mechanism of virucidal Action	General usage	Limitations	Strengths
Alcohols Isopropyl alcohol (isopropanol) Ethyl Alcohol (Ethanol)	Disrupts cell envelope, coagulates and denatures proteins. Isopropyl alcohol is lipophilic disrupting lipid membrane.	Skin antisepsis (ca 70% v/v) Small equipment disinfection, for example, thermometers, critical tools, non-invasive probes	Not sporicidal Prolonged and repeat usage affects integrity of materials such as plastics. Flammable	No-staining, low toxicity, mild pleasant odour
Cationic surfactants — QAC such as BZK, MBAT, DDA	Mostly disrupt by solvating or disrupting cell envelope — cationic ammonium groups with hydrophilic heads	Fomites (200 ppm),	Require warmer temperature and longer periods for achieving MEC Low affinity against non-enveloped viruses	Nontoxic, colourless and odourless — retain activity in hard water, high tolerance to organic matter
Oxidising agent — Sodium hypochlorite	Oxidation of cell envelope	Household bleach — dissolves in water to form hypochlorous acid — used in clinical area for fomites, non-critical surfaces where there is blood spillage or vomit	Sensitive to presence of organic matter and porous material — can range from <1000 pm to 10,000 ppm depending on organic material — cleaning step and ventilation needed	Fast acting at low concentrations — inactivates envelope and non-envelope viruses
Oxidising agent Hydrogen peroxide	Hydroxyl free radicals cleave or crosslink biomolecules including proteins, nucleic acids, an lipids	Skin antisepsis (0.125% v/v); contact surfaces (35% v/v)	Limited information. Concentration of 0.5% effective against enveloped and non-envelop viruses.	Decomposes to form water and oxygen — effective against SARS-CoV-2 and surrogates — can be used on stainless steel
Halogenated compounds — Povidone iodine and Povidone Iodone (PVP-1)	Possibly blocking receptor for viral binding. Iodine can inhibit viral enzymes (neuraminidase) essential for viral release from host	PVP-1 (0.23%) used for rapid skin, oral cavity, nasal disinfection. Povidone iodine used at 7.5–10% pre-operative skin disinfection, antiseptic hand washes, scrubs, ointments	Can be cytotoxic and cause skin irritancy — Is an iodophor is mixture of iodine and carrier polymerpolyvinl pyrrolidone — not suitable for use with silicone products	PVP-1 water soluble, stains can be removed by washing. Substitute or used in combination with for alcohol-based disinfection products.
Aldehydes Glutaraldehyde. Formaldehyde and OPA	Chemically alkylating the amino and sulfhydryl groups of proteins and amino groups of nucleic acid bases	High level broad spectrum virucidal disinfection — vaccine production — decontaminates of surgical equipment, endoscopes, dialysers.	High reactivity, hazardous to health — irritant. Apart from OPE, more reactive at alkaline conditions. Pungent odour <1 ppm, monitoring.	Rubber, plastics, lensed instruments are tolerant. OPA chemically stable over pH 3–9, non-irritant, stains skin wear PPE.

QAC – Quaternary ammonium compounds; BZK - benzalkonium chloride; mon; MBAT - biz(tri-methyl ammonium methylene chloride)-alkaly ($C_{9\text{-}15}$) toluene; DDA – didecyldimethyl ammonium chloride; OPA – Ortho-phthalaldehyde or 1,2-dicarboxybenzaldehyde.

MEC –lowest concentration of biocide that reduces virus titre by 99.9% or greater compared to control reactions. Adapted from Lin [ ], Dev Kumar [ ].

Table 4

Use of different disinfection approaches for inactivating SARS-CoV-2 and its' surrogate indicators.

Disinfectant		Parameters	SARS-CoV-2 & Surrogate species	Reduction Assay used
Chemical	Ethanol	60–70%, 1 min, hard surfaces, ceramic and porcelain tiles -carrier test.	hCoV (HCoV-229e)	3 - 4 log, $TCID_{50}$ assay [53]
	H_2O_2	0.5%, 15 min, surface carrier test	SARS-CoV-2	6 log plaque assay using Vero E6 cells [52]
		1–6%, 30 s, suspension testing of oral mouth wash	SARS-CoV-2, USA–WA1/2020 strain	1–1.8 log TCID assay using Vero 76 cells [54]
	QAC – BAC QAC – DDAC	0.04% w/v, 1 min, steel surface. quantitative carrier test	Parainfluenzavirus type 3 (HPIV-3) and human coronavirus 229-E (HOV-229E)	3 log Plaque assay using MA-104 line of rhesus monkey kidney cells
		0.025%, 3 days, suspension test	Canine Coronavirus	4 log TCID assay using A72 fibroma cell line [55]
	Sodium hypochlorite	0.1%, 1 min, suspension test.	SARS-CoV-2	4 log [39]
		6%, 30 s surface carrier test.		TCID assay [40]
	IPA	70–90%, 30 s	SARS-CoV-1	4 log,
		70–80%, 15 s, ceramic and porcelain tiles -carrier test.	hCoV (HCoV-229e)	TCID assay [53]
	Acetic acid	6%, 5 min, aqueous suspension test.	SARS-CoV-2 (Hu/DP/Kng/19–020 strain)	4 log $TCID_{50}$ assay using VeroE6-TMPRSS2 cells [43]
	Glutaraldehyde	0.5%, 2 min, suspension test	SARS-CoV	3 log
	Formaldehyde	0.7–1%, 2 min, suspension test	isolate FFM-1	TCID using human embryonic lung fibroblasts [23]
	Povidone iodine	1–2.5%, 15 s, suspension testing of oral mouth wash	SARS-CoV-2, USA-WA1/2020 strain	4 log TCID assay using Vero 76 cells [54]
Technologies	Steam sterilisation	121 °C, 5 min, medical masks, N95 respirators	Avian coronavirus (H120)	2 log inoculation of embryonated eggs, real-time TaqMan RT-PCR assay [56]
	Heat	56 °C, 30 min, 65 °C, 15 min, 98 °C 2 min, suspension test.	SARS-CoV-2	5 log $TCID_{50}$ assay using Vero E6 cells [57],
	Deep UV LED	265, 280, and 300 nm, 20 s, hard surfaces, carrier test	SARS-CoV-2	3.3 log Plaque assay using Vero E6 cells [58]
	Simulated sunlight	60 min on hard surfaces, carrier test on surface dried droplets.	SARS-CoV-2 USA-WA1/2020	4 log. $TCID_{50}$ assay using Vero cells (ATCC CCL-81) [60]
	UVC	254 nm, 4–9 s, wet and dried droplets	SARS-CoV-2	3 log Plague assay [61]
	Ozone	30 ppmv, 40 min 100 ppmv, 30 min, 1000 ppmv, 20 min on surfaces, carrier test.	hCoV 229E (HuCoV-229E)	95% reduction (1 log) HEK-293 cells and imaging using IncuCyte ZOOM system [36]
	Vapourised H_2O_2	0.5%, 60 s, surface of stainless steel disks, carrier test.	feline calicivirus, human adenovirus type 1, avian and swine influenza virus	4 log TCID50 assay using A-549 cells, MDCK cells [59]
	Chlorine dioxide gas (ClO2)	30–300 ppm, 25 °C to 30 °C, 1.5–3 h, in vivo.	avian infectious bronchitis coronavirus	infected chick embryos as models [62]
	Gamma radiation (cobalt-60)	1–5 MRads, suspension test.	arenavirus, bunyavirus, coronavirus, filovirus, flavivirus, orthomyxovirus, paramyxovirus	6 log $TCID_{50}$ assay Vero cells [63]

$TCID_{50}$ assay - Tissue Culture Infectious Dose assay, QAC quaternary ammonia compound, BAC benzalkonium chloride, DDAC didecyl dimethyl ammonium chloride.

or $TCID_{50}$ assay) or by performing a viral plague assay; however, reverse transcriptase - polymerase chain reaction (RT-PCR) using threshold (Ct value) is also used to determine viral load through detection of specific genes. Determining factors influencing biocides efficacy has traditionally been conducted to evaluate minimum inhibitory concentrations or lethal effects such as European suspension test, rate-of-kill test, and in-use test that are more suitable for anti-bacterial agents [29]. The Sterilization industry relies upon 12 log_{10} reductions of recalcitrant bacterial spores as biological indicators or surrogates, such as *Geobacillus stearothermophilus*, *Bacillus atrophaeus*, for determining sterility assurance levels for different sterilants where there is significant overkill to ensure validation of processes [25,30,31,32]. Thus, existing disinfection processes are ultimately based upon the probability of viral reduction where there is a pressing need to elucidate robust real-time inactivation enumeration methods [such as 31], which is likely to be informed by predictive modelling and may create opportunities for machine learning and artificial intelligence.

Disinfection of SARS-CoV-2

As an enveloped virus, SARS-CoV-2 is susceptible to commercial disinfectant chemicals, technologies, and physical disinfection methods [33,34] (Table 4). Recent studies have detected SARS-CoV-2 RNA on surfaces in isolation wards 28-days following exposure via RT-PCR methods [35], where the infectivity ability of viral RNA is unknown. However, it is unlikely that undamaged viral RNA realized on treated surfaces would remain a significant risk because of its inability to enter human lung cells as RNA only. Determination of biocidal efficacy against SARS-CoV-2 is not always feasible as the virus requires biosafety level 3 or higher; therefore, fewer pathogenic viruses as surrogate indicators of infectivity are frequently used [36]. Virucidal efficacy is determined by quantitative suspension tests, namely EN 14476 requiring 4 log reduction using surrogate enveloped species such as polio, adenovirus and murine norovirus, where efficacy against SARS-CoV-2 has yet to be elucidated experimentally. The framework of the European Committee for Standardization outlines surrogate species suitable for disinfection studies for many microorganisms. For virucidal activity against enveloped viruses, including SARS-CoV-2, the vaccinia virus has been specified as the relevant test organism according to this framework [37]. In clinical settings, SARS-CoV-2 has been detected on surfaces in intensive care units (4.4−5.2 log_{10}), in isolation rooms, and on general wards (2.8−4.0 log_{10}) [38]. While the viral load of SARS-CoV-2 on fomites directly following contact with infected persons is currently unknown, it is known that the virus remains infectious on surfaces for up to 9 days [39,40] depending on the surface material, pH, temperature, and humidity [40]. The suitability of suspension tests to determine efficacy on surfaces is unknown, particularly where the organic matter may be present such as in healthcare settings. ISO 18184 is a surface carrier test for virucidal activity; at present, no studies have demonstrated biocidal efficacy against SARS-CoV-2 using this method. The disinfection of surfaces and hand sanitation is of paramount importance in controlling viral transmission as recommended by the WHO. Disinfectant efficacy is affected by viral type, organic matter, viral titre, pH, viral clumping, biocide contact time and concentration. As such, cleaning is an essential prerequisite for disinfection to remove contaminating organic matter. In healthcare settings, disinfection agents in use include high-pressure steam sterilization, dry heat, UV-light, ethylene oxide (EtO) gas, hydrogen peroxide gas plasma, and biocidal chemicals [41] (Table 4).

The environmental protection agency (EPA) has approved various chemical biocide for use domestically and clinically to reduce coronavirus transmission, including quaternary ammonium (QACs), hydrogen peroxide (H_2O_2), peroxyacetic acid, isopropanol (IPA), ethanol, sodium hypochlorite, octanoic acid, phenolic, among others [41]. Viral inactivation is resultant from disruption of the cell structure, destruction of the lipid envelope, protein coagulation, nucleic acid, and protein denaturation [23] (Tables 3 and 4). Studies assessing disinfection efficacy are difficult to compare because of innate experimental variations and lack of standardized procedures, including test material, varied exposure times, viral load, test chemical or combinations and the organic inhibitor used [40] (Table 4). Studies report efficacy of 70−90% IPA with 30 s exposure against SARS-CoV with 1−3% H_2O_2 demonstrating efficacy after 1 min exposure [42], while 0.1% sodium hypochlorite was effective in 1 min [39] (Table 4). A concentration of 6% acetic acid reduced human coronavirus (hCo-V) viability by 3.5 log_{10} after 1 min contact time [43] on surfaces. 60−70% ethanol reduced surface viral load by >3 log_{10} after 1 min exposure in healthcare settings [44]. For the inactivation of SARS-CoV-2, the most common disinfectants used are 62−70% ethanol, 0.5% H_2O_2, and 0.1% sodium hypochlorite, which are effective with 1 min exposure via oxidative reactions [42]. Pulsed plasma gas discharge has also produced biocidal water comprising short-lived oxygenated free radicals that has potential contact surface disinfection leaving no unwanted chemical residues [45]. For hand disinfection, the WHO recommends the use of 75% IPA or 80% ethanol hand rubs for 30 s to inactivate SARS-CoV-2 [46]. However, there is increasing opportunities to exploit advances in digitization and modelling to inform the future efficacy of disinfectants, including combining biocidal approaches and to hurdle limitations for broad applications [9,47]. Material compatability and functionality are important factors to consider when using new eco-alternatives to conventional biocides that includes combinational treatments [64].

Conclusion

Variant strains of SARS-CoV-2 are constantly emerging due to innate mutagenic changes in the viral genome, primarily altering structural components such as spike proteins. Although the efficacy of vaccinations program against these variants is unknown, such structural changes are unlikely to confer disinfection resistance as non-specific destruction of proteins and lipids of the viral capsid occur. Frontline biocides appear to be affective against VOC, but factors governing usage needs careful consideration. Vigilance is needed to protect our environment during the COVID-19 era, particularly by avoiding injudicious use of biocide that may negatively impact our agroecosystems [48]. Prolonged and excessive biocide use may give rise to situations that potentially promote cross-antimicrobial resistance in problematical bacteria to frontline antibiotics [49]. Adaptive resistance to frontline biocides has been reported since the early 1990s such as against bisphenol, triclosan, glutaraldehyde, and oxidising agents [22]. Paul et al. [48] highlighted that extensive application of biocides affects microbial flora that may lead to a decrease in the number and diversity of beneficial microbes that may directly affect the functioning of nutrient cycles; thus, careful considerations should be given to biocide neutralization, environmental management and sustainability [50,51]. Understanding these factors is important for the training of end-users, (e.g. healthcare, industry and community), to ensure the efficacy of biocidal product is maintained and effectively neutralized, along with monitoring policy for effective and responsible deployment of biocides. This current opinion supports Article 18 of the European Union's biocidal products regulation that directs the European Commission to issue a report on how the biocidal products regulation contributes to the sustainable use of biocidal products to reduce the risks posed to human health, animal health, and the environment by biocidal products. The aforementioned also recommends a series of actions to be completed by 2024, including investing additional resources in enforcement activities; developing the best available techniques reference documents that can be relevant for biocidal products used in industrial processes, and encouraging the development and implementation of standards that could contribute to the sustainable use of biocidal products and alternatives to biocidal products.

Funding

No funding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. ** Rowan NJ, Laffey JG: **Challenges and solutions for addressing critical shortage of supply chain for personal and protective equipment (PPE) arising from Coronavirus disease (COVID19) pandemic–Case study from the Republic of Ireland**. *Sci Total Environ* 2020, **725**:138532, https://doi.org/10.1016/j.scitotenv.2020.138532.
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Review

Mycotherapy: Potential of Fungal Bioactives for the Treatment of Mental Health Disorders and Morbidities of Chronic Pain

Elaine Meade [1], Sarah Hehir [1,2], Neil Rowan [3] and Mary Garvey [1,2,*]

[1] Department of Life Science, Sligo Institute of Technology, F91 YW50 Sligo, Ireland; elaine.meade@mail.itsligo.ie (E.M.); hehir.sarah@itsligo.ie (S.H.)
[2] Centre for Precision Engineering, Materials and Manufacturing Research (PEM), Institute of Technology, F91 YW50 Sligo, Ireland
[3] Bioscience Research Institute, Technical University Shannon Midlands Midwest, N37 HD68 Athlone, Ireland; neil.rowan@tus.ie
* Correspondence: garvey.mary@itsligo.ie; Tel.: +353-071-9305529

Abstract: Mushrooms have been used as traditional medicine for millennia, fungi are the main natural source of psychedelic compounds. There is now increasing interest in using fungal active compounds such as psychedelics for alleviating symptoms of mental health disorders including major depressive disorder, anxiety, and addiction. The anxiolytic, antidepressant and anti-addictive effect of these compounds has raised awareness stimulating neuropharmacological investigations. Micro-dosing or acute dosing with psychedelics including Lysergic acid diethylamide (LSD) and psilocybin may offer patients treatment options which are unmet by current therapeutic options. Studies suggest that either dosing regimen produces a rapid and long-lasting effect on the patient post administration with a good safety profile. Psychedelics can also modulate immune systems including pro-inflammatory cytokines suggesting a potential in the treatment of auto-immune and other chronic pain conditions. This literature review aims to explore recent evidence relating to the application of fungal bioactives in treating chronic mental health and chronic pain morbidities.

Keywords: fungi; biologics; mushrooms; chronic pain; mycotherapy; mental health

Citation: Meade, E.; Hehir, S.; Rowan, N.; Garvey, M. Mycotherapy: Potential of Fungal Bioactives for the Treatment of Mental Health Disorders and Morbidities of Chronic Pain. *J. Fungi* **2022**, *8*, 290. https://doi.org/10.3390/jof8030290

Academic Editor: Laurent Dufossé

Received: 24 February 2022
Accepted: 10 March 2022
Published: 11 March 2022

1. Introduction

There are many recognised mental health disorders or mental illnesses including (but not limited to) anxiety disorders, mood disorders (depression, bipolar disorder, and cyclothymic disorder) psychotic disorders (schizophrenia), eating disorders (anorexia nervosa, bulimia nervosa), impulse control and addiction disorders, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD) and personality disorders [1]. Each mental illness manifests with a variable array of symptoms that differ depending on the illness present; it is accepted, however, that a person's mood, thinking, perceptions, anhedonia (pleasure sensation) and behaviours are affected. According to the 2013 Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), "A mental disorder is a syndrome characterized by clinically significant disturbance in an individual's cognition, emotion regulation, or behaviour that reflects a dysfunction in the psychological, biological, or developmental processes underlying mental functioning" [1]. These issues are undoubtedly proliferated by the stigma toward mental disorders such as anxiety, clinical depression (major depressive disorder), and OCD where a lack of understanding in society prohibits afflicted persons from seeking help or speaking out [2]. Stigma where mentally ill persons are perceived as dangerous, unpredictable, or as having a weakness of character has excluded them from society to an extent, further exasperating the issue [2]. The use of the DSM-5 in the diagnosis of mental illness, however, remains under scrutiny as understanding and expansion of mental illness progresses [3]. The World Health Organisation (WHO, Geneva, Switzerland) created the WHO International Consortium in Psychiatric Epidemiology

(ICPE) in 1998 with the aim of gathering data on mental illness via a diagnostic instrument, i.e., the WHO Composite International Diagnostic Interview (CIDI) surveys [4]. The WHO states that mental health illness is increasing yearly across the globe with approximately 20% of children and adolescents being diagnosed with a mental illness where suicide is the second leading cause of death in 15- to 29-year-olds [5]. In Europe, mental health disorders affect approximately 165 million people yearly with anxiety, mood and addictive disorders being most common [6]. Suffering a mental health disorder is devasting for the individual afflicted but also impacts the family unit as substance abuse and suicidal idealisation often manifests. Considering the wider burden of mental health disease and its associated functional impairment, the socio-economic impact must also be considered due to their increasing prevalence and high disability rate where treatment options often remain unmet [7].

The direct economic burden mental illness relates to diagnosis, treatment, or hospitalisation costs with indirect costs associated with the impact on economic growth including loss of income due to disability, absence from work and cost of additional care [6].

The impact of chronic pain co-morbidities associated with mental health must also be considered including functional disorders such as fibromyalgia and other functional somatic syndromes (FSSs). The treatment of mental health disorders is typically of combination of drug therapy based on psychoactive drugs (Table 1), mood stabilisers or antipsychotic drugs, and psychotherapeutic treatments. In many cases, however, mentally ill persons are non-responsive to therapy, termed treatment resistant [8]. Currently, 30% of clinically depressed are treatment resistant with this cohort having increased issues with social interactions, occupational difficulties, declining physical health and often suicidal thoughts [9]. Additionally, therapeutics currently in use are prone to numerous side effects where treatment is ultimately discontinued in patients (Table 1). To provide therapeutic options for such persons, and to reduce the burden of disease, it is imperative that we seek effective alternatives. The use of psychedelics, such as psilocybin (magic mushrooms), Lysergic acid diethylamide (LSD), and ayahuasca have drawn attention due to their noticeable ability to alter consciousness in personally meaningful, therapeutic, and spiritual ways [10]. Indeed, clinical trials on psilocybin demonstrated its efficacy in reducing cancer-related anxiety and depression, treatment-resistant depression, major depressive disorder (MDD), and substance misuse [11]. This review aims to highlight and discuss the potential of fungal biologics including psilocybin, LSD and others as therapeutic alternatives (mycotherapy) for alleviating the symptoms of mental health disorders. The current knowledge underlining the potential of fungal biologics is highlighted and discussed in the context of common mental health disorders including addiction, MDD and anxiety where current treatment is ineffective or causes significant undesirable side effects. Additionally, this review will review the potential of said biologics to also alleviate the symptoms of chronic pain conditions commonly associated with mental illness including fibromyalgia and irritable bowel syndrome.

Table 1. Treatment of mental health disorders.

Disorder	Treatment	Mode of Action	Efficacy	Side Effects
Anxiety disorders *	SSRIs e.g., sertraline, escitalopram	Inhibit the reuptake of 5-HT	First-line treatments for PD, GAD, SAD, and PTSD [12]	GI problems (nausea, diarrhoea, dyspepsia, bleeding), dry mouth, headaches, dizziness, anxiety, insomnia, and sexual dysfunction [13]
	SNRIs e.g., venlafaxine, duloxetine	Inhibit the reuptake of NE and 5-HT (and/or DA)		
	TCAs e.g., amitriptyline, imipramine	Inhibit the reuptake of NE and 5-HT	Equivocal efficacy with SSRIs; however, cause more adverse side effects due to their anticholinergic activity [14]	Nausea, dry mouth, constipation, weight gain, blurred vision, light-headedness, confusion, sedation, urine retention and tachycardia [15]
	MAOIs e.g., moclobemide, phenelzine	Inhibit the mitochondrial enzyme monoamine oxidase	Third-line treatment for refractory SAD and PD, i.e., considered for patients who are non-responsive to other treatments [16]	Dry mouth, nausea, diarrhoea, constipation, drowsiness, insomnia, dizziness/or light-headedness, fatigue, urinary problems, sexual dysfunction, hypertensive crisis reaction, and serotonin syndrome [17]
	Benzodiazepines e.g., alprazolam, diazepam, clonazepam	Positive allosteric modulators of GABA-A, resulting in increased frequency of chloride channel opening	Effective and fast acting in the treatment of GAD. Recommended as second-line therapy and for short duration use due potential risks of tolerance, dependence, abuse, or misuse [13]	Drowsiness, lethargy, fatigue, and potential for dependence. Higher doses can cause impaired motor coordination, dizziness, vertigo, slurred speech, blurry vision, mood swings, euphoria and hostile or erratic behaviour
	Pregabalin	Calcium channel modulator [18]	Effective as a monotherapy for GAD, or as an adjunct to SSRIs/SNRIs in treatment-resistant GAD [19,20]	Sedation, dizziness, somnolence, dry mouth, amblyopia, diarrhoea, weight gain and potential for dependence [12]
	Buspirone	High affinity for 5-HT_{1A} receptors [21]		Nausea, headaches, dizziness, and fatigue [13]

Table 1. *Cont.*

Disorder	Treatment	Mode of Action	Efficacy	Side Effects
Mood disorders *	Lithium	Multiple mechanisms including modulation of (GABA)-ergic and glutamatergic neurotransmission, and alteration of voltage-gated ion channels or intracellular signalling pathways [22,23]	First-line treatment for prevention of manic and depressive episodes of bipolar disorder (BD) [24]	Cardiac problems, cognitive problems, acne, psoriasis, thyroid problems, nausea, vomiting, weight gain, hyponatremia, sedation, decreased libido, and teratogenic [25]
	valproic acid		First-line treatment for acute mania and maintenance of BD [26]	Cardiac problems, cognitive problems, hair loss, hypothyroidism, aplastic anaemia, Leukopenia, increased transaminases, hepatitis, SLE-like syndrome, hyponatremia, tremor, decreased libido, infertility and teratogenic [25]
	Carbamazepine		Effective as a monotherapy to treat manic symptoms of bipolar or as adjunct to lithium or valproic acid [27]	Cardiac problems, cognitive problems, acne, hair loss, hypothyroidism, PCOS. diarrhoea, nausea, vomiting, pancreatitis, increased transaminases, metabolic syndrome, weight gain, sedation, tremor, decreased sexual function, infertility and teratogenic [25]
Psychotic disorders	First-generation antipsychotics (FGA) e.g., Chlorpromazine, haloperidol	D2 antagonists: work by inhibiting dopaminergic neurotransmission [28]	Effective in the treatment and maintenance of schizophrenia, acute mania with psychotic symptoms, major depressive order with psychotic features, and delusional disorder [28]	Adverse effects are drug specific and include anticholinergic effects (dry mouth, blurry vision, tachycardia, constipation), sedation, distonias, weight gain, increased lipids, parkinsonism (tremor, rigidity, bradykinesia), akathisia tardive dyskinesia, sialorrhea, orthostatic hypotension, neuroleptic malignant syndrome, sexual disfunction, neutropenia/agranulocytosis, and myocarditis [29]
	Second-generation antipsychotics (SGA) e.g., quetiapine, aripiprazole	Serotonin-dopamine antagonists: work by blocking D2 dopamine receptors as well as serotonin receptor antagonist action [28]	Same clinical efficacy as FGA, with the exception of clozapine, which has unique efficacy against treatment resistant schizophrenia [30]	

Table 1. *Cont.*

Disorder	Treatment	Mode of Action	Efficacy	Side Effects
Eating disorder	Olanzapine (SGA)	Block dopaminergic (D1-4 antagonism) and serotonergic (5-$HT_{2A/2C}$ antagonism) receptors [31]	Effective as an adjunctive therapy in treatment of AN, increasing appetite and decreasing anxiety and ruminating thoughts involving body image and food [32]	Dizziness, orthostatic hypotension, hypercholesterolemia, hypertriglyceridemia, hyperglycaemia, weight gain, extra-pyramidal symptoms, dry mouth, hyperprolactinemia, and insomnia [32]
	Antidepressants(SSRIs, SNRIs, TCAs, MAOIs)	Defined above	Effective as an adjunctive therapy in treatment of BN and BED, reducing the crisis of binge eating, purging phenomena and improving mood and anxiety [33]	Listed above
	Mood stabilizers e.g., topiramate	Blocks voltage gated sodium channels, enhances GABA-A receptor activity, reduces membrane depolarization by AMPA/Kainate receptors and is a weak inhibitor of carbonic anhydrase [34]	Shown efficacy in treatment of BN and BED, reducing the crisis of binge eating, purging phenomena and promoting weight loss (in overweight or obese patients) [33]	Paraesthesia, fatigue, cognitive problems, dizziness, somnolence, psychomotor slowing, memory/concentration difficulties, nervousness, confusion, weight loss [34]
Impulse control, addiction, and obsessive-compulsive disorders	Antidepressants e.g., SSRIs and clomipramine (TCA)	Potently inhibit the reuptake of 5-HT	Effective as a monotherapy or as an augmentation agent in the treatment of impulsive (PG, KM, TTM, IED and pyromania), addiction and compulsive disorders [35–39]	Listed above
	Mood stabilisers e.g., olanzapine, carbamazepine	Defined above		
	Naltrexone	Non-specific competitive opioid antagonist with highest affinity for the mu-opioid receptors in the CNS [39]		Nausea, vomiting, abdominal pain, decreased appetite, dizziness, lethargy, headaches and sleep disorders [40]
Personality disorders	Antidepressants (SSRIs, SNRIs) Quetiapine Naltrexone	Defined above	Shown efficacy in the treatment of BPD [41,42]	Listed above

Abbreviations: SSRI = selective serotonin reuptake inhibitors, SNRI = serotonin and norepinephrine reuptake inhibitor, TCA = Tricyclic antidepressants, MAOIs = Monoamine oxidase inhibitors, FGA = first generation antipsychotics, SGA = second generation antipsychotics, 5-HT = 5-hydroxytryptamine, NE = norepinephrine, DA = dopamine, GABA = γ-AMPA = aminobutyric acid, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, PD = panic disorder, GAD = generalised anxiety disorder, SAD = social anxiety disorder, PTSD = post-traumatic stress disorder, BD = bipolar disorder, AN = anorexia nervosa, BN = bulimia nervosa, BED = binge eating disorder, PG = pathological gambling, KM = kleptomania, TTM = trichotillomania, IED = intermittent explosive disorder, BPD = Borderline Personality Disorder, SLE = systemic lupus erythematosus, PCOS = Polycystic ovary syndrome, CNS = central nervous system. * Many drugs listed for the treatment of anxiety are also employed for the treatment of mood disorders including SSRIs, NSRIs and TCAs.

2. Mental Health Disorders

The incidence of mental health disorders is increasing globally, yet a fundamental understanding of the aetiology of mental health disease remains elusive. Assumptions relate severe mental illness to a small number of genes with a specific relationship between genotype and mental illness where biological pathways and environmental factors impact on illness [43]. Studies have demonstrated a strong relationship between severe trauma such as childhood abuse and/or abandonment with adult mental health disorders and a diminished quality of life [44]. The most widely discussed theories of depression are based on the regulation of the monoamines, norepinephrine (NE), dopamine and serotonin. The catecholamine hypothesis of depression developed in the 1960s remains the basis of treatment of mental health disorders such as MDD and anxiety disorder. Based on the theory that such mental illness is a result of a NE or dopamine deficiency at central nervous system (CNS) synapses, treatment aims to increase dopamine or NE levels to alleviate symptoms [45]. The serotonin hypothesis of depression also from the 1960s relates mood disorders to a deficiency in serotonin (5-hydroxytryptamine, 5-HT) a neurotransmitter involved in regulating emotion and mood [46]. There are currently 14 5-HT receptor subtypes identified having varying affinities for serotonin, present presynaptically, postsynaptically in the CNS and throughout the brain being predominately G protein coupled receptors (except 5-HT3 receptor) [47]. Agonism of the 5-HT_{2A} receptors is involved in increasing cortical glutamate levels [48]. The neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis a natural pathway activated in times of stress also becomes hyperactive in states of depression. The HPA axis is involved in maintaining blood glucocorticoid levels, where elevated levels can disrupt HPA function [49]. Studies have shown that cortisol elevation long term results in cognitive and other medical issues in patients and exacerbate depressive symptoms as shown in mice [50]. Thus, it is generally accepted that neuropsychiatric diseases such as PTSD, MDD, anxiety etc are a result of a chemical imbalance in neural circuits distributed across the brain and CNS related to a loss of neuro plasticity [51]. Consequently, treatment of mental health disorders is based on prescribing anti-depressants namely selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), dopamine reuptake inhibitors (DRIs) and tricyclic antidepressants (TCAs) which aim to increase neurotransmitter levels at the synapses and increase neuroplasticity. While the efficacy of this approach remains under question the side effects associated with the varying therapeutics in these categories are undeniable. Serotonin syndrome for example which is a potentially life-threatening condition can be caused by using serotonergic drugs and overactivation of 5-HT_{1A} and 5-HT_{2A} receptors [52]. Additionally, antidepressants such as TCAs and SSRIs are known to increase the concentration of neurotransmitters at synapses within minutes, yet it takes weeks to months for mental illness symptoms to regress in patients [53]. Additional theories of depression include the neurogenic hypothesis of depression and inflammatory hypothesis based on an increase in proinflammatory cytokines in the nervous system [7]. The 5-HT receptors are known to regulate the release of cytokines interleukin (IL) and tumour necrosis factor (TNF) and modulate immune macrophage and dendritic cell function and aid in maintaining an anti-inflammatory state in vivo [54]. With increasing rates of mental illness and suicide ideation and tendencies, it is imperative that a more rapid acting treatment option is established.

Mental Health and Co-Morbidities of Chronic Pain

Functional somatic syndrome is the term used for conditions that do not appear to have an underlying biological pathology [55]. These debilitating conditions are becoming increasingly frequent imposing a significant economic burden. Chronic fatigue syndrome (CFS), irritable bowel syndrome (IBS) and fibromyalgia (FM) amongst others are FSSs where pain, fatigue, muscle, and joint aches often present without an identifiable cause. Theories have arisen (the 'Lumpers' vs. 'Splitters' debate) suggesting that certain FSSs have one underlying aetiology and are associated with psychiatric disorder's including anxiety and depression. While the pathophysiology of FM remains unknown it has been postulated that

thinly myelinated and unmyelinated C-nerve fibres have been reported in FMS patients [56] suggesting a link between loss of nerve myelin and chronic pain. Additionally, studies suggest that a dysfunction of dopamine is present in FM patients where dopamine agonists appear effective in treating symptoms [57]. Studies have demonstrated decreased cortical dopamine receptor binding in FM patients [57]. FM characterised by extreme hyperalgesia is highly prevalent (3 times more prevalent) in persons with major depressive disorder than in patients without, studies also show high rates of MDD amongst family members of FM patients suggesting a genetic aspect [58]. Additionally, sleep abnormalities in FM are similar to those present with a 5-HT dysfunction in depressed individuals [58]. CFS appears less linked to mental health disorders where psychiatric status is not considered an important causal contributor to CFS [59]. IBS is a painful syndrome characterized by chronic abdominal pain, altered bowel habits, correlated to significant psychological distress and psychiatric comorbidities anxiety, panic disorder, MDD and suicidal ideation [60]. Polymorphisms in the serotonin transporter gene (SERT) has been associated with higher risk of depression in IBS patients with a dysfunction of serotonin transporters impacting the emotion regulating regions of the brain [61,62]. At present, treatment of FM and IBS is also reliant on the use of antidepressant drugs TCAs and SNRIs where successful therapy has supported the belief that a dysfunction of serotonin and NE is present in FSSs similarly to MDD [58].

The relationship between mental health disorders and autoimmune conditions (including multiple sclerosis, rheumatoid arthritis, colitis, and lupus erythematosus) has been well established where there is increased risk of MDD, anxiety and schizophrenia in autoimmune patients. Theories suggest the excessive inflammation characteristic of autoimmune diseases may be a casual factor impacting the CNS or via cytokine interactions between nerve and immune cells of the patient [54]. The relationship between increased levels of cytokines including tumour necrosis factor-α (TNF-α) and IL-6 and major depression disorder is now recognised [47]. This raises the question if fungal extracts showing promise in treating mental health disorders may also alleviate the symptoms of certain FSSs namely FM and IBS and auto immune conditions.

3. Fungal Biologics: Unlocking the Potential of Eastern Practice into Western Medicine

There has been an increasing interest in translating potential medicinal cures derived from medicinal mushroom used for centuries in Eastern practice into Western medicine [63,64]. Modern medical practice relies heavily on the use of highly purified pharmaceutical compounds whose purity can be easily assessed and whose pharmaceutical activity and toxicity show clear structure-function relationships. In contrast, for decades, many mushroom-derived medicines contain mixtures of natural compounds had not undergone detailed chemical analyses and whose mechanism of action had not been elucidated [65]. With the decline in the number of new therapeutics produced from the pharmaceutical industry, novel biologic agents are being sought from traditional medicine. Translating traditional Eastern practices into acceptable evidence-based Western therapies is challenging given different manufacturing standards, criteria of purity, and under-powered clinical trials making assessment of efficacy and toxicity by Western standards of clinical evidence difficult. Purified bioactive compounds derived from medicinal mushrooms using appropriate drug discovery programs are a potentially important new source of psychoactive agents. For example, the therapeutic potential of medicinal mushrooms is evidenced by the fact that two glucan isolates were licensed as drugs in Japan as immune-adjuvant therapy for cancer in 1980. Moreover, Murphy et al. (2021) noted that approximately 200 registered clinical trials have appeared in the literature focusing on use of beta-glucans extracted from a diversity of medicinal mushrooms with a focus on cancer treatments using mainly oral administration [65]. These authors observed significant challenges exist to further clinical testing and translation of mushroom-derived biologics. The diverse range of conditions for which biologics from medicinal fungi are in clinical testing underlines the incomplete understanding of the diverse mechanisms of action that is a key knowledge gap.

By far the greatest mechanistic information relates to elucidating immunomodulatory potential of medicinal mushrooms [66,67]. Furthermore, important differences appear to exist in the effects of apparently similar mushroom-derived preparations, which may be due to differences in sources and extraction procedures, another poorly understood issue [65]. In general, there is a dearth in evidence-based knowledge from registered clinical trials on potential use of biologics derived from medicinal mushrooms for alleviating mental health conditions. Therefore, there is growing interest in adopting a Quadruple Helix approach (academia–industry–policy–society) to support emerging industries, including medicinal fungi companies, in the 'bioprospecting' and development of new therapeutic products informed by compliance with appropriate regulatory framework and clinical trials; moreover, this multi-actor framework also enables greater societal awareness of the potential for higher fungi to meet a diversity of emerging needs [68–70].

Outside of the use of extracts and along with the beta-glucans, a plethora of fungal bioactives have been isolated and fully purified including further polysaccharides, peptides and polyphenols, medium-sized macromolecules such as triterpenoids and the small molecule type structures of the indole alkaloids including tryptamines. Yildiz et al. (2015), for example, reports on the potent antioxidant action of phenol compounds in many medicinal mushrooms [71]. The focus here will be on the bioactives with low molecular weights which can cross the BBB and have structural features and effects similar to the common neurotransmitters. The neurotransmitters, serotonin, dopamine and NE are monoamines derived from either tyrosine or tryptophan. Some of the most interesting and potent compounds discussed herein are derived from the same precursors. Indeed, serotonin itself and psilocybin are in fact both substituted tryptamines (Figure 1).

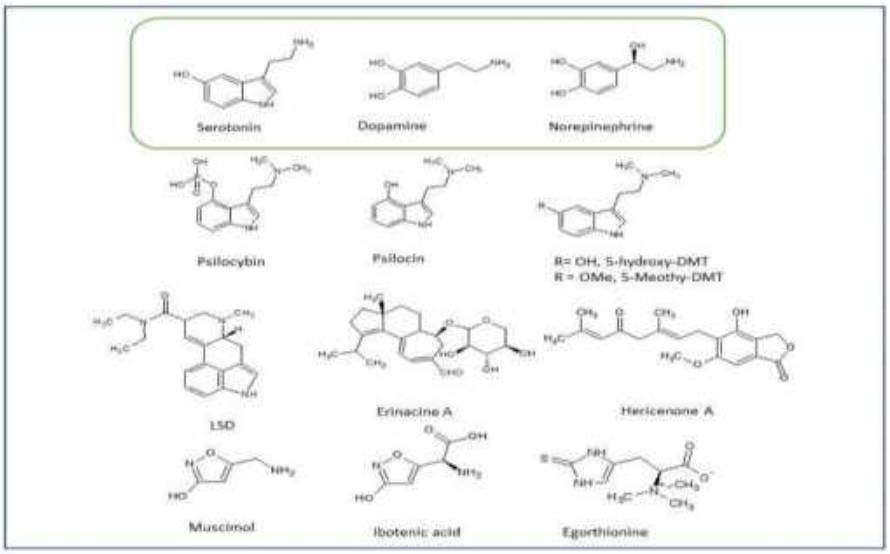

Figure 1. Monoamine neurotransmitters and fungal bioactives.

Psychedelics or serotonergic hallucinogens are natural substances which have agonism for the 5-HT receptors and include psilocybin, dimethyltryptamine (DMT) from the chacruna (ayahuasca brew) and jurema plants and mescaline from the peyote and San Pedro cacti [72]. Psychedelic compounds produce hallucinogenic experiences via activity in brain regions regulating cognition, emotions, self-awareness and perception of pain [73]. Psychedelics are included in the psychoplastogens class of compounds which have effect on neural plasticity in key circuits involved in brain health [53]. By definition psychoplastogens produce a quantifiable change in plasticity within a short time frame (>72 h) post administration [74]. Agonism of the 5-HT receptor group is associated with neuroplastic changes potentially reducing depression and anxiety symptoms [75]. Studies show that psychoplastogens have a range of therapeutic potential in treating neuropsychiatric diseases including anxiety disorder, mood disorders, PTSD, and addiction [74].

The 5-HT receptor group also has an important role in the gut-brain axis and is linked to the pathophysiology of IBS via dysregulated serotonin signalling [76]. Mushrooms containing psychoplastogens have been in use as traditional medicine in numerous cultures as healing rituals for millennia. Fungal biologics of therapeutic potential include lysergic acid diethylamide (LSD) a synthetic derivative of the precursor LSA (d-lysergic acid) or ergine from the rye ergot fungus *Claviceps purpurea*, muscimol and a DMT derivative from *Amanita muscaria* mushroom (amongst others) and psilocybin, a hallucinogenic pro-drug of psilocin found in Psilocybe mushrooms (magic mushrooms) (Table 2). Psilocybe alkaloids are more recently obtained from magic truffles (fungis sclerotia) as this species is currently overlooked in the prohibition laws banning sale [48]. Non hallucinogenic fungal biologics showing potential for mental health treatment include compounds from the edible mushroom *Hericium erinaceus* and ergothioneine from *Pleurotus cornucopiae*.

Table 2. Fungal biologics of therapeutic potential.

	Mescaline	Psilocybin/Psilocin	LSD
Pharmacodynamics	Naturally occurring substituted phenethylamine extracted from the peyote cactus	Naturally occurring indole-alkylamine (tryptamine) extracted from Psilocybe mushrooms	Semisynthetic indole-alkylamine (ergoline) derived from lysergic acid found in *Claviceps purpurea*
	5-HT releasing agent, catecholamine-like structure [77]	Close structural analogue of 5-HT	Close structural analogue of 5-HT
	Primarily interacts at 5-$HT_{2A/2C}$ and α_2-adrenergic receptors [78]	Primarily interacts at 5-$HT_{2A/2C}$ and 5-HT_{1A}, 5-HT_{2C} [79]	Mixed 5-HT_2/5-HT_1 receptor partial agonist [80]
	Low binding affinity at dopaminergic and histaminergic receptors [78]	Indirectly increases DA concentration but has no affinity for D2 receptors [81]	High affinity dopaminergic, adrenergic, and histaminergic receptors [82,83]
Pharmacokinetics	Can be ingested orally, smoked, or insufflated	Can be ingested orally or intravenously	Can be ingested orally, smoked, injected, or snorted
	Relatively low-potency: active doses in the 200−400 mg range) [84]	20× more potent than mescaline: active doses in 10–30 mg range [81]	2000× more potent mescaline: active doses in 25–200 µg range [85]
	Rapidly absorbed in GI and distributed to the kidneys and liver	Rapidly absorbed and dephosphorylated to psilocin (bioactive form) [81]	Rapidly absorbed in GI and distributed to tissues and organs
	Low lipid solubility, weak ability to penetrate BBB [77]	Lipid soluble, can easily cross BBB [86]	Can easily cross BBB [87]
	Detoxification via oxidative deamination	Detoxification via demethylation and oxidative deamination	Detoxification via N-dealkylation and/or oxidation processes
	Long-lasting, half-life of 6 hrs	Half-life of 3 hrs	Half-life of 3.6 hrs
	Eliminated in urine mainly in the unchanged form (81.4%) and the remaining as the metabolite TMPA [88]	Eliminated in urine mainly as glucuronidated metabolites (80%) as well as unaltered psilocybin (3–10%) [89]	Eliminated in urine mainly as metabolites, only 1% of the dose is excreted unchanged) [87]
Efficacy	Acute experiences of psychological insight during mescaline use are associated with self-reported improvements in anxiety disorders, depression, and substance abuse [78,84]	Therapeutic efficacy in treating mood and anxiety disorders, depression, cluster headaches, chronic pain, intractable phantom pain, obsessive-compulsive disorder, and substance abuse [79,81,90]	Therapeutic efficacy in treating anxiety disorders, depression, cluster headaches, obsessive-compulsive disorder, substance abuse, psychosomatic illnesses, and anxiety in relation to life-threatening diseases [91,92]

3.1. Psilocybe Mushrooms

Undoubtedly psychedelics from the Psilocybe species are currently the more commonly investigated fungal compounds for their biological and therapeutic potential. Psychedelic compounds are known to exert their effects via activation of serotonergic 5-HT receptors dispersed throughout the central and peripheral nervous system impacting on cognition, mood and emotion [93]. Psilocybin is a potent agonist of 5-HT_{2A} receptors and moderate agonist of 5-HT_{1A} and 5-HT_{2C3} receptors present in the thalamus and cortex

of the brain [79]. These receptors are also associated with peripheral and central nervous system pain perception [73] justifying the use of SSRIs in the treatment of chronic pain conditions such as FM. Psilocybin has also demonstrated affinity for dopamine receptors and serotonin transporter protein [94]. Both psilocybin and its metabolite psilocin, pass through the blood–brain barrier (BBB) where psilocin is 1.5 times more potent. Indeed, between the 1950s and 1970s, researchers actively investigated the potential of psilocybin in the treatment of OCD, addiction, and neurotic behaviours [72] but studies were inhibited by prohibition until the early 1990s. Psychedelics are now believed to have additional beneficial biological activities including promoting neural plasticity and immune modulation [95]. Additionally, psilocybin positively impacted symptoms of MDD, treatment resistant depression, substance addiction and anxiety (anxiolytic) in afflicted persons and terminal cancer patients [96]. Comparative studies where one dose of ca. 30 mg/70 kg psilocybin was administered to terminally ill patients reduced symptoms of depression and anxiety and improved optimism in 51 cancer patients for up to 6 months post administration [97]. In one trial study, psilocybin reduced symptoms of MDD, anxiety and anhedonia as early as 1 week post administration of 10 mg and 25 mg (seven days apart) with no observed side effects to the patient [75]. Studies assessing the impact of psilocybin on alcohol addiction showed patients had significantly fewer drinking days in the treatment period of 8 weeks at 300 µg/kg 4 weeks apart [79]. Promising results were also observed with the use of psilocybin in conjunction with cognitive behavioural therapy (CBT) to aid in smoking cessation [98]. A long term follow up study reported 60% abstinence rates after more than 12 months compared with a 31% anstinence rate at 12 months using conventional therapies. Small scale clinical studies also demonstrate the efficacy of psilocybin at reducing the symptoms of OCD where SSRI treatment failed [99]. Similar to the action of SSRIs on 5-HT receptors alleviating chronic pain in patients with neuropathic, musculoskeletal pain and fibromyalgia, psilocybin may reduce chronic pain symptoms [73]. Clinical trials are warranted on the administration of psilocybin to chronic pain patients including FSSs comparative to current treatment options such as SSRIs and opioids.

3.2. *Claviceps purpurea*

The fungus *Claviceps purpurea* is extensively known for its production of ergot alkaloids having activity on the nervous system and smooth muscles where therapeutic application includes treating Parkinson's disease, cluster headaches and migraine [100]. LSD is a semi-synthetic derivative of lysergic acid found in fungus *Claviceps purpurea* [73]. LSD was also a psychedelic compound highly researched in the 1950s until the Controlled Substances Act was introduced in 1970 [72]. Like psilocybin, LSD has activity on brain processes involved in cognition, emotion, and perception via affinity for serotonin receptors [101]. LSD is an agonist of 5-HT with high affinity for 5-HT2A and 5-HT2C and is small enough to pass the BBB being approximately 100 times more potent than psilocybin [73]. Interestingly, LSD is also an agonist of the dopaminergic receptors with relatively high affinity for dopamine 2 receptors [102] and an agonist of the trace amine associate receptor 1 (TAAR1) [103]. Currently, in the treatment of psychotic illness the therapeutic efficacy of active pharmaceutical ingredients (APIs) is required to be greater than 70% occupancy of D2 receptors [103]. TAAR1 is essential in the regulation of monoamines (dopamine, NE, serotonin) in neurons of the CNS and neuro immune modulation [104] via agonism of trace amines (β-phenylethylamine, p-tyramine, and tryptamine). Dysregulation of trace amines and TAAR1 receptor dysfunction has been identified in psychotic disorders including MDD, bipolar disorder and schizophrenia [103]. Additionally, the TAAR1 receptors are associated with the pathophysiology of IBS [76]. Studies have demonstrated the positive effects of LSD on anxiety relating to terminal illness [93] with positive affects also observed for PTSD and addiction. The analgesic activity of LSD has also been described in terminally ill patients lasting up to 12 h post administration [73]. While reports on pain management of headaches are self-reported, patients state that sub hallucinogenic concentrations (micro dosing) of LSD prophylactically and metaphylactically alleviated migraine and cluster

headaches [105]. One clinical study determined that 20 µg LSD increased pain tolerance and reduced pain perception in patients compared to placebo with similar outcomes to oxycodone and morphine [106]. Such analgesic effects may be attributed to the dopaminergic activity of LSD suggesting positive effects on neurological disease such as FM. LSD's mechanism of action is pleiotropic affecting serotonin (5-HT), dopamine and TAAR1 receptor pathways potentially alleviating symptoms of mental illness and chronic pain conditions FM and IBS. It is important to note that LSD is a complex molecule, effecting many receptor pathways, where long-term administration may result in psychotic-like symptoms or Hallucinogen Persisting Perception Disorder (HPPD) or flashbacks in the user [103]. Such effects, however, are deemed low risk, uncommon and mostly associated with recreational use [107]. Additionally, secalonic acid A and its isomers are also found in Claviceps purpurea which has anti-cancer activity via topoisomerase I and II inhibition [108].

3.3. Amanita muscaria

A. muscaria contains many biologically active compounds including the psychoactive alkaloids: muscarine, ibotenic acid and muscimol [109] where ibotenic and muscimol are structurally similar to gamma-aminobutyric acid (GABA) having effect on glutamate receptors in the CNS [110] and can cross the BBB. Ibotenic acid is a potent neurotoxin via activation of N-methyl-d-aspartate (NMDA) receptors where muscimol has a strong psychoactivating action [111]. The alkaloid muscarine is an acetylcholine agonist of the parasympathetic nervous system typically negatively impacting the functioning of numerous organs but cannot pass the BBB [109]. Over stimulation of muscarinic cholinergic system and acetylcholine is associated with the aetiology of depression suggesting that the agonist muscarine will elevate depressive symptoms in patients. Studies by Corbett 1991, demonstrate that muscimol improved social behaviour and had anxiolytic-like effects post systemic administration to test rats, similar to diazepam [112]. Additionally, muscimol when used as a combination therapy with endomorphin-1 reduced the symptoms of neuropathic pain due to spinal cord injury when administered for 7 consecutive days [113]. Bufotenine (5-HO-DMT) is an alkaloid and a substituted tryptamine similar to serotonin and psilocin, also present in the Amanita mushroom. As such, it has the ability to cross the BBB having agonistic effects on 5-HT1A and 5-HT1B receptors [114]. Another substituted tryptamine,5-MeO-DMT, requires enzymatic activity of a monoamine oxidase (MAO) inhibitor to induce its psychedelic effects orally [48]. Recently, β-carbolines, which are well-known MAO inhibitors have been isolated directly from psilocybe mushrooms. β-carbolines, as well as psilocybin itself, are derivatives of tryptophan. This represents an interesting natural product pathway where two different types of products from the same precursor contribute to the same pharmacological effect [115]. Tryptamines have been used to treat symptoms of PTSD, MDD, addiction and anxiety [116] and show potential as treatment with low risks for addiction.

3.4. Hericium erinaceus (H. erinaceus)

The medical benefits of *H. erinaceus* have been well documented with anticancer, antioxidative, anti-inflammatory and antimicrobial activity amongst others [7] where neurotropic compounds (Figure 1) present in the mushroom are more recently being considered for neuropsychiatric disorders. Hericenones and erinacines (cyathane derivatives) are biological neurotropic compounds found in the fruiting body and mycelium of *H. erinaceus*. These compounds can pass the BBB due to their low molecular weight [117] which may beneficially impact on Alzheimer's and Parkinson's disease due to their impact on nerve growth factor (NGF) [118]. Indeed, many biological active compounds are present in the fruiting bodies of *H. erinaceus* including aromatic compounds, diterpenoids, steroids, and polysaccharides [119]. NGF is essential for protecting nerve tissue and maintaining neuron functionality where studies have shown NGF levels in MDD patients is also significantly reduced [120]. Interestingly, NGF itself is unable to pass the BBB highlighting the benefits of these compounds which stimulate NGF production within the brain and

encourage nerve myelination throughout the CNS [121]. Perhaps such myelination effects may also aid in alleviating the symptoms of FM in patients where improper myelination of nerves is a possible issue [56]. Initial studies suggest that the NGF enhancing activity of these extracts may reduce MDD and anxiety [122]. Elevated levels of NGF are associated with neuroplasticity and neurogenesis which may improve mood in MDD patients based on the neurogenic hypothesis of depression [7]. Additionally, in vivo studies on depressed animals demonstrated that when fed with *H. erinaceus* levels of the monoamine's serotonin, dopamine and NE were elevated [123]. In vivo studies report that erinacine can increase monamine expression and modulate anti-inflammatory brain-derived neurotrophic factor (BDNF) signalling in depressed animals [124]. Amycenone, a proprietary mixture of compounds which are present in the fruiting body of *H. erinaceus*, demonstrated anti-inflammatory activity against cytokines TNF and IL and reduced inflammatory induced depression in test animals [119] a similar activity observed with SSRIs and SNRIs. Such anti-inflammatory activity may also prove beneficial in alleviating symptoms of autoimmune co-morbidities of mental illness. Studies show when consuming H. erinaceus adults with mild cognitive impairment and menopausal women were less depressed, anxious with improved cognitive abilities compared to placebo control groups [121]. Studies are warranted however, confirming the efficacy of biological extracts from *H. erinaceus* mushrooms comparative to consuming the entire fruiting body of the mushroom at a therapeutic level. Additionally, while these compounds have been shown to affect the levels of monoamines, the mechanism of modulation requires further investigation.

3.5. Pleurotus cornucopiae

One of the fungal bioactives with the most diverse effects and potential uses currently under widespread investigation is Ergothionine. Found in large amounts in oyster mushrooms (*Pleurotus cornucopiae* var. *citrinopileatus*), Ergothione is a metabolite of the aromatic amino acid histidine. It has been shown to have neuroprotective and anti-inflammatory effects as well as being potentially involved in neurogenesis, all of which are characteristics of compounds which may be useful in the treatment of the aforementioned disorders [125]. Dietary ergothionine was shown to significantly reduce immobility time in forced swim test (FST) and tail spin test (TST) which are used as models of depression in mice [126]. Additionally, oral administration of ergothioneine prior to and during another mouse model of depression (Social Defeat Stress (SDS) paradigm) had a preventative effect on depressive behaviours such as social avoidance and sleep abnormalities [127]. Ergothioneine is a substrate of carnitine/organic cation transporter OCTN1 and this has led to investigation of the potential targeting of such atypical monoamine transporters as a strategy for the development of new anti-depressants [128].

4. Pharmacological Consideration of Mycotherapy

Bioactive compounds which promote neuro plasticity and induce long term changes in mood, emotion and cognition may offer therapeutic options to chronically mentally ill patients [95]. When considering the administration of psychedelic compounds as therapeutics the efficacy of single dosing versus micro-dosing must be considered as current studies vary between self-medicating/micro dosing patients and single dose clinical trials. The time frame required for onset of action is an important consideration as current drug therapy (SSRI, NSRIs) for mental illness requires weeks to months for therapeutic effect. Studies indicate the effects of the fungal bioactives described occur rapidly and have a prolonged lasting effect on the symptoms of the diseases investigated [93]. Additionally, there appears to be no, or limited side effects or withdrawal symptoms associated with acute therapy or micro-dosing. Tolerance can develop however, relating to down regulation or de-sensitization of the 5-HT receptors and a cross tolerance with other psychedelic compounds may occur [47]. Psilocybin has low toxicity with chronic exposure and moderate toxicity in cases of acute exposure and has low addiction and dependence issues [75]. LSD which is more potent than psilocybin induced some dose dependent physical and

psychological symptoms including derealization, depersonalization and dissociation [106]. Psilocybin has pharmacodynamic effect lasting between 4–6 h with LSD lasting up to 12 h, an elimination half-life of 3.5 h and 3 h for LSD [129] and psilocybin [130] respectively, has been established. Route of administration is an important formulation consideration for psychotherapy as tryptamine and its derivates are prone to first pass metabolism. The addition of a monoamine oxidase inhibitor may be needed for oral delivery formulations [48]. Such an inhibitor would increase bioavailability and pharmacological efficacy of bioactive amines including psilocybin and LSD. The administration of such compounds is not straight forward as each has specific receptor affinities, duration of action and potency. The therapeutic index for each compound must be established to fully determine the safety profile as long-term treatment may be required for chronic mental health illness or chronic pain conditions. Currently, mood and behavioural studies are conducted in animals where analysis of symptoms and extrapolation to humans is difficult. Consuming such compounds may affect the patient's consciousness and provide insights and existential and spiritual questions [99]. For example, at 12 month follow up in the smoking cessation work by Johnson et al., 86.7% of the participants rated their psilocybin experience among the 5 most personally meaningful and spiritually significant of their lives. Administration of these compounds, therefore, must be in a controlled manner to avoid the risk to persons prone to psychosis and to determine the patient's mindset prior to administration. To this end, many of the studies involving the psychedelics and compounds which alter consciousness have been carried out in the presence of psychiatrists and counselling professionals specifically trained in psychedelic psychotherapy. Some alternative methods of determining activity have been utilized based on gene ontology, predictive analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to investigate fungal biologics [124].

There is a pressing need to reach international consensus and agreement on the defined production methods for bioactives produced from medicinal fungi for this potential purpose as batch-to-batch variation in production methods and types of mushrooms may generated different structural-bioactives that may lead to vary functional activities [67,131,132]. This situation will be addressed by promoting international collaborations between academia and industry partners in the areas of biotechnology, mycology, toxicology, bioinformatics and biopharma.

5. Conclusions

Bioactives found in higher fungi such as mushrooms have chemical structures similar to neurotransmitters and can act as agonists of receptor pathways involved in psychiatric conditions. Harnessing this activity as therapy for chronic mental health and pain diseases may offer benefits where therapeutic needs are currently unmet. Considering the high morbidity and mortality rates associated with these disorders and the economic burden placed on health systems, it is imperative competent treatment options are investigated. Bioactives such as psychedelics effect a person's cognition, emotion and mood often having a long-lasting effect on symptoms of mental illness. Interest has arisen in tryptamine derivatives LSD and psilocybin and actives including hericenones and erinacines for the treatment of mood disorders, anxiety disorders, PTSD and addiction. Additionally, compounds acting as agonists of serotonin receptors may aid in alleviating symptoms of inflammatory conditions by regulating pro-inflammatory cytokines TNF and IL. While the potential benefits of such mycotherapy emerge, studies are warranted to determine their full pharmacokinetic and pharmacodynamic profiles before implementation as alternative drug therapy can be considered. While addiction and dependence do not appear to be an issue with such compounds, tolerance and cumulative effects must be considered. The complete aetiology of mental health disorders remains undetermined with the relationship between the mind, the brain and CNS a multifaceted interaction. As knowledge emerges giving a better understanding of mental illness, advances in neuropharmacology will undoubtedly follow. Perhaps such advances will include mycotherapy. Currently, the biggest challenge associated with psychedelic pharmacology studies is prohibition

where psychedelics are classed as a Schedule 1 drug and the social stigma associated with their use. Chong et al., 2021 implied a predictive analysis method to investigate the molecular mechanisms of *H. erinaceus* [124], perhaps this offers a novel means of determining efficacy prior to clinical trials in line with prohibition. Other methods including ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis to investigate fungal biologics may also be implemented. Using animal species to model depression system in humans is currently standard protocol. Such assays allow researchers to investigate neural circuitry and molecular and cellular pathways in acute and chronic states. While such models off advantages, perhaps going forward inclusive studies of depression may employ several strains and multiple testing models. Where studies must compare genetic and epigenetic expressions, and activity to better extrapolate to human patients. Until a more comprehensive understanding of the aetiology of depression is established critical analysis of current drug therapy and fungal bioactives is warranted on existing animal and clinical studies to accurately determine the exact mode of action of fungal biologicals. Adopting 'Quadruple Helix' framework that unites academia, industry, policy, and society will further enable and support bioprospecting, development, testing and validation of bioactives of interest extracted from higher fungi (mushrooms) such as to advance the field of chronic pain and mental health disorders and conditions. Currently, international drug policies are slowly evolving as the potential benefits of these compounds are being revealed; however, care must be taken at clinical therapy stage.

Author Contributions: Conceptualization of this article was by M.G. and S.H. Design, research, and writing of this article was conducted by E.M., S.H., N.R. and M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: M.G., S.H. and E.M. would like to acknowledge the PEM centre and IT Sligo.

Conflicts of Interest: The author declares no conflict of interest.

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Section Five – Elucidating and Developing New Sustainable Technologies and Frameworks

The **final section** describes some of my latter years research as Director of the Bioscience Research Institute in Ireland at TUS on the holistic development of sustainable innovation at demonstration sites. These studies with and without novel processing or disease-mitigating technologies where emphasis is on developing a new integrated, multitrophic aquaculture system (IMTA) that recirculates clean water based on use of fish waste stream by microalgae and duckweed present in the ponds. Studies report on the first use IMTA in the peatlands as alternative means of sustainable land use aligned to zero waste, zero pollution and climate action principles. Studies showed that an understanding of key disease preventative approaches was critical to informing reliable and repeatable operation of the IMTA site where pulsed UV was removed as an end-of-pipe solution (as no discharge of aquaculture effluent to receiving water occurs negating PUV or other technology use). Studies report on the simultaneous use of hand-held diagnostic equipment with 'in field' sensors for monitoring fish culture ponds, fish health and disease mitigation. Bioinformatics with next-generation sequencing are also used to support and inform traditional enumerating techniques for entire ecosystem in IMTA peatland site. Studies revealed that climate change produced three successive storm events that flooded this IMTA system causing significant fish mortality where there was a requirement to introduce disease mitigation approaches (non-chemical solutions), as site is organic status. Studies describe first use of multi-actor hubs including triple to quintuple helix frameworks for integrated development of innovation. Studies address modelling and geo-mapping of chemicals of emerging concerns on the European Watch List in water and the importance of understanding and identifying sensitive detection methods and having appropriate disinfection assets at waste water treatment facilities as critical control points. Studies describe life cycle assessment and ecological tools to confirm efficacy and appropriateness of innovation for food and environmental deployment. Studies also compare and address large scale-disinfection technologies for effective treatment and enumeration of food and water-borne parasites in their different life-cycle development/growth phases as these are particularly complex, recalcitrant and fastidious pathogens.

Aquaculture 526 (2020) 735377

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Synchronizing use of sophisticated wet-laboratory and in-field handheld technologies for real-time monitoring of key microalgae, bacteria and physicochemical parameters influencing efficacy of water quality in a freshwater aquaculture recirculation system: A case study from the Republic of Ireland

Sarah Naughton[a,*], Siobhán Kavanagh[a,b], Mark Lynch[a], Neil J. Rowan[a,c]

[a] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[b] Department of Life and Physical Science, Athlone Institute of Technology, Ireland
[c] Centre for Disinfection, Sterilisation and Biosecurity, Athlone Institute of Technology, Ireland

ARTICLE INFO

Keywords:
Microalgae
Freshwater aquaculture
Waste treatment
Resource utilization
Sustainability

ABSTRACT

There has been growing interest in exploiting microalgae as a natural process for low cost wastewater treatment and for water quality control and remediation in aquaculture. This constitutes the first study to report on a strong relationship between use of sophisticated wet-laboratory flow cytometry equipment and in-field AlgaeTorch® technologies for determining microalgae and bacteria population dynamics in a freshwater pill-pond aquaculture farm over a 10-month monitoring period producing Eurasian Perch, *Perca fluviatilis,* in the Republic of Ireland. Nitrate levels and temperature were the most significant factors influencing microalgae numbers in rearing and treatment ponds as determined by Principle Component Analysis. Variance in climate, namely drought conditions that occurred during monitoring period, did not affect microalgae or microbial numbers. *Chlorophyta, Bacillariophyta* and *Cryptophyta* were the most dominant algal divisions observed in this recirculating aquaculture system, many of these are recognized as a natural source of beneficial prebiotics for fish. Determining baseline microalgal profiles in rearing water, followed by elucidating physicochemical parameters governing wastewater treatment performance, can inform future intensification and diversification of freshwater aquaculture by exploiting and replicating knowledge of favourable algal-microbial ecosystems. Furthermore, holistic datasets can be utilised for smart agriculture by way of informing management tools for future remote monitoring and decision-making by producers.

1. Introduction

Aquaculture has become the fastest-growing food sector in the world (FAO, 2018; Ruis-Salmon et al., 2020). During the period 2010 to 2030, global aquaculture production needs to increase threefold in order to meet the demands for fish and food (DAFM, 2015; O'Neill et al., 2019; O'Neill et al., 2020). In the aquaculture industry, water quality needs to be closely monitored in order to maintain, as closely as possible, the optimal growth conditions for a given cultured fish, and consequently to ensure optimal production (European Food Safety Authority (EFSA), 2008). Commensurately, water pollution has become a concern, posing threats to environmental protection that will retard intensive sustainability of aquaculture globally (Tahar et al., 2018; Tahar et al., 2019). To overcome these challenges, significant effort has been devoted to control wastewater pollution and to improve water quality control in aquaculture (Han et al., 2019). However, traditional environmental remediation approaches, such as aeration, filtration and other physical technologies, require high energy consumption or substantial add to the investment that increases total cost and financial burden of the industry (Longo et al., 2016; Tahar et al., 2019). These traditional technologies are often unable to fully utilise and recycle resources such as nutrients (including nitrogen, phosphorous and carbon) along with producing large amounts of CO_2 and sludge that cause secondary environmental pollution (Lu et al., 2019a). Moreover, antibiotics and medicines are frequently used in aquaculture to mitigate against disease and to reduce risk of outbreaks, which adds to growing

* Corresponding author.
E-mail address: s.naughton@research.ait.ie (S. Naughton).

https://doi.org/10.1016/j.aquaculture.2020.735377
Received 21 November 2019; Received in revised form 4 March 2020; Accepted 14 April 2020
Available online 16 April 2020

concerns over the antibiotic-resistance crisis globally (Muziasari et al., 2016). Consequently, there has been growing interest in the development of alternative or complementary environmental-friendly and economically feasible solutions to advance aquaculture (O'Neill et al., 2020).

In aquaculture, fish are reared at high densities for increased productivity, which can lead to the build-up of in-organic nutrients, excreted waste and feed remnants that can lead to unwanted eutrophication in the receiving aquatic environment (Bentzon-Tilia et al., 2016). When conditions are optimal, namely high nutrient loads, high temperature and sunlight, algae can grow to an excessive number forming either harmful or beneficial blooms in the rearing water that can effect fish health (Drikas et al., 2001). With high density rearing practices, harmful pathogens also have a greater chance of rapid circulation and persistence resulting in the potential to cause a disease outbreak. There is pressing need to monitor and manage microalgae, bacteria and key parameters in rearing water on fish farms that both detects and mitigates for the emergence of pathogens in order to make remedial management actions in real time (O'Neill et al., 2020). However, biomass analysis tends to be an insufficient method for speciation of microalgae as they lack distinctive features (Li et al., 2012). This highlights the importance of traditional time-consuming microscopic analysis in aquaculture.

There are many approaches to enumerating microalgae that have been traditionally limited to using conventional slide methods, such as the Sedgewick-Rafter slide, the Palmer-Maloney slide, the Petroff-Hausser slide and the standard haemocytometer method (Guillard, 1978; Han et al., 2019). The most common method for phytoplankton enumeration, especially for multispecies communities, is the Utermöhl method that requires an inverted microscopic with sophisticated optics in order to ensure reliable results (Vuorio et al., 2007). There is also considerable inter-variation between operators of the method, and variation between microscopes used that affects reliability and robustness (Vuorio et al., 2007). The use of real-time enumeration methods in marine aquaculture, such as flow cytometry (FCM), enables rapid-counting and differentiation of more than 10,000 cells or targets per second that can add to accuracy of algal determinations in a non-destructive manner (Endo et al., 2010). Recently, researchers have reported on the use of handheld monitoring devices, such as AlgaeTorch®, for taxonomic classification of algae through measuring fluorescence (Szymański et al., 2017). However, comparative use of conventional and sophisticated wet-laboratory equipment (such as flow cytometry) with on-farm monitoring devices (such as AlgaeTorch®), has yet to be reported for characterization and monitoring of freshwater aquaculture that relies on using natural biological processes for controlling water quality.

Thus, the aim of this timely study was to gain a comprehensive understanding of the role and relationship of microalga species with key bacterial and physicochemical parameters in freshwater aquaculture over a 10-month monitoring period using both wet-laboratory and on-farm measurements in order to define and enhance freshwater aquaculture.

2. Materials and methods

2.1. Study site and sampling

Samples were collected from a freshwater fish farm located in Boyle, Co. Sligo, Republic of Ireland that produces Eurasian perch (*Perca fluviatilis*). It contains broodstock tanks, a hatchery for eggs and larvae and a nursery for juvenile growth. All of these indoor systems are based in tanks and operate under recirculating aquaculture systems (RAS). There are three grow-out ponds for the larger fish. These are earthen pill-pond systems based on low surface flow water sourced from a local stream. This study focused on one of the grow out ponds, designated Pond 1 that is denoted by the red perimeter shown in Fig. 1. Pond 1 is divided into two connecting ponds; the fish pond (FP) that is stocked with adult perch, and the treatment pond (TP) devoid of fish. Flow is circulated in and out of the FP region using paddle wheels guided by walls to aid aeration. The TP supports the growth of microalgae that also provides oxygenation and wastewater remediation. A schematic is also inserted into Fig. 1 to help contextualize the operational concept. Sampling occurred in triplicate from March 2018 to November 2018 in order to capture seasonality as briefly outlined in Table 1. Samples were transported in a cooler box to the laboratory for analysis. Preserved samples were stored at 4 °C until further analysis was carried out.

2.2. Physicochemical parameter measurement

Physicochemical parameters that were measured in the laboratory included nitrates, nitrites, ammonium and phosphorus concentrations, pH and carbonate hardness. Whilst nitrates, nitrites, ammonia, phosphate, pH, oxygen and turbidity were measured *in situ* on the farm by standard methods. All parameters measured in the lab were carried out using individual test kits for each specific parameter as outlined in Table 2. After initial preservation with sulphuric acid, the pH was increased to between 6 and 7, which was required for all tests. Each test was carried out as per the individual kit instruction manual in duplicate. A Jenway UV–Vis Spectrophotometer was used for the spectrophotometric tests.

2.3. Use of flow cytometry to enumerate microalgae

The MACSQuant® Analyzer 10 (Miltenyi Biotec) flow cytometry (FCM) system was used for this study. Samples were centrifuged at 3500 xg for 20 mins, thereafter the cell pellet was resuspended in running buffer (PBS with 1 mM EDTA, 0.2% Tween, 0.1% sodium azide, 0.2 μm filtered). Samples were stained with nucleic acid dye SYBR Green (1:10,000×), to separate the DNA-containing cells from cellular debris and sedimentation present in the pond. SYBR Green fluorescence was detected on the B1 channel. An unstained algal control was required to eliminate natural auto-fluorescence in the B1 channel caused by excitation by the blue laser. Relative Cell Size and granularity was determined by forward scatter (FSC) and side scatter (SSC) channels. The blue laser stimulates chlorophyll (Chl) fluorescence on the B3 channel, and red laser stimulates phycocyanin (PC) fluorescence (R1 channel). Each sample was analysed in triplicate.

The gating method to obtain and enumerate the desired population, *i.e.* algae excluding cyanobacteria, was adapted from Moorhouse et al. (2018), Haynes et al. (2015) and Read et al. (2014) for phytoplankton and plankton analysis. This process is explained in Fig. 2 and involved running an unstained representative for each sample for the elimination of auto-fluorescence. This gate was used to determine DNA-containing cells in the SYBR Green-stained samples, without natural auto-fluorescence interference. Fig. 2(B) illustrates the distribution of cells present with all auto-fluorescent cells or unwanted material falling outside of the area/gate of interest. The Chl^+ population was isolated by acquiring the cells that fluoresced in the B3 channel. Fig. 2(C) illustrates the Chl^+ population with FSC on the x-axis relating to cell size and SSC on the y-axis relating to cell granularity and complexity. Cyanobacteria were present in this population as they also possess chlorophyll. This group was eliminated by graphing B3 against R1, and gating around the Chl^+/PC^- population. This is illustrated in Fig. 2(D) The final population depicted in Fig. 2(E) is the population of interest viewed under FSC and SSC. The isolated cyanobacterial populations from each sample were also analysed and enumerated to determine the trends over the duration of the study. All flow cytometry data gating was carried out using FlowJo software package.

2.4. Microalga profile development and in-field AlgaeTorch monitoring

Identification using a standard inverted microscope for

Fig. 1. Perch farm flow through ponds - aerial view, with insert schematic of Pond 1 layout.

Table 1
Sampling regime for obtaining rearing water profile at the perch farm.

Sample type	Volume	Application	Frequency	Preservative
Algal	100 ml	Enumeration, Identification & Profile Development	Biweekly	1% Lugol's Iodine
Bacterial	500 ml	Enumeration	Biweekly	1% Formaldehyde
Physicochemical	500 ml	Parameter Measurement	Biweekly	Sulphuric Acid

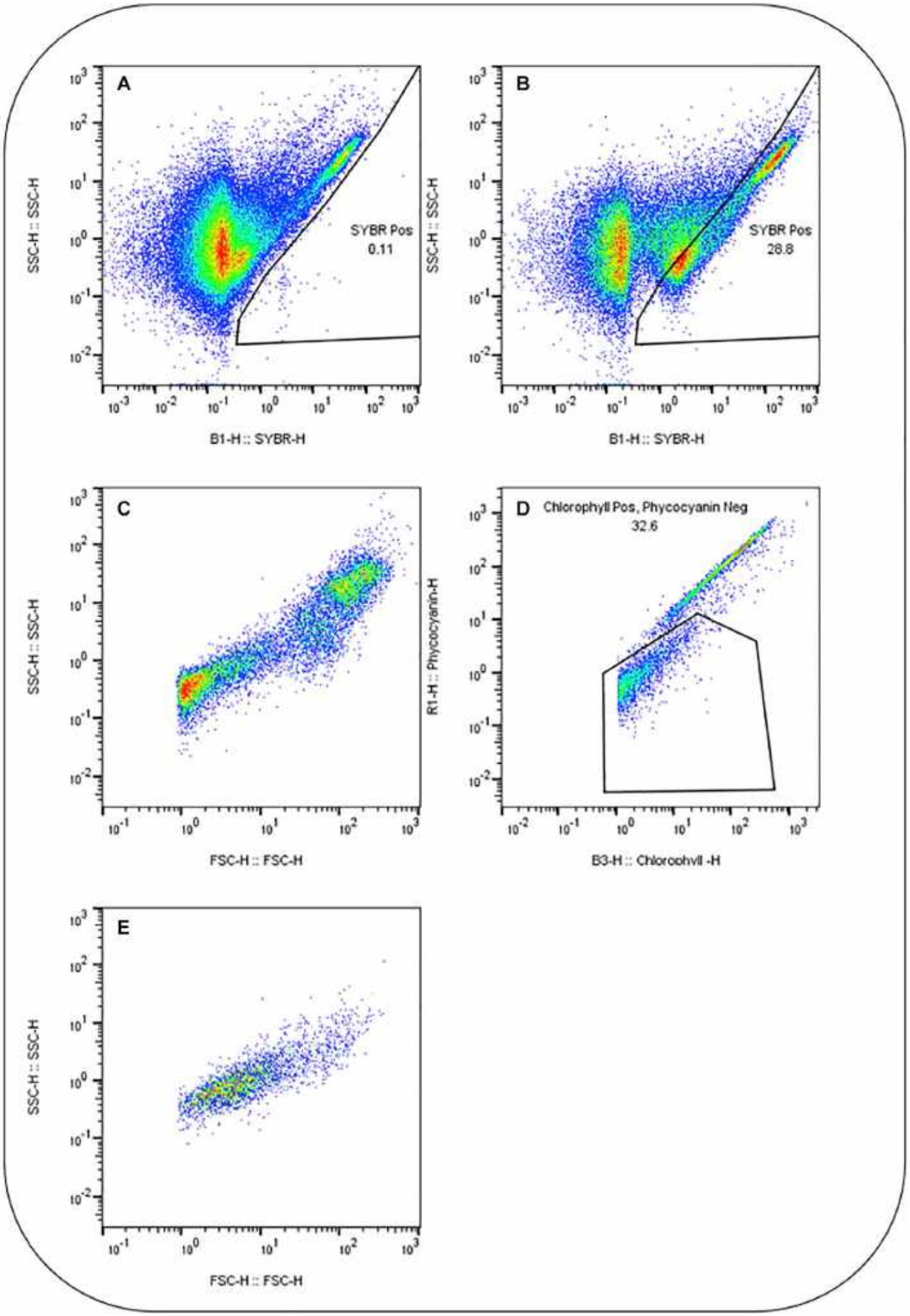
Table 2
Test kits and devices for physicochemical parameter analysis at the perch farm including acceptable limits.

Parameter	Measurement	R^2 Coefficient	Result Ranges (mg/l)		MAC (mg/l)	
			Fish pond (FP)	Treatment pond (TP)	SI No. 77 (2019)	Boyd (1998)
Nitrates	Photometric [Spectroquant® Nitrate Test – 1.09713]	0.998	< 0.01–3.27	< 0.01–3.25	50	0.2–10.0
Nitrites	Photometric [Spectroquant® Nitrite Test – 1.14776]	1	< 0.01–0.12	< 0.01–0.10	0.03	< 0.3
Ammonium	Photometric [Spectroquant® Ammonium Test – 1.14752]	0.998	0.12–1.89	0.08–1.69	–	0.2–2.0
Ammonia	Calculation	–	< 0.01	< 0.01	< 0.03	< 0.1
Phosphates	Photometric [Spectroquant® Phosphate Test – 1.14848]	0.999	0.17–1.66	0.10–2.20	0.025	0.005–0.2
Carbonate Hardness	Titration [MColortest™ Carbonate Hardness Test – 1.08048	–	70.06–650.57	50.04–630.55	–	50–200

morphological analysis and photographic identification keys were used in conjunction with FCM for enumeration to maximise the information obtained. In addition to FCM analysis of microalgal populations, the chlorophyll *a* and Cyanobacteria populations were also measured *in situ* using the AlgaeTorch® (bbe Moldaenke). The AlgaeTorch® is based on real-time *in vivo* fluorescent measurement upon excitation of the microalgal cells in response to six LEDs of three different wavelengths, 470, 525 and 610 nm. The measurement analysis carried out is in accordance with ISO 10260:1992 Water quality – Measurement of biochemical parameters – Spectrometric determination of the chlorophyll-*a* concentration and DIN 38412/16:1985 German standard methods for the examination of water, waste water and sludge; Test methods using water organisms (Group L); Determination of chlorophyll *a* in surface water (L 16). Both chlorophyll *a* and cyanobacterial content were measured in µg chl-a/l (bbe Moldaenke, 2017).

2.5. Bacterial enumeration using epifluorescent microscopy

Sample preparation for bacterial enumeration was carried out as per Bloem and Vos (2004). SYBR Gold was used to stain the microbial cells. Bacteria were filtered using Sartorius filtration apparatus onto 0.22 µm isopore membrane filters and a support Whatman™ filter was used to enhance vacuum distribution filtered onto the membrane. Residual background staining was removed using distilled deionised water. The filter was placed on a glass slide and counted immediately to avoid fading of the stain (Kumaravel et al., 2009).



(caption on next page)

Fig. 2. Flow cytometry algal gating process involving (A) Gating for autofluorescence in the unstained sample, (B) Gating for relevant living organisms in the SYBR Green stained sample, (C) Chlorophyll positive population of the living cells viewed under FSC *vs* SSC, (D) Gating to eliminate cyanobacterial population and (E) Algal population of interest for enumeration viewed under FSC *vs.* SSC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. AlgaeTorch diagram outlining the main components for operation (Source: bbe Moldaenke).

For each filter, a selection of random fields were counted until a total of at least 300 cells were counted over a minimum five fields. Counting was facilitated *via* the use of an epifluorescent microscope under oil immersion (100× objective lens). The samples were observed under a blue optical filter, which has an excitation from 465 to 505 nm, 510 nm cut-off; emission from 515 to 565 nm as adopted from Shibata et al. (2006). Bacterial cells were visualised as green dots or lines. Once a count was obtained, the following formula was used for enumeration of bacterial cells per millilitre:

$$\frac{Count\ x\ 176}{Dilution\ factor\ x\ No.\ of\ fields\ counts\ x\ 0.186}$$

where 176 was the area of the field of view on the microscope, and 0.186 was the filter area. Each sample was counted in triplicate and where numbers exceeded 100 cells per field of view, a minimum of 4 counts were obtained or a further dilution was made.

2.6. Statistical analysis

All statistical analysis for physicochemical measurements, algal enumeration and bacterial enumeration was carried out using GraphPad Prism 8. A D'Agostino-Pearson normality test was carried out to test the normality of all data. Results from this informed the use of a parametric unpaired *t*-test or a nonparametric Mann-Whitney test for comparison of results between the FP and the TP. A value of $p \leq 0.05$ indicated a significant difference at the 95% level of confidence. FlowJo software package was used for analysing flow cytometry data and for the generation of cytograms in order to establish an algal cell count. XLSTAT was used to generate the Principle Component Analysis (PCA) using Pearson correlation matrix scores to compare all parameters analysed. The closer the score to 1 or (−) 1 the greater the positive or negative correlation between two parameters, respectively. In the case where correlations existed between parameters, yellow scores denote a moderately strong correlation, red scores denote a strong correlation and blue scores denote a very strong correlation.

3. Results

3.1. Physicochemical parameter analysis

All physicochemical measurements analysed in the lab are displayed in Table 2. Fig. 4 displays the physicochemical parameter trends for the FP with similar results achieved for the TP. Parameter levels were assessed according to Boyd (1998) and SI No. 77 of 2019 – European Union Environmental Objectives (Surface Waters) (Amendment) Regulations 2019. Nitrates, nitrites, ammonium and phosphates results were expressed in mg/l, with water hardness expressed in the most common form as calcium carbonate hardness ($CaCO_3$) as per Wurts and Durborow (1992). In all cases an R^2 value of >0.99 was achieved for each standard curve, indicating the reliability of the test method with very little variation. The nitrate concentration peaked in October reaching levels of 3.27 mg/l and 3.25 mg/l for the FP and TP, respectively. The levels were lowest at the end of May with levels below the limit of detection recorded for both the FP and TP (Fig. 4). Nitrite levels ranged from below the level of detection in March to 0.118 mg/l and 0.103 mg/l in June, for the FP and TP, respectively (Fig. 4). The ammonium trends were lowest at levels of 0.12 and 0.08 mg/l for the FP and TP in April, respectively. The highest ammonium concentration levels were in June for both ponds, with a concentration of 1.89 mg/l in the FP and 1.69 mg/l in the TP (Fig. 4). The highest phosphate concentrations were measured at 1.66 mg/l in the FP and 2.20 mg/l in the TP (Fig. 4). Water hardness levels ranged from 70.06 to 650.57 mg $CaCO_3$/l in the FP and 50.04 to 630.55 mg $CaCO_3$/l in the TP (Fig. 4). The water hardness was highest in September, with average hardness levels reaching 157.95 mg $CaCO_3$/l in FP and 129.53 mg $CaCO_3$/l in TP (Fig. 4), when the two high outliers reached in September were excluded.

There were no statistically significant differences between the data obtained for both sections of the pond regardless of the test used for analysis, as reported for nitrate (p = .9455), nitrite (p = .9347), ammonium (p = .7567), phosphate (p = .9215) and carbonate hardness (p = .0619).

3.2. Microalgal enumeration

The algal numbers from March until November of 2018 in the perch farm are displayed in Fig. 5 expressed as algal cells per ml, which was measured using FCM. As stated in the gating methodology for algal enumeration, the desired population included the chlorophyll population excluding cells positive for PC, therefore the graphs below do not include the majority of the Cyanobacteria numbers. The algal population in terms of enumeration remained steady for March and April and

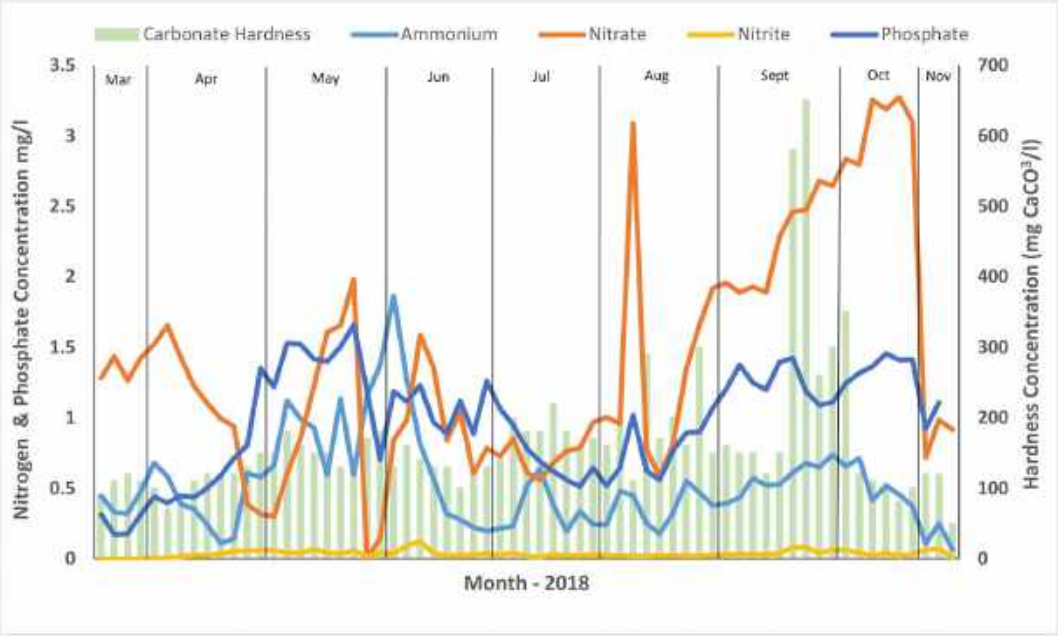


Fig. 4. Trends for nitrogen, phosphate and carbonate hardness for the FP at the perch farm, 2018.

then increased in May when the light levels increased and temperatures increased to highs of 19 °C and 21 °C in May and July. Algal numbers peaked in late June, with counts of 1.54×10^5 cells/ml and 1.57×10^5 cells/ml observed for the FP and TP, respectively (Fig. 5). Lowest numbers were detected in the winter months *via* flow cytometry analysis of less than 100 cells/ml. There was a slight decrease in the algal numbers in August in the TP compared with the FP, a trend that was reversed in September, when a higher algal count was observed for the TP (Fig. 5). A *p*-value of 0.821, following a Mann-Whitney test indicated that there was no significant difference between the values obtained for both the FP and TP in terms of enumeration.

3.3. Cyanobacterial population enumeration

The total number of chlorophyll-containing cells was determined *via* flow cytometry. The PC^+ population was assumed to closely represent the cyanobacterial population in this study. This data was used to establish the cyanobacterial population from the total number of chlorophyll positive cells. Figs. 6 and 7 illustrate the total chlorophyll positive population and the number of cells in this population that represent algal cells compared to cyanobacterial cells for the FP and TP, respectively. On average, Cyanobacteria accounted for 80% of the chlorophyll-containing cells present in the rearing water, whereas the algal cells only accounted for 20% of the chlorophyll-containing cells.

3.4. Microalgal community profiling

Through use of the inverted light microscope, the microalgal profile and species diversity for each month was determined. The dominant species for each month are presented in Table 3 for the FP and Table 4 for the TP. The most numerous species are listed with the dominating phylum for each month shaded in pink. Over 40 different algal species were observed, with the majority identified to species level using photographic identification keys. Chlorophyta was the most dominant phylum for the FP and the TP overall, with the most commonly occuring species including *Chlamydomonas* sp., *Chlorella* sp., *Dictyosphaerium* sp., *Monoraphidium* sp., *Pandorina* sp., *Scenedesmus* sp., *Selenastrum* sp., *Tetraspora* sp. and *Westella* sp. In the FP the most dominant phylum for June and September was Bacillariophyta (diatoms). In June this was mainly *Stephanodiscus* sp., and *Cyclotella* sp. In September when the algal number decreased in the FP compared to the TP, the dominant species in the FP were *Aulodiscus* sp., *Hyalodiscus* sp. and *Cyclotella* sp., whereas, the TP was completely dominated by *Cryptomonas* sp., of the Cryptophyta phylum.

3.5. Bacterial enumeration

Bacterial counts were conducted in order to provide supplementary data to algae enumeration and profiling for correlation purposes. The overall average count of total bacteria for the pond was 6.33×10^6

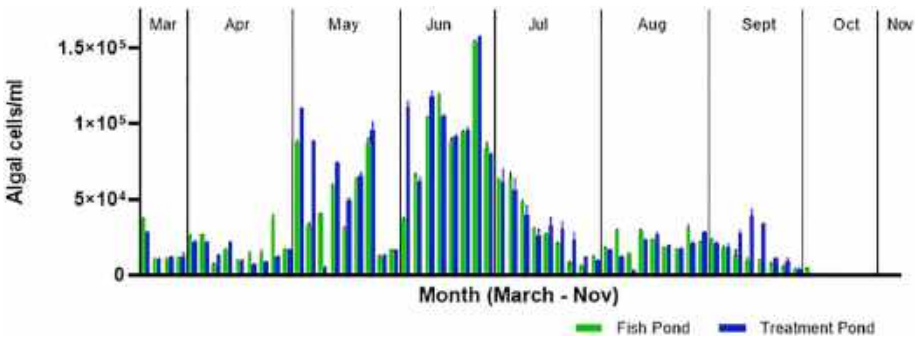


Fig. 5. Algae cells/ml in from March to November 2018 in Pond 1 at the perch farm.

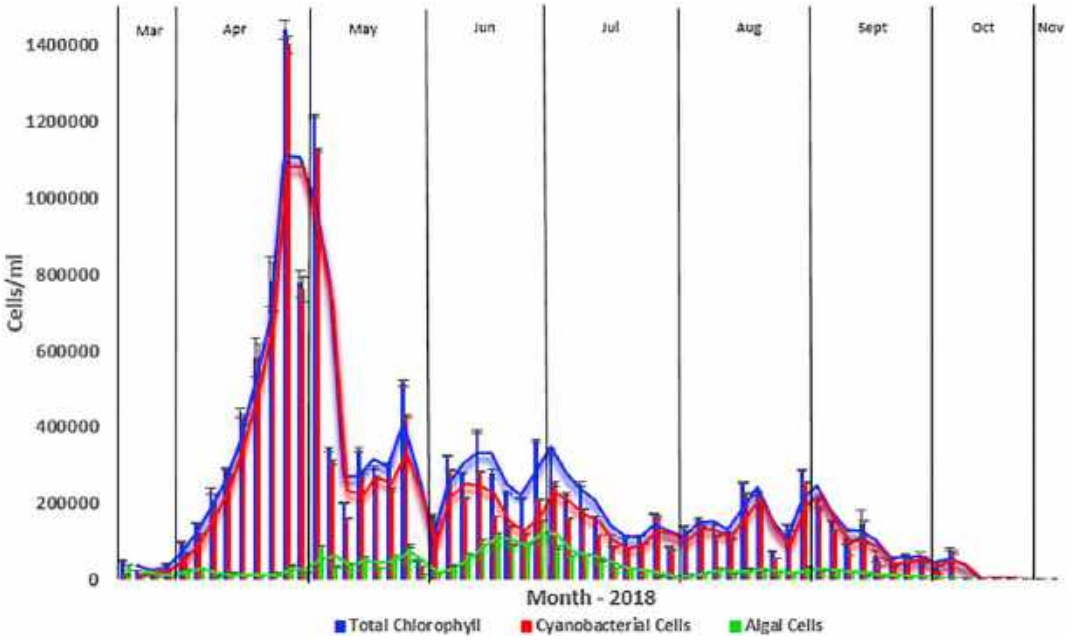


Fig. 6. Enumeration of total chlorophyll-containing cells consisting of cyanobacterial and algal cells in the FP at the perch farm, 2018.

cell/ml. The trend for this analysis from March to November 2018 is displayed in Fig. 8 expressed as $\log_{10}$ cells/ml.

3.6. Principle component analysis

In order to analyse the large volume of data, PCA analysis was conducted in Excel using the XLSTAT software to observe any correlations and or variability between parameters, which can be difficult to ascertain from individual and even overlaid parameters. This type of analysis provides tables and graphs through which observations on the relationships between parameters were made. The parameters analysed included a combination of variables measured in the laboratory and measured on site. The lab parameters analysed were nitrate concentration, nitrite concentration, ammonium concentration, phosphate concentration, water hardness (mg $CaCO_3$/l), bacterial numbers, algal numbers, chlorophyll containing cells and cyanobacterial cells, with the latter four parameters measured in cells/ml. The parameters analysed that were recorded on site included pH, oxygen (mg/l), turbidity (FTU), feeding rate and total chlorophyll-*a* and Cyanobacteria measured using an AlgaeTorch® that is in good agreement with FCM determinations, with results in µg/l. Tables 5 and 6 display the correlation matrix scores obtained for the parameters analysed in this study.

3.7. Weather conditions

The Republic of Ireland experienced one of its hottest summers on record in 2018 that coincided with the sampling period in this study (Met Éireann, 2019). Drought conditions and national hosepipe bans were put in place for most of the country up to the end of August 2018 (O'Neill et al., 2019). As a result of these unusual weather conditions,

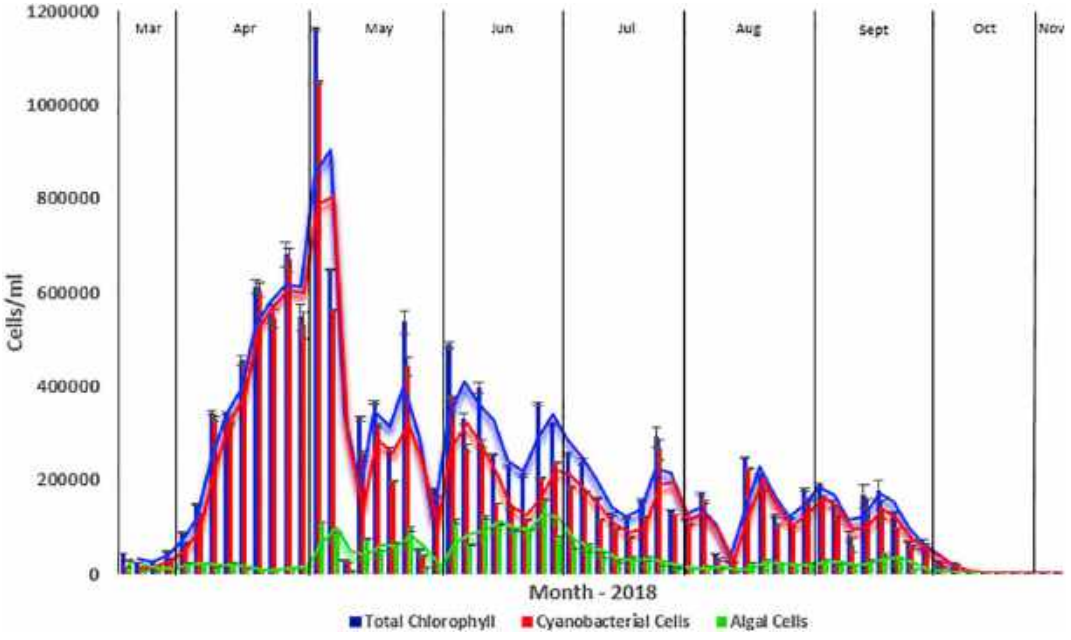


Fig. 7. Enumeration of total chlorophyll-containing cells consisting of cyanobacterial and algal cells in the TP at the perch farm, 2018.

Table 3
Dominant algal species from March to November 2018 in the FP of Pond 1 at the perch farm.

	Most to least dominant in each group	* Shading indicates the dominant group	
Month	**Species**		
March	Chlorella, Monoraphidium - Chlorophyta		
April	Chlamydomonas, Chlorella, Scenedesmus, Monoraphidium - Chlorophyta	Chroomonas, Cryptomonas - Cryptophyta	Trachelomas - Euglenophyta
May	Dictyosphaerium, Pandorina, Chlamydomonas, Tetraspora, Westella, - Chlorophyta		
June	Dictyospaherium, Westella, Chlamydomonas, Chlorella, Tetraspora - Chlorophyta	Stephanodiscus, Cyclotella - Bacillariophyta	
July	Dictyosphaerium, Westella, Chlamydomonas, Chlorella, Sphaerocystis - Chlorophyta		
August	Westella, Dictyosphaerium, Chlamydomonas, Scenedesmus - Chlorophyta	Cryptomonas - Cryptophyta	
September	Dictyosphaerium, Selenastrum - Chlorophyta	Aulodiscus, Hyalodiscus, Cyclotella - Bacillariophyta	
October	Selenastrum, Dictyosphaerium, Chlorella - Chlorophyta		
November	Chlorella, Dictyosphaerium, Chlamydomonas - Chlorophyta	Cryptomonas - Cryptophyta	Chroococcus - Cyanophyta

Table 4
Dominant algal species from March to November 2018 in the TP of Pond 1 of the perch farm.

	Most to least dominant in each group	* Shading indicates the dominant group	
Month	**Species**		
March	Chlorella, Monoraphidium - Chlorophyta		
April	Chlorella, Monoraphidium, Dictyosphaerium, Scenedesmus, Chlamydomonas - Chlorophyta	Trachelomas - Euglenophyta	Aphanocapsa - Cyanophyta
May	Chlamydomonas, Chlorella, Pandorina, Westella, Tetraspora - Chlorophyta	Stephanodiscus, Cyclotella - Bacillariophyta	Cryptomonas - Cryptophyta
June	Dictyosphaerium, Westella, Chlorella, Chlamydomonas, Tetraspora - Chlorophyta	Cyclotella - Bacillariophyta	
July	Dictyosphaerium, Westella, Chlamydomonas - Chlorophyta	Snowella - Cyanophyta	
August	Westella, Chlamydomonas, Tetraspora - Chlorophyta		
September	Chlorella - Chlorophyta	Cryptomonas - Cryptophyta	
October	Dictyosphaerium, Selenastrum, Chlorella - Chlorophyta	Aphanocapsa - Cyanophyta	
November	Dictyosphaerium, Westella, Chlamydomonas - Chlorophyta		

mean rainfall and temperature data collected at three Met Eireann weather stations surrounding and closest to the fish farm in the Republic of Ireland, were observed. The stations were located in Knock, Markree and Mount Dillion. Decreases in the average monthly rainfall (Fig. 9**(A)**) and increases in temperature (Fig. 9(B)) were observed across the weather stations.

4. Discussion

4.1. Physicochemical parameter analysis

The highest nitrate concentrations of 3.27 mg/l and 3.25 mg/l observed for the FP and TP, respectively are below the 50 mg/l maximum acceptable limit of SI No. 77 of 2019. When the levels were lowest at the end of May there was a water change in the pond, therefore the fish were removed. This process may have reduced the level of nitrates, with one of the main sources being fish waste, as well as decaying

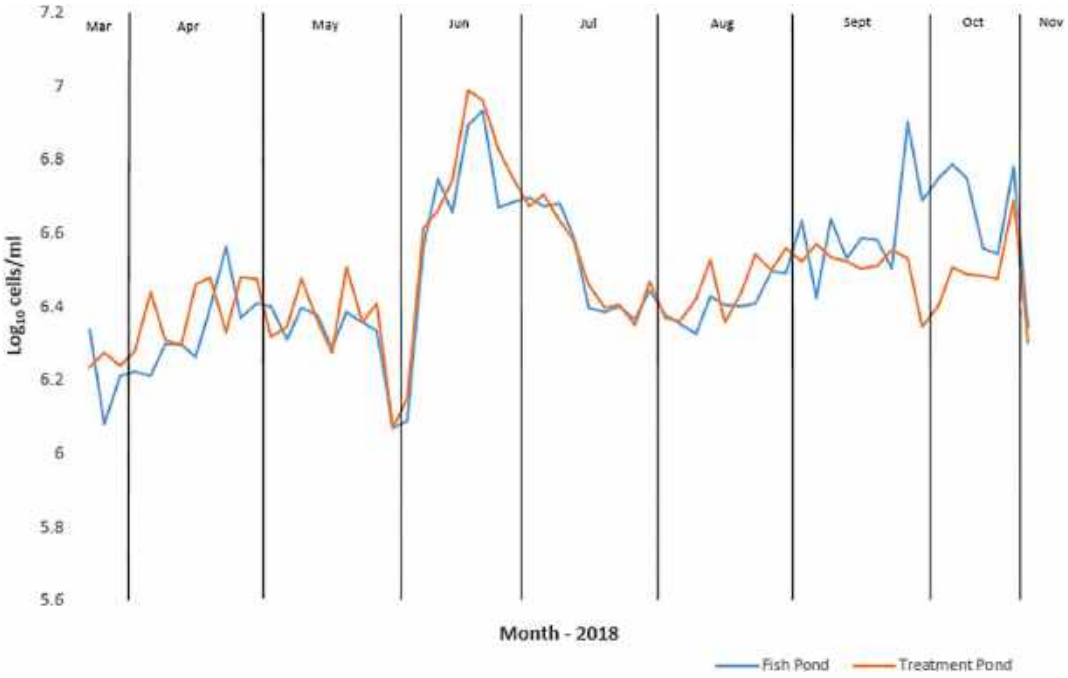


Fig. 8. Bacterial enumeration (log10 cells/ml) from March to November 2018 in Pond 1 at the perch farm.

organic matter in the water (Jiménez-Montealegre et al., 2002; Thajuddin and Subramanian, 2005), which would have also been partially removed in the process. Nitrite levels were below the limit of detection in March and highest in June with concentrations of 0.118 mg/l and 0.103 mg/l for the FP and TP, respectively. The temperature was highest at this point ranging between 19 °C and 21 °C and the bacterial counts were also highest in June overall. As the initial steps in this process involves the oxidation of ammonia to nitrite (Hargreaves, 1998; Helfrich and Libey, 1990), Fig. 4 illustrates the decrease of ammonium levels at the start of June, followed by an increase in the nitrite levels, respectively, which may in part account for this increase in nitrite concentration. The partial change of water in the pond at the end of May could have contributed to the rise in ammonium. A sudden decrease in bacterial and algal numbers evident from Figs. 5 and 8 may have impacted the nutrient recycling process. The ammonium ion tends to be harmless to fish unless extremely high concentrations are reached (Boyd and Lichtkoppler, 1979). The highest phosphate concentrations were detected in May for both ponds. Lund (1965) stated that phosphorus levels can decrease in the summer, and as the summer months progressed, the phosphate concentrations measured during this study did in fact decrease. This may in part be due to the high levels of bacteria in the summer which are major competitors of algae for the

Table 5
Correlation coefficient scores for PCA analysis carried out on TP parameters at the perch farm using XLSTAT (Chl = chlorophyll; Ctn. = containing; Cyano = cyanobacterial).

Variables	Nitrate	Nitrite	Ammonium	Phosphate	Temperature	pH	Oxygen	Turbidity	Hardness	Feeding Rate	Bacteria	Algae	Chl-Ctn Cells	Cyano Cells	Chl (Torch)	Cyano (Torch)
Nitrate	1															
Nitrite	0.200	1														
Ammonium	0.118	0.342	1													
Phosphate	0.261	0.550	0.426	1												
Temperature	-0.173	0.249	0.026	0.268	1											
pH	-0.433	-0.203	-0.331	-0.251	0.616	1										
Oxygen	-0.483	-0.234	-0.120	-0.292	-0.338	0.195	1									
Turbidity	-0.167	0.268	0.421	0.493	-0.001	-0.267	0.161	1								
Hardness	0.240	0.512	0.099	0.171	0.108	0.005	-0.220	-0.145	1							
Feeding Rate	-0.093	-0.091	-0.076	0.068	0.420	0.367	-0.292	-0.060	0.030	1						
Bacteria	-0.030	0.216	-0.080	0.257	0.402	0.304	-0.002	-0.121	0.100	0.235	1					
Algae	-0.369	0.230	0.043	0.190	0.329	0.264	0.336	0.319	-0.160	0.108	0.555	1				
Chl-Ctn Cells	-0.463	0.366	0.065	0.284	0.069	-0.043	0.327	0.484	-0.151	-0.115	0.004	0.441	1			
Cyano Cells	-0.435	0.350	0.063	0.270	0.023	-0.084	0.296	0.463	-0.137	-0.137	-0.082	0.314	0.990	1		
Chl (Torch)	-0.539	0.211	-0.073	0.286	0.731	0.490	0.057	0.357	-0.131	0.308	0.339	0.511	0.555	0.508	1	
Cyano(Torch)	-0.568	0.175	-0.206	0.060	0.445	0.580	0.377	0.100	-0.103	0.151	0.489	0.804	0.527	0.466	0.652	1

0 = No Relationship
> 0.2 = Very Weak
0.2 – 0.4 = Weak
0.4 – 0.6 = Moderately Strong
0.8 – 1.0 = Very Strong
1.0 = Perfect Linear Relationship

Table 6
Correlation coefficient scores for PCA analysis carried out on FP parameters at the perch farm using XLSTAT (Chl = chlorophyll; Ctn. = containing; Cyano = cyanobacterial).

Variables	Nitrate	Nitrite	Ammonium	Phosphate	Temperature	pH	Oxygen	Turbidity	Hardness	Feeding Rate	Bacteria	Algae	Chl Ctn. Cells	Cyano Cells	Chl (Torch)	Cyano (Torch)
Nitrate	1															
Nitrite	0.282	1														
Ammonium	0.150	0.296	1													
Phosphate	0.398	0.624	0.438	1												
Temperature	-0.261	0.234	0.029	0.276	1											
pH	-0.500	-0.215	-0.338	-0.263	0.615	1										
Oxygen	-0.494	-0.211	-0.150	-0.354	-0.338	0.195	1									
Turbidity	-0.155	0.264	0.391	0.399	-0.001	-0.267	0.161	1								
Hardness	0.246	0.424	0.325	0.209	-0.065	-0.138	-0.079	0.087	1							
Feeding Rate	-0.061	-0.105	-0.081	0.048	0.420	0.367	-0.292	-0.060	-0.052	1						
Bacteria	-0.110	0.173	-0.068	0.233	0.384	0.282	0.019	-0.100	-0.099	0.276	1					
Algae	-0.357	0.281	0.252	0.229	0.370	0.187	0.256	0.377	-0.030	0.140	0.539	1				
Chl Ctn. Cells	-0.481	0.321	0.119	0.101	0.131	-0.029	0.270	0.496	0.014	0.019	0.067	0.607	1			
Cyano Cells	-0.459	0.303	0.085	0.069	0.069	-0.076	0.247	0.477	0.023	-0.010	-0.032	0.475	0.988	1		
Chl (Torch)	-0.599	0.199	-0.084	0.174	0.731	0.490	0.057	0.357	-0.169	0.308	0.352	0.502	0.567	0.526	1	
Cyano (Torch)	-0.573	0.179	-0.257	0.024	0.445	0.580	0.377	0.100	-0.159	0.151	0.480	0.548	0.481	0.421	0.852	1

0 = No Relationship
> 0.2 = Very Weak
0.2 – 0.4 = Weak
0.4 – 0.6 = Moderately Strong
0.8 – 1.0 = Very Strong
1.0 = Perfect Linear Relationship

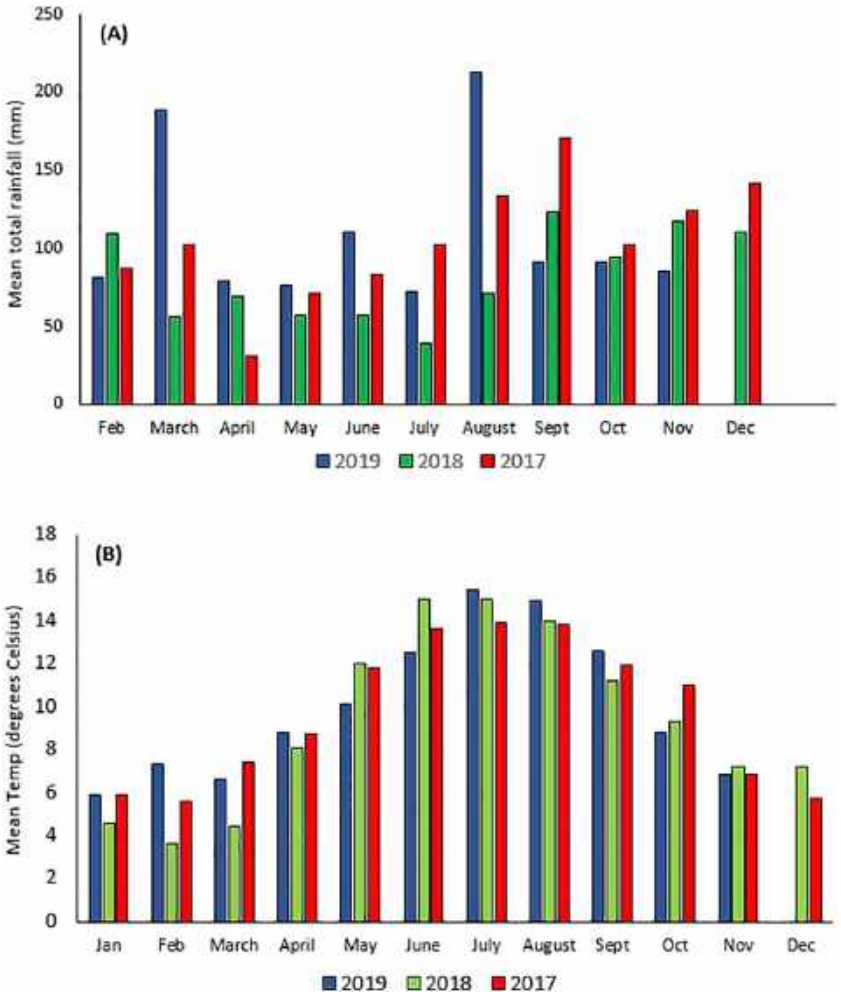


Fig. 9. (A) Mean total rainfall (mm) for three met offices nearby to sampled fish farm, and (B) mean temperature in degrees Celsius recorded at same three met offices, over periods 2017 to 2019.

uptake and utilization of inorganic phosphorus (Lund, 1965).

4.2. Algal enumeration

The algal population remained steady across the sampling months but peaked when the temperatures were at the highest (19 °C and 21 °C) between May and July. The decrease in the algal numbers in the TP compared to the FP in August may have been due to the removal of weeds and duckweed from the outer area of the TP which may have led to a dilution of the algal population. It is a common trend that algal cells reach highest concentrations during the summer months due to increasing irradiance, longer daylight hours and higher temperatures. Li et al. (2012) found that algae numbers were higher in summer than winter as is the general trend of algal growth in lakes or ponds. However, the decrease from summer to winter was only marginal, from 3.45×10^3 to 1.46×10^3 cells/l, whereas in this project cell numbers declined to much lower levels in winter, decreasing to 9 cells/ml in the first week of November.

PCA analysis indicated a strong correlation between temperature and the chlorophyll content measured using the AlgaeTorch®, with a coefficient score of 0.791. This would be expected as temperature and daylight are two of the most important factors in terms of phytoplankton growth, so an increase in temperature will lead to an increase in phytoplankton. This correlation was not, however, present for the chlorophyll analysis carried out in the lab, even though this parameter correlated to the on-site chlorophyll measurements. This result may imply that although certain parameters have been proven to share relationships, this may not always be reflected in the data as other variables may interfere or an increased number of data points may be required.

There was a moderately strong positive correlation between algae and bacterial enumeration data in both the FP and TP, with coefficient scores of 0.555 and 0.539, respectively. This correlation is commonly observed due to the symbiotic relationship shared between these two biological entities. Algae require nitrogen as an essential element for building structural and functional proteins (Hu, 2004). It is available in the soil organic matter (SOM); however, nitrogen is not in a bioavailable form for algae to utilise. Nitrogen-fixing bacteria convert the nitrogen (nitrogen fixation) into a form that can then be utilised by algae (Neospark, 2014; Thajuddin and Subramanian, 2005). The rate of nitrogen fixation largely depends on the bacterial species present in the water and the concentration of ammonia (Hargreaves, 1998). This nitrogen fixation process highlights the important interdependent relationship that exists between algae and bacteria. Another aspect of this dynamic relationship involves organic matter, on which bacteria thrive (Amon and Benner, 1996; Baines and Pace, 1991; Blancheton et al., 2013). One of the principal sources of organic matter in the rearing water is primary production by microalgae, followed by excreta and feed pellets (Baines and Pace, 1991; Moriarty, 1997). Aerobic bacteria present in the water body break down this organic matter into CO_2 and ammonia (Phang, 1991). Algae then utilise the CO_2 for photosynthesis and release oxygen during the process, which in turn oxygenates the water for the fish (Neospark, 2014). Algae also uptake the ammonia as well as heavy metals, reducing the availability of toxic substances for fish to consume (Neori et al., 2004).

Phosphorus was also a determining factor for plankton richness in the study carried out by Li et al. (2012) on an artificial lake, whereas transparency negatively correlated with plankton communities. This negative correlation was related to the negative correlation achieved between transparency and the presence of algae, indicating that algae have a major impact on the turbidity of water (Li et al., 2012). This finding agrees with the moderately strong positive correlation achieved between turbidity and chlorophyll-containing cells in both the FP and TP in this study.

Microalgae are capable of assimilating nutrients in eutrophic water bodies (Leng et al., 2018) along with wastewater remediation from many sources including food industry, agriculture and municipal effluents (Han et al., 2019). *Chlorella* sp. and *Scenedesmus* sp. have been previously reported to positively contribute to the natural treatment of wastewater due to their efficiency at nutrient, antibiotic and heavy metal removal (Chen et al., 2012; Choi and Lee, 2012; Delgadillo-Mirquez et al., 2016; Delrue et al., 2016; de Godos et al., 2012; Min et al., 2011; Nurdogan and Oswald, 1995). In addition to treating wastewater, microalgae can also synthesize value-added components such as proteins, lipids and natural pigments (Han et al., 2019). Previous researchers have reported on the value-added biomass derived from microalgae activities that could contribute to aquaculture feed along with augmenting immunity of farmed fish (Sirakov et al., 2015; Ansari et al., 2017). Microalgae-assisted aquaculture generates oxygen as a natural aeration process that can also influence and adjust microbial communities, which, if effectively controlled, could be applied to negate oxygen depletion and unwanted algal blooms (Han et al., 2019; Lu et al., 2019b).

A biotic balance should be achieved to ensure that the algal and bacterial numbers and populations are having this positive influence on productivity, rather than negatively influencing rearing water quality and consequently the production and efficiency of an aquaculture farm. Findings from this present study provides knowledge regarding microbial interactions, and ecology of these systems, that prevents the utilization of microbial communities in the assessment, improvement and control of aquaculture farms. There is increasing evidence to suggest that a co-dependent relationship exists between phytoplankton and nutrients in rearing water, as phytoplankton abundance depends on nutrient availability and nutrient cycling depends majorly on the presence of phytoplankton (Li et al., 2012; Bentzon-Tilia et al., 2016). This highlights how important the microalga balance is in rearing water, as not only do the algae produce oxygen during daylight (Moriarty, 1997), but also recycle metabolites that would otherwise build up in the water. Moriarty (1997) also highlights the importance of nitrogen and phosphorus on microalgae productivity.

4.3. Cyanobacterial population enumeration

The Cyanobacteria population accounted for the majority of the chlorophyll-containing cells detected/recorded throughout the sampling period in both ponds. This observation is highlighted further with the use of the moving average trend line in Figs. 6 and 7. While certain species of Cyanobacteria, for example *Microcystis* sp., can release toxins into the water and exhert detrimental effects on other organisms, Cyanobacteria are also beneficial for processes such as nitrification. Throughout this process, the nutrients are taken up by the cells and are therefore removed from the water, increasing the water quality in terms of nutrient pollution. In a study carried out by Liu et al. (2018) for the treatment of aquaculture using Chlorophyta, prior to inoculation with the green microalgae, Cyanobacteria were responsible for the partial removal of the pollutants from the aquaculture water, highlighting the importance of Cyanobacteria in the rearing water.

Following the PCA analysis, the highest correlation for the FP and the TP was the positive one observed between chlorophyll-containing cells and the cyanobacterial cells with coefficient scores of 0.990 and 0.988 for the FP and TP, respectively. This clearly corresponds to the data displayed in Figs. 5 and 6, reiterating the observation that the Cyanobacteria phylum represented most of the chlorophyll source present in pond 1 at the perch farm compared to algae. There was a strong positive correlation between the same two parameters when measured on site using an algal torch with a score of 0.652, which indicates that both on site and in lab measurements of phytoplankton produced similar trends. There was a moderately strong positive correlation between algal counts and chlorophyll-containing cells for the FP with a score of 0.441. Whereas a strong positive correlation was established for the same parameters in the TP, with a score of 0.607. This would suggest that the trends for algal numbers in the TP were

more in line with the overall trends for chlorophyll-containing cells compared to the algal numbers in the FP. This may be due to the presence of fish in the FP, which would have impacted the algal numbers to a higher extent, by uptake into the diet, for example.

In the case of nitrates, the data for nitrate concentration in both the FP and the TP was negatively correlated with chlorophyll and cyanobacterial parameters measured. This would indicate that the presence of chlorophyll-containing cells/pigment, the majority of which corresponded to Cyanobacteria, had a negative impact on nitrate levels. Phytoplankton are known for the uptake and removal of certain nutrients from the water and the coefficient scores reflect this fact. This finding is comparable to results determined in a study carried out by Choi and Lee (2010), where the growth of Cyanobacteria and algae inhibited the maximum nitrification rate by a factor of 4 in an autotrophic bioreactor. Hu et al. (2000) also established similar results in an assessment of the removal of nitrate from groundwater by Cyanobacteria, with *Synechococcus* sp. displaying the highest rate of nitrate removal.

4.4. Algal community profiling

Chlorella and *Monoraphidium* were the most common algae present in both the FP and TP in March and both species remained quite dominant in April. *Chlorella* has been reported as one of the most effective algal species at nutrient uptake, particularly nitrogen and phosphorus (Wang et al., 2010). In this study, the nitrate, nitrite and ammonium concentrations all decreased in early/mid-June when the *Chlorella* population remained prevalent. The effectiveness of *Chlorella* sp., in particular *Chlorella vulgaris*, at nutrient removal is also evident from its common use in the treatment of wastewater (Abdel-Raouf et al., 2012; Choi and Lee, 2012; Delrue et al., 2016; de Godos et al., 2012; Min et al., 2011). *Chlorella kessleri,* synonymous with *Parachlorellakessleri,* has also shown great potential for pollutant removal from aquaculture wastewater. Liu et al. (2018) inoculated aquaculture wastewater with five Chlorophyta species with *P. kessleri* exhibiting the greatest rate of nutrient uptake in terms of COD, nitrogen and total phosphorus. *Monoraphidium* sp. have also been reported for successful nutrient uptake. In a biodiesel production study carried out by Holbrook et al. (2014) *Monoraphidium* reduced concentrations of nitrates and phosphates to < 5 mg/l and < 1 mg/l, respectively. Therefore, *Monoraphidium* sp. are potentially useful organisms for phytoremediation of aquaculture water if the cell densities are increased. Sanchis-Perucho et al. (2018) discovered that the nutrient removal efficiency of a consortium of *Monoraphidium* and *Scenedesmus* sp. was more effective than the removal of nitrogen and phosphorus compared to *Chlorella*.

There was an increase in algal diversity for the month of April, with Chlorophyta dominating in both the FP and TP. The most numerous Chlorophyta observed, other than *Chlorella* and *Monoraphidium*, included *Pandorina* sp., *Chlamydomonas* sp., *Dictyosphaerium* sp., *Kirchneriella* sp. and *Scenedesmus* sp., with *S. obtusus*, *S. quadricauda*, *S. obliquus*, *S. opaliensis* and *S. acuminatus* all identified. The presence of different clonal populations for some algae species, such as a four- and eight-colony formation of *Scenedesmus* sp., compared to single-celled organisms may be attributed to the selective pressures in aquatic environments. For example, a study carried out by Zhu et al. (2015) demonstrated that upon exposure to *Daphnia* filtrate, acting as a predator, the *Scenedesmus* sp. increased the rate of the formation of four- and eight-celled populations. The presence of both the four- and eight-celled *Scenedesmus* sp. at the collaborating farm in this study may be indicative of the selective pressure that was present in the rearing water for the duration of the study, due to the abundant diversity that was evident from all the samples analysed. There may also have been selective pressures due to the dramatic changes in meteorological and environmental conditions, ranging from snow in March to drought in the summer months. Crytophytes and Euglenophyta were also observed in April in the form of *Chroomonas* sp. and *Cryptomonas* sp., and *Trachelomonas* sp., respectively. *Pediastrum* sp. was observed in the sample for May, which was not present prior to then. This species remained present until October, after which it was not observed.

Diatoms, mainly *Cyclotella* and *Stephanodiscus* sp., were the most frequent species observed in the month of May for the TP and June for the FP and the TP. According to Stoermer and Julius (2003) diatoms tend to be specific to certain habitats which allows for their use as indicators of water quality, with *Stephanodiscus* considered to be one of the most common and ubiquitous freshwater diatoms. In July, *Merismopedia* sp. was present which had not been present prior to then. *Synura* sp. were quite dominant in July for both the FP and TP. There was an increase in the presence of *Scenedesmus* sp. in August compared to any other month.

The species diversity for both the FP and the TP was very similar every month, however with one major exception. In the month of September, the TP was completely dominated by *Cryptomonas* sp. Contrastingly, very large diatoms, which resembled *Aulacodiscus*, *Hyalodiscus* and *Cyclotella* sp. dominated the FP. The presence of these diatoms seemed to cause a decrease in other species present, possibly due to feeding or out-competing with other species for nutrients. In fact, the algal counts for mid-September were lower for the FP compared to the TP.

According to Vuorio et al. (2007), when analysing multispecies communities of phytoplankton, enumeration procedures can be complicated and more information regarding water quality can be determined by phytoplankton community analysis compared to basic nutrient or chlorophyll *a* measurements. Therefore, it was important to perform a two-step algal analysis procedure in the form of flow cytometry and microscopy. Other factors can also be problematic for the algal identification process. Stoermer and Julius (2003) state that the average size of diatomic cells decreases after each vegetative life cycle, which can lead to variability in cell morphology of the same species. Environmental conditions such as salt levels can also alter diatom morphology (Stoermer and Julius, 2003). As well as that Small et al. (2016) stated that in terms of the capacity of photoautotrophic systems, such as algae, to remove nutrient waste from the water depends largely on energy uptake from sunlight, which is very unpredictable in a climate such as the one in Ireland. With variations in climate change and increasing temperatures worldwide, certain microalgae species may not be able to grow, and as they are a source of oxygen in the ponds, the use of a natural means of producing oxygen for biological processes may no longer be an option.

Resident bacteria and other microbes, which can limit or influence microalga growth due to availability of nutrients, contaminated microalgae populations in the pill pond. Compared with previously described closed photo-bioreactors (Han et al., 2019), a pill pond system has a much lower investment and operational cost but higher volume, making it more suitable for treatment of aquaculture wastewater. Han et al. (2019) indicated that two critical factors should be considered for advancing this type of aquaculture process. Firstly, location and pond depth need to be properly evaluated so as to improve light transmittance and photosynthesis rate. Secondly, the relationship between microalgae and bacteria should be thoroughly investigated to be fully elucidated. Van den Hende et al. (2014) reported that microalgae-bacterial flocs contributed to the removal of 28% COD, 53% BOD_5, 31% Total Nitrogen, and 64% Total Phosphorus in aquaculture wastewater (12 m^3 raceway pond), suggesting that the threat of bacteria to microalgae is putatively low when a beneficial cooperation is established.

5. Conclusion

The results of this study conducted at an aquaculture perch farm in the Republic of Ireland provide a baseline for the rearing water microalgae and physicochemical ecosystem interactions. In pond aquaculture that often relies upon natural wastewater treatment processes,

there is much less control over environmental conditions compared to closed tanks, which is why the ecosystem dynamic needs to be understood and possibly manipulated for successful and sustainable fish production. Identification of the most influential biological species in more depth would provide the opportunity of transplantation of specific microbial assemblages when required for certain processes, *i.e.* the addition of a specific bacteria for nitrification or the fertilisation of a specific algal species for nutrient removal or oxygen supplementation during the daytime. However, in order for this to be possible and beneficial, the function of each species needs to be determined. In addition, more sustainable and effective disease control measures need to be implemented for successful management and eradication of unwanted pathogens and possibly for control of the algal population, once identified. Without the baseline information, the required knowledge to inform prevention measures rather than undergoing treatment processes would be unfeasible. This constitutes the first study that reported good agreement between use of real-time laboratory-based techniques and in-field monitoring technologies for enumerating microalgae and bacterial communities, where it is envisaged that use of these combinational approaches will aid the future development of aquaculture processes. Limitations associated with findings relate to the fact that flow cytometry is highly specialised and not broadly available to support the aquaculture industry, but could be provided as a specialist contract service. Having an in-depth knowledge of this characterization would also be the basis for future diagnostic applications such as the design and development of diagnostic molecular kits.

Credit author statement

CRediT roles:

Sarah Naughton: Data curation, formal analysis, investigation, methodology, software, validation, writing original paper and revision.

Siobhan Kavanagh: funding acquisition, investigation, methodology, supervision, writing original manuscript and editing revision.

Mark Lynch: investigation, methodology, supervision, writing original paper and making edits to revision.

Neil J Rowan: Conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing orginal draft and revision with editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to thank the collaborating aquaculture farm, Bord Iascaigh Mhara (BIM) and European Monetary Fund (EMF) for co-funding this research under the Knowledge Gateway Scheme (17KGS004).

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Longitudinal evaluation of the impact of traditional rainbow trout farming on receiving water quality in Ireland

Alexandre Tahar[1], Alan M. Kennedy[2], Richard D. Fitzgerald[3,†], Eoghan Clifford[2] and Neil Rowan[1]

[1] Bioscience Research Institute, Athlone Institute of Technology, Athlone, Ireland
[2] Civil Engineering, National University of Ireland, Galway, Ireland
[3] Ryan Institute, National University of Ireland, Galway, Ireland
[†] Deceased.

ABSTRACT

In the context of future aquaculture intensification, a longitudinal ten-year evaluation of the current traditional rainbow trout production in Ireland was performed. Publically available and independent data obtained from local authorities were gathered and analysed. Inlet and outlet concentrations of parameters such as BOD_5, ammonium, nitrite, dissolved oxygen and pH for four consecutive flow-through fish farms covering the four seasons over a ten-year period (2005–2015) were analysed. The objectives of the study were (i) to characterize the impact of each fish farm on water quality in function of their respective production and identify any seasonal variability, (ii) to quantify the cumulative impact of the four farms on the river quality and to check if the self-purification capacity of the river was enough to allow the river to reach back its background levels for the analysed parameters, (iii) to build a baseline study for Ireland in order to extrapolate as a dataset for expected climate change and production intensification. For most of the parameter analysed, no significant impact of the fish farming activity on water quality/river quality was observed. These results, the first ones generated in Ireland so far, will have to be completed by a survey on biodiversity and ecotoxicology and compared after production intensification and the likely future introduction of water treatment systems on the different sites.

Subjects Aquaculture, Fisheries and Fish Science, Ecology, Aquatic and Marine Chemistry, Environmental Contamination and Remediation, Environmental Impacts
Keywords Pond-based, Freshwater aquaculture, Environmental impact, Rainbow trout, Receiving water, Ireland

INTRODUCTION

With the wild fish catching capacities almost reached and with the overall human population increase on the planet, fish from aquaculture is and will increasingly become a more important food source for human consumption in the near future (e.g., *Donnely, 2011*; *Guilpart et al., 2012*). Aquaculture production increased more than five-fold from 1990 to 2012, while the world capture fisheries increased with only 8% at the same time (*Krause et al., 2015*). Furthermore, it is planned that this increase in production will be sharper in the next decades in Ireland, notably with the objective of increasing food export (including

Submitted 8 February 2018
Accepted 14 June 2018
Published 24 July 2018

Corresponding author
Alexandre Tahar, atahar@ait.ie

Academic editor
Marcia Marques

Additional Information and Declarations can be found on page 19

DOI **10.7717/peerj.5281**

How to cite this article Tahar et al. (2018), Longitudinal evaluation of the impact of traditional rainbow trout farming on receiving water quality in Ireland. **PeerJ 6:e5281; DOI 10.7717/peerj.5281**

aquaculture fish products) by 85% by 2025 through the application of the Food Wise 2025, the strategic plan for the development of agri-food sector (*DAFM, 2015*). The future and current intensification of this activity is associated with meeting more stringent environmental regulations to ensure a sustainable and environmentally friendly production. At the European scale, freshwater fish farming is essentially regulated through the Water Framework Directive (WFD) that aims at providing the good chemical and ecological status of the rivers (*Aubin, Tocqueville & Kaushik, 2011*; *European Commission, 2000*; *Guilpart et al., 2012*). Currently, there is a dearth in scientific knowledge on the potential impact and environmental risk of aquaculture farming practices on receiving water quality as it relates to both WFD compliance and related EU Rivers Basin Management Plan 2016–2021.

Various anthropogenic activities can cause the deterioration of river quality including point source pollution such as wastewater treatment plants (WWTPs) with the discharge of partially treated effluents or non-point sources such as agricultural activities (e.g., *Papatryphon et al., 2005*). The traditional freshwater fish farming industry generally operates using flow-through systems (FT) without any water treatment, with the oxygen levels being maintained by relatively high volume water abstraction & flow through the farm. Fish farming activity generates wastes from fish excreta and uneaten feed which if discharged untreated could potentially impact the water quality of the discharge receiving water (*Caramel et al., 2014*; *Garcia et al., 2014*; *Lalonde, Ernst & Garron, 2015*; *Lazzari & Baldisserotto, 2008*; *Sindilariu, Brinker & Reiter, 2009*; *Verdegem, 2013*). The potential impacts of such intensive FT fish farming on receiving river quality can include increased concentrations of five-day biochemical oxygen demand (BOD_5), a drop in dissolved oxygen concentrations (DO), an enrichment of total suspended solids (TSS) content in the receiving water, in nutrients such as nitrogen generally characterised by total ammonia nitrogen (TAN), and phosphorus generally characterised by orthophosphate (PO_4-P) that could both potentially lead to surface water eutrophication (e.g., *Boaventura et al., 1997*; *Caramel et al., 2014*; *Garcia et al., 2014*; *Lazzari & Baldisserotto, 2008*; *Teodorowicz, 2013*). This impact will depend on the production system employed, the production intensity and on the type of feed but also on the assimilative capacity of the receiving water (*Boaventura et al., 1997*; *Caramel et al., 2014*).

A number of studies have focused on the characterization of freshwater fish farm effluents across the world (e.g., *Boaventura et al., 1997*; *Guilpart et al., 2012*; *Lalonde, Ernst & Garron, 2015*; *Neto, Nocko & Ostrensky, 2015*; *True, Johnson & Chen, 2004*). Most of these studies have inherent drawbacks due to their respective framework; some studies focused only on a short period of time of one year or less (e.g., *Caramel et al., 2014*; *Všetičková et al., 2012*; *Živić et al., 2009*) or relied on individual water samples (e.g., *Caramel et al., 2014*; *Hennessy et al., 1996*; *Lalonde, Ernst & Garron, 2015*; *Noroozrajabi et al., 2013*; *Všetičková et al., 2012*), while the impact of aquaculture should be assessed using longer term evaluation process encompassing production, fish life stages and river characteristics variations (*Aubin, Tocqueville & Kaushik, 2011*; *Hennessy et al., 1996*). Other studies (e.g., *Koçer et al., 2013*; *Sindilariu, Brinker & Reiter, 2009*; *Všetičková et al., 2012*; *Yalcuk, Pakdil & Kantürer, 2014*) have focused on fish farm gates (comparison of farm inlet and outlet water quality) and did not aim at evaluating the impact they might have on receiving water. Some limited studies

(*Aubin, Tocqueville & Kaushik, 2011*; *Boaventura et al., 1997*; *Pulatsu et al., 2004*) analysed the impact of freshwater fish farming on the receiving water quality during a long period of time (i.e., at least two years duration), allowing one to fully understand the impact of specific farms on specific rivers and in specific places. However, data from these studies are difficult to extrapolate and apply into different contexts in light of the parameters and conditions such as species, temperature, aquaculture practices, chemicals used, receiving water features (e.g., flow, hydromorphology) and local environmental conditions that may be very specific to individual farms and locations (*Lalonde, Ernst & Garron, 2015*).

With the increasing demand for fish and fish products worldwide (e.g., *Guilpart et al., 2012*), the freshwater aquaculture industry is facing the challenge of finding the way to produce more without any associated environmental degradation (*Martins et al., 2010*; *Sturrock et al., 2008*; *Teodorowicz, 2013*). In some countries such as Denmark, freshwater aquaculture practices have recently evolved to model trout farms, systems that were able to fulfil both production and environmental objectives (*Jokumsen & Svendsen, 2010*; *Lalonde, Ernst & Garron, 2015*; *Teodorowicz, 2013*). However, some other countries such as Poland (*Teodorowicz, 2013*), France (*Papatryphon et al., 2005*) and Ireland still require a drastic evolution of the aquaculture practices through more advanced systems in order to fulfil those ambitious objectives. To the best of our knowledge, there is no existing data about the impact of freshwater fish farming on downstream river quality in Ireland. There is, therefore, a pressing need to evaluate the impact of aquaculture activity on a longer-term basis using evidence-based data that are publically available, and to ascertain if there is any accumulation of pollution in receiving water. Thus, the main aim of the present study was to evaluate such data in terms of reported aquaculture influent and effluent parameters over a ten year period in order to establish relationship (if any) with receiving water quality in Ireland. In that aim, the impact of four consecutive FT traditional freshwater rainbow trout (*Oncorhynchus mykiss*) farms on the receiving water quality was assessed through publically available historical data analysis (2005–2015). The objectives of the study were (i) to characterize the impact of each fish farm on water quality in function of their respective production and identify any seasonal variability, (ii) to quantify the cumulative impact of the four farms on the river quality and to check if the self-purification capacity of the river was enough to allow the river to reach back its background levels for the analysed parameters, (iii) to build a baseline study for Ireland in order to extrapolate as a dataset for expected climate change and production intensification.

MATERIALS AND METHODS

Fish farms description

The studied facilities are all Irish traditional rainbow trout fish farms, all operating in a FT system during the studied period. The schematic representation of fish farms in Fig. 1 shows the very specific configuration of the area with four different farms, all abstracting the water needed and having their discharge into the same river, all in a relative small area (about 4 km between the first and the last farm). This specific configuration allowed the study of the potential cumulative impact the fish farms might have on river quality.

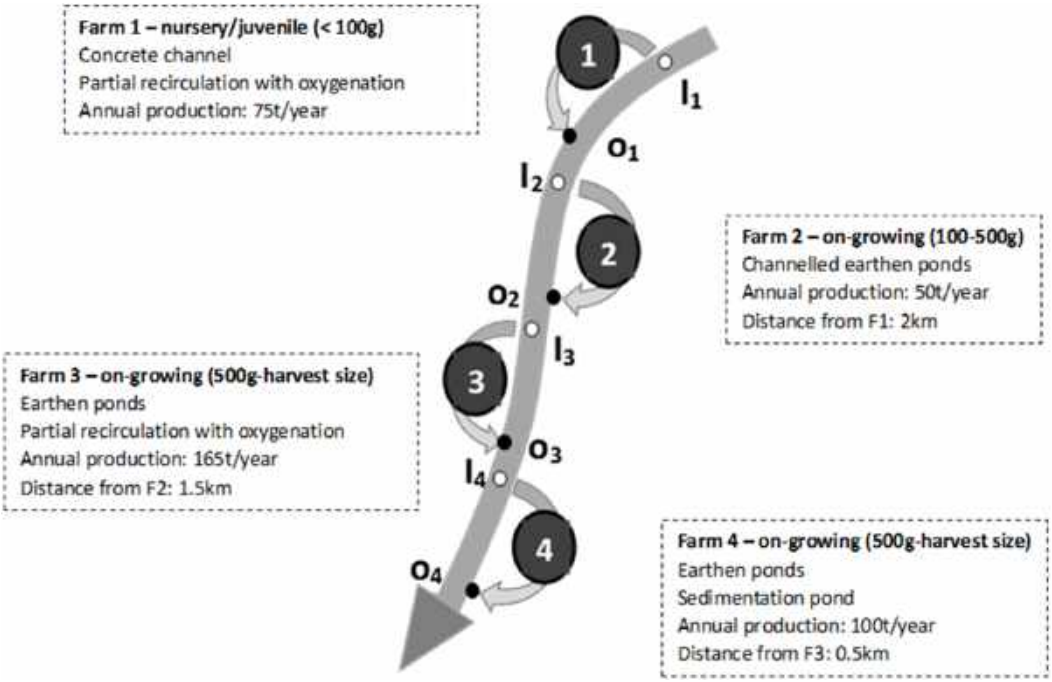

Figure 1 Schematic representation of the river, the four studied fish farms and of the associated monitoring points (inlets and outlets). The main characteristics of each farm are also presented.
Full-size DOI: 10.7717/peerj.5281/fig-1

Farm 1 (F1) was a hatchery and juvenile (i.e., <100 g) production site. It was operating in FT in a concrete channel separated in different consecutive sections and had a partial recirculation of the water associated to an oxygenation step (no water treatment). As a hatchery site, this farm was the most sensitive to water quality, and which is why it was historically located at the most upstream location where the water quality was not theoretically affected by the fish farm activity yet. Farm 2 (F2) was about 2 km downstream from F1 and was a medium size (i.e., 100–500 g) on-growing site that was operating in FT in an earthen pond. Farm 3 (F3) was about 1.5 km downstream from F2 and was an on-growing site (i.e., 500 g to harvest size) that was operating in FT in earthen ponds with a partial recirculation of the water associated to oxygenation (no water treatment). Farm 4 (F4) was about 500 m downstream from F3 and was an on-growing site (i.e., 500 g to harvest size) that was operating in FT in an earthen pond associated with a sedimentation pond aiming at removing settleable solids from the discharge water.

On average, the four farms produced 75, 50, 165 and 100 tons a year respectively during the investigated period (2005–2015).

Considering that there was no or limited form of water treatment on these farms (Fig. 1), the objective was to evaluate the impact of the whole pool of farms, and of successive individual farms, on the water quality in order to check if there was any accumulation of pollution (e.g., TSS, BOD_5, TAN, PO_4-P) within their receiving water. According to Ireland's EPA (http://www.epa.ie/hydronet/#Water%20Levels), the river 95% percentile flow was calculated as being 1.1 m^3/s (average over 30 years of 5.5 m^3/s). It is noteworthy

that there was no agricultural activity or WWTPs within the area (i.e., upstream and in between the different farms) and that therefore, in the present study, any significant modification of the river quality downstream from the farms will be attributed to the fish farming activity and will represent its impact on the river quality.

The farms water intake flow was measured at regular intervals by the fish farmer and were about 0.4 m^3/s on average on each farm (range 0.1–0.6 m^3/s depending on the farm and the flow conditions in the river).

The discharge licence

Each Irish County Council (Local Authority) require two licences to operate—an aquaculture licence issued by the Department of Agriculture, Food & the Marine and a Trade Effluent Discharge Licence issued by the relevant Local Authority.

For these four farms the discharge licences states both (i) a regulation on the maximum water abstraction rate that the farm cannot exceed depending on the river flow, and (ii) a maximum differential concentration (or value) between fish farms influent and effluent waters for a range of parameters (i.e., 1 mgO_2/L for BOD_5, 10 mg/L for TSS, 5 NTU for turbidity, 0.4 mgN/L for TAN, 0.002 mgN/L for nitrite (NO_2-N) and 0.2 mgP/L for PO_4-P). For three other parameters, the regulation defines absolute limit values for the farm effluent (i.e., 60% saturation for DO, range 6–9 for pH and ambient temperature). The discharge licence specified that each fish farm had to be sampled by the regulatory agency for both inlet and outlet water at a frequency of four times a year (i.e., generally one sampling per season).

The parameters monitored

A full ten-year record of historical data (2005–2015) generated by an independent and accredited water analysis lab was gathered for this study. The present study focused on the parameters that were monitored and analysed during the studied period. Hence regulated parameters (presented in the previous section) and total oxidised nitrogen (TON), representing the sum of NO_2-N and nitrate (NO_3-N), were considered for the present study.

Surprisingly, according to the publicly available documents that were consulted for this study, ammonium (NH_4-N) was monitored instead of TAN (regulated parameter) that represents the sum of NH_4-N and ammonia (NH_3-N), the latter being much more toxic for fish (*Tomasso, 1994*). The predominance of one ammonia form or another depends on pH and temperature with NH_3-N predominating at high temperature and pH values (*Emerson et al., 1975*). Therefore, for the present study, pH and temperature values were used to enable an estimation of TAN concentrations from NH_4-N measurements.

Validated standard methods (*APHA, 2012*; *APHA, 2005*) were employed by the accredited labs for the analysis of each monitored parameter.

Sampling

All samples were performed as spot samples and no composite samples were taken. According to the Local Authority responsible for the water monitoring, all the inlet sampling locations (i_{1-4}, see Fig. 1) were located in the river itself immediately upstream

from the inlet channel of each farm (taken every time at the same section at the centre of the river). The outlet sampling spots (o_{1-4}, see Fig. 1) were directly located in the outlet channels of each farm (farm effluents) and therefore do not include any dilution by the downstream river.

The discharge licence did not specify a sampling method and thus it is likely that spot samples were employed as they were considered the most efficient and cheap sampling method. Samples (i.e., 1L in PET bottles) were stabilized in acidic conditions (to reach a pH value below 2 in order to avoid any transformation of the nitrogenous compounds) and brought to the labs where they were kept refrigerated before analysis. Dissolved oxygen saturation levels, pH and temperature values were obtained *in-situ* before sample stabilization by a multi-parameter sensor (YSI 51B oxygenmeter and WTW pH 330 pH meter). Separate samples were taken for the analysis of BOD_5 parameter (not acidified).

Approach employed for the quantification of the fish farms impacts

The following range of assessments were considered when evaluating the impacts of the farms on the receiving river water—individual farm impact on water quality, and cumulative impact of the four fish farms on river water quality. Additionally, in order to check a potential higher impact during summer condition (as observed in *Lalonde, Ernst & Garron, 2015*), the seasonal variability of the impacts was also studied by considering the evolution of the effluents, quality and the impacts for the different seasons during the year. For this purpose, data obtained from a monitoring in December, January and February were classified as "winter data"; the ones from the months of March, April and May as "spring data"; the ones from the months of June, July and August as "summer data" and the ones from the months of September, October and November as "autumn data".

As discussed above, the four fish farms were abstracting the water from and discharging into the same river (Fig. 1). Therefore, the fish farms inlet analysis results (i.e., I_1, I_2, I_3 and I_4) were employed to study the evolution of the water quality along the river across the 4 farms. We noticed that this method was used to study the impact of the three first farms (i.e., farms 1–3). The evaluation of the cumulative impact of the three first farms (F1, F2 and F3) on the river quality was achieved by the comparison of the results obtained in i_1 and i_4 sampling locations. This allowed us to take account of the dilution of the effluent of each farm by the river and therefore to take account of the pollution load emitted by the farms. The impact of F4 was not possible to assess this way because river quality downstream from this farm was not monitored (i.e., the only location of these farm effluent monitoring points that does not take account of the dilution by the river, see Fig. 1).

Unfortunately, those results were not correlated with the fish parameters because we could not have access to accurate records of these biological data but only of average yearly production. Therefore, this study only focused on water quality parameters.

Data treatment

The following equation was used to calculate the differential concentrations (D_i) for each monitored farm (i) and parameter introduced above.

$$D_i = O_i - I_i.$$

With O_i and I_i the outlet and inlet concentrations at the farm i for a given parameter on a given sampling event.

Only concentration values were considered and treated although load values (taking farm outlet flow values in consideration) would have been a better way to characterize the impact of the different farms. However, flow values were not measured continuously on the different sites and this did not allow for an accurate calculation of the loads for the different parameters at different times. Also, river concentrations were employed to characterize the impact of the different farms on river quality; those river concentrations are taking river dilution into account and were therefore considered as a good approach to consider the load of each of parameters considered.

Statistical tests were performed using MATLAB software (MathWorks, Natick, MA, USA) in order to assess if datasets means were significantly different (e.g., concentrations measured upstream and downstream from each farm) and if the fish farms had an impact on the water quality. Box plot representations of the data were chosen in order to show the dispersion of the data and to avoid any misinterpretation due to the occurrence of extreme values. Two samples Student's t-tests were employed to compare datasets after checking the normal distribution of each dataset (validity domain of this statistical test). A confidence interval of 95% was systematically employed.

Many results from the datasets obtained from the regulation body were below the limit of quantification (LOQ) associated to the analytical method employed. In this case, for the calculations performed in the present study and because the sampling and analysis were not performed by our team, the data was considered as equal to the LOQ (substitution approach). The real value of the data could be lower than LOQ but this "conservative" approach of "below LOQ = LOQ" was chosen here, although other approach could have been used such as "below LOQ = LOQ/2" or "below LOQ = LOQ/$\sqrt{2}$" (*Hornund & Reed, 1990*; *US Environmental Protection Agency, 2003*), to avoid any misinterpretations.

RESULTS AND DISCUSSION

General overview of the results—outcome of inlet and outlet values for each parameters

More than 1,000 inlet/outlet couples of data were gathered overall for all the parameters and the four fish farms investigated. The full dataset as well as the representation of the data for each parameter and each farm and the monthly distribution of the data are presented in supplementary materials sections (SP1, Tables S1 and S2).

Figure 2 gives an overview of the gathered data for all farms and presents a global comparison of inlet and outlet water quality observed globally and for all the parameters monitored.

Globally, by comparing inlet and outlet values for each parameter, Fig. 2 shows the mean increase or decrease of the monitored parameters values caused by the four farms without distinction. No significant differences between farms inlets and outlets were observed for TON, temperature, BOD_5, turbidity, TSS and NO_2-N (P value > 0.05) demonstrating that the investigated fish farms might have no impact on downstream river quality for these

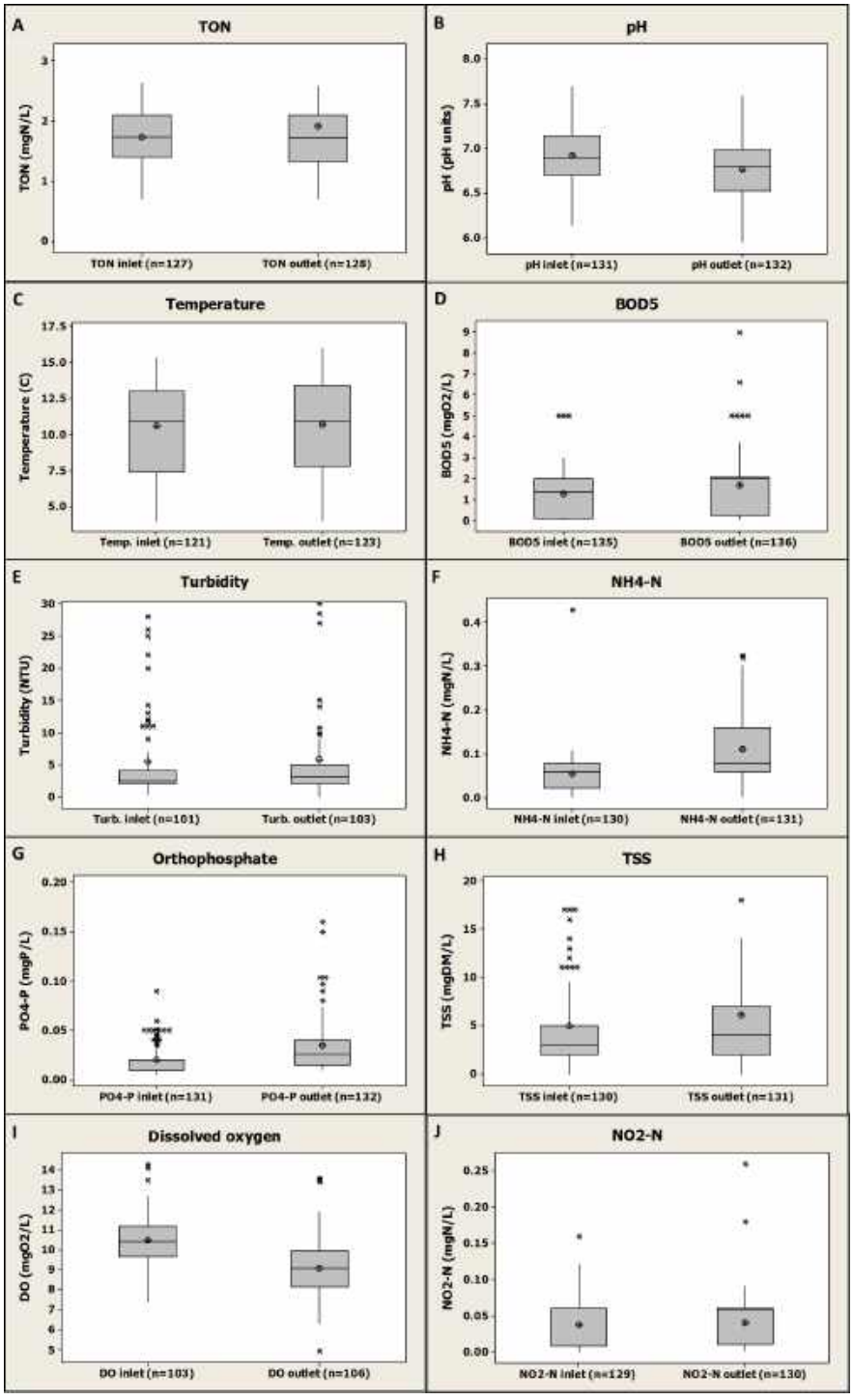

Figure 2 **Box-plot global representation of inlet and outlet values compiled and gathered for the four fish farms and for all the monitored parameters (2005–2015).**

Full-size DOI: 10.7717/peerj.5281/fig-2

parameters. However, a significant increase from fish farms inlets to outlets was observed for NH_4-N (P value < 0.001), PO_4-P (P value < 0.001) and BOD_5 (P value < 0.01) demonstrating that the investigated fish farms might have an impact on the downstream river quality through the release of those compounds. However, the increase in NH_4-N was only about 0.08 mgN/L on average ($\pm$0.08 mgN/L, $n = 130$, see Fig. 2F), which can be considered as a relatively low value compared to values found in other studies such as a 1.46 mgN/L increase (*Boaventura et al., 1997*). The same observation can be done for PO_4-P with an average increase of about 0.015 mgP/L ($\pm$0.03 mgP/L, $n = 130$, see Fig. 2G) found in the present study and a range of 0.06–0.58 mgP/L increase found in another study where three different rainbow trout farms were investigated during one year (*Boaventura et al., 1997*). Some other studies observed some increase from rainbow trout farms inlets to outlets for NH_4-N (*Caramel et al., 2014*; *Guilpart et al., 2012*; *Kırkağaç, Pulatsu & Topcu, 2009*; *Lalonde, Ernst & Garron, 2015*), BOD_5 (*Teodorowicz, 2013*), total nitrogen (*Caramel et al., 2014*; *Lalonde, Ernst & Garron, 2015*), total phosphorus (*Caramel et al., 2014*; *Kırkağaç, Pulatsu & Topcu, 2009*; *Lalonde, Ernst & Garron, 2015*) and PO_4-P (*Caramel et al., 2014*; *Guilpart et al., 2012*). Furthermore, a significant decrease was observed in the present study at fish farms outlet compared to inlets for both DO (P value < 0.001) and pH (P value $= 0.001$) showing, as expected, (i) a global oxygen consumption due to fish metabolism (*Boyd & Tucker, 1998*), and (ii) a production of carbon dioxide by fish with the consequence of lower pH value at the outlets (*Boyd & Tucker, 1998*). An average decrease in DO of about 1.42 mgO_2/L ($\pm$1.28 mgO_2/L, $n = 101$, see Fig. 2I) from farms inlets to farms outlets was observed, that is in agreement with previously published results such as a study where a DO decrease of 0.7–2.4 mgO_2/L depending on the fish farm investigated was found (*Boaventura et al., 1997*). A deeper focus on these data, with an emphasis on the inlet and outlet of each farm, will be presented in the following sections of this study as to whether or not they confirm these global trends. An evaluation of potentially significant impacts of those individual fish farms on the receiving water quality for the monitored parameters will be provided.

Impact on water quality—individual farms level

The objective of this section are (i) to quantify the impact of each farm on water quality and (ii) to address their compliance to their discharge licences criteria.

Parameters regulated in term of differential concentrations

Here we present an outcome of the differential concentrations for five regulated parameters (i.e., BOD_5, NO_2-N, PO_4-P, TSS and turbidity) and for ammonium (NH_4-N) for each farm (Fig. 3).

A first general observation of the results is that for most of the regulated parameters (i.e., turbidity, TSS, PO_4-P, NH_4-N, BOD_5) neither the average nor median values are higher than their associated differential limit value. The different farms were therefore globally in compliance with their discharge licence for those parameters. An exception is NO_2-N, with an average differential concentration higher than the limit value (i.e., 0.006 $\pm$ 0.025 mgN/L, $n = 40$ vs 0.002 mgN/L, see Fig. 3C) for F4. However, the median value was equal

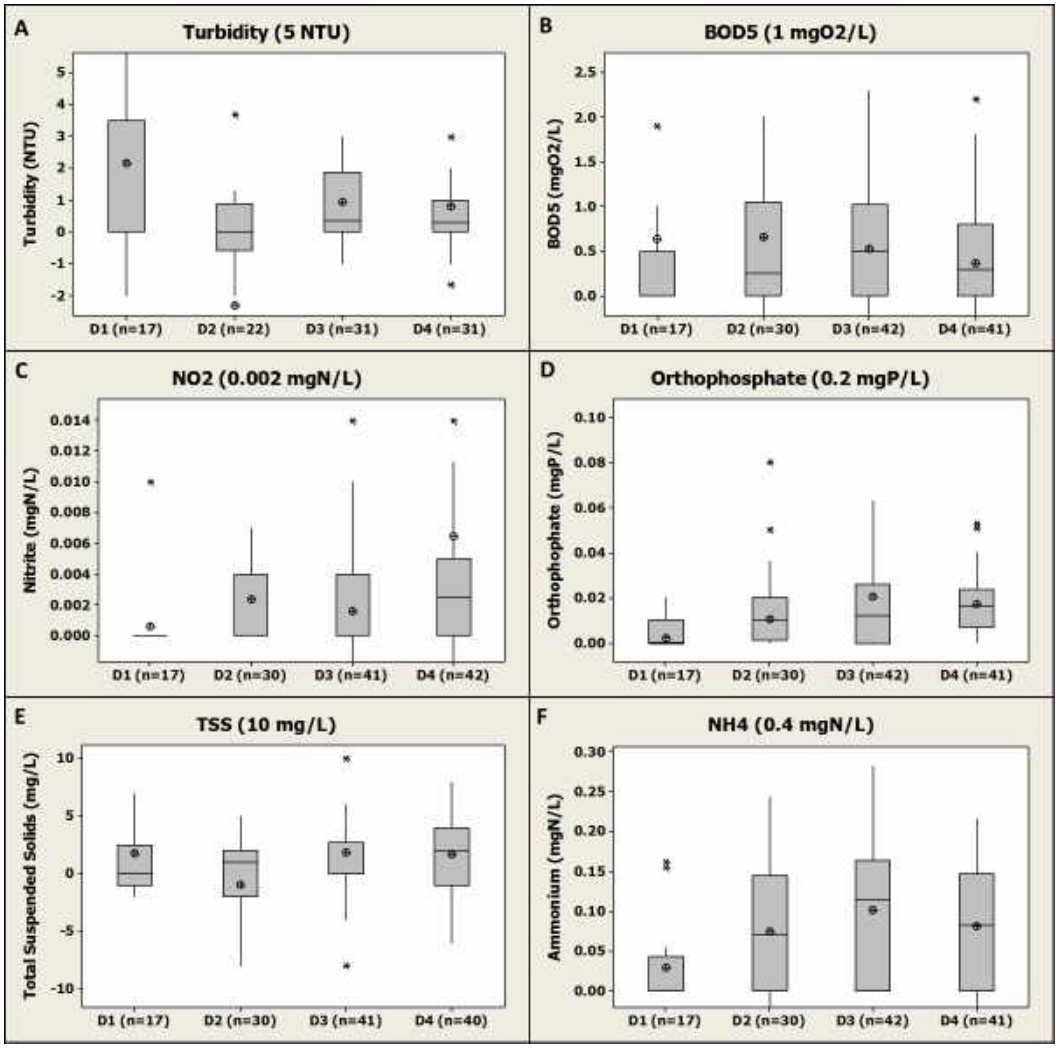

Figure 3 Box-plot representation of the differential concentrations (D_i) between inlet and outlet of each of the four fish farms and for each regulated parameter (2005–2015). Numbers 1, 2, 3 and 4 represent the consecutive fish farms in the order of the river flow direction. In brackets, the discharge licence limit value for each regulated parameter as mentioned in the discharge licence. Median, average, Q1 and Q3 are presented. For NH_4-N, the limit value associated is for total ammonia (TAN).

Full-size DOI: 10.7717/peerj.5281/fig-3

to the limit value (i.e., 0.002 mgN/L) underpinning the influence of extreme values on the average calculation. For this parameter, a large number of data were <LOQ and as stated above were considered as equal to the LOQ; for F1 a very large proportion of the data (i.e., about 95%) were <LOQ at both inlet and outlet giving resulting differential concentrations of 0 mgN/L according to the equation presented in the materials and method section (it is noticed that the same result would have been obtained with a different choice of values for analysis below the LOQ). Surprisingly, the LOQ for NO_2-N was generally 0.06 mgN/L which is higher than the differential concentration limit of 0.002 mgN/L. This shows the limit of the approach set by the Local Authority to set some very low differential limits

that cannot actually be addressed by the chemical analysis procedure typically applied in accredited chemical analysis labs.

For BOD_5, a global compliance with the discharge licence was observed with both average and median values below the differential limit value (i.e., 1 mgO_2/L, see Fig. 3B) for the four farms. However, for F2, F3 and F4 a substantial number of individual differentials values was above the limit value (i.e., about a quarter of the values for the three farms) demonstrating that even if a general compliance was observed the compliance was not achieved all the time. The low differential limit value for this parameter (i.e., 1 mgO_2/L) compared to other fish farms in the country (i.e., generally 2 mg/L) might explain this relative high number of "non-compliance" data. Furthermore, according to the statistical analysis performed, no significant difference were observed between the different farms for BOD_5 differential values (i.e., P values > 0.05).

For TAN (i.e., NH_4-N + NH_3-N), the compliance cannot be directly assessed by the available NH_4-N monitoring; however, considering that pH values were always below 8 (i.e., range 6.5–7.5 pH units, see Supplementary Material SP1) and the temperature range of 5–15 °C during all the monitoring period (see Supplementary Material SP1), NH_4-N was highly predominating over NH_3-N and therefore TAN concentrations were similar to NH_4-N (about 0.1 mgN/L depending on the farm, see Fig. 3F). Therefore, the conclusion is that all farms were in compliance with the differential concentration limit for TAN of 0.4 mgN/L. Furthermore, according to the statistical analysis performed, some significant differences were observed between the different farms in terms of NH_4-N differential values; mean NH_4-N differential value for F1 was revealed to be significantly lower than for F2 (P value > 0.05), F3 (P value < 0.001) and F4 (P value < 0.01). No significant difference was observed between F2, F3 and F4 for this parameter.

Farm 2 and F4 showed a substantial proportion of negative differential values for turbidity and TSS (i.e., nearly half of the total number of data for these farms, see Fig. 3E) demonstrating that those farms were polishing the water regarding solids. The earthen pond configuration and low water velocity for F2 and the presence of a sedimentation pond in F4 could explain this observation (Fig. 1). However, the presence of such solid removal processes did not enhance the removal of other compounds such as BOD_5 and PO_4-P that could have been reduced by the presence of a sedimentation pond (*Teodorowicz, 2013*). The statistical analysis of the data showed that the TSS differential mean value was significantly lower for F2 than for F3 (P value < 0.01). No other significant difference between farms was observed for both TSS and turbidity.

Farm 3 was the farm with the highest average yearly production of 165 tons (Fig. 1) and was also the one associated to the highest average differential values for PO_4-P and NH_4-N (i.e., 0.02 ± 0.042 mgP/L, $n = 42$ and 0.1 ± 0.08 mgN/L, $n = 42$, respectively, see Figs. 3D and 3F), even if the difference with the other farms were not significant according to the statistical analysis. In the present study, a higher fish production led to a higher release of phosphorus and nitrogenous wastes as was observed in other studies such as *Guilpart et al. (2012)* where a proportionality relationship was observed between nutrient release and farm production. For the same set of parameters, F1 was revealed to be less impacting than the other farms with on average lower differential values than any other farm. This might

be due to a relative low yearly production in this farm (i.e., 75 tons, see Fig. 1) and the fact that this was a hatchery and juvenile production farm as it was observed in another study (*Guilpart et al., 2012*; *Teodorowicz, 2013*). The statistical analysis revealed that mean PO_4-P differential value for F1 was significantly lower than for F3 (P value $= 0.01$) and for F4 (P value < 0.001). No other significant difference between the different farms was observed for PO_4-P.

Globally, a general compliance with the discharge licence for all the regulated parameters was observed for the four farms and therefore the studied individual fish farms were not substantially impacting water quality. However, it was also observed that the F3 and F4 had on average higher differential values than the other farms. This fact could be due to higher yearly production for F3 and F4 compared to the other farms (Fig. 1) (*Boaventura et al., 1997*; *Guilpart et al., 2012*; *Teodorowicz, 2013*).

Parameters regulated in term of absolute values

Three parameters were regulated in term of absolute effluent values (i.e., DO, pH, temperature). The limit value for DO was 60% saturation in water and it appeared in the present study that no value was found to be below this threshold (average value for all farm outlets of 80% saturation, see Supplementary Materials SP1). The accepted range for pH was 6–9 pH units and no value was found to be out of this range, with an actual range of 6.5–7.5 pH units for all farms (see Supplementary Materials SP1). For temperature, the discharge licence stated that the effluent had to be ''ambient'' and no significant differences were observed between farms inlets and outlets (see Supplementary Materials SP1) meaning that the outlet water was actually at ambient temperature. Therefore, the four different farms were in compliance with the discharge licence for these three parameters and the farms were not substantially impacting water quality for these parameters.

The statistical analysis revealed that for temperature, the mean differential value for F3 was significantly higher than for F4 (P value < 0.05). This could be due to the different configuration of those farms; F3 being a multi-pond based farm with an assumed (not measured) associated relatively high hydraulic retention time (HRT), allowing a higher water heating potential than F4 which was assumed to be associated to a lower HRT due to its different configuration (i.e., only two ponds) and therefore a lower heating potential than F3. The statistical analysis for the other parameters (i.e., pH and DO) mean differential values did not reveal any significant differences between the four farms.

Cumulative impact of the fish farms on the river quality

The evolution of the quality of the inlet water through the four consecutive fish farms is presented in Fig. 4.

Upstream water quality

Background river quality (i.e., I_1, see Fig. 4) was on average very suitable for rainbow trout production with temperature range of 5–15 °C (see Supplementary Materials SP1), high level of DO (i.e., about 10.3 ± 1.29 mgO_2/L, $n = 17$ on average, see Fig. 4G), low levels of BOD_5 (i.e., about 2.06 ± 0.95 mgO_2/L, $n = 17$ on average, see Fig. 4B), NH_4-N (i.e., 0.08 ± 0.004 mgN/L, $n = 17$ on average, see Fig. 4D), NO_2-N (i.e., 0.06 ± 0.012 mgN/L, $n = 17$

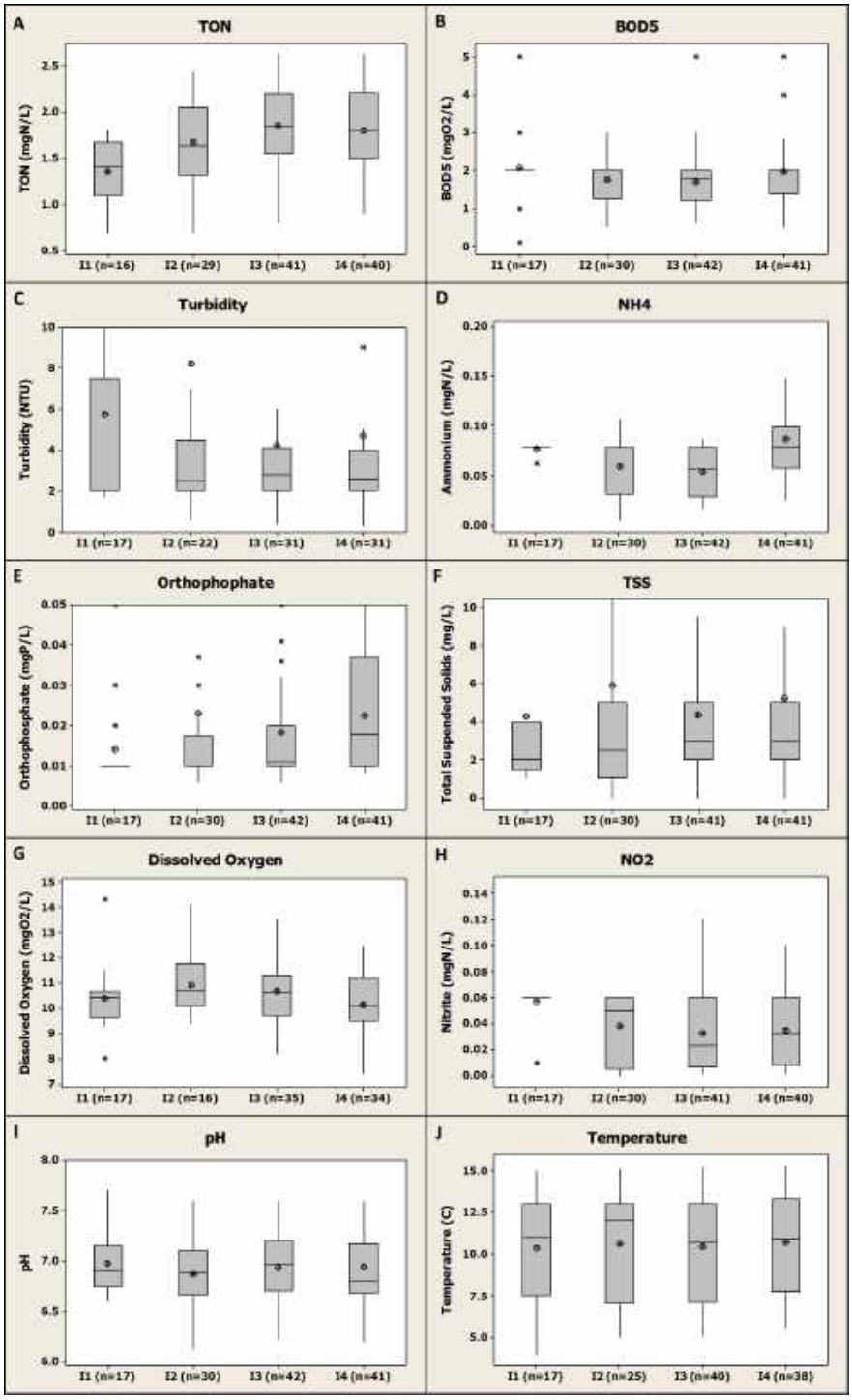

Figure 4 Box-plot representation of the water quality evolution through the four different fish farms inlets showing the cumulative impact of the fish farms on river quality for the monitored parameters. Numbers 1, 2, 3 and 4 represent the consecutive farms in the order of the river flow. Median, average, Q1 and Q3 are presented.

Full-size DOI: 10.7717/peerj.5281/fig-4

on average, see Fig. 4H) and TON (i.e., 1.36 ± 0.32 mgN/L, $n = 16$ on average, see Fig. 4A). Overall, these levels are all suitable for fish growth and all below the proven chronic toxicity levels for salmonids (*EEC, 1978*). Furthermore, these values confirmed that there was no potentially polluting activity (e.g., agriculture, WWTP) upstream from the fish farms that would have degraded the river quality for the parameters monitored.

BOD_5/dissolved oxygen (DO) patterns through the river flow

Overall there was no negative impact observed on river water quality due to the farms in terms of BOD_5 and DO concentrations which remained at about 2 mgO_2/L and 10 mgO_2/L respectively across the four different farms inlets and therefore along the river.

For DO, these results demonstrated that even if a significant decrease was observed at farm scale (as mentioned before), the river had the potential to reoxygenate between the different farms to reach its background level. In a similar study dealing with the cumulative impact of five different rainbow trout farms in Turkey, *Pulatsu et al. (2004)* observed an impact of fish farming that significantly decreased the DO levels in the river at 100 m downstream from the last farm. This relative short distance compared to distances between the different farms in the present study might explain why an impact was observed in this study.

For BOD_5, no significant difference in the inlet mean concentrations for the four farms was observed in the present study according to the statistical test applied (P value > 0.05) (Fig. 4B). This demonstrated that even if some BOD_5 differential concentrations were higher than the limit values for F2, F3 and F4, this had globally no impact on the river quality. A possible explanation for this observed trend could be a combination of (i) a relative high river flow compared to average fish farms water uptake flow providing a high dilution capacity to the river, (ii) a relative low differential concentrations for BOD_5 associated to river self-cleaning potential for this compound as it was observed in a study dealing with nine different rainbow trout and carp farms in Poland (*Teodorowicz, 2013*). On the contrary, *Pulatsu et al. (2004)* observed a significant impact of trout farming on the BOD_5 concentration in the river. However, in this study the downstream monitoring station was only at 100 m from the last farm investigated that might not be enough distance for the river to clean itself or to reoxygenate as it was observed in other studies (e.g., *Boaventura et al., 1997*; *Teodorowicz, 2013*). Depending on river/farm flow and river characteristics, a distance of 2–3 km downstream the fish farm is considered as being necessary to allow a self-purification of the river regarding BOD_5 (*Boaventura et al., 1997*).

Ammonium pattern through the river flow

Slight variations were observed for NH_4-N concentrations across the four fish farms inlets and therefore along the river. However, upstream and downstream farms inlets (i.e., F1 and F4 respectively) were associated to similar NH_4-N mean concentrations of 0.08 mg/L (no significant differences between those two farms, P value > 0.05) (Fig. 4D). Thus, as for BOD_5 parameter, we can assume that the distance between the different farms in the present study was long enough to allow the river to purify itself regarding NH_4-N and to get back to its initial background concentration. Therefore, there was not any impact of the fish farms on the river quality in term of NH_4-N concentrations. However a significant difference

was observed between F3 and F4 for this parameters (P value < 0.01) with a higher value at F4 inlet meaning that F3 might have had an impact on river quality for NH_4-N. The relative high average yearly production in F3 (i.e., 165 tons, see Fig. 1) associated to the relative short distance between those two farms (i.e., 500 m, see Fig. 1) might explain this observation due to a limited self-cleaning potential by the river within this relative short distance (*Boaventura et al., 1997*; *Lalonde, Ernst & Garron, 2015*). Depending on farm production and dry weather flow for the receiving water, *Boaventura et al. (1997)* estimated at 3–12 km the distance necessary for the river to get back to its initial organic chemical compound (including NH_4-N) concentration downstream from a given trout farm. With such low differential concentrations in F4 (0.08 ± 0.08 mgN/L, $n = 41$, on average, see Fig. 3F) and the self-cleaning potential of the river for this compound (*Boaventura et al., 1997*; *Lalonde, Ernst & Garron, 2015*), it appears very unlikely that this fish farm would have any impact on the downstream river quality in term of NH_4-N concentration.

Nitrite pattern through the river flow

A decrease in NO_2-N concentrations was observed across the fish farm inlets and therefore along the river (Fig. 4H). Median NO_2-N concentration was revealed to be 0.06 and 0.03 mgN/L at the upstream (i.e., F1 inlet) and downstream (i.e., F4 inlet) monitoring locations respectively. Therefore, considering this observation, the fish farm activity might have had a positive impact on the river quality regarding the NO_2-N concentration. To our knowledge, this potential "purification" of a river by trout farms regarding NO_2-N was never observed in fish farms where no denitrification step is applied, as was the case for the investigated fish farms in the present study where no water treatment processes were applied.

However, this result might be an artefact because as observed before most of the results obtained for F1 inlet location were below the LOQ of generally 0.06 mgN/L. As previously mentioned, in these cases, a value equal to the LOQ was considered for the present study but the real values could have been lower. The analytical LOQ was generally lower for F4 than for F1; this could explain why this "apparent dilution" of the river by the farms effluent was obtained for this parameter. The relative small dataset for F1 ($n = 17$) compared to the other farms ($n = 30$–41) might also explain the differences observed between upstream and downstream locations (i.e., F1 and F4 respectively). Other studies demonstrated the potential impact of trout farming on NO_2-N levels in river. *Pulatsu et al. (2004)* observed a significant increase from 0.019 to 0.581 mgN/L from upstream to 100 m downstream from five different trout farms. This could confirm the occurrence of an artefact with the results of the present study.

Total oxidized nitrogen pattern through the river flow

A slight increase was observed across the fish farms inlets and therefore along the river for TON with median concentrations of 1.4 and 1.8 mgN/L for the upstream (i.e., F1) and downstream (i.e., F4) locations respectively (Fig. 4A). Therefore, fish farming might have a slight impact on the river quality considering this parameter and might be responsible for a release of NO_3-N (not directly monitored). However, according to statistical analysis, no significant difference was observed between F1 and F4 inlets for this parameter average

values (P value > 0.05). This result is in agreement with the literature on this topic; in one study, NO_3-N mean concentrations were observed to increase from 0.13 mgN/L to 0.43 mgN/L from upstream to downstream of freshwater salmonids farms in Canada but no significant difference was observed (*Lalonde, Ernst & Garron, 2015*). In another study, a significant impact of trout farming on river quality for both NO_3-N and NO_2-N was demonstrated (*Pulatsu et al., 2004*). Another study dealing with the potential impact of eight different trout farms in France demonstrated that there was no trend for NO_3-N and that fish farms could be either responsible of an increase or a decrease of NO_3-N concentrations in the receiving water (i.e., 100, 1,000 m downstream from the farms) (*Guilpart et al., 2012*).

Orthophosphate pattern through the river flow

Stable PO_4-P concentrations were observed from F1 inlet to F3 inlet. Then, an increase was observed on PO_4-P concentration from 0.01 mgP/L to almost 0.02 mgP/L between F3 and F4 inlets respectively (i.e., median values, see Fig. 4E). Therefore, and considering that there was not any other potentially polluting activity in the area (i.e., WWTP, agriculture), F3 had an impact on the river quality in term of PO_4-P concentration. This was confirmed by the statistical analysis revealing that mean PO_4-P inlet concentrations for F3 and F4 were significantly different (P value < 0.001) and that PO_4-P concentrations were significantly higher at the F4 inlet than at F3 inlet. As for NH_4-N this trend might be due to both a relative high production in F3 and short distance between F3 and F4. Considering similar PO_4-P differential concentrations for F3 and F4 of about 0.02 mgP/L (see Fig. 3D) and a similar water uptake flow both farms, it is highly possible that an impact of F4 would have been observed if the river was monitored directly downstream from this fish farm. However, considering the self-cleaning potential of the river, the river might have got back to its PO_4-P background concentration of about 0.01 mgP/L (see Fig. 4E) a few kilometres downstream from F4 (*Boaventura et al., 1997*). In another study, an impact of trout farming on total phosphorus (TP) on the river quality was observed with an increase from 0.069 mgP/L upstream to 0.117 mgP/L downstream from the fish farms (*Pulatsu et al., 2004*). It is assumed that this increase in TP is likely to be due to an increase in the reactive form, PO_4-P, which was the monitored parameter in the present study.

Total suspended solids/turbidity patterns through the river flow

A slight TSS (and turbidity) increase were observed all along the river from 2 mg/L (2 NTU) to 3 mg/L (2.5 NTU) for upstream (i.e., F1 inlet) and downstream (i.e., F4 inlet) locations respectively (Figs. 4F and 4C). This demonstrated that the four fish farms had a very limited impact on the river quality in term of TSS (and turbidity). This limited impact was confirmed by the statistical analysis that demonstrated that there was no significant differences between TSS and turbidity average values between F1 and F4 inlet values (P value > 0.05). Considering relative low TSS and turbidity differential values observed for F4 (see Fig. 3E), it is unlikely that this fish farm would impact the downstream river quality for these parameters. This result is in agreement with the literature (*Lalonde, Ernst & Garron, 2015*; *Pulatsu et al., 2004*) where a non-significant impact of trout farms on receiving water quality was observed for TSS.

Temperature/pH patterns through the river flow

No significant impact of fish farms on both river temperature and pH was observed in this study. This result is in agreement with *Pulatsu et al. (2004)*, who did not observe any impact of trout farms on the receiving water quality for these parameters.

Seasonal variability (influent/effluent quality, impact)

Differential values were gathered by season with no distinction between the different farms. The results are presented in Fig. 5.

The results were compared statistically season by season in order to show any significant difference from one season to another for each parameter. Overall, except for NH_4-N (Fig. 5C), no significant difference was observed between the average results obtained for each season for all the parameters monitored. Even if differential values were on average higher in summer for TON, NH_4-N, PO_4-P and NO_2-N, the statistical analysis revealed that no significant differences were observed with mean values calculated for the other seasons. The analysis of the results obtained for NH_4-N revealed that mean values obtained for the spring season were significantly lower (P value < 0.01) than the results obtained for any other season. No significant difference was observed when the other seasons were compared. These results demonstrate that the season change had an overall limited impact on the polluting potential of the fish farms investigated and on the parameters monitored and that, despite apparent higher differential values for nutrient during summer, fish farming was not significantly more impacting during this season than during any other seasons in terms of discharge. This observation is in agreement with another study where a negligible impact of season on 15 trout farms effluent quality was observed for all the parameters monitored except for TP (*Lalonde, Ernst & Garron, 2015*). However, other studies demonstrated that the impact of trout farms on receiving water quality was higher in summer conditions when stocks are high, and both river flow and DO levels are low (*Boyd & Tucker, 1998*). In the future, climate change might increase the gap between winter and summer conditions with potential higher temperature/lower flows in rivers during the summer; therefore, the impact on aquaculture and on effluent water quality might be more and more significant.

CONCLUSION

A ten-year longitudinal survey (2005–2015) of the impact of four consecutive FT trout farms was performed for the first time in Ireland based only on publically available and independently generated data analysis. First, it was demonstrated that publically available data, although not always of very good quality, can be used to reliably assess the impact of fish farming on receiving water quality. The impact of each farm on water quality was assessed, and it was demonstrated that the impact was significant for NH_4-N and DO and more important for the farms which produced the most during the investigated period. Those results were expected in regard to other studies performed on the impact of traditional trout production across the world (*Boaventura et al., 1997*; *Caramel et al., 2014*; *Garcia et al., 2014*; *Teodorowicz, 2013*). The cumulative impact of the fish farms on receiving water quality was also assessed and it was demonstrated that the distance between

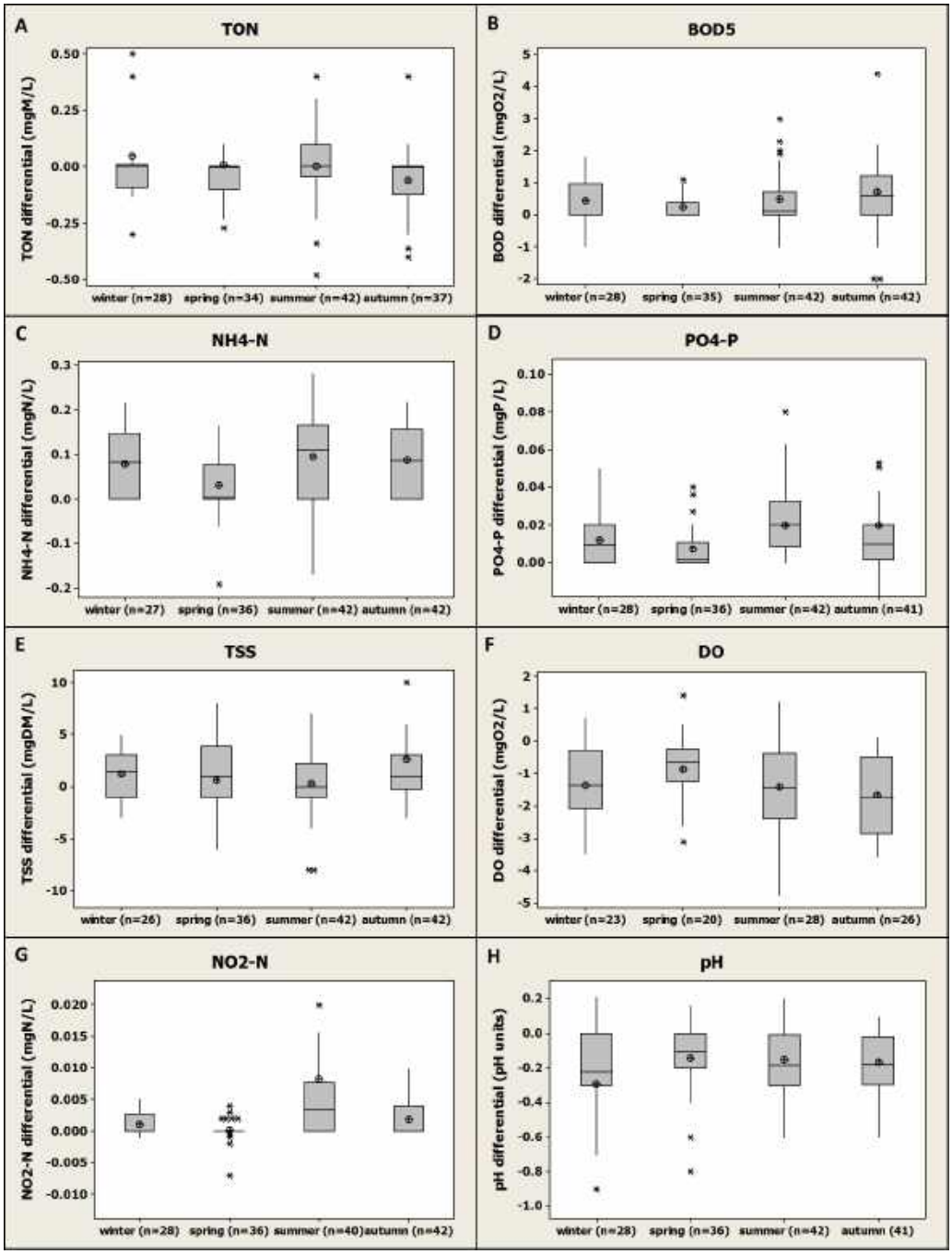

Figure 5 Box-plot representation of the seasonal variation of the differential values compiled and gathered for the four fish farms for the monitored parameters. Median, average, Q1 and Q3 are presented.

Full-size DOI: 10.7717/peerj.5281/fig-5

the different fish farms was globally sufficient to allow the river for self-purification regarding the parameters analysed and no overall cumulative impact was observed for the parameters considered. However, considering the relative high water volumes extracted by the studied farm, an increase production would not be possible without the addition of water treatment technologies to apply water reuse and therefore to reduce the demand on water without impacting receiving water quality. The present study represents the first benchmarking of the freshwater fish farming industry in Ireland and will be used as a baseline study, along with a study of the potential impacts on river's hydromorphology, for comparison before the evolution through more advanced practices and the expected implementation of water treatment processes in a near future due to the more and more stringent legislative framework. However, only the relevance of using the water quality parameters can be discussed. Indeed, notions such as the impact on biodiversity and ecotoxicology were not assessed in the present study and will represent a challenge for future studies in order to fully take account of the impact of the aquaculture industry in regard to the implication of the WFD and the expected future intensification of the production. The present study will provide a basis for policy and commensurate decisions on future fish farm licencing and for meeting good quality status under WFD as it pertains to the operation of fish farming facilities.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by Ireland's Department of Agriculture, Food and the Marine through the Morefish project and by Bord Iascaigh Mhara though the EcoAqua project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Ireland's Department of Agriculture, Food and the Marine.
Bord Iascaigh Mhara.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Alexandre Tahar conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Alan M. Kennedy and Richard D. Fitzgerald conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

- Eoghan Clifford and Neil Rowan conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data are provided as Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5281#supplemental-information.

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Article

Full Water Quality Monitoring of a Traditional Flow-Through Rainbow Trout Farm

Alexandre Tahar [1,*], Alan Kennedy [2], Richard D. Fitzgerald [3], Eoghan Clifford [2,3] and Neil Rowan [1]

1 Athlone Institute of Technology, Dublin Road, Athlone, County Westmeath, Ireland; nrowan@ait.ie
2 Civil Engineering, College of Engineering and Informatics, National University of Ireland, Galway, Ireland; michaelalan.kennedy@nuigalway.ie (A.K.); eoghan.clifford@nuigalway.ie (E.C.)
3 Ryan Institute, National University of Ireland, Galway, Ireland; richard.fitzgerald@nuigalway.ie
* Correspondence: atahar@ait.ie

Received: 10 May 2018; Accepted: 11 July 2018; Published: 17 July 2018

Abstract: Traditional freshwater rainbow trout farms are still popular in some European countries such as Poland, France and Ireland. These systems generally operate in flow-through configuration. The impact such production systems might have on water quality remains mostly unknown. The present study was set up to fulfil this objective of monitoring water quality on different fish farm locations in order to identify the impacts of the whole farm (comparison of farm inlet and outlet) and at pond scale in order to understand the water quality dynamics and to better understand the impact of multiple water reuse (water passes) in a given pond on water quality. In the absence of any sort of water treatment, an increase in the number of water passes was shown to create an increase in ammonium concentration along the farm. Finally, this traditional flow-through rainbow trout production system was revealed to almost be at its full carrying capacity with respect to internal water quality parameters and fish welfare. To increase fish production, some water treatment techniques (solid/liquid separation, nitrification) would have to be introduced in order to optimize the rearing water quality for fish growth and to minimize the release of pollutants in the receiving water to limit the impact on the environment.

Keywords: rainbow trout; traditional flow-through; pond-based; rearing water quality; environmental impact

1. Introduction

With the wild fish catching capacities reached and the overall human population increase, fish produced through aquaculture is and will be more and more an important food source for human consumption in the near future [1,2]. Aquaculture production increased more than five-fold from 1990 to 2012, while the world capture fisheries increased by only 8% at the same time [3]. The future and current intensification of this activity is associated with more and more stringent environmental regulations to ensure a sustainable and environmentally friendly production process/model [4–6]. At European scale, freshwater fish farming is essentially regulated through the water framework directive (WFD), which aims at achieving good chemical and ecological status of the rivers [1,7,8].

The traditional freshwater fish farming industry generally operates using flow-through systems (FT) without any water treatment, the dissolved oxygen (DO) levels in the culture water being maintained by relatively high volumes of water abstracted and flowing through the fish farm. The oxygen loss due to both fish activity and sediments is generally compensated by mechanical aerators such as paddle-wheels. In traditional FT systems, this aeration allows the fish farmers to use the water several times in different ponds before release (multi-pass); however, the impact of

this water reuse on water quality and the potential accumulation of waste in the rearing water has not been clearly identified so far. When the amount of DO is too low in the fish farm inlet water, fish farmers generally use supplementary oxygenation systems such as oxygenation cones in order to maintain optimum DO levels. Furthermore, fish farming activity generates dissolved and solid wastes such as nutrients (nitrogen and phosphorus) and organic matter from fish excreta and uneaten feed. Therefore, if the effluent is discharged untreated, it could potentially impact the discharge water quality compared to make-up water, and as a consequence impact the receiving water quality [9–14].

In Ireland, a large proportion of the freshwater production is still carried out using traditional FT systems without, or with very limited, water treatment before discharge. To the best of our knowledge, there is no existing published data on the effluent quality and flow, allowing estimation of pollutants fluxes and the potential impact the fish farming activity might have on water quality in Ireland. Models could be employed for the evaluation of organic matter (OM), nitrogen and phosphorus fluxes from feed composition and fish data (stocking density, average fish mass, overall fish mass) by the nutritional method [15]. However, missing data on fish/feed (especially on feed composition, ingested feed quantity, digestibility coefficients and the proportion of uneaten feed must be available according to [15]) forced us to evaluate the different fluxes by the hydrobiological method (involving flow measurements and water quality analysis).

Traditional flow-through systems generally involve a multi-use (or multi-pass) of water, with water passing through different ponds with no water treatment before being released to the receiving water, although some reuse might involve a supplementary oxygenation step. The objective of this study was to assess the water quality at different farm levels (pond, 1-pass, 2-pass and 3 or more pass ponds, whole farm) of a traditional rainbow trout farm in Ireland. To that aim, the whole farm (inlet/outlet and strategic fish farm locations) was monitored during two consecutive dry days. The monitoring program included water physico-chemical quality (dissolved oxygen, turbidity, nitrate, ammonium, and pH) and flow measurement in order to determine the flow dynamic of the whole farm and its different ponds. Each parameter was monitored on an hourly basis at the different selected farm locations in order to study fluxes and to identify any diurnal variation.

2. Results and Discussion

2.1. Fish and Flow Parameters

The table below summarizes the data related to (i) the biomass for each pond (i.e., total biomass, number of fish, average fish weight and stocking densities) that were obtained from the fish farmers, and (ii) flow values that were measured for each monitored pond (Tables 1 and 2).

Table 1. Outcome of fish data for the whole fish farm at the moment of the experiment. The figures 0, 1, 2 and 3 for ponds number are theoretical numbers of water uses (see Figure 7).

FISH					
Pond	**Volume**	**No. of Fish**	**Average Weight**	**Biomass**	**Stocking Density**
	m^3		**g/Fish**	**kg**	**kg/m^3**
1_1	121	3000	667	2000	17
1_2	144	3069	558	1713	12
1_3	147	12,000	170	2040	14
1_4	153	7696	610	4695	31
1_5	140	9736	800	7789	56
1_6	159	12,000	170	2040	13
1_7	122	5409	980	5301	43
1_8	86	15,750	200	3150	37
1_9	68	0	/	0	0
1_{10}	56	10,500	260	2730	49
1_{11}	44	10,500	260	2730	62
1_{12}	41	0	/	0	0
1_{13}	33	10,080	240	2400	73
after harvest		nd		600	19
1_{14}	25	10,080	220	2218	89
1_{15}	32	8000	280	2240	70
1_{16}	39	8000	220	1760	45
1_{17}	36	8000	220	1760	49
1_{18}	16	0	/	0	0
2_1	592	6000	1600	9600	16
2_2	207	6500	1300	8450	41
2_3	256	11,041	1330	14,685	57
3_2	301	1800	1100	1980	7
3_3	435	1283	1400	1796	4

Table 2. Outcome of flow data measured on the selected fish farm locations at the moment of the experiment. Standard deviation (St. Dev.); Minimum (Min.); Maximum (Max.).

FLOW						
Pond	**Mean Value**	**St. Dev.**	**Min.**	**Max.**	**Number**	**Comment**
	L/s	**L/s**	**L/s**	**L/s**		
1_5	19.9		17.5	24.5	35	
1_{13}	7.3	0.1	7.3	7.4	10	
1_{15}	6.8	0.7	5.9	7.5	39	
2_1	−1.8	0.2	−1.3	−2.1	11	2_1 outlet 1
	35.2	2.3	29.8	37.2	42	2_1 outlet 2
	66.1	4.3	0.0	96.3	370	recirculation
2_2	6.0	0.8	4.4	7.7	23	2_2 outlet 1
	34.5	0.8	33.2	35.7	21	2_2 outlet 2
2_3	76.1	7.8	57.6	148.5	473	Farm outlet 1
3_2	50.3	7.7	34.1	63.9	20	
3_3	58.1	21.5	0.0	112.7	565	Farm outlet 2

Table 1 shows the range of fish sizes being reared at the time of the experiment (170–1600 g/fish). Different stocking densities were also observed in the different ponds (4–89 kg/m^3).

The flow measurements showed a flow dynamic slightly different than what was expected initially in terms of number of water passes. For example, a negative flow value was obtained between 2_1 and 2_2 ponds (Table 2). This might be a consequence of the recirculation pump in operation in 2_1 that would have applied a suction of the water from 2_2 (average recirculation flow of 66 L/s, see Table 2). This demonstrated that 2_2 was completely isolated from 2_1 and therefore isolated from any recirculated water. This is the reason why, contrary to initial expectations where it would have been a "3-pass

pond", 2_2 was considered as a "2-pass pond", as it only received water from $1_{8–12}$ ponds (i.e., "1-pass ponds"). Furthermore, we observed that the average flow from 2_2 to 2_3 pond was very limited (i.e., 6 L/s, see Table 2). This is why 2_3 pond was considered a "mostly 2-pass pond", as it received water from six "1-pass ponds" (i.e., $1_{13–18}$ ponds at 7 L/s each, see Table 2) and only one "2-pass pond" (i.e., 2_2 pond at 6 L/s, see Table 2).

Temperature was monitored in different ponds during the experiment and water temperature was 8–9.5 °C in all ponds during the whole experiment.

2.2. Impact of the Whole Fish Farm on Water Quality

The objective was to assess the overall impact of the farm on water quality (i.e., farm influent vs. effluent water quality). Farm inlet (before oxygenation, feeding channel, i.e., 0_0) and farm outlet water quality were compared. Differential concentrations ($\Delta_C(t)$, see Equation (1)) were calculated between inlet and mean outlet concentrations between the two outlets (considering the two farm outlets from 2_3 and 3_3 ponds had similar flows, see Table 2) monitored during the two days of experiment for all the studied parameters (see Section 3.2).

The figure below presents an outcome of the differential values obtained between the fish farm outlets and inlet for five parameters (i.e., DO, turbidity, nitrate (NO_3–N), ammonium (NH_4–N) and pH) during the two days of monitoring (Figure 1).

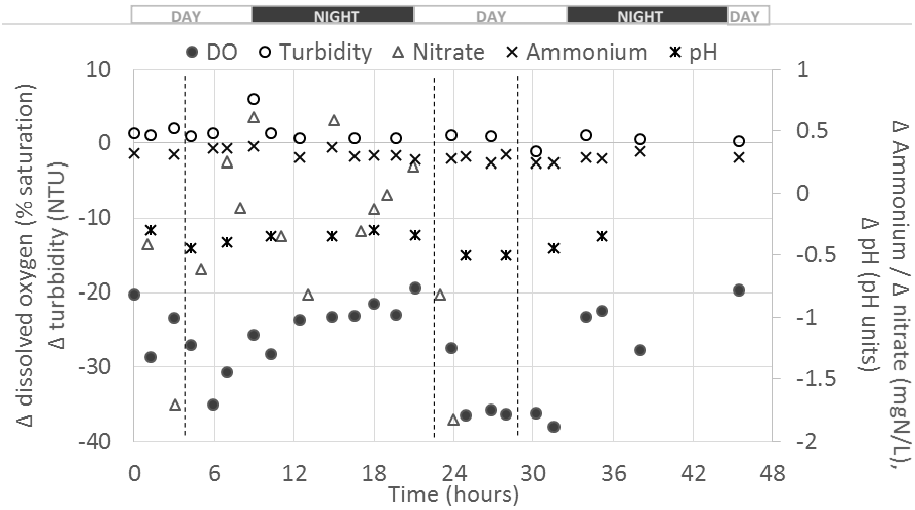

Figure 1. Whole farm differential values between outlets and inlet of dissolved oxygen (DO), turbidity (in NTU (Nephelometric Turbidity Unit)), nitrate, ammonium and pH during the monitoring program. Vertical dash lines represent feeding times.

Globally, negative Δ_C were observed for DO, pH and NO_3–N for the duration of the monitoring. Such results were expected for DO and pH due to O_2 consumption by fish and sediments and the CO_2 production leading to a pH drop, as observed on other studies [16,17]. However, positive Δ_C were observed for turbidity and NH_4–N, meaning that the farm activity "created" solids and NH_4–N. Except for NO_3–N and DO, $\Delta_C(t)$ appeared relatively stable all along the experiment and no diurnal effect was observed at this overall farm scale. For DO, higher absolute $\Delta_C(t)$ (i.e., down to almost −40%) were observed during the days compared to night times (i.e., about −20%); this might be due to the higher O_2 consumption by fish during the day due to the occurrences of feedings and higher fish activity [18].

The table below summarizes these data by providing average and associated standard deviation of the overall farm $\Delta_C(t)$ values (Table 3). This presentation of the overall farm mean Δ_C shows a global picture of the influence of the fish farm on water quality. Associated fluxes calculated as presented in Section 3.4 (Equations (2) and (3)) are also presented.

Table 3. Summary of the overall farm differential data between outlets and inlet. Turbidity (Turb.); nitrate (NO_3–N); ammonium (NH_4–N); not applicable (NA).

	DO		Turb.	NO_3–N	NH_4–N	pH
	%	mg/L	NTU	mgN/L	mgN/L	
mean difference	−27.39	−3.09	1.19	−0.36	0.30	−0.39
st. dev.	6.05	0.67	1.44	0.72	0.04	0.07
min.	−38.10	−4.33	−1.10	−1.83	0.24	−0.50
max.	−19.40	−2.25	6.01	0.61	0.38	−0.30
count	24		16	15	21	11
flux (mg/s) *	NA	−413.9	159.3	−48.6	40.4	NA
flux (g/h) *		−1489.9	573.5	−174.8	145.4	
flux ($g/h/kg_{fish}$) **		−0.018	0.007	−0.002	0.002	
flux ($mg/h/kg_{fish}$) **		−18.37	7.07	−2.16	1.79	

* fluxes calculated at farm scale using the average cumulated outlet flow rate of 134 L/s (see Table 2); ** fluxes calculated considering a total biomass reared on farm of 81,094 kg (see Table 1).

One of the parameters most affected by the farm was the DO saturation rate, with an average global "loss" of about 27% between farm inlet and outlet despite supplementary aeration/oxygenation steps on the farm (see Figure 7). Considering the total biomass and the outlet flows measured, mean DO F value was $-18.4\ mgO_2/h/kg_{fish}$.

An increase in turbidity values from farm inlet to outlet was observed with an average positive Δ_C of about 1.2 Nephelometric Turbidity Unit (NTU). This value appears relatively low compared to values measured at the outlets of some ponds (up to 20 NTU), and this may be due to the sedimentation of suspended solids in ponds, which would have lowered the release of suspended solids at the farm outlet. A peak in turbidity increase (i.e., $\Delta_C(t)$ of 6 NTU) was observed at the beginning of the first night of the monitoring (Figure 1) which could be due to fish metabolism and the release of faeces a few hours after the afternoon feeding [16–18]. In terms of flux, average F value was $7.1\ NTU/h/kg_{fish}$.

A global average drop in NO_3–N concentrations of about 0.36 mgN/L between farm inlet and outlet was observed (Table 3). As shown on Figure 1, and regarding the relatively high standard deviation associated to the average differential value for this parameter (i.e., 0.72 mgN/L), a high variability of NO_3–N Δ_C during the experiment (i.e., range −1.83–+0.61) was observed. A negative value of average flux of $-2.2\ mgN/h/kg_{fish}$ was obtained.

As expected, NH_4–N appeared to be "produced" by the farm, with a positive average Δ_C of 0.30 mgN/L (see Table 3). This represents a significant increase regarding the average farm inlet concentration of about 0.30 mgN/L (see Supplementary Materials). This Δ_C remained stable all along the experiment, with a relatively narrow range observed (0.24–0.38 mgN/L). Therefore, the release of NH_4–N by the farm seemed not be affected by light and feeding regimes. It is noticed that this apparent NH_4–N "production" might be an artefact due to the overall pH drop of 0.39 units (Table 3) that would have changed the ammonia equilibrium through the ionized form, NH_4–N. An average F value of $1.8\ mgN/h/kg_{fish}$ was obtained.

An average pH decrease of about 0.39 pH units between farm inlet and outlet was observed (Table 3) that might a consequence of a CO_2 production by fish metabolism [16–18]. This Δ_C remained stable all along the experiment with a relatively narrow range observed (−0.50–−0.30 pH units, see Table 3).

2.3. Comparison of the Water Quality from Inlet/1-Pass/2-Pass/3 Pass Ponds

The objective of this part of the study was to identify trends between the water quality of the farm inlet, 1-pass, 2-pass and 3-pass waters in order (i) to check if fish farm inlet water impacted water quality flowing through the farm and (ii) to identify potential trends in water quality parameters throughout the farm.

According to the flow measurements performed, only 2_2 and 2_3 ponds were considered as "2-pass ponds" (see Table 2 and Section 2.1). Ponds 3_2 and 3_3 were considered as "at least 3-pass ponds"

because, as explained before, they received some recirculated water from 2_1 (see Table 2 and Section 2.1). It is noticed that 2_1 pond was kept alone for this analysis because it had the specificity to receive directly recirculated water from $1_{1–7}$. This made the number of water passes of this pond difficult to evaluate from the flow measurements performed.

Average values for inlets (after supplementary oxygenation, i.e., 1_{13} and 1_{15} inlets), 1-pass (1_{13} and 1_{15} outlets), 2-pass (or "mostly 2-pass ponds", i.e., 2_2 and 2_3 outlets) and "at least 3-pass ponds" (i.e., 3_2 and 3_3 outlets), 2_1 outlet and associated standard deviation were calculated. The two "1-pass ponds" monitored (i.e., 1_{13} and 1_{15}) were considered separately because of the 1_{13} pond harvesting that occurred after 24 h into the monitoring period. For more simplicity, in the following paragraphs the terms "2-pass ponds" and "3-pass ponds" will be employed instead of "mostly 2-pass ponds" and "at least 3-pass ponds", respectively. The overall results for all the different pond categories (number of water passes) and for all the monitored parameters are presented on the figure below (Figure 2).

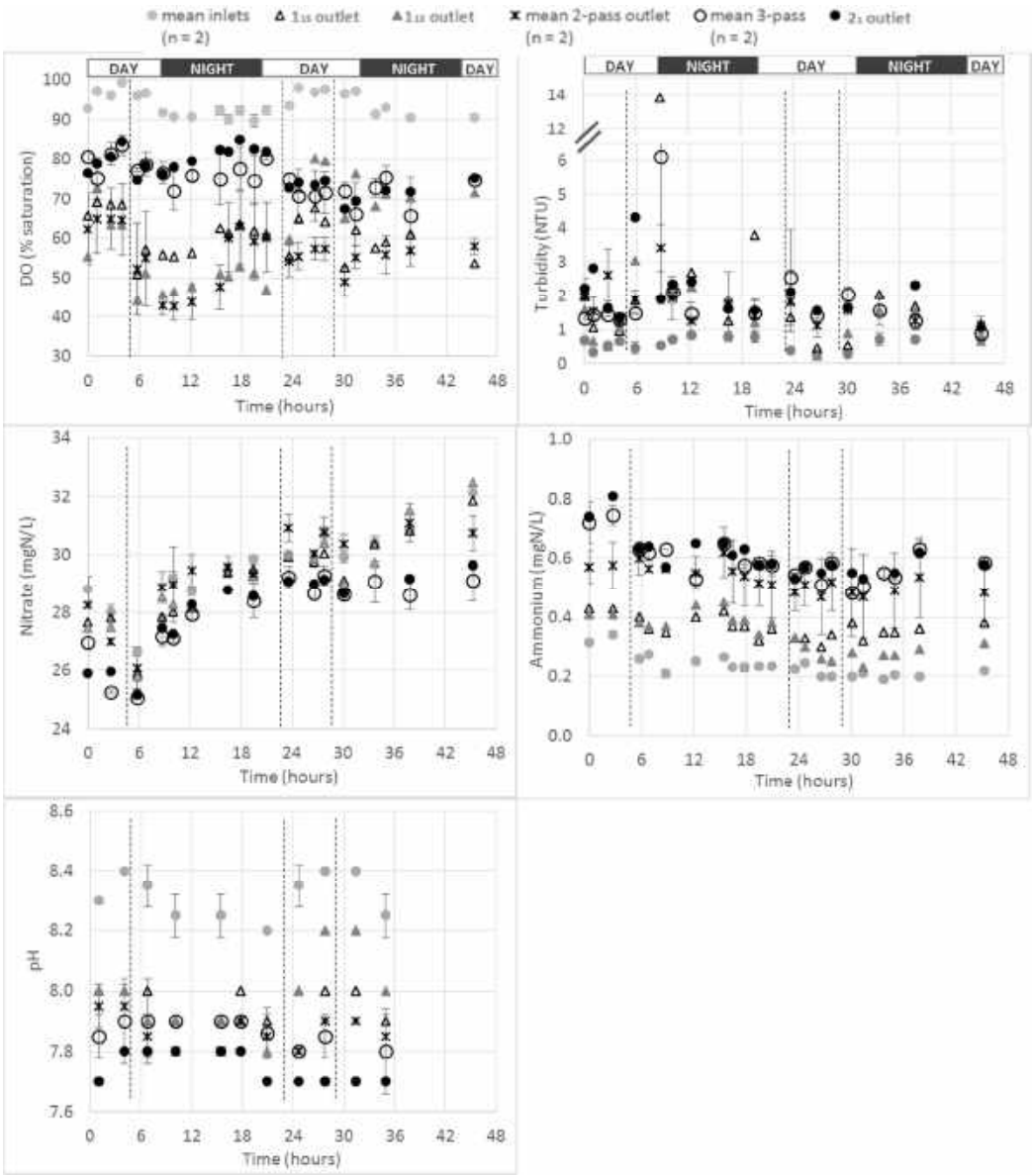

Figure 2. Average evolution of water quality (i.e., dissolved oxygen saturation rate, turbidity, nitrate, ammonium and pH) at the farm inlets and in "1-pass", "2-pass", "3-pass" and 2_1 ponds outlets. Vertical dash lines represent feeding times.

2.3.1. Dissolved Oxygen

As expected, over the whole farm, the most oxygenated waters were the fish farm inlet waters, with saturation values in the range 85–100%. A higher DO saturation rate was measured during day time compared to night time (average DO values of 96% and 84%, respectively). These higher DO values during the day might be a consequence of the plants and phytoplankton photosynthesis occurring with sunlight and producing O_2 [17]. The same trend was observed at the inlets of $1_{13}/1_{15}$ ponds after supplementary oxygenation by the two oxygenation cones located in the inlet channels 0_2 and 0_3 (see Figure 7) and that were in operation continuously during the whole monitoring program. However, the impact of the oxygenation cones on the level of DO saturation of the water appeared to be negligible during day time (i.e., 2–3% higher in the oxygenated water (i.e., $1_{13}/1_{15}$ ponds inlets) than in the inlet water from the inlet channel 0_0, see Supplementary Materials). This impact was revealed to be higher at night time with a difference between oxygenated and non-oxygenated water up to 10% (i.e., 84% and 94% for non-oxygenated and oxygenated water respectively, see Supplementary Materials).

The monitoring of the three "1-pass ponds" showed that a systematic drop in the DO level was observed after every feeding event. This drop appeared to occur immediately after feeding. This would coincide with an increase in fish activity and in the metabolic demands associated with digestion [18].

Interestingly, the "3-pass ponds" did not show the least oxygenated water, with values in the range 65–85% of DO saturation at their outlets. According to the flow measurement performed (see Table 1), it was demonstrated that those ponds were fed more or less directly by recirculated water (i.e., water from 2_1 pond). This demonstrated the efficiency of applying recirculation/supplementary oxygenation and the associated impact on DO saturation rate observed all along the farm from 2_1 pond to the 3_3 farm outlet (see Figure 7).

The least oxygenated ponds were the "2-pass ponds", with values in the range 45–65%. These values were in the same range than the ones observed for "1-pass ponds" water from 1_{15} (i.e., 50–70%), demonstrating the ability of paddle-wheel aerators to compensate for the loss of oxygen that occurred in those "2-pass ponds" due to fish/sediments consumption. Furthermore, the two "2-pass ponds" were not fed in any way by recirculated water (aeration of these ponds relied only on paddle-wheel aerators, see Table 2 and Section 3.1), and that could explain the relative low oxygen saturation levels observed in those ponds compared to the "3-pass ponds". Among the two "2-pass ponds", 2_3 pond had a higher DO saturation at its outlet than 2_2 pond during the whole monitoring period (see Supplementary Materials); this might be due to the differences in aeration systems from one pond to another (i.e., 2_2 being aerated with two paddle-wheel aerators and 2_3 with only one paddle-wheel, see Figure 7). This observation would confirm the ability of paddle-wheel aerators in compensating for the DO loss due to fish and sediments. Furthermore, 2_1 and 3_2 ponds outlets had similar DO profiles during the experiment (see Supplementary Materials). Thus, the aeration systems in operation in 3_2 (i.e., two paddle-wheels see Figure 7) were efficient enough to maintain the DO saturation levels higher than 70%. However, it was observed that DO levels were generally lower in 3_3 than in $2_1/3_2$ ponds showing that the aeration system (i.e., one paddle-wheel, see Figure 7) was not efficient enough to maintain the same DO level in this pond.

2.3.2. Turbidity

As expected, the lowest turbidity levels and variability were observed for the farm inlet waters. However, the comparison of the different pond classes is not straightforward. The potential sedimentation of solids along the farm might be a potential explanation of the difficulty to analyze the results for this parameter [16,18]. The relatively low turbidity values measured (a few NTU) and the potentially high uncertainty associated with turbidity measurements at these low levels might be another potential explanation. However, the same peak of turbidity was observed for the two "1-pass ponds" (1_{13} and 1_{15}) on the first day of monitoring (at about 8 h monitoring time) and the same general turbidity profiles were observed for those two ponds (Figure 2). The fact that the turbidity measurements were repeatable from those two ponds with similar fish characteristics (i.e., same total

biomass and average fish weight, see Table 1) indicates that the turbidity measurements performed were accurate and reliable. The greater variability in turbidity was observed for the 1-pass ponds outlets (i.e., 1_{13} and 1_{15}). This may be due to the sedimentation of suspended solids all along the farm that would have smoothed the associated turbidity profiles [16,18].

2.3.3. Nitrate

The same profile was observed for NO_3–N concentrations at any farm level for the duration of the monitoring. The general increase of concentration observed at farm inlet was also observed at each level of water passes. However, the ponds associated to the highest levels of passes (i.e., "3-pass ponds" and 2_1) were associated to the lowest NO_3–N concentrations (i.e., in the range 25–29 mgN/L) while the farm inlet concentrations were in the range 26–32 mg/L. It is noteworthy that the "3-pass ponds" and 2_1 pond (all fed with recirculated water) showed similar NO_3–N profile. This demonstrated that, as for the DO profile, 2_1 pond could not be considered a "2-pass pond" for the NO_3–N profile. Additionally, the "2-pass ponds" were not affected in terms of the NO_3–N concentration profile by the fish farming activity. This was highlighted by the comparison with the farm inlet NO_3–N concentrations that were observed to be in similar range than 2-pass ponds all along the experiment (range of 2-pass ponds 27–32 mgN/L).

2.3.4. Ammonium

A clear distinction between NH_4–N levels at the farm inlets, "1-pass", "2-pass" and "3-pass ponds" outlets was observed. As expected, the higher the number of passes is, the higher the NH_4–N concentration. This demonstrates the progressive accumulation of NH_4–N across the whole farm due to fish excretion and/or to progressive dissolution of sediments [16,18].

Farm inlet NH_4–N concentrations were in the range 0.20–0.34 mgN/L.

The "1-pass pond" outlets' NH_4–N concentrations were in the range 0.30–0.45 mgN/L (before 1_{13} harvest). It is noteworthy that almost the exact same NH_4–N profile was obtained at the outlet of both "1-pass ponds" (i.e., 1_{13} before harvest and 1_{15}); then, lower values were observed after 1_{13} harvest during the second day of monitoring. This highlighted both (i) the good quality and reproducibility of the data generated regarding the use of the multisensor probes in terms of NH_4–N monitoring and (ii) the impact of fish in NH_4–N production in a pond.

The "2-pass ponds" average NH_4–N outlet concentrations were in the range 0.47–0.62 mgN/L, which is a higher level than the ones observed for "1-pass ponds". A great variability between the values measured for the two "2-pass ponds" considered (i.e., 2_2 and 2_3) was observed; the NH_4–N concentrations were indeed systematically higher for 2_2, by about 0.1 mgN/L. This could be due to the different receiving waters for those two ponds (2_2 received water from the five $1_{8–12}$ "1-pass ponds" (not monitored during this study) and 2_3 pond received water from the six $1_{13–18}$ "1-pass ponds"). This could also be due to the different biomass reared in both 2_2 and 2_3 ponds (see Table 1).

The "3-pass" and 2_1 ponds NH_4–N outlet concentrations were in the range 0.49–0.81 mgN/L, which was the highest range recorded over the whole farm. This observation is in agreement with both (i) the expected NH_4–N accumulation across the whole farm and (ii) the water recirculation without any nitrification step to oxidize NH_4–N (into NO_2–N and NO_3–N) leading to an NH_4–N accumulation within the recirculation loop. This underpinned the impact of recirculation on the 2_1 pond water quality. It is noticed that the 2_2 pond outlet NH_4–N concentrations were in the same range that those measured at "3-pass pond" outlets.

2.3.5. pH

As expected, an impact of the farm on pH values was observed with a general decrease of pH across the whole farm, likely due to the CO_2 production by fish metabolism [17].

The inlet mean values were in the range 8.2–8.4 pH units with lower values generally observed at night. The "1-pass pond" outlets' pH values were measured in the range 7.9–8.0 (before harvest for 1_{13}

pond). It is interesting to observe values up to 8.2 pH units after harvest at the 1_{13} pond outlet (similar to the values observed at the inlet). This highlights the impact of fish on pH variation. The "2-pass ponds", "3-pass ponds" and 2_1 pond outlets pH values were in the range 7.8–7.95 and 7.8–7.9 and 7.7–7.8 pH units, respectively. The lowest values were therefore observed for the recirculated water from the 2_1 pond. This again demonstrated the impact of recirculation on water quality. This could be due to the recirculation loop that could have brought back water with low pH into this pond. As mentioned before, this could explain the high NH_4–N concentration measured in this pond.

Of note is that the ponds with the highest number of water passes had the lowest pH values and the highest NH_4–N concentrations. At low pH (i.e., <8), and at the temperatures measured during this monitoring program, the ionized form of ammonia (NH_4–N, relatively non-toxic to fish) is highly predominating compared to the toxic unionized form (NH_3–N). Therefore, the highest NH_4–N concentrations observed in the high number of passes ponds could be just an artefact due to pH variation and not due to an accumulation of fish wastes.

2.4. Impact of 1-Pass Ponds on Water Quality

Three different "1-pass ponds" were intensively monitored during the experiment (i.e., 1_5, 1_{13} and 1_{15}, see Figure 7). As for the whole farm, $\Delta_C(t)$, calculated as explained in Section 3.4 and using Equation (1), are presented. Fluxes, calculated as explained in Section 3.4 (Equations (2) and (3)), are also presented. The flux values will be specifically discussed in Section 2.5 below.

2.4.1. 1_{15} Pond

The figure below presents the pattern of Δ_C between the outlet and inlet of 1_{15} pond during the monitoring program (Figure 3).

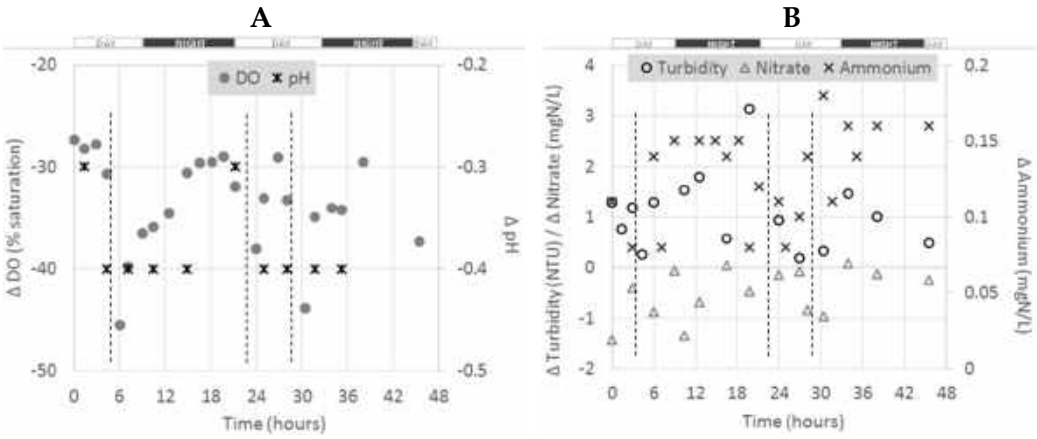

Figure 3. Pond 1_{15} differential values between outlet and inlet for dissolved oxygen and pH (**A**) and for turbidity, nitrate and ammonium (**B**) during the monitoring program. Vertical dash lines represent feeding times.

As for the whole farm scale, different trends were observed, depending on the parameter considered at this pond scale. Negative Δ_C were obtained for pH, DO and NO_3–N. Positive values were obtained for turbidity and NH_4–N. For NO_3–N, a general increase in Δ_C from the beginning to the end of the experiment (from -1.5 to about 0 mgN/L) was observed. The Δ_C for NH_4–N was higher during night times. Dissolved oxygen showed diurnal variation also, with higher consumption (or lower Δ_C) during day time with drops likely to be due to feedings [19]. No clear trends or diurnal variation were observed for the other parameters.

The Δ_C data are summarized in the table below, which provides average values, associated standard deviation and the calculated fluxes for each parameter (Table 4). Fluxes data, as described in Section 3.4 are also presented.

Table 4. Summary of the 1_{15} pond differential data and fluxes between outlets and inlet.

	DO		Turb.	NO_3–N	NH_4–N	pH
	%	mg/L	NTU	mgN/L	mgN/L	
mean difference	−33.50	−3.80	1.85	−0.50	0.13	−0.38
st. dev.	4.88	0.53	3.15	0.50	0.03	0.04
min.	−45.50	−5.04	0.18	−1.43	0.08	−0.40
max.	−27.30	−2.92	13.32	0.08	0.18	−0.30
count	24		16	15	21	10
flux (mg/s) *	NA	−26.6	12.9	−3.5	0.9	NA
flux (g/h) *		−95.7	46.5	−12.7	3.2	
flux ($g/h/kg_{fish}$) **		−0.043	0.021	−0.006	0.001	
flux ($mg/h/kg_{fish}$) **		−42.74	20.76	−5.65	1.44	

* fluxes calculated at 1_{15} pond scale (including the impact of fish and sediments) with the average flow rate of 7L/s (see Table 2); ** fluxes calculated considering a total biomass reared in 1_{15} pond of 2240 kg (see Table 1).

2.4.2. 1_{13} Pond

As for 1_{15} pond, the figure below presents the evolution of Δ_C between outlet and inlet of 1_{13} pond during all the monitoring program (Figure 4). It is noticed that this pond was partially harvested on day two of the experiment (after about 24 h of monitoring) (see Table 1).

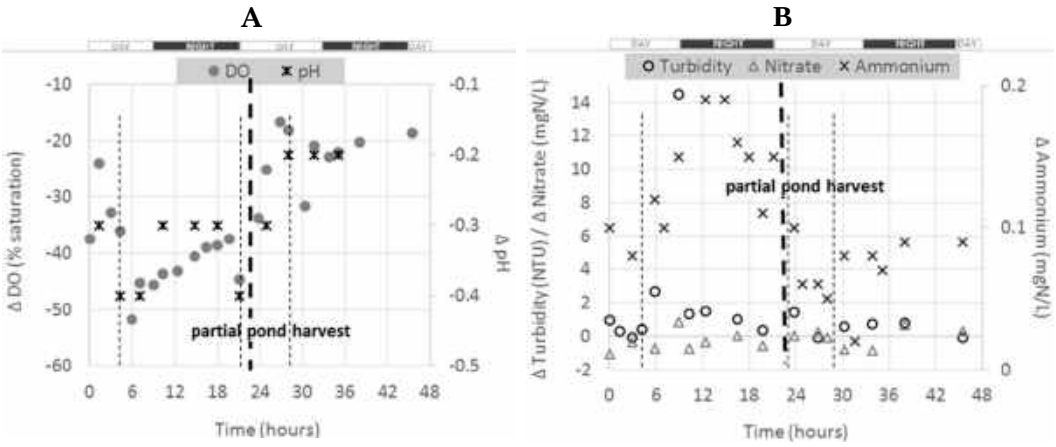

Figure 4. Pond 1_{13} differential values between outlet and inlet for dissolved oxygen and pH (**A**) and for turbidity, nitrate and ammonium (**B**) during the monitoring program. Vertical light dash lines represent feeding times.

As for the whole farm and the 1_{15} pond, negative Δ_C values were observed for NO_3–N, pH and DO and positive ones for turbidity and NH_4–N. Before harvest, Δ_C for NH_4–N were higher at night, confirming the observations made for 1_{15} pond (see Section 2.4.1). After harvest, all Δ_C profiles changed for all parameters and all the absolute values were lower. As expected, the effect of the fish activity in this pond was therefore reduced after harvesting, as the total biomass was reduced (see Table 1).

The Δ_C data are summarized in the table below, which provides average values, associated standard deviation and the calculated fluxes for each parameter before and after harvest (Table 5). Fluxes data, as described in Section 2.4 and according to Equations (2) and (3) are also presented.

Table 5. Summary of the 1_{13} pond differential data and fluxes between outlets and inlet before and after harvest.

	Before Harvest						**After Harvest**					
	DO		**Turb.**	**NO_3–N**	**NH_4–N**	**pH**	**DO**		**Turb.**	**NO_3–N**	**NH_4–N**	**pH**
	%	**mg/L**	**NTU**	**mgN/L**	**mgN/L**		**%**	**mg/L**	**NTU**	**mgN/L**	**mgN/L**	
mean difference	−40	−4.55124	2.295	−0.37125	0.136364	−0.34286	−23.01	−2.607	0.561667	−0.06714	0.07	−0.225
st. dev.	6.685	0.708759	4.367466	0.590507	0.036952	0.053452	5.641208	0.630768	0.579738	0.568997	0.02357	0.05
min.	−51.8	−5.73	−0.06	−1.06	0.08	−0.4	−33.7	−3.83	−0.08	−0.85	0.02	−0.3
max.	−24.1	−2.87	14.52	0.86	0.19	−0.3	−16.8	−1.92	1.45	0.67	0.1	−0.2
count	14		10	8	11	7	10		6	7	10	4
flux (mg/s) *	NA	−31.8587	16.065	−2.59875	0.954545	NA	NA	−18.249	3.931667	−0.47	0.49	NA
flux (g/h) *		−114.691	57.834	−9.3555	3.436364			−65.6964	14.154	−1.692	1.764	
flux ($g/h/kg_{fish}$) **		−0.04779	0.024098	−0.0039	0.001432			−0.10949	0.02359	−0.00282	0.00294	
flux ($mg/h/kg_{fish}$) **		−47.788	24.0975	−3.89813	1.431818			−109.494	23.59	−2.82	2.94	

* fluxes calculated at 1_{13} pond scale (including the impact of fishes and sediments) with the average flow rate of 7L/s (see Table 1); ** fluxes calculated considering a total biomass reared in 1_{13} pond of 2400 kg and 600 kg before and after harvest respectively (see Table 1).

The comparison of 1_{15} and 1_{13} ponds before harvest (similar total and average fish weight in those two ponds as presented in Table 1) showed very similar values, demonstrating the reproducibility of our approach. The effect of harvesting on the differential values appears very clear when results before and after harvest are compared. Mean DO consumption dropped from 40 to 23%, the mean pH difference dropped from 0.34 to 0.23 pH units, mean turbidity production dropped from 2.30 to 0.56 NTU and the mean NH_4–N production dropped from 0.14 to 0.07 mgN/L.

It is noteworthy that despite the fact that the 1_{13} pond was harvested and that only 600 kg of fish remained after harvest, the pond fluxes in terms of DO and NH_4–N are not negligible. This underpins the potential impact of the sediments accumulated at the bottom of the tank might have on DO consumption or NH_4–N production. The role of sediments on the residual Δ_C values obtained after harvest will be discussed in Section 2.5.

2.4.3. 1_5 Pond

The figure below presents the evolution of Δ_C between outlet and inlet of 1_{13} pond during the entire monitoring program (Figure 5).

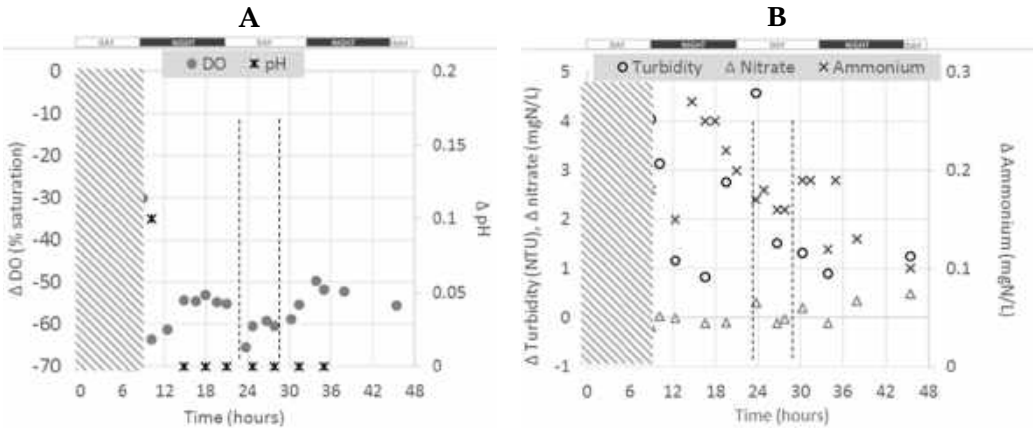

Figure 5. Pond 1_5 differential values between outlet and inlet for dissolved oxygen and pH (**A**) and for turbidity, nitrate and ammonium (**B**) during the monitoring program. The inlet monitoring location was changed after 8 h of monitoring. Vertical dash lines represent feeding times.

This pond had three main particularities compared to $1_{13}/1_{15}$ ponds regarding DO:

(1) It was supplied with super-saturated water from the recirculation loop (average 140% saturation rate, see Supplementary Materials),
(2) It had a paddle-wheel aerator running during the experiment (see Figure 7),
(3) Bigger fish than in $1_{13}/1_{15}$ ponds were reared (average weight of 800 g and 220–240 g respectively, see Table 1)

The table below presents a summary of the Δ_C values observed for all parameters during the whole experiment (Table 6). The amount of data is lower for this pond because the initial inlet monitoring location had to be changed for another during the experiment as the initial one was observed to be unsuitable for monitoring.

Table 6. Summary of the 1_5 pond differential data and fluxes between outlets and inlet.

	DO		Turb.	NO_3–N	NH_4–N	pH
	%	mg/L	NTU	mgN/L	mgN/L	
mean difference	−56.82	−6.41	1.49	0.08	0.18	0.01
st. dev.	4.42	0.49	1.87	0.22	0.05	0.04
min.	−65.50	−7.39	−2.57	−0.12	0.10	0.00
max.	−49.70	−5.48	4.58	0.49	0.27	0.10
count	17	17	10	11	16	8
flux (mg/s) *	NA	−128.1	29.8	1.6	3.7	NA
flux (g/h) *		−461.2	107.1	5.8	13.2	
flux ($g/h/kg_{fish}$) **		−0.059	0.014	0.001	0.002	
flux ($mg/h/kg_{fish}$) **		−59.21	13.75	0.75	1.69	

* fluxes calculated at 1_5 pond scale (including the impact of fishes and sediments) with the average flow rate of 20 L/s (see Table 2); ** fluxes calculated considering a total biomass reared in 1_5 pond of 7789 kg (see Table 1).

A relatively high DO consumption rate of 59 $mgO_2/h/kg_{fish}$ compared to the ones found for $1_{13}/1_{15}$ ponds (43 and 48 $mgO_2/h/kg_{fish}$, respectively; see Tables 3 and 4) was observed. The NH_4–N production rate was found to be similar to what was found for the other "1-pass ponds" and for the whole farm (Table 3).

Contrary to the other "1-pass ponds" monitored (i.e., 1_{13} and 1_{15}), a paddle-wheel aerator was employed in this pond for supplementary aeration (Figure 7) and thus, had to be considered in the DO mass balance for this 1_5 pond. However, the impact of the paddle-wheel aerator in increasing DO levels in the pond could not be assessed.

These results regarding the higher DO consumption rate in 1_5 compared to $1_{13}/1_{15}$ ponds was unexpected for at least two reasons:

(1) The presence of a paddle-wheel aerator should have increased the DO amount received by the pond, and therefore decreased the overall consumption rate (because the calculation did not into account the O_2 input from the paddle-wheel aerator).
(2) From the literature [16], bigger fish are supposed to consume less O_2 by mass unit than smaller ones, and the comparisons of results obtained for 1_5 and $1_{13}/1_{15}$ ponds appeared not to confirm this statement.

The different hypotheses that might explain these results could be:

- In relation to the very high initial DO saturation rate at the inlet of this 1_5 pond (i.e., 140% on average) that would have modified the O_2 liquid/air equilibrium and therefore could have caused a faster decrease in DO concentration within the pond (which would have caused the O_2 loss by liquid/air transfer higher and give a higher apparent consumption rate).
- In relation to sediment amount at the bottom of this pond that could be higher per square meter than in $1_{13}/1_{15}$ ponds that could have caused a higher sediment DO consumption rate for this 1_5 pond.

2.5. Outcome on Fluxes

The figure below presents the mean $F(t)$ values calculated from Equation (3) for (i) the three "1-pass ponds" monitored (i.e., 1_5, 1_{13} before and after harvest and 1_{15} ponds) and (ii) the whole farm (Figure 6).

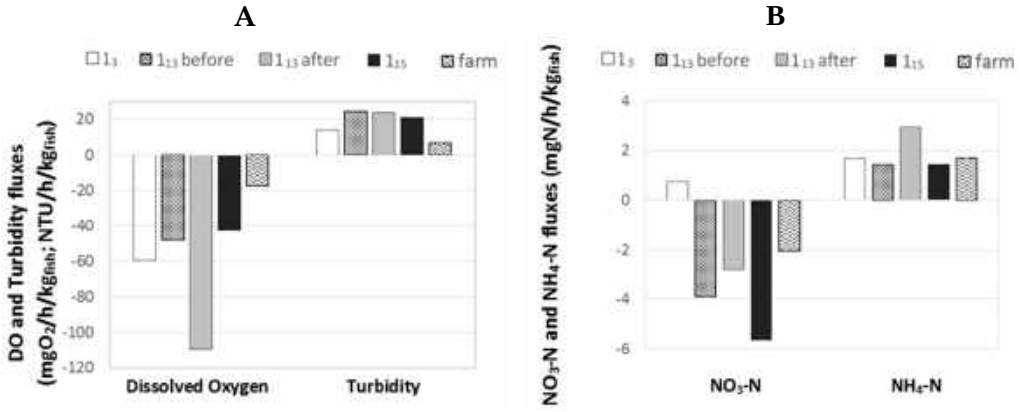

Figure 6. Mean fluxes calculated for DO, turbidity (**A**), NO_3–N and NH_4–N (**B**) at different farm levels (1-pass ponds and whole farm).

The differences in DO fluxes between 1_3 and the other "1-pass ponds" were already discussed in a previous Section (Section 2.4.3) and it was assumed that the higher fluxes observed in 1_3 pond were due to a higher initial DO saturation rate. It is confirmed here that 1_{13} pond before harvest and 1_{15} pond had similar DO fluxes, with F values being −48 and −43 $mgO_2/h/kg_{fish}$ respectively. The lower F value observed for the whole farm (i.e., F = −18 $mgO_2/h/kg_{fish}$) can be explained by the supply of oxygen given by both aeration and oxygenation devices across the farm that would have lowered the apparent loss of O_2 at farm scale. The highest F value observed for 1_{13} pond after harvest (i.e., −109 $mgO_2/h/kg_{fish}$) could be an artefact due the impact of sediments (fish faeces and uneaten feed) on the overall DO consumption. The same observation can be done for NH_4–N flux with a very high F value observed for 1_{13} after harvest compared to other ponds. It is noteworthy that no data were generated in order to quantify the potential impact of other compartments (plants, algae, phytoplankton, soil etc.) on the DO/NH_4–N levels in ponds. However, no diurnal variation was observed in DO consumption after harvest in pond 1_{13} (Figure 5); this observation would confirm that the impact of phytoplankton and plants was negligible compared to the impact of sediments and fish.

For turbidity, F values for 1_{13} before and after harvest and for 1_{15} were similar (i.e., 24.1, 23.6 and 20.8 $NTU/h/kg_{fish}$ respectively). The F value for 1_5 pond was slightly lower (i.e., 13.8 $NTU/h/kg_{fish}$), which could be caused by the higher size of this pond and lower water velocity, which would enhance the settling capacities of this pond, increasing the amount of solids removed from water and thus the F value for turbidity. At farm scale, F value was even lower (i.e., 7.1 $NTU/h/kg_{fish}$); this observation could be due to the settling capacity of the whole farm [16,18].

For NO_3–N, a negative flux was observed for all ponds except 1_3 pond. This pond was the only one to receive some recirculated water which could have created this flux difference and this apparent NO_3–N production by the pond by bringing back some low NO_3–N content water into the loop.

3. Materials and Methods

3.1. Farm Description

The studied fish farm was an earth pond-based rainbow trout farm operating mostly in FT configuration with an additional recirculation loop involving a supplementary oxygenation step. The general layout of the fish farm and the different monitoring locations are presented on the figure below (Figure 7). Fish of different growth stages were distributed into the different ponds according to their weight. Fish were fed manually twice a day until apparent satiation with manufactured pellets (Efico Alfa from Biomar, Brande, Denmark, 41−44% protein, 25−28% lipid, 16−19% carbohydrate, 0.8−2.4% fiber, 4–7% ash, 0.9% total phosphorus, according to the supplier) of different size (pellets diameter) according to the size/weight of fish to be fed in each of the different ponds (see Table 1).

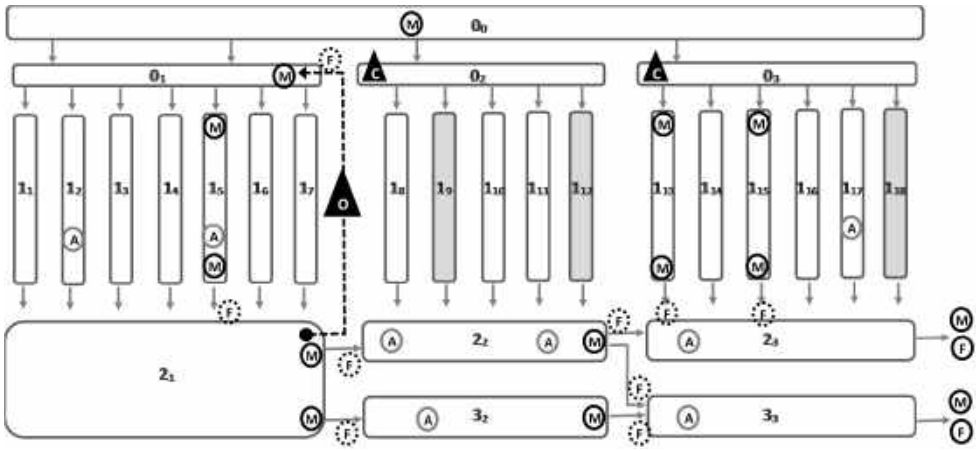

Figure 7. Fish farm general layout (A: paddle-wheel aerator, C: oxygen cone; O: high pressure oxygenator; M: physico-chemical monitoring location; F: water flow monitoring location). Some ponds (i.e., 1_9, 1_{12} and 1_{18}) were not stocked at the moment of monitoring, they are represented in grey. The figures 0, 1, 2 and 3 are theoretical numbers of water uses.

Supplementary oxygenation of the inlet water was achieved using oxygenation cones with supplementary aeration provided within the ponds by mechanical aerators (paddle-wheels) (Figure 7). Additionally, there was a partial recirculation of the water from 2_1 pond to 0_1 pond also involving a supplementary oxygenation step. It is noteworthy that this recirculation was applied without any water treatment system (i.e., neither solid removal nor nitrification step applied), hence this farm would still fall within the classification of "flow-through farm".

According to the farm layout and the flow dynamic we initially defined the expected number of water passes for each pond. For one given pond, the number of water passes was defined as the number of ponds the water flowed through. As an example, all the ponds labelled $1_{1–18}$ were considered as 1-pass ponds.

3.2. Water Quality and Flow Monitoring

Water quality parameters (pH, DO, NO_3–N, NH_4–N, temperature) were analyzed in situ using a multi-sensor probe (YSI, Xylem Inc., Tunbridge Wells, United Kingdom).

Flows were monitored in different farm locations (Figure 7) using ultrasonic flow meters (Nivus PCM4 portable ultrasonic flow measurement devices, Eppingen, Germany). Both 2_3 and 3_3 ponds outlets were monitored continuously and some other locations (1_5, 1_{13}, 1_{15}, 2_1, 2_2 and 3_2 ponds outlets) were monitored at set intervals during the monitoring program in order to understand the whole farm dynamic and to be able calculate/estimate fluxes at (i) farm outlet, and (ii) within the farm. It is noteworthy that the assumption was made that farm inlet flow (not measured) was equal to the farm outlet flow and that no water loss occurred within the farm.

3.3. Experimental Set-Up

The objective was to assess flows and water quality dynamic over the whole fish farm. As all the fish farm locations could not be monitored continuously due to lack of both time and human resources, choices of the farm locations to monitor were made in order to cover most of the farm operation and to fulfil the objectives presented in Section 1. To that aim, the farm locations monitored were the following:

(1) The general inlet channel 0_0 as the fish farm inlet (i.e., before supplementary oxygenation by the cones, see Figure 7).

(2) The inlets and outlets of three "1-pass ponds" (i.e., 1_5, 1_{13} and 1_{15}, see Figure 7) that were chosen both to represent the diversity of the farm (i.e., different average fish weights, total biomass in

1_5 and $1_{13}/1_{15}$ ponds with larger fish in 1_5 as presented in Table 1) and to generate results from similar ponds in order to obtain a duplication of the results and confirm that the protocol set-up was accurate (i.e., 1_{13} and 1_{15} with similar average fish weight and total biomass, see Table 1).

(3) The outlets of all the other ponds (i.e., 2-pass, 3-pass and more) in order to identify the dynamic of the farm and to assess the influence of the number of water passes on water quality.

(4) The recirculated water (from 2_1 to inlet channel 0_1, see Figure 7) in order to assess the impact of the recirculation loop on water quality.

Due to limited personnel resources and time operational constraints, only the ponds outlets' water quality was monitored. For simplification purposes, outlet concentrations were considered as equal to those encountered within the pond themselves.

A period of two consecutive dry-weather days of monitoring was considered in order to avoid any effect on flows that would have been created by rain. It was checked that no rain event was observed upstream during the whole experiment and the week before.

3.4. Data Treatment

Differential concentrations ($\Delta_C(t)$) were calculated as being the difference between outlet and inlet concentrations for a given parameter at a given time. These concentrations were calculated using the following equation (Equation (1)):

$$\Delta_C(t) = C_{outlet}(t) - C_{inlet}(t) \quad (1)$$

where $\Delta_C(t)$, generally expressed in mg/L (except for turbidity and pH), represents the differential concentration of a given parameter at the given time t.

These $\Delta_C(t)$ were calculated at (i) farm scale ($C_{outlet}(t)$ being the mean value calculated for the two farm outlets (i.e., 2_3 and 3_3) for a given time and $C_{inlet}(t)$ the value measured at farm inlet (i.e., 0_0) at the same time for a given parameter) in order to study the impact of the whole farm on water quality, and (ii) at pond scale in order to study the impact of each monitored pond on water quality and to assess the influence of water passes on water quality. The $\Delta_C(t)$ were calculated for all monitored times and also averaged for the duration of the monitoring. Hence, a positive value for $\Delta_C(t)$ would mean an increase in the concentration of a given parameter from inlet to outlet, and therefore a "production" by the farm (or by the pond considered); similarly, a negative $\Delta_C(t)$ value would mean a decrease in the concentration of a given parameter from inlet to outlet and therefore a "consumption" by the farm (or by the pond considered).

From the obtained $\Delta_C(t)$ values, flow values were used to calculate fluxes ($f(t)$, generally expressed in mg/h, except for pH and turbidity) both at farm and pond scales for all the monitored parameters. The $f(t)$ were calculated as follow (Equation (2)):

$$f(t) = \Delta_C(t) \times Q_{outlet}. \quad (2)$$

where $f(t)$ (mg/h) is the flux of a given parameter from the whole farm or from a given pond and Q_{outlet} (L/h) the flow at the outlet of the farm or of a given pond.

Those fluxes were converted using the total biomass in presence in each pond (see Table 1) to obtain fluxes in mg/h/kg_{fish}. according to the following equation (Equation (3)):

$$F(T) = \frac{f(t)}{m_{T,pond}} \quad (3)$$

With $m_{T,pond}$ (kg_{fish}), the total biomass in the pond considered.

All $F(t)$ values were averaged for the farm and for the monitored ponds for all the monitored parameters to obtain comparable mean flux values between the different ponds monitored.

4. Conclusions

A traditional flow-through rainbow trout farm was monitored extensively for the first time in Ireland. It is recalled that traditional flow-through systems still represent 90% of the trout production at the country scale today, and even if practices are evolving through recirculated aquaculture systems (RAS), this type of practice remains very common at the EU scale (France, Poland, Czech Republic, etc.). Therefore, the results and observations from this study are very relevant to the industry.

A combination of flow and water quality monitoring made it possible to understand the dynamics of water and its nutrient and dissolved oxygen content. To allow for a better understanding and analysis of the results, the concept of "multi water passes" was defined as the number of ponds the water flows through before release at the farm outlet. The flow measurements performed illustrated the difficulty in assigning a number of water passes to each pond, and the results obtained were not in agreement with the expected number of water passes (negligible or even negative flows observed at some farm locations). Thus, this study gives the impact and limits of multi water reuse on water quality and of the implementation of supplementary aeration/oxygenation, still practiced broadly in Ireland and in Europe. Furthermore, this study made it possible to attain proper and independent monitoring of the effluent water quality of the farm effluent and of the impact it might have on the receiving water quality.

The impact of water passes on water quality was identified, with a notable increase in NH_4–N concentrations when the number of water passes increases. However, the loss in DO was globally compensated by the oxygenation/aeration technologies (oxygenation cones, recirculation loop and paddlewheel aerators) employed onsite. The role of sediments in DO consumption and NH_4–N production was clearly identified as being substantial, and sediments could be responsible for a significant DO consumption, which has to be compensated by the operation of some oxygenation/aeration devices with their associated costs (energy, investment). It is, therefore, recommended for fish farmers still employing traditional flow-through systems to "de-sludge" their ponds more frequently in order to limit DO loss and NH_4–N increase in the rearing water.

The industry has to produce more to be economically viable in the future [20]. However, it was shown during the present study that ammonium levels increased with the number of water passes, and that it could reach concentrations up to almost 1 mgN/L in ponds. This shows that increasing production in the same farm configuration would increase nutrients concentration even more, which could hamper production due to an expected impact on fish health from this concentration [21]. Thus, with regards to fish welfare, the outcomes of this study make it possible to identify areas and practices within the FT systems that show water quality levels close to threshold levels for fish welfare. From this, appropriate actions and practices can be implemented. Therefore, if this fish farm were to produce more, some water treatment processes (solid/liquid separation, nitrification) would have to be set up in order to both limit the build-up of nutrients in the rearing water and to reduce the nutrient release in the effluent to fulfil the WFD objectives. It also identifies performances of the industry with respect to the WFD, criteria thereby facilitating gap analysis for the implementation of water treatment technologies. Finally, this study also acts as a benchmark for the industry to define what improvements are required and develop a roadmap for the sustainable development of the freshwater aquaculture sector in Ireland and beyond (specifically for trout production).

Supplementary Materials: The following are available online at http://www.mdpi.com/2410-3888/3/3/28/s1, raw data excel files.

Author Contributions: Conceptualization, A.T. and R.D.F.; Data curation, A.T. and A.K.; Formal analysis, A.T., R.D.F. and E.C.; Funding acquisition, A.K. and R.D.F.; Investigation, E.C. and N.R.; Methodology, A.T. and A.K.; Project administration, A.K. and E.C.; Supervision, E.C. and N.R.; Validation, A.K., E.C. and N.R.; Visualization, R.D.F.; Writing—original draft, A.T.; Writing—review & editing, A.T.

Funding: The authors express gratitude to Ireland's Department of Agriculture, Food and the Marine for supporting this study through the MOREFISH project and Bord Iascaigh Mhara for supporting the EcoAqua project.

Acknowledgments: The authors would like to acknowledge Ronan Cooney (MSc. NUIG) and Conor Behan (MSc. NUIG) for their active participation to the monitoring campaigns.

Conflicts of Interest: The authors declare no conflict of interest.

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Case Report

Development of a novel recirculatory multitrophic peatland system for the production of high-value bio-based products at scale embracing zero waste and pollution principles to unlock sustainable development goals

Neil J. Rowan [a,b,*], Antoine Fort [a,b], Emer A. O'Neill [a,b], Eoghan Clifford [c], Marcel Jansen [d], Markus Helfert [e], Damien Toner [f], Julie Maguire [g], Brijesh Tiwari [h]

[a] Technological University of the Shannon, Midlands Campus, University Road, Athlone, Co Westmeath, N37 HD68, Ireland
[b] Empower Eco Sustainability Hub, Technological University of the Shannon, East Campus, University Road, Athlone, Co Westmeath, N37 F6D7, Ireland
[c] Civil Engineering, School of Engineering, University of Galway, University Road, Galway, H91 TK33, Ireland
[d] School of Biological, Earth and Environmental Sciences & Environmental Research Institute, University College Cork, Distillery Fields, North Mall, Cork, T23 N73K, Ireland
[e] School of Business Maynooth, Innovation Value Institute, Maynooth University, Maynooth, Co Kildare, W23 F2H6, Ireland
[f] Bord Iascaigh Mhara, Crofton Road, Dún Laoghaire, Co Dublin, A96 E5A0, Ireland
[g] Indigo Rock Marine Research Centre, Gearhies, Bantry, Co Cork, P75 AX07, Ireland
[h] Teagasc Food Research Centre, Moorepark, Moorepark West, Fermoy, Co Cork, P61 C996, Ireland

ARTICLE INFO

Keywords:
Bioeconomy
Peatland
Commercial demonstrator
Aquaculture
Change of land use
Sustainability

ABSTRACT

This case study describes the novel development and demonstration of commercial, green, bio-based products using a peatland based recirculating integrated multi-trophic aquaculture (IMTA) system in the Irish Midlands. This site enables the transition from traditional peat harvesting for energy generation towards alternative sustainable employment. The system effectively addresses sustainable in-land freshwater aquaculture development. It also demonstrates value-chain products at scale for new feeds arising from the fish waste-stream by exploiting cascades from the fish culture waste-stream (bio-fertiliser) for cultivating duckweed and macroalgae. These plants can then be bio-refined and valorised to produce new products. The system also provides a circular demonstrator site that will facilitate industry and entrepreneurs to develop and test new innovations and ideas. By providing this open-access site to support companies in testing, financial constraints such as access to specialist equipment and technical expertise will be off-set thus enabling entrepreneurs and industry to develop new commercial products at scale. Additionally, the outputs from this system will help address and inform several United Nations sustainable development goals.

1. Introduction

The steady growth in global population coupled with the commensurate demand for edible protein has contributed to an agricultural intensification that presents challenges for the sustainability of livestock farming [1]. Specifically, there is a need to reduce the production and release of agriculture waste products to protect our fragile environment [2]. Conversely, new opportunities are arising to valorise agricultural waste, for example; through the co-production of different bio-based products from raw materials (biomass) that can be used for the production of food, feed and biofuels [3]. Such a circular bioeconomy is aligned with a renewed focus on rural resilience and regional development across Europe [1] that seeks to promote zero-carbon changes in land use, including rewetting peatland [4]. The bioeconomy offers new sustainable and climate-resilient pathways for economic development aiding a fair and just transition (JT) from a fossil fuel driven economy [5]. However, there remains pressing technical and financial challenges that includes scalability and delivering clear messaging where solutions can be met through pilot commercial demonstration activities. Consequently, this case study describes the use of a peatland-based, recirculating integrated multi-trophic aquaculture (IMTA) system in the Irish JT Territory to overcome some of these hurdles through organic fish-production and culturing duckweed as a protein crop. This does not just include technical advances, but also

* Corresponding author. Technological University of the Shannon, Midlands Campus, University Road, Athlone, Co Westmeath, N37 HD68, Ireland.
E-mail address: neil.rowan@tus.ie (N.J. Rowan).

https://doi.org/10.1016/j.cscee.2024.100763
Received 17 March 2024; Received in revised form 13 May 2024; Accepted 16 May 2024
Available online 16 May 2024

embraces, for example, multi-actor stakeholder engagement using a quadruple helix approach [6,7] that integrates industry, policy-makers, society and academics.

2. IMTA process at Mount Lucas wind farm in Irish peatlands

The IMTA site at Mount Lucas in the Irish midlands is a former commercial peat-harvesting site that has operated several years as a successful organic fish farm using the principle of integrated multi-tropic aquaculture i.e., where two or more organisms are cultured or farmed together [8]. Building on that core principle, this case study describes the development and future trajectory of the site. Ziegler et al. [9] reported that key challenges affecting the development of peatland-based innovation include the lack of shared systems of data generation for meta-analysis, corporate strategy, risk-mitigation and business disruption. In Ireland, there is nearly 100,000 ha. degraded peatland used for commercial peat cutting thus presenting scope to develop and demonstrate an IMTA approach to deliver solutions at scale for the bioeconomy. The IMTA farm is located in the Irish Midlands on a wind farm (53°17′3″ N – 7°11′45″ W) and is a cut-away rewetted glacial till site, from which peat was previously extracted [9,10]. Four split (pill) aquaculture ponds were dug-out on the infertile cut-away substrate and used for culturing of rainbow trout (*Oncorhynchus mykiss*) and European perch (*Perca fluviatilis*) [10]. The system is circulatory and fishponds are connected by means of channels to an algae and duckweed lagoon that serves as a natural fish effluent waste treatment system (see Fig. 1). Energy is locally generated by wind turbines. Water is rarely taken into (only done so to compensate for evaporation) or released from (only during times of excessive rainfall, the system to a nearby bog river.

3. Use of freshwater macroalgae for nutrient recycling and biomass production

Early studies have revealed the presence of a large variety of algae in the IMTA system at Mount Lucas [11]. As part of the IMTA process, the fish cultivation waste stream will be used to cultivate macroalgae in land-based tanks. Freshwater macroalgae, unlike their marine counterparts, are a relatively overlooked group of plants that, to date, have not been fully harnessed at scale for their nutrient removal potential or their biomass composition in aquaculture systems. Several species have been shown to be indicators for eutrophication through their sustained growth in nutrient-rich freshwater bodies [12]. Such characteristics will be leveraged in Mount Lucas to efficiently recycle the fish effluent (which mostly contains ammonium and phosphates) into useable biomass. Macroalgae have distinct advantages over freshwater microalgae, namely their containment within the site, ease of harvest, and higher resistance to bacterial contamination in the environment [13].

Determining the diversity and performance of local freshwater macroalgae species under high nutrient load and/or a seasonal pattern has yet to be characterized. Screening for growth and nutrient removal potential of Irish freshwater macroalgal species, particularly Chlorophyta such as *Cladophora*, *Oedogonium*, *Monostroma*, *Pithophora* and freshwater *Ulva* genera will advance system performance [8]. Some species of these genera have been shown to grow in freshwater at very low to no salinity conditions and sustain high growth in high-nutrient environments [14,15]. Growth and nutrient uptake kinetics will be interpreted in the context of continuous monitoring of nutrient levels using sensors. An on-site nursery informing production of fresh macroalgae biomass in land-based tanks will be deployed with focus on determining seasonality of species to ensure an all year-round process. Harvested macroalgae will be bio-refined for bioactives such as proteins, carbohydrates, lipids and antioxidants. For example; it has already been shown that *Oedogonium* represents a high-quality source of proteins for animal feed [16], while *Cladophora* biomass has been shown to possess a wide range of possible applications from feed, nutraceuticals and composites to bio-stimulants [17].

Fig. 1. Aerial view of the full IMTA system. Culture ponds (blue squares), reservoir (orange square), treatment lagoon (green square) and neighbouring wetland (yellow triangle) are visible. Airlift (white squares) and paddlewheel (orange circles) locations have been indicated. Peatland/bog river (blue dashed lines) has been included along with the intake point (grey circle) and overflow point (grey rectangle). Red arrows indicate water flow in and around the system. Wind turbine (WT) 19 which provides all electrical needs for the farm can be seen in the bottom left-hand corner. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Duckweed for sustainable valorisation of aquaculture wastewater

Duckweed (*Lemnaceae*) species have gained considerable attention as a sustainable source of high-quality nutrition, biofuels, and pharmaceuticals, as well as effective organisms for the phytoremediation of wastewaters [18]. A protein content of up to 45 % makes duckweed biomass nutritionally interesting as an ingredient for animal feeds, or for human use [18]. Previous work conducted on the pilot IMTA site at Mount Lucas has demonstrated the potential to generate high biomass yields from duckweed, with yields in excess of 30 tonnes [19] containing some ten tonnes of protein. Such yields are substantial in comparison with those of traditional crops such as soybean that rarely exceed five tonnes per year. The duckweed protein content was found to range between 20 % and 25 % [19], but there is scope to increase the protein content to about 40 % [20], through an increase in nitrogen in the water column. Thus, integrated management involving fish-husbandry, nutrient monitoring and duckweed manipulation can increase the economic case for the cultivation of duckweed as a novel protein crop. The strength of the IMTA is that it comprises of an integrated system in which fish, duckweed and algae species engage in mutually beneficial, as well as antagonistic interactions which need to be aligned with stakeholder interests (i.e., protein extraction technology) in accordance with priorities for a JT.

Moreover, outdoor duckweed cultivation has gained in popularity in recent decades, but it can be challenging to optimise, to control operationally and to integrate into a more comprehensive system such as an IMTA. Substantial challenges remain with respect to scaling up small-scale systems (up to several m^2) to semi-commercial, large-scale systems. This includes understanding the relevant importance of the diffusion and mixing processes in facilitating plant nutrient supply to the thin surface layer of the floating duckweed plant. Optimising recirculating flow through technology, as described in this IMTA peatland model, coupled with sensor supports systems with artificial intelligence (AI) and machine-learning growth models, and monitoring of growth using an UAV, can maximise biomass yields. The duckweed performance data will be used to infer performance modelling of the farm and identify positive and negative drivers of duckweed growth *in-situ* (e.g., nutrient levels, flow rates, weather conditions), but also to calculate the capacity of the duckweed system to manage water nutrient levels that are safe for diverse fish species. Furthermore, such monitoring will facilitate accurate timing of autonomous harvesting, optimised to maximise yield. To avoid harvesting becoming a bottleneck to commercial exploitation, a SWOT analysis of key activities, including biomass cultivation, harvesting, biomass pre-treatment and protein extraction, will be undertaken.

5. Conversion systems, bio-refinery and valorisation

Bio-refinery concepts comprise of an integration of processes to make high-value-added products such as food or feed ingredients (e.g., proteins, fibres), biomaterials, nutraceuticals (e.g., bioactives), and lower-value-market products (e.g., biogas). The Mount Lucas IMTA system addresses production of macroalgal and duckweed with a biorefinery approach that will yield marketable products without producing unused waste or side streams (Fig. 2). The key challenges associated with the development of sustainable zero-waste value chains are; (1) post-harvest interventions for biomass; (2) on-site preservation; (3) pre-treatment technologies; and (4) robust, energy-efficient conversion systems (Fig. 3). For industry, two aspects of biomass processing methods can be considered. One is on-site preservation using ensiling. The more preferred but energy-intensive method currently employed is hot air drying. Currently, most large-scale biomass drying is carried out using conventional hot air dying (10–15 h. at 50°C) without pre-treatments. Macroalgal and duckweed biomass will need to be intensively dried post-harvest in order to reduce their moisture content from 70-80 % to

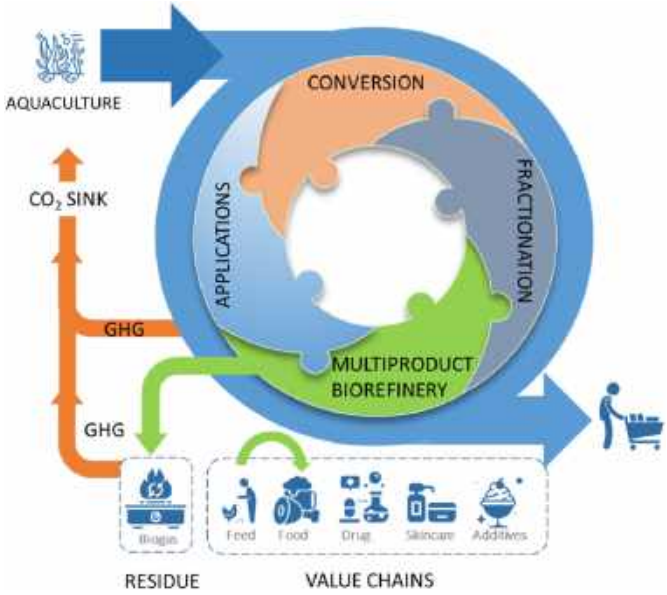

Fig. 2. Zero waste approach for Biorefineries'.

8–10 % ahead of further fractionation. From a cost perspective, it is preferable to avoid processing such as intensive drying, which is expensive to run, especially when drying large volumes of biomass that has a very high moisture content (>70 %), such as macroalgal and duckweed biomass. However, the application of alternative novel drying techniques, such as microwave-assisted and ultrasound-assisted drying, can improve drying efficiencies and product quality. Alternatively, establishing bio-refineries from fresh biomass will limit the degradation of valuable unstable bioactive compounds such as polyphenols; however, this puts a strain on the producer, as transporting fresh biomass is logistically challenging, expensive and inefficient. In an ideal situation, the bio-refinery processing plant would be located close to the biomass production site, as is the vision for the IMTA system.

One of the key bottlenecks of bio-refinery has been the separation of some fractions without wasting other fractions through simple, cost-effective and scalable processes with low-energy requirements [21]. Hence, key steps in establishing a zero-waste bio-refinery for the Mount Lucas site can be adapted from IEA Bioenergy (https://www.ieabioenergyreview.org/) (Table 1). Novel extraction and processing technologies play an important role in establishing energy-efficient bio-refineries by reducing dependencies on solvents and improving extraction efficiencies while preserving desired functional properties. The application of a range of new technologies for valorisation of biomass has been reviewed extensively [22]. Table 2 outlines the key features of novel technologies for the extraction of compounds of interest. One of the best strategies for improving extraction efficiencies is to develop a combination of sequential extraction technologies to maximise the synergies [23]. Such sequential extraction of ingredients (proteins, fibres and bioactives) has been established for macroalgae [24]. The zero-waste potential of macroalgal bio-refinery has been realised in the production of several products for food and pharmaceutical applications [25], and the potential to integrate advances in bio-refinery technologies with a commercially viable business has been highlighted [26].

6. From innovation to sustainable business ideas

The pilot IMTA demonstrator site will facilitate industry and entrepreneurs to develop and test innovations and ideas matching business canvas model. Environmental sustainability (including risks, threats and bottlenecks) of new products and value chains, demonstrated on-site, will be determined using life cycle assessment (LCA). Appropriate LCA

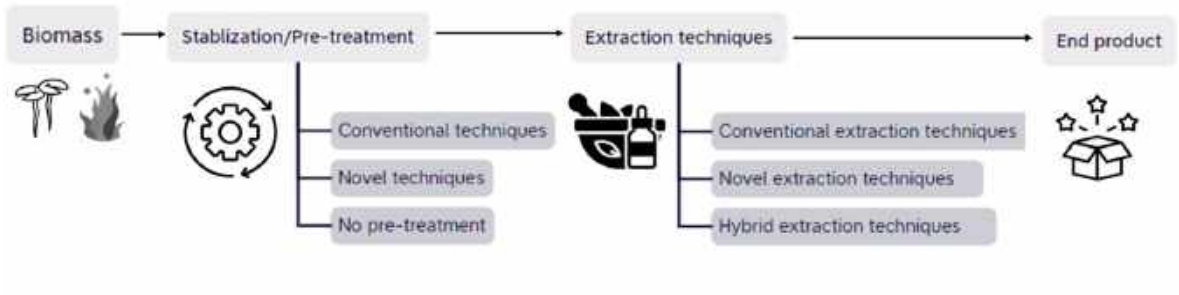

Fig. 3. Valorisation of biomass.

Table 1
Key steps for developing energy efficient zero waste biorefineries.

1	Determining chemical composition and physicochemical properties of target biomass (e.g., macroalgae, duckweed)
2	Define the target bio-products and physicochemical properties required for potential applications (e.g., agri-food, nutraceuticals, energy)
3	Develop an inventory of technologies or any interventions required to produce targeted bio-products (*e.g.*, pre-treatments, processing technologies, solvents)
4	Inventory of input materials (*e.g.*, solvents, energy, biomass)
5	Data collection for each unit operation (*e.g.*, conversion, fractionation, processing
6	Determine the chemical compounds to be transformed via pre-treatment/processes (e.g., side streams generated)
7	Carry out cost-benefit analysis (LCA/LCCA) to meet targets (*e.g.*, economic, environmental and social indicators)

Table 2
Comparison of selected extraction techniques.

	Hydrodynamic Cavitation	Ultrasound cavitation	Microwave	Pulsed electric field	High pressure processing	Enzymatic processes	Chemical processes
Scalability	Medium	Low	Medium	Medium	High	Low	Medium
Operating cost	Medium	Medium	Medium	Medium	High	High	Low
Efficiency	High	Medium	Medium	Medium	High	Medium	Medium
Energy requirement	Low	Low	Medium	Medium	High	Low	Low
Residue					Particulates	Enzymatic residue	Chemical residue
Harsh condition			High temperature		High pressure		
Selectivity	Low	Low	Low	High	Low	High	High

and life-cycle costing analysis (LLCA) will be conducted for value chains across the IMTA, supporting optimal engagement with stakeholders/beneficiaries with a commercial market orientation [27]. Ecological and ecotoxicological assessments that also embrace the impact of weather variance and climate change will be conducted in accordance with the methods of O'Neill et al. [28]. A go-to-market strategy will assist the transition from pilot ideas to market-ready innovations using appropriate business analysis and modelling tools (e.g., SWOT, PESTELE, PM-CANVAS). Innovation studies thus far at this IMTA site (such as aquaculture and protein harvesting from duckweed) [8,18,28,29] have implemented proven methodologies such as the 'innovation sweet-spot' i.e., the optimal intersection at which feasibility (what is technically possible), viability (what can be sustainable for a business) and desirability (what users or customers need and want) converge, leading to successful and impactful innovations [30].

7. Summary

The development of a fully monitored and characterised IMTA site in the peatland at Mount Lucas presents a timely opportunity to demonstrate high-value bio-based products at scale. This peatland recirculatory site will be informed by an integrated multi-actor stakeholder approach that will help identify and overcome technical and economic challenges for viable new products and services that embrace appropriate change of land use. The value stream generated from the fish culture waste stream presents many exciting business opportunities that will be balanced by conducting an appropriate environmental LCA. Fish production (freshwater aquaculture) in the rewetted peatland is an important activity for food security. However, an on-site hatchery and mesocosm are needed to increase nutrients in the waste stream so that sufficient amounts of biomass are produced for duckweed and macroalgae bio-refinery activities, leading to high-value products and services. This IMTA model provides an open-access site for supporting companies in the testing and development of new green products. Additionally, financial constraints in terms of access to specialist equipment and technical expertise are off-set. This IMTA site will operate at the critical interface between top-down government policies (and strategies) for informing effective bioeconomy activities to meet end-user/beneficiary needs and addressing a fair and JT for communities pivoting to low-carbon economies. Current and future outputs from this IMTA site will help address and inform several Unite Nations Sustainable Development Goals (UNSDGs) including; No Poverty (SDG 1), Zero Hunger (SDG 2), Good Health and Wellbeing (SDG 3), Responsible Consumption and Production (SDG 12), and Life Below Water (SDG 14).

CRediT authorship contribution statement

Neil J. Rowan: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Conceptualization. **Antoine Fort:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Emer A. O'Neill:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Eoghan Clifford:** Writing – review & editing, Writing – original draft, Methodology. **Marcel Jansen:** Writing – review & editing, Writing – original

draft, Methodology, Funding acquisition, Conceptualization. **Markus Helfert:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition. **Damien Toner:** Writing – review & editing, Writing – original draft, Methodology. **Julie Maguire:** Writing – review & editing, Writing – original draft, Methodology. **Brijesh Tiwari:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge funding support from the Department of Agriculture, Food and the Marine (2022 DAFM PSS125 'BioDMJT' project).

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Science of the Total Environment 802 (2022) 149800

Contents lists available at ScienceDirect

Science of the Total Environment

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Microalgae as a natural ecological bioindicator for the simple real-time monitoring of aquaculture wastewater quality including provision for assessing impact of extremes in climate variance – A comparative case study from the Republic of Ireland

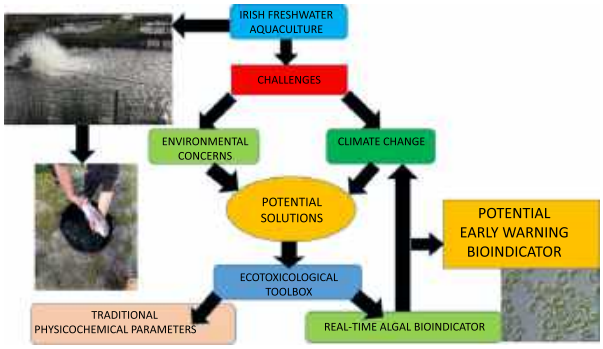
Emer A. O'Neill [a,b,*], Neil J. Rowan [a,b]

[a] Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland
[b] Empower Eco Sustainability Hub, Boora, Co. Offaly, Ireland

HIGHLIGHTS

- Traditional monitoring of aquaculture outputs using physicochemical parameters has limited efficacy.
- Complementary use of algae supports environmental monitoring of aquaculture.
- Duckweed supports and improves efficacy of aquaculture wastewater treatment.
- Algae are a potentially rapid and sensitive bioindicator of aquaculture water quality.
- Algae are a potential early warning tool for assessing impacts of climate change in aquaculture.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 July 2021
Received in revised form 16 August 2021
Accepted 17 August 2021
Available online xxxx

Editor: Damia Barcelo

Keywords:
Algae
Bioindicator
Ecotoxicology
Climate change
Freshwater aquaculture
Sustainability

ABSTRACT

Aquaculture is one of the fastest growing food producing industries globally, providing ~50% of fish for human consumption. However, the rapid growth of aquaculture presents a range of challenges including balancing environmental impact that can be influenced by variations in climatic conditions. Monitoring of physicochemical parameters is traditionally used to evaluate aquaculture output quality; however, this approach does not indicate the cumulative ecotoxicological effects on receiving waters. Specifically, this case study investigated the relationship between measuring traditional physicochemical parameters and the health of the alga *Pseudokirchneriella subcapitata* in order to evaluate the potential ecotoxicological effects of freshwater aquaculture on the receiving aquatic ecosystem in the Irish midlands. This constituted the first 2-year longitudinal study conducted in 2018 and 2019 that reports on the efficacy of using algae as a natural bioindicator to monitor and assess freshwater aquaculture wastewater from a traditional flow-through fish farm producing Eurasian Perch (*Perca fluviatilis*); monitoring was compared over a same six-month period in the same location each year. Findings demonstrated significant differences between the two monitoring periods when using *P. subcapitata* for assessing the quality of aquaculture intake ($P = 0.030$) and output ($P = 0.039$). No stimulatory effects were observed during 2019 unlike >50% rates experienced the previous year. These observations coincided with changes in climatic conditions whereby the 2018 period experienced extended levels of drought; whereas non-drought conditions were observed during 2019. Findings suggest that reliance upon traditional monitoring techniques may not provide

* Corresponding author at: Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland.
E-mail address: e.oneill@research.ait.ie (E.A. O'Neill).

https://doi.org/10.1016/j.scitotenv.2021.149800

sufficient robustness or versatility to address emerging issues, such as extremes in climate variance, which may influence the future intensive sustainability of freshwater aquaculture. This research supports the complementary use of *P. subcapitata* as a rapid and simple early-warning bioindicator for measuring aquaculture output quality on receiving aquatic ecosystems.

1. Introduction

The depletion of wild capture fishery practices has resulted in the rapid development of aquaculture (Han et al., 2019) making it the fastest growing food producing industry worldwide (Ottinger et al., 2016; O'Neill et al., 2019, 2020). According to the FAO (2018), aquaculture now accounts for ~50% of fish produced for human consumption; this figure is expected to rise to ~62% by 2030 (Fredricks et al., 2015; Liu et al., 2017). The dramatic increase in aquaculture production is attributed to over exploitation of wild fisheries that are now at their maximum sustainable yields, along with increased consumer demand for fish (Tahar et al., 2018a, 2018b). Farmed fish is rich in protein and is also a more efficient protein utilisation and feed conversion source than other animals destined for protein production (Tschirner and Kloas, 2017). However, despite its numerous advantages, the rapid increase in aquaculture production has resulted in the emergence of several issues within the industry that include limitations in water and space, increased incidences of disease and increased environmental concerns (Ngo et al., 2016; Troell et al., 2017; Han et al., 2019; O'Neill et al., 2019). Stenevik and Sundby (2007) have also indicated that variations in climatic conditions have demonstrated substantial effects on increases as well as decreases in stocking densities; therefore, the success of fish stock assessment depends to a large extent on the ability to predict impacts climate change has on the dynamics of aquatic ecosystems. These treats have hindered the sustainable development and expansion of the industry (Han et al., 2019).

The ecological importance of algae have received consideration in studies focusing on natural approaches to wastewater remediation in freshwater aquaculture (Naughton et al., 2020) including potential influence of climate variance on process performance (O'Neill et al., 2019). Previous researchers have also noted the potential of algal communities to exhibit many attributes as biological indicators of spatial and temporal environmental change (Omar, 2010); additionally, microalgae have been reported as potentially useful monitoring quality of water bodies (Zaghloul et al., 2020; Parus and Karbowska, 2020; Kadam et al., 2020; Tsarenko et al., 2021). Parus and Karbowska (2020) recently reported on the possibility of using the algae *Ulva* and *Cystoseira* as natural indicators of environmental cleanliness given that these species where shown to accumulate metals. Parmar and Rawtani (2016) described several potential advantages for use of bioindicators, namely (1) biological impacts can be determined; (2) potential synergetic and antagonistic impacts of various combined pollutants on ecosystems can be exhibited; (3) early stage diagnosis of putative harmful effects of toxins on human and animal health can be monitored; and (4) can be considered as a potentially viable economic alternative to use of conventional sophisticated methods.

According to Rindi (2014), terrestrial algae (green algae and diatoms) are more directly affected by climate change and can therefore respond in a more immediate way. This is attributed in part to the fact that algae have short generations, fast turnovers and respond quickly to changes in environmental conditions. Sarmaja-Korjonen et al. (2006) demonstrated that algae appeared to be comparatively good indicators of environmental conditions by representing productivity disparities during changing climatic conditions. Hallegraeff (2010) has also indicated that changes in algal communities can putatively provide a sensitive early warning for climate-driven uncertainties in aquatic ecosystems. There has been increased interest in alternative uses for microalgae within aquaculture to assist with sustainability, in addition to enabling ecotoxicological assessment and water quality control (Han et al., 2019; O'Neill et al., 2019). According to Han et al. (2019), microalgae can also be utilised in aquaculture for wastewater assimilation, oxygen production and partial feed replacement. The microalgae *Pseudokirchneriella subcapitata* (*P. subcapitata*) has previously been suggested as a potential early warning indicator for altering issues associated with in aquaculture processing due to environmental variances, including climate change (O'Neill et al., 2019).

Fish farm wastewater is traditionally high in nutrient rich products (Ngo et al., 2016; Sikder et al., 2016). Nitrogen, phosphorus and organic matter are characteristic of this nutrient rich waste which is normally as a result of metabolic waste products and left over food (Jegatheesan et al., 2011; O'Neill et al., 2019). If this is released into a water body untreated, water pollution will develop leading to issues that may include eutrophication in that aquatic system (Martinez-Porchas et al., 2014). Eutrophication occurs when a water body is put under pressure with large levels of organic matter and nutrient waste that is taken in and biologically processed which in turn leads to algal blooms (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Sikder et al., 2016). Algal blooms in turn can lead to decreases in light and oxygen production, which can suffocate aquatic life (Jegatheesan et al., 2011; Chislock et al., 2013; O'Neill et al., 2019). Organic matter and nutrient waste is typically as a result of the application of artificial feed supplementation which is necessary in order to increase and maintain yields to meet the increased demands (Kolarevic et al., 2014; Feucht and Zander, 2015; O'Neill et al., 2019).

Water quality is typically assessed to determine the potential effects it may have in its receiving system; this is traditionally conducted by means of physicochemical analysis (da Silva et al., 2017). The use of these parameters alone will only provide a limited window in time of the water quality for a system (O'Neill et al., 2019; O'Neill et al., 2020). Inclusion of bioassays to assess the potential effects on aquatic ecosystems and the organisms therein will provide a broader scope on the quality of water. Microalgae are primary producers and are keystones in aquatic food chains. They represent an imperative group of highly sensitive photosynthetic organisms frequently used to assess aquatic systems (Rodgher et al., 2012). *Pseudokirchneriella subcapitata* (*P. subcapitata*) is unicellular green algae most commonly used and recommended for ecotoxicological assessment due to its being inexpensive, and both highly reliable and reproducible (ISO, 2012).

The hypothesis of this study is that algal traditionally used in ecotoxicology bioassays can be further utilised for the real-time sustainable enhancement of aquaculture as it provides a potential means of monitoring the influence of adverse environmental effects caused by extreme weather events attributed to variances in climate. Thus, the aim of this research is to determine the robustness of *P. subcapitata* as a putative early warning bio-indicator for monitoring impact of climate variance using an Irish freshwater aquaculture farm as a case study.

2. Materials & methods

2.1. Sampling

Intake and output water samples were collected from a freshwater fish farm located in Boyle, Co. Sligo (Fig. 1). The farm cultures European perch (*Perca fluviatilis*) and consists of three culture ponds that use a flow through system, a hatchery and nursery that use a recirculating aquaculture system (RAS) and a constructed wetland that is used for culture water treatment. Grab samples were collected

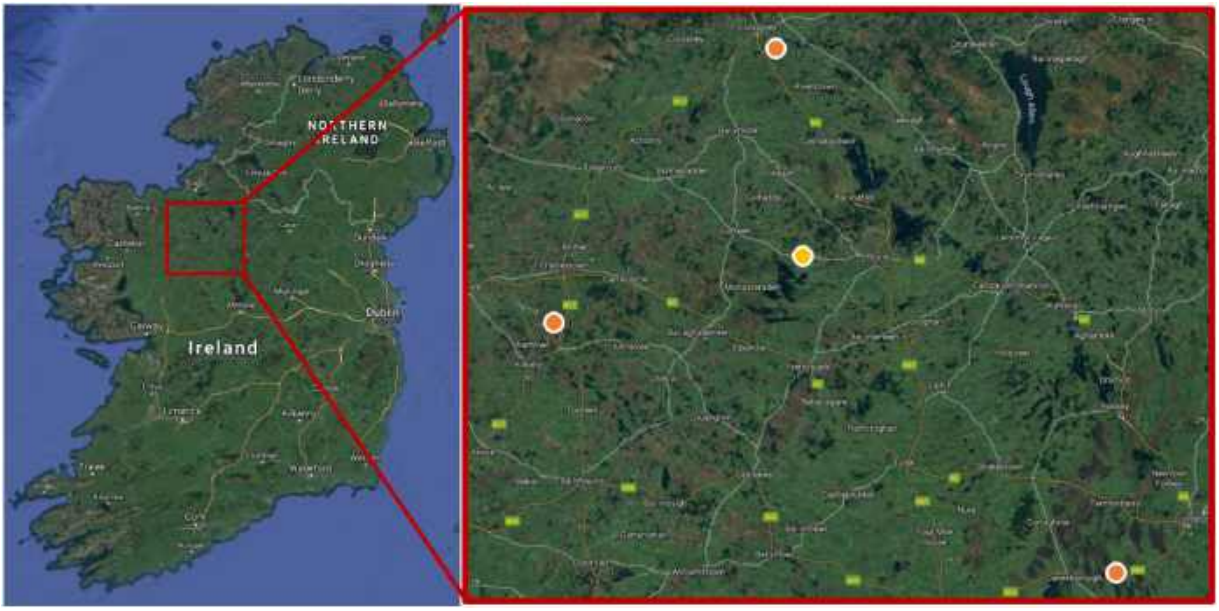


Fig. 1. Map of Ireland indication the approximate location of the freshwater fish farm (53°58′16″ N, 08°24′44″ W) indicated with yellow, and the three closest Met Eireann weather stations (Markree – 54°10′30″ N, 08°27′20″ W; Mount Dillon – 53°43′37″ N, 07°58′51″ W; Knock – 53°54′22″ N, 08°49′4″ W) surrounding the farm, indicated by orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in 5 L octagonal carboy HDPE bottles (Lennox) and transported directly to the lab via car approximately 70 km away. Samples were collected directly from the intake and output sources once a month from March 2019 to August 2019 as this was the time period analysed during the previous study at same freshwater aquaculture farm reported in O'Neill et al. (2019). Samples from the settlement pond were also collected for analysis in order to determine the treatment efficacy of the constructed wetland, which was not fully operational until June 2019 reflecting period of maintenance. Wastewater collection occurred on the same day, at approximately the same time, during each month of monitoring where sampling points are displayed in Fig. 2. Triplicate samples were analysed from the same 5 L grab sample.

2.2. Physicochemical analysis

The Statutory Instrument (S.I.) 77/2019, S.I. 272/2009, and the Irish Environmental Protection Agency's (EPA) water quality parameters (Environmental Protection Agency, 2001; Irish Statutory Office, 2009; Irish Statutory Office, 2019) were followed to measure water quality parameters. Discharge licensing in Ireland is currently based on an individual basis. Grab samples collected represented 30 min of the 24 h period; composite sampling was not possible. To compensate for the latter, results complied in this study were also compared to previous research studies conducted on a range of aquaculture facilities (Table 7 in the Supplementary data).

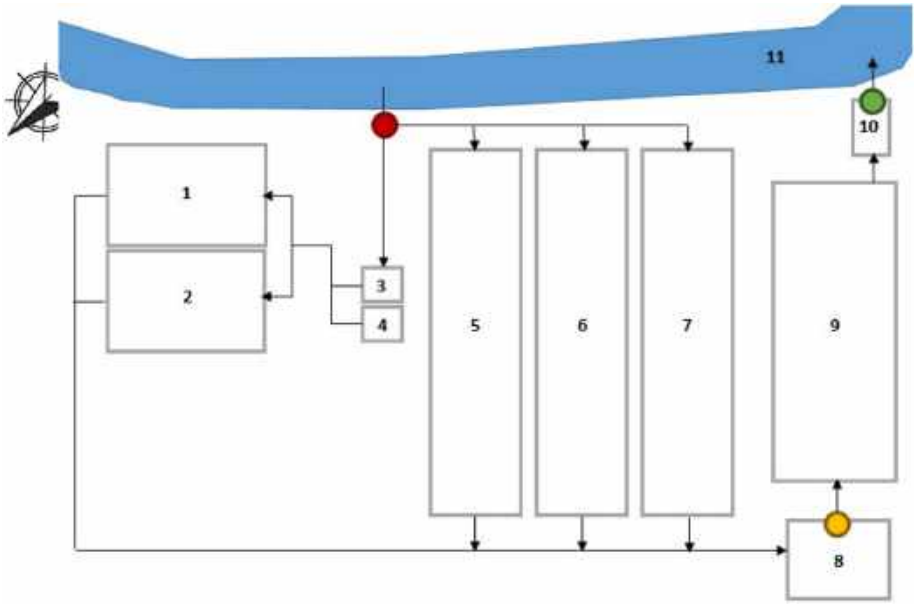


Fig. 2. Schematic of the Irish freshwater fish farm layout indicating the locations of the collection points for the intake (red), output (green) and settlement pond (yellow) water samples. 1) hatchery, 2) nursery, 3-4) mesocosms, 5-7) culture ponds, 8) settlement pond, 9) constructed wetland, 10) holding tank, 11) river. Black arrows indicate flow of water. Note: Schematic is not to scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of the methods used to assess the physicochemical parameters investigated on the Irish freshwater aquaculture intake, output and settlement pond water samples. The method employed, detection limit for all kits used and standard water and wastewater analysis methods numbers have been included.

Physicochemical parameter	Method	Detection limit (mg L^{-1})	Standard method number
Alkalinity	Titrimetric	–	2320-B
BOD	Membrane electrode	–	5210-B
COD	Photometric	0-150 15-300	5220-D
DO	Membrane electrode	–	4500-O G
Hardness	Titrimetric	–	2340-C
NH_4^+	Photometric	0.013-3.86 2.6-193.0	4500-NH_3-F
NO_2^-	Photometric	0.007-3.28	345-1
NO_3^-	Photometric	0.4-110.7	4500-NO_3
pH	Membrane electrode	–	2310-B
PO_4^{3-}	Photometric	0.007-15.3 1.5-92.0	4500-P-C
Suspended solids	Gravimetric	–	2540-D
Temperature	Thermometer	–	2550-B

NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand.

Physicochemical parameters – temperature, pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, DO, BOD, COD, suspended solids, hardness and alkalinity were analysed within 24 h of collection to remove the need for preservation. Spectroquant® kits (Sigma Aldrich) were used as per the manufacturer's instructions to assess NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and COD levels. Temperature and pH were analysed using the VWR pHenomenal™ MU 6100 L meter and VWR 111662-1157 pH probe. DO and BOD_{5day} were assessed using the Jenway 9500 DO_2 meter and probe. The suspended solids were analysed by filtration using a Buchner flask and funnel. Alkalinity was assessed via titration using phenolphthalein indicator, methyl orange indicator and hydrochloric acid. Hardness was analysed via titration using pH 10 buffer, erichrome black and EDTA. A summary of all physicochemical methods employed in this study, including each standard method number, are shown in Table 1.

2.3. Ecotoxicity analysis

The unicellular freshwater green algae *P. subcapitata* was used to determine the quality of the water. A culture was obtained from The Culture Collection of Algae and Protozoa (CCAP 278/4; SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland, U.K.) and grown in standard Jarworski's culture medium at 23 °C ± 2 °C exposed to continuous illumination (lux 6000-10,000). Additionally, starter cultures of *Asterionella formosa* (CCAP 1005/9) and *Monoraphidium contortum* (CCAP 245/2) were obtained from The Culture Collection of Algae and Protozoa (SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland). *P. subcapitata* was compared with *A. formosa* and *M. contortum* to ensure that *P. subcapitata* was representative of Irish aquatic algae (Table 2). Algae were sub-cultured every three days to ensure the growth rate remained in the exponential phase. Analysis was conducted as per the Water quality – Fresh water algal growth inhibition test with unicellular green algae ISO (8692:2012) guidelines. The *P. subcapitata* was exposed to the intake and output samples for 72 h at 23 °C ± 2 °C exposed to continuous illumination under static conditions. The percent of algal growth rate inhibition (E_rC_{50}) was calculated by comparing samples to a negative control containing just the Jarworski's culture medium. The E_rC_{50} is the concentration at which the has been a 50% reduction in the growth rate relative to the control within 72 h (ISO, 2012). Eqs. (1), (2) and (3) were taken directly from the ISO (8692:2012) guidelines (ISO, 2012) and calculations were conducted as follows;

$$\text{Algae cells mL}^{-1} = \frac{n}{0.02} \times 10^3 \quad (1)$$

where

n = the number of cells counted using a haemocytometer.

$$\text{Average specific growth rate } (\mu) = \frac{\ln X_n - \ln X_0}{T_n - T_0} \quad (2)$$

where

ln = natural log of
X_n = Algae cells mL^{-1} at 72 h
X_0 = Algae cells mL^{-1} at 0 h
T_n = Duration of test
T_0 = Time zero.

$$\text{Percent growth rate inhibition} = \frac{C\mu - T\mu}{C\mu} \times 100 \quad (3)$$

where

Cμ = Average specific growth rate for control
Tμ = Average specific growth rate for treatment.

Table 2
Mean concentrations calculated for each parameter investigated in this study conducted in 2019 (intake and output water 2019) and the previous study conducted in 2018 on the same fish farm (intake and output water 2018) by O'Neill et al. (2019). All data is based on the average across six months. S.D. has been indicated.

Parameter	Intake water		Output water	
	2018	2019	2018	2019
NH_4^+ (mg L^{-1})	0.16 ± 0.18	0.06 ± 0.09	1.16 ± 0.64	0.53 ± 0.53
NO_2^- (mg L^{-1})	0.02 ± 0.01	0.01 ± 0.01	0.32 ± 0.38	0.10 ± 0.07
NO_3^- (mg L^{-1})	3.62 ± 1.60	1.81 ± 1.27	5.29 ± 5.56	1.74 ± 1.10
PO_4^{3-} (mg L^{-1})	1.76 ± 0.84	0.63 ± 1.14	3.78 ± 2.00	0.77 ± 0.51
DO (mg O_2 L^{-1})	10.31 ± 0.87	10.76 ± 2.75	5.10 ± 2.85	7.66 ± 3.06
BOD (mg O_2 L^{-1})	2.27 ± 1.47	2.68 ± 0.70	3.24 ± 1.95	2.80 ± 0.96
COD (mg O_2 L^{-1})	45.91 ± 40.81	25.97 ± 9.98	76.44 ± 59.06	19.24 ± 11.68
Temperature (°C)	14.76 ± 2.53	13.85 ± 1.35	15.53 ± 2.66	14.23 ± 1.48
pH	7.76 ± 0.19	7.70 ± 0.14	7.11 ± 0.18	7.14 ± 0.06
Suspended solids (mg L^{-1})	40.17 ± 79.08	20.50 ± 8.00	83.67 ± 144.33	19.22 ± 9.23
Hardness (mg $CaCO_3$ L^{-1})	100.49 ± 9.22	106.24 ± 12.18	116.03 ± 16.80	111.58 ± 22.45
Alkalinity (mg $CaCO_3$ L^{-1})	122.55 ± 17.71	135.03 ± 20.49	128.91 ± 18.19	129.47 ± 17.98
P. subcapitata (% growth rate inhibition)	43.14 ± 18.47	13.66 ± 1.44	-2.70 ± 20.41	9.73 ± 2.03

NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand.

2.4. Statistical analysis

Statistical analyses were conducted using MINITAB 18 and GRAPHPAD PRISM 8. The generated data were grouped and subjected to normality testing (Anderson-Darling) to ensure all samples were normally distributed. Unpaired *t*-tests and ANOVA were used to identify any significant differences in the variables. $P < 0.05$ indicated a statistically significant difference. Pearson's correlation (r) was used to assess if any correlations between the algae and/or the physicochemical parameters existed (Fig. 3).

3. Results

3.1. Physicochemical analysis

Results determined for the physicochemical parameters investigated in this study on Irish freshwater fish farm intake, output and settlement pond water samples are displayed in Fig. 4. Increases in NH_4^+, NO_2^-, PO_4^{3-}, BOD and temperature, along with decreases in DO, COD, pH and alkalinity occurred when comparing the intake and output water from the fish farm. Fluctuations from month-to-month in NO_3^- and

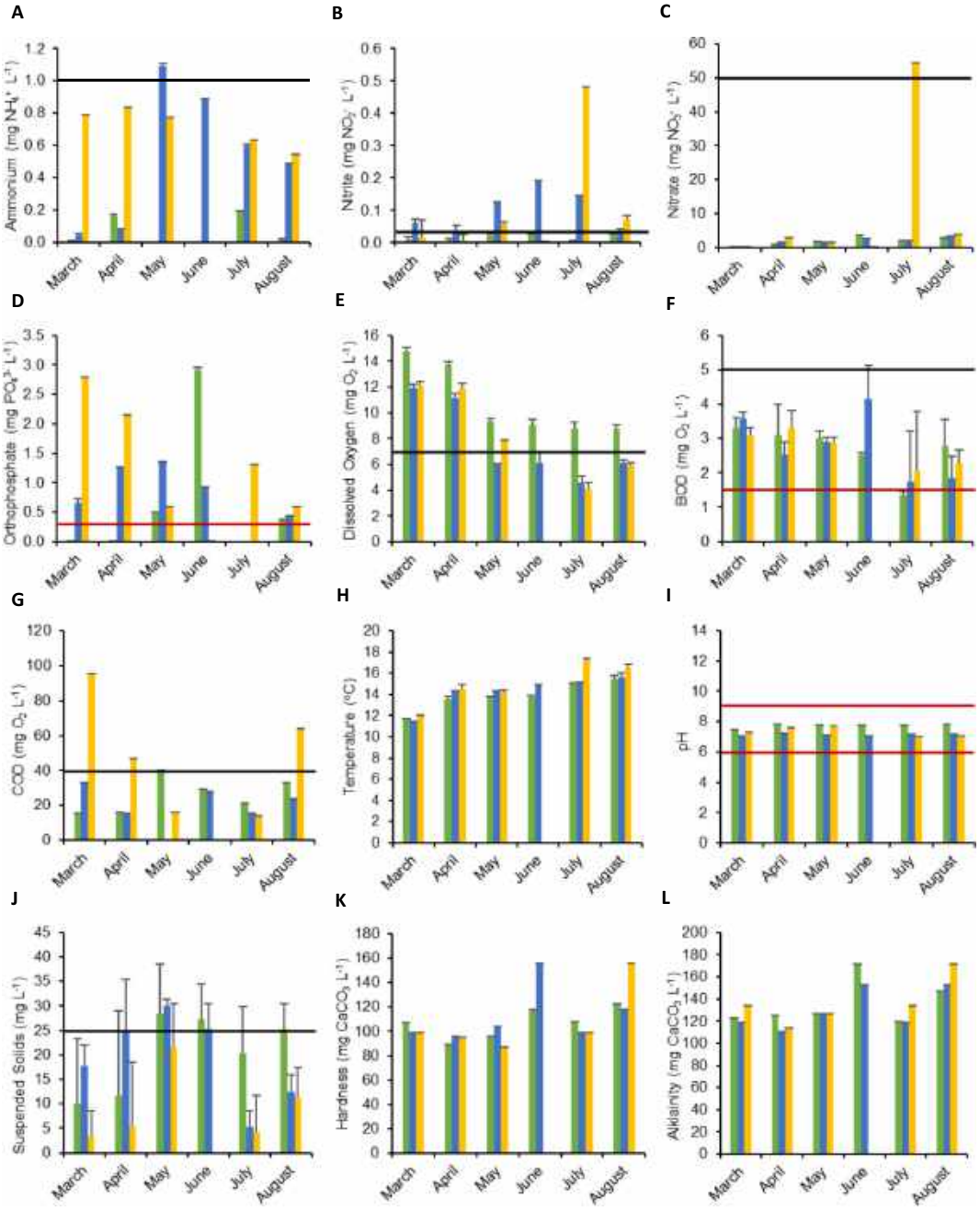


Fig. 3. Breakdown for the physicochemical parameters investigated on Irish freshwater aquaculture intake (green), output (blue) and settlement pond (yellow) water samples from March 2019 to August 2019. Parameters investigated were A) NH_4^+, B) NO_2^-, C) NO_3^-, D) PO_4^{3-}, E) DO, F) BOD, G) COD, H) temperature, I) pH, J) suspended solids, K) hardness and L) alkalinity. Red lines indicate levels set out by S.I. 272 of 2009 and 77 of 2019. Black lines indicate levels set out by the Irish EPAs parameters for water quality. NOTE: Dilution factor of the receiving water body has not been included. Lines do not appear on temperature and CaCO3 as no limits were indicated. S.D. indicated, $n = 9$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

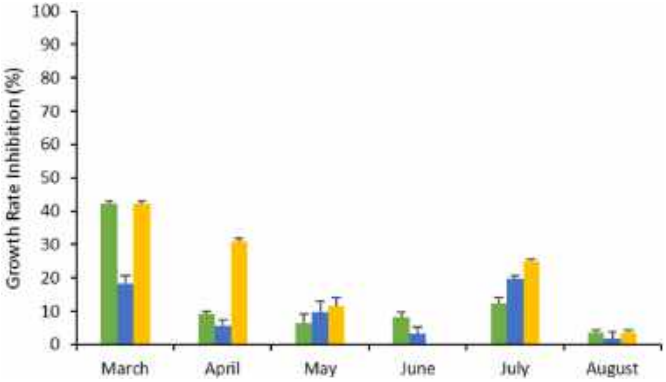


Fig. 4. Percentage growth rate inhibition observed in *P. subcapitata* proceeding exposure to Irish freshwater aquaculture intake (green), output (blue) and settlement pond (yellow) water samples for 72 h at 23 °C ± 2 °C under continuous illumination from March 2019 to august 2019. S.D. indicated, $n = 9$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hardness were also observed. With the exception of NO_2^- ($P = 0.011$) and pH ($P = 0.025$), no statistically significant (one-way ANOVA) differences were indicated. With the exception of NH_4^+ and NO_2^- levels in May and June, decreases were observed in the physicochemical parameters between the settlement pond and output water. With the exception of suspended solids ($P = 0.044$), no statistically significant (one-way ANOVA) differences were observed; one-way ANOVA was conducted across all three sampling points and no statistically significant differences were observed. Two-way ANOVA was conducted in order to take the sampling month into consideration; with the exception of BOD ($P = 0.083$) and suspended solids ($P = 0.150$), a statistically significant difference was observed in all parameters.

3.2. Algal bioassay analysis

The percentage growth rate inhibition observed in the intake, output and settlement pond water are displayed in Fig. 5. With the exception of samples for May and July, a decrease in growth rate inhibition between the intake and output samples was demonstrated. A decrease was also observed in all samples between the settlement pond and output water. No statistically significant (one-way ANOVA) differences were observed for either set of samples. One-way ANOVA was conducted across the three sampling points and no statistically significant differences were indicated. Two-way ANOVA was conducted to determine whether any statistically significant differences were observed when the sampling month was taken into consideration. A statistically significant difference ($P = 0.001$) was indicated when the sampling month was included.

3.3. Comparative study

Table 3 summarises an average of all results obtained during a previous study conducted at a similar time of year on the same fish farm during times of extreme weather conditions (heat wave and drought) by O'Neill et al. (2019) and those determined in this study which were conducted under normal weather conditions for the Republic of Ireland. With the exception of the dissolved oxygen, pH, alkalinity and hardness, all physicochemical concentrations decreased during the similar time periods of 2018 and 2019 in both the intake and output water samples. The pH, alkalinity and hardness remained similar whilst dissolved oxygen levels increased. For the *P. subcapitata*, considerable decreases in inhibition toxicity were observed in the intake water and no stimulation was observed in the output water from this study compared to 2018. Both of which demonstrated a statistically significant (*t*-tests) difference ($P = 0.030$ for the intake water and $P = 0.039$ for the output water).

3.4. Correlation studies for monitoring periods of freshwater aquaculture farm

Correlation studies were conducted between all parameters investigated at the three sampling points. A positive correlation between two parameters indicates that as one parameter increases or decreases, so too does the other parameter. A negative correlation between two parameters indicates that as one parameter increases or decreases, the opposite occurs with the other parameter i.e., an inverse relationship. All results for the intake, output and settlement pond water samples are displayed in Tables 3, 4, 5, and 6 respectively. In the intake samples a negative correlation was observed between *P. subcapitata* and temperature as well as pH. A negative correlation was indicated between NH_4^+ and NO_2^-. A positive correlation was identified between NO_2^- and alkalinity. The NO_3^- demonstrated a negative correlation with DO and a positive correlation with suspended solids. A positive correlation was observed between PO_4^{3-} and alkalinity. A negative correlation was identified between DO and suspended solids. In the output samples a positive correlation between temperature and NO_3^- was indicated. A negative correlation was identified between DO and NH_4^+ as well as NO_2^-. Hardness displayed a positive correlation between PO_4^{3-} and alkalinity. In the settlement pond a positive correlation between *P. subcapitata* and PO_4^{3-} was observed. Temperature demonstrated a positive correlation with pH, NH_4^+, hardness and alkalinity. The pH indicated a positive correlation with NH_4^+, BOD hardness and alkalinity.

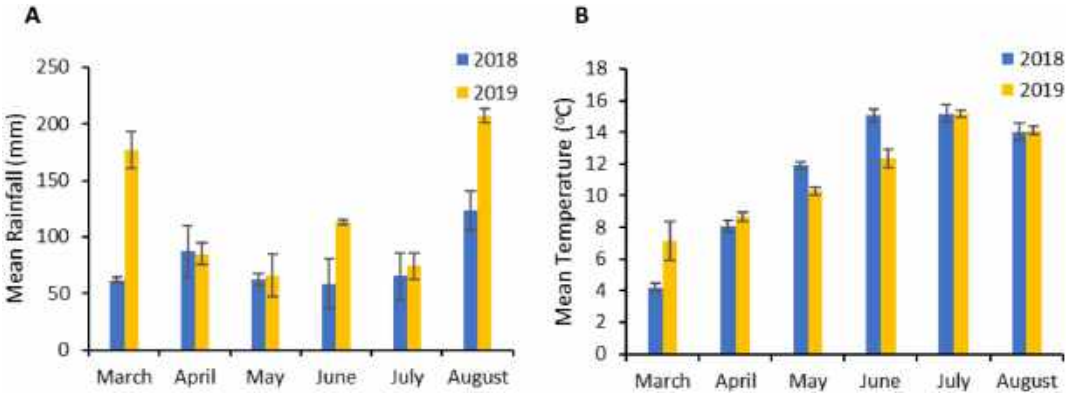


Fig. 5. Average A) rainfall and B) temperature recorded for 2018 (blue) and 2019 (yellow) at three Met Eireann weather stations surrounding the freshwater fish farm during the sampling period of March 2019 to August 2019. Stations were located at 1) Markree, Co. Sligo, 2) Knock, Co. Mayo and 3) Mount Dillon, Co. Roscommon. Stations were located north-west, south-west and south-east of the fish farm, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Correlation matrix for *P. subcapitata* and all physicochemical parameters investigated on the Irish freshwater aquaculture intake water samples. Bold figures indicate where statistically significant differences were observed. Breakdown of correlation ranges are also indicated.

	P. sub	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H	A
P. sub	1.000								0 = No relationship				
T	**-0.830**	1.000							>0-0.3 = Weak relationship				
pH	**-0.951**	0.794	1.000						0.3-0.5 = Moderately weak relationship				
NH_4^+	-0.183	0.360	0.451	1.000					0.5-0.7 = Moderately strong relationship				
NO_2^-	-0.260	0.037	-0.020	**-0.845**	1.000				0.7-<1 = Strong relationship				
NO_3^-	-0.727	0.687	0.557	-0.173	0.656	1.000			1 = Perfect linear relationship				
PO_4^{3-}	-0.286	0.024	0.133	-0.444	0.785	0.728	1.000						
DO	0.726	-0.809	-0.553	0.057	-0.468	**-0.876**	-0.400	1.000					
BOD	0.307	-0.627	-0.351	-0.582	0.232	-0.465	-0.073	0.625	1.000				
COD	-0.623	0.448	0.372	-0.523	0.712	0.623	0.353	-0.749	0.007	1.000			
SS	-0.702	0.594	0.466	-0.380	0.730	**0.855**	0.559	**-0.915**	-0.295	0.924	1.000		
H	-0.061	0.326	-0.138	-0.426	0.588	0.638	0.471	-0.502	-0.264	0.254	0.438	1.000	
A	-0.394	0.201	0.218	-0.497	**0.831**	0.800	**0.916**	-0.440	0.017	0.400	0.577	0.659	1.000

P. sub = *P. subcapitata*, T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, SS = suspended solids, H = hardness, A = alkalinity.

A positive correlation was identified between NH_4^+ and DO as well as BOD. A positive correlation was observed between NO_2^- and NO_3^-. DO demonstrated positive correlations with PO_4^{3-} and BOD. Finally, a positive correlation was identified between hardness and alkalinity.

3.5. Weather conditions influencing water quality on monitored aquaculture farm

Due to observations determined in the previous study, conducted by same authors (O'Neill et al., 2019), as a result of dramatic weather conditions experienced during 2018 in the Republic of Ireland that coincided with occurrence of the hottest summer recorded to date by Irish Meteorological Office (Met Eireann) resulting in a nationwide hosepipe ban due to limit negative impacts of drought; weather conditions during the sampling period have been included. For continuity, mean temperature and rainfall data collected by Met Eireann at the same three weather stations surrounding the fish farm as the previous study have been included for this sample period (Met Éireann, 2019). Stations were situated at Markree Co. Sligo, Knock Co. Mayo and Mount Dillon Co. Roscommon, as shown in Fig. 1. Increases in the mean rainfall (Fig. 5A) and decreases in the mean temperature (Fig. 5B) were observed for 2019 versus the same time period in 2018 across the three stations. Maxima temperatures had also decreased (Fig. 6 – Supplementary). Statistical analysis found that the relationships between the algae and the rainfall and temperature switched. A moderately strong inverse relationship (r = -0.559) between the algae and temperature, and a weak inverse relationship (r = -0.209) between the algae and rainfall now existed.

4. Discussion

4.1. Physicochemical evaluation

In order to ascertain whether the processes conducted in the fish farm altered the quality of the water, the physicochemical results determined in the intake and output water were compared firstly to one another and then to the previous study conducted. It should be appreciated that the dilution factor of the receiving river on potential impact of aquaculture effluent has not been considered in this research. The presence of NH_4^+, NO_2^- and NO_3^- in the output water suggested that the nitrification process (enzymatic oxidation of NH_4^+ to NO_3^- by way of NO_2^-) was occurring. Increases in these parameters between the intake and output samples suggested that, for the most part, their production was due to practices within the farm. These were most likely due to the presence of fish waste and uneaten artificial pelleted feed used in the cultural process. Increases above guidance levels (1 mg NH_4^+ L^{-1} and 0.03 mg NO_2^- L^{-1}) in the parameters were only observed between May and June, which suggested a potential cause for concern. However, this was most likely attributed to the constructed wetland, which was not functioning to its optimal capacity due to undergoing maintenance works: no discharge of aquaculture effluent was released during these times. Levels of monitored physicochemical parameters dropped below

Table 4
Correlation matrix for *P. subcapitata* and all physicochemical parameters investigated on the Irish freshwater aquaculture output water samples. Bold figures indicate where statistically significant differences were observed. Breakdown of correlation ranges are also indicated.

	P. sub	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H	A
P. sub	1.000								0 = No relationship				
T	-0.537	1.000							>0-0.3 = Weak relationship				
pH	-0.332	0.419	1.000						0.3-0.5 = Moderately weak relationship				
NH_4^+	-0.210	0.496	-0.262	1.000					0.5-0.7 = Moderately strong relationship				
NO_2^-	0.131	0.213	-0.645	0.733	1.000				0.7-<1 = Strong relationship				
NO_3^-	-0.627	**0.929**	0.234	0.439	0.161	1.000			1 = Perfect linear relationship				
PO_4^{3-}	-0.488	0.139	-0.500	0.433	0.656	0.213	1.000						
DO	0.296	-0.670	0.370	**-0.816**	**-0.811**	-0.700	-0.612	1.000					
BOD	-0.102	-0.518	-0.702	0.128	0.431	-0.405	0.766	-0.112	1.000				
COD	0.007	-0.362	-0.505	-0.529	-0.092	-0.070	0.252	0.095	0.385	1.000			
SS	-0.442	-0.194	-0.004	0.304	0.124	-0.267	0.525	0.033	0.634	-0.324	1.000		
H	-0.574	0.344	-0.514	0.494	0.603	0.522	**0.902**	-0.769	0.558	0.364	0.246	1.000	
A	-0.639	0.479	-0.327	0.494	0.299	0.736	0.544	-0.701	0.197	0.296	0.019	**0.839**	1.000

P. sub = *P. subcapitata*, T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, SS = suspended solids, H = hardness, A = alkalinity.

Table 5
Correlation matrix for *P. subcapitata* and all physicochemical parameters investigated on the Irish freshwater aquaculture settlement pond water samples. Bold figures indicate where statistically significant differences were observed. Breakdown of correlation ranges are also indicated.

	P. sub	T	pH	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	H	A
P. sub	1.000								0 = No relationship				
T	0.386	1.000							>0-0.3 = Weak relationship				
pH	0.566	**0.938**	1.000						0.3-0.5 = Moderately weak relationship				
NH_4^+	0.714	**0.816**	**0.958**	1.000					0.5-0.7 = Moderately strong relationship				
NO_2^-	0.133	0.464	0.229	0.107	1.000				0.7-<1 = Strong relationship				
NO_3^-	0.168	0.417	0.182	0.079	**0.992**	1.000			1 = Perfect linear relationship				
PO_4^{3-}	**0.976**	0.393	0.580	0.715	-0.004	0.032	1.000						
DO	0.784	0.525	0.765	**0.883**	-0.288	-0.287	**0.847**	1.000					
BOD	0.679	0.786	**0.945**	**0.986**	-0.034	-0.060	0.714	**0.927**	1.000				
COD	0.593	0.362	0.523	0.532	-0.322	-0.336	0.730	0.744	0.616	1.000			
SS	-0.255	0.468	0.514	0.438	-0.085	-0.183	-0.271	0.201	0.441	-0.077	1.000		
H	0.251	**0.898**	**0.843**	0.677	0.205	0.147	0.341	0.512	0.709	0.609	0.412	1.000	
A	0.356	**0.942**	**0.920**	0.781	0.279	0.212	0.407	0.571	0.785	0.582	0.488	**0.974**	1.000

P. sub = *P. subcapitata*, T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, COD = chemical oxygen demand, SS = suspended solids, H = hardness, A = alkalinity.

guidance values, once the wetland was fully functional where low levels were also observed in the intake water. It is likely that agricultural processes (cattle and sheep farming) and forestry processes (tree felling) occurring upstream of the fish farm contributed to these measured physicochemical parameters.

The PO_4^{3-} levels in the output water was greater than that of the input as a result of the processes within the aquaculture farm. However, levels observed in the intake suggested that agricultural and forestry processes upstream of the farm could have also contributed to levels. Concentrations of PO_4^{3-} were greater than guidance levels (0.35 mg PO_4^{3-} L^{-1}) suggesting a potential cause for concern as excess levels can result in the promotion of algal blooms leading to potential hypoxic conditions in the water body (O'Neill et al., 2019). However, once maintenance was completed on the constructed wetland, levels detected in the output water were reduced to guidance levels indicating no foreseen issues.

A decrease in oxygen levels was observed between the intake and output water due to the aquaculture process. This decrease may also have been due to changes in seasonality. According to Alam et al. (2007), da Silva et al. (2017) and O'Neill et al. (2019) oxygen levels ≥4 mg O_2 L^{-1} are sufficient to maintain aquatic life. Although levels in the output water dropped just below the guidance value (7 mg O_2 L^{-1} for cyprinid waters), levels remained above the critical 4 mg O_2 L^{-1} level and as a result had indicated no cause for concern. BOD levels between the intake and output water fluctuated i.e., BOD increased between the intake and output water during March, June and July, whilst decreases were observed in April, May and August. Despite fluctuations, BOD levels remained below the guidance value of 5 mg O_2 L^{-1} for cyprinid waters suggested by the Irish EPA. Additionally, the dilution factor of the receiving water system has not been included therefore BOD levels would further decrease upon release. With the exception of March, COD levels decreased between the intake and output water. This suggested that the COD levels were not due to processes within the farm and were more likely due to works being conducted upstream. Despite this, COD levels were well below the guidance value of 40 mg O_2 L^{-1}.

Suspended solids levels were greater between the intake and output water during the months of March, April and May which were most likely due to increases in production processes in the farm. However, this trend reversed for the months of June, July and August. This was most likely due to high levels of tree felling being conducted in the forestry upstream of the farm during this time. Levels were greater than the guidance level of 25 mg L^{-1} during May and June but this was most likely due to the constructed wetland maintenance work as once the wetland became fully functional again after the June maintenance, concentrations dropped well below this level. Once again, it should be noted that water did not leave the farm during this time.

Temperature between the intake and output water samples remained consistent with increases observed during the summer months, as would be expected. Fish farms must not release water that is greater than 20 °C. At no point during the study did temperatures rise to this level. Aquaculture waters are recommended to have a pH of between 6 and 9 (EPA, 2001). All samples remained within this range. The intake samples were slightly more alkaline than the output samples. Output samples had greater $CaCO_3$ levels and therefore a greater buffering capacity which may account for pH levels of just about neutral (pH 7) in the output water. $CaCO_3$ levels were measured for hardness. Results suggested that the water was slightly to moderately hard. This correlates with water hardness demonstrated around Boyle, Co. Roscommon (O'Neill et al., 2019).

All parameters were then compared to the previous year's study. In 2018, Ireland experienced its hottest summer on record whereby the country experienced long periods of drought. The physicochemical parameters were greater in 2018 than that of this study (2019) for the similar time period (O'Neill et al., 2019). This was most likely due to increased flow rates as a result of increased rainfall resulting in no drought conditions being observed in 2019. As this research only focused on one type of fish farm culturing one specific species of fish (European Perch) results from this study were also compared to previous aquaculture studies. These studies were located worldwide and encompassed a range of different aquaculture systems culturing several different species of fish, as shown in Table 6. The studies reviewed demonstrated similar or higher levels than the concentrations observed in this study.

4.2. Algal bioassay evaluation

Inhibition of the growth rate of the *P. subcapitata* was observed in both the intake and output water samples. The presence of growth rate inhibition suggested that algal blooms downstream of the fish farm would be unlikely. However, growth rate inhibition is still demonstration of a toxic effect. This inhibition may result in loss of biodiversity in the receiving water body (Rabalais, 2002; O'Neill et al., 2019). Exclusive of the months of May and July, the percentage of growth rate inhibition was found to decrease between the intake and output water samples. The inhibition toxicity detected throughout the study was at sub-lethal levels. Additionally, toxicity was reduced once the water had passed through the fish farm's constructed wetland. This suggested that the farm itself was successfully improving the water quality.

When results were compared to the previous study of 2018, a statistically significant difference was observed in both the intake

($P = 0.030$) and the output ($P = 0.039$) water samples. Unlike the previous study, no growth rate stimulation was observed in the output water. Equally, considerably lower levels (sub-lethal) of toxicity were observed in the intake water, e.g. levels of up to 75% growth rate inhibition were observed during the drought conditions of 2018 (O'Neill et al., 2019). This reduction was most likely due to the reduced temperatures and resulting increased flow rates.

Results were than compared to previous studies that utilised *P. subcapitata* to assess fish farm output water. Miashiro et al. (2012) demonstrated similar results in a Brazilian study (with a traditionally much warmer climate than Ireland) to the previous study conducted on the fish farm by O'Neill et al. (2019) during the heat wave and drought conditions, where by similar levels of growth rate stimulation were observed. The current study however, conformed to most of the available research on the effects of fish farm output water on *P. subcapitata*. Guéguen et al. (2004), Ivanova and Groudeva (2006) and Ma et al. (2006) all observed similar growth inhibition levels to those demonstrated. These studies were also conducted in countries (Poland and Bulgaria) with similar temperate weather conditions to those normally experienced in Ireland.

4.3. Constructed wetland evaluation

The previous study conducted by O'Neill et al. (2019) indicated that there may have been issues with the constructed wetland due to increased concentration of nitrogenous and phosphorus waste in the output water samples. However, it was unclear whether this issue may have been due to the extreme weather conditions experienced during 2018 in Ireland. As a result, samples were included at the exit point of the settlement pond to ascertain the efficacy of the wetland. This was the point at which the wastewater entered the constructed wetland. Evaluation of the settlement pond demonstrated that, when fully functional after the June maintenance, the constructed wetland was effective in the removal of waste products from the water. This efficacy may also be due in part to the re-introduction of duckweed (*Lemna minor*). The previous study found spikes in nitrogenous waste concentrations when the duckweed was removed from the farm. Duckweed has the ability to use NO_3^- as a nutrient source (O'Neill et al., 2019) and research is ongoing in this area.

4.4. Climate change

According to the Intergovernmental Panel on Climate Change (2020), the momentum of climate change had greatly increased in 2019. Climate change is the most troubling scientific issues of our time (Bulkeley and Newell, 2015). The Bulletin of the Atomic Scientists (2020) has now moved the hypothetical Doomsday Clock to 100 s to midnight which is the closest it's ever been to the "point of no return" represented by midnight. Originally introduced in 1947 due to the threat of nuclear weapons, climate change is now considered an equal threat to that (Weisberger, 2020). This research has further indicated that climate change has a direct impact on fish farming, as suggested by the lack of algal growth stimulation or high levels of growth inhibition due normal weather conditions reported in this study. Algal growth and temperature still demonstrate a strong correlationship ($r = -0.830$) in the intake samples. This research has further demonstrated the ability of *P. subcapitata* to be utilised as an early warning indicator for climate change ambiguity in freshwater aquaculture.

5. Conclusion

- The findings of this timely study responds to the main tenets of the recent intergovernmental report on global climate change (IPCC, 2021) that seeks urgent viable and resilient technological solutions to help future proof for a climate-smart environmentally friendly agri-food sector, including fisheries. Moreover, this 'code red for humanity' IPCC report on climate change clearly highlights that human or anthropogenic activity has contributed greatly to greenhouse gas levels in the atmosphere where there is a pressing need to reduce carbon dioxide and methane emissions, and to stall rising global temperatures that leads to extreme weather events.
- Regarding the latter, there is pressing need for countries to use innovative approaches to support and to develop sustainable food systems delivering benefits for the sector, for society, and for the environment. The findings of this present study will support and enable viable and resilient primary producers to provide food that are safe, nutritious and appealing; thus, using eco-technologies and talent to inform innovative, competitive and resilient agri-food sector regionally, and internationally (Rowan and Pogue, 2021).
- Specifically, this study revealed that the freshwater microalga *P. subcapitata* can be used for the real-time prediction of potential adverse environmental issues associated with freshwater aquaculture wastewater, which can be seen as complementary to relying upon using traditional physicochemical parametric measurements.
- As this research focused exclusively on one type of fish farm in the Republic of Ireland, use of this algal bioindicator technique should be also applied to evaluate different types of aquaculture farms including pond-based, flow-through, and recirculation in order to ensure harmonised results across a range of culture systems and fish species.
- Inclusion of additional ecotoxicological bioassays *such as* a full test battery, encompassing different trophic levels (e.g., *Daphnia magna* – primary consumer, *Vibrio fischeri* – decomposer) (Garvey et al., 2013) within the aquatic ecosystems should also be considered for future studies in order to develop a better understanding of the potential environmental effects' aquaculture processes could have on water bodies.
- There is merit in conducting molecular profiling of naturally occurring microalgae in order to incorporate these as a cocktail of native species representative of local natural aquatic ecosystems, which will support and inform biodiversity, conservation management along with enhanced bioindicator performance. For example, Kadam et al. (2020) identified 33 Taxa belonging to 27 genera of microalgae when they considered development of a putative 'Algal Genus Pollution Index' for potentially assessing water bodies in the Doon valley, India.
- The constructed wetland servicing this aquaculture farm needs to be increased in size in order to be effective in treating volume of the wastewater effluent where efficacy of treatment can be also influenced by extreme weather events that influence flow rates.
- The lack of growth rate stimulation and decrease in growth rate inhibition when compared to the previous study (O'Neill et al., 2019) supports future use of *P. subcapitata* as an early warning indicator to potential issues in fish farms associated with climate change where unpredictable and more erratic weather conditions may become more frequent. It is appreciated that there a dearth in evidence-based literature on the use of microalgae as a bioindicator for monitoring impact of climate change and its potential effects in aquaculture.
- Whilst this present research has demonstrated interested findings, there is a need to pursue catchment based-studies that incorporates an extended number of locations and inter-laboratory evaluations for to improve technological rigor and stakeholder acceptance including policy-makers.
- Increasing NH_3 levels in the monitored fish ponds can be potentially toxic to fish that require further investigation.
- There are emerging opportunities for use of natural microalgae in the development of predictive environmental risk models that will help inform the quality status of water catchments, along with evaluating commensurate efficacy of intervention strategies, such as municipal wastewater treatment plants (Tahar et al., 2017).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149800.

CRediT authorship contribution statement

Emer A. O'Neill: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Neil J. Rowan:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no competing interests or conflict of interest with respect to the publication of this article.

Acknowledgements

The authors would like to thank Athlone Institute of Technology (President Seed Fund), for providing financial support for this research. The authors would also like to acknowledge and thank Paul Kearney for access to his freshwater aquaculture facility in order to conduct this research.

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Science of the Total Environment 819 (2022) 153073

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Effects of climate and environmental variance on the performance of a novel peatland-based integrated multi-trophic aquaculture (IMTA) system: Implications and opportunities for advancing research and disruptive innovation post COVID-19 era

E.A. O'Neill [a,*], A.P. Morse [b], N.J. Rowan [a]

[a] Bioscience Research Institute, Technological University of the Shannon – Midlands and Midwest, University Road, Athlone, Ireland
[b] Department of Geography and Planning, School of Environmental Sciences, University of Liverpool, UK

HIGHLIGHTS

- Integrated multi-trophic (IMTA) aquaculture offers a sustainable food process.
- IMTA can be affected by extremes in weather/climate events.
- IMTA balance can be disrupted by emergence of toxigenic algae.
- Future need for in-farm real-time monitoring of IMTA systems

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:
Received 23 November 2021
Received in revised form 7 January 2022
Accepted 8 January 2022
Available online 14 January 2022

Editor: Damià Barceló

Keywords:
Sustainable aquaculture
climate change
food security
environmental indicators
disruptive innovation
paludiculture

ABSTRACT

Advancing wet peatland 'paludiculture' innovation present enormous potential to sustain carbon-cycles, reduce greenhouse-gas (GHG) gas emissions and to transition communities to low-carbon economies; however, there is limited scientific-evidence to support and enable direct commercial viability of eco-friendly products and services. This timely study reports on a novel, paludiculture-based, integrated-multi-trophic-aquaculture (IMTA) system for sustainable food production in the Irish midlands. This freshwater IMTA process relies on a naturally occurring ecosystem of microalgae, bacteria and duckweed in ponds for managing waste and water quality that is powered by wind turbines; however, as it is recirculating, it does not rely upon end-of-pipe solutions and does not discharge effluent to receiving waters. This constitutes the first report on the effects of extreme weather events on the performance of this IMTA system that produces European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*) during Spring 2020. Sampling coincided with lockdown periods of worker mobility restriction due to COVID-19 pandemic. Observations revealed that the frequency and intensity of storms generated high levels of rainfall that disrupted the algal and bacterial ecosystem in the IMTA leading to the emergence and predominance of toxic cyanobacteria that caused fish mortality. There is a pressing need for international agreement on standardized set of environmental indicators to advance paludiculture innovation that addresses climate-change and sustainability. This study describes important technical parameters for advancing freshwater aquaculture (IMTA), which can be future refined using real-time monitoring-tools at farm level to inform management decision-making based on evaluating environmental indicators and weather data. The relevance of these findings to informing global sustaining and disruptive research and innovation in

* Corresponding author.
E-mail address: e.oneill@research.ait.ie (E.A. O'Neill).

http://dx.doi.org/10.1016/j.scitotenv.2022.153073

paludiculture is presented, along with alignment with UN Sustainable Development goals. This study also addresses global challenges and opportunities highlighting a commensurate need for international agreement on resilient indicators encompassing linked ecological, societal, cultural, economic and cultural domains.

1. Introduction

Unlocking the challenges of climate-orientated peatland management will enable conservation of their important carbon stocks (Wichmaan et al., 2016; Ziegler et al., 2021). However, some 15% of peatlands globally are degraded due to drainage-base agriculture and forestry that are key contributors to greenhouse gas emissions (GHG) (Uràk et al., 2017; Ziegler et al., 2021). The controlled rewetting of peatlands offers solutions to preventing GHG emissions and biodiversity loss (Ziegler et al., 2021), and presents opportunities for farming and innovative use of these rewetted peatlands that is termed 'paludiculture' (O'Neill et al., 2019; Ziegler et al., 2021). There is strong interest in developing commercial paludiculture activities including fuel, horticulture, aquaculture, and construction material; however, there is a need to address knowledge gaps that will influence the economic viability of paludiculture-derived products and services (Ziegler et al., 2021; O'Neill and Rowan, 2022). Factors affecting effective deployment of paludiculture are complex as fostering sustainable green innovation requires a holistic strategic response that can be effectively facilitated through the 'quadruple helix' approach of industry, academia, government and society (Rowan and Casey, 2021; Tan et al., 2021).

Key challenges affecting the development of paludiculture-based innovation include the lack of shared real-time systems of data generation for met-analysis, corporate strategy, risk-mitigation, and business disruption (Ziegler et al., 2021). There is a lack of consensus on international standardization of paludiculture innovation in terms of outcome sets, and agreement on sustaining measured variables that must encompass protecting biodiversity (Ziegler et al., 2021). Unlocking the disruptive potential of paludiculture innovation can be met by collaborate use of in-field environmental test-bed facilities (Rowan and Casey, 2021).

Aquaculture is the fastest growing food producing industry in the world that accounts for half of the fish produced globally for human consumption (Nielsen et al., 2016; Liu et al., 2017; O'Neill et al., 2019, O'Neill et al., 2020; O'Neill and Rowan, 2022). Farmed fish is considered to be a more efficient protein utilisation and feed conversion source than other animals destined for protein production (Tschirner and Kloas, 2017). According to the United Nations (UN), aquaculture now provides fish availability to countries and regions that would have previously been limited or non-existent, often at affordable prices; thus, providing improved nutrition and food security (Fish Farming Expert, 2020; Rowan and Casey, 2021). The increasing interest in exploiting low-cost environmentally-friendly 'natural' processes in aquaculture has led to the timely development of integrated multi-trophic aquaculture systems or IMTA, along with accelerating efforts to implement eco-innovation and to improve the monitoring of traditional processes (Granada et al., 2016; Tahar et al., 2018a, 2018b; Naughton et al., 2020). However, advances in aquaculture must also be balanced with the commensurate need to meet commitments as set out by many European directives for environmental protection as well as the Water Framework Directive (WFD), where the latter aims to achieve good water status in all waters across all EU member countries (Voulvoulis et al., 2017; WFD Ireland, 2018).

In addition to these environmental concerns, extreme weather events, such as drought have displayed substantial effects on variances in stocking densities within aquaculture facilities therefore, successful stock assessments will be influenced by our ability to understand, monitor and to predict variances in weather and climate change on aquaculture ecosystem dynamics (Rowan and Pogue, 2021; O'Neill and Rowan, 2022). An increasing number of studies have intimated that climate vulnerability and climate change can have adverse impacts on global food production and food security (Iizumi and Ramankutty, 2015). These authors reported that ongoing climate change, and associated variances in intensity, frequency, and duration of weather/climate extremes, in conjunction with growing populations and dietary requirements, further complicate this drive to improve food sustainability and security. Therefore, stakeholders including policymakers, urgently require better estimates of the likely incidence of extreme weather events, their impact on food production and security, and the commensurate consequential impact in terms of socio-economic losses (Chavez et al., 2015). There is a pressing need to address the uncertainties in climate model predictions not only over years, but also at regional and local scale to inform efficacy of food production systems, and interventions. Mowi Ireland, a coastal aquaculture farmer, recently lost approximately 80,000 salmon due to toxigenic plankton bloom where the rearing water was 13 °C compared to typical 11.5 °C, which was potentially attributed to climate change (Moore, 2021).

This constitutes the first study to report on the potential effects of extreme weather events on the technical performance of a novel peatlands-based IMTA process located in the Republic of Ireland that lead to fish mortality, which coincided with occurrence of COVID-19 pandemic. It describes the worldwide relevance of data generated by using a Quadruple Helix (academia-industry-government-society) approach to advancing paludiculture innovation along with challenges and opportunities. It describes measurable technical and environmental variables for the development of this IMTA system that embraces extreme weather events; it describes smart tools to inform technical, policy and societal readiness level of paludicutlure innovation aligned with U.N.s' sustainable development goals.

2. Materials and methods

2.1. Sampling

Oasis fish farm is an innovative peatland cut-away integrated multi-trophic aquaculture (IMTA) system process set in the middle of Mount Lucas Wind Farm, Co. Offaly (53°17′3″ N – 7°11′45″ W). This IMTA holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), common duckweed (*Lemna minor*) and gibbous duckweed (*Lemna gibba*) and exploits use of microalgae for waste removal. The aquaculture system consists of four split (pill) ponds connected to an algae and duckweed lagoon with 16 channels serving as a treatment system. Fish are kept at a density that does not exceed the organic farming standard (e.g., <20 kg/m^{-3} for perch), using screens at the D-ends of each split pond. The space between two D-end fish culture areas is also used to treat waste with free living algae in suspension. Flow in each split pond is generated and water is circulated using an airlift. Each D-end fish culture area is equipped with oxygen and temperature probes connected to paddlewheels to provide extra oxygen when necessary. The farm is designed to hold a maximum of 32,000 kg of fish.

Water samples were collected from the Oasis farm in five liter octagonal carboy HDPE bottles (Lennox) and transported in insulated cool boxes directly to the lab, 62 km away, via car. Samples were collected every two weeks from December 2019 to February 2020 and then once a week until October 2020. Samples were taken from each on the culture ponds, the entry and exit points of the duckweed lagoons and the reservoir during this study. Samples were also taken from the overflow tank during times when there may have been a potential for discharge. Refer to Fig. 1 for the locations of all sample points within the farm.

2.2. Physicochemical analysis

Good water quality is critical for the cultivation of fish as well as for the receiving ecosystems attached to aquaculture facilities. Analysing the physicochemical parameters is the most common method of determining the

Fig. 1. Aerial view of Oasis Fish Farm, located in Ballycon, Co. Offaly. The culture ponds, water reservoir, algae & duckweed wastewater treatment channels, overflow tank and bog river are all visible. Blue lines indicate the direction of the flow of water. The green squares indicate all sampling points within the farm to monitor the IMTA process.

current water quality (Shukla et al., 2013). No definite set of physicochemical parameters specific for Irish aquaculture water and wastewater could be found. Therefore, the physicochemical parameters routinely monitored in Irish fish farms to assess water quality were applied. Additionally, a range of previous studies conducted on aquaculture facilities across the world were researched. The range of parameters investigated in these studies were also applied to this research. As no values for the individual physicochemical parameter levels could be found in relation to Irish aquaculture, the standard Irish EPA water quality parameters based on the Freshwater Fish Directive [78/659/EEC], Surface Water Regulations [1989] and surface water regulations [SI 272 of 2009] and amendments [SI 77 of 2019] were used as guidance (EPA, 2001; Irish Statutory Office, 2009; Irish Statutory OfficeOffice, 2019).

Water parameters; temperature, pH, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), orthophosphate (PO_4^{3-}), dissolved oxygen (DO), biochemical oxygen demand (BOD), suspended solids (SS), dissolved solids (DS), hardness, and alkalinity – were investigated in the laboratory within 24 h of collection to prevent the need for preservation. The Standard Methods for the Analysis of Water and Wastewater were used for all of the parameters employed. See Table 1. Spectroquant® photometric kits were used to assess the NH_4^+, NO_2^-, $NO3^-$ and PO_4^{3-}. Analysis was conducted as per the manufacturer's instructions. Absorbance was analysed using a Shimadzu UV-2250 spectrophotometer. Temperature, pH, dissolved solids and conductivity were analysed using a VWR pHenomenal™ MU 6100 L meter, VWR 111662–1157 pH probe and VWR CO11 conductivity probe. DO and BOD_{5day} were analysed using a Jenway 9500 DO_2 meter and probe. Suspended solids were analysed via filtration using a Buchner flask, Buchner funnel and Whatman 0.45 μm pore membrane filter. Hardness was assessed via titration using pH 10 buffer, Erichrome black and EDTA. Alkalinity was analysed by titration using phenolphthalein indicator, methyl orange indicator and hydrochloric acid.

Table 1
Summary of all physicochemical methods applied to this research. All parameters/variables, their respective methods, standard analysis of water and wastewater method numbers and the detection limits for parameters where photometric test kits were employed have been included.

Parameter/variable	Analytical method	Standard analysis method number	Detection limit (mg L^{-1})
Alkalinity	Titrimetric	2320-B	–
Ammonium (NH_4^+)	Photometric	4500-NH_3-F	0.013–3.86 2.6–193.0
Biochemical oxygen demand (BOD)	Membrane electrode	5210-B	–
Dissolved oxygen (DO)	Membrane electrode	4500-O G	–
Dissolved solids (DS)	Electrode	2540-C	–
Hardness	Titrimetric	2340-C	–
Nitrate (NO_3^-)	Photometric	4500-NO_3	0.4–110.7
Nitrite (NO_2^-)	Photometric	345–1	0.007–3.28
Orthophosphate (PO_4^{3-})	Photometric	4500-P-C	0.007–15.3 1.5–92.0
pH	Membrane electrode	2310-B	–
Suspended solids (SS)	Gravimetric	2540-D	–
Temperature	Thermometer	2550-B	–

2.3. Algae enumeration

Algae cells were both manually and automatically counted. All manual enumeration was conducted using a Superior Marienfeld Neubauer Improved Haemocytometer (0.1 mm, 0.0025 mm^2, Tiefe Depth Profondeur No: 717810) and a Nikon YS100 light microscope. The Miltenyi Biotec MACSQuant® Analyser 10 Flow Cytometer (FCM) was used for the automated enumeration of the algae. Preparation of phytoplankton samples for flow cytometry was adapted from Naughton et al. (2020). A ten millimeter aliquot of each sample preserved with Lugols Iodine were centrifuged at 3500 $\times g$ for 20 min. The supernatant was removed and the algae pellet was re-suspended in flow buffer. The flow buffer was prepared by adding 1 mM EDTA, 0.2% Tween and 0.1% NaN_3 to 1 L phosphate saline buffer or PBS (Merck). The buffer was filtered using a 0.20 μm filter (Sigma-Aldrich) to remove impurities which may interfere with the flow cytometer. The re-suspended sample was divided into two aliquots (one three milliliter aliquot and one seven milliliter aliquot). The three milliliter aliquot was used for the unstained negative control samples. The seven milliliter aliquot was used for the stained samples. Two hundred microliters of 10X SYBR Green was added to the seven milliliter aliquot and incubated for 15 min in the dark at room temperature. Using two milliliter Eppendorf's (Merck), 1.5 mL from the unstained aliquot and three 1.5 mL's of the stained aliquot were centrifuged at 3500 $\times g$ for 15 min. The supernatant was removed and the pellets were re-suspended in 1.5 mL of fresh flow buffer. Samples were then loaded onto a round-bottomed 96 well plate. Two hundred microliters of each aliquot was loaded onto the plate i.e., four wells containing one unstained and three (triplicate) stained aliquots were loaded for each sample.

The instrument was set to uptake 100 μL of each sample for analysis. The trigger point for the FSC laser was set at 1.0 to eliminate the detection of as much debris as possible in the samples. The FlowJo™ v10.7 software program was used for the analysis of the data generated from the FCM. Gating was used to enumerate the algal and cyanobacterial populations. The gating method was adapted from Haynes et al. (2016), Moorhouse et al.

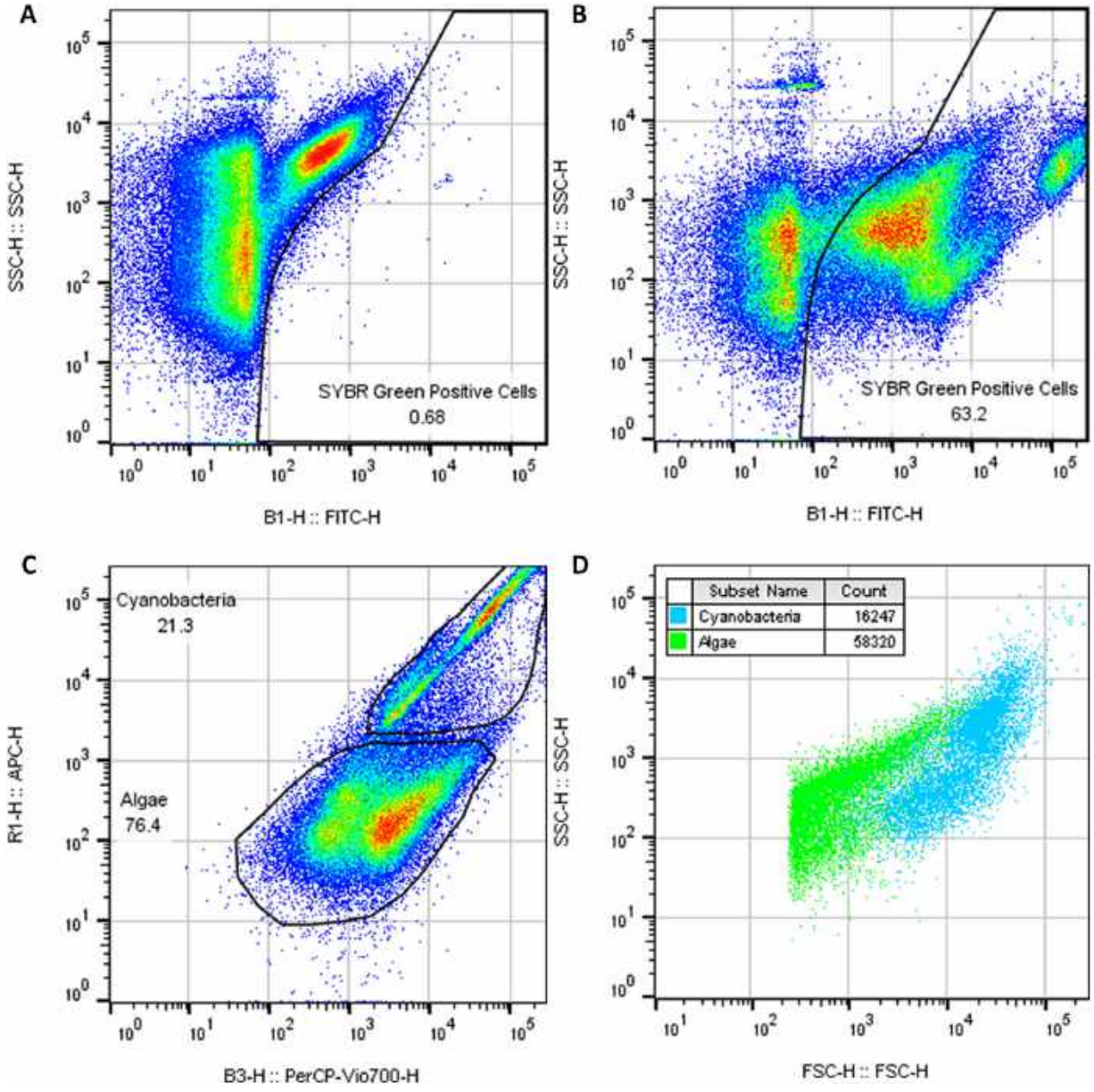

Fig. 2. Flow cytometry dot diagrams for the enumeration of algae and cyanobacteria. A) Unstained samples to eliminate autofluorescence interference, B) cells stained with SYBR Green for the enumerations of algal and cyanobacterial populations, C) chlorophyll and phycocyanin levels used to distinguish between algae and cyanobacteria, D) enumeration of both the algae and the cyanobacteria populations.

(2018), Naughton et al. (2020) and Read et al. (2014). The unstained sample (negative control) was first gated (Fig. 2A) to eliminate as much autofluorescence interference as possible. This gate was then applied to the stained samples (Fig. 2B) in order to identify and enumerate the cells present in each sample. As per Moorhouse et al. (2018) and Naughton et al. (2020), the blue B3 channel, which was used to identify chlorophyll positive cells, was plotted against the red R1 channel, which was used to identify phycocyanin positive cells to distinguish between algae and cyanobacteria. (Fig. 2C). Enumeration of the algal and cyanobacterial populations were then established (Fig. 2D).

2.4. Statistical analysis

All statistical analysis and construction of dose response curves, standard curves, etc. were performed on GRAPHPAD PRISM 7, 8 and 9, and MINITAB 18 and 19. The data generated were grouped and subject to normality tests (Anderson-Darling), to determine if samples were from a normal distribution ($p > 0.05$ = normal distribution). This in turn would establish whether parametric or non-parametric testing was to be conducted on results. As there was normal distribution, parametric testing was applied. *t*-tests and ANOVA were used to determine if any significant

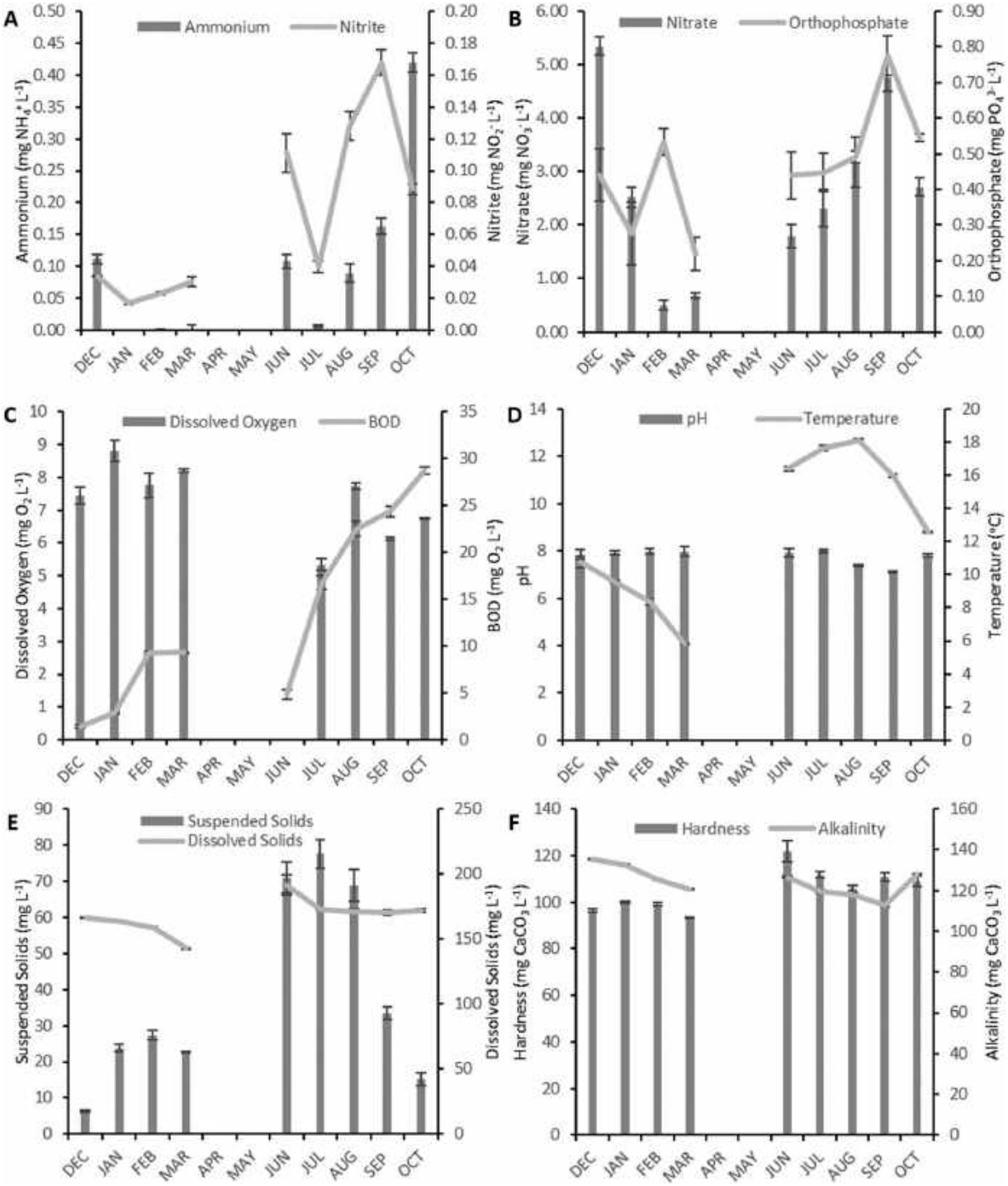

Fig. 3. Breakdown of all physicochemical parameters investigated on the novel trial fish farm between December 2019 and October 2020. Parameters investigated were A) ammonium and nitrite, B) nitrate and orthophosphate, C) dissolved oxygen and biochemical oxygen demand, D) pH and Temperature, E) suspended and dissolved solids, and F) hardness and alkalinity. S.D. indicated, $n = 8$. Samples missing from April and May 2020 due to COVID-19 lockdown and restrictions in the Republic of Ireland.

differences were observed in the variables ($p < 0.05$ = significant difference). Unpaired tests were used as different sets of samples were analysed to assess the quality of the aquaculture water samples. For the correlation studies, the Pearson's correlation coefficient (r) was used to determine whether any relationships existed between any of the parameters investigated.

3. Results & discussion

3.1. Effects of extreme weather variance on the technical performance of a new recirculating IMTA process in the Irish peatland

This timely study describes the effects of extreme weather events experienced in Spring period of 2020 on the performance of a fully-integrated multi-trophic aquaculture process developed in the Irish peatlands. This IMTA process uses a balanced ecosystem of naturally-occurring microalgae, bacteria and duckweed to regulate waste and maintain water quality; in addition, it uses wind turbines as a renewable source of energy to operate aeration systems in the circulatory freshwater aquaculture ponds (O'Neill et al. 2020). This study characterises this IMTA process by way of physicochemical and algae monitoring over a year-long case study. As no statistically significant differences were observed between each of the sampling points results were averaged for ease of reporting (refer to Fig. 3.).

3.1.1. Physicochemical analysis

Prior to the first COVID-19 national lockdown in the Republic of Ireland in March 2020, very little to no NH_4^+ levels were detected within the IMTA farm. This was believed to be due to a combination of issues associated with cyanobacteria levels and increased levels of rainfall experienced in February 2020. NH_4^+ levels increased across all sampling points from the end of July 2020 with spikes of up to 0.90 mg NH_4^+ L^{-1} observed at the beginning of October 2020. However, levels did not rise to greater than the guidance values of 1 mg NH_4^+ L^{-1} (EPA, 2001). Additionally, no NH_4^+ was detected at the discharge point during times of possible overflow and release indicating no potential issues associated with NH_4^+ for the receiving peatlands. Levels of NH_4^+ also decreased between the culture ponds and the duckweed lagoon (treatment lagoon) suggesting that the treatment process was effective at reducing NH_4^+ levels in wastewater.

Before the lockdown period, NO_2^- levels fluctuated between 0 mg NO_2^- L^{-1} and 0.03 mg NO_2^- L^{-1}. However, levels increased greatly after this period, with concentrations spiking to between 0.25 mg NO_2^- L^{-1} and 0.30 mg NO_2^- L^{-1} in mid-July and mid-September, respectively. This was a tenfold increase on previous levels as well as being tenfold greater than the guidance value of 0.03 mg L^{-1} (EPA, 2001). NO_2^- is highly toxic to aquatic life (O'Neill et al., 2019; Pollice et al., 2002) but is extremely unstable and would not remain in this form for long as it would be quickly transformed to NO_3^- (Durborow et al., 1997; O'Neill et al., 2019; O'Neill et al., 2020). As no overflow and release occurred during the times of high levels, the NO_2^- would not cause issues within the bog.

NO_3^- levels dropped considerably the month prior to lockdown (February 2020) going from >8 mg NO_3^- L^{-1} to 0 mg NO_3^- L^{-1}. This coincided with changes in weather conditions and excessive rainfall experienced throughout the month. Once analysis recommenced after the lockdown period, NO_3^- levels slowly increased reaching levels >8 mg NO_3^- L^{-1} in September before dropping back to between 2 mg NO_3^- L^{-1} and 4 mg NO_3^- L^{-1} in October. Levels were well below the guidance value of 50 mg L^{-1} (EPA, 2001). The increased levels in NO_3^- observed in this study may be due to the increased levels of NO_2^- also observed.

PO_4^{3-} levels were observed across all sampling points within the farm. Statistical analysis demonstrated no significant difference between the different sampling points ($p = 0.2160$). PO_4^{3-} levels detected were above the guidance value of <0.035 mg L^{-1} (Irish Statutory OfficeOffice, 2019, 2009) indicating there may be potential issues within the farm and additional treatment process to reduce PO_4^{3-} levels may need to be considered as the algae and duckweed lagoon is not effectively removing it.

Variations in DO levels and BOD levels were observed across the eight sampling points. No statistically significant differences were observed across both parameters (DO $p = 0.1421$, BOD $p = 0.5464$). DO levels fluctuated between 4 mg O_2 L^{-1} and 10 mg O_2 L^{-1}. The recommended DO concentration present in salmonid waters is ≥9 mg O_2 L^{-1} and in cyprinid waters is ≥7 mg O_2 L^{-1} (EPA, 2001). Levels continually increased and decreased above and below these recommended levels. However, they did not drop below the threshold of 4 mg O_2 L^{-1} required for sufficient maintenance of aquatic life (Alam et al., 2007; da Silva et al., 2017; O'Neill et al., 2019; O'Neill et al., 2020).

The SI 272/2009 and SI 77/2019 recommended a mean BOD concentration of 1.30 mg O_2 L^{-1} for high water status and 1.50 mg O_2 L^{-1} for good water status (Irish Statutory Office, 2009; Irish Statutory OfficeOffice, 2019). However, the EPA suggested ≤3 mg O_2 L^{-1} and ≤6 mg O_2 L^{-1} for salmonid and cyprinid waters, respectively (EPA, 2001). Issues were indicated with the BOD levels observed across all sampling points. The BOD is caused by microorganisms using O_2 when consuming organic matter therefore organic matter needs to be reduced in order to decrease BOD levels (EPA, 2001; Gupta et al., 2017; Kasuya et al., 1998; Lee and Nikraz, 2015; Mcintosh and Fitzsimmons, 2003; Sultana et al., 2017). Increasing O_2 levels and the addition of filtration to remove some of the organic matter have been found to decrease BOD levels (Gupta et al., 2017; Lee and Nikraz, 2015). Work is ongoing to reduce BOD levels. However, as no water was released from the farm during times of increased BOD levels, no concerns associated with this issue affecting the surrounding peatland habitat were foreseen.

Fluxes in the levels of suspended solids were observed across all sampling points, while dissolved solid concentrations remained more consistent throughout the study. The levels of suspended solids observed throughout the summer months (June, July, August) were well above the guidance value of 25 mg L^{-1} (EPA, 2001) reaching highs of >120 mg L^{-1}. Given that suspended solids can cause gill irritation, signs of which were observed in some of the fish, this was considered to be a major issue. It was also believed that this issue was linked to the issues with BOD previously mentioned. After filtration methods were applied to different areas of the farm, suspended solid levels dropped back to below the MAC level by September and remained so until the end of the study. Dissolved solid concentrations observed in the study were well below the suggested concentration of <300 mg L^{-1} for excellent water status (WHO, 2003).

Fluctuations were indicated in the temperature range and the pH range observed across all of the sampling points. The elevations in temperature were observed between June and September as would be expected given the season (summer). Although no specific guidance value for temperature could be established as all species of fish have a slightly different optimum temperature, any water released into an aquatic system must be <20 °C (EPA, 2001). Temperatures were >20 °C only once in mid-July. However, as water was not released from the system, this was not deemed to be an issue. Recommended pH levels of between pH 6 and pH 8 were suggested (EPA, 2001; Irish Statutory Office, 2009; Irish Statutory OfficeOffice, 2019). The pH levels remained within this range throughout the study. The pH levels were just below pH 8 however, levels dropped to just above pH 7 from August 2020 to September 2020. Although levels remained within the recommended range, the alteration in pH levels may have had an effect on the BOD issues observed in the farm. Alterations in pH can decrease the rate of organic removal rates thus affecting BOD measurements (Mukherjee et al., 1968).

$CaCO_3$ levels were measured in the eight sampling points in order to determine hardness and alkalinity levels. Statistical analysis was conducted for each parameter and no significant differences were observed (hardness $p = 0.5237$, alkalinity $p = 0.4806$). Hardness levels observed suggested that the water was slight to moderately hard. This correlated with water hardness maps of Ireland which demonstrated water in the midlands around Co. Offaly were also slight to moderately hard. It has been suggested that fish prefer a minimum of 20 mg $CaCO_3$ L^{-1} alkalinity levels. Levels recorded within the farm remained above this optimum threshold throughout

the study (Boyd and Tucker, 2015; EPA, 2001). Alkalinity levels observed in this study demonstrated similar results to those reported by Stephens and Farris (2004b).

All physicochemical findings were then compared to previous research. Results were similar to those observed in other studies with the exception of NO_2^-, suspended solids and BOD (Boaventura et al., 1997; Camargo, 1994; Cao et al., 2007; Caramel et al., 2014; Costanzo et al., 2004; da Silva et al., 2017; Fadaeifard et al., 2011; Guilpart et al., 2012; Lalonde et al., 2014; Moreira et al., 2010; Namin et al., 2013; Noroozrajabi et al., 2013; O'Neill et al., 2019; Pulatsü et al., 2004; Stephens and Farris, 2004a, 2004b; Ziemann et al., 1992; Živić et al., 2009). A ten-fold increase was detected in the NO2-levels and suspended solids levels were higher than the majority of the previous studies, as too were BOD levels. Correlations were indicated between BOD levels and a range of parameters including pH, temperature, dissolved oxygen, alkalinity and suspended solids. This demonstrated the importance of maintaining high DO levels as oxygen is vital for the BOD process. Abnormal or irregular pH levels, which were observed for a time in the farm, can decrease the rate of removal of organic compounds which affect BOD levels. By proxy, changes in alkalinity will also have an impact (Chinedu et al., 2015). Small amounts of all suspended solids are considered volatile suspended solids and exert greater pressures on the oxygen demand thus increasing BOD levels (Gerardi and Lytle, 2015). Finally, as temperatures increase so too does BOD removal rates as higher temperatures enhance microbes respiration rates (Lim et al., 2001). The range of correlations with BOD has demonstrated how complex the process is and may be why issues were encountered in controlling the BOD levels within the farm.

3.1.2. Algae & cyanobacteria analysis

Lower levels of algae were observed during the winter months which is to be expected as temperatures are lower and less sunlight is experienced. The spring month displayed a rise in levels which corresponded with the increase in light and temperature. However, a drop in algal numbers were observed just prior to the first COVID-19 national lockdown that occurred in March 2020. This drop was most likely due to excessive levels of rain fall experienced during the month of February. Algae numbers consistently remained between 1×10^5 and 5×10^5 cells mL^{-1} after the lockdown period until the end of the study. This suggested the stabilisation had occurred. Moderately strong correlations were observed with most of the nitrogen nutrients indicating that NH_4^+, NO_2^- and NO_3^- play a vital role in maintaining optimum algae levels in the novel IMTA process. Results also found that the higher and more stable the levels of NO_3^- present, the more stable the algae numbers. Given that NO_3^- is algae's preferred form of nutrient, and NH_4^+ and NO_2^- are necessary for the natural production of NO_3^- via the nitrification process, this result was expected.

Cyanobacteria levels were also monitored in parallel to the algal numbers, as shown in Fig. 4. Although many species of cyanobacteria can provide beneficial elements (e.g., *Spirulina*) the presence of increased levels cyanobacteria was found to have a negative impact on the novel IMTA system. Increased incidences of mortality were observed as cyanobacterial levels rose. Much like with freshwater bodies, cyanobacteria numbers were always present in the system. They remained below the level of algae being reported highlighting algae's ability to control cyanobacteria levels as both are competing for the nitrogen nutrient source. However, cyanobacteria levels were found to increase just before the lockdown period, demonstrating an inverse relationship with the algae. This suggested that the cyanobacteria were out competing the algae for nutrients. Again, this coincided with extreme weather conditions. Once levels stabilised after the lockdown period, they were once again consistently below the level of algae, remaining between 1×10^4 and 1×10^5 cells mL^{-1}. Reducing in mortality levels also coincided with the stabilisation of cyanobacteria levels.

Correlation studies were conducted between the algae and cyanobacterial levels and all of the physicochemical parameters. In addition to a correlation observed between the algae and the cyanobacteria themselves, correlations were observed between both counts and a range

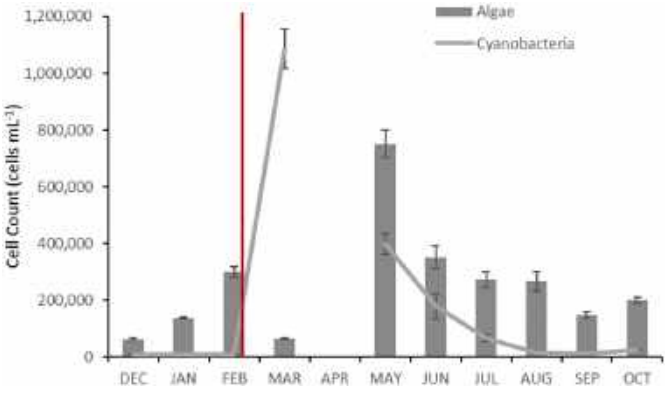

Fig. 4. Cell counts in cells mL^{-1} of algae (bar) and cyanobacteria (line) observed throughout the novel trial fish farm between December 2019 and October 2020. Cell counts conducted via flow cytometry. S.D. indicated, $n = 8$. Samples missing from April 2020 due to COVID-19 lockdown in the Republic of Ireland. Red line indicates when excessive levels of rainfall were observed.

of physicochemical parameters. A correlation was observed with the N parameters (NH_4^+, NO_2^- and NO_3^-) as well as with the P (PO_4^{3-}). As N and P are both necessary for algal growth this was expected. It also highlighted the need to ensure these nutrients were present in the system to ensure the continued presence of algae that was necessary for the novel IMTA process to be effective. The correlation between the pH and algae was also expected as it is well known that although algae can tolerate small fluctuations in pH, increased and more frequent fluctuations can slow down growth rates (Dubinsky and Rotem, 1974).

3.1.3. Climate variance

During this research process, algae demonstrated the potential to be used as an early warning indicator for highlighting issues associated with climate change having experiences a range of different weather conditions including flooding conditions in 2020 where record levels of rain fell due to the development of a number of storm systems in close proximity to one another. February 2020 was one of the wettest on record for the Republic of Ireland. This high level of rainfall was as a result of two extratropical cyclone storms hitting Ireland in that month and in close proximity to one another. Storm Ciara (formed 7th February 2020, dissipated 16th February 2020) and Storm Dennis (formed 11th February 2020, dissipated 18th February) affected Ireland less than a week apart. Just after this weather event cyanobacteria levels began to rise within the farm, as shown in Fig. 4, as well as fish mortalities (up to 44%) which had up until that point remained consistently low (3%). Veterinary post-mortems found signs of hepatotoxicity (liver necrosis) and high instances of gill irritation. Unfortunately, the first lockdown period began in March 2020 and as a result no samples could be analysed after this point until May 2020. Literature searches were conducted remotely to find an appropriate action of reducing or removing the cyanobacterial levels without having additional consequences on the fish health. Both Iredale et al. (2012) and Rajabi et al. (2010) demonstrated successful cyanobacterial control and removal from freshwater bodies with the application of barley straw. This method was suggested and then subsequently applied to the culture ponds at which point the farm reported a reduction in mortalities and cyanobacterial levels. Findings from the overarching Bord Iascaigh Mhara (BIM, 2020) project subsequently revealed that 3402 species of bacteria and microalgae were occurring in the IMTA ecosystem that were identified using next generation sequencing and bioinformatics, of which 1864 are algal. This demonstrated the complexity of the study given the high level of algal diversity within the farm. Of these 1864; 1551 species or sub-species of algae were identified across 210 genera, 60 were identified on family as opposed to genus or species, 42 were classified as uncultured.

In order to determine whether the excessive levels of rainfall indirectly caused issues in February and the lack there of caused issues in May, Met

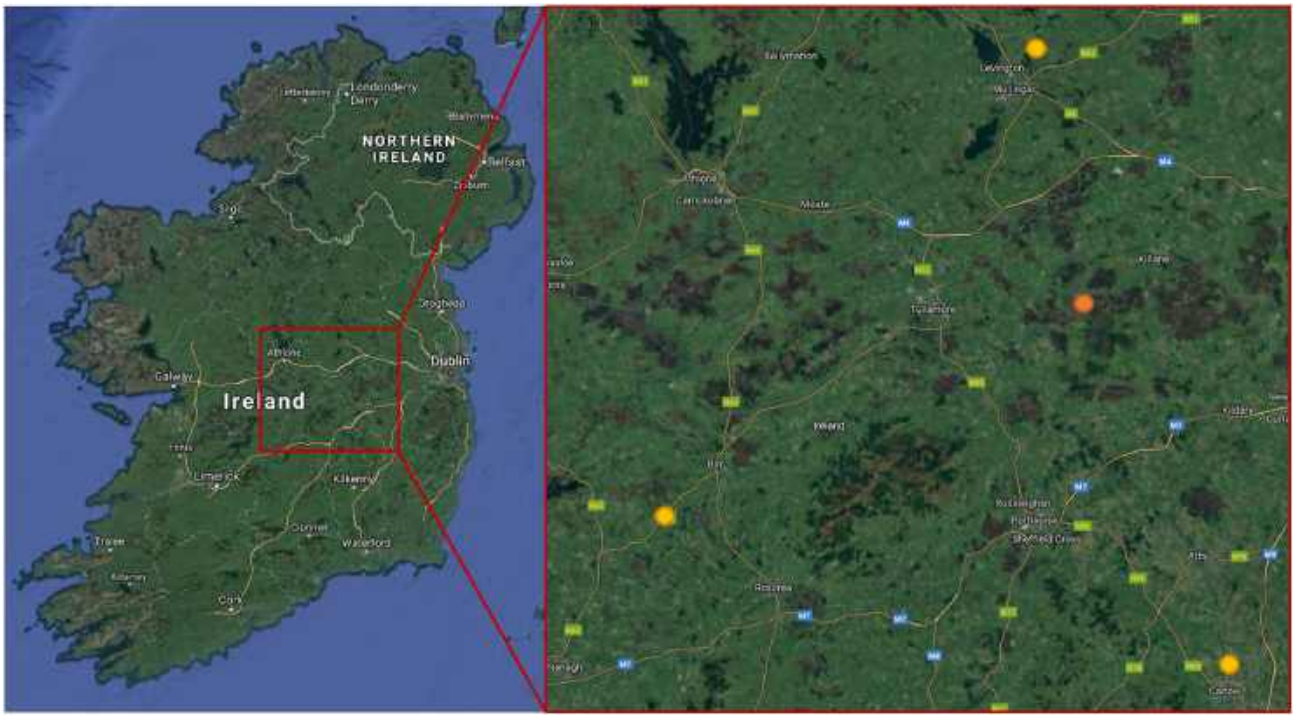

Fig. 5. Map of Ireland indication the approximate location of Oasis fish farm (53°17′03″ N, 07°11′45″ W) indicated with orange, and the three closest Met Eireann weather stations (Gurteen – 53°02′24″ N, 08°00′36″ W; Oak Park – 52°51′36″ N, 06°55′36″ W; Mullingar – 53°33′36″ N, 07°20′24″ W) surrounding the farm, indicated by yellow.

Éireann metadata was once again investigated. The three closest weather stations surround Oasis fish farm at Mount Lucas were used (Mullingar, Co. Westmeath; Oak Park, Co. Carlow; and Gurteen, Co Tipperary), as indicated in Fig. 5. The Irish midlands traditionally get an average of 70.3 mm of rainfall for the month of February. However, according to Met Éireann, 197.7 mm of rainfall fell for that month (Met Éireann, 2021), as shown in Fig. 6. This subsequently would have diluted down all nutrient levels and reduced algal / cyanobacterial numbers within the farm. As nutrients build back up, the ammonium, which is cyanobacteria's preferred source of nutrients, is used by the cyanobacteria before it has a chance to be converted to nitrate via the nitrification process (Herrero et al., 2001) which is the algae's preferred form. This in turn allows the cyanobacteria to grow and out compete the algae. Normally the higher levels of algae and increased levels of nitrate control the levels of cyanobacteria. Leachate or runoff from the bog itself many also be contributing to the issues however no studies on this have been conduct to date. Cyanobacteria levels rose again in May with similar levels or mortality begin observed again. However, there were no instances of rainfall during this instance (Fig. 6.). In fact, May 2020 was considered one of the driest in recent year's (Met Éireann, 2021). No physicochemical analysis could be conducted at this time due to COVID-19 restrictions still being in place. Again, Veterinary post-mortems found signs of hepatotoxicity and gill irritation. The Barley straw had been removed from the ponds prior to this event and was therefore though to be the main cause for the problem. It may also be as a seasonal event as cyanobacteria have been known to "bloom" during the spring and early summer months as temperatures increase. However, as this was the first study ever conducted on a peatland IMTA system additional research and analysis would need to be conducted.

Overland flow of water from rainfall events can impact on infrastructure and cause flooding, which is different to riverine flooding. Overland flow

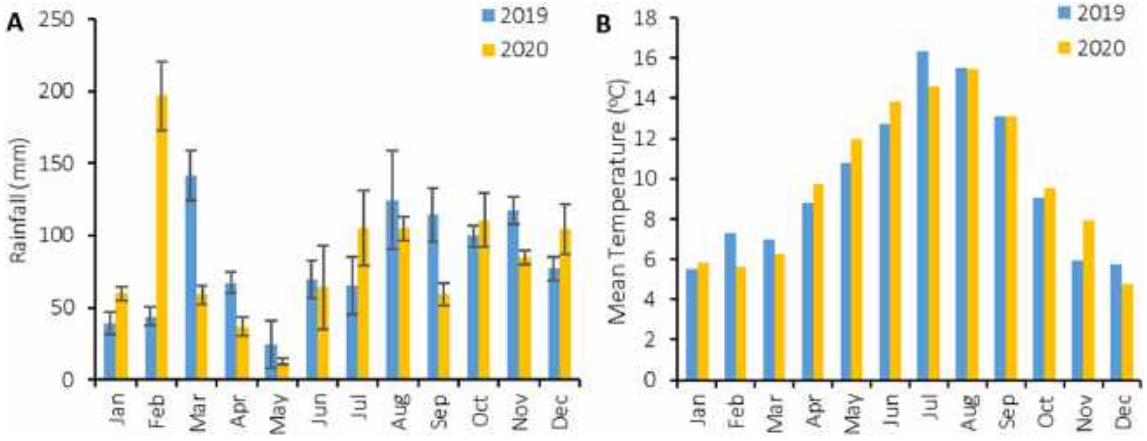

Fig. 6. Average A) rainfall and B) temperature recorded for 2019 (blue) and 2020 (yellow) at three Met Eireann weather stations surrounding the Oasis fish farm during the sampling period of December 2019 to October 2020. Stations were located at 1) Mullingar, Co. Westmeath, 2) Oak Park, Co. Carlow and 3) Gurteen, Co Tipperary. Stations were located north, south-west and south-east of the fish farm, respectively.

Table 2
Relevance of this 'paludiculture-based' IMTA system to enabling the advancement of adjacent and emerging worldwide cross-cutting research and innovation including UN SDGs.

Core topics and enabling innovation, products and services	UN SDG number
Paludiculture 'wet peatland' innovation *Collaborative advancement and standardization of innovation for direct economic viability including horticulture, food production and bio-based industry (Tan et al., 2021; Ziegler et al., 2021) *Ecotoxicology, biodiversity and safety evaluation of emerging innovation and services (Rowan and Galanakis, 2020; Wan Mohtar et al., 2021; Usuldin et al., 2021) *Sustainable Carbon Cycles - supporting the European Commission in reaching climate neutrality through specific focus on quality, credibility and certification of carbon removals in land sector using IMTA system (European Commission, 2021). *Development of novel multimodal sensing technologies, IoT devices, Microsoft Azure Cloud and AI models to inform effective paludiculture innovation and management including carbon reduction (Science Foundation Ireland (SFI), 2021) *Ecosystem Service Management and Pollination, including social enterprises (Goblirsch et al., 2021) *Biorefinery of bio-based products from IMTA-generated microalgae for one-health applications (Wichmaan et al., 2020; Rowan and Pogue, 2021; McKeon-Bennett and Hodkinson, 2021) and bioplastics (Silva, 2021) *Real-time monitoring, testing and development of in-field technologies with sophisticated laboratory equipment, such as flow cytometry with in-field algaTorch™ (Naughton et al., 2020) *Advancement of bioinformatics, next generation sequencing and machine learning (BIM, 2020) *Nexus between water – food – energy for fisheries development including development of LCA, MFA and PCA tools for informing sustainability of products and services (Ruiz-Salmón et al., 2020a,b) *Social marketing, awareness and stakeholder behavioural change towards low-carbon food security including circular bioeconomy (Domegan, 2021) *Effective communication strategies for seafood to promote health and sustainability (Sacchetttini et al., 2021) *Testing of new environmentally-efficient aquafeeds through use of multi-criteria decision-support tools (Cooney et al., 2021). *Sustainable recirculating alternatives to traditional 'end-of-pipe' technologies that negates unwanted pollution to receiving water (Tahar et al., 2017; Rowan, 2019)	1. 2. 3. 6. 7. 8. 9. 11. 13. 14. 15.
Quadruple helix hub concept and approach to sustainable research and innovation *Trans-regional development of Quadruple Helix Hubs (industry-government-academia-society) for advancing low carbon innovation and supporting communities and stakeholders (Rowan and Casey, 2021) *Development of living lab linked to environmental test beds for advancing research, enterprise, entrepreneurship and innovation with nexus to education, training and job creation (Li et al., 2018); Blue Hatch Aquaculture Accelerator Program, 2020: Rowan and Casey, 2021; Rowan and Galanakis, 2020) *Investing and enabling eco-innovation including green finance, design thinking and high potential start up accelerator investment linked to stage gate needs (Rowan and Pogue, 2021) *Early-technical validation (test-the-tech, experimentation/validation in pre-pilot); scaling to real-life setting *Transnational modelling and cluster development including forging European Innovation HUBs such as (Sharebiotech, 2021) and Regional University Network-European Universities (RUN-EU, 2020).	2. 4. 5. 9. 11. 12. 13.
Climate strategy, risk management, awareness and transparency *Publically-backed climate risk insurance for farmers that considers weather indexing for protection against extreme weather events (Doherty et al., 2021). *Risk modelling and assessment of key variables affecting asset and innovation performance and sustainability including use of EPA's SPR model (Tiedeken et al., 2017; Tahar et al., 2017; Tahar et al., 2018) *Supporting sustaining and disruptive innovation and services that also embraces technological, political and societal readiness levels (Geels, 2018; Rowan and Casey, 2021; Schuelke-Leech, 2021) *Current limited short-term focus on risk management, transparency post review of 1168 companies. Also, a lack of understanding of climate risk with few companies having climate strategies (Coppola et al., 2019) *International consensus on best indicators reflecting climate resilience, economy, society and culture (Barry and Hoyne, 2021).	4. 9. 11. 12. 13.
COVID-19 pandemic *Food and supply chain disruption including research provision (Guan et al., 2020; Herreor et al., 2020; Galanakis et al., 2021; Rowan and Galanakis, 2020) *Biorefining alternative sustainable materials for multiple-use PPE (Rowan and Laffey, 2020a,b; Rowan and Moral, 2021) *Food security and safety (Galanakis et al., 2021)	1. 2. 3. 9.
International policy and cohesiveness *Informing EU Green New Deal; UN Sustainable Development Goals; Paris Climate Agreement (Galanakis et al., 2022)	1. 2. 4. 9. 11. 16.
Climate change and action plans *Weather events and disease mitigating simulation and predictive models (Jenkings and Kane, 2019) *Development of in-field biosensors (and digital-sensors) to monitor performance of agri-food security linked to extreme weather events (Naughton et al., 2020; O'Neill and Rowan, 2022). *Informing multi-disciplinary and cross-functional international projects to seek agreement on climate change indicators that addresses ecosystem resilience with nexus to economic and sociocultural indicators (Barry and Hoyne, 2021).	3. 4. 13. 14. 15.
Digital transformation *Development of drones and satellites, IoT devices, AI and cloud-enabled technologies to monitor carbon sequestration of peatlands and other land types to improve understanding on human activity on land use and how it relates to climate change (Science Foundation Ireland (SFI), 2021) *ArcGIS mapping to inform restoration and rehabilitation of peatlands linked to ecology/biodiversity to inform carbon cycles (Rowan and Casey, 2021) *Development of digital twin that includes ARVR (Quality of Experience) for remote specialist virtual training (Braga Rodriguez et al., 2020; Rowan and Galanakis, 2020) *Precision agriculture including blockchain, AI, robotics, along with combining machine learning with bioinformatics to future proof environmental test beds and validate new products and services including ecological/biodiversity forecasting (Rowan and Pogue, 2021; Rowan et al., 2021; George et al., 2021) *Rainfall radar data and rainfall information collected from commercial microwave mobile phone backbone transmission networks (Chwala and Kunstmann, 2019); worldwide there is an interest in changes to sub-daily rainfall regimes for example through the INTENSE project (Blenkinsop et al., 2018) *Agriculture IoT, construction of agriculture IoT infrastructures, data security and data sharing, sustainable energy solutions, economic analysis and operation management in agriculture IoT, and IoT-based agriculture financing and e-business modes. IoT-based precision agriculture (Ruan et al., 2019).	1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 13. 14.

United Nations Sustainable Development Goals (UN SDGs); Augmented Reality Virtual Reality (ARVR); Life Cycle Analysis (LCA); Material Flow Analysis (MFA); Principle Component Analysis (PCA); Artificial Intelligence (AI); Internet of Things (IoT)

occurs when either the ground is already saturated, due to previous rainfall, and the new rain falling can only run off; or when the rainfall intensity exceeds the infiltration rate of the ground and again the rain will run off, these situations are often called a flash flood (Dimitriou, 2011). Detecting trends in long-term rainfall records is difficult due to the highly variable nature of rainfall and scarcity, often, of long records. Information on rainfall intensity, explicitly sub-daily and sub-hourly records, is much more sparse and short term in its record; although, data generated from growing hobby weather station networks can be potentially used to help fill this gap. Other sources could include processed rainfall radar data and rainfall information can be gathered from commercial microwave mobile phone

backbone transmission networks (Chwala and Kunstmann, 2019). Worldwide, there is an interest in changes to sub-daily rainfall regimes; for example, through the INTENSE project (Blenkinsop et al., 2018).

Extratropical cyclones, produce more than 70% of the winter rainfall in north-west Europe. The islands of Ireland and Great Britain had their stormiest winter on record during 2012/2013 with more than two intense cyclones per week (Priestley et al., 2017). It is plausible that impacts due to global climate change in the sea surface temperatures in the Western tropical West Pacific along with the reduction in Arctic sea-ice may have allowed this stormy period of weather to occur. Noone et al. (2016) working on a recently homogenised Irish rainfall data set from 1850 to 2010 found there was a positive trend for the winter months and a negative trend for the summer months. In Ireland there are interests in the impact of unusual rainfall events on infrastructure. The impact of rainfall events and the destabilisation of railway embankments in Ireland was studies leading to an intensity and duration curve for significant failures (Martinović et al., 2018). A similar approach may be able to be taken in safeguarding other assets including aquaculture. There is also interest in blanket peat failures on slopes in Ireland, due to excess rainfall (Jennings and Kane, 2019). Flood impacts on aquaculture have been studied in the Czech Republic where during flooding in 2002, 2006, 2009, and 2013 some 54% of fish in pond-based system were lost (Rutkayová et al., 2018). Many of these losses seem to be from flash flood events attributed to riverine and overland flooding. Rutkayová et al., 2018 found the impacts to be different between juvenile and adult fish, and by species. These researchers also noted the importance in term of impacts of what they term the 'train effect', which is a repeated series of large rainfall events over the same area in a short space of time. This is a classic high impact situation where a series of non-record breaking, but high events when combined over a short space of time can have extreme impacts. Commensurate use of tools to inform management and decision-making at the farm level informed by life cycle assessment, material flow analysis and risk modelling will support and enable solutions to these complex challenges (Tahar et al., 2017; Ruiz-Salmón et al., 2020a; Ruiz-Salmón et al., 2020b).

4. Implications and opportunities of IMTA findings for informing emerging paludiculture and adjacent research and innovation

Findings from this fully-recirculating IMTA study have broader implications for informing worldwide collaborative research and innovation in many multidisciplinary and cross-functional domains beyond the initial focus of paludiculture (Table 2), and alignment with United Nations' Sustainable Development Goals (Table 3). Traditionally, there remains reliance on flow-through aquaculture processes that relies upon bespoke understanding of process performance and interventions including end-of-pipe solutions to safeguard receiving waters from polluted effluents (Rowan, 2011; Tahar et al., 2017). The reliable and repeatable operation of this freshwater IMTA process will enable generation of data that can inform the international development and standardization of paludiculture innovation (Tan et al., 2021; Ziegler et al., 2021). This wet-peatland aquculture innovation can be used for informing efficacy of carbon sequestration, GHG emission reductions and carbon cycles (Science Foundation Ireland (SFI), 2021), ecotoxicology and biodiversity (Rowan and Galanakis, 2020), food production, safety and security (Galanakis et al., 2021; Sacchetttini et al., 2021), ecosystem service management and pollination (Goblirsch et al., 2021), real-time monitoring of in-field technologies linked to living labs (Naughton et al., 2020); suitability of new environmental-friendly aquafeeds (Cooney et al., 2021), digital transformation including novel multimodal sensing technologies, IoT devices, AI and cloud-based innovation to inform effective management of carbon reduction (Science Foundation Ireland SFI, 2022); along with use of biorefinery concept to extract high value bio-based materials from microalgae and duckweed for one health applications (Rowan and Pogue, 2021) (Table 2).

Table 3
Indicative examples of IMTA activities and tools to support, enable and accelerate potential green innovation disruption under United Nations' Sustainable Development Goals (SDGS*).

UN sustainable development goal*		Sustaining or potentially disruptive activity
1	No poverty	Food production and value chain. Food security and managing climate events to avoid supply chain disruption. Production of high protein sustainable foods via IMTA system. Researcher mobility creating opportunities in education.
2	Zero hunger	Development and future climate-proofing sustainable agri-food processes and crops along with alternative sources for protein that includes training.
3	Good health and wellbeing	Adopting One Health approach to informing eco-green innovation community transition and social enterprise for health including COVID-19 that considers bio-refinery concept (Rowan and Galanakis, 2020; Galanakis et al., 2021)
4	Quality education	Openly sharing knowledge, discoveries and growing collaborations in academia, that cross-cuts STEM with Social Science and humanities, to inform behavioural change. Use of IMTA for specialist training linked to new eco innovation education that encompass green finance, food disruption, climate change.
5	Gender equality	IMTA framework is aligns with gender equality focused with equal representation in research, innovation, entrepreneurship, social enterprise, outreach, education.
6	Clean water and sanitation	Innovative green research and enterprise to promote natural resources for water quality and mitigate waste that moves beyond end-of-pipe solutions
7	Affordable and clean energy	IMTA powered by wind turbines, but also enables development of LCA tools for investing nexus between energy, water and food systems. This also enables commensurate research on sustainable carbon cycles (European Commission, 2021) and green energy using paludiculture platform.
8	Decent work and economic growth	IMTA adopts Quadruple Helix Hub approach that connects industry, government, academic and society – this will lead to informing new job creation and new eco-focused Start Ups along with risk management, transparency for climate action governance in business beyond short-term needs (Rowan and Casey, 2021). This will inform future economic indicators and growth via international collaboration.
9	Industry, innovation, infrastructure	IMTA is trigger new eco or green innovation and research that considers future infrastructure to provide standardization of outcome sets for climate change and sustainability using living labs and environmental demonstrator facilities – this will pump-prime new bio-based research, food production and circularity.
10	Reduced inequalities	Adopting a Quadruple Helix approach will enable broad stakeholder engagement to ensured equalities are met.
11	Sustainable cities and communities	IMTA system is a green innovation aligned with supporting needs of low-carbon communities for regional development and regeneration. This is aligned with European Just Transition and European Green Deal initiatives. This will be informed by social marketing and appropriate communication strategies (Domegan, 2021; Sacchetttini et al., 2021).
12	Responsible consumption and production	IMTA system will support digital transformation of what is a defined sustainable process to support responsible consumption, and production that will inform global needs. Future disruptive innovation likely to emerge from digital domain.
13	Climate action	IMTA system can inform efficacy of future food production processes as it pertains across addressing climate events and inform environmental, ecological, social, political and cultural indicators as state-of-the-art collaborative international demonstrator facility.
14	Life below water	IMTA supports freshwater aquaculture and studies on biodiversity related to natural aquatic ecosystems in peatlands
15	Life on land	IMTA supports biodiversity, pollination and ecosystem service management
14	Peace, justice, strong inst.	IMTA has core tenets that blends academia, industry, authority with communities.
17	Partnerships for the goals	IMTA supports and enable national and international partnerships aligned with UN SDGs that includes mobility and training.

This IMTA process will also support and enable development of low-carbon community and social enterprises promoted through the European Just Transition and Green Deal Initiatives (Rowan and Galanakis, 2020), which can be effectively managed through the use of 'Quadruple Helix Hubs' that converge academia, industry, government and society (Rowan and Casey, 2021). This Quadruple Helix Hub framework will also support and enable the commensurate delivery of paludiculture-based and adjacent research, innovation, entrepreneurship linked to education and specialist training of stakeholders and communities, which are essential converging activities underpinning trans-regional European Innovation Hubs. The development and deployment of a validated IMTA process in the peatlands will also support future sustaining and disruptive innovation, where the design, applicability and maturity of the innovation, product or service may be informed by use of various smart evaluation tools including technical, political and societal readiness levels (Fig. 7), life cycle assessment, material flow analysis, principle component analysis, ecotoxicology and so forth. For example, Ruiz-Salmón and co-workers (2020b) used LCA to highlight the important nexus between water, food and energy for development of fisheries including aquaculture across the European Atlantic Area. The open knowledge sharing of IMTA data will also support research into new green business development and transnational modelling of research and innovation (Rowan and Casey, 2021), which will include climate-related awareness, risk management and transparency (Tahar et al., 2019; Coppola et al., 2019) (Table 2). IMTA process and environmental data that can inform future risk mitigation for innovation will be particularly relevant. For example, Coppola et al. (2019) reported that Deloitte asked 1168 Chief Financial Officers (CFOs) what their companies are doing to help address climate change where a thorough understanding of climate risks was rare, few companies had governance mechanisms in place to implement comprehensive climate strategies, and targets for carbon emission reductions were usually not aligned with the Paris Agreement. Coppola et al. (2019) also noted that more than US$30 trillion in funds were held in sustainable or green investments in the five major markets tracked by the Global Sustainable Investment Alliance. The measured effects of extreme weather events, using this technically-defined IMTA process, will also inform development of predictive and simulated disease models influenced by climate change (Interreg Neptunas Project, 2019).

Barry and Hoyne (2021) reported that changes in weather systems, such as increased precipitation, snow and ice events, heatwaves and storms, have led the European Commission to develop new policies and strategies to deal with extreme events. Findings from, and future use of this IMTA system, will help inform a robust set of appropriate indicators to assess the impacts of climate change on adaptive food production and security at local and national levels. Barry and Hoyne (2021) noted that these indicators must encompass a multidisciplinary scope that also embraces ecological, social, cultural and economic changes, with greater awareness within all areas of society (Table 2). Process technical parameters used in this IMTA study will provide a useful environmental case study to help evaluate impact of climate change on paludiculture and other food systems beyond baseline observations of rainfall, temperature, GHG emissions, sea level rise/flooding and soil degradation (Aguiar et al., 2018). There is a pressing need for international agreement of relevant indicators existing outcome set used for scientific audiences that also address public interest and effects of climate change; these should allow public consumption and education through clear and concise communication standards (Williams and Eggleston, 2017). These IMTA findings contribute to climate resilience that relates to the capacity for ecosystems to respond to impacts, events or disturbances that are associated with climate change (Baho et al., 2017).

Moreover, Barry and Hoyne (2021) suggested seeking international agreement on indicators to inform ecological resilience, along with economic (such as number of new SME creation, innovation, investment in training, and specialist upskilling), social enterprise and cultural indicators (such as diversity of youth initiatives to increase civil action, solidarity and engagement). This IMTA system will also support need to inform natural capital and improvements in biodiversity, such as by promoting

Technology Readiness Levels* (TRL)*		Society Readiness Levels** (SRL)		Policy Readiness Levels*** (PFL)	
TRL 1 – **Basic Research -** Principles postulated and observed –no experimental proof (Discovery)	Knowledge development Academia	SRL 1 – **Basic Research** - identifying problem and identifying societal readiness (Discovery)	Knowledge development Academia	PRL 1 –**Basic Research** - identifying issue/problem and identifying policy readiness (Discovery)	Knowledge development Academia
TRL 2 – **Technology Concept Formulated** – concept and application defined (Concept Definition)		SRL 2 – **Formulation** of problem, proposed solution(s) and potential impact, expected societal readiness; identifying intended stakeholders for project (Concept Definition)		PRL 2 – **Formulation** of issue/problem, proposed solutions and potential impact; expected policy readiness; concept identification relevant to stakeholders (Concept Definition)	
TRL 3 –**Experimental Applied Research Concept** – first laboratory tests completed (Proof of Concept)		SRL 3 – **Applied Research** - initial testing of proposed solution(s) with intended stakeholders (Proof of Concept)		PRL 3- **First testing** of proposed solution(s) with relevant stakeholders; modelling, consultations, feedback, development complete (Proof of Concept).	
TRL 4- **Technology Validated in Lab** - Small scale prototype – built and tested in lab (lab validation)		SRL 4 – **Pilot-Test Scale** - concept validated through pilot testing in relevant environment to substantiate proposed impact and societal readiness (Concept Validation)		PRL 4 – **Problem validated "in lab"** through pilot testing in intended environment to substantiate proposed impact, policy readiness, feedback development (lab validation)	
TRL 5 **Large-Scale Prototype tested** in intended environment (test facility validation	Technology Development Collaboration	SRL 5 – **Large Scale Test/system -** proposed solution(s) validated; with intended stakeholders ("open water" validation)	Societal Development Collaboration	PRL 5 – **Proposed solution(s) validated**; now by intended stakeholders in the area for application ("open water" validation)	Policy Development Collaboration
TRL 6 – **Technology demonstrated in intended environment** – close to expected performance ("open water" validation)		SRL 6 – **Demonstrated system** - solution(s) demonstrated in relevant environ and with intended stakeholders for feedback on policy		PRL 6 – **Demonstration system** in intended environ & with intended stakeholders at pre-role out scale for feedback on impact (system demo)	
TRL 7 – **System prototype demonstration in operational environment** – at pre-commercial scale (system demo)		SRL 7- **System Refinement** - refinement of product, and/or solution(s), and if needed, retesting in intended environment with stakeholders (refinement)		PRL 7 – **System refinement** of scheme and/or solution(s), and possibly, retesting in intended environment with intended stakeholders to gain feedback (refinement)	
TRL 8 – **First system complete, qualified, verified** – First commercial system – manufactured issues solved (verification)	Business Development Industry	SRL 8 – **First System** – issues solved, proposed solution(s), as well as plan for societal adaption complete, and qualified (verification)	Stakeholder Development Governing	PTL 8 – **First System** - proposed solution(s), as well as plan for policy **adaptation complete, and qualified** (verification)	Scheme development Government
TRL 9 – **Actual Full commercial system proven in operational environment** – technology available for beneficiaries (deployment)		SRL 9 – **Full Social System** - actual project solution(s) in intended or relevant environment (deployment)		PRL 9 – **Policy Implementation** - actual project solution(s) proven in relevant environment. Issues solved, continued monitoring, evaluation, and review of scheme/solution (deployment)	
*TRL – are indicators of status or maturity level of particular technology been researched and commonly used for European Commission in context of Horizon Europe.					
**SRL – Used to assess the level of societal adaptation to project, technology, product, process or management practice , or innovation to be integrated into society					
***PRL – Used to assess the level of societal adaptation to project, technology, product, process or management practice or innovation to be integrated into policy					

Fig. 7. Applying IMTA ecosystem to develop and track new eco-innovation to align and commensurate with technology, societal and policy readiness levels (adapted from Rowan and Casey, 2021).

agroecological farming, re-establishing organic carbon and microbiota in the soil and land, the potential use of biochar (Galanakis et al., 2022). Funding instruments, such as the European Just Transition Fund with an overall budget of €17.5 billion will help with a fair and equitable transformation to low-carbon communities where challenges will create opportunities through digitalisation that will boost employment and growth (Table 2). George et al. (2021) reported that digital sustainability and entrepreneurship can help tackle climate change and sustainable development (Table 2). Findings of this study will also support research informing sustaining and disruptive innovation (Schuelke-Leech, 2021), where greater awareness will be enabled through social marketing (Domegan, 2021). Bio-based products harvested and refined from microalgae occurring in this IMTA ecosystem can contribute to one-health solutions, including potentially contributing to alleviating COVID-19 (Murphy et al., 2020, 2022; Pogue et al., 2021).

There is a commensurate need to develop tailored communication strategies for promoting greater awareness of health and sustainability in seafood consumption given the diversity of attitudes and perceptions reported among Italian consumers by Sacchetttini et al. (2021) (Table 2). Moreover, Domegan (2021) highlighted the pivotal role of social marketing in critically examining the interface of human and natural systems and their interconnected dynamic forces as a powerful means of influencing behaviours for the accorded transformation and betterment of individuals, communities, society and the planet. In pursuit of Green Deal Innovations, such as embodied in this IMTA process, critical emerging trends in social marketing embrace important systems science, stakeholder engagement and digital technologies.

5. Conclusion

- Peatlands-based IMTA constitutes a potentially important sustainable and resilient system for advancing aquaculture, which will also support and enable development and validation of other sustaining and disruptive innovation, products and services.
  - While this IMTA model successfully produces commercial fish, and typically does not discharge to receiving water, this present study highlighted the challenges of operating a recirculating system with increased, and unexpected rainfall due to storms.
  - This IMTA system can help with improved understanding of environmental, social, cultural and economic indicators for broader appreciation of climate impacts based upon the people who are directly affected by the changes at local and national levels.
  - There is a pressing need to develop real-time approaches to monitor algae species that are potentially toxigenic, and to thoroughly investigate the impact of extreme weather events on balanced algal and microbial population in freshwater aquaculture ecosystem.
  - Given that there are potentially 1864 species of algae occurring in this peatland IMTA system that were identified using next generation sequencing and bioinformatics, there are pressing opportunities to exploit advances in flow-cytometry combined with machine learning to help unlock the challenge of purposeful monitoring.
  - Accessing and using weather data will present using opportunities to inform in field monitoring tools; for example, Ireland has a long-term data set of daily rainfall across 25 stations.
  - COP26 highlighted the pressing importance of enlisting a global response to curb carbon emissions so at to limit temperature rises to 1.5 °C; therefore, there is a commensurate need to investigate and test new sustainable food production systems (such as IMTA) that considers and hurdles extreme weather/climate events.
  - Biorefining products from this IMTA system using circular concept will help advance adjacent innovation, such as developing and testing fish feed improved for immune-stimulation to boost fish immunity to disease may help with priming against unforeseen events.
  - Further development and use of this IMTA process will help communities transition to low-carbon economies; however, there is a commensurate need to improve awareness in sustainable eco-innovation that can be improved by increasing social enterprises and by supporting the creating of new businesses such as through the Quadruple Helix Hub concepts that aligns with collaborative European Innovation Hubs (EIH), European Green deal and European Just Transition progamme initiatives.
  - A future climate-proofed IMTA system will also support and help meet the United Nations Development Goals, particularly alleviating against poverty, hunger along with fostering quality education, economic growth and innovation.
  - There are pressing opportunities to advance paludiculture through digital transformation, which may lead to business model or green-disruption.
  - Use of IMTA-system data to inform IoT-based precision paludiculture and agriculture.

CRediT authorship contribution statement

E. A. O'Neill, **N. J. Rowan**: Conceptualization. **E. A. O'Neill**: Data Curation. **E. A. O'Neill**, **A. P. Morse**, **N. J. Rowan**: Formal Analysis. **N. J. Rowan**: Funding Acquisition. **E. A. O'Neill**: Investigation. **E. A. O'Neill**: Methodology. **E. A. O'Neill**: Project Administration. **E. A. O'Neill**, **N. J. Rowan**: Resources. E. **A. O'Neill**: Software. **N. J. Rowan**: Supervision. **E. A. O'Neill**: Validation. **E. A. O'Neill**: Visualization. E. **A. O'Neill**, **A. P. Morse**, **N. J. Rowan**: Roles/Writing – Original Draft. **E. A. O'Neill**, **A. P. Morse**, **N. J. Rowan**: Writing – Review & Editing.

Declaration of competing interest

The authors declare that there are no competing interests or conflicts of interest with respect to the publication of this article.

Acknowledgements

The authors would like to thank Bord Iascaigh Mhara (Project BIM 19-KGS-008) and Interreg Atlantic Area Neptunus (Project EAPA_576/2018) for funding support. We thank Gustavo W. Fehrenbach for assistance with microscopic analysis of samples during COVID-19 lockdown period.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153073.

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Science of the Total Environment 692 (2019) 209–218

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Novel use of the alga *Pseudokirchneriella subcapitata*, as an early-warning indicator to identify climate change ambiguity in aquatic environments using freshwater finfish farming as a case study

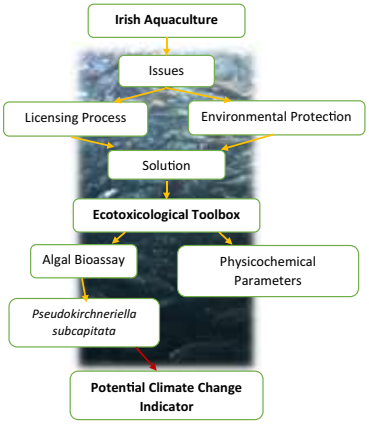
Emer A. O'Neill *, Neil J. Rowan, Andrew M. Fogarty

Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co., Westmeath, Ireland
Department of Life & Physical Science, Faculty of Science & Health, Athlone Institute of Technology, Dublin, Road, Athlone, Co. Westmeath, Ireland

HIGHLIGHTS

- Fish farm effluent monitored using algal bioassay and physicochemical parameters.
- Alga (*Pseudokirchneriella subcapitata*), more responsive model than physicochemical parameters alone.
- Standard water quality parameters are not applicable to fish farm wastewater.
- Duckweed and constructed wetlands were under appreciated in wastewater treatment.
- Alga demonstrated potential use as early warning system for climate change.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 May 2019
Received in revised form 15 July 2019
Accepted 15 July 2019
Available online 17 July 2019

Editor: Damia Barcelo

Keywords:
Ecotoxicological
Bioassay
Physicochemical
Fish
Monitoring
Sustainability

ABSTRACT

Aquaculture is one of the fastest growing food producing industries in the world. This dramatic increase in growth has raised many environmental concerns. Evaluation of fish farm effluent is frequently assessed by physicochemical parameters. This approach indicates potential degradation caused by the effluent and not cumulative effects on aquatic ecosystems. This study investigated relationships between physicochemical parameters (temperature, pH, conductivity, nitrogen, phosphorus, oxygen and suspended solids), typically used to assess water quality with the *Pseudokirchneriella subcapitata* algal bioassay, which evaluated the potential ecotoxicological effects that freshwater fish farm effluent has on its receiving ecosystems and organisms. Influent and effluent samples were collected from a freshwater farm facility every two weeks from April 2018 to October 2018 in the Republic of Ireland. This monitoring period coincided with one of the warmest and driest periods recorded by meteorological stations in the Republic of Ireland. Physicochemical analyses were found to be similar to those in other farm studies. After exposure of algae to the effluent, stimulation of algal growth rates increased by >50%. This stimulation was observed during periods of increased temperatures which were as a result of heat wave and drought conditions experienced during monitoring. Correlation studies identified a moderately strong relationship between algal stimulation and temperature ($r = -0.619$). This study discovered that removal of *Lemna minor* (aquatic plant), impacted strongly on the freshwater farm pond-process to cope with nitrates.

* Corresponding author at: Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co., Westmeath, Ireland.
E-mail address: e.oneill@research.ait.ie (E.A. O'Neill).

https://doi.org/10.1016/j.scitotenv.2019.07.243

The constructed wetland system was unable to efficiently treat nitrates and phosphates during conditions of drought. These findings indicate that standard water quality parameters may not be applicable to inform appropriate suitability of fish farm effluent for discharge to receiving water. The research conducted in this study has suggested a potential toolbox that includes *P. subcapitata* may provide an early warning system for adverse effects as a result of climate change.

1. Introduction

Aquaculture is one of the fastest growing food producing industries in the world (Fečkaninová et al., 2017; Liu et al., 2017), and provides one of the most sustainable forms of edible protein (Liu et al., 2017; Yue and Wang, 2017). The dramatic increase in the growth of global aquaculture production indicate its importance in modern day food supply (Jegatheesan et al., 2011), by providing a means to meet the growth in global demand (Seoane et al., 2014). However, despite this and many other advantages (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014), there are many environmental concerns thought to be associated with aquaculture (Martinez-Porchas et al., 2014; Ngo et al., 2016; Troell et al., 2017), and in particular to the impacts fish farm effluent is thought to have on the receiving aquatic ecosystem (Jegatheesan et al., 2011).

In 2017, the Irish aquaculture sector produced 47,147 t of fish valued at €208.4 M which was an increase of almost €100 M since 2014 (Bord Iascaigh Mhara, 2018). The Irish aquaculture sector is primarily based in coastal areas but land-based recirculating aquaculture systems (RAS), and more traditional freshwater and land based systems are also used (Department of Agriculture Food and the Marine, 2015), and are projected to potentially grow rapidly over the next 10 years. The sector provides valuable employment on a year round basis and aids in the preservation of viable local and rural communities (Department of Agriculture Food and the Marine, 2015). The Irish aquaculture sector can be divided into shellfish and finfish culture with rope mussels, bottom grown mussels, Gigas oysters, salmon and trout the main species cultured. (Department of Agriculture Food and the Marine, 2015; Bord Iascaigh Mhara, 2018). Irish aquaculture also produces a wide range of novel species, both in marine and freshwater, such as Abalone, sea urchins, seaweed and perch (Bord Iascaigh Mhara, 2018).

Fish farm effluent typically contains nutrient rich waste products (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; Sikder et al., 2016), which if released untreated into water bodies can lead to water pollution (Jegatheesan et al., 2011; Sikder et al., 2016). It may cause direct negative effects such as eutrophication (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; Sikder et al., 2016; Troell et al., 2017), which is one of the greatest concerns (Ngo et al., 2016). Eutrophication is a process by which a water body receives large levels of nutrients and organic matter that can be taken in and biologically processed (Martinez-Porchas et al., 2014; Sikder et al., 2016), which in turn can result in increased levels of algal blooms and decreased levels of oxygen which can suffocate aquatic life in the water body (Jegatheesan et al., 2011; Chislock et al., 2013; Ngo et al., 2016). Fish farm effluent is commonly characterised by rich levels of nutrients, such as nitrogen and phosphorous, and organic matter (Jegatheesan et al., 2011; Ngo et al., 2016). These characteristics are as a result of uneaten or left over food and metabolic waste products such as faeces and urea (Jegatheesan et al., 2011; Martinez-Porchas et al., 2014; Ngo et al., 2016; Troell et al., 2017). In fact, the primary source of nitrogen, phosphorous and organic matter in the water is derived from the application of feed (Jegatheesan et al., 2011), which is necessary to maintain high production yields in order to meet demands (Kolarevic et al., 2014; Feucht and Zander, 2015). Another source of organic matter is the influent used to fill ponds or raceways and maintain the farms water levels (Jegatheesan et al., 2011).

The evaluation of fish farm effluent water quality is frequently assessed by the measurement of physicochemical parameters (da Silva et al., 2017), which include nitrogen, phosphorus, oxygen, suspended solids, temperature, pH, alkalinity, hardness and conductivity. Ammonium (NH_4^+) is highly toxic to aquatic life (Zhang et al., 2011), and requires treatment before its release into its receiving water body (Celik et al., 2001). Nitrite (NO_2^-), is very toxic to aquatic life (Pollice et al., 2002), but highly unstable and only remains in this form during for a short period of time during the transformation of NH_4^+ to nitrate (NO_3^-), (Durborow et al., 1997). Orthophosphate (PO_4^{3-}), a reactive form of phosphorus (Brogan et al., 2001), is one of the main causes of algal blooms and the hypoxic conditions which may occur in water bodies (Brogan et al., 2001; Barcellos et al., 2019). Biochemical oxygen demand (BOD), is the amount of oxygen used by bacteria in breaking down organic matter in the water (Lee and Nikraz, 2015). Chemical oxygen demand (COD), measures the stress a quantity of organic matter puts on a receiving water body (Lee and Nikraz, 2015). Suspended solids often consist of organic matter and elevated levels can be an indicator of eutrophic conditions (Bilotta and Brazier, 2008). Temperature is a critical environmental factor for fish farming due to its effect on growth, metabolism, survival, immune responses and oxygen consumption (Ferreira et al., 2011). Calcium carbonate ($CaCO_3$), improves conditions for benthic animals and microbial activity and increases CO_2 (Ferreira et al., 2011). The alkalinity is the buffering capacity of the water body and is related to important factors in fish farming and water hardness is the amount of dissolved calcium and/or magnesium present in the water (Ferreira et al., 2011). Investigation of these parameters alone only indicate the potential degradation caused by the effluent at a given time, not their effects on aquatic ecosystems and organisms (Stephens and Farris, 2004a, 2004b; da Silva et al., 2017). Thus, ecotoxicological bioassays are used in conjunction with physicochemical analysis however, there are few studies that assess the toxic effects of fish farm effluent on aquatic ecosystems and organisms (da Silva et al., 2017).

Planktonic microalgae are primary producers and are a key component in the food chains of aquatic ecosystems (Aruoja, 2011). Sphaeropleales are one of the most dominant groups of green microalgae in the world and contain species that are considered to be very important to freshwater ecosystems (Suzuki et al., 2018). According to Rodgher et al. (2012), these microalgae constitute an important group of highly sensitive photosynthetic organisms that are frequently used to assess aquatic ecosystems. One such species in this group is *Pseudokirchneriella subcapitata* (*P. subcapitata*), CCAP 278/4, also commonly known as *Raphidocelis subcapitata* or *Selenastrum capricornutum* (Aruoja, 2011; Rodgher et al., 2012; Yamagishi et al., 2017; Suzuki et al., 2018). *P. subcapitata* is a unicellular algae and is consider the most widely known and used bio indicator in ecotoxicological assessments of freshwater ecosystems due to its high growth rate, high sensitivity and high reproducibility (Suzuki et al., 2018). As such, *P. subcapitata* has become one of the species of choice for primary producer assessment in multi-trophic testing and is the main algae recommended by the ISO (6892:2012), guidelines (International Organisation for Standardisation, 2012).

Globally, aquaculture is dominated by freshwater farming and the bulk of the production is finfish (Wang et al., 2015). The aim of this

study was to conduct an ecotoxicological evaluation of Irish freshwater finfish farm effluent using the *P. subcapitata* algal bioassay ISO (8692:2012), in conjunction with traditional physicochemical analysis. Correlation studies were also conducted to determine any potential relationships between the algae and the physicochemical parameters.

2. Materials & methods

2.1. Sampling

Water samples were collected from a freshwater fish farm located in Boyle, Co. Roscommon (Fig. 1). The farm, which cultures perch (*Perca fluviatilis*), consisted of an RAS used for the hatchery and nursery, three culture ponds for the adult fish that uses a flow through system (FTS), a settlement pond and a constructed wetland for wastewater treatment. Samples were collected in 5 L octagonal carboy HDPE bottle (Lennox), and transported directly to the lab, 70 km away, via car. Samples were taken directly from the effluent source of the farm every two weeks from April 2018 to October 2018. Sampling could not be conducted in June due to unforeseen circumstances. Collection occurred on the same day (Thursdays), and at approximately the same time (10:30 a.m.). Influent samples were also collected and analysed so that any potential issues caused by works upstream of the fish farm and not as a result of works within the facility itself could be taken into consideration. Influent and effluent sampling points are shown in Fig. 2.

2.2. Physicochemical analysis

Water parameters – temperature, pH, NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, dissolved oxygen (DO), BOD, COD, suspended solids (SS), hardness, alkalinity and conductivity – were investigated in the laboratory within 24 h of collection to prevent the need for preservation. Table 1 summarises the physicochemical methods employed in this study. Spectroquant® photometric kits were used to assess the NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and COD. Analysis was conducted as per the manufacturer's instructions. Temperature, pH and conductivity were analysed using a VWR pHenomenal™ MU 6100 L meter, VWR 111662–1157 pH probe and VWR CO11 conductivity probe. DO and BOD_{5day} were analysed using a Jenway 9500 DO_2 meter and probe. Suspended solids were analysed via filtration using a Buchner flask and funnel. Hardness was assessed via titration using pH 10 buffer, Erichrome black and EDTA.

Fig. 1. Map of Ireland indicating the location of the fish farm (yellow) and the closest weather stations (orange).

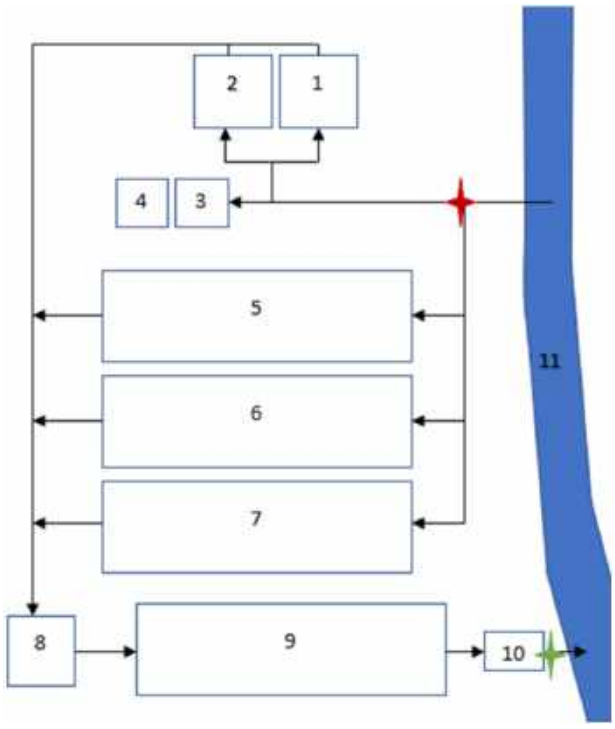


Fig. 2. Schematic of the fish farm layout. Location of the collection point for the influent (red X), and effluent (green X), are indicated. 1) Hatchery, 2) Nursery, 3–4) Mesocosms, 5–7) Culture ponds, 8) Settlement Pond, 9) Constructed Wetland, 10) Holding Tank, 11) River.

Table 1
Methods, including detection limits, used for the analyses of water quality parameters in the influent and effluent. Figure in brackets indicate the standard method number.

Parameter/variable	Method	Detection limit ($mg\ L^{-1}$)
Temperature	Thermometer (2550-B)	–
pH	Membrane Electrode (2310-B)	–
Ammonium (NH_4^+)	Photometric (4500-NH_3-F)	0.013–3.86 2.6–193.0
Nitrite (NO_2^-)	Photometric (345–1)	0.007–3.28
Nitrate (NO_3^-)	Photometric (4500-NO_3)	0.4–110.7
Phosphate (PO_4^{3-})	Photometric (4500-P-C)	0.007–15.3 1.5–92.0
Dissolved oxygen	Membrane Electrode (4500-O G)	–
Biochemical Oxygen demand (BOD)	Membrane Electrode (5210-B)	–
Chemical oxygen Demand (COD)	Photometric (5220-D)	0–150 15–300
Suspended solids	Gravimetric (2540-D)	–
Hardness	Titrimetric (2340-C)	–
Alkalinity	Titrimetric (2320-B)	–
Conductivity	Electrical Conductivity (2510-A)	–

Alkalinity was analysed by titration using phenolphthalein indicator, methyl orange indicator and hydrochloric acid.

2.3. Toxicity testing

The freshwater unicellular green algae *P. subcapitata,* was used in the toxicity test. A starter culture of the *P. subcapitata* (*Raphidocelis subcapitata*), was obtained from The Culture Collection of Algae and Protozoa (CCAP 278/4; SAMS Limited, Scottish Marine Institute, Oban, Argyll, Scotland, U.K.), and grown in standard Jarworski's Medium under controlled conditions of 23 °C ± 2 °C exposed to continuous illumination (lux 6000–10,000). Sub-culturing was conducted every two to three days to ensure the growth rate remained in the exponential phase. Toxicity testing was conducted as per the Water quality – Fresh water algal growth inhibition test with unicellular green algae ISO (8692:2012), guidelines. The *P. subcapitata* was exposed to the influent and effluent samples for 72 h under static conditions at 23 °C ± 2 °C exposed to the continuous illumination. The algae growth rate inhibition and stimulation, in percent, was calculated by comparing the samples to a negative control containing just the Jarworski's medium. Calculations were conducted as follows;

$$\text{Algal cells mL}^{-1} = \frac{n}{0.02}\ x\ 10^{3.}$$

where n = number of cells counted using a haemocytometer

$$\text{Average specific growth rate } (\mu), = \frac{\ln X_n - \ln X_o}{T_n - T_o}$$

where ln = natural log of
X_n = Algae cells mL^{-1} at 72 h
X_o = Algae cells mL^{-1} at 0 h
T_n = Duration of test
T_0 = Time zero

$$\text{Percent inhibition in growth rate} = \frac{C\mu - T\mu}{C\mu}\ x\ 100$$

where Cμ = Average specific growth rate for control
Tμ = Average specific growth rate for treatment

2.4. Statistical analysis

All statistical analyses were conducted using GRAPHPAD PRISM 8 and MINITAB 18. The data generated were grouped and subject to normality test (Anderson-Darling). *t*-tests and one-way ANOVA with Tukey were used to identify significant differences in the variables. Pearson's correlation was used to assess any correlations between the algae and/or the physicochemical parameters.

3. Results

3.1. Physicochemical analysis

Mean concentrations determined for the physicochemical parameters investigated on Irish freshwater fish farm influent and effluent samples over the entire testing period are summarised in Table 2 and Fig. 3 provides a monthly breakdown of the results. With the exception of the pH, increases in concentrations between the influent and effluent were observed in all parameters. When the influent and effluent were compared via statistical analysis, a significant difference was observed in the NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} and DO levels ($p < 0.05$).

Table 2
Physicochemical parameters investigated on Irish freshwater finfish aquaculture influent and effluent samples from April 2018 to October 2018. Results are presented as means ± S.D. *Significant differences, where $p < 0.05$, are indicated (n = 12).

Parameter	Influent	Effluent	*P* value
Temperature (°C)	14.76 ± 2.53	15.53 ± 2.66	0.854
pH	7.76 ± 0.19	7.11 ± 0.18	0.709
Ammonium (mg NH_4^+ L^{-1})	0.16 ± 0.18	1.16 ± 0.64	0.001*
Nitrite (mg NO_2^- L^{-1})	0.02 ± 0.01	0.32 ± 0.38	<0.001*
Nitrate (mg NO_3^- L^{-1})	3.62 ± 1.60	5.29 ± 5.56	0.006*
Orthophosphate (mg PO_4^{3-} L^{-1})	1.76 ± 0.84	3.78 ± 2.00	0.013*
DO (mg O_2 L^{-1})	10.31 ± 0.87	5.10 ± 2.85	<0.001*
BOD (mg O_2 L^{-1})	2.27 ± 1.47	3.24 ± 1.95	0.185
COD (mg O_2 L^{-1})	45.91 ± 40.81	76.44 ± 59.06	0.230
Suspended Solids (mg L^{-1})	40.17 ± 79.08	83.67 ± 144.33	0.073
Hardness (mg $CaCO_3$ L^{-1})	100.49 ± 9.22	116.03 ± 16.80	0.092
Alkalinity (mg $CaCO_3$ L^{-1})	122.55 ± 17.71	128.91 ± 18.19	0.915
Conductivity (μS cm^{-1})	247.18 ± 57.82	298.17 ± 57.12	0.241

DO = Dissolved Oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand.

3.2. Algal bioassay

The *P. subcapitata* algal bioassay ISO (8692:2012), was performed on influent and effluent samples from the Irish freshwater fish farm every two weeks to determine whether or not the rate of growth inhibition or stimulation were observed as a result of exposure to either sample. The growth rate inhibition and stimulation observed in the influent and effluent can be found in Fig. 4. Statistical analysis was conducted between the influent and effluent and a significant difference was observed ($p \leq 0.0001$ where <0.05 indicates a significant difference).

3.3. Correlation studies

The Pearson's correlation test (Table 3), demonstrated that in the effluent, the algae (*P. subcapitata*), was negatively correlated with temperature and suspended solids and positively correlated with alkalinity. Nitrite was positively correlated with orthophosphate. Temperature was negatively correlated with dissolved oxygen and positively correlated with conductivity. The parameters hardness and alkalinity were negatively correlated with orthophosphate. Dissolved oxygen was negatively correlated with conductivity and positively correlated with nitrate. Hardness was positively correlated with alkalinity and a negative correlation with nitrite. The pH had a negative correlation with conductivity.

3.4. Weather conditions

The Republic of Ireland experienced one of its hottest summers on record in 2018 that coincided with the sampling period in the study (Met Eireann, 2018b). Drought conditions and a national hose pipe ban were put in place for most of the country up to the end of August 2018 (Irish Water, 2018). As a result of these unusual weather conditions mean rainfall and temperature data collected at three Met Eireann weather stations surrounding and closest to the fish farm in the Republic of Ireland (Fig. 1), were observed. These stations were located in; Markree, Knock and Mount Dillon. Decreases in the average monthly rainfall and increases in temperature were observed across the weather stations (Fig. 5). Statistical analysis suggested that moderately strong inverse relationship existed between the algae and the rainfall ($r = -0.562$), and a weak relationship between the algae and the temperature ($r = 0.276$). Fig. 6 compares the average temperatures observed across the stations and the average maximum temperatures experienced. When the maximum temperatures were taken into account, a moderately strong relationship between the algae and temperature was observed ($r = 0.505$).

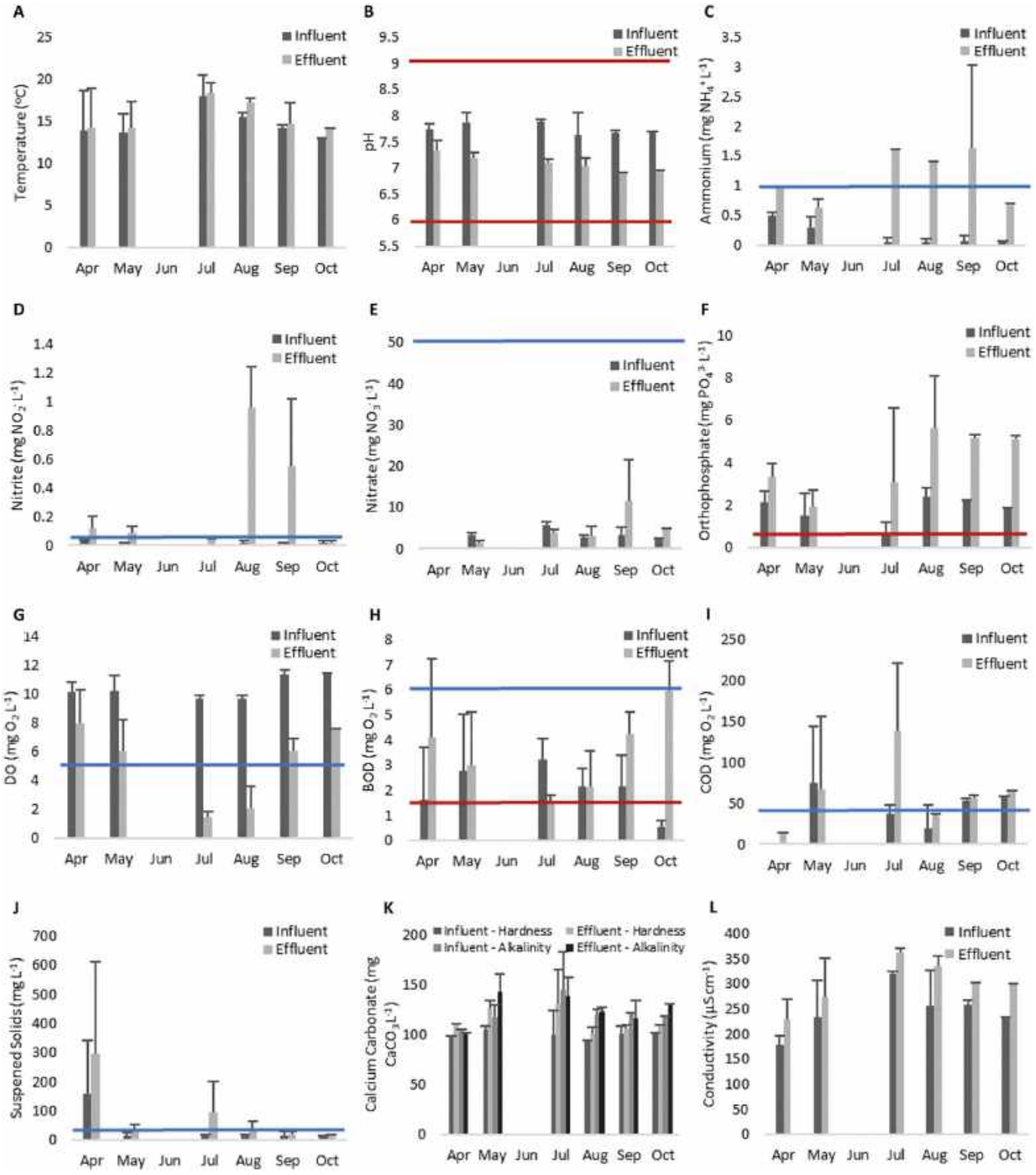
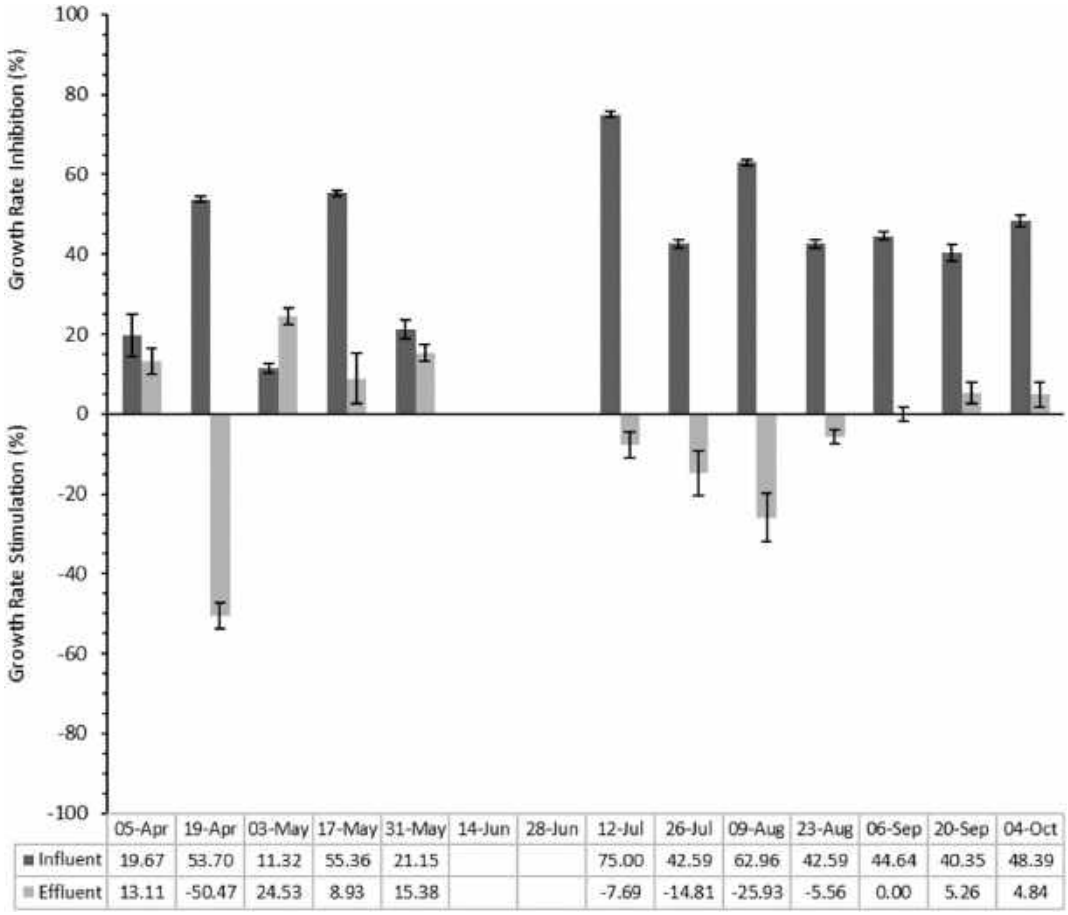


Fig. 3. Bar charts displaying monthly means for the physicochemical parameters investigated on Irish freshwater finfish aquaculture influent and effluent from April 2018 to October 2018. Parameters investigated were A) temperature, B) pH, C) NH_4^+, D) NO_2^-, E) NO_3^-, F) PO_4^{3-}, G) dissolved oxygen, H) Biochemical Oxygen Demand, I) Chemical Oxygen Demand, J) suspended solids, K) hardness and alkalinity via $CaCO_3$ content, and L) conductivity. S.D. indicated, $n = 12$. Red lines indicate levels set out by S.I. 272 of 2009, which is the European Communities Environmental Objective (Surface Waters) Regulations of 2009. Blue lines indicated levels set out by the Irish EPAs Parameters for water quality, which are based on Freshwater Fish Directive [78/659/EEC], Irish Salmonid Waters Regulations [1988], and Irish Surface Water Regulations [1989]. NOTE: The dilution factor of the receiving water body has not been taken into consideration. Lines do not appear on temperature, $CaCO_3$ content and conductivity as no limits were indicated and/or the limit is well above the range of the graph.



	05-Apr	19-Apr	03-May	17-May	31-May	14-Jun	28-Jun	12-Jul	26-Jul	09-Aug	23-Aug	06-Sep	20-Sep	04-Oct
Influent	19.67	53.70	11.32	55.36	21.15			75.00	42.59	62.96	42.59	44.64	40.35	48.39
Effluent	13.11	-50.47	24.53	8.93	15.38			-7.69	-14.81	-25.93	-5.56	0.00	5.26	4.84

Fig. 4. The percentage growth rate inhibition (positive y scale), and stimulation (negative y scale), observed in *Pseudokirchneriella subcapitata* after exposure to the freshwater finfish aquaculture influent and effluent for 72 h at 23 °C ± 2 °C under continuous illumination. Samples were collected and analysed from April 2018 to October 2018. Dates of collection appear at the bottom of each pair of results. Individual results appear in the table. Positive figures indicate inhibition and negative figures indicate stimulation. ($n = 3$, SD indicated).

4. Discussion

4.1. Physicochemical evaluation

As far as the authors are aware, water quality parameters specific for fish farm effluent in Irish water are not currently available and the Irish EPA are currently investigating the regulation of Irish freshwater fish farm effluent. Recommended water quality parameters set out by the Statutory Instrument (S.I.), 272/2009 (European Communities Environmental Objectives – Surface Waters – Regulations 2009), and the Irish Environmental Protection Agency's (EPA), parameters of water quality were therefore used as guidance (Environmental Protection Agency, 2001; Irish Stationery Office, 2009a, 2009b). The parameters set out in the above-mentioned Irish EPA document are based on the Freshwater Fish Directive [78/659/EEC] and/or Irish Surface Water Regulations [1989].

Presence of the highly toxic NH_4^+ (Zhang et al., 2011), is an indicator of recent pollution. When comparing both samples, the concentration of

Table 3
Correlation matrix for the algae (*Pseudokirchneriella subcapitata*), and physicochemical parameters investigated on the Irish freshwater finfish aquaculture effluent. Bold figures indicate where significant differences ($p < 0.05$), have been observed. Breakdown of correlation figures are indicated in box. Breakdown of correlation values is based on Ratner (2009), and his work correlation coefficient values.

	ALGAE	PH	TEMP	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	DO	BOD	COD	SS	HARD.	ALK.
ALGAE	1							***0 = No relationship***					
PH	0.112	1						***>0–0.3 = Weak relationship***					
TEMP	**−0.619**	−0.480	1					***0.3–0.5 = Moderately weak relationship***					
NH_4^+	−0.285	−0.437	0.414	1				***0.5–0.7 = Moderately strong relationship***					
NO_2^-	−0.307	−0.389	0.213	**0.554**	1			***0.7 - < 1 = Strong relationship***					
NO_3^-	0.164	−0.468	−0.475	−0.346	−0.100	1		***1 = Perfect linear relationship***					
PO_4^{3-}	−0.393	−0.435	0.209	0.308	**0.651**	0.320	1						
DO	0.356	0.408	**−0.846**	−0.492	−0.298	**0.578**	−0.028	1					
BOD	−0.197	−0.307	0.008	−0.065	0.048	0.251	0.310	0.422	1				
COD	0.270	0.310	−0.216	−0.083	−0.430	−0.003	−0.088	0.004	−0.495	1			
SS	**−0.727**	0.228	0.244	−0.064	−0.191	−0.121	−0.139	0.069	0.399	−0.345	1		
HARD.	0.343	0.393	−0.021	−0.095	**−0.580**	−0.335	**−0.885**	−0.156	−0.199	0.085	0.115	1	
ALK.	**0.607**	0.224	0.034	−0.408	−0.493	−0.228	**−0.657**	−0.240	−0.359	0.098	−0.368	**0.701**	1
COND.	−0.292	**−0.547**	**0.841**	0.394	0.251	−0.485	0.138	**−0.851**	−0.227	−0.083	−0.174	0.101	0.344

Temp = Temperature, DO = Dissolved Oxygen, BOD = Biochemical Oxygen Demand, COD = Chemical Oxygen Demand, SS = Suspended Solids, Hard. = Hardness, Alk. = Alkalinity, Cond. = Conductivity.

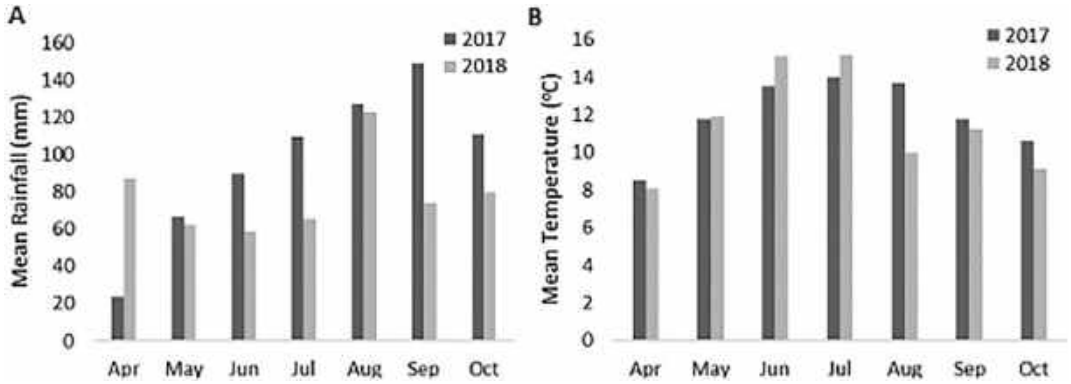


Fig. 5. Mean rainfall in mm (A), and temperature in °C (B), collected from three Met Eireann weather stations located at; 1) Markree, Co. Sligo, 2) Knock, Co. Mayo, and 3) Mount Dillon, Co Roscommon. These stations were selected as they were located to the north-west, south-west and south-east of the fish farm investigated in this study. Data from April to October in 2017 (dark grey), and 2018 (light grey), were examined.

NH_4^+ present in the influent is lower than that of the effluent. This suggested that the levels of NH_4^+ detected are being generated within the farm itself. However, the small amount detected in the influent also suggests some form of pollution is being generated upstream of the farm. The concentrations observed in the effluent were more than five times greater than the 1 mg L^{-1} suggested by the Irish EPA (Environmental Protection Agency, 2001). NO_2^- levels detected were very low mainly due to its transient nature and quick transformation from NH_4^+ to NO_3^- (Durborow et al., 1997). Similar with NH_4^+, the presence of NO_2^- is an indication of recent pollution. When comparing the NO_2^- levels in both samples, levels in the effluent were much higher than that of the influent. The small amount detected in the influent suggests some form of pollution is being generated upstream of the farm. The higher levels detected in the effluent are generated within the farm itself. The concentrations detected in the effluent were one log dose greater than the 0.03 mg L^{-1} for cyprinid waters, as per the Irish EPAs suggested water quality parameters (Environmental Protection Agency, 2001). NO_3^- levels observed in the influent indicated that NO_3^- may have been entering the river upstream of the fish farm as well as its expected generation within the farm. The aquatic plant *Lemna minor* (duckweed), was present in the farm and can use NO_3^- as a source of nutrients (Stewart and Rhodes, 1976). A spike in the effluent at the end of September 2018 was observed and may have been due to the fact that the *Lemna minor* within the farm had been removed. NO_3^- levels were well below the guidance value of 50 mg L^{-1} suggested by the Irish EPA (Environmental Protection Agency, 2001). *Lemna minor*'s ability to use NO_3^- as a nutrient source and the spikes observed in the farm after its removal highlighted that the duckweed holds great potential as a wastewater treatment option for fish farms and further research is currently being conducted.

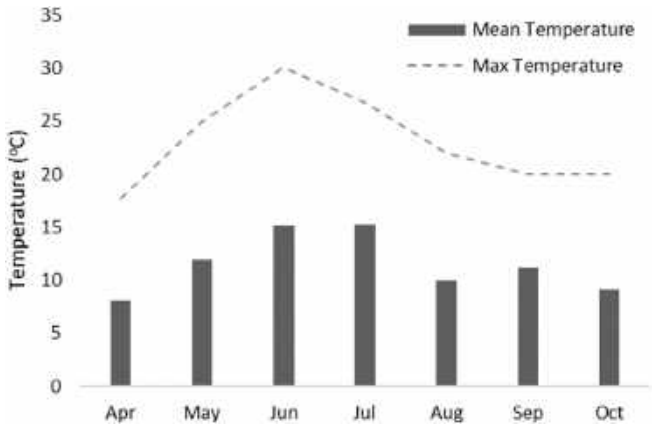


Fig. 6. Mean temperatures (Bar Chart), and maximum temperatures (Line Chart), observed at three Met Eireann weather stations located closest to the fish farm, from April 2018 to October 2018. Data was based on all temperature readings collected at the three weather stations by Met Eireann (Met Eireann, 2018a).

PO_4^{3-} was detected in both the influent and effluent. The levels observed in the influent were less than that in the effluent. This suggested that phosphorus pollution is entering the river upstream of the fish farm, as well as being generated within the farm itself. Levels in both the influent and effluent may be cause for concern due to its ability to cause algal blooms and the hypoxic conditions which may occur thereafter in water bodies. Concentrations detected were just over two log doses greater than the recommended value of 0.035 mg L^{-1} set out by the S.I. 272/2009 for good water status (Irish Stationery Office, 2009b). The farm uses a constructed wetland pond for treatment of nitrates, phosphates and so forth, before being released. Further research is currently being conducted in the efficacy of the constructed wetland by way of expansion of treatment to address culture area. This has been the subject of previous research (Jegatheesan et al., 2011; Sharrer et al., 2016), however it still remains unclear as to the governing factors underpinning the effective ratio for culture treatment of fish farm effluent.

The recommended DO concentration in salmonid waters should be ≥9 mg L^{-1} and cyprinid waters (e.g. perch) should be ≥7 mg O_2 L^{-1} (Environmental Protection Agency, 2001). There are no issues with the DO levels present in the influent however there may be cause for concern with levels observed in the effluent as they were well below the recommended concentration of ≥7 mg O_2 L^{-1}. Levels below this concentration were only observed during the heat wave and drought conditions, and the unusual weather conditions may have played a role. Conditions began to improve once weather conditions had returned normal. Alam et al. (2007), and da Silva et al. (2017), have suggested that oxygen concentrations of ≥4 mg L^{-1} are sufficient for maintenance of aquatic life. S.I. 272/2009 recommend a mean BOD concentration of 1.3 mg L^{-1} for high water status and 1.5 mg L^{-1} for good water status. However, the Irish EPA has suggested ≤3 mg L^{-1} and ≤ 5 mg L^{-1} for salmonid and cyprinid waters, respectively (Environmental Protection Agency, 2001). The current BOD levels detected in the influent suggested no issues. The concentration of BOD detected in the effluent may be cause for concern. Although the level was below that suggested by the Irish EPA for cyprinid water, it was greater than that suggested in the S.I. 272 of 2009. COD was detected in both the influent and effluent. The levels observed in both sets of samples may be cause for concern. The mean concentration was almost double the suggested 40 mg L^{-1} set out by the Irish EPA (Environmental Protection Agency, 2001).

Two concentrations of suspended solids have been suggested by the Irish EPA (Environmental Protection Agency, 2001). Fifty mg L^{-1} as per the Surface Water Regulations [1989], and 25 mg L^{-1} as per the Freshwater Fish Directive [78/659/EEC], and Salmonid Waters Regulations

[1988]. Suspended solids can increase gill irritation and blanket the benthos (Bilotta and Brazier, 2008), therefore the lower concentration of 25 mg L^{-1} was taken as the maximum allowable concentration (MAC), in this study of an Irish freshwater finfish farm. The average levels detected in both the influent and effluent may be cause for concern as they were above the 25 mg L^{-1}.

With growing concerns associated with climate change and global warming, increases in temperatures may become more frequent. Fluctuations in temperature were observed in both sets of samples. These rises in temperatures were only observed during the elevated temperatures experienced in Ireland in 2018. The results for the pH indicated that the influent was slightly more alkaline than the effluent, which the pH just above neutral (pH 7). The slight difference in pH levels in the samples may have been due to the alkalinity levels observed in the samples. The effluent had a higher level of $CaCO_3$ and thus a better buffering capacity. The recommended pH levels should be between 6 and 9. Levels in both the influent and effluent are well within this level and therefore are present no issues.

Alkalinity has been measured as $CaCO_3$. For hardness, $CaCO_3$ levels were also measured. Results suggested that the water is slight to moderately hard. This correlates with water hardness maps of Ireland which demonstrated water around Boyle, Co. Roscommon was slightly too moderately hard.

Some of the results observed (NH_4^+, NO_2^-, PO_4^{3-}, COD and suspended solids), suggested potential issues however, the dilution factor of the receiving river has not been included and needs to be taken into consideration. Equally, the condition of the influent needs to be considered. Additionally, as this research only focused on one fish farm, the results observed in this study were compared to previous studies conducted on a range of different farm effluents including; brown and rainbow trout, Atlantic salmon, catfish, shrimp and prawns. All results obtained were similar to other research studies conducted (Ziemann et al., 1992; Camargo, 1994; Boaventura et al., 1997; Mcintosh and Fitzsimmons, 2003; Biao et al., 2004; Pulatsü et al., 2004; Stephens and Farris, 2004a, 2004b; Costanzo et al., 2004; Živić et al., 2009; Moreira et al., 2010; Guilpart et al., 2012; Namin et al., 2013; Noroozrajabi et al., 2013; Herbeck et al., 2013; Caramel et al., 2014; Lalonde et al., 2014; Ferreira et al., 2015; da Silva et al., 2017), with the exception of BOD. Although not many studies included BOD, those reviewed demonstrated higher levels than the concentrations detected in this study (Boaventura et al., 1997; Mcintosh and Fitzsimmons, 2003; Ansah et al., 2012; Miashiro et al., 2012).

4.2. Ecotoxicological bioassay evaluation

Growth stimulation was observed in the effluent. Stimulation occurred in mid-April and then again from July to September. This coincided with the elevated temperatures and drought conditions experienced in the Republic of Ireland in the summer of 2018. Ireland's mean summer maxima temperature is between 18 °C and 20 °C (Walsh, 2012). In 2018, temperatures exceeded 30 °C (Met Eireann, 2018b). This resulted in low rainfall levels, e.g. the three weather stations (Markree, Knock and Mount Dillion), measured an average total rainfall of only 61.9 mm for the months of May, June and July 2018 compared to 88.9 mm for the same three months in 2017 (Met Eireann, 2018a), leading to a national hose pipe ban and water restrictions (Irish Water, 2018). The ability of the effluent to cause growth stimulation suggested that the possibility of algal blooms (resulting in eutrophication), downstream of the fish farm are more likely to occur. This may result in loss of biodiversity, habitat and submerged aquatic vegetation, disruption of the ecosystems functionality, deficiencies in oxygen and modifications in food webs (Rabalais, 2002).

A higher level of growth inhibition was observed in the influent compared to the effluent. This suggested that the influent would seem unlikely to cause issues such as algal blooms. However, the high level of growth inhibition in the influent also indicated toxicity and may result in losses to the biodiversity of the receiving water body (Rabalais, 2002). This toxicity may result in the loss of primary producers (e.g. algae), in the aquatic ecosystem. This may subsequently cause indirect adverse effects on the aquatic food chain, e.g. microcrustaceans feed on algae and fish in turn, feed on the microcrustaceans. Loss of the algae removes the food source for the microcrustaceans, resulting in their potential loss. This in turn, could result in the removal of a valuable food source for the fish. It should be noted that this toxic effect does not occur within the fish farm itself and suggests potential issues upstream of the farm. This issue may also affect the health and welfare of the fish within the farm. This is being closely monitored by the owner and no adverse effects have been observed with the fish. Further investigation into the water quality entering the fish farm has also been conducted. An old school is situated just upstream of the fish farm that has an older waste water treatment system which may have resulted in some waste entering the river. As a result of this, water is now piped from upstream of the school directly into the farm in order to assist in determining the exact cause for the poor water quality.

Most of the available research involving *P. subcapitata* focused on inhibition of growth (Guéguen et al., 2004; Ivanova and Groudeva, 2006; Ma et al., 2006). One study involving *P. subcapitata* and fish farm effluent was published by Miashiro et al. (2012), demonstrated similar results to this study, i.e. growth stimulation instead of inhibition was observed. Miashiro et al. (2012), suggested that the stimulated algal growth may have been due to the high concentration of nutrients that were observed. High levels of nutrients, were also observed in this study. The lack of available research suggested an under use of *P. subcapitata* as an early indicator of potential issues in fish farming. Stimulation of *P. subcapitata* in the effluent may be indicative of potential eutrophication downstream of the fish farm.

4.3. Climate change

"There's one issue that will define the contours of this century more dramatically than any other, and that is the urgent threat of a changing climate. Climate change is no longer some far-off problem; it is happening here, it is happening now" (Obama, 2015). Climate change is considered to be one of the most troubling, challenging and unrelenting scientific issues of our time (Bulkeley and Newell, 2015). Climate change, including global warming, is a complicated and increasingly problematic challenge causing changes to rainfall and hydrology, e.g. extensive summer droughts caused by changes in rainfall (Paerl and Scott, 2010; Paerl et al., 2016).

The results observed in this study have demonstrated that climate change may have a direct impact on fish farming, as suggested by the moderately strong relationship ($r = -0.619$), observed between the increases in temperature and the stimulation of algal growth rates, which could lead to increased instances of eutrophication downstream of the fish farm. *P. subcapitata* should be investigated further for its ability to be utilised as a potential early warning indicator of climate change.

5. Conclusion

The use of *P. subcapitata* as an early warning indicator to potential environmental issues associated with fish farms has been demonstrated as a more responsive model than physicochemical parameters alone. Evaluation of farm effluent could include ecotoxicological bioassays in order to determine any potential effects the effluent may have on the receiving aquatic ecosystem. Inclusion of the *P. subcapitata* Algal Bioassay ISO (8692:2012), in this study has demonstrated the potential eutrophication implications as a result of releasing untreated effluent from fish farms. It should be noted that this research focused on only one fish farm and therefore additional research is currently being conducted. Other bioassays that focus on different trophic levels should also be

considered in order to develop a broader picture of the potential effect's fish farm effluent poses on its receiving ecosystems.

Water quality parameters specific for fish farm effluent has not yet been established in Ireland. The Irish EPA has begun the process of regulating fish farm effluent. Results observed in this study have demonstrated that water quality parameters suggested by S.I. 272/2009 and the Irish EPA may not be applicable to fish farm effluent. The dilution factor of the receiving aquatic ecosystem is important and therefore also needs to be taken into consideration when these water quality parameters are to be determined. Results have also indicated that influent water quality is also as important when assessing fish farm effluent as it may indicate potential environmental issues as a result of activities upstream and not that which is occurring within the farm.

The study has highlighted that on review of the performance of the constructed wetland, it is envisaged that it needs to be increased in order to be effective. Also highlighted is the importance and potential use of *Lemna minor* as a waste water treatment system within fish farming facilities.

Although this study focused on the ecotoxicological effects freshwater fish farm effluent may induce on its receiving aquatic ecosystem, the research suggested that the changes in temperatures that were observed during the heat wave and drought conditions experienced in the summer months of 2018 had a direct relationship with the increased levels of algal growth stimulation detected. With irregular weather patterns becoming more frequent, especially rises in mean temperatures due to global warming, further research into the effects of climate change on aquatic ecosystems, fish farm effluent and the effects of effluents on its receiving ecosystem will need to be conducted and is currently being investigated. The research conducted in this study has suggested a potential toolbox that includes *P. subcapitata* may provide an early warning system for adverse effects as a result of climate change.

CRediT authorship contribution statement

Emer A. O'Neill: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Neil J. Rowan:**Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Andrew M. Fogarty:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no competing interests or conflicts of interest with respect to the publication of this article.

Acknowledgements

The authors would like to thank Athlone Institute of Technology (President Seed Fund), for providing financial support for this research. The authors would also like to thank Paul Kearney and Keywater Fisheries for access to his freshwater aquaculture facility in order to conduct this research.

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Science of the Total Environment 851 (2022) 158392

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Use of next generation sequencing and bioinformatics for profiling freshwater eukaryotic microalgae in a novel peatland integrated multi-trophic aquaculture (IMTA) system: Case study from the Republic of Ireland

Emer A. O'Neill [a,*], Gustavo Fehrenbach [a], Emma Murphy [a], Sérgio A. Alencar [b], Robert Pogue [a,b], Neil J. Rowan [a]

[a] Bioscience Research Institute, Technological University of the Shannon: Midlands Midwest, University Road, Athlone, Co. Westmeath, Ireland
[b] Universidade Católica de Brasília, QS 7 LOTE 1 - Taguatinga, Brasília, DF 71966-700, Brazil

HIGHLIGHTS

- Integrated multi-trophic aquaculture (IMTA) will sustain food systems for peatlands.
- IMTA needs better understanding of algae for bioremediation and water quality.
- Reliance on traditional monitoring methods limits innovation and sustainability.
- Use of next generation sequencing for algae profiling will advance paludiculture.
- IMTA system delivers new 'Green' innovation balanced with environmental protection.

GRAPHICAL ABSTRACT

ARTICLE INFO

Editor: Damià Barceló

Keywords:
Algae
Aquaculture
Food security
Ecosystem
Sustainability
Next-generation sequencing

ABSTRACT

Development of integrated multi-trophic aquaculture (IMTA) systems constitutes a step change in the sustainable production of freshwater fish to meet emerging needs for high-protein foods globally. Recently, there has been a paradigm shift away from harvesting peat as a fuel towards the development of wettable peatland innovation (termed 'paludiculture'), such as aquaculture. Such eco-innovations support carbon sequestration and align with a balanced environmental approach to protecting biodiversity. This novel peatland-based IMTA process in the Irish midlands relies upon natural microalgae for waste treatment, recirculation and water quality where there is no use of pesticides or antibiotics. This novel IMTA system is powered with a wind turbine and the process has 'organic status'; moreover, it does not discharge aquaculture effluent to receiving water. However, there is a significant lack of understanding as to diversity of microalgae in this 'paludiculture'-based IMTA processes. This constitutes the first case study to use conventional microscopy combined with next-generation sequencing and bioinformatics to profile microalgae occurring in this novel IMTA system from pooled samples over a 12 month period in 2020. Conventional microscopy combined with classic identification revealed twenty genera of algae; with Chlorophyta and Charophyta being the most common present. However, algal DNA isolation, 16 s sequencing and bioinformatics revealed a combined total of 982 species from 341 genera across nine phyla from the same IMTA system, which emphasized a significant underestimation in the number and diversity of beneficial or potentially harmful algae in the IMTA-microbiome. These new methods also yield rich data that can be used by digital technologies to transform future monitoring and performance of the IMTA system for sustainability. The findings of this study align with many sustainability development goals of the United Nations including no poverty, zero hunger, good health and well-being, responsible consumption and production, climate change, and life below water.

* Corresponding author.
E-mail address: emer.oneill@ait.ie (E.A. O'Neill).

http://dx.doi.org/10.1016/j.scitotenv.2022.158392
Received 19 May 2022; Received in revised form 25 August 2022; Accepted 25 August 2022
Available online 30 August 2022

1. Introduction

Aquaculture is the fastest growing food producing industry in the world (O'Neill et al., 2019, 2020; Naughton et al., 2020). It now dominates aquatic food production (Tacon, 2020) with >50 % of all fish produced for human consumption coming from aquaculture (FAO, 2020; O'Neill et al., 2022). This is expected to exceed 62 % by 2030 (O'Neill and Rowan, 2022). Depletion of wild fishery practices as a result of over exploitation of wild fish stocks, which are now at their maximum sustainable yields, and increased consumer demand as a result of a growing global population has attributed to the dramatic increase in aquaculture (Tahar et al., 2018a, 2018b; Han et al., 2019; O'Neill and Rowan, 2022). Aquaculture is also providing an essential and important means of food security, both directly and indirectly (O'Neill et al., 2022). Farmed fish are a highly rich source of protein and provide a more efficient protein utilisation and feed conversion source than other animals destined for protein production (Tschirner and Kloas, 2017; O'Neill et al., 2022). Previously limited or non-existent access to fish is now available in under-developed countries and regions, often at cheap prices; thus, providing improved nutrition and food security in addition to increased employment (Rowan and Casey, 2021; O'Neill et al., 2022).

At the beginning of 2020, the Irish aquaculture industry produced just over 38,238 t of fish valued at €173.5 Million for the national economy (Dennis et al., 2020). Food Wise 2025 is a strategy developed by the Department of Agriculture, Food and the Marine (DAFM) for the Irish agri-food sector. This ten-year plan set out and underlines the sectors position in the Irish economy. It also illustrated the potential for expansion within the sector (DAFM, 2015a). Food Wise 2025 predicted that the Irish agri-food sector has the potential to increase exports to €19 Billion, *per annum* by 2025. As part of this prediction, it proposed that the Irish aquaculture industry, or more specifically aquaculture production, could be increased to 81,700 t by 2023 in order to assist in meeting this goal (DAFM, 2015a). However, issues with the Irish aquaculture licensing process, associated with the adoption of European Union environmental protection directives resulting in space limitations have hampered the growth and the development of the industry (Moylan et al., 2017). There is now therefore, an increasing interest in exploiting low-cost environmental-friendly 'natural' processes in aquaculture (Han et al., 2019). For example, the aforementioned aquaculture issues have led to an increased research focus on developing integrated multi-trophic aquaculture systems or IMTA which many believe can help mitigate these impacts (Granada et al., 2016; Ingle et al., 2022; O'Neill et al., 2022) along with eco-innovation and monitoring of traditional processes (Tahar et al., 2018a, 2018b; Rowan, 2019). Advances in aquaculture must also be balanced with the need to meet commitments as set out by the Water Framework Directive (WFD), which aims to achieve good water status in all waters across all EU member countries (Voulvoulis et al., 2017; WFD Ireland, 2018a, 2018b; O'Neill et al., 2022). Development of new high protein fish production process using natural resources also offers potential solutions to meet many of the sustainable development goals of the United Nations (Rowan and Casey, 2021).

As part of Ireland's Strategic Plan for Sustainable Aquaculture Development, and in addition to their research into further sustainable development of traditional aquaculture processes such as flow-through systems (FTS), Bord Iascaigh Mhara (BIM), Ireland's seafood development agency, undertook a feasibility study to assess the novel use of peatlands for aquaculture diversification (DAFM, 2015b). The urgent threat of climate change, in addition to some of these peatlands now being listed as important habitats under the EU's Birds and Habitats Directives due to their scarcity, have resulted in dramatic changes in the peat industry including conversion of peatland usage to wind energy, forestry, biodiversity, amenity and waste management (Toner, 2018; Bord na Móna, 2019a: 2019b; Irish Peatland Conservation Council, 2019; O'Neill et al., 2019; Ward et al., 2019). With that, BIM further expanded the potential use of these cutaway bogs to develop Ireland's first IMTA system adhering to organic principles in order to assist in developing the Irish freshwater aquaculture industry in a sustainable manner. This trial IMTA holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), common duckweed (*Lemna minor*) and gibbous duckweed (*Lemna gibba*) and exploits use of microalgae for waste removal (Bord na Mona, 2019b). This IMTA process differs from traditional aquaculture practices that use water from rivers and lakes where the latter traditional systems must consider potential pollutants from agricultural runoff, industry, waste-water treatments plants and so forth. (Rowan, 2011; Tahar et al., 2017; Tiedeken et al., 2017; Tahar et al., 2018c). This IMTA process consists of four culture ponds with eight compartments for fish and a duckweed lagoon that has sixteen channels where there are airlifts for water movement and oxygen supply and paddlewheel aerators move water between fish culture ponds and the duckweed areas (O'Neill et al., 2020).

It is only in recent years that studies have been conducted, confirming the potential beneficial roles of microalgae in aquaculture (Gao et al., 2016; Ansari et al., 2017; Han et al., 2019). Microalgae could efficiently assimilate nutrients providing a good method for wastewater remediation (Wang et al., 2015a, 2015b; Leng et al., 2018; Han et al., 2019) in aquaculture, having already demonstrated promising performances in the food and agriculture industries, and in municipal wastewater treatment (De-Bashan et al., 2004; Lu et al., 2015, 2017; Han et al., 2019). Microalgae synthesise high value compounds *e.g.*, proteins, lipids and pigments (Han et al., 2019). Studies conducted by Ansari et al. (2017), Lu et al. (2017) and Sirakov et al. (2015) have also demonstrated the application of various microalgal species for the production of biomass which could be exploited as a partial feed replacement and to enhance aquatic animal immunity. Due to the potential of these benefits, the use of microalgae in aquaculture has recently emerged into the forefront. However, the role of algae in aquaculture is still lacking (Han et al., 2019; O'Neill et al., 2019, 2020).

The overarching aim of this study was to conduct a preliminary study to determine what populations of algae were present within the novel IMTA system in order to establish what species of algae could be utilised to benefit the system and what species could potentially contribute to adverse effects so as to better inform the management and development of the process. Therefore, the objectives were (a) to establish ability of traditional approaches in determining numbers and types of microalgae in this IMTA process; and (b) to compare the efficacy of next-generation sequencing and bioinformatics to discern diversity and numbers of microalgae from pond microbiome in same system. The hypothesis is that traditional approaches used for monitoring algae in novel IMTA underestimate actual diversity and numbers of algae that limits intensive sustainability of this process.

2. Methods

2.1. Study site

'Oasis' fish farm is an innovative peatland cut-away integrated multi-trophic aquaculture (IMTA) system process set in the middle of Mount Lucas Wind Farm, Co. Offaly (53°17'3" N - 7°11'45" W). The closed/semi-closed aquaculture system consists of four split (pill) earthen ponds, culturing European perch (*Perca fluviatilis*) in one and rainbow trout (*Oncorhynchus mykiis*) in three, utilizing glacial till that were connected to an algae and duckweed lagoon with sixteen channels serving as a treatment system. See Fig. 1. Fish are kept at a density that does not exceed the organic farming standard (*e.g.*, < 20 kg/m^{-3} for perch), using screens at the D-ends of each split pond. An average of approximately 3000 kg of feed was applied per 10,000 kg biomass per month. The space between two D-end fish culture areas is also used to treat waste with free living algae in suspension. Flow in each split pond is generated and water is circulated using an airlift. Each D-end fish culture area is equipped with oxygen and temperature probes connected to paddlewheels to provide extra oxygen when necessary. The farm is designed to hold a maximum of 32,000 Kg of fish. As this is a cutaway site, the peat was previously removed by harvesting over many years. What is left underneath is the original glacial till from which the aquaculture ponds were created; therefore, to the best of our knowledge, no oxygenation of peat occurs. The algae and aquatic plants that grow in the cultured fish ponds sink to the bottom sequester carbon and create a new sink.

Fig. 1. Ariel view of trial fish farm. Sampling points have been indicated (green). Flow of water throughout the system has been included (blue). Image shows the four D-end culture ponds, the reservoir and the 16-channel duckweed and algae treatment lagoon. The turbine, which provides all electrical inputs for the farm is also visible.

2.2. Sample collection, preservation and preparation

Five litre water samples were collected from the trial IMTA fish farm in 5 L octagonal carboy HDPE bottles (Lennox) and transported directly to the lab, 62 km away, *via* car in insulated boxes. Samples were collected bi-weekly from December 2019 to February 2020 and then once a week from March 2020 to October 2020 for the identification of algal species within the novel IMTA system. Samples were collected on the same day (Wednesday) at approximately the same time (8:30 a.m.). The sampling regime changed due to the development of unforeseen technical issues within some of the culture ponds and increased monitoring was required. Samples were taken from each of the culture ponds, the entry and exit points of the treatment lagoon. Samples from the reservoir began in June due to the commencement of culturing in it as a result of issues being observed in the culture ponds. See Fig. 1 for sampling locations. Testing began within one hour of collection.

Samples were preserved in order to minimise the loss of the biological composition and maintain as close to *in-situ* conditions as possible (Nachimuthu et al., 2020). Based on the success Naughton et al. (2020), Guillard and Sieracki (2005) and, Noble and Fuhrman (1998) observed with preservation methods, 1 % Lugol's iodine was used to preserve the samples. For each of the five L grab samples taken from the individual locations within the farm, 500 mL samples were placed into 500 mL carboy HDPE bottles (Lennox) and 1 mL of the 1 % Lugol's iodine was added. All samples were mixed well and stored at 4 °C until analysis was conducted. Analysis was conducted on both fresh and preserved samples.

2.3. Physicochemical measurement

The physicochemical parameters (temperature, pH, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), orthophosphate (PO_4^{3-}), dissolved oxygen (DO), biochemical oxygen demand (BOD), suspended solids (TSS), dissolved solids (TDS), hardness, alkalinity and conductivity) were investigated in the laboratory within 24 h of collection to prevent the need for preservation. Temperature, pH, dissolved solids and conductivity were analysed using a VWR pHenomenal™ MU 6100 L meter, VWR 111662–1157 pH probe and VWR CO11 conductivity probe. DO and BOD_{5day} were analysed using a Jenway 9500 DO_2 meter and probe. Spectroquant® photometric kits were used to assess the NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}. Analysis was conducted as per the manufacturer's instructions. (See Supplementary Data 1 – Method Breakdown). Absorbance was analysed using a Shimadzu UV-2250 spectrophotometer. Suspended solids were analysed *via* filtration using a Buchner flask, Buchner funnel and Whatman 0.45 μm pore membrane filter. Hardness was assessed *via* titration using pH 10 buffer, Erichrome black and EDTA. Alkalinity was analysed by titration using phenolphthalein indicator, methyl orange indicator and HCl.

2.4. Microscopic analysis

Microscopic analysis was conducted as per Naughton et al. (2020) with some modifications. Six 5 mL aliquots were taken from the sample and placed into the wells of a six well plate. This was conducted for each sampling point. Analysis was also conducted on both the fresh and preserved samples as Lugol's iodine can cause changes to cells size (Hawkins et al., 2005) and abundance (Zarauz and Irigoien, 2008). During times of high algal concentration, aliquots were diluted to 1:2, 1:5 and 1:10. The plates were left to sit for 48 h which allowed for the algae to settle out. Plates were then examined extensively using an Olympus CKX41 inverted microscope. Images were viewed at 100× and 400×. Twelve images per well were taken using the ISCapture software to ensure as many algae as possible could be observed and identified. These images were then analysed as per Bellinger and Sigee (2015a, 2015b, 2015c) whereby the physical features, including; size, shape, colour, morphology and motility, were recorded and then identified using identification keys and cross comparison images from the Algae Base data bank (Algae Base, 2022).

2.5. Isolation of algal DNA, 18S sequencing and bioinformatics

Algal samples were centrifuged and re-suspended in 1 mL of water, before sonication at 120 W/40 kHz in a sonicator bath (Cuyson) at 50 °C for 15 min. Algal genomic DNA was isolated using the High Pure PCR Template Preparation kit (Roche). Molecular profiling was carried out by two different methods. First, 18S DNA was sequenced commercially (Macrogen, South Korea) on the HiSeq platform (Illumina), using a 300-cycle paired-end protocol, yielding an average of ~100,000 reads per sample.

Secondly, 50–100 ng of DNA was used in a PCR reaction for amplification of a 1800 bp segment of the 18S ribosomal DNA sequence, using primers described by Khaw et al. (2020), modified with adapter sequences for subsequent sequencing on the Oxford Nanopore MinIon platform (adaptor sequences underlined):

(F): TTTCTGTTGGTGCTGATATTGCGGTGATCCTGCCAGTAGTCATATGCTTG
(R): ACTTGCCTGTCGCTCTATCTTCGATCCTTCCGCAGGTTCACCTACGGAAACC

Following quality assessment of the Illumina fastq files using FastQC tool (http://www.bioinformatics.babraham.ac.uk), low quality reads were trimmed with Trimmomatic (Bolger et al., 2014). Then the Qiime 2 taxonomic classification pipeline was used in order to obtain classifications at species level (Bokulich et al., 2018).

Sequencing on the MinIon platform was carried out using the PCR Barcoding Amplicons (SQK-LSK-109) protocol, according to the manufacturer's instructions. The average yield obtained was 154,374 per sample.

Quality assessment of the fastq files obtained from MinIon Nanopore sequencing data was carried out using the FastQC tool (http://www.bioinformatics.babraham.ac.uk). Adapters were removed using Porechop (v0.2.4, https://github.com/rrwick/Porechop), which trims off adapters on the ends of reads, and when a read has an adapter in its middle, it is treated as chimeric and chopped into separate reads. The Fastp tool (PMID: 30423086) was then used in order to remove low quality reads, followed by quality assessment once again with FastQC, which confirmed that poor quality bases were removed. The resulting high-quality reads

Table 1
Average concentrations for all physicochemical parameters monitored in Mount Lucas fish farm from December 2019 to October 2020. Measuring units and standard error of the mean (SEM) have been included, $n = 10$. P value <0.05 indicates a statistically significant difference. Water quality guidance values from Irish EPA water quality, Statutory Instrument (SI) 272/2009 and SI 77/2019 have been included.

Parameter	Average	SEM	P Value	Guidance Value
Alkalinity (mg $CaCO_3$ L^{-1})	124.33	1.41	0.4806	–
Ammonium (mg NH_4^+ L^{-1})	0.10	0.03	0.5520	< 1
BOD (mg O_2 L^{-1})	13.36	1.97	0.5464	< 6
Conductivity (μS cm^{-1})	261.44	3.69	0.3271	< 1000
Dissolved Oxygen (mg O_2 L^{-1})	7.26	0.22	0.1421	> 7
Dissolved Solids (mg L^{-1})	167.78	2.54	0.3172	< 300
Hardness (mg $CaCO_3$ L^{-1})	105.26	1.77	0.5237	–
Nitrate (mg NO_3^- L^{-1})	2.65	0.32	0.1000	< 50
Nitrite (mg NO_2^- L^{-1})	0.07	0.01	0.4867	< 0.03
Orthophosphate (mg PO_4^{3-} L^{-1})	0.05	0.03	0.2160	< 0.03
pH	7.77	0.06	0.9952	> 6 - < 9
Suspended Solids (mg L^{-1})	38.46	5.24	0.8604	< 25
Temperature (°C)	12.81	0.87	0.1671	< 20

were then clustered using the isONclust software (v0.0.4, https://github.com/ksahlin/isONclust). Each cluster, representing all reads that came from a gene, was then aligned against the National Center for Biotechnology Information (NCBI) nucleotide database (excluding fungi data in the search) using BLASTn with an e-value of 0.05.

2.6. Statistical analysis

Statistical analysis was conducted in order to determine whether any statistically significant differences were observed between any of the variables monitored throughout the study. All statistical analysis were performed on GRAPHPAD PRISM 9, and MINITAB 18. The data generated were grouped and subject to normality tests (Anderson-Darling), to determine if samples were from a normal distribution ($p > 0.05$ = normal distribution). Parametric testing was then applied. One-way and two-way ANOVA were used to determine if any significant differences were observed in the parameters investigated ($p < 0.05$ = significant difference). Unpaired tests were used as different sets of samples were analysed to assess the quality of the aquaculture water samples. Grubbs test was used to determine if any outliers were indicated.

3. Results

3.1. Physicochemical analysis

Bi-weekly physicochemical analysis was conducted. Statistical analysis indicated no significant differences within each set of physicochemical parameters (Table 1). Therefore, results were grouped together for ease of reporting. An average of all physicochemical parameters monitored throughout the preliminary study on the trial fish farm can be found in Table 1. Established parameters were compared to guidance water quality parameters as none currently exist in Ireland based on Irish freshwater aquaculture. However, the Irish Environmental Protection Agency (EPA) are now actively reviewing this. Due to limitations, composite sampling could not be conducted. All parameters, with the exception of BOD (13.36 mg O_2 L^{-1}, $P = 0.5464$), nitrite (0.07 mg NO_2^- L^{-1}, $P = 0.4867$), orthophosphate (0.05 mg PO_4^{3-} L^{-1}, $P = 0.2160$) and suspended solids (38.46 mg L^{-1}, P = 0.2160) were within the guidance values parameters (Table 1). Additional aeration and a filtration system was added to aid in reducing these parameters, after which the farm workers reported a reduction in BOD, nitrite and suspended solids. The orthophosphate remained just above the guidance value. Additional research is currently being conducted in order to reduce levels in an environmentally sustainable manner. Ideally, composite samples taken every hour across a 24 h period would provide a much clearer picture of the physicochemical composition of the farm. This should be noted when interpreting these results.

3.2. Microscopic identification

Partial speciation was first conducted on all samples collected using microscopy. As similar findings were observed at all of the sampling points, results were grouped together as a whole for ease of reporting. Approximately 20 genera of algae were identified using microscopy and classic identification keys (see Table 2). The most common type of algae present was green algae (Chlorophyta and Charophyta) with a minimum of 12 genera observed (*Scenedesmus, Monoraphidium, Micractinium, Chlorella, Chlamydomonas, Pediastrum, Dictyosphaerium, Closterium, Actinastrum, Pandorina, Oocystis* and *Ankistrodesmus*). A total of 5 genera of brown algae (Ochrophyta) were observed, 4 of which were diatoms (*Cyclotella, Nitzschia, Stephanodiscus* and *Tabellaria*). Finally, *Euglena* (Euglenophyta) which are commonly referred to as naked algae, the dinoflagellate *Peridinium* (Pyrrophyta)

Table 2
Breakdown of the genera of algae easily identifiable under microscopic examination. Breakdown includes all sampling points collected from the novel IMTA fish farm between December 2019 and October 2020. The genus name, the individual algal phylum and types (based on pigmentation) to which they belong, the month their presence was observed and the recording of multiple species have been included.

Genus Identified	Phylum	Algae Type	Month Present	Multiple Species
Actinastrum	Chlorophyta	Green Alga	6–10	Unknown
Ankistrodesmus	Chlorophyta	Green Alga	3–9	Unknown
Chlamydomonas	Chlorophyta	Green Alga	1–4, 7–11	Unknown
Chlorella	Chlorophyta	Green Alga	1–11	Yes
Closterium	Charophyta	Green Alga	1–11	Unknown
Cyclotella	Bracillariophyta	Diatom	1–4	Unknown
Dictyosphaerium	Chlorophyta	Green Alga	1–4, 6	Unknown
Euglena	Euglenozoa	'Naked' alga	3	Unknown
Mallomonas	Ochrophyta	Brown Alga	7	Unknown
Micractinium	Chlorophyta	Green Alga	1–11	Unknown
Monoraphidium	Chlorophyta	Green Alga	1–11	Yes
Nitzschia	Bracillariophyta	Diatom	1–11	Yes
Oocystis	Chlorophyta	Green Alga	3–4, 6–8	Unknown
Pandorina	Chlorophyta	Green Alga	2–4, 6–8	Unknown
Pediastrum	Chlorophyta	Green Alga	2, 7–8, 10	Unknown
Peridinium	Miozoa	DinoflagellateYellow-Brown Alga	1–4, 6–7, 10	Unknown
Rhodomonas	Cryptophyta	Nearly Brown Alga	1–4, 6–8	Unknown
Scenedesmus	Chlorophyta	Green Alga	1–11	Yes
Stephanodiscus	Bracillariophyta	Diatom	1–10	Unknown
Tabellaria	Bracillariophyta	Diatom	6–7, 10	Unknown

1 = Dec, 2 = Jan, 3 = Feb, 4 = Mar, 5 = Apr, 6 = May, 7 = Jun, 8 = Jul, 9 = Aug, 10 = Sep & 11 = Oct.

and *Rhodomonas* (Cryptophyta) were also observed. The majority of the species were observed throughout most the entire year. *Cyclotella* and *Dictyosphaerium* were only observed between December and March / May respectively. *Pandorina, Oocystis* and *Ankistrodesmus* were found between February and August. *Tabellaria* and *Actinastrum* were observed between May and September. *Mallomonas* was only found in June and *Euglena* only in February.

Microscopic analysis also indicated that at least 4 genera (*Chlorella, Monoraphidium, Nitzschia* and *Scenedesmus*) had multiple species present. Due to the similarities and complexities of the algae when observing them under the microscope, and the high volume and variation within the individual samples, an indication of whether multiple species were present for all genera could not be immediately established. Additionally, physiological similarities within individual genera also hindered the determination of full speciation, *e.g.* many species of *Scenedesmus* appeared to be visually very similar making it very hard to differentiate between the individual species. As such, molecular identification was subsequently conducted in order to; 1) confirm the presence of the genera identified using microscopy, 2) determine whether multiple species for the respective genera were also present, and 3) identify all species present in the system, including those that were not identifiable using microscopy.

3.3. Molecular identification

Crossing the Illumina and MinION data, a combined total of 982 species from 341 genera across nine phyla were identified, as shown in Fig. 2. Nine species across two genera (0.92 %) of Haptophyta, 44 species across nine genera (4.48 %) of Cryptophyta (four of which had multiple species), 38 species across nine genera (3.87 %) of Euglenophyta or Euglenozoa (with only one indicating multiple species), two species across two genera (0.20 %) of Glaucophyta and four species across three genera (0.41 %) of Rhodophyta were identified. The four remaining phyla displayed the greatest populations. Fifty species of Pyrrophyta (Miozoa), or more specifically dinoflagellates, were identified across nineteen genera (5.09 %), three genera of which were found to have multiple species. A total of 304 species of Ochrophyta were identified across 85 genera (30.96 %). Of that, 177 species across 53 genera (18.02 %) were found to be diatoms (Bracillariophyta). Six genera of Ochrophyta and nine genera of diatoms had multi species. Some 45 species of Charophyta were identified across 21 genera (4.58 %), and 486 species of Chlorophyta were identified across 191 genera (49.49 %) making it the most common phylum of algae present in the system. Only two genera of Charophyta had multi species found. However, Chlorophyta had nineteen genera with multi species.

Given the high volume of species identified, only genera where multiple species (>4) were included in the phylogenetic tree (Fig. 2) for ease of reporting. Again, for ease of reporting, only the most common genera and species, as well as those considered to potentially be the most beneficial / potential hazardous were included (Zhou et al., 2009; Lucas et al., 2019; Yarnold et al., 2019; Lee and Ryu, 2021; Al-Hussieny, 2022). A full breakdown of all species can be found in the Supplementary Data 2 – Species Breakdown. Due to limitations and restrictions as a result of the COVID-19 pandemic, samples could not be analysed for each individual month as well as for each location (culture ponds, treatment lagoon and reservoir). Therefore, samples for each location were pooled together. Samples had to be sent away for analysis which severely limited how much analysis could be requested.

As no variation was observed between the four culture ponds with respect to the genera and species identified, results were grouped together. The same applied for the entry and exit points of the treatment lagoon. See Table 3 for a breakdown of the most common genera identified in their respective locations. The culture ponds demonstrated the greatest variation of algal populations with all of the most common genera, with the exception of *Frustulia*, *Halamphora* and *Lagerheimia*, identified. The treatment lagoon was found to have 37 of the 46 common genera and half (23) of the most common genera identified throughout the farm were found in the reservoir.

4. Discussion

Although research into the use of algae in aquaculture is still limited, many of the most abundant species identified during this preliminary IMTA peatland study hold great potential for their application to organic, sustainable aquaculture. These included *Scenedesmus, Monoraphidium, Micractinium, Chlorella, Chlamydomonas, Pediastrum, Dictyosphaerium, Closterium, Actinastrum,* and *Ankistrodesmus*. The majority of the identified genera, including those previously mentioned, have not been known to cause any adverse effects on their ecosystems. However, two species have been previously found to contribute to adverse effects. Some *Pandorina* species have been known to excrete toxic compounds that inhibit the growth of other algae and higher plant life (Patterson and Harris, 2007). Some species of *Oocystis* are well known to cause harmful algal blooms or HAB's (Pal et al., 2020). With the exception of *Peridinium*, which is also well known to be an instigator of HAB's (Hallegraeff et al., 2004; Ki and Han, 2007), the rest of the genus identified in the samples are not known to cause negative effects on their environment. Although *Pandorina* can exhibit potentially negative effects by inhibiting beneficial algae and plants, it has been reported to also be an inhibitor of *Peridinium*, making its presence in the system potentially advantageous (Patterson and Harris, 2007). There is a commensurate interest on the impact of extreme climate events that may affect aquaculture processes where microalgae have a key role in process performance and regulation (Naughton et al., 2020; O'Neill et al., 2022).

Microalgae have been extensively studied as a means to support waste process remediation along with production of bioactives for OneHealth applications (Naughton et al., 2020; Rowan and Pogue, 2021). Satyanarayana et al. (2011) highlighted that microalgae are a promising resource due to their high production capacity of vegetable oils. These authors highlight that microalgae possess a high growth rate, need abundantly available solar light and CO_2, and thus are more photosynthetically efficient than oil crops. Also, they tolerate high concentration of salts allowing the use of any type of water for the agriculture and the possibility of production using innovative compact photobioreactors. Tan et al. (2020) revealed the potential enormity of high-value bio-compounds derived from microalgae such as lipids, carbohydrates, proteins, and other bioactive compounds from microalgae; however, large scale commercial production and extraction processes have only recently becoming established.

There has been growing interest in exploiting microalgae as a natural process for low-cost wastewater treatment and for water quality control and remediation in aquaculture (Naughton et al., 2020). These authors evaluated an Irish based freshwater flow-through aquaculture process for production of Eurasian Perch (*Perca fluviatilis*) in the Republic of Ireland and revealed the predominance of microalgae and cyanobacteria where *Chlorophyta*, *Bracillariophyta* and *Cryptophyta* were the most dominant algal phyla present. They showed that use of flow-cytometry correlated with in-field AlgaeTorch™ for analysing microalgae in aquaculture. Findings from this present study however highlighted the enormous variability in microalgae species present that may be under appreciated by use of the aforementioned conventional microscopy, flow cytometry and in field AlgaeTorch handheld sensor. However, there is also significant merit in exploring the role of Artificial Intelligence and machine learning in the real-time analysis of large next-generation sequencing data sets that will support and enable decision making on the farm. Preventive risk mitigation to combat occurrence of unwanted cyanobacteria could entail growth of beneficial helper microalgae (such as *Chlorella*) in bioreactors and re-introducing these into the pond as preventive or counter-measure to combat emergence of undesirable cyanobacteria that can cause fish death.

In terms of this IMTA production process supported by microalgae and nexus to pond water quality, the established physicochemical parameters were compared to water quality parameters from the Irish EPA, which were based on the Freshwater Fish Directive [78/659/EEC] and Surface Water Regulations [1989] (EPA, 2001), and the Statutory Instrument (SI) European Communities Environmental Objectives (Surface Waters) Regulations 2009 [SI 272 of 2009] and amendments [SI 77 of 2019]

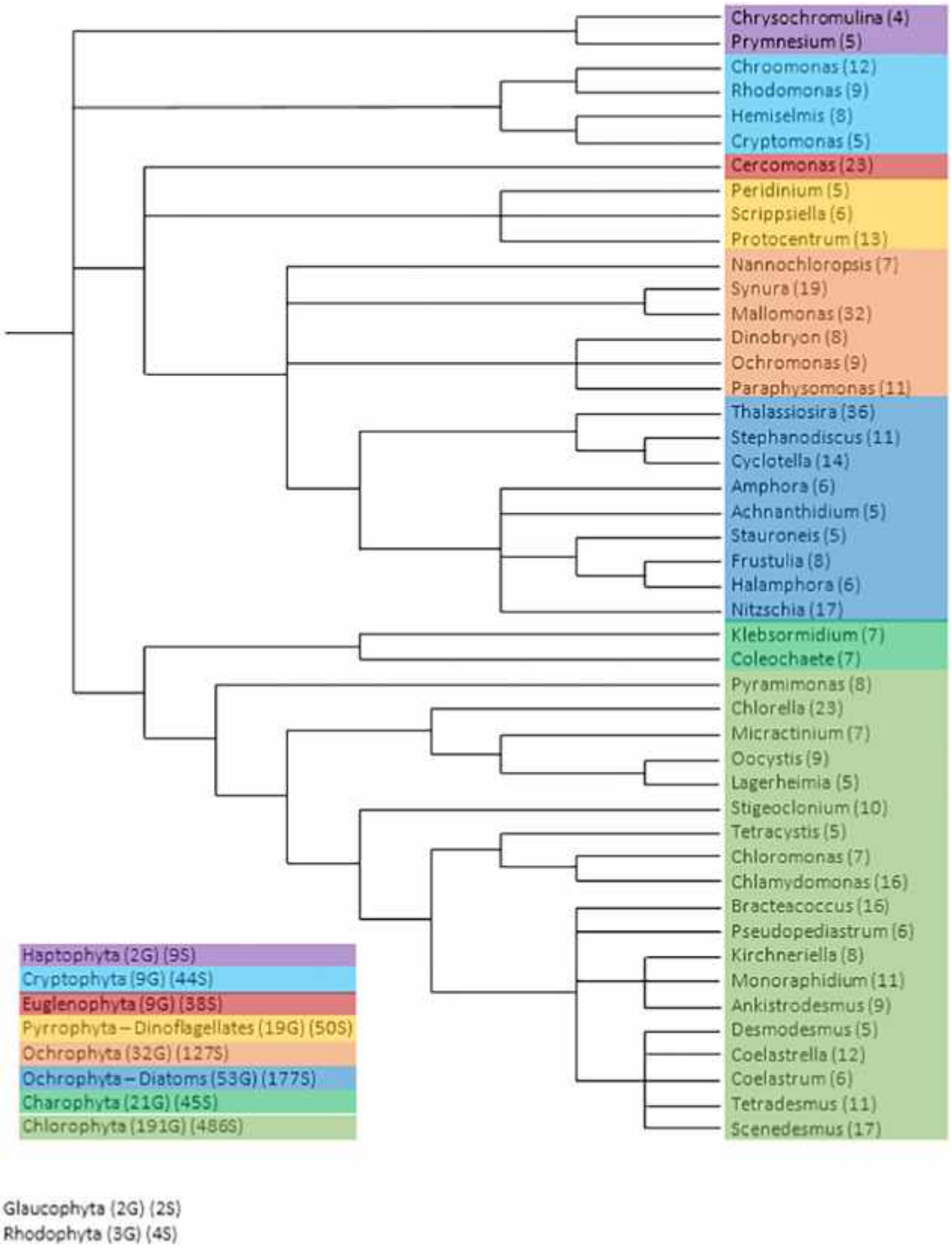

Fig. 2. Phylogenetic tree identifying genera of algae found in the novel freshwater IMTA system between December 2019 and October 2020. (n) = number of species for that specific genera, (nG) = number of genera for that specific phylum, (nS) = number of species for that specific phylum. For ease or reporting only genera with multiple species have been included. Euglenophyta = Euglenozoa, Pyrrophyta = Miozoa, Ochrophyta (Diatoms) = Bracillariophyta.

(Irish Statutory Office, 2009, 2019). With the exception of four parameters (nitrite, orthophosphate, BOD and suspended solids), all other parameters were within range of the guidance and recommended values. The orthophosphate was above the guidance value of <0.03 mg PO_4^{3-} L^{-1}, as too was the nitrite guidance value of <0.03 mg NO_2^- L^{-1}. An increase in these parameters should increase algal growth within the system. However, issues with both BOD and suspended solid levels may prevent this. The BOD levels were well above the <3 mg O_2 L^{-1} guidance value for salmonid waters and suspended solid levels were above the guidance value of <25 mg L^{-1}. Excess levels of BOD in the system reduces the level of oxygen available for both the algae and the fish. Whilst higher than normal levels of suspended solids affect the level of sunlight available for algae to photosynthesise and well as induce gill irritation within the fish. Mitigation levels were applied within the farm to aid in these issues. Additional aeration for oxygen generation was added by the introduction of more paddle wheels and air lifts, and filtration systems were introduced to reduce the level of suspended solids. Reduction in levels were observed after these changes were applied.

Table 3
Breakdown of the location of where the most common genera with multiple species that were identified during molecular analysis. • indicates presence of genus, grey indicates absence of genus.

Genera	Location			Genera	Location		
	Pond	Lagoon	Reservoir		Pond	Lagoon	Reservoir
Achnanthidium	•			*Mallomonas*	•	•	•
Amphora	•	•		*Micractinium*	•	•	•
Ankistrodesmus	•	•		*Monoraphidium*	•	•	•
Bracteacoccus	•	•	•	*Nannochloropsis*	•	•	
Cercomonas	•	•		*Nitzschia*	•	•	•
Chlamydomonas	•	•	•	*Ochromonas*	•	•	•
Chlorella	•	•	•	*Oocystis*	•	•	•
Chloromonas	•	•		*Paraphysomonas*	•		
Chroomonas	•	•	•	*Peridinium*	•		•
Chrysochromulina	•	•		*Prorocentrum*	•		
Coelastrella	•	•	•	*Prymnesium*	•		
Coelastrum	•	•	•	*Pseudopediastrum*	•	•	•
Coleochaete	•	•		*Pyramimonas*	•	•	
Cryptomonas	•	•		*Rhodomonas*	•	•	
Cyclotella	•	•	•	*Scenedesmus*	•	•	•
Desmodesmus	•	•		*Scrippsiella*	•		
Dinobryon	•	•	•	*Stauroneis*	•	•	
Frustulia		•	•	*Stephanodiscus*	•	•	•
Halamphora		•		*Stigeoclonium*	•		
Hemiselmis	•	•	•	*Synura*	•	•	
Kirchneriella	•	•		*Tetracystis*	•		•
Klebsormidium	•	•		*Tetradesmus*	•		•
Lagerheimia		•		Thalassiosira	•	•	•

Increasing trends in global warming already evident, the likelihood of further rise continuing, and their impacts give urgency to addressing carbon sequestration technologies more coherently and effectively (Rowan and Galanakis, 2020; Galanakis et al., 2021). Carbon dioxide (CO_2) is responsible for over half the warming potential of all greenhouse gases (GHG), due to the dependence of world economies on fossil fuels. Peatlands represent an important carbon sink (Rowan and Galanakis, 2020). However, increasing the trends in global warming are already evident where scientists predict that we have a fifty-fifty chance of experiencing a 1.5 °C rise in temperature by 2026 (Frost, 2022). Thus, the unprecedented impacts of same gives urgency to addressing carbon sequestration technologies more coherently and effectively. The processes involving CO_2 capture and storage (CCS) are gaining attention as an alternative for reducing CO_2 concentration in the ambient air (Iglina et al., 2022); however, these technologies are considered as short-term solutions, as there are still concerns about the environmental sustainability of these processes (Singh and Ahluwalia, 2012). A promising technology could be the biological capture of CO_2 using microalgae due to its unmatched advantages over higher plants that can be pursued through this peatland based IMTA system. Microalgae are phototrophic microorganisms with simple nutritional requirements that have strong potential to support carbon sequestration for our planet (Singh and Ahluwalia, 2012). Iglina et al. (2022) recently reported *Chlorella* species is the best microalgae they studied at capturing CO_2 using vertical tubular bioreactors. These authors noted that CO_2 emission accounts for about 77 % of all greenhouse gases, and the calculation of greenhouse gas emissions is 56 % of all CO_2 imports.

Development of new sustainable food production systems as described in this case study are aligned with many of the sustainable development goals of the United Nation's (Rowan and Casey, 2021). Given recent flux in food production and security brought on by stressors such as climate change, COVID-19 and conflict in Ukraine, there is likely to be a food supply chain challenge where development and replication of such IMTA systems across the peatlands globally will help address this challenge using a green solution (Galanakis et al., 2021). Digital transformation of IMTA process will improve monitoring, performance and protection of innovation for future sustainability that includes internet of things (IoT), cloud-edge enablers for remote sensing including use of drones and robots, artificial intelligence, immersive technologies for training, and use of

blockchain for cybersecurity and business model development (Rowan et al., 2022; Rowan, 2022). However, there is also significant potential for future use of artificial intelligence with machine learning for evaluating NGS and bioinformatics data for performance and trends (Liakos et al., 2018; Sharma et al., 2020; Benos et al., 2021; Meshram et al., 2021). For example, digital technologies may also inform and enable fish pumping, fish counting, automated feeding along with real-time monitoring of physicochemical parameters such as free ammonia, temperature, oxygen and pH at this IMTA site. These findings will inform the adjacent Terrain-AI (2022) that uses drone and satellite technologies to uncover new insights into supporting effective climate change decision-making across peatlands with comparisons made to grasslands, cropland and forestry.

5. Conclusion

Microalgae constitute an important element of the microbiome in aquaculture ponds where there is a reliance on the efficacy of these to maintain water quality in a novel IMTA system situated in the Irish peatlands. Detailed studies revealed significant difference in the abundance of microalga based upon use of conventional microscopy compared to that of using next generation sequencing and bioinformatics for the same ponds. Given the broad diversity, there is an opportunity to exploit AI and machine learning to monitor microalgae in this IMTA system that will inform real-time decision making. There is also a pressing need to understand better the dynamic interaction between microalgae and bacteria that includes provision for modelling and predicting the impact of extreme weather events on fish biomass production and disease mitigation. This IMTA system offers potential new solution towards high protein production that aligns with many of the UN's sustainable development goals.

6. Future research

The preliminary study has demonstrated how even more complex the system is than originally thought. Therefore, the next phase of research will include; Analysis of each location on a monthly basis in order to establish a better understanding of the seasonal relationship between the populations of algae present in the system *versus* the physicochemical parameters necessary for growth, as well as the potential impacts the changes to weather will have. Analysis into what other organisms are present within the system in order to establish their role within the system and their relationship, if any, whether it be beneficial, hazardous or unaffected, to the growth and performance of algae *e.g.*, microscopic analysis indicated the presence of rotifers within the system. A similar month-to-month profile of all cyanobacterial species present needs to be conducted, as well as the development of mitigation procedures in order to eliminate hazardous species. Potential combined use bioinformatics with artificial intelligence including machine learning to understanding the occurrence and variance of different microalgae in this IMTA pond in real time based on operational parameters and environmental conditions including extreme weather events given that this is an open plan recirculating aquaculture system. This is aligned with precision aquaculture and will inform decision making on process adjustment and farm management. Apply digital technologies to improve real-time monitoring and performance of IMTA process that includes data-analysis and protection for improved decision making. Future research will also focus on the combined use of massive sequencing data from this IMTA system to inform the importance of phytoplankton generated in the food chains of the peat bogs and if they serve as food for fish. This approach will also be expanded to include autotrophic species comprising periphyton that can potentially represent primary productivity, such as presence of cyanoprokaryotes in freshwater environments that are possibly toxic. Finally, an investigation into the development of organic and sustainable methods to help mitigate issues that arise from unwanted species, as well as a means to limit their future presence from the system without impacting the beneficial species present.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158392.

CRediT authorship contribution statement

Emer A. O'Neill: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gustavo Fehrenbach:** Data curation, Methodology. **Emma Murphy:** Data curation, Methodology. **Sérgio A. Alencar:** Data curation, Formal analysis, Methodology, Software, Writing – review & editing. **Robert Pogue:** Data curation, Formal analysis, Methodology, Software, Writing – review & editing. **Neil J. Rowan:** Conceptualization, Formal analysis, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Data availability

The authors are unable or have chosen not to specify which data has been used.

Declaration of competing interest

The authors declare that there are no competing interests of conflicts of interest with respect to the publication of this article.

Acknowledgements

The authors acknowledge provision of aerial image of Mount Lucas peatland site furnished by Bord Iascaigh Mhara (BIM). The authors would like to thank Interreg Atlantic Area Neptunus (Project EAPA_576/2018), Regional University Network European University (RUN-EU Project), and Bord Iascaigh Mhara (Project 2019 BIM-KGS-008) for funding support.

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Science of the Total Environment 868 (2023) 161495

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Potential disruptive effects of zoosporic parasites on peatland-based organic freshwater aquaculture: Case study from the Republic of Ireland

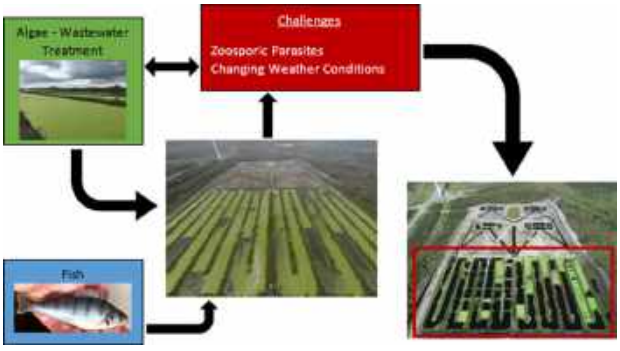
Emer A. O'Neill *, Neil J. Rowan

Centre for Sustainable Disinfection and Sustainability, Bioscience Research Institute, Technological University of the Shannon: Midlands Midwest, Athlone Campus, University Road, Athlone, Co. Westmeath, Ireland
Faculty of Science & Health, Technological University of the Shannon: Midlands Midwest, Athlone Campus, University Road, Athlone, Co. Westmeath, Ireland

HIGHLIGHTS

- Novel aquaculture system offers sustainable fish production.
- System depends on microalgae for waste bioremediation and water quality.
- Algal presence is important for organic processes.
- Zoosporic parasites may cause collapse of algae population.
- Climate change may contribute to zoosporic parasite issues.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
Microalgae
Cyanobacteria
Next generation sequencing
Recirculating aquaculture multi-trophic pond system
Climate
Sustainability

ABSTRACT

Irish freshwater aquaculture holds great potential for aiding food security. However, its necessary expansion has been hampered by the adoption of important environmental EU directives. A novel peatland-based recirculating aquaculture multi-trophic pond system (RAMPS) was developed to assess its potential to assist in the sustainable development of industry whilst remaining aligned with environmental protection by adhering to organic aquaculture practices. Microalgae play a pivotal role in the farms' wastewater bioremediation. However, a collapse of the algal population within the system towards the end of the pilot study was observed. No relationship between physicochemical fluctuations and the collapse were indicated. Further investigations into the potential presence of biological agents were then conducted and fourteen species of zoosporic parasites from five different genera (*Labyrinthula, Vampyrella, Amoeboaphelidium, Paraphelidium* and *Aphelidium*) were identified after conducting next-generation sequencing (MinION). The presence of these species indicated the potential cause of algal collapse. Additionally, changes in weather conditions may have also contributed to the issue. Given the lack of data available on zoosporic parasites and their potential impact on organic aquaculture practices, additional research needs to be conducted. Developing a means to monitor and mitigate against these complex zoosporic parasites will inform food security, it will particularly help safeguard "organic" freshwater aquaculture where there is a reliance on using natural-based approaches to address disease mitigation. This information will in turn inform the replication of this RAMPs system in peatlands internationally creating local employment in green technologies, as communities' transition away from burning peat as fossil fuel. Also, zoosporic parasites may reduce important microalgae in peatland-based culture ponds that serve as exceptional sequesters of carbon. Findings of this study will inform related research that focus on the emergence of microbial pathogens in local aquatic ecosystems brought on by variances in climate.

* Corresponding author.
E-mail address: emer.oneill@ait.ie (E.A. O'Neill).

http://dx.doi.org/10.1016/j.scitotenv.2023.161495
Received 21 October 2022; Received in revised form 5 January 2023; Accepted 5 January 2023
Available online 10 January 2023

1. Introduction

Aquaculture has become the fastest growing food producing industry in the world (O'Neill et al., 2019, 2020; Houston et al., 2020) as a result of capture fisheries reaching the maximum sustainable yields, and in some instances surpassing it (Tsikliras and Froese, 2019; O'Neill and Rowan, 2022). It provides an important source for food security, both directly as a means of meeting the ever growing demand for food from an expanding global population and indirectly by providing a means of employment opportunities (Golden et al., 2021; Garlock et al., 2022). Fish produced *via* aquaculture is now available to countries that would have previously been limited or non-existent, often at cheaper prices, thus providing improved nutrition and food security (Rowan and Casey, 2021; Galanakis et al., 2021; O'Neill et al., 2022a, 2022b). However, growth of the industry has begun to slow down (Edwards et al., 2019; Mirto et al., 2022).

In Ireland, issues with licensing, environmental concerns and limited space / resources have hampered the growth of the industry (O'Neill et al., 2020, 2022b). To respond to these challenges, Bord Iascaigh Mhara (BIM), Ireland's state agency for developing the national marine fishing and aquaculture industries, developed Ireland's first trial peatland based, recirculating aquaculture multi-trophic pond systems (RAMPS) adhering to 'organic' principles, as peatlands now being listed as important habitats under the EU's Birds [79/409/EEC & 2009/147/EC] and Habitats [92/43/EC] directives. The peatland RAMPS system holds European perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiis*), common duckweed (*Lemna minor*) and gibbous duckweed (*Lemna gibba*) and exploits the use of microalgae for waste removal (Bord na Móna, 2019; O'Neill et al., 2022c; Rowan et al., 2022). The process differs from traditional aquaculture practices that use water from rivers and lakes where traditional systems must consider potential pollutants from agriculture, industry, wastewater treatments plants, *etc.* (Rowan, 2011; Tahar et al., 2017; Tiedeken et al., 2017; Tahar et al., 2018a, 2018b, 2018c).

The potential beneficial roles of microalgae in aquaculture has only recently been confirmed (Gao et al., 2016; Ansari et al., 2017; Han et al., 2019; Nagarajan et al., 2021). For example, microalgae could efficiently assimilate nutrients providing a good method for wastewater remediation (Wang et al., 2015; Leng et al., 2018; Han et al., 2019) in aquaculture, having already demonstrated promising performances in the food and agriculture industries, and in municipal wastewater treatment (DeBashan et al., 2004; Lu et al., 2015, 2017; Han et al., 2019; Nagarajan et al., 2021; O'Neill et al., 2022b). As a result of this and other emerging benefits, the use of microalgae in aquaculture has recently emerged into the forefront. However, research into the role of microalgae in aquaculture is still lacking (Han et al., 2019; O'Neill et al., 2022a).

The development of microalgae derived products has greatly increased in recent years however, sustainable production faces biological challenges, such as the presence of zoosporic parasites (Höger et al., 2021). Zoosporic parasites are facultative parasites that produce motile spores as their infective propagules causing frequent epidemics in aquatic ecosystems (Scholz et al., 2016) as many of these parasites are known to infect vertebrates, invertebrates, vascular plants, macroalgae, phytoplankton, fungi and protists (Gleason et al., 2014a). Additionally, it is very difficult to differentiate between divergent species of zoosporic parasites as many exhibit very similar morphological characteristics. As such, it is therefore possible that the profile of zoosporic parasites present in aquatic habitats may be undescribed. Given these adversities, it is also possible that a general lack of research in this area may result in infection incidences within the susceptible algal populations of these aquatic habitats being underreported (Scholz et al., 2016). They are believed to be major drivers of phytoplankton succession, and as a consequence, infections can alter the composition of algal species in aquatic ecosystems (Scholz et al., 2016). Zoosporic parasites have received increased attention in recent years; however, research is still greatly lacking (Gleason et al., 2014b; Scholz et al., 2016).

The hypothesis behind this research evolved around the fact that dramatic losses in the microalgae populations within the peatland based trial fish farm could not be explained through use of traditional monitoring techniques and approaches. This intimated that there was other unexplained agent(s) potentially present in the RAMS system causing the observed disruptive issues. Therefore, the aim of this research was to determine whether issues associated with the loss of important algal and cyanobacterial populations within this unique peatland-based aquaculture farm may have been due to the presence and deleterious activities of zoosporic parasites and if so, what potential effects could their presence induce on this novel primary food production system and its connected aquatic environment.

2. Materials & methods

2.1. Study site

Oasis fish farm is an innovative trial peatland based recirculating aquaculture multi-trophic pond system (RAMPS) process set in the middle of Mount Lucas Wind Farm, Co. Offaly (53°17′3″ N - 7°11′45″ W). The RAMPS system combines elements from a recirculating aquaculture system (RAS) and an integrated multi-trophic aquaculture (IMTA) system). The RAMPS consists of four split (pill) ponds connected to a microalgae and duckweed lagoon with sixteen channels serving as an organic treatment system. See Fig. 1. Fish are kept at a density that does not exceed the organic farming standard (*e.g.*, <20 kg/m^{-3} for perch), using screens at the D-ends of each split pond. The space between two D-end fish culture areas is also used to treat waste with free living microalgae in suspension. Flow in each split pond is generated and water is circulated using an airlift. Each D-end fish culture area is equipped with oxygen and temperature probes connected to paddlewheels to provide extra oxygen when necessary. Flow in the lagoon is generated with paddle wheels. The farm is designed to hold a maximum of 32,000 Kg of fish. European perch (*Perca fluviatilis*) is cultured in one pond and rainbow trout (*Oncorhynchus mykiis*) is cultured in the other three. No effluent is released from the farm unless excessively high levels of rainfall is experienced (O'Neill et al., 2022b; Rowan et al., 2022).

2.2. Sample collection and preservation

Collection and preservation of samples were conducted as per O'Neill et al. (2022a, 2022b) with some modifications (samples were collected on different days to the previous studies conducted in parallel). Five litres (grab) water samples were collected from the trial RAMPS fish farm in octagonal carboy HDPE bottles (Lennox) and transported directly to the lab *via* car, 62 km away, in insulated boxes in order to maintain as close to *in-situ* conditions as possible. Samples were collected once a week between July 2020 and October 2020. Samples were taken from each of the culture ponds and, the entry and exit points of the treatment lagoon. See Fig. 1 for an overview of the farm sampling locations. One hundred mL of each sample was stored at -20 °C until molecular analysis was conducted. Physicochemical analysis was conducted immediately upon arrival from the fish farm.

2.3. Microalgae and cyanobacteria monitoring

The microalgae and cyanobacteria levels were monitored on the farm in real time using the AlgaeTorch® (ISO 10260:1992; DIN 38412-16). The hand help device measurement is based on fluorescence which is proportional to the chlorophyll present in the microalgae and cyanobacteria (Naughton et al., 2020). Measurements were recorded in the same locations where the environmental samples were taken (Fig. 1). The device was placed into the water for approximately 15 s and the readings indicated on the device's display were recorded.

2.4. Physicochemical monitoring

Physicochemical analysis was conducted as per O'Neill et al. (2022a, 2022b) with some modifications. Temperature, pH, ammonium (NH_4^+),

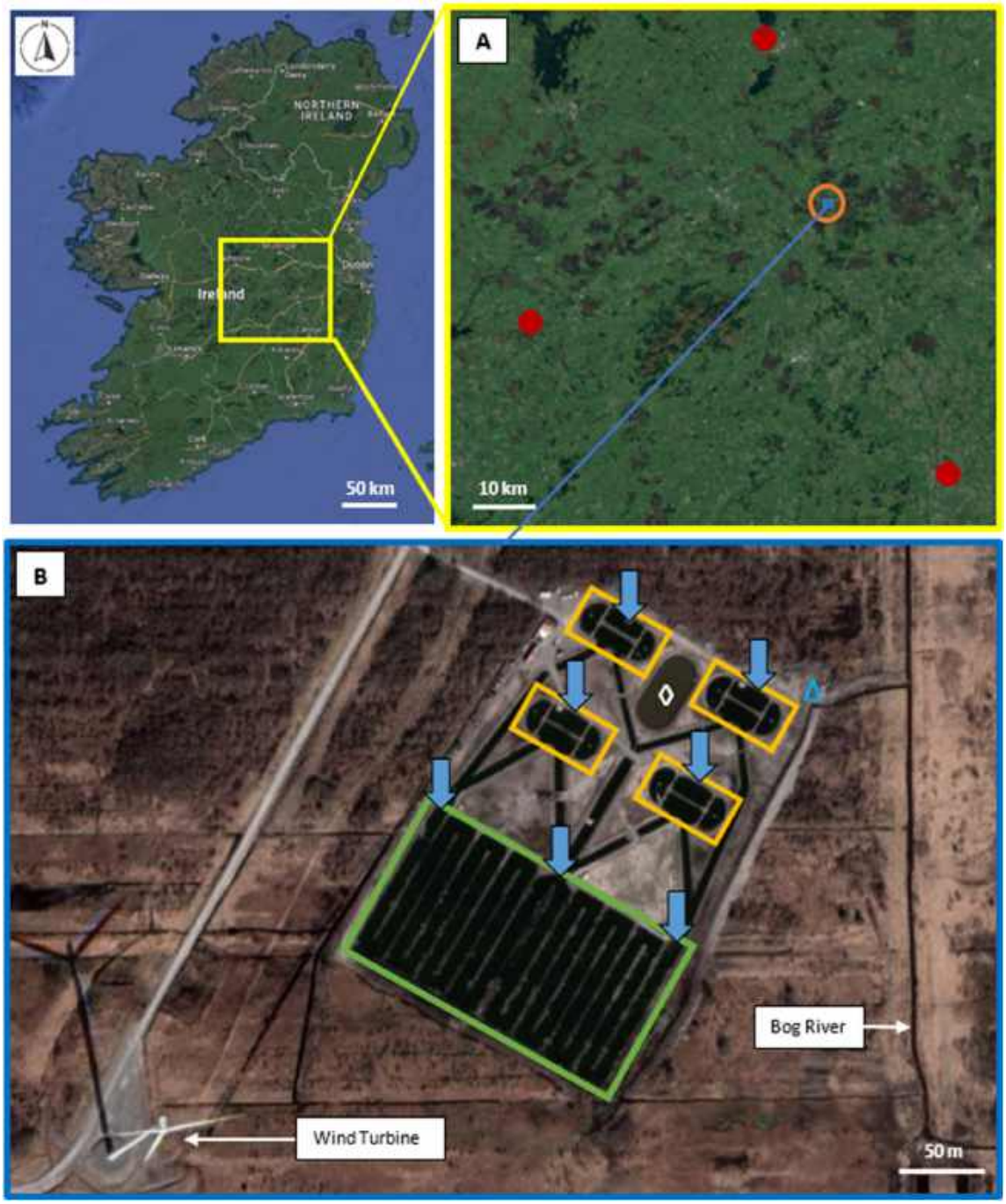


Fig. 1. A) Location of the three meteorological weather stations (yellow box); Gurteen – 53°02′24″ N, 08°00′36″ W; Oak Park– 52°51′36″ N, 06°55′36″ W; Mullingar – 53°33′36″ N, 07°20′24″ W, (red •) surrounding the trial fish farm (orange **O**). Meta data from these three stations were used to analyse any potential impacts changing weather conditions may have had on the system. B) Ariel view of the trial fish farm (Oasis) at Mount Lucas. Culture ponds (orange box), algae and duckweed lagoon (green box), reservoir (white ◊) and water intake location (blue **Δ**) displayed. Blue arrows indicate location of sampling points. Wind turbine which generates all electricity for the farm and the bog river which supplies water for the farm are also included. Figures adapted from O'Neill et al., 2022a. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nitrite (NO_2^-), nitrate (NO_3^-), orthophosphate (PO_4^{3-}), dissolved oxygen (DO), biochemical oxygen demand (BOD), total suspended solids (TSS) and total dissolved solids (TDS) were investigated in the laboratory within 24 h of collection to prevent the need for preservation. Temperature, pH and TDS were analysed using a benchtop pH and conductivity meter (VWR pHenomenal™ MU 6100 L meter, VWR 111662–1157 pH probe, VWR CO11 conductivity probe). DO and BOD_{5day} were analysed using a benchtop DO_2 meter and probe (Jenway). Photometric kits were used to assess the NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} (Merck), as per the manufacturer's (Spectroquant®) instructions. The TSS were analysed *via* filtration using a Buchner flask, Buchner funnel and 0.20 μm pore membrane filter (Whatman).

2.5. Molecular analysis

Isolation of DNA, 18S sequencing and bioinformatics were conducted as per O'Neill et al. (2022b) with some modifications. The frozen water samples were first thawed, then centrifuged and the resulting pellet

re-suspended in 2 mL of dH_2O, before sonication at 40 kHz in a sonicator bath (Cuyson) at 45 °C for 20 min. DNA was isolated using the Roche High Pure PCR Template Preparation kit (Merck). Between 100 and 150 ng of DNA was used in a PCR reaction for the amplification of an 1800 base pair segment of the 18S ribosomal DNA sequence that was modified with adapter sequences (O'Neill et al., 2022b) for subsequent sequencing on the Oxford Nanopore MinION platform (adaptor sequences in red and underlined):

(F): 5′- TTTCTGTTGGTGCTGATATTGCGGTGATCCTGCCAGTAGTCATATGCTTG -3′.

(R): 3′- ACTTGCCTGTCGCTCTATCTTCGATCCTTCCGCAGGTTCACCTACGGAAACC -5′.

Sequencing was conducted using the Ligation Sequencing Kit SQK-LSK114 (Oxford Nanopore Technologies) protocol, as per the manufacturer's instructions. The quality of the sequencing was assessed using the FastQC tool (v0.11.9). The adapters were removed using the Porechop adaptor trimmer tool (v0.2.4). The FastQ tool (v0.14.0) was then used to eliminate low quality reads, followed by quality assessment once again to confirm its success. The resulting high-quality reads were then grouped together using the isONclust software (v0.0.4). Each group, representing all reads that came from an individual gene, was then aligned against the National Center for Biotechnology Information (NCBI) nucleotide database using the BLASTn tool (v2.13.0) with an *E*-value of 0.05.

2.6. Statistical analysis

Statistical analysis were performed on GRAPHPAD PRISM 9.3. The data generated were grouped and subject to normality tests (Anderson-Darling), to determine if samples were from a normal distribution ($P > 0.05$ = normal distribution). Parametric testing was then applied as a normal distribution was indicated. Grubbs test was used to determine if any outliers were indicated. *t*-tests and ANOVA (one-way with Tukey and two-way with Sidak) were used to determine if any significant differences were observed between the variables ($P < 0.05$ = significant difference). Unpaired tests were used as different sets of samples were analysed. Pearson's coefficient was applied to determine whether any relationship correlations (r) were indicated.

3. Results

In order to obtain a more comprehensive analysis of the water quality conditions, ideally a composite sampler would be used to collect approximately 100 mL of water every hour for 24 h. However, due to technical difficulties and limitations in obtaining composite samplers, grab samples were collected. A larger volume (5 L) was collected as slowly as possible in order to aid in compensating for the lack of access to composite samples. This should be noted when interpreting these results.

3.1. Microalgae and cyanobacteria

Microalgae and cyanobacteria readings across the entire system indicated no statistically significant difference (microalgae $P = 0.8976$, cyanobacteria $P = 0.9380$). Therefore, results were grouped together for ease of reporting. Microalgae levels fluctuated between 170 μg L^{-1} and 280 μg L^{-1} between December 2019 and August 2020, as shown in Fig. 2. However, levels dropped dramatically between August 2020 and September 2020 (Fig. 2), falling to 31.65 μg L^{-1} before rising slightly to 95.83 μg L^{-1} the following month (October 2020). Cyanobacteria levels remained consistently between 20 μg L^{-1} and 40 μg L^{-1} from January 2020 to April 2020 before spiking in May to 140 μg L^{-1} (Fig. 2), as would be expected given the time of year. Levels dropped back to 37.45 by July 2020, as shown in Fig. 2. However, similarly with the microalgae, cyanobacteria levels dropped to 1.45 μg L^{-1} during September 2020 before making a slight increase tom 8.83 μg L^{-1} in October 2020 (Fig. 2). Similar but not identical trends were observed in a separate study conducted in parallel with this research at the same site where direct algal and cyanobacterial counts were established using flow cytometry (O'Neill et al., 2022a).

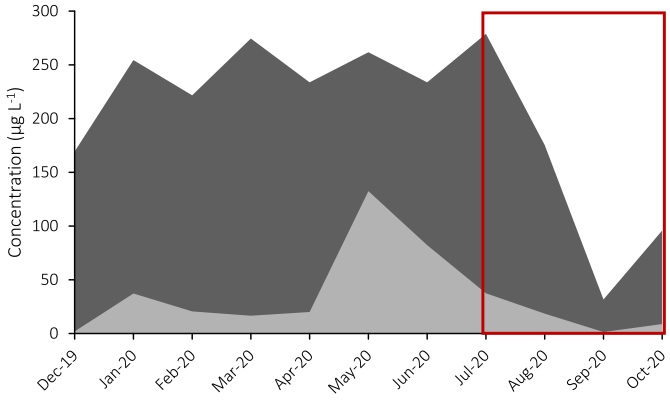


Fig. 2. Concentrations of algae (dark grey) and cyanobacteria (light grey) in μg L^{-1} detected on the trial fish farm between December 2019 and October 2020 using the AlgaeTorch®. Red box indicates when issues were observed within the system resulting in the loss of both algal and cyanobacterial levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Weather conditions

Research conducted by O'Neill et al. (2022a) at the same location indicated changing weather conditions, most likely as a result of climate change, played a contributing factor in issues associated with fish mortalities. Therefore, Met Éireann (The Irish Meteorological Service) meta data was subsequently investigated during the time period where issues with the loss of algal and cyanobacteria populations were experienced to determine whether any relationships existed. Meta data from the three closest Met Éireann weather stations surrounding the fish farm (Mullingar, Co. Westmeath 53°33′36″ N, 07°20′24″ W; Oak Park, Co. Carlow 52°51′36″ N, 06°55′36″ W; Gurteen, Co. Tipperary 53°02′24″ N, 08°00′36″ W) were investigated. See Fig. 1 for the location of all weather stations with respect to the trial fish farm. The average monthly rainfall and temperature between July 2020 and October 2020 were included, as well as rainfall and temperature long term averages (LTA) for those months (Met Éireann, 2022), as shown in Fig. 3.

The average rainfall for the time period remained between 104 mm and 111 mm with the exception of the month of September 2020 where an average of only 59.33 mm was recorded. When data from the 2020 averages were compared to the LTA, rainfall was found to increase for the months of July, August and October but decrease for the month of September (Fig. 3). However, this was not statistically significant ($P = 0.3124$). The average temperature for the time period ranged between 15.43 °C and 9.57 °C. When the 2020 average data was compared to the LTA, similar trends were observed ($P = 0.9830$), as shown in Fig. 3. A correlation study then was conducted to determine whether the issues with algal and cyanobacterial populations (Fig. 2) were as a result of changing temperature or rainfall levels. Although moderate to strong relationships were observed for the microalgae and cyanobacteria with regards to both the rainfall and temperature data, none were statistically significant, as shown in Fig. 4 (microalgae v rainfall $r = 0.647$, $P = 0.3527$; microalgae v temperature $r = 0.534$, $P = 0.4664$; cyanobacteria v rainfall $r = 0.583$, $P = 0.4165$; cyanobacteria v temperature $r = 0.517$, $P = 0.4827$).

3.3. Physicochemical analysis

The physicochemical results were compared to water quality parameters set out by the Irish Environmental Protection Agency (EPA) and the Irish Statutory Office [SI 272/2009 and SI 77/2019] for guidance

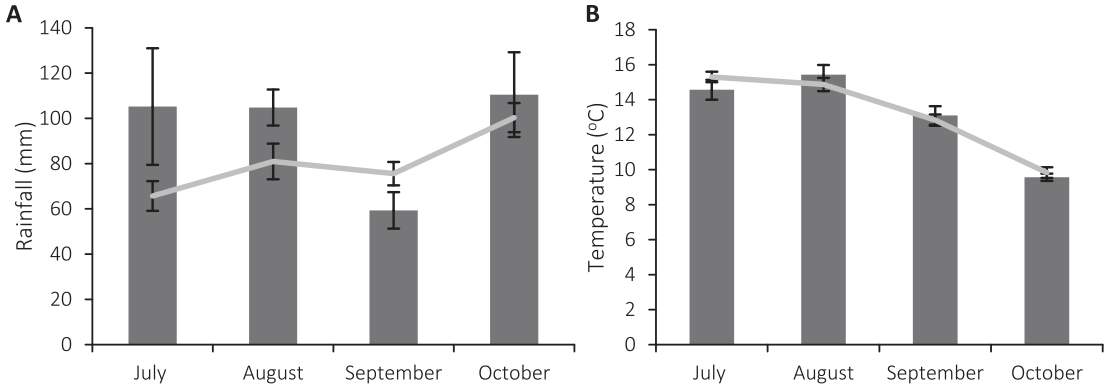


Fig. 3. A) Average rainfall (mm) and B) average temperature (°C) for 2020 (bar chart) and the long term average or LTA (line chart) records across the three meteorological weather stations (Gurteen; Oak Park; Mullingar) surrounding the Mount Lucas trial fish farm (Oasis) during the period (July 2020 – October 2020) where issues were observed within the system. SD indicated.

(EPA, 2001; Irish Statutory Office, 2009, 2019) as no physicochemical values specifically for Irish aquaculture were available (O'Neill et al., 2022a), as shown in Table 1. No issues were observed after physicochemical analysis, with the exception of NO_2^-, PO_4^{3-}, DO and TSS. Both NO_2^- and PO_4^{3-} were consistently above the guidance value of 0.03 mg L^{-1} which may be due to the reducing levels of microalgae and cyanobacteria present in the system and the nutrients were not therefore being consumed by the organisms. The BOD levels were consistently above the guidance value of 6 mg O_2 L^{-1}. This was an on-going problem and mitigation techniques were applied (additional aeration and filtration) to aid in reducing this issue. The TSS levels were also consistently above the guidance value of 25 mg L^{-1}. However, once additional filtration was applied TSS dropped below the guidance value. Fluctuations in oxygen were continually observed however, paddle wheels and airlifts were present in the farm to ensure oxygen levels remained above the guidance values. Oxygen levels may have also dropped during the transfer of environmental samples from the farm to the lab. Results were consistent with physicochemical analysis observed in studies conducted in parallel with this research at the same site during 2020. A correlation study was then conducted to determine whether fluctuations in the physicochemical parameters may have contributed to the loss of microalgae and cyanobacteria populations. No statistically significant correlations were observed between any of the physicochemical parameters and the microalgae or cyanobacteria results, as shown in Fig. 4.

3.4. Zoosporic parasites

Samples for each month and for each location were pooled together. After analysis of the MinION data, fourteen species from five genera (*Labyrinthula, Vampyrella, Amoeboaphelidium, Paraphelidium* and *Aphelidium*) of zoosporic parasites were identified across all of the sampling locations within the system (Fig. 5). All fourteen species were identified in every month (July to October).

4. Discussion

The novel RAMPS, which follows organic aquaculture principles, was initially designed to aid in alleviating issues surrounding the development of the Irish freshwater aquaculture industry as a result of the adoption of important environmental EU directives that have delayed the licensing process, as well as highlighted limitations in space and resources for the industry. Part of the organic process is the use of microalgae for wastewater bioremediation making it an important factor within the farm. This is additionally important as peatlands are now protected habitats given their increasing scarcity. Therefore, the unforeseen and unexpected loss of microalgal populations within the system, particularly around the month of September 2020 was highly problematic. Physicochemical results suggested that the fluctuations in the parameters were unlikely the cause of

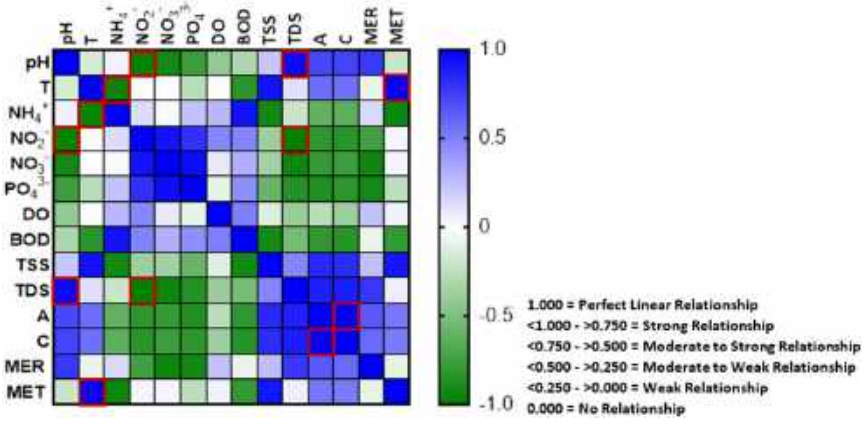


Fig. 4. Correlation matrix used to determine whether any relationships existed between physicochemical analysis, algal levels and cyanobacterial levels observed in the trial fish farm (Oasis) and, the average rainfall and the average temperatures recorded in the surrounding meteorological weather stations between July 2020 and October 2020. Positive correlations indicated by blue, inverse correlations indicated by green. Red box indicates statistically significant results where $P < 0.05$. T = temperature, NH_4^+ = ammonium, NO_2^- = nitrite, NO_3^- = nitrate, PO_4^{3-} = orthophosphate, DO = dissolved oxygen, BOD = biochemical oxygen demand, TSS = total suspended solids, TDS = total dissolved solids, A = algae, C = cyanobacteria, MER = Met Eireann Rainfall Data, MET = Met Eireann Temperature Data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Breakdown of the physicochemical analysis conducted on the trial fish farm (Oasis), during the period between July 2020 and October 2020, where issues within the system were observed. Values from the Irish Environmental Protection Agency and the Irish Statutory Office with regards to water quality were included for guidance as no physicochemical values specifically for Irish aquaculture were available. * indicates when results were beyond the guidance values. S.D. indicated, $n = 6$.

	July	August	September	October	Guidance Values
pH	8.11 ± 0.08	7.03 ± 0.01	7.48 ± 0.06	7.70 ± 0.04	>6 - < 9
Temperature (°C)	18.00 ± 0.46	17.46 ± 0.39	13.42 ± 0.16	12.16 ± 0.10	<25
Ammonium (mg NH_4^+ L^{-1})	0.01 ± 0.01	0.12 ± 0.03	0.10 ± 0.01	0.33 ± 0.22	<1
Nitrite (mg NO_2^- L^{-1})	0.05 ± 0.01*	0.13 ± 0.01*	0.10 ± 0.01*	0.08 ± 0.01*	<0.03
Nitrate (mg NO_3^- L^{-1})	1.39 ± 0.12	2.72 ± 0.34	4.88 ± 0.30	2.60 ± 0.77	<50
Orthophosphate (mg PO_4^{3-} L^{-1})	1.11 ± 0.07*	1.81 ± 0.06*	1.72 ± 0.07*	1.68 ± 0.11*	<0.03
Dissolved Oxygen (mg O_2 L^{-1})	5.13 ± 0.20*	7.07 ± 0.19	5.59 ± 0.17*	6.49 ± 0.11*	>7 (C) >9 (S)
BOD (mg O_2 L^{-1})	21.98 ± 1.50*	28.87 ± 2.32*	22.42 ± 2.26*	26.05 ± 1.94*	<6 (C) <3 (S)
Suspended Solids (mg L^{-1})	72.33 ± 7.53*	40.00 ± 2.53*	23.68 ± 5.28	14.00 ± 5.51	<25
Dissolved Solids (mg L^{-1})	170.00 ± 2.77	169.00 ± 4.33	172.00 ± 4.51	171.00 ± 2.47	<300

C = Cyprinids, S = Salmonids, BOD = Biochemical Oxygen Demand.

the problem especially given the fact the fluctuations were consistently observed previously without the loss of microalgal (microalgae and cyanobacteria) numbers (O'Neill et al., 2022b). The changes in weather conditions may be potentially contributing to the issues, as moderately strong relationships were indicated, although not statistically significant. This in turn led the authors to consider that an unforeseen deleterious agent may have been the cause of the previously unexplained disruption and reduction in algal in this RAMPs system. Zoosporic parasites were subsequently investigated as they have been previously reported to cause sudden and considerable cellular death in microalgae in both natural and industrial settings (Höger et al., 2021). The presence of five known genera of zoosporic parasites genera (*Labyrinthula, Vampyrella, Amoeboaphelidium, Paraphelidium* and *Aphelidium*) was confirmed.

Labyrinthula are a fungal like aquatic protist found in a diverse range of habitats (both freshwater and marine) and are opportunistic zoosporic parasites known to infect diatoms (Popova et al., 2020), which were routinely observed in the system (O'Neill et al., 2022b). *Vampyrella*, which generally occur in freshwater systems, are naked amoebae that are known as 'vampire' amoebae. They puncture hole in the host's cell wall and 'suck up' the cytoplasm causing deterioration and collapse of the cell, most

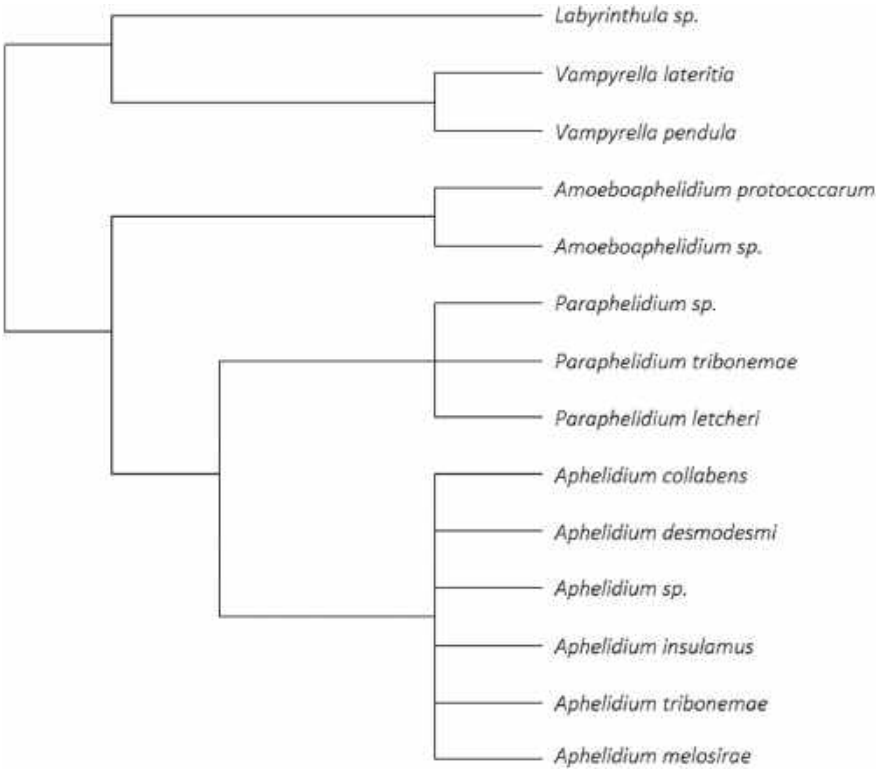


Fig. 5. Phylogenetic tree indicating all zoosporic parasites identified in the trial fish farm (Oasis) between July 2020 and October 2020. See supplementary data for breakdown.

often targeting green microalgae such as *Scenedesmus* (Gong et al., 2015) which was one of the most common green microalgae in the system (O'Neill et al., 2022b). The three remaining genera identified (*Aphelidium, Paraphelidi*um and *Amoeboaphelidium*) are all aphelids which are intracellular parasitoids of many groups of microalgae (Karpov et al., 2017). *Aphelidium* form cysts that penetrate the host cell and migrate the cysts contents into the cell causing deterioration and subsequent death (Karpov et al., 2020) and are commonly found to infect *Scenedesmus* (Gleason et al., 2014c). *Parapheilidium* penetrate and digest the cell wall by both mechanical and enzymatic means, targeting a range of different algal groups (Torruella et al., 2018). *Amoeboaphelidium* attaches itself to the host cell wall then encapsulates it and ultimately causes cell death by engulfing it (Höger et al., 2021). They have been found to infect *Ankistrodesmus, Desmodesmus, Chlorella* and *Scenedesmus* (Gleason et al., 2014c), all of which were routinely observed within the farm, with *Chlorella* being the most common and potentially most beneficial to the system (Ahmad et al., 2020; Arguelles, 2021; Arteaga Quico et al., 2021; O'Neill et al., 2022b). Although it cannot be inferred directly from the results, this suggested that the collapse of the microalgal populations may potentially be linked to the presence of the reported zoosporic parasites as they have the capacity to and are known to infect algal cells. Therefore, additional research is required from both an aquaculture and environmental point of view (as indicated in the future work). Not only would the presence of zoosporic parasites have a huge impact on organic aquaculture that strongly relies on microalgae, but they could also have knock on effects on surrounding aquatic ecosystems. Although this would unlikely be an issue with this system as water is very rarely released, should such parasites accidently be released from other aquaculture facilities that are directly connected to freshwater systems, the loss of microalgae (primary producers) within an aquatic ecosystem could instigate the collapse of the aquatic food chain. These findings also align with related research that considers a relations between climate variance and the emergence of infectious agents and vectors (El-Sayed and Kamel, 2020).

5. Conclusion

Research from this pilot study has indicated that the present of zoosporic parasites may, in part, have great adverse effects on the organic aquaculture system given their ability to demolish microalgal populations so quickly. Additionally, the changes in weather conditions, most likely due to climate change, may also be potentially contributing. The identification and real-time profiling of zoosporic parasites is significantly underappreciated for sustainable-food based systems given that this also has implications for green-industries and entrepreneurs who are focusing on developing large pond-based systems for the growth of microalgae as new biomass with a view to the bio-refining of bioactive-products for potential food, feed and health applications. Also, the microalgae in ponds serve as exceptional sequesters of carbon that could be disrupted. Ultimately, more focused research of the potential effects the presence of zoosporic parasites on organic aquaculture processes needs to be conducted, as well as an increased focus on their effects on the surrounding environments and the potential effects climate change may contribute. As such, during the next phase of the project, the following research will be conducted;

6. Future work

- Given that the collapse of the algal population was not consistently observed, research into the origin of the zoosporic parasites presence in the system needs determined in order to aid in the prevention of future occurrences.
- As molecular analysis could not be conducted on a week-to-week basis, additional research is needed to determine what time period throughout the year zoosporic parasites are most prevalent. This will include an extended monitoring period (12 months). Additionally, research needs to be conducted as to determine which species, and at what quantity (Real-Time PCR / qPCR), are occurring *i.e.*, are different species more prevalent throughout different periods of the year.
- Given that some species of microalgae are more beneficial than other, analysis on which species of microalgae are predominantly affected needs to be determined in order aid in preventing their entire collapse.
- Mitigation measures needs to be investigated in order to reduce and eliminate the zoosporic parasites presence without causing adverse effects on the microalgae, the fish or the surrounding system in general.
- Although the novel RAMPS is ultimately a closed system in so far as no discharge is released from the system unless excessive levels of rainfall is experienced causing the lagoon to overflow; given that the surrounding peatlands are a very important ecosystem, analysis into the potential effects zoosporic parasites on the surrounding peatland environment needs to be conducted in order to ensure all systems are protected.
- Finally, additionally research on any potential links with zoosporic emergence and changes in weather conditions needs to be considered, especially given the fact that climate change has increased the occurrence of more changing and erratic weather conditions.

CRediT authorship contribution statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Science of the Total Environment.

CRediT roles:

Conceptualization	E. A. O'Neill, N. J. Rowan
Data Curation	E. A. O'Neill
Formal Analysis	E. A. O'Neill, N. J. Rowan
Funding Acquisition	N. J. Rowan
Investigation	E. A. O'Neill
Methodology	E. A. O'Neill
Project Administration	E. A. O'Neill
Resources	E. A. O'Neill, N. J. Rowan
Software	E. A. O'Neill
Supervision	N. J. Rowan
Validation	E. A. O'Neill
Visualization	E. A. O'Neill
Roles/Writing – Original Draft	E. A. O'Neill, N. J. Rowan
Writing – Review & Editing	E. A. O'Neill, N. J. Rowan

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that there are no competing interests or conflicts of interest with respect to the publication of this article.

Acknowledgements

The authors acknowledge provision of aerial image of Mount Lucas peatland site furnished by Bord Iascaigh Mhara (BIM). The authors would like to thank Interreg Atlantic Area Neptunus (Project EAPA_576/2018), Regional University Network European University (RUN-EU Project), and Bord Iascaigh Mhara (Project 2019 BIM-KGS-008) for funding support. The authors would also like to thank Niamh Higgins for advice using the Nanopore MinION platform.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161495.

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Science of the Total Environment 838 (2022) 156328

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Digital transformation of peatland eco-innovations ('Paludiculture'): Enabling a paradigm shift towards the real-time sustainable production of 'green-friendly' products and services

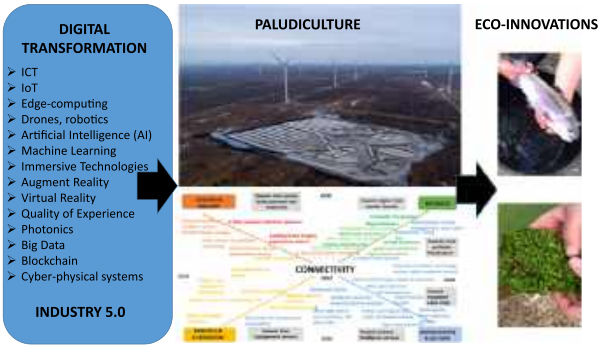
Neil J. Rowan [a,b,*], Niall Murray [c], Yuansong Qiao [c], E. O'Neill [a,b], Eoghan Clifford [d], Damià Barceló [e], Deborah M. Power [a,f]

[a] Bioscience Research Institute, Technological University of the Shannon Midlands Midwest (TUS), Dublin Road, Athlone, Ireland
[b] Empower Eco™ Sustainable Hub, Technological University of the Shannon Midlands Midwest (TUS), Dublin Road, Athlone, Ireland
[c] Software Research Institute, TUS, Athlone, Ireland
[d] School of Engineering and Informatics, NUI, Galway, Ireland
[e] Catalan Institute for Water Research, Faculty of Chemistry, University of Bacrelona, (ICRA), Spain
[f] Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas 8005-139, Faro, Portugal

HIGHLIGHTS

- Paludiculture is an emerging sector transitioning towards a sustainable green sector
- Harnessing digital transformation will enable peatland eco-innovations across the full value chain
- Mount Lucas is a paludiculture demonstrator for digital transformation
- Digital technologies developed for Agriculture 4.0 and Industry 5.0 will advance paludiculture
- Digitisation will future-proof paludiculture for climate change and sustainable eco- intensification

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Frederic Coulon

Keywords:
Digital transformation
Peatlands
Climate change
Disruption
Sustainability innovation
Natural-based solutions

ABSTRACT

The world is heading in the wrong direction on carbon emissions where we are not on track to limit global warming to 1.5 °C; Ireland is among the countries where overall emissions have continued to rise. The development of wettable peatland products and services (termed 'Paludiculture') present significant opportunities for enabling a transition away from peat-harvesting (fossil fuels) to developing 'green' eco-innovations. However, this must be balanced with sustainable carbon sequestration and environmental protection. This complex transition from 'brown to green' must be met in real time by enabling digital technologies across the full value chain. This will potentially necessitate creation of new green-business models with the potential to support disruptive innovation. This timely paper describes digital transformation of paludiculture-based eco-innovation that will potentially lead to a paradigm shift towards using smart digital technologies to address efficiency of products and services along with future-proofing for climate change. Digital transform of paludiculture also aligns with the 'Industry 5.0 - a human-centric solution'. However, companies supporting peatland innovation may lack necessary standards, data-sharing or capabilities that can also affect viable business model propositions that can jeopardize economic, political and social sustainability. Digital solutions may reduce costs, increase productivity, improve produce develop, and achieve faster time to market for paludiculture. Digitisation also enables information systems to be open, interoperable, and user-friendly. This constitutes the first

* Corresponding author at: Bioscience Research Institute, Technological University of the Shannon Midlands Midwest (TUS), Dublin Road, Athlone, Ireland.
E-mail address: neil.rowan@tus.ie (N.J. Rowan).

http://dx.doi.org/10.1016/j.scitotenv.2022.156328
Received 19 April 2022; Received in revised form 25 May 2022; Accepted 25 May 2022
Available online 29 May 2022

study to describe the digital transformation of paludiculture, both vertically and horizontally, in order to inform sustainability that includes process automation via AI, machine learning, IoT-Cloud informed sensors and robotics, virtual and augmented reality, and blockchain for cyber-physical systems. Thus, the aim of this paper is to describe the applicability of digital transformation to actualize the benefits and opportunities of paludiculture activities and enterprises in the Irish midlands with a global orientation.

Contents

1. Introduction

The onset of COVID-19 pandemic and commensurate disruption to supply chains worldwide has created opportunities for developing new solutions to pressing societal challenges (Rowan and Laffey, 2020a; Rowan and Laffey, 2020b; Rowan and Galanakis, 2020). However, a panoply of factors impact the sustainability of green innovations, but could be ameliorated by enabling real time data management, usage and protection by engaging and implementing the vision of Industry 4.0 (a.k.a the fourth industrial revolution, digital transformation) in the agriculture and food sectors. The occurrence of COVID-19, an unpredictable and unexpected event that had severe worldwide consequences, coincided with the launch of the European 'Green New Deal', which was aimed at implementing solutions to combat climate change by supporting the sustainable intensification of eco-innovation, products and services (Rowan and Pogue, 2021). The European Just Transition initiative embraces and develops the vision of sharing knowledge openly to enhance societal inclusiveness, regenerate communities and ultimately promote a low carbon economy (Rowan and Casey, 2021). These ambitious programs and the attainment of their goals will be shaped and impacted by other mitigating factors including extreme weather events brought on by climate change (Weiskopt et al., 2020), the global energy crisis brought on by the Ukraine war and threat to the "breadbasket" of Europe and food production and security (http://www.dw.com). Despite the slowdown in carbon emissions caused by successive "lockdowns" worldwide, the current global models for business and production means the world continues to head in the wrong direction on carbon emissions in spite of the narrowing window of less than a decade to contain a catastrophic rise in global temperatures (Intergovermental Panel on Climate Change (IPCC), 2022). The world is not on track to limit warming to 1.5 °C and only a major transition in the energy sector including a substantial reduction in fossil fuel use, widespread electrification, improved energy efficiency and the use of alternative fuels can halt what is becoming an irreversible warming trend (O'Sullivan, 2022). Mondejar et al. (2021) noted that changing climatic conditions are accomplished by high and low stress, altered rainfall patterns, elevated carbon dioxide, increased frequency of extreme weather events like flooding, droughts, cyclonic disturbances and increased saline soils. Okolo et al. (2019) reported that increased stress negatively influences agroecosystems' natural resilience; this is expected to cause dramatic environmental changes on a world scale that can affect supply chains for food, feed and clean energy. The reader is also directed to the published work of Mondejar et al. (2021) for applications of digital technologies that provide sustainable solutions to different agriculture problems.

Peatlands are a unique ecosystem that constitute an important carbon sink globally (Ziegler et al., 2021; O'Neill et al., 2022) and also represents an environment that may potentially provide a win-win scenario for new activities that could bring economic and social benefits while simultaneously contributing to reduce the harm to the climate (Rowan and Galanakis, 2020; Ziegler et al., 2021). The globalization of the current crisis (climate change, pandemics, population growth and food security) emphasise that solutions can only be found through global actions to co-create and implement solutions for these complex societal challenges and make the United Nations' Sustainability Development Goals set for 2030 a reality (Rowan and Casey, 2021).

Peatlands are a type of wetland that covers 3% of global land surface and currently approximately 15% of peatlands are degraded due to drainage for agriculture, forestry, and peat mining as a fossil fuel (Urák et al. 2017; Ziegler et al., 2021; O'Neill and Rowan, 2022), Ziegler et al. (2021) reported that the adverse consequences of intensive peatland degradation can contribute to inter alia undesirable greenhouse gas emissions, biodiversity loss and pollution of receiving waters. However, "rewilding" peatlands through controlled wetting can promote biodiversity and sustainable economies through the unique practice of paludiculture (production under permanently wet, peat-conserving and potentially peat-forming conditions, https://www.eurosite.org/). Indeed, peatlands are one of the world's most vital ecosystems, supporting a range of rare and stress-hardened

plants and species, cleaning and filtering water, and mitigating flooding (Rowan and Casey, 2021). Peatlands cover just 3% of the planet, but store twice as much carbon as all the world's forests combined (Sargent, 2022). Ireland is rich in peatlands, which cover 20% of the land mass with near perfect conditions for peat soils and lock in 75% of all soil organic carbon (Rowan and Casey, 2021). Irish peatlands are estimated to actively capture around 57,000 t of carbon per year (Sargent, 2022) and constitute an important resource for carbon removal from the atmosphere and for enhancing sustainable carbon cycles (O'Neill et al., 2019).

Traditionally, use of wet peatlands (or 'paludiculture') is a potentially exciting means of alternative land use worldwide as this can lead to new sustainable employment opportunities for farmers and create opportunities for communities to fairly transition to a low carbon economy (Ziegler et al., 2021). However, promotion of paludiculture needs robust, evidence-based, collaborative research from pilot (pre-commercial) studies across a diversity of paludiculture activities to inform appropriate sustainable business models, such as for production of, fuel, fodder, food and construction materials (Wichmaan et al., 2016; Ziegler et al., 2021). Ziegler et al. (2021) and others (Tan et al., 2021; O'Neill et al., 2022) noted that support for the sustainable intensification of wet peatlands is a novel concept. O'Neill and co-workers (2019) reported for the first time the development of an innovative integrated multi-trophic aquaculture (IMTA) system for sustainable foods in peatlands of the Irish midlands. This freshwater IMTA system exploited a naturally occurring ecosystem of bacteria, algae and duckweed in ponds for managing waste and ensuring water quality and was powered by wind turbines. The described recirculating aquaculture process does not rely on complex end-of-circuit solutions for maintaining waste effluent treatments (Rowan and Galanakis, 2020; Galanakis et al., 2021).

Current freshwater aquaculture production systems are resource-constrained and the production systems are unlikely to meet the increasing demand for aquatic foods, which is foreseen as one solution for feeding the growing world populations that is estimated to reach approximately 10 billion by 2050 (Rasmussen et al., 2018; Xia et al., 2022). Moreover, there is increasing anthropogenic-mediated destruction of natural resources caused by increasingly frequent extreme weather events that will affect the supply of safe, affordable, and nutritious food (Weiskopt et al., 2020). Aquatic ecosystems encompass about 75% of planet's surface, including that represented by the wet peatlands, which present new opportunities to intensify and diversify eco-innovation transnationally (Rowan and Casey, 2021; FAO, 2020). A knowledge gap that will have to be filled is the development and implementation of disruptive technologies in aquaculture to promote green, sustainable and profitable production models (Xia et al., 2022). Digitalization of aquaculture systems represents a new frontier and will be transformative by allowing development of precision farming solutions improve yields of food rich in high quality protein and omega-3 fatty acids (Mondejar et al., 2021; Rowan and Casey, 2021). The development and adaption of sophisticated innovations, along with open knowledge and technology exchange, is required to meet hurdles presented by low-environmental impact aquatic-based innovations (O'Neill et al., 2022).

There is increasing interest in adapting digital technologies such as artificial intelligence (AI) and machine learning, the Internet of Things (IoT), sensors, drones, blockchain for improving adjacent industries (Hrustek, 2020; Mondejar et al., 2021). Indeed, the disruptive approach of Industry 4.0 has started to be felt in some traditional agri-food activities and cyber-physical systems, IoT, AI and machine learning, big-data and analytics, and cloud technology have been integrated with agricultural machinery with exciting possible benefits (Tsolakis et al., 2019; Hrustek, 2020; Arvanitis and Symenonaki, 2020) (Table 1). Collectively, application of these next-generation digital technologies can support and accelerate Open knowledge exchange worldwide and can inform real time development of exciting new technologies to meet the challenges of today; although, the diversity and complexity of agri-food activities makes sector-customized solutions a challenge and a priority (Mondejar et al., 2021). Hrustek (2020) noted that digital transformation will help inform stability for the sustainable development of the global economy including preventing or mitigating against the impact of uncertainties created by increased frequency of extreme weather events and new global crisis (financial, health, war, food and water). Hrustek (2020) also advocated that organisations must understand the key drivers of digital transformation that affect technology development and industry. Similarly, for development and future intensification of peatland innovation, a balance must be achieved between striding towards a high rate of economic progress and protecting the environment that enables delivering a climate friendly and sustainable production sector (Mondejar et al., 2021; Weizkopt et al., 2020).

The vision outlined by the authors in the present commentary is that digital transformations can inform technological, societal and political drivers of peatland, as it has for agricultural innovation. Moreover, 4IR should be deployed with the objective of enhancing the quality of life of the billions of people worldwide through creating a system that is secure and fair and establishing a framework that monitors and promotes sustainable practices (Shamin et al., 2019; Hrustek, 2020). Moving forward, peatland innovations should become an integral component of the value chain in the economy, providing products and services to society and informing food security and stability while promoting environmental, economic and social sustainability (Rowan and Galanakis, 2020). However, implementing innovative sustainable technologies typically requires far-reaching changes of the macro environment in which innovating companies operate where there is a need to strategically create an appropriate supportive external environment, such as collective system building (Planko et al., 2016; Zeigler, 2020) and quadruple helix innovation hub approach (Rowan and Casey, 2021). Commensurately, there will be a focus on achieving compliance with environmental and quality regulations as seen in agriculture sector (Hrustek, 2020). The aforementioned also aligns with the tenets of Industry 5.0 that provides a vision of the industry that looks beyond efficiency and productivity as the sole goals, but reinforces the role and the contribution of industry to society (Skobelev and Borovik, 2017a, 2017b; Nahavandi, 2019). It complements the fourth industrial revolution named Industry 4.0 by using digital transformation to place research and innovation at the service of the transition to a sustainable, human-centric and resilient industry. Industry 4.0 combines physical world of real things with their 'virtual twins' (Table 1).

Peatland innovation will also potentially mitigate against impact of reduced water supply and the environmental impact of intensive plant and livestock production to meet increased animal and human needs for high quality food production (Rowan and Casey, 2021; O'Neill et al., 2022), where the Food and Agriculture Organization (FAO, 2020) advocates adoption of digital technologies to enhance productivity and ensure food safety. Hrustek (2020) highlighted similarities to applying digital technologies to the agriculture sector where it is also envisaged that peatland innovation will require standardization and will be made available across the entire value chain where creative and adaptable approaches will be required to support sustainable development of paludiculture. It is likely that peatland innovation will also be advanced by applying smart and precise processes including process automation and robotics, peatland applications and information systems, cyber-physical systems, related tools and machines, and collection and evaluation of large amounts of data (Mondejar et al., 2021). Lessons learnt from sustainable practices in adjacent agriculture infers that sustainability across the peatlands will be centred on delivering flexibility in tandem with efficiency where future digital transformative activities will help unlock complex economic and societal challenges (Rowan and Pogue, 2021). However, the complexity and diversity of such data is likely to be vast and will require systematic approaches to make digitalization sustainable, including life cycle assessments (LCA) (Ruiz-Salmón et al., 2020; Ruiz-Salmon et al., 2021; Laso et al., 2022), material flow analysis (MFA, Abualtaher and Bar, 2019), principle component analysis (Naughton et al., 2020), bioinformatics to mitigate mislabelling in worldwide seafood market and to protect consumer health (Vindigni et al., 2021) and so forth. Cooney and co-workers

Table 1
Digital technologies – definitions and applications in peatland ecosystems.

Digital technologies	Peatlands use
Information and communications technology (ICT) encompasses the capture, storage, retrieval, processing, display, representation, presentation, organization, management, security, transfer, and interchange of data and information.	Connects Peatlands ecosystem
Internet of things (IoT) – network of smart, interconnected devices and services capable of sensing or listening to requests and perform actions using actuators. IoT enables network sensors to remote connect, track and manage products and systems.	Aquatic systems Agroforestry Vertical farming
Cloud computing – use of tools and applications (such as data storage, servers, databases, software) based on a network of severs through the internet. It enables user to rent computer resources on demand to store files and applications in a virtualised servers and access all data via the internet.	Connects peatland ecosystem
Artificial Intelligence (AI) defines machines achieving human-like cognitive functions (ex. learning, reasoning, interacting) that comprises different forms of cognition and meaning understanding (such as speech recognition) and human interaction (signal sensing, smart control, simulators) rooted in algorithms and software.	Sensors, chips, robots, logistics, autonomous machines
Machine learning (ML) – a subset of AI, use and development of computer systems that learn and adapt without following explicit instructions, by using algorithms and statistical models to analyse and draw inferences from patterns in data.	Automated processes across peatland
Big data – continuous increase in data & technologies that needs to be collected, stored, managed and analysed. Complex and multidimensional that impacts processes, technologies. Characterised by Volume (amount of data sets), Velocity (speed of data processing), Variety (types/sources of data), Veracity (quality of data analysed).	Full peatland innovations and value chain
Blockchain is a shared digital, immutable ledger that facilitates the process of recording transitions and tracking assets in a business network using cryptographic algorithms). Blockchain protocols aggregate, validate, and relay transactions within the blockchain network. The blockchain system records the transactions in sequence. A transaction may contain a value transfer or a smart contract invocation. Almost anything of value can be tracked or transacted on a blockchain network, reducing risk and costs in business.	Traceability, security of processes across peatlands, novel business models
Photonics is a multidisciplinary field related to light including energy generation, detection, and process management. Photonics is the scientific study or application of electromagnetic energy whos basic unit is the photon, incorporating optics, laser technology, electrical engineering, material science, and information storage and processing. Photonic applications use the photon in the same what that electronic applications use the electron. Agri-photonics is a growing area of precision agriculture (Massaro et al., 2020).	Disease mitigation. Optic sensing to for protein levels in foods, water quality and fish health
Augmented reality – a technology that superimposes a computer-generated image on a user's view of the real world; thus, provide a composite view.	Training
Virtual Reality – the computer-generated simulation of a 3D image or environment that can be interacted with in a seemingly real or physical way by a person using special electronic equipment, such as helmet with screen inside or gloves fitted with sensors.	Training
Quality of Experience (QoE) – is the degree of delight or annoyance registered by the use of an application or service.	Training
Logistics – the detailed organization and implementation of a complex operation.	Peatlands eco management
Robotics – a branch of technology that deals with the design, construction, operation and application of robots. In multi-robot or swarm robot systems, the robot collaborate to complete predefined tasks.	Aquaculture feed, monitoring
Cobot, or collaborative robot, is a robot intended for direct human robot interaction with a shared space, or where humans and robots are in proximity.	Food processing, in field logistics
Digital twin – a digital win is a virtual model designed to accurately reflect a physical object.	Wind turbine fitted with various sensors for control
Edge Cloud – Edge computing is developed as complement to cloud computing, encompassing storage and compute assets located at the edge and interconnected by a scalable, application-aware network that can sense and adapt to changing needs, securely & in real time	Peatland ecosystem networking
Cybersecurity or information technology (IT) security – is the practice of protecting critical systems and sensitive information from digital attack. It is how individuals and organisations reduce the risk of a cyber attack where cyber security code function protects the devices (smartphones, laptops, tables).	Security of production, products and services
Cyber-physical systems refer to systems where software and hardware components are seamlessly integrated towards performing well defined tasks.	Security of processes.

(2021) investigated impact categories that can also be applied to evaluate emerging peatland aquaculture systems, namely using LCA and included global warming potential (GWP), acidification potential (AP), eutrophication potential (EP), freshwater and marine ecotoxicity potential (EAETP and MAEPT), cumulative energy demand (CED), net primary production use (NPPU) and water use. Rowan and Casey (2021) evaluated the maturity of paludiculture eco-innovation in terms of technology, society and policy readiness levels (O'Neill et al., 2022). The present article expands on the scope of the previous articles by:

- Addressing what digital transformation of the peatlands simply means and its' application to supporting sustainability in order to embrace wide opportunities for the development of green enterprises, job creation and to improve competitiveness of the region with global orientations.
- Describes and explores current state-of-the-art knowledge on sustainability across a diversity of peatland innovation, and adjacent activities, framed upon the use case of a novel freshwater paludiculture site in the Irish midlands and digital transformation.
- Unlocks challenges and opportunities for the development of peatland innovation as it relates to environmental, economic and societal sustainability informed by digital transformations. This includes the likely impact of climate change and the uncertainty arising from extreme weather events, habit loss or biodiversity disruption and mitigation using effective precision systems.

2. Development of aquaculture, aquaponics and adjacent green innovation in the Irish peatlands

Aquaculture is the fastest growing sector in agriculture and has a long history of contributing high-quality proteins to humans (O'Neill et al., 2022). Aquaculture is diversifying compared to other agriculture sectors in terms of fish species, feeds, production systems, diseases, products, business sectors and marketing (FAO, 2020; Yue and Shen, 2022). Developing live feeds, including microalgae, rotifers, and brine shrimp in hatcheries have bridged the bottleneck in aquaculture of some marine species (Yue and Shen, 2022). Selective breeding has enabled progression of quantitative genetics that has improved traits in over 60 commercial aquaculture species (Gjedrem and Robinson, 2014). This includes QTL (quantitative trait locus) mapping and maker assisted selected (MAS) that have been a basis for trait selection. Improved feed formulations to meet the nutritional requirements of each fish species have improved feed conversation rates, reduced costs and improved product quality (Tacon and Metian, 2015). Technologies and innovations for disease mitigation and management have controlled diseases in aquaculture (Kelly and Renekdas, 2020; Pogue et al., 2021). However, it will be very challenging to meet increasing demand for seafood seen as a viable option with a low environmental impact for feeding the growing world population (FAO, 2020). It is important to support and enable product diversification from aquaculture and adjacent activities that are likely to be impacted by disruptive environmental

conditions, reduced supply of fish meal and oils and extreme weather events brought on by climate change (Shen et al., 2020). These challenges, and opportunities have stimulated innovations in aquaculture (Ab Rahman et al., 2017; O'Neill et al., 2022), such as genomic selection (Houston et al., 2020; genome editing (Gratacap et al., 2019), information and digital technology (Hassan and De Filippi, 2021), recirculating aquaculture systems (RAS), development of renewable energies such as solar (Aich et al., 2020) and wind (O'Neill and Rowan, 2022; O'Neill et al., 2022), vaccines (Shefat, 2018), and novel business strategies with blockchain (Anderson et al., 2019).

The Mount Lucas peatland site covers ca. 4 ha in the Irish midlands, has also a strong trajectory to support and enable green innovation and social enterprises including vertical farming, honey production, exotic fungi/mushroom cultivation, agro-forestry including provision for dark-sky ecotourism (Fig. 1). An advantage of peatland-based aquaculture is that it harnesses water from rivers and lakes and doesn't have the possible threats from potential pollutants in raw, untreated wastewater, such as agricultural run-off (O'Neill et al., 2020). The emphasis is on creating a balanced system in the four 1000m^3 fish ponds, which were created using a natural glacial till (Fig. 1). The duckweed area is 1.2 ha, linked by channels to the pond. The full volume of water is exchanged every 4 h. Among the many green boxes the project ticks is low freshwater use, with any additional water requirements normally taken care of by the Irish weather (O'Neill et al., 2019). The Mount Lucas site has a licence for 25 t of fish biomass, which works out at 13 t perch and 12 t of trout that are produced for high value markets across Europe (O'Neill et al., 2020). The underlying principle is define process for replication across peatland where the limited tonnage of fish biomass is dictated by 'organic status'. Thus, Mount Lucas hosts a blend of traditional social enterprises with emerging green technologies, products and services that requires digital transformation to effectively manage their potential along the full value chain that includes efficient wastewater recirculation and bioprospecting of bioactives. Digital transformation can also highlight the maturity of key innovations that will potentially meet several sustainable development goals of the United Nations (Galanakis et al., 2021; Rowan and Casey, 2021; O'Neill et al., 2022).

3. Exploiting digitization to integrate and unlock vertical and horizontal value chains

A definition of key terms and technologies that underpin digital transformation can be found in Table 1 that also connects with the envisaged peatland aquaculture innovations.

Vertical integration of processes crosscut the whole value peatland ecosystem from procurement of monitoring equipment and smart feeding regimes to optimised fish biomass to diversification of activities to include recycling of wastewater for irrigation of vertical farming and exploitation of the extensive "wilded" ecosystem for pollination/ecosystem service management. Essentially, all data generated from production processes is considered from the perspective of real-time efficiency, quality, risk mitigation and organization planning by digital technologies that will be optimised into an integrated system (Savastano et al., 2018).

Horizontal integration extends beyond the internal operations at Mount Lucas peatland site from suppliers to end-users including all key stakeholders. In recent times, seamlessly connecting this broad holistic ecosystem can be potentially met by the Quadruple Helix 'Empower Eco™' Concept that unites academia, industry, government and society regionally (Rowan and Casey, 2021). Digitization is the foundation for the development of new strategic plans and enable opportunities in real-time through specialist training across the water-energy and food nexus that will also stimulate new social enterprises, greater community engagement, and job creation in paliduculture. For example, exploiting the European Digital Innovation Hub (EIDH) concept at Mount Lucas will manage the digital transformation of bespoke peatland eco-innovations, along with connecting this site trans-regionally with complementary EIHDs to create and add value (Galanakis et al., 2021). Funding instruments such as Horizon Europe MSCA RISE, Erasmus Plus, European Just Transition and Interreg

Fig. 1. Aerial view of Mount Lucas peatland site in the Republic of Ireland for development of aquatic innovations powered by wind turbines. The wind turbine indicated (WT) provides all electrical requirements for the production site (red). The site consists for 4 D-end split culture ponds (blue), a 16-channel treatment lagoon (green) which contains algae and duckweed, and a water reservoir (yellow) for the system. Water is taken is from an adjacent bog river (— — -). This occurs only to compensate for loss of water from the system via evaporation. Discharge from the system only occurs during times of excessive rainfall whereby filling the overflow tank (orange). The site is situated in the middle of a peatland which is undergoing natural restoration.

programmes can support and accelerate horizontal integration and at Mount Lucas will be matched with balanced rural enterprise and growth for economic, political and societal sustainability (Rowan and Casey, 2021; Rowan and Pogue, 2021).

Digitization provides access to an integrated network of unexploited big data with potential benefits to society and to the environment (Mondejar et al., 2021); moreover, it is the integration of digital technologies into everyday life. Appio et al. (2021) noted that our ability to make well-informed decisions underpinning how to efficiently use natural resources and services has a significant impact on sustainability and equal access. The Food Standards Agency UK (2021) highlighted the role of digital innovation as emerging technologies that will positively impact on the UK food system. Moreover, the Food Standards Agency UK (2021) stated "digital technologies are been adopted rapidly across the value chain that includes discrete applications at the field, farm and factory level that includes automation, robotics and performance monitoring – mostly aiming at process optimisation; at the consumer level with a multitude of innovative new internet-enabled food distribution platforms and services; and increasingly at an integrated system level, connecting actors at all stages of the value chain, including supply chain management, and secure and gap-less digital traceability of food items from farm to fork". It loosely grouped digital technologies (DT) across three main categories, (a) DTs applied directly to food production processes (such as sensor-based agriculture, traceability, monitoring of production and delivery) where resulting flow of information is based on input data from the food; (b) DTs that generate information relevant to food from input data, but not directly gathered from the actual food (such as social marketing for customer awareness, behaviour and choice); (c) DT platforms used to aggregate and transmit data securely (cyberphysical), record keeping and for making decisions either automomously or with human input. It is appreciated that digitization is a vast topic; consequently, this article will provide examples of digital technologies that are currently applied to, or will future enable peatland innovation with particular relevance to the development of the Mount Lucas site in the Republic of Ireland.

3.1. Robotics

No robotics are currently deployed at Mount Lucas; but, are being used and developed elsewhere for feeding, cleaning ponds (Osaka et al., 2010; Yue and Shen, 2022), oral or injecting vaccines (Lee et al., 2013), monitoring behaviours and removing diseased fish (Antononucci and Costa, 2020), all labour intensive and costly processes (Lucas et al., 2019). For the first time, consumption of farmed fish has exceeded that of wild-caught fish, and by 2030, digitization of aquaculture (such as by use of robotics) and aquaculture production will potentially contribute for two-thirds of fish that humans consume (Connolly, 2018). Robotics can facilitate risk mitigation and improve profit margins and sustainability (Table 1). For example, in the salmon industry automated underwater robots have been deployed for cleaning and inspecting nets and have reduced human operations (Paspalakisk et al., 2020) and have been used to evaluate fish health and monitor and prevent escape of farmed fish (Ohrem et al., 2020). Some of the advantages of robots is that they more profitable as they can work continuously and can be substituted to address none-ideal human workforce conditions, labour intensity and sophistication. The high density of fish during aquaculture increases the risk of disease, which reduces production and profits. Cermaq are overcoming this problem using robotics to sort diseased or harmed fish from good quality fish for processing (Connolly, 2018). SINTEF (an independent research organization in Norway) is developing underwater robots to examine and repair nets for marine aquaculture and contributing to the cost-effectiveness and sustainability of the salmon industry (Connolly, 2018). A survey of the literature revealed that several academic/industry partnerships, Innovasea (https://www.innovasea.com/), Cermaq (https://www.cermaq.com/), Robotfish (https://www.edlbf.com/), SeaVax (https://theexplorer.no/), and Sublue (https://www.sinfet.no/en/) are developing robotics for aquaculture (Yue and Shen, 2022). Moreover, with the development of Industry 5.0 human centric technologies (Xu et al., 2021), collaborative robots (cobots) will play vital roles in agriculture, paludiculture and food production along the full value chain. These cobots will work closely with human operators to perform dangerous and less popular menial tasks along with more labor-intensive, monotonous and repetitive jobs (Wong et al., 2022). Cobots can also be used as transportation for in-field logis0ics applications (Ducket et al., 2018).

3.2. Drones and satellites

In terms of aquaculture, drones can function above and below the water (Sousa et al., 2019; Yue and Shen, 2022). Connolly (2018) noted that drones can be used for monitoring offshore fish farms such as for inspecting under water cages for damage and holes. Drones can be used to monitor duckweed coverage and production in the ponds of the Mount Lucas peatland site where they can contribute to increase operational efficacy of wastewater treatment, quality and recirculation (O'Neill et al., 2019). Yue and Shen (2022) noted research institutes and industries developing drones for deployment in aquaculture include Subblue (https://www.subblue.com/), Apium Swarm Robotics (http://apium.com), Blueye Pioneer (https://www.avetics.com/) and SeaDrone (https://seadronepro.com/). Connolly (2018) reported that drones produced by Apium Swam Robotics can be deployed en masse to survey the oceans and use sensors for analysis. Whereas Blueye Pioneer produced drones that provide live streaming of underwater exploration using a Blueye app on a smartphone, computer or headset/goggles. Drones can be used to collect data that would be challenging for humans to obtain and can contribute data that can be used to generate new algorithms for developing technologies (Yoo et al., 2020). Drones enable surveying of large areas and could be used to survey and monitor the status of the entire Irish peatlands (ca. 80,000 ha) and the collected data used to assist planning and monitoring of rewetting, rewilding and to support eco-innovation developments. Salidrone (https://salidrone.com/) is collecting data for evaluating fish stocks and environmental conditions and by integrating AI and cloud computing the cost of aquaculture can be reduced and the operational performance of aquaculture improved (Chen and Zhang, 2017). The potential of drones for aquaculture is enormous and currently the drone market is estimated to be worth $5.1 Billion by 2025 (Yue and Shen, 2022).

Other adjacent studies have reported on the use of sensors for measuring water parameters can advance aquaculture (Xing et al., 2019; Su et al., 2020). Science Foundation Ireland (2021) reported on new 'Terrain_AI' project between academic researchers and Microsoft Ireland focused on digital transformation that includes novel multimodal sensing technologies, IoT devices, Microsoft Azure-informed AI and cloud-based innovation to monitor carbon sequestration of peatlands and other land types to improve our understanding of the relationship between human activity and land use and how this may relate to climate change. Terrain-AI will build artificial intelligence (AI) models that can inform more effective and sustainable management practices, leading to significant carbon reduction. Data will be captured from satellites, airborne platforms, as well as in-field instruments, from 14 test sites strategically located across Ireland. This digital project will help improve our understanding of the interactions between the land and human activities that lead to carbon emissions that will also support and enable comparison between peatlands and all land types including grasslands, croplands, forestry, wetlands, to urban areas. This will integrate the aforementioned-generated data into a modelling framework that will inform more effective policies to reduce carbon emissions. Terrain-AI will also help to inform future land use practices that will achieve reduced carbon outputs such as, precision aquaculture and farming, carbon sequestration of grassland, and new approaches to public transport, along with other adjacent needs such as tree planting in urban areas. Terrain-AI will design a cloud platform that can use the insights from these Irish findings that will be shared with other countries to enable everyone to explore land usage and carbon reduction in their respective geographical jurisdictions. Notable, Microsoft has been carbon neutral across the world since 2012 and is committed to achieving carbon negative

status by 2030; it seeks to promote sustainable development and low-carbon business practices through the use of cloud-enabled technologies (Science Foundation Ireland, 2021). This builds upon delivery of Airband project in partnership with Teagasc (https://www.teagasc.ie/) tohelp the farming community to stay connected, where Terrain-AI will explore how we can leverage technology to reduce carbon emissions across different land types. This Triple HUB approach combines Earth Observation, Geocomputation and Climate Modelling with stakeholders that will collect high quality data, extract verifiable information and generate the facts to enable society make informed decisions about changing how we manage our climate and environment. It will deliver unique insights to help landowners and planners make better informed decisions to reduce carbon emissions. This platform leverages off the latest in AI and IoT technologies where open sharing of data and insights will collectively inform new solutions to reduce carbon emissions globally and will support the delivery of a net zero future.

3.3. Sensors

Many of the drones and robots use sensors to navigate underwater to collect data such as physicochemical parameters, or above water for geo-survey and referencing (Yue and Shen, 2022). Connolly (2018) noted the biosensors, such as those produced by Sense-T can improve efficiencies across he salmon to oyster industry by analysis of oxygen levels and water temperature; even extended to measuring heart rate and metabolism. Sensorex devices are used to monitor dissolved oxygen levels and pH to create appropriate environment for improve shrimp efficiency and yields (Connolly, 2018). The company YSI has also developed handheld sensing devices for automatic feeding technology and transportation tanks that maintains ideal environment for fish (Connolly et al., 2018). Recent studies at Mount Lucas has focused on the monitoring water physicochemical parameters along with matching use of handled algal-Torch for monitoring algal populations to that of using flow cytometry for real time determinations in living laboratories (O'Neill et al., 2022).

Biosensors have been exploited to advance the aquaculture industry for determining DO levels, water salinity and temperature (Antononucci and Costa, 2020). Svendsen et al. (2020) has used biosensors to monitor heart rate and other physiological conditions in salmon. While Zhou et al. (2019) described the potential of using underwater sensors linked to the internet to inform efficacy of feeding based upon hunger status of cultured fish in various aquatic environments including ponds and rivers. Yue and Shen (2022), reported on a European consortium project comprising academia and aquaculture companies that are developing an automated/integrated platform to detect and monitor chemical contaminants, harmful algal blooms, pathogens and toxins. Mount Lucas aquaculture can be advanced by developing and testing sensors combined with cloud management along with mobile phone apps to help inform the establishment of an optimised environment for fish and feeding; Yue and Shen (2022) also advocate need for sensors to monitor stress levels in individual fish species and emergence of pathogens in water, where devices could be inserted into live fish in such a manner to support detection on land, boats or satellites. Measurement of changes in microalgae species in the aquaculture pond (O'Neill et al., 2022; O'Neill and Rowan, 2022), and disruption brought on by flooding to IMTA system due to frequent storms were attributed to climate change (O'Neill et al., 2022).

3.4. Photonics

Optical and photonic technologies are currently adopted to measure crop health and agri-food quality using remote sensing data in the visible, near-infrared, and thermal-infrared wavebands (Massaro et al., 2020) (Table 1). Agri-photonics constitutes a new area of research that encompasses electronic and opto-electronic technological advances implemented on unmanned aerial vehicle (UAV), decision support systems (DSS), multispectral imaging, and precision agriculture sensing. Traditional methods in agriculture cultivation is labour-intensive and struggles to meet the increasing demands of our growing populations. Optics and photonic technologies have been proven as state-of-the-art-solutions for helping with crop production and harvesting technologies (Yeong et al., 2019); thus, have potential applicability for transforming peatland innovations.

3.5. Artificial intelligence and machine learning

Artificial intelligence (AI) can help inform reliable and appropriate decision making based upon large data sets measured using digital devices, such as drones, robots, and sensors (Yue and Shen, 2022) (Table 1). An Australian company, the Yield, provides a diverse suite of technologies for all types of agriculture which it uses Sensing + Aqua technology to enable predictive analytics for enhanced data-driven decision- making (Connolly, 2018). This author also noted that nearly 32% of wild-fish caught are procured unsustainability where AI through cameras and data collection can identify species, reduce overexploitation of fish species, and enable greater accountability of harvesting methods. It is envisaged that research from this peatland site will be linked to the Terrain-AI project (Science Foundation Ireland, 2021). Indeed, the need to rapidly develop aquaculture and adjacent innovations is reflected in many global partnerships between academia and aquaculture industry. Such efforts are deploying AI to inform decision making (Evensen, 2020) that can reduce reliance on labour intensive practices such as feeders, water quality, harvesting and processing. Josthiswaran et al. (2020) highlighted the potential of using AI in enhancing control systems in aquaculture, such as in the area of reducing waste streams and improving costs. However, there is commensurate opportunities to use machine learning and development of algorithms based on increased data sets through Open Innovation and knowledge exchange for advancing the industry linked to academia and digital companies. One research effort is to apply deep learning technologies in aquaculture, e.g. for fish classification, counting, behaviour monitoring, and fish fillet defect detection (Sun et al., 2020, Yang et al., 2021a, 2021b). Deep learning has outperformed the traditional machine learning algorithms in many application areas. However, one major drawback of deep learning is that it requires a large dataset to train the model, which is a significant challenge for applying deep learning in aquaculture.

Digital transformation can also advance bioprospecting of key bioactives from microalgae, duckweed and other peatlands resources for one health application. For example, AI can be used to potentially screen and inform the appropriate type of bioactives that elicit pro- and anti-inflammatory responses for fish welfare with view to fortification of feed (Murphy et al., 2020a; Murphy e al., 2020b; Murphy et al., 2022; Pogue et al., 2021). Masterson et al. (2020) reported on the use of lentinan from Shiitake mushroom to ameliorate against clinical isolates of *Klebsiella pneumoniae* exhibiting antimicrobial resistance using novel lung infection models. Commensurately, Felix and Angnes (2018) also highlighted increased interest in electro-chemical immunsensors that may potentially disruptive the bioprospecting of such high value peatland products; such immunosensors explore measurements of an electrical signal produced from an electrochemical transducer. Moroever, this signal can be voltammetric, potentitiometric, conductiometric, or impedimetric that can be harnessed as tools since they are specific, simple, portable, generally disposable, and can carry out in situ or automated detection (Felix and Angnes, 2018). Use of bioinformatics linked to machine learning can be applied to understand diversity and richness of microbial and algal species in the aquaculture ponds (O'Neill et al., 2022). Use of drones and satellites can support and enable digital transformation of in situ living labs connected to environmental test beds at Mount Lucas (Rowan and Casey, 2021).

3.6. Immersive technologies, augmented reality (AR), virtual reality (VR)

Augmented reality is an interactive experience within which digital and context-based content is overlayed upon real-world objectives (Egan et al., 2016; Braga Rodrigues et al., 2020). As such, AR could be employed to inform aquaculture activities by their nature are highly variable and labor-intensive that are frequently influenced by species, location and

aquaculture process (FAO, 2020; Yue and Shen, 2022). Connolly (2018) reported that the U.S.Navy uses Divers Augmented Vision Display (DAVD) that superimposes high-resolution sonar imagery on a diver's visual experience. AR can be used to improve efficiency of aquaculture production, monitor mortalities and welfare status of fish under a plethora of environmental conditions. This was also noted by Yue and Shen (2022) that the use of AR, in combination VR, for training and education as it pertained to fish welfare, disease prevention, escaping fish and dangerous working conditions. Yue and Shen (2022) highlighted the capability of AR to inform efficacy and economics of deploying underwater drones and robots that encompassed monitoring fish behaviour and mortality. Yue and Shen (2022) have reported on the use of AR with a cloud system to advance aquaculture to increase fish biomass, and to monitor fish health linked to water parameter determinants. Augment and Virtualh Reality in may be possible to review and evaluate appropriate locations across ca. 80,000 ha of peatland for both sustainable carbon cycles and deployment of aquaculture systems that includes risk mitigation.

3.7. Georeferencing and mapping

ArcGIS mapping can be used to inform restoration and rehabilitation of peatlands linked to ecology/biodiversity to inform carbon cycles (Rowan and Casey, 2021). ArcGIS mapping of the 80,000 ha of peatlands under the management of Ireland's Bord Na Mona (State Body) would enable profiling of peatlands to match locations for development of aquatic innovations in terms of desirable production sites balanced with environmental protection and ecology. ArcGIS mapping of peatlands, linked to ecology/biodiversity, ensures appropriate use of carbon cycles. Bord na Móna is rehabilitating and restoring bogs as part of its Peatlands Climate Action Scheme with the aim of reducing carbon emissions and the eventual creation of carbon sinks. This is complex as raised bog is very dependent on sphagnum moss activity that prefers acidic conditions; however, the ground water in cutaway bog from peat harvesting will have become alkaline. Thus, it is important to map the peatlands linked to physiochemical profiles that would be extremely challenging without using digital tools such as ArcGIS. For example, given differences in peatland acidity, solutions are more so focused not on bog restoration, but bog rehabilitation, which depends on the peatland providing a suite of habitat types, such as wetlands, fens, scrub and woodland that would benefit from end-to-end monitoring and use of cloud edge computing (Table 1). Bord na Móna, with Ireland's Economic and Social Research Institute (ESRI) has a trajectory to actively restore and rehabilitate 33,000 ha of peatlands. It uses ArcGIS to design the most appropriate rehabilitation measures and then implements a wide range of measures in real time; moreover, ArcGIS rapidly visualises the existing conditions across thousands of hectares of bogs using numerous datasets that informs design and implementation of the most appropriate rehabilitation measures to restore peatland function and deliver climate action benefits. For example, for each bog identified for rehabilitation, GIS specialists and ecologists use the desktop solution ArcGIS Pro and 3D spatial analysis tools to examine the ground level and create detailed, map-based rehabilitation plans.

Tahar et al. (2018a, 2018b, 2018c) reported on the use of ArcGIS to enable monitoring and mapping of emerging contaminants of concern in aquatic environments for subsequent risk mitigation and management decision making (Tahar et al., 2017). Effective risk assessment and prediction for deployment of appropriate interventions to mitigate pollutants in waste water is at best 'semi-quantitative' given the enormous number of contributory factors and variables to inform management decision making (Tahar et al., 2017). Tahar et al. (2018a, 2018b, 2018c) highlighted that occurrence and geodatabase mapping of contaminations of emerging concern onto appropriate river basin catchment management tools will inform predictive and simulated risk determinations to inform strategic investment in necessary mitigation infrastructure to protect rivers and economic activities that rely on clean water. The medium to longer term ambition would be to utilize relevant European software and models for the development of spatially explicit Geography-Referenced Regional Exposure Assessment Tool for Peatlands to manage these resources, similar to what is been achieved for European River Basins.

Digital transformation of data sets would enable real-time and improve reliability of water quality determinants for risk management and policy decision making. There is help identify key constraints, such as knowledge underpinning sensitivity of existing sophisticated analytical equipment to measure low-level pollutants in real time; therefore, the commensurate development of appropriate risk management models will also inform future intensification and diversification of aquatic industries including peatland-based innovation. For example, Tahar et al. (2017) developed a semi-quantitative risk assessment model for evaluating the environmental threat posed by three EU watch list pharmaceutials namely, diclofenac, 17-beta-estradiol, and 17 alpha-ethinyestradiol, to aquatic ecosystems using Irish data; this model adopts EPA's Source-Pathway-Receptor concept to define relevant parameters including low, medium and high risk score for each agglomeration of waste water treatment plants, including catchment, treatment, operational and management. It is envisaged that a similar type semi-quantitative RA approach may aid development of peatlands globally in terms of screening for potential risks where there is a need to measure or predict environmental pollutant concentrations and where hydrological data are available. This approach is semi-quantitative, as other factors such as climate change will need to be considered for estimating and predicting risks with new aquatic innovations. Nair and Domnic (2022) noted the influence of machine leaning in advancing many aspects of this industry'; specifically, these authors described a combined strategy including non-learning enhanced method and deep CNN (convolution neural networks) for picture reduction and reconstruction in underwater imagine in aquaculture. These authors suggested that this model outperforms existing methods in terms of picture enhanced, compression, and reconstruction quality.

3.8. Edge-internet of things (IoT) systems

Globalization has radically informed the development of sustaining and disruptive technologies (Schuelke-Leech, 2018), where traditional industries such as agriculture and aquaculture employ vanguard technologies to expand upon opportunities that has enabled smart farming and the agri-food industry 4.0 (Klerkz et al., 2019). Perez-Pons et al. (2021) highlighted pressing need to make farms more profitable and sustainable via the analysis of data envelopment analysis and the application of the Internet of things and Edge computing; this approach allows monitoring environmental conditions with real-time data from the different sensors installed on the farm; thus, minimizing costs and achieving robustness by way of transitioning important data to the cloud after edge computing. Essentially, the edge devices process the data and then decide either to send this data to the cloud for further processing or make decision locally at the edge (Table 1). Edge computing can also be applied to sensor fault diagnosis and data repair (Wang et al., 2021). Technology requirements are increasingly important for agri-food industry with particular emphasis on meeting challenges faced by producers along value chain that also reflects a diversity of types of farms such as crop-cultivating or mixed farms that grow crops and produce livestock. These also rely upon fragile water resources; moreover, Eurostat noted that that total irrigable are in the EU-28 was ca. 15.5 Mha (8.9% of the total) whereas only 10.2 Mha (5.9% of the total) was irrigated thus highlighting the opportunities for implementing low-cost technological solutions (Fleming et al., 2016). The Industrial Internet of Things (IIoT) potentially enables technologies focused on implementation of monitoring and resource management solutions across many Industry 4.0 applications that embraces cloud computing, big data, AI or distributed ledger technologies (such as blockchain) that will improve traceability and productivity of commercial processes (Yu et al., 2017).

Perez-Pons et al. (2021) noted that when transmitting data to the cloud, there remains several challenges including data privacy, energy consumption, or costs associated with cloud services. Essentially, service providers charge users relative to the amount of data transferred, stored or processed in the cloud. However, by using Edge Computing technologies, one can

decrease the amount of data transferred between the IoT layer and the cloud that will also allow for deployment of machine learning models at the edge of the network reducing response time and enabling service provision even if communication with the cloud is interrupted (such as rural areas associated with peatlands) (Alonso et al., 2020). Perez-Pons et al. (2021) had also reflected on the findings of Pedra-Munoz et al. (2016) who reviewed years of improvements of applying technologies at family-farm level with sustainability, where the former reported that IoT and Edge computing can present a competitive advantage when measuring efficacy of decision making units. Perez-Pons et al. (2021) reported on different variables from the Environmental Performance Index with real-time sensors and the application of Edge-computing platforms that can reduce the data traffic to the cloud. In addition, 5G is a key enabler of edge computing, which provides low latency and high bandwidth communication services between sensors/edge devices and the cloud, and as well as direct device to device communication (Wang et al., 2021).

4. Pollination and ecosystem service management

Mount Lucas is an abundant source of heather and other peatland flowering plants, this provides a rich opportunity for honey production from nectar or from secretions from living part of plans by native bees; this can be addressed in on site living labs. The stressful environment created by the peatlands can influence emergence of novel bioactive properties produced in heather-honey. Shafiee et al. (2013) has previously reported on the use of machine learning to differentiate and classify polyfloral from monofloral honey where latter has a higher commercial value. There is a correlation between honey colour and its floral origin and some chemical parameters that can be discerned using image analysis and algorithms. Different monofloral honeys have distinctive flavour and colour due to variance in their main nectar sources (Escriche et al., 2011). Use of imagine analysis is an area of emerging importance that reflects a method that supports rapid, real-time, simple, selective and low-cost properties appropriate for honey characterization. Moreover, honey industry requires simple, non-invasive, fast and economic technologies for characterizations – other digital approaches include an electronic tongue and data fusion of electronic nose (Escriche et al., 2011) where there is good correlation evident by applying data fusion. The latter aids human panels in making decisions for application to honey quality evaluation that captures adulteration, classification of flora types along with their geographical sources. Image analysis can help discern honey colour that depends on a plethora of factors including phenolic and flavonoid contents, mineral contents and antioxidant activity (Dominiguez and Centurion, 2015). Machine learning as an innovation has been shown to enable objective assessment of visual attributes of food quality including colour. There are other multiple opportunities for machine learning for informing pollination; for example, Goblirsch et al. (2021) describe the potential of electron-beam for low-temperature treatment of pollen contaminated by complex bee parasites and viruses that may be used for commercial bumble bee purposes. The complexity of pollen contamination can be unravelled be potentially unravelled by use of flow cytometry using a suite of specific biomarkers in real time- where this non-invasive enumeration approach is potentially appropriate for machine learning and automation applications. The Irish company Apis Protect developed a digital platform using sensors and machine learning for monitoring hives; specifically, key parameters encompassing temperature, sound, humidity and temperature are recorded suing a wireless in-hive sensor device (Robb, 2021).

5. Waste water recirculation – nexus between monitoring, treatment and energy using a paludiculture framework

The Farm to Fork Strategy which is at the heart of the EU Green Deal is a key driver for efficiency within the nexus of food production, water and wastewater resources and treatment and broad sustainability issues. Indeed across the OECD Farm to Fork strategy reflects the requirements of food systems in terms of the "triple challenge" of food security and nutrition, livelihoods, and environmental sustainability (Rowan and Casey, 2021). While the concept of Industry 4.0 is now well established, when evaluating food production systems in terms of the use and reuse of resources such as water it is appropriate to consider the newer concept of Industry 5.0. Industry 5.0 sees digitisation as not just about productivity and growth; but is also part of a broader need for sustainable, human-centric and resilient industry. In the context of the positive transformation of peatland ecosystems, digitisation, applied to water resources can be seen in the context of Industry 4.0 and 5.0 concepts. The "Digital Water" programme within the International Water Association offers key lessons on how the water industry (or industry's where water is a key resource) can uptake and integrate next generation digital technologies. A series of white papers describe the various opportunities and challenges associated with the Digital Water Concept. Challenges with adoption include technical issues such as integrating smart actuators, sensors, and autonomous control systems in a sensible and transparent manner, cybersecurity issues, human resources issues and, crucially ensuring the need to have a clear value proposition.

Water management is key in paludiculture and should (i) maintain appropriate water levels for the activity in question and (ii) may be required to enable the supply of nutrients through the water inlet (Vroom et al., 2018). This may be required to address nitrogen losses after rewetting due to denitrification processes and anaerobic ammonium oxidation. Indeed this can provide an opportunity for local reuse of N rich agricultural wastewaters (Vroom et al., 2018). Recent work in the UK, analysed water management requirements through pumping of water to rivers when inputs to peatlands, due to rainfall, exceed evapotranspiration (Mulholland et al., 2020). Despite this there can be significant reductions in energy use, costs and associated greenhouse gas emissions for paludiculture when compared to arable activities on deep fen peat (Mulholland et al., 2020). Real time control of storm water control measures has been shown to have significant potential for urban water management (Xu et al., 2021) and reinforcement learning has also potential to mitigate flooding when compared to passive control and rule-based control systems (Bowes et al., 2021). Such innovation could be adapted for water management in paduciculture activities Furthermore, a digital transformation of how water and wastewater resources are managed, can impact both the use of water abstracted directly for paladiculture activities (e.g. for aquaculture) and the management from wastewaters resulting from such activities. Recent work in the water and wastewater treatment sectors can point the way forward in terms of digital adoption for efficient management of water resources in paludiculture. This can also help reduce concerns in terms of digital adoption through visibility of related case studies.

Artificial intelligence can be applied within the water sector under three headings namely (i) modelling, prediction and forecasting, (ii) decision support and operational management, and (iii) optimization. A broad digital transformation would also impact these areas but also a fourth, namely system and infrastructure design could be considered. There are lessons for paduliculture that can be gleaned from other sectors such as storm and flood management, wastewater treatment and the water treatment and distribution sectors. Table 2 summarises, in the context of paludiculture how a digital transformation could impact the sector. Developments in remote sensing could have significant implications for paludiculture. Weiss et al. (2020) presented a meta-review of remote sensing for agricultural applications. Technological improvements have meant that global, regional and local data on crop mapping, yield forecasting, biodiversity loss, water and soil impacts are, in many situations, readily available. Chawla et al. (2020) reviewed remote sensing products for analysis of water quality (e.g. surface water), water quantity (e.g. river or stream flow) and extremes (e.g. flooding and drought impacts). Remote sensing also provides an alternative for locations where in-situ sensing is problematic where satellite remote sensing can now monitor in near-real-time retrievals, most components of the terrestrial water cycle. Challenges remain relates to accuracy, data consistency, utility and also in retrieving data related to groundwater, water quality, surface water levels, and river flows and in relation to the products themselves (Chawla et al., 2020).

Table 2
Examples of impacts of digital transformation on water resources in paludiculture.

Modelling, prediction and forecasting	System and infrastructure design	Decision support and operational management	Optimization
• Scenario analysis to support design (e.g. robust design under varying conditions) • Links to process optimisation and potential for real--time process modelling using digital twins.	• Optimise design to enable future expansion and digitisation • Reduce life cycle costs through development of digital models and/or digital twins • Design to enable future expansion	• Management of water levels • Real-time control of waste-water treatment processes • Fault detection and diagnosis on water systems • Regulatory compliance	• Optimised pumping regimes for water level management • Minimise energy consumption • Optimised system maintenance (e.g. preventative maintenance) • Optimise on-site productivity

Data collection in the water sector has long been recognised as a key challenge. In many cases the may be collected from relatively harsh environments which results in added maintenance. Furthermore there can be concerns including (i) lack of trust in data veracity, (ii) poor data management systems and (iii) the use of systems that are over-complicated and unoperable for end-users. Therrien et al. (2020) also highlighted key steps and ways forward for ensuring the steps from adequate data collection to action can be completed. Corominas et al. (2018) conducted a comprehensive review of computer based techniques for data analysis to improve operation of wastewater treatment plants. The EU have led in terms of research in this area with the most cited techniques including artificial neural networks, principal component analysis, independent component analysis and partial least squares. However the review acknowledged a lack of objective comparison of techniques, the need for guidelines, the requirement for validation at full-scale, and the limited options for active optimization of data information content and quality. Clifford et al. (2017) proposed an approach balancing spatial resolution of data with the costs involved in collecting such data and It is clear the use of real-time monitoring and control systems has significant potential but further case-specific validation is necessary.

Newhart et al. (2019) comprehensively reviewed specific applications of various real-time control in wastewater treatment facilities and presented examples of on-site applications across various industrial and municipal sectors. While Newhart et al. (2019) pointed out the potential for such control to be more difficult to implement in decentralised wastewater treatment facilities, Fox et al. (2022) demonstrated how data driven real-time control of a decentralised wastewater treatment system (in this case a sequencing batch reactor) can be optimised to achieve regulatory compliance, reduce energy consumption and increase system throughput. Both standard statical approaches and more advanced approaches using neural networks and regression modelling (Fox et al., 2022) were implemented. Key to these approaches was their compatibility with standard (low-cost) programmable logic controllers and enabling the end-user optimise the control approach.

Fault detection and diagnosis, while common across various engineering sectors, has focused mainly on leak detection at a municipal water supply level rather than building or industrial settings (Seyoum et al., 2017; Hashim et al., 2020). Fault detection in these settings can reduce leaks but also enable efficient monitoring and preventative maintenance of equipment such as sensors, valves, pumps and motors used for water and wastewater management. There are various approaches that can be used to leverage data from water systems in industrial processes. Mulligan et al. (2021) developed a series of water distribution system performance assessment rules and demonstrated how these would result in significant energy and water savings and associated greenhouse gas emissions. Hashim et al. (2020) used principal component analysis and support vector machine techniques to enable accurate detection of various faults in a large public building and an industrial setting. The problem of false alarm moderation (false alarms can undermine user confidence) has also received recent attention using both modelled data (Chen, 2010) and using case-study data from industrial settings and large buildings (Hashim et al., 2020). Fault detection in wastewater treatment also requires further testing in full-scale facilities and a pathway forward of using hybrid models such as linking gaussian process regression, artificial neural networks with principal component analysis or reinforcement learning to challenges associated with issues around sensor accuracy and transient operational conditions (Sundui et al., 2021).

Nature based solutions (NBS) have been identified as key in addressing challenges in water management across urban, agricultural and ecological settings. In the agricultural landscape, NBS can be applied for soil health, carbon mitigation, downstream water quality protection, biodiversity benefits as well as assisting agricultural production and supply chains to achieve net-zero environmental emissions (Rowan and Casey, 2021). Evaluting the ecotoxicological safety of NBMS will also be importnat moving forward (Garvey et al., 2015). Examples of recent applications include wastewater treated using naturally occurring algae, bacterial and duckweed using IMTA process (O'Neill et al., 2020), use of earthworms based technology for composing and wastewater treatment applications (Cooney et al., 2021; Hylton et al., 2022; Arora and Saraswat, 2021), zooplankton for tertiary wastewater treatment to enable wastewater reuse (Pous et al., 2021). The INNOQUA (H2020) project presented reviewed and demonstrated various pathways for nature based treatment of wastewater and options for enabling wastewater reuse (Bumbac et al., 2021). The study reviewed constructed wetlands, waste stabilisation ponds, anaerobic treatment systems and vermifilters and presented details of performance, design and maintenance requirements and ability to meet various regulatory standards across a wide variety of applications. NBS technology can be underpinned by models and tools that support better land use, enable accurate nutrient flow modelling and support the development of sustainability metrics via life cycle assessment; moreover, while NBS are designed to minimise technological requirements, there are significant opportunities to further enhance their benefits and enable process flexibility through targeted real-time control (Basil et al., 2021).

6. Aquaculture and other aquatic systems

Peatlands presents an opportunity to establish new food production systems that can offset negative environmental consequences of resource constrained conventional terrestrial farming. Peatlands aquaculture can fulfil consumer needs by both intensifying and diversifying production of new freshwater areas and fish species. The emerging nature of the industry provides an opportunity to build in digital solutions that can enable high technological developments tailored to the specific needs of the production model. Technology interfaced with digital solutions can be used to address the challenges of low impact paludiculture (such as low-trophic IMTA, RAS, organic) and environmental services. Digital technologies can support efficient development of paludiculture products and can be used to develop climate-friendly, sustainable production systems generating high quality proteins and other products.

Essentially, Mount Lucas peatland demonstrator is ideal for establishing digital systems to allow digitalization and use of collected data across the full value chain to inform new eco-innovation balanced with environment protection. Digital tools such as Edge-cloud sensors, AI, machine learning and augmented reality can be used to build models from collected data and facilitate implementation of a circular strategy to produce biomass of high value fish (such as trout and perch). At the same time models that integrate biological constraints (microbiome, fish growth and health), system functioning, and effluent outputs can improve biomass productivity and represent a step towards precision aquaculture. Moreover, the integration of customized management systems throughout the supply chain will

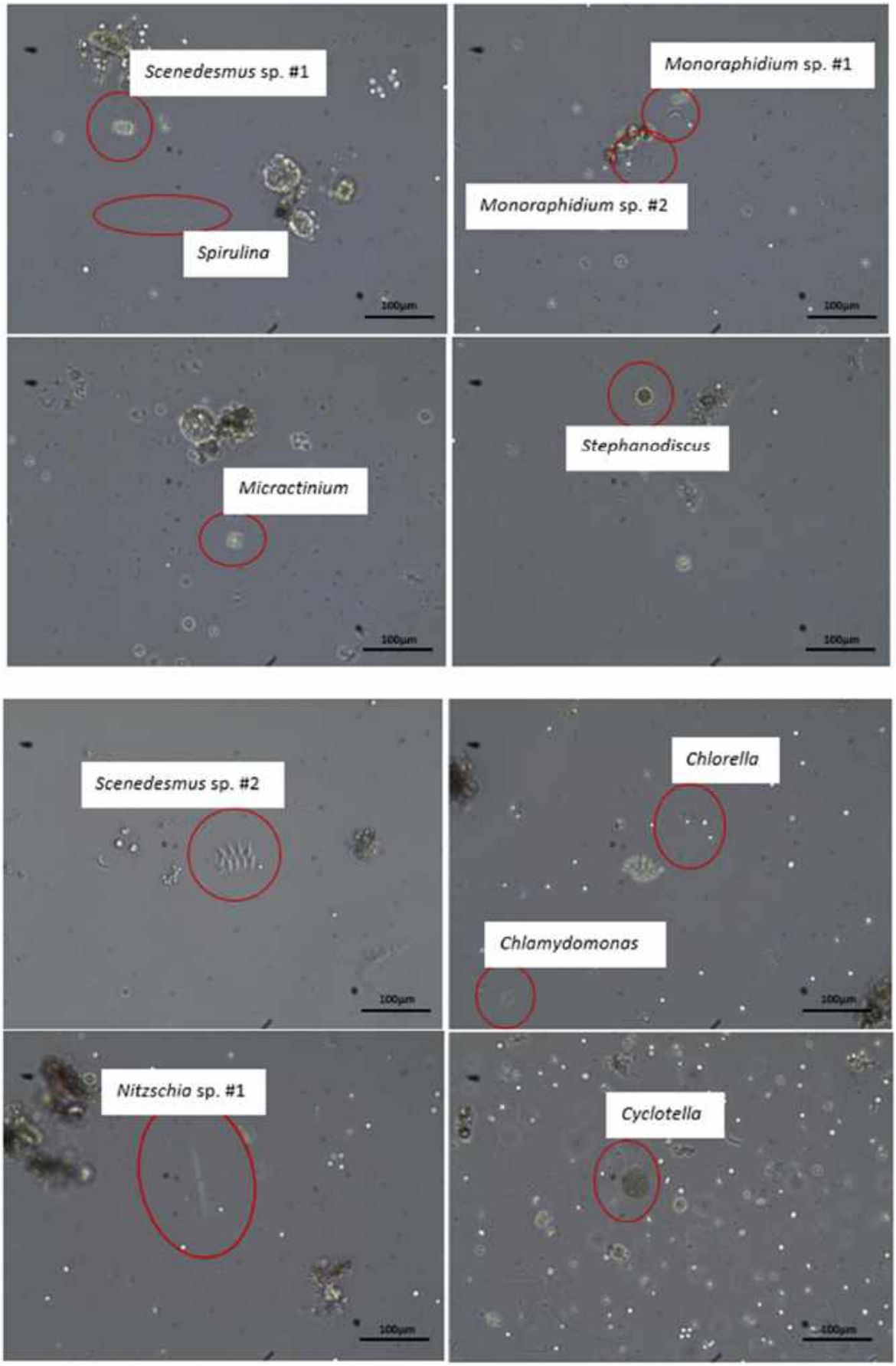


Fig. 2. Imagery of microalgae from the genera Scenedesmus, Nitzschia, Monoraphidium, Chlorella, Chlamydomonas and Cyclotella identified in Mount Lucas IMTA.

enable a well-managed, responsive and crisis proof system with improved traceability and authentication (Fig. 2). Such a digitally transformed, intelligent management system will enable development, testing and validation of optimised integrated multi-trophic aquaculture and recirculating aquaculture systems (IMTA/RAS) on the peatlands by integrating multi-sensing (heterogenous sensors), multifunctional real-time modelling for decision making with provision for climate resilience through full system monitoring (microbiome, water physicochemical parameters).

There is a pressing need to design, implement and deploy ICT technologies to provide end-to-end monitoring systems for aquatic-based foods produced in the peatlands (O'Neill et al., 2022). Integrated software, and establishment of smart sensors for real-time monitoring and remote data logging of production, is being implemented at the Mount Lucas peatland site. LCA, PCA, MFA models can contribute to green business models and the standardization of emerging peatland aquaculture and associated businesses globally. The latter also includes use of AI, machine learning and Edge – cloud to develop and evolve strategies to reduce waste and to consolidate gains achieved in aquaculture supply chain management. On a related point, Ruiz-Salmón et al. (2020) noted that the water-energy-food nexus allows assessment of the life cycle of seafood products that enables clustering and knowledge transferring to add value in the European Atlantic region. LCA can be applied to help understanding the benefits of ecolabelling and eco-design in aquculture under a circular economy approach (Ruiz-Salmón et al., 2020). Open knowledge sharing can also help advance paludiculture, such as software that integrates sensors (cameras, remote sensing), and predictive analysis (biomass estimation and forecasting, water quality monitoring) to enable improved operational decisions for optimised production balanced with environmental protection. Combined use of augmented reality and virtual reality can be used for training to promote human resource development, so it accompanies 4IR and becomes part of the digital transformation and Just Transition. ICT can be used to create a quality of experience (QoE) that enables specialist training in paludiculture on site and remote by linking to living labs. Hatch blue accelerator is gaining in popularity as a novel sustainable aquaculture and innovation programme offered globally for innovators that includes those converging disciplines (https://www.hatch.blue/accelerator).

Traditionally, there has been worldwide reliance upon 'end-of-pipe' engineering solutions for discharge wastewater control to safeguard water resources (Barrett et al., 2016; Tahar et al., 2017). This IMTA system of Mount Lucas will provide solutions and data for wastewater management and be a model system for social marketing and studies of consumer awareness and acceptance of aquatic paludiculture processes (Domegan, 2021) that may lead to disruptive innovation (Schuelke-Leech, 2018; Schuelke-Leech, 2021a, 2021b). Mount Lucas will also enable emerging innovations such as oral vaccine testing and the potential use of other safety indicators (Taufek, 2020; Usuldin et al., 2021; Wan-Mohtar et al., 2021). The digital transformation "Terrain-AI" project uses drones across the peatlands for monitoring carbon sequestration as a means for sustaining carbon sinks (SFI, 2021). ICT systems coupled to drones for data collection can provide real-time ecosystem management to promote and preserve pollinators across the peatlands by preserving and enhancing habitats, improving food sources through rewilding and decrease bee-disease through use of innovative technologies (Goblirsch et al., 2021).

The development of fully recirculated systems, such as this aforementioned IMTA system, that relies upon natural processes for remediating water quality, and do not discharge to receiving water presents a step change or potential disruptive sustainable solution. O'Neill et al. (2020) described the first IMTA system developed in the Irish peatlands that uses a balanced ecosystem of naturally occurring microalgae, bacteria and potentially duckweed that regulates waste and maintains water quality. An on-site wind turbine provide a renewable source of energy to operate aeration systems in the circulatory aquaculture ponds. However, global warming has created greater opportunities for extreme weather events that can influence vital food production, by way of droughts and flooding. The ability to monitor and predict the impact of extreme weather events through digitalization of the pilot IMTA processes will help future proof and protect food supplies globally. Naughton et al. (2020) highlighted the potential benefit of digital technologies for connecting 'in field' monitoring devices with living lab sophisticated equipment for real-time decision making in freshwater aquaculture.

Previous researchers have also highlighted that microalgae species that constitute a major proportion of the peatland aquatic-biome are excellence candidates for CO_2 bio-capture (Lopez-Pacheco et al., 2021; Wang et al., 2021); thus, use of end-to-end monitoring linked to sensors that will enhance efficacy will be important going forward. The microalgae in the aquaculture ponds constitute potentially thousands of species (O'Neill et al., 2022) where there is a pressing use machine learning with bioinformatics to unlock their real-time monitoring and occurrence. O'Neill et al. (2022) has also reported on using the occurrence of key microalgae in aquaculture ponds as potential biosensors for assessing the impact of climate change on IMTA process, which can be informed by using Cloud-edge computing. It is notable that microalgae represent a superior option for carbon fixation than terrestrial plants for higher growth and faster biomass production, doubling their biomass in less than 24 h for most species (Farrelly et al., 2013; Guo et al., 2017; Lopez-Pacheco et al., 2021). Fig. 2 illustrates the different microalgae represented of the genera *Chlorella, Raphidocelis, Scendesmus, Desmodesmus, Monaraphidium and Graesiella* that were isolated from Mount Lucas aquatic environment and were previously noted to exhibit CO_2 capture abilities (Lopez-Pacheco et al., 2021). Tabatabaei et al. (2011) noted that microalgae use carbon dioxide for energy conversion while producing approximately half of the atmospheric oxygen. While Zhao and Su (2020) estimated that microalgae can capture a maximum of 2.35 $GtCO_2$ in 100,000 km^2 culture area, which shows the potential of using these organisms that naturally occur in the peatlands for CO_2 capture (Ramaraj et al., 2014). Use of microalgae in this IMTA closed system have many advantages, including easy control, insufficient space required, high CO_2 sequestration rate, and no contamination risk, nearby all microalgae species may be cultivated, and high biomass density (Lopez-Pacheco et al., 2021). Lopez-Pacheco et al. (2021) also noted that through photosynthesis microalgae can fixate CO_2 by what is known as phyco-capture process. Thus, there is a pressing need to use digital tools to enable end-to-end monitoring and to optimise ponds systems to cultivate microalgae for CO_2 bio-capture.

7. European Digital Innovation Hubs – Quo Vadis

Digital Innovation Hubs (DIHs) are one-stop shops that help companies to become more competitive with regard to their business/production processes, products or services using digital technologies, while remaining environmentally sustainable and reducing greenhouse gas emissions. DIHs are based on technology infrastructure (Competence Centre) and provide access to the latest knowledge, expertise and technology to support their customers with piloting, testing and experimenting with digital innovations. DIHs also provide business and financing support to implement these activities, if needed across the value chain. As proximity is considered essential, DIH act as first regional point of contact; consequently, a DIH is a regional multi-partner corporation (RTOs, universities, industry associations, chambers of commerce, incubator/accelerators, regional development agencies, and potentially governments), and can also provide strong nexus with other service providers outside their region supporting companies with access to their services. The rationale behind this DIH initiative is to help European industry, small or large, high-tech or not, to grasp the digital opportunities. The EC will focus 500 M€ over the next 5 years from Horizon Europe budget to support the development of DIHs as the level of digitalisation remains uneven, depending on the sector, country and size of company: only 20% of SMEs in the EU are highly digitised. This challenge is particularly pertinent for the digital transformation of peatland eco-products and services. Key assets aligned with DIHs include (a) information on infrastructures; (b) expertise; (c) network contacts of key players and communities at large; (d) expertise in initiating robust collaborations of key stakeholders to meet specialist USP offerings; (e) access to financial capital; and (f) effective digital marking to ensure trustworthy

brand that attracts stakeholders and ensures high quality delivery. Thus, digital transformation of a peatland-focused hub will enable real-time access to networks; upskill and satisfy R&D opportunities; track and communicate technical expertise and consultancy, enable seamless and managed access to facilities; and support the contribution to policy measures.

Currently, there are 706 DIHs registered on the Catalogue of Candidate European DIHs tool that are in following evolutionary stages; fully operational (413), in preparation (223), and potential new DIHs from H2020 (70) (https://europa.eu/!NX87WD). The purpose of this European catalogue is to support networking of DIHs and to provide an overview of the landscape of DIHs in Europe supported by regional, national and European initiatives for the digitization of the industry. European DIHs (EDIHs) will play a central role in the Digital Europe Programme to stimulate the broad uptake of AI, high performance computing (HPC) and cybersecurity, as well as other digital solutions/interoperability for the public sector. EDIHs will support companies, and the public sector organisations, in the use of digital technology to improve the sustainability of their processes and products, in particular with regard to energy consumption and reduction in carbon emissions. The 325 candidate EDIHs are the DIHs (existing or not) that are assigned by Member States to participate in the Call for Proposal of the EC to obtain funding to become European DIHs; a network of 200 EDIHs will be financed in Europe via the new Digital Programme 2021–2027. Currently, the EC is verifying all entries in the catalogue tool based on information provided by each DIH as to whether or not the comply with 4 criteria; (1) be part of a regional, national, or European policy initiative to digitise the industry; (2) be a non-profit organization; (3) have a physical presence in the region and present an updated website clearly explaining the DIHs' activities and services provided for the digital transformation of SMEs/Midcaps or industrial sectors currently insufficiently taking up digital technologies; and (4) have at least 3 examples of how the DIH has helped a company with their digital transformation, referring to publicly available information, identifying for: client profile, client need, and solution provided to meet the needs. Generally, the main functions of EDIHs include (1) Test before invest, (2) Skills and training, (3) Support to Find Investments, and (4) Innovation ecosystem and networking. EDIHs are embedded in a local economy; for example, if manufacturing is important, the hub will enable companies in adopting Industry 4.0 and circular economy methods. Traditional ICT method, such as simulation and supply chain integration, will take on an important role where these are becoming more AI and HPC orientated. In addition, by introducing digital manufacturing, cybersecurity becomes a prerequisite. .

It is likely that the applicability and digital maturity of entities supported by Digital Innovation Hubs alone or embedded in Quadruple Helix concepts will be profiled using the categories described in Table 3. Innovation Radar methodology is an approach adopted by the European Commission to assess the impact of “test before invest” and “support to find investments” services of EDIHs. Mount Lucas peatland HUB will support and enable ‘train the trainer’ developing ways to transfer knowledge generated in a HPC, AI and Cybersecurity through regular workshops. This will also enable community building for stakeholders that includes agriculture, horticulture, health, public administration and so forth. Other activities to be adopted, aligned with EDIH framework, digital matchmaking marketplace, InvestEU, short term training courses, engagement with regional and national policy makers, deploy effective media presence to highlight peatland activities across the network, and impact assessment of activities that includes analysis of indicators, and KPIs, developing targets, generating new knowledge including Open access provision to support benchmarking and policy recommendations.

8. Business modelling and sustainability for enabling accelerating Mount Lucas green innovation

Ziegler et al. (2021) highlighted that despite increasing sustaining or disruptive potential, it remains challenging for value propositions and value network for new paludiculture products and services remains to be hurdled, which presents a barrier for commercial applicability. Several authors with subject matter-expertise in peatland innovation have appropriately noted that digital transformation of paludiculture products and services will have limited utility in the absence of a robust and valid green business model that delivers scale. Ziegler et al. (2021) described paludiculture to be an emerging, science driven, and collaborative innovation that are rarely directly commercially viable, where the authors focused on fuel, fodder, horticulture substrate and construction material. These peatland products are currently not under development at Mount Lucas. Moreover, Ziegler et al. (2021) reported that paludiculture faces significant, adverse path-dependency due to subsidies and regulations that appear to preferentially support agriculture on drained peatlands. Paludiculture initiatives to date typically involve landowners and users where further economic models supporting experimentation and scaling up of paludiculture products and services are required (Ziegler et al., 2021). Zeigler (2020) also reported that paludiculture is the productive use of wet and rewetted peatlands and proposed a 3Ms-schema of mission, modes and making innovation as a device to create space for a wide and inclusive discussion of paludiculture. The reader is directed to the published work of Planko et al. (2016) for key processes for building up a technological innovation system (that includes paludiculture) and arguments supporting a system building model approach in strategic management.

Entrepreneurs and SMEs at Mount Lucas are supported and enabled through a quadruple helix hub approach under an Empower Eco sustainability framework, which connects stakeholders across the business ecosystem including academia, industry, government and society (end-users). This reflects that need to adopt far-reaching changes of the macro environment in order to implement innovative sustainable technologies (Planko et al., 2015; Planko et al., 2016). This Empower Eco holistic approach aligns with previous scholars who reported on the combined insights from the strategic management literature and the technological innovations system (TIS) literature in order to provide a strategy framework for entrepreneurs to collectively build a favourable environment for their sustainable technology (Planko et al., 2016). It is notable that insights from system-building literature originate mainly from the systems perspective (Musiolik et al., 2012) that had previously not considered specific subject-matter insights from the company perspective (Planko et al., 2016). The holistic system building approach, aligned strongly with Quadruple Helix Hub concept depicted by Empower Eco, connects actors across their ecosystem to collaborate strategically in order to shape their environment (Rowan and Casey, 2021). Planko et al. (2016) noted that term ‘system building’ originates from the TIS literature and is defined as the “deliberate creation or modification of broader institutional or organizational structures in a technological innovation system carried out by innovative actors” (Musiolik et al., 2012). While this system building approach harnesses the use of a diverse suite of assets, facilities and expert in collaborating universities for addressing broad ranging enterprise needs, this approach as supports entrepreneurs and start-ups in co-creation and testing of low cost green solutions (Table 4).

For example, Accelerate Green is the first Irish accelerator dedicated to scaling companies based in the peatlands with a particular focus on climate action and sustainability by developing eco-products and services based on green innovation with a base in Boora, which is adjacent to Mount Lucas (Rowan and Galanakis, 2020). Accelerate Green combines Bord Na Móna’s commercial expertise and Resolve Partners expertise in building innovative companies, and is an equity-free scaling accelerator designed to create step change in green innovation (Bord Na Móna, 2022). It delivers eight 2-day deep progamme sessions that action planning, offsite innovation, strategic and business planning along with working with a peer group of successful entrepreneurs or ‘green-change leaders'. Accelerate Green also provides an ‘invest and enable’ funding strategy with an initial focus on established scaling climate tech companies, high-growth SMEs pivoting to climate change economy, earlier stage innovative-driven enterprises, renewables/carbon reduction, waste/circular, and AgTech.

Use of this systems building approach has also supported UNIVIV OneHealth/Emerald SME partners to map a business canvas that includes the economic feasibility for commercially producing high value protein

from duckweed (Fig. 3) harvested from the aquaculture ponds at Mount Lucas that includes scalability. Irish demand for plan protein in animal feeds is approximately 900,000 tons, where current protein rich native crops (peas and beans) provide approximately 52,000 tons on 10,000 ha. The National Protein Stakeholders Group aspires to increase this production to 120,000 on 20,000 ha. Notably, 3000 ha of rewetted peatland ponds will produce 5000 tons of protein concentrate with zero artificial inputs including fertiliser and pesticide. Some 5000 tons of duckweed protein concentrate would displace 50% of imported protein concentrate Irish organic salmon industry. Thirty thousand hectares of duckweed on marginal lands would produce 50,000 tons of 65% protein concentrate that is approximately equivalent to 20,000 ha of pea and bean protein at approximately 30% to 35% protein. The direct commercial value is in excess of €50 m; thus, potentially displacing €50 m of imports from an Irish feed production and security perspective. Thus, rewetting peatland is commercially feasible from a farmers' perspective, if duckweed is cultivated as an alternative sustainable crop. Topics that require attention include processing duckweed to produce animal feed, optimizing duckweed growth conditions, and identifying lands most suitable for rewetting and change of use. The Mount Lucas site is licensed for fish farming where the fish waste stream supports the linked commercial production of duckweed that can be potentially used in the animal feed industry (O'Neill et al., 2022). O'Neill et al. (2019) also reported that duckweed is important for waste aquaculture stream quality and recirculation at Mount Lucas peatland site where duckweed works in concert with algae and microbes for this purpose. De Beukelaar et al. (2018) reported that duckweed is considered to be a promising source of protein for human food products due to its high protein content and environmentally friendly production properties. Albeit not peatland focused, Bonomo et al. (1997) had previously described the use of a pilot duckweed as biological approach to produce reliable, simple and cost-effective small wastewater treatment system. In spite of the profitable characteristics of duckweed (high productivity, high protein content, wide geographic distribution, control of negative impacts from conventional wastewater treatment ponds), the results intimate that extensive use in Italy seems difficult due to the high requirement of land area and the ceasing of growth in winter months (at least in Northern Italy). However, the peatlands represents a large expansive area in many countries internationally, including Ireland. In temperate climates, a reasonable use of duckweed looks to be the production of good quality secondary effluents (BOD and SS removal) from small communities, especially in seasonal (summer) wastewater treatment plants. Duckweed is the smallest and fastest-growing aquatic plant, and has advantages including simple processing and the ability to grow high biomass in smaller areas. Therefore, duckweed could also be used as a new potential bioreactor for biological products such as vaccines, antibodies, pharmaceutical proteins, and industrial enzymes. Moreover, Yang et al. (2021a, 2021b) recently reported on plant bioreactors have flourished into an exciting area of synthetic biology due to their product safety, inexpensive production cost, and easy scale-up.

Currently, the commercial production of high value 'organic' cultured perch and trout at Mount Lucas is no likely to be commercially viable given the low fish biomass or tonnage on a 4 ha site, but this also provides an excellent innovation system for education, training and research given it's state-of-the-art IMTA system focus. Fish health/welfare and the linked microbiome profile in cultured pond water will also strongly contribute as indicators or tools for studying impact of climate change on sustainable food production systems (O'Neill and Rowan, 2022; O'Neill et al., 2022), where these activities will also support social enterprises (Rowan and Casey, 2021), and the development of digital technologies.

The EIDH smart specialisation strategy for structured support and development of peatland innovation includes provision for Entrepreneurial Discovery process using bottom up approach. Typically, this embraces five categories– (1) launching strategic initiatives; (2) re-entering existing programmes; (3) updating stakeholder strategic agencies; (4) aligning infrastructure; and (5) setting up strategic fora. Digitising Peatland activities to manage projects, networks, skills and training, tech transfer, consultancy and strategy; transnational connectivity and communication; profile and embed regional actors that can promote diffusion of best practices and knowledge transfer; expand project opportunities; facilitate learning, experimentation and capacity building; manage workshops, SAPs and supporting specialist modules for PhDs; promote and foster ad-hoc alliances to enable open innovation; broker/match make academia with industry; accelerate entrepreneurial activities; align interests to local industries and opportunities for converging these; connecting with complementary EIDH for information flow and sharing on EU topics; support trusted partners that will provide subject-matter expertise and complementary services; develop successful business models for collaborations; an efficient channel to other EIHs, regional and markets that includes access to capacities, best practices and skills.

Table 3
Categories used to describe common functions of European Digital Innovation Hubs.
Source: https://europa.eu/!NX87WD

Category	Description
INTELLIGENCE	are intelligent systems used for decision making that both understand and adjust to specific circumstances; these are systems than can predict or plan to improve quality and to optimise capacity
CONNECTIVITY	the ability to access data in a secure and real-time manner; appropriate systems and machine will exchange data that may also be an integrated part of the business process.
FLEXIBIITY	the ability to adapt and customise systems and business processes to specific needs so that personalized products can be produced at affordable, mass-production prices?
AUTOMATION	can repetitive task be automated in a reliable way.
SUSTAINABILITY	are natural resources used in a sustainable manner, whereby not wasting fragile resources, also ensuring that no harm is done to the environment nor quality of life of citizens. SERVICES – are new sustaining or disruptive business models used where products are offered as a service.
SOCIAL	are workers motivated, engaged and empowered to carry out their work in an autonomous manner when working within the new systems.

Mount Lucas has focused on development innovations to support primary production of freshwater fish and to improve ecological performance with future provision for secondary process and diversification towards reduced waste and improving shelf-life. Development of customized ICT solutions for production and supply chain management to improve safety, quality and awareness towards enabling transition to eco-friendly aquaculture and adjacent innovations. This will be achieved by digitally connecting the living labs with in field aquatic and adjacent innovation in the peatlands (Fig. 1). Mount Lucas will support multi-stakeholder engagement to stimulate creation of innovative and disruptive solutions for eco-friendly aquatic food systems. Met through generation, validation and application of new knowledge at both pilot-scale and commercially in order to sustainably improve performance by enabling knowledge and technology exchanges that considers Open innovation. Means to consolidate sustainability include establishing and deploying best practice, quality assurance and risk management that can be met by specialist training and infrastructure with partnering companies. Essential is the development of effective and appropriate ICT management and related innovative tools throughout the supply chain that will embrace traceability and safety.

Mount Lucas will rely upon digital tools to develop, optimise, automate and validate land-based IMTA-RAS (IMRAS) models that include high protein foods that have a low environmental footprint or impact. Peatland-focused models to satisfy a circularity approach to produce high-value fish (such as Perch and Trout) along with low trophic plant species, such as duckweed. Monitor and control physicochemical parameters of culture ponds to reflect optimised ratio of microalgae and bacteria with duckweed (*Lemna minor*) to produce biomass of fish along with using this natural ecosystem to regulate waste and maintain water quality through recirculation. Develop sensors that will respond to key performance indicators in the pond to match aquaculture effluent and biomass productivity, along with

commercial production of duckweed that feeds of aquaculture waste stream.

Integrated digital (intelligent) management system for IMTA that includes provision for multi-sensing technologies aligned with multi-functional management platforms (advanced monitoring, modelling, data analytics, and decision making); this system should also measure and model variances in climate and other disruptive fluxes to system through real-time system monitoring (microbiome, water physicochemical parameters, toxic microalgae) and modelling.

Schuelke-Leech (2021a, 2021b) recently described disruptive innovation and how it applies to the changes proposed for the Green New Deal era, which is also highly relevant to digital transformation of the peatlands. This author noted that achieving the ambitions of the Green New Deal will require social, economic, and industrial revolutions, rarely experienced in our history. However, climate change is going to force monumental changes; the latter has already decoupled the end-to-end value chain at Mount Lucas where O'Neill et al. (2022) recently reported that IMTA production for trout and perch was affected by extreme weather events that lead to fish mortality. O'Neill and co-workers (2022) also reported also noted that the emergence of different species of microalgae in this IMTA peatland process can be used as an early warning tool for disruptive climate variance events. Schuelke-Leech (2021a, 2021b) reported that the Green New Deal offers a vision for controlling and directing society's transformation through the development of new green technologies and public policies that mitigate and support the transformation to a sustainable and just society. The reader is directed to the model of Schuelke-Leech (2018) for an understanding of magnitudes of disruptive technologies and applicability. Rowan (2019) also provided examples of potential disruptive technologies for disease mitigation that can be applied to safeguard peatlands aquatic products. A summary of potential key emerging innovative activities, models, products and services arising from the convergence of biological with digital domains over the next decade is illustrated in Fig. 4; it noticeable that some of these may lead to disruptive innovation.

Table 4
Digital transformation of the multi-actor ecosystem (Quadruple Helix Hub) along with commensurate infrastructure, supports and enterprise activities.

Concentrated single-access supports for industry, entrepreneurs, disruptors	Linked acceleration activities	Digitisation
Step-Change Physical Infrastructure & Systems Supports	R&D Collaborative Facilitation	End-to-end Edge computing AI, ARVR, QoE, blockchain, cyber-physical systems
Pre-start Ups	Design Maturation Activities	ARVR, QoE, AI.
Ideation & Design Thinking	Technical Maturation Activities	AI, machine learning, augment reality, IoT
Market Research	Financial Planning	Blockchain
-Early Needs Analysis	Legal Assistance	Blockchain
-Product Market Fit Analysis	Social and Digital Marketing (including informing customer behavioural change)	ARVR, IoT
Early Technical Validation	Networking	Edge – IoT systems
-Test the Tech	Dedicated Grant Writing/Reporting	End-to-end monitoring, Edge computing (sensors, robotics), photonics.
-Experimentation/Validation in Pre-Pilot	Connection to Academic Staff/Expertise and	Edge computing,
-Scaled to Real-Life Setting	Equipment to support commercialisation	ARVR (QoE).
"Living lab" – specialist equipment usage for data generation (real-time analysis)	Test beds in the environment (traditional slow analysis and hand-held devices)	Big data, machine learning AI, ARVR.
Conduit to State Financial Supports/Agencies	Enable Social Enterprise - Outreach Functions	Blockchain, cyper-physical systems, AI.
Specialist training	Technical training - ecotourism	ARVR/QoE; digital twin

9. Conclusions

There is a strong trajectory towards development of wet peatland innovation for new green job creation where this novel aquatic ecosystem must balance economic, environmental and social sustainability. Peatlands represents an important carbon sink where it is commensurately imperative to protect the environment, ecology aligned with regulatory policies. Digital transformation has radically changed adjacent Agriculture 4.0 that has been mainly driven new business models focusing on local productivity. Green innovation and social enterprises across the peatlands value chain will be accelerated through digital transformation, which will be harnessed through EDIH model structure that is complementary to that of the quadruple helix hub (academic-industry-government-society) concept. This new paludiculture enterprise ecosystem will test-the-tech and digitally connect green-minded companies, stakeholders, enables and end-users. It is advocated that future strategic orientation of digital policies and plans will be aimed at ensuring and achieving international consensus and agreement on harmonization of processes. For example, digital tools for enabling future strategic development of this Irish Mount Lucas peatland demonstrate will include focusing on protecting biodiversity; monitoring and ensure sustaining carbon sink with net zero gas emissions; developing new aquatic green-innovations that includes high quality protein sources, fully

Fig. 3. Duckweed harvested from the IMTA peatland site at Mount Lucas in the Republic of Ireland.

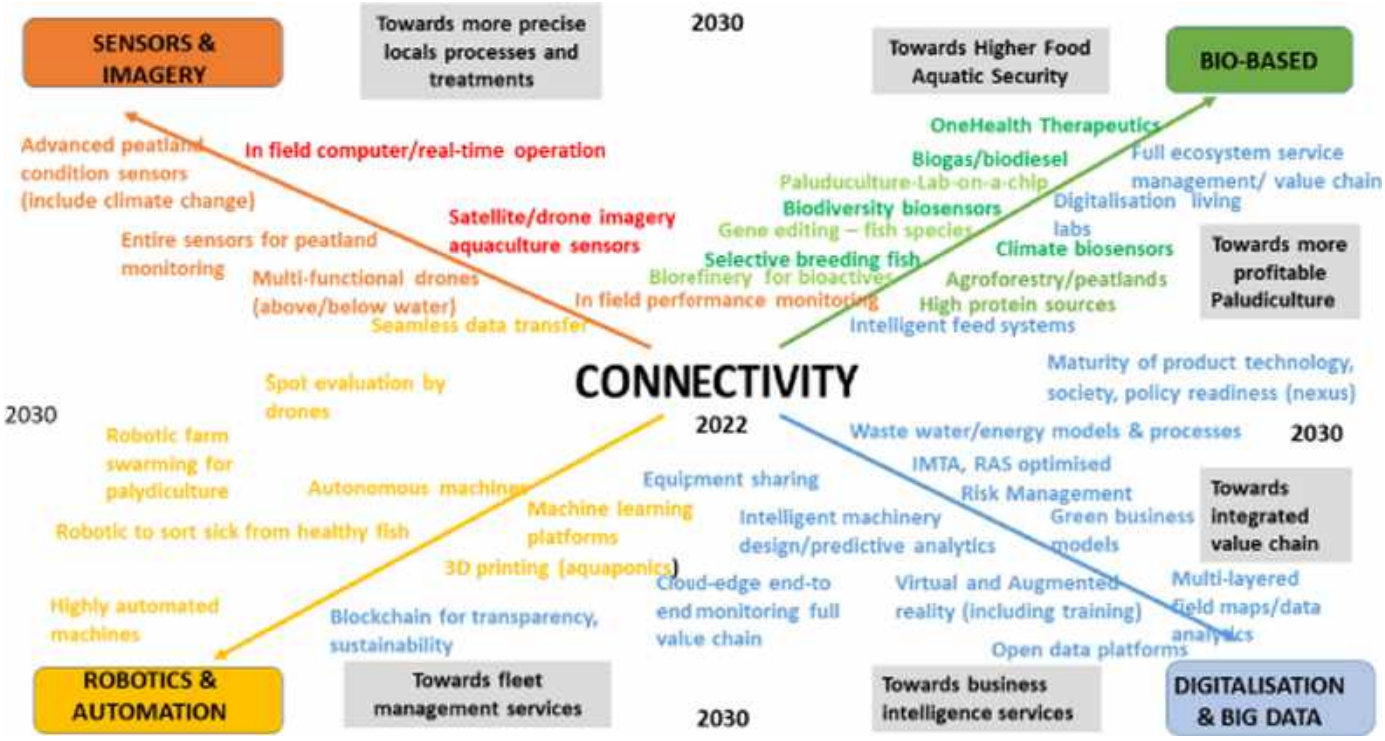


Fig. 4. Summary of potential key emerging innovative activities emerging from peatlands enabled by digital transformation over next decade.

recirculated waste water without need for end-of-pipe disease mitigation treatments, development of renewable energies, bioprospecting of bioactives from waste streams, in field testing of sensors and robotics, smart development of vertical farming and exotic mushrooms, agro-forestry, and pollination and ecosystem service management. Peatlands innovation will be advanced through digital solutions that address process automation, data analytics and processing, control and management systems; moreover, these activities reflect those applied to inform the 4th technological revolution under Industry 4.0 along with alignment with the main tenets of Industry 5.0 human-centric model, and with many of the UN's sustainable development goals. Digital transformation of paludiculture products and services as described herein will yield the next generation of multidisciplinary-trained researchers equipped with the necessary cross-cutting knowledge and skills to advance sustainability and climate action agendas for society with a global orientation. Funding instruments such as the European Just Transition, Interreg, and Horizon Europe will advance these opportunities by fostering and supporting key partnerships with stakeholders for the betterment of society.

9.1. Implications and recommendations

- There is much to be learnt by generating a deeper appreciation of the central role of key digital technologies supporting Agriculture 4.0 for unlocking adjacent and cross-cutting opportunities for the peatlands that will be met by more collaborate projects aligned with EIDH and Quadruple Helix Hub concepts. For example, this holistic approach should also focus on gaining a better understanding of the impacts of climate change for the intensive sustaining of peatland innovations at Mount Lucas (Ireland), which will potentially provide a useful blueprint to replicate across peatlands globally using an Open access knowledge-sharing model.
- There remains a lack of clear understanding as to what the concept of sustainability means for industries supporting paludiculture; therefore, it is envisaged that digital technologies will help meet this gap by way of promoting and implementing virtual training, management and creating greater awareness of these emerging eco-enterprises, products and services for end-users. Examples include using AI, machine learning, immersive technologies (AR/VR)
- Over the past 5 years there has been an accelerated transition from 'Brown to Green' that reflects what has become the paradigm shift away from harvesting peat as a fossil fuel to developing sustainable eco-innovations on the same peatlands. Therefore, there is a pressing need to understanding the roles of local government, industry and society in the management development of paludiculture innovations, which will inform new policies and job creation.
- It is important to create an awareness surrounding Peatlands innovations and to understanding how the digital transformation of such enterprises can powerful contributors to new business models and regional economies, which includes meeting several of the United Nations' sustainable development goals.
- Big data created from digital transformation of peatlands using drones will inform geo-referencing of sites with view to optimised usage balanced with protecting environment and biodiversity including ecosystem service management.
- There is a trend to develop new regional digital innovation hubs to meet community transitions to low carbon economy; however, there is a pressing need to provide clear guidance on the roles of each of the separate 706 DIHs across Europe so as to optimise cross-cutting usage by stakeholders that includes provision for integration trans-regionally.
- There are growing opportunities to support the swarm of new green start-ups companies that will be enabled through digitised living labs connected to environment test beds that will include AI/ML informed process automation, blockchain and cyber-physical systems for transparency, safety and security, IoT Cloud based approach to optimise sensors, drones and satellites for carbon sequestration and development of aquatic innovations ranging from aquaculture to vertical farming.
- Digital technologies will be enable Peatland enterprises to become more efficient through effective business models in real time; moreover, lessons learnt from Agriculture 4.0 suggests that key activities will include process automation and robotics, information systems and applications, cyber-physical systems, data analytics and logistics.
- Digital technologies will unlock societal challenges for rural and poor

regions as attested by the Irish peatland – midlands that will inform a fair and just transition across Europe.
- Digital technologies will help develop green innovation that will contribute to strategic management in order to mitigate against disruption in supply chains; this topic including mitigating against emerging risks including climate change, global conflict, and pandemics.
- Digital tools will help us appreciate key data outputs across several environmental test bed, such as use of LCA, PCA, MLF for generating data to help inform societal, political and technological readiness levels (or maturity) of green innovation across the aquatic peatlands ecosystem. Digital technologies will help define international standards for peatland innovation balanced with environmental protection including new policies, plans and licencing.
- Digital transformation can expedite the transition from 'brown to green'; for example, the trend away from peat harvesting as a fossil fuel to supporting and enabling the development of sustainable eco-innovations such as e-aquaculture, aquaponics, vertical farming, agroforestry and social enterprises such as honey production, exotic mushroom cultivation, pollination and ecosystem service management, eco- and darksky tourism.
- There is a need to reach an agreed consensus from stakeholders on the implementation of appropriate digital strategies to develop the peatlands including use of georeferencing, drones and satellites to map the peatlands matched with physiochemical parameters, carbon sink and biodiversity.
- Digital transformation to wettable peatlands products and services will also inform a new generation of researcher and promote job creation for rural areas that were strongly relient-upon peat harvesting as a means of producing fossil fuels for their livelihoods that disturbed carbon sink. Notably, the adjacent Agriculture and food sector are responsible for 19.29% of global gas emissions.
- Use of blockchain will help local farmers to directly reach customers in a secure and efficient manner.
- AI, virtual reality and augmented reality will enable a quality of experience for specialist training in the peatlands living laboratories (wet-labs, ecotoxicology, biodiversity) and provide a connected experience to the suite of environmental test beds that will showcase new eco-innovation, products and services; such as through Erasmsus +, MSCA RISE programmes.
- Digital transformation of peatland research and enterprise will inform new specific polices met through Horizon Europe, European Just Transition, European Green Deal, UN Sustainable Development Goals and so forth
- Digital transformation of peatlands will inform a fair and just transition of communities that are pivoting to low carbon economies along with enabling and accelerating green innovation.

CRediT authorship contribution statement

N. J. Rowan, Deborah Power: Conceptualization. E. A. O'Neill, Eoghan Clifford, Niall Murray, N.J. Rowan: Data Curation: N. J. Rowan, D. Barcelo, E. Clifford, Y. Qiao, D.M. Power, E. A. O'Neill: Data analysis; N. J. Rowan: Funding Acquisition; E.A. O'Neill, E. Clifford, N. J. Rowan; Investigation; N. Rowan; N. Murray, Y. Qiao, E. Clifford, D. Barcelo: methodology; E. A. O'Neill: Project Administration; N. Rowan, E. Clifford; D. Power: Resources; N. Murray, Y. Qiao; software; E.O'Neill, Y. Qiao, N. Rowan, N. Murray: validation; N. Rowan, N. Murray, Y. Qioa, D. Barcelo, E. Clifford, D. Power, E. O'Neill: roles/writing – original draft. N. Rowan, N. Murray, Y. Qioa, D. Barcelo, E. Clifford, D.M. Power, E.O'Neill: Writing – Review and Editing.

Declaration of competing interest

The authors declare that there are no competing interests or conflicts of interest with respect to the publication of this article.

Acknowledgments

The author acknowledges provision of aerial image of Mount Lucas peatland site furnished by Bord Iascaigh Mhara (BIM). The authors would like to thank Interreg Atlantic Area Neptunus (Project EAPA_576/2018), Regional University Network European University (RUN-EU Project), and Bord Iascaigh Mhara (Project 2019 BIM-KGS-008) for funding support. We thank the SME, UNIVIV OneHealth Solutions, for providing business model information informing the commercial development of their their Green paludiculture innovation.

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Trends in Food Science & Technology 110 (2021) 193–200

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Innovations and technology disruptions in the food sector within the COVID-19 pandemic and post-lockdown era

Charis M. Galanakis [a,b,c,*], Myrto Rizou [a], Turki M.S. Aldawoud [b], Ilknur Ucak [d], Neil J. Rowan [e,f]

[a] Research & Innovation Department, Galanakis Laboratories, Chania, Greece
[b] College of Science, King Saud University, Riyadh, Saudi Arabia
[c] Food Waste Recovery Group, ISEKI Food Association, Vienna, Austria
[d] Nigde Omer Halisdemir University, Faculty of Agricultural Sciences and Technologies, Nigde, Turkey
[e] Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Ireland
[f] Centre for Disinfection, Sterilization and Biosecurity, Athlone Institute of Technology, Athlone, Ireland

ARTICLE INFO

Keywords:
SARS-CoV-2
COVID-19
Food innovation
Lockdown transitioning
Disruptions
Sustainability

ABSTRACT

Background: COVID-19 pandemic has caused a global lockdown that has abruptly shut down core businesses and caused a worldwide recession. The forecast for a smooth transition for the agri-food and drink industry is, at best, alarming. Given that COVID-19 shutdown multiple core services (such as aviation, food services, supply chains, and export and import markets), there is an enormous deficiency in critical information to inform priority decision making for companies where this uncertainly is likely to impact negatively upon recovery.
Scope and approach: The current article investigates potential innovations within the era of the COVID-19 crisis after framing them within the four issues of the food sector (food safety, bioactive food compounds, food security, and sustainability) that are directly affected by the pandemic. The prospect of foreseen innovations to disrupt the food sector during lockdown periods and the post-COVID-19 era is also discussed.
Key findings and conclusions: Internet and Communication Technologies, blockchain in the food supply chain and other Industry 4.0 applications, as well as approaches that redefine the way we consume food (e.g., lab-grown meat, plant-based alternatives of meat, and valorization of a vast range of bioresources), are the innovations with the highest potential in the new era. There is also an equally pressing need to exploit social marketing to understand attitudes, perceptions, and barriers that influence the behavior change of consumers and the agri-food industry. Subsequently, this change will contribute to adapting to new norms forged by the COVID-19 pandemic, where there is a significant gap in knowledge for decision making.

1. Introduction

The COVID-19 pandemic led to millions of infections and deaths worldwide, changing dramatically what we perceived as norms and impacting society, health systems, governmental policies, and businesses. The food sector is no exception, as the consequences of this "*Black Swan*" socio-economic event (Reid et al., 2020) has changed the way we think, buy and consume food by accelerating pre-existing innovation trends (Askew, 2020f), marking a "before" and "after" period. In the short term, the pandemic affected the sector by causing labor problems (e.g., lack of workers due to illness and quarantine measures), the shutdown of factories, food shortages on shelves, and some cases stress of cash flow for the active businesses (Reid et al., 2020). We are also on the verge of a significant global recession (Guan et al., 2020), lacking critical information for recovery. The role of mapping trends and predicting consumer behavior towards new technologies, services, and products for transitioning beyond COVID-19 will be highly beneficial as per approaches described previously by Busse and Siebert (Busse & Siebert, 2018) and Suanda et al. (Suanda et al., 2018). On a long term basis, the pandemic affects the whole food sector into four main domains: food safety, bioactive food ingredients, food security, and sustainability (Galanakis, 2020).

Overcoming the pandemic's obstacles will not be achieved through austerity but by strong leadership, inspiration, and ambition. Even though vaccination to protect against COVID-19 has begun, the second pandemic wave has affected many countries. Thus it is impossible to

* Corresponding author. Research & Innovation Department, Galanakis Laboratories, Chania, Greece.
E-mail addresses: cgalanakis@chemlab.gr (C.M. Galanakis), myrtorizou@chemlab.gr (M. Rizou), tdawoud@ksu.edu.sa (T.M.S. Aldawoud), ilknurucak@ohu.edu.tr (I. Ucak), nrowan@ait.ie (N.J. Rowan).

https://doi.org/10.1016/j.tifs.2021.02.002
Received 20 July 2020; Received in revised form 22 January 2021; Accepted 5 February 2021
Available online 10 February 2021

exclude the possibility of repeated infection and lockdown waves, as the pandemic might continue up to the end of 2021, establishing further uncertainty to the food sector (Rowan & Laffey, 2020).

Subsequently, the pandemic's most significant impact may be from the changes induced during the following recovery period (Askew, 2020f). Today, it is uncertain with any degree of confidence what specific impact the global recession will have on the economy, as it relates to particular opportunities and needs encountered by emerging technologies in the food sector.

Through a long reflective lens, lessons have been learned from previous catastrophic global events, such as the Spanish Flu or Black Death, where inspiration has led to paradigm shifts in disruptive technologies. Subsequently, there is an increasing focus on innovative technologies to make the food sector sustainable to meet the opportunities arising from the COVID-19 pandemic (Munekata et al., 2020). The current perspective article explores relevant innovations within the era of the COVID-19 pandemic and post-lockdown era. The foreseen innovations are framed as two more dimensions within the boundaries of the four domains above (Fig. 1) before discussing their prospects of being implemented during expected lockdown periods and eventually to disrupt the food sector after the end of the pandemic crisis.

1.1. Implications of the pandemic in the food sector

As a '*Black Swan*' socio-economic event occurring extremely rarely, the COVID-19 pandemic resulted in catastrophic consequences. In this kind of crisis, companies would not have predicted and planned accordingly (Reid et al., 2020). Food and food supply chain safety was the first emergent issue under consideration, requiring an increasing number of precautionary measures as long as we move from farm to fork (Rizou et al., 2020). Sustaining food production through COVID-19 brought challenges, including clustering of cases in agricultural food production, slaughterhouses, and food processing industries (Ecdc, 2020). . This fact revealed operational issues of the food industry new era trying to maintain food supply balanced with social distancing. Likewise, the unified social solidarity to protect frontline workers from fighting COVID-19 has resulted in the abrupt mass closure of services and businesses globally, which has prompted governments to launch commensurate 'staggering' economic recovery packages to support a beleaguered industry (Govie, 2020; IBEC, 2020). COVID-19 pandemic has reset the new norm for society, with the frenzy of uncertainty fueling many companies to scramble to maintain functionality within their marketplace (Guan et al., 2020). Work practices have changed in terms of remote operation, including working from home and digital communication platforms and Internet and Communication Technologies (ICT) (Del Rio & Malani, 2020). What became evident during this crisis is that advanced and more digital traceability is a powerful tool in comprehending the implications of the supply chain in the case of a public health emerging event (Hahn, 2020).

Over the last year, boosting the immune system was a priority for consumers. This trend has accelerated within the COVID-19 era, and consumers' interest in sustainable, healthy, organic, and functional foods has been increasing rapidly (Askew, 2020a, 2020d; Galanakis, 2015, 2021; Zinoviadou et al., 2015). Moreover, products that are considered by consumers to boost their immune system (e.g., camomile, kombucha) have experienced a 3- to 4-fold increase in sales (Askew, 2020f). A recent survey of 23,000 European shoppers indicated that 72% of consumers would change their eating habits in the post-lockdown era to follow more healthy patterns (Fmcggurus, 2020). Following consumers' needs, food companies are commercializing products with bioactives, creating a trend towards seeking recognition of food bioactives as immune-boosting agents (Galanakis et al., 2020) (Daniells, 2020) that encompass forging more collaborations between governmental bodies and academic institutions to address this need (Koe, 2020a, 2020b).

On the other hand, the pandemic led to an instantaneous lack of critical information about consumers' preferences, attitudes, and bottlenecks in the post-lockdown period (Rowan & Laffey, 2020). The

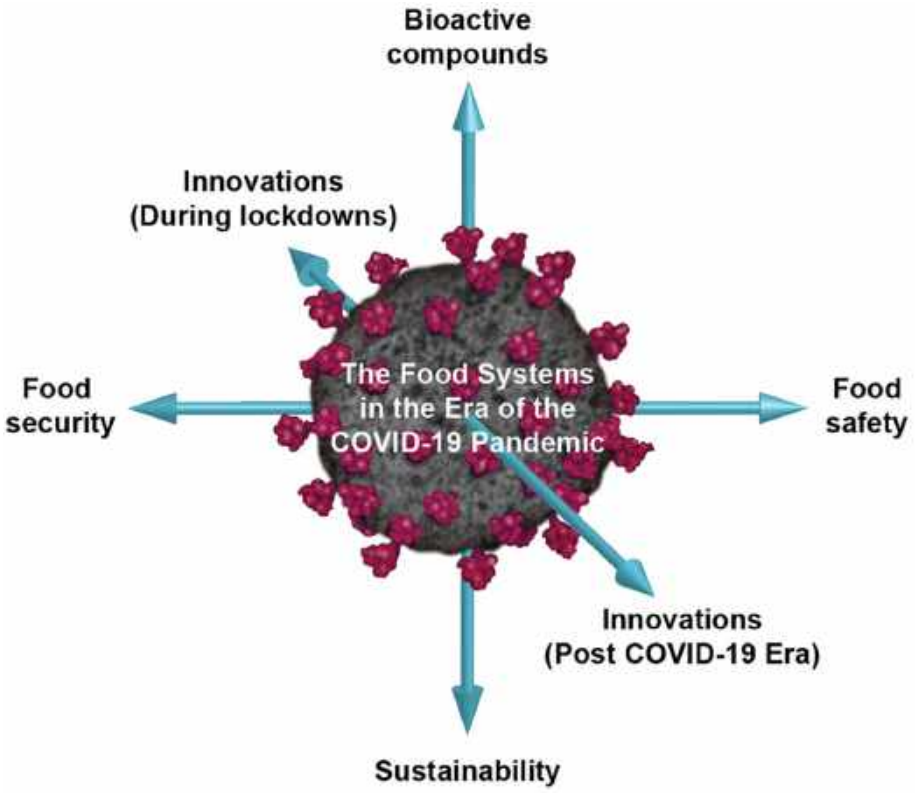

Fig. 1. The food systems in the era of the COVID-19 pandemic and the prospect of implementing innovations during lockdowns and post COVID-19 era.

induced disruptions in the food supply chain have increased the risk for food fraud, whereas finding convincing evidence about the real health benefits of nutraceuticals and functional foods became critical. This gap also highlighted the emerging role of digitization using data analytics and Artificial Intelligence (AI) to support the real-time needs (e.g., remote monitoring and management decision tools) of the food industry, smart agriculture, supply chain, and food security (Bacco et al., 2019). Moreover, the proliferation of new technologies helped digitize the food supply chain and increased traceability systems' investments to mitigate risk, improve efficiency, and underpin sustainability initiatives (Kennedy et al., 2020).

Sectors benefiting from the current challenges of COVID-19 include companies producing non-perishable foods and processed food companies. For example, panic buying during the lockdown has driven consumers to develop new habits and taste products that they have not purchased before, e.g., freeze-dry ready meals. Since consumers have tasted rehydrated products and compared them with fresh food, it is expected that the demand for this kind of product will grow over the next years (Askew, 2020b). Companies are also experiencing a rapid increase in the need for plant-based ready meals (Askew, 2020e) and alternative protein sources to meat and dairy products (Foodnavigator, 2019). Direct-to-consumer services have rapidly increased, whereas many traditional grocery brands try to develop an easy route to market and become online first brands (Askew, 2020f). The online food trade has witnessed an explosive increase in demand, whereas there is a warning for sustainable packaging solutions due to the rapidly increased need for packaged food. Although the measures needed to ensure food safety, security, and sustainability converge more than ever before, it is essential to avoid hazardous and illegal food products reach the market due to shortages, false claims, or other reasons (e.g., economic). For example, food recalls due to authenticity concerns are likely to be reduced (Everstine, 2020). Since the online order of nutraceuticals has rapidly increased, attention should be given to these products' adulteration and safety (Bullimore, 2020). The second wave of infection that has already been witnessed worldwide will create additional opportunities for innovators and services such as online retail and deliveries (Rowan & Laffey, 2020).

2. Innovations and disruptions in the new era

COVID-19 pandemic has accelerated innovation all around the world. This change has already been seen in places where the virus hit first (e.g., in China), where large companies such as Huawei have increased their expenses for research and development activities. There is also reasoning behind this trend as companies that invested in the 2008 economic crisis (instead of just cutting costs) grew fast during the recession (Davey, 2020). On the other hand, defining and forecasting what constitutes a disruptive technology is complicated as the impact is more likely to be measured from a retrospective downstream perspective. Since the 1990s, researchers have referred to disruptive technologies as whirlwind, ground-breaking, game-changing, earth-quake, and emergent technologies that typically cause a substantial disturbance in established market structure and prominent companies. This disturbance is generated by producing highly efficient products and services that are more competitively priced, less complicated, and more accessible than established innovations (Christensen, 1997; Christensen & Bower, 1996; Schuelke-Leech, 2018). Disruptive technologies can substantially cause localized change within a market or industry (e.g., first-order disruption) or cause ground-breaking changes across many cross-cutting domains (e.g., second-order disruption) over a period that substantially influences societal norms. The challenges for technological forecasters and investors are those disruptive technologies are by their nature nascent – meaning that they can only be proven as disruptive in hindsight based upon demonstrating evidence-based impact. The uncertainty arising from COVID-19 will shape future disruptive technologies that may emerge from entrepreneurs, start-ups, small and medium-sized enterprises (SMEs), and larger established companies. These companies are willing to integrate new solutions at providing smaller, lighter, more flexible, cheaper, and more convenient products (Rowan, 2019).

Fig. 2 illustrates the different innovations within the COVID-19 pandemic era and their application's field(s). The chord diagrams of Fig. 3 demonstrate the prospect of implementing the foreseen innovations in the food sector within the era of the COVID-19 pandemic and respective applications during lockdown periods and within the post-COVID-19 era. The chords start from the potential innovations during the lockdowns and end up in the post-COVID-19 era. The thicker the chords are, and the higher are the prospects of the new era's innovations. These innovations may be enhanced by adjacent industries and services dealing with digitization, using meteorological data linked with climate modeling (Ruiz-Salmón et al., 2020). Overall, it is challenging to predict the holistic consequences of this COVID-19 pandemic and at what time point we will emerge from it, and which innovations and technologies will disrupt the food sector.

2.1. Bioactive compounds

The pandemic generated opportunities and challenges for the commercialization of innovative functional foods and nutraceuticals containing target bioactive compounds (e.g., Vitamins and antioxidants) and highlighted the advances of personalized nutrition to boost consumers' immune system and improve their overall health (Galanakis et al., 2020). These prospects are expected to remain high within the post-lockdown and post-pandemic era due to the increased interest of health-conscious individuals. Subsequently, companies will seek new information and knowledge about consumer needs toward these products to address the pandemic's challenges. Nowadays, the development of these kinds of products are proposed with the simultaneous valorization of bioresources, e.g., the recovery of high added-value compounds from food waste (Galanakis, 2012, 2018; Galanakis et al., 2018; Nagarajan et al., 2019; Roselló-Soto, Barba, et al., 2015; Ruiz-Salmón et al., 2020; Wong et al., 2015), and the utilization of microalgae and plant foods (Ananey-Obiri et al., 2018; Bursać Kovačević et al., 2018;

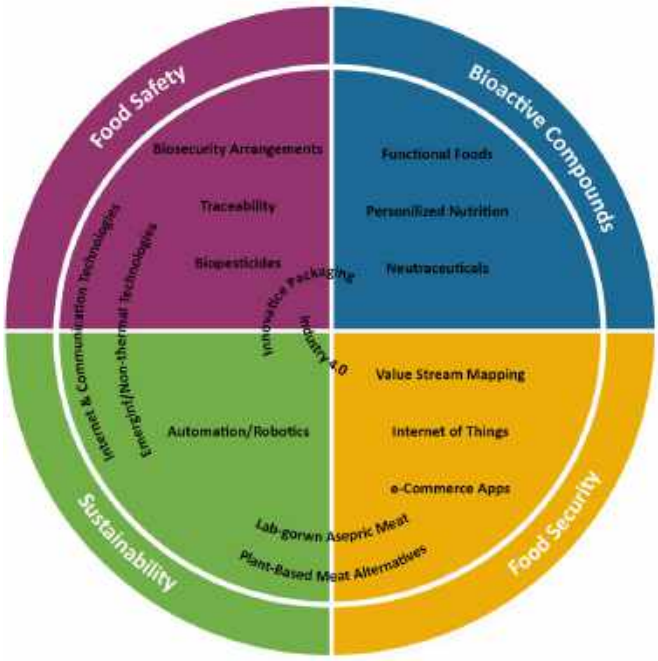

Fig. 2. Foreseen innovations and disruptive technologies to tackle challenges of the four directions affected by the COVID-19 pandemic: food safety, bioactive compounds, food security, and sustainability.

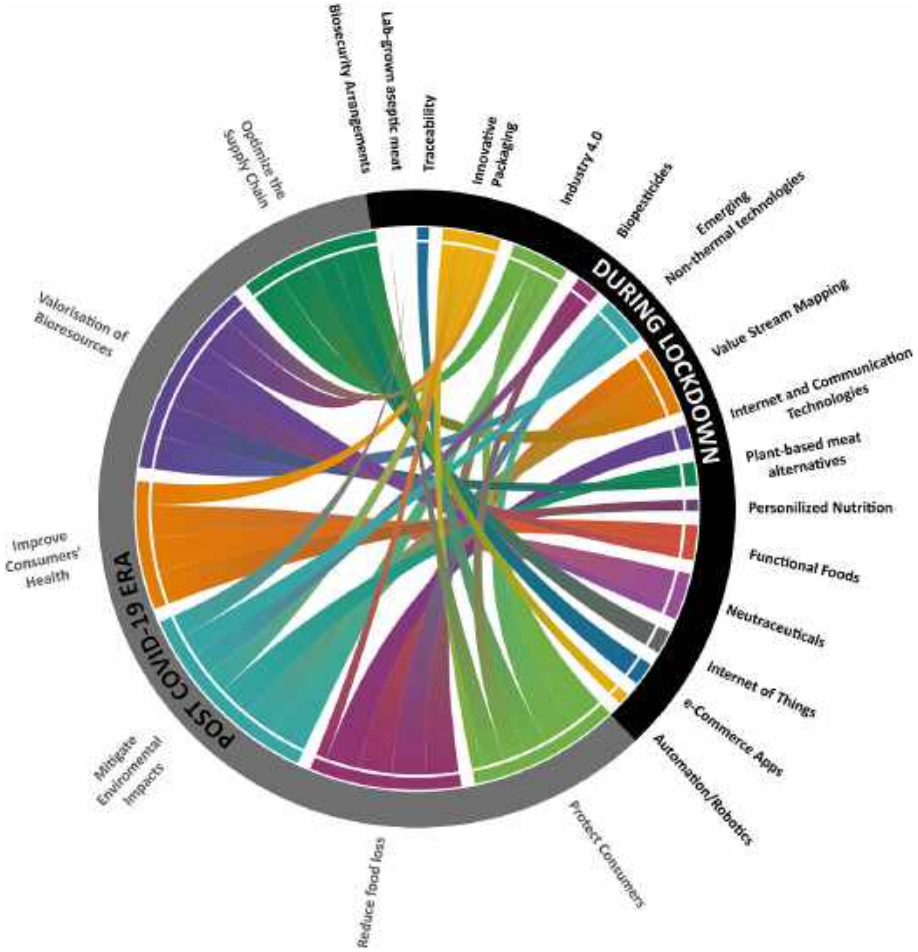

Fig. 3. The prospect of implementing the foreseen innovations in the food sector within the era of COVID-19 pandemic and respective applications during lockdown and post-COVID-19 periods.

Galanakis, 2013; Roselló-Soto, Galanakis, et al., 2015). Adjacent advances will also be made in immune-boosting animal feed products such as recently reported by Taufek and co-workers (Taufek et al., 2020), who described the performance of mycelial biomass and exopolysaccharide from Malaysian edible mushroom *Ganoderma lucidum* for the fungivore red hybrid Tilapia (Oreochromis sp.) in Zebrafish embryo. Carballo and co-workers (Carballo et al., 2019) also reported on the use of β-glucans from yeast combined with microalgae extracted digest to improve the health of gut microbiome of high-value fish that prevented bacterial infection.

2.2. Food safety

On the other hand, innovations such as smart and active packaging, advanced traceability systems (e.g., using blockchain technology), new biosecurity arrangements (e.g., promoting a food safety culture in food processing facilities and farms), the application of biopesticides to agriculture and industry 4.0 (e.g., blockchain technology) are expected to grow substantially in the new era. The ultimate goal is to protect consumers by ensuring the food and food supply chain's safety and reduce food loss and the environmental impact of the food sector. These innovations may lead to new business models that could disrupt the food supply chain and the market of food products in a techno-socioeconomic way. By achieving international consensus on datasets, priority should be given to reliable processing and critical information for clinical trials (Rowan, 2019) by promoting open access to findings.

For instance, the FDA is planning to release a relevant blueprint targeting the development of traceable food systems and safer, more digital, and more secure food supply (FDA, 2020). Technologies such as artificial intelligence, blockchain, the Internet of Things (IoT), and sensor technology would allow the direct tracking of foods and commodities from farm to fork. The combination of advanced traceability systems with modern analytical and smart tools (e.g., remote or virtual inspections, root cause analyses) would reduce the response in foodborne outbreaks by using data streams. The latest could make the supply chain more visible, reducing the time between tracking the contamination origin of food and responding with mitigating actions.

These kinds of technologies would also assist in imbalances caused by panic buying and spot shortages due to extreme events and help comprehend the causes of food contamination and interpret predictive analytics. The latest use data to predict the contamination possibility and ultimately reduce food loss and waste, e.g., when lockdowns temporarily disrupt the chain of producers and customers in public places (e.g., schools and restaurants). New biosecurity arrangements will help to promote a food safety culture in food processing facilities and farms. There is also a pressing need for education and social enterprise to support the community transitioning to emerging changes and embrace new approaches for food sustainability and security that will accelerate consumer acceptance of green innovation.

2.3. Sustainability

With the changing lifestyle and rapid urbanization of the global population that has been accelerated by behavior changes arising from COVID-19, there is an increased generation of food waste from various industrial, agricultural, and household sources (Sharma et al., 2020).

From price spikes and panic buying to the acceleration of food waste, sustainability, and other economic implications, the COVID-19 crisis (as a real exercise) has reminded us that the current food systems are fragile. Thus, they should become more resilient, ensuring food security in future crises such as new pandemics and extreme events due to climate change. Two overarching priorities are to ensure that producers and processors can continue to operate effectively and keep supply lines open and that most services can work as effectively as possible during the pandemic. These are on top of the pressing need to develop innovative means to increase food production to meet growing populations internationally informed by digital technologies. Priorities also include the likely consolidation of significant industries with secure packaging and capacity for research and innovation that will potentially flourish during and post COVID-19 era, when socio-economic norms have been reset and countries quickly deploy economic recovery plans.

The dominant linear economy system, which is mainly based on increasing production to address increased consumption, has proved inefficient for the sustainable management of our resources (Hetemäki et al., 2017; Stegmann et al., 2020). On the other hand, the transition of the current development model to a circular bioeconomy approach could enhance resilience by converting biomass into various biobased products (Farcas et al., 2020; Mak et al., 2020). Thus, a more exceptional drive for innovations in this direction will balance the food supply chain's impact on the environment with the emergence of less-energy intensive, eco-friendly processes, products, and services (O'Neill et al., 2019). For instance, the traditional approaches for managing food waste include land-filling and incineration that generate toxic gases, causing severe environmental and human health hazards. The circular bioeconomy provides opportunities to valorize food waste utilizing biorefineries that produce biofuels, electrical energy, biosurfactants, biofertilizers and so forth (Mordorinelligence, 2020; O'Neill et al., 2019; Rahmanian et al., 2014). The use of biobased packaging materials and non-thermal disinfection technologies for packaging, such as pulsed light (Rowan, 2019), are two examples that could accelerate green innovation in the new era. Biobased materials have been developed to mitigate the complications instigated by conventional plastics. The bioplastic packaging market has been driven by the increasing awareness of traditional plastics' adverse effects, which has steered both consumers and regulatory bodies to opt for biobased materials in place of conventional plastic. The bioplastics packaging market was valued at USD 14.85 billion in 2019 and is expected to reach USD 39.37 billion by 2025 (Mordorinelligence, 2020).

Seafood and aquaculture sectors of Europe, are encounter also significant challenges concerning environmental threats (climate change, marine debris, resource depletion), social development (worker rights, consumer's awareness), or economic growth (market and nonmarket goods and services, global competitiveness). These issues are pressuring all stakeholders, from policy-makers to citizens and industries, to adopt more sustainable policies, practices, and processes. For example, O'Neill (O'Neill et al., 2020) reported Trout and Perch's organic fish farm production on the Irish peatlands using an aquaculture recirculation system (powered with wind energy) where water quality and waste remediation are controlled naturally by using indigenous microalgae and bacteria. Moreover, collaborations among different parties and beyond borders should be improved, aiming to create more efficient networks along the seafood and aquaculture sectors' supply chain. To achieve this, a "nexus thinking" approach (i.e., the analysis of actions in connected systems) combined with a life cycle thinking appears like an excellent opportunity to facilitate the transition to a circular bioeconomy. The emergence of centers of research excellence linked to enterprise and education with a global orientation that seeks to exploit added value to products derived from food waste will increasingly come to the fore. Future research would consider the impact of climate change on food supply chains, including circular bioeconomy, as recently demonstrated by O'Neill et al. (O'Neill et al., 2019), who showed the use of naturally occurring biological indicators to assess the environmental impact of sustainable periods of drought on Irish freshwater aquaculture.

Besides, aquaculture is an emerging high-protein low carbon emission process that is of interest globally for intensive food sustainability (Tahar et al., 2018a, 2018b). The generated opportunities will be met in part by advances in the digitization of food technologies, innovation in manufacturing (e.g., emerging non-thermal technologies), and services for a diversity of markets and commensurate sustaining disruptive innovation in the adjacent manufacturing and materials sectors. Although most innovations (e.g., lab-grown aseptic meat, plant-based meat alternatives, biobased packaging, automation of food production, and robotics) targeting the sustainability of the food sector are today in a nascent development stage, they are expected to disrupt the food industry in the years to come (Galanakis, 2020). Also, exploiting advanced manufacturing (such as digitization, big data, ICT, blockchain, artificial intelligence, non-thermal technologies, robotics, augment and virtual reality, and 3D food printing) will address sustainability and security qualitative standards, and traceability along the entire food supply chain.

2.4. Food security

Disruptive innovation in digitization is transforming the pace and scale of the food and drinks industry globally, ensuring food safety and increasing food sustainability and food security applications. For instance, advanced digital solutions (e.g., IoT, blockchain in the supply chain) to ensure 24/7 order taking are expected to grow, whereas companies will have to promote their values and brand and their quality commitments (Askew, 2020c). The adaptation to new business models that allow the modernization of retail would also contribute to this direction. These models include, e.g., IoT and ICT (e.g., online delivery for supply chain), or e-commerce that utilizes mobile apps for shopping purposes, helping smallholders and producers to find different customers in small city centers (Askew, 2020f) (FDA, 2020; Galanakis, 2020). To this line, Naughton et al. (S. Naughton et al., 2020) demonstrated the need for real-time digitization and ICT in aquaculture to connect complex laboratory data analysis with in-field physicochemical measurements. These tools should determine optimum feeding rates at a fish farm or conditions' adjustments for disease mitigation.

The application value stream mapping is also expected to grow fast in the new era. This digital tool allows the proper management of the supply chain from agricultural production to processing, retail, and consumption. Likewise, it reduces food loss and mitigates environmental impacts (Wesana et al., 2019). Use of potential disruptive non-thermal technologies, such as pulsed light, for food packaging disinfection that also has potential for second-order disruption in areas of waste effluent treatment and critical ecosystem service management such as treatment of pollen frequently contaminated complex parasites and viruses for farmed bumblebees used in buzz pollination of soft fruits and crops across Europe (Garvey & Rowan, 2019; I. M. T. Murray et al., 2018; J. Naughton et al., 2017; Rowan, 2019). Disruptive technologies for promoting pollination security can be extended to commercial electron beam or x-ray treatments for large scale throughput, where the benefits of these emerging solutions have been comprehensively reviewed by (McEvoy & Rowan, 2019). MacFadden and co-workers (Mcfadden et al., 2016) also reported comparing the efficacy of using commercial electron beam and pulsed light for novel sterilization of Irish retailed infant milk formulae to further enhance safety and security from a non-thermal processing perspective. Traditional and emerging technologies were also comprehensively reviewed to help future-proof food systems contaminated with complex foodborne parasites (Franssen et al., 2019; Gérard et al., 2019; Herrero et al., 2020).

There is also a growing potential to exploit immersive Industry 4.0 advances (e.g., virtual and augmented reality) to advance remote workforce training that can embrace the need for social distance to prevent transmission of COVID-19 (N. Murray et al., 2019). Training and

competence development remains mostly grounded in traditional practice methods against a backdrop of highly innovative processes and technology advancements (N. Murray et al., 2019; Rowan, 2019). Likewise, the provision of specialist training in the agri-food workforce merits innovative approaches using technology as a tool in competence development. In the context of specialist training and educational programs, Immersive Multimedia (I.M.) technologies such as virtual reality are emerging as potential platforms based on their delivery of 360° visuals, spatial audio, and allowing the learner to move beyond the passive mode towards an active participant in their learning experience (Braga Rodrigues et al., 2020). In conjunction with wearable sensor technologies (capturing different user physiological metrics), task performance, and user interaction, these technologies facilitate a right "human--in-the-loop" system that supports adaptive, personalized while maintaining context-based learning. Combined educational (such as Cognitive Load Theory) and immersive training capture, at the individual level, critical abilities of the learner, which then informs how the presentation system challenges the learner, thus optimizing the learner experience (Braga Rodrigues et al., 2020; N.; Murray et al., 2019).

3. Conclusion

The COVID-19 pandemic has led to historical changes in our society's norms and the way people interact. It also showed direct and high impacts on the food sector, affecting mainly bioactive compounds, food safety, food security, and sustainability. The lockdown of billions of people during the last winter and spring and the lockdown waves that are expected to come in the next months/years led to different innovations in the food sector. Among them, Industry 4.0 applications (IoT, ICT, and blockchain technology) and innovations that disrupt the way we consider and consume food (e.g., lab-grown meat, plant-based alternatives of meat, and valorization of a vast range of bioresources) are the ones with the highest potential in the new era. Niche applications such as the development of nutritional and immune-boosting products to support the health and recovery of COVID-19 patients will become popular. There is also a trend towards intensive sustainable food production systems (such as digitization, AI, and automation in smart agriculture) with future-proofing for the potential impact of security risks and climate change through the supply chain to mitigate critical needs embrace opportunities. Education and training for the next generation of workforce in emerging technologies and accelerating initiatives that will foster behavior change of consumers to the merits of these new business services, technologies, and products will be becoming increasingly important. Globally, there will be a pressing focus on food security regionally and nationally to mitigate against challenges presented by the potential occurrence of future viral pandemics such as that caused by SARS-CoV-2 to protect vulnerable critical supply chains.

Acknowledgenments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Environmental Science and Pollution Research (2023) 30:70771–70782
https://doi.org/10.1007/s11356-023-27286-6

RESEARCH ARTICLE

Pulsed ultraviolet (PUV) disinfection of artificially contaminated seawater seeded with high levels of pathogen disease indicators as an alternative for the shellfish industry depuration systems

Gustavo Waltzer Fehrenbach[1] · Emma Murphy[1,2] · Robert Pogue[3,4] · Frank Carter[5] · Eoghan Clifford[6,7] · Ian Major[1] · Neil Rowan[4]

Received: 11 January 2023 / Accepted: 24 April 2023 / Published online: 8 May 2023

Abstract
The increase in pathogen levels in seawater threatens the safety of entire aquatic ecosystems. Foodborne pathogens can potentially accumulate in shellfish, especially in filter feeders such as bivalves, requiring an efficient depuration process before consumption. Alternative approaches to promote a cost-efficient purge at depuration plants are urgently needed. A small prototype pulsed ultraviolet (PUV) light recirculation system was designed, and its depuration potential was tested in a seawater matrix artificially contaminated with high levels of microbial pathogens *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus* and *Candida albicans*. The analysis of treatment parameters including voltage, number of pulses and duration of treatment was performed to ensure the highest reduction in contaminant levels. Optimal PUV disinfection was attained at 60 pulses/min at 1 kV for 10 min (a UV output of 12.9 J/cm^2). All reductions were statistically significant, and the greatest was observed for *S. aureus* (5.63 $\log_{10}$), followed by *C. albicans* (5.15 $\log_{10}$), *S. typhimurium* (5 $\log_{10}$), *B. cereus* (4.59 $\log_{10}$) and *E. coli* (4.55 $\log_{10}$). PUV treatment disrupted the pathogen DNA with the result that *S. aureus*, *C. albicans* and *S. typhimurium* were not detectable by PCR. Regulations were reviewed to address the applicability of PUV treatment as a promising alternative to assist in the reduction of microbial pathogens at depuration plants due to its high efficiency, short treatment period, high UV dose and recirculation system as currently employed in shellfish depuration plants.

Keywords Disinfection · Foodborne pathogens · Seafood · Depuration · Ultraviolet

Introduction

Seafood is an important food source and includes finfish, crustaceans, echinoderms and molluscs obtained from fresh and saltwater. Seafood represents approximately 7% of the global food market and is projected to reach a value of $336

Responsible Editor: Sami Rtimi

✉ Gustavo Waltzer Fehrenbach
gfehrenbach@ait.ie

[1] Materials Research Institute, Technological University of the Shannon – Midlands Campus, N37 HD68, Athlone, Ireland

[2] LIFE – Health and Biosciences Research Institute, Technological University of the Shannon – Midwest Campus, V94 EC5T, Limerick, Ireland

[3] Post-Graduate Program in Genomic Sciences and Biotechnology, Catholic University of Brasilia, 71966-700, Brasilia, Brazil

[4] Center for Sustainable Disinfection and Sterilization, Bioscience Research Institute, Technological University of the Shannon – Midlands Campus, N37 F6D7, Athlone, Ireland

[5] Coney Island Shellfish Ltd., F91 YH56, Sligo, Ireland

[6] School of Engineering, National University of Ireland Galway, H91 HX31, Galway, Ireland

[7] Ryan Institute, National University of Ireland Galway, H91 HX31, Galway, Ireland

billion US dollars by 2025 (Shahbandeh, 2021). Global seafood consumption has increased at an average rate of 3% a year from 1961 to 2019, almost twice the annual world population growth for the same period (FAO, 2022). Consumption per capita grew from 9 kg in 1961 to 20.2 kg in 2020, and the seafood production chain evolved to meet the increased demand. One of the main changes was the rapid development of aquaculture systems with the use of technology such as automated feeding process and traceability of development to maximize yields and increase sustainability, expanding production from 19 million tonnes in 1950 to 179 million tonnes in 2018 (FAO, 2016). Shellfish is a seafood widely cultured in sea-based systems, relying on suitable water and environmental conditions. Bivalves are the most abundantly produced shellfish in Europe and will be referred to in this research. As a result of their filter-feeding mechanism, bivalves are susceptible to water impurities, which may lead to the accumulation of high levels of microbial pathogens (Martinez-Albores et al. 2020). Its filter physiology will also purge pathogens if placed in uncontaminated water and is often realized in depuration plants to purge it to levels within safe limits (EC, 2019).

After harvesting, the live bivalve is washed/cleaned in a processing plant to remove mud and debris, and graded by weight, a process whereby the underweight bivalve is returned to cages in water until achieving the commercial weight. The product is then transported to a depuration facility where it will remain for the depuration period. Seawater is mainly treated on recirculation and flow-through systems in large tanks of usually 1–3 m^3 and often pre-treated before usage due to the presence of contaminants. The type of depuration system deployed depends on factors such as availability and the average weight of the bivalve treated. Water quality is assessed by the presence of contaminants, phytoplankton concentration and parameters such as temperature, dissolved oxygen, turbidity and salinity. Re-circulation systems rely on water consistency since they can recirculate the same water for at least 24 h (Schneider et al. 2009). Such systems (Fig. 1a) usually consist of a pump that pumps water from the tank, recirculates it through a UV treatment and sprays it on the bivalve seated on trays. Chlorine, iodophors and ozone are also used in the depuration process to deactivate pathogens such as *E. coli* and NOV released from the bivalve and in the pre-treatment of seawater. In flow-through systems (Fig. 1b), seawater is continuously pumped through the depuration system and will discharge fractions of the water and replenish it during the depuration process. A clean and reliable source of seawater is necessary to provide water free of contaminants, and UV or membrane filtration can enhance seawater quality prior to pumping into the depuration system.

The disuse of chlorine treatment is due to several effects on bivalves such as shutting down the filter-feeding activity on oysters and carcinogenic potential of chlorinated metabolites, requiring a post-treatment with vigorous agitation and degassing with thiosulphate (Schneider et al. 2009; Martinez-Albores et al. 2020). Iodophors are substances whereby their bactericidal activity is based on elemental iodine, which penetrate the cell walls and membranes interfering with DNA synthesis. Their efficiency and safety for bivalve's depuration system have not been widely researched. Ozone is a strong oxidizing agent that inactivates contaminants by attacking the double bonds of organic compounds, supporting its use on lipophilic toxins. It has an increased operation cost and can generate cancerous co-products such as bromates. Of the available techniques, UV disinfection is distinct due to significant reductions in the viability of waterborne pathogens without leaving residuals and is the most widely used method in the UK, the USA and Australia (McLeod et al. 2017) and partially used in China where ozone is also applied (Lee et al. 2008).

The treatment efficacy is due to severe damage to cell structure from the activation of the photoreactive potential of purines and pyrimidines in DNA that triggers the

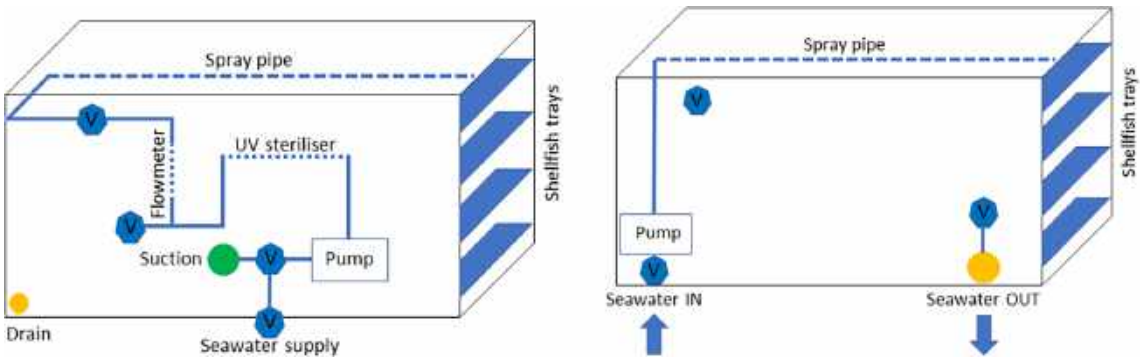

Fig. 1 Shellfish depuration system. **a** Recirculation system. **b** Flow-through. V: valves

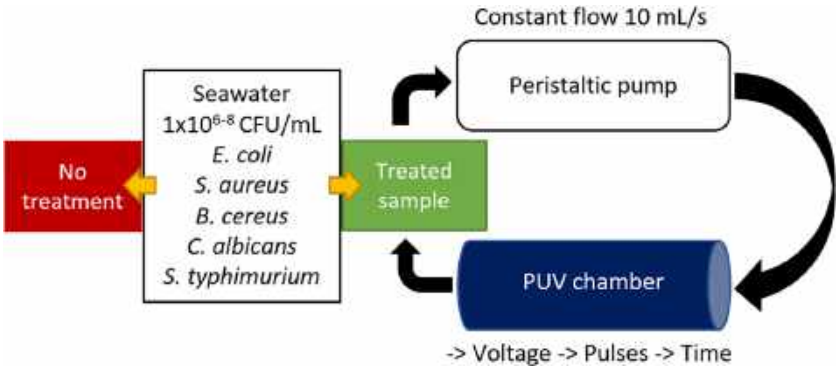

Fig. 2 PUV treatment, food-borne pathogens and main parameters associated with the treatment

formation of mutations, oxidative stress and production of reactive oxygen species (ROS). These ROS attack nucleotide pools and stress-mediated mutagenesis (Ikehata and Ono, 2011). The current UV systems operate continuously and inactivate pathogens in recirculated water from depuration tanks. They are typically low-pressure mercury UV bulbs working mostly monochromatically from 100 to 400 nm and approximately 40 W. Treatment efficacy results from severe damage to a pathogen's cell structure. The efficacy of the method may also be reduced when enteric viruses (such as NOV) are present, because higher UV doses and extended retention times are required to reach safety levels. In this case, however, the use of high-power systems with wider wavelengths and UV doses can overcome these challenges, reducing the average time a certain amount of water stays in the depuration plant (hydraulic retention time) and ensuring food safety.

Pulsed ultraviolet (PUV) light treatment is based on high peak power pulses of > 1 kW/cm^2 generated by xenon lamps and delivered at short intervals (100 ns to 2 ms) within a broad wavelength spectrum ranging from 100 to 1100 nm. Its disinfection efficacy was reported for different pathogens and areas such as the packaging industry (Garvey and Rowan, 2015; Chen et al. 2015), surfaces in an animal laboratory (Li et al. 2020), artificially contaminated wastewater (Fitzhenry et al. 2021), different types of milk (Ansari et al. 2019) and cheese (Ricciardi et al. 2020). PUV is a promising alternative for the food industry as there is no by-product identified for PUV treatment (Rowan, 2019), and its application in food products is authorized by the United States Food and Drug Administration (FDA, 1999). Its high power and UV dose can potentially inactivate pathogens in the shellfish depuration system.

In Europe, the level of contaminants is constantly monitored in designated shellfish production areas which are classified as A, B and C. Bivalve molluscs from class A shall not exceed 230 *Escherichia coli* per 100 g of flesh and intravalvular liquid in 80% of samples collected, considered safe to consume if no other health risk is present. Class B is designated for areas where *E. coli* abundance does not exceed 4600 in 90% of the samples per 100 g of flesh and intravalvular liquid and 46,000 per 100 g of flesh and intravalvular liquid for class C. *E. coli* is still the main parameter for monitoring as most of the shellfish contamination arises from human and animal faeces, which suggests the presence of other contaminants also present in faeces but in lower concentrations. When harvested from class B and C areas, shellfish must be purged in depuration plants. Other contaminants such as paralytic shellfish poison (PSP) and norovirus (NOV) are also periodically monitored, and regulations follow the basic principles of food law to protect human health and consumer interests as set by Regulation (EC) No. 178/2002 (EC, 2002). The Centre for Environment, Fisheries and Aquaculture Science (CEFAS, 2022) recommends a minimum of 42 h to depurate the shellfish from class A and B harvesting areas. Then, it can be commercialized if it is within the limits established for the following parameters: organoleptic characteristics (1), biotoxins (2), *E. coli* (3), *S. typhimurium* (4), norovirus (5) and hepatitis A (6). The first parameter requires an absence of dirt on shells, a normal amount of intravalvular liquid and adequate response to percussion. Biotoxins must be within limits, such as paralytic shellfish poison (< 800 µg/kg), amnesic shellfish poison (< 20 mg of domoic acid/kg), yessotoxins (1 mg/kg) and azaspiracods (160 µg/kg). *Salmonella* should not be detected in 25 g of bivalve tissue (EC, 2004). Hepatitis A and norovirus (NOV) are highly contagious, and there is no consensus

Table 1 PUV preliminary experiments to identify the best treatment conditions

Treatment	Voltage (V)	Number of pulses per min	Duration (min)
1	500	6	1
2	1000	6	1
3	500	6	10
4	1000	6	10
5	500	60	10
6	1000	60	10

Table 2 Operating parameters of PUV lamp at 500 and 1000 V

Discharge voltage (V)	Energy per pulse (J)	Peak current (A)	Peak power (kW)	Peak admittance (S)	Current rise/fall time (µs)
500	5	443	175	1.10	12/36
1000	20	1173	985	1.46	7/27

or regulations for limits of RNA copies in shellfish which are monitored and controlled in a pro-active way to preserve consumer safety (FSAI et al. 2018).

In this paper, we describe and report the test results from a small prototype pulsed ultraviolet recirculation system for purification of seawater artificially contaminated with high levels of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Candida albicans* and *Salmonella typhimurium*. For example, *Escherichia coli* and *Salmonella* species were specifically chosen because they are used as index pathogens of faecal contamination for water quality assessment (Holcomb and Stewart, 2020). *Candida albicans* was chosen because it is representative of a clinical yeast occurring in wastewater (Babič et al. 2017). *Staphylococcus aureus* was chosen because it is associated with foodborne intoxications (FSAI, 2011). These organisms are also representative of Gram-positive and Gram-negative pathogens, which allows for verifying the effectiveness of PUV in different cellular structures. The proposed treatment described here was developed to be further tested in and ultimately employed by the shellfish industry at depuration plants to assist in the disinfection from common foodborne pathogens.

Material and methods

Pulsed-ultraviolet treatment

A small pulsed ultraviolet (PUV) light reactor was built to be further tested in the depuration process of the shellfish industry. The 60-kPa low-pressure xenon-filled flash lamp (Heraeus Noblelight XAP type NL4006) was fixed in stainless steel chamber of 100 mL volume internally walled with glass. The system was powered by a 1000-V scale power source (Samtech Pulsed UV 2018) equipped with an automatic trigger system. Purification was performed in a recirculation regime, and a schematic is presented in Fig. 2. A peristaltic pump (ColeParmer) was employed to recirculate 200 mL of contaminated seawater of salinity of 35 ppt and a temperature of 18 °C at a constant flowrate of 10 mL/s with hydraulic retention time (HRT) of 0.10 s. Using the same inoculum, no treatment (Fig. 2) was performed but not exposed to PUV, and each pathogen was recirculated through the system under the same conditions. The efficacy of PUV treatment was then compared to no treatment.

PUV treatment conditions were selected based on log reduction after treatment at different voltage intensities (V), number of pulses per min and time (min) as presented in Table 1.

Energy, peak current, peak power, peak admittance and current rise/fall time at 500 and 1000 V are presented in Table 2. Values are available in the PUV manufacturer's manual. Energy (J) corresponds to release per pulse in the flash lamp. Peak current (A) and power (kW) are the maximum current and power at the peak of pulsed energy, respectively, while peak admittance (S) is a measure of how easily the current will flow through the system and reach the lamp. Current rise/fall corresponds to time (µs) until it reaches the peak current and returns to 0.

UV output was determined by the product of UV irradiance (mJ/cm^2), therefore a wavelength < 300 nm, and exposure time (s). The calculations are presented below.

1) Energy output (Eo) was determined by the product of energy per pulse (J or W/s) and pulses per second (0.1 and 1).
2) Energy intensity (Ea) was determined by the division of Eo by the lamp surface area (170 cm^2 - height: 26 cm and radius: 1 cm).
3) Considering the ratio of energy generated at the UV range (< 300 nm) as 0.18 (Appendix 1), the product of Ea by 0.18 resulted in the energy intensity in the UV region (EaUV).
4) Finally, UV output (J/cm^2) after complete treatment was calculated by the product of exposure time (s) and EaUV (Table 3).

Table 3 UV output (J/cm^2) at 500 and 1000 V when applying 0.1 and 1 pulses per second for 1 and 10 min

Discharge voltage (V)	Energy per pulse (J)	Pulses per second	Eo (W)	Ea (mW/cm^2)	EaUV (mW/cm^2)	Exposure time (s)	UV output (J/cm^2)
500	5	0.1	0.5	2.941	0.529	60	0.0324
		0.1	0.5	2.941	0.529	600	0.324
		1	5	29.41	5.29	600	3.24
1000	20	0.1	2	11.76	2.11	60	0.129
		0.1	2	11.76	2.11	600	1.29
		1	20	117.6	21.1	600	12.9

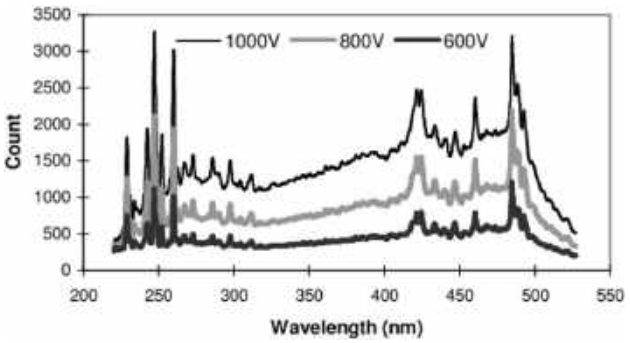

Fig. 3 Wavelength spectrum from emitted PUV flash (Samtech Pulsed UV 2018)

The spectrum emitted from the flash lamp is presented in Fig. 3. The voltages of 500 and 1000 V have similar spectra, with high output in the UVC region (220–80 nm) and visible light (430, 460 and 500 nm).

Foodborne pathogens and culture conditions

Bacterial species detected in shellfish and yeast associated with foodborne illness were selected to simulate a severe contamination of seawater. Contaminant levels in the seafood industry are much lower than the levels tested here; however, this approach was chosen to test the robustness of the PUV method. *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC29213), *Bacillus cereus* (ATCC11778), *Candida albicans* (ATCC10231) and *Salmonella typhimurium* (IMD121) were selected and cultivated at under specific conditions and with specific media. *E. coli and S. aureus* were cultivated in Luria Bertani (10 g/L peptone, 5 g/L yeast extract and 5 g/L chloride), while *B. cereus*, *C. albicans* and *S. typhimurium* in Brain Heart Infusion (200 g/L calf brain, 250 g/L beef heart, 10 g/L proteose peptone, 5 g/L sodium chloride, 2.5 g/L sodium phosphate, 2 g/L dextrose), Potato Dextrose Broth (200 g/L potato infusion, 20 g/L dextrose) and Tryptic Soy Broth (17 g/L peptone from casein, 3 g/L peptone from soymeal, 2.5 g/L D(+) glucose monohydrate, 5 g/L sodium chloride, 2.5 g/L di-potassium hydrogen phosphate), respectively. Flasks containing media and respective strains were then cultivated in a shaker at 28 °C (*C. albicans*) and 37 °C (*E. coli*, *S. aureus*, *B. cereus*, *S. typhimurium*) to exponential phase and used as inoculum. Seawater was collected at Sligo Bay (54.3432° N, 8.5728° W) and autoclaved for 15 min at 121 °C to be used as a treatment matrix. A salinity of 35 ppt was observed, and pH was 7.6. The spiking was performed at room temperature (18 °C), and strains were treated in separate reactions at 10^{6-8} CFU/mL. Experiments were run as presented in Fig. 2. The PUV system was washed and sanitized with isopropanol 70° several times to ensure complete cleaning. The average initial concentration of pathogens in preliminary assays was $1.8 \times 10^8 \pm 1.68$ CFU/mL (Fig. 4a–e) and $2 \times 10^9 \pm 2.31$ CFU/mL (Fig. 4f) when re-testing treatment 6 conditions.

Cell viability

A spread plate method was used to count the number of viable cells of no treatment (NT) and treated (T) with PUV. Samples were collected, serially diluted, spread onto agar plates and incubated at 28 °C (*C. albicans*) and 37 °C (other pathogens) for 36 and 24 h, respectively. Cell viability reduction (CVR) was calculated as presented in Eq. 1.

$$\mathrm{CVR} = \log_{10} \frac{\mathrm{NT}}{\mathrm{T}}$$

where NT is no treatment, and T is treated

DNA extraction

Bacterial genomic DNA was extracted as described on the GenElute kit (Sigma-Aldrich) with modifications. Samples of 2 mL were collected before and after PUV treatment and non-treated used as a positive control. Samples were centrifuged at 13,000 rpm for 2 min, and pellets of *B. cereus*, *S. aureus* and *C. albicans* were pre-treated with 200 µL of lysozyme for 30 min at 37 °C, while *E. coli* and *S. typhimurium* incubated with 20 µL of proteinase K for 30 min at 55 °C. To assist with DNA extraction, *C. albicans* was sonicated (35 W/40 °C) for 20 min prior to incubation with lysozyme. Proteinase K was then added to Gram-positive species, and 200 µL of lysis solution C was added to all strains and incubated for 10 min at 55 °C. The subsequent steps of column preparation, binding and washing steps were performed as described in the kit. DNA retained in the column was eluted with elution solution (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) and stored at − 20 °C.

Amplification conditions and gel electrophoresis

Polymerase chain reaction (PCR) was performed to verify the integrity and viability of pathogens after PUV treatment. The extracted DNA from *E. coli*, *S. aureus*, *B. cereus*, *C. albicans* and *S. typhimurium* were thawed, and 1 ng was mixed with the master mix according to Table 4. Bacterial 16S rDNA was amplified with the primers Pro341F and Pro805R (Table 5), with an expected product of 464 bp (Takahashi et al. 2014). For *C. albicans*, 18S rDNA primers SS5F and SS3R were used, and a product of 1800 bp was expected from amplification (Matsumoto et al. 2010).

PCR products were loaded with agarose 1X onto 1.5% agarose gel stained with SyBR green. Amplicons were

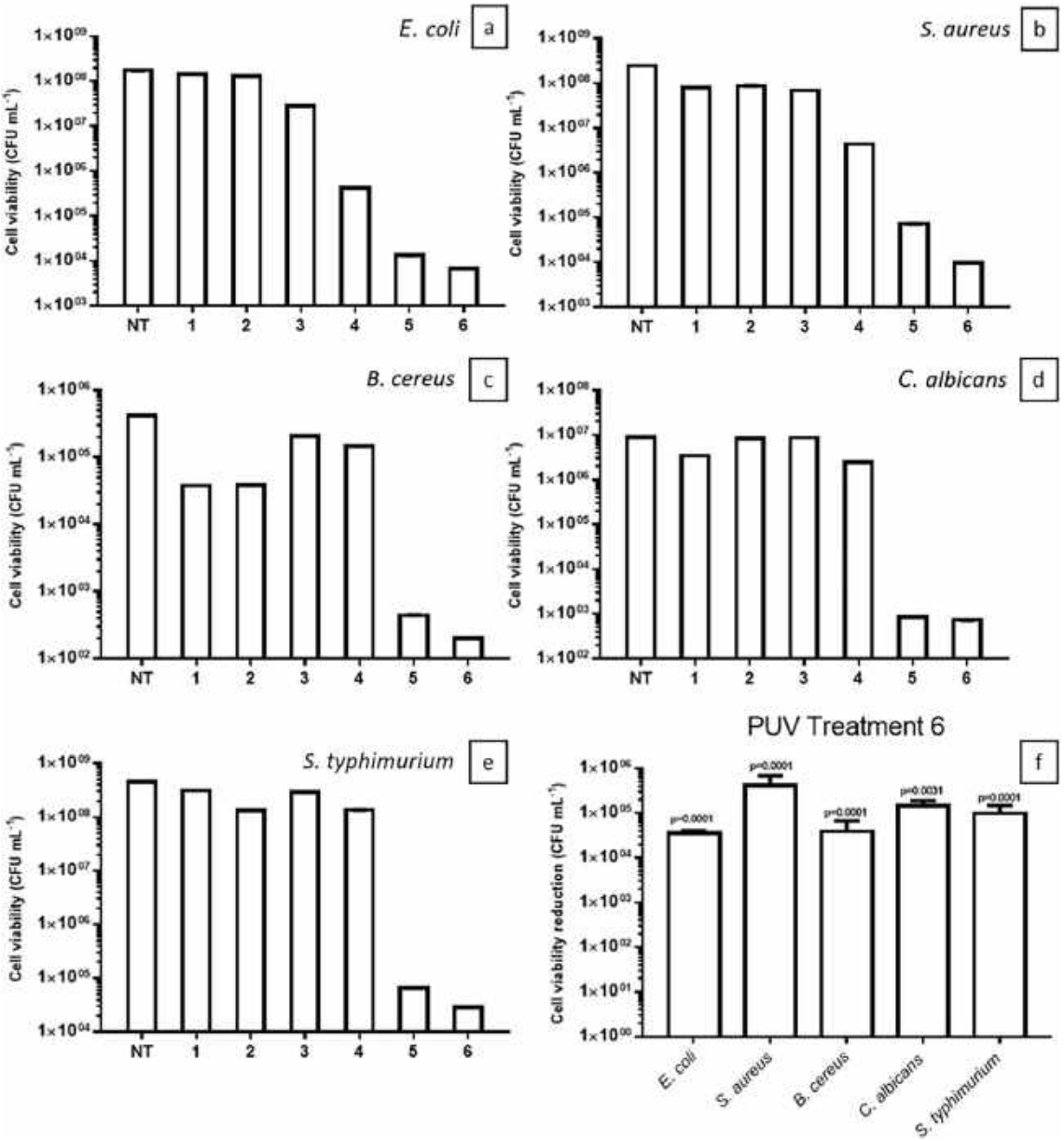

Fig. 4 Cell viability (CFU/mL) of *Escherichia coli* (**a**), *Staphylococcus aureus* (**b**), *Bacillus cereus* (**c**), *Candida albicans* (**d**) and *Salmonella typhimurium* (**e**) not treated (NT) and after PUV treatment at different conditions (**f**). CVR of pathogens under optimal conditions identified on repeated treatment 6 (1000 V, 60 pulses/min and 10-min treatment). Standard deviation bars are presented in ± CFU/mL. Statistical significance ($p < 0.05$) was observed in all pathogens treated with PUV at optimal conditions when comparing viabilities after treatment with no treatment levels

Table 4 PCR reagents and cycling conditions

	Molarity	Cycling conditions	Molarity	Cycling conditions
5X Platinum Buffer	1X	94 °C – 2’ *94 °C – 15” *60 °C – 15” *68 °C – 15” *25 cycles	1X	95 °C – 1’ *95 °C – 15” *55 °C – 15” *72 °C – 30” 72 °C – 7’ *25 cycles
2 mM dNTP Mix	0.2 µM		0.2 µM	
Forward primer	0.2 µM		0.265	
Reverse primer	0.2 µM		0.265	
Taq polymerase	0.04 U/µL		0.0275	
Water	-		-	
Genomic DNA	1 ng		1 ng	

Table 5 Primer sequence and expected product in base pairs (bp)

Primers	Sequence	Product (bp)
Pro341F	AATGATACGGCGACCACCGAGATC TACACTCTTTCCCTACACGACGCT CTTCCGATCTCCTACGGGAGGCAG CAGCCTACGGGNBGCASCAG	464
Pro805R	CAAGCAGAAGACGGCATACGAGAT NNNNNNGTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTGACTACN-VGGGTATCTAATCC	
SS5F	GGTGATCCTGCCAGTAGTCATATG CTTG	1800
SS3R	GATCCTTCCGCAGGTTCACCTACG GAAACC	

visualized in a UV chamber, and band pixels were quantified on the software ImageJ (Rasband, 2018) to estimate the cDNA concentration.

Statistical analysis

Results were analysed on the *Statistica* software version 10 (Statsoft, USA) by Student's *t*-test comparing means, and $p < 0.05$ was considered significant. All experiments were done at least three times in triplicates.

Results and discussion

The objective of this paper was to construct and test a small prototype pulsed ultraviolet (PUV) light system to demonstrate how this method might assist the shellfish industry in the depuration processes. The analysis of treatment parameters such as voltage, number of pulses and duration of treatment was performed to ensure a high reduction in contaminant levels. The recirculation system was based on current depuration plants in the shellfish industry where animals like oysters, for example, are kept in recirculating water treated by stationary UV systems to reduce the level of the pathogens purged from the oysters. The initial concentration of pathogens in treatment tanks ranged from 10^5 to 10^8 CFU/mL, simulating a highly contaminated environment. These levels are not usually found in seawater; however, the parameters were chosen to test the robustness and efficacy of PUV treatment in critical conditions, for example, a high level of bacteria can interfere with UV penetration depth. The preliminary tests to identify the voltage, number of pulses and duration of treatment were done to ensure high reduction and avoid excessive wear and tear of the PUV system.

The results from PUV treatment are presented in Fig. 4. Treatments 1 and 2, at 500 and 1000 V for 1 min, respectively, were the shortest and had fewer pulses (Table 1), resulting in microbe cell viability reductions ranging from 0.08 $\log_{10}$ for *E. coli* to 1.04 $\log_{10}$ for *B. cereus* (Fig. 4c) compared to the level in not treated (NT). The cell viability reduction (CVR) was not statistically significant between treatments 1 and 2 (Fig. 4a–e), showing the need for a prolonged treatment for improvement. Treatment 3 was prolonged to 10 min and the PUV operated at 500 V; however, reductions remained between 0 $\log_{10}$ for *B. cereus* and 0.79 $\log_{10}$ for *E. coli* compared to NT. A major increase in lethality was only observed when 1000 V pulses were applied for 10 min on treatment 4 (Fig. 4a), reducing the level of *E. coli* by 2.62 $\log_{10}$. The relation between the number of pulses, duration and voltage was also observed for treatments 5 and 6 with a total of 60 pulses per minute applied in each treatment. Considering the results of all pathogens, lower CVR reductions from 2.97 $\log_{10}$ to 4.12 $\log_{10}$ were obtained by applying 60 pulses per minute and 500 V when compared to the PUV unit operating at 1000 V and 60 pulses per minute, with a CVR from 3.32 $\log_{10}$ to

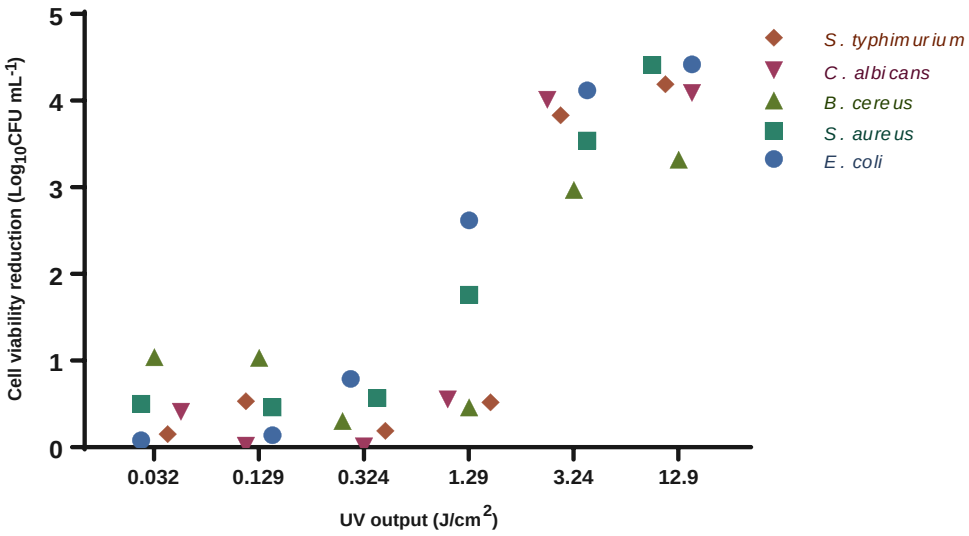

Fig. 5 Cell viability reduction of *E. coli*, *S. aureus*, *B. cereus*, *C. albicans* and *S. typhimurium* by UV output of 0.032 J/cm^2 for treatment 1, 0.129 J/cm^2 for treatment 2, 0.324 J/cm^2 for treatment 3, 1.29 J/cm^2 for treatment 4, 3.24 J/cm^2 for treatment 5 and 12.9 J/cm^2 for treatment 6

Table 6 Average peak area of amplicons bands from PCR of *E. coli*, *S. aureus*, *B. cereus*, *C. albicans* and S. typhimurium

	Pathogen	Average peak area		Ratio
		Non-treated	Treated	
1	*E. coli*	10,492.42	1692.26	6.2
2	*S. aureus*	10,111.01	0.00	-
3	*B. cereus*	11,770.74	796.48	14.78
4	*C. albicans*	12,607.45	0.00	-
5	*S. typhimurium*	8431.78	0.00	-

4.42 $\log_{10}$. This fact is explained by the higher energy and UV output of 1000 V pulses delivering 20 J and 12.9 J/cm^2, respectively, while 5 J and 3.2 J/cm^2 were delivered by 500 V pulses (Tables 1 and 2). CVR averages were statistically lower with 1000 V treatment than with 500 V for *S. aureus* ($p = 0.0019$), *B. cereus* ($p = 0.0377$) and *S. typhimurium* ($p = 0.0036$). Therefore, the conditions of treatment 6 were chosen, and 1000 V, 60 pulses/min and 10 min employed in further assays. In terms of pathogen resistance to PUV treatment, the bacterium *E. coli* was the most affected. Reductions ranged from 0.08 (T1) to 4.42 $\log_{10}$ (T6) followed by *S. aureus* from 0.46 (T2) to 4.41 $\log_{10}$ (T6), *S. typhimurium* from 0.15 (T1) to 4.19 (T6), *C. albicans* from 0.02 (T2) to 4.09 $\log_{10}$ (T6) and *B. cereus* from 0 (T3) to 3.32 (T6). Therefore, the highest CVR reductions were observed for all pathogens when 1000 V, 60 pulses and 10 min treatment were applied.

The optimal conditions for the PUV treatment were verified, and CVR is presented in Fig. 4f. All CVR were statistically significant; the highest observed was 5.63 $\log_{10}$ for *S. aureus*, followed by 5.15 $\log_{10}$ for *C. albicans*, 5 $\log_{10}$ for *S. typhimurium*, 4.59 $\log_{10}$ for *B. cereus* and 4.55 $\log_{10}$ for *E. coli*. These results are in accordance with the same treatment tested in preliminary assays as average CVR were not statistically different ($p = 0.1044$).

Log reduction of cell viability by UV output of treatments 1 to 6 is presented in Fig. 5. A UV output between 0.032 and 0.324 J/cm^2 resulted in the reduction of cell viability of approximately 1 $\log_{10}$ to all tested strains. *E. coli* and *S. aureus* were more sensitive to a UV output of 1.29 J/cm^2 and presented a 1.76 and 2.62 $\log_{10}$ reduction, respectively (Fig. 5). To achieve reductions from 3 to 4, a UV output from 3.24 to 12.9 J/cm^2 was required for all strains.

To ensure bivalve safety for consumers, alternative depuration methods and immune system stimulation approaches have been explored. A biological approach was reported by Jun et al. (2014) applying bacteriophage against *V. parahaemolyticus* in artificially contaminated oysters. Bacterial growth inhibition of 7.4×10^5 CFU/mL after 12 h of phage application was reported. Probiotic bacteria can also be used to facilitate the depuration process and stimulate the shellfish immune system. Fajardo et al. (2014) obtained an isolate from a total of 365 bacteria from the shellfish digestive gland with in vitro antibacterial and antiviral activities. Ahmadi et al. (2015) reported a bio-physical method applying bacteriophages and high hydrostatic pressure for the inactivation of *S. flexneri* and *V. cholerae* in salmon and mussels. Complete inactivation of pathogens was achieved at 550 MPa for 5 min followed by the addition of a bacteriophage suspension at 10^9 PFU/mL.

The treatment system described in this study was developed to be easily installable at current depuration plants without the need for modifications. The true challenge for such complex alternatives such as those already mentioned (biological and bio-physical) emerges when upscaling. For a flow rate of 10 mL/s (36 L treated per hour) and 0.2 L of water, a real scale depuration tank of 500 L would require 14 h of operation. The usual levels of contaminants are

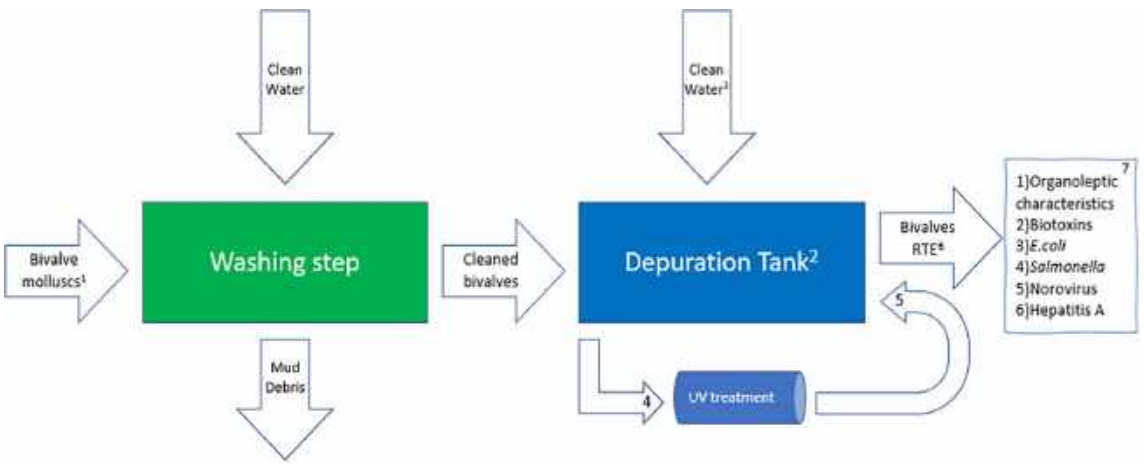

Fig. 6 Usual depuration system for bivalves

approximately 10^1–10^3 CFU/mL, reaching 10^4 in class C areas, much lower than the $10^{8–9}$ CFU/mL tested here, and would require a shorter period of treatment. Furthermore, additional PUV chambers can be set together in parallel to increase the depuration efficiency and also get benefited by operating with the current low-pressure UV system installed in most of the depuration plants. UV efficacy was reported by Garcia et al. (2015) with a 99% reduction of recombinant adenovirus and murine norovirus levels after 24 h of treatment by a low-pressure UV lamp and UV output of 6.4 J/cm^2. Low-pressure efficacy in *S. aureus* and *E. coli* was also observed by Fitzhenry et al. (2021) with 5.3 and 6 $\log_{10}$ and 14 mJ/cm^2. The economics of running costs and capital equipment compared with UV have yet to be calculated accurately; however, pilot PUV systems will cost more until full optimization is made. PUV can be considered as a bolt-on system for depuration for the shellfish in terms of sharing cooperative functionality. Shutdown of a processing plant and relocation of shellfish result in significant financial losses, affecting logistics and deliveries, requiring a re-validation of the depuration facility which takes weeks to complete, and massive damages to the reputation of the shellfish industry sector, causing foodborne infections in local and abroad consumers. PUV can be employed in terms of risk mitigation and food security.

The results of DNA integrity assessed qualitatively by gel electrophoresis and semi-quantitatively by analysing band pixels are presented in Table 6. No amplification was observed in *S. aureus*, *C. albicans* and *S. typhimurium*. We believe that PUV treatment has affected the availability and integrity of DNA while the same amount of template was used for PCR and quantified in high-accuracy fluorometer Qubit (Invitrogen), and it did not amplify. The disruptive and genotoxic effects of UV light are widely described. UV light induces the photoreactive potential of purines (adenine and guanine) and pyrimidines (cytosine, thymine and uracil) in DNA, triggering the formation of mutagenic DNA lesions and inactivating the replication, leading to a reduction in CVR. Oxidative stress is also observed in cells exposed to UV, leading to produce reactive oxygen species (ROS) that attack nucleotide pools and stress-mediated mutations (Ikehata and Ono, 2011).

Pullerits et al. (2020) investigated the effect of UV doses of 250, 400 and 600 J/m^2 on bacterial communities in water from a water treatment plant treated in a low-pressure UV system consisting of 10 rows of four UV lamps. The authors identified a long-term effect of UV irradiation that continued influencing the microbial dynamics after treatment and amplicons with greater guanine/cytosine contents were more resistant to UV treatment. In this study, *E. coli* and *B. cereus* had significantly lower amplification rates of 6.2 and 14.78-fold reduction, respectively. It was not possible to correlate; however, the lower amplification with lower CVR shown in Fig. 4 as the same amount of DNA to PCR was used for all pathogens and resulted in amplifications similar to those observed in non-treated samples (Table 6).

B. cereus is Gram-positive spore-forming bacterium widely distributed in nature and has been frequently reported to be the causative agent of food poisoning (FSAI, 2016; Rowan, 2019). *Bacillus* endospores are tolerant of environmental stresses and are often used as bioindicator organisms for evaluating the efficacy of disinfection and sterilization modalities. Garvey and Rowan (2015) previously reported that pulsed UV can inactivate 1.5 $\log_{10}$ *B. cereus* and *B. megaterium* endospores in a flow-through system using a UV dose of 21.6 µJ/cm^2 and 6.46 µJ/cm^2, respectively. A spore, *B. cereus* and its toxins can be heat resistant, requiring an extended pre-cooking procedure to reduce it to safe levels. Its resistance was observed by gel electrophoresis and detection of amplification bands post-PUV treatment and reduction in 4.59 log of *B. cereus* cells in a 10-min treatment. The PUV treatment method must be modified to better address the contaminant resistance. Taylor et al. (2020), for example, reported higher reductions of *B. cereus* spores when narrowing the UV to 222 nm, a peak also emitted in the present PUV protocol (Fig. 2). *E. coli* is a Gram-negative bacterium that is frequently associated with causing human and animal infections and can harbour multiple antibiotic resistance (AMR) genes. Zhang et al. (2017) reported its resistance when assessing the effect of a low-pressure UV treatment on antibiotic-resistant *E. coli* (AREC) isolated from a wastewater treatment plant. AREC required a higher UV dose of 20 mJ/cm^2 to cause reduction when compared to antibiotic-sensitive strains (8 mJ/cm^2).

The periodical monitoring of seawater and farm areas is important for safeguarding and for helping consumers helping the industry in decision-making (Fehrenbach et al. 2022). For shellfish producers, the water quality in these areas has been threatened by the expansion of cities and agriculture to shoreline. Irregular discharges of leachates and urban effluents represent a direct source of faecal contamination, mainly monitored and detected by *E. coli* levels, and this is a parameter considered when assessing the need for post-harvest manipulation. Other contaminants can also be present as chemicals and biotoxins that must also be periodically analysed. The location and boundaries of production areas are classified according to the level of faecal contamination. Regulation (EC) No. 854/2004 establishes the requirements for production areas in the European Union. Class A areas have the lowest risks and can be collected for direct human consumption if in accordance with health standards for live bivalves. Classes B and C cannot exceed 4600 and 46,000 *E. coli* per 100 g of flesh and intravalvular liquid, respectively, in the most probable number (MPN) test (EC, 2019). Classes B and C must be depurated at purification facilities to reduce the level of contaminants. For example, guidance for local action

on handling high *E. coli* results, pollution events and biotoxin results was released by Food Standards Agency (FSA) – England and Wales (2021). Levels of *E. coli* above 700, 18,000 and 46,000 per 100 g of shellfish for classes A, B and C, respectively, will require immediate action, from downgrading to temporary closure (FSA, 2021).

The usual system employed at purification facilities for bivalves is presented in Fig. 6. It consists of two main steps of washing and depuration. First, bivalves are transported by conveyor to a cleaning facility where they are graded according to size and serially cleaned in washing equipment. Then, the bivalves are placed into depuration tanks with clean water that recirculates in a purification system equipped with a UV system for disinfection. This system is usually effective for low contamination levels. The development of new alternatives is necessary to ensure efficient depuration, especially during winter when contaminants are higher and norovirus, for example, can reach 20,000 genome copies g^{-1} of digestive tissue (Rajko-Nenow et al. 2012). When this occurs, bivalves must be depurated for longer periods and/or also combined with higher water temperatures which can overload the facility period. Alternatively, they can be reintroduced into areas of cleaner water (EC, 2004; FSAI et al. 2018). These alternatives not only increase the production costs but also affect the production chain, possibly leading to delivery delays and affecting quality decrease to stressing of shellfish as well as increasing costs to the consumer. PUV treatment reached a bacterial removal of 5.63 log in highly contaminated artificial seawater after 10 min of treatment and could potentially support the removal of other contaminants such as NOV. It also supports the depuration of shellfish cultured in highly contaminated waters such as class C, where *E. coli* levels can reach 46,000 MPU per 100 g of flesh and intravalvular liquid (EC, 2019). Considering 46,000 MPU per 100 g as the *E. coli* load in shellfish, the PUV treatment can reduce it to safe consumption levels < 230 *E. coli*/100 g. However, it is necessary to consider the required incubation period for shellfish to depurate and shellfish load per batch. Figure 6 presents the usual depuration system for bivalves: (1) bivalves harvested from class B and C production areas or a purification centre or another dispatch centre. Production areas are classified according to the level of contamination, where A is the least and C the most contaminated; (2) bivalves must be kept at stressless conditions that support the filter-feeding act; (3) clean seawater collected and analysed before usage, it can be treated for safety measures; (4) water from depuration tanks is pumped through a UV chamber to inactivate pathogens; (5) treated water returns to depuration tank; (6) bivalves clean and ready-to-eat (RTE) are immediately packed and kept at 4 °C; and (7) parameters that must be verified in the bivalves before commercialization.

In this research paper, we present a PUV system of broad efficacy reducing in 4.5–5.6 $\log_{10}$ the initial level of 5 common foodborne pathogens of different complexity and persistence. PUV treatment is a promising alternative to assist in the reduction of pathogen levels at depuration plants. Other pathogens, such as *Vibrio* sp. and norovirus, will also be tested in future studies to assess the PUV efficacy. The high disinfection efficiency and short treatment duration support the upscaling of PUV at the pilot-scale level, as well as the modelling of the treatment.

Conclusions

- The main parameters for PUV treatment such as voltage, number of pulses and duration successfully led to higher reductions in cell viability, as verified by posterior treatment.
- A UV output of 12.9 J/cm^2 at 1000 V, 60 pulses/min for 10 min of treatment reduced the levels of common foodborne pathogens to a maximum of 5.63 $\log_{10}$ compared to no treatment.
- PUV treatment disrupted the pathogen DNA where *S. aureus*, *C. albicans* and *S. typhimurium* were not amplified by PCR; *E. coli* and *B. cereus* bands were detected by the software ImageJ, however, reduced by 6.2- to 14.8-fold compared to before treatment.
- Excellent depuration results were achieved with the same treatment conditions for all tested microorganisms without requiring specific UV conditions.
- The efficient depuration of different pathogens and simple set-up of the PUV system favours future testing at the pilot scale.

Appendix

See Table 7.

Table 7 Spectrum output (μJ/cm^2) at different distance from the flash lamp (cm) and bandwidth wavelengths

Distance (cm)	< 300 nm	300–400 nm	400–500 nm	500–600 nm	600–700 nm	> 700 nm
10			630	370	444	1877
15	346	222	295	156	191	778
20	166	140	168	93	112	462
25	129	84	114	67	73	302
30	76	57	83	42	54	215

Distance (cm)	< 300 nm	300–400 nm	400–500 nm	500–600 nm	600–700 nm	> 700 nm
35	62	43	57	38	40	158
40	40	39	48	26	30	122
45	38	27	40	20	24	98
50	34	21	33	16	20	79

Authors' contributions GWF: conceptualization, methodology, investigation, writing—original draft preparation, and visualization. EM: methodology, writing—review and editing, supervision, and project administration. RP: conceptualization, methodology, writing—review and editing, supervision, and project administration. FC: writing—review and editing. EC: conceptualization, writing—review and editing, supervision, and project administration. IM: conceptualization, methodology, writing—review and editing, supervision, and project administration. NR: conceptualization, writing—review and editing, supervision, and project administration.

Funding The research conducted in this publication was funded by the Irish Research Council under grant number GOIPG/2020/807 and Department of Agriculture, Food and the Marine under grant number PSS125.

Data Availability Data and materials for this study are available and will be provided to the journal upon request.

Declarations

Ethical approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Science of the Total Environment 844 (2022) 157067

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Implications for the seafood industry, consumers and the environment arising from contamination of shellfish with pharmaceuticals, plastics and potentially toxic elements: A case study from Irish waters with a global orientation

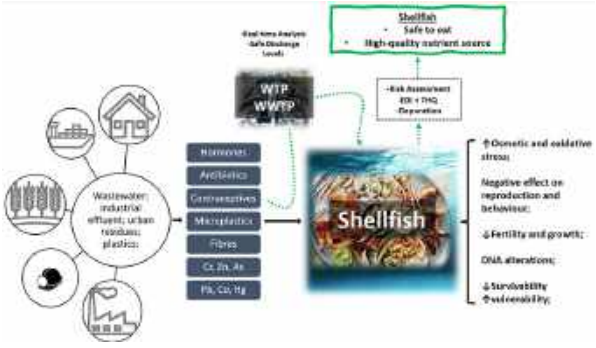
Gustavo Waltzer Fehrenbach [a,*], Robert Pogue [a,b], Frank Carter [c], Eoghan Clifford [d,e], Neil Rowan [a,f]

[a] Bioscience Research Institute, Technological University of the Shannon – Midlands Midwest, N37 F6D7, Ireland
[b] Post-Graduate Program in Genomic Sciences and Biotechnology, Catholic University of Brasilia, 71966-700, Brazil
[c] Coney Island Shellfish Ltd., Sligo F91YH56, Ireland
[d] School of Engineering, National University of Ireland Galway, H91HX31, Ireland
[e] Ryan Institute, National University of Ireland Galway, Ireland
[f] Empower Eco™ Sustainability Hub, Technological University of the Shannon – Midlands Midwest, N37F6D7, Ireland

HIGHLIGHTS

- Increased presence of pharmaceuticals, plastics and potentially toxic elements in shellfish waters
- Potential implications for industry and consumers arising from contaminated seafood
- Main contaminants and pressure points are reviewed offering potential solutions.
- Need for increased stakeholder awareness along with commensurate risk mitigation
- Risk assessment to safeguard shellfish industry and to protect food supply chain

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
Contamination
Shellfish
Plastics
Pharmaceuticals
Risk mitigation
Potentially toxic elements

ABSTRACT

Shellfish are a rich source of minerals, B-vitamins and omega-3 to the human diet. The global population is expected to reach 9.6 billion people by 2050 where there will be increased demand for shellfish and for sustained improvements in harvesting. The production of most consumed species of shellfish is sea-based and are thus susceptible to *in situ* environmental conditions and water quality. Population growth has contributed to expansion of urbanization and the generation of effluent and waste that reaches aquatic environments, potentially contaminating seafood by exposure to non-treated effluents or inappropriately discarded waste. Environmental contaminants as microplastics (MP), pharmaceuticals (PHAR) and potentially toxic contaminants (PTE) are being identified in all trophic levels and are a current threat to both shellfish and consumer safety. Immunotoxicity, genotoxicity, fertility reduction, mortality and bioaccumulation of PTE are representative examples of the variety of effects already established in contaminated shellfish. In humans, the consumption of contaminated shellfish can lead to neurological and developmental effects, reproductive and gastrointestinal disorders and in extreme cases, death. This timely review provides insights into the presence of MP, PHAR and PTE in shellfish, and estimate the daily intake and hazard quotient for consumption behaviours. Alternatives approaches for seafood depuration that encompass risk reduction are addressed, to reflect state of the art knowledge from a Republic of Ireland perspective. Review of best-published literature revealed that

* Corresponding author.
E-mail address: gfehrenbach@ait.ie (G.W. Fehrenbach).

http://dx.doi.org/10.1016/j.scitotenv.2022.157067
Received 30 March 2022; Received in revised form 9 June 2022; Accepted 26 June 2022
Available online 30 June 2022

MP, PHAR and PTE contaminants were detected in commercialised species of shellfish, such as *Crassostrea* and *Mytilus*. The ability to accumulate these contaminants by shellfish due to feeding characteristics is attested by extensive *in vitro* studies. However, there is lack of knowledge surrounding the distribution of these contaminants in the aquatic environment their interactions with humans. Preventive approaches including risk assessment are necessary to safeguard the shellfish industry and the consumer.

Contents

1. Introduction

Shellfish are aquatic invertebrates that have been used as a source of food by humans for over 160,000 years — and were one of the last additions to the human diet before the revolutionary culture of plants and domestication of animals (Marean et al., 2007). Shellfish can be classified as crustaceans or molluscs. There are approximately 40,000 known species of crustaceans, characterized by a segmented body protected by a hard shell, as observed in well-known species such as shrimp, crabs and lobsters (Hobbs, 2012; Prié, 2019). In the molluscs group, around 85,000 species such as slugs, snails, mussels, clams and octopuses, are grouped by distinctive characteristics including a muscular foot, and a fleshy mantle covering the viscera (Hobbs, 2012).

The importance of shellfish to the human diet and the rising demand for shellfish products has led to the development of production systems to increase availability and support harvesting from natural sources. In 2012, aquaculture (fish, shellfish, aquatic plants and molluscs) surpassed wild catches and production increased by 250 % between 2000 and 2015 (FAO, 2016; Hannah and Max, 2021). In 2018, global seafood production reached 179 million metric tons. Shellfish are a rich source of nutrients for the human diet. Benefits include the presence of a wide variety of essential amino acids, B-vitamins, and minerals in relatively high concentrations. They are an important source of omega-3,6 fatty acids, high levels of easily digestible protein, have low levels of fat and calories, and contain nutraceuticals with antioxidant, antiobesity, antidiabetes, and immunomodulatory activities (Venugopal and Gopakumar, 2017). However, the presence of contaminants in water and production plant systems can lead to harmful effects and illnesses, including disruptions in the human endocrine system, and immuno-genotoxicity in shellfish (Álvarez-Muñoz et al., 2015; Gadelha et al., 2019; Lacaze et al., 2015).

Most shellfish are cultivated in sea-based systems which rely on good water quality and environmental conditions. Geographical information, water quality reports from monitoring programs and risk modelling are important resources in the selection of appropriate sites for shellfish farms (Silva et al., 2011). Nonetheless, the discharge of industrial, domestic and agricultural effluents are unpredictable factors and result in chemical pollution, including pharmaceuticals, pesticides, metabolites, organic compounds and personal care products (Fairbairn et al., 2016). The growing population and agricultural demand for food has increased the use of pesticides and the discharge of domestic effluents, which also contaminate the seafood industry (Gadelha et al., 2019).

There is a constant requirement to remove persistent pharmaceuticals (PHAR) from the effluent matrix, including stimulants, anti-inflammatories, antibiotics and many other drugs (Hernando et al., 2006). If not removed, these PHAR will end up in the environment, reaching the shellfish and ultimately being consumed through the human diet. Potentially toxic elements (PTE) are naturally present in the environment at non-hazardous concentrations. However, these elements have been detected in toxic levels in industrialized areas such as ports, areas with mining activity, post-environmental accidents, as well as in discharge of effluent into rivers and seas (Bayen et al., 2005). The persistence and high accumulative potential of PTE have been extensively detected in a wide variety of shellfish species (Wang et al., 2018). The intensive use of plastics and poor management of waste have also contributed to their dispersal in terrestrial and aquatic environments. Nowadays, plastic materials and microplastics (MP) can be found in the seabed and riverbed, suspended in water or deposited at coastal areas; studies have also demonstrated MP translocation to the circulatory system in mussels (Browne et al., 2008; Hantoro et al., 2019).

The interactions of the shellfish industry and environmental conditions are not only evident through contamination and pollution issues — but shellfish farms must also deal with the uncertainty arising from climate change. Ocean acidification and changing temperature are examples of processes that can impact plankton levels and shellfish metabolism, leading to complete decimation of cultures (Froehlich et al., 2018). The physiological effects of temperature and contamination on to the bivalve *M. arenaria* were reported by Greco et al. (2011). Increased temperature altered the response to a mixture of herbicides, enhancing the malondialdehyde content, cytochrome C oxidase and superoxide dismutase activity. Shellfish farms must also deal with natural predators, and the challenge of protecting the shellfish without affecting the ecological balance (Rheault, 2012). The survivability of shellfish farms is essential as a source of food and protection of wild stocks, and the dependence on a healthy environment for shellfish production stimulates the protection of cultured areas. Understanding the risks associated with shellfish production is the first step towards ensuring food safety and quality, therefore, a risk assessment (RA) strategy can represent an important resource to support the identification of risks, effects, and development of preventive strategies. The long coastline of Ireland, together with its importance as a marine habitat to many finfish and shellfish species, implies the need for new studies in preservation and protection. The Irish Marine Institute, an Irish state agency, has been providing essential monitoring of a wide range of contaminants in Irish waters. These studies were reviewed with available literature to diagnose the current situation in Ireland.

The objective of this review paper is to discuss the presence, in shellfish, of PHA, PTE and MP that represent a threat to the host and human safety, with insights into contaminants present in Irish waters, and state of the art. The regulations for maximum limits will be discussed in a way as to be used as a guide for researchers and for the shellfish farming industry. This paper will also address the concept and main steps involved in implementing a risk assessment to support the development of protective strategies, and also a guide to estimate the ingestion of contaminants and hazard quotient based on consumption profile and contaminant concentration.

2. Pharmaceuticals in shellfish

The rate of increase of available medicines to treat and assist society is remarkable. Nonetheless, a wide variety of compounds produced for medical purposes have been identified as contaminants in different environments (Alygizakis et al., 2016; Mezzelani et al., 2018). The low degradation rate and high persistence of pharmaceutical (PHAR) compounds under environmental conditions represents a risk to aquatic environments, wildlife, and human safety (Almeida et al., 2020). Wastewater treatment plants are able to partially reduce the levels of PHAR from domestic effluent, however, the constant discharges and inadequate disposal methods increase their abundance in the environment, reaching aquatic organisms through the food chain, from phytoplankton, bivalves and crustaceans, to fish (Álvarez-Muñoz et al., 2015; Jjemba, 2018). The environmental concentration of PHAR in the marine environment can be found at ng L^{-1} to μg L^{-1} levels, and can lead to sub-lethal or chronic toxicity, while higher concentrations induce acute effects on reproduction and development (Capolupo et al., 2018; Fabbri and Franzellitti, 2016). When absorbed, PHAR compounds can lead to alterations in DNA, gene expression, antioxidant activity and immune responses, reflecting negatively on growth, behaviour and reproduction (Almeida et al., 2020; Lacaze et al., 2015).

Tiedeken et al. (2017) reported that pollution of European receiving waters with PHAR, such as with 17-beta-estradiol (a natural estrogenic hormone, E2), along with the pharmaceutically-active compounds diclofenac (an anti-inflammatory drug, DCL) and 17-alpha-ethynylestradiol (a synthetic estrogenic hormone, EE2) is a ubiquitous phenomenon. These authors noted that European surface water concentrations of DCL are typically reported below the proposed annual average environmental quality standard (AA EQS) of 100 ng L^{-1}, but that exceedances frequently occur. E2 and EE2 surface water concentrations are typically below 50 ng L^{-1} and 10 ng L^{-1} respectively, but these values greatly exceed the proposed AA EQS values for these compounds (0.04 and 0.035 ng L^{-1} respectively). Levels of these PHARs are frequently reported to be disproportionately high in EU receiving waters, particularly in effluents at control points that require urgent attention. Overall, it was found that DCL and EE2 enter European aquatic environments mainly following human consumption and excretion of therapeutic drugs, and by incomplete removal from influent at urban wastewater treatment plants (WWTPs) (Tiedeken et al., 2017). E2 is a natural hormone excreted by humans, which also experiences incomplete removal during WWTPs treatment (Tahar et al., 2018). Current conventional analytical chemistry methods are sufficiently sensitive for the detection and quantification of DCL, but not for E2 and EE2, thus alternative, ultra-trace, time-integrated monitoring techniques such as passive sampling are needed to inform water quality for these estrogens (Tiedeken et al., 2017). DCL appears resistant to conventional wastewater treatment while E2 and EE2 have high removal efficiencies that occur through biodegradation or sorption to organic matter (Tahar et al., 2018).

The presence of PHAR in Irish surface waters has been reported by McEneff et al. (2014). Carbamazepine, diclofenac, gemfibrozil, mefenamic acid and trimetrophim were found in >85 % of the samples collected from two exposure sites in the West and East of Ireland, and two areas close to effluent treatment stations. These drugs were also detected at slightly lower concentrations in the marine surface water and can be accumulated in the shellfish, as observed for the marine mussels.

Beyond the importance highlighted by consumption, molluscs, especially bivalves, have also been used as bioindicators of pollution since they exhibit the required characteristics to evaluate an environment: they are filter feeders, show bioaccumulation, have a wide distribution, exhibit slow movement and have well-characterized life-cycles (Almeida et al., 2020; McEneff et al., 2014). Capolupo et al. (2018) address this characteristic by reporting the impact of contraceptive PHAR on molluscs in a study of *in vitro* inhibition of mussel (*Mytilus galloprovincialis*) gamete fertilization induced by 500 ng L^{-1} of contraceptive 17-α ethinylestradiol (EE2), and also a reduction of fertility in sea urchins (*Paracentrotus lividus*) at 5000 ng L^{-1} of the lipid regulator gemfibrozil. Analyses of exposure during early phases are required to evaluate the effects of contaminants on animal development. EE2 and other active substances such as flame retardants, pesticides and fungicides are also considered emergent pollutants due to their endocrine-disruptive activity (Álvarez-Muñoz et al., 2015; Fehrenbach et al., 2021). Purdom et al. (1994) first registered the feminisation of male fish after exposure to treated effluent water, identifying the female hormone vitellogenin, naturally produced by the liver in females under use of estrogens. The occurrence of endocrine-disrupting compounds (EDC) is associated with human presence, and not restricted to contaminated areas or effluent discharges. As reported by Álvarez-Muñoz et al. (2015), in a study of 19 EDCs, 10 were present in bivalves and/or fish from the Tagus estuary (Portugal), Scheldt estuary (Netherlands), Po delta (Italy) and Ebro delta (Spain). The highest level of an EDC was observed in mullet, a fish found worldwide, containing 98.4 ng g^{-1} dw of flame retardant Tris(2-butoxyethyl) phosphate. Chiu et al. (2018) deployed the native mussels *M. galloprovincialis*, *M. coruscus* and *P. viridis* and semipermeable membrane devices in highly industrialized coastal areas to assess the presence of EDCs. In one month of exposure, the concentration of EDCs ranged from 99.4 to 326 ng g^{-1} dw and a correlation between the membranes and mussels was observed, confirming the potential of shellfish as indicators due to filter feeding ability.

In vitro studies with blue mussel (*Mytilus edulis*) using the psychotropic drugs paroxetine and venlafaxine at 1.5 μg L^{-1}, generated DNA breaks in hemocytes, and caused immunomodulation, respectively; the antibiotic trimethoprim was genotoxic at 200 μg L^{-1} and immunotoxic at 20 μg L^{-1}, and the same effects were reported for erythromycin at concentrations higher than 20 μg L^{-1} (Lacaze et al., 2015). The ability of the organism to retain the tested compounds, together with high consumption of blue mussel worldwide, reveal the risk of consuming this filter feeder produced/caught in contaminated environments. The presence of pharmaceuticals (PHAR) in the marine environment occurs uninterrupted (Capolupo et al., 2018), as demonstrated by Álvarez-Muñoz et al. (2015) with the identification of 15 out of 23 tested PHAR in *Mytilus* spp., *Chamalea gallina* and *Crassostrea gigas* collected in Portugal, Italy and Spain. The same authors identified 10 EDCs out of 20 compounds analysed in the mullet (*L. aurata*) and flounder (*P. flesus*) in Portugal and the Netherlands, respectively. On the Arabian Sea coast, the exposure to polycyclic aromatic hydrocarbons (PAH) and potentially toxic elements available in the water has induced DNA damage in the oyster *Sacccostrea cucullate* (Sarker et al., 2018).

The wide variety of drugs present in different environments and shellfish species, and their effects, are described in Table 1.

PHARs can complex with organic compounds present in complex matrices as wastewater, resulting in higher persistence and accumulation in receiving waters. Wastewater treatment plants (WWTP) are responsible for reducing the impact of urban and industrial effluent through the removal of nutrients and contaminants. Mechanical, chemical, physical and biological processes are applied in the preliminary, primary, secondary and tertiary treatment steps (McEneff et al., 2015). PHAR are mostly removed in the tertiary treatment by biodegradation with adapted and capable bacteria in bioreactor and/or physical and mechanical as filtration and ozonation. Angeles et al. (2020) reported >95 % overall removal for 14 out of 11 PHAR compounds detected in WWTP, while biological treatment provided negative to <50 %. Higher removal efficiencies employing physical and mechanical as filtration are usually associated to increased treatment costs and maintenance.

Tahar et al. (2018) reported that of approximately 1000 WWTPs in the Republic of Ireland, only 16 have been monitored for PHARs. Diclofenac is

Table 1
Pharmaceuticals and respective drugs detected in shellfish, marine water or tested in *in vitro* studies. The main effects observed are addressed with the duration of exposure.

Drug	Concentration and exposure time	Species	Environment or assay	Effect	Ref.
EE2	5 and 50 ng L^{-1}; 39 d	*M. edulis*	Marine	Reproductive system	(Blalock et al., 2018)
	5 to 425 ng L^{-1}; 20 h	*C. gigas*	Marine	Embryotoxicity	(Wessel et al., 2007)
	1 μg L^{-1}; 10 exp + 8 dep	*C. virginica*	Marine	Osmotic and oxidative stress, bioconcentration, metabolism	(Brew et al., 2020)
	5 ng L^{-1}; 12 d	*E. complanata*	Freshwater	Reproductive behaviour and biochemical parameters	(Leonard et al., 2017)
	5, 50 and 500 ng L^{-1}; 48 to 96 h	*M. galloprovincialis*, *P. lividus* and *S. aurata*	*In vitro*	Alteration in gamete fertilization and morphological abnormalities in *M. galloprovincialis* and *P. lividus*. Decreased survival of *S. aurata*.	(Capolupo et al., 2018)
IBP	1 and 100 μg L^{-1}; 7 d	*C. gigas*	*In vitro*	Increased transcription of certain genes and alterations in auxiliary enzymes and antioxidants	(Serrano et al., 2015)
	0.1 to 50 μg L^{-1}; 14 d	*R. philippinarum*	*In vitro*	Decrease in lysosomal membrane stability; induced detoxification metabolism and oxidative stress; genotoxic effects	(Aguirre-Martínez et al., 2016)
	15 ng g^{-1}; 48 h	*P. perna*	Marine sediment	Development affected; decrease in lysosomal membrane stability	
DCF	100 and 600 μg L^{-1}; 7 d	*M. galloprovincialis*	*In vitro*	Identification of 13 metabolites from DCF spiking	(Bonnefille et al., 2017)
	2.5 μg L^{-1}; 60 d	*M. galloprovincialis*	*In vitro*	IBU and KTP presented the same effects as DCF. Genotoxic, modulation of lipid metabolism, alterations of immunological parameters, and changes in cellular turn-over	(Mezzelani et al., 2018)
	0.5 μg L^{-1}; 96 h	*R. philippinarum*	*In vitro* flow through system	Mortality increases for larvae, shell alterations, increase in lactase activity	(Munari et al., 2016)
PAR	0.5, 5, 50 and 500 μg L^{-1}; 96 h	*Mytilus* spp.	*In vitro*	Reduce of food intake, increase in glycogen levels, metabolic changes	(Piedade et al., 2020)
	40, 250 and 100 μg L^{-1}; 7 d	*M. edulis*	*In vitro*	Alterations in gene expression affecting the reproductive system	(Koagouw and Ciocan, 2020)
	1 and 100 μg L^{-1}; 1, 4 and 7 d	*C. gigas*	*In vitro*	Alterations at transcriptional level	(Bebianno et al., 2017)
PROP	500, 5000 and 50,000 ng L^{-1}; 48 to 96 h	*M. galloprovincialis,* and *S. aurata*	*In vitro*	Fertilization and survival reduction in *P. lividus* and morphological abnormalities in *M. galloprovincialis*	(Capolupo et al., 2018)
TCS	75 ng g^{-1}; 24 h	*L. variegatus*; *M. charruana*	Marine sediment	Development affected; decrease in lysosomal membrane stability	(Pusceddu et al., 2018)
	0.007, 0.014, and 0.036 mg L^{-1}; 20 d	*C. catla*	*In vitro*	Enhanced GOT, GPT, and GST enzyme activity; physiological alterations	(Hemalatha et al., 2019)

EE2: 17alpha-ethinylestradiol; IBP: ibuprofen; DCF: diclofenac; KTP: ketoprofen; PAR: paracetamol; PROP: propranolol; TCS: triclosan; GOT: glutamate oxaloacetate transaminase; GPT: glutamate pyruvate transaminase; GST: glutathione-S-transferase; dep: depuration; exp: exposure.

found in treated effluents from 5 WWTPs at levels at least as high as other European WWPTs, and sometimes higher. Measurements of E2 and EE2 in WWPT effluents were rare and effluents were more often evaluated for total estrogens; these PHARs were generally not detected using conventional analytical methods because of limits of detection being too high compared to environmental concentrations and WFD environmental quality standards (Tiedeken et al., 2017; Tahar et al., 2018). Tahar et al. (2018) reported a correlation between occurrence of these PHAR and regional drug dispensing data in Ireland. However, mapping the aforementioned data onto appropriate river basin catchment management tools will inform predictive and simulated risk determinations to inform investment in infrastructure that is necessary to protect rivers and beaches and economic activities that rely on clean water.

Adams et al. (2002) spiked sterile river water with common antibiotics to evaluate the effectiveness of conventional water treatment processes on antibiotic removal. They reported an effective removal when applying chlorine, ozone, powdered activated carbon and reverse osmosis, however, only powdered activated carbon (PAC) effectively removed the antibiotics at typical plant dosages of PAC and at a frequency that increases the treatment costs. However, matrices with higher concentrations of PHAR, such as urban wastewater, can make removal of PHAR more challenging. In shellfish, (McEneff et al., 2013) investigated the concentration of PHAR after a cooking process, spiking *Mytilus* spp. and artificial seawater with several PHAR at concentrations 1000 times greater than those observed in marine surface waters. The authors observed an increase of pharmaceutical residues in seawater and shellfish tissue after cooking by steaming. Currently, there is no standard method for PHAR removal in shellfish and seawater, as this depends on several parameters. The continuous water quality monitoring at shellfish farms and surroundings is essential to monitor the PHAR level. An early detection can reduce the bioconcentration and possibly the natural depuration of contaminants in controlled conditions.

3. Plastics and microplastics in shellfish

Plastics are polymeric materials of highly diverse composition and uses, moulded under specific pressure and temperature to provide adequate resistance and performance (FAO, 2018). The demand for plastic materials has been constantly increasing over the past decades and reached a worldwide production of 368 million tonnes in 2019 (PlasticsEurope, 2020). The intensive usage of plastic materials and inadequate management to control its life cycle have led to dispersal in the terrestrial and aquatic environments, expanding to shorelines, open ocean and reaching the deep seas. It has been estimated that between 4.8 and 12.7 million tons of plastic entered the oceans in 2010, not including abandoned equipment and fishing nets (Jambeck et al., 2015). Contamination occurs on different levels, where low-density plastics are dominant and float at the top layers, and high-density plastics combined with biofouled low-density material sink and remain on the sea bottom (Andrady, 2011; Bellas et al., 2016). The physical properties of plastic materials, such as resistance, durability, light weight, and generally low cost make them appropriate for use in ropes, cages, tanks and general materials associated with the fish and shellfish industry. However, this can also threaten shellfish and finfish safety as these materials are not completely inert, where degradation under environmental conditions such as ultraviolet radiation and/or physical abrasion can release plastic fibres and particles in even higher concentrations than external contaminants (Hantoro et al., 2019).

The ingestion of plastic materials occurs involuntarily in finfish and shellfish; however, filter feeders are more susceptible to accumulating higher concentration of plastics than finfish due to non-selective feeding. Plastics can reach shellfish directly as primary plastic of specific size and composition, or as secondary materials of different sizes and composition, products of exposure to environmental conditions and ultraviolet radiation (Hantoro et al., 2019). The secondary plastics are mostly present as fibres,

powders, and pellets, classified by size as meso (>5 mm), micro (1000 nm) and nanoplastics (1–100 nm). In invertebrates, once the microplastic is ingested it can be transferred to body tissues though the gut epithelial lining or egested in faeces (Browne et al., 2008). To investigate the presence of plastic materials in the oyster *C. gigas* and mussel *M. edulis*, van Cauwenberghe et al. (2015) performed an overnight digestion of shellfish wet tissue using nitric acid (69 %) followed by a boiling step, where samples from a mussel farm and local supermarket resulted in 0.36 and 0.47 particles/g of tissue, respectively. Using the same digestion protocol, van Cauwenberghe and Janssen (2014) detected microplastics (MP) in all animal tissues and faeces collected from *M. edulis* and *A. marina* and an increase of energy consumption in the exposed *M. edulis* when compared to control, linked to increase of stress. Browne et al. (2008) used *M. edulis* as a model organism to investigate the route and biological consequences of ingesting microparticles of polystyrene, a polymer that reached a worldwide production of 15.61 million metric tons in 2021 (Statista, 2020). They identified a correlation between smaller particles and high accumulation in tissues. In 3 days, particles moved from the gut to the circulatory system and remained for 48 h, reaching their greatest level on the 12th day. Ingested microplastics can also work as a carrier of other contaminants to seafood and not only a direct contaminant. Polychlorinated biphenyls and bacteria were reported on polystyrene surface of eight of the 14 species analysed by Carpenter et al. (1972).

The accidental ingestion of plastics due to similarity to food can be summed up with the contamination of lower trophic levels and subsequent trophic transfer, as observed by the same authors in zooplankton samples. Crustaceans, for example, are non-selective and can have a wide variety of food sources. This fact might explain the presence of microplastics in the gastrointestinal tract of the crustacean *Nephrops norvegicus*, collected in Irish prawn grounds and presented by Hara et al. (2020). Fibres were predominant (98 % of plastic constituent) in the collected plastic and a positive correlation was observed between the prawn carapace condition and microplastic. Devriese et al. (2015) reported the natural ability for the shrimp *Crangon crangon* to ingest microplastics, reaching an uptake of 0.68 g w w^{-1}. The role of shrimps in the trophic transfer of microplastics was also discussed as the variable diet of shrimps and the importance as food for a large range of predators contributes to transfer.

On the Irish continental shelf, an extension from the coastline of 200 nautical miles under the sea, microplastics were detected within superficial sediments and bottom water (Martin et al., 2017). The depth and environmental conditions at these regions slow the breakdown of plastic materials, contributing to their persistence and accumulation. Woodall et al. (2014) reports the high concentration of microplastics in samples collected from the North Atlantic Ocean, Mediterranean Sea and SW Indian Ocean. Microplastics were mostly in the form of fibres of 2–3 mm in length and <0.1 mm in diameter in deep-sea sediments and coral samples, regions widely habited by commercially explored finfish and shellfish. Lusher et al. (2018) reported the presence of macro-debris and micro-debris in Irish cetaceans, which shows trophic transfer as another route of contamination. The authors also observed a higher incidence of macrodebris ingestion in deep-diving species, however, it was not possible to investigate the relationship to habitat. Ireland is not located in accumulation zone of debris, however due to an extension of 7524 km, a combination of weather, season and geographical position can lead to high accumulation levels (Lusher et al., 2018). Concentration of microplastics such as fibres, fragments, films and filaments detected in shellfish species at different locations are presented in Table 2.

An alternative for reducing the levels of plastic in shellfish is a longer depuration process keeping the shellfish in tanks with recirculating water that pass through a treatment system, allowing the excretion of plastic materials from the gut. Depuration processes are designed to provide optimum conditions such as temperature, salinity and oxygenation, reducing the impact on organoleptic properties and increasing the filtering activity. van Cauwenberghe and Janssen (2014) reported a reduction in the levels of particles in *M. edulis* and *C. gigas* after depurating for three days. Alternatively, a change in the mode of consumption can be made by removing parts of the shellfish or cooking. However, this can impact on consumer experience and affect important characteristics to shellfish as freshness. Daniel et al. (2020) suggested the consumption of *F. indicus* in whole dried by cooking or peeled form to reduce the exposure to microplastics owing to the concentration in the foregut and midgut. However, more studies are needed to improve the depuration and cleaning processes while preserving the organoleptic properties.

4. Potentially toxic elements in shellfish

Potentially toxic elements (PTE) are pollutants of high persistence and toxicity that contaminate a wide variety of ecosystems and organisms (Wang et al., 2018). The term PTE describes metals and metalloids that naturally present in soil, and some are considered essential nutrients, required at low doses and participating in biochemical and physiological functions (Tchounwou et al., 2012). Others, such as lead, mercury, and cadmium, are on a list of ten chemicals of major public concern (World Health Organization, 2020a). Anthropogenic activities such as general industrial processes, mining, agriculture, and domestic use of PTE have been associated with environmental contamination and human exposure (Tchounwou et al., 2012). Marine and freshwater ecosystems are highly affected by PTE, where shellfish can accumulate these compounds to toxic concentrations, releasing them back into the water after death, or transporting them to the next trophic level (Djedjibegovic et al., 2020). The ability of shellfish species to bioaccumulate chemicals, their long-life span, and high density, have supported their use as local bioindicators of contamination, where the determination of PTE is determined directly from the shellfish tissue (El-Shenawy et al., 2016).

The most common method to verify the presence of metals in shellfish is by total concentration, extracting all the contaminants from tissues and analysing by specialized techniques; they can also be determined in terms of bioacessibility, which represents the fraction of contaminants that is

Table 2
Concentration of microplastics in shellfish species and major findings.

Microplastic	Concentration	Specie	Comments	Location	Reference
Fibres, fragments and films	1.75 ± 2.01 items per shrimp	*N. norvegicus*	Most common range of 1 to 2 mm	Irish waters	(Hara et al., 2020)
Filaments	83 % contained plastic filaments	*N. norvegicus*	Fishing waste was probably the source for microfilaments	Clyde Sea	(Murray and Cowie, 2011)
Fibres	0.68 ± 0.55 microplastics/g ww	*C. crangon*	The analysed specie was able to ingest plastic particles from natural habitat	Southern North Sea and Channel area	(Devriese et al., 2015)
Microplastics	0.2 ± 0.3 microplastics/g; 1.2 ± 2.8 particles/g	*M. edulis*; *A. marina* (annelid)	Microplastics were present in all organisms collected in the field	French, Belgian and Dutch North Sea coast	(van Cauwenberghe et al., 2015)
Microplastics (LDP, HDP and POS)	0.36 ± 0.07 particles/g (ww); plastic load 0.47 ± 0.16 particles/g ww	*M. edulis*; *C. gigas*	Annual dietary exposure of 11,000 microplastics	North Sea and Atlantic Ocean	(van Cauwenberghe and Janssen, 2014)
Microplastics	128 microplastics (83 % were fibres)	*F. indicus*	Contamination significantly higher in Monsoon season	Coastal waters off Cochin, India	(Daniel et al., 2020)

g: gram; ww: wet weight;

Table 3
Potentially toxic elements quantified in shellfish and seawater. General comments were added to address the main findings of studies.

Metal	Concentration	Specie	Comments	Reference
Cd	2.0–12.4 μg g^{-1} dw	*C. angulate* oysters	Bio-accessibility from 13 to 58 %	(He et al., 2016)
	1.7–5.1 μg g^{-1} g	*C. hongkongensis*		
	0.644 mg kg^{-1} ww	*D. gahi*	TWI would be reached in a 70 kg adult by a weekly consumption of 274 g of *M. edulis* or 272 g of *D. gahi*	(Djedjibegovic et al., 2020)
	0.015	*P. monodon*		
	0.002	*P. indicus*		
	0.062	*M. edulis*		
	0.17/0.1/0.135 μg g^{-1} ww	*R. decussatus*	Concentration in three different locations in the Lake Timsah; PTWI: 0.01–0.06 μg kg^{-1}/day) - below the PTWI of 7 μg kg^{-1}/week for both groups of consumers	(El-Shenawy et al., 2016)
	0.23/0.23/0.23	*P. undulate*		
	0.635 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	0.41 mg kg^{-1} dw	*O. glomerate*	Concentration in seawater 0.08 mg kg^{-1} dw	(Yuan et al., 2020)
	0.31	*P. viridis*		
	0.07	*C. scripta*		
	0.06	*M. edulis*		
	1.14	*G. divaricatum*		
	0.66	*B. virescens*		
	0.03–0.14 mg kg^{-1} ww	Blue mussels	Exceeded the 1881/2006/EC max limit in oyster	(Irish Marine Institute, 2018)
	0.02	Clams		
	0.09–1.25	Oysters (Pacific and native)		
Cu	173–2212 μg g^{-1} dw	*C. angulate oysters*	Bio-accessibility from 42 to 95 %	(He et al., 2016)
	1063–5314 μg g^{-1}	*C. hongkongensis*		
	32.2 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	12.04 mg kg^{-1} dw	*O. glomerate*	Concentration in the seawater 1.48 mg kg^{-1} dw	(Yuan et al., 2020)
	1.58	*P. viridis*		
	0.9	*C. scripta*		
	1.49	*M. edulis*		
	1.1	*G. divaricatum*		
	1.32	*B. virescens*		
	2.53/3.79/3.16 μg g^{-1} ww	*R. decussatus*	Concentration in three different locations in the Lake Timsah; EDI was lower than the FAO/WHO guidelines, meaning that is safe for consumption;	(El-Shenawy et al., 2016)
	2.26/2.64/2.45	*P. undulate*		
	0.38–1.32 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	0.77	Clams		
	1.81–22.2	Oysters (Pacific and native)		
Zn	1173–6050 μg g^{-1} dw	*C. angulate* oysters	Bio-accessibility from 13 to 58 %	(He et al., 2016)
	2103–7021 μg g^{-1}	*C. hongkongensis*		
	154.29 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	10.59 mg kg^{-1} dw	*O. glomerate*	Concentration in the seawater 12.25 mg kg^{-1} dw	(Yuan et al., 2020)
	11.95	*P. viridis*		
	9.79	*C. scripta*		
	10.36	*M. edulis*		
	10.64	*G. divaricatum*		
	11.32	*B. virescens*		
	9.87–21 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	11.4	Clams		
	91.8–448	Oysters (Pacific and native)		
Hg	0.02 mg kg^{-1} ww	*D. gahi*		(Djedjibegovic et al., 2020)
	0.044	*M. edulis*		
	0.058	*P. monodon*		
	0.037	*P. indicus*		
	0.01 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	0.02 mg kg^{-1} dw	*O. glomerate*	Concentration in seawater 0.007 mg kg^{-1} dw	(Yuan et al., 2020)
	0.02	*P. viridis*		
	0.02	*C. scripta*		
	0.01	*M. edulis*		
	0.02	*G. divaricatum*		
	0.03	*B. virescens*		
	0.01–0.03 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	0.01	Clams		
	0.01–0.04	Oysters (Pacific and native)		
Pb	0.003 mg kg^{-1} ww	*D. gahi*	TWI would be reached in a 70 kg adult by a weekly consumption of 274 g of *M. edulis* or 272 g of *D. gahi*	(Djedjibegovic et al., 2020)
	0.014	*P. monodon*		
	0.013	*P. indicus*		
	0.161	*M. edulis*		
	0.47 mg kg^{-1} dw	*O. glomerate*	Concentration in seawater 0.77 mg kg^{-1} dw	(Yuan et al., 2020)
	0.48	*P. viridis*		
	0.34	*C. scripta*		
	0.44	*M. edulis*		
	0.24	*G. divaricatum*		
	0.83	*B. virescens*		
	0.23 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	0.05–0.37 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	0.11	Clams		
	0.02–0.13	Oysters (Pacific and native)		
Cr	0.16 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	0.76 mg kg^{-1} dw	*O. glomerate*	Concentration in seawater 0.72 mg kg^{-1} dw	(Yuan et al., 2020)
	0.78	*P. viridis*		

Table 3 (*continued*)

Metal	Concentration	Specie	Comments	Reference
	1.79	*C. scripta*		
	1.08	*M. edulis*		
	0.78	*G. divaricatum*		
	0.23	*B. virescens*		
	0.08–0.35 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	0.07	Clams		
	0.04–0.9	Oysters (Pacific and native)		
Ni	0.26 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	0.94/0.9/0.92 μg g^{-1} ww	*R. decussatus*	Concentration in three different locations in the Lake Timsah; EDI between 0.01 and 1.26 μg kg^{-1}/day for both bivalves	(El-Shenawy et al., 2016)
	1.04/1.1/1.07	*P. undulate*		
	0.08–0.26 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	0.05	Clams		
	0.03–0.1	Oysters (Pacific and native)		
As	1.27 mg kg^{-1} ww	Bivalve molluscs		(Wang et al., 2018)
	0.48 mg kg^{-1} dw	*O. glomerate*	Concentration in seawater 0.77 mg kg^{-1} dw	(Yuan et al., 2020)
	0.50	*P. viridis*		
	0.29	*C. scripta*		
	0.22	*M. edulis*		
	0.50	*G. divaricatum*		
	0.39	*B. virescens*		
	1.03–1.82 mg kg^{-1} ww	Blue mussels		(Irish Marine Institute, 2018)
	1.65	Clams		
	1.12–2.69	Oysters (Pacific and native)		

ww: wet weight; dw: dry weight.

released from the food matrix and interacts with the human digestive tract (Brandon et al., 2006; He et al., 2016). Quantification by analytical techniques can be diverse. Wang et al. (2018) applied atomic absorption spectrophotometry to measure Cd, Pb, Cr, Ni, Cu, Zn, and atomic fluorescence spectrophotometry for Hg and As, while Djedjibegovic et al. (2020) analysed Cd and Pb by graphite furnace atomic absorption spectrometry and Hg by flow-injection cold vapour atomic absorption spectrometry. *In vitro* models can also be used to provide analysis of specific routes of consumption such as ingestion and sucking. As an example, He et al. (2016) investigated the bio-accessibility of Cd, Cu and Zn in *Crassostrea angulate* (green oyster) and *Crassostrea hongkongensis* (blue oyster). The oysters were collected from a station along the Jiulong River Estuary, China, and digested by an *in vitro* process that mimics the mouth, stomach and small intestine of humans. The total concentration was higher than maximum levels established by USEPA, and the bioacessibility range from 13 to 95 % highlighted the potential threat to human health and safety. The authors also found a correlation between the shellfish tissue colour and concentration of Cu, Cd and Zn.

The main exposure routes of humans to PTE occurs by ingestion of contaminated food and water. Due to their resistance to relatively polluted environments, shellfish species can bioaccumulate and biomagnify PTE through the food chain. PTE toxic effects are associated with gastrointestinal and kidney dysfunction, vascular damage, birth effects, bloody diarrhoea, and many other disorders (Balali-Mood et al., 2021). When exposed to low PTE doses, the effects can be more complicated to diagnose and correlate with source of intake after a long period of exposure. Mazumdar et al. (2011) found that lead exposure in childhood predicts intellectual function in young adulthood. Therefore, a long-term diet containing low doses can be a silent threat. In shellfish, the exposure to low doses of PTE can lead to incorporation of Pb and Zn instead of Ca, as they are incorporated by the same pathways. Stewart et al. (2021) recently reported the correlation between the weakening of shell strength in *Pecten maximus* and metal pollution in sediments, increasing the vulnerability of the bivalve in the environment.

In Irish Coastal Waters, the levels of PTE in waters and shellfish have been monitored mainly by state agencies such as the Irish Marine Institute (Ireland), and the Centre for Environment, Fisheries and Aquaculture (United Kingdom). A review of the contaminant status of the Irish Sea was reported by Kenny et al. (2005). This 100,000 km^2 area is bounded by Scotland, England, Wales and Ireland. In dredged samples collected from 1995 to 2005, zinc, lead and arsenic were present at higher concentrations than other PTE such as Cu, Ni and Cr. A variation of PTE levels was also observed, allowing the researchers to associate the presence of these chemicals in the environment as a consequence of society, industry and weather particularities. In mussel samples collected from inshore sites of the Irish Sea between 1999 and 2001, the authors also identified levels of Cd (208–465.69 μg kg^{-1} wet weight), Cu (923–1987 μg kg^{-1} wet weight), Pb (352–1668 μg kg^{-1} wet weight), Zn (12,673–37,527 μg kg^{-1} wet weight), Hg (18–70 μg kg^{-1} wet weight) and Ag (50–260 μg kg^{-1} wet weight). The concentrations were below the maximum acceptable limits. Another study by the Irish Marine Institute (2018) quantified several metals in bivalve molluscs collected in the Irish seawater in 2015 — from oysters, blue mussels and clams; only Cd in oysters was above the maximum limit established by 1881/2006/EC. The determination was also carried in seawater and the levels of metals complied with the maximum limits established by the Statutory Instruments and Shellfish Waters Directive. However, the presence of these elements at non-natural levels alerts to an intoxication risk as the accumulation of PTE will depend on consumption habits and local body weight, discussed in the risk assessment.

In Table 3 are presented the main potentially toxic elements and their respective concentrations in shellfish tissues, seawater and after *in situ* studies, during bioacessibility and toxicology assays.

Han et al. (1993) reported one of the first depuration studies of metals in shellfish. *C. gigas* and *M. smarangdium* were collected from a region contaminated with copper and zinc and transferred to natural clean seawater for depuration. A 351 μg g^{-1} day^{-1} depuration rate of cooper was reported for the first 6 days, approximately 67 % of the total in *C. gigas*, and only 36 % of accumulated zinc. Longer depuration periods are associate with increased costs of treatment and infrastructure. FAO (2008) describes the fundamentals and practical aspects of bivalve depuration. A minimum depuration period of 42 h is determined and extended according to contamination/removal levels.

Anacleto et al. (2015) evaluated the effect of depuration process on the levels of S, Cl, K, Ca, Fe, Zn, Br, Cu, Se, Rb and Sr in the bivalves *R. philippinarum*, *M. galloprovincialis*, and *S. plana*, collected at different sites of Tagus estuary. Depuration was performed in recirculating tanks set according to European Guidelines (European Commission, 2004). The level of toxic elements (Hg, Cd, Pb and As) after depuration were reduced in *R. philippinarum* (Hg, Cd, Pb and As), *M. galloprovincialis* (Pb) and *S. plana* (Pb).

Currently, there is no consensus about mitigation methods for removing/reducing PTE in shellfish. More studies are necessary to address effective technologies and factors associated with reduction and quality. The continuous monitoring of PTE levels in the full shellfish farming environment is still the main recommendation.

5. Regulation and international guidelines

In order to protect consumer health and ensure product quality, international commissions have elaborated guidelines and limits for a wide diversity of contaminants. These limits were established to balance the potential benefit of consuming food with even a low concentration of contaminants that would not lead to serious effects, and also to keep the production/depuration costs affordable. European Commission Regulations and the U.S. Food and Drug Administration are the main references for maximum levels of contaminants (Table 4). The limits established by each regulatory agency also set the tolerance for legal actions such as recall of products from the market, shut down of production plants, and fines.

6. Risk assessment and management for addressing contamination in shellfish

The analysis of risks associated with an activity, or a hazard is a holistic approach involving all available knowledge and data that can support a prediction mode (Tahar et al., 2017). Besides the diversity of approaches, the risk assessment (RA) modelling is based on the main steps of hazard identification, exposure assessment, and hazard characterization, resulting in a broad risk characterization (Oscar, 2012; Tiedeken et al., 2017). RA can be applied to different environments, also delineating critical control points along the production and supply chain for industry — every stage of processing, production, storage, marketing and consumption is considered in an industrial environment.

Table 4
Environmental chemical contaminants and tolerable levels allowed in shellfish according to international agencies.

Substance	Group/species	Maximum level (concentration of contaminant per wet weight)
European Commission Regulation[1]		
Lead	Crustaceans	0.5 mg kg^{-1}
	Bivalve molluscs	1.5 mg kg^{-1}
	Cephalopods	0.3 mg kg^{-1}
Cadmium	Bivalve molluscs	1 mg kg^{-1}
	Crustaceans	0.5 mg kg^{-1}
	Cephalopods	1 mg kg^{-1}
Mercury	Crustaceans	0.5 mg kg^{-1}
Dioxins and PCBS	Crustaceans	3.5 pg/g (sum of dioxins), 6.6 (sum of dioxins anddioxin-like PCBs); 75 ng g^{-1} (PCB28, PCB52, PCB101, PCB138, PCB153 and PCB180)
Benzo(*a*)pyrene, benzo(*a*)anthracene, benzo(*b*) fluoranthene and chrysene	Smoked crustaceans	2.0 μg kg^{-1}
	Smoked bivalve molluscs	6 μg kg^{-1} Benzo(*a*)pyrene, 35 μg kg^{-1} Sum of benzo(a)-pyrene, benz(*a*)anthracene, benzo(*b*) fluoranthene and chrysene)
U. S. Food and Drug Administration[2]		
Aldrin/Dieldrin	Shellfish and finfish	0.3 ppm
Chlordane	Shellfish and finfish	0.3 ppm
Chlordecone	Shellfish and finfish	0.3 ppm
	Crabmeat	0.4 ppm
DDT, TDE, DDE	Shellfish and finfish	5 ppm
Heptachlor/Heptachlor Epoxide	Shellfish and finfish	0.3 ppm
Mirex	Shellfish and finfish	0.1 ppm
Arsenic	Crustacea	76 ppm
	Molluscan bivalves	86 ppm
Cadmium	Crustacea	3 ppm
	Molluscan bivalves	4 ppm
Chromium	Crustacea	12 ppm
	Molluscan bivalves	13 ppm
Lead	Crustacea	1.5 ppm
	Molluscan bivalves	1.7 ppm
Nickel	Crustacea	70 ppm
	Molluscan bivalves	80 ppm
Methyl Mercury	Shellfish and finfish	1 ppm
Diquat	Shellfish and finfish	0.1 ppm
Fluridone	Crayfish	0.5 ppm
Glyphosate	Shellfish	3 ppm
PCBs	Shellfish and finfish	2 ppm
2,4-D	Shellfish and finfish	1 ppm
Chinese National Food Safety Standard Maximum Levels of Contaminants in Foods[3]		
Lead	Fish, crustacean	0.5 mg kg^{-1}
	Bivalves	1.5 mg kg^{-1}
Cadmium	Crustacean	0.5
	Bivalves, gastropods, cephalopods, echinoderms	2 (viscera removed)
Methyl mercury	Aquatic animals	0.5 mg kg^{-1}
As	Aquatic animals and its products, not including fish	0.5 mg kg^{-1}
Cr	Aquatic animal and its products	2 mg kg^{-1}
Benzo(*a*)pyrene	Aquatic animals and its products	5 μg kg^{-1}
N-Nitrosodimethylamine	Aquatic animals and its products	4 μg kg^{-1}
Polychlorinated biphenyl	Aquatic animals and its products	0.5 mg kg^{-1}[a]

[1] European Comission Regulation (ECR, 2006).
[2] U.S Food and Drug Administration (USDA, 1996a, 1996b, 1993).
[3] Chinese National Food Safety Standard Maximum Levels of Contaminants in Foods (NHFPC and CFDA, 2017).
[a] Polychlorinated biphenyl is calculated by total of PCB28, PCB52, PCB101, PCB118, PCB138, PCB153 and PCB180.

In the food safety area, RA is based on standard protocols established by governmental and international agencies, focused on standard methods and contaminants limits. However, Tahar et al. (2017) highlighted that developing and deploying an RA model for evaluating environmental threat posed by emerging contaminants of concern is challenging given the multiplicity of influencing and controlling factors to be addressed. The authors defined a semi-quantitative RA model that adopted the Irish EPA's Source-Pathway-Receptor concept to define relevant parameters for calculating low, medium or high-risk scores for each agglomeration of WWTP, which included catchment, treatments, operational and management factors. This RA model may be potentially applied on a transnational scale to (i) identify WWTPs that pose a particular risk as regards releasing disproportionally high levels of contaminants that may reach shellfish, and (ii) to identify priority locations for introducing or upgrading control measures (*e.g.*, tertiary treatment, source reduction). This RA was deemed semi-quantitative as other influencing factors such as influence of climate change, hydrological data, pollutant usage or occurrence dose to be considered in a future point for estimating and predicting risk.

A general model and steps for conducting a risk assessment study is presented in Fig. 1. Correlating for the needs of the shellfish industry, the first step is the identification of hazards, where physicochemical contaminants, biotoxins, processing fails, inadequate handling and short depuration process are frequently observed (Fazil, 2005). Post identification, the contaminants and processing failures are measured and analysed to verify the severity of the potential risk; for example, if the contaminants are PTE that occur at high concentrations in shellfish waters, then represents an increased potential risk of exposure, that may also impact upon subsequent consumer safety. Next, the hazard characterization provides a better understanding of the problem and supports the development of mitigation alternatives. The fourth step in the proposed RA model reflects the development of an action plan and the main implementation of targeted measures. In the shellfish industry, the depuration process is one of the major mitigation strategies, and is mainly applied to reduce the shellfish bacterial and viral loads. Finally, the last step reflects the need for continuous monitoring of hazards identified and review of appropriate response(s); for example, Tahar et al. (2018) and Tiedeken et al. (2017) reported on the challenges limited data and lack of sufficiently sensitive analytical detection methods to inform robust RA modelling. Physical assets, such as depuration facilities, may also require upgrading to enable installation of more effective disinfection technologies for complex viral threats such as human norovirus. There is also potential to use the online graphic information system, ArcGIS, to inform the occurrence and geomapping for these targeted contaminants of concern (Tahar et al., 2018) that will inform location of concern with a view to upgrading mitigation infrastructure and assets.

In Europe, RA can be performed by permanent bodies or decentralised EU agencies and scientific communities (necessarily set up by the European Commission). A guidance to authorities is provided in the Article 20 of Regulation (EC) No 765/2008, and an implement to the risk assessment methodology described in 2015-IMP-MSG-15 (European Commission, 2016). In the United States of America, the Environmental Protection Agency (EPA) and National Research Council (NRC) provides the main guidance for risk assessment studies, separately according to the hazard and activity. International bodies such as the World Health Organization (WHO) and Food and Agriculture Organization (FAO) are also major references for risk assessment studies (Fazil, 2005; World Health Organization, 2020b). The International Organization for Standardization (ISO) is an important non-governmental organization responsible for standards, where the ISO 31000 is the international certification for risk management (ISO, 2018). In Ireland, with reference to the European and ISO guidelines, the Health Safety Authority (HSA) provides the guidance to protect employees and identify hazards (HSA, 2022).

A risk assessment is not only interesting to evaluate an ongoing activity, but essential to evaluate the environmental impact of new activities. In a recent report, the Irish Marine Institute (2020) provided a full analysis of a conservation area and its potential use for aquaculture and fishing projects. The description of *in site* activities, risks, effects and assessment through a score system were considered to endorse or reject the setting and usage of the natural habitat. The RA can be also focused on one contaminant/activity, or effect in shellfish/humans.

7. Estimated daily intake and targeted hazard quotient of contaminants in shellfish

The health risk due to contaminants in shellfish can be estimated by calculating the estimated daily intake (EDI) and targeted hazard quotient (THQ) (USEPA, 2000). First, EDI is calculated considering the concentration of contaminant in the shellfish (Eq. 1), usually expressed in $\mu g\ g^{-1}$; the daily

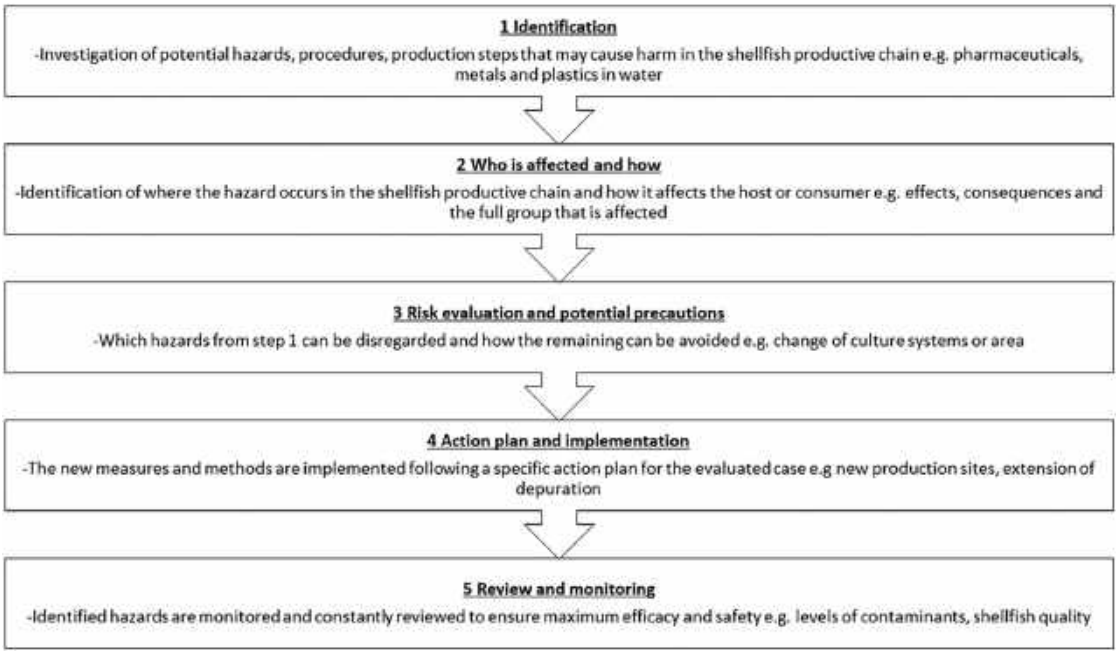


Fig. 1. General steps of a risk assessment study.

mean ingestion (DMN) expresses the daily consumption of >shellfish *per capita* in the region/area where the shellfish is mainly consumed; and the average body weight of consumer (BW).

Then, THQ (Eq. 2) is obtained considering the oral reference dose (RFd) of the contaminant, an estimate of daily exposure to low risk of deleterious effects during a lifetime (USEDA, 1991)

For estimated daily intake (EDI), concentration of contaminant in shellfish is multiplied by the daily mean ingestion and divided by average body weight:

$$EDI = \frac{C.Contaminant \times daily\ mean\ ingestion}{Body\ weight} \quad (1)$$

For targeted hazard quotient (THQ), estimated daily intake is divided by oral reference dose, resulting in a constant value for THQ:

$$THQ = \frac{EDI}{RFd} \quad (2)$$

Concentration of PHAR, PTE and MP in shellfish (Tables 1, 2 and 3) were used to simulate the EDI for adults in Europe, Africa, Asia, America and Oceania based on average population weight and shellfish consumption *per capita* (Appendix A) (Rodriguez-Martinez et al., 2020). EDI and THQ were estimated based on two scenarios: continent-specific consumption, *per capita*, of crustaceans and molluscs reported by FAO (2020), where Europe, Africa, Asia, America and Oceania consume mean values of 10.16 ± 0.23, 0.55 ± 0.2, 16.24 ± 1.77, 1.56 ± 2 and 14.24 ± 1.8 g day^{-1}, respectively; and according to USDA and USDHHS (2020) in a guide for fish and shellfish consumption that classifies species that can be consumed in 2–3 servings (maximum 340 g) a week based on low mercury levels, and 1 serving (maximum 28 g) for species with higher levels. The consumption advice released by USDA and USDHHS (2020) intends to stimulate the healthy consumption of non-contaminated seafood due to benefits as part of a balanced eating pattern. An ingestion of 3 servings of 4 oz. (340 g) of shellfish (non-pregnant adults) was chosen in a hypothetical scenario to estimate the exposure to PHAR, PTE and MP in shellfish (Tables 1–3) when consuming the recommended amount, *per capita*, of shellfish. Therefore, 48.5 g was used as reference for daily consumption. The oral RFd for metals were based on USEPA (1991, 2000), a database platform part of the Integrated Risk Information System (IRIS) to provide the risk information and assessment of chemicals. For PHAR without a RFd, the parameter (Eq. 2) was replaced by the acceptable daily intake (ADI), corresponding to the lowest daily therapeutic dose without harmful effect. Currently, RFd availability is limited to PHAR applied on agriculture and/or animals. The daily intake of MP was estimated considering the number of units and particles/g and due to absence of reference values for MP to calculate the THQ.

EDI was calculated based on PHAR, PTE and MP concentrations reported in the literature. Then, THQ of PHAR and PTE were calculated and categorized according to levels and risk: no hazard (THQ ≤ 0.1), low hazard (THQ 0.1–1), moderate (THQ 1–10) and high hazard (THQ > 10) (Lemly, 1996). The total analysis for a specific contaminant, for example EDI and THQ of Cd in male and female according to FAO and USDA daily intakes, was considered the total (100 %), and each hazard level summed to calculate the specific percentage of the total. In Figs. 2 and 3 is presented the percentage of each hazard level per contaminant. The complete dataset is available in Appendix A.

EDI and THQ (Fig. 2) were higher for simulations with USDA and USDHHS (2020) consumption profile than FAO due to recommended ingestion of shellfish being higher than current mean. The lowest daily consumption of shellfish was identified in Africa (0.55 g day^{-1}) and highest in Asia (16.24 g day^{-1}).

THQ for potentially toxic elements (PTE) is presented in Fig. 2. Highest THQ were observed in Asia, and to Cu based on the PTE concentrations reported in shellfish. THQ was lower for As and Cr, with a moderate/low risk/no hazard levels. EDI and THQ were expressively higher in studies where shellfish were sampled from severely contaminated areas as reported by He et al. (2016). The authors calculated the HQ for Cu, Cd, and Zn in *Crassostrea angulata* and *Crassostrea hongkongensis*, based on the reference doses (RfD) and estimated daily intake (EDI) as established by the USEPA (2005), considering the average body weight of a person from China and specific metal bioaccessibility (<100 %). THQ reported agreed with the

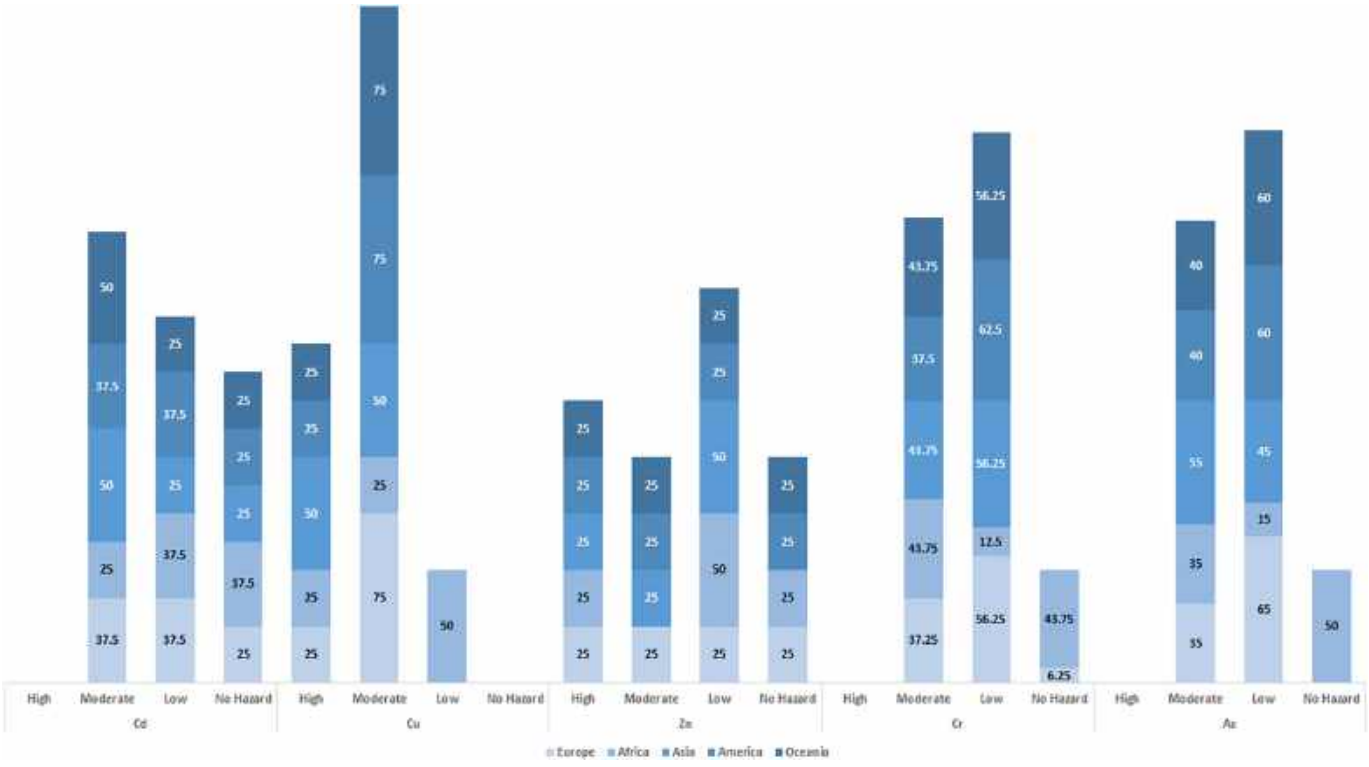


Fig. 2. Distribution of potentially toxic elements hazards (%) by continent.

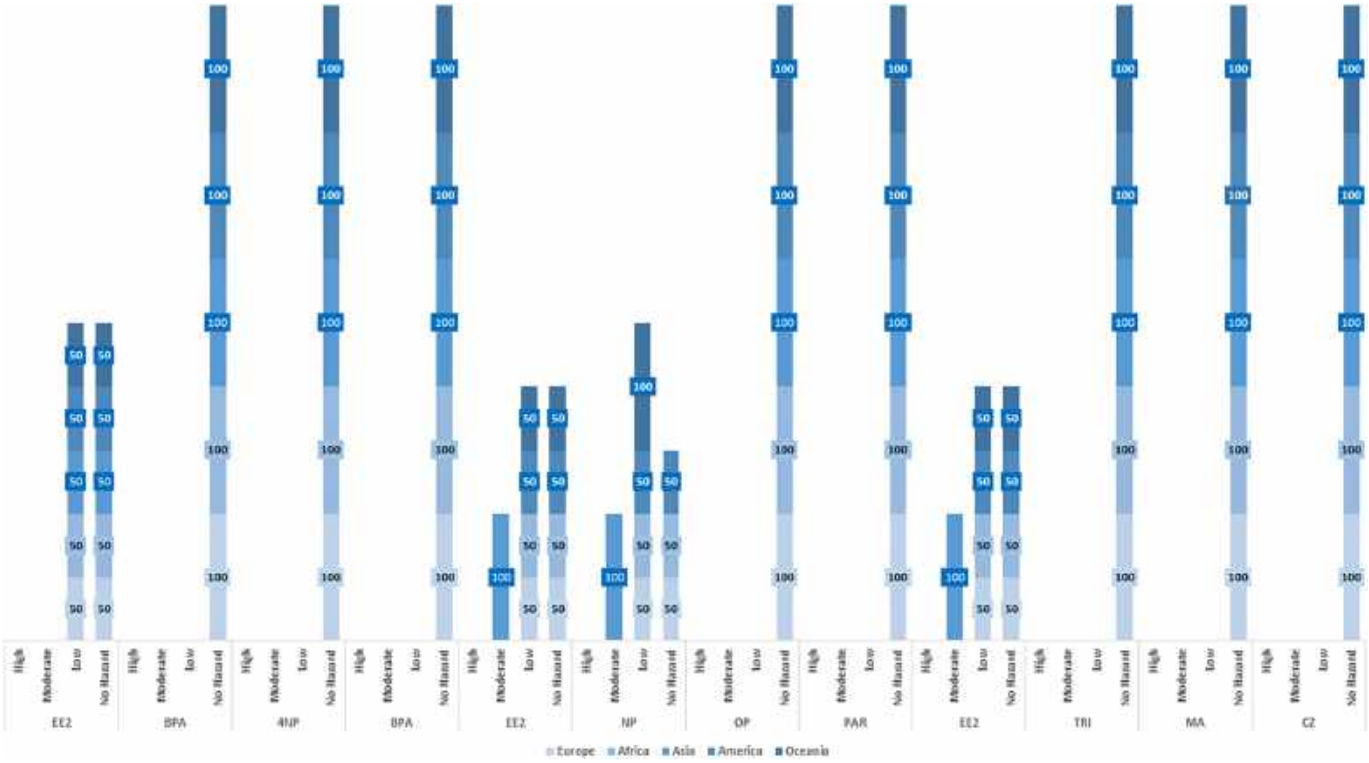


Fig. 3. Distribution of pharmaceuticals hazards (%) by continent.

authors, signalling a high/moderate risk for Cu, Cd and Zn from consuming *C. angulate* and *C. hongkongensis* with contamination levels from reported waters. Cu concentration reported by the authors led to the highest THQ (Fig. 2), with Asia having 50 % of population in a potential scenario of high exposure. Yuan et al. (2020) considered the average weight of a person from China in their study and determined the HQ, bioconcentration factor (BF), cumulative risk (AR) and carcinogenic risk (CR) of Cu, Pb, Zn, Cd, Cr, Hg, and As in *P. viridis*, *M. edulis*, *O. glomerate*, *B. virescens*, *G. divaricatum*, and *C. scripta*. The authors identified high BF for Cu and Zn, calculated by dividing the concentration of PTE in bivalves by concentration of PTE in the sampled seawater.

Yuan et al. (2020) In another study in Bosnia and Herzegovina, Djedjibegovic et al. (2020) determined the health risk of exposure to Cd, Hg, and Pb in commercial seafood products by estimating the weekly intake, hazard index, target hazard quotients, and percentage of tolerable weekly intake by adults of average weight of 70 kg over a lifetime of 70 years. No risk was identified for shellfish based on the consumption profile of adults in Bosnia and Herzegovina. El-Shenawy et al. (2016) estimated the exposure to Al, Cd, Cr, Cu, Co, Fe, Mn, Mo, Ni, Pb, Sr, V, and Zn for local consumers of *Ruditapes decussatus* and *Paphia undulata* shellfish in Ismailia, Egypt, considering the consumption rate of a specific body weight group. Fe, Al, Zn, and Sr had the highest concentration in the bivalves, and Pb was nearly two times higher than the maximum limit; however, the HQ was lower than the limit for all metals.

THQ of PHARs values are presented in Fig. 3. Highest continental exposure was identified in Asia and a similar exposure between Europe, Africa, America and Oceania. Nonylphenol (NP) was the highest hazard PHAR, with moderate to no hazard THQs. NP is a synthetic organic compound widely used in the production of surfactants with extensively estrogenic activity reported. Its persistence and high accumulation in sediments was reported by Zhang et al. (2011) with a concentration of 1964.8 ng g^{-1} in Yundang Lagoon of Xiamen, China. Asia would be more affected to pharmaceuticals in shellfish with a moderate exposure to ubiquitous contaminants such as EE2 and NP, although Europe, Africa, America and Oceania had a THQ < 1. BPA, OP, PAR, TRI, MA and CZ were not present in hazard levels (THQ ≤ 0.1) and no high hazard (THQ > 10) was observed for listed PHAs. Interestingly, concentration of BPA in different shellfish species and locations reported by Chiu et al. (2018) and Zhang et al. (2011) resulted in the same exposure level in all continents. A similar THQ for EE2 was observed by Chiu et al. (2018), Zhang et al. (2011) and McEneff et al. (2014) at different locations and shellfish species. A variety of PHARs are usually present at same time in water and shellfish. If combined and calculated as hazard index, PHARs can potentially threat consumer safety and increase its hazard level.

Mello et al. (2022) reported the occurrence of PHAR in highly consumed bivalves and at Parnaiba River Delta (Brazil) and Sepetiba Bay (Brazil). Risk assessment and human exposure were also assessed. The presence of furosemide (FUR), carbamazepine (CBZ), ketoprofen (KET), bezafibrate (BZF), ibuprofen (IBU), gemfibrozil (GFB), diclofenac (DIC), simvastatin (SIM) was identified in the bivalve species *A. brasiliana* and *M. edulis*, with a consumer daily exposure to SIM, FUR and IBU up to 10 ng kg^{-1} body weight of bivalve. *A. brasiliana* had the highest human exposure of 20.3 ng kg^{-1} body weight when considering all PHARs analysed. The estimated exposure was considered safe as target hazard quotient and hazard index were below 1 for all species and PHARs. However, only one metabolite was analysed in the study and the exposure could be underestimated. After ingestion, a drug can be metabolised by hydrolysis, oxidation, reduction and several reactions before reaching the shellfish. The constant monitoring of coastal areas and surroundings of shellfish farms is necessary to evaluate the level of contamination and register possible alterations from constant exposure to low concentrations.

The highest estimated daily exposure (EDI) to microplastics (MP) in shellfish was observed in Asia. Devriese et al. (2015) reported the presence of 0.68 microplastics/gram of shrimp. The consumption of 48.6 g of shellfish would result in the ingestion of 0.53 g of microplastics a day — approximately 1 % of shrimp weight corresponding to microplastics. Levels of microplastics in the papers analysed were not distinct by species, van Cauwenberghe and Janssen (2014) and Devriese et al. (2015) reported 0.2 and 0.36 microplastics/g of *M. edulis*, respectively. As observed in Appendix A, EDI ranged from 0.005 to 0.53 microplastics/g, an annual maximum ingestion of 193 microplastics/g of shellfish. A RA of microplastics in bivalve molluscs was recently published by Ding et al.

(2022). The authors analysed the quantitative and qualitative data from 52 peer-reviewed papers and estimated the chemical composition risk, annual dietary intake and load index of microplastics by consumption of bivalve molluscs. Polyethylene terephthalate was the most abundant polymer in bivalves (20.4 % ± 21.2 %) followed by polyethylene (13 % ± 19.2 %), rayon (9 % ± 15.6 %), polypropylene (8.9 % ± 13.3 %), cellophane (8 % ± 17.7 %), polyester (7 % ± 12.4 %) and polyamide (5.3 % ± 14.8 %). The global abundance was in the range of 0.04–20 microplastics/g. Iran, Greece and China were the three countries with the highest levels. Median intake of microplastics *via* mollusc consumption was 751 microplastics per person. Differences in mollusc consumption, microplastics abundance, culture and geography were aspects associated with the wide intake range of 15–7333 microplastics/person.

The estimation of EDI and TDH for contaminants in shellfish based on levels available in publications, reports and papers allowed the construction of two consumption scenarios: first with the recommended portion of shellfish (USEPA), and the current mean in each continent (FAO). However, there is a significant variability in the shellfish consumption between countries of the same group (continent) and it would be necessary to calculate separately to provide accurately the status of each region. The objective of calculating EDI and THD showed step-by-step how risks can be easily estimated, allowing the small shellfish producers to estimate the local exposure and support decision-making. Many contaminants can be accumulated by shellfish when farmed in contaminated areas, a higher risk when compared to single analysis. The constant monitoring of water, soil and shellfish is essential to provide accurate assessment of shellfish risk and quality and must be associated to bioavailability studies to inform the %contaminant directly available. Most of the RA conducted for the shellfish industry are related to PTE (Hantoro et al., 2019). There remains a wide diversity of contaminants (*e.g.* plastics and pharmaceuticals) that must be comprehensively studied in order to ensure development of appropriate RA to inform management and where applicable, changes to policies.

8. Conclusions

- In the present study, the environmental contaminants microplastic, pharmaceuticals and potentially toxic elements that threaten the shellfish industry were widely discussed.
- The effects and consequences in the most consumed shellfish species were highlighted and promising mitigation alternatives registered.
- Regulation and international guidelines were provided that can potentially assist decision making and contamination control.
- A panorama of Irish waters was provided. As noted in other countries, there is a gap in understanding the presence, extension of contamination and effects of the contaminants discussed.
- There is a need for the development of techniques for monitoring, standardization of risk assessments and detection of emerging contaminants in the environment.
- The reduction of contamination level in shellfish waters and employment of a risk assessment (RA) approach is essential to ensure consumer safety.
- International consensus must be reached on oral reference dose and exposure limits. There is a pressing need to simultaneously evaluate, model and predict the plethora of influencing factors governing the efficacy of RA models to inform decision making in real time.

CRediT authorship contribution statement

Gustavo Waltzer Fehrenbach: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **Robert Pogue:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Frank Carter:** Methodology, Writing – review & editing. **Eoghan Clifford:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Neil Rowan:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

Gustavo Waltzer Fehrenbach reports financial support was provided by Irish Research Council.

Acknowledgements

We would like to thank the Technological University of The Shannon – Midlands Midwest. The research conducted in this publication was funded by the Irish Research Council under grant number GOIPG/2020/807.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157067.

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Journal of Dairy Research (2018) **85** 366–374. © *Hannah Dairy Research Foundation 2018*
doi:10.1017/S0022029918000614

DairyWater: striving for sustainability within the dairy processing industry in the Republic of Ireland

William Finnegan[1,2], Eoghan Clifford[1,2], Jamie Goggins[1,2,3], Niall O'Leary[4], Alan Dobson[4], Neil Rowan[5], Liwen Xiao[6], Song Miao[7], Kelly Fitzhenry[1,2], Peter Leonard[1,2], Emma Tarpey[1], Beatriz Gil-Pulido[4], Fei Gao[6] and Xinmin Zhan[1,2]*

[1] College of Engineering and Informatics, National University of Ireland, Galway, Ireland
[2] Ryan Institute for Environmental, Marine and Energy Research, National University of Ireland, Galway, Ireland
[3] Centre for Marine and Renewable Energy (MaREI), Galway, Ireland
[4] School of Microbiology, University College Cork, Ireland
[5] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[6] Department of Civil, Structural and Environmental Engineering, Trinity College Dublin, Ireland
[7] Teagasc Food Research Centre, Moorpark, Fermoy, Co. Cork, Ireland

Received 19 October 2017; accepted for publication 15 July 2018

This Review describes the objectives and methodology of the DairyWater project as it aims to aid the Irish dairy processing industry in achieving sustainability as it expands. With the abolition of European milk quotas in March 2015, the Republic of Ireland saw a surge in milk production. The DairyWater project was established in anticipation of this expansion of the Irish dairy sector in order to develop innovative solutions for the efficient management of water consumption, wastewater treatment and the resulting energy use within the country's dairy processing industry. Therefore, the project can be divided into three main thematic areas: dairy wastewater treatment technologies and microbial analysis, water re-use and rainwater harvesting and environmental assessment. In order to ensure the project remains as relevant as possible to the industry, a project advisory board containing key industry stakeholders has been established. To date, a number of large scale studies, using data obtained directly from the Irish dairy industry, have been performed. Additionally, pilot-scale wastewater treatment (intermittently aerated sequencing batch reactor) and tertiary treatment (flow-through pulsed ultraviolet system) technologies have been demonstrated within the project. Further details on selected aspects of the project are discussed in greater detail in the subsequent cluster of research communications.

Keywords: Dairy processing, Ireland, sustainability, tertiary treatment, wastewater treatment.

Ireland has a long tradition of dairy farming and the production of dairy products for international markets, primarily butter and cheese. Today, Ireland is one of Europe's largest producers of cow's milk and a globally recognised producer of dairy products and ingredients. Over the last two decades, Ireland has become one of the world's leading producers of infant nutritional products with the presence in Ireland of a number of leading infant nutrition companies and accounts for 15% of the global supply of infant formula (Barry, 2012).

In 2016, there were approximately 1·295 million dairy cows on over 17 000 dairy farms in the Republic of Ireland, which produced 6654 million litres of milk with a fat content of 4·1% and a protein content 3·45% (CSO, 2017). Unlike most of Ireland's competitors, the vast majority of cows in Irish dairy herds are fed on grass; for up to 300 d a year this is fresh pasture grazing, while, for the remainder of the year, the main source of fodder is grass silage. However, since milk production is essentially grass-based, it is very seasonal with a milk production ratio of 7 to 1, when comparing May to January (IFA, 2012). Irish milk is produced mainly by three cattle breeds; British Friesian, Holstein Friesian and Jersey. In 2016, an additional 813 million litres of milk was imported from Northern Ireland into Irish dairies, bringing the total amount of milk processed in the Republic of Ireland to 7467 million litres (CSO, 2017).

Currently, dairy ingredients and products comprise almost 30% of the Irish food and drink export market. In 2013, dairy ingredients and products surpassed €3 billion for the first time, making it Ireland's largest indigenous

*For correspondence; e-mail: xinmin.zhan@nuigalway.ie

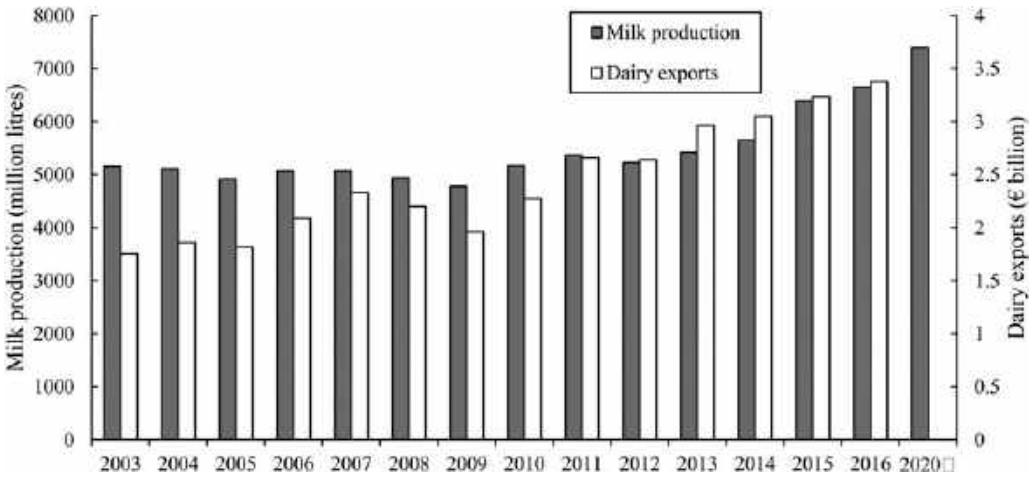

Fig. 1. Domestic milk production in the Republic of Ireland between 200 and 2016 and included the projected volume ([a]) for 2020; data obtained from CSO (2017) and NMA (2017).

industry that reached €3·4 billion in 2016 (NMA, 2017), which can be seen in Fig. 1. The increasing value of Irish dairy produce is also evident from this graphic, as Irish dairy processors move towards high value dairy products and ingredients. Traditionally individual dairy processing factories would have produced either cheese and whey products or butter and powder products (Geraghty, 2011). However, with the growth and development of the industry, certain dairy processing factories now specialise in the production of powders, cheeses or whey products. Approximately 7% of Ireland's milk is used for liquid milk consumption with the remainder (93%) manufactured into a variety of products, including butter, cheese, milk powder, whey and proteins (NMA, 2017).

At present, the Republic of Ireland is on the brink of a new era for the dairy industry as European milk quotas, which restricted milk production since 1984, were abolished in March 2015. As a result, milk production is expected to increase by 50% by 2020, based on the reference years 2007 to 2009 (Farrelly et al. 2014). Since their abolition, an immediate increase in milk production has been instigated, which is evident from Fig. 1.

This increase in the volume of milk being processed, together with stringent measures on emissions from the industry and growing commercial drive for operational efficiencies is driving the need for innovative technological and operational solutions within the dairy processing industry. In this context DairyWater, a multi-stakeholder research project, is developing innovative solutions for the efficient management of water consumption, wastewater treatment and the resulting energy use within the country's dairy processing industry. This project has the potential to position Ireland at the forefront of European, or indeed international, research in this sector as it strives to make the Irish dairy processing industry more efficient and environmentally sustainable by reducing carbon footprints, energy and water use. This will, in turn, lead to greater potential for exports, increased international competitiveness for Irish products and stimulate job creation.

This paper outlines the project aims and technologies that are being explored in the DairyWater project. Much of the data presented in this paper has been compiled through direct contact with the Irish dairy processing industry, due to the lack of published data available. However, although the presented study is based on the Irish dairy industry, it has relevance internationally as processing technologies are similar to those employed in other developed countries, in particular those European countries whose milk production was previously restricted by the milk quotas. The study would be particularly comparable to other countries with pasture based milk production, such as New Zealand, the United Kingdom, Galicia in Spain and Michigan in the United States of America. The overarching aim of the project is to aid the Irish dairy processing industry in becoming more sustainable. In order to achieve this the project intends to efficiently and effectively treat wastewater effluent from dairy processing factories using a range of innovative biological, nanomaterial-based and disinfection technologies. In parallel, the efficient use of water (and resulting energy costs) within the factories is also being explored. Additionally, a study on the environmental impact of the Irish dairy industry, along with an assessment of the contribution water and wastewater treatment at the dairy processing factory make to this, is performed. Selected aspects of the project have been discussed in greater detail as a cluster of research communications, which are entitled:

- Efficient treatment of dairy processing wastewater in a laboratory scale Intermittently Aerated Sequencing Batch Reactor (IASBR) (Leonard et al. 2018a)
- Efficient treatment of dairy processing wastewater in a pilot scale Intermittently Aerated Sequencing Batch Reactor (IASBR) (Leonard et al. 2018b)

https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

- Dominance of the genus *Polaromonas* in the microbial ecology of an Intermittently Aerated Sequencing Batch Reactor (IASBR) treating dairy processing wastewater under varying aeration rates (Gil-Pulido et al. 2018)
- Potential of using synthesised nano-zeolite for ammonium and phosphate immobilisation in dairy wastewater (Gao et al. 2018)
- Microbiological characterisation and impact of suspended solids on pathogen removal from wastewaters in dairy processing factories (Fitzhenry et al. 2018)
- What is the environmental impact of the dairy processing industry in the Republic of Ireland? (Finnegan et al. 2018)

The main aim of the DairyWater project is to aid the Irish dairy processing industry in becoming more sustainable. In order to ensure that the research remains relevant to the Irish dairy processing industry throughout the project, the research team worked closely with leading industry stakeholders and a project advisory board is in place, which included leading members of the dairy processing industry and government funded bodies. Due to the fact that there is very little detailed information available in the published literature, it was essential for the industry partners to provide data, host site visits and facilitate pilot-scale activities, during the project; thus enabling potential commercial benefits of this research to be realised. The project can be divided into the following main research areas, which are discussed in the remainder of this section:

- water consumption and dairy wastewater characterisation;
- dairy wastewater treatment technologies and microbial analysis;
- water re-use and rainwater harvesting; and
- environmental assessment.

Water consumption and dairy wastewater characterisation

Initially, in order to gain a greater insight into the Irish dairy processing industry, a series of site visits, which included site data surveys and sampling of water and wastewater streams, took place. The feedback from these visits and surveys further reinforced the need to a reduction in water consumption at the factories and for more efficient and environmentally friendly solutions for wastewater treatment. Grab samples were used to investigate the water and wastewater streams within the dairy processing factories, which were taken during site visits due to time and resource constraints. The results of part of the survey, relating to the on-site water consumption and a breakdown of water usage, is given in Table 1 for 5 Irish dairy processing factories. Overall, the largest consumer of water on-site is related to cleaning, where a summary of the main water users within a dairy processing factory is shown graphically in Fig. 3. The wastewater produced, as a result of its high nutrient and microbial quantities, needs to be treated to reduce its environmental impact (such as eutrophication) on receiving waters. Therefore, a nutrient and microbial analysis was performed on samples of this wastewater to give an indication of the typical concentrations of key nutrients, where the results are given in Table 2. However, it can be seen in Table 2 that these nutrients vary greatly from site-to-site and, based on discussions with site personnel, can vary greatly throughout the day at a single site. For example, in this analysis, ammonium nitrogen, ortho-phosphate and coliforms vary between 0·9–184·2 mg/l, 5–102 mg/l and 3– 450 000 CFU/ml, respectively. The details relating to the operation of the facility and the results of the site data surveys were used as direct inputs in order to perform the environmental assessment of the Irish dairy processing industry.

Dairy wastewater treatment technologies and microbial analysis

One of the most central aspects of the project is to investigate the potential for the intermittently aerated sequencing batch reactor (IASBR) technology as solution for treating dairy wastewater. This technology has been selected as it previously showed potential for treating high strength wastewater and the resources of the project only allow for the complete investigation of one wastewater treatment technology, although the research team acknowledges that there may be other suitable solutions. Its viability within the Irish dairy industry, along with the development of an operating procedure for the IASBR technology to allow it to perform efficiently when treating dairy wastewaters, is investigated during the project. This technology has a distinct advantage over current systems as it is a biological wastewater treatment system in which wastewater is completely mixed with the microorganisms in the system for the duration of the react phase in order to remove nutrients and treat the wastewater. Previously, the IASBR technology has been applied in the treatment of municipal wastewater (Henry et al. 2013), high strength slaughterhouse wastewater (Henry, 2014; Pan et al. 2014) and, at laboratory-scale, dairy wastewater (Tarpey, 2016). Since wastewaters with high biodegradability are suitable for treatment by biological processes (Pan et al. 2014), the IASBR system has the potential to treat dairy wastewater efficiently. Additionally, the technology has been shown, if managed correctly, to be superior in certain industries in the removal of nitrogen and phosphorus than the more traditionally used sequencing batch reactor technology for the treatment of the wastewater (Pan et al. 2013; Henry, 2014).

In order to develop the technology, experimental procedures at both laboratory-scale and pilot-scale IASBR systems will be investigated. Nutrients which are commonly known to be responsible for eutrophication, primarily nitrogen and phosphorus, (Pan et al. 2013) will be removed and their removal efficiencies will be monitored, as well as the operating cost and energy demand. This will aid in determining the most efficient and effective solution when compared technologies currently employed in the Irish dairy processing industry. The initial step of the project was to

https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

Table 1. Results from a survey of 5 Irish dairy processing factories relating to water consumption, including a breakdown in water usage within the factory

	Site 1		Site 2		Site 4	Site 5		Site 6
Products processed	Fluid milk	Cream	Milk powder	Butter	Milk powder	Milk powder	Butter	Infant formula
Milk processed (million litres)	50		206		80	587		125
Total water consumption (m^3)	58 000		155 000		82 000	500 000		438 000
Water consumption (litre per litre milk)	1·16		0·75		1·03	0·85		3·5
CIP	56%	1·7%	33%	10%	75%	53%	7%	15%
Boilers & flushing	3%	0·1%			25%	13%	2%	2%
Cooling						22%	3%	
Intake	14%	0·5%						
Separators				4%				
Butter factory				8%				
Deaerator				8%				
Dryers			14%					24%
Evaporators			14%					
Tanker wash			7%	2%				
WWTP	7%	0·2%						
Other	17%	0·5%						59%

Legend: clean-in-place system (CIP); wastewater treatment plant (WWTP).

Table 2. Results from a nutrient and microbial analysis of samples of wastewater (prior to on-site wastewater treatment) at 6 Irish dairy processing factories

	pH	TSS (mg/l)	COD	BOD_5	NH_4	Nitrite	PO_4	Coliforms (CFU/ml)	*E. coli*
	–								
Site 1	N/A	2370	9770	4900	7·8	0·144	5·3	$1·00 \times 10^2$	$8·35 \times 10^1$
Site 2	N/A	750	1430	N/A	0·9	4·5	5·0	N/A	N/A
Site 5	9·8	915	3310	3300	184·2	0·1	8·1	$4·50 \times 10^4$	$0·00 \times 10^0$
Site 7	5	490	3270	1900	20·1	0·065	31·7	$1·35 \times 10^5$	$2·55 \times 10^1$
Site 8	6·3	311	1210	1200	6·1	0·0	6·6	$4·50 \times 10^5$	$5·63 \times 10^2$
Site 9	N/A	205	1171	375	1	0	102	$3·00 \times 10^0$	$0·00 \times 10^0$

Legend: ammonium nitrogen (NH_4); 5-day biochemical oxygen demand (BOD_5); chemical oxygen demand (COD); colony-forming unit (CFU); *Escherichia coli* (*E. coli*); ortho-phosphate (PO_4); test not performed (N/A); total suspended solids (TSS).

replicate the technology in a laboratory setting (using a laboratory-scale system containing three 8 l cylindrical units, which is shown in Fig. 2, to gain a greater understanding of the technology and to create parameters from which a pilot-scale system could be designed. This laboratory-scale system not only proved the validity of the technology for the treatment of dairy wastewater, but also determined initial operating parameters for the pilot-scale IASBR system. The pilot-scale IASBR system has been designed and installed on-site at the wastewater treatment plant of an Irish dairy processing factory and has a working volume of 3000 l, which is shown in Fig. 2. The system is supplied with wastewater from the processing factory and is monitored daily using an on-site automated refrigerated sampler. These samples are tested weekly in order to monitor the performance of the system. This pilot-scale system gives a greater insight into the performance of the IASBR technology at a near commercial-scale.

In order to gain a greater understanding about the performance of the IASBR technology, the bacterial communities underpinning nutrient removal efficiencies arising from the laboratory-scale and pilot-scale systems have been profiled. Microorganisms are they key contributors in biological wastewater treatment processes and the knowledge of bacterial community structures contributes to the development of strategies for process optimisation (Daims et al. 2006). Therefore, during this research, next generation sequencing (NGS) methods will be applied to IASBR biomass samples. Sample collection is intended to provide representation of varying operational parameters at laboratory-scale (e.g. aeration rates), pilot-scale (e.g. cycle length) and varying influent compositions (e.g. synthetic *vs.* industrial influent). NGS outputs were subjected to comprehensive *in silico* analyses to determine microbial community structures and to facilitate correlations between relative microbial abundance and reactor performance (Zhang et al. 2012; Weissbrodt

https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

Fig. 2. Experimental systems used as part of the DairyWater project. Clockwise (from top left): laboratory-scale intermittently aerated sequencing batch reactor (IASBR) system; laboratory-scale pulsed ultra violet (PUV) system; low pressure ultra violet (LPUV) system; pilot-scale IASBR system.

et al. 2014). In silico metabolic profiling of the bacterial community was also performed to predict the abundance of functional gene families and to identify potential, key contributors to nutrient bioremediation (Ahmed et al. 2017). In an effort to corroborate the modelled outputs with respect to NGS determined dominant groups and metabolic functionalities, spatial distribution analyses was also performed. Fluorescence in situ Hybridisation (FISH) and quantitative polymerase chain reaction (qPCR) will be used for the enumeration of specific targeted microbial groups of interest and their spatial organisation within the representative biomass samples (Nielsen et al. 2009; van Loosdrecht et al. 2016). This study seeks to address the knowledge gap which exists in relation to the microbial community structures underpinning IASBR application. Such knowledge is critical to the optimisation of the IASBR technology, where a reliance on key microbiota can be demonstrated. While the target application of this project is the dairy processing sector, the findings have potential significance in the treatment of other agri-industrial wastewaters.

The use of nanomaterials to improve the efficiency of wastewater treatment processes in this sector has not previously been well explored. Therefore, a selection of nanomaterials have been tested in order to seek an appropriate one for simultaneous removal and recovery of nitrogen, phosphorus and other contaminants for the treatment of dairy wastewater. The nanomaterials tested in the study include nano-zeolite, surface modified nano-zeolite, carbon nanotubes and activated charcoal (Camblor et al. 1998). The physicochemical characteristics of nano-zeolite, such as high mechanical and chemical resistance and its high surface area, have formed the basis for its widespread use in catalysis, separation, and ion-exchange (Song et al. 2005). In this study, the feasibility of using coal fly ash to synthesise nano-zeolite was studied, along with the nitrogen and phosphorus adsorption efficiencies of the nano-zeolite. In addition, surface modifications were conducted by adhering Cu, Mg, Fe, Zn, Ca ions and Hexadecyltrimethylammonium (HDTMA) onto the surface of nano-zeolite in order to change the surface properties and enhance the contaminants removal efficiency.

https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

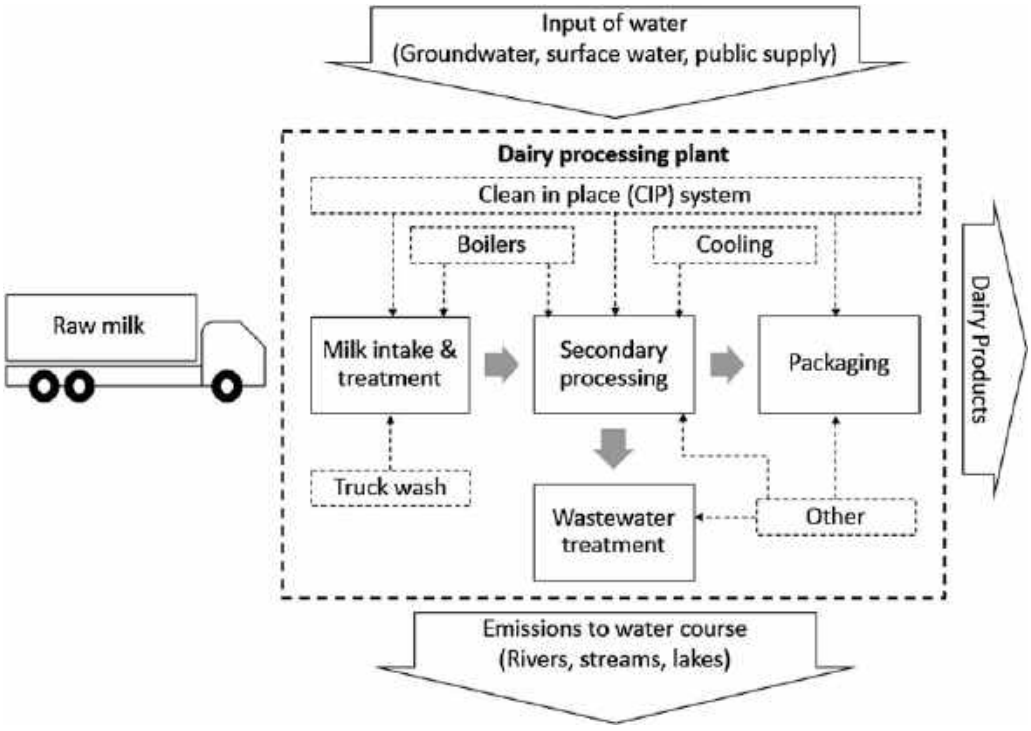

Fig. 3. Schematic summarising processing within a dairy processing factory and the main water users within the manufacture of dairy products.

Comparative batch experiments were conducted to study the effect of sorption time, pH values and dosage of nanomaterials on the contaminant removal efficiency. Additionally, in order to minimise the operation cost, the regeneration of nanomaterials after use has been considered. A microbial fuel cell (MFC) has been chosen instead of typical acid/alkaline wash, as MFCs possess the ability of generating electricity from organic matter using exoelectrogenic bacteria during wastewater treatment (Logan, 2008). The possibility of regenerating the nanomaterials and energy recovered by using MFCs will be examined further in this research.

Water reuse and rainwater harvesting

Given the significant quantities of water consumed by the Irish dairy processing industry, the reuse of wastewater within the sector may be necessary for economic, regulatory and sustainability purposes (Geraghty, 2011). A key barrier to water reuse within this sector (and indeed other industries) is the implementation of pathogen removal. This study compares a flow-through pulsed ultraviolet (PUV) system (Barrett et al. 2016) and a continuous low-pressure UV (LPUV) system, which are both shown in Fig. 2, as potential technologies for (i) tertiary wastewater treatment plant effluent disinfection and (ii) disinfection systems to restore dairy wastewater to reusable levels for certain dairy processing factory practices. The two systems were compared in terms of energy usage and bacterial UV dose response and the impact of dairy wastewater parameters, primarily suspended solids (SS), on the system, along with the inactivation efficiency of *E. coli*.

Preliminary work included a nationwide dairy processing factory site survey followed by the microbiological characterisation of water/wastewater streams. The sample streams tested included cooling waters, condensate water and wastewater treatment plant (WWTP) influent and effluent. Feedback from this survey analysis indicated low levels of water reuse practices at dairy processing factories however the introduction of microbiological effluent discharge standards is likely in the near future. The key findings from the microbiological characterisation included the detection of faecal indicators in 11 out of the 12 samples collected including all three WWTP effluent samples. Pathogenic bacteria typically associated with the dairy industry were also detected; *Listeria monocytogenes* was present in all samples at two sites, while *Campylobacter* spp. was also present in cooling waters and wastewater treatment plant effluent at one of the sites. *Bacillus pumilus* and *Bacillus subtilis* endospores have been investigated as they are noted to exhibit increased resistance to LPUV in comparison to vegetative cells (WRF, 2010; Boczek et al. 2016).

From the results, inert suspended solids do not appear to inhibit PUV or LPUV efficiency at high SS concentrations (30 mg/l and above) while organic particles impacted on the PUV and to a lesser extent on LPUV efficiency. A PUV dose of approximately 2000 mJ/cm^2 was required for a 2

https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

log inactivation of *B. pumilus* endospores while a LPUV dose of 30 mJ/cm^2 was required for a 5 log inactivation. The effect of agar supplement manganese sulphate ($MnSO_4$) on the enhanced UV resistance of endospores has also been analysed. Cultivation agar supplemented with $MnSO_4$ appears to increase the UV resistance of *Bacillus* spp. endospores when exposed to both PUV and LPUV. Preliminary investigations into the energy efficiency of both systems indicated that the LPUV system exhibits a higher efficiency of converting electrical energy to UV energy in comparison to the PUV system. The photoreactivation potential of dairy pathogens post PUV and LPUV flow-through disinfection is to be investigated, along with 'on-site' dairy wastewater been incorporated into UV disinfection trials to evaluate the potential of UV treatment for low-level water reuse at dairy processing factories.

As dairy production increases, so too will the vast volumes of water required for dairy product output, which currently stands at 2·5 m^3/m^3 of milk processed (Geraghty, 2011). Legislation regarding the discharge limitations of dairy wastewater effluent is becoming increasingly stringent along with initiatives to conserve and reuse water within the industry. Thus, alongside the potential for reusing treated wastewaters, there is significant potential to harvest and use rainwater. A key consideration for the industry in implementing rainwater harvesting is the potential for a positive and benefit to cost ratio and a relatively short pay-back time. Given the relatively low costs of water in Ireland (in some cases where water is withdrawn from private sources there may not be any charge associated with water use) this project focused on the development of design tools that would enable the industry determine the viability of implementing rainwater harvesting at a number of case-study sites. The toolkit also includes a life cycle assessment module which modelled expected life cycle emissions from the construction and operation/maintenance of the rainwater harvesting system. The tool was trialled, based on case-study data from a number of dairy facilities in Ireland. In general it was shown that given current water tariffs, the costs of deploying rainwater harvesting at each site and the potential end-uses for rainwater such systems might not at this time be economically viable.

Environmental assessment

Since the main aim of the DairyWater project is to help form a more sustainable Irish dairy processing industry, particularly from an environmental point-of-view, it is first necessary to establish a benchmark for the industry. This study will also aid in determining the true impact of water and wastewater treatment technologies on the overall environmental impact associated with the manufacture of dairy products. Therefore, life cycle assessment (LCA) is being used in order to estimate the current environmental impact of the Irish dairy industry. Additionally, LCA will be used to evaluate the environmental impact of the technologies developed during the DairyWater project in order to ensure that they will have a positive impact on the environment. In order to ensure the accuracy of this study, it is structured in accordance with the LCA guidelines of the International Organisation for Standardisation (ISO, 2006) and the LCA methodology for the dairy industry published by the International Dairy Federation (FIL-IDF, 2015).

Initially, a macro-scale LCA of the Irish dairy industry, which assessed the global warming potential associated with the main Irish dairy products, was performed (Finnegan et al. 2017a). The life cycle stages included in this study are raw milk production, raw milk transportation and dairy processing and publically available data, along with national statistics, were used. This study identified the critical processes, inputs and emissions that are necessary in delivering an accurate LCA. The main LCA performed during the study is an environmental LCA of selected dairy products in the Republic of Ireland, which included a number of environmental impact categories. This analysis was performed for a number of dairy products, including milk powder, butter, fluid milk, cream, infant formula, cheese and whey powder. So as to perform this analysis, data was collected from 11 dairy processing factories within the Republic of Ireland for 2013, which process approximately 49% of the total raw milk processed. Due to the availability of data, comprehensive studies relating to the production and manufacture of milk powder and butter have been carried out (Finnegan et al. 2017b, c). From these studies, the environmental impact of these two products have been estimated and the processes (within the dairy processing factories) that are the most significant contributor to the impact have been identified.

Along with performing an assessment of Irish dairy products, both an economic cost–benefit analysis and an environmental assessment (using LCA) of the potential positive impact of novel technologies investigated in this project will be carried out. The results of this analysis will be compared to existing dairy water and wastewater treatment technologies in order to demonstrate how sustainability may be improved.

The technologies being developed in this project have the potential to greatly increase the sustainability of the Irish dairy processing industry, while also returning a financial benefit. For example, the proposed IASBR system is a biological wastewater treatment system and, thus, does not require any chemicals, which is one of the largest expenses when treating wastewater. Additionally, as Ireland's agricultural and agri-food sector continues to grow, it has been suggested that a carbon tax on dairy processors, similar to the carbon tax paid by motorists for their cars and householders for their fuel bills, may be introduced as an incentive to reduce emissions (Melia, 2015).

At current milk processing levels, many dairy processors in Ireland are at the limits of their emissions to water, as set by the Irish environmental protection agency (EPA). As a result of the increase in raw milk production due to the European milk quotas being abolished, processors will be

https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

under increased pressures to remain within these limits. Additionally, in 2015, domestic water charges were introduced in the Republic of Ireland for the first time. Even though this didn't affect the Irish dairy processing industry, as groundwater and surface waters are the main source, it may in the future if extraction charges are introduced. Therefore, water reuse, particularly the reuse of wastewater as process water, may be necessary to reduce the water footprint of dairy processing factories and to reduce costs associated with production. The tertiary treatment systems proposed in this project, PUV and LPUV systems, would be vital in exploiting the potential of water reuse together with meeting the requirements of dairy processing factories to comply with strict regulations when emitting treated effluent to rivers and lakes, such as those implemented by the Water Framework Directive (WFD – 2000/60/EC), Surface Waters Regulations (S.I. No. 272 of 2009) and the European Community Shellfish Waters Directive (2006/113/EC).

In conclusion, this article outlines the project aims and technologies that are being explored in the DairyWater project, where the main aim is to aid the Irish dairy processing industry in becoming more sustainable. As Europe moves into a post-quota era, it will be essential for Ireland to improve its sustainability and maintain its 'green' image if it is to remain competitive internationally. Therefore, the DairyWater project has the potential to position Ireland at the forefront of European, or indeed international, research in this sector. The project strives to make the Irish dairy processing industry more efficient and environmentally sustainable, which will lead to greater potential for exports and increased international competitiveness for Irish products, along with stimulating job creation. In addition, the results of the study are suitable to be applied internationally as, although milk production conditions may not be pasture based, processing, and many of the associated environmental impacts and challenges that arise, are generally the same when using modern processing technologies.

The authors would like to acknowledge the funding provided by the Department of Agriculture, Food and the Marine for the DairyWater project (Ref.: 13-F-507); for additional details see http://www.dairywater.ie. Additionally, the DairyWater research team would like to the members of the Irish dairy sector for their cooperation and contributions throughout the research project.

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https://doi.org/10.1017/S0022029918000614 Published online by Cambridge University Press

Science of the Total Environment 574 (2017) 1140–1163

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Monitoring, sources, receptors, and control measures for three European Union watch list substances of emerging concern in receiving waters – A 20 year systematic review

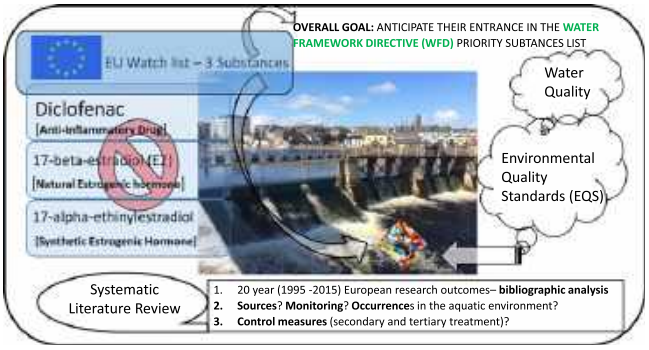
Erin Jo Tiedeken [a], Alexandre Tahar [a], Brendan McHugh [b], Neil J. Rowan [a,*]

[a] Bioscience Research Institute, Athlone Institute of Technology, Co. Westmeath, Ireland
[b] Marine Institute, Rinville, Oranmore, Co. Galway, Ireland

HIGHLIGHTS

- Three EU watch list substances of emerging concern in receiving waters are reviewed
- Pharmaceuticals diclofenac and EE2 along with natural hormone E2 reported above environmental quality standards
- Under monitoring of these substances of emerging concern in many EU member countries
- Need for more sensitive estrogen detection methods to meet WFD limits
- Control measures frequently do not fully remove these harmful chemicals.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 July 2016
Received in revised form 25 August 2016
Accepted 11 September 2016
Available online xxxx

Editor: D. Barcelo

Keywords:
Water framework directive
Diclofenac
17-Beta-estradiol (E2)
17-Alpha-ethynylestradiol (EE2)
Sources
Receptors
Control
Watch list

ABSTRACT

Pollution of European receiving waters with contaminants of emerging concern (CECs), such as with 17-beta-estradiol (a natural estrogenic hormone, E2), along with pharmaceutically-active compounds diclofenac (an anti-inflammatory drug, DCL) and 17-alpha-ethynylestradiol (a synthetic estrogenic hormone, EE2)) is a ubiquitous phenomenon. These three CECs were added to the EU watch list of emerging substances to be monitoring in 2013, which was updated in 2015 to comprise 10 substances/groups of substances in the field of water policy. A systematic literature review was conducted of 3952 potentially relevant articles over period 1995 to 2015 that produced a new EU-wide database consisting of 1268 publications on DCL, E2 and EE2. European surface water concentrations of DCL are typically reported below the proposed annual average environmental quality standard (AA EQS) of 100 ng/l, but that exceedances frequently occur. E2 and EE2 surface water concentrations are typically below 50 ng/l and 10 ng/l respectively, but these values greatly exceed the proposed AA EQS values for these compounds (0.04 and 0.035 ng/l respectively). However, levels of these CECs are frequently reported to be disproportionately high in EU receiving waters, particularly in effluents at control points that require urgent attention. Overall it was found that DCL and EE2 enter European aquatic environment mainly following human consumption and excretion of therapeutic drugs, and by incomplete removal from influent at urban wastewater treatment plants (WWTPs). E2 is a natural hormone excreted by humans which also experiences incomplete removal during WWTPs treatment. Current conventional analytical chemistry methods are sufficiently sensitive for the detection and quantification of DCL but not for E2 and EE2, thus alternative, ultra-trace, time-integrated monitoring techniques such as passive sampling are needed to inform water quality for these estrogens. DCL appears

* Corresponding author at: Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland.
E-mail address: nrowan@ait.ie (N.J. Rowan).

http://dx.doi.org/10.1016/j.scitotenv.2016.09.084

resistant to conventional wastewater treatment while E2 and EE2 have high removal efficiencies that occur through biodegradation or sorption to organic matter. There is a pressing need to determine fate and behaviour of these CECs in European receiving waters such as using GIS-modelling of river basins as this will identify pressure points for informing priority decision making and alleviation strategies for upgrade of WWTPs and for hospital effluents with advanced treatment technologies. More monitoring data for these CECs in receiving waters is urgently needed for EU legislation and effective risk management.

Contents

1. Introduction

Water is an essential resource, crucial to all living organisms and for a diversity of human activities (Barbosa et al., 2016). Drinking and food preparation, support of the natural environment and a growing economy all require a healthy and secure water supply (Rowan, 2011). Unfortunately, there are significant pressures on this fragile resource. Natural and along with other anthropogenic substances such as pharmaceuticals, pesticides, industrial compounds, personal care products, steroid hormones, drugs of abuse and others, end up in surface water, ground water and vital drinking water (Ribeiro et al., 2015) Specifically, this review focuses on monitoring, sources, receptors and control measures for addressing the pharmaceutically-active compounds diclofenac (DCL) and the synthetic hormone 17-alpha-ethinylestradiol (EE2) along with the natural hormone 17-beta-estradiol (E2), which hereafter will be referred to as contaminants of emerging concern (CECs). Pharmaceuticals and their pharmaceutically active metabolites/transformation products are a class of CECs that are widely used in human and veterinary medicine and are essential to modern healthcare (Fent et al., 2006; Nikolaou et al., 2007). Nevertheless, there are growing concerns about the negative impacts that may result from continuous contamination of the environment with pharmaceutically-active compounds (Barbosa et al., 2016; Verlicchi and Zambello, 2016). This research is important because of the potential toxic effects for aquatic biota and human health that may result from chronic exposure to such CECs (Fent et al., 2006; Kümmerer, 2009; Nikolaou et al., 2007). Characteristics specific to this class of environmental contaminants can however present significant challenges for research. For example, CECs exhibit wide variation in function, chemical structure and physiochemical properties, making it difficult to generalize about their behaviour, persistence or impact in the environment. CECs are also designed to be biologically active, have a specific mode of action and to be persistent in the body, meaning they can impact humans and wildlife at trace concentrations which are often hard to detect and quantify using traditional analytical methods (Fent et al., 2006).

Although there are no legal discharge limits for micropollutants into the environment, some regulations have been published in the last few years (Barbosa et al., 2016). CECs in the aquatic environment primarily originate from use in human medicines, however certain classes are also heavily used in veterinary practices (e.g. anti-inflammatory drugs, antibiotics) (Fent et al., 2006; Zhou et al., 2009). A large number of CECs have been detected in WWTPs influents and effluents and surface, ground and drinking water worldwide in recent years (Barreiros et al., 2016; Heberer, 2002; Nikolaou et al., 2007; Ternes, 1998; Zhou et al., 2009; Verlicchi and Zambello, 2016). In fact, it is now established that throughout the developed world, CECs are ubiquitous at μg to ng per

litre levels in the aquatic environment (Nikolaou et al., 2007), although the concentrations of specific compounds depend on usage patterns in different countries and can vary temporally (Verlicchi et al., 2012). The impacts of chronic exposure to trace concentrations of many CECs on wildlife and human health may be severe (e.g. Verlicchi et al., 2012), thus it is critical to limit as much as possible the concentrations of this class of contaminants in our waterways. Certain CECs can specifically impact the endocrine system of humans or wildlife; such chemicals are part of emerging pollutants known as endocrine disrupting chemicals (EDCs). Much of the growing interest in this field of research stems from fears that chronic exposure to EDCs (in bathing or drinking water, for example) may be linked to adverse human health conditions such as declining male fertility, birth defects, and breast and testicular cancer (Nikolaou et al., 2007). Furthermore negative impacts of EDCs exposure on wildlife may include severe consequences such as feminisation in fish (Sumpter and Johnson, 2008). Similar to CECs as a whole, EDCs are mainly thought to be transported into the aquatic environment via incomplete removal at WWTPs (Nikolaou et al., 2007).

Until recently, environmental regulations worldwide had not required explicit testing for any CECs in water bodies. However given the growing concern about contamination of the aquatic environment with these compounds, legislation has recently begun to acknowledge this potential problem. The Water Framework Directive (WFD, 2000/60/EC) is an overarching piece of European environmental legislation aimed at protecting and improving water quality throughout the EU. The WFD committed EU Member States to achieve good qualitative and quantitative status of all water bodies by 2015. In order to reach this goal, certain chemicals identified by Annex X of the WFD have been deemed priority substances; these chemicals (e.g. some pesticides, metals such as lead or mercury, organic volatile compounds and other organics such as polycyclic aromatic hydrocarbon) must be monitored by all member states and cannot exceed specific concentration thresholds in surface waters (defined by the legislation as Environmental Quality Standards, or EQSs). Furthermore, article 16(4) of this legislation requires that the list of priority substances must be reviewed and adjusted as appropriate at regular intervals. As such, directive 2013/39/EU of 12 August 2013 added a further 12 substances to Annex X of the WFD. In addition, Article 8b of Directive 2013/39/EU states that "the Commission shall establish a watch list of substances for which EU-wide monitoring data are to be gathered for the purpose of supporting future prioritisation exercises." In response to growing EU concern about the release of untreated CECs into the aquatic environment, three compounds were included in the first watch list in 2013: diclofenac (CAS# 15307-79-6, hereafter referred as DCL), 17-beta-estradiol (CAS# 50-28-2, hereafter referred as E2) and 17-alpha-ethinylestradiol (CAS# 57-63-6, hereafter referred as EE2). It is relevant to note that the European Commission implemented decision 495 of 20 March 2015 that expanded substances or groups of substances on the watch list to 10 in the field of water policy. Besides the two pharmaceuticals (DCL and EE2) and the natural hormone (E2) that were previously recommended to be included by the Directive 39/2013/EU, the first watch list of 10 substances/groups of substances also refers to the three macrolide antibiotics (clarithromycin, azithromycin and erythromycin) other natural hormone (E1), some pesticides (oxadiazon, methiocarb, imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid and triallate), a UVB filter (2-ethinylhexyl 4-methoxcinnamate) and an antioxidant (2,6-di-tert-butyl-4-methylphenol) commonly used as a food additive. This review focuses solely on the first three substances DCL, E2 and EE2 as there is a requirement to investigate policy implications for Ireland of these PhACs in receiving waters in the first instance. An overview of the EU policy in the water field as it relates to commonly used conventional and advanced treatment processes in the aqueous matrices of these 10 substances/groups can be found in Barbosa et al. (2016). The EU-wide monitoring data that will be produced in the next few years will help legislators determine whether or not these compounds are ultimately added to the list of priority substances from Annex X of the WFD. The WFD requires that all EU member states prepare river basin management plans (RBMPs) to address the many issues relating to water quality and protection in a holistic manner. These RBMPs identify the main pressures and activities affecting water status and propose environmental objectives that must be achieved during certain time periods. The recent European legislation on DCL, E2 and EE2 mentioned above has been identified as potentially significant water management issue that may need to be addressed in the next round of RBMPs (due for publication in 2017).

The overall aim of this literature review was to identify and evaluate all previous relevant EU-wide studies on contamination of the aquatic environment with the three watch list pharmaceuticals DCL, E2 and EE2 in order to anticipate their entrance in the WFD priority substances list and to identify gaps in knowledge aiming at guiding future research. This review is directed towards at-risk industries, companies, researchers, regulators and any sectors that would be affected by the addition of these compounds to future iterations of the WFD priority substance list (toxicology, water treatment, chemical analysis, biology, regulation). Risk assessment was not included in this literature review as it has been addressed by other authors (Camacho-Muñoz et al., 2012; Ferrari et al., 2004; Futran Fuhrman et al., 2015). However, this systematic literature review addresses four main research questions for each compound:

1) What are the likely sources/entry points of these CECs into European aquatic environment?
2) What are the likely receptors and loadings in European waters?
3) What monitoring methods are currently employed to measure aquatic concentrations of these CECs, and what are the current limits of detection/quantification?
4) What control measures (including both source control and treatment options) are effective (or potentially effective) and employed for lowering concentrations of these compounds in the aquatic environment?

2. Materials and methods

2.1. Systematic review protocol and defining search parameters

Even a cursory search of the literature reveals a vast amount of published material regarding the sources, receptors, monitoring and control measures of DCL, E2 and EE2 (Fatta-Kassinos et al., 2011; Johnson et al., 2013; Qian et al., 2015). Consequently this literature review was carried out using a defined systematic approach that answers research questions based on the published evidence, which is gathered using a predefined protocol that was adapted from the Centre for Evidence-Based Conservation's (CEBC) "Guidelines for Systematic Review in Conservation and Environmental Management" (Pautasso, 2013; Pullin and Stewart, 2006). The protocol comprised defining search parameters (databases to be searched, search times, types of publications), selecting search terms, developing eligibility (inclusion/exclusion) criteria, and conducting the literature search and carrying out the article review and selection process to produce publication database and bibliographic analysis. The article review was a two-step process including both a title and abstract filterStudies on the sources, receptors/monitoring and control measures of DCL, E2 and EE2 were identified using the Scopus database and from professional networks that included grey literature sources or sources that would not be returned by the database search (such as PhD theses or government reports) (Pullin and Stewart, 2006).The search was limited to literature published from 1995 to 2015 to ensure the publications included in the final database were up-to-date. The mid 1990s reflected time period when this field of research was in its infancy (Qian et al., 2015). Search terms were selected to ensure all potentially relevant articles were returned from the database searches. Two separate searches were run, one for DCL and one combined search for E2 and EE2. The E2 and EE2 searches were

combined due to a high percentage of overlap in these search results. For both final searches, results were limited to articles, articles in press or review papers. All 28 EU member states were included, as well as Switzerland, Norway and Turkey. Terms for each of the two searches included "water" and "wastewater" in order to focus on articles considering the CECs in aquatic matrices. In order to cover all relevant research, search terms included the class of CECs describing each drug of interest (i.e. "NSAID" or "estrogen") and all relevant synonyms for each specific compound. In order not to miss articles considering the veterinary usage of DCL, which can be a significant source of environmental pollution (Boxall, 2010; Hunt et al., 2015), the term "veterinary" was also included. A list of eligibility criteria was developed so that once all of the potentially relevant articles were located through the searches described above, articles for inclusion in the database could be distinguished (Table 1).

2.2. Article review and selection

Once all potentially relevant articles were identified through the searches, a selection process was undertaken to find articles for inclusion in the final database framed upon meeting eligibility criteria (Table 1). Title and abstract review were undertaken by two researchers with 10% overlap in order to validate consistent choices. During the abstract review, additional fields were added to the spreadsheet by the reviewer (Fig. 1), which were organised into six domains namely topic of article, monitoring type, compounds studies, analytical methods used, study type, and country study was performed in. Articles with authors or fieldwork from multiple countries were counted as full publications for each country, rather than fractionally (Qian et al., 2015). These additional fields were filled in by reading the abstract, or if necessary, by downloading and reading the full-text of the article. The only exception was the analytical method employed for detection; this field was only filled out if the method was specified in the abstract. These additional fields, as well as the bibliographic information provided by Scopus, were utilized to conduct the bibliographic analysis (Section 3.2).

3. Results and discussion

The aim of this systematic literature review was to evaluate current state of knowledge on contamination of the European aquatic environment with DCL, E2 and EE2, especially in regards to sources, receptors, monitoring and control measures. The following sections address the specific research questions this systematic review was concerned with: Sections 3.1 and 3.2 report the results from the bibliographic analysis, Section 3.3 details the key findings on the sources of these CECs in the aquatic environment; Section 3.4 discusses the receptors and concentrations of these CECs in a European context; and Section 3.5 discusses the effectiveness and challenges associated with monitoring methods used to detect these compounds. Finally, Section 3.6 discusses DCL, E2 and EE2 current and potential control measures.

Table 1
Eligibility criteria for systematic literature review; used for title and abstract filter.

Eligibility criteria
- Must specifically discuss at least one of the three compounds of interest
- Cannot focus exclusively on impacts of compound for human/animal/plant health
- Exclude papers that focus only on ecological/environmental/toxicological impacts unless they also discuss relevant sources, receptors/monitoring or control measures
- Exclude clinical trial studies
- Must include some specific information on sources, receptors/monitoring or control measures
- Cannot focus on exposure routes other than water
- Study cannot be purely chemical, i.e. determining a chemical coefficient
- Exclude any papers on leaching of chemicals from bottled water/plastics
- Must be peer reviewed original article or review, or article in press
- Must be published between 1995-May 2015
- Research must be conducted in Europe or by at least one author affiliated with a European country
- Article must be written in English
- Full text must be available

3.1. General overview of the database

Even following strict exclusion criteria (see Section 2.1), the systematic review identified a large number of peer-reviewed publications on the sources, receptors/monitoring and control measures for DCL, E2 and EE2. Fig. 2 demonstrates the enormous number of articles returned by our searches, and the number of articles excluded (and reasons for exclusion) during the title and abstract filters. The database of publications and the summary information regarding this database (bibliographic analysis, Section 3.2) include 1268 publications deemed eligible by the systematic review protocol. Published review studies were analysed for data on monitoring, source, receptors and control measures for Sections 3.3 to 3.6 where summary data on three topics was extracted: (i) concentrations of DCL, E2 or EE2 in influent or effluent, and their removal efficiencies during various wastewater treatments; (ii) concentrations of these three CECs in surface, ground or drinking water; and (iii) methods of detection and limits of detection (LODs) for each of the three compounds.

3.2. Bibliographic analysis: State of European research on DCL, E2 and EE2

Bibliographic analyses are particularly useful for fields with large bodies of research that are difficult or impossible to summarize via traditional, full-text review studies (Belter & Seidel, 2013). They are also important for defining gaps in the literature and directing future research (Qian et al., 2015). This bibliographic analysis originates from the database of publications created during the systematic review; it summarizes the state of European research on DCL, E2 and EE2 from 1995 to May 2015 (details of the methodology used to create the database are provided in Section 2).

3.2.1. Pharmaceuticals studied

EU database constituted of 628, 697, and 665 EU studies reported on DCL, E2 and EE2 respectively as per alignment with eligibility criteria (Fig. 1). Many of the individual studies in the database reported on more than one of these CECs. In particular, studies that investigated hormones tended to include both the natural steroid estrogen E2 as well as the synthetic EE2. There are a large number of total studies (>600) focused on each of these three CECs, however slightly more research has been published on E2 and EE2 when compared with DCL; that may be due to particular concerns regarding environmental contamination with hormonal EDCs. Fig. 3 demonstrates the total annual number of published articles from the database that include information on each CEC of interest. It is clear that a large increase in research on the contamination of aquatic matrices with DCL, E2 and EE2 has occurred since the early 2000s. The annual counts of articles increased from 0 for all three CECs in 1995 to 76, 50 and 51 for DCL, E2 and EE2 respectively in 2014. The maximum number of annual publications on DCL sources, receptors or control measures occurred in 2012 (83), while E2 and EE2 reached a maximum in 2013 (68 and 67 respectively). This figure also demonstrates that most years, slightly more articles are published on E2 and EE2 when compared with DCL, although this trend reversed itself from 2011 onwards. Finally, the majority of publications (>84%) on these three CECs have occurred from 2005 onward. This trend likely relates to the recent increased concern regarding DCL, E2 and EE2 in regards to EU legislation (specifically via the WFD). The apparent sharp decrease in publications from 2014 to 2015 is an artefact as the search was conducted in May of 2015, thus presumably many more articles were published on these CECs in the second half of the year.

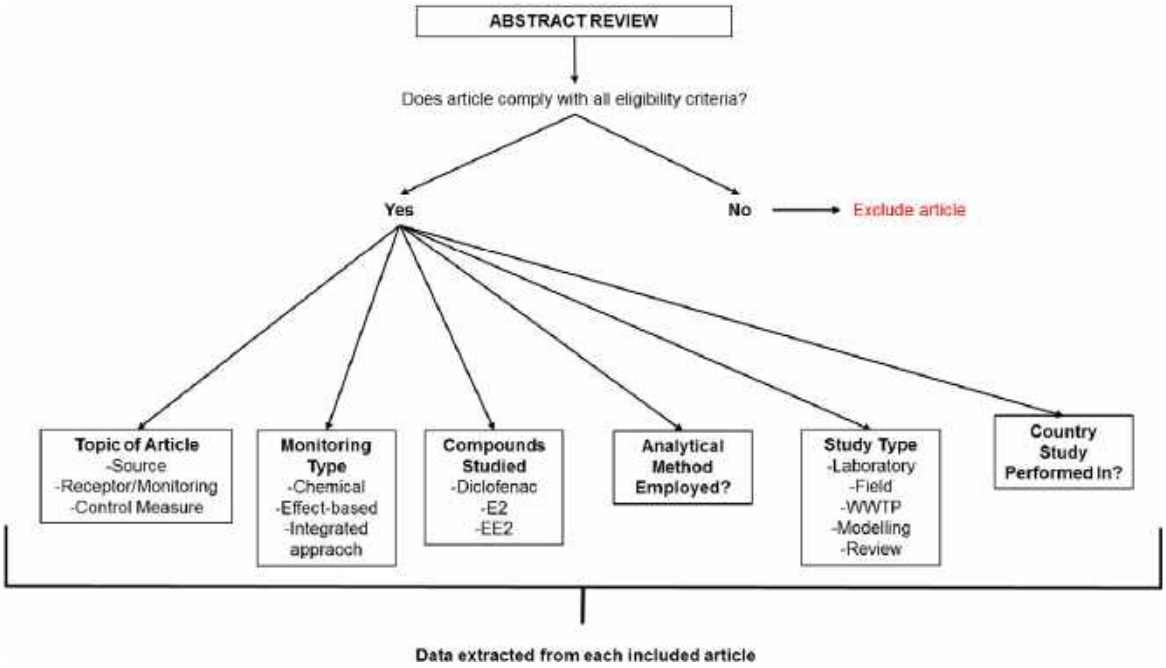


Fig. 1. Multi-step abstract review and data extraction approach used in the abstract filter step of the systematic literature review. Extracted data was used to carry out the bibliographic analysis.

3.2.2. Research theme studied

This systematic review investigated three general themes regarding research on DCL, E2 and EE2 and found 595 studies for sources of contamination (Section 3.3); 775 studies for receptors or monitoring methods used to measure the levels of these compounds in the aquatic environment (Sections 3.4–3.5); and 651 for control measures for reducing contamination (Section 3.6). Studies often focus on more than one of these themes, for example, some monitoring studies also discuss removal of CECs via wastewater treatment. Furthermore, studies focused on receptors or monitoring methods outnumber source studies by nearly 200 articles and control measure studies by over a hundred articles. Many of these monitoring articles describe analytical methods and conditions used to detect low levels of the CECs of interest, but they often also report detected concentrations in wastewater influent and effluent; surface, ground and drinking water or other environmental matrices for validation of the developed analytical protocols (e.g. Ben

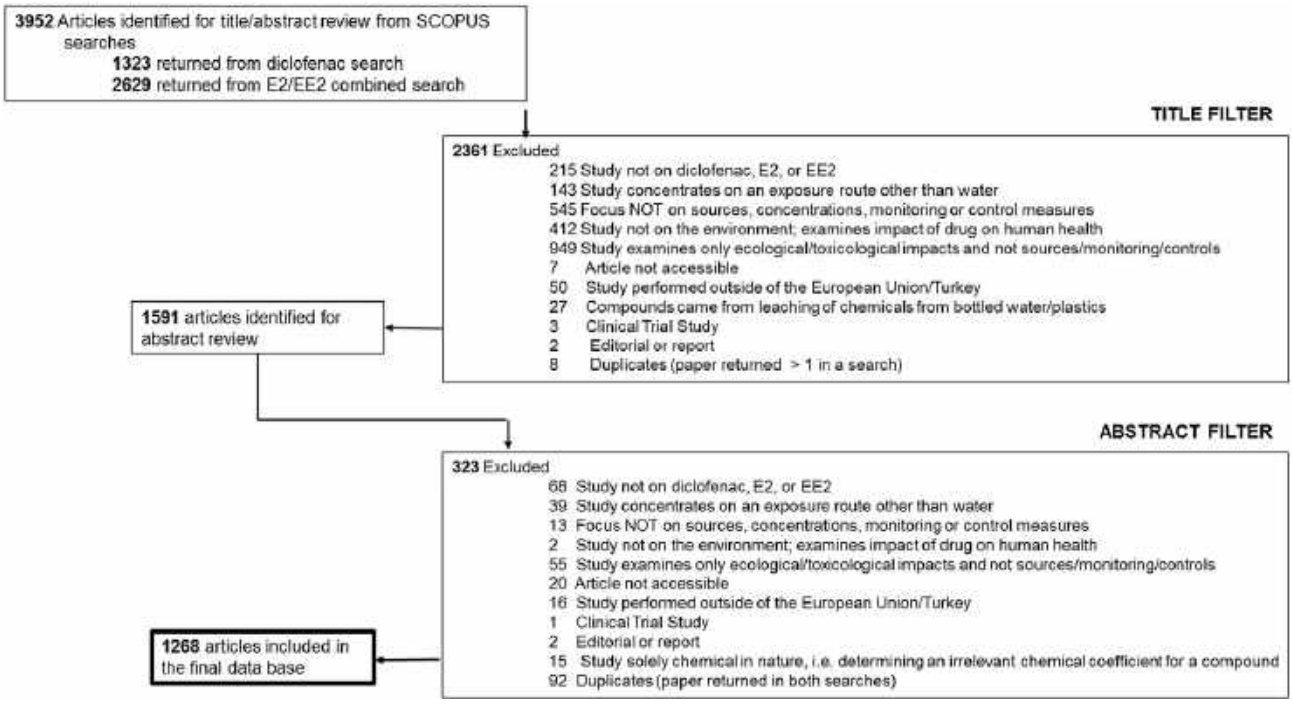


Fig. 2. Publications (articles) returned from the systematic review searches; the figure demonstrates the number of publications excluded plus reasons for exclusion during the title and abstract filter, as well as the total number of publications included in the final database.

Fig. 3. Total combined number of EU studies on sources, receptors or control measures for each DCL, E2 and EE2 from 1995 to May 2015, by year.

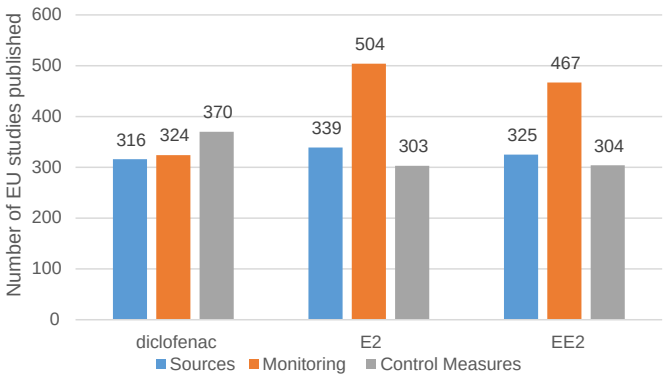


Fig. 5. Total number of EU studies on each pharmaceutical of interest investigating sources, of contamination, monitoring data or techniques, or control measures, from 1995 to May 2015.

Fredj et al., 2015; Lacey et al., 2008; Ronan and McHugh, 2013). Studies on sources are the least common of the three research themes and often focus on consumption rates, the contribution of municipal vs industry wastewater to total CECs load, or contributions via agricultural or veterinary practices (e.g. Kümmerer, 2009; Rivera-Utrilla et al., 2013; Santos et al., 2010). Finally, studies on control measures occur frequently in the database, but these publications represent studies carried out on a variety of scales, from laboratory experiments, to pilot scale studies, to whole WWTP-level studies. They also include investigations of removal via primary, secondary and tertiary treatment technologies. Fig. 4 demonstrates the total number of studies from each research theme carried out each year, from 1995 to May 2015. While publications on all three themes of research have increased dramatically during this time period, the graph demonstrates that since 2010 studies on sources of contamination have become less popular and have begun to level out. Commensurately, the number of publications on monitoring methods has been slightly lower than the number of publications on control measures in recent years (2012 to 2015). This may indicate that while monitoring methods are still being developed and measurements of these CECs in water matrices are still taking place, the research community is increasingly concerned with investigating mitigation methods for CEC contamination. Given the potential for increased regulations regarding aquatic contamination with DCL, E2 and EE2, a further increase in control measure studies is expected.

In order to understand if source, monitoring and control measure studies are conducted equally for each CEC, Fig. 5 shows the number of each type of study conducted for each compound. The difference in monitoring studies compared with source or control measure studies is accentuated for the two hormones, while DCL studies are more evenly split between the three research themes. The number of source studies is approximately equal for each of the three CECs, however control studies are conducted more frequently for DCL. The inability of conventional WWTPs processes to remove this NSAID (see Section 3.6) has likely led to more investigations of alternative or advanced treatments that may improve removal efficiencies.

3.2.3. Hormones: chemical vs biological monitoring method

In addition to traditional chemical monitoring, a variety of in vitro, effect-based monitoring assays can be used to identify the total estrogenic activity in environmental samples (Kunz et al., 2015). Fig. 6 compares the number of E2/EE2 monitoring studies that used traditional chemical (concentration) methods vs those that used biological effects monitoring. There is also a category for integrated or combined monitoring methods. Clearly chemical methods are much more common than biological effects monitoring. This trend is apparent both during the early years of research (1999–2001) and in more recent years (2007–2015). More information on these monitoring methods are presented in sections 3.5.2 and 3.5.3. The recent spike in concentration studies is likely related to an increase in the sensitivity of recent analytical approaches for measuring estrogens. Nevertheless, detecting environmentally relevant, low concentrations of estrogens remains a challenge, thus biological effect monitoring has become more popular as the field has developed.

3.2.4. Scale of the studies

Studies on DCL, E2 and EE2 can be conducted on a variety of scales. Some studies take place at the field level, measuring CEC concentrations in various aquatic matrices such as surface or ground water (e.g. Camacho-Muñoz et al., 2013; McEneff et al., 2014). Some take place on a laboratory scale, e.g. measuring the removal or effectiveness of monitoring methodologies of spiked water samples in the lab (e.g. Rizzo et al., 2015; Zhou and Jiang, 2015). Others are conducted on a full WWTP level, where influent/effluent concentrations and removal efficiencies are measured at specific WWTPs (e.g. Clara et al., 2005b; Lacey et al., 2012). The concentrations of CECs in different matrices can also be modelled (Balaam et al., 2010; Johnson et al., 2007), and

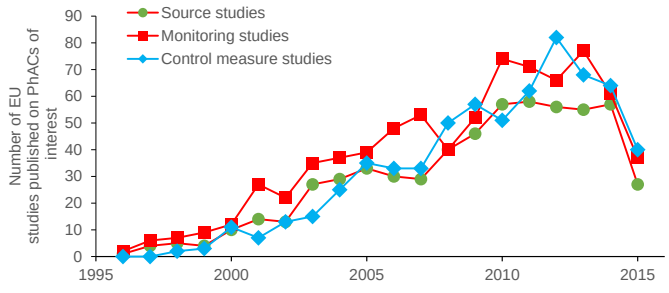


Fig. 4. Number of EU studies on at least one of the three pharmaceutically active compounds (DCL, E2 or EE2) investigating: sources of contamination, monitoring data or techniques, or control measures, from 1995 to May 2015, by year.

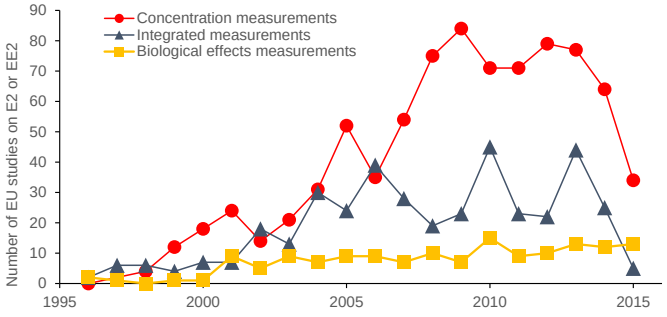


Fig. 6. EU studies investigating E2 and EE2 using concentration measurements, biological effects measurements, or an integrated approach by year, 1995–May 2015.

many studies are reviews of recent literature (see Sections 3.3–3.6). The number of each of these study types published annually from the database results is presented in Fig. 7 below. This figure demonstrates that by far, laboratory scale studies are the most common type of investigations on DCL, E2 or EE2. Laboratory studies are manageable, have controlled conditions, and can be done relatively quickly, all factors that likely contribute to the high frequency of this study type. Field studies can be more time intensive and expensive as they involve travel to a variety of locations for the collection of samples; nevertheless these types of studies have occurred with increasing frequency in the past two decades as people become more concerned with the levels of these three CECs in the aquatic environment. WWTPs scale studies have increased slowly but steadily in frequency, and now >20 tend to be published each year on just these three CECs alone. Such studies contribute to valuable meta-analyses which can provide important information regarding removal efficiencies via various wastewater treatments (Miège et al., 2008; Verlicchi et al., 2012). Modelling studies have increased recently (from 2007 onward) as more data have become available in this field, and a further increase in this study type is likely. As total number of primary publications on these CECs increases, so does the number of reviews including data on these compounds.

3.2.5. Repartition of the studies by country

This bibliographic analysis identified which European countries are producing the majority of research regarding contamination of the aquatic environment with DCL, E2 and EE2 (Table 2). As stated in Section 2.2, articles with authors from multiple countries were counted as full publications for each country, rather than fractionally (Qian et al., 2015). Spain and Germany effectively contribute 528 (35.5%) of total studies where review papers evaluating such national studies have been published (González et al., 2012; Jurado et al., 2012). The top 6 EU countries including Switzerland listed in Table 2 collectively published 971 (65%) studies where metadata on these CECs informs baseline and predictive modelling such as for river basins and catchments. However, the majority of EU countries have limited studies reported and will require substantial monitoring to effectively inform decision making and policy.

3.3. Sources and vectors of DCL, E2 and EE2

As the bibliographic analysis above demonstrates, contamination of the environment with CECs is a relatively recent research field with the majority of studies conducted in the past 15 years (Qian et al., 2015; Rivera-Utrilla et al., 2013; Santos et al., 2010). Now that researchers have been able to identify and quantify a large number of potentially harmful CECs in the aquatic environment (Santos et al., 2010), there is increased interest in identifying sources and vectors of these compounds. Only when the sources and pathways of CEC contamination are understood can opportunities to reduce the input of these substances into the aquatic environment be identified (Jurado et al., 2012; Kümmerer, 2010). The main sources and vectors discussed by these articles are reviewed below in a general manner, because many of them are applicable to DCL, E2 and EE2, as well as other CECs (Section 3.3.1). However, sources and vectors specific to each of the three compounds of interest are also addressed below (Section 3.3.2).

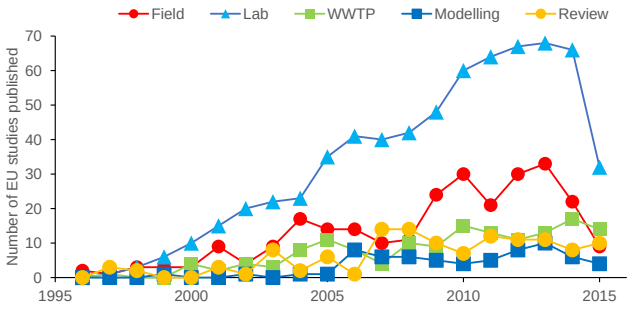


Fig. 7. Number of studies on three CECs (DCL, E2 and/or EE2) published in the EU from 1995 to May 2015 broken down by type of study: field, laboratory scale, WWTP, modelling and review.

Table 2
Number of articles produced by each EU country along with Switzerland, Norway and Turkey on sources, monitoring or control measures for DCL, E2 or EE2: 1995–May 2015.

Country	Total number (%) of studies
Spain	285 (19.2)
Germany	243 (16.3)
United Kingdom	179 (12.0)
France	93 (6.3)
Switzerland	87 (5.8)
Italy	84 (5.7)
The Netherlands	57 (3.8)
Sweden	51 (3.4)
Portugal	50 (3.4)
Greece	43 (2.9)
Belgium	42 (2.8)
Denmark	37 (2.5)
Poland	37 (2.5)
Czech Republic	26 (1.7)
Austria	24 (1.6)
Finland	23 (1.5)
Norway	21 (1.4)
Slovenia	21 (1.4)
Turkey	19 (1.3)
Ireland	17 (1.2)
Cyprus	14 (0.9)
Hungary	11 (0.7)
Romania	7 (0.5)
Luxembourg	6 (0.4)
Croatia	3 (0.2)
Slovakia	3 (0.2)
Bulgaria	2 (0.1)
Estonia	2 (0.1)
Northern Ireland	2 (0.1)
Lithuania	1 (0.06)
Latvia	0 (0)
Malta	0 (0)

3.3.1. General sources of CECs

The largest source of environmental contamination with CECs comes from human use of therapeutic drugs (Kümmerer, 2009; Rivera-Utrilla et al., 2013; Santos et al., 2010). After consumption, unaltered CECs can enter the environment via excretion in urine and faeces (Santos et al., 2010). Medicines containing the CECs of interest in this study are almost exclusively prescription medications; this allows for relatively easy measurement of drug usage or consumption (Clouzot et al., 2008; Wise et al., 2011; Zhang et al., 2008), a critical factor for predicting the ultimate levels of environmental contamination in an area. Furthermore, review studies have noted that consumption of CECs varies temporally and spatially. For example, significant differences in consumption of individual compounds can occur from one country to another, often due to cultural or economic factors (Kümmerer, 2009). In addition to excretion of unaltered CECs, parent compounds can also be converted to metabolites or conjugates through various reactions in the body. These metabolites/conjugates are then excreted and can be harmful to aquatic organisms themselves, or can be transformed or deconjugated back into the parent compound in environmental matrices (Santos et al., 2010).

CECs that are excreted by humans will ultimately end up in wastewater, and will potentially receive treatment at a municipal WWTP or via a domestic treatment system (e.g. septic tank). However, WWTPs and domestic treatment systems are generally not designed to treat CECs (e.g. Verlicchi et al., 2012) (see Section 3.6). If incomplete removal of CECs during municipal or domestic wastewater treatment occurs, the

compounds will enter the aquatic environment via WWTPs effluents discharged into receiving waters.

Another potential source of environmental contamination with CECs comes from household disposal of unused or out-of-date medications (Kümmerer, 2009; Santos et al., 2010). These medications are either discarded through the sink/toilet, in which case, they go directly to WWTPs via sewage influent, or they are disposed of via household waste. If household waste containing unused drugs is landfilled, CECs can enter the landfill effluent (Kümmerer, 2009; Santos et al., 2010) and consequently the aquatic environment. In addition to household waste, sludge from WWTPs can also be brought to landfills. In this case, leaching of CECs that were removed from wastewater via sorption to sludge could further increase the CEC content of landfill effluents (Santos et al., 2010). Treated sludge (biosolids) may also be applied to soil and recent studies have documented that CECs may also reach the environment by this entry route (Verlicchi et al., 2012).

Industrial effluent can be another significant source of pharmaceutically-active chemicals (PhACs) contamination (Kümmerer, 2009; Rivera-Utrilla et al., 2013; Santos et al., 2010). The effluents of pharmaceutical production facilities in particular can contain high levels of bioactive compounds (Santos et al., 2010). However, although very limited data exist, good manufacturing practices, regulatory requirements and the high value of the active ingredients in most pharmaceuticals have often led to the assumption that such emissions are negligible in a European context (Kümmerer, 2009). Another type of wastewater that could contain high levels of CECs is hospital effluent (Kümmerer, 2009; Rivera-Utrilla et al., 2013; Santos et al., 2010). Reviews studies indicate that while CEC concentrations in hospital wastewater tend to be much higher than those in municipal sewage, the total contribution of this source to environmental contamination with CECs is low because of the relatively lower volume of hospital effluent (Kümmerer, 2009). Verlicchi and Zambello (2016) studied chemical characterisation of hospital effluents in terms of predicted and measured concentrations in Italy. It was found that uncertainties in predictions are mainly attributed to the wastewater volume and extraction factor, whereas for measured concentrations, uncertainties are mainly due to the sampling mode. In the last fifteen years, Verlicchi and Zambello (2016) remarked that investigations and studies have focused on chemical characterisation of hospital effluents in terms of detection of a selection of CECs including diclofenac (Verlicchi et al., 2012) in order to inter alia; estimate the contribution of a hospital to the influent CEC load of a municipal WWTP (Herrmann et al., 2015); analyse the most appropriate hospital effluent management (Verlicchi et al., 2015); ascertain removal efficacy of conventional and advanced treatments with regards to selected CECs (Gautam et al., 2007); determine environmental risk evaluation posed by CEC in hospital effluent (Mendoza et al., 2015); establish ecotoxicology (Perrodin et al., 2015), define framework for proposing proper management and treatment (Al Aukidy et al., 2014; Daouk et al., 2016); prioritise compounds to monitor (Daouk et al., 2015). Verlicchi and Zambello (2016) noted that annual consumption data and weight percentage in the investigated hospital for DCL was 1.07 kg/year and 0.63% respectively. The concentration mean value for DCL in hospital effluent was found to be 223 ng/L and 510 ng/L for summer and winter monitoring periods respectively.

The use of CECs in agriculture and aquaculture can also be sources of environmental contamination, particularly in rural environments (Boxall, 2010; Rivera-Utrilla et al., 2013; Santos et al., 2010). First, CECs given to grazing or outdoor animals are excreted directly onto the ground or into surface waters without receiving any wastewater treatment. Furthermore, disposal of farmyard manure, slurry or litter containing unmetabolized CECs via application onto agricultural land can lead to leaching of compounds into groundwater, or runoff into surface water (Rivera-Utrilla et al., 2013). Municipal sewage sludge is also often spread on agricultural land as a fertilizer, and can contain CECs that were removed from wastewater during the treatment process (Santos et al., 2010). In the case of aquaculture, CECs can be used as veterinary medicines and may be applied through many routes, including via feed, topical application or injection; all of these uses have potential to lead to contamination of surface waters (Boxall, 2010).

3.3.2. Specific sources of DCL, E2 and EE2

3.3.2.1. Sources of diclofenac. Sources of DCL were specifically addressed by two review articles in the database of publications (Vieno and Sillanpää, 2014; Zhang et al., 2008). DCL is an arylacetic acid NSAID. It is prescribed as oral tablets or a topical gel, and it is sold under many commercial names including Dicloabac, Diclofenbeta, Diclomex, Voltaren, among others (Vieno and Sillanpää, 2014). Vieno and Sillanpää (2014) comprehensively reviewed the human metabolism of this PhAC. They found that studies generally report that only 6–7% of the topical gel is absorbed, while the rest is washed off the skin or attaches to clothing. This is significant in regards to environmental contamination because a large percentage of topically applied DCL will end up washed down household drains, ultimately ending up in WWTP influent. Vieno and Sillanpää (2014) also summarized the metabolism of the tablet form; the studies they reviewed found that between 65 and 75% of the orally administered dose is excreted through urine and 20–30% is excreted in faeces as the parent drug or metabolites. This review also reports that both the topical and oral forms of DCL undergo almost complete biotransformation in the body, with <1% of the orally administered dose being excreted as unmetabolized DCL. The World Health Organization defined daily dose for DCL as 100 mg, of which less than 1 mg is eliminated as DCL and 11 mg as DCL conjugates. The rest is excreted as metabolites of DCL or their conjugates (Vieno and Sillanpää, 2014). This finding demonstrates the importance of analyzing environmental matrices for metabolites and conjugates, as well as the parent drug. Diclofenac is one of the most widely used NSAID, and consumption of this compound in a variety of regions is reviewed by Zhang et al. (2008) and Ziylan and Ince (2011). They summarized the annual consumed volumes of DCL for some European countries including Austria, France, Germany, and England. Consumption in the Zhang et al. (2008) study was compared using dose per capita, or the annual consumption of the drug in an area divided by that area's population. The authors reported that Germany had the highest dose per capita (915 mg), followed by Austria (750 mg), England (531 mg) and France (271 mg). The authors also calculated a simplified estimate of annual global DCL consumption of 940 tons. Such estimations of human consumption are critical for understanding the concentrations of this CEC expected in aquatic matrices.

Treated municipal wastewater effluent is considered to be the major vector of contamination of the aquatic environment with DCL (Vieno and Sillanpää, 2014). DCL is considered as a recalcitrant compound, meaning its removal efficiency during conventional wastewater treatment is poor (Miège et al., 2008; Verlicchi et al., 2012 and see Section 3.6). Thus, concentrations of this compound in effluent are generally high (Table 6), and DCL is commonly released via this pathway into surface waters. This compound is hydrophilic, meaning it dissolves in water and does not significantly sorb onto sludge during wastewater treatment to any significant extent (Vieno and Sillanpää, 2014 and Section 3.6). It is thus unlikely that DCL contamination will result from the spreading of sewage sludge on agricultural land. DCL may be found in landfill effluent, though only via disposal of the compound through household wastes, and not from sewage sludge deposited in landfills. To our knowledge, the removal of DCL in domestic treatment systems has not been investigated yet, but this could be another potential vector of environmental contamination. Veterinary use of DCL in Europe is a potential source of contamination with this CEC. Nevertheless, the European Medicines Agency (2014) reports that DCL is authorized for veterinary use in many member states. Increased regulations and risk assessments associated with veterinary use of DCL have been suggested, and may be implemented on a European level (European Medicines Agency, 2014).

3.3.2.2. Sources of E2. E2 is one of three naturally occurring steroid estrogens produced by the human body. Females excrete on average more E2 than males (males = 1.6 μg/day), and menstruating and pregnant women excrete particularly large amounts of this natural estrogenic compound (3.5 and 259 μg/day respectively) (reviewed in Wise et al., 2011). This natural CEC can also be used in prescribed drugs, including hormone replacement therapy and to treat infertility in women or advanced prostate and breast cancer (reviewed in Kunz et al., 2015). Compared with the other natural estrogenic hormones, E2 has the highest potency and levels of aquatic contamination of this CEC are therefore of great concern (Wise et al., 2011). Given that E2 is a naturally produced compound, humans represent one of the most important sources of contamination of the environment with this CEC. Similar to DCL, effluents from WWTPs are still one of the most important vector of aquatic contamination with E2 (Burkhardt-Holm, 2010; Hecker and Hollert, 2011; Verlicchi et al., 2012; Wise et al., 2011). This compound is easily eliminated during wastewater treatment (see Section 3.6), nevertheless removal of E2 is usually incomplete (Table 3). Trace concentrations of E2 are therefore released into surface waters via WWTPs effluents. E2 is also excreted by livestock, which in general excrete the same natural hormones as humans (Burkhardt-Holm, 2010; Wise et al., 2011). Research has demonstrated that surface waters downstream of agricultural land or farms often have relatively elevated levels of estrogens, including E2 (Wise et al., 2011). Sewage sludge is not thought to be a significant source of E2 contamination, again because the compound is readily biodegradable (see Section 3.6). Domestic treatment systems and landfill effluent can contribute to environmental contamination of E2 according to a review by Burkhardt-Holm (2010). Finally, E2 has been used as a veterinary medication for livestock, although determining the contribution of natural versus pharmaceutical estrogens to total livestock excretions is difficult (Wise et al., 2011).

3.3.2.3. Sources of EE2. The structure of the synthetic estrogen EE2 is more similar to E2 than any other natural estrogen (Clouzot et al., 2008). EE2 is the main estrogenic ingredient in oral contraceptive pills taken by women of reproductive age (Clouzot et al., 2008; Wise et al., 2011). It is also found in other prescription medications including hormone replacement therapies, palliative treatments for breast and prostate cancer, and lotions used to prevent androgen-dependent hair loss in women (reviewed in Kunz et al., 2015). Estimation of consumption of this CEC can be difficult because it is usually prescribed as a combination drug (usually in combination with a progestin). Wise et al. (2011) reviewed studies on the excretion of EE2, and they report that the average daily dose of this compound is 30–35 μg of EE2 per pill, and that women on oral contraceptives fully metabolize 20–48% of this dose. The rest is excreted in either its original form or as EE2 sulfate or glucuronide conjugates, but these conjugates are mostly deconjugated back to its original form in the environment (Clouzot et al., 2008; Wise et al., 2011). As with E2, effluent from municipal WWTPs is often considered to be the most important vector of environmental EE2 contamination (Burkhardt-Holm, 2010; Hecker and Hollert, 2011; Verlicchi et al., 2012). EE2 is prone to biodegradation during wastewater treatment (see Section 3.6), but it is significantly more recalcitrant (and therefore has lower removal rates, see Table 3) than E2 (Miège et al., 2008; Verlicchi et al., 2012). Because this CEC is not completely removed by conventional wastewater treatment, it enters surface waters via WWTP effluent discharge. Unlike E2, EE2 is not produced by livestock. Sewage sludge transferred to landfills or spread on agricultural land may contain traces of EE2, but this compound is thought to biodegrade readily and thus these practices also may not represent significant sources of EE2 contamination. As for E2, domestic treatment systems and landfill leachate may present pathways to groundwater contamination with EE2, again, related back to human usage of this compound (Burkhardt-Holm, 2010).

3.4. Receptors and occurrence of diclofenac, E2 and EE2 in European waters

There is now evidence of contamination of the aquatic environment with hundreds of different CECs (Kümmerer, 2010) from a variety of therapeutic classes, including antibiotics, lipid regulators, psychiatric drugs, and of course, NSAIDs (e.g. DCL) and hormones (e.g. E2 and EE2) (Verlicchi et al., 2012). Levels of CECs in the aquatic environment can vary dramatically, but are usually present in low concentrations from the nanogram to microgram per litre range depending on the location and the aquatic matrix considered (Kümmerer, 2010; Verlicchi et al., 2012). In addition to global reviews (e.g. Ratola et al., 2012; Verlicchi et al., 2012; Vieno and Sillanpää, 2014) there are now also several studies summarizing the findings of CECs occurrence in particular European countries such as Spain (González et al., 2012; Vazquez-Roig et al., 2013) and Italy (Meffe and de Bustamante, 2014). Given the importance of WWTPs as point sources of CEC contamination (as mentioned in Section 3.3), it is essential to understand the levels of compounds entering the system via influent, as well as the final concentrations in treated effluent. Many of the published review studies are devoted specifically to evaluating the typical occurrence of CECs in WWTPs influents and effluents (e.g. Miège et al., 2008; Verlicchi et al., 2012; Vieno and Sillanpää, 2014). Here only inlet and outlet WWTPs concentrations for the three CECs of interest will be discussed; some more specific information on the removal efficiencies obtained with different treatment processes and potential interpretations of the encountered removal efficiencies for DCL, E2 and EE2 will be given in the next chapter (i.e. 3.6). Other reviews focus instead on the reported concentration of CECs in surface, ground and drinking water, as concentrations in these aquatic matrices ultimately have the most relevance for animal and human health (e.g Petrie et al., 2013; Lapworth et al., 2012; Martin and Voulvoulis, 2009). Generally, many more reviews summarize surface water concentrations than ground or drinking water concentrations, due to the low number of primary studies that consider the two later matrices. Tables 3 and 4 summarize the findings from recent review papers regarding the occurrence of DCL, E2 and EE2 in these aquatic matrices in Europe and internationally. Although concentrations of DCL, E2 and EE2 can vary a great deal in each of these aquatic matrices (even when considering each compound individually), typical concentrations (including averages and ranges) in each matrix are compared and contrasted below.

3.4.1. Occurrence of diclofenac

Compared with E2 and EE2, DCL tends to be present in high concentrations in WWTP influents. This finding is common for compounds in this therapeutic class; in a recent meta-analysis Miège et al. (2008) found that NSAIDs had the highest WWTPs influent concentrations when compared with other drug classes (e.g. antibiotics, beta-blockers, lipid regulators, vasodilators). In the review papers evaluated, average DCL values varied from 80 to 2100 ng/l in this aquatic matrix (Table 3). The minimum DCL influent value reported by any of the reviews was 2 ng/l (Santos et al., 2010), while the maximum was 203,000 ng/l (Ratola et al., 2012). These large variations in reported influent concentrations may be partially explained by differences in consumption of DCL between and within countries (see Section 3.3.1), and also by the differences in analytical methods employed (see Section 3.5). Such differences can make describing or predicting DCL influent concentrations difficult (Zhang et al., 2008).

Meta-analyses that evaluate multiple PhACs repeatedly found that DCL is among the most frequently detected compound in WWTPs effluents (Miège et al., 2008; Verlicchi et al., 2012). DCL is rarely completely eliminated during wastewater treatment, especially using conventional treatment processes (Table 3). As a result, this recalcitrant compound rarely falls below the LODs of a few ng/l in WWTPs effluents (Zhang et al., 2008). In the reviews evaluated, mean DCL concentrations in effluents varied widely, from <2 to 2500 ng/l. These values do tend to be slightly lower than the average influent values reported in Table 3. Nevertheless, it is clear that high nanogram to microgram per litre levels of

Table 3
Summary of influent and effluent concentrations and removal efficiencies following various wastewater treatments throughout Europe. All values for influent and effluent concentrations reported in ng/l. Values reported as minimum, maximum, range or mean, depending on what was reviewed by the reference. Removal efficiencies could be determined using lab, pilot or whole plant scale studies. Removal efficiencies are also given for a variety of secondary or tertiary treatments. Note: removal efficiencies represent global removal, and are not based on direct comparisons between the listed influent and effluent concentrations. These values do not represent central tendencies of removal efficiencies unless specified; furthermore they may be influenced by factors such as artefacts of the analytical (detection) methods used. Data originate from summary information provided by review studies from published database: specific references cited for each PhAC.

Drug	Influent concentration (ng/l)				Effluent concentration (ng/l)				Removal efficiency (%)		Comment	Reference
	Mean	Max	Min	Range	Mean	Max	Min	Range	Mean	Range		
Diclofenac										90–100	Ozonation	Jekel et al., 2015
										91–99	Bank filtration and soil aquifer treatment [Germany]	
	80–2300	150–7100			<2–2500	120–4700			36		CAS	Vieno and Sillanpää, 2014
									36		Activated sludge with BNR	
									48		MBR	
	250				215					28–46	Activated carbon [Italy, Belgium, UK, Ireland, Germany]	Rivera-Utrilla et al., 2013
									100		O3 based AOPs	
									>80		AOPs based on UV radiation [the Netherlands]	
										62.9–85	Gamma radiation, various parameters [Italy]	
				nd–203,000				nd–19,200				Ratola et al., 2012
										43–77	Generally <50% DCL removed (CAS at varying SRTs) [Austria]	Petrie et al., 2013
										23–76	Biofiltration processes [Spain]	
										92–99	Ozonation [Spain, Austria]	
										69–98	Sorption processes [UK]	
				105–4110				5–5450		9–60	Activated sludge plants, various treatment operations	Ziylan and Ince, 2011
									96		Ozonation	
									29		UV radiation	
				2–3600				0.3–2400			Luxembourg	Santos et al., 2010
									<30		Conventional wastewater treatment, 66% of reviewed studies found removal rates of <30%	Oulton et al., 2010
										6–96	Removal efficiency of CW; WWTP removal for comparison was 24% [Spain]	Matamoros and Bayona, 2008
	2400							670	45		15 WWTPs designed for up to 756,000 population [Portugal]	Pereira et al., 2015
										59–75	Full scale WWTPs, treatments not specified; high removal rates due to elimination of sludge during primary treatment and/or enhanced sorption to sludge during secondary treatment upon addition of inorganic salts for P precipitation [Spain]	Suárez et al., 2008
	882	4110	105		477	1720	35		35		WWTPs with activated sludge processes {France]	Miège et al., 2008
								<100–1750		0–80	Mainly 21 to 40% (European studies, various treatment processes including Austria, Denmark, France, Greece, Italy, Spain, Sweden, UK)	Zhang et al., 2008
	1000	1200			800	1100			29		International studies, CAS	Verlicchi et al., 2012
									60		International studies, MBR	
E2								2.7–48			UK	Kralchevska et al., 2013
				2.5–125				0.3–30				Ratola et al., 2012
				4–30				0.1–60		39–100	Removal of various estrogens via various oxidative treatments	Pereira et al., 2011
										94–100	Removal of various estrogens via ozonation [Spain]	
				0.3–102				<0.3–85			Luxembourg	Santos et al., 2010
				10–31				3–8			Italy	
									>90		CAS, 69% of reviewed international studies found removal rates of >90%	Oulton et al., 2010

(continued on next page)

Table 3 (*continued*)

Drug	Influent concentration (ng/l)				Effluent concentration (ng/l)				Removal efficiency (%)		Comment	Reference
	Mean	Max	Min	Range	Mean	Max	Min	Range	Mean	Range		
								1–10			Germany, UK	Burkhardt-Holm, 2010
									36		Removal efficiency of constructed wetlands; WWTP removal for comparison was 85–99%) [Spain]	Matamoros and Bayona, 2008
								0–50			Median = 2 ng/L [UK]	Martin and Voulvoulis, 2009
										30–100	Full scale WWTPs, treatments not specified [Spain]	Suárez et al., 2008
	27.4	48.4	2.5		1.8	5.2	0.3		85		WWTPs with activated sludge processes [France]	Miège et al., 2008
	250	3000			10	80			80 99		International studies, CAS International studies, MBR	Verlicchi et al., 2012
EE2								0.1–8.9			Germany	Kralchevska et al., 2013
										7–82 >50–>66 >43	Biofiltration processes [Sweden] Ozonation [Sweden] Sorption processes [UK]	Petrie et al., 2013
				1.5–17.2				0.1–3.1				Ratola et al., 2012
				7–50				2–60		39–100	Removal of various estrogens via various oxidative treatments	Pereira et al., 2011
										94–100	Removal of various estrogens via ozonation [Spain]	
				<1.6–24				<1.1–1.7				Santos et al., 2010
									41		Removal efficiency of constructed wetlands; WWTP removal for comparison was 71–78%) [Spain]	Matamoros and Bayona, 2008
								nd–10			Germany, UK	Burkhardt-Holm, 2010
										(−18)–98	Full scale WWTPs, treatments not specified [Spain]	Suárez et al., 2008
								0–25			Median = 1 ng/L [UK]	Martin and Voulvoulis, 2009
	1.5	2.8	0.8		0.6	1.4	0.2		95		WWTPs with activated sludge processes [France]	Miège et al., 2008
		13	<0.2	1.4–6.1		42	<0.02	<0.2–9		<80	Activated sludge processes [Denmark]	Clouzot et al., 2008
	20	50			3	10			78 60		International studies, CAS International studies, MBR	Verlicchi et al., 2012

CAS = conventional activated sludge; BNR = biological nutrient removal; AOP = advanced oxidation process; SRT = solids retention time; MBR = membrane bioreactor; nd = not detected.

DCL in WWTPs effluents are common throughout Europe. Occasionally individual studies found that DCL showed negative removal rates during WWTPs treatment, i.e. concentrations are actually higher in effluent than influent (e.g. Clara et al., 2005b; Lacey et al., 2012; Lacey et al., 2008). Besides the impact of analytical uncertainty, two mechanisms have been proposed to explain this phenomenon, deconjugation of glucuronidated or sulfated DCL, or desorption of this compound from particles (Verlicchi et al., 2012; Vieno and Sillanpää, 2014). It should be noted that many review papers do not include these negative removal rates when calculating average removal via WWTPs processes (see removal efficiency, Table 3). Verlicchi et al. (2012) conducted the most comprehensive, recent meta-analysis of CEC concentrations in municipal WWTPs found in our database, and DCL was one of the compounds included. CEC concentrations of raw influent at >200 municipal WWTPs (all utilizing conventional activated sludge (CAS) systems) were compared with the concentrations in secondary effluents in order to calculate global removal efficiencies. The average concentration of DCL in influent was 1.0 μg/l, but even in this one review, the minimum and maximum reported values varied over an order of magnitude. The average concentration of DCL in effluent was 0.8 μg/l, but again the values ranged greatly; in one study, DCL was found in WWTP effluent at 11 μg/l, one of the highest absolute effluent concentrations found for all 118 CECs included in the study. In another study, Loos et al. (2012) analysed effluents from 90 WWTPs across Europe for 156 polar organic chemical contaminants and showed that DCL had a frequency of detection of 89%. The maximum concentration of DCL found was 174 ng/l and the median concentration was 43 ng/l. These levels are relatively low when compared with levels found in similar studies; the most recent review of DCL found that mean concentrations in wastewater effluents were usually above 100 ng/l, however mean values as low as 2 ng/l have been found also (Vieno and Sillanpää, 2014). Loos et al. (2012) hypothesize that the low levels could have been due to problems with different analytical standards.

DCL is frequently detected in surface waters throughout Europe (Table 4). This fact is not surprising given the high levels often found in WWTPs effluents. According to the most recent review, DCL concentrations in surface waters are generally reported below 100 ng/l (Vieno and Sillanpää, 2014). Other reviews however include maximum values as high as 1030 ng/l (Ziylan and Ince, 2011) or 1200 ng/l (Rivera-Utrilla et al., 2013). Still, such high levels are the exceptions rather than the rule in regards to concentrations of DCL in surface waters. Surface waters in the UK range in DCL concentrations from <0.5 to 261 ng/l while the same author reports a range of <12 to 154 ng/l for mainland Europe (Petrie et al., 2013). Similarly, an Italian review study found a maximum

Table 4
Concentrations of each CEC of interest in EU waters, including surface, ground and drinking water. Data originate from summary information provided in review studies from publication database; specific references listed for each CEC. All values reported in ng/l. Values reported as minimum, maximum, range or mean, depending on what was reviewed by the reference.

Drug	Concentrations in water matrices (ng/l)[a]			Comment	Reference
	Surface water	Ground water	Drinking water		
Diclofenac	158	nd		Max in Italian studies	Meffe and de Bustamante, 2014
	<100	<LODs	<LODs	Surface water: generally below 100 ng/l, almost always below 500 ng/l [UK, Spain, Italy, Germany, Sweden, Finland) Ground water: generally low or below detection limits, max = 380 ng/l [Spain, Italy, UK) Drinking water: generally low or below detection limits, range = 1–7 ng/l (Italy, Spain, France)	Vieno and Sillanpää, 2014
	1200			Max of international studies between 1999 and 2004	Rivera-Utrilla et al., 2013
	<0.5–261			Range in UK	Petrie et al., 2013
	<12–154			Range in Austria	
	1–90			Range in Spanish protected areas (wetlands, estuaries, watersheds)	Vazquez-Roig et al., 2013
		477		Max in Spanish studies	Jurado et al., 2012
		121		Mean value, the Netherlands (max = 590 ng/l, min = 2.5 ng/l)	Lapworth et al., 2012
	1–1030			Range i	Ziylan and Ince, 2011
	0.3–147	10–50	<0.25–7	Range in UK, Germany, Slovenia	Santos et al., 2010
	15–135			Range in Germany	Díaz-Cruz and Barceló, 2008
	<50–290			Mean value, range of international studies	Zhang et al., 2008
E2	12.9			Max in Italian studies	Meffe and de Bustamante, 2014
	0.11		0.2–2.1	European studies [Italy and Germany]	Kralchevska et al., 2013
	nd			Studies in Llobregat River (Spain)	González et al., 2012
		nd		Max in Spanish studies	Jurado et al., 2012
		31		Mean value, the Netherlands (max = 120 ng/l, min = 0.79 ng/l)	Lapworth et al., 2012
	0.2–50	0.08–2	nd	Range of international studies, excluding outliers	Pereira et al., 2011
	<0.2–100	0.3–1.3	nd	in Germany France	Santos et al., 2010
	nd–200	nd–45	nd–2	Range in the Netherlands, France, Germany	Martin and Voulvoulis, 2009
	0.15–17		0.2–17	Germany, UK, the Netherlands	Wise et al., 2011
EE2	2.7	nd		Max in Italian studies	Meffe and de Bustamante, 2014
	4.3			Max of international studies between 1999 and 2004	Rivera-Utrilla et al., 2013
	0.04		0.15–2.4	Italy, Germany	Kralchevska et al., 2013
	nd			Studies in Llobregat River (Spain)	González et al., 2012
		nd		Max in Spanish studies	Jurado et al., 2012
	0.5–50	0.7–5	1–3	Range of international studies, excluding outliers	Pereira et al., 2011
	<0.2–73	0.5–3	<0.1	Germany, France	Santos et al., 2010
	nd–831		nd–0.5	the Netherlands, UK, France	Martin and Voulvoulis, 2009
	<0.1–5.1		0.15–1.4	Germany	Wise et al., 2011

[a] Indicates value published from cited references.

concentration of 158 ng/l of DCL in surface waters (Meffe and de Bustamante, 2014). Levels in protected areas may be lower, as was demonstrated by a review of DCL levels in Spanish wetlands, estuaries and watersheds where levels ranged from 1 to 90 ng/l (Vazquez-Roig et al., 2013). Given that the predicted no effect concentration (PNEC) for DCL is reported in the literature as approximately 14 μg/l (Santos et al., 2007), the data in Table 4 suggest that typical surface water concentrations in Europe do not usually pose a significant environmental threat. However, point sources of pollution can lead to concerning levels of DCL contamination in European surface waters.

Levels of DCL in groundwater tend to be much lower than those in surface water (Table 4). The most recent review of DCL states that levels in groundwater are typically low or below LODs (of generally a few ng/l for this type of water, See Section 3.5) (Vieno and Sillanpää, 2014). According to a recent review, no Italian study has detected DCL in groundwater to date with LODs generally in the ng/l range (Meffe and de Bustamante, 2014, see Section 3.5.1), however Spanish studies have found a maximum concentration of 477 ng/l in groundwater (Jurado et al., 2012). In a review of international studies, Lapworth et al. (2012) found a mean groundwater concentration of 121 ng/l, while Santos et al. (2010) found values ranging from <10 to 50 ng/l. Finally, concentrations in drinking water appear to be even lower; only two review studies report on DCL levels in drinking water, and they state that international studies demonstrate levels between 1 and 7 ng/l (Vieno and Sillanpää, 2014) and <0.25 to 7 ng/l (Santos et al., 2010).

3.4.2. Occurrence of E2

Levels of E2 in WWTPs influents tend to be in the nanogram per litre range (Miège et al., 2008; Pereira et al., 2011; Ratola et al., 2012; Verlicchi et al., 2012). Of the reviews evaluated, mean E2 concentrations in influents ranged from 27.4 to 250 ng/l, considerably lower than those reported for DCL (Table 3) (Miège et al., 2008; Verlicchi et al., 2012). Similar to DCL, however, the range of E2 values the reviews report for influents are high; the lowest reported influent value in any review paper was 0.3 ng/l (Santos et al., 2010) and the highest was 3000 ng/l (Verlicchi et al., 2012). In the Verlicchi et al. (2012) meta-analysis, E2 in influent presented the highest absolute concentration and the highest average observed value among any of the hormones studied. In contrast, in a meta-analysis performed by Miège et al. (2008), the mean E2 value was lower, 27.4 ng/l, and the range was much smaller (min = 2.5 to 48.4 ng/l). The Verlicchi et al. review included three studies with extremely high E2 influent concentrations (>1000 ng/l), which drove the overall reported mean value up considerably. In general however, European influent concentrations of E2 are much <1000 ng/l.

A greater number of reviews provide summary information on E2 concentrations in WWTP effluents than in influents (Table 3). These reviews demonstrate that levels of E2 in WWTPs effluents are also usually found in the low nanogram per litre range. Furthermore, reported E2 concentrations in effluents are generally lower than average influent concentrations. For example, the Verlicchi et al. (2012) meta-analysis reported a mean E2 concentration of 10 ng/l in effluent, 25 times less than the mean concentration in influent. Similarly the Miège et al. (2008) meta-analysis reported a decrease in E2 effluent concentrations when compared with influent concentrations (1.8 ng/l vs 27.4 ng/l respectively). This decrease in E2 concentrations in effluent is likely due to the high removal rates of E2 during many wastewater treatment processes (often >90%, see Table 3 and Section 3.6.2). In contrast, Pereira et al. state in their 2011 review paper that estrogen concentrations in

effluent wastewaters are similar to those found in influent wastewaters; however, the values they report for each matrix do indicate a slight decrease in effluent levels for E2 specifically (Table 3).

The presence of estrogenic compounds (including E2) in surface water has been widely investigated (Table 4), supposedly largely due to concerns about the endocrine disrupting effects of these compounds. We found that the majority of recent review studies report surface water E2 concentrations of <50 ng/l (Meffe and de Bustamante, 2014; Pereira et al., 2011), although in some studies the maximum values extend as high as 200 ng/l (Martin and Voulvoulis, 2009; Santos et al., 2010). E2 surface water concentrations can reach these high levels of >100 ng/l when measurements are taken directly downstream from WWTPs effluent discharge (Pereira et al., 2011). However it is also not uncommon for studies to report that E2 is below the LOD in surface waters (generally a few ng/l or below in this type of water, see Section 3.5). For example, in a review of studies conducted in the Llobregat River (Spain), González et al. (2012) find no reports of E2 exceeding LODs (generally in the ng/l range, see Section 3.5.2). Similarly Santos et al. (2010) and Martin and Voulvoulis (2009) report that some of the studies they reviewed did not detect E2 in surface waters. Nevertheless, very low concentrations (i.e. sub ng/l range) of EDCs such as E2 can have a negative impact on aquatic organisms, especially via chronic exposure; thus even though on average, surface water concentrations of E2 are lower than many other CECs, the environmental impact of this compound should not be underestimated (Burkhardt-Holm, 2010; Abargues Llamas et al., 2012). Reviews examining the occurrence of E2 in the aquatic environment often consider levels in groundwater, but less frequently discuss levels in drinking water (Table 4). Measuring the low concentrations in drinking water can present a serious analytical challenge in terms of the sensitivity of the method (see Section 3.5), thus, there are not as many primary studies that are able to investigate this aquatic matrix. Out of all of the reviews evaluated, the highest E2 concentration reported for groundwater was 120 ng/l (Lapworth et al., 2012), however, most values were much lower than this (i.e. a few nanograms per litre), especially in reviews that excluded outliers (Pereira et al., 2011; Santos et al., 2010). Several reviews reported that E2 is often present in concentrations below detection levels in groundwater (Jurado et al., 2012; Martin and Voulvoulis, 2009; Pereira et al., 2011). Concentrations of E2 in drinking water usually are reported as even lower, reaching only a few ng/l according to most reviews (Table 4).

3.4.3. Occurrence of EE2

Reviews that consider occurrence of E2 in aquatic matrices often also include figures for the synthetic estrogen EE2 (Pereira et al., 2011; Ratola et al., 2012; Verlicchi et al., 2012). According to reviews included in our study, EE2 concentrations in WWTPs influents range from <0.2 to 50 ng/l, with mean values ranging from 1.5 to 20 ng/l (Table 3). As with E2, two recent meta-analyses provide the best information on likely concentrations of these compounds in European WWTPs influents and effluents (Miège et al., 2008; Verlicchi et al., 2012). Verlicchi et al. (2012) include a small number of studies in their analyses with higher EE2 influent values (>10 ng/l), whereas Miège et al. (2008) report a maximum concentration of 5.2 ng/l EE2 in WWTPs influents. Both meta-analyses report lower concentrations of EE2 in influents compared with the natural hormone E2. The low concentrations of EE2 in WWTPs influents, as well as other aquatic matrices, makes it difficult to quantify or even detect this compound using standard analytical methods; this can limit the discussion about EE2 levels and removal during wastewater treatment (Clouzot et al., 2008 and see Section 3.5). Similar to influent concentrations, effluent concentrations are usually just a few nanograms EE2 per litre (Table 3). In the review studies evaluated, mean EE2 effluent concentrations ranged from 0.6 ng/l (Miège et al., 2008) to 3 ng/l (Verlicchi et al., 2012). As with influent concentrations, these values are lower than the corresponding mean E2 effluent concentrations. The minimum reported value in effluent is <0.02 ng/l (below LOD) (Clouzot et al., 2008), while the maximum value is 60 ng/l (Pereira et al., 2011). Generally average effluent concentrations are less than influent concentrations, however EE2 is known to be slightly more recalcitrant than E2, especially in regards to conventional WWT processes (Petrie et al., 2013).

Generally reviews of EE2 indicate that surface water concentrations are very low, often below LODs (González et al., 2012; Jurado et al., 2012; Martin and Voulvoulis, 2009). According to the reviews evaluated (Table 4), surface water concentrations of EE2 range from 0.04 ng/l (Kralchevska et al., 2013) to as high as 831 ng/l (Martin and Voulvoulis, 2009). The Martin and Voulvoulis (2009) review, which reported the highest EE2 surface water concentration of all the studies evaluated, is the only review to report a maximum value above 100 ng/l. In contrast, most reviews state that EE2 concentrations in surface waters do not exceed 10 ng/l (Clouzot et al., 2008; Meffe and de Bustamante, 2014; Rivera-Utrilla et al., 2013; Wise et al., 2011). Compared with other steroid estrogens such as E2 and estrone (E1), EE2 is detected in surface waters with the lowest frequency and at the lowest concentrations (Wise et al., 2011). Nevertheless, extremely low concentrations of EE2, even levels below most LODs, are known to cause endocrine disruptions such as intersex fish or vitellogenin induction (Clouzot et al., 2008). Thus similar to E2, the environmental risk of EE2 should not be underestimated just because surface water levels are low compared with other CECs.

As of 2011, only a small number of studies had measured EE2 in drinking water (Wise et al., 2011). Wise et al. (2011) reviewed these studies and found that in the UK, the EE2 levels were usually below reported LODs. Since then, a few more studies have reviewed concentrations of EE2 in drinking water and have found similarly low levels, ranging from 0.15 to 3 ng/l (Kralchevska et al., 2013; Pereira et al., 2011). Groundwater concentrations of EE2 have been reviewed by four studies; two reviews found no studies that detected EE2 in groundwater (Jurado et al., 2012; Meffe and de Bustamante, 2014), while two found values that ranged from 0.5 to 5 ng/l (Pereira et al., 2011; Santos et al., 2010).

3.5. Monitoring for DCL, E2 and EE2

The majority of the review studies evaluated by this systematic literature review were summaries of various methods for monitoring CECs in different environmental matrices. Variation in monitoring techniques can greatly influence the results of studies that report levels of CECs in the aquatic environment (Vazquez-Roig et al., 2013). This variation is certainly one component responsible for the wide range of DCL, E2 and EE2 concentrations in different matrices reported in section 3.4 above. The following section is by no means a comprehensive review of CEC monitoring and analyse techniques; instead, it specifically focuses on some of the most common methods and problems for evaluating the presence, the concentrations and effects of DCL, E2 and EE2 in the context of WFD monitoring. It also addresses some major issues and concerns related to monitoring techniques for priority substances in general.

In order to evaluate and regulate the levels of priority substances in water, the WFD has defined environmental quality criteria (Environmental Quality Standards, EQSs) (European Parliament and Council of the EU, 2008). Two forms of EQSs are used, the annual average (AA) EQS and the maximum allowable concentration (MAC) EQS (units of both are μg/l or ng/l). The arithmetic mean of the concentrations of a given priority substance recorded during all representative monitoring points in a water body for a given year must not exceed the defined AA-EQS. In contrast, the measured concentration at any monitoring point within a water body may not exceed the WFD-defined MAC-EQS. EQS values can be proposed for inland surface waters (which encompass rivers and lakes and related artificial or heavily modified water bodies) as well as "other" surface waters (European Parliament and Council of the EU, 2008). To date, AA-EQS values for both inland

and other surface waters have been proposed by the WFD for DCL, E2 and EE2 (European Commission, 2011). Compliance with EQSs is necessary to achieve a good chemical status of surface waters with regards to the chemicals on the EU list of priority substances, which could soon include DCL, E2 and EE2. The EQS values set by the WFD legislation will therefore directly impact which monitoring techniques will be acceptable for reporting purposes for a given compound, and will dictate the required level of sensitivity of those monitoring methods (Kunz et al., 2015).

3.5.1. Monitoring of diclofenac

The AA-EQS values proposed by the European Commission for DCL are 100 ng/l for inland surface waters and 10 ng/l for other surface waters (European Commission, 2011). The methods for detecting NSAIDs such as DCL were recently reviewed by Olives et al. (2012). These authors report that common identification and quantification methods include gas chromatography–mass spectrometry (GC–MS) and liquid chromatography (LC) coupled with a variety of detection methods, including ultraviolet (UV) detection, diode array detection, florescence detection and tandem MS. Because DCL is a polar compound, it is more suitable for analysis by LC as opposed to GC (Vazquez-Roig et al., 2013). Furthermore, in the review studies evaluated, LC was most often coupled with MS, a highly specific technique which can detect target compounds with high accuracy (Fischer et al., 2012; Hernández et al., 2014; Vazquez-Roig et al., 2013). Another recent review from the database of publications states that there is a clear trend towards the use of LC-MS over alternative detection methods for this class of emerging contaminants (Hernández et al., 2014). LC-MS/MS (liquid chromatography with tandem MS/MS detection) is preferred over LC-MS because the former method has greater analytical sensitivity and selectivity in the analysis of drug residues in complex samples (Olives et al., 2012). Table 5 shows that recent reviews from the database of publications indicate that when using these state-of-the-art analytical methods, the LOD for DCL are typically only a few nanograms per litre (Vieno and Sillanpää, 2014). It is therefore the case that current chemical analysis techniques can usually achieve the sensitivity required to detect DCL at the concentrations required for WFD reporting.

3.5.2. Monitoring of E2 and EE2

The AA-EQS values for E2 are 0.4 ng/l in inland surface waters and 0.08 ng/l in other surface waters (European Commission, 2011). For EE2, the AA-EQS values are even lower, 0.035 ng/l and 0.007 ng/l in inland and other surface waters respectively (European Commission, 2011). The WFD-proposed AA-EQS values are derived based on species sensitivity distribution studies using the most sensitive taxonomic groups, which in this case are fish and amphibians (Kunz et al., 2015). Because even very low concentrations of E2 and EE2 can have endocrine disrupting effects for some aquatic organisms (reviewed in Burkhardt-Holm, 2010), the proposed AA-EQs values for these two compounds are low in order to provide adequate protection for the aquatic environment and human health (Kunz et al., 2015). The implications of these low standards for monitoring methods and reporting, however, are significant.

In comparison to DCL, many more review studies in our database of publications focused on monitoring methods for measuring the effects and concentrations of estrogens in aquatic matrices (Briciu et al., 2009; Kozlowska-Tylingo et al., 2010; Kunz et al., 2015; Simon et al., 2015; Sosa-Ferrera et al., 2013; Streck, 2009; Tomšíková et al., 2012). Similar to DCL, techniques for the separation of steroid estrogens are usually based on LC or GC (Briciu et al., 2009; Streck, 2009; Tomšíková et al., 2012). Detection of these compounds is also carried out using various techniques, including UV detection, florescence detection, diode detection, MS detection and tandem MS (MS/MS) (Streck, 2009; Tomšíková et al., 2012). It is difficult to achieve the required sensitivity with UV, diode or florescence detection, whereas GC–MS, GC–MS/MS, LC-MS and LC-MS/MS have much lower LODs (Briciu et al., 2009; Streck, 2009; Tomšíková et al., 2012). The specificity and sensitivity of LC-MS/MS techniques are especially required for analysis of environmental samples with steroid estrogens because of the presence of endogenous steroids in biota; that LC-MS/MS can accurately identify endogenous and exogenous estrogens is a major advantage of this technique, and has led to it being the preferred method of choice for steroid-hormone analysis (Briciu et al., 2009; Sosa-Ferrera et al., 2013; Tomšíková et al., 2012). Even LC-MS analyses typically fail to provide the required level of sensitivity to detect and quantify trace concentrations of these compounds in environmental samples (Streck, 2009), thus the most sensitive methodology for the identification and quantification of steroid estrogens is widely recognized as LC-MS/MS.

Table 5 contains summary information about the LODs for E2 and EE2 from the recent review articles in the database of publications. It is clear from this summary table that oftentimes, even when using the advanced analytical detection methods described above, current monitoring techniques are not sensitive enough to detect E2 and EE2 levels in the low ng/l or pg/l range. This can result in many studies reporting no detects for these two compounds, which makes discussions of their levels and removal rates in environmental matrices difficult. What is especially problematic is that the LODs for the most advanced analytical detection methods are usually higher than the proposed WFD EQS values. This results in a serious problem regarding monitoring and reporting of E2 and EE2 concentrations in surface waters for WFD compliance. In fact a recent review study demonstrated that only 35% of published methods are able to detect E2 at the AA-EQS value of 0.4 ng/l, and only one published method exists that can detect EE2 at the AA-EQS value of 0.035 ng/l (Kunz et al., 2015; Tomšíková et al., 2012).

3.5.3. Possible alternatives in the monitoring of PhACs

Unlike the situation for DCL, current analytical detection methods are often insufficiently sensitive or robust for monitoring E2 and EE2 given the proposed WFD standards. Under this directive, methods of analysis must be able to achieve limits of quantitation (LOQ) equal to or below 30% of the associated EQS. For these emerging compounds extremely low EQS values, especially for marine waters, have been set which provide a great challenge to the analyst. One potential support technique for future monitoring lies in the application of passive sampling (PS) techniques in investigative and surveillance monitoring. Passive samplers are specifically designed to be deployed over a period of days to weeks, so that time-weighted average (TWA) concentrations of compounds in aquatic environments can be obtained (Wille et al., 2012). PS as a technique is based on the free flow of analyte molecules from a medium being sampled to a receiving medium due to a difference in chemical potentials (Miège et al., 2009).

PS is proving to be a valuable tool for the monitoring of a range of priority substances in water, sediment and biota, and can generally provide more representative profile information than infrequent spot sampling on the concentrations of pollutants in water bodies, particularly where concentrations fluctuate markedly in time. PS is rapidly gaining general acceptance as being applicable to monitoring the behaviour and (eco)toxicological effects and fate of polar compounds including, DCL, E2 and EE2 in the water column and generally can often enable much greater analytical sensitivity than can be achieved by "traditional" spot-sampling, potentially improving detection capabilities by orders of magnitude. While a variety of PS devices are now commercially available, several review studies describe the use of the Polar Organic Chemical Integrative Sampler (POCIS) (Buchberger, 2011; Vermeirssen et al., 2008; Wille et al., 2012). The POCIS has a polymer component sandwiched between two thin polythersulfone membranes. CECs with particular physiochemical properties will sorb onto this polymer while the device is deployed, and can then be extracted and analysed in the laboratory using analytical techniques (Buchberger, 2011; Vermeirssen et al., 2008). In addition to providing estimates of TWA concentration of compounds, passive samplers can be a potential solution for the

Table 5
Proposed WFD annual average environmental quality standard (AA EQS) values for each of the three CECs of interest vs current detection limits reported in literature reviews. Data originate from review studies from publication database, specific references are listed for each CEC (LOD/MDL values in ng/l). If no method of detection is provided with a LOD value, values represent summary (range or average) LODs for a variety of different methods.

Drug	Proposed AA-EQS (ng/l)[a]	LOD (ng/l)	Analytical method	Comment	Country	Review reference
DCL	100	A few	LC-MS	WWT effluent		Vieno and Sillanpää, 2014
		6	Immuno-assay	Surface water; WWT effluent	Germany	Buchberger, 2011
		–0.3	LC-MS	STP effluent	Luxemburg	Santos et al., 2010
		20	LC-MS	STP influent	UK	
		7	HPLC-MS	STP effluent	Spain	
		30	HPLC-MS	Hospital effluent	Spain	
		29	GC–MS	Groundwater	Germany	
		100	GC–MS	STP effluent	Spain	
		6	GC–MS	STP effluent	Switzerland	
		1	GC–MS	STP effluent	Greece	
E2	0.4	0.008–40	In vitro bioassays	Surface water	Switzerland	Kunz et al., 2015
		0.6–3.5	LC-MS	Sewage sludge	Spain	Sosa-Ferrera et al., 2013
		0.04–2.01	LC-MS	Environmental water	Spain	Tomšíková et al., 2012
		0.3–2	LC-MS	Surface water; STP effluent	Luxembourg	
		0.6–1.6	LC-MS	STP effluent	Germany	
		0.01–2	LC-MS	Groundwater	France	Santos et al., 2010
		1	LC-MS	STP effluent	Luxembourg	
		1.6	LC-MS	STP influent	Italy	
		1.1	LC-MS	STP effluent	Italy	
		0.4	LC-MS	Surface water	Italy	
		1.6	LC-MS	STP influent	Germany	
		0.4	LC-MS	STP effluent	Germany	
		0.2	LC-MS	Surface water	Germany	
		0.003–15	LC-MS	STP effluent, River Waters	Italy	Briciu et al., 2009
		2.3–10.6	LC-MS	River water	Belgium	
		0.1–0.2	HPLC-MS	WWT effluent	UK	
EE2	0.035	0.01–50	In vitro bioassays	Surface water	Switzerland	Kunz et al., 2015
		−0.04–2.01	LC-MS	Environmental water	Spain	Tomšíková et al., 2012
		0.3	LC-MS	STP effluent	Luxembourg	
		0.6–1.6	LC-MS	STP effluent	Germany	
		0.01–0.2	Immuno-assay	Surface water; WWT effluent	Germany	Buchberger, 2011
		2.0–	LC-MS	STP effluent	Luxembourg	Santos et al., 2010
		2.0	LC-MS	STP influent	Germany	
		0.4	LC-MS	STP effluent	Germany	
		0.2	LC-MS	Surface water	Germany	
		1.6	LC-MS	STP influent	Italy	
		1.1	LC-MS	STP effluent	Italy	
		0.4	LC-MS	Tibre river water	Italy	
		0.2	LC-MS	Groundwater	France	
		0.6–3.5	LM-MS	Sewage sludge	Spain	Sosa-Ferrera et al., 2013
		0.02–15	LC-MS	STP effluent; river water	Italy	Briciu et al., 2009
		2.3–10.6	LC-MS	River water	Belgium	
		0.1–0.2	HPLC-MS	WWT effluent	UK	
		0.3–4.1	various	WWT effluents	Denmark	Clouzot et al., 2008

LOD = limit of detection. Sensitivity of various analytical techniques deployed is influenced by sample preparation method and volume used for extraction.
[a] AA EQS values are annual average environmental quality standards for inland surface waters, which according to WFD legislation encompass rivers and lakes and related artificial or heavily modified water bodies. (Carvalho et al., 2015)

problem presented by the low AA-EQS values for E2 and EE2. These compounds may accumulate in passive sampling devices over time, allowing for current analytical techniques to detect and quantify E2 and EE2 levels. Several recent review studies refer to the use of passive sampling to monitor various environmental matrices for DCL, E2 and EE2, especially as a potential useful screening method in regards to WFD monitoring (Buchberger, 2011; Vermeirssen et al., 2008; Wille et al., 2012).

While passive sampling shows potential in future monitoring of concentrations and fate of emerging contaminants, application of the technique (particularly in the case of polar compounds) does face some obstacles before passive sampling is considered as a viable sampling method for the WFD or other legislation. Although the risk of toxicity for aquatic organisms is based on the bioavailable, or dissolved pollutants in a water body, the EQS set out in the WFD for the priority substances, (with the exception of trace metals), are expressed as concentrations in 'whole water'. This means that current analysis must include both the dissolved fraction and any suspended matter when used in compliance monitoring. However, for samples in which the level of suspended solids are low, it is often very difficult to reach the required limits of detection (LODs) by conventional means, and in this situation passive sampling could provide a useful alternative since they will take up the freely dissolved analytes in the water and have been shown to reach generally lower LODs than conventional grab samples. PS is also affected by environmental variables (temperature, water flow rate, salinity) and on the development of biofilms on the surface of the device which as an external factor can impede the uptake rate. Ongoing research is required to further develop the area of performance reference compounds (PRCs) to generally account for such effects however currently in the case of polar compounds the reliability of PRC information is limited and thus use of polar passive samplers is primarily restricted to use as a screening tool. As noted throughout this review, generation of accurate concentration information on levels of pharmaceuticals and NSAIDs in aquatic environments is becoming much more relevant in respect of greater legislative monitoring requirements and/or in terms of the generation of accurate data to support consumer or ecosystem risk exposure assessments. Passive sampling exhibits great potential for application in future monitoring

programs for the screening of current priority and emerging compounds in water, identification of "new" pollutants of concern, source identification and its potential role in operational, investigative and surveillance monitoring under the WFD and for other legislation source attribution and fate studies of any other potential solution to the problem presented by the low EQS.

Another potential solution to the problem presented by the low EQS values of E2 and EE2 is the use of biological effects monitoring techniques (Kunz et al., 2015; Simon et al., 2015; Streck, 2009). In the case of the estrogens in particular, a variety of in vitro assays for effect monitoring can identify the total estrogenic activity in environmental samples, which is reported as E2 equivalent (or EEQ) concentrations (Kunz et al., 2015). Streck (2009) reviewed several in vitro bioassays to measure endocrine disruption and he categorizes them into three groups: ligand-binding assays; recombinant receptor-reporter assays; and assays based on the measurement of cell proliferation induced by endocrine active compounds. Effect-based monitoring techniques are particularly useful in the context of the WFD for two reasons: (i) they could be used in future elaborations of monitoring programs to provide a link between chemical and ecological assessments of water quality, and (ii) they are an excellent method for analyzing the overall impact of mixtures of xenoestrogens present in many water bodies (Kunz et al., 2015; Streck, 2009). Furthermore, for compounds with extremely low EQSs, effect-based techniques can provide increased sensitivity, and may be used as a screening tool in monitoring programs. Integrated monitoring is currently the recommended approach according to experts in the field, and future iterations of European and national CEC monitoring programs will thus likely incorporate both chemical and biological monitoring techniques (Hecker and Hollert, 2011; Kunz et al., 2015; Simon et al., 2015). Miège et al. (2015) reviewed outcomes of discussions at a NORMAN Network-supported workshop in Lyon (France) in November 2014 that aimed at providing a common position on PS community experts regarding concrete actions required to foster use of PS techniques in support of contamination risk assessment and management and for routine monitoring of contaminants in aquatic environment. Agreed list of recommendations focused on improving the acceptance of PS by policy makers; drafting of guidelines, quality assurance and control procedures; developing demonstration projects where biomonitoring and PS were undertaken alongside each other; organising proficiency testing schemes; and inter-laboratory comparison and establishing passive sampler-based assessment criteria in relation to existing EQS. This was in agreement with international findings reported by Booij et al. (2016) who reviewed compliance monitoring requirements in the European Union, the United States, and the Oslo-Paris Convention for the protection of marine environment of the North East Atlantic, and evaluated if these are met by PS methods for non-polar compounds. International experts concluded the existence of several knowledge gaps outlined above, PS presently is best available technology for chemical monitoring of non-polar compounds.

3.6. Control Measures

This section reviews how the specific physiochemical properties of DCL, E2 and EE2 impact their removal from wastewater. It also discusses the control measures found to be effective for removal of these specific CECs. This section addresses three main issues: (i) how the chemical properties of DCL, E2 and EE2 impact their removal during wastewater treatment, (ii) how these three CECs respond during conventional secondary wastewater treatment (specifically in CAS plants) where we focused on the main elimination pathways (i.e. sorption and biodegradation), and (iii) which tertiary or advanced treatments are effective against each CEC of interest. For the later, we focused on the 4 main categories of advanced treatments, oxidation technologies, membrane technologies, activated carbon (AC) technologies and constructed wetlands (CWs).

3.6.1. Control measures of diclofenac

3.6.1.1. Chemical properties of diclofenac impacting removal. Diclofenac is weakly soluble in water (water solubility = 2.37 mg/L at 25 °C, (DrugBank, 2015a)), with an octanol–water coefficient ($\log K_{ow}$) of 4.51 (SRC, 2013). The pK_a of DCL is 4.15 (DrugBank, 2015a; SRC, 2013). DCL has a carboxylic acid portion in its molecular structure, and this region becomes negatively ionized at a neutral pH. At acidic pH, DCL becomes electronically neutral, which increases its capacity for sorption (Vieno and Sillanpää, 2014). Thus, DCL is a compound for which D_{ow} is a better predictor of hydrophobicity. Because D_{ow} is pH dependent, the matrix in which it is measured must be specified. The $\log D_{ow}$ value for DCL at a pH typical of wastewater treatment (approximately 8) is 2.51 (De Ridder et al., 2011). $\log K_d$ values for DCL are reviewed in Vieno and Sillanpää (2014), and listed in Table 6 As these values are typically less than three, very little removal of DCL due to sorption is predicted by this physiochemical property (Ternes et al., 2004). According to biodegradability studies, DCL biodegradation is slow or non-existent (Joss et al., 2005; Quintana et al., 2005). Studies investigating the biodegradation constant of DCL conclude that it is almost always $<0.1\ l\ g^{-1}\ ss\ d^{-1}$, indicating no substantial biodegradation (Joss et al., 2005; reviewed in Vieno and Sillanpää, 2014).

3.6.1.2. Removal of diclofenac during secondary treatment. In general, the physiochemical properties of DCL (summarized above in Section 3.6.1.1) lead to low removal via sorption (Joss et al., 2005; Martín et al., 2012; Radjenović et al., 2009; Suárez et al., 2012; Ternes et al., 2004). On average, DCL's sorption to secondary sludge is <5%, while its sorption to primary sludge is in the region of 5%–15% (Ternes et al., 2004). These removal percentages are actually often lower than would be predicted based on $\log D_{ow}$ values (De Ridder et al., 2011). Furthermore, DCL is poorly biodegradable (Joss et al., 2005; Joss et al., 2006a, 2006b; Quintana et al., 2005). As a result of its low removal via sorption and biodegradation, incomplete elimination of DCL can be expected during conventional activated sludge treatment (Table 7, Luo et al., 2014; Vieno and Sillanpää, 2014). A study by Patrolecco et al. (2015) identified DCL as one of the PhACs that exhibited the most persistence to removal at four WWTPs in Rome. Mainly primary and CAS secondary treatments were performed at the plants investigated. DCL showed high concentrations at the four treatment plants tested in both the influent and effluent samples (range = 519–2230 ng/l in influent and 321–1424 ng/l in effluent), and had the lowest removal efficiency out of all of the PhACs studied. Mean removal efficiencies for DCL were 36% removal in spring and 39% removal in winter. These values are consistent with other CAS plants according to a recent review (Vieno and Sillanpää, 2014). Similarly to the Patrolecco et al. (2015) study, Martín et al. (2012), found that DCL had the poorest removal of any of the NSAIDs studied (mean removal efficiency = 14%). The authors hypothesized that the poor removal could be due to DCL's poor degradation in wastewater. They also hypothesized that low removal efficiencies could be a consequence of the release of further DCL molecules by de-conjugation of glucuronidated or sulfated DCL and/or desorption from particles. Furthermore, in this study the PhACs that were detected in the wastewater were also detected in the sludge, indicating partial removal from wastewater through sorption; DCL, however, was only detected in wastewater confirming its low potential for sorption onto sludge. Studies have shown that elimination of DCL can be enhanced during secondary treatment by changing process configuration (reviewed in Vieno and Sillanpää, 2014). There is limited evidence that membrane bioreactors (MBRs) can increase removal efficiencies compared with CAS (Radjenović et al., 2009). This may be due to the higher biomass content and longer sludge retention time (SRT) applied in MBR. However, some studies show no increase in removal of DCL from wastewater when comparing MBR to CAS (e.g. Clara et al., 2005a). CAS with biological nutrient removal (BNR) utilizes a combination of aerobic, anaerobic and anoxic treatment units in

Table 6
Chemical parameters of DCL, E2 and EE2 potentially impacting removal from wastewater.

Drug	Chemical formula	CAS no	Molecular mass	Water solubility (experimental)	pK_a	Log K_{ow}	Log K_d	Proposed WFD AA EQS (inland surface waters)	Summary
Diclofenac	$C_{14}H_{10}Cl_2NO_2$ (DrugBank, 2015a)	15307-86-5 15307-79-6 (disodium salt)	296.15 (DrugBank, 2015a)	2.37 mg/L (at 25 °C) (DrugBank, 2015a)	4.15 (DrugBank, 2015a; SRC, 2013)	4.51 (SRC, 2013)	$logK_{d,primary\ sludge}$ 2.7 (Ternes et al., 2004) 2.3 (Radjenović et al., 2009) $logK_{d,secondary\ sludge}$ 1.2 (Ternes et al., 2004) 2.1 (Radjenović et al., 2009)	100 ng/l (European Commission, 2011)	Fairly soluble in water, moderately low octanol–water coefficient; ionization at neutral pH, becomes electronically neutral at acidic pH (reviewed in Vieno and Sillanpää, 2014)
E2	$C_{18}H_{24}O_2$ (DrugBank, 2015b)	50-28-2	272.38 (DrugBank, 2015b)	3.6 mg/L (at 27 °C) (DrugBank, 2015b)	10.4 (Joss et al., 2006b) (DrugBank, 2015b)	4.0 (Joss et al., 2006b)	$logK_{d,\ sludge}$ 2.3–2.8 (Carballa et al., 2008)	0.4 ng/l (European Commission, 2011)	E2 is weakly soluble in water, has high pK_a, fairly hydrophobic (Ben Fredj et al., 2015)
EE2	$C_{20}H_{24}O_2$ (DrugBank, 2015c)	57-63-6	296.40 (DrugBank, 2015c)	11.3 mg/L (at 27 °C) (DrugBank, 2015c)	10.4–10.7 (DrugBank, 2015c; Joss et al., 2006b)	4.2 (Joss et al., 2006b)	$logK_{d,primary\ sludge}$ 2.28–2.67 (Martín et al., 2012) $logK_{d,secondary\ sludge}$ 2.77–3.54 (Martín et al., 2012)	0.035 ng/l (European Commission, 2011)	EE2 is weakly soluble in water, has high pK_a; high $logK_d$ indicates it tends to be retained onto sludge, consistent with high pK_a value (Martín et al., 2012)

order to remove excess nutrients from wastewater. The use of BNR processes has been shown to sometimes increase removal of DCL from wastewater. However it should be noted that in a recent review of the impact process configuration has on DCL removal, MBR, BNR and CAS average removal efficiencies were very similar (48%, 36% and 36% average removal efficiencies respectively (Vieno and Sillanpää, 2014).

Elimination of DCL during conventional secondary treatment can also be enhanced by altering process parameters such as hydraulic retention time (HRT) and SRT. Increasing HRT to >2–3 days would increase the contact time of water with the biomass, leading to higher removal efficiencies (Suárez et al., 2012). However such an alteration would is likely to be unrealistic at an operational level due to the resulting need of increasing the volumetric capacity of the WWTP and high investment and operating costs associated. Moreover, enriching the bioreactor with DCL degrading microbes may also enhance elimination. This could be achieved by applying an SRT of >150 days; however, this may also not be a realistic option at full scale WWTPs (Fernandez-Fontaina et al., 2012). Bioaugmentation, which is the addition of cultured microbes possessing the ability to degrade DCL into the biological process, could be used, but this approach requires further research (Vieno and Sillanpää, 2014).

Table 7
Impact of conventional activated sludge on removal of CECs of interest during wastewater treatment.

PhAC	Sorption to sludge	Degradation potential	HRT	SRT	Removal efficiency (conventional activated sludge)
Diclofenac	Low potential Sorption to sludge observed to a low degree (Martín et al., 2012; Patrolecco et al., 2015; Radjenović et al., 2009; Suárez et al., 2012; Ternes et al., 2004)	Low potential Poorly biodegradable (Joss et al., 2005; Joss et al., 2006a, 2006b; Quintana et al., 2005)	Elimination of diclofenac could be enhanced by increasing HRT to > 2–3 days; would increase the contact time of water with the biomass (Suárez et al., 2012)	Enriching the bioreactor with diclofenac degrading microbes may enhance elimination; could be achieved by applying a SRTs >150 days (Fernandez-Fontaina et al., 2012)	Variable but generally poorly removed; 0–81.4% (Luo et al., 2014) Mean concentrations in European municipal influents between 0.11 and 2.3 μg/l (110 and 2300 ng/l), effluents between 0.002 and 2.5 μg/l (2 and 2500 ng/l) (Vieno and Sillanpää, 2014)
E2 & EE2	Moderate potential Susceptible to removal by sorption (Ben Fredj et al., 2015; Carballa et al., 2008; Martín et al., 2012; Joss et al., 2006a; Zhang and Zhou, 2008)	High potential Generally biodegraded very effectively in WWTP processes under aerobic and anaerobic conditions (Abargues Llamas et al., 2012; Alvarino et al., 2014; Petrie et al., 2014)	Biodegradation was increased when the HRT was optimised by extending it from 8 to 24 h (Petrie et al., 2014)	Maximum achievable removal when at the maximum SRT studied (27 days) (Petrie et al., 2014) Critical SRT of 10 days for removal of natural estrogens and some micropollutants suggested (Clara et al., 2005a)	Highly removed: E2: 92.6–100% (Luo et al., 2014) EE2: 43.8–100% (Luo et al., 2014) Reduced by ~85%. Final effluents normally contain nanogram per litre concentrations (Griffith et al., 2014) EE2 typically more recalcitrant than E2 (Petrie et al., 2014)

3.6.1.3. Removal of diclofenac during tertiary treatment. The recalcitrant nature of DCL during conventional wastewater treatment has led to a large body of research investigating further removal of this compound from treated wastewater via tertiary treatments. Much of this research has focused on oxidation technologies, which have been found effective at mineralizing many NSAIDs (Malato, 2008; Oulton et al., 2010; Suárez et al., 2008; Ziylan and Ince, 2011). Ziylan and Ince (2011) compared the relative efficiencies of some basic advanced treatment processes and found that ozonation was among the most effective in terms of achieving the complete disappearance of NSAIDs, including DCL; they report that 95–100% of residues can be destroyed using this treatment. Some oxidation technologies that have been found to effectively degrade DCL in treated wastewater are gamma ray irradiation (Liu et al., 2011), ionizing radiation (Kimura et al., 2012) and UV or UV/H_2O_2 (Lekkerkerker-Teunissen et al., 2012), among others (reviewed in Ziylan and Ince, 2011). Operating conditions, however, can impact DCL removal efficiencies when considering oxidation technologies (Malato, 2008; Ziylan and Ince, 2011). For example, initial DCL concentration (Liu et al., 2011) operation pH (Malato, 2008), TSS loading (Oulton et al., 2010) and oxidant dose and contact time (Oulton et al., 2010; Rivera-Utrilla et al., 2013) have all been shown to impact DCL removal efficiencies. Differences in such operating conditions can explain the range of removal efficiencies reported for a vast number of oxidation technologies. Combined homogenous advanced oxidation processes (AOPs) in particular (for example, UV/H_2O_2, O_3/UV, Fe^{2+}/H_2O_2 (Fenton) and UV/Fenton oxidation) are thought to be very promising, and have shown great efficacy for DCL removal from treated wastewater (Ribeiro et al., 2015; reviewed in Ziylan and Ince, 2011). However, the major drawback of oxidation technologies for treating PhACs remains the potential formation of toxic or persistent by-products if a compound fails to be completely mineralized (Oulton et al., 2010). DCL is one of the compounds that has specifically been shown to produce by-products after treatment, especially if the oxidant dose or contact time are not adequate (Sein et al., 2008). The toxicity of these compounds must be evaluated in order to fully assess the potential of any oxidation technology for treating this particular CEC (Andreozzi et al., 2004).

The use of membrane filtration technologies has also been explored as a possibility for removing DCL from treated wastewater (Kimura et al., 2003; Snyder et al., 2007; reviewed in Suárez et al., 2008; Xu et al., 2005). In general, the effectiveness of membrane filtration for DCL removal greatly depends on the type of technology considered. For example, it has been shown that DCL is poorly eliminated by microfiltration or ultrafiltration membranes (Snyder et al., 2007), making these technologies poor choices for the removal of this PhAC from treated wastewater. However studies show that nanofiltration and reverse osmosis membranes can eliminate DCL very effectively (i.e. >90%) (Kimura et al., 2003; Snyder et al., 2007; Suárez et al., 2008), although lower elimination efficiencies (60%) have also been reported (Röhricht et al., 2009). Snyder et al. (2007) specify that charged compounds (including DCL), had high rejection efficiencies for the nano and reverse osmosis membranes utilized in their study due to electrostatic exclusion between the anionic compounds and the negatively charged membranes. Nevertheless, rejection efficiency via membrane filtration has been found to decrease as the concentration of DCL in treated wastewater decreases (Kimura et al., 2003). Biofouling of membranes can also impact the rejection efficiencies of some organic compounds; however, the physiochemical properties of contaminants can have an impact on their behaviour in regards to biofouling. In one study Botton et al. (2012) found that the rejection efficiencies of negatively charged compounds (including DCL) were no different when comparing virgin and biofouled nanofiltration membranes. Although the use of membrane filtration processes for removal of DCL is technically feasible and effective, some studies report that it may not be economical for wastewater treatment given high operational and investment costs (Röhricht et al., 2009; Suárez et al., 2008).

The use of both powdered AC (PAC) and granular AC (GAC) can also result in the removal of many PhACs - including DCL- from water (Delgado et al., 2012; Rivera-Utrilla et al., 2013; Snyder et al., 2007). Because removal via this technology type is based largely on sorption, the physiochemical properties of specific compounds influences their removal efficiencies (Baccar et al., 2012). For example, as sorption mechanisms are mostly hydrophobic when using AC materials (Delgado et al., 2012), logD_{ow} values can sometimes be good indicators of compound removal by AC. Although this type of tertiary treatment can partially remove DCL from water, in a recent review by Delgado et al. (2012), DCL was repeatedly cited as one of the most difficult compounds to remove using AC (e.g. below 85% at 35 mg PAC/L in Snyder et al., 2007). Removal efficiencies for DCL are also variable and can depend on factors such as contact time, pH, concentration of natural organic matter and AC dose (Baccar et al., 2012; Delgado et al., 2012; Snyder et al., 2007). Removal efficiencies of DCL can be enhanced when AC is used in combination with other technologies, such as AOPs. In this case, by-products or intermediates produced from the oxidation process can be removed via sorption onto the AC (Rivera-Utrilla et al., 2013).

The ability of CWs to remove CECs like DCL has been studied more extensively in the past decade (Hijosa-Valsero et al., 2010; Hijosa-Valsero et al., 2011; Matamoros and Bayona, 2008; Matamoros and Bayona, 2006). Although many CECs can be removed from wastewater extremely efficiently through the use of CWs, DCL is commonly cited by studies as a particularly recalcitrant compound in these systems (Hijosa-Valsero et al., 2010; Hijosa-Valsero et al., 2011; Matamoros and Bayona, 2008; Matamoros and Bayona, 2006; Oulton et al., 2010). Mean removal efficiencies for DCL in CW systems are very variable, ranging in just one study from 0 to 45%. This variability is similar to removal efficiencies for this compound in conventional wastewater treatment (Matamoros and Bayona, 2006 and see review in Section 3.6.1.2). Many factors can impact DCL removal in CWs, including process configuration (surface vs subsurface designs (Matamoros and Bayona, 2008; Oulton et al., 2010)); design parameters (water depths, presence of vegetation, plant species, etc. (Hijosa-Valsero et al., 2011; Matamoros and Bayona, 2006)); and environmental parameters (initial concentration of the compound, oxygen availability or the season (Hijosa-Valsero et al., 2011; Matamoros and Bayona, 2008)). These variables are obviously not independent as configuration and design parameters will impact many of the environmental conditions at a given treatment site. If these systems are going to be utilized with the aim of achieving significant DCL removal from wastewaters, specific parameters that have been shown in the literature to increase removal efficacy should be implemented. For example, recent research has demonstrated that high redox potential and the presence of plants appears to favour DCL removal (Hijosa-Valsero et al., 2011). It should also be kept in mind that removal efficiencies of PhACs at CWs can vary seasonally, with some evidence of lower removal in winter months due to lower bacterial activities at low temperatures (Hijosa-Valsero et al., 2011). Furthermore, the use of low-cost alternative sorbent materials (e.g. expanded clay, zeolite), as opposed to conventional inert materials such as sand and gravels or advanced materials such as AC, was shown to have a great potential for the removal of DCL in CW with removal efficiencies up to 90% (Dordio and Carvalho, 2013); however despite a great potential these studies were performed at pilot scale and the results need to be confirmed in real scale experiments.

3.6.2. Control measures of E2 and EE2

3.6.2.1. Chemical properties of E2 and EE2 impacting removal. EDCs such as E2 and EE2 are mostly hydrophobic organic molecules, meaning they have a tendency to distribute in organic phases (Ben Fredj et al., 2015). E2 has a logK_{ow} of 4.0 (Joss et al., 2006b) and EE2 has a logK_{ow} of 4.2 (Joss et al., 2006b). The logK_d values for E2 fall between 2.3 and 2.8 (Carballa et al., 2008) and as high as 3.54 for EE2 (Table 6) (Martín

et al., 2012). When $\log K_d$ values are approximately 3–5, the compounds can be expected to have moderate potential for sorption to sludge (Ternes et al., 2004); even though values below three have been reported for these estrogens under specific conditions, they are close enough to this threshold that moderate sorption potential can be expected for E2 and EE2. Indeed, studies found that these two hormones tend to gather on underwater fauna, sediments or WWTP sludge when in aquatic matrices (Zhang and Zhou, 2008). Nevertheless, biodegradation is accepted as their foremost removal pathway (Petrie et al., 2014); the K_{biol} constants for E2 and EE2 are much higher than that of DCL (300–800 and 7–9 respectively according to a review by Suárez et al. (2008)).

3.6.2.2. Removal of E2 and EE2 during secondary treatment. E2 and EE2 are both generally biodegraded very effectively in WWTPs under aerobic and anaerobic conditions (Table 7, Abargues Llamas et al., 2012). According to Alvarino et al. (2014), higher biodegradation of both compounds is achieved under aerobic conditions. Cometabolism (i.e. when an organic compound is transformed by microorganisms that cannot use the compound or its transformation products as a source energy (Grady et al., 1999)) has been shown to be the main mechanism in the removal of EE2 under nitrifying conditions, through the enzyme ammonium monooxygenase. Alvarino et al. (2014) found that in addition, other aerobic bacteria could be contributing to EE2 removal via biodegradation. As well as removal via biodegradation, the low polarity of these estrogenic compounds means sorption onto sludge may also be partially responsible for their removal from wastewater (Martín et al., 2012).

In a study by Petrie et al. (2014), the potential of CAS processes to simultaneously remove multiple micropollutants was evaluated. The study utilized a pilot-scale activated sludge plant in order to ensure process control and avoid variations in receiving sewage composition and flow; they then controlled SRT and HRT in order to evaluate the impact of these process parameters on micropollutant (including the estrogens E2 and EE2) removal. First, they evaluated whether an increase in SRT had an influence on removal at a fixed HRT of 8 h. The authors recorded maximum achievable micropollutant removal for all chemicals, including the estrogens, when at the maximum SRT studied (27 days). Furthermore, removal efficiencies were increased when the HRT was optimised by extending it from 8 h to 24 h. Most notably in the study was the improvement in the removal of the persistent EE2 (increased from 41% to 65% ± 19% at the 24 hour HRT). Improved removal of E2 (≥93%) was also demonstrated following this operational process change. Lengthening of the HRT saw a decrease in the food to microorganism ratio (F: M). A lower F: M ratio is indicative of a substrate limitation which in turn can lead to less-favoured carbon substrates like steroid estrogens being biodegraded as the primary food source (Cho et al., 2014). This together with an increased contact time for biodegradation might explain the improvement in the observed biodegradation at the longer HRTs (Petrie et al., 2014).

Similar to DCL, process configuration can have an impact on E2 and EE2 removal. In a 2011 study, the removal of E2 and EE2 was determined in four different WWTPs in the UK (Ifelebuegu, 2011). Removal ranges were 83–97% for E2 and 41–58% for EE2, demonstrating again that EE2 is often more persistent when compared with other estrogenic compounds. In this study, activated sludge plants that were configured for BNR showed better removal of the estrogens compared with other CAS plants. Again, both biodegradation and sorption to sludge were recognized as the primary pathways for removal (Ifelebuegu, 2011).

Jarošová et al. (2014) conducted a pan-European monitoring campaign of WWTPs effluents which included an effect-based assessment to determine estrogenicity. They found that one third of the tested municipal WWTPs effluents had EEQ values >0.5 ng/l, and that the values ranged from 0.53 to 17.9 ng/l EEQ. Overall this study shows that although removal efficiencies of E2 and EE2 (and other estrogenic compounds) are usually quite high, incomplete removal could still pose a threat to the environment; thus everything possible should be done at conventional wastewater treatment facilities to increase removal of these potent estrogenic compounds.

3.6.2.3. Removal of E2 and EE2 during tertiary treatment. Unlike DCL and as discussed above, E2 and EE2 are not particularly resistant to conventional wastewater treatment; however, tertiary treatment options for removing trace concentrations of these compounds are still being investigated because of their potential to negatively impact wildlife and humans even at the low levels found in conventional wastewater effluent (Burkhardt-Holm, 2010).

According to the literature, oxidative treatments (including ozonation and AOPs) are extremely efficient at eliminating estrogens from treated wastewater (Clouzot et al., 2008; Oulton et al., 2010; Pereira et al., 2011; Pereira et al., 2012; Ribeiro et al., 2015; Suárez et al., 2008). In a recent review, Pereira et al. (2011) found that estrogenic compound levels (including E2 and EE2) can be reduced between 94 and 99% using various AOP technologies. In fact, ozone is even more reactive with E2 and EE2 than with DCL, thus almost complete transformation of these compounds is expected following treatment (Suárez et al., 2008). However, removal of estrogens from treated wastewater through ozonation is pH dependent, and higher pH values reportedly lead to better reactivity of these compounds with ozone (Pereira et al., 2011). Furthermore the ozone reaction slows down at estrogen concentrations <100 ng/l, which is significant because such low levels are often found in water requiring treatment. In addition the presence of other compounds in treated wastewater can reduce reaction efficiencies between estrogenic compounds and oxidants (reviewed in Koh et al., 2008; Pereira et al., 2011). According to Pereira et al. (2011) some of the best oxidative technologies for the removal of estrogens are ozonation, ferrate oxidation, and disinfection with chlorine dioxide. In contrast, E2 and EE2 are poorly removed via direct phototransformation (Oulton et al., 2010). The presence of toxic by-products and intermediates produced through the treatment of estrogens with oxidation technologies is a growing concern. Although some studies suggest that the by-products produced are less estrogenic than the parent compounds (reviewed in Clouzot et al., 2008), more work is needed to identify and evaluate these by-products. If oxidation technologies are used to reduce the estrogenicity of wastewater, at the very least operating parameters (such as oxidant dose, contact time, and water pH) should be evaluated and adjusted in order to reduce by-product production (Pereira et al., 2011).

The ability of membrane filtration technologies to remove estrogenic compounds has also been investigated, and similar to DCL, removal efficiencies depend on the technology employed (reviewed by Koh et al., 2008; Oulton et al., 2010; Suárez et al., 2008). One in depth study found that microfiltration and ultrafiltration, while inefficient for the removal of most CECs, were very effective at removing steroid hormones (including E2 and EE2 (Snyder et al., 2007)). In general, however, microfiltration and ultrafiltration are not thought to perform as well as nanofiltration and reverse osmosis membranes (Koh et al., 2008), which are considered effective at removing estrogenic hormones from water (Braeken and Van der Bruggen, 2009; Dudziak and Bodzek, 2009; Koh et al., 2008; Snyder et al., 2007). In a review of the treatment and control strategies for removing estrogens from wastewater, Koh et al. (2008) state that nanofiltration and reverse osmosis can achieve up to 90% removal of estrogens. These figures however are variable, and lower estrogen removal via nanofiltration has been reported in other studies (between 60 and 85%) (Braeken and Van der Bruggen, 2009; Dudziak and Bodzek, 2009). This variability can be caused by differences in the properties of specific membranes (e.g. molecular weight cut-off, hydrophobicity, surface roughness or charge); the physiochemical properties of specific compounds (e.g. molecular size/weight, the acid dissociation constant, $\log K_{ow}$ values or polarity); or the characteristics of the wastewater (e.g. concentration of the compound, pH, presence of additional organic matter) (Dudziak and Bodzek, 2009; reviewed by Oulton et al., 2010). Finally, when considering E2 and EE2 in regards

to membrane filtration technologies, both hydrophobic adsorption and size exclusion should be considered as potential mechanisms of removal, and if ultra or microfiltration are used in MBR, biodegradation may also play an important role in the removal process (Koh et al., 2008; Oulton et al., 2010).

The removal of estrogenic compounds using AC has also been shown to be very effective (Clouzot et al., 2008; Delgado et al., 2012; Koh et al., 2008; Snyder et al., 2007; Suárez et al., 2008). In a comprehensive study by Snyder et al. (2007), both PAC and GAC were capable of removing E2 and EE2 to high levels (up to 100%). It is also specified in this study and elsewhere that the efficacy of AC for removing estrogens is reduced when natural organic matter is present, because it competes for binding sites thereby limiting removal (Koh et al., 2008; Snyder et al., 2007).

Finally, constructed wetlands (CWs) have been evaluated for their ability to remove estrogenic compounds from wastewater. A comprehensive review on this subject was carried out by Matamoros and Bayona (2008). They report that CWs can remove estrogens to similar extents as conventional wastewater treatment plants, and that certain configurations achieve >90% removal. The authors also suggest that the main mechanism of estrogen removal in CWs is sorption, and therefore subsurface flow configurations will be preferable to surface flow systems.

3.6.3. Practicalities of implementing various tertiary technologies for wastewater treatment

All four of the tertiary treatment types discussed above (oxidation technologies, membrane filtration, AC and CWs) have shown some efficacy for the removal of DCL, E2 and/or EE2 (Table 8). However, whether or not tertiary treatments will ultimately be implemented at WWTPs depends on a number of factors besides their efficacy for removing micropollutants. Instillation and running costs, increases in consumer payments, overall environmental footprint, stage of development of different technologies, and general drawbacks associated with each type of treatment must be considered and weighed against potential benefits (Jones et al., 2007). Life cycle analysis (LCA) is currently a popular tool for evaluating the costs and benefits of products, services or processes in a number of sectors, and it recently has been applied to the wastewater treatment industry (reviewed in Corominas et al., 2013). LCA is unique in that it allows for a "cradle-to-grave" analysis of the technologies in question. LCA has been used to compare emerging technologies with conventional wastewater treatments (e.g. Igos et al., 2012; Kalbar et al., 2013; Machado et al., 2007). Researchers have also implemented it to evaluate the use of advanced treatment options for micropollutant (including CECs) removal (Hoibye et al., 2008; Rodríguez et al., 2012; Wenzel et al., 2008). This field of research is still relatively new (for example no LCA studies evaluating constructed wetlands in this context were found), but LCA studies for oxidation technologies (Hoibye et al., 2008; Rodríguez et al., 2012; Wenzel et al., 2008), AC (Igos et al., 2013) and membrane filtration technologies (Hoibye et al., 2008; Wenzel et al., 2008) regarding micropollutant removal do exist. A recent review of such studies states that overall, most findings indicate that there is little to no environmental benefit from the removal of CECs achieved by most advanced treatment technologies (Corominas et al., 2013). However, this is largely due to the uncertainty regarding the environmental impacts of CECs at very low concentrations; a better understanding of the implication of contamination of waters with trace concentrations of CECs is therefore necessary to improve LCA studies in this field. Additionally, economic analyses have also indicated that treating wastewater with advanced technologies for the purposes of micropollutant removal may not be feasible; Jones et al. (2007) suggest that the high costs of adopting tertiary treatments at wastewater facilities would most likely be passed onto industrial and domestic consumers. To avoid this phenomenon, they suggest that instead, parameters in conventional wastewater treatment plants should be adjusted to maximize CECs removal.

Barbosa et al. (2016) recently reviewed the occurrence and removal of organic micropollutants that comprise the complete watch list of EU Decision 2015/495. It was concluded that the efficiency of the treatment processes can decrease considerably when realistic water matices are used instead of simulated ones. As reported above, these reviewers also commented, since multiple factors can affect the efficiency of treatments, experiments should be performed as close as possible to the real conditions. Additionally, the formation of intermediates should be further investigated, considering that the produced by-products may be more toxic and/or persistent that the parent compounds. Barbosa et

Table 8
Impact of various tertiary treatment types on removal of CECs of interest during wastewater treatment.

Technology	Diclofenac removal	E2/EE2 removal	Costs	By-product danger
Membrane filtration technologies	Highly dependent on filtration technology; poor for micro and ultrafiltration, can be efficient for nano and reverse osmosis (Kimura et al., 2003; Snyder et al., 2007; Suárez et al., 2008)	Variable depending on technology; removal via nanofiltration ranges from > 50%–90% (Braeken and Van der Bruggen, 2009; Dudziak and Bodzek, 2009; Koh et al., 2008)	Capital costs include construction, engineering, materials costs, operational and management costs include replacing membranes and power to pump wastewater (US EPA, 1999a)	No (US EPA, 1999a)
Activated carbon	Can be efficient, depending on operational variables (Delgado et al., 2012)	Can be efficient, depending on operational parameters and wastewater characteristics (Delgado et al., 2012; Koh et al., 2008)	Dependent on different carbon contactor configurations and the cost of on-site vs off-site regeneration, as well as site and wastewater characteristics Capital costs include carbon contactors, storage tanks, regeneration systems (etc.) and operational costs include purchase of carbon, electrical power, flushing of carbon slurry piping, etc. (US EPA, 2000b)	No (Rivera-Utrilla et al., 2013)
Oxidation Technologies	Highly efficient processes for DCL removal (>90%) (Ribeiro et al., 2015; Ziylan and Ince, 2011)	Highly efficient process for estrogen removal (94–99%) (Pereira et al., 2011)	Dependent on technology type, capacity of the plant, wastewater characteristics, manufacturer and the site; e.g. ozonation costs generally high compared with other technologies, while UV can be competitive (US EPA, 1999b, 1999c)	High for DCL (Sein et al., 2008) and estrogenic compounds (Pereira et al., 2011)
Constructed wetlands	Very variable removal efficiencies, DCL considered recalcitrant compound (Matamoros and Bayona, 2008; Oulton et al., 2010)	Variable removal efficiencies but can be effective (>90%) depending on configuration and design parameters (Matamoros and Bayona, 2008)	Major capital costs include purchasing land, liner costs, engineering, etc., but both capital and operational and management costs tend to be much lower than conventional wastewater treatments (US EPA, 2000a, 2000c)	

al. (2016) also commented on the affordability of scale up of wastewater treatment options for tackling CECs, particularly, both in the implementation and management. Fate and behavioural geographical mapping and modelling of CEC hot spots can inform priority of decision making for upgrade of wastewater treatment plants and other pressure points of pollution, such as hospital effluent outputs, with advanced treatment technologies Aldekoa et al. (2013).

HRT: hydraulic residence time; SRT: solids retention time.

4. Conclusions and future research needs

The overall aim of this study was to provide a baseline study for Europe exploring the implications of the addition of the three watch list compounds DCL, E2 and EE2 to the Water Framework Directive (WFD) priority substances list. This study utilized a systematic literature review to summarize the European state of knowledge in regards to the sources and prevalence of these CECs. Finally, a critical analysis of the effectiveness of potential control measures was carried out based on best-published information. Below, the main conclusions from each of these components of the study are summarized and future research needs are established.

The bibliographic analysis carried out by this study determined that the annual output of European research on DCL, E2 and EE2 has increased steadily from 1995 to 2015, with approximately 84% of all articles on aquatic contamination with these CECs published since 2005. More studies are performed on the estrogens than DCL annually, and studies focused on monitoring are more common than those on sources of contamination or control measures, though control measure studies are on the rise in recent years. Laboratory scale studies are the most common, while more realistic field and wastewater treatment plant (WWTP) level studies are rarer. This can likely be attributed to a lack of sensitive analytical techniques or accurate sensors. Spain and Germany are the European leaders in the field. The systematic literature review conducted by this study investigated the sources, receptors and monitoring methods for the three CECs of interest. Overall it was found that DCL and EE2 enter the European aquatic environment mainly following human consumption and excretion of therapeutic drugs, and incomplete removal from influent at urban WWTPs. E2 is a natural hormone excreted by humans which also experiences incomplete removal during WWTPs treatment, although livestock populations in Europe are also a significant non-point source of E2 contamination. In regards to receptors, throughout Europe DCL has on average higher concentrations (high ng/l or μg/l levels) in all aquatic matrices compared with the hormones E2 and EE2 (ng/l range); however, this does not necessarily translate to higher negative environmental impact/risk to aquatic organisms. Diclofenac concentrations found in European surface waters are generally below the annual average environmental quality standard (AA EQS) proposed by the WFD (100 ng/l), but several review studies report values exceeding this limit in the UK, Italy and other mainland European countries. E2 European surface water values are usually <50 ng/l but nevertheless such values still greatly exceed the proposed AA EQS value (0.04 ng/l) of this bioactive compound. Similarly, EE2 is either not detected or found at low levels in European surface waters (usually below 10 ng/l), but reported values are often still higher than the proposed AA EQS value (0.035 ng/l). Finally, current standard, laboratory-based analytical chemistry methods are sufficiently sensitive for the detection and quantification of DCL, but limits of detection for E2 and EE2 are often higher than proposed AA EQS values, presenting serious analytical challenges in regards to chemical monitoring methods and reporting for these two CECs.

The systematic literature review results were expanded to analyse potential control measures that may be implemented at WWTPs to decrease levels of DCL, E2 and EE2 in final effluents. The review revealed that physiochemical properties or experimentally determined constants that can be used to predict the removal of CECs during wastewater treatment include the octanol–water partition coefficient (K_{ow}), n-octanol–water partition coefficient (D_{ow}), solid-water distribution coefficient (K_d), half-life and the biodegradation constant, (K_{bio}). CECs with high water solubility and low biodegradability are the most recalcitrant during wastewater treatment. Studies showed that DCL is poorly removed during conventional wastewater treatment; removal percentages are variable but generally fall between 21 and 40%. Mean concentrations in European municipal influents are between 0.11 and 2.3 μg/l (110 and 2300 ng/l) and effluents between 0.002 and 2.5 μg/l (2 and 2500 ng/l). In contrast, E2 and EE2 are generally highly removed during conventional wastewater treatment: removal percentages generally are 85% or greater. Final European effluents normally contain nanogram per litre concentrations of these compounds, but EE2 is consistently more recalcitrant than E2.

Where secondary treatments are deemed insufficient, tertiary treatments may be used to further improve the quality of the final effluent. In particular, recent studies have mostly investigated four types of tertiary treatment technologies for removal of CECs from treated wastewater including oxidation technologies, membrane filtration, the use of activated carbon (AC) and constructed wetlands (CW). Oxidation technologies are considered highly efficient at DCL removal. Membrane filtration can also be efficient at removing DCL from treated wastewater, but it depends on the technology used; micro and ultra-filtration are typically ineffective while nano and reverse osmosis filtration are very efficient for this particular CEC. The application of AC can effectively reduce DCL concentrations in treated wastewater, but removal efficiencies largely depend on operational variables. Finally, CWs demonstrate variable removal of DCL, but this compound is generally considered recalcitrant in these systems. For E2 and EE2 removal, oxidation technologies are considered very effective treatments. Membrane filtration technologies, while exhibiting more variation than oxidation technologies, can also be extremely effective. The use of AC is also appropriate for estrogen removal, but similar to DCL removal efficiencies depend on operational variables and wastewater characteristics. Finally, CWs can perform estrogen removal, but the effectiveness depends upon the configuration and design of the system. Although more information is needed to accurately model the benefits of using tertiary treatments to reduce CEC concentrations in treated wastewaters, in general the literature suggests that the environmental benefits may not outweigh the costs. Some sources suggest that it may currently be more economically to adapt conventional wastewater treatment operational variables to decrease CEC emissions, rather than incur the costs/complications of adding tertiary treatments.

This study has highlighted areas for future research attention to include (a) development of more sensitive and validated analytical methods for different environmental samples (especially for the steroid estrogens) in order to be able to comply with WFD reporting requirements. Given the extremely low AA EQSs proposed for these compounds, it is also necessary to continue to investigate alternatives to chemical analyses, such as passive sampling, use of appropriate biological surrogates or effect-based monitoring techniques; (b) intensification of technology-focused studies for effective and efficient control measures for CECs removal particularly at areas showing disproportionally high levels of these CECs in terms of load; (c) evaluation of validated, bolt-on or mobile technologies effective for the removal of CECs in wastewater, (d) identify and quantify population level effects of wild biota from endocrine disrupting chemical exposure; and (e) investigate seasonal variations in CEC loading and removal efficiencies in future studies with particularly addressing influence of climate change.

Acknowledgements

The authors express gratitude to Ireland's Environmental Protection Agency STRIVE Programme for supporting this study (Project 2014-W-DS-18).

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Science of the Total Environment 603–604 (2017) 627–638

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Development of a semi-quantitative risk assessment model for evaluating environmental threat posed by the three first EU watch-list pharmaceuticals to urban wastewater treatment plants: An Irish case study

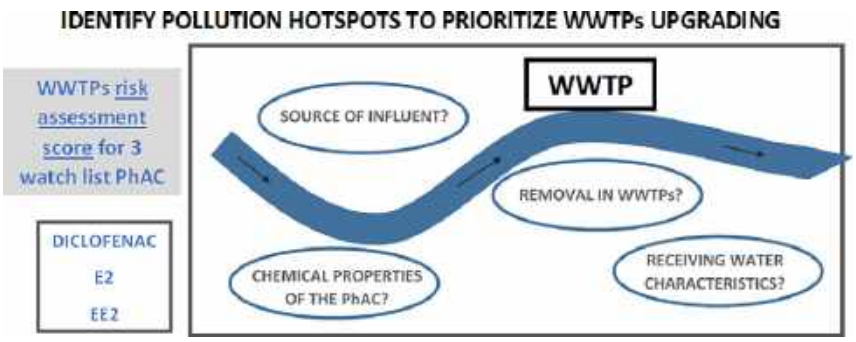
Alexandre Tahar [a,*], Erin Jo Tiedeken [a,b], Eoghan Clifford [c], Enda Cummins [d], Neil Rowan [a]

[a] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[b] National Biodiversity Data Centre, Waterford, Ireland
[c] College of Engineering and informatics, Department of Civil Engineering, National University of Ireland Galway, Ireland
[d] School of Biosystems and Food Engineering, University College Dublin, Ireland

HIGHLIGHTS

- Lack of data regarding the likelihood of one given pharmaceutical to be found in river
- Semi-quantitative risk assessment (RA) proposed for three watch-list PhACs
- The RA is based on influent source, PhACs properties, WWTP removal and receiving water
- A method to identify pollution hotspots and WWTPs responsible for PhACs release is provided

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 February 2017
Received in revised form 23 May 2017
Accepted 24 May 2017
Available online 26 June 2017

Editor: D. Barcelo

Keywords:
Pharmaceutical compounds
EU-watch list
Receiving water
Risk assessment
Wastewater treatment plants

ABSTRACT

Contamination of receiving waters with pharmaceutical compounds is of pressing concern. This constitutes the first study to report on the development of a semi-quantitative risk assessment (RA) model for evaluating the environmental threat posed by three EU watch list pharmaceutical compounds namely, diclofenac, 17-beta-estradiol and 17-alpha-ethinylestradiol, to aquatic ecosystems using Irish data as a case study. This RA model adopts the Irish Environmental Protection Agency Source-Pathway-Receptor concept to define relevant parameters for calculating low, medium or high risk score for each agglomeration of wastewater treatment plant (WWTP), which include catchment, treatments, operational and management factors. This RA model may potentially be used on a national scale to (i) identify WWTPs that pose a particular risk as regards releasing disproportionally high levels of these pharmaceutical compounds, and (ii) help identify priority locations for introducing or upgrading control measures (e.g. tertiary treatment, source reduction). To assess risks for these substances of emerging concern, the model was applied to 16 urban WWTPs located in different regions in Ireland that were scored for the three different compounds and ranked as low, medium or high risk. As a validation proxy, this case study used limited monitoring data recorded at some these plants receiving waters. It is envisaged that this semi-quantitative RA approach may aid other EU countries investigate and screen for potential

* Corresponding author at: Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland.
E-mail address: atahar@ait.ie (A. Tahar).

http://dx.doi.org/10.1016/j.scitotenv.2017.05.227

risks where limited measured or predicted environmental pollutant concentrations and/or hydrological data are available. This model is semi-quantitative, as other factors such as influence of climate change and drug usage or prescription data will need to be considered in a future point for estimating and predicting risks.

1. Introduction

Pollution of European receiving waters containing pharmaceuticals is a ubiquitous phenomenon (Barbosa et al., 2016; Verlicchi and Zambello, 2016; Tiedeken et al., 2017). Pharmaceuticals are a class of emerging environmental contaminants that are widely used in human and veterinary medicine and are essential to modern healthcare (Streck, 2009; Kosma et al., 2014). From here on, these compounds will be referred to as pharmaceutically active chemicals (PhACs). Nevertheless, there are growing concerns about the negative impacts that may result from continuous contamination of the environment with PhACs. This research is important because of the potential toxic effects for aquatic biota and human health that may result from chronic exposure to PhACs (Miege et al., 2008; Streck, 2009; Kosma et al., 2014). PhACs exhibit wide variation in function, chemical structure and physiochemical properties, making it difficult to generalize about their behaviour, persistence or impact in the environment. PhACs are also designed to be biologically active, have a specific mode of action and to be persistent in the body, meaning they can impact humans and wildlife at trace concentrations that are often hard to detect and quantify using traditional analytical methods (Kosma et al., 2014). A large number of PhACs have been detected in wastewater treatment plants (WWTPs) influents and effluents and surface, ground and drinking water worldwide in recent years (Nikolaou et al., 2007; Cirja et al., 2008; Streck, 2009; Verlicchi and Zambello, 2016). The impacts of chronic exposure to trace concentrations of many PhACs on wildlife and human health may be severe (Verlicchi et al., 2012b), thus it is critical to limit as much as possible the concentrations of this class of contaminants in aquatic environments.

Until recently, environmental regulations worldwide had not required explicit testing for any PhACs in water bodies. However, given the growing concern about contamination of the aquatic environment with these compounds, legislation has recently begun to acknowledge this potential problem. The Water Framework Directive (WFD) requires that all EU member states prepare river basin management plans (RBMPs) to address the many issues relating to water quality and protection in a holistic manner. In response to growing EU concern about the release of untreated PhACs into the aquatic environment, three compounds were included on in the first EU watch list in 2013: diclofenac (CAS# 15307–79-6, hereafter referred as DCL), 17-beta-estradiol (CAS# 50–28 − 2, hereafter referred as E2) and 17-alpha-ethinylestradiol (CAS# 57–63-6, hereafter referred as EE2). E2 and EE2 can impact the endocrine system of humans or wildlife where there is growing fears that chronic exposure to these endocrine disrupting chemicals or endocrine disrupting chemicals (EDCs) even at low concentrations (ng/L) (in bathing or drinking water, for example) may be linked to adverse human health conditions (Hernando et al., 2006; Streck, 2009). Similar to PhACs as a whole, EDCs are generally thought to be transported into the aquatic environment mostly via incomplete removal at wastewater treatment plants (WWTPs) (Streck, 2009). It is relevant to note that the European Commission implemented decision 495 of 20 March 2015 that expanded substances or groups of substances on the watch list to 10 in the field of water policy (Barbosa et al., 2016). This review focuses solely on the first three substances DCL, E2 and EE2 as there is a requirement to investigate policy implications for Ireland of these PhACs in receiving waters in the first instance.

Tiedeken et al. (2017) conducted a 20 year systematic review of 3945 potentially relevant articles published between 1995 and 2015 that produced a EU-wide database consisting of 1268 publications on DCL, E2 and EE2 in receiving waters. Overall, European surface water concentrations of DCL are typically reported below the proposed annual average environmental quality standard (AA EQS) of 100 ng/L with only a few extreme values exceeding this threshold (up to 1200 ng/L according to Rivera-Utrilla et al. (2013)). E2 and EE2 surface water concentrations are typically below 50 ng/L and 10 ng/L respectively, but these concentrations greatly exceed the proposed AA EQS values for these compounds (0.4 and 0.035 ng/L respectively). Furthermore, levels of these PhACs are frequently reported to be disproportionately high in EU receiving waters (up to 200 ng/L and 831 ng/L respectively in surface water according to Tiedeken et al. (2017)), particularly in effluents at control points that require urgent attention. Furthermore, the number of articles produced by each of the 28 EU countries along with Switzerland, Norway and Turkey varied greatly on sources, monitoring or control measures for DCL, E2 and EE2 over this 20-year systematic review period, where Spain, Germany and the United Kingdom contributed 707 (56%) of all reports. However, 24 and 16 EU countries produced under 50 and 20 reports respectively on these PhACs in their national receiving waters; consequently, very few countries have reported on use predicted or measured environmental concentrations to underpin modelling or to inform risks in their river basins (ter Laak et al., 2010; Guillén et al., 2012). Overall, it was found that DCL and EE2 enter European aquatic environment mainly following human consumption and excretion of therapeutic drugs, and by incomplete removal from influent at urban WWTPs. E2 is a natural hormone excreted by humans, which also experiences incomplete removal during WWTP treatments. Thus, WWTPs (initially not designed to remove PhACs) are considered as pressure point for control of PhACs in aquatic environment (Tiedeken et al., 2017). Current conventional analytical methods (i.e. LC-MS/MS) are sufficiently sensitive for the detection and quantification of DCL, but generally not for E2 and EE2 (at the very low EQS levels proposed for those compounds, i.e. 0.4 and 0.035 ng/L respectively, levels that cannot be reached for most of the standards chemical analysis labs) (Streck, 2009), thus alternative, ultra-trace, time-integrated monitoring techniques such as passive sampling are needed to inform water quality for these estrogens (Buchberger, 2011; Wille et al., 2012). Another emerging potential solution to the problem of low EQS values of E2 and EE2 is the use of biological effects monitoring techniques (Streck, 2009; Kunz et al., 2015; Simon et al., 2015). WWTPs are today widely considered as the main vector of PhACs through the aquatic environment (Hernando et al., 2006; Tiedeken et al., 2017). However, in Ireland as well in numerous other EU countries the number of reliable WWTPs and surface/ground waters monitoring data is still limited (Tiedeken et al., 2017) making difficult the direct and quantitative risk analysis associated to the selected PhACs. The aforementioned limitations of reliable data at WWTPs makes it difficult to directly quantify risks for aquatic environment. As an alternative, the application of a risk assessment (RA) method appeared to be the best way to obtain a full picture of the potential contamination of surface waters by the three selected PhACs and to identify PhACs emission hotspots. Other RA models related to PhACs in the aquatic environment aiming at: (i) prioritize certain compounds (Guillén et al., 2012), (ii) the evaluation of the ecological/environmental risk related to the presence of PhACs in rivers downstream from WWTP (e.g. Hernando et al., 2006; Ginebreda et al., 2010; Gros et al., 2010; Kosma et al., 2014; Pereira et al., 2015), (iii) the evaluation of PhACs concentrations in surface water based on their physico-chemical characteristics (Lindim et al., 2017), or on their

consumption (ter Laak et al., 2010). These models are generally "compound related" and focused on a certain number of compounds, never specifically on the WFD watch-list compounds. These studies generally aimed at the estimation of risk based the comparison of on predicted environmental concentration (PEC) (from WWTP monitoring) with predicted no effect concentrations (PNEC) (generally calculated from acute toxicity tests). To the best of our knowledge, no "WWTPs related" RA was developed so far.

This article aims to develop a semi-quantitative RA model for evaluating the environmental threats posed by Irish urban WWTPs in regards to PhACs contamination of aquatic ecosystems. As a case study, this risk screening model identified urban WWTPs in Ireland that are at risk of negatively impacting the aquatic environment via contamination with the three targeted WFD watch list PhACs of interest: namely DCL, E2 and EE2. While focused on Irish data, this RA model offers a promising tool to be used to identify WWTPs that pose a particular risk in regards to these PhACs, and to therefore prioritize the adoption of control measures at pollution 'hot-spots' (e.g. the application of efficient tertiary treatment such as advanced oxidation processes, activated carbon or membrane bioreactors or a combination of these). The model also identifies risk factors relevant to a variety of human pharmaceuticals, an emerging class of pollutants that is of ever-growing concern. Therefore, this study should be of interest to the international reader trying to protect the environment from pollution and to examine efficacy of WWTPs as critical pressure points for governing water quality in river catchment basins.

2. Materials and methods

2.1. Risk assessment model development

The model was designed following the risk screening guidelines of Ireland's Drinking Water Safety Plans (EPA, 2010) where the "Risk Screening Methodology for *Cryptosporidium*" was adapted to consider risk factors specific to the discharge of the PhACs of interest in this study. Information was also drawn from national and international peer-reviewed research in this field of study, such as Tiedeken et al. (2017) that constituted the foundation systematic bibliographic within this project. The general principles of this RA model align with this environmental protection agency (EPA)-sanctioned risk screening methodology, which uses the Source-Pathway-Receptor (SPR) concept to define relevant input parameters. A scoring system was employed that enables determination of each agglomeration as low, medium or high risk for each PhAC of interest with threshold values being as one third and two thirds of the maximum score respectively (Table 1). A risk score was calculated for each input based on specific factors; for this preliminary study and in order to build a semi-quantitative model, the risk scores associated to each input were defined as the sum of the score given to each factor. The final score was defined as the sum of the scores for each of the 4 inputs, allowing for WWTPs to be ranked relative to each other. The four input scores, considered being representative of the different sources of the targeted PhACs through the aquatic environment, were (i) source of influent, (ii) removal due to treatment, (iii) chemical properties of PhAC and (iv) fate of effluent. Where the level of uncertainty regarding an input parameter is high, a precautionary approach is adopted and the highest score is used; however, in such a case the source of uncertainty is noted and further review or monitoring for the factor is recommended. Due specifically to different substance characteristics, 3 separate RA are described within this same model.

Table 1
Maximum possible risk score for each PhAC of interest, and relevant scoring system enabling the classification of each WWTP as high risk, medium risk or low risk. Scores originate from four input parameters which are added to determine the final risk category.

	Diclofenac	E2	EE2	Risk classification
Input 1	5	13	6	
Input 2	0	0	0	
Input 3	11	11	11	
Input 4	8	8	8	
Total	24	32	25	Highest possible score
	≥17	≥21	≥17	High risk classification
	9 – 16	11 – 20	9 – 16	Medium risk classification
	≤8	≤10	≤8	Low risk classification

2.2. Input 1 - source of influent entering wastewater treatment plants

Agglomeration generated load (AGL) was included as a measure of the size of the population that is serviced by each WWTP. This factor assumes that the greater the AGL, the higher the potential for receiving influent contaminated with the PhAC of interest, i.e. higher population leads to higher levels of drug usage. According to the Terms and Definitions of the Urban Wastewater Treatment Directive 91/271/EEC, AGL is the organic biodegradable load of a WWTP expressed in population equivalents (PE), and consists of all urban wastewater requiring collection under Article 3(1) of the Directive. Furthermore, one PE is equal to the organic biodegradable load having a five-day biochemical oxygen demand (BOD_5) of 60 g oxygen/day (Santos et al., 2010). AGL was grouped into 5 categories (Table 2), with risk scores increasing with higher PE values. AGL data were obtained from agglomeration specific 2015 annual environmental reports (AERs) submitted to the EPA. Some WWTPs are responsible for taking in sludge or effluent from domestic septic tanks and/or various industries. These data were obtained from agglomeration specific 2014 AERs submitted to the EPA. Drug utilization data was available in Ireland through Heath Service Executive (HSE) Primary Care Reimbursement Services to supplement AGL determinations, however as this data only covered 70% of Irish population it was not factored into this RA model. Agglomeration size can be indicatively used as a substitute to drug utilization data in RA models (EPA, 2010), particularly those that consider emissions of PhACs from specific WWTPs (Boxall et al., 2014). Domestic effluent and sludge could be a significant source of pharmaceuticals, especially the natural steroid estrogen E2 (UWWTD-REP Working Group, 2006; Gill et al., 2009). Industrial and healthcare inputs could potentially include effluent or sludge

Table 2
Source of influent factors used in risk assessment model to calculate input one risk score. White indicates factor considered for all 3 compounds, light grey indicates factor considered only for E2 and EE2, dark grey indicates parameter considered only for E2. The colour of the risk score indicates whether there is increased risk (positive values, red), no impact on risk (zero values, blue) or decreased risk (negative values, green).

Factor	Source factor description	Risk score
1. Agglomeration generated load (AGL)	PE served <500	1
	PE served 500–50,000	2
	PE served >50,000	3
2. Domestic septic tank sludge/effluent received?	No	0
	Yes	1
3. Industrial sludge/ effluent received?	No	0
	Yes	1
4. Gender ratio in county, women/men	≤1	0
	>1	1
5. Cattle score	No cattle/calves in region	0
	≤80 livestock unit per ha forage area in region	2
	>80 livestock unit per ha forage area in region	3
6. Sheep score	No sheep/lambs in region	0
	≤70 livestock unit per ha forage area in region	1
	>70 livestock unit per ha forage area in region	2
7. Pig score	No pigs in county	0
	≤20 livestock unit per ha forage area in region	1
	>20 livestock unit per ha forage area in region	2
Total for input 1		

from hospitals or pharmaceutical manufacturers. Previous studies have shown that effluent and sludge from hospitals and pharmaceutical manufacturing facilities can contain high levels of DCL, with concentrations up to 3000 ng/L (Fent et al., 2006; Kümmerer, 2009b) and estrogens, with concentrations up to 70 ng/L (Kümmerer, 2009b; Santos et al., 2010). As a result, if a WWTP is required to receive either one of these additional inputs, a point is awarded towards the final risk score for this factor (Table 2).

For the E2/EE2 models in terms of specific sources of hormones, additional factors are considered for input one. The natural steroid estrogen E2 is mainly excreted by humans and excretion efficiencies are especially high in pregnant women (Qian et al., 2015); this natural PhAC can also be used in prescribed drugs, including hormone replacement therapy and to treat infertility in women or advanced prostate and breast cancer (reviewed in Ben Fredj et al., 2015). EE2 is the main estrogenic ingredient in oral contraceptive pills taken by women of reproductive age (Verlicchi et al., 2012a; Tiedeken et al., 2016). Therefore, EE2 is mainly excreted by the portion of the population who ingest the contraceptive pill, i.e. women. It is also found in other prescription medications including hormone replacement therapies, palliative treatments for breast and prostate cancer, and lotions used to prevent androgen-dependent hair loss in women (reviewed in Ben Fredj et al., 2015). When considering E2 and EE2 (mainly excreted by women), the gender ratio of the county in which the agglomeration is located is considered. An additional point is therefore awarded towards the final risk score if the ratio of women: men is greater than one (Table 2). It is noticed that the score of locations where gender ratio is lower that one was kept to zero whereas such population will still emit some E2 and EE2; it was considered that it was sufficient accuracy for the development of such a semi-quantitative model as the one presented here. Population and gender information were obtained through the Central Statistics Office (CSO) 2011 census data. Agriculture is known to be another potential source of natural steroid estrogens (Vieno and Sillanpää, 2014). In particular dairy cows are known to be major contributors in comparison to sheep and pigs (Santos et al., 2010). For that reason cattle density in the regional authority in which the agglomeration and its primary discharge point are located is included as a factor in the RA model (as in EPA, 2010). Pig and sheep density were also considered, however a lower risk score is associated with these livestock compared with cattle (Table 2). Data on livestock numbers were obtained through the CSO StatBank online resource. The number of livestock in June for the year 2014 (file AAA07) was considered for total cattle, total sheep and total pigs. The spatial resolution of these data were obtained from the corresponding regional authority (NUTS3 classification), and the numbers were divided by the total land area of each region, as defined by the CSO. The excretions from livestock will not enter the WWTP for treatment; however high densities of livestock may lead to high background levels of estrogens in receiving waters. This factor is only considered when modelling for E2 as synthetic estradiol products (e.g. EE2) are banned in the EU as growth promoters, thus no products containing them are authorized by the Health Products Regulatory Authority for use in animals. To the best of our knowledge, despite authorized by the European Medicines Agency (2014), DCL is not used as a veterinary drug in Ireland and was therefore excluded from the livestock section of the model.

2.3. Input factor 2 - removal of compounds due to wastewater treatment

Removal of PhACs from wastewater due to treatment can occur to varying extents (de Graaff et al., 2011); however, it is dependent upon a number of critical factors concerning the treatment, operation and management of WWTPs for each agglomeration (EPA, 2010). If not treated, a PhAC will end up in the environment making WWTPs the main vectors of PhACs for unwanted pollution. The first factor considered under input two is whether or not the WWTP effluent undergoes tertiary treatment. In Ireland, the majority of WWTPs do not include tertiary treatment (EPA, 2015). However, a limited number that discharge at/near a sensitive area employ tertiary treatments all year round, while others only treat for part of the season (i.e. the bathing season) in order to compensate the increased effluent load and to maintain a certain level of treatment during the tourism season. Advanced treatment technologies can increase removal efficiencies of PhACs, specifically E2 and EE2 (Boxall, 2010) and DCL (Joss et al., 2006). Therefore, if tertiary treatments is implemented for the whole year, the risk score associated with this factor is negative, because increased removal efficiencies of these three PhACs at such facilities would be expected (Table 3). The treatment technologies for the specific WWTPs of interest were identified through 2014 AERs and communication with the EPA.

The second factor under input two is the type of secondary treatment employed by the WWTP. Conventional WWTPs generally include both a primary physico-chemical and a secondary treatment characterized by biological treatments via activated sludge. Activated sludge plants have a limited capacity to remove many PhACs because most of these compounds cannot be metabolized by microorganisms (Joss et al., 2006). However, activated sludge plants including nutrient removal by extended aeration in the secondary treatment step have been shown to more effectively remove PhACs (including E2 and EE2 (Santos et al., 2010) and DCL (Kümmerer, 2009b; Lacey et al., 2012)). According to the Urban Wastewater report published by the EPA, 25.7% of Irish wastewater load (in PE) received secondary treatment plus biological nutrient reduction in the year 2014 (EPA, 2015). The risk score for this factor therefore decreases when the most recent AER indicates that a WWTP employs biological nutrient removal (Table 3). There is no additional or reduced risk when the plant employs conventional treatments without nutrient removal (no extended aeration) or sequence batch reactors (with or without phosphorous removal), as studies indicate these are not influencing the removal efficiencies of the PhACs of interest (Joss et al., 2006; Lacey et al., 2012). The type of secondary treatment technology employed by each plant was identified from the 2015 AERs for each agglomeration of interest.

The third factor taken into consideration for input two is a general measurement of the performances of WWTPs assuming that if standard

Table 3
Treatment, operation and management factors used in risk assessment model to calculate input two risk score. The colour of the risk score indicates whether there is increased risk (positive values, red), no impact on risk (zero values, blue) or decreased risk (negative values, green).

Factor	Treatment factor description	Risk score
1– Tertiary treatment	Present year round	−2
	Implemented seasonally	0
	Absent year round	0
2– Type of secondary treatment (including nutrient removal)	Extended aeration (N removal)	−1
	Conventional activated sludge/sequence batch reactor (with or without P removal)	0
3– WWTP quality measurement	Pass most recent UWWTD compliance criteria	−1
	Fail most recent UWWTD compliance criteria	0
Total for input 2		

parameters (e.g., BOD_5, COD, TSS, nitrogen and phosphate compounds) are not efficiently eliminated then the PhACs won't be eliminated either. National compliance measures recorded by the EPA can have different requirements depending on the specifications in WWTP licenses. If a WWTP passed the most recent UWWTD compliance criteria, a negative score was awarded to the risk score for this factor (Table 3). Unpublished compliance data were provided by the EPA and originate from Irish Water reported values.

2.4. Input factor 3 - chemical properties of compounds

The chemical properties of the specific PhACs of interest will play a contributing role in their persistence in water during the wastewater treatment process. For example, a characteristic specific to individual PhACs is metabolism in the human body. The rate of excretion, combined with drug usage data, will ultimately determine how much of the compound ends up in wastewater and enters a plant for treatment (Oaks et al., 2004; Santos et al., 2010) excluding some potential degradation/metabolization in the pipework leading to the WWTP (considered negligible in the present study due to low biomass content and short hydraulic residence time). Thus, the metabolism of the compound of interest was considered (Table 4). As the rate of excretion of the parent compound increases, so does the risk score. It is noticed that only excretion via urine was considered as the targeted PhACs residues or metabolites are demonstrated to be mostly excreted via urine compared to faeces (Lienert et al., 2007).

Efficiencies of excretion for DCL, E2 and EE2 were determined from the literature and with an EPA technical report (Tiedeken et al., 2016; Tiedeken et al., 2017). The main removal pathways for the PhACs of interest during wastewater treatment are sorption to sludge and biotransformation/biodegradation (Kümmerer, 2009b; Santos et al., 2010; Lacey et al., 2012). Sludge is a complex material comprised of live and dead microorganisms which provide a large surface area for PhACs to sorb onto; however, sorption is a complex process, which can depend on hydrophobicity, functional group polarity, ion exchange, chelation or other factors (Rivera-Utrilla et al., 2013). In this RA model, compounds that are recognized in the literature as having low water solubility/high hydrophobicity have a lower risk score because they will theoretically tend to sorb onto sludge and therefore not be discharged in the WWTP effluent (Table 4). It is noteworthy that some initially sorbed compounds could reach the aquatic environment through an overflow event that could accidentally cause the release of some untreated wastewater; this potential alternative route was considered negligible and therefore ignored in the design of the present model. The hydrophobicity and therefore the likelihood of a compound to sorb to sludge is often correlated with physico-chemical parameters such as a compound's octanol-water partition coefficient (K_{ow}) (Joss et al., 2006) or its pH-dependent equivalent, D_{ow} (Lapworth et al., 2012). It was suggested that Log (K_{ow}) values could be related to organic compounds sorption potential on sludge and that this phenomenon could be considered as "not expected", moderate and high for values ≤2.5, 2.5–4 and ≥4, respectively (Cirja et al., 2008). As a result, Log (K_{ow}) (or Log (D_{ow}) for DCL that is an ionisable compound, pKa = 4.15) values from the literature were considered when determining the likelihood of sorption for each PhAC of interest (Tiedeken et al., 2017) and were considered as threshold values for associated risk score assignments.

Similar to sorption, biodegradation and biotransformation are complex processes, and no single physico-chemical parameter can predict a compound's potential for elimination via this removal pathway (Kümmerer, 2009b). Again, information from the literature was used to determine whether the potential of each PhAC to experience biodegradation or biotransformation was high or low (Table 4). A property that helped make this determination was the compound's half-life in the environment; however, this value can vary widely depending on the environmental matrix being studied. The experimentally determined biological degradation constant (K_{biol}) was therefore taken into consideration (Ziylan and Ince, 2011). Joss et al. (2006) suggested that the biological degradation constant K_{biol} ($L/g_{ss}/d$) could be used to estimate the biodegradation potential of a compound, where values <0.1 translate to no substantial biodegradation, values between 0.1 and 10 indicate partial biodegradation and values above 10 indicate ready degradation (Table 4).

Some full scale WWTP studies report negative removal efficiencies of PhACs; in other words, the concentration of the PhAC of interest is higher in the effluent than in the influent (Thomas et al., 2007; Kümmerer, 2009b). Apart from an analytical artefact, it has been suggested that this phenomenon occurs because of deconjugation of conjugated metabolites of the PhACs of interest (Thomas et al., 2007; Lacey et al., 2012). To account for this, the final factor associated with this input was assigned a point towards the risk score if the literature

Table 4
Chemical properties of compounds used in risk assessment model to calculate input three risk score. The colour of the risk score indicates whether there is increased risk (positive values, red), no impact on risk (zero values, blue) or decreased risk (negative values, green).

Factor	Chemical properties factor description	Risk score
1– Metabolism	Rate of excretion 0–25%	1
	Rate of excretion 26–50%	2
	Rate of excretion 51–75%	3
	Rate of excretion 76–100%	4
2– Sorption potential	Low water solubility/high hydrophobicity $Log(D_{ow}) \geq 4$	1
	Medium water solubility/low hydrophobicity $Log(D_{ow}) = 2.5–4$	2
	High water solubility/low hydrophobicity $Log(D_{ow}) \leq 2.5$	3
3– Degradation potential	High degradation through photolysis, hydrolysis or other mechanisms $K_{biol} > 10\ L/g_{ss}/day$	1
	Medium degradation through photolysis, hydrolysis or other mechanisms $K_{biol} = 1–10\ L/g_{ss}/day$	2
	Low degradation through photolysis, hydrolysis or other mechanisms $K_{biol} < 1.10\ L/g_{ss}/day$	3
4– Potential for deconjugation of conjugated metabolites during treatment	Not found to occur in the literature	0
	Deconjugation potential identified through literature	1
Total for input 3		

indicated that deconjugation of conjugated metabolites during wastewater treatment was proven (Table 4).

Specific supporting information underpinning RA model for targeted PhACs is presented as follows.

Diclofenac: in regards to factor number one (metabolism), in this RA model only excretion via urine is considered (Table 4). Of the orally administered dose of DCL, between 65 and 70% is excreted in urine as the parent compound or metabolites (and Fent et al., 2006; reviewed in Lacey et al., 2012; Vieno and Sillanpää, 2014). According to the RA model methodology, DCL is thus assigned a risk score of three for this factor (Table 4). Removal efficiencies of DCL during wastewater treatment can vary widely depending on both the process configuration and parameters utilized by a treatment plant (reviewed in Lacey et al., 2012). In general however, DCL has only a weak ability for removal through sorption to sewage sludge (Zhang et al., 2008; Boxall, 2010; Wise et al., 2011; Vieno and Sillanpää, 2014). This is due in part to its physico-chemical properties; DCL is soluble in water and has a low Log(D_{ow}) (2.50 at a pH value of 8, typical of wastewater treatment conditions) (Kümmerer, 2009a). As expected of compounds with low Log(D_{ow}) values, sorption of DCL to sludge is unlikely. Due to these reports from the literature, in the RA model DCL is considered to have low sorption potential and is assigned a risk score of three (Table 4). Similar to the potential for sorption, biodegradation of DCL during wastewater treatment is generally accepted in the literature as being poor (Zhang et al., 2008; Vieno and Sillanpää, 2014). The K_{biol} threshold values of 0.1 and 10 were used as suggested in Joss et al. (2006). The biodegradation values measured for DCL in the Joss et al. (2011) study were below 0.1, regardless of the biological process configuration. Additional evidence of the poor biodegradation of DCL is reviewed in Vieno and Sillanpää (2014), hence the risk score associated with this factor is three (Table 5). Finally, numerous studies (including one carried out in Ireland) have noted higher concentrations of DCL in WWTP effluent than influent (e.g. Thomas et al., 2007; Zhang et al., 2008; Santos et al., 2010). These studies have suggested that deconjugation of glucuronide or sulphate conjugates of DCL might occur during the wastewater treatment process, leading to negative removal efficiencies. Thus, DCL is assigned a risk score of two for the final factor in input three (Table 4).

E2 and EE2 - in this RA model, only excretion of the parent metabolite or conjugates via urine is considered. Literature indicates that 22–50% of the daily dose of EE2 is excreted in urine (Verlicchi et al., 2012a). Thus, EE2 is assigned a risk score of 2 for factor one, input three (Table 4). Metabolism for E2 is evaluated slightly differently; because this is a natural hormone, rate of excretion represented as percent of the daily dose ingested is inappropriate. Instead quantities (μg) of E2 excreted in the urine as conjugated or unconjugated forms must be considered. The literature reports that 14 μg and 2.4–3.0 μg of conjugated and unconjugated E2 respectively are excreted by women in urine per day (Verlicchi et al., 2012a; Petrie et al., 2013; Rivera-Utrilla et al., 2013). In order to assign a risk score for the model, these values were compared with the excretion efficiencies of other natural estrogenic hormones (estrone E1 and estradiol E3). In the studies examined, E2 was always the natural hormone with the lowest daily excretion rate (Petrie et al., 2013; Rivera-Utrilla et al., 2013), therefore it is assigned a risk score of two in our model (Table 4). The use of μg excreted per day (as opposed to percent of the daily dose excreted) prevents direct comparison of the E2 input 3 score with that of the other targeted PhACs.

Both E2 and EE2 have low polarity and like most other EDCs, are moderately hydrophobic organic molecules; this means they have a tendency to distribute into organic phases (Kunz et al., 2015). The compounds E2 and EE2 are not ionisable and log(K_{ow}) values of 4.01 and 3.67 respectively are therefore taken into account. Sorption onto sludge is therefore considered partially responsible for removal of E2 and EE2 from wastewater, and they are assigned a score of 1 and 2 respectively in the RA model (Table 4). Compared with DCL, E2 and EE2 have a much higher potential for biodegradation (Burkhardt-Holm, 2010; Hecker and Hollert, 2011; Wise et al., 2011). However, studies comparing the removal efficiencies of estrogens indicate that EE2 is more recalcitrant than E2 (Wise et al., 2011; Kunz et al., 2015). K_{biol} values for E2 range from 300 to 800 L/g_{ss}/d in the literature, whereas values for EE2 are between 7 and 9 L/g_{ss}/d (Verlicchi et al., 2012a). The risk score for this factor is thus classified as one for E2 and two for EE2 (Table 4). Finally, in regards to factor four (potential for deconjugation of conjugated metabolites during treatment), the same approach was taken as previous RA models in assuming that all the steroids excreted as conjugates are de-conjugated in the sewers before they reach the treatment plant (Wise et al., 2011). Therefore the risk score assigned for this factor is 0 for both E2 and EE2 (Table 4).

The total input three scores for DCL, E2 and EE2 are 12, 4 and 5 respectively (Table 5). These scores can be used to compare the risk associated with DCL, E2 and EE2 in regards to aquatic persistence during wastewater treatment. According to our model, of these three compounds, DCL presents approximately twice the risk of being found in receiving waters compared with E2 or EE2. This finding is consistent with reports from the literature as it demonstrates that DCL is among the most recalcitrant pharmaceuticals (Wise et al., 2011), and that its removal percentages during wastewater treatment, though variable, are on average much lower than those of E2 or EE2 (Lacey et al., 2012; Kunz et al., 2015; Tiedeken et al., 2017). The concentrations of DCL in effluent and receiving waters are generally in the high nanogram-microgram per litre range, whereas E2 and EE2 are almost always reduced to nanogram per litre levels after wastewater treatment (Burkhardt-Holm, 2010; Lacey et al., 2012). In addition, the input three scores for E2 and EE2 indicate that the latter is slightly more persistent after wastewater treatment; this is also consistent with findings reported in the literature, which show that EE2 is considerably more recalcitrant than E2 and its removal efficiencies in activated sludge treatment plants are more variable (Wise et al., 2011). The congruence of these input three scores with the literature indicates the factors for this input are scored appropriately.

Table 5
Factor scoring for each PhAC of interest for risk assessment model, input three. Final score assigned in model for each factor is in colour and in bold.

	Factor 1 Metabolism	Factor 2 Sorption	Factor 3 Degradation	Factor 4 Conjugation	Total score
Diclofenac	3[a]	3[b]	3[c]	1[d]	10
17-beta-estradiol (E2)	2[e]	1[f]	1[g]	0[h]	4
17-alpha-ethinylestradiol (EE2)	2[i]	2[f]	2[g]	0[h]	6

[a] (Vieno and Sillanpää, 2014) reviewed in (Fent et al., 2006; Lacey et al., 2012).
[b] (Zhang et al., 2008; Boxall, 2010; Wise et al., 2011; Vieno and Sillanpää, 2014) reviewed in Vieno and Sillanpää (2012).
[c] (Zhang et al., 2008; Ziylan and Ince, 2011; reviewed in Vieno and Sillanpää, (2014)
[d] (Thomas et al., 2007; Santos et al., 2010).
[e] (Verlicchi et al., 2012a; Petrie et al., 2013; Rivera-Utrilla et al., 2013).
[f] (Wise et al., 2011; Kunz et al., 2015).
[g] (Burkhardt-Holm, 2010; Hecker and Hollert, 2011; Wise et al., 2011; Verlicchi et al., 2012a).
[h] (Wise et al., 2011).
[i] (Verlicchi et al., 2012a).

2.5. Input factor 4 - fate of treated effluent

Models of the risk and impact of PhACs in surface waters typically use information not only on drug usage, metabolism, and fate in WWTPs, but also on fate in receiving waters. Consequently, the final input used to calculate the overall risk score for this model considers factors specific to the receiving water the treated effluent from a WWTP is discharged to. For this RA study, only the primary discharge site of the WWTPs of interest and its potential PhACs dilution were considered. The self-purification potential of the receiving water was ignored for this preliminary study due to the pseudo-persistence concept and the fact that PhACs of interest are constantly introduced into receiving water via WWTPs (Hernando et al., 2006). The first factor associated with input four is the type of receiving water the treated effluent is discharged to. Receiving water is classified as one of four types: 1.) coastal; 2.) transitional, estuary, river, or lake; 3.) stream; or 4.) ground (Table 6). No WWTP evaluated released treated effluent directly to ground water, but theoretically this situation may occur, for example when considering domestic wastewater treatment systems (septic tanks) (UWWTD-REP Working Group, 2006; Gill et al., 2009; Ronan and McHugh, 2013). Dilution of the treated effluent is very high when released to coastal environments, hence, a low risk score is associated with this type of receiving water. Dilution will vary considerably in transitional/estuary/river/lake receiving waters (depending on their size, flow, etc.), but generally should be lower than in coastal receiving waters, hence, the risk score is increased. The risk score is the highest if the receiving water is classified as a stream, where dilution could be very low, especially during periods of low rainfall (Table 6). Data on the type of receiving water of each of the WWTP of interest were obtained from the 2015 AERs. Another factor is whether or not the primary discharge point is located at or near a sensitive area (Table 6). Sensitive areas are identified in the 2001 and 2010 Urban Wastewater Treatment Regulations. If the primary discharge point from a plant was reported in the 2015 AER as being directly into a sensitive area, an additional point is given to the risk score for this factor. The flow of the receiving water is considered in order to evaluate the fate of the treated effluent (Table 6). The hydrometric monitoring station closest to the primary discharge location of each WWTP was identified, and the 95 percentile flow (m^3/s) was obtained from the Water Data Unit of the EPA. The flow for each receiving water is classified as high (>10 m^3/s), medium (1–10 m^3/s) or low (<1 m^3/s). This information is used to assign the risk score for this factor, which increases with decreasing flow. Several of the WWTPs used in the case study discharge into estuarine waters; where there is no estimate of 95 percentile flow available. These locations were thus assigned a risk score value of one, the lowest score available for this factor.

2.6. Validation of the model

In order to validate the developed approach, the values obtained regarding the three RA models were compared to Irish monitoring data of the water downstream from each WWTP. Monitoring data for DCL, E2 and EE2 are not yet legally required in Ireland; thus existing data originate from independent research projects. All such monitoring data of DCL, E2, EE2 or EEQ at Irish WWTPs were compiled during the literature review (Tiedeken et al., 2016). For each publication identified as containing relevant Irish DCL, E2 or EE2 monitoring data, several parameters were extracted. First, the date water samples were taken was identified (including day, month and year if available, but year at minimum). The type of study (i.e. a study measuring the concentration of a compound or a study using effect-based methods) as well as the compound analysed (DCL, E2, EE2 or EEQ) was identified. The method of sampling (grab, passive, or the type of assay utilized) was identified, as was the matrix studied (marine water, lake water, ground water, effluent, etc.). The concentration of the compound recorded during each sampling event was also recorded if it was available. If multiple samples were taken at the same location during a study (i.e. repeat sampling over time), each sampling event was listed separately. However, if multiple analyses were run on the same samples (i.e. tests run in duplicate or triplicate) the individual results were not reported; instead the final value as presented by the authors of the study (usually an average) was utilized. For the concentration data, effect-based studies were ignored and only concentration studies were considered. The remaining concentration data were then sorted by compound, location and value, and for each unique location the highest concentration recorded for each compound was identified for mapping. These concentrations therefore represent the worst case scenario as recorded by monitoring studies to date at each location. It must be noted that this does not mean that these sites will always have such high concentrations of the compounds of interest; additional sampling events may have recorded lower values or non-detects. It should also be noted that just because a compound has not been detected at a site does not mean it will not exceed WFD proposed limits; it is possible (especially for the estrogens) that the LOD may exceed the WFD limit. Nevertheless, these monitoring data still provide an indication of which areas could be pollution "hotspots".

These data were used to test the effectiveness of the RA model developed. The distribution of those data and underpinning the scarcity of such data across Ireland (only a few data on 16 WWTPs) is presented in supplementary materials. This justifies the necessity and the relevance of the RA approach in order to get a full picture of the contamination by the three targeted PhACs at the country scale.

For the purposes of this RA model, monitoring data falls into one of two classes: 1.) PhAC concentrations are below WFD limits or below PNECs, and 2.) PhAC concentrations are above WFD proposed limits or equivalent to or higher than PNEC values. Although sparse, this pool of monitoring data was used to have an idea of the validity of the approach employed. Some of the WWTPs did not have some monitoring data for each PhAC of interest.

Table 6
Fate of treated effluent factors used in risk assessment model to calculate input four risk score. The colour of the risk score indicates whether there is increased risk (positive values, red), no impact on risk (zero values, blue) or decreased risk (negative values, green).

Factor	Fate factor description	Risk score
1– Type of receiving water	Coastal	1
	Transitional/estuary/river/lake	2
	Stream	3
	Ground	4
2– Proximity to sensitive area	Primary discharge location not at/near sensitive area	0
	Primary discharge location at/near sensitive area	1
3– Flow of receiving water	High (>10 m^3/s)	1
	Medium (1 – 10 m^3/s)	2
	Low (<1 m^3/s)	3
Total for input 4		

2.7. Details on the urban wastewater treatment plants used for this Irish case study

Sixteen urban WWTPs were identified for which there were some existing monitoring data on DCL, E2, EE2 or EEQ (see a map in Supplementary materials).

Details on the location and treatment characteristics for each WWTP is presented in Table 7. Coordinates for the 16 Irish urban WWTPs were recorded as the primary discharge point listed in the EPA wastewater license. These included WWTPs of a range of sizes and treatment technologies, which are distributed relatively evenly throughout the country. The evaluation of these 16 WWTPs was used as a case study to test the effectiveness of our semi-quantitative model.

3. Results

3.1. Case study to evaluate RA model using urban WWTPs in Ireland

The required data for each factor in all four inputs of our RA model was compiled for each of the 16 investigated Irish WWTPs, and the cumulative risk score for each was calculated (Table 1) and used to rank the agglomerations from highest to lowest risk of contamination of receiving waters with DCL, E2 and EE2 (Table 8). Seven agglomerations received the highest risk score for DCL, E2 and EE2 namely, Ringsend (East), Leixlip (East), Osberstown (East), Kilkenny (South), Killarney (South), Fermoy (South) and Longford (midlands) (Table 8). These seven plants vary in terms of their spatial distribution; Ringsend, Leixlip and Osberstown are eastern WWTPs, Kilkenny is located in the southeast of the country, Longford is in the midlands and Killarney and Fermoy are in the west. These high scores can be attributed to the following WWTP characteristics: they have no tertiary treatment and no nutrient removal (lack of extended aeration); they have a primary discharge point near/at a sensitive area; and/or they are discharging into receiving waters with relatively low 95 percentile flow. All of these aspects are relevant to the factors throughout input one, two and four, and as a result these WWTPs received high scores in all models corresponding to the three selected PhACs.

Compared with the high-risk agglomerations, the WWTPs with the lowest risk scores demonstrated more variation between the DCL and the E2/EE2 models (Table 8). Nevertheless, Ballincollig, Swords and Tralee consistently received some of the lowest scores in all models corresponding to the three selected PhACs. These three agglomerations received lower overall scores as the process configuration of the plants, i.e. Tralee plant utilizes tertiary treatment and Ballincollig/Swords plants employ extended aeration. These treatment processes lead to decreased levels of PhACs in receiving waters, and thus warrant a reduced risk score via the RA methodology. Tullamore WWTP, even if ranked high risk for DCL, was consistently ranked in the middle of the agglomerations in the E2 and EE2 models. This middle risk ranking is due to a relatively low input one score in all three models, contrasted by a high input four score. The low input one score was a result of the agglomeration not receiving any domestic or industrial inputs, while the high input four score was a result of discharging effluent into a low-flow receiving water (i.e., 95% percentile flow of 0.002 m^3/s) that is near a sensitive area. The lower cumulative risk score for Swords is due to a relatively low input one score as well as the use of extended aeration, which decreases the cumulative risk score of Swords WWTP further.

For DCL, the maximum cumulative risk score achieved by a WWTP was 19 and the minimum was 14 (Table 8). According to the risk classification scheme defined by our RA model (Table 1), this range spanned two risk classifications, high risk (≥17) and medium risk (9–16). None of the plants from the case study were categorized as low risk for DCL contamination. A similar range in WWTPs scores was observed for E2 (maximum of 20 and minimum of 14); however, all of the agglomerations are classified as medium risk (11 − 20) for E2 contamination (Table 8). EE2 scores were in general much lower than those for DCL and E2, with a maximum value of 16 and a minimum of 11. Again, all 16 Irish WWTPs were classified as medium risk (9–16) for contamination with EE2 (Table 1).

Even though the risk classifications assigned to WWTPs in this study were always high or medium, the overall distribution of risk scores was sufficient to allow for a meaningful risk ranking of these

Table 7
Characteristics of the 16 WWTPs used to pilot the risk assessment model and corresponding data. Includes all WWTPs for which published monitoring data on diclofenac, E2, EE2 or estradiols equivalents is available.

WWTP	Size (PE)	Type of secondary treatment (final)	Domestic effluent	Industrial effluent	Gender ratio	Livestock density (ind/ha)			Tertiary treatment	WWTP quality test	Monitoring data	Receiving water	Flow of receiving water (m^3/s)	Sensitive area?
						Cattle	Sheep	Pig						
Athlone	21,155	Extended aeration	No	Yes	>1	122	48	45	no	Pass	EEQ	River	18.2	Yes
Ballincollig	27,697	Extended aeration	Yes	No	>1	115	61	26	no	Pass	EEQ	River	2.65	No
Carlow	39,043	Extended aeration	No	Yes	≤1	141	73	34	no	Pass	EEQ	River	4.3	Yes
Clonmel	34,909	Extended aeration	Yes	Yes	≤1	141	73	34	no	Pass	EEQ	River	11.4	Yes
Fermoy	18,608	CAS	Yes	Yes	>1	115	61	26	no	Pass	EEQ	River	6.8	Yes
Galway	213,424	CAS	Yes	No	≤1	68	99	3	no	Pass	DCL	Coastal water	nd	No
Kilkenny	51,988	CAS	Yes	Yes	≤1	141	73	34	no	Pass	EEQ	River	3.72	Yes
Killarney	41,836	CAS	Yes	Yes	>1	115	61	26	no	Pass	EEQ	River	0.057	No
Leixlip	100,309	CAS	Yes	Yes	>1	85	112	12	no	Pass	EEQ, DCL	River	2	Yes
Longford	11,672	CAS	yes	Yes	≤1	122	48	46	no	Pass	EEQ	River	0.348	Yes
Osberstown	104,723	Sequence batch reactor	Yes	Yes	>1	85	112	12	no	Pass	EEQ, DCL, E2, EE2	River	2.6	Yes
Ringsend	2,124,000	Sequence batch reactor	No	Yes	>1	85	112	12	UV (part of the year)	Fail	EEQ, DCL, E2, EE2	Estuarine	nd	Yes
Roscommon	6989	CAS	No	No	≤1	68	99	3	no	Pass	EEQ	River	0.2	Yes
Swords	77,014	Extended aeration	No	No	>1	85	112	12	no	Pass	DCL	Estuarine	nd	Yes
Tralee	35,149	CAS	Yes	Yes	>1	115	61	26	UV (year-round)	Pass	EEQ	Estuarine	nd	Yes
Tullamore	24,055	CAS	No	No	≤1	122	48	46	no	Pass	EEQ	River	0.002	Yes

Table 8
Results of case study evaluating 16 Irish WWTP using the developed risk assessment model for diclofenac, E2 and EE2.

WWTP	Input 1			Input 2			Input 3			Input 4			Total		
	DCL	E2	EE2	DCL	E2	EE2	DCL	E2	EE2	DCL	E2	EE2	DCL	E2	EE2
Athlone	4	11	5	−2	−2	−2	10	4	6	4	4	4	16	17	13
Ballincollig	3	10	4	−2	−2	−2	10	4	6	4	4	4	15	16	12
Carlow	3	7	3	−2	−2	−2	10	4	6	5	5	5	16	14	12
Clonmel	4	11	4	−2	−2	−2	10	4	6	4	4	4	16	17	12
Fermoy	4	11	5	−1	−1	−1	10	4	6	5	5	5	18	19	15
Galway	4	9	4	−1	−1	−1	10	4	6	2	2	2	15	14	11
Kilkenny	5	12	5	−1	−1	−1	10	4	6	5	5	5	19	20	15
Killarney	4	11	5	−1	−1	−1	10	4	6	5	5	5	18	19	15
Leixlip	5	12	6	−1	−1	−1	10	4	6	5	5	5	19	20	16
Longford	4	11	4	−1	−1	−1	10	4	6	6	6	6	19	20	15
Osberstown	5	12	6	−1	−1	−1	10	4	6	5	5	5	19	20	16
Ringsend	4	11	5	0	0	0	10	4	6	3	3	3	17	18	14
Roscommon	2	7	2	−1	−1	−1	10	4	6	6	6	6	17	16	13
Swords	3	10	4	−2	−2	−2	10	4	6	3	3	3	14	15	11
Tralee	4	11	5	−3	−3	−3	10	4	6	3	3	3	14	15	11
Tullamore	2	8	2	−1	−1	−1	10	4	6	6	6	6	17	17	13

WWTPs (Tables 1 and 8). One reason that no agglomerations were classified as low risk was the size of the chosen WWTPs; the smallest plant had a PE of 6989, thus all plants received a relative high risk score for this factor (Tables 2, 7). This is also significant because PhACs contamination of effluents and receiving waters has yet to be considered for the many small WWTPs throughout the country. In addition, several of the plants classed as medium risk by this model could achieve low risk status if they were to employ process configurations known to reduce levels of pharmaceuticals in the effluent. For example, if a tertiary treatment and extended aeration (N removal) during secondary treatment were employed by some plants (i.e. Galway, Swords and Tralee for the EE2 model), they would have received low risk classification. Finally, obtaining higher quality or better resolved data for input into the model would increase the range of the final cumulative risk scores, and could lead to some WWTPs being classified as low risk. Overall, our model for DCL is likely to be a worse predictor of risk than the models for E2 and EE2. Input one, which considers the source of the contaminant being investigated by the RA model, has only three factors for DCL. In contrast, this input has four and seven factors associated with it for EE2 and E2 respectively. Although preliminary, this case study demonstrates the usefulness of this basic, semi-quantitative RA model for determining the relative risks posed by WWTPs in regards to environmental consequences of contamination with the PhACs of interest. It also identifies seven WWTPs with particularly high scores in regards to DCL, E2 and EE2 contamination (Table 8). Future monitoring studies should be considered including these WWTPs in order to better evaluate the levels of DCL, E2 and EE2 in their treated effluent/receiving waters. This kind of methodology allows the highest risk WWTPs to be prioritized for improvements such as the implementation of extended aeration and/or tertiary treatment in order to mitigate the impacts of PhACs pollution in the aquatic environment. It is essential, however, that this model be improved and additional sources of data be utilized in order to provide stakeholders with the most relevant and realistic RA possible. The following discussion section identifies areas for improvement which would increase the usefulness of this RA model or future models used for environmental risks regarding contamination with PhACs.

3.2. Validation of the model

With such a semi-quantitative RA model, a quantitative validation would be a non-sense, especially with the low number of Irish monitoring data found (both WWTPs efficiency and corresponding downstream surface water). This is the reason why the following validation will only be assessed qualitatively.

Existing monitoring data from 5 of the 16 WWTPs included in this study (i.e. Ringsend, Swords, Galway, Leixlip and Osberstown), indicate that DCL is found in treated effluents as in other European WWTPs (Thomas et al., 2007; Kümmerer, 2009b; Wille et al., 2012). Furthermore, the level of accuracy of the analytical methods employed for the analysis of DCL was generally low, with LODs in the μg/L range (Lacey et al., 2008; Lacey et al., 2012). As a consequence, the monitoring data for Leixlip, Osberstown and Swords revealed only concentrations below LODs (Thomas et al., 2007; Kümmerer, 2009b; Wille et al., 2012) that limits the possible interpretation or correlation study with the scores from the present RA model. No data on concentrations of DCL in surface water downstream from the studied WWTPs were found. A comparison between the results from our RA model and existing DCL monitoring data is difficult as DCL levels in WWTPs effluent are unknown for the remaining eleven WWTPs included in this study (Table 7).

In regards to E2 and EE2, existing monitoring data are more complete (Table 7); measures of EEQ in treated effluent exist for all but two of the 16 WWTPs included in this case study (Swords and Galway) (Table 7). These monitoring data, however, were generated in different studies with different methods applied and are therefore difficult to compare; this is why we focused only on comparison of results from the same study where data were generated in the same condition (sampling, methodology, analytical methods, duration, etc). One study focused on the comparison of estrogenic responses upstream and downstream from two WWTPs considered in the present study, Osberstown and Ballincollig, by performing vitellogenin assays on

wild brown trout (Tarrant et al., 2005). For Osberstown, it was demonstrated an impact of the WWTP and an evidence of estrogenic exposure of the brown trout was observed by a significant increase in plasma vitellogenin concentration from upstream to downstream from the plant. For Ballincollig, no impact of the WWTP on the fish vitellogenin concentration was observed and the authors conclude to an absence of estrogenic exposure that could potentially occur downstream from the plant (Tarrant et al., 2005). The respective E2/EE2 scores from the RA model were 20/16 and 16/12 for Osberstown and Ballincollig respectively and Osberstown plant was therefore given a higher score than Ballincollig one. In the same study, some EEQ values from downstream other WWTPs (e.g. Leixlip, Clonmel, Fermoy) were also given but the range obtained (1–3 ng/L) was deemed too narrow to be used in the present study as a qualitative validation tool. Another study quantified the EEQ for different locations situated downstream from some Irish WWTPs during a 2-years period using grab samples (Quinn-Hosey et al., 2012). The mean EEQ values obtained ranged from 2.7 ng/L to 16.2 ng/L for Roscommon and Longford respectively; the corresponding E2/EE2 scores for these WWTPs were 16/13 and 20/15 respectively, showing a correlation between monitoring and RA model data. These observations, even qualitative, confirm the potential of such RA model to assess which WWTP could be considered as hotspot for the release of the targeted PhACs, especially in cases where monitoring data are scarce as it is the case for most of EU countries (Tiedeken et al., 2017).

4. Discussion

This model allows for a preliminary RA evaluating the environmental threat posed by Irish WWTPs in regards to PhACs contamination of aquatic ecosystems. The case study results demonstrate that some improvements should be made before this model is fully implemented on a national/European scale, or used to make decisions regarding regulations or infrastructure and paving the way to the development of a fully quantitative model. Firstly, the risk classification system used in this model could be adapted. Currently, the three risk categories (high, medium and low) were split equally based on the highest possible score for each PhAC (Table 1). Redefining the risk categories so that the threshold for a "low risk" categorization is increased could be appropriate. Before taking this step, however, additional reliable studies evaluating the concentrations of the three selected PhACs and their main metabolites in WWTPs effluents and receiving waters should be carried out. How such monitoring data correlate with the cumulative risk scores from this model, and how they compare to PNEC and WFD proposed limits, will provide the best information on how to define the risk classifications. This would provide a reliable dataset that could be used as a real validation dataset for the future developments of the current RA model. To this aim, best practices in terms of analytical methods and sampling should be employed as it was noticed that most of Irish based studies found were still using non-representative grab samples and analytical methods not sensitive enough to quantify or even detect PhACs at environmental concentrations. Secondly, the same risk classification system could have been applied to all three drugs (i.e. DCL, E2 and EE2). For example, when scoring for DCL we chose to exclude factors in input one that were deemed irrelevant to this particular PhAC (i.e. livestock densities and gender ratios). Instead, these irrelevant factors could have received a risk score of zero. This alternative method assumes that if one compound has an additional source, it should be higher risk than compounds with fewer sources. Such a methodology would allow for a comparison between compounds at each WWTP, however it was deemed inappropriate due to lack of data. The best solution to this issue is to obtain drug usage data instead of relying on proxies (e.g. gender ratios) for information on compound consumption.

An additional change that could be applied to this RA model is the weighting of certain critical risk factors or inputs. For example, whether or not a plant utilizes a tertiary treatment will have a substantial impact on the removal efficiencies of DCL, E2 and EE2 during treatment (Joss et al., 2006; Boxall, 2010). It is thus possible that this factor should be considered to a greater extent than the rest of the risk factors, or that the input that this factor contributes to (input two) should be weighted. Because this was a preliminary study, we left all inputs and factors unweighted, but future iterations should consider the impact weighting could have on the model's predictive ability. In addition to structural changes to the existing RA model, the data sources for most of the factors could be improved, and additional data sources could be added as factors to relevant inputs. First, input one (source of the influent) is lacking drug utilization data, arguably one of the most important inputs to a RA model for any prescription drug (Oaks et al., 2004; Santos et al., 2010). Drug utilization data for DCL, E2 and EE2 are available for Ireland from the Health Service Executive (HSE) via the Primary Care Reimbursement Services (PCRS) sector, but is incomplete as approximately 80% and 60% are missing for DCL and estradiol respectively. Furthermore, these drug utilization data should be completed by data on legal or illegal internet purchase that could represent a substantial addition to the overall PhAC consumption. Thus, although drug utilization is important to include in future models, data quality for these three compounds in Ireland is imperfect and other relevant factors will need to be considered in order to ensure the predictive power of an RA model. In particular, the use of AGL as a factor in input one should not be abandoned, even if drug utilization is incorporated into future models (EPA, 2010; Boxall et al., 2014).

Factors two and three from input one (the addition of domestic or industrial septic tank sludge/effluent) would benefit from higher quality data. In regards to industrial effluent and sludge, the type of industry should be taken into account. Sludge that is likely to contain the PhACs of interest (e.g. from a pharmaceutical manufacturer or a hospital) should be assigned a higher risk score. Hospitals can be significant contributors to influent loads of PhACs (Fent et al., 2006; Kümmerer, 2009b; Boxall, 2010), thus an additional factor that could be added to input one of the RA model is data on the distribution of hospitals throughout the country. This factor should consider not just the location of hospitals, but also whether or not they have independent discharge licenses, whether they treat effluent before release, or whether any discharges are trucked to WWTPs. For the fifth, six and seventh factors in input one (livestock densities), data that were freely available from the CSO's online database StatBank were used. Additional factors could be considered regarding livestock, including whether sewage sludge/slurry spreading or slurry/dung stores occur in the catchment (EPA, 2010).

In regards to input two, removal of compounds due to treatment, there are many additional process parameters and configuration variables that could be considered in order to improve this RA model. At minimum, the solids retention time (SRT) and hydraulic residence time (HRT) utilized at each plant should be taken into consideration. SRT especially may impact PhACs removal because a higher value leads to increased microbial diversity, which could increase the metabolising and transforming capabilities of the sludge in general (Clara et al., 2005; Kümmerer, 2009b). An increased HRT increases the contact time of water with the biomass, potentially leading to higher removal efficiencies of PhACs (Gros et al., 2010; Vieno and Sillanpää, 2014). These variables are specific to each WWTP and can vary over time, thus information would need to be gathered through WWTPs staff and facilitated by the corresponding public authority.

In future RA models, input four (fate of the effluent) could consider secondary in addition to primary discharge points. In addition, only the dilution capacity of the receiving water was considered in the present model but the self-purification capacity of the receiving water should be taken into account considering that features such dissolved oxygen content or depth could impact the removal of PhACs once discharged through a river/lake/sea.

One general drawback of the current RA model is the scale of the data for many of the factors included in the various inputs. For example, gender ratio was calculated at the county level, and livestock densities

were calculated for each regional authority. Ideally, all factors would consider data based on the population or land area served by the WWTP, instead of at the county or regional authority level. Gender ratio would thus consider the people living within the area serviced by a WWTP, and livestock density would be calculated based on the catchment in which the primary discharge point falls. These types of data manipulation are possible but time consuming, and often require permission from various national authorities. Future models should consider fully quantitative approach that is validated using standardized monitoring data (Oaks et al., 2004; Petrie et al., 2013; Rivera-Utrilla et al., 2013) and to consider potential ecotoxicological and biodiversity decline. In this perspective, potential mixture effect should be considered. This RA model should also be adapted to consider climate change such as large uncertainties associated with projections of precipitation variations at the river catchment scale reflecting the need to integrate hydrological data derived from application of catchment models (Bastola et al., 2011).

5. Conclusion

A preliminary semi-quantitative RA model aiming at identifying WFD watch-list compounds pollution hotspots was developed. This model is based on a scoring system that encompasses different inputs that could potentially impact the presence of the three first watch list compounds in WWTPs receiving waters. The model focuses on (i) an estimation of the load of each PhAC in WWTPs influent, (ii) WWTPs and their potential for PhACs elimination from the water phase, (iii) on physico-chemical characteristics of the compounds that could impact this removal and (iv) on the dilution potential of the receiving water. Depending of the different scores obtained, a panel of 16 Irish WWTPs was ranked as low, medium or high risk for the three targeted compounds. The approach was tested using actual monitoring data found for the considered WWTPs; unfortunately, the low number of monitoring data found and their relative unreliability prevented from an actual validation of the RA model. However, the present work, although preliminary, is paving the way through the future development of a quantitative model. Indeed, some recommendations and potential improvements that could be used to strengthen the approach an being able to identify which WWTPs would necessitate some improvements as regards to the application of the WFD and specifically to reduce the presence of watch list PhACs in their receiving waters.

Acknowledgements

The authors express gratitude to Ireland's Environmental Protection Agency STRIVE Programme for supporting this study (Project 2014-W-DS-18).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.05.227.

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Science of the Total Environment 616–617 (2018) 187–197

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Review

Occurrence and geodatabase mapping of three contaminants of emerging concern in receiving water and at effluent from waste water treatment plants – A first overview of the situation in the Republic of Ireland

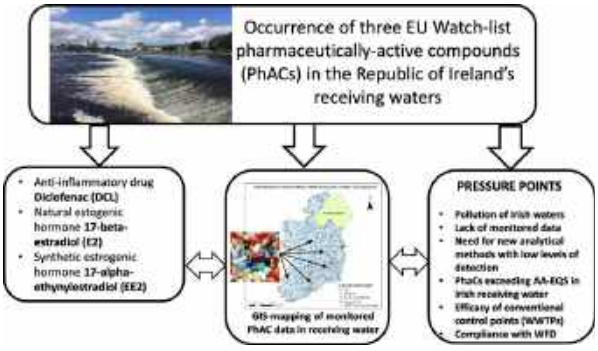
Alexandre Tahar [a], Erin Jo Tiedeken [a,b], Neil J. Rowan [a,*]

[a] Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland
[b] School of Science, National College of New Jersey, Pennington Road Ewing, NJ 08628-0718, USA

HIGHLIGHTS

- Occurrence and mapping of 3 EU Watch list substances in Irish aquatic environment
- Lack of monitored data for CECs given number of river basin catchments and control points
- Need for new analytical techniques with low appropriate levels of detection to meet WFD limits
- Control measures frequently do not fully remove these harmful chemicals.
- Mapping of CECs will strategically inform future upgrades to important control points.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 August 2017
Received in revised form 30 October 2017
Accepted 2 November 2017
Available online xxxx

Editor: D. Barcelo

Keywords:
EU watch-list substances
Diclofenac
Estrogens
Occurrence
Monitoring
Receiving water
Control points
Republic of Ireland

ABSTRACT

This constitutes the first study to address occurrence and geodatabase mapping of the anti-inflammatory drug diclofenac (DCL) and the natural (17-beta-estradiol or E2) and synthetic (17-alpha-ethynylestradiol or EE2) estrogenic hormones in Republic of Ireland receiving waters over the period 1999 to 2015. Among these data, 317 samples came from concentration studies, while 205 were from effect-based studies. Monitoring data came from 16 waste water treatment plants (WWTPs), 23 water bodies (including rivers, lakes, marine and transitional waters) and 7 from domestic locations. Out of approximately 1000 WWPTs in the Republic of Ireland, only 16 have been monitored for at least one of these compounds of emerging concern (CECs). Diclofenac is found in treated effluents from 5 WWTPs at levels at least as high as other European WWPTs, and sometime higher. Measurements of E2 and EE2 in WWPT effluents were rare and effluents were more often evaluated for total estrogens; these CECs were generally not detected using conventional analytical methods because of limits of detection being too high compared to environmental concentrations and WFD environmental quality standards. There was good agreement between occurrence of these CEC and regional drug dispensing data in Ireland. Mapping the aforementioned data onto appropriate river basin catchment management tools will inform predictive and simulated risk determinations to inform investment in infrastructure that is necessary to protect rivers and beaches and economic activities that rely on clean water. There is a pressing commensurate need to refine/develop new analytical methods with low levels of detection for future CEC intervention.

* Corresponding author.
E-mail address: nrowan@ait.ie (N.J. Rowan).

https://doi.org/10.1016/j.scitotenv.2017.11.021
0048-9697/

Contents

1. Introduction

Pollution of European receiving waters containing pharmaceuticals is a ubiquitous phenomenon (Verlicchi and Zambello, 2016; Barbosa et al., 2016; Tiedeken et al., 2017). Until recently environmental regulations worldwide had not required explicit testing of these contaminants of emerging concern in water bodies. However, given concern about contamination of aquatic environment with these substances, legislation such as the Water Framework Directive (WFD) and the Environmental Quality Standards Directive (EQSD) at a European level and associated legislation at a local level has recently begun to acknowledge this problem (Tiedeken et al., 2017; Tahar et al., 2017) The identification of these contaminants and associated analytical methods may inform pressure points and efficacy of appropriate interventions for consideration in future WFD-monitoring programmes and regulations. Pharmaceuticals are a class of emerging environmental contaminants that are widely used in human and veterinary medicine (Tahar et al., 2017). From here on, these substances of emerging concern will be referred to as pharmaceutically active chemicals (PhACs) that includes not just pharmaceuticals but also their pharmaceutically active metabolites/transformation products. This research is important because of the potential toxic effects for aquatic biota and human health that may result from chronic exposure to PhACs (Streck, 2009; Kümmerer, 2009; Kosma et al., 2014). PhACs exhibit wide variation in function, chemical structure and physiochemical properties, making it difficult to generalize about their behaviour, persistence or impact in the environment. PhACs are also designed to be biologically active, have a specific mode of action and to be persistent in the body, meaning they can impact humans and wildlife at trace concentrations which are often hard to detect and quantify using traditional analytical methods (Kosma et al., 2014). A large number of PhACs have been detected in wastewater treatment plants (WWTP) influents and effluents and surface, ground and drinking water worldwide in recent years (Cirja et al., 2008; Streck, 2009; Zhou et al., 2009; Verlicchi and Zambello, 2016). It is now established that throughout the developed world, PhACs are ubiquitous at μg to ng per litre levels in the aquatic environment (Streck, 2009). The impacts of chronic exposure to trace concentrations of many PhACs on wildlife and human health may be severe (Verlicchi et al., 2012); thus, it is critical to limit as much as possible the concentrations of this class of contaminants in our waterways.

Until recently, environmental regulations worldwide had not required explicit testing for any PhACs in water bodies. However given the growing concern about contamination of the aquatic environment with these compounds, legislation has recently begun to acknowledge this potential problem. The WFD requires that all EU member states prepare river basin management plans (RBMPs) to address the many issues relating to water quality and protection in a holistic manner. In response to growing EU concern about the release of untreated PhACs into the aquatic environment, three compounds were included on in the first EU watch list in 2013: diclofenac (CAS# 15307-79-6, hereafter referred as DCL), 17-beta-estradiol (CAS# 50-28-2, hereafter referred as E2) and 17-alpha-ethinylestradiol (CAS# 57-63-6, hereafter referred as EE2) (Barbosa et al., 2016). Annual average environmental quality standard (AA-EQS) were defined for these 3 compounds as being the concentrations defining the boundary between good and moderate WFD status. The respective AA EQS in surface water for DCL, E2 and EE2 are 100 ng/L, 0.035 ng/L and 0.4 ng/L. EE2 and E2 can impact the endocrine system of humans or wildlife (Verlicchi et al., 2012). There are growing fears that chronic exposure to these endocrine disrupting chemicals or EDCs (in bathing or drinking water, for example) may be linked to adverse human health conditions such as declining male fertility, birth defects, and breast and testicular cancer. Similar to PhACs as a whole, EDCs are mainly thought to be transported into the aquatic environment via incomplete removal at WWTPs (Streck, 2009). It is relevant to note that the European Commission implemented decision 495 of 20 March 2015 that expanded substances or groups of substances on the watch list to 10 in the field of water policy (Barbosa et al., 2016). Also, following the timetable for the common implementation of the WFD, the first management cycle ended in 2015, and the second river basin management plan combined with the first flood management plan is due to end in 2021.

A systematic review of these three first EU watch list PhACs in receiving waters was recently published, which reviewed 3945 potentially relevant articles over period 1995 to 2015 publications on uses, sources, monitoring and control measures to produce a EU-wide database (Tiedeken et al., 2017). Overall, European surface water concentrations of DCL are typically below reported annual proposed AA EQS of 100 ng/L, but exceedances frequently occur. E2 and EE2 surface water concentrations are typically below 50 ng/L and 10 ng/L respectively, but these values greatly exceed the proposed AA EQS values for these compounds (0.4 and 0.035 ng/L respectively). However, levels of these PhACs are frequently reported to be disproportionately high in EU receiving waters, particularly in effluents at control points that require urgent attention. In addition, the EPA reported in October 2017 that in 42 locations in the Republic of Ireland, sewage is discharged untreated, putting rivers and bathing areas at risk of pollution: 44 of 170 large urban areas did not comply with EU water quality standards (EPA, 2017). The review of Tiedeken et al. (2017) highlighted that there is a pressing need to conduct detailed mapping of the occurrences and control measures for CECs at a national scale that provides a platform for EU orientation so as to inform policy and decision-making on improving and protecting water quality. Thus, the aim of this case study was to evaluate best-published data on these three EU Watch list PhACs so as to geographically map their occurrence in Irish receiving waters and in effluent at WWTPs and to compare with regional drug dispensing data.

2. Materials and methods

For each publication identified as containing relevant Irish DCL, E2 or EE2 monitoring data, several other parameters were extracted for use in mapping. First, the date water samples were taken was identified (including day, month and year if available, but year at minimum). The type of study (i.e. a study measuring the concentration of a compound or a study using effect-based methods) as well as the compound analysed (DCL, E2, EE2 or estradiols equivalents, EEQ) was identified. The method of sampling (grab, composite, passive, or any type of assay utilised) was identified, as was the matrix studied (marine water, lake water, ground water, WWTP influent/effluent). The name and GPS coordinated of the specific location where the sampling took place as well as the county was listed. If the sampling location was a WWTP, its coordinates were recorded as the primary discharge point listed in the EPA wastewater license (http://www.epa.ie/terminalfour/wwda/index.jsp?disclaimer=yes&Submit=Continue#.VpPcJPmLTIX). The concentration (in ng/L) of the compound recorded during each sampling event was also recorded if it was available. If multiple samples were taken at the same location during a study (i.e. repeat sampling over time), each sampling event was listed separately. However, if multiple analyses were run on the same samples (i.e. tests run in duplicate or triplicate) the individual results were not reported; instead the final value as presented by the authors of the study (usually an average) was utilised. The publication (reference) associated with each data point was recorded. Frequently some of the data described above were not available from the publications, in which case corresponding authors were emailed or otherwise contacted and a data request was made.

Two aspects of these data were mapped for this report, (i) the distribution of the sampling events (or sampling effort) for each compound and (ii) the highest recorded concentrations of each compound at a sampling location. In order to map sampling events, the data were divided into three 5–6 year intervals: 1999–2004, 2005–2009, and 2010–2015. Results for samples that were taken at the same location, analysed for the same compound and sampled during the same interval were consolidated, and the number of sampling events at each location was specified. This allowed the sampling effort at each location to be mapped for each compound of interest. For the concentration data, effect-based studies were ignored. For some of these studies, the concentration values were not available. This was because either the study reported only presence/absence of the compound or reported average values instead of raw data. In the latter case, authors were contacted to attempt to obtain raw data values, however a small number of authors did not respond to data requests. These studies were thus excluded from concentration mapping. The remaining concentration data were then sorted by compound, location and value, and for each unique location the highest concentration recorded for each compound was identified for mapping. These concentration maps therefore represent the worst case scenario as recorded by monitoring studies to date at each location. It must be noted that this does not mean that these sites will always have such high concentrations of the compounds of interest; additional sampling events may have recorded lower values or non-detects. It should also be noted that just because a compound has not been detected at a site does not mean it will not exceed WFD proposed limits (EQS); it is possible (especially for the estrogens) that the limit of detection (LOD) may exceed the EQS limit. Nevertheless, these maps still provide an indication of which areas could be pollution "hotspots" based on currently available monitoring data.

Data were mapped using ArcGIS Desktop software (Arc Catalog and ArcMap 10.3.1, Environmental Systems Research Institute, via an ESRI single-user, one-year license). Additional data used to create the file geodatabase were downloaded from the Central Statistics Office database "StatBank Ireland," (http://www.cso.ie/px/pxeirestat/statire/SelectTable/Omrade0.asp?Planguage=0, utilised for county boundaries, city locations and population density) and the EPA's Geo Portal (http://gis.epa.ie/GetData/Download, utilised for river basin catchments, WFD river basin districts, WWTP locations and attribute data, and WFD protected areas). A map of the Republic of Ireland river basin catchment is provided in Supplementary materials (Fig. S1).

Reported drug utilisation or dispensing data is an important source of information on the quantities of PhACs that ultimately are released into the environment (Boxall, 2010; Rowan, 2011). Therefore, with the objective to correlate the concentration data gathered and to provide a proxy for the region where no environmental data were found, drug utilising dispensing data was provided by Ireland's Health Service Executive (HSE) for the 32 Local Health Offices (LHOs) for DCL and E2. The total volume (kg) of DCL and E2 dispensed to patients in all LHOs from 2009 to 2012 was provided, however data for years 2008 and 2013 were excluded as these periods were not full calendar years. EE2 is often prescribed as a combination drug, thus obtaining and summarizing dispensing data is more difficult than for DCL and E2 and was therefore not included in this study.

3. Results and discussion

3.1. GIS mapping – an overview of Irish DCL, E2 and EE2 data (1999–2015)

From the literature review performed, a total of 522 unique Irish monitoring data points were identified for DCL, E2, EE2 or EEQ concentrations. Of these samples, 151 were measurements of DCL concentrations, 83 each were measurements of E2 and EE2 concentrations and 205 were measurements of EEQ concentrations. These monitoring data include samples from 50 unique locations, comprised of influent or effluent from 16 Irish WWTPs, samples from 23 unique water bodies (including rivers, lakes, marine and transitional waters) and domestic effluent from 7 locations. Fig. 1 demonstrates the distribution and frequency of the national monitoring data for DCL, E2, EE2 and EEQ concentrations over the entire period reviewed by this report, from 1999 to 2014 (the different maps for the periods 1999–2004, 2005–2009 and 2010–2015 are presented in Supplementary material). Several patterns regarding the overall distribution of these monitoring data can be observed. First, the monitoring data are distributed relatively evenly throughout the country, though they do tend to be focused around population centres (Galway, Dublin and Cork). EEQ sampling is particularly evenly distributed, having been taken for many inland and coastal surface waters, including the east, south east, south west, and west coasts, as well as one location in the north of the country. Much of the concentration data for all three compounds, however, is carried out in coastal water bodies (marine or transitional waters), rather than in inland surface waters. This is particularly true of DCL, which has mainly been sampled in coastal waters surrounding Dublin and Galway. Concentration measurements of DCL, E2 and EE2 are particularly lacking in the midlands region. Finally, it is clear from this figure that all monitoring data on these watch list PhACs are lacking from the north, north east and northwest coasts of the republic of Ireland. A summary of all the monitoring studies across the country is provided in Supplementary materials (Figs. S3–S5).

In general, the estrogens/estrogenicity is better studied in the Republic of Ireland in terms of monitoring data when compared with DCL. Furthermore, in regards to the estrogens, effect data (measured by EEQ) are more common than concentration data. This is likely due to difficulties associated with obtaining sufficiently sensitive analytical methods of detecting trace amounts of estrogens in water samples. While data from effect-based studies reporting EEQ concentrations can help give an indication of the overall estrogenicity of Irish waters, the compounds responsible usually are not identified. Thus, in regards to quantifying contamination from these two specific estrogenic compounds, effect-based sampling is not as useful as concentration studies that report specific levels of E2 or EE2. Nevertheless, given the lack of sensitivity of current analytical methods, effect-based studies may have a place regarding reporting for WFD monitoring. Fig. 1 illustrates that in addition to surface water samples, monitoring data also came from measurements of WWTP influent or effluent.

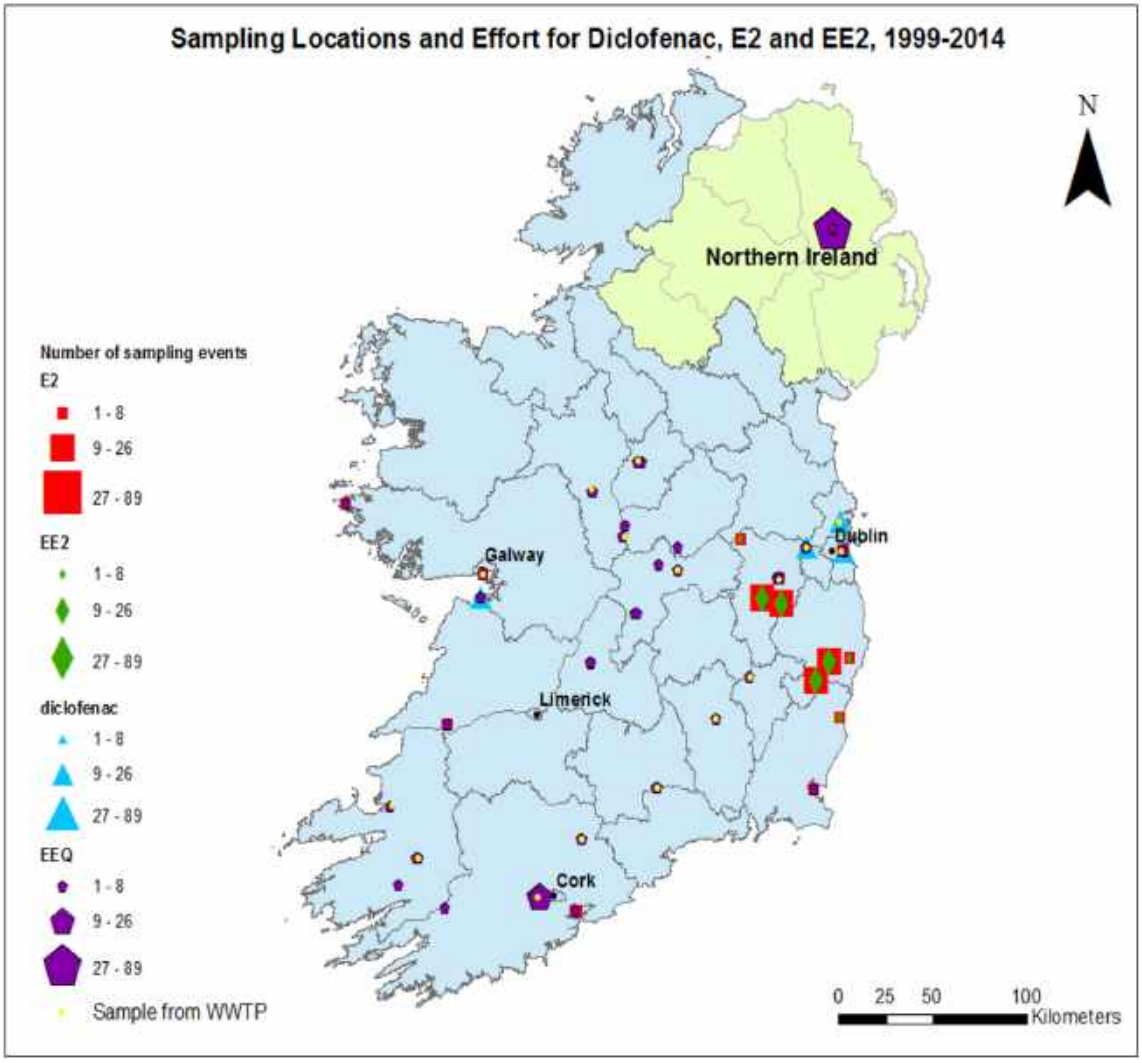


Fig. 1. Summary of national monitoring distribution and frequency for diclofenac (blue triangles), E2 (red squares), EE2 (green diamonds), and estradiols equivalents (purple pentagons) in Ireland from 1999 to 2015. Symbol size increases with increasing number of samples taken at each location. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the period 1999–2004, studies of endocrine disrupting chemicals and pharmaceutical contamination of aquatic environments were just beginning to gain international popularity during this time. Irish monitoring data during these early years consist mostly of results from effect-based studies, reporting EEQ concentrations. They were carried out largely in the east and southwest coasts and midlands by a project focusing on the Shannon International River Basin District (Tarrant et al., 2005; Tarrant et al., 2008). Some E2 and EE2 concentration measurements were also taken for domestic effluent at sites in the south east of the country, but LODs for these compounds were still too high to compare to WFD proposed limits (Kelly et al., 2010; Quinn-Hosey et al., 2012). No DCL data were found for this period. In the second time period, from 2005 to 2009, the first DCL sampling took place on the east coast of the country, although all three sampling locations were samples from WWTP influent and effluent as opposed to surface waters (Lacey et al., 2008; Lacey et al., 2012). EEQ sampling for estrogenicity continued for the Shannon International River Basin District project during this time period (Quinn-Hosey et al., 2012) and EEQ sampling began on east, south east and west coast locations for the SeaChange project (Giltrap et al., 2013). The project with the most individual EEQ sampling events (89) also took place on a dairy farm in the north of Ireland during this time period (Cai et al., 2012). In the final and most recent time period, from 2010 to 2015, a shift in estrogen sampling is apparent; instead of sampling more inland freshwater sources using largely effect-based studies (EEQ measurements), coastal locations are more frequently monitored. This means that instead of freshwater samples there was a focus on marine and transitional waters. This is because the SeaChange project, which was meant to rectify the lack of estrogen concentration data in marine waters, took place largely during this time period. Less individual samples tend to be taken during concentration studies, because analyses are more expensive and time intensive than many effect-based studies; this is indicated by the smaller symbol sizes in Fig. 4, as opposed to 2 and 3. For DCL, more samples were taken in surface waters in both the east and west coasts of the country (McEneff et al., 2014), these data represent the best information to date on DCL levels in surface waters in Ireland. The GIS maps associated to these periods are presented in Supplementary material.

3.2. Monitoring studies

3.2.1. WWTPs monitoring and locations in Republic of Ireland

Fig. 2 highlights the monitoring data which originate from the 16 sampled Irish WWTPs, and demonstrates the distribution of these sampling locations in regards to all Irish WWTPs. Details of the 16 WWTPs is shown in Supplementary material (Table S1). In total, 178 (34%) of the national monitoring data points from the reviewed publications were

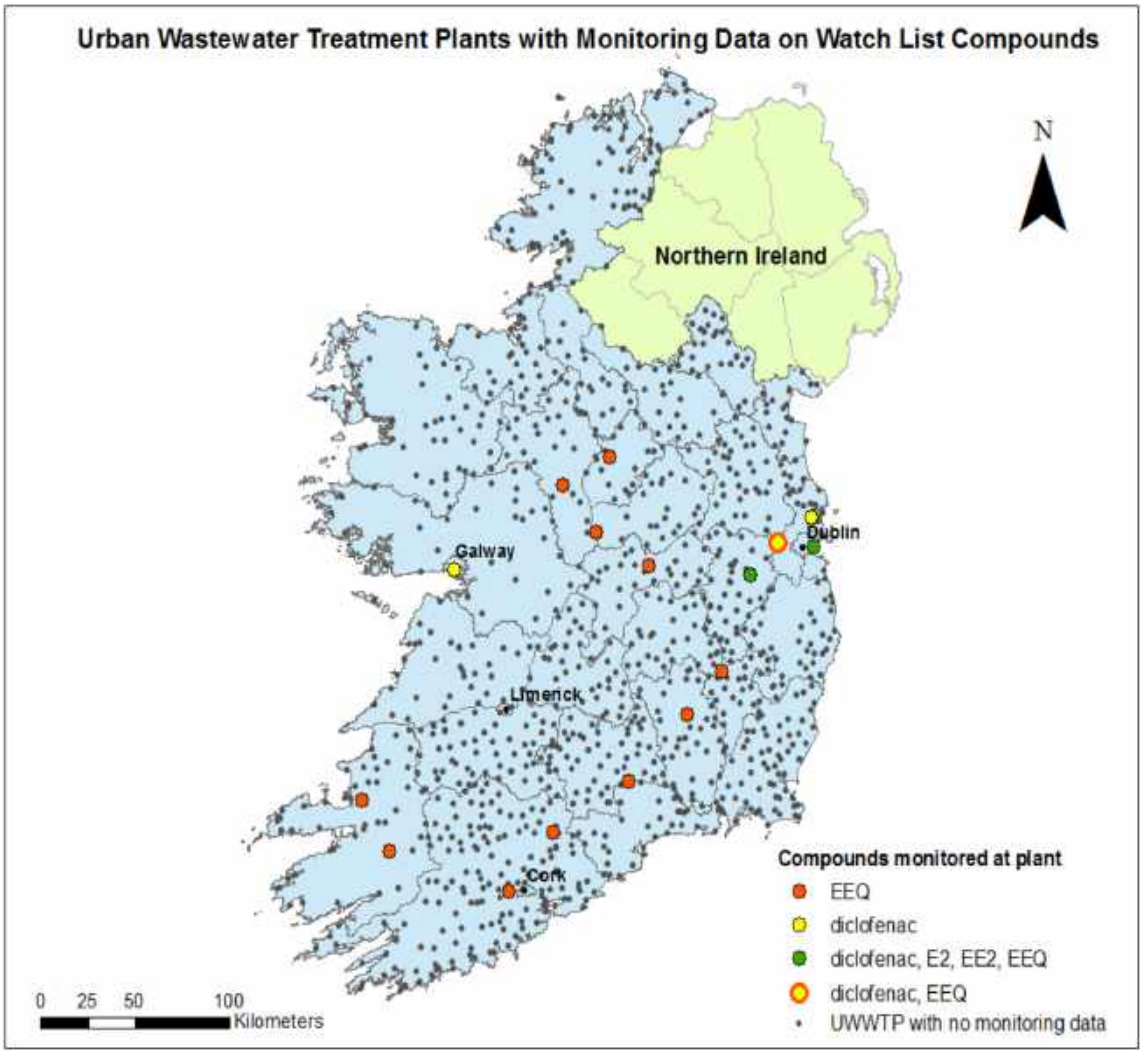


Fig. 2. Distribution of urban wastewater treatment plants (UWWTPs) with existing monitoring data on diclofenac, EE2 and/or estradiol equivalents (EEQ). Orange dots represent agglomerations with only EEQ measurements; yellow dots represent plants with only diclofenac monitoring data; green dots represent plants that have been monitored for all four compounds; yellow dots with orange outlines represent plants that have been monitored for diclofenac and EEQ only; and black dots represent the locations of agglomerations with no monitoring data for these compounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measurements of one of the four investigated parameters (E2, EE2, DCL or EEQ concentrations) from WWTP influent or effluent. Most WWTPs were sampled for estrogenicity only. Fourteen WWTPs were sampled for estrogenicity in total, including Athlone, Ballincollig, Carlow, Clonmel, Fermoy, Kilkenny, Killarney, Leixlip, Longford, Osberstown, Ringsend, Roscommon, Tralee and Tullamore. Only two WWTPs were sampled for E2 and EE2 specifically, Ringsend and Osberstown. Five plants were monitored for DCL levels including Galway, Leixlip,

Table 1
Sixteen WWTPs for which published monitoring data on diclofenac, E2, EE2 or estradiols equivalents is available in Republic of Ireland.

WWTP name	County	Catchment	WFD river basin district	Size (PE)	Type of secondary treatment
Carlow	Carlow	Barrow	South-eastern	39,043	Extended aeration
Ballincollig	Cork	Lee	South-western	27,697	Extended aeration
Fermoy	Cork	Blackwater	South-western	18,608	CAS
Ringsend	Dublin	Coastal	Eastern	2,124,000	Sequence batch reactor
Swords	Fingal	Broad Meadow Water	Eastern	77,014	Extended aeration
Galway	Galway	Coastal	Western	213,424	CAS
Killarney	Kerry	Laune	South-western	41,836	CAS
Tralee	Kerry	Coastal	Shannon	35,149	CAS
Leixlip	Kildare	Liffey	Eastern	100,309	CAS
Osberstown	Kildare	Liffey	Eastern	104,723	Sequence batch reactor
Kilkenny	Kilkenny	Nore	South-eastern	51,988	CAS
Longford	Longford	Shannon Upper	Shannon	11,672	CAS
Tullamore	Offaly	Shannon Lower	Shannon	24,055	CAS
Roscommon	Roscommon	Shannon Upper	Shannon	6989	CAS
Clonmel	Tipperary	Suir	South-eastern	34,909	Extended aeration
Athlone	Westmeath	Shannon Upper	Shannon	21,155	Extended aeration

CAS = conventional activated sludge; PE = population equivalents.

Ringsend Osberstown and Swords. The two WWTPs that were sampled for all 4 compounds were Ringsend and Osberstown. The 16 WWTPs for which monitoring data for at least one of these four compounds exists make up approximately 1.45% of all of Ireland's WWTPs, indicating that more data on the concentrations of these compounds in Irish agglomerations would be useful. The WWTPs with EEQ sampling data are distributed evenly throughout the country, though measurements are missing from the northwest. DCL again has only been sampled in WWTPs surrounding the major population centres of Dublin and Galway. Data on DCL levels in WWTPs influent and effluent in the midlands, south and north are severely lacking (Table 1).

3.2.2. Highest monitored concentrations in Republic of Ireland for the 3 targeted compounds

Figs. 3–4 and Supplementary material (Fig. S2) were created by mapping the highest concentration value recorded for each drug at each site where it was monitored. These figures thus allow for a comparison of the maximum recorded concentrations of each particular compound at the various sites for which monitoring data currently exist. These maps provide an indication of which areas could be pollution "hotspots" based on currently available monitoring data. The maximum E2 concentrations ranged from not being detected in any samples at a site to 15.2 ng/L. The AA EQS proposed for E2 is 0.4 ng/L (European Commission, 2011); it is clear from Fig. 3 that several locations had maximum values which exceed this limit, including locations near Cork, Galway and Dublin city. Given that E2 is a natural steroid estrogen, it is unsurprising that high concentrations are found near these population centres. Out of a total of 12 sites with reliable quantitative monitoring data, 7 had maximum values that exceeded the proposed AA EQS value. Of those 7, two were domestic wastewater (that are not to be compared with AA EQS that refer to surface water after mixing), but the rest were surface waters distributed throughout the country; future studies should consider these water bodies as potential monitoring sites as it is possible their E2 levels may exceed WFD limits.

EE2 could not be detected at 9 out of 11 sites where sampling occurred, and the maximum concentration recorded for EE2 at any site was 0.32 ng/L. This relatively high value was found in a sample of domestic effluent from a home in County Wicklow as opposed to in a surface water sample. The AA EQS value proposed for EE2 is 0.035 ng/L (European Commission, 2011). From the data presented in Fig. 4, it may seem like only two locations may exceed this AA EQS; however, this interpretation cannot be trusted because the sensitivity of many of the analytical methods used to detect and quantify EE2 was insufficient. Few methods exist that can reach the required LOD of 0.035 ng/L, hence it is

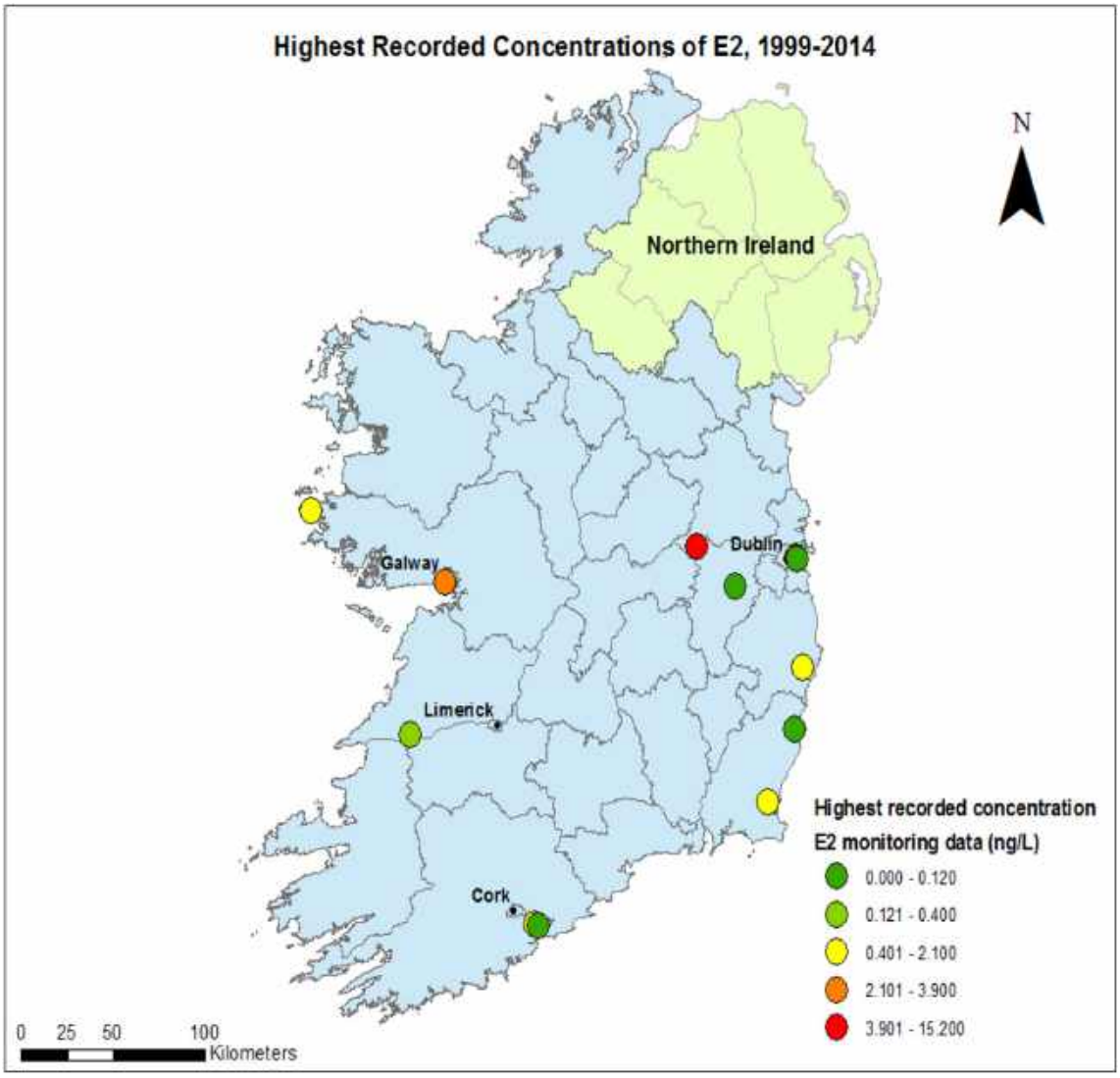


Fig. 3. Highest recorded concentrations (ng/L) of E2 at each sampling site where concentration monitoring data were collected. Relative concentration values are indicated by the symbol colour, where low concentrations are indicated by greens and high by reds. Yellow, orange and red indicate sites where the highest recorded concentration was greater than the proposed WFD AA-EQS value for E2 (0.4 ng/L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

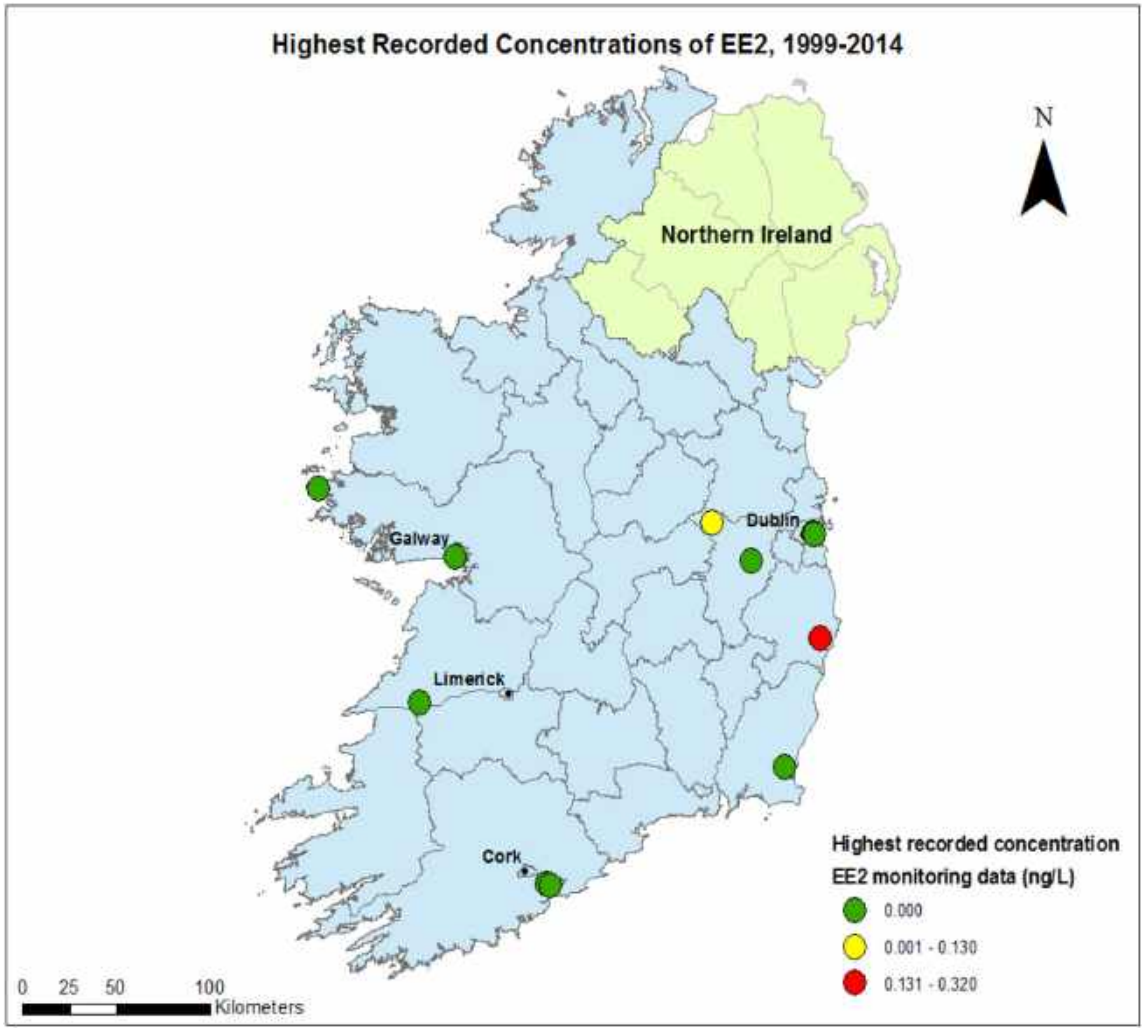


Fig. 4. Highest recorded concentrations (ng/L) of EE2 at each sampling site where concentration monitoring data were collected. Relative concentration values are indicated by the symbol colour, where low concentrations are indicated by greens and high by reds. Zero values represent no detects. There are two green dots near Cork and Dublin respectively, although only one is visible at this scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

impossible to state with certainty that EE2 is below the WFD proposed limit if the compound is not detected at any of these locations. Additional sampling of surface waters for EE2 using sufficiently sensitive analytical methods is therefore of critical importance to our understanding of how concentrations of this compound in Irish waters compare to WFD limits.

For DCL there were clearly less quantitative available data in surface waters, even though analytical methods used to detect this compound are more easily available than for the estrogenic compounds. The AA EQS value proposed for DCL is 100 ng/L, and the maximum recorded concentration at every surface water sampling location except for one exceeded this value, including samples from Galway and Dublin Bay. Among all monitoring locations, the highest recorded values at two locations actually exceed 1000 ng/L, however these were effluent samples from WWTPs; however, these effluent waters are diluted in receiving waters and the expected concentrations in receiving water would be reduced. Nevertheless, it appears that certain locations in Ireland can have very high levels of DCL at times, indicating that additional monitoring for this compound is necessary.

3.2.3. Wastewater treatment plants and receiving waters monitoring studies

Table 2 gives a summary of the monitoring data found for the Republic of Ireland for the three compounds of interest. The first study investigated DCL in WWTPs for three agglomerations in the greater Dublin area (Lacey et al., 2008). Twenty-four hour composite samples were collected from the influent and effluent of each WWTP. The authors report that DCL was not detected in the influent samples (limit of detection or LOD = 0.855 μg/L), but was present in effluent samples (LOD = 0.743 μg/L), although not above the study's limit of quantification (LOQ) for this compound (LOQ in effluent = 2.478 μg/L). A second study by the same research group analysed monthly influent and effluent samples in three WWTPs for a full year (Lacey et al., 2012). Diclofenac was again detected in at least one effluent sample from each plant (detected in 5 effluent samples in total, LOD ranged from 0.5–2.95 μg/L). Similar to the previous study however, DCL was not detected in any influent samples (Lacey et al., 2012). The authors suggest this could be due to deconjugation of conjugated metabolites during the treatment process, which has been observed in other studies (Zhang et al., 2008), this could also be due to possible higher interferences in the analysis of the inlet water due to higher organic matter content that could have reduced the sensitivity of the chemical analysis; however, it is possible that DCL was present in the influent, but at levels below the LOD. In fact in comparison to other European studies, the LODs for both of the Lacey et al. (2012) studies were high. LOD in the ng/L range for DCL are currently standard in the international literature. These were the only studies found that investigated removal efficiencies of DCL, E2 or EE2 in Irish WWTPs.

Table 2
Summary of monitoring data for the three compounds of interest in the Republic of Ireland.

	Concentration (ng/L)	Matrix	Experiment	Reference
DCL	nd (LOD = 855 ng/L)	WWTP influent	3 WWTPs sampled once	Lacey et al. (2008)
	nd (LOD = 743 ng/L)	WWTP effluent		
	nd (LOD = 855 ng/L)	WWTPs influent	3 WWTPs sampled monthly during a year	Lacey et al. (2012)
	Detected in 5 samples (LOD = 500–2950 ng/L)	WWTP effluent		
	2630 (max value over a year long study)	WWTP effluent	2 WWTPs sampled (East and West coast) + receiving sea waters + mussels	Schmidt et al. (2013)
	550 (max value over a year long study)	WWTP receiving sea water		
	nd (LOD = 29 ng/g)	Mussels tissues		
	80.3	WWTP effluent	Pan EU campaign	Loos et al. (2013)
	144.3			
E2	nd (LOQ = 10 ng/L)	WWTP effluent (2 plants)	Pan EU campaign	Jarošová et al. (2014)
EE2	nd (LOQ = 10 ng/L)	WWTP effluent (2 plants)	Pan EU campaign	Jarošová et al. (2014)
EEQ	17.2	WWTP effluent (Co. Kildare)	YES assay	Tarrant et al. (2005)
	3.2	WWTP effluent (Co. Cork)		
	1.1–16.0	WWTPs effluents (8 plants)	YES assay	Tarrant et al. (2008)
	0.9–2.9	WWTPs receiving waters		
	0.53–2.67	WWTPs receiving waters + 2 control locations	YES assay	Tiedeken et al. (2016)
	16.21 (max value over a 2-years survey)	WWTPs receiving waters		
	<0.05	WWTP effluent (2 plants)	Pan EU campaign	Jarošová et al. (2014)

nd: not detected.

However, some studies of E2 and EE2 in Irish WWTPs used biomarkers or in vitro bioassays to quantify total estrogenic activity (reported as estradiols equivalents, EEQ) instead of direct chemical analysis. The first study to evaluate Irish WWTP effluent for estrogenicity was carried out in 2004 by Quinn et al. (2004). An effluent sample from Athlone WWTP was analysed for the presence of estrogenic chemicals using the yeast estrogen screen (YES) assay, and HPLC and GC–MS were used for chemical analyses. Lake water from a marina in Killenure Lough, Lough Ree (Co. Westmeath) was also sampled and analysed (Quinn et al., 2004). This study confirmed the presence of a complex mixture of compounds with estrogenic activity in the WWTP effluent. This estrogenicity was attributed mostly to E2, EE2 and bisphenol A. This was also the first study to demonstrate the presence of EDCs in lake water in Ireland. However, results in both effluent and lake water were limited; attempts to identify E2 and EE2 with GC–MS were unsuccessful due to relatively high LOQs associated to the chemical analysis, and the authors stated that further work was necessary to confirm their presence in these aquatic matrices. At approximately the same time that the Quinn study was performed (Quinn et al., 2004), the Tarrant et al. (2005) also began investigating Irish WWPT effluent for estrogenicity. They used the YES assay to screen undiluted effluent from Osberstown (Co. Kildare) and Ballincollig (Co. Cork) WWTPs, and found estrogen levels at 17.2 ± 3.8 and 3.2 ± 1.1 ng/L EEQ respectively. Ballincolig effluent levels were diluted upon entering the River Lee to a level that was below the threshold required to induce vitellogenesis in fish. The effluent form Osberstown WWTP, however, was not diluted enough by the River Liffey to reach levels below this threshold. Accordingly, the study also found raised vitellogenin in male brown trout from this study site, indicating the water was estrogenic enough to have an impact on wild fish (Tarrant et al., 2005). It should be noted however, that the compounds responsible for the estrogenicity of the water were not identified analytically; however, the authors hypothesized EE2 might be responsible because a component of Osberstown WWTP industrial effluent came from a manufacturer of the contraceptive pill. In the work described in Tarrant et al. (2008) estrogenicty using the YES assay was also measured for 8 additional Irish WWTPs and some of their receiving waters. Again, results are reported in EEQ; effluents ranged from 1.1–16.0 ng/L and receiving waters ranged from 0.9–2.9 ng/L. These results indicated that (besides a few notable exceptions in Co. Dublin), levels of estrogenic compounds were high in some Irish WWTP effluents, but that they were sufficiently diluted in most receiving waters so as not to cause an immediate threat to aquatic wildlife (Tarrant et al., 2008).

Given that previous studies mostly focused on surface waters near Dublin or Cork, in 2010 a study investigating estrogenicty in the Shannon International River Basin District (SIRBD) was carried out (Kelly et al., 2010). Five rivers were sampled and estrogenicity was assessed using the YES assay. Not only was estrogenicity evident in the rivers at all sites downstream of WWTPs, it was also present at two control locations. The EEQ values ranged from 0.53–2.67 ng/L, within the concentration range of E2 required to induce vitellogenesis in male rainbow trout (Kelly et al., 2010). This study also demonstrated that levels of EDCs in Irish rivers were elevated in comparison to EEQ values reported by previous studies (Tarrant et al., 2005). An additional study was carried out by the same research group further investigating EDCs in the Border, Midland and Western (BMW) region of Ireland (DoELG, 1999). In this work, influent and effluent samples were collected from inlet and outlet pipes at 4 different WWTPs, and corresponding upstream (control) and downstream water samples were taken from the Rivers Hind, Camlin, Shannon (at Athlone) and Silver (at Tullamore). Grab samples were taken over two year period to investigate seasonal changes. The results demonstrated that some WWTPs in this region were highly efficient in removing estrogen contamination; EEQ removal achieved was 98% and 92% respectively for Tullamore and Longford WWTPs. However, the EEQ levels in receiving water samples from downstream and upstream of the WWTPs demonstrated that this region of Ireland can obtain heavy loads of EDCs (maximum EEQ = 16.21 ng/L in a downstream sample from the River Camlin) (DoELG, 1999).

The SeaChange project provides the most recent monitoring data of E2 and EE2 in Irish waters (Giltrap et al., 2013). For example, in one study, water and mussel samples were collected from three locations on the Irish coast in June 2010: (i) in the estuary of the River Liffey (Dublin site), (ii) in Galway Bay (Galway site) and (iii) in Redbank hatchery, Aughinish Co. Clare (Clare site, mussels only analysed). Samples were analysed by LCMS/MS for estrone (E1), E2 and EE2. This study provided the first detection of E1 by LC/MS/MS in Irish marine waters (Dublin Bay, 0.76 ng/L) (Kunz et al., 2015). E2 and EE2 were not detected in the water samples from Galway Bay or in any mussel samples from

the Dublin, Galway or Clare sites (Kunz et al., 2015). E2 was detected in a sample from Dublin Bay, but levels were low (0.13 ng/L) and three additional samples were non-detects. Therefore the annual average value was below the proposed WFD AA EQS·In general, the authors of the report concluded that the levels and associated endocrine disrupting effects were generally low at their study sites, and there was likely a low risk of estrogen-caused endocrine disruption to resident species; however areas with significant anthropogenic pressures are at higher risk and additional monitoring was suggested by the authors at such sites (e.g. Dublin Bay) (Giltrap et al., 2013). Further sampling for estrogens at additional sites along the Irish coastline was carried out during the SeaChange project using passive sampling and effect-based monitoring, however these results are not as easily compared with the proposed standards for the WFD.

The most recent data evaluating DCL levels in Irish wastewater effluent or surface waters come from Schmidt et al. (2013). In a year-long study, sewage effluent, receiving marine waters and marine bivalves were analysed for DCL and other pharmaceutical residues. Effluent was sampled (24-h composite samples) from one WWTP on both the east and west coast of Ireland. Mussels were deployed in a year-long cage experiment in a control site and two effluent exposure sites of the east and west coast, and grab samples of surrounding marine surface waters were also analysed. LOQ for DCL were 225 ng/L, 22 ng/L, and 29 ng/g respectively in effluent, marine water, and mussels. The highest DCL concentrations detected in effluent were 1.69 and 2.63 μg/L in the eastern and western WWTP respectively. In marine surface waters the highest values were 0.46 and 0.55 μg/L in the east and west. Diclofenac was not detected in mussels (Wille et al., 2012). The concentrations of DCL in effluents and surface waters are comparable to those found in other EU monitoring programmes, but surface water values in particular are on average higher than DCL concentrations detected in other European marine waters (Wille et al., 2012). Furthermore, the highest marine surface water value detected in this study was approximately 5 times higher than the current proposed AA EQS for DCL. In a later experiment associated with the same project, wild mussels from a pristine site off the west of Ireland and a highly contaminated site on the east coast were analysed for several PhACs; again, DCL was not detected in any samples (McEneff et al., 2013). An additional study investigated PhACs residues (including DCL) in cooked and uncooked marine mussel tissue, and found that cooking increased PhACs residues in contaminated tissue. Although this does provide evidence for the potential exposure of humans to DCL via bioaccumulation, mussels in this experiment were artificially exposed in the lab and so this study did not provide monitoring data.

3.2.4. Monitoring of on-site non collective treatment systems

In Ireland the domestic wastewater of more than one-third of the population is treated by on-site systems (DoELG, 1999). Considering that human excretions represent a major source of PhACs contamination (Buchberger, 2011) on-site wastewater treatment systems could thus be an important source requiring consideration in Ireland. Work reported by Gill et al. (2009) which investigated the effectiveness of septic tank and secondary treatment on-site wastewater systems, produced two publications specific to EDC removal. The first study sampled domestic effluent at 4 sites in Ireland, two with effluent discharged following primary treatment (i.e. septic tank) and two with secondary treatment (i.e. peat filter) systems (Ó Súlleabháin et al., 2009). EDCs were found at all 4 sites, but E2 and EE2 were each found at two out the four sites with no straightforward relationship with the type of treatment. The sensitivity of the chemical analysis was poor in this study; EE2 was not determined quantitatively and E2 had a high LOD (2 μg/L). The second study aimed to answer similar questions using analytical methods with increased sensitivity (e.g. LOD for E2 = 0.05 μg/L). Gill et al. (2009) again investigated the natural attenuation of EDCs in the most common on-site treatment system in Ireland, the septic tank and subsoil percolation area. The study focused on the transport of EDCs, including E2 and EE2, through the soil at three sites in the east of Ireland. Overall, the authors found that E2 and EE2 were significantly degraded with depth to sub ng/L levels at all sites investigated. These results are the only indication of how efficient on-site systems are at EDC removal in Ireland, and no equivalent study has investigated DCL removal.

3.2.5. Studies on alternative control measures

Studies investigating DCL, E2 and EE2 in wastewater effluent such as those reviewed above (Quinn et al., 2004; Tarrant et al., 2005; Tarrant et al., 2008; Lacey et al., 2008; Gill et al., 2009; Lacey et al., 2012; Wille et al., 2012; McEneff et al., 2013; Loos et al., 2013; Schmidt et al., 2013; Jarošová et al., 2014; Tiedeken et al., 2017) demonstrate that these compounds are often not removed completely during treatment at Irish WWTPs. Some Irish studies have therefore examined advanced treatment options which may remove DCL, E2 or EE2 from water more efficiently. For example, a study in 2010 examined the feasibility, kinetics and efficiency of using liquid-core microcapsules as a novel methodology for removal of DCL from water (Whelehan et al., 2010). The work demonstrated that liquid-core microcapsules are capable of rapid extraction of DCL (100% within 50 min of capsule addition to contaminated water) and other common PhACs. Another study investigated the temporal removal of estrogenic activity of several estrogens (including E2 and EE2) by UVA irradiation over an immobilised titanium dioxide (TiO_2) catalyst (Coleman et al., 2004). UVA photolysis over the catalyst was equally effective at removing estrogenic activity of E2, EE2 and E1; the study demonstrated a 50% reduction in estrogenicity in samples treated for 10 min. Also, the work reviewed above by Cai et al. (2013) investigated removal of hormones from dairy farm wastewater with the aim of evaluating the efficiency of CWs to reduce estrogenic hormone concentrations in dairy wastewater as it was demonstrated in a study carried out in the UK that dairy cows were identified as the largest contributors to excreted estrogens (Johnson et al., 2006). Over the course of a year, monthly samples were taken at seven locations on the farm (i.e. the inflow pond, a plant covered area, close to the outlet of ponds, a lake on the farm, the receiving river, and a groundwater monitoring well) and analysed via a reporter gene assay (RGA). An average removal efficiency for estrogenic compounds of 95.2% was found, indicating that the CW was efficient at removal of such compounds; however, the lowest removal rate during the year was 83.7%, and the concentration at the final pond was 18.8 EEQ ng/L, which is above the proposed lowest observable effect concentration of 10 ng/L (Cai et al., 2012). These authors found that CWs currently employed in Ireland can eliminate hormones in dairy wastewater to low levels often acceptable for effluent. Cai et al. (2013) was also reported that advanced treatments, such as the employment of reactive and sorptive materials (granulated activated carbon, organoclay, etc.), can further improve CWS treated dairy farm wastewater quality, which may be particularly important in enhancing removal efficiency of peak hormone concentrations Additional studies on effective treatment options that can function in an Irish context are needed and could improve the effluent quality in regards to DCL, E2 and EE2 levels.

3.2.6. Comparison of Irish and EU monitoring data

In 2010, two Irish WWTPs (one in Co. Kildare and one in Co. Dublin) were surveyed (via grab samples) for a wide range of PhACs, including DCL, E2 and EE2. This monitoring was part of a Joint Research Centre (JRC) pan-European campaign designed to provide the first concise overview of concentrations of many emerging pollutants in WWTP effluents across Europe (Jarošová et al., 2014). Along with conventional analytical techniques, effect-based monitoring was also carried out in order to determine total estrogenicity of the effluents, reported as EEQ. The study's results for Ireland found no steroid estrogens above their LOQ (i.e. 10 ng/L for steroidal estrogens) via chemical monitoring. In addition, the detected EEQ for both Irish WWTPs were <0.5 ng/L, even though one third of the municipal WWTP effluents from across

Europe that were included in the study had values >0.5 ng/L. This finding indicates that the two Irish WWTP effluents have relatively low estrogenicity in comparison to many European countries. One of the Irish WWTPs was one of only 4 municipal WWTPs in the study that utilised a tertiary treatment step (UV light); this advanced treatment could have contributed to the observed low levels of estrogenic contamination (Loos et al., 2013). The same year, another study based on the same Irish sampling events was published which analysed effluents for additional PhACs, including DCL (Loos et al., 2013). The study found that throughout Europe, DCL was among the compounds with the highest median concentration levels (it was found in 89% of all samples, max = 174 ng/L, median = 43.3 ng/L). In the Irish samples specifically, DCL was detected at concentrations of 80.3 and 144.3 ng/L for the two investigated WWTPs respectively (Loos et al., 2013).

Another European level study in 2013 used the GWAVA model to predict water concentrations of DCL, E2 and EE2 in rivers throughout Europe. The study recognized that the levels of these compounds found in receiving waters would vary considerably between European nations depending on available dilution of sewage effluent. Overall, the model predicted that 12%, 1% and 2% by length of Europe's rivers would exceed the EE2, E2 and DCL proposed annual average EQSs. For all three compounds however, <10% of Irish river length was predicted to exceed EQSs. There are significant sources of uncertainty in the model that should be noted; as far as we can tell, estimates are based on Northern Irish data only, and the parameters determining effluent concentrations, a critical component of estimating river concentrations, have additional uncertainty (Johnson et al., 2013).

3.3. Relationship with drug utilising dispensing data

Annual dispensing data for each LHO throughout the country is mapped for DCL and for E2 (heat maps and dispensing data presented in Supplementary material. Similar trend emerged where LHOs exhibiting higher dispensing data for DCL also matched locations reported previously for upper concentrations of the same compound using monitoring techniques (Supplementary material). However, this observation should be taken with care considering the low number of monitoring data referenced for DCL in surface waters. This trend was also evident for E2 where mapped baseline drug dispensing data from LHO's exhibiting upper concentrations of this estrogen were similar to locations identified for upper values using monitoring techniques (Fig. 3). While this proxy baseline dispensing data is useful for possibly intimating indicator areas that make disproportionately high contributions to overall pollution load, these “hotspots” may vary spatially and temporality and may occur due a variety of other contributing sources such as discharge points of high-risk WWTPs, high densities of livestock near water sources, effluent from hospitals and residential homes, and/or pharmaceutical producers (Verlicchi et al., 2012; Tiedeken et al., 2017).

3.4. Conclusion and perspectives

The Water Framework Directive (WFD) and Irish River Basin Management Plans (RBMPs) establish both legal and operational frameworks to protect and restore clean water and to ensure its long-term, sustainable use. These goals require an integrated approach to the sustainable management and protection of water resources. Critical shortfalls in existing Irish RBMPs highlight the importance of affordability and prioritization considerations, particularly given the economic and social value of a clean and protected water supply. Therefore, the overall aim of this study was to provide a baseline study for Ireland exploring the implications of the addition of DCL, E2 and EE2 to the WFD priority substances list. This study mapped all national concentration data and concludes that DCL concentrations found in surface waters are generally below the limits proposed by the WFD, but that exceedances have occasionally been reported as it is the case in several European countries. In comparison, E2 and EE2 surface water concentrations are generally much lower, however reported values still commonly exceed the WFD proposed limits for these bioactive compounds. Perhaps most notably, while current standard, laboratory-based analytical chemistry methods are sufficiently sensitive for the detection and quantification of DCL, limits of detection for E2 and EE2 are often higher than proposed EQSs. This issue presents serious analytical challenges in regards to chemical monitoring methods and reporting for these two PhACs, and impacts Ireland's ability to meet European reporting requirements for these estrogenic compounds. The mapping work conducted during this study demonstrated that more monitoring data on DCL, E2 and EE2 in Irish waters is required. Nevertheless, based on the limited Irish data extracted from the literature and mapped in this study, it appears that the majority of Irish surface waters may not exceed WFD proposed limits for DCL, E2 and EE2, but that point sources of pollution could lead to occasional hotspots exceeding European limits. It must be noted that this prediction is based upon the use of very limited data, and is especially uncertain because of a lack of sufficiently sensitive analytical detection methods. This observation will resonate with the majority of EU countries in terms of current levels of CEC monitoring in respective receiving waters and at control points. Conventional analytical methods are sufficiently sensitive for the detection and quantification of DCL, but not for E2 and EE2, thus alternative, ultra-trace, time-integrated monitoring techniques such as passing sampling are needed to inform water quality for these estrogens. Another emerging potential solution to the problem of low EQS values of E2 and EE2 is the use of biological effects monitoring techniques (Streck, 2009; Kunz et al., 2015; Simon et al., 2015).

In comparison to other 28 EU countries along with Switzerland, Norway and Turkey, Ireland produced 21 studies on these three contaminants of emerging concern over 15-year systematic review period (Tiedeken et al., 2017) where Spain, Germany and the United Kingdom contributed 707 (56%) of all reports. However, 24 and 16 EU countries produced under 50 and 20 articles respectively on these PhACs in their national receiving waters; consequently, very few countries have reported on use predicted or measured environmental concentrations to underpin modeling or to inform risks in their river basins (ter Laak et al., 2010; Guillén et al., 2012; Murphy et al., 2017). Overall, this Irish study supported main tenets of Tiedeken et al. (2017) which found that DCL and EE2 enter European aquatic environment mainly following human consumption and excretion of therapeutic drugs, and by incomplete removal from influent at urban wastewater treatment plants. E2 is a natural hormone excreted by humans, which also experiences incomplete removal during WWTP treatments.

Future Irish-specific work in this research field is essential in order to ensure PhACs do not threaten our water supplies. Irish studies evaluating PhACs levels in WWTPs influents and effluents are also lacking; these are needed in order to develop effective and economic control measures for PhAC removal from wastewaters. The present study also found that on-site treatment systems could potentially be major sources of PhACs contamination in an Irish context, thus future research should address this issue. Given the positive results and outcomes from studies that utilise effect-based (biological) monitoring, passive sampling or an integrated monitoring approach (combined use of chemical and biological monitoring methods), thus Ireland along with other EU member-states must consider broader acceptance of these types of methodologies for WFD priority substance reporting. Future projects evaluating concentrations in aquatic and other environmental matrices (sludge, sediment, biota) must be supported, particularly for compounds that are not yet considered priority substances (current and potential future watch list compounds). Furthermore, more data should be collected on prescriptions written and dispensed by public and private health agencies in Ireland, and such data should be made available to research projects. Another issue is the unavailability of commercially sensitive data such as PhAC sales/production information. This information would facilitate a more accurate determination of emission sources in different

catchments. Now that the limitations of these existing monitoring data are understood, additional types of data that can help inform regulators and policy makers about the levels of these substances in Irish waters should be explored. In particular, it would be useful to apply established European models for predicting fate, behaviour and concentrations of chemical pollutants in surface waters in an Irish context including considerations for influence of climate change given that 2015 was the wettest year recorded in Ireland over 250 years of annual measurements (Murphy et al., 2017). To this aim, longer term projects that utilise European software and models to predict fate of watch list compound concentrations in whole Irish watersheds should be carried out such development of spatially explicit Geography-Referenced Regional Exposure Assessment Tool for European River Basins (GREAT-ER).

Acknowledgements

We also acknowledge Environmental Protection Agency STRIVE Programme for funding this research (Project 2014-W-DS-18).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.11.021.

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Science of the Total Environment 874 (2023) 162380

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Current decontamination challenges and potentially complementary solutions to safeguard the vulnerable seafood industry from recalcitrant human norovirus in live shellfish: Quo Vadis?

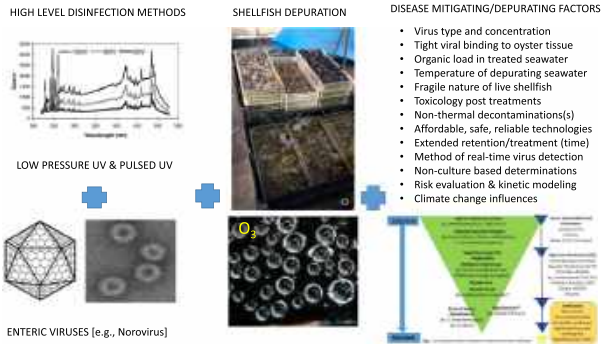
Neil J. Rowan

Centre for Sustainable Disinfection and Sterilization, Bioscience Research Institute, Technological University of the Shannon Midlands Midwest, Athlone Campus, Ireland

HIGHLIGHTS

- Choice of appropriate non-thermal intervention(s) is limited as shellfish are consumed raw.
- Depuration using standard UV-irradiation effectively addresses bacterial contaminants in live shellfish.
- Additional non-thermal mitigation strategies are required to completely destroy norovirus in live oysters.
- New diagnostic method(s) are required to confirm non-viable norovirus post depuration.
- Global warming of coastal growing environments may increase risks for commercial shellfish producers.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
Norovirus
Food security
Sustainability
Decontamination
Shellfish
Diagnostics

ABSTRACT

Safeguarding the seafood industry is important given its contribution to supporting our growing global population. However, shellfish are filter feeders that bioaccumulate microbial contaminants in their tissue from wastewater discharged into the same coastal growing environments leading to significant human disease outbreaks unless appropriately mitigated. Removal or inactivation of enteric viruses is very challenging particularly as human norovirus (hNoV) binds to specific histo-blood ligands in live oyster tissue that are consumed raw or lightly cooked. The regulatory framework that sets out use of clean seawater and UV disinfection is appropriate for bacterial decontamination at the post-harvest land-based depuration (cleaning) stage. However, additional non-thermal technologies are required to eliminate hNoV in live shellfish (particularly oysters) where published genomic studies report that low-pressure UV has limited effectiveness in inactivating hNoV. The use of the standard genomic detection method (ISO 15, 216–1:2017) is not appropriate for assessing the loss of infectious hNoV in treated live shellfish. The use of surrogate viral infectivity methods appear to offer some insight into the loss of hNoV infectiousness in live shellfish during decontamination. This paper reviews the use of existing and potentially other combinational treatment approaches to enhance the removal or inactivation of enteric viruses in live shellfish. The use of alternative and complementary novel diagnostic approaches to discern viable hNoV are discussed. The effectiveness and virological safety of new affordable hNoV intervention(s) require testing and validating at commercial shellfish production in conjunction with laboratory-based research. Appropriate risk management planning should encompass key stakeholders including local government and the wastewater industry. Gaining a mechanistic understanding of the relationship between hNoV response at molecular and structural levels in individually treated oysters as a unit will inform predictive modeling and appropriate treatment technologies. Global warming of coastal growing environments may introduce additional

E-mail address: nrowan@ait.ie.

http://dx.doi.org/10.1016/j.scitotenv.2023.162380
Received 11 January 2023; Received in revised form 16 February 2023; Accepted 17 February 2023
Available online 24 February 2023

contaminant challenges (such as invasive species); thus, underscoring need to develop real-time ecosystem monitoring of growing environments to alert shellfish producers to appropriately mitigate these threats.

Contents

1. Introduction

The seafood industry is an important sector of food production worldwide (Ruiz-Salmön et al., 2020). An estimated 18 million tons of marine molluscs are globally harvested each year with an estimated value of $35 billion, comprising 9 % of the value of fisheries worldwide (Sharp et al., 2021). Shellfish consumption is an increasingly important part of human diet and is an emerging area for economic growth worldwide (Ruiz-Salmön et al., 2020; Ruiz-Salmön et al., 2021; Laso et al., 2022; Cooney et al., 2023). This is particularly relevant given that our growing global population recently reached eight billion people. Shellfish filter hundreds of litres of coastal water for nutrients and can bioaccumulate human pathogens including norovirus from their growing marine environment, if contaminated with faecal material (Compos and Lees, 2014). Human norovirus is found in high concentrations in faeces (10^{11} virus/g) (La Rosa et al., 2012); consequently, this virus is recognized as a high risk for environmental transmission (McLeod et al., 2017: Sharp et al., 2021). Pilotto et al. (2019) reported concentrations of 10^{11} genome copies/per gram (cg/g) of murine norovirus (MNV1) that were achieved during 24 h bioaccumulation in the Pacific oyster *Crassostrea gigas*; whereas, Maalouf et al. (2011) noted that both human norovirus genogroups GI and GII were shown to simultaneously bioaccumulate in *C. gigas* oysters to 10^6 cg/g. Contamination of raw seafood with pathogenic microorganisms occurs through primary production and from infected food handlers (McLeod et al., 2017; Sharp et al., 2021).

In many parts of the world, microbiological pollution of coastal areas with human sewage, and agricultural run-off, can readily occur in commercial harvesting such that shellfish bioaccumulate large amounts of bacterial and/or viral pathogens (Bosch et al., 1995; Winterbourn et al., 2016); thus, requiring to be appropriately cleaned (depurated) (Rupnik et al., 2018; Razafimahefa et al., 2020; Rupnik et al., 2021). The faecal indicator organism *Escherichia coli* is frequently used as a general indicator of sewage contamination and for assessing the effectiveness of shellfish depuration processes (Sharp et al., 2021). These authors reported that non-pathogenic *E. coli,* pathogenic *E. coli* O157:H7 and hNoV GII RNA accumulate rapidly in mussels using simulated water contamination after a point-source release from a combined sewer overflow (CSO) and untreated wastewater released directly into the coastal zone. "All three microbiological indicators reached close to maximum concentration within 3 h of exposure, demonstrating that short CSO discharges pose an immediate threat to shellfish harvesting areas" (Sharp et al., 2021). Depuration in clean seawater proved partially successful at removing pathogenic and non-pathogenic *E. coli* from shellfish tissue, but failed to eradicate hNoV GII RNA. The authors concluded that current EU standards for evaluating microbiological risk in shellfish are inadequate for protecting consumers against exposure to hNoV; thus, intimating a need to improve depuration efficiencies including developing new appropriate mitigation technologies (McLeod et al., 2017; McMenemy et al., 2018).

It is notable that hNoV is the leading viral cause of human gastroenteritis, where consuming contaminated shellfish contributes as a vector in this transmission (Yu et al., 2015). In developed countries, it accounts for 95 % of non-bacterial foodborne outbreaks, and over 50 % of all microbial outbreaks (Dewey-Mattia, 2018; Wikswo et al., 2021). In the US alone, noroviruses are responsible for ~20 million cases and >70,000 hospitalizations of infected children, annually (Smith and Smith, 2019). Oysters are frequently implicated as the source of this human gastroenteritis illness posing a particular risk to young, elderly and immunocompromised (Centers for Disease Control and Prevention (CDC), 2020). While most adults recover from viral diarrhoea, such illness in young children can lead to hospitalization and life-threatening dehydration (Centers for Disease Control and Prevention (CDC), 2020). Notably, contaminated shellfish are estimated to cause between 9 and 34 % of all foodborne norovirus cases in the US (Pouillot et al., 2021), and similar etiological ranges have been reported internationally (Havelaar et al., 2008; Davidson et al., 2011; Advisory Committee on the Microbiological Safety of Food, 2015). No vaccine currently exists that can prevent hNoV infection (Centers for Disease Control and Prevention (CDC), 2020). Best published evidence suggests that oysters are a main source of foodborne hNoV-transmission due to (a) the mode of transmission (as they mainly consumed raw or lightly cooked) (Rupnik et al., 2018); (b) production of oysters in same intertidal waters where human sewage is discharged as a source of hNoV (Rupnik et al., 2018; Sharp et al., 2021); and (c) specific retention of hNoV strains in oysters through binding to ligands enabling lengthy persistence (McLeod et al., 2017).

Oysters are premium food products of economic importance that are consumed globally (McLeod et al., 2017); however; failure to appropriately depurate contaminated shellfish can lead to reputational damage to the exporting industry and a commensurate loss of confidence for importing countries (Younger et al., 2020). Currently, it is technically challenging to confidently address the effective removal or destruction of recalcitrant enteric viruses, particularly from live contaminated shellfish (Leduc et al., 2020; Younger et al., 2020; Rupnik et al., 2021). The Pacific oyster (*C. gigas*) is the most commonly produced oyster globally; but other species are also commercially harvested including *Crassostrea viginica* (the Eastern oyster) in the US, *Saccostrea glomerata* in Australia, and flat oysters (*Ostrea edulis*) that are produced in many countries including Ireland, the United Kingdom, and Croatia (McLeod et al., 2017).

Norovirus is a small (approximately 30 nm in diameter) non-enveloped, single-stranded RNA virus that belongs to the family Caliciviridae (Sharp et al., 2021). Norovirus was named after the original Norwalk strain, which caused an outbreak of gastroenteritis in a school in Norwalk, Ohio in 1968. Noroviruses are now classified into ten genogroups (GI-GX) and 48 genotypes where typically GI and GII predominate (McLeod et al., 2017; Chhabra et al., 2019; Rupnik et al., 2021). While wastewater treatment plants (WWTPs) can contribute to enteric virus removal, it appears that improvements in the effectiveness of technologies or processes are required to fully remove or inactivate enteric viruses from human sewage (Barrett et al., 2016). Data published in a recent EFSA baseline survey highlights that peak hNoV concentration and prevalence in contaminated oysters was observed in the months of January and February when almost 65 % of samples tested were positive for hNoV [mean concentration of 661 genome copies/g] (EFSA, 2019).

Hepatitis A Virus (HAV) is also a small (approximately 30 nm in size), non-enveloped, icosahedron-shaped, RNA enteric virus that contaminates shellfish in polluted seafood leading to human illness (Woods and Burkhardt III, 2010). A vaccine exists for HAV (Centers for Disease Control and Prevention (CDC), 2020). Coincidently, viruses with icosahedral-shaped capsids efficiently package their RNA (Martin-Bravo et al., 2021), which will be discussed later in this review in the context of a focus for real-time detection and as a structural target for decontaminating enteric viruses of similar geometry (such as hNoV and HAV). Notably, researchers have reported on geometric defects and icosahedral viruses that may influence assembly, dissociation, or accessibility of cellular proteins to virion components (Wang et al., 2018). Many complex shellfish pathogens, such as these enteric viruses and waterborne protozoan enteroparasites (such as *Cryptosporidium* oocysts, and *Giardia* cysts), do not grow on standard laboratory based culture media, and require use of more sophisticated enumerations methods post-treatment, such as quantitative PCR (ISO standard) (Garvey et al., 2010; Garvey et al., 2013; Hayes et al., 2013; Gerard et al., 2019; Franssen et al., 2019; Sharp et al., 2021; Rupnik et al., 2021). There are in vitro cell culture methods to study the viability of treated surrogate enteric viruses (Barrett et al., 2016) and for waterborne parasites (Garvey et al., 2014a; Garvey et al., 2014b), which can be combined with the standard qPCR method to inform decontamination. Currently, there is no appropriate *in vitro* model for studying the infectivity of hNoV strains that has hindered development, testing and standardization of treatment approaches for the shellfish industry, particularly at commercial depuration phase (McLeod et al., 2017; Rupnik et al., 2018; Rupnik et al., 2021). Sophisticated diagnostic techniques are not routinely available in standard food-testing laboratories for enteric viruses; for example, hNoV and HAV are classified as belonging to Human Pathogen Hazard Group II viral pathogens necessitating use of more specialized Cat II facilities (Centers for Disease Control and Prevention (CDC), 2020).

These enteric viruses have a low infectious dose and may remain infectious for weeks in the environment or on food surfaces (Nasheri et al., 2021). However, limited physiological or mechanistic information is available regarding viral survival, persistence and transmission in contaminated shellfish. Interestingly, Kokkinos and co-workers (2021) noted that "the vast majority of viral agents, which are transmitted via the faecal-oral route are non-enveloped, highly stable under environmental conditions, characterized by extremely small size, and include emerging and re-emerging Caliciviridae, Adenoviridae, Hepevirdae, Picornaviridae and Reoviridae. The enteric viruses of human stool and urine belong to more than 140 types (Kokkinos et al., 2011) where untreated wastewater has been identified as the most diverse viral metagenome examined thus far. Most sequence reads have little or no sequence relations to known problematic viruses, underscoring that most of the viruses have yet to be characterized", and are underestimated in prevalence (Cantalupo et al., 2011).

Previous researchers have reported that under EU law, sanitary classification of shellfish production areas is recognized based on the presence and concentration of the faecal bacterium *Escherichia coli* as designated EU Regulation 627/2019 (Rupnik et al., 2021; Hunt et al., 2023). Currently, the minimum time and water temperature used for commercial depuration are not stipulated in EU Regulation, however, Rupnik et al. (2021) had noted that such depuration should be performed for a minimum of 42 h with a water temperature of no <8 °C in Ireland. Specifically, shellfish harvesting waters are classified as A, B, or C, which is based on increasing *E. coli* concentrations measured in shellfish flesh and fluid (Hunt et al., 2023). Consequently, within each class, specific post-harvest decontamination methods such as depuration and relaying are mandated before any live product can be sold (Rupnik et al., 2018; Hunt et al., 2023). However, studies have indicated that monitoring faecal indicator bacteria in shellfish may be a poor indicator of water pollution and the risk of human exposure to pathogens from consuming shellfish (Romalde et al., 1994; Younger et al., 2018). Essentially, "bacterial species are traditionally used as indicators of faecal contamination of agricultural products, shellfish and shellfish waters" (Garcia et al., 2020). This led to the aforementioned formulation of legislation based on the measurement of faecal indicator bacteria (EU, 2020). Previous researchers have reported that there is a poor correlation between concentrations of *E. coli* and norovirus, making it less relevant as an indicator organism (Flannery et al., 2009; Lowther et al., 2019; Hunt et al., 2023). Although methods for detection and quantification of problematical pathogens (e.g., *Vibrio* spp.) in shellfish exist, these also have yet to be incorporated into EU legislation due to the lack of robust evidence and non-consensus agreements over what new standards should be incorporated (Hassard et al., 2017; EFSA, 2019; Sharp et al., 2021).

Post-harvest disease mitigation typically occurs in land-based tanks containing clean seawater. Duration of treatment is governed by several biological and environmental factors that are informed by the monitoring of enteric virus concentration (or viral load) (Rupnik et al., 2021). Thus, oysters and other bivalve molluscan shellfish harvested from class B category waters (accounting for the majority of overall oyster production from European countries) (McLeod et al., 2017), must undergo appropriate depuration before human consumption (EFSA, 2019). These regulations have informed the effective decontamination of bacterial-associated illness caused by contaminated oysters; however, despite same, there are numerous reports of enteric viral outbreaks caused by depurated oysters (Rajko-Nenow et al., 2013). For example, Doré et al. (2010) noted that illnesses have also been reported for the consumption of contaminated oysters that were harvested from category A waters where post-harvest decontamination is not mandatory. Consequently, in order to mitigate against the occurrence of enteric viral illness and to avoid the loss of consumer confidence in shellfish products, many commercial producers apply depuration treatment for oysters harvested from category A growing waters as part of their risk management procedures (Rupnik et al., 2018).

The most widely practised post-harvest treatments is depuration, whereby bivalve shellfish undergo self-purification in land-based tanks containing clean seawater (McLeod et al., 2017). However, the effect of depuration is reducing harmful norovirus in live shellfish is less well established compared with treating *E. coli*, particularly at the standard conditions of 48 h at 8 to 15 °C (McLeod et al., 2017; Hunt et al., 2023). The use of viral surrogates is a common approach to studying depuration efficacy such as using F + RNA bacteriophage type I or II (designated FRNA or FRNAPII) where there are established infectivity assays to offset this technical problem for assessing hNoV post treatments using the ISO standard detection method (Polo et al., 2014; Rupnik et al., 2018; Leduc et al., 2020; Rupnik

et al., 2021). "F-specific coliphages (F+ coliphages) are bacteriophages that infect *Escherichia coli* cells possessing F pili. F+ coliphages are classified into FDNA phages (FDNAPHs) or FRNA phages (FRNAPHs), depending on whether their genomes consist of single-stranded DNA or single-stranded RNA, respectively" (Hata et al., 2016). Oysters can bioaccumulate high concentrations of enteric viruses after a few hours (Flannery et al., 2012; Pilotto et al., 2019) which promotes rapid contaminated with potentially several hNoV strains. Optimal reduction of norovirus load was previously reported in the region of 1 $\log_{10}$ (McLeod et al., 2017). Other experiments have reported marginal improvements in norovirus reductions, particularly using elevated seawater temperature (>11 °C) during depuration (Rupnik et al., 2021).

Oysters retain smaller particles, such as hNoV that bind to these particles depending on their isoelectric point (McLeod et al., 2017). However, it is also appreciated that the recognition of hNoV persists for longer periods than bacteria where oysters are depurated due to specific hNoV-ligand mechanisms (McLeod et al., 2017) as also reported in humans (Hutson et al., 2002). This is one of the main reasons for viral persistence in oysters compared to bacteria where contaminated shellfish can retain virus copies for weeks or months after initial exposure; thus, acting as a reservoir for foodborne transmission (Maalouf et al., 2010; Mathijs et al., 2012). For example, hNoV GI.1 strain binds to the midgut and digestive diverticula of Pacific oysters, but not to other tissues (Le Guyader et al., 2012). While hNoV GII strain binds to various tissue types including digestive diverticula, midgut (intestine), gills, mantle, and labial palps, McLeod et al. (2017) has provided a comprehensive review of factors affecting hNoV binding in oysters including seasonal variations where these authors note that published findings support strain-specific variations in hNoV binding patterns. Oysters retain hNoV through specific ligand-binding in tissues that affects selective accumulation and persistence that may help explain their long retention in oysters (as observed in depuration, McLeod et al., 2017). However, there appears to be a lack of published studies on the ability of oysters to specifically bind surrogate viruses that would inform the comparative ability for infectious viral removal or deactivation at depuration. Hunt et al. (2023) advocated the need for new monitoring methods and regulatory regimens for the specific hazard of enteric viruses in oysters to better manage this risk. EFSA (2019) recommends better understanding of the actual risk associated with positive hNoV test results from contaminated production areas for all genogroups. However, Hunt et al. (2023) stated that "where to apply quantitative thresholds remains a core question in addition to what post-harvest or other interventions could be applied to further manage the risk of norovirus contamination in oysters. To determine the best methods for controlling shellfish virus risk is currently a matter of live discussion in the EU".

Consequently, this constitutes the first review to compare the effectiveness of different established and emerging technologies affecting the decontamination of enteric and surrogate viruses in live oysters. It also considers other complementary novel approaches for the real-time detection of viable and infectious hNoV strains to that of using a standard genomic method (RT-qPCR) that may potentially inform their effectiveness and advance studies underpinning the virological safety of shellfish, particularly addressing oysters.

2. Methods for human norovirus removal or destruction in contaminated live shellfish

A review of publications from PubMed and Scopus database over period 1981 to 2023 was used to address this research topic using PRISMA guiding framework. Of the combined keywords, norovirus (n = 7781) and "depuration" (n = 50), 43 publications were deemed eligible based on criteria: on the follow; (a) norovirus detection, persistence, and accumulation in shellfish, (b) decontamination technologies and operational factors affecting effectiveness for live shellfish applications; (c) use of surrogate microorganisms; (d) kinetic inactivation modeling and (e) foodborne transmission outbreaks. Seven publications were excluded for the reason that they addressed post processing of non-living shellfish; use of chlorine and heating in shellfish, food distribution chain and studies on norovirus aerosols in wastewater production plants. Combining "depuration"-based publications with "enteric viruses" (n = 10,305) over the period 1988 to 2023 revealed 22 matching publications where 6 were different to the aforementioned "norovirus" publication list. These addressed treatment of hepatitis A virus (HAV) in mussels using a closed circulatory system, treatment of the surrogates HAV, poliovirus type 1 and coliphage MS2 in shelled clambs, public health implications of viral-contaminated mussels, and development of diagnostic tests. One study was excluded for the reason that it addressed virological control of contaminated ground water that is not the subject of this review.

Decontamination of live shellfish is mainly carried out at the commercial depuration phase post-harvest using clean seawater where in-line or bolt-on solutions must be non-thermal in nature to mitigate against damage to the treated live oysters (Rupnik et al., 2021). "Acceptable post-harvest treatments available to ensure oysters meet the *E. coli* standard include self-purification in land-based tanks containing clean seawater by a process called depuration or relaying bivalve shellfish to clean marine locations for an extended period (four weeks)". Norovirus-related gastroenteritis outbreaks have occurred even when oysters have been demonstrated to be fully compliant with regulatory end-product standards. Therefore, the combination of marine harvest area controls and post-harvest treatments as currently practised does not completely protect consumers from the virological safety risk associated with norovirus-contaminated shellfish (Rupnik et al., 2018). Findings from various researchers would support the idea that shorter, less-intense solar irradiation in the winter (such as in Northern hemisphere countries) may also contribute substantially to the environmental persistence of human noroviruses in shellfish (Younger et al., 2020; Rupnik et al., 2021).

Typically, solutions for live shellfish decontamination must consider inter alia: the volume of live seafood products to be treated; the cellular and molecular mechanism(s) of action of the applied decontamination approach(es) ensuring irreversible inactivation, or removal; the microbial load or initial starting population including composition, such as the type of pathogen(s) present as these frequently differ in behaviour and level of susceptibility to decontamination methods, particularly hNoV (Fig. 1); the amount of decontaminant applied or intensity of removal/disinfection methods; the exposure or treatment time (hours, days); the appropriate inactivation kinetic performance of applied decontamination approaches (log linear, or bi-/tri-phasic adaptive microbial responses); the environmental parameters (seawater depuration temperature, salinity, pH, presence of interfering suspended solids or turbidity), nutritional factors (oysters are consumed raw; thus nutritional and organoleptic characteristics must be maintained post non-thermal treatments); scalability; affordability; the availability of subject-matter technical assistance for equipment operation; operator safety (UV-irradiation), detection and infectivity enumeration methods; and biocompatibility post treatments including environmental (ecotoxicological) compliance. The sequence of susceptibility of treated microorganisms (and surrogates) to applied disinfection technologies, as shown in Fig. 1, is indicative as it is appreciated that the mode of action of biocidal action may differ depending upon the type of treatment methods. For example, specific hNoV strains (such as G1) may generally be more tolerant to a variety of technologies as specifically bind to ligands in oyster tissues that may confer greater protection (Leduc et al., 2020; Rupnik et al., 2021) (Table 1); thus, they are potentially more recalcitrant compared to treating similar hNoV genotypes under less complex planktonic situations as often replicated in laboratories. Waterborne parasites (such as *Cryptosporidium parvum* oocysts or *Giardia lamblia* cysts) appear to exhibit greater tolerance to reactive oxygen species (ROS) associated with advanced oxidative processes (AOP) yet appear to be more susceptible to UV-irradiation (Garvey et al., 2014a; Garvey et al., 2014b). However, combined use of UV/H_2O_2 appears more effective for microbial disinfection compared to separately using the treatment technologies.

Effective post-harvest processing technologies that are recognized by regulators (such as the US FDA) have had limited acceptance in the domestic industry due to the high initial capital equipment costs and the

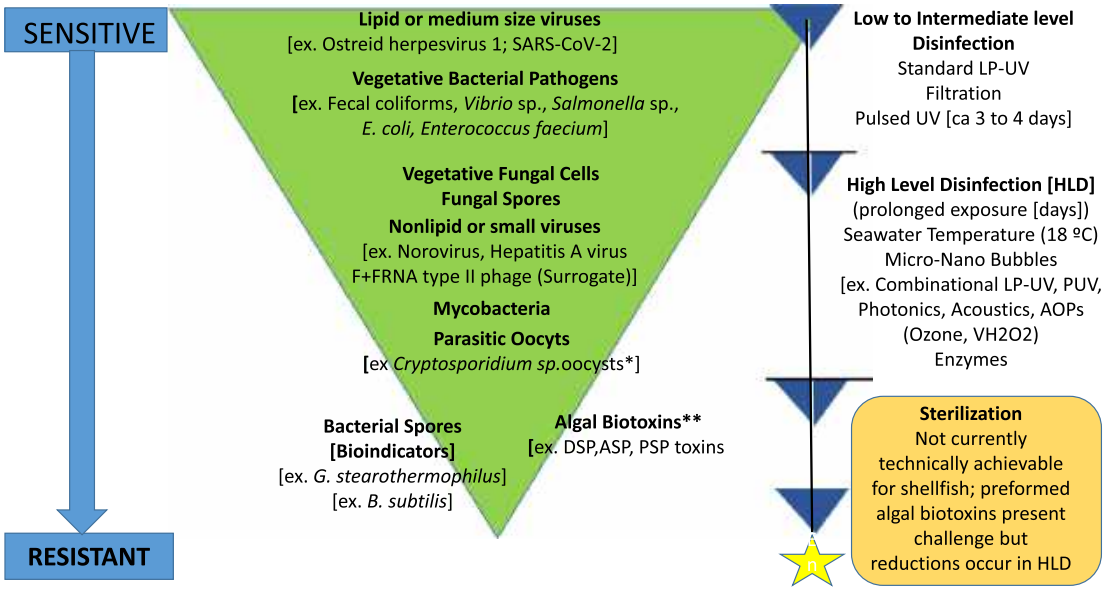


Fig. 1. Increasing microbial resistance to decontamination methods.

economics of transporting and storing shell oysters. While advances have been made to try to standardize or harmonize non-thermal disinfection technologies for food-based processes including packaging (Gómez-López et al., 2022), there is a marked knowledge gap in the development and reporting of appropriate treatment technologies (used alone or combined) for commercial-scale seafood applications, particularly, for technologies or approaches that can address complex challenges including enteric viruses, parasitic oocysts, algal toxins and other potential contaminants of concern (McLeod et al., 2017; Fehrenbach et al., 2022). However, the majority of physical and chemical disinfection technologies used in food and adjacent medical device processing industries would not be deemed appropriate for live shellfish decontamination. For example, researchers have highlighted similar challenges to effectively process and sterilize heat-sensitive medical devices containing complex materials; however, such Medtech-approved chemical disinfectants (such as glutaraldehyde), and terminal sterilization modalities (gamma, x-ray, electron beam, ethylene oxide are not appropriate for use in seafood depuration due to killing of live shellfish, cost of equipment, and general non-practicalities. However, the use of vaporized hydrogen peroxide by the medical device industry may have a future role in commercial shellfish decontamination. In addition, there is a pressing commensurate need to establish the safety of new technologies including their environmental impact. For example, Hayes et al. (2013) reported that pulsed-plasma gas-discharge (PPGD) in oxygen-sparged water generated a range of short-lived highly-oxidative biocidal properties killing enteric bacterial pathogens and recalcitrant waterborne parasitic oocysts. This PPGD technology has been applied for contact food surface decontamination studies (Rowan et al., 2007, However, despite demonstrating biocidal efficacy, this PPGD system was reported to produce unwanted toxicological endpoints post treatments making it unsuitable for the intended use (Hayes et al., 2013). This is also relevant given scalability issues surrounding the transition from early discovery (lab-based) to full commercial testing of technologies at depuration where there are limited published studies addressing shellfish decontamination. Such an innovative disease mitigation topic is underappreciated when considering the combined technological (TRL), societal (SRL) and policy readiness level (PRL) framework for evaluating new decontamination approaches ranging from discovery to full commercial deployment (as exemplified by Rowan and Casey, 2021), particularly for safely and affordably treating live shellfish. Given the amount of key governing factors, complexity of data generated and the need to achieve real time outcomes for effective decision-making (Naughton et al., 2020), potential practical disease mitigation solutions will be informed, supported and enabled by digital technologies (Rowan, 2022; Rowan et al., 2022).

2.1. Using conventional low-pressure UV irradiation and filtration methods

Reference to the use of UV has appeared in 198,506 journals since 1946 to present day (combined PubMed and Scopus), where 117 journals combine UV with norovirus. A total of 48 papers were excluded from consideration in this review as they focused on the combined use of UV with other approaches (such as heating, H_2O_2 generating wipes, peracetic acid, TiO2) for treating strawberries, onions, and lettuce or treating reclaimed water a golf course, simulating vomiting, SARS-CoV-2 (COVID-19) applications, therapeutic applications for intestinal microbiome, solar radiation, electrochemical paper-based devices, and chlorination

UV processes have been developed for food and water disinfection applications and have been divided into three main categories (a) low pressure/low intensity lamps (b) low pressure/high intensity (c) medium pressure/high intensity lamps (Fitzhenry et al., 2021). Barrett et al. (2016) noted that these UV systems vary with respect to operating pressure and output level; for example, it is low and medium pressure assets that are deployed for treating wastewater. Also, turbidity and suspended solids can affect UV disinfection efficiency by decreasing transmissivity (i.e., the transmission of UV light through the water body) (Barrett et al., 2016). Yet, these factors are not routinely considered when monitoring UV performance. UV light technologies have supported the effective treatment of drinking water for decades. Low pressure (LP) UV disinfection results in photochemical damage to viral RNA thus inhibiting viral reproduction (Fitzhenry et al., 2021). Advances have been recently made in the reduction of norovirus in contaminated oysters at the depuration phase using filtration and LP-UV (Rupnik et al., 2018; Leduc et al., 2020; Younger et al., 2020; Rupnik et al., 2021) (Table 1).

Previous researchers have reported that depuration time and seawater temperature are parameters that may influence human viral decontamination efficacy (Lees and Cen, 2010; Rupnik et al., 2018), but these parameters are not currently stipulated in EU regulation (Rupnik et al., 2021). Rupnik et al. (2021) reported that laboratory-based studies revealed up to 74 % of initial norovirus GII concentrations were achieved after three days of 17–21 °C [designated 'high' depuration temperature], and after 11 °C to 15 °C [designated 'medium' depuration temperature], compared to 44 % reductions at 7 °C to 9 °C [designated 'low' depuration temperature] where artificial seawater (mimicking water salinity of estuary) was treated

Table 1
Examples of the application of LP-UV or Pulsed UV irradiation for the decontamination of norovirus with relevance to shellfish.

Target	Source	Treatment	Method	Characteristics	Duration	Pre-depuration	Post-depuration Log10 reduction		Reference
NoV GI	WWTP effluent	LP-UV	RT-qPCR*	Flow through 60s, 120 S	24 h	10^6/100 mL	0.09 ± 0.04 to 0.52 ± 0.06 [60s]	0.15 ± 0.12 to 0.7 ± 0.6 [120 s]	Barrett et al. (2016)
NoV GII	WWTP effluent	LP-UV	RT-qPCR*	Flow through 60s, I20 S	24 h	10^6/100 mL	−0.11 ± 0.55 to 0.4 ± 0.5 [60s]	−0.11 ± 0.65 to 0.7 ± 0.1 [120 s]	Barrett et al. (2016)
FRNAPH	WWTP effluent	LP-UV	RT-qPCR	Flow through 60s; 120 s	24 h	106/100 mL	−0.43 to 1.07 ± 0.27 [60s]	−1.27 ± 2.31 to 1.1 ± 0.2 [120 s]	Barrett et al. (2016)
FRNAPH	WWTP effluent	LP-UV 117 mJ/cm^2	Plaque Assay	HRT (0.23 m^3/h	24 h	10^5 PFU/mL	Ca 2 log		Barrett et al. (2016)
FRNAPH	WWTP effluent	LP-UV 51.6 mJ/cm^2	Plaque Assay	HRT (0.52 m^3/h	24 h	10^5 PFU/mL	Ca 2 log		Barrett et al. (2016)
FRNAPH	WWTP effluent	LP-UV 31.6 mJ/cm^2	Plague Assay	HRT (0.85 m^3/h	24 h	105 PFU/mL	Ca 1.5 log		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 3.4 J/cm^2	Plague Assay		24 h	10^7 PFU/mL	1.24 to 2.21		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 6.9 J/cm^2	Plague Assay		24 h	10^7 PFU/mL	1.4 to 3.14 ± 0.03		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 1.2 J/cm^2	Plague Assay	120 s [900 V, 1 pps]	24 h	10^7 PFU/mL	1.3		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 0,55 J/cm^2	Plague Assay	120 s [600 V, 1 pps]	24 h	10^7 PFU/mL	1.0		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 0.14 J/cm^2	Plague Assay	HRT 120 s [300 V, 1 pps]	24 h	10^7 PFU/mL	0.4		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 3.4 J/cm2	Plague Assay	60s, SS (57.5 mg/L); ToC 8 mg/L; TIC 24 mg/L	24 h	10^7 PFU/mL	2.2 ± 0.2		Barrett et al. (2016)
FRNAPH	WWTP effluent	PUV 6.9 J/cm2	Plague Assay	120 s, SS (57.5 mg/l); ToC 8 mg/l; TIC 24 mg/L	24 h	10^7 PFU/mL	3.1 ± 0.2		Barrett et al. (2016)
FRNAPH	Unfiltered WWTP effluent	PUV 3.4 J/cm2	Plague Assay	60s	24 h	10^6 PFU/mL	2.3 (Site 1)	Barrett et al. (2016)	Barrett et al. (2016)
FRNAPH	Unfiltered WWTP effluent	PUV 6.9 J/cm2	Plague Assay	90s	24 h	10^6 PFU/mL	<1 PFU/ml (6 log) Site 1)	Barrett et al. (2016)	Barrett et al. (2016)
NoV GI	*G. gigas*	Not stated	RT-PCR [LoQ 100 gcg^{-1}]	Commercial Production Site A 12–16 °C	7 d	251 gcg^{-1}	121 (0.4 log or 67.5 % reduction)		Rupnik et al., 2021.
NoV GI	*G. gigas*	Not stated	RT-PCR [LoQ 100 gcg^{-1}]	Commercial Production Site B 18 °C	3 d	241 gcg^{-1}	129 (0.4 log or 66.4 % reduction)		Rupnik et al., 2021.
NoV GII	*C. gigas*	Not Stated	RT-PCR [LoQ 100 gcg^{-1}]	Commercial Production Site A 12–16 °C	7 d	281 gcg^{-1}	45 (0.79 log or 83.95 % reduction)		Rupnik et al., 2021.
NoV II	*C. gigas*	Not Stated	RT-PCR [LoQ 100 gcg^{-1}]	Commercial Production Site B 18 °C	3 d	526 gcg^{-1}	66 (0.90. log or 87.47 reduction)		Rupnik et al., 2021.
NoV GII	C.gigas	LP-UV (3 W) No fluence	RT-PCR [LoQ 100 gcg^{-1}]	Lab; D.O. 80 % **8 °C**, 2000 L/h	1–7 d	290 gcg^{-1} [example]	310 gcg^{-1} (d1); 284 (d2); 142 (d3); <LoQ (d4); 249 (d5); 147 (d6); 225 (d7)		Rupnik et al., 2021
NoV GII	C.gigas	LP-UV (3 W) No fluence	RT-PCR [LoQ 100 gcg^{-1}]	Lab; D.O. 80 % **12 °C**, 2000 L/h	1–7 d	452 gc/g$^-$ (example)	143 gcg^{-1} (d1); 231 (d2); 202 (d3); < LoQ (d4); <219 (d5);181 (d6); <LoQ (d7)		Rupnik et al., 2021
NoV GII	C.gigas	LP-UV (3 W) No fluence	RT-PCR	Lab; D.O. 80 % **18 °C**, 2000 L/h	1–7 d	405 gc/g$^-$	236 (d1); 147 (d2); <LoQ (d3); <LoQ d4) < LoQ (d5) < LoQ (d6); <LoQ (d7)		Rupnik et al., 2021
NoV GII	C.gigas	LP-UV (3 W) No fluence	RT-PCR [LoQ 100 gcg^{-1}]	Lab; D.O. 80 % **20 °C**, 2000 L/h	1–7 d	290 gcg^{-1} [example <1000 gc/g	165 (d1); <LoQ (d2); <LoQ (d3); <LoQ (d4); <LoQ (d5); <LoQ (d6); <LoQ (d7)		Rupnik et al., 2021
NoV GII	C.gigas	LP-UV (3 W) No fluence	RT-PCR [LoQ 100 gcg^{-1}]	Lab; D.O. 80 % **20 °C**, 2000 L/h	1–7 d	5573 gcg^{-1} [example >1000 gc/g]	4670 (d1); 5896 (d2); 2296 (d3); 2414 (d4); 1769 (d5); 1513 (d6); 2054 (d7)		Rupnik et al., 2021
NoV GI	Oyster digestive tissue	LP-UV (80 to 90 mJ/cm^2)	RT-qPCR	10 °C	1 to 43 d	293 gcg^{-1}	47.8 days (Time for 1 log reduction in genomic material)		Leduc et al., 2020
NoV GI	Oyster digestive tissue	LP-UV (80 to 90 mJ/cm^2)	RT-qPCR	10 °C	1 to 43 d	802 gcg^{-1}	26.7 days (Time for 1 log reduction in genomic material)		Leduc et al., 2020
FRNAPH	Oyster	LP-UV (80 to 90 mJ/cm^2)	RT-qPCR	10 °C	1 to 43 d	463 gcg^{-1}	43.9 days (Time for 1 log reduction in genomic material)		Leduc et al., 2020
NoV GI	Oyster	LP-UV (80 to 90 mJ/cm^2)	**ICC**-qRT-PCR	10 °C	1 to 43 d	463 gcg^{-1}	26.7 days (Time for 1 log reduction in infectious particles)		Leduc et al., 2020

Low Pressure UV (LPUV); Pulsed UV (PUV); Suspended Solids (SS); Total Organic Carbon (TOC), Total Inorganic Carbon (TIC), Waste Water Treatment Plant (WWTP).

with a 36 W UV-C lamp (fluence was not reported) (Table 1). Rupnik et al. (2021) determined norovirus GI and GII concentrations in contaminated oysters using standardized quantitative real-time reverse transcription PCR (RT-qPCR) in accordance with ISO, 2017. Seawater was circulated and maintained within 1 °C of the target depuration temperature and bar sprinklers were deployed for aeration where dissolved oxygen levels were kept in excess of 80 % in all trials. "Oysters were analysed for the presence of norovirus GI and GII before depuration where norovirus GII concentrations in environmentally contaminated oysters ranged from 178 to 16,426 norovirus gc/g (noting, the limit for quantification [LOQ] and limit of detection [LOD] for Nov GII were ≤100 genome copies/g and 20 genome copies/g respectively)" (Rupnik et al., 2021). Results showed that heavily initial norovirus GII concentrations (≥850 genome copies/g) remained above 300 genome copies/g in oysters irrespective of depuration water temperature after seven days of treatment (Rupnik et al., 2021). For example, samples were reduced from 1739 to 1248 norovirus GI concentration (genome copies/g) at 14 °C [medium temperature]; and from 5573 to 2054 norovirus GII concentration (genome copies/g) at 20 °C [high temperature], after seven days depuration. The authors noted that the ability to reduce hNoV concentration in oysters to <LOQ (100 genome copies/g, or 2

log hNoV g^{-1} potentially remaining) using this LP-UV treatment method differed when contaminated below or above 1000 genome copies/g (or 4 log hNoV g^{-1} present).

These important findings have potentially profound implications for determining the choice of disinfection technology for complementary or alternative use to LP-UV to improve human NoV removal or inactivation efficiencies in oysters that can be informed by viral kinetic inactivation modeling data (Rowan et al., 2015). In addition, the potential use of viral monitoring and screening approach for contaminated oysters (≤1000 NoV genome copies/g) may inform suitability for achieving appropriate levels of disinfection treatment based on current disinfection methods at depuration, or for relaying to clean seawater for four weeks (>1000 genome copies/g) from a risk-based assessment and virological safety perspective. However, the RT-qPCR detection method was solely used by Rupnik et al. (2021) where there is no infectivity assay for NoV; therefore, it is uncertain if the genomic copies of NoV per gram detected in oyster post these LP-UV treatments were viable where the use of ISO 15216-1 standard may underestimate the level of NoV lethality or removal achieved. It is appreciated that the combinational laboratory-commercial decontamination study of Rupnik et al. (2021) has made a significant contribution to advancing this technical NoV challenge in oysters. This is timely given that the EFSA has published a baseline survey on comprehensive scientific data for norovirus prevalence in European oyster harvesting areas that will inform future safety limits on norovirus concentration in oysters.

Rupnik et al. (2021) also noted that norovirus reductions were also assessed in two Irish commercial depuration systems that are routinely used to produce oysters. The authors reported up to 68 % reduction for hNoV GI and up to 90 % for hNoV GII reduction. This finding also supports the general observation by other researchers that intimates hNoV GI exhibits greater tolerance or persistence over hNoV GII strain possibly due to the specific behaviour of ligand binding in oyster tissue (Leduc et al., 2020; Younger et al., 2020). Additionally, other researchers have also deployed standard UV decontamination for removing norovirus and FRNAPII from oysters during depuration using genome and infectivity assays (Leduc et al., 2020; Younger et al., 2020). Specifically, these studies intimate that standard UV irradiation of seawater under aerated depuration circulatory conditions either inactivates human norovirus GI and GII genogroups (Leduc et al., 2020 [UV dose, 80 to 90 mJ/cm^2], or, destroys and removes this enteric virus (Younger et al., 2020 [2 × 25 W lamps, no UV dose was reported]); thus, highlighting that greater studies are required to understand the mechanism of viral reduction and residual potential to cause infection at low concentrations (Table 1). Younger et al. (2020) found approximately 46 % removal of hNoV GII at 18 °C after two days and 60 % after five days compared with a maximum of 16 % hNoV GI removal. These researchers noted that "twice the rate of NoV GII removal was achieved at 18°C compared with 8°C after five days. Younger et al. (2020) also found that FRNAP-II was more readily removed than noroviruses. Notably, no significant difference was found between the rate of removal (as measured by RT-qPCR) and inactivation (as measured by bioassay) of FRNAPII". Younger et al. (2020) inferred from their results that the reduction in FRNAPII may be primarily due to physical removal (or destruction) rather than in situ inactivation of the virus. Also, the efficacy of RT-qPCR method to confirm the viability of the remaining norovirus post treatments remains questionable given that this molecular approach does not distinguish between live or dead viruses. Leduc et al. (2020) reported that FRNAPII infectivity bioassay (presence of viable phage) may be appropriate for informing disinfection effectiveness of viable hNoV given that a 1 log reduction in FRNAPII infectivity occurred after 20.6 days treatment using LP-UV (80 to 90 mJ/cm^2) compared to its genome (43.9 days) and NoV GI genome (47.8 days). Leduc et al. (2020) reported that "FRNAPII and NoV genomes may display similar behaviours with low kinetic removal from the oysters under all purification conditions tested". In terms of surrogate representation of hNoV, Lowther et al. (2019) reported that "both viruses were in high concentrations in outbreak-related samples and that infectious FRNAPII were detected in all outbreak samples (n = 9)". Leduc et al. (2020) and Lowther et al. (2019) suggest combining RT-qPCR testing with a test for infectious FRANPII detection in order to improve the hNoV risk assessment in shellfish. Leduc et al. (2020) also highlighted the importance of developing appropriate testing to identify new strategies for the effective elimination of hNoV, as addressed in this review. FRNAPII bacteriophages have been studied for shellfish decontamination due to their structural similarity to waterborne viruses and proof of faecal pollution coming from urban areas (Leduc et al., 2020).

Recent results underscore the importance of determining the typical range of norovirus prevalence and concentration in contaminated oysters to deploy appropriate decontamination methods. However, the best practice would be 'a multi-actor approach to strategically manage this problem by working with local government authorities and the water industry to reduce the occurrence of human enteric viruses in sewage effluent that may be discharged into the same intertidal growing areas where commercial oyster production occurs. Thus, placing greater emphasis on disease prevention, rather than relying on introducing complex removal strategies at the depuration phase for these enteric viruses in live oysters where there are currently limited appropriate solutions.

Jeong et al. (2021) also reported that after 60 h of depuration equipped with a standard UV light source *Vibrio vulnficus* cell numbers were reduced by <4.0 log MPN/g in Pacific oyster tissue from an initial population of ca. 8 log MPN/g. Lee (2020) reported that the use of standard UV for treating seawater in depuration tanks could extend the shelf life (two to three days) of raw oysters with minimal changes in food quality with faecal coliforms maintained at or below 2 log/g compared to non-depurated and generally packaged oysters. Depuration at temperatures between 7 °C and 15 °C using UV-treated seawater reduced *V. parahaemolyticus* populations in oysters by >3 log MPN/g after five days with no loss of live oysters (Phuvasate et al., 2012). The US National Shellfish Sanitation Program established time/temperature regulations that limit maximum hours of holding shellfish from harvest to refrigeration (≤10 °C) to reduce the risk of infections from *Vibro* spp. associated with shellfish consumption (Reid and Durance, 2000).

2.2. Pulsed light (PL) technology

Only 6 of 4239 "Pulsed UV" and "Pulsed Light" publications [2010 to 2023] have focused on treating norovirus or enteric viruses when assessing PubMed and Scopus databases. One study was excluded for the reason that it focused on PPE decontamination. Ten publications were also included that provided supporting context to background technology. Pulsed light (PL) technology is an exciting approach that delivers ultra-short bursts of broad spectrum (200 nm to 1100 nm light) (Rowan, 2019) and is commercially deployed by companies such as Claranor (France, https://www.claranor.com/en/) for food packaging sterilization with over 500 units installed in 52 countries. The benefits of using PUV reflect the potentially ultra-short disinfection against viruses, waterborne protozoan parasite oocysts (*Cryptosporidium parvum*) (Garvey et al., 2014a, 2014b) and cysts (*Giardia lamblia*) (Garvey et al., 2014b), and biological endospore indicators using laboratory-based static or limited flow-through treatment configurations (Garvey et al., 2010; Rowan, 2011; Hayes et al., 2012; Garvey et al., 2013; Garvey and Rowan, 2015). PL has been referred to as high-intensity pulsed UV light (HIPL), pulsed UV (PUV), high-intensity broad spectrum UV light (BSPL), intense light pulsed (ILP) and pulsed white light (PWL) (Rowan et al., 1999; Rowan, 2019). PL has been approved by the US FDA in the production, processing and handling of foods since 1996 up to cumulative UV dose (or fluence) of 12 J cm^{-2} where emission spectra are to be kept between 200 and 1100 nm and pulsed duration ≤2 ms (Rowan, 2019). The technological principle of PL disinfection is based upon the accumulation of high discharge voltage in a capacitor where the stored energy is delivered in ultra-short pulses through a light source filled with xenon gas. The xenon-light source emits a broad spectrum light flash typically in the range of ca. 200 to 1100 nm with approximately 25 % in the UV range (Gómez-López et al., 2022). PL disinfection efficiency is higher compared with continuous-wave low-pressure UV irradiation (CW-UV) due to its high peak power along with the ability to deliver stored

energy over short durations, typically 1 to 10 pulses per second (Garvey and Rowan, 2019). The main reporting parameters governing effective PL operation for disinfection are the fluence [J cm^{-2}], exposure time [s], number of pulses applied [*n*], pulse width [τ], frequency [Hz], and the peak power [W] (Rowan et al., 2015; Gómez-López et al., 2022). Garvey et al. (2015) reported on a satisfactory ecological assessment of pulsed UV light treated water containing microbial species and *Cryptosporidium parvum* using a microbiotest test battery.

Very limited studies have been conducted on the inactivation of pulsed light for inactivating enteric viruses that have focused on bench-scale applications (Barrett et al., 2016). Barrett et al. (2016) compared the efficiency of pulsed light irradiation and low-pressure UV irradiation as a means of hNoV and FRNA bacteriophage using secondary treated wastewater effluent. While hNoV GI and GII inactivation could not be determined, it was found that a maximum UV dose of 6.9 J/cm^2 (at a hydraulic residence time of 120 s in a flow-through system) achieved 2.4 log $_{10}$ reduction of FRNA bacteriophage, which indicates the need for high pulsed UV doses to fully remove NoV (Table 1). Vimont et al. (2015) reduced murine NoV using pulsed UV by 3 log_{10} in <3 s (fluence, 3.45 J/cm^{-2}), while Jubinville et al. (2022) reported on the efficacy of pulsed UV against HAV on berries. Three studies have discussed the potential of using pulsed UV for enteric-virus decontamination on foods or for environmental applications (Jean et al., 2011; Pexara and Govaris, 2020).

All pulsed UV dosage rates related to wavelengths <300 nm and the average initial FRNAPII concentration was 10^6 PFU/mL (Barrett et al., 2016). The authors also found that increasing concentration of suspended solids impacted PUV disinfection efficiency. The use of LP-UV reduced FRNAPII phage by ca. 2 log_{10} using a significantly reduced UV dose of 31 mJ/cm^2; however, the combined use of LP-UV and pulsed light for enteric virus reductions were not considered. Results also indicate that absorption of viral particles to solids in wastewater occurs; therefore, it would be prudent to also introduce a barrier process such as a tangential flow filtration system that filters the particulate matter and the flow through when using UV-irradiation. Interestingly, PUV was capable of achieving up to 3 log_{10} reduction in FRNAPII infectivity within 24 h treatment under varying conditions (such as flow rates [120 s], total suspended solids [57.5 mg/L]) in unfiltered secondary wastewater effluent with enhanced destruction (up to 6 log_{10} PFU/ml) achieved in filtered secondary wastewater effluent (Table 1). This finding contrasts with other previously reported LP-UV studies that required up from 3 to 47 days to achieve a l log reduction in NoV and FRANPII in shellfish during depuration (Table 1). Thus, there is potential to augment enteric viral destruction in contaminated shellfish using a combinational LP-UV and PUV approach. PUV offers the benefits of delivering a broad high-intensity light spectrum that includes wavelengths in the blue light spectrum (such as 406 nm) that has been previously reported to generate hydroxyl radicals from oxygen in water via an advanced oxidation process (AOP) (Kingsley et al., 2018). Barrett et al. (2016) also noted that as RT-qPCR provided inconsistent results; therefore it was deemed not an appropriate method for assessing the inactivation of NoV and FRNAPII via pulsed light after treatments. Other researchers also observed this discrepancy (Pecson et al., 2011). Baert et al. (2009) found RT-qPCR results were unable to distinguish between infectious and non-infectious NoV using murine norovirus (MNV) as surrogate post-heat treatments. Uslu et al. (2016) employed PUV as a wastewater disinfection tool and indicated that in addition to pathogen removal/inactivation, it also reduces the organic load of municipal wastewater effluent by reducing chemical oxygen demand and total organic carbon.

However, Fitzhenry et al. (2021) recently reported that LP-UV is superior to that of using pulsed light for converting energy through the light source to UV dose for submerged wastewater-treatments. Thus, advances in PUV design is required (such as improving light source including the use of light emitting diodes, introducing more light sources such as in parallel; reflective surfaces for light scattering, and inclusion of smart materials that includes photocatalysis of TiO_2 for localized ROS generation) in order to realize the dual benefits of combining PUV (broad spectrum) and fixed-wavelength LP-UV for improved decontamination effectiveness. The authors report that a pulsed UV system output of 2052 mJ/m^{2-} (energy below 300 nm) was required for a 2 log inactivation of *Bacillus pumilus*, where low a lower LPUV system output of 12 mJ/cm^2 produced a similar level of inactivation in flow-through water systems. Complete inactivation of *B. pumilus* was achieved via LP-UV disinfection using a UV output of 30 mJ/cm^2. Fitzhenry et al. (2021) reported that "while a typical xenon gas PUV system is comprised of light emissions within the broad spectrum range of UV, and infrared light, it may be an important consideration to prioritize UV dose/output calculations in terms of 'biocidal PUV dose/out' (i.e., the energy applied from wavelengths below 300 nm ahead of the 'total PUV dose/output', which infers the total energy applied across the whole spectrum output). This has been previously demonstrated in a number of studies with the aid of spectrometer/pyroelectric detectors and in some cases the UV dose/output from PUV systems in within the same order of magnitude as LPUV dose/outputs such as 1 – 100 mJ/$m^{2"}$. However, measurements from Fitzhenry et al. (2021) indicated that only 26 % of the lamp energy reached the same sample (at 900 V and a distance of 10.75 cm), and of that, only 8 % was within the UV wavelength range.

Interestingly, the PUV and flow-through treatment configuration used by Fitzhenry et al. (2021) is the same system that was used by Barrett et al. (2016) to inactivate NoV GI and GII and FRNAPII phage with moderate success (such as 2.4 log reduction with 6.8 J/cm^2) (Table 1). This outcome could be improved by replacing the xenon source with a specific wavelength light-emitting diode (LED). For example, Wen et al. (2022) report UV-LEDs are safe algicidal technologies for inactivating the marine microalgae *Tetraselmis* sp. These authors showed that the wavelength of 265 nm exhibited maximum inactivation efficiency, whereas 285 nm achieved optimal energy efficiency. UV irradiation is also affected by turbidity where viruses can be protected on particles, which must also be considered at the depuration stage, particularly in developing countries where water quality per se may not be appropriate for depuration.

Garvey and Rowan (2015) reported a reduction of 4.23 log_{10} in *Bacillus megaterium* vegetative cells using a UV dose of 6.48 μJ/cm^2 with no significant further microbial reduction after doubling the UV dose to 12.96um in a flow-through PUV system at a retention time of 60s and flow rate of 30 L/h. However, only a 1.48 log_{10} and 1.43 log_{10} a reduction occurred of *B. megaterium* and *B. cereus* endospores respectively at 12.98 μJ/L (RT 60s), indicating reduced efficacy of PUV in treating recalcitrant pathogens in submerged flow-through treatment configuration. The presence of inorganic contaminants did not significantly reduce PUV efficacy at the concentrations used.

2.3. Potential combined use of advanced oxidative processes (AOPs)

Of the 1764 published studies focusing on advanced oxidation processes or "AOPs" appearing in PubMed and Scopus databases over period 1981 to 2023, only 3 addressed norovirus. The reason for including AOPs in this review reflected the emergence of AOPs as non-thermal approaches to improve and enable decontamination performances for food treatments and for environmental applications. One was excluded for the reason that it was COVID-19 orientated. However, there is an increased sharing of knowledge on the potential for using advanced oxidation processes (or AOPs) for enteric viral destruction. This aligns with the need for new or more efficient methods that will destroy NoV in shellfish (Gerba et al., 2018; Kokkinos et al., 2021). AOPs relay on the in situ formation of chemical oxidants to disinfect liquids and degrade diverse harmful organic contaminants (Shabat-Hadas et al., 2017). Kokkinos et al. (2021) noted that "AOPs are, in practice, redox technologies that encompass different processes such as ozonation, ozonation coupled with hydrogen peroxide, and/or UV radiation, Fenton and alike reactions, photocatalysis activated by semiconductors, sonolysis, electrochemical oxidation, and various combinations of these." They are based on the generation of highly reactive oxygen species (ROS), characterized by the non-selectivity of the target and can be deployed before or after treatment of a biological process (Galeano et al., 2019).

The principle oxidizing agent is the hydroxyl radical; however, other ROS may be produced including hydroperoxyl radicals and superoxide radical anions (Shabat-Hadas et al., 2017). Kokkinos et al. (2021) noted that photo-Fenton AOP in which hydroxyl radicals are produced from light, iron and H_2O_2 is a well-studied, environmentally-friendly, simple, low-cost process that inactivates complex or resistant microorganisms (Giannakis et al., 2017). Enteric viruses and a wide range of recalcitrant microorganisms are inactivated through the action of ROS such as singlet and triplet oxygen, anion-radical superoxide, hydroxyl and hydroperoxyl radical, and H_2O_2. ROS are recognized oxidants for inactivating a variety of molecules including proteins, lipids and nucleic acids. "When considering the lethal action of ROS on treated enteric viral nucleic acids, this AOP can change the nucleotides, break important phosphodiester bonds, enhance the formation of pyrimidine dimers, change the tri-dimensional structure, and affect RNA replication" (Kokkinos et al., 2021). Recently, photo-Fenton and alike processes have been developed as a 'green' alternative to chemical disinfection (such as the use of chlorine) for water and wastewater applications (Giannakis, 2018). Evaluation of the disinfection efficacy using light-mediated AOPs has been based on using varying methods such as computational fluid dynamics, chemical actinometry or biodosimetry (Shabat-Habas et al., 2017). An understanding of these assessment methods is critical to informing the efficacy of shellfish decontamination processes from a standardization, reliability and repeatability perspective (as per Rowan, 2019).

The utility of UV technologies has been enhanced through the combinational use of AOPs over the last few decades (Timchak and Gitis, 2012); particularly as an emerging high-efficiency technique for disinfecting enteric viruses leaving no unwanted disinfection by-products (Chu et al., 2012). Microbial inactivation is achieved through UV-induced photochemical reactions on genetic material (Rowan, 2019). Endogenous (direct) inactivation encompasses the absorbance of UVB light by the treated viral genome that causes its degradation. While the full antiviral mechanistic process has yet to be fully elucidated at molecular or structural levels, it is appreciated that UVC/UVB are strongly absorbed by enteric viral RNA with additional decontamination effects. "UVA cannot damage RNA and has no direct photochemical reactions; but it can produce reactive intermediates such as ROS (such as hydroxyl and superoxide radicals, hydrogen peroxide and so forth), which in turn can damage critical microbial targets (such as proteins, nucleic acids)" (Kokkinos et al., 2021). Thus, the role of using an optimised pulsed light technology that produces an intense broad spectrum (200 nm to 1100 nm) may potentially enhance AOP efficacy for enteric virus disinfection. Interestingly, compared to DNA, RNA is known to be more susceptible to the lethal action of UV irradiation (Galeano et al., 2019). The UVB/UVA and visible light wavelengths are absorbed by different water sensitizers through exogenous (or indirect) disinfection processes, such as organic matter, nitrate, and iron-containing complexes. Thus, the type of virus, the suspension menstruum (including suspended solids) and conditions (such as pH, temperature) are important governing factors affecting disinfection efficacy for MS-2 virus (Kosel et al., 2017).

Various AOP approaches have been studied including combined UV with H_2O_2 for treating complex wastewater such as in the meat processing industry (Yapıcıoğlu, 2018). Another technique potentially applicable for the treatment of circulating water in the depuration tanks is pulsed-plasma gas-discharge. Such an AOP produces hydrodynamic cavitation that causes viral disinfection through photochemical (production of hydroxyl radicals), and physical mechanisms (pressure gradients, shock and acoustic waves, shear forces, and very high local temperatures) (Kosel et al., 2017). The mechanisms of hydrodynamic cavitation have yet to be elucidated, but it is theorized that it causes disruption to the viral capsid (icosahedral) and destabilizes recognition receptors.

Mycoystin_LR (MC-LR) is produced by cyanobacteria that attract attention due to its high toxicity and high concentration in aquatic systems. Lu et al. (2018) showed that the combined use of UV/H_2O_2 process and O_3/H_2O_2 were effective methods to remove MC-LR from water and they performed better that UV-, O_3-, H_2O_2 alone processes under the same conditions. However, UV dosage of 1800 mJ/cm^2 was required to remove 90 % Of 100 mg/L MC-LR, where the amount significantly decreased to 500 mJ/cm^2 when 1.7 mg/L H_2O_2 was added. Murray et al. (2017) reported that the use of pulsed light reduced the toxicity of the dinoflagellate algal toxin okadaic acid where ecotoxicological assessments were also performed using a miniaturised format of the conventional in vivo freshwater crustacean *Daphnia* sp. acute toxicity test. Findings revealed a 24-h EC50 of 25.87 μg/L for PL-treated okadaic acid at a UV dose of 12.98 μJ/cm^2 compared to a 24-h EC50 of 1.68 μg/L for the untreated okadaic acid control, suggesting a 15-fold reduction in toxicity to *Daphnia pulex*.

Despite positive observations as a clean decontamination technology, it remains uncertain as to the mechanism by which cavitation generated bubbles clean, disinfection and kill microbial organisms including viruses and enhance chemistry activity (Zupanc et al., 2019). Zupanc et al., 2019 also reported that "cavitation describes the formation of small vapour bubbles (cavities) inside an initially homogeneous liquid medium. It is a rapid physical phenomenon triggered by a sudden decrease in pressure. As the pressure recovers the bubble goes through a violent collapse and possible rebounds. By bubble growth, an energy from the surrounding liquid is collected and released by bubble collapsation, where extreme conditions can be formed locally. Bubble collapse can cause pressure shocks up to several 100 MPa and if the bubble collapses asymmetrically the so-called microjets with high velocities above 100 m/s can form". These observations are also aligned with the related studies of Chahine and Hsiao (2015). In addition, the so-called hot spots with extreme temperatures in order of several 1000 K can form at the centre of the bubble at its collapse, which can cause the formation of highly reactive radicals (Koda et al., 2003). Zupanc et al. (2019) also stated that the "exact manifestation of cavitation is influenced by liquid properties (temperature, density, viscosity and surface tension) and quality (number of solid particles and amount of dissolved gasses, which can both act as a nuclei). In general, two types of cavitation are recognized, hydrodynamic and acoustic cavitation. The difference is in the mechanism, which causes the local pressure to drop, while the principles which govern the hydrodynamic bubble and the acoustic bubble are basically the same".

The effects of cavitation including mechanical and thermal effects are: (a) microsteaming that can damage microorganism – together with shockwaves generated by bubble collapse; (b) chemical effects (implosion of bubbles and formation of hot spots for homolytic cleavage of H_2O molecules and formation of highly oxidizing free radicals (*OH and *H) - *OH readily oxidize particulate matter and also form H_2O_2 – many other species can form ($*O_2H$, *N, *, $1O_2$) where different gases air/oxygen are dissolved in water; (c) oxidation of miroorganism's constituents (AC process produces a level of ROS beyond microbial antioxidant stress response capabilities) and kills a broad range of pathogens including viruses (McDonnell and Russell, 1999). Also different ROS affects polysaccharides, proteins, lipids and RNA/DNA); (c) oxidation of lipids, polysaccharides and nucleic acids by oxidative stress initiative by (ROS). High-frequency ultrasound inactives *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Legionella pneumophila, Enterobacter aerogenes, Vibrio cholera, Salmonella enterica*, coliforms and *Mycobacterium* species albeit at different rates. However, different cellular and molecular mechanisms were postulated including cell wall breakage for *Bacillus subtilis* where this was the only study to report on simultaneous and sequential mechanisms using a pulsed plasma-gas-discharge approach (Hayes et al., 2013), where a similar approach was used to explain the lethal action of pulsed light (Farrell et al., 2011). Variations in effectiveness were also evident between using cavitation treating microalgae including *Scendeesmus* sp. The majority of researchers noted that the effect of cavitation on microalgae and microorganisms depends on the ultrasound frequency and that higher intensities and longer exposures correlate to more effective lethality (Wu et al., 2012). MS2 virus appears to be very susceptible to inactivation with all types of cavitation (Su et al., 2010) regardless of the initial concentration and medium tested only that in the case of higher concentrations more time for inactivation was needed. The exact mechanism of how cavitation causes virus inactivation is not yet elucidated. Su et al. (2010) suggested that the damages

inflicted during cavitation on the outer protein capsid itself or recognition sites on the capsid surface could be the reason for virus inactivation.

2.4. Photonics

There is also a potential role for developing photonics as a combined method for destroying enteric viruses, such as using visible blue light wavelengths (380–480 nm) that have been safely deployed for the inactivation of microbial pathogens in healthcare settings; thus, enabling the simultaneous activities of healthcare workers (Tomb et al., 2018). Maclean et al., 2009 have postulated that blue (405 nm) light inactivates bacteria via its interactions with porphyrins (five and six carbon multi-ring contain alternating single and double bonds) within the membrane of bacteria. The Irish SME, Atlantic Photonic Solutions Ltd., is developing innovative photonic solutions for the aquaculture Industry (APS, 2023). This company has identified specific wavelengths that can eliminate complex parasites without harming the hosts, such as high-throughput removal of sea lice from salmon in commercial aquaculture processes.

Kingsley et al. (2018) reported on oxygen-depended laser inactivation of murine norovirus using visible light lasers. Kingsley et al. (2018) used a tuneable mode-locked Sapphire laser where the frequency was doubled to generate femtosecond pulses at wavelengths of 400, 408, 450, 465 and 510 nm. >3 log murine NoV were achieved after 3 h exposure at 408, 425 or 450 femtosecond light pulses; thus, viral destruction was not wavelength specific. The use of photosensitizers, such as riboflavin, rose Bengal or methylene blue that generates singlet oxygen substantially improved the efficiency of inactivation. Findings indicate "a photochemical mechanism of the laser-induced inactivation where the action of relatively low-power blue laser light generates singlet oxygen" (Kingsley et al., 2018).

Kingsley et al. (2018) also noted that atmospheric sunlight at sea level contains visible light (400–700 nm), but also contains a UV component that is predominately 300–400 nm (Bird et al., 1983) which does not substantially penetrate water as UV is strongly absorbed. Recent research has indicated that sunlight can interact with dissolved organic molecules in aqueous settings to catalyse the formation of O_2 ($a^1\Delta_g$), commonly referred to as singlet oxygen, as well as other reactive oxygen species (Rosado-Lausell et al., 2013). These complex organic molecules act as sensitizers that become energetically excited by UV–Vis light and then transfer electronic energy to oxygen molecules producing singlet oxygen. Ultrashort pulse laser (USPL) light treatments were previously shown to be capable of inactivating murine norovirus (MNV) and other viruses (Tsen et al., 2007; Tsen et al., 2012); where impulsive stimulated Raman scattering (ISRS) was the postulated inactivation mechanism (Tsen et al., 2014; Tsen et al., 2014). Essentially the ISRS hypothesis was that high-frequency resonance vibrations are potentially induced by the 425 nm USPL, with a bandwidth of 420–430 nm, which may be capable of causing vibrations of sufficient strength such that the icosahedron capsid is destroyed after short nonthermal treatments (Kingsley et al., 2018). However, Kingsley et al. (2018) demonstrated that the inactivation of MNV by the USPL with variable wavelengths of blue light (400–450 nm) and by 408 nm CW indicated the inactivation mechanism was not specific to the 425 nm wavelength and, although more substantial inactivation was achieved with femtosecond pulse light, was not dependent on light pulses. This suggested that inactivation may have been via a non-ISRS mechanism. The addition of sodium bisulfate, an oxygen scavenger, substantially reduced CW laser inactivation, strongly implicating singlet oxygen as the cause of visible laser light-induced virus inactivation.

Kingsley et al. (2018) noted that as a priori, one would not expect vibrational resonance induced by ISRS to necessarily be wavelength-dependent. However, due to pulse-width dependence, it is well understood that ISRS cannot be induced using CW lasers since they do not generate light pulses. The authors demonstrated that CW lasers can inactivate MNV, which potentially conflicts with the hypothesis that ISRS is the mechanism of inactivation as proposed by Tsen et al. (2014) although it remains formally conceivable that both ISRS and singlet oxygen mechanisms could both contribute to laser inactivation observed by the USP laser. Also, it is difficult to envision a scenario in which the low concentrations of sodium bisulfate that reacts with, and sequesters, dissolved oxygen molecules within the MNV sample would inhibit a laser-induced vibrational mechanism. Indeed it was noted that purified MNV and MNV from a cell lysate had roughly equivalent inactivation rates (2014) suggesting that singlet oxygen enhancers are not substantially present in virus stocks, which are derived from virus-infected cell lysate. This suggests that either the virus capsid itself may function as an endogenous enhancer molecule, or that an enhancer may not be strictly required to produce singlet oxygen when interacting with intense blue light. This finding offers a potential explanation as to why norovirus illness was originally termed the 'winter vomiting disease.' The use of photonics has significant potential for nonthermal inactivation of microbial and parasitic pathogens, including, potentially, seafood.

2.5. Use of micro- and nano-bubble technology

Increased aeration and mixing can be potentially achieved in seawater depuration tanks using bulk micro-nanobubble (NB) water using hydrodynamic cavitation (Zhou et al., 2022). Nanobubbles (NBs) show technological potential in commercial applications including wastewater treatment, floatation, agriculture, nanoscope cleaning, and biological and medical applications (Ghadimkhani et al., 2016; Sun et al., 2022). The gas mix could be adjusted for delivery of hydrogen peroxide or ozone to accelerate disinfection of NoV deep in tissue. Such applications are attributed to the unique properties of NBs including low ascending velocity, long longevity retention time, massive interfacial surface area, high internal gas pressure, and negatively charged surface characteristics. Ultrafine NBs can form at the solid-liquid interface and in solutions and have a diameter of between 10 and 100 nm (Alheshibri et al., 2016). For commercial depuration of contaminated oysters, bubble concentrations and sizes in bulk NB water under different conditions should be evaluated along with the putative relationship between dissolved oxygen concentration and cavitation behaviours of bubbles used for this purpose. Thus, NBs have received attention for their unique physicochemical characteristics, including the previously mentioned large specific surface area, long residence time, high gas-liquid mass transfer efficiency, high zeta potential, and reactive oxygen species production (Zhang et al., 2022a, 2022b). Notably, NBs have exhibited outstanding performance, particularly in food safety and quality. Jaykus and Green (2015) describe a funded pilot-scale project addressing the efficacy of ozonated microbubbles for shellfish indicator organisms, Vibrio bacteria and NoV reduction in post-harvest depuration processing of Eastern oysters. The company ScanAqua now provides advanced nanobubble oxygenated technology with 'game-changing integrated engineering services and world-wide support for improved productivity, better water quality and fish welfare in related aquaculture.

The gas composition can be adjusted for NB generation to enhance antiviral activities, such as introducing reactive oxygen species (ROS) (Hayes et al., 2013). There is also potential to combine cold plasma with the nano-bubble biocide delivery concept. Despite positive observations as a clean decontamination technology, it remains uncertain as to the mechanism by which cavitation-generated bubbles clean, disinfect and kill microbial organisms including viruses and enhance chemistry activity (Zupanc et al., 2019) (Table 2). Cavitation describes the formation of small vapour bubbles (cavities) inside an initially homogeneous liquid medium. It is a rapid physical phenomenon triggered by a sudden decrease in pressure. As the pressure recovers the bubble goes through a violent collapse and possible rebounds. By bubble growth, energy from the surrounding liquid is collected and released by bubble collapses, where extreme conditions can be formed locally. Bubble collapse can cause pressure shocks up to several 100 MPa and if the bubble collapses asymmetrically the so-called microjets with high velocities above 100 m/s can form (Chahine and Hsiao, 2015). In addition, the so-called hot spots with extreme temperatures in order of several 1000 K can form at the centre of the bubble at its collapse, which can cause the formation of highly reactive radicals (Koda et al., 2003). Exact manifestation of cavitation is influenced by liquid properties (temperature, density, viscosity and surface tension) and quality

(number of solid particles and amount of dissolved gasses, which can both act as nuclei) (Zupanc et al., 2019). In general, two types of cavitation are recognized, hydrodynamic and acoustic cavitation. The difference is in the mechanism, which causes the local pressure to drop, while the principles which govern the hydrodynamic bubble and the acoustic bubble are the same.

The effects of cavitation including mechanical and thermal effects are: (a) microsteaming that can damage microorganism – together with shockwaves generated by bubble collapse; (b) chemical effects (implosion of bubbles and formation of hot spots for homolytic cleavage of H_2O molecules and formation of highly oxidizing free radicals (*OH and *H) - *OH readily oxidize particulate matter and also form H_2O_2 – many other species can form ($*O_2H$, *N, *, $1O_2$) where different gases air/oxygen are dissolved in water; (c) oxidation of microorganism's constituents (AC process produces a level of ROS beyond microbial antioxidant stress response capabilities) and kills a broad range of pathogens including viruses (McDonnell and Russell, 1999). Also different ROS affects polysaccharides, proteins, lipids and RNA/DNA); (c) oxidation of lipids, polysaccharides and nucleic acids by oxidative stress initiative by ROS. High-frequency ultrasound inactives *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Legionella pneumophila*, *Enterobacter aerogenes*, *Vibrio cholera, Salmonella enterica*, coliforms and *Mycobacterium* spp. albeit at different rates However, different cellular and molecular mechanisms were postulated including cell wall breakage for *Bacillus subtilis* where the study my laboratory was the only one to report on simultaneous and sequential mechanisms using a pulsed plasma-gas-discharge approach (Hayes et al., 2013), where similar approach was used to explain lethal action of pulsed light (Farrell et al., 2011).

Binding behaviour of noroviruses in oyster tissue may contribute to persistence and further challenge effectiveness of appropriate decontamination.

Careful considerations should be given to applying solutions that address the bioaccumulation of different NoV strains in oysters. This potentially contributes to the lengthy persistence of NoV in depurated oysters (McLeod et al., 2017). For example, immunochemistry studies carried on using Pacific oyster tissues, along with confirmatory tests using monoclonal antibodies and NoV particles with mutated capsids of similar tissues, established that GI·I binds specifically to the midgut and digestive tissue via an A-like carbohydrate, similar to the HBGAs used for NoV attachment to human epithelial cells (Le Guyader et al., 2006). In contrast, NoV GII binds to various tissue types in oysters, including the digestive tract, diverticula, midgut (intestine), gills, mantle, and labial palps (Wang et al., 2008; McLeod et al., 2009). Collectively, McLeod et al. (2017) noted that these findings intimate strain-specific variations in NoV binding patterns in contaminated oysters that can be seasonal due to higher bioaccumulation efficiency (GI·I strain), or no variations in seasonal patterns (GII strains). There is a need to understand typical viral load levels to deploy appropriate counter-measures; for example, there have been NoV outbreaks from depurated oysters containing ca. 10^3 genome copies/g oyster tissue (far exceeding the median infectious dose [ID50]) (McLeod et al., 2017). Yet, the authors noted that "half of the published NoV reduction studies reported no decrease in NoV during depuration, and in the remaining studies it took between 9 and 45.5 d for a 1 – log reduction – significantly longer than commercial time frames".

3. Use of safe enteric surrogates to monitor and assess disinfection of shellfish

McLeod et al. (2017) noted that comparative elimination studies to date have shown that surrogate viruses (including phage, Feline calicivirus, Tulane virus, Mengovirus) are more rapidly depurated than NoV under a variety of depuration conditions, including temperatures of 8 to 25 °C, times varying between 23 h and 8 wk., and using both recirculating and flow-through systems that have UV and/or filtration disinfection. The rapid reductions noted for surrogate viruses may be partly attributable to the quantitation of infectious virions in some studies, whereas hNoV quantitation is based on genome detection; however, several studies (Ueki et al., 2007; Drouaz et al., 2015) have used genome detection for the analysis of both hNoV and surrogate viruses and large differences in reduction rates were still observed. McLeod et al. (2017) reported that "for a surrogate virus to provide useful information on hNoV infectivity, it is important that the characteristics of hNoV and the surrogate virus are similar within oyster tissues, including the way in which they interact with ligands, the stability of the virus capsid, and their persistence". Given that some researchers have reported more rapid depuration of certain surrogate viruses tested to date (such as FRNAPII), they may not be suitable for assessing the virological safety of depurated oysters when used alone without other confirmatory method. This is challenging given that there is currently no infectivity method for hNoV.

However, it is frequently challenging to use actual enteric viruses for developing and testing the efficacy of established or emerging disinfection technologies due to their infectious nature, the need to use Cat 2 facilities and the lack of a simple in vitro cell culture model. In addition, there is uncertainty surrounding the use of RT-qPCR for amplifying genomic material from treated enteric pathogens as this molecular approach does not distinguish between live or dead viruses. This is further complicated by the fact that using infectious hNoV at commercial plant level would not be appropriate for validating new technologies due to concerns over introducing pathogens into live shellfish. It is not uncommon to deploy different yet safe microorganism(s) as representative of the target pathogen of interest from a disinfection perspective; for example, *Geobacillus stearothermophilus* and *Bacillus atrophaeus* endospores are used as bioindicators for the terminal sterilization industry (McEvoy and Rowan, 2019), which exhibit a greater level of resistance to the applied lethal stress (such as UV) compared

Table 2
Examples of cavitation effects of treated microbial and algal species (adopted from Zupanc et al., 2019).

Target organism	Characteristic size and shape	Treatment medium	Pre-treatment concentration	Ultrasound frequency (kHz)	Effectiveness (% reduction)	Reference
Enterobacter aerogenes	0.7 × 3.5 μm (rod)	SS	10^6 CFU mL^{-1}	20 [LFUS]	4.4 % after 20 min	Gao et al. (2014)
Haemophilus influenza	0.5 μm diameter (spherical)	BS	10^3 CFU mL^{-1}	20 [LFUS]	99 % after 10 min	Monsen et al. (2009)
Klebsiella pneumoniae	0.7 × 1.6 μm (rod)	BS	10^3 CFU mL^{-1}	20 [LFUS]	90 % after 15 min	Al Bsoul et al. (2010)
Legionella pneumophilia	0.5 × 2 μm (rod)	BS	1.5×10^3 CFU mL^{-1}	33 [LFUS]	20 % after 60 min	Šarc et al., 2014
Vibrio cholerae	0.4 × 3.1 μm (rod)	ASW	$2–5 \times 10^6$ CFU mL^{-1}	19 [LFUS]	90 % after 0.9 min	Holm et al. (2008)
Coliforms		SUW	250 CFU mL^{-1}	20 [LFUS]	70.8 % after 15 min	Al-Juboori et al. (2015)
Escherichia coli	0.5 × 1.5 μm (rod)	ASWS	10^6 CFU mL^{-1}	19 [LFUS]	90 % after 1.4 min	Holm et al. (2008)
Enterococcus faecalis	1 μm (oval)	DW	10^6 CFU mL^{-1}	–	100 % after 10 mins	Cerecedo et al. (2018)
Microcystis sp. (Algae)	4–5 μm (Oval)	GM	10^9 cell mL^{-1}	11 [LFUS]	5 % after 155 min	Zhang et al., 2006
Chlamydomonas sp. (Algae)	3–10 μm (spherical)	GM	5.5×10^7 cells ml^1	1100 [HFUS]	85 % after 7 min	Bigelow et al. (2014)
Scendesmus sp. (Algae)	6–8 μm (bean shaped)	GM	5.2×10^4 cells ml^{-1}		85 % after 60 min	Batista et al. (2017)
MS2 bacteriophage	24–27 nm	BS	10^6 PFU mL^{-1}	20 [LFUS]	4.62 % after 30 min	Su et al. (2010)
Feline calicivirus	27–40 nm	BS	10^6 PFU mL^{-1}	20 [LFUS]		Su et al. (2010)
Murine norovirus	28–35 nm	BS	10^6 PFU mL^{-1}	20 [LFUS]	>3.8 % after 30 min	Su et al. (2010)

Artificial seawater (ASW); Surface Water (SUW); Salt Solution (SS); Buffer Solution (BS), Distilled Water (DS); Growth Medium (GM); Low Frequency ultrasound (LFUS); high frequency ultrasound (HFUS); Plague Forming Units (PFU); Colony Forming Units (CFU).

to similarly-treated microbial pathogens there is also a commensurate need to propagate high population levels of microorganisms (typically ≥ 6 $\log_{10}$ microbes/ml) in order to measure, standardize and to model the inactivation rate and kinetic shape to ensure sufficient disinfection performance. This is known as 'sterility assurance level' or SAL in the adjacent terminal sterilization industry that is used to inform appropriate modalities for the medical device industry. Several safe viral surrogates (or bioindicators [BIs]) have been used for assessment removal or disinfection performance of pathogenic hNoV in live shellfish, such as FRNAPII.

Norovirus is a fastidious virus as it does not grow on culture-based media where there is a reliance on molecular-based approaches to quantify the removal of norovirus and other enteric viruses post treatments; but, molecular approaches do not confirm if the material is alive or dead. Indeed, many existing molecular-based detection methods have great limitations and it is necessary to find alternative or complimentary methods for rapid multi-species and strain testing (Mi et al., 2021). Researchers have highlighted potential misalignment on the use of standard RT-qPCR that may be amplifying residual genetic determinants in the treatment process along with intact virion; which was informed by comparative studies that used FRNA surrogate virus (Rupnik et al., 2021). Plante et al. (2021) reported on improvements in efficiency to standard ISO 15216-1 genomic method using a Droplet-Digital PCR approach.

4. Advanced real-time diagnostics: future use of combined confocal microscopy and RAMAN spectroscopy (CRS)

Of the 53,612 publications that featured RAMAN in title, abstract or main body of the text in PubMed and Scopus during period 1995 to 2023, 12 focused on detecting norovirus and enteric viruses. The reason for including RAMAN spectroscopy reflected increasing evidence-based research that supported use of this technology for rapidly identifying and differentiating microorganisms including viruses. The development of rapid, cost-effective decontamination approaches for foodborne pathogens including viruses is of great importance for food safety, early detection of disease, and environmental monitoring (Yin et al., 2020).

There is a pressing opportunity to exploit the fact that hNoV strains and some surrogates (such as FRNAPII) have similar icosahedron-shaped capsids. Detection, and potentially differentiation between viable and inactivated (non-infectious) NoV and surrogates can be potentially achieved using Confocal Raman spectroscopy (CRS) combined with chemometrics as a novel assessment tool to inform disinfection efficacy. CRS is a fast, reliable and efficient method for detecting and identifying microorganisms without laborious pre-treatments. Raman spectroscopy is already accepted as a "powerful analytical technique for the rapid characterization of bacteria without external labels or tedious preparation. Raman spectrum, deriving from molecular vibrations, can be considered as a typical whole organism fingerprint of the biochemical composition of microorganisms where these vibrational spectra could show differences of molecular composition in various bacterial pathogens at the molecular level" (Lin et al., 2019). Thus, Raman spectra has been used to discern strain-specific physiological, metabolic and phenotypic states in microorganisms (Chisanga et al., 2020).

Huang et al. (2021) recently provided a comprehensive review addressing the use of Raman spectroscopy for fast and accurate detection of viruses; it also offers hand-held instruments for aiding practicality for point-of-use or care applicability. Mori et al. (2018) described the use of RAMAN spectroscopy for studying norovirus encapsulation. Other researchers have also exploiting use of this surface-enhanced scattering technology (SERS) for ultrasensitive and rapid identification of norovirus including; use of molybdenum trioxide nanocubes with graphene oxide (Achadu et al., 2020); molybdenum-trioxide quantum dots used with naonogels (Achadu et al., 2021), peroxidase-like graphene-gold particles (Ahmed et al., 2017), polyhedral Cu nanoshells (Kim et al., 2017) combined use of sulfur-doped carbon dots polydopamine-functionalized magnetic silver nanomaterials (Achadu et al., 2021), and plasmonic biosensing (Mauriz, 2020). Zhang et al. (2022a, 2022b) reported the use of CRS to identify and differentiate several types of bacterial pathogens (*Vibrio cholera*, *Salmonella flexneri*, *L. monocytogenes*, *S. aureus*, *S. typhimurium*, and *Clostridium botulinum*) based on characteristic peaks and peak intensity ratio. Principal component analysis (PCA), decision tree, artificial neural networks and Fisher's discrimination analysis were used to investigate differences in microbial detection. Combing Raman spectroscopy with confocal enables the identification of single bacterial cells in high spectral resolution, combining the power of 3D analysis with focused biological component in aqueous medium (Vlasov et al., 2020). The combined use of chemometrics to spectral data enables the analysis of nucleic acids, proteins, lipids, and carbohydrates for distinguishing different bacterial species (Zhang et al., 2022a, 2022b). The use of CRS may also be relevant for ensuring loss of all pathogens of concern including *Vibrio* species – it is particularly relevant to include a battery of test reference strains (*E. coli, Vibrio* sp., hNoV and surrogates) during the development of testing so as to ensure that bacterial and viral pathogens of concern are both addressed in live shellfish. For example, the use of pulsed light offers broader light spectrum targeting cellular constituents (cell membrane, lipid peroxidation and so forth) in Gram negatives bacteria; however, a focus on hNoV destruction from best-published data intimates delivering a short intensity of fixed wavelengths <300 nm that specifically targets viral RNA (and bacterial DNA) (Farrell et al., 2011; Hayes et al., 2012).

5. Advanced real-time diagnostics: future use of flow cytometry

Of the 253,560 publications cited in PubMed and Scopus on using "flow cytometry" of FCM, 26 papers described the combined use of FCM with noroviruses between period 1974 and 2023. Sixteen papers were excluded for the reason that they focused on non-enteric virus detection in shellfish or food systems, namely immune dysregulation associated with villous atrophy; NoV infection or Primary B Cell immune activation in vitro; interactions with *Cryptosporidium parvum* during infection of HCT-8 cells; identification of human NoV CD8+ T Cell restricted to HLA-A*0201 allele; experimental inoculation of juvenile rhesus macaques; and bacterial-linked analysis of geothermal bathing pools. Detection or loss of important epitopic sites on NoV capsids by exposure to treatment technologies may affect the binding of these antibodies that can be rapidly determined in real-time using flow cytometry (FCM) – this is particularly relevant given that an intact capsid retaining surface receptors are critical for enabling viral attachment to specific human histo-blood group antigen (HGBA) sites to cause infection (Esseli et al., 2019). Razafimahefa et al. (2021a, 2021b) described the development of a specific anti-capsid antibody and magnet bead-based immunoassay to detect human NoV in stool samples and spiked mussels. Ponterio et al. (2013) reported on the phenotypic analysis use of FCM to detect pattern of human antigen presenting cells by genotype GII.4 norovirus. Hamza et al. (2011) noted that not all viruses are able to produce cytopathic effects where use of FCM may help with human NoV detection that have no available cell line for propagation. Annamalai et al. (2019) used FCM to evaluate infectivity of GII.4 human norovirus where the authors showed that this approach can inform differentiation between T-B-NK+ severe combined immunodeficiency (SCID) and non-SCID gnotobiotic pigs, implicating the role of NK cells in mediation of human NoV infection.

Thus, use of flow cytometry (FCM) is already an established immunological tool (Cossarizza et al., 2021) including for use in fisheries and shellfish research, where it has been applied to bivalve molluscan shellfish to analyse haemocytes (Van Nguyen and Alfaro, 2019). Cossarizza et al. (2021) noted that "FCM is a laser-based technique that is used to analyse physical characteristics of cells or particles in heterogenous fluid mixtures as they pass through the light source. When a sample of interest is injected into the FCM, targeted components are excited by the laser which emits light in a band of wavelengths. Here, the fluorescence intensity is measured for each particular target cell/particle (virus) at a rate of thousands per second. FCM provides a fast, accurate, convenient, simple to use and affordable tool that can achieve the desired simultaneous measurements of 'multiple' cellular or viral components in real time. This includes inter

alia total cell or particle counts, cell viability including assessing sub-cell populations, quality of genomic content, phagocytosis, oxidative stress and apoptosis (Van Nguyen and Alfaro, 2019). Molecular targets of interest are typically labelled with fluorescent reagents that may include a broad range of dyes, stains, monoclonal antibodies or quantum dots (Van Nguyen and Alfaro, 2019). FCM also enables specific gating of plotted data for a more fine-grain focus on characteristic(s) of interest. For example, my research team (McEvoy et al., 2021) used flow cytometry as a real-time quantitative tool to enumerate biological indicators (*Bacillus atrophaeus* and *Geobacillus stearothermophilus*) in a commercial vaporized hydrogen peroxide (VHP) process used by the terminal sterilization industry.

FCM has been used for studying and quantifying viruses including clinical and aquatic applications for four decades (Hercher et al., 1979; McSharry, 1994; Marie et al., 1999; Kraus et al., 2007; Yang et al., 2008; Brussaard et al., 2010); for example, Marie et al. (1999) reported that FCM can successfully be used to enumerate viruses in seawater after staining and noted that the technique was first optimised by using the phaeocystis lytic virus PpV-01. Yang et al. (2008) advanced the use of microfluidic FCM for virus detection by introducing and integrating several functional micro-devices including antibody recognition and capturing of target viruses. Kraus et al. (2007) compared plaque and FCM-based methods for measuring dengue virus neutralization.

Given that this approach is based on the loss or reduction of a measured signal from fluorophore conjugated antibody that targets treated hNoV capsid – FCM can be deployed as a tool for determining the removal and inactivation modalities including assessing hNoV infectivity post treatments. FCM can be used to compare hNoV detection and quantitative efficiency of the established RT-qPCR genomic method at the laboratory bench (such as discovery phase, TRL 1–3) to compare and inform effectiveness of technologies at commercial plant level (TRL 6 to 9). Thus, and in theory, rapid detection (or loss of detection) of a cocktail of specific monoclonal antibodies matching epitopic sites on hNoV capsid may be used to directly inform quantification of inactivated hNoV by FCM (such as Van Nguyen and Alfaro, 2019). FCM can also be used to simultaneous assess the immunological status of treated live oysters in terms of vitality indicators (Van Nguyen and Alfaro, 2019). Commensurately, loss of specific spectral signals from combined use of CRS method will inform physical disruption of hNoV capsids post treatments, to be supplemented with use of scanning electron microscopy (SEM). For example, such approaches have also contributed to a generation of unique data in our laboratory that elucidates simultaneous and sequential changes at molecular and cellular level informing mechanistic of irreversible lethal action of pulsed light technology against clinical strains of *Candida albicans* (Hayes et al., 2013).

It is envisaged that this advanced suite of imaging techniques (FCM, CRS, SEM) will be centrally located in regional research and enterprise hubs linked to academic experts and industry (Rowan and Casey, 2021), where end-users can access and use these tools affordably that includes offering bespoke training to industry, as available in my laboratories. For example, this suite of advanced imaging tools will be initially used to compare effectiveness for rapid detection of hNoV methods including the provision of new real-time data to help understand the loss of hNoV infectivity during depuration treatments. This combined approach giving techniques that produce real-time spectral measurements would also be appropriate for future automation including the development of deep learning/machine learning algorithms. The Raman community needs to encourage the deposition of virus spectral findings to a central database so as to grow this technique for the food and healthcare sectors.

6. Modeling of enteric viral removal and inactivation and risk mitigation

Of the 1,973,267 papers published on mathematical "modeling" that appear in PubMed and Scopus databases [1933–2023], 26 addressed modeling for detecting and enumerating norovirus in shellfish [1996–2023]. Twelve papers were excluded for the reason that they focused on risk assessment and modeling of cooked or high-pressure-processed shellfish, and development of a model virus or model system. The majority of modeling studies estimated distribution of norovirus or enteric viruses in shellfish, particularly oysters (Hunt et al., 2020) that included sewage impact (Winterbourn et al., 2016). For example, Campos et al. (2017) deployed modeling to determine the zone of impact of norovirus contamination in shellfish production areas through microbiological monitoring and hydrographic analysis. Whereas Razafimahefa et al. (2021b) used this approach to optimize a PMAxx™ -RT-qPCR assay and the preceding extraction method to selectively detect infectious murine norovirus particles in mussels.

Kinetic modeling of viral removal or inactivation performance is important in order to understand the behaviour of treated viruses to the applied treatment technology (Polo et al., 2014; Rowan et al., 2015; Polo et al., 2015; Qin et al., 2022). Qin et al. (2022) noted that a framework is needed to describe the complex nonlinear virus-oyster interactions that included a mathematical model addressing key processes for this viral dynamics, such as oyster filtration, viral replication, the antiviral immune response, apoptosis, autophagy, and selective accumulation. This has implications for informing the non-homogenous distribution of norovirus in oysters and effectiveness of appropriate depuration or relaying. Ideally, a first-order log-linear inactivation plot is achieved over the treatment regime that eliminates or destroys the target pathogens of concern exponentially. However, depending on the efficacy of the treatment modalities, the resistance or tolerance potential of the microorganisms (Fig. 2), and other governing or interfering parameters (such as the presence of organic matter and particulates or biofilms, pH, salinity, dissolved oxygen and so forth), prior microbial stress adaption to applied lethal technologies, the shape of the inactivation plot may differ (Fig. 2). Microbial kinetic inactivation plots may exhibit an initial protective 'shoulder' effect followed by a linear kinetic plot, and/or a pronounced adaptive or resistant 'tailing' effect that may be evident after longer exposure times. These are referred to as bi-phasic if the shoulder or tail effect accompany a log linear kinetic plot, or triphasic if both the shoulder and tailing effects are present. For example, the absence of a shoulder and tailing effect in microbial inactivation plots for the adjacent sterilization industry is essential as medical devices must achieve a 12 log reduction in bacterial endospore numbers where the first 6 logs are enumerated, but the next 6 logs are predicted based on the probability of a linear plot from the first 6 log (half-cycle). If the tailing effect is evident in first 6 log half-cycle plot, then it cannot be assumed that linearity occurred; thus, introducing the possibility of microbial survivors in the latter treatment stages (McEvoy et al., 2023). For food treatments, it is desirable to have an initial starting microbial population of 5 to 6 log orders in order to standardize treatments between laboratories (Rowan, 2019; Gómez-López et al., 2022) and to achieved a discernible number of D-value reductions (a D value relates to the time taken for a 1 log reduction in microbial numbers achieved under a fixed treatment regime) (Rowan et al., 2000). US FDA also recommend using a 6 log starting population for PUV irradiation studies for food surface decontamination to include natural microbial contaminants during testing and validation (Rowan, 2019).

However, determining accurate viral kinetic modeling for a complete norovirus reduction in shellfish is challenging due to: (a) there is no infectivity assay that would allow the propagation of human NoV to artificially high levels (viral load) such as log 6 gcg^{-1} for kinetic disinfection studies; (b) contamination of shellfish such as oysters appear to be typically contaminated at significantly less viral loads (such as $\leq$5 log); (c) is its uncertain if the RT-qPCR alone will provide data on individual virus post treatments including potential for amplifying genomic artefacts in whole or disintegrated capsid; (d) the LOQ for RT-qPCR (100 gcg^{-1}; Rupnik et al., 2021); (e) use of existing surrogates such as FRNAP II may be less resistant to actual hNoV strains in contaminated live virus due in part to differences in behaviour or non-ligand binding to oysters; and (f) as yet, there is no universally accepted model for determining kinetic destruction of hNoV in shellfish with particular relevance to commercial depuration deployment.

There is a marked gap in data on hNoV inactivation modeling for shellfish. However, Rupnik et al. (2021) reported that norovirus GII exhibited bi-phasic viral reduction kinetic performance using LP-UV irradiation in

contaminated Pacific oysters. After the initial rapid linear reduction of GII, the rate of depuration decreased with a pronounced tailing effect; no further reduction was observed between days 3 and 7, or days 4 and 7 for high and medium depuration temperatures. Interestingly, the two phase viral reduction kinetics was not evident at low temperature trials, with no oyster mortalities observed during the first two weeks of depuration. This pronounced hNoV 'tailing' effect is a potential concern where there is a pressing need to test and develop complementary treatment technologies (such as possibly pulsed light, AOPs, photonics, micro- nano-bubbles) and improved hNoV detection techniques. Potential use of complementary approaches to assess effectiveness of hNoV removal or destruction below the existing LoQ or LoD for RT-qPCR method would be beneficial (as proposed and discussed in the next section). Also, for future modeling studies, it will be relevant to include a test reference hNoV strain, along other microbial reference strains (*E. coli*, *Vibrio* sp), in order to compare the broad-spectrum antimicrobial action of treatment technologies through collaborative inter-laboratory validation trials with commercial producers of oysters.

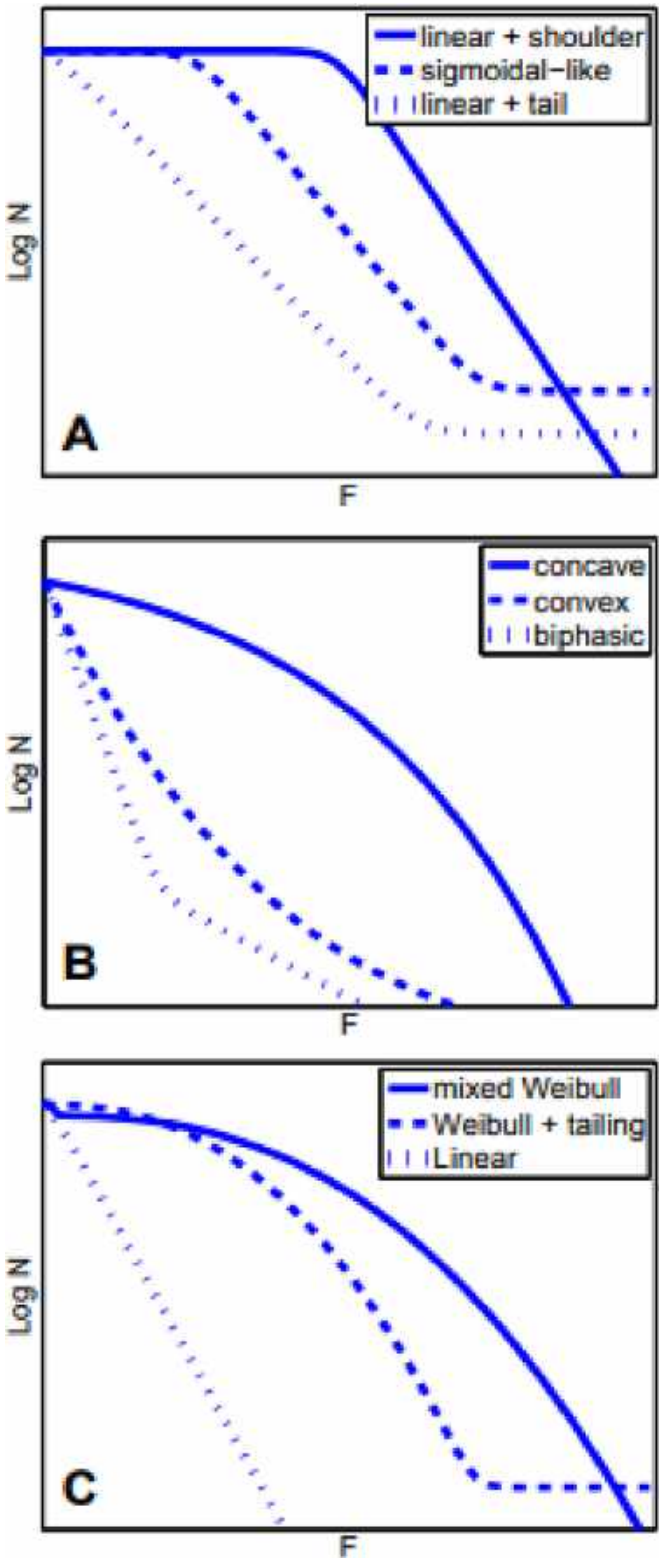


Fig. 2. Commonly observed types of inactivation curves during non-thermal UV processing expressed as $\log_{10}$ N versus F (fluence, J/cm^2). Plot A: sigmoidal-like, linear with a preceding shoulder, log-linear with a tailing. Plot B: biphasic, concave upwards or downwards. Plot C: Linear, Weibull incorporating a tailing effect, two mixed Weibullian distributions (adopted from Rowan et al., 2015).

Thus, lessons can be learnt from the adjacent Medtech domain where complex disinfection and sterilization challenges to address disinfection and sterilization are met in tandem consultation with regulators (such as the USF FDA) for sterility assurance levels. Starting viral populations of 6 $\log_{10}$ for NoV GI and GII (and surrogates such as FRNAPII) are required in order to model inactivation or removal profile to confirm efficiency of modalities where it is assumed that inactivation is exponential (or log-linear). McMenemy et al. (2018) proposed a mathematical model for estimating pathogen variability (including *E. coli*, hNoV) in shellfish and predicting minimum depuration times (including FRNAPII). This interesting model assumes a 'worst case scenario' for viability of pathogens and is then used to predict minimum depuration times to achieve hNoV levels which fall within possible risk management levels, which is a logical approach to mitigating this challenge as a tool to assist in future control strategies given its complexity. The authors state that this model is based on documented assumptions that hNoV is log-normally distributed throughout a population of oysters, and that the pathogen load decay during depuration is exponential. This model requires the "input of four parameters: i) the initial average hNoV load, ii) the depuration efficacy, iii) the desired assurance level (i.e., proportion of shellfish pathogen in depuration which must have pathogen loads less than pathogen threshold limit at the end of the depuration), and iv) the required hNoV threshold" (McMenemy et al., 2018).

However, observations from best-fit linear plots of hNoV and FRNAPII inactivation data in treated oysters appear to frequently show distinct initial shoulder and latter tailing effects that are associated with bi and triphasic viral survivor plot responses (Leduc et al., 2020; Rupnik et al., 2021); therefore, not all documented decontamination studies appear to exhibit viral data that are log-linear in performance. This is important as the pronounced tailing of the hNoV survivor plot would necessitate longer or alternative treatment approaches to ensure complete decontamination of enteric viruses in live shellfish (Rowan et al., 2015). The initial shoulder or increase in hNoV numbers after the onset of depuration (Rupnik et al., 2021) may be attributed to the release of viruses from more concentrated locations in oysters or possibly due to variance in treatment methods and conditions.

In the context of related modeling in adjacent domains, McEvoy et al. (2021) recently reported that vaporized hydrogen peroxide effectively kills recalcitrant bioindicators (*Geobacillus stearothermophilus* and *Bacilllus atrophaeus*) in a commercial process where the inactivation produces linear death rate kinetics. This provides confidence for the industry where there were prior assumptions made through the half-cycle sterilization process (6-D log10 reductions) that linear inactivation occurs in a closed box end-to-end monitoring treatment system. This is particularly important as the terminal sterilization makes inferences about the probability of linear inactivation in order to deliver treatments for 12 $\log_{10}$ reductions in *Bacillus* endospores. Modeling of kinetic data for norovirus destruction (such as longitudinal models for Covid-19, Rowan and Moral, 2021), along with gaining an understanding of the mechanistic destruction of this virus at a molecular level will also help inform reliable and repeatable destruction of this food-borne pathogen for the seafood industry. Interestingly, several publications refer to sterilization (Cong et al., 2021), when it is a 'high-level disinfection' process for human norovirus decontamination as one cannot

assume the destruction of all microbial life that is defined by 'sterilization'. There is a pressing need to develop an appropriate risk mitigation approach using a source-pathway-receptor model in order to determine the efficacy of hNoV removal or destruction from live shellfish. This is likely to be a quasi-quantitative approach given the complexity of the challenge as there is likely to be a residual risk to consumers and commercial seafood producers given their current understanding of existing treatment combinations. The question remains as to what is the acceptable risk given the uncertainly in diagnostic (RT-qPCR) for determining viable hNoV and the limited data available on the efficacy of established and emerging disinfection technologies for full removal or inactivation of enteric viruses deployed at commercial depuration for the shellfish industry. The quasi-quantitative, risk assessment-based approach of Tahar et al. (2017) may inform the aforementioned given that this study addressed the efficacy of WWTPs to remove complex contaminants of emerging concern including risks associated with discharge to receiving waters.

Modeling may also inform previous researcher observations that noted hNoV depuration to adopt a 'two-phase' response with elimination in the first few days being more rapid than subsequent days (Love et al., 2010; Polo et al., 2014, 2015). The first rapid phase of viral depuration is likely related to extracellular digestion and purging of the digestive tract; the speed of purging is governed by physiological traits of the shellfish species concerned, including filtration and clearance rates, digestion rate, and enzymatic activity. Thus, optimizing the physiological state of shellfish through adjusting different parameters such as temperature and salinity contributes to maximizing hNoV reductions in the first phase of depuration (that is, gut purging). However, the persistence of hNoV in shellfish during the second slower phase of elimination indicates that other properties are at play. Indeed, the binding of hNoV to HBGA-like ligands present on oyster gastrointestinal cells, gills, and mantle represents a major barrier to enhancing depuration and will be a key point to address in future studies (Maalouf et al., 2011; Polo et al., 2014). McLeod et al. (2017) also introduced the novel concept of enzymatic pre-treatment to help remove hNoV that could also be modelled for effectiveness.

Hunt et al. (2023) reported for the first time on a quantitative exposure assessment model to predict variations in per-serving norovirus consumption using results from the standard SIO 15216–1:2017 detection method. This model recognises that norovirus is infectious at low doses intimating that small variations in human exposure can potentially lead to significant differences in consumer health. Hunt et al. (2023) findings show "the boundaries for potential exposure following a given ISO detection result, and the relative importance of mean concentration, serving size, and oyster grade. This is directly relevant to potential regulatory thresholds being considered in the EU". However, improvements in the application of appropriate decontamination technologies would potentially impact positively on reduced risk in this model for consumers as these may remove or deactive noroviruses. Addressing norovirus reduction is a complex challenge for risk mitigation with reliance on the LoQ for the ISO method (i.e., 100 gc/g), with a LoD of 20 gc/g. Hunt et al. (2023) noted that the EFSA baseline survey (EFSA, 2019) defined LoQ of 300 gc/g, as achievable for routine monitoring EU. Findings published by Hunt et al. (2023) show that a mean of 200 g/g predicts 95 % consumption range of 57 to 338 gc in a serving of a single oyster. However, the median infectious dose attributed to oysters must be below 2020 genomic copies of hNoV; thus, implying a high probability of infection even at levels lower than the LoQ. Hunt et al. (2023) noted that the median illness-causing does is higher, with a median predicted value of 260 copies needed, as calculated from outbreak events. Additional factors need to be considered to fully progress this QMRA model given the large difference between proportion of oyster batches testing positive for hNoV (such as 76.2 %, [Lowther et al., 2012]), and the proportion of positive samples associated with illness, which is estimated at <0.28 % (Lowther et al., 2010) in the UK. Future use of this QMRA model that incorporates additional factors such as disinfection potential of technologies can also potentially address challenges for hNoV risk assessment.

7. Climate change influences

Climate change is increasing seawater temperatures that can cause disruption in aquatic microbiome enabling the emergence of toxigenic phytoplankton and invasive species, toxic algal blooms, and survival of *Vibrio parahaemolyticus* (Winder and Sommer, 2012; Duchenee-Moutiner and Netto, 2021) in marine environments (Noorian et al., 2023); thus, presenting additional shellfish depuration or relaying challenges. Moreover, 2022 was a year of climate extremes with record high temperatures and rising concentrations of greenhouse gas (Copernicus, 2023). This report highlighted that the "last eight years have been the eight warmest on record globally. La Niña conditions persisted during much of the year. The annual average temperature was 0.3°C above the reference period of 1991-2020, which equates to approximately 1.2°C higher than the period 1850-1900. Europe saw its second warmest year on record, exceeded by 2020 and only slightly warmer than 2019, 2015 and 2014. Europe experienced its hottest summer ever recorded in 2022, where autumn was the third warmest on record. The world's oceans reached temperatures in 2021 despite a La Niña event that typically has a cooling influence". In 2021, the upper 2000 m of the ocean, where most of the warming occurs, absorbed 14 more zettajoules (a unit of electrical energy equal to one sextillion joules) than it did in 2020. This amount of extra energy is 145 times greater than the world's entire electricity generation which, by comparison, is about half of a zettajoule (Milman, 2022). U.S. EPA (2021) reported that "sea surface temperature has been consistently higher during the past three decades than at any other time since reliable observations began in 1880". Seawater temperature rises have been attributed to changes in marine ecosystems such as the recent toxic algae bloom that caused significant losses at commercial salmon sites (Mowi) in Ireland (Intrafish, 2021).

Numerous reports have connected climate change impacts to influencing infectious diseases due to increased hazard, exposure and vulnerability, such as through modeling (Semenza and Paz, 2021; O'Neill et al., 2022a, 2022b). Extreme precipitation events have caused waterborne outbreaks and longer summer seasons (Semenza and Paz, 2021). Researchers have also noted that project risks from infectious disease can be reduced in the future by acting on hazard, exposure and vulnerability through appropriate mitigation and adaption, such as a described in this review (Semenza and Paz, 2021). Carlson et al. (2022) reported that climate change increases cross-species viral transmission risk where there is a mechanistic link between global environmental change and disease emergence. Notably, these authors stated that "this ecological transition may already be underway and holding warming under 2°C within the twenty-first century will not reduce future viral sharing. Our findings highlight an urgent need to pair viral surveillance and discovery efforts with biodiversity surveys tracking the range shifts of species". O'Neill et al. (2022b) reported on the role of using next-generation sequencing and bioinformatics to profile freshwater aquaculture-biome to understand the impact of climate variance of key algae and bacteria including the emergence of waterborne parasites (O'Neill and Rowan, 2023). Duchenne-Moutien and Neetoo (2021) also intimated a link between climate change and food safety issues.

Rindi (2014) reported that terrestrial algae (green algae and diatoms) are more directly affected by climate change and can therefore respond in a more immediate way. This is attributed in part to the fact that algae have short generations, fast turnovers and respond quickly to changes in environmental conditions. Sarmaja-Korjonen et al. (2006) demonstrated that algae appeared to be comparatively good indicators of environmental conditions by representing productivity disparities during changing climatic conditions. Hallegraeff (2010) has also indicated that changes in algal communities can putatively provide a sensitive early warning for climate-driven uncertainties in aquatic ecosystems. There has been increased interest in alternative uses of microalgae within aquaculture to assist with sustainability, in addition to enabling an ecotoxicological assessment and water quality control (O'Neill et al., 2019; O'Neill and Rowan, 2021). Eutrophication occurs when a water body is put under pressure with large levels of organic matter and nutrient waste that is taken in and biologically processed which in turn leads to algal blooms (Jegatheesan et al., 2011;

Martinez-Porchas et al., 2014; Sikder et al., 2016). Algal blooms in turn can lead to decreases in light and oxygen production, which can suffocate aquatic life (Jegatheesan et al., 2011; Chislock et al., 2013; O'Neill et al., 2019) and can be exacerbated by increased marine and freshwater temperatures.

Barry and Hoyne (2021) reported that changes in weather systems, such as increased precipitation, snow and ice events, heatwaves and storms, have led the European Commission to develop new policies and strategies to deal with extreme events. Moreover, Barry and Hoyne (2021) suggested seeking international agreement on indicators to inform ecological resilience, along with economic (such as a number of new SME creations, innovation, investment in training, and specialist upskilling), social enterprise and culture (such as diversity of youth initiatives to increase civil action, solidarity and engagement). Several researchers have also reported on the impact of climate change shifts on receiving waters including flooding (Blöschl et al., 2019).

8. Quadruple Helix Hub Approach

The role of Quadruple Helix HUB (academia-industry-regulators-society) to unlock challenges and to provide subject-matter inputs and shared use of complex or sophisticated equipment (such as novel treatment and diagnostic technologies) from an educational, training and innovation perspective (Rowan and Casey, 2021; Rowan, 2022) is vital. This approach can concentrate single-access supports for industry, entrepreneurs and disruptors; such as step-change in physical infrastructure and systems supports, pre-start-ups; ideation and design thinking to inform technology readiness level (TRLs); market research and enterprise support including early needs analysis and product market fit analysis; early technical validation (such as test the tech, experimentation and validation of pre-pilot and scale to commercial setting); digital transformation (including end-to-end monitoring, AI, remoting immersive training); and a conduit for grant or funding support (Rowan, 2022; Rowan et al., 2022). There is a pressing need for real-time monitoring and confirmation of disease mitigation efficacy – possibly through loss of essential housekeeping or virulence molecular determinant(s) in treated hNoV. This could be elucidated through combinational bioinformatics and next-generation sequencing (NGS). There is an enormity of potential parameters that require simplification that will inform decision making on technology adoption and deployment (Rowan and Galanakis, 2020). There is pressing need for concerted multi-actor collaborations that address the open sharing of data and knowledge that involves hurdling IP discovery phase in TRLs from the screening pilot phase studies for realistic translation to commercial depuration (O'Neill and Rowan, 2022c; O'Neill et al., 2022c). This could all be accelerated through digital transformation that can also address the sustainability of the applied monitoring and disease mitigation technology from a commensurate energy, carbon footprint and risk evaluation perspective that will also help with investment in solutions. Studies conducted in the Hub linked to industry can inform toxicology (Usuldin et al., 2021; Wan-Mohtar et al., 2012

There is a need for a new generation of workers cross-trained in different converging topics to enable sustaining, and potentially, disruptive approaches to tackling industry and societal challenges. This can be met through the Quadruple Helix Hub concept that can also help streamline top-down policy with a bottom-up user understanding at a critical interface to inform decision-making. Testing across independent laboratories and pilot/commercial plants internationally for validation and to support investment in solutions. The greatest challenge is time, as effective solutions that includes confirmation of disease mitigation efficacy are required now.

9. Conclusions and implications for future research

Shellfish are an important source of nutritious food that will contribute to feeding our increasing global populations. However, bivalve molluscan shellfish (particularly oysters) are filter feeding animals and can bioaccumulate pathogenic microorganisms in their digestive tissue that lead to outbreaks of foodborne illness and damage the reputation of the seafood industry. Existing disease mitigation measures rely upon either relaying contaminated shellfish to intertidal locations of clean water for four weeks; or more commonly, depuration using clean seawater combined with a treatment technology (such as UV-irradiation) at the commercial shellfish production site. Growing intertidal environmental locations for shellfish production are graded A to C to dictate requirements for treatment based on microbiology quality; category B growing locations require mandatory depuration. However, many European shellfish producers operate depuration from category A sites that have resulted in contaminated oysters. While bacterial pathogens can be effectively removed at the depuration phase, there is a pressing need to improve existing technologies to address complex human NoV that can persist for lengthy periods due to specific ligand-binding of NoV strains to bivalve molluscan tissues (where the type of tissue for viral attached varies depending on the strain). Assessing the effectiveness of the removal or an inactivation approach for hNoV in live oysters is further complicated by the fact that there is no infectivity assay for this enteric pathogen with reliance upon the use of genomic RT-qPCR technique that does not distinguish between live or dead hNoV. Consequently, the use of surrogate viruses (seen as representative of hNoV to the applied treatment technologies) is adopted such as using the FRNA bacteriophage. However, best-published evidence suggests that existing depuration practices remove viral surrogates faster than targeted hNoV intimating differences in behaviour in viral binding to oyster tissue, and possibly the variance in enumeration methods. Therefore, there is a pressing need to improve both hNoV removal and inactivation methods at depuration along with implementing more appropriate detection and enumeration approaches for hNoV at the commercial depuration phase. There is also uncertainty regarding the appropriateness of using standard molecular-based method for assessing hNoV post treatments compared with surrogate infectious virus values, thus, it is potentially plausible that the level of viral lethality achieved is underestimated given current diagnostic challenges.

The following recommendations are proposed to help with this challenge:

- Further investigate and potentially apply combinational decontamination and treatment technologies for hNoV elimination in live shellfish such as PUV, AOPs, photonics, nano-bubbles and potentially enzymology.
- Develop appropriate laboratory-based diagnostic methods to focus on the destruction of icosahedron-shaped viral capsid, specifically addressing enteric viruses (noroviruses) and appropriate viral surrogates (such as FRNAPII).
- Compare the effectiveness of genomic RT-qPCR (ISO standard), and infectivity assays (such as FRNPH plaque method) against the aforementioned capsid-stability diagnostic method(s) (such as immuno-phenotypic profiling using FCM, confocal-RAMAN spectroscopy) pre and post treatment.
- Enhance support and engagement with the shellfish industry such as through the Quintuple Helix HUB framework (academia-industry-government-environment-society) that addresses access to specialist equipment, subject-matter experts and contract-service provision. This can inform testing and translation of new decontamination technologies to support commercial oyster producers in addition to implementing a practical strategy for unlocking affordable effective solutions.
- Determine actual representative hNoV viral load for testing and validating decontamination technologies.
- Determine the link between detection (ISO, 2017), disinfection efficacy and consumer exposure for norovirus contamination in oysters that is particularly relevant to commercial producers and for risk managers in EU.
- Develop appropriate inactivation kinetic models that embrace circumstances where there are deviations from exponential log-linear plots (such as bi-and tri-phasic performances associated with protective shouldering and tailing) that will inform decision-making for shellfish managers.
- Develop an appropriate holistic 'multi-actor' risk mitigation strategy for preventing hNoV contamination of shellfish including local government

and wastewater industry. This can be combined with new ecological profiling of the same intertidal growing environments as commercial production shellfish production that will address early detection of established recalcitrant hNoV strains and emerging microbial or toxicological contaminations (such as algal biotoxins and invasive species that may be influenced by climate change/variance).
- Translate findings into supporting and facilitating new policies on hNoV in shellfish.
- Develop appropriate training that embraces new techniques including digital technologies.
- Given the complexity of multiple factors potentially governing reliable and repeatable hNoV decontamination of shellfish, introduce edge-computing for end-to-end monitoring and AI/machine learning and blockchain to evaluate and analyse datasets for real-time decision-making.
- Routinely include standard microbial reference strains and for stakeholders to reach consensus on an agreed harmonized method for hNoV decontamination (for example, UV/PUV dose, viral load, depuration temperature, flow rate, duration and so forth).
- Enhance social marketing to help inform stakeholders in real-time and for behavioural change.

CRediT authorship contribution statement

Neil Rowan designed and wrote this paper solely.

Funding acknowledgement

The author acknowledges funded support from the MSCA RISE ICHTHYS Project [No. 872217] and Interreg Atlantic Area NEPTUNUS Project [No. EAPA_576/2018].

Data availability

Data will be made available on request.

Declaration of competing interest

The author declares no conflict of interest.

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Science of the Total Environment 761 (2021) 144094

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Life cycle assessment of fish and seafood processed products – A review of methodologies and new challenges

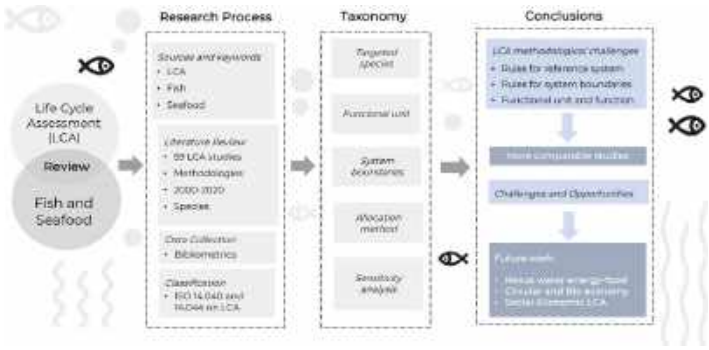
Israel Ruiz-Salmón [a,*], Jara Laso [a], María Margallo [a], Pedro Villanueva-Rey [b], Eduardo Rodríguez [b], Paula Quinteiro [c], Ana Cláudia Dias [c], Cheila Almeida [d], Maria Leonor Nunes [d,e], António Marques [d,e], Antonio Cortés [f], María Teresa Moreira [f], Gumersindo Feijoo [f], Philippe Loubet [g], Guido Sonnemann [g], Andrew P. Morse [h], Ronan Cooney [i,j], Eoghan Clifford [i,j], Leticia Regueiro [k], Diego Méndez [k], Clémentine Anglada [l], Christelle Noirot [l], Neil Rowan [m], Ian Vázquez-Rowe [n], Rubén Aldaco [a]

[a] Departamento de Ingenierías Química y Biomolecular, Universidad de Cantabria, Avda. de Los Castros, s.n., 39005 Santander, Spain
[b] EnergyLab, Fonte das Abelleiras s/n, Campus Universidad de Vigo, 36310 Vigo, Galicia, Spain
[c] Centre for Environmental and Marine Studies (CESAM), Department of Environment and Planning, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[d] IPMA - Instituto Português do Mar e da Atmosfera (IPMA), Divisão de Aquacultura, Valorização e Bioprospeção, Avenida Doutor Alfredo Magalhães Ramalho 6, 1495-165 Lisboa, Portugal
[e] CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal
[f] Department of Chemical Engineering, Institute of Technology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Galicia, Spain
[g] Univ. Bordeaux, CNRS, Bordeaux INP, ISM, UMR 5255, F-33400 Talence, France
[h] School of Environmental Sciences, University of Liverpool, Liverpool, UK
[i] School of Engineering, NUI Galway, Galway H91 HX31 j Ryan Institute, NUI Galway, H91 TK33
[j] Ryan Institute, NUI Galway, Ireland
[k] ANFACO-CECOPESCA, Campus University 16, 36310 Vigo PO, Spain
[l] VertigoLab, Darwin Ecosystème, 87 Quai de Queyries, 33100 Bordeaux, France
[m] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[n] Peruvian LCA Network (PELCAN), Department of Engineering, Pontificia Universidad Católica del Perú, Avenida Universitaria 1801, San Miguel, 15088 Lima, Peru

HIGHLIGHTS

- The review dissects 59 LCA studies about seafood, 90% of them over the last decade.
- LCA methodologies, the origin of the research centres and fish species are reviewed.
- LCA is key to climate change mitigation, energy and food sustainability and security.
- Challenges and potential opportunities for the seafood sector are addressed.
- Nexus water-energy-food to reduce environmental impact getting positive synergies.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 September 2020
Received in revised form 17 November 2020
Accepted 22 November 2020
Available online 14 December 2020

ABSTRACT

Life cycle assessment (LCA) has been widely applied in many different sectors, but the marine products and seafood segment have received relatively little attention in the past. In recent decades, global fish production experienced sustained growth and peaked at about 179 million tonnes in 2018. Consequently, increased interest in the environmental implications of fishery products along the supply chain, namely from capture to end of life, was recently experienced by society, industry and policy-makers.

* Corresponding author.
E-mail address: Israel.ruizsalmon@unican.es (I. Ruiz-Salmón).

https://doi.org/10.1016/j.scitotenv.2020.144094

Editor: Damia Barcelo

Keywords:
Life cycle assessment
Seafood
Fisheries
Nexus
Environmental impacts
Sustainability

This timely review aims to describe the current framework of LCA and its application to the seafood sector that mainly focused on fish extraction and processing, but it also encompassed the remaining stages. An excess of 60 studies conducted over the last decade, along with some additional publications, were comprehensively reviewed; these focused on the main LCA methodological choices, including but not limited to, functional unit, system boundaries allocation methods and environmental indicators.

The review identifies key recommendations on the progression of LCA for this increasingly important sustaining seafood sector. Specifically, these recommendations include (i) the need for specific indicators for fish-related activities, (ii) the target species and their geographical origin, (iii) knowledge and technology transfer and, (iv) the application and implementation of key recommendations from LCA research that will improve the accuracy of LCA models in this sector. Furthermore, the review comprises a section addressing previous and current challenges of the seafood sector. Wastewater treatment, ghost fishing or climate change, are also the objects of discussion together with advocating support for the water-energy-food nexus as a valuable tool to minimize environmental negativities and to frame successful synergies.

Contents

1. Introduction

The 17 Sustainable Development Goals (SDG), launched by the United Nations (UN) for the 2030 agenda, seek "*a shared blueprint for peace and prosperity for people and the planet, now and into the future*" (Sustainable Development Goals, 2020). Among these, SDG-14, "Conserve and sustainably use the oceans, seas and marine resources for sustainable development", specifically addresses the priceless environmental, cultural and social wealth of these water bodies that produce half of the oxygen we breath and provide 16% of the animal protein we eat (European Commission, 2019a). Therefore, addressing these goals are essential for human survival, not only from a biological, but also from a sustaining socio-economic perspective. More specifically, goal SDG-14 focuses on the widely known 'blue economy'; that is, all activities related to oceans, seas and littoral environments including marine living resources, marine extraction of non-living resources, maritime transport, port activities, shipbuilding and repair, and coastal tourism.

In 2018, the production of global capture fisheries reached the highest level ever recorded at 96.4 million tonnes (live weight); whereas, the aquaculture sector attained another all-time record high of 82.1 million tonnes. The labour force involved in the primary sector for fisheries and aquaculture is represented by a total of 59.5 million people (FAO, 2020). China leads the sector as attested to accounting for 35% of the global fish production that is followed by other Asian countries (34%), America (14%), Europe (10%), Africa (7%) and Oceania (1%). In general terms, total fish production followed an increasing trend but was geographically differenced: Asia and Africa have almost doubled their production in the past two decades and America fluctuated since the peak of the mid-1990s due to the strong influence of El Niño – Southern Oscillation on the abundance of anchoveta (*Engraulis ringens*). Whereas Europe suffered a gradual decline since the late 1980s that was buffered in recent years (FAO, 2020).

Beyond the macro data, the sustainability of the seafood sector involves maintaining a complex and dynamic equilibrium: namely, to: i) guarantee social protection for fishermen and fish farmers (Maritime Labour Convention, 2006) and subsidisation (Sumaila et al., 2019); ensure the periodical renewal of fishing grounds and the biodiversity preservation (Johnson et al., 2019), iii) keep the quality and security ruled by the standards of the food supply chain (Gephart et al., 2017); and, iv) face climate change consequences (Peck and Pinnegar, 2018) and other environmental negativities related to captures (e.g., ghost fishing, fuel leaks) or processing stages (e.g., waste streams).

To deal with these complex issues, significant efforts have been made to establish an appropriate legal framework at different levels, particularly the need to progressively apply an environmental approach across many different sectors. Notably, the inclusion of environmental public criteria in decision-making is of paramount relevance, which address the provision of important information about the use of resources from raw materials to water and energy. Although the Blue Economy only represents 2.2% of employment in the European Union (EU)-28 along with a gross domestic product estimated at €15,900 billion (€13,500 without the UK) in 2018 (European Commission, 2020a), the continent has a long history of applying high standards related to environment, food production and consumption policies. The European Commission implemented a shared-management perspective of the oceans and seas through the Common Fisheries Policy (CFP) (European Commission, 2020b) in the 1970s and, more recently, a Green Deal to combat climate change and decouple economic growth from resource use. The implementation of the Circular Economy Action Plan reported in 2019 (European Commission, 2019b), the obligation of landing all discards in 2020 (European Commission, 2018a) or the ban of single-use plastics to minimize marine litter starting in 2021 (European Commission, 2018b) are some of the latest policy strategies in this regard (Ruiz-Salmón et al., 2020). In addition, EU members are also forced to promote policies under this framework and some of them are in place. For instance, Spain, with a coastline of almost 8000 km and the largest fishing industry in the EU, recently designated the agri-food and seafood sector as a priority intervention in the Spanish Circular Economy Strategy (SCES) (Spanish Government, 2020).

A quarter of global greenhouse gas emissions related to food production (Poore and Nemecek, 2018). Consequently, a grand challenge is to effectively promote the production of high-quality food at it' origin that have affordable prices along with commensurately reducing the impact derived from its production, both in terms of emissions, water use, non-valued waste. Also, emphasis should be placed on reducing the impact derived from the generation of waste both in the field of production and arising from consumption. Notably, the SCES strongly recommends an increase in energy efficiency and a reduction of carbon dioxide (CO_2) emissions from the seafood sector: thus, promoting efficient control of fisheries and data collection through improving knowledge in decision-making that underpin adaptation of products to consumer demand, reduce waste or improve recycling will add significant value. These recommendations will be enabled and advanced through applying life cycle assessment (LCA). LCA is the most established scientific tool for environmental analyses as it can fully address the quantification of footprints (e.g., energy, water, carbon) and environmental impact categories (e.g., global warming, eutrophication, human toxicity, etc.) along the life cycle of products and processes by studying the inputs (e.g., resources) and outputs (e.g., residues, by-products) of the system (ISO, 2006a, 2006b). Briefly, an LCA follows 4 inter-related stages: 1) statement of the goal and scope of the study, including the limits of the system and the functional unit; 2) development of inventory flows (inputs and outputs); 3) evaluation of the potential environmental impacts; and, 4) interpretation of the results drawing conclusions and recommendations.

Although LCA has been applied for decades, initially it focused on packaging, energy use and emission reductions in the 1960s. Following a gap in 1970s that coincided with methodological development, the first standardization occurred before the end of the millennium. Notable developments in the early 2000s included the release of the first datasets that emphasised increasing interest in the use of LCA as a potentially disruptive tool applied to national energy systems and waste management systems, among others. In the last two decades, LCA has experienced a methodological consolidation and a commensurate international collaboration across many sectors, such as business, research and innovation, product or process design, education, policy development, labelling, food and agriculture, consumer goods and energy industries (Hauschild et al., 2018). Nowadays, LCA is already part of the Single Market for Green Products Initiative launched by the European Commission (European Commission, 2013). Moreover, Product Environmental Footprint Category Rules (PEFCRs) are currently being updated with marine fish, including both fisheries and aquaculture production of live, fresh, chilled and frozen fish, as well as manufactured products, processed and preserved fish, crustaceans and molluscs (EC, 2020c). LCA has also been highlighted as an important tool to help companies pivot beyond COVID-19, including transitioning for uptake of new green deal innovations and services (Rowan and Galanakis, 2020).

In parallel, scientific LCA publication linked to the capture, farming and processing of fish started in mid-2000 where the LCA-related publications strongly increased from less than 20 cited in 2010 to more than 90 in 2019. Avadí and Fréon (2013) published one of the first reviews on LCA as applied to fisheries that was based on 16 studies issued in the first decade of 2000: these included the typical LCA phases: goal and scope definition, inventory analysis, impact assessment and interpretation. Other LCA reviews published by Vázquez-Rowe et al. (2012b) and Ziegler et al. (2016) also delved primarily into the fisheries sector and associated supply chain, whereas other groups focused on the aquaculture sector (Henriksson et al., 2012; Bohnes et al., 2019). More recently, Avadí et al. (2020) published another LCA review where the main objective was to present the first effort to aggregate and standardize seafood-related datasets in the Ecoinvent database. This LCA also explained the main data sources and commensurate methodological choices used in the building of the datasets. In this context, the current LCA review advances this topic by addressing the whole supply chain of the seafood sector with a particular focus on the fisheries sector. More specifically, the aim of the review is to assemble LCA-based studies published in the past decade, to analyse the evolution in research and innovation, and to describe the database and assessment models. Moreover, hotspots, challenges and opportunities arising from the current seafood sector are discussed from different perspectives including as climate change, economic market and environmental protection as well as the nexus between food, energy and water. No prior published literature review focused on this LCA-nexus integration has been reported. Overall, given the nature of bibliometric analysis performed, the expected audience of this review study are LCA practitioners. However, given the description of the utility and scientific challenges, this review will also appeal to broad range of stakeholders in the seafood sector, especially fish managers in the public and private sectors, and NGOs, which will help future proof the sector.

2. Materials and methods

The importance of the eco-perspective has been introduced in an increasing number of frameworks due to the historic push of the environmental movements and the multiple responses given by the rest of the actors. Occasionally, it is reflected in an eco-friendly commitment from policy-makers, companies and citizens. There are also instances of an eco-style approach to informing communities or green-washing campaigns from industries and big corporations. Nowadays, environmental awareness is a prominent cross-cutting key topic that features strongly in an increasing number of scientific publications along with permeating and influencing adjacent disciplines. In this context, this review addresses LCA studies applied to the fishing sector that focuses mainly on wild fish capture fisheries. It also commensurately addresses seafood processing and other stages of its life cycle based on their inclusion as topics appearing in system boundaries in publihed literature reviewed.

The bibliometric analysis was conducted using Scopus, which is the largest abstract and citation database of peer-reviewed literature; it addressed over 20,000 journals (Geng et al., 2017). This approach provides several options to make the search more accurate and reliable including use of keywords. Thus, three kinds of searches were applied using Scopus as a first step to appraise the breadth of LCA application in marine products and processes during the period 2010 to 2019: 1) using

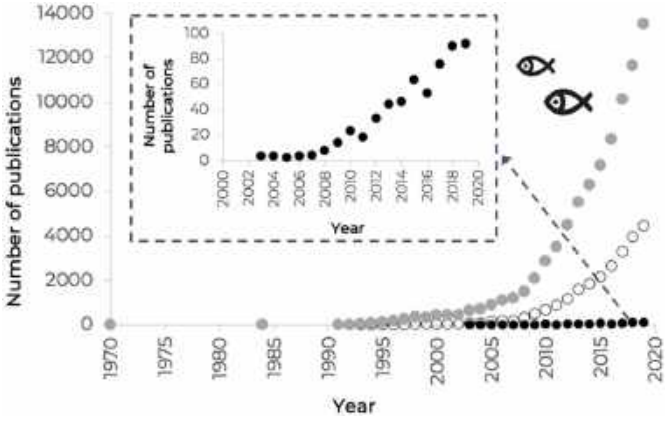


Fig. 1. Number of publications per year in scientific journals that include the search terms "life cycle assessment" (grey circles), plus "food" (white circles) or plus "fish" and "seafood" (black circles) accessed in Scopus in June 2020.

the search term "life cycle assessment" and combining this with 2) "food" and 3) "fish" and "seafood". A total of 537 files were found in the literature search for the period analysed. Fig. 1 highlights an increasing use of LCA studies over the last decade where it appeared in almost 100 publications in both 2018 and 2019. Notwithstanding same, the seafood sector still represents a very low percentage of LCA-published studies that focused on food. Although the search included all kinds of publications, i.e., articles, reviews, conference papers, books, reports, etc., most of them (67-70%) were articles and about 76-83% involved articles or reviews. Thus, a refinement in the literature search was made combining "life cycle assessment" and "fish" or "seafood" terms only on titles, abstracts and keywords. The scope was reduced to 243 documents (198 English full-length articles). Finally, other papers were excluded because the subject area was out of scope -health, mathematics or engineering- (73) or addressed LCA related to diets (15), packaging and food waste (7), only aquaculture species (7), or did not include a case study.

In brief, 69 LCA-related publications have been extensively analysed for this review. Fig. 2 shows the 20 scientific journals where these articles were published. Two of these, the International Journal of Life Cycle Assessment and the Journal of Cleaner Production, represented 43% of the LCA publications that were included in this work. From all the publications assessed, 59 were case studies that included LCA impact categories along with occasional fisheries management indicator work. The other 10 studies encompassed two studies of Avadí and co-authors; namely, a general disposition to LCA (Avadí and Fréon, 2013), and a partial life cycle inventory review (Avadí et al., 2020). Other publications related to the best available techniques (BATs) in the fishing sector (Barros et al., 2009; Bello Bugallo et al., 2013), ecolabeling and certification (Thrane et al., 2009b; Vázquez-Rowe et al., 2016), best practices in LCA implementation and guidelines for managers and policy-makers (Vázquez-Rowe et al., 2012b; Vázquez-Rowe and Benetto, 2014), or regional context of fisheries in Peru (Fréon et al., 2014a) and seafood processing in Denmark (Thrane et al., 2009a). Among the 59 case studies, almost 90% were published after 2010 with only 7 appearing before that year; thus, making this present review representative of the previous decade.

This review does not include aquaculture despite its products representing approximately 15% of the worldwide fish production between 1986 and 1995: where the present day level of production is approximately 46% (FAO, 2020). Advances in science and technology have mirrored economic and labour growth in terms of rate of growth. A number of studies have comprehensively reviewed state-of–the-art in LCA for enhancing aquaculture over the past two years (Bohnes et al., 2019; Bohnes and Laurent, 2019; Philis et al., 2019). However, aquaculture was not a major focus of the current study, as mentioned in Section 1, although this particular sector is partially addressed in the discussion. Studies linked to the production of mussels in mussel rafts, along the coast of Galicia (Spain), were included within the scope of the current review given the fact that an auxiliary fishing fleet is needed to attend the production of this oceanic infrastructure.

The review follows the structure of the ISO standards 14,040 and 14,044 on LCA (ISO, 2006a, 2006b), also suggested by Avadí and Fréon (2013), yet advances this area much further by way of addressing new concepts linked to LCA in cross-cutting fields of study along with embracing current challenges facing the fishing sector. Firstly, a discussion focusing on the main LCA principles and requirements is addressed through use of several tables linked to text in which case studies were desegregated for ease of comparison: these included goal and scope, boundaries of the system, functional unit and life cycle impact assessment methods, impact categories and so forth. Thereafter, this

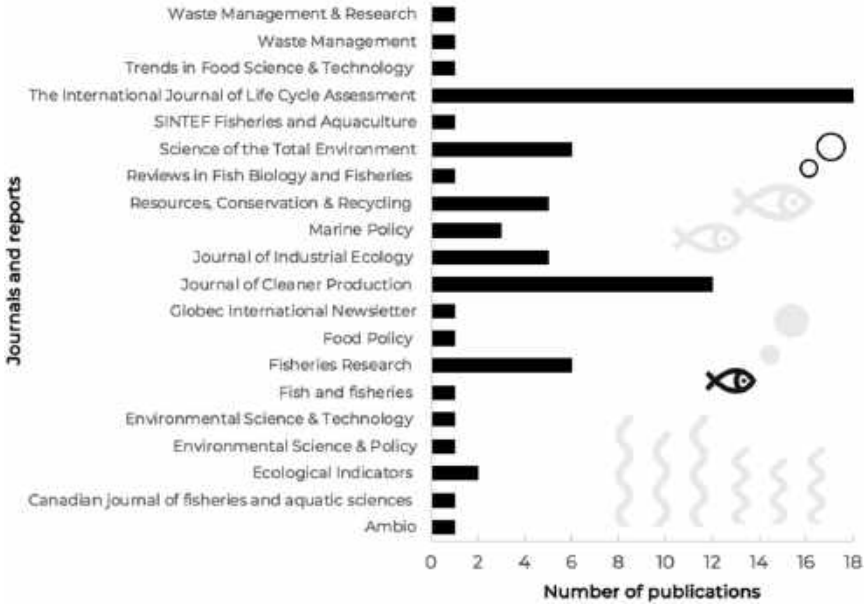


Fig. 2. Distribution of the 69 publications analysed according to the journal of publication.

constitutes the first study that comprehensively reviews use of LCA research for addressing the development of the fishing sector from a global perspective. Finally, the relationship or nexus between energy, water and food is discussed in addition to articulating previous and ongoing implications that hinder technical and environmental progress across the sector, such as marine debris, ghost shipping and climate change.

3. Results and discussion

3.1. Goal and scope definition

The goal definition is framed on addressing key questions, such as, "why do we perform an LCA?", "who are the target audiences?" and "what is the product under study?" However, the scope definition is more complicated as it includes the definition of the system boundaries, the functional unit, the allocation strategy and other relevant hypotheses and assumptions (ISO, 2006a. 2006b). Table 1 presents some of the main characteristics of the reviewed LCA studies: namely, targeted species, functional unit, system boundaries, allocation method and sensitivity analysis.

3.1.1. Functional unit definition

The functional unit (FU) is the element that quantifies the function of the system, the calculation basis for which all inputs and outputs of the system must refer. The selection of a coherent FU is a fundamental step in order to perform a robust and comparable LCA. Choosing an appropriate FU allows direct comparison between alternative scenarios that perform the same function. However, FUs vary greatly, which highlight the difficulties of comparing results reported across many different articles. In this study, the choice of different FUs in the reviewed studies was assessed in detail. Around 30% of analysed papers focused their research on the extraction phase and use the amount of product landed in a port as the FU, typically 1 kg or 1 t. For instance, Abdou et al. (2018) defined their FU as 1 t of landed seafood (shrimp, demersal finfish, mullets, rays and sharks) by demersal trawlers in the Gulf of Gabes. Avadí et al. (2015) used 1 ton of landed fish by the Peruvian anchoveta purse seining fleet in the period 2005-2010 as the study FU. This FU was also used by Lourguioui et al. (2017) to assess mussel cultivation, Avadí et al. (2018) to address Peruvian hake capture, Fréon et al. (2014b) to analyse the Peruvian anchoveta caught in the north-centre fishing zone of Peru and Gonzalez-García et al. (2015) and Villanueva-Rey et al. (2018) to assess European pilchard capture. However, in some cases, such as Driscoll et al. (2015), this perspective includes other life cycle stages in the FU, such as transportation to the processing plant.

An alternative FU was used when studies focused on the processing stage. For example, Barros et al. (2009) analysed the operation of a mussel processing plant for one year, while Bello Bugallo et al. (2013) sought to characterise the operation of different seafood processing plants by the degree of implementation of BATs and an output-based FU was used. A similar case was analysed in Denham et al. (2016), where 1 t of processed fish sold at retail was used as the FU. Mass of packaged product ready for dispatch was a FU used by several authors. Findings of Ziegler et al. (2011), which was replicated in 2012 and 2016 but not included in the tables, defined the FU as 1 kg of shrimp and the accompanying packaging material ready to import to Europe. Almeida et al. (2015) used 1 kg of edible product (i.e., canned sardine with olive oil) as FU, whereas Avadí et al. (2014b) used 1 kg of fish (Peruvian anchoveta) in the final product. Other authors defined the FU as the commercial or serving fish product (Ziegler et al., 2003; Ziegler and Valentinsson, 2008). For example, Iribarren et al. (2010d) used one triple pack of round cans of canned mussels composed by 129 g of canned mussel flesh, 120 g of sauce, 81 g of tinplate can and 12.73 g of cardboard as the FU. Similarly, Laso et al. (2017a) selected one "octavillo" (i.e., a special can with the right amount of product, usually served as individual ration) of canned anchovy as the FU, which was composed by 30 g of canned anchovy, 20 g of extra virgin oil, one aluminium can and cardboard. Parker and Tyedmers (2012a) defined three FUs in accordance with the three Antarctic krill-derived products studied: 1 kg of krill meal, 1 L of krill oil and 1 consumer-ready bottle of 60 omega-3 krill oil capsules. On the other hand, Svanes et al. (2011) defined a specific FU for each of the four-derived cod products under study – wetpack, burger, loin and processing residues to animal feed. Vázquez-Rowe et al. (2011a, 2013a) analysed two types of hake: production of 500 g of raw gutted fresh hake fillet reaching the household including packaging and 1 package of frozen fish sticks containing 10 fish sticks, which corresponds to approximately 320 g of edible product. Based on a nutritional point of view, some authors defined the FU as the amount of protein supplied to consumer. For instance, Vázquez-Rowe et al. (2014b) used the amount of protein (17.26 g) supplied by one can of sardines (85 g) in olive oil produced by a Galician canning industry as the FU, whereas Fréon et al. (2010) defined the FU as 100 g of animal protein of anchoveta or derivative product on the consumer plate.

However, another trend has been to analyse a feedstock-based FU. Hospido et al. (2006) analysed the production of canned tuna and the FU evaluated was 1 t of raw tuna entering the factory. Similarly, Laso et al. (2017b) assessed the production of canned anchovy and the selected FU was also 1 kg of raw anchovy entering the factory. Both options present different advantages that are worth emphasizing. Choosing an input-based FU allows for comparison of the environmental performance of different processes, which simultaneously determines the strengths and weaknesses of these processes. In contrast, selecting an output-based FU allows for detailed product analysis, including its use as a key step in eco-labelling. Fréon et al. (2017) evaluated two scenarios: firstly, the production of fishmeal and fish-oil as by-products of fishing for which an output-based FU (one tonne of by-product produced) was used. However, Laso et al. (2016) evaluated the valorisation of anchovy residues by means of the production of fishmeal and fish oil defining an input-based FU (1 t of anchovy residues). Similarly, Iribarren et al. (2010b) addressed the valorisation of mussel shells and mussel organic remains using an input-based FU (100 t of each residue). Alternatively and secondly, Fréon et al. (2017) aimed at analysing the intrinsic characteristics of the processing process, for which a feedstock-based FU was chosen (1 ton of raw material). Even though they do not follow a conventional LCA approach, the studies by Hallstrom et al. (2019) and Hélias et al. (2018) are also noteworthy. Hallstrom et al. (2019) sought to establish a nexus between the carbon footprint and the nutritional impact of Swedish seafood consumption, for which the author makes a relative comparison between the results and their variation from the median value. Hélias et al. (2018) used fishery operations to develop characterization factors that allow determining biotic resource depletion.

3.1.2. System boundaries

The reasons that influence an author to decide which processes should be included within the boundaries of the system must be duly justified, including what are the clearly defined criteria that govern this decision, and justify same. Studies included in this LCA review show some variability in the definition of the system boundaries. Thus, the scope of the analysis will depend on the approach of the system (Fig. 3): "cradle to grave", involving all stages of the life cycle; "cradle to gate" or "gate to grave", including limits from the beginning of the cycle to a specific "gate" (e.g., from capture to landing, from capture to get the product, etc.) or from a midpoint to the end of life stage, respectively; "gate to gate", for intermediate stages; or specific parts of the life cycle, such as the end of life, product components, etc. (ISO, 2006a, 2006b). Regarding fishery-related LCA studies, system boundaries usually include at least the use and maintenance of the vessel (Almeida et al., 2014; Driscoll and Tyedmers, 2010), while in other cases construction (Abdou et al., 2018; Avadí et al., 2014b; Avadí et al., 2018; Driscoll et al., 2015; Gonzalez-García et al., 2015; Hospido and Tyedmers,

Table 1
Main characteristics of the reviewed LCA studies: Functional Unit, System Boundaries, Allocations and Sensitivity analysis.

Reference	Targeted species	Functional unit	System boundaries	Allocation method	Sensitivity analysis
Abdou et al., 2018	Shrimp and demersal finfish (*Sparidae Diplodus annularis, Sparus aurata*), mullets (*Mullus barbatus, M. surmuletus*), rays (e.g. *Raja clavata*) and sharks (e.g. *Mustelus mustelus*).	1 t of landed seafood	Cradle to gate	No	No
Abdou et al., 2020	Shrimp and demersal finfish (*SparidaeDiplodus annularis, Sparus aurata*), mullets (*Mullus barbatus, M. surmuletus*), rays (e.g. *Raja clavata*) and sharks (e.g. *Mustelus mustelus*).	1 t of landed seafood	Cradle to gate	No	Yes, management scenarios: establishment of marine protected areas, extension of the biological rest period, and decrease in the number of demersal trawlers.
Almeida et al., 2014	European pilchard (*Sardina pilchardus*)	1 kg of landed sardine,	Cradle to gate	Mass	Yes, gear and time lapse comparison.
Almeida et al., 2015	European pilchard (*Sardina pilchardus*)	1 kg edible of canned sardine with olive oil	Cradle to gate	Mass	Yes, packaging analysis and comparison with other seafood products.
Avadí et al., 2014a	Peruvian anchoveta *(Engraulis ringens)*	1 kg of fish in the final product	Cradle to gate	Mass	Yes, electricity reduction, packaging material and reduction in-plant discards.
Avadí et al., 2014b	Peruvian anchoveta *(Engraulis ringens)*	1 t of landed fish	Cradle to gate	No	Yes, Data Envelopment Analysis (DEA) to determine the relative efficiencies of multiple comparable units.
Avadí et al., 2015	Ecuadorian tuna yellowfin *(Thunnus albacares)*, skipjack *(Katsuwonus pelamis)* and bigeye *(Thunnus obesus)*	1 t of tuna product	Cradle to gate	Mass	Yes, fuel use intensity and packaging material.
Avadí and Fréon, 2015	Peruvian anchoveta *(Engraulis ringens)*, trout *(Oncorhynchus mykiss)*, tilapia *(Oreochromis spp.)* and black pacu *(Colossoma macropomum)*	1 t of edible fish in a Direct Human Consumption product in the case of anchoveta and fresh fish edible portion for cultured species.	Cradle to gate	Mass	No
Avadí et al., 2018	Peruvian hake (*Merluccius gayi peruanus*)	1 t of whole hake landed	Cradle to gate	Mass	Yes, fuel use intensity and comparison with published results from other hake fisheries and with another Merlucciidae fish, the Patagonian grenadier (*Macruronus magellanicus*).
Avadí et al., 2020	South Pacific anchovies and hake (including *Patagonian grenadier-Macruronus magellanicus*) and Pacific tunas (*Thunnus spp.*), tilapia (*Oreochromis spp.*) and trout (*Oncorhynchus mykiss*)	N/A	Cradle to grave	Mass, economic	No
Denham et al., 2016	Different species of finfish: Crimson snapper (*Lutjanus erythropterus*), Bluespotted emperor (*Lethrinus punctulatus*) and Rosy threadfin bream (*Nemipterus furcosus*) among others.	1 t of processed fish sold at retail	Gate to gate	Mass	Yes, cleaner production strategies: solar electricity, biogas electricity, reduction of GHG emissions from refrigeration, and utilizing waste to develop by-products.
Driscoll and Tyedmers, 2010	Atlantic herring (*Clupea harengus*)	1 t of fish landed	Cradle to gate	N/A	Yes, variations of Total Allowable Catch and purse seine fishing effort.
Driscoll et al., 2015	American lobster (*Homarus americanus*)	1 t of live lobster	Cradle to gate	Mass	Yes, comparison of different scenarios: no allocation between the main product and co-products, electricity use for storage, fuel use in vessel, different database for fuel combustion and post-capture mortality rate.
Farmery et al., 2015	White banana prawn (*Fenneropenaeus merguiensis*)	1 kg of frozen prawn	Cradle to gate	Mass	Yes, comparison of impact method (IPCC 100 years, CML 2 Baseline 2000 and ReCiPe). Also catch variation: 10% increase and decrease in catch with the same number of boat days.
Fréon et al., 2010	Peruvian anchoveta (*Engraulis ringens*)	100 g of protein	Cradle to gate	No	No
Fréon et al., 2014b	Peruvian anchoveta (*Engraulis ringens*)	1 t of fresh fish	Cradle to gate	No	No
Fréon et al.,	Peruvian anchoveta (*Engraulis*	1 t of fresh fish	Cradle to gate	Mass and economic	Yes, simulations of fuel use

Table 1 (*continued*)

Reference	Targeted species	Functional unit	System boundaries	Allocation method	Sensitivity analysis
2014c	*ringens*)				variations of ±20% and recomputing single scores considering mass allocation.
Fréon et al., 2017	Peruvian anchoveta (*Engraulis ringens*)	(i) 1 t of fish oil or fishmeal at the gate of the plant and (ii) 1 t of raw material at the plant	(i) Cradle to gate and (ii) Gate to gate	Gross energy content, economic value and mass	Yes, cleaner production strategies: using natural gas instead of heavy fuel.
Gonzalez-García et al., 2015	European pilchard (*Sardina pilchardus*)	1 t of landed pilchard	Cradle to gate	No	No
Hallstrom et al., 2019	Alaskan pollock (*Theragra chalcogramma*), Arctic char (*Salvelinus alpinus*), Cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), Atlantic salmon (*Salmo salar*), Cape hake (*Merluccius capensis*), Cephalopods (*Cephalopoda spp.*), European eel (*Anguilla anguilla*), European flounder (*Platichtys flesus*), Hake (*Merluccius merluccius*), Seabass (*Dicentrarchus labrax*), Sprat (*Sprattus sprattus*), Gilt-head seabream (*Sparus aurata*), Haddock (*Melanogrammus aeglefinus*), Hoki (*Macruronus novaezelandiae*), Lobster (*Homarus gammarus*), Northern prawn (*Pandalus borealis*), Norway lobster (*Nephrops norvegicus*), Oyster (*Ostreidae spp.*), Pangasius (*Pangasius hypophthalmus*), Perch (*Perca fluviatilis*), Pike (*Esox lucius*), Pike--perch (*Sander lucioperca*), Pink salmon (*Oncorhynchus gorbuscha*), Plaice (*Pleuronectes platessa*), Rainbow trout (*Oncorhynchus mykiss*), Saithe (*Pollachius virens*), Scallop (*Pecten maximus*), Tilapia (*Oreochromis niloticus*), Trout (*Salmo trutta*), Turbot (*Scophthalmus maxima*), Whitefish (*Coregonus spp.*), Whiting (*Merlangius merlangus*)	N/A. Results of GHG emissions and nutritional score are presented as a variation of the median of the entire analysed sample.	Cradle to gate	No	Yes, variation in nutritional results when using different methods.
Hélias et al., 2018	Black-bellied anglerfish (*Lophius budegassa*), White anglerfish (*Lophius piscatorius*), Cod (*Gadus morhua*), Roundnose grenadier (*Coryphaenoides rupestris*), Haddock (*Melanogrammus aeglefinus*), Hake (*Merluccius merluccius*), Greenland halibut (*Reinhardtius hippoglossoides*), Herring (*Clupea harengus*), Ling (*Molva molva*), Blue ling (*Molva dypterygia*), Mackerel (*Scomber scombrus*), Horse mackerel (*Trachurus trachurus*), Megrim (*Lepidorhombus whiffiagonis*), Four-spot megrim (*Lepidorhombus boscii*), Plaice (*Pleuronectes platessa*), Beaked redfish (*Sebastes mentella*), Golden redfish (*Sebastes norvegicus*), Saithe (*Pollachius virens*), Sandeel (*Ammodytes spp.*), Seabass (*Dicentrarchus labrax*), Sole (*Solea solea*), Sprat (*Sprattus sprattus*), Tusk (*Brosme brosme*), Whiting (*Merlangius merlangus*) and Blue whiting (*Micromesistius poutassou*)	N/A	N/A	No	No
Hospido and Tyedmers, 2005	Skipjack tuna (Katsuwonus pelamis) and Yellowfin tuna (*Thunnus albacares*)	1 t of frozen fish landed	Cradle to gate	No, they consider various target species within their global FU	Yes, increase and decrease fuel inputs by one standard deviation and the use of alternative emission factors from different sources.
Hospido et al., 2006	Tuna (*Thunnus albacares*)	1 t of frozen fish entering the factory	Gate to grave	Economic (for transport from retailers to	Yes, improvement actions: recycled percentage of

(*continued on next page*)

Table 1 (*continued*)

Reference	Targeted species	Functional unit	System boundaries	Allocation method	Sensitivity analysis
				households)	packaging materials, substitution of packaging materials.
Iribarren et al., 2010a[a]	Mussel (*Mytilus galloprovincialis*)	(i) 1 kg of fresh mussels and (ii) 1 kg of canned mussels' flesh	Purification/transformation and consumption stages. Excluded mussel culture and valorization of mussel organic waste and shells	Mass	No
Iribarren et al., 2010b[a]	Mussel (*Mytilus galloprovincialis*):	(i) 100 t of mussel shells and (ii) 100 t of mussel organic remains	Grave to grave: valorization of shells to calcium carbonate and organic waste to fish meal	System expansion for waste valorization	Yes, differences between current methodology of valorization (producing calcium carbonate) and others (landfilling, incineration) are considered. Also, similar differences between current organic waste valorization to fish meal and alternative production of mussel pate are analysed.
Iribarren et al., 2010c[a]	Mussel (*Mytilus galloprovincialis*)	100 kg of cultured mussel: 40 kg for fresh, 35 canning, 20 frozen in cooking-freezing plants and 5 from cooking plants for cannery. For comparative effects 1 kg of fresh, canned or frozen mussel	Cradle to grave named business-to-consumer (B2C)	System expansion for waste valorization (same procedure that Iribarren et al., 2010b)	Yes, regarding analysis based on 1 kg of protein supplied comparing mussels and chicken and canned tuna.
Iribarren et al., 2010d[a]	Mussel (*Mytilus galloprovincialis*)	One triple pack or rounds cans format (129 g of canned mussels, 120 g of sauce, 81 g of primary packaging and 12.73 g of secondary packaging)	Cradle to grave (B2C)	System expansion for waste valorization (same procedure that Iribarren et al., 2010b)	No
Iribarren et al., 2010e	Species from coastal fishing (horse mackerel, Atlantic mackerel, European pilchard and blue whiting), offshore fishing (european hake, megrim and anglerfish), deep-sea fishing (skipjack and yellowfin tuna), extensive aquaculture (mussels) and intensive aquaculture (turbot)	1 t of fish	Cradle to gate	Economic and mass (sensitivity analysis)	Capital goods are relevant in carbon footprint results for extensive aquaculture species but not for the others.
Iribarren et al., 2011	Species from coastal fishing, offshore fishing, deep-sea fishing, extensive aquaculture and intensive aquaculture (same species that Iribarren et al., 2010e)	1 t of fish	Cradle to gate	Economic and mass	Yes, based on the type of cooling agents.
Laso et al., 2016	Anchovy *(Engraulis encrasicolus)*	For heads and spines: 1 t of fish meat entering the plant. For the remaining and broken fish: 1 t paste processing	Gate to grave.	Economic and system expansion for anchovy waste valorisation	No
Laso et al., 2017a	Anchovy *(Engraulis encrasicolus)*	1 can of fish in extra virgin olive oil.	Cradle to gate (from fish to factory), gate to gate (factory process and canned products) and gate to grave (distribution and use and EoL)	System expansion for anchovy waste valorisation	Yes, different scenarios: packaging recycling is proposed -Application of BATs for canned anchovy industry such as recycle process water, recycle cardboard boxes, separate possible valorization streams, dry cleaning…
Laso et al., 2017b	Anchovy *(Engraulis encrasicolus)*	1 kg of fish entering the canning plant	Cradle to grave	System expansion, mass and economic	Yes, sensitive analysis based on mass or economic allocation.
Laso et al., 2018a	Anchovy *(Engraulis encrasicolus)*	1 kg of processed fish	Cradle to grave	System expansion	Yes, Green protein footprint according the packaging type or no packaging.
Laso et al., 2018b	Anchovy *(Engraulis encrasicolus)*	1 kg of fish	Cradle to gate	System expansion	No
Laso et al., 2018c	Anchovy *(Engraulis encrasicolus)*	1 t fish food loss (FL)	2 scenarios: Food waste-to-energy-to-food" and "Food-waste-to-food".	System expansion	No
Lourguioui et al., 2017	Mussel *(Mytilus galloprovincialis)*	1 t of mussels	Cradle to gate	No	Scenarios for mussel farms management and uncertainty analysis/Monte Carlo
Lozano et al.,	Mussel	1 t of mussels for each	Cradle to gate	No	No

Table 1 (*continued*)

Reference	Targeted species	Functional unit	System boundaries	Allocation method	Sensitivity analysis
2010		raft			
Parker and Tyedmers, 2012a	Antarctic krill (*Euphausia superba*)	1 kg of krill meal 1 L of krill oil 1 consumer-ready bottle of 60 omega-3 krill oil capsules	(i) Krill meal and oil: cradle to consumer (ii) Krill omega-3 capsules: cradle to retailer	(i) Krill meal and oil: energy content in fishing and primary processing (ii) Krill meal and oil: mass in transport to port (iii) Krill omega-3 capsules: system expansion in the secondary processing	Application of three allocation scenarios to omega-3 capsules. Scenario analyses of different parameters for krill meal and omega-3 capsules.
Parker and Tyedmers, 2012b	Peruvian anchovy *(Engraulis ringens)*, Atlantic herring *(Clupea harengus)*, Gulf menhaden *(Brevoortia patronus)*, blue *whiting (Micromesistius poutassou)* and Antarctic krill *(Euphausia superba)*	For each species: 100 GJ of combined meal and oil products, respecting the species-specific yields of meal and oil	N/A	Output nutritional energy of meal and oil products	Uncertainty analysis/Monte Carlo Sensitivity analysis to the FU (basis of comparison between species): ▪ 100 GJ of energy from meal and oil (baseline); ▪ 1 t of protein from meal and oil ▪ 1 t wet weight biomass
Parker et al., 2015	4 tuna species: skipjack (*Katsuwonus pelamis*), yellowfin (*Thunnus albacores*), albacore (*Thunnus alalunga*), bigeye (*Thunnus obesus*)	1 t of landed fish	Cradle to gate	Mass	No
Ramos et al., 2011	North East Atlantic Mackerel (NEAM) *(Scomber scombrus)*	1 t of landed round fish	Cradle to gate	Temporal allocation	No
Svanes et al., 2011	Cod *(Gadus morhua)*	1 kg cod wetpack, frozen, in 400 g packages, delivered to retailer in Sweden 1 kg cod burger, frozen, in 5 kg packages, delivered to institutional buyer in Sweden 1 kg processed cod loin product in 2 kg package, delivered to distribution centre in the UK 1 kg processing residue, frozen, going to animal feed production in Norway	Cod wetpack and cod burger: cradle to consumer Processed cod loin: cradle to distribution Cradle to gate (arrival at the processing plant)	Mass and economic	Sensitivity analysis based on either mass and economic allocation. Scenario analyses on different parameters.
Van Putten et al., 2016	Tropical rock lobster (TRL, *Panulirus ornatus*) and southern rock lobster (SRL, *Jasus edwardsii*)	1 kg of lobster	Cradle to consumer	a) mass, assuming heads are wasted; b) mass, assuming heads are used; c) nutritional value (total MJ of edible product); d) economic (ex-vessel price)	Scenario analyses, using base case mass allocation on different parameters.
Thrane, 2004	Codfish, flatfish, prawn, shrimp, Norway lobster, mussels, herring, mackerel, industrial fish	1 kg of fish	Cradle to gate	System expansion and mass and economic allocation	No
Thrane, 2006	Flatfish	1 kg of frozen fish filet	Cradle to cradle	System expansion and mass and economic allocation	No
Vázquez-Rowe et al., 2010a	Atlantic horse mackerel (*Trachurus trachurus*)	1 t of round fish	Cradle to gate	Mass and economic	Yes
Vázquez-Rowe et al., 2010b	European hake (*Merluccius merluccius*), horse mackerel (*Trachurus trachurus*), Atlantic mackerel (*Scomber scombrus*), blue whiting (*Micromesistius potassou*)	1 kg of fish	Cradle to gate	No, global catch value was considered as FU	No
Vázquez-Rowe et al., 2011a	European hake (*Merluccius merluccius*)	500 g of gutted fish fillet	Cradle to grave	Mass and economic	No
Vázquez-Rowe et al., 2011b	Broad number of vessels within selected Galician fishing fleets (target species are quite varied, depending on the gear type and geographical zone where they fish)	1 t of landed fish	Cradle to gate	Mass	No
Vázquez-Rowe et al., 2012a	Common octopus (*Octopus vulgaris*)	24 kg of frozen octopus up to the point of import	Cradle to gate	Mass	No
Vázquez-Rowe	Hake (*Macruronus magellanicus*) fish	1 package of 10 frozen	Cradle to gate	Mass	No

(*continued on next page*)

Table 1 (*continued*)

Reference	Targeted species	Functional unit	System boundaries	Allocation method	Sensitivity analysis
et al., 2013a	sticks produced in a processing plant in Spain	fish sticks			
Vázquez-Rowe et al., 2013b	Hake (*Macruronus magellanicus*) fish sticks produced in a processing plant in Spain	1 package of 10 frozen fish sticks	Gate to grave	Mass	No
Vázquez-Rowe et al., 2013c	Goose barnacle (*Pollicipes pollicipes*)	1 kg of barnacles	Cradle to gate	No, due to the lack of co-products	No
Vázquez-Rowe et al., 2014a	Seafood species landed in Galician ports	1 of fish	Cradle to gate	Mass	No
Vázquez-Rowe et al., 2014b	European pilchard (*Sardina pilchardus*)	Amount of protein (17.26 g) supplied by one can of sardines (85.0 g) in olive oil	Cradle to gate	Mass, economic and energy	No
Villanueva-Rey et al., 2018	European pilchard (Sardina pilchardus)	1 t of fish	Cradle to gate	Mass	Yes, different assessment methods, allocation approach (economic), fishing gear life span, base port influence, engine type.
Winther et al., 2009	Norwegian seafood supply chain: a) aquaculture: Atlantic salmon (*Salmo salar*) and Blue mussels (*Mytilus edulis*); b) fishing: cod (*Gadus morhua*), saithe (*Pollachius virens*), haddock (*Melanogrammus aeglefinus*), herring (*Clupea harengus*) mackerel (*Scomber scombrus*)	1 kg of edible product	Cradle to gate	Mass	Yes, different scenarios were analysed: electricity mix, product waste, edible yield, allocation approach (economic), utilization of processing stage by-products, feed conversion ratio, refrigerant agent, etc.
Ziegler et al., 2003	Cod (*Gadus morhua*)	400 g of fish fillets	Cradle to gate	Economic	Yes, different scenarios were considered for fishing based on fishing gear.
Ziegler and Valentinsson, 2008	Norway lobster (*Nephrops norvegicus*)	300 g of lobster tails	Cradle to gate	Economic	Yes, fuel use, allocation choice, product yield, impact assessment method and background data.
Ziegler et al., 2011	Southern pink shrimp (*Penaeus notialis*)	1 kg of shrimp	Cradle to gate	Economic	No

[a] The production of mussels (*Mytilus galloprovincialis*) in Galicia, Spain, corresponds to extensive aquaculture. However, an important auxiliary fishing fleet (1267 vessels according to the 2020 regional census) supports the cultivation of mussels in mussel rafts along the Galician rias (Pesca de Galicia, 2020).

2005; Ramos et al., 2011; Vázquez-Rowe et al., 2010a; Vázquez-Rowe et al., 2010b; Vázquez-Rowe et al., 2011b) and end of life stages (Fréon et al., 2014b; Laso et al., 2018b) are also included. Only two studies assessed the consumption of fuel within the fishing activity (Parker et al., 2015; Thrane, 2004), distinguishing between fuel used during propulsion to reach fishing grounds and fuel used during the actual extraction of fish. Alternatively, Lozano et al. (2010) considered mussel cultivation farming, including the construction, operation and maintenance of mussel rafts. However, these authors also addressed the environmental impact of the auxiliary boats used by the sector to reach the rafts, which are usually located several miles away from the coast. Beyond vessel and farming-related activities, the systems commonly end at the harbour, when fish is landed. Within the sample analysed, a small number of studies included fish transport to the processing plant -e.g. Farmery et al. (2015), while others included transport to retail (Driscoll et al., 2015).

With regard to studies focusing on processing, the most recurrent feature is that the system boundaries include activities carried out directly in the processing plant, in addition to ancillary operations such as power or steam production, excluding the fishing or farming stage (Iribarren et al., 2010a). On the other hand, some authors considered the fishing stage together with processing (e.g. Almeida et al., 2015; Avadí et al., 2018). Fréon et al. (2017) also included the fishing stage within the boundaries of fishmeal and fish-oil production, which allowed performing an exhaustive study of the environmental impacts related to the production of these co-products. Svanes et al. (2011) and Vázquez-Rowe et al. (2012a) added one additional step along with including the distribution to retailer of cod and octopus' products, respectively. Winther et al. (2009) and Ziegler et al. (2012) also included the transportation of Norwegian seafood products to the wholesaler. Other authors advanced thee activities further; for instance, Hospido et al. (2006) also analysed transportation stages to wholesale and consumption at the households that followed a gate-to-grave approach. Similarly, Fréon et al. (2010) and Laso et al. (2017a) assessed the whole life cycle of Peruvian and European anchovy, respectively, considering anchovy capture, production, transport, use and disposal. Vázquez-Rowe et al. (2011a) analysed the life cycle of fresh hake including the household consumption. Denham et al. (2016) included the transportation of all consumable items to city and regional retailer and waste disposal to landfill. In summary, the majority of studies were cradle-to-grave analysis, with some studies adopting either gate-to-gate or gate-to-cradle type approaches.

3.1.3. Allocations

Multifunctional processes are those economic systems that i) produce more than one valuable output (multi-output) (Huijungs and Guinée, 2007), ii) have more than one input (multi-input), such as waste treatment processes with a mixture of input waste flows (Iribarren et al., 2010c); or iii) transform a product into another product (open-loop recycling). In all these systems, the environmental burdens associated with a particular process must be partitioned over the various functional flows of that process (Margallo et al., 2014).

To handle this multifunctional problem, the ISO standard (ISO, 2006a, 2006b) establishes a preference order that consists on dividing processes into sub-processes, or expanding system boundaries to include the additional functions. If that is not possible, then the allocation problem must be solved by using physical causation or other relationships, including the economic value, mass or energy content of the functional outputs (Azapagic and Clift, 1999). In other circumstances, allocations are not needed as authors focus on the contribution of each production stage or fishing gear to the environmental impacts,

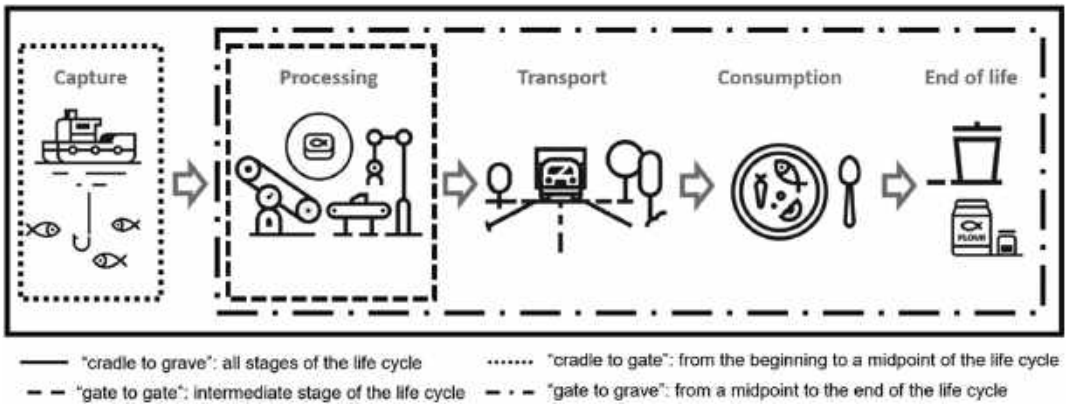


Fig. 3. System boundaries applied in LCA for the seafood sector.

instead of the impact associated to the different species landed (Abdou et al., 2018; Lozano et al., 2010).

Most of the LCA studies analysed in this review can be classified as multi-output process. The most common need for allocation arises when fishing fleets land by-catch (organisms inadvertently captured while fishing for more valuable or legally permitted species) or target multiple species. In these cases, the use of system expansion is not usually implemented due to the lack of fisheries where only the by-catch species are caught (Ayer et al., 2007). Exceptionally, Thrane (2004, 2006) applied a combination of technical subdivision along with system expansion, mass and economic allocation to conduct an energy analysis and an LCA, respectively. In mass and economic allocation, the fuel consumption and impacts were distributed to each species based on the proportion to their weight and the catch value. Ideally, the system expansion should consider that a by-catch of a particular species affects the fishing vessels targeting that species, as their quotas are reduced proportionally to meet the overall quota -in those countries that fix it- (Thrane, 2004). Hence, the by-catch substitutes catch (or quota) in other Danish fisheries, which targets these species (Thrane, 2006).

Nonetheless, in most studies, multi-output fishery systems are solved using mass, energy or economic allocations. Despite that, some authors define mass allocation as arbitrary and unjustified; however, this procedure has been widely applied (e.g. Avadí et al., 2015; Avadí and Fréon, 2015; Avadí et al., 2018; Driscoll et al., 2015; Parker et al., 2015; Vázquez-Rowe et al., 2014b; Vázquez-Rowe et al., 2016; Villanueva-Rey et al., 2018; Winther et al., 2009) because there is no other way to particularize inputs to specific species during fishing operations. On the other hand, economic allocation has generally been defended as a reasonable and more socially relevant approach (Ayer et al., 2007). However, several authors point out that the main problem of this approach is the highly volatile economic price of the product, which depends on the season, freshness of the product and many other market factors (Vázquez-Rowe et al., 2013a; Vázquez-Rowe et al., 2011a), making it difficult to establish a stable allocation over time (Winther et al., 2009). Pelletier and Tyedmers (2011) warn about the difficulties of applying economic criteria to partition natural systems.

Contrasting several allocation methods provides more robust and precise LCA results Avadí and Fréon, 2013). This type of approach, using economic and mass allocation was conducted in several studies (Fréon et al., 2017; Vázquez-Rowe et al., 2011a). Vázquez-Rowe et al. (2011a) compared the use of mass and economic allocation for fresh hake fillet captured by the Galician, founding similar results since the average sale price for European hake does not entail major differences. However, the use of economic allocation was shown to be preferred to mass allocation in mixed fisheries where the landed species have great differences in economic value (Ayer et al., 2007). Ramos et al. (2011) proposed a temporal allocation for the evaluation of fishing of North East Atlantic Mackerel (NEAM). This procedure was applied to construction and maintenance materials, as the Basque coastal purse-seining fishing fleet presents three distinct fishing seasons. Fishing activity is focused on NEAM and on the anchovy during the first half of the year, while the albacore fishing season takes place in the second half of the year.

In the case of fish processing, the main allocation problem is obtaining data of secondary co-products. In these studies, the use of system expansion is more common, since it is easier to find an alternative by-product. Additionally, the substitution by another fishery or non-fishery protein source is always possible (Avadí and Fréon, 2013). The valorisation of shells and mussel meat by-product was allocated by substitution to alternative products. Mussel shells can be used as raw material for calcium carbonate production and it was assumed that 100 t of shells avoids the conventional production of 65 t of calcium carbonate (Iribarren et al., 2010b). Similarly, the valorisation of organic waste from anchovy canning plants results in anchovy paste (from remaining anchovies) and fishmeal and fish oil (from head and spines). The impact of these co-products was allocated using a system expansion based on substitution for alternative products (Laso et al., 2016; Laso et al., 2017a; Laso et al., 2017b; Laso et al., 2018a; Laso et al., 2018b; Laso et al., 2018c). Particularly, these authors assumed that 1 t of anchovy paste substituted the production of 1 t of tuna pâté given its similar protein content, whereas 1 t of heads and spines replaced the production of 212 kg of fishmeal and 108 kg of fish oil from fresh anchovy (Laso et al., 2016). This assumption was compared with mass and economic allocation (Laso et al., 2017b). Similarly to fisheries, if there is a lack of alternative production systems for the by-product under analysis, causality or non-causality allocation were applied (Vázquez-Rowe et al., 2013a; Winther et al., 2009).

Mass allocation was used for processed finfish (Denham et al., 2016), tuna (Avadí et al., 2014a), frozen prawn (Farmery et al., 2015), frozen common octopus (Vázquez-Rowe et al., 2012a), and frozen hake sticks (Vázquez-Rowe et al., 2013a; Vázquez-Rowe et al., 2013b). The use of mass or economic allocation was analysed for the Galician fishing activity (Iribarren et al., 2010e; Iribarren et al., 2011) providing changes in the carbon footprint from 0.3% to 57%, denoting the importance of the allocation method.

Svanes et al. (2011) evaluated the environmental performance of cod caught by the autoline fleet in Norwegian territorial waters and its processing in four cod-derived products: wetpack, burger, loin and processing residues to animal feed. Using mass allocation, the main differences were found in the choice of whether the head was considered a co-product or a waste, while low variations were observed with economic allocation.

Economic allocation was the preferred option for packaged cod fillets (Ziegler et al., 2003) as they were dominating both in quantity and in gross sales and for Norway lobster (Ziegler and Valentinsson,

2008) and Southern pink shrimp (Ziegler et al., 2011) due to its high economic value.

Allocation based on the energy content has been traditionally downplayed as an arbitrary method that does not generally reflect the relationship between the inputs and outputs of a studied system (Ayer et al., 2007). This is common for other non-causality allocations, such as mass or economic allocation. To analyse the impact of Peruvian fishmeal or fish oil, Fréon et al. (2017) applied gross energy content, as well as economic value and mass. In addition, Parker and Tyedmers (2012a) applied allocations based on energy content, mass allocation and system expansion. This study defined three functional units: 1 kg of krill meal, 1 L of krill oil and 1 consumer-ready bottle of 60 omega-3 krill oil capsules. The authors used energy allocation for fishing and primary processing, mass allocation for transport of meal to port, and system expansion to allocate between omega-3 capsules and lower grade meal. A sensitivity analysis evaluated three allocation scenarios to omega-3 capsules: i) energy allocation for fishing and primary processing; ii) mass allocation for transport of meal to port, and iii) system expansion to allocate between omega-3 capsules and lower grade meal.

LCA practitioners may avoid allocation, if the environmental impacts are assigned to the fishing gear or the production step, instead of the species. Nevertheless, when the species and by-products are considered, it is first recommended to expand the boundaries of the system prior to allocation and, if possible, use both mass and economic allocation for the same case study in order to compare approaches. Generally, mass allocation gives initial accuracy on data (widely known) if the energy content of all species is within the same range. If the variability in nutrient content of the species landed is high or will be transformed into sub-products (e.g., fish oil), energy allocation is suggested (Avadí and Fréon, 2013) together with the use of economic variables. However, as mentioned above, sometimes this information is not available and is strongly influenced by market fluctuations.

3.1.4. Sensitivity analysis

When sensitivity analysis was conducted for the studies reviewed mainly discussed the fuel use of vessels (Avadí et al., 2015, 2018; Fréon et al., 2014c; Hospido and Tyedmers, 2005), packaging and cleaner production strategies. Although the manufacture of primary packaging for marine products is not the main contributor to the total environmental impact along it's life cycle (Molina-Besch, 2016), several authors evaluated the kind of material and the recycling percentage of the packaging material used (Almeida et al., 2015; Avadí et al., 2014a, 2015; Hospido et al., 2006).

Linked to cleaner production strategies, Laso et al. (2017a) proposed some BATs for canned anchovy industry, such as reuse of process water, recycling of cardboard boxes, separate possible valorisation streams or dry cleaning. Other authors also applied sensitivity analysis for BATs. For instance, Avadí et al. (2014a) and Driscoll et al. (2015) evaluated the use of electricity and, concretely, Denham et al. (2016), focused on solar electricity and biogas electricity, and other scenarios such as the reduction of greenhouse gas (GHG) emissions from refrigeration and utilizing waste to develop by-products. Meanwhile, Iribarren et al. (2010b) considered differences between current methodology of mussel valorisation (producing calcium carbonate) and others (landfilling, incineration) and concluded that the contribution of environmental impacts coming from valorisation processes are lower compared to mussel culture, fresh mussel purification or mussel transformation in cannery factories.

Finally, some studies collected the impacts of the fishing gear (Almeida et al., 2014; Driscoll and Tyedmers, 2010; Ziegler et al., 2003), catch variation (Driscoll and Tyedmers, 2010; Farmery et al., 2015), or multiple variables. For instance, Abdou et al. (2020) addressed management scenarios, varying the marine protected areas or the extension of the biological rest period, as well as evaluated a decrease in the number of demersal trawlers (Abdou et al., 2020). Driscoll et al. (2015) studied the reduction of refrigerant leakage of the fishing fleet, the fuel consumption to wetpack and the use of energy, packaging and water, and the increment of gutted fish parts to human consumption. Winther et al. (2009) discussed electricity mix, product waste, edible yield, by-products and refrigerant agents.

3.2. Life cycle impact assessment (LCIA)

Regarding life cycle impact assessment (LCIA) methods, CML-IA (Guinée, 2001) was the most widely used representing approximately 50% of the studies reviewed. There was a marked decline in the use of its versions (2000, 2001 and 2002), as evident in Table 3. The impact categories included in this method are those used in many LCA studies. The baseline indicators, which are the standard, are based on the best practice principle available and are category indicators at the outcome level (also referred to as the problem-oriented approach). LCA practitioners rarely applied the non-baseline, which addresses 11 impact categories in CML 2001, disaggregated into 50 subcategories, against the 8 general impact categories of the baseline (acidification, climate change, depletion of abiotic resources, ecotoxicity, eutrophication, human toxicity, ozone layer depletion and photochemical oxidation).

Likewise, more recent assessment methods were applied, such as ReCiPe (Huijbregts et al., 2017) that was implemented in approximately 25% of the publications analysed. This method can be described as an update and combination of 18 midpoint indicators of the CML 2001 and the three endpoint indicators (i.e., damage to human health, ecosystem quality, and resource availability) of the Ecoindicator 99 methodologies. In addition, utilization of other assessment methods addressing specific impacts, such as the carbon footprint that was updated through the 2001, 2007 and 2013 versions of the IPCC, the PAS 2050 British specification published in 2008 (Iribarren et al., 2010e, 2011), and the GHGs emission factors (Denham et al., 2016; Driscoll and Tyedmers, 2010; Hallstrom et al., 2019); or the energy consumption through the Cumulative Energy Demand (CED) (Vázquez-Rowe et al., 2014a), aiming at energy return on investment (EROI) calculation.

Some of the aforementioned methods were used for the same studies, or in combination with others. For instance, UseTox, developed under the United Nations Environment Program and the Society for Environmental Toxicology and Chemistry Life Cycle Initiative (UNEP-SETAC) (Rosenbaum et al., 2008), was applied together with ICLM and ReCiPe to evaluate the air, agricultural soil, natural soil, freshwater and seawater dimension of the impacts (Avadí et al., 2014a; Avadí and Fréon, 2015; Fréon et al., 2014b), while the ILCD recommendation (European Commission, 2011) was also implemented with ReCiPe and CED by Winther et al. (2009).

3.2.1. Impact categories in LCIA

Almost 50 impact categories indicators were found in the 59 studies reviewed (see Supplementary Material). However, several indicators refer to the same environmental mechanism impact (midpoint) or damage (endpoint). In order to simplify the analysis, we were able to identify 17 types of impacts analysed by the papers, which can be computed by different methods: climate change, indicator of potential global warming due to GHG emissions; ozone depletion, for air emissions destroying the stratospheric ozone layer; photochemical oxidant formation, focused on the photochemical ozone created in the lower atmosphere (smog) catalysed by sunlight; particulate matter formation, for assessing damage to human health due to primary PM2.5 and PM2.5 precursor emissions; ionizing radiation, related to the damage that emissions of radionuclides produce in human health and ecosystems; acidification, of soils and water due to the release of gases such as nitrogen and sulphur oxides; eutrophication, measuring the abnormal nutrient enrichment of aquatic ecosystems due to nitrogen or phosphor containing compounds; ecotoxicity, which evaluates the toxic emissions on freshwater, sea water or land; human toxicity, based on the potential harm of emitted substances in people; land use,

addressing the damage for occupation; abiotic depletion, referred to the consumption of non-biological resources; water use; and energy demand, indicator to quantify the primary energy usage (Acero et al., 2017).

Table 2 shows this classification and to which acronym the impact categories type refer (for: CML-IA, ReCiPe, ESA/IChemE). It also shows the number of studies (and the associated percentage) that consider each type of impact. The detail of the main impact categories studied in each study is shown in Table 3. These LCIA indicators also involved more than 20 indicators related to specific issues (specific to fisheries or socio-economic topics) that are discussed in the Section 3.2.2.

Focusing on emission related impacts, 54 studies (92%) computed climate change impacts as it constituted the most scrutinized impact in LCA. It should be noted that 6 studies measured carbon footprint exclusively and, therefore, only assessed climate change impacts. Ozone depletion (53%), photochemical oxidant formation (42%), acidification (64%), eutrophication (66%) and ecotoxicity (46%) were the 5 next most represented impact categories that were computed in most of the LCIA methods reviewed. Surprisingly, human toxicity impact category was only found in 32% of the studies reviewed, despite the fact that it is included in most of LCIA methods. The lack of consensus behind this impact category due to the uncertainty and variability related to both the health and ecotoxic effect data or the limited data on bioconcentration factors for fish or chemical degradation rates, among other parameters, could be one of the main reasons explaining its underrepresentation in the sample assessed (Rosenbaum et al., 2008). Finally, the two other emission-related impact categories, namely particulate matter formation and ionizing radiation, were found in only 14% and 10% of the studies, respectively. Such impact categories could be of interest for the fishery and seafood sector, more singularly particulate matter formation due to the influence of fishing boats to the ambient air and human health near the coast (Zhang et al., 2018).

Impact categories related to resource use and energy indicators were less represented in the studies reviewed. Abiotic depletion (including fossil and metal) and land use categories were found in 41% and 22% of the studies, respectively. Water use related impacts were computed in only 17% of the studies, which can be justified by the fact that methodology development in this field is recent. Indeed, the water foot printing was coined as "virtual water" in 1997, later renamed to the current terminology at the beginning of the millennium and finally adopted in the LCIA methodologies in 2010s (Pfister et al., 2017). Specific biotic resources use and sea use impact categories for fisheries have been applied in the reviewed papers and are more specifically analysed in the Section 3.3.2. Notwithstanding this, energy demand indicators are assessed in nearly half of the studies (46%). Even if such indicators are not part of LCIA methods as they are rather synthetic LCI indicators, it highlights that they are frequently used as complementary information.

Recent publications that describe the use of up to date and innovative methodologies, such as ReCiPe, suggests that it is worth including a large set of environmental impacts that may be of importance for the fisheries sector. This would also enable the computation of endpoint damage and would provide synthetized information. Modelling of endpoint damages was found in 8 studies (Laso et al., 2018c; Vázquez-Rowe et al., 2014b; Avadí and Fréon, 2015; Avadí et al., 2014a; Avadí et al., 2014b; Fréon et al., 2014b; Gonzalez-García et al., 2015) using ReCiPe. ReCiPe aggregates 18 midpoint impact categories in three endpoint damage categories or areas of protection, namely human health, ecosystem quality and resources. These 8 studies also compute single score that weights and normalizes the three areas of protection. Studies highlight the benefit of single score for communication, however, the large uncertainty associated with such metrics was evident in some studies (Laso et al., 2018a, 2018b, 2018c).

Another trend in LCIA is the regionalization of impacts in order to better represent site-specific environmental interventions (Patouillard et al., 2018). This may enhance the relevance of LCIA for seafood products because they usually generate direct impacts that are space dependant (e.g., impacts associated to toxic or eutrophying substances emitted in the marine environment or to water use in the supply chain). However, none of the reviewed studies used spatially differentiated LCIA methodologies (such as LC-Impact (Verones et al., 2020)), as they are too recent and not yet implemented in LCA software.

3.2.2. Fishery-specific impacts categories

Other impact categories related to the fish and seafood sector are assessed in the studies reviewed. Although this kind of indicators were rarely the main target of studies, 25 different indicators related to biotic (fish) resource, sea use, nutritional and socio-economic approaches were analysed (see Supplementary Material).

Impacts related to the removal of fish stocks have been estimated by several different indicators in 37% of the studies reviewed. A significant

Table 2
Frequency of LCIA impact categories and methods application in the reviewed studies.

Impact categories	CML-IA	ReCiPe	ESA IChemE	Others methods/ indicators	N studies	%
Climate change	GWP	CC	GWP	–	54	86%
Ozone depletion	ODP	OD	SOD	–	31	49%
Photochemical oxidant formation	POFP	POF	POF	–	25	40%
Particulate matter formation	–	PMF	–	–	8	13%
Ionizing radiation	–	IR	–	–	6	10%
Acidification	AP	TA	AA	–	38	60%
Eutrophication	EP	FE, ME	E, AOD	–	39	62%
Ecotoxicity	METP, TETP	TE, FE, MET	EAL, EAL2	–	27	43%
Human toxicity	HTP	HT	–	–	19	30%
Land use	LOP	ALO, ULO, NLT	–	–	13	21%
Abiotic depletion	ADP	MD, FD	–	–	24	38%
Water use	–	WD	–	–	10	16%
Energy demand	–	–	–	CED, TCED, EU	27	43%
Biotic (fish) resources use	–	–	–	see detailed analysis	22	37%
Nutritional impact	–	–	–	see detailed analysis	7	12%
Sea-bed damage	–	–	–	see detailed analysis	1	2%
Socio-economic	–	–	–	see detailed analysis	3	5%

GWP: Global Warming Potential; CC: Climate Change; ODP: Ozone Depletion Potential; OD: Ozone Depletion; SOD: Stratospheric Ozone Depletion; POFP: Photochemical Oxidant Formation Potential; POF: Photochemical Oxidant formation; PMF: Particulate matter formation; IR: Ionizing radiation; AP: Acidification Potential; TA: Terrestrial acidification; AA: Atmospheric acidification; EP: Eutrophication Potential; FE: Freshwater eutrophication; ME: Marine eutrophication; E: Eutrophication; AOD: Aquatic oxygen demand; METP: Marine Eco-Toxicity Potential; TETP: Terrestrial Eco-Toxicity Potential; TE: Terrestrial ecotoxicity; FE: Freshwater ecotoxicity; MET: Marine ecotoxicity; EAL: Ecotoxicity to aquatic life (metals to seawater); EAL2: Ecotoxicity to aquatic life (other substances); HTP: Human Toxicity Potential; HT: Human toxicity; LOP: Land Occupation Potential; ALO: Agricultural land occupation; ULO: Urban land occupation; NLT: Natural land transformation; ADP: Abiotic Depletion Potential; MD: Metal depletion; FD: Fossil depletion; WD: Water depletion; CED: Cumulative energy demand; TCED: Total Cumulative Energy Demand; EU: Energy use.

Table 3
Impact categories per study.

Study	LCIA Method	Typical LCIA indicators												LCI	Fishery specific			
		Climate change	Ozone depletion	Photochemical oxidant formation	Particlaute matter formation	Ionizing radiation	Acidification	Eutrophication	Ecotoxicity	Human toxicity	Land use	Abiotic depletion	Water use	Energyu demand	Biotic (fish) resources use	Sea use	Nutritional	Socio-economic
Abdou et al., 2018	CML baseline 2000	X	X	X			X	X	X	X	X	X		X	X	X		
Abdou et al., 2020	Ecopath with Ecosim (EWE)	X	X	X			X	X	X	X	X	X		X	X			
Almeida et al., 2014	CML baseline 22,002	X	X				X	X						X	X			
Almeida et al., 2015	CML-IA baseline	X	X	X			X	X	X			X		X				
Avadí et al., 2014a	ReCIPE, CML baseline 2000 and USEtox	X					X	X		X	X	X	X	X	X			
Avadí et al., 2014b	ReCIPE	X	X	X	X	X	X	X	X	X	X	X	X					
Avadí et al., 2015	ReCiPe	X		X	X			X		X		X		X				
Avadí and Fréon, 2015	ReCIPE, CML-IA baseline 2000 and USEtox	X	X	X	X	X	X	X	X	X	X	X	X	X	X			X
Avadí et al., 2018	ReCiPe	X		X	X			X	X	X	X	X	X	X	X			
Denham et al., 2016	GHGs emission factors	X																
Driscoll and Tyedmers, 2010	GHGs emission factors	X													X			
Driscoll et al., 2015	CML baseline 22,000	X	X				X					X		X	X			
Farmery et al., 2015	Australian impact method	X						X	X				X	X	X			
Fréon et al., 2010	N/A																	X
Fréon et al., 2014b	ReCiPe, USETox, CML 2000 and 2001	X	X	X	X	X	X	X	X	X	X	X	X					
Fréon et al., 2014c	ReCiPe	X					X	X						X	X			X
Fréon et al., 2017	ReCiPe	X	X	X	X	X	X	X	X	X	X	X	X					
Gonzalez-García et al., 2015	ReCiPe	X																
Hallstrom et al., 2019	GHGs emission factors	X																
Hélias et al., 2018	N/A														X			
Hospido and Tyedmers, 2005	CML baseline	X	X	X			X		X	X								
Hospido et al., 2006	CML baseline	X	X	X			X	X				X						
Iribarren et al., 2010a	CML 2000	X	X	X			X	X	X	X	X	X						
Iribarren et al., 2010b	CML 2001	X	X	X			X	X	X	X	X	X						
Iribarren et al., 2010c	CML 2001	X	X	X			X	X	X	X	X	X						
Iribarren et al., 2010d	PAS 2050 and IPCC, 2007.	X																
Iribarren et al., 2010e	PAS 2050 and IPCC, 2007.	X																
Iribarren et al., 2011	PAS 2050 and IPCC, 2007.	X																
Laso et al., 2016	ESA with metrics from IChemE 2002.	X	X				X	X	X									
Laso et al., 2017a	ESA with metrics from IChemE 2002.	X	X				X	X	X									
Laso et al., 2017b	ESA with metrics from IChemE	X	X				X	X	X									

	2002.																	
Laso et al., 2018a	JRC of EC. IPCC, 2013, ReCiPe, CML-IA	X					X	X										
Laso et al., 2018b	JRC of EC. ReCiPe, CML-IA	X					X	X										
Laso et al., 2018c	IPCC, 2013 and specific calculations	X											X	X			X	
Lourguioui et al., 2017	CML baseline 2000	X					X	X						X				
Lozano et al., 2010	CML baseline 2000	X	X	X			X	X	X	X		X			X			
Parker and Tyedmers, 2012a	CML 2 baseline 2000	X	X				X	X						X	X			
Parker and Tyedmers, 2012b	Marine footprint														X			
Parker et al., 2015	IPCC 2007 characterization factors	X												X	X			
Ramos et al., 2011	CML baseline 2000	X	X				X	X	X			X						
Van Putten et al., 2016	CML baseline 2000	X	X				X	X						X				
Svanes et al., 2011	CML 2 baseline 2000	X	X	X			X	X						X				
Thrane, 2004	ISO 14040-43													X	X	X		
Thrane, 2006	Danish EDIP 97	X	X	X			X	X	X	X				X				
Vázquez-Rowe et al., 2010a	CML baseline 2000	X	X	X			X	X	X	X		X						
Vázquez-Rowe et al., 2010b	CML baseline 2000	X	X	X			X	X				X						
Vázquez-Rowe et al., 2011a	CML baseline 2000	X	X				X	X	X			X			X			
Vázquez-Rowe et al., 2011b	EMEP Corinair, CML baseline 2000	X					X	X	X			X		X				
Vázquez-Rowe et al., 2012a	CML baseline 2000	X	X	X			X	X	X			X			X	X		
Vázquez-Rowe et al., 2013a	IPCC 2001	X												X				
Vázquez-Rowe et al., 2013b	IPCC 2001	X																
Vázquez-Rowe et al., 2013c	IPCC 2001	X												X				
Vázquez-Rowe et al., 2014a	Cumulative Energy Demand													X				
Vázquez-Rowe et al., 2014b	ReCiPe	X	X	X	X	X	X	X	X	X	X	X	X		X	X		
Villanueva-Rey et al., 2018	ILCD, ReCiPe, CED	X	X	X	X	X	X	X	X	X	X	X	X	X				
Winther et al., 2009	IPCC 2007; CED	X												X				
Ziegler et al., 2003	CML baseline 2001	X		X			X	X						X	X	X		
Ziegler and Valentinsson, 2008	CML baseline 2001, Ecoindicator 99	X	X	X			X	X	X						X	X		
Ziegler et al., 2011	CML baseline 22,000	X	X	X			X	X	X	X				X	X	X		
	Number of studies	54	31	25	8	6	38	39	27	19	13	24	10	27	22	7	1	3
	%	92%	53%	42%	14%	10%	64%	66%	46%	32%	22%	41%	17%	46%	37%	12%	2%	5%

number of studies assesses this impact given that overfishing ranks as one the most important threat to biodiversity loss in global marine ecosystems (Millennium Ecosystem Assessment, 2005; Emanuelsson et al., 2014). Such indicators include mean trophic index, lost potential yield, biotic natural resources index, discards and fish biomass extraction, among others. Avadí and Fréon (2013) already extensively discuss these indicators and their relevance, indicating that most of them are stand-alone indicators that are not part of LCIA methods. The diversity of indicators used shows the importance of setting up consensual or harmonized indicators to address the challenge of biotic resource use, a task that has been led by the Life Cycle Initiative through its Global Guidance on Environmental Life Cycle Impact Assessment Indicators (GLAM) project (LCI, 2020). It should be noted that there have been attempts to better integrate such indicators in LCIA framework, leading to natural resources area of protection (Hélias et al., 2018; Hélias and Heijungs, 2019).

Seabed damage is also a predominant driver for biodiversity loss in oceans worldwide (Millennium Ecosystem Assessment, 2005; Woods and Verones, 2019). Twelve percent of the studies consider this impact with various indicators that are discussed in Avadí and Fréon (2013) and Woods et al. (2016). Meanwhile, only one study considers nutritional indicator, through the use of protein content (Laso et al., 2018c). Nutritional impacts are more and more assessed in LCA of food and new methods are being developed in this area (Stylianou et al., 2016). Phase 3 of GLAM should focus on biotic resources and nutritional impact and therefore give guidance to the fishery and seafood sectors to address these important impact and damage categories (UNEP, 2020). Moreover, Woods et al. (2016) recommend the inclusion of (over)exploitation of fish and seabed damage in order to have a meaningful assessment of marine ecological impacts in LCA.

Other pathways recommended by Woods et al. (2016) include marine plastic debris related impacts. This was not found in the reviewed papers whereas fisheries can contribute to this impact category. This is because no operational methods exist yet to integrate such impacts in LCA. New methods are being developed in the frame of the Marine Impacts in Life Cycle Assessment initiative (MarilCA, 2020), following the call of the Medellin Declaration to develop new impact pathways to account for this environmental hazard (Sonneman and Valdivia, 2017).

LCA traditionally focuses on the evaluation of the environmental impacts of processes or products. Thus, discussion of socio-economic issues has been minimal in the context of fisheries LCA literature (Pelletier et al., 2007). All of the 59 reviewed studies focus on environmental impacts of production systems, and only three address socio-economic indicators in fishery-specific impacts categories. Yet, in order to address sustainability objectives, assessments need to consider not only environmental aspects but also social and economic impacts (Kruse et al., 2008).

Avadí and Fréon (2015) proposed to complete LCA indicators with a set of other indicators to evaluate the sustainability performance of anchoveta fisheries and freshwater aquaculture industries. The set included nutritional profiling, energy and socio-economic assessments. The socio-economic indicators include production costs, added value, gross profit generation and employment. This approach allows accurate comparisons of different products by bringing an added value to LCA and gives a concrete perspective of sustainability by incorporating the social and economic perspectives together with the environmental one.

Other life cycle methods, namely Social LCA (SLCA) and Life Cycle Costing (LCC), have been developed as necessary complements for capturing trade-offs between environmental, social and economic aspects along the life cycle of production systems (Dreyer et al., 2006; Guinée et al., 2011). For instance, Soltanpour et al. (2020) used SLCA to analyse a case of fisheries management. However, SLCA approaches show that the perception of social impacts is highly variable, and the methodology is often debated, in particular regarding data frames (Jørgensen et al., 2008). Moreover, the Life Cycle Sustainability Assessment (LCSA) framework combines LCA, SLCA and LCC. LCSA evaluates environmental, social and economic negative impacts and benefits on decision-making processes towards more sustainable products throughout their life cycle (Valdivia et al., 2013). LCSA has also been applied to fisheries management research. For instance, Kruse et al. (2008) attempted to apply this approach in a seafood context.

Beyond life cycle methods, a great variety of system analysis tools have been developed, focusing on diverse types of impacts and dimensions of sustainability. Some of those methods could complement fisheries LCAs for wider, more holistic studies (Avadí and Fréon, 2013). By using an input-output model to test the socio-economic impacts on a few case studies in the Atlantic coast, the Interreg Neptunus project will also study economic implications of seafood circular economy, as well as economic benefits and drawbacks of implementing actions for proposed strategies under a circular and NEXUS eco-labelling approach (Neptunus, 2020). There is also scope to assign risk assessment categories for input-outputs model that will contribute to LCA knolwedge for seafood sector as described recently by Tahar et al. (2017) who focused on the waste water industry.

3.3. Seafood LCA practitioners: who and why

From the revision of the LCA studies, the location of the research institutions involved and the species studied offers a good overview of the investigation on seafood. Fig. 4 qualitatively represents the geographic distribution of the authorship of the studies per country and fishing ground (area from which the studied species are caught). Most of the research was carried out by one or several institutions from only one territory, while 14 had international collaboration and involved researchers from two to four different countries. Geographically, Europe concentrates the largest number of research centres practicing LCA, constituting the FAO fishing area 27 and the most evaluated oceanid territory (FAO, 2015). Institutions from Spain participated in 33 studies collected in this review, followed by France and Peru (9 each), Sweden (6), Canada (5), Australia (4), Denmark and Portugal (3 each), Norway, Tunisia and United States of America (2 each) and, finally, Algeria, Ecuador, Italy, Luxembourg, Switzerland and United Kingdom (1 each). Regarding the fishing zones or waters, LCA studies in the 2000s focused on Atlantic and Pacific fisheries (Vázquez-Rowe et al., 2012b). However, to date, almost all major waters have been part of at least one article including -Mediterranean, Caribbean, Baltic, Tasman or North seas- but excluding, for instance, the Black or Caspian seas and Eastern Asia. This is notable as Asia is the largest worldwide fish producer: China remains a major fish producer, accounting for 35% of global fish production (FAO, 2020). Although numerous studies on aquaculture LCA have been also published in Asia in recent years (Henriksson et al., 2018; Järviö et al., 2018). It's appreciated that LCA typically remains a Western tool in this field of study, which is probably due to the higher environmental requirements for food production in European countries and others belonging to the Organization for Economic Co-operation and Development (EOCD, 2020). This unbalanced distribution can also be related with persistent differences in fish consumption levels. In developed countries the apparent fish consumption is 26.4 kg, 22% above global average (20.5 kg) while in developing countries it is considerably lower, 19.4 kg (FAO, 2020).

With respect to the analysed species, most studies focused on a single species or several similar species (Hospido and Tyedmers, 2005; Parker et al., 2015; Avadí et al., 2015). Typically, these species are emblematic of the regions analysed, such as anchovy in Peru (Fréon et al., 2010, 2014a, 2014b, 2014c, 2017) and Cantabria (Laso et al., 2016, 2017a, 2017b, 2018a, 2018b, 2018c), mussels in Galicia (Barros et al., 2009; Iribarren et al., 2010a, 2010b, 2010c, 2010d), pilchard in Portugal (Almeida et al., 2014, 2015; Gonzalez-García et al., 2015), white banana prawn in Australia (Farmery et al., 2015) or lobster in the United States (Driscoll et al., 2015). Few authors addressed the analysis of multiple species in the same study (Parker and Tyedmers, 2012b; Vázquez-Rowe et al., 2012b; Hélias et al., 2018; Avadí et al., 2020) and

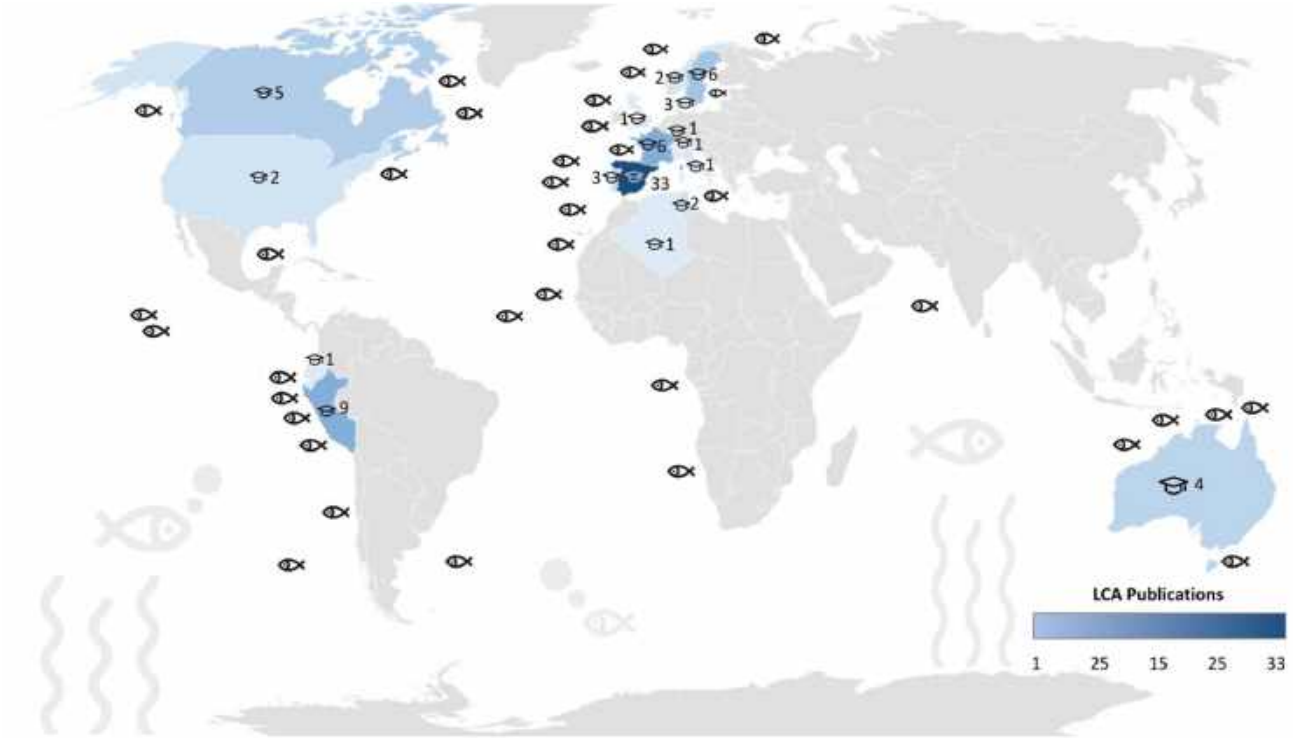


Fig. 4. Geographic distribution of LCA publications per country (cap) and fishing ground (fish). The most intense colour in a territory indicates the largest number of studies.

some included species from coastal fishing, offshore fishing, deep-sea fishing, extensive aquaculture and intensive aquaculture (Iribarren et al., 2010e, 2011; Winther et al., 2009; Avadí and Fréon, 2015).

Almost all studies have been conducted by public bodies, such as universities, institutes and schools with the purpose of characterizing the seafood sector to address the potential environmental impact of the seafood supply chain, from fishing through processing up to consumers, as well as to identify hotspots and evaluate improvement opportunities to promote the sustainability of this part of the Blue economy. Research focused on evaluating the marine species stock, eventual increase of the wild-caught fishery, potential expansion of aquaculture, ecolabelling, effects of climate change in ocean ecosystems, and use of different fishing gears were issues that were the topic in these LCA studies. In contrast, very few LCA articles shared authorship with private companies, industries or consultancies (Laso et al., 2017a, 2017b, 2018a, 2018b, 2018c; Avadí et al., 2018). Only one research article was attributed to industry, which analysed and compared (with competing products from EU market) the carbon and energy footprint of Norwegian seafood supply chain (Winther et al., 2009). The remaining studies were developed by LCA practitioners in research centres from a theoretical perpsective based on direct enquiries to stakeholder and producers, official inventories, and so forth. This is probably due to scientific interest as the public and/or market relevance in their countries; or simply, ease of data collection.

The scientific and technical knowledge of some papers provides valuable inputs for fishery management and future regulations (Ziegler et al., 2016; Villanueva-Rey et al., 2018). For instance, fuel use and packaging in canning products were identified as important shortcomings to be addressed in the extraction and processing phases, respectively. These implications may guide policymakers and stakeholders about where best to make improvements that will lead to sustaining processes and design strategies, including behavioural change. Moreover, several papers promoted new approaches for the first time including 'geographically' focusing on LCA for aquaculture (Lourguioui et al., 2017) and fisheries (Abdou et al., 2018) in the Mediterranean or making a comprehensive LCA of the entire Peruvian anchovy fleet (Fréon et al., 2014b). Other studies report on the 'timely' development of the first fishery LCA study with inventory data for the period 2001 to 2008 (Ramos et al., 2011; Almeida et al., 2014). 'Technically', the first LCA study of fishmeal plants considered construction and maintenance phases (Fréon et al., 2017) or applying the 'water-energy-food-climate nexus" index to a case study for fisheries (Laso et al., 2018c). "Managerially', the first ecolabel in the Spanish fishing sector has been based on life cycle approaches for seafood products (Vázquez-Rowe et al., 2016).

3.4. Nexus water-energy-food in the seafood sector

The assessment of individual environmental impact categories in LCA offers high added-value information, such as providing insights for further improvements in processes, products and services, as well as support in policy-making and environmental certification schemes. However, such an assessment gives a perspective that is circumscribed to the link between each impact category and the specific environmental problem that it represents. Although in some cases the environmental impacts of these impact categories are aggregated in endpoint damage indicators, which provide a more direct connection with the tecnosphere and the environment, in the case of seafood LCA studies, as mentioned above, these were only applied in 8 studies. Moreover, even the computation of endpoint damages with current LCIA methods lacks a strong interconnection between variables to reach robust results that allow a holistic analysis of production systems.

In this context, the nexus approach arises as a perspective that recognizes the fact that water, energy, food production systems (in this case, wild fish capture) and natural ecosystems show a series of robust and indivisible multi-dimensional interlinkages. We argue that when referring to life cycle studies in the seafood sector, nexus thinking is needed to appropriately transition to a circular economy (Ruiz-Salmón et al., 2020). This is essentially a transformative approach to governance, and also requires substantial changes in individual behavior. Therefore, the nexus implies how to govern such transformations, and the policy tools that will be required, including behavioural change interventions that go beyond mere education to influence how people make decisions regarding

the purchase and consumption of marine products. In contrast, a traditional fragmented approach, that obviates this nexus perspective while attempting to achieve resource security independently, will not only generate sub-optimal outcomes, but may also endanger food security and sustainability (Staupe-Delgado, 2020).

Water, energy and food are basic requirements for everyday life and are key activities advancing the seafood sector. In this sense, the lack of a secure and economical provision of one of them might lead to disruption in the supply and accessibility of the two others (Machell et al., 2015). Thus, the application of a water-energy-food nexus under a holistic approach appears to cover the gap of the isolated impact categories aforementioned. LCA is particularly important for understanding the interconnections in the nexus, as it enables the consideration of entire supply chains. In fact, Hamiche et al. (2016) highlighted LCA as a valuable tool to shed light on the links between the water and energy sectors, since it is able to account for direct and indirect consumption. Similarly, De Laurentiis et al. (2016) and Mannan et al. (2018) also considered LCA as the best available tool to enable the developing of the nexus framework, driving the shift towards sustainable food systems thereby.

Having said this, a nexus approach using life cycle methods would have to redefine the selection of environmental impact categories that are traditionally used (see Table 3). For instance, the quantification of freshwater consumption by the seafood sector has received little attention (Vanham, 2016). This is remarkable considering that freshwater aquaculture, as well as fish processing in general, is a water-intensive industry and a large discharge of organic material. Therefore, we consider that new water consumption metrics, currently available in LCIA methods (e.g., AWARE), as well as a wider array of water degradation categories should be included in seafood LCA studies in order to enhance the meaningfulness of these under a nexus approach. Moreover, this would allow determining whether the planetary boundary of freshwater use is below its breaching risk in the studies conducted, ensuring, this way, clean, affordable and accessible energy generation and sustainable freshwater consumption to support food supply (Steffen et al., 2015).

In terms of energy, it must be noted that a high correlation exists between food and energy prices. This connection is even more pronounced for intensive capture fisheries, which are usually fully dependent on fossil fuels (Parker and Tyedmers, 2015). In contrast to water, energy has been repeatedly reported and included in seafood LCA studies, directly, through the use of the CED impact category or the EROI indicator, or indirectly, by monitoring environmental impacts that are heavily dependent on energy intensity (Weidema et al., 2008).

Finally, focusing on the food component of the nexus, seafood products are widely accepted to be an essential component of a balanced and healthy diet because they have a high "good fat" content and provide high quality proteins and many micronutrients, such as vitamins and minerals (Larsen et al., 2011; WHO, 2020). For instance, the NRF9.3 score, i.e., a nutritional index based on 9 nutrients to encourage and 3 nutrients to limit (Drewnowski, 2009) has been combined in the literature with LCA, mainly from a human diet perspective, in an effort to analysed the sustainability of diets across the world and determine, through linear programming or other methods (Vázquez-Rowe et al., 2019; Larrea-Gallegos and Vázquez-Rowe, 2020), future healthy diets. In this context, Castañé and Antón (2017) identified a considerable environmental impact due to a high global warming potential per kg of seafood produced due to energy consumption, but no details were given on the specific seafood products that were included in the assessment. Similarly, some attempts have been made to include a ranking for fish species based on nutrient density, climate impact and their combination (Hallstrom et al., 2019), but the nexus between the potential of the nutrients from seafood and environment effects is yet to be deeply researched.

Overall, the nexus approach can support the identification of synergies and trade-offs between water and energy systems and food systems aiming at resources efficiency and environmental impacts –production and consumption– reduction (Mannan et al., 2018). Advancements towards a greater linkage between terrestrial and marine systems, however, are necessary for fishing activities within a nexus framework. Freshwater consumption should be considered throughout the full supply chain of seafood products (Gephart et al., 2017; Vanham, 2016), as well as energy consumption produced by water resources (D'Odorico et al., 2018). The water dimension of the nexus should consider the water used during on-board activities (e.g. ice and water) and on-land activities where several processing activities consume freshwater and use energy produced by water resources (Gephart et al., 2017; Vanham, 2016). Some of the freshwater used in these processing activities can result in wastewater that, in turn, also consumes energy for its treatment (Vanham, 2016). Furthermore, there are also seafood processed-derived products that use crop ingredients (Vázquez-Rowe et al., 2013a), which, in turn, also consume freshwater that needs to be accounted for (Salmoral and Yan, 2018). Moreover, within a global context of increasing population and growth of water and energy use, the production, consumption and waste of food raises social questions, such as global food insecurity, food prices and international food shortages. Consequently, reducing food losses and waste is necessary to meet forecasted nutritional needs and to enhance the link with the energy and water implications.

All in all, a standardization for the nexus variable which defines, evaluates and modifies future strategies is lacking. The existence of this framework would allow the analysis of the interacting governing forces and balance the nutritional, economic and energetic value of the seafood sector to foster informed decisions, engaging industry stakeholders and consumers. Thus, the development of a unified water-energy-food nexus framework (Endo et al., 2017) would address the opportunity to strengthen the social, economic and environmental aspects of this specific but worldwide system. Furthermore, the nexus also helps international policies and scientific researchers to better adapt local and regional commerce for new perspectives. Finally, there are clear links between the SDGs of the Agenda 2030 of the UN and the water-energy-food nexus. Hence, this nexus can support the achievement of the SDGs when taking a transdisciplinary approach (Ghodsvali et al., 2019).

3.5. Challenges and opportunities for LCA in the seafood sector

The application of LCA in the broader seafood sector presents a number of key challenges and opportunities, which in many cases are related to the circular economy and the management of process waste streams. Some of the most pressing challenges facing the sector are the: (i) proliferation of marine debris comprised of plastics and other artificial materials (Maximenko et al., 2019); (ii) generation of waste and its valorisation, and (iii) climate change (Ruiz-Salmón et al., 2020). These are challenges that LCA can help address in particular when evaluating the nature of the challenge, analysing how various parts of the seafood sector contribute to these issues and identifying where targeted improvements to the sector can yield most sustainability gains while transitioning to a CE.

3.5.1. Marine debris

It is estimated that there are 120 million tonnes of plastic in the oceans and between 11.6 and 21.1 t in the Atlantic Ocean alone (Jambeck et al., 2015; Pabortsava and Lampitt, 2020). These plastics are non-biodegradable and breakdown into smaller pieces due to weakening by ultraviolet light and the motion of the ocean. They can impact on marine wildlife and trophic levels, where organisms mistake the plastics for food resulting in ingestion which may cause physical impairment or death (Maximenko et al., 2019; Provencher et al., 2019). The use of additional substances (e.g. colorants and stabilisers) in the production of these plastics, may make them toxic, further exacerbating their impacts. There remains, however, a significant gap of knowledge in the understanding of these toxicological and ecotoxicological impacts.

Derelict and lost fishing gear, as well as shipwrecks, is the archetype of how seafood supply chains and processes can contribute to marine debris. It is estimated that lost gear makes up 46% of the Great Pacific Garbage Patch (Lebreton et al., 2018; Maximenko et al., 2019). As well as contributing to marine debris, lost fishing gear, commonly referred to as ghost nets, can continue to capture and kill fish and other organisms for many years after being lost. While switching to biodegradable nets is being evaluated, it has been reported that these nets may have a lower catch efficiency when compared to conventional nylon nets (Grimaldo et al., 2019). These losses in efficiency and a possible increase in fishing effort to offset this loss, is something which would benefit from an LCA perspective; particularly with regard to changes related to fuel use, the largest contributor to the impacts (Avadí et al., 2020), but also in other inventory items, such as refrigerating agents (Vázquez-Rowe et al., 2012a).

While the impact of ghost fishing (Vázquez-Rowe et al., 2012c) and seabed disturbance (Woods and Verones, 2019) has been described in some fishery LCA studies, current LCIA methods do not consider certain marine environmental impacts, such as biotic depletion or the degradation of the marine environment due to plastics accumulation in the ocean or damage to seafloor (Avadí et al., 2020), despite certain methodological advancement in recent years (Hélias et al., 2018). Furthermore, there is concern over the trophic transfer of these substances due to bioaccumulation and biomagnification (Maximenko et al., 2019; Provencher et al., 2019). Although Woods et al. (2019) have already developed effect factors due to entanglement from macroplastics, research into accounting for these impacts in LCA is ongoing through the MariLCA working group (Marilca, 2020). Finally, inventory flows linked to the presence of nano-, micro- and macroplastics, which can be potentially ingested, remain scarce, although certain initiatives, such as the study by Stefanini et al. (2020), or the PLP report recently published by Quantis (2020).

Similarly, the environmental consequences of the release of plastic debris to water bodies has arisen the interest of the LCA community, since this environmental dimension is not included in current metrics. In fact, Saling et al. (2020) have recently proposed a characterization model regarding the relationship between degradation and fragmentation of plastics in the marine environment. Moreover, Woods et al. (under review), have produced a detailed framework in which they describe the main cause-effect pathways that must be considered in LCIA in order to account for the different damages to human health (mainly through seafood), ecosystem quality (e.g., entanglement or invasive species) (Woods et al., 2019) and other endpoint damages.

3.5.2. Waste valorisation

Another challenge the seafood sector is facing is the issue of seafood waste, which can indirectly increase pressure on fisheries through increased fishing effort to supplement this waste. It is estimated that around 40% of the total food supply is lost or wasted between harvesting, production and processing (Laso et al., 2016; Love et al., 2015). In Europe it estimated that seafood losses and wastage rates are greater than 30% (FAO, 2011). This loss and the knock-on effect of triggering increased fishing effort to meet market demand perpetuates a linear economy of use and waste in food production. The adaptation of a circular economy based on business models which replace the 'end-of-life' concept with reducing, alternative reuse, recycling and recovering materials in production/distribution and consumption processes, at the micro, *meso* and macro level can promote the minimisation of food loss and wastes through the development of more sustainable production loops (Kirchherr et al., 2017). One of the main avenues in closing the loop in seafood production is through the valorisation of classical waste streams and their utilization in other industries while eventually being fed back to the original industry (de la Caba et al., 2019). An example of this would be sludge from finfish aquaculture and its use as a substrate for anaerobic digestion and electricity generation. Examples of valorised waste which were mentioned in this review article were: trimmings, such as heads and spines, as fishmeal and fish oil, waste meat or flesh as a paste (Laso et al., 2016) and mussel shells as a source for calcium carbonate (Iribarren et al., 2010a). A circular economy approach applied to these waste streams can highlight management and valorisation opportunities for sectors, processors and companies. Examples of waste or co-product valorisation were previously discussed in Section 3.1.3.

Emerging valorisation strategies include the use of by-products as bio-based materials (García-Santiago et al., 2020). Fish waste has been used for biodiesels and activated carbon production mainly via extractions from fish oil (Fadhil et al., 2017). Blood waters from pelagic processing plants have been shown to be a potential source of proteins, amino acids and vitamins which can be used as ingredients for bio-based materials such as fuels, inks and feeds (Barr and Landis, 2018; Fadhil et al., 2017; Hayes and Gallagher, 2019). A similar approach of extracting glycogen from wastewater has been put forward for mussel processing sites (Barros et al., 2009), where it may be used for lactic acid production. In the instance of fish skin and bone, collagen from these wastes can be valorised as fish gelatine and in the manufacturing of active packaging (de la Caba et al., 2019).

3.5.3. Packaging

A key area in which LCA was applied and valorisation of waste streams can play a role is that of seafood packaging. Several studies reviewed in this article have identified the issue of packaging as being a hotspot in the environmental impact of seafood products. The majority of these studies have focused on canned products, such as European anchovies (Laso et al., 2016, Laso et al., 2017b; Laso et al., 2018a, 2018b, 2018c), Peruvian anchovies (Avadí and Fréon, 2015; Avadí et al., 2014a), sardines (Almeida et al., 2015; Hospido et al., 2006; Vázquez-Rowe et al., 2014b; Laso et al., 2016) with several studies also considering fresh and frozen products, mussels (Iribarren et al., 2010; Svanes et al., 2011; Thrane, 2006; Van Putten et al., 2016). The studies have focused on the use of conventional packaging techniques and materials, but a benchmarking and deeper investigation of these materials from a life cycle perspective including their production, recyclability prospects, or substitution by innovative packaging materials (de la Caba et al., 2019) as well as on the packaging design optimization can help in reducing the environmental challenge associated with this stage of the product life cycle.

3.5.4. Best available techniques

Other means of implementing LCA and circular economy philosophies in the seafood sector can be reached through BATs (Barros et al., 2009; Laso et al., 2016; Laso et al., 2017a; Morris et al., 2019). A number of studies (Barros et al., 2009; Laso et al., 2017a, 2017b) have demonstrated that BATs and the use of environmental management tools such as maintaining an accurate inventory and promoting recycling of by-products and wastes could reduce the environmental burden on environmental aspects such as energy, water and raw material consumption.

As these processes and valorisation strategies exit the research phase and enter validation and trial stages, it will be important to consider their influence on the life cycle impacts of seafood products. There is an opportunity to implement circular economy and life cycle techniques in the seafood sector, which can help it to become an example of how a transition from a linear to a circular economy may be achieved.

3.5.5. Climate change

Finally, climate change can interact with fisheries in many different ways, through the increase in water temperatures, extreme water flow events (floods and droughts), and warming of maritime environments (Ruiz-Salmón et al., 2020). Regarding the sea surface temperatures (SST), it was found that warming is not homogeneous, and the pattern is complex, as observed in the Atlantic Ocean. Garrett et al. (2018) noted that data were not uniform in 2017 at Malin Head (north of Ireland), and were the highest on record at 0.89 °C above the

1981-2010 average. Nevertheless, some areas of the North Atlantic show a slight cooling, such as the subpolar gyre close to 50°N. This location, sometimes referred to as the "cold blob" (Rahmstorf et al., 2015), has cooled by about 0.9 °C since 1900 (Allan and Allan, 2019). In the mid-Atlantic (longitude approx. of Reykjavik and latitude approx. of Liverpool/Galway) seasonal cycle ranges from less than 10 °C to almost 15 °C (NOAA, 2019). Additionally, in this location a general increase in annual SST has been observed, especially through the last 20 years with 2015 and 2018 being cooler than the long-term average (Rayner et al., 2003).

These changes may impact migration routes of fish, such as mackerel, and stock recovery. Fish migrate between different regions in part based on water temperature through the year as fish species have evolved narrow temperature tolerances (Cheung et al., 2016). This will also adapt their cellular machinery to tolerate a wider range of temperatures, which demands a lot of energy. Fish bodies start to fail when they find themselves in warmer water, so they have to use their energy to move to cooler waters instead of breeding or searching for food. This problem was studied by Pinnegar et al. (2017) for mackerel. The authors set out a series of impacts of climate on UK fisheries, noting the spread of this species into Icelandic and Faroese waters, impacting quota allocation between nations and fleets and governance. Although these aspects are yet to be included in LCA studies, it is possible that the impact of plastic litter working as a vector for invasive species will be included within the Marilca framework (Marilca, 2020).

Moreover, energy use in the capture stage of fishing is large especially when compared to the nutritional energy that is gained. Trawling for small pelagic fish, for instance, result in about 12.5 kg fish per kg of fuel, whereas when trawling for shrimp the fuel to fish ratio is can be between 3 and 4 kg fish per kg of fuel (Furuya et al., 2011; Parker et al., 2018). Despite the improved efficiency of midwater trawling as compared to bottom trawling, however, a wide range of LCA studies demonstrate the better energy performance of pelagic nets, such as purse seines (Driscoll and Tyedmers, 2010; Vázquez-Rowe et al., 2010a; Avadí and Fréon, 2013; Gonzalez-García et al., 2015). This excludes further energy costs in on shore processing, distribution or storage. In fact, for fisheries, EROI or edible protein EROI have shown to be highly relevant when examining the fishery stage, which as previously explained, is highly energy intensive (Vázquez-Rowe et al., 2014a). EROI considers the energy extracted from the edible content of the fish divided by the energy consumed in the production process (e.g., fuel use in the harvesting stage). Guillen et al. (2016) analysed the EROI of selected European fishing fleets in 2008. They estimated the total EROI average (here presented as a decimal) as being 0.11: the energy content of fuel burnt was 9 times greater than the edible energy content of the catch, with gear-specific EROI values ranging from 0.02 for beam trawl to 1.12 for pelagic trawlers and seiners capturing low value species (e.g. herring, mackerel, sand eels and sprats). In addition, they compared the EROI of fish species to those of other food systems. For instance, the EROI for soybeans was estimated at 4.92, corn 0.81, wheat 0.89, beef (pastured-based) 0.05, and broiler poultry 0.177. Strategies to improve the performance of the fishing industry should include behavioural, technological and managerial efforts since the potential to reduce fuel consumption varies substantially between fisheries (Parker et al., 2018).

4. Conclusions

LCA has emerged as a powerful methodology for the environmental evaluation of the seafood sector along its supply chain. In fact, the increasing number of studies published demonstrate the interest of researchers, decision makers and stakeholders in the seafood sector to use LCA for the decision-making process. In fact, the seafood sector has used LCA to address many environmental sustainability challenges and, in turn, the studies carried out by LCA practitioners have served to reinforce LCA as a valid tool to deal with sustainability challenges for seafood and other sectors. Both parties need to continue this path together in order to increasingly sustain the level of innovation necessary for the sector to grow in the current context. The common challenge is to continue enriching each other and move forward in attaining improved sustainability in the sector. To this end, there are a number of key areas to be addressed.

The main methodological challenges for the LCA tool applied to the seafood sector lie in: (i) identifying and defining the reference system so that different studies are more comparable and harmonized; (ii) establishing consensual rules for defining system boundaries, and (ii) clearly defining not only the functional unit, but also the function of the system. In terms of Life Cycle Inventory, the availability of inventory flows for nations and fisheries in the developing world are still substantially lower than for the developed world, especially Europe, where most seafood LCA studies have been conducted. In fact, most studies linked to developing countries have covered the extraction of fish resources that are ultimately exported to developed markets. Moreover, while certain databases have provided an upgrade of their inventory flows in recent years, some if which include fisheries and seafood products (e.g., Ecoinvent), there is still a lag between seafood-based products and other food commodities. From an LCIA perspective, the main challenges are linked to providing a more complete scope of environmental impacts and damages related to the marine environment. While other methodological challenges remain when assessing the impact of ocean-related activities, including fisheries, the addition of impact categories linked to plastic debris in the ocean and fisheries depletion would potentially enhance the utility and visibility of LCA within fisheries management authorities and scientists.

The main outlook of this review is that a life cycle approach is essential for understanding the nexus along the whole supply chains. The water–energy–food nexus approach appears to be crucial to monitoring of the SDGs, since it considers intersectoral synergies and complementarities that will be crucial to improve the sustainability of the fish and seafood processing sector within the CE framework and according to the 2030 EU agenda. Recommended future work should, therefore, include the development of guidelines adapted to the seafood sector, as well as additional empirical case studies that quantify in addition to the environmental impacts, social and economic impacts caused by the new challenges of the circular economy and the bio-economy. For the former, the release of the PEFCR for the fishing sector, which is expected in upcoming years, may shed light on a more harmonized way of conducting LCA studies for the sector. The development of nexus guidelines for the sector would also allow an enhanced interconnection of seafood LCA with other pillars of sustainability. For the latter, further integration of LCA with a wide array of economic, social, nutritional methods, or its integration with machine learning models may enhance the utility of the tool in the future. In this sense, we argue that although LCA must continue to expand its holistic perspective by updating and upgrading its inventory databases and impact assessment methods, it cannot cover the entire set of sustainability indicators, especially beyond those that are purely environmental, that fisheries managers seek to respond. Consequently, beyond a PEFCR for the sector, higher levels of harmonization between LCA and other management support tools must be undertaken to foster the utility of life cycle methodologies in the sector. The aforementioned may be also informed by carrying out risk assessment modelling, which together with LCA, will help future proof the seafood sector for sustainable development along with mitigating against uncertainties.

Accurate assessment by means of LCA based tools represents an excellent opportunity to contribute to the economic, social and environmental development of the seafood sector, but also implies a high responsibility that needs to be articulated through tangible mid and long-term actions. LCA can jointly address a global concern and interest in terms of policies and strategies aimed at climate change mitigation, energy and food security, marine debris or treatment of wastewater. To address the challenges posed by these objectives, sustainable and

multilateral research cooperation is needed to define integrated methodologies and strategies, such as the water-energy-food nexus, a valuable tool to minimize environmental negativities and get successful synergies. Furthermore, the added methodological challenge is to integrate environmental, social and economic variables that meet national needs through transnational strategies. The establishment of synergies in knowledge and experiences and challenges at the local level will help overcome challenges at a global level.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by the EAPA_576/2018 NEPTUNUS project. The authors would like to acknowledge the financial support of Interreg Atlantic Area.

Ana Cláudia Dias and Paula Quinteiro acknowledge FCT/MCTES for the financial support to CESAM (UIDB/50017/2020 + UIDP/50017/2020), through national funds, and to the research contracts CEECIND/02174/2017 and CEECIND/00143/2017, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144094.

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Science of the Total Environment 863 (2023) 160846

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Seafood consumers engagement in reducing environmental impacts from packaging

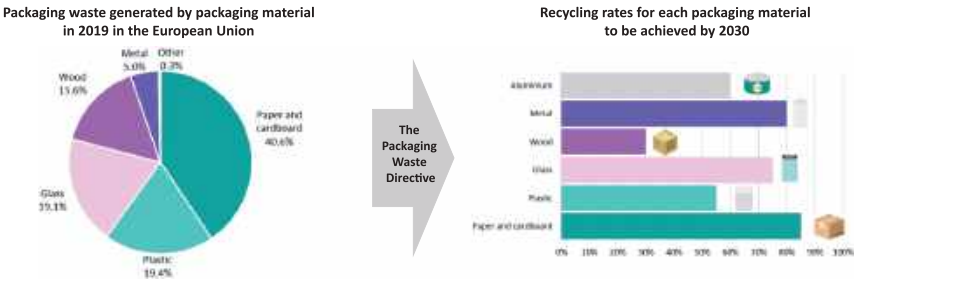
Cheila Almeida [a,b,*], Jara Laso [c], David Baptista de Sousa [d], Ronan Cooney [e,f], Paula Quinteiro [g], Neil Rowan [h], Ana Cláudia Dias [g], Eoghan Clifford [e,f], Rodrigo G. Reboredo [d], María Margallo [c], Maria Leonor Nunes [b], António Marques [a,b]

[a] Instituto Português do Mar e da Atmosfera (IPMA), Divisão de Aquacultura, Valorização e Bioprospeção, Avenida Doutor Alfredo Magalhães Ramalho 6, 1495-165 Lisboa, Portugal
[b] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal
[c] Departamento de Ingenierías Química y Biomolecular, Universidad de Cantabria, Avda. de Los Castros, s.n., 39005 Santander, Spain
[d] ANFACO-CECOPESCA, Campus University 16, 36310 Vigo, PO, Spain
[e] School of Engineering, National University of Ireland Galway, H91 HX31, Ireland
[f] Ryan Institute, National University of Ireland Galway, H91 HX31, Ireland
[g] Centre for Environmental and Marine Studies (CESAM), Department of Environment and Planning, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[h] Bioscience Research Institute, Technological University of the Shannon Midlands Midwest, Ireland

HIGHLIGHTS

- Household practices on packaging waste relate with environmental improvements.
- Consumers are not fully aware about what type of plastics can be recycled.
- Recycling information specific for packaging materials can help consumers' behaviour.
- Consumers were willing to pay more for products with more sustainable packaging.

GRAPHICAL ABSTRACT



ARTICLE INFO

Guest Editor: Ruben Aldaco

Keywords:
Packaging
Fish
Survey
Recycling
Plastic
Consumer behaviour

ABSTRACT

Packaging is essential to protect food, inform consumers, and avoid food waste, yet it can also contribute to the environmental footprint of products. Recycling waste treatment potentially provides more environmental benefits than other options (e.g., landfill), but only 66 % of packaging waste goes to recycling in the European Union. However, the prevention of packaging production with greater reuse, while extending the lifetime or improving packaging design should be firstly encouraged. This highlights the need to assess the willingness of consumers in reducing the environmental impact of seafood products from packaging. An online questionnaire was conducted in three countries (Portugal, Spain, and Ireland), composed of four sections: (i) seafood consumption, (ii) waste separation to be sent recycling, (iii) willingness to purchase seafood products with packaging designed to reduce environmental impact, and (iv) sociodemographic characteristics. Findings revealed that respondents from Spain and Portugal reported a slightly higher frequency of waste sent to recycle compared to Ireland. Irish respondents appear to have more difficulties about the type of plastic materials that can be sent to recycling due to Irish waste management capabilities; whereas Spanish and Portuguese respondents were not fully aware that packaging does not need to be washed prior to recycling. The most popular alternatives to improve the sustainability of seafood packaging were the use of reusable packaging, compostable packaging material, glass jars for canned seafood instead of cans, and intelligent packaging.

* Corresponding author at; Instituto Português do Mar e da Atmosfera (IPMA), Divisão de Aquacultura, Valorização e Bioprospeção, Avenida Doutor Alfredo Magalhães Ramalho 6, 1495-165 Lisboa, Portugal.
E-mail address: cheila.almeida@ipma.pt (C. Almeida).

http://dx.doi.org/10.1016/j.scitotenv.2022.160846
Received 29 July 2022; Received in revised form 27 September 2022; Accepted 7 December 2022
Available online 13 December 2022

Most respondents were willing to pay more for seafood products that use more sustainable packaging (62 % for Spain, 68 % for Ireland, 70 % for Portugal) and half of the respondents intimated that they avoid seafood products due to excessive packaging. With more detailed information on the waste management of packaging, seafood consumers could actively contribute with their attitudes where commensurate changes can improve environmental assessment of seafood.

1. Introduction

While the primary function of packaging is to protect the product, less attention has been paid to packaging functions that reduce food waste. Such measures on packaging ability to reduce waste can be more important than the type of packaging material (Wikström et al., 2014; Williams and Wikström, 2011). Packaging effectively decreases the environmental burden of the product when considering the food loss related to injury during transportation and the environmental burden of additional production to compensate for the food loss (Sasaki et al., 2021). To fully assess the environmental impacts of food packaging, the trade-off between investment in packaging and potential reduced food waste should be quantified (Molina-Besch et al., 2019). Furthermore, the type of end-of-life treatment will compromise the contribution of the packaging to the total environmental assessment of the product. For example, packaging materials can be recycled into new products, produce energy if incinerated, or, in the worst-case scenario, end up in landfill (Wikström et al., 2019).

The current recycling rate of overall packaging waste in the European Union (EU) is around 66 % (European Environment Agency, 2021). In a production-based approach, the responsibility for packaging waste management is on the countries and producers, encouraging them to improve the recyclability of their packaging materials (Gao et al., 2022). However, for many years EU countries have shipped their plastic waste to other countries to meet their recycling targets, until 2017, when China refused to take any new waste (Brooks et al., 2018). This constraint reinforced the motivation to domestically recycle plastic waste and introduce more returnable or refillable containers in food packaging.

The transition towards a low-carbon footprint and circular economy, by extending the useful life of materials and encouraging recycling whilst reducing resource use, has become a priority in the EU (Tallentire and Steubing, 2020). The packaging sector is one of the major contributors to waste generation. In the EU, about 80 million tonnes of packaging waste was generated in 2019 (Eurostat, 2022). Paper and cardboard represented the main waste materials contributing 41 % of the total packaging waste generated, while plastic and glass represented both 19 %, wood 16 %, and metal 5 % (Eurostat, 2022). The Packaging Waste Directive contains updated measures to prevent the production of packaging waste, and promote its reuse, recycling, and other forms of recovery instead of final disposal (European Commission, 2018). It established the following recycling targets for each packaging material to be achieved by 2030: 85 % for paper/cardboard; 80 % for metal; 75 % for glass; 60 % for aluminium, 55 % for plastic; and 30 % for wood. These recycling targets should be calculated with the weight at the point where packaging waste enters the recycling operation (European Commission, 2018). Such targets are linked to the Circular Economy Action Plan which aims to make all packaging fully recyclable by 2030 (European Commission, 2020). In this sense, EU countries are required to adopt appropriate measures such as enhancing the circularity of waste systems, reduce the complexity of packaging, set up a minimum percentage of reusable packaging, design for bio-based materials application and re-use, and define deposit-return schemes. All these measures should be aligned with national programmes and economic instruments as incentives (e.g., taxes, subsidies, and charges) to achieve the established targets.

Studies on the environmental impacts of food production have shown that later stages in the supply system, such as packaging, retail, and transport combined contribute <14 % of greenhouse gas (GHG) emissions (Poore and Nemecek, 2018). However, in the case of specific types of products such as bottled drinks or canning products, packaging can contribute significantly to a product's GHG emissions since the production of glass and metal requires a high level of energy (Poovarodom et al., 2012). Hence, packaging can contribute within a large range of values to the environmental burden of seafood products. The carbon footprint (CF) of packaging goes from almost zero up to 19 kg CO_2 eq per kg of seafood, corresponding to <1 % to 89 % of the total CF of seafood products, differing substantially depending on the type of material used and its processing (Almeida et al., 2021). For example, the best case scenario is freezing lobster in a waxed cardboard box (van Putten et al., 2016) and the worst case is canned mussels in tinplate (Iribarren et al., 2010).

Most seafood studies focused on the production stage, whereas few have considered the complexity of seafood processing considering the supply chain from an integrated perspective. Packaging can have different levels, including primary packaging that is in direct contact with the product (e.g., aluminium can), secondary packaging corresponding to subsequent layers of material that contain one or more primary packaging (e.g., cardboard box), or tertiary packaging for transport, handling, and distribution (e.g., pallets) (ISO, 2016). The resources used along the seafood supply chain require consistent management to reduce waste and improve efficiency (Liu et al., 2020). However, seafood is highly prone to spoilage compared to other food (Love et al., 2015). As an example, canning allows for the preservation of perishable products such as small pelagic fish (e.g., sardines) caught in large volumes, thus increasing the proportion of fish available for human consumption (Almeida et al., 2015b). Viewed through recycling commitments, aluminium cans perform relatively well due to the 81 %[1] recycling rate for aluminium in the EU. However, plastic appears more favourable than aluminium if we prioritize climate impact since aluminium production relies on a more carbon-intensive production processes and is difficult to offset even with higher overall recycling rates. Therefore, given that frozen, chilled or cooked seafood packaging consists mainly of paper, plastic and wood, on average it contributes to <5 % of the CF, corresponding to <1 kg CO_2 eq per kg of seafood (Almeida et al., 2021). Plastics could also present an advantage in food distribution if their reuse is considered, being the most environmentally friendly option when compared to single-use cardboard boxes (Abejón et al., 2020). Expanded polystyrene (EPS) packaging represents one of the most widely used materials in seafood, due to its resistance to temperature changes during transport or storage and a significant environmental impact reduction is obtained when EPS is recycled and/or reused (Konstantinidis et al., 2021).

Plastic packaging waste has the lowest recycling rate compared with other materials such as glass, paper, and metals (Gao et al., 2022). Only 9 % of plastic waste is recycled worldwide, with the overwhelming majority of global plastic waste being landfilled or ending up contaminating the environment (Geyer et al., 2017). Plastic recycling represents a challenge due to the wide variety of additives and blends used in a multitude of products and the variation of material properties (e.g., shortening of the polymer chains) that limit the number of times products can be recycled (Geyer et al., 2017). A quality drop in the plastic recycled material reduces its application options, typically leading to down-cycling and delaying final disposal, but not avoiding it (van der Harst et al., 2016).

Measures to reduce the environmental impact of packaging, for example, switching from single-use to multi-use packaging (e.g., reusing a glass jar after the main product has been consumed) would further cut the carbon footprint, while also reducing waste. Reusable packaging systems are more often found in business-to-business markets for transport (e.g., boxes, pallets) while in business-to-consumer system addresses refillable-by-bulk-dispenser

[1] https://international-aluminium.org/resource/aluminium-recycling-fact-sheet/

or parent packaging (e.g., refill packaging made with less material than parent packaging), and returnable packaging (Coelho et al., 2020). Other measures can be applied to reduce the environmental footprint of packaging such as the removal of excessive packaging, redesigning packaging to use less material, and plain packaging so that it can be easily recycled (Gao et al., 2022). Nevertheless, a shared responsibility approach is needed with incentives for producers and suppliers but also a consumer responsibility when the environmental impact of products includes consumption and end-of-life. As an example, consumers can pay a tax for the management of waste they generate, irrespective of where the packaging was produced, to guarantee that end-of-life of packaging waste follows the best interest of all (Gao et al., 2022).

Household waste sorting is an essential aspect of the waste management system; moreover, correct sorting of food packaging waste is cost-effective since it facilitates recycling and enhances the quality of the recycled materials (Nemat et al., 2019). However, environmental studies with data arising specifically on consumer behaviour from pre-purchase to post-consumption are scarce. There has been limited investigation into the relationship between sustainable packaging and consumer purchasing behaviour, and more studies should be conducted to cover the different products and consumer perspectives (Martinho et al., 2015).

The seafood industry is considered to be less innovative in new packaging development compared to other adjacent industries such as meat and poultry (Olsen et al., 2017). Furthermore, seafood packaging attributes appear to be underappreciated by researchers (Carlucci et al., 2015). Most of the research related to seafood consumption focus on consumers' preferences, motives and barriers (e.g., Cardoso et al., 2016; Pieniak et al., 2007; Verbeke et al., 2005). Attributes such as taste, appearance, freshness, and health benefits are often valued over production-related features (e.g., sustainability, production method) (Witter et al., 2021). Consumers value, above all, packaging attributes associated with quality, including the possibility to visualize the product (Olsen et al., 2017). To our knowledge, no research has been performed yet on the willingness of consumers in reducing the environmental impact of seafood products from packaging and if they proceed correctly at home when dealing with different seafood packaging waste materials. To collect survey data, consumer studies can be developed using web-based questionnaires (e.g., Altintzoglou et al., 2010a; Minnens et al., 2020), consumer panels (Altintzoglou et al., 2010b; Kole et al., 2009) or recruitment companies (Altintzoglou et al., 2021).

In this study, an online survey was developed to approach consumers in three countries, Spain (ES), Portugal (PT), and Ireland (IE). These countries were selected because they are European Atlantic countries, and the NEPTUNUS project, the framework where this research was developed, had data available. The main goals of this study were to establish among consumers from the three countries: 1) the reported rate of household waste sent to recycling for different seafood packaging types and associated materials; 2) if a relationship exists between seafood consumption frequency and the rate of seafood packaging separation for recycling; 3) knowledge regarding the recycling practices of different types of seafood packaging or materials; and 4) the willingness of consumers to purchase seafood products that use packaging with lower environmental impacts.

2. Material and methods

The data collection was carried out online and it was preferred over other sampling methods (e.g., face-to-face questionnaires) based on convenience. Respondents can connect to the internet using various types of mobile devices and it allows instant access to a wide audience, irrespective of their geographical location, which makes it appropriate for cross-sectional studies and/or international comparisons (Evans and Mathur, 2018; Ilieva et al., 2002). A web-based questionnaire is easily accessible by a broader range of consumers and has the advantage to facilitate data entry, and overcoming time and budget constraints (Rogers, 2007). The anonymity possible by the internet is believed to help respondents in sharing their experiences and opinions (Van Selm and Jankowski, 2006). This sampling method has also disadvantages since only respondents with access to computer technology and the internet will be able to participate and the answering instructions could be unclear (Callegaro et al., 2014). There is also a problem with sampling bias in non-probability panels (i.e., when people select themselves into the panel) with the impossibility to know in advance the probability of respondents' characteristics (Evans and Mathur, 2018). When results of online surveys cannot be defined as representative of the population, non-probability samples can be valuable as they may be representative of a subgroup of the total population (Van Selm and Jankowski, 2006). Therefore, a web-based questionnaire was a suitable sampling method for the explorative aim of this analysis. The survey questions were prepared in English, tested in an online form, and translated into other three languages: Portuguese, Spanish, and Gaelic. Adaptations were made in the different versions to better represent the specificities of each country (e.g., the recycling bin where plastic and metal should be discarded in Portugal and Spain is "yellow" and in Ireland is not associated exactly with a colour).

Data were collected through an online survey created on the LimeSurvey online tool. The links of four surveys were released corresponding to each language and shared via social media (e.g., Twitter, Facebook, LinkedIn) and email, with 640 validated and complete responses obtained: 228 from Portugal (PT), 234 from Spain (ES), and 154 from Ireland (IE). Additionally, seven answers were received from other countries and 17 more that did not present socio-economic data were eliminated. For those who agreed to participate, written informed consent was given at the beginning of the survey according to the General Data Protection Regulation (GDPR). Data collection was conducted from July to September 2021.

The questionnaire was structured in three parts (supplementary material): the first part collected information on seafood consumption patterns; the second section included questions about recycling and willingness to accept seafood products with packaging improvements to reduce environmental impacts; and the last section collected data on individuals' sociodemographic characteristics, such as gender, age, level of education completed, occupation, number of household members, living with children, living distance from the coast, and type of living place (e.g., rural versus city and inland versus coastal). Questions on seafood consumption habits referred explicitly to the period before the COVID-19 pandemic. Also, it was clarified in the consumption group of questions that "seafood" was meant as "fisheries and aquaculture products including fish, crustaceans and molluscs" and seafood consumption frequency should relate to an average weight per meal of a "portion of around 150 g or about the size and thickness of your hand".

Data were analysed by country; for the seafood consumption frequency, a cluster diagram was prepared to represent the respondents with high consumption which corresponded to more than twice a week, and low consumption which corresponded to equal or less than twice a week. Statistical differences were verified for socio-economic characteristics, seafood consumption frequencies, and the reported rate of household waste sent to recycling for different seafood packaging types with Pearson's chi-squared test for contingency tables. For tests of associations between gender and answers related to seafood consumption and packaging, we used Chi-squared, Spearman's correlation, Kruskal-Wallis, Two-sided Student's *t*-test, and Wilcoxon signed-rank tests.

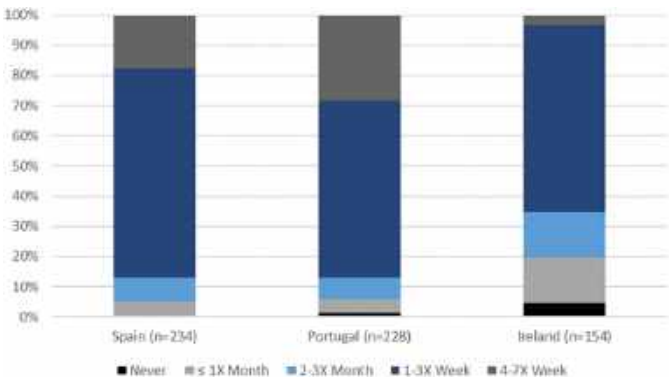


Fig. 1. Relative frequency of seafood consumption in the three countries.

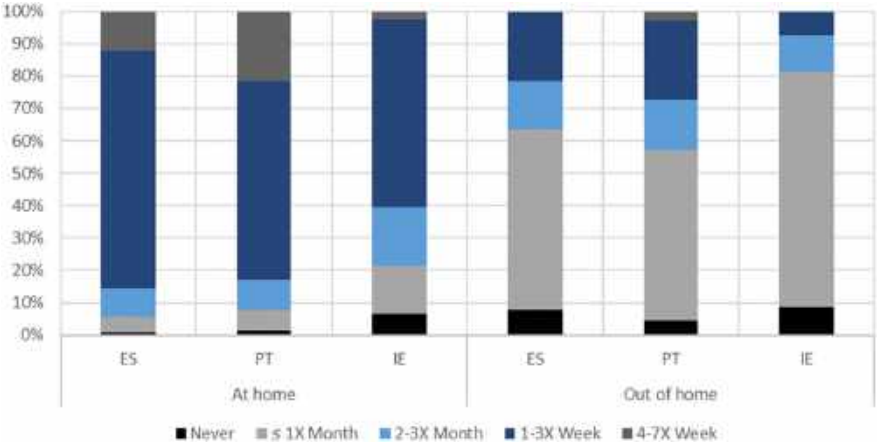


Fig. 2. Relative frequency of seafood consumption in the three countries at home and out of home.

3. Results

3.1. Seafood consumption frequency of the respondents

Regarding the seafood consumption frequency (Fig. 1), most of the respondents reported consuming seafood between one to three times a week (ES – 68.8 %, PT – 57.0 %, IE – 61.0 %). However, significant differences were found between participating countries ($p < 10^{-10}$, Chi-squared test), which can be explained by a much higher percentage of Spanish and Portuguese who consumed seafood four to seven times a week (ES – 17.5 %, PT – 27.6 %) compared to Irish consumers (IE - 3.2 %), and a much higher percentage of Irish people who answered that they never consumed seafood (IE – 4.5 %). When analysing the frequency of seafood consumption in and outside the home (Fig. 2), similarities between Portugal and Spain were observed. For consumption at home, a higher percentage of respondents ate one to three times a week in both countries when compared to Ireland (ES - 72.6 %, PT - 61.4 %, IE - 58.8 %). For consumption of seafood outside the home, more respondents from Ireland eat seafood only once a month or less than compared to Portugal or Spain (ES - 53.0 %, PT - 48.7 %, IE - 72.1 %).

When the analysis of the consumption frequency was by type of seafood product (Fig. 3), there was a higher consumption of fresh/chilled products in the three countries, with a higher number of respondents answering that they consumed these products one to three times a week (ES – 60.3 %, PT – 54.8 %, IE – 41.6 %). For frozen and canned seafood, the highest number of respondents occurred from Spain and Portugal who answered that they consumed these products one to three times a week (ES – 35.9 % and 49.1 %; PT – 35.9 % and 34.2 %, respectively). However, it was once or less a month for Irish respondents (IE – 45.5 % and 41.6 %). Seafood products that were marketed as pre-cooked or ready-to-eat were less consumed in the three countries since the majority of respondents never consumed these products (ES – 58.1 %, PT – 47.4 %, IE – 46.8 %). Smoked products were consumed in general by most of the respondents from the three countries once or less a month (ES – 48.3 %, PT – 55.7 %, IE – 57.8 %) while dried and salted products were also eaten in Portugal once or less a month (PT – 48.2 %); but, in the case of Spain and Ireland most people never eat such products (ES - 43.6 %, IE – 76.0 %).

3.2. Household waste sent to recycling and knowledge of recycling guidelines

In the case of Spain and Portugal, the largest number of respondents answered that they "always" send their non-organic waste (i.e., glass, paper, plastic waste) to specific bins to collect waste for recycling (Fig. 4), as reflected by 40.6 % and 42.3 % response rates respectively. In the case of respondents from Ireland, the largest number answered that they separate non-organic waste "often" (75 % of the times) to send it to recycling, corresponding to 29.5 % of the respondents. When the question was specific to seafood packaging types, in the case of Spain and Portugal the largest number of respondents answered that they "always" sent metal cans, and plastic package including expanded polystyrene, paper package, and glass jars to recycling. In the case of Ireland, a different pattern was observed where the largest number of Irish respondents answered that they "always" sent

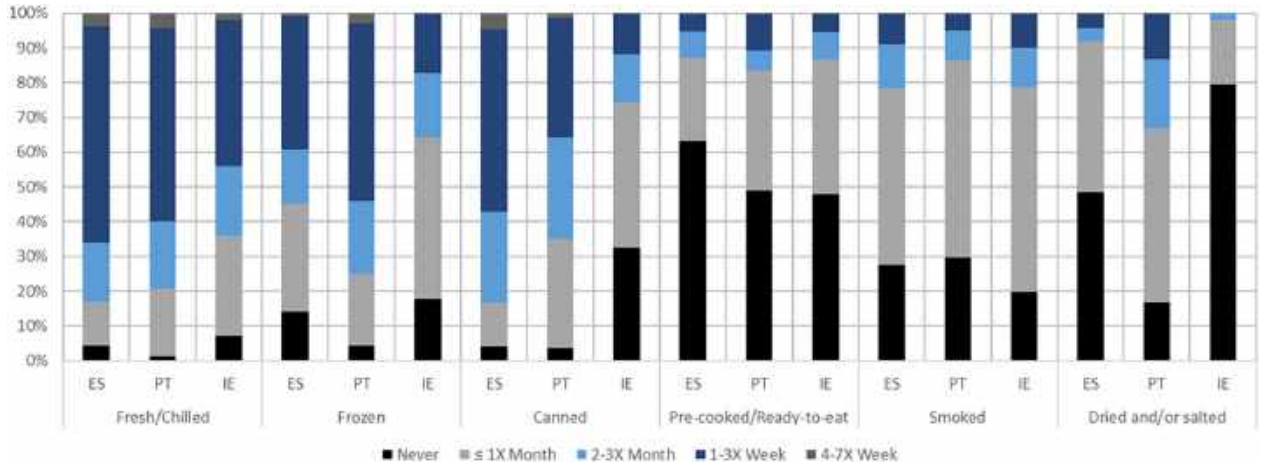


Fig. 3. Realtive frequency of seafood consumption for different post-harvest processing products.

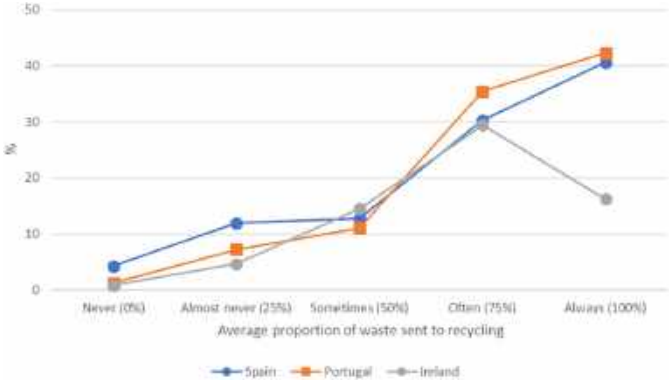


Fig. 4. Percentage of respondents that perform seperation of household waste of non-organic waste(i.e. glass, paper, plastic waste) to be sent to recycling in the three countries.

metal cans, paper packaging, and glass jars to recycling. However, for plastic packaging, the majority answered "often" and expanded polystyrene was "almost never" sent to recycling (Table 1).

When the respondents were divided into groups according to their seafood consumption frequency, a statistically significant difference related to the behaviour of sending household waste to recycling was found ($p < 0.006$, Chi-squared test). Respondents who self-reported higher consumption of seafood also self-reported higher intention of sending non-organic waste for recycling (Table 2).

By quantifying correct answers to the statements (Table 3) (question number 10 in the questionnaire available in the supplementary material), it was possible to characterize in part the knowledge of recycling practices when consumers deal with different types of packaging or materials. The answer that provided the greatest agreement by the three participating countries related to the statement that "metal cans from canned seafood packaging should be put in the yellow waste container or recycling bin" (TRUE was the correct answer in all cases). It received the highest number of correct answers from the three countries and significant differences were found between countries ($p = 0.02$, Chi-squared test), driven by the higher number of correct answers from PT. The answer that "empty plastic bags used for fresh seafood packaging should be put in yellow waste container

Table 2
Percentage of respondents that separate household packaging waste to recycling associated with their level of seafood consumption (%).

	Rate of household waste sent to recycling				
	0 %	25 %	50 %	75 %	100 %
High consumption (> 2× week)	1.3	7.7	11.1	34.2	45.7
Low consumption (≤ 2× week)	3.3	10.1	16.7	38.0	31.9

to recycle or recycling bin" (TRUE was the correct answer to ES and PT; FALSE was the correct answer to IE) received the second highest number of correct answers from the three countries and differences between countries were significative ($p = 0.0028$, Chi-squared test). In terms of whether it is necessary to wash metal cans from canned seafood packaging before they are sent to recycling (FALSE in the case of ES and PT, and TRUE in the case of IE), it received the lowest number of correct answers from Spain (47.9 %), less than half, and highest from Ireland (76.0 %). Differences between countries were significant ($p = 0.00000008$, Chi-squared test), driven by a higher number of correct answers from IE and lower from ES. The statement saying that "it is not necessary to wash plastic packaging from frozen seafood products before they are sent to waste" (TRUE was the correct answer in all cases) got overall the lowest number of correct answers and differences between countries were significant ($p = 10^{-13}$, Chi-squared test). Ireland is the country that overall had a lower average (63.9 %) when compared to Spain (68.1 %) and Portugal (78.5 %).

3.3. Willing to consume seafood products that use more sustainable packaging or avoid products with excess packaging

The most accepted alternatives for more sustainable seafood packaging according to responses from the three countries were (i) reusable packaging (e.g., return glass jar from canned seafood to the producer/retailer), (ii) a compostable type of packaging material (e.g., flexible film made from crustacean shells), (iii) "glass jar packaging for canning seafood instead of metal cans, and (iv) intelligent packaging (which extends shelf-life and maintains the quality of the food) (Table 4) (question number 11 in the questionnaire available in the supplementary material). Between 81.6 % and 96.5 % of the respondents answered that they are receptive to these practices. Only 37.0 % to 59.1 % of the respondents were willing to use a squeeze-tube type packaging instead of metal cans and plastic pouch packaging for

Table 1
Percentage of respondents that perform separation of the different seafood packaging types/materials to be sent to recycling in the three countries (%).

		Spain ($n = 234$)	Portugal ($n = 228$)	Ireland ($n = 154$)
Metal cans	Never (0 %)	12.0	4.4	9.7
	Almost never (25 %)	8.1	8.3	5.2
	Sometimes (50 %)	9.0	6.6	6.5
	Often (75 %)	14.1	11.0	11.0
	Always (100 %)	56.8	69.7	67.5
Plastic package	Never (0 %)	7.3	3.1	7.1
	Almost never (25 %)	6.8	4.8	10.4
	Sometimes (50 %)	8.1	9.6	16.9
	Often (75 %)	21.8	22.4	37.0
	Always (100 %)	56.0	60.1	28.6
Paper package	Never (0 %)	8.5	7.0	9.1
	Almost never (25 %)	12.4	9.2	9.7
	Sometimes (50 %)	11.1	12.3	7.8
	Often (75 %)	20.9	20.6	16.2
	Always (100 %)	47.0	50.9	57.1
Glass jar	Never (0 %)	9.4	4.8	13.0
	Almost never (25 %)	6.8	5.3	1.3
	Sometimes (50 %)	9.0	6.6	1.3
	Often (75 %)	13.7	9.6	9.1
	Always (100 %)	61.1	73.7	75.3
Expanded polystyrene	Never (0 %)	19.2	16.2	44.2
	Almost never (25 %)	12.4	10.5	14.9
	Sometimes (50 %)	14.5	8.8	13.6
	Often (75 %)	19.2	11.8	9.7
	Always (100 %)	34.6	52.6	17.5

Table 3
Percentage of respondents from each country that gave the correct answer about recycling guidelines statements (%).

Countries version/statement [correct answers]	Spain (ES) (n = 234)	Portugal (PT) (n = 228)	Ireland (PT) (n = 154)
ES, PT / Metal cans from canned seafood packaging should be put in yellow waste container to recycle [TRUE] – IE / Metal cans from canned seafood packaging should be put in the recycling bin [TRUE] *	88.0	94.7	87.7
ES, PT / It is necessary to wash metal cans from canned seafood packaging before they are sent to waste [FALSE] - IE / It is not necessary to wash metal cans from canned seafood packaging before they are sent to waste [FALSE] *	47.9	64.5	76.0
ES, PT / The plastic packaging from frozen seafood products should be put in normal/undifferentiated waste container [FALSE] - IE / The plastic packaging from frozen seafood products should be put in the general waste [FALSE] *	77.8	93.0	46.8
ES, PT, IE / It is not necessary to wash plastic packaging from frozen seafood products before they are sent to waste [TRUE] *	57.7	72.4	32.5
ES, PT / Expanded polystyrene boxes used for fresh seafood should be put in yellow waste container to recycle [TRUE] - IE / Expanded polystyrene boxes used for fresh seafood should be put in the recycling bin [FALSE]	70.5	75.4	73.4
ES, PT, IE / It is necessary to wash expanded polystyrene boxes before they are sent to waste [FALSE] *	61.5	70.6	58.4
ES, PT / If empty, plastic bags used for fresh seafood packaging should be put in yellow waste container to recycle [TRUE] - IE / If empty, plastic bags used for fresh seafood packaging should be put in the recycling bin [FALSE] *	77.8	88.2	72.1
ES, PT / It is not necessary to wash plastic bags used for fresh seafood packaging before they are sent to waste [TRUE] - IE / It is not necessary to wash plastic bags used for fresh seafood packaging before they are sent to waste [FALSE]	63.7	68.9	64.3
Average	68.1	78.5	63.9

* Statistically significant ($p < 0.05$, Chi-Squared test) differences were found between countries.

canning seafood instead of metal cans, which demonstrates that these should be considered less successful measures to improve seafood packaging from the consumers' point of view.

The avoidance of seafood products due to excess packaging (e.g., a package that has a secondary layer of carton) was confirmed by around half of the respondents, with Portuguese respondents having the highest rate (56.6 %) and Irish the lowest with 44.8 % of the respondents (Table 5). The dried and/or salted products (e.g., salted and dried cod) were considered to have the least excessive packaging for a post-harvest processed seafood product (ES - 5.1 %, PT - 4.8 %, IE - 3.2 %). The opposite trend was found for pre-cooked/ready-to-eat type of products, whereby significant numbers considered packaging excessive (ES - 37.2 %, PT - 39.5 %, IE - 30.5 %).

The majority of respondents, between 62.0 % for Spain, 68.8 % for Ireland, and 70.2 % for Portugal are willing to pay more for seafood products that use more sustainable packaging (Table 6). Of these respondents, the largest number answered that would be willing to pay 10 % more (ES - 43.6 %, PT - 53.1 %, IE - 46.1 %).

3.4. Socio-economic characteristics

The socio-economic characteristics of the sample (Table 7) were statistically different between countries for all characteristics except gender. The percentage of female respondents was slightly greater in the three countries (between 64.5 % to 67.1 %). The sample from Spain presented a younger population (more in 21–40 years old), fewer people lived with parents (25.2 %), a greater percentage of households without children (46.2 %), and more people that lived in a small town (51.7 %) compared to the other two countries. The majority of respondents from Portugal were between 41 and 60 years old (64.9 %) and, compared to the other two countries, had a higher level of education (30.7 % of respondents had a PhD), the majority lived in <50 km distance from the coast (93.9 %) and in a large town (60.5 %) and also did not live with parents (89.9 %). The Irish sample had the highest percentage of people with three or more children (13.6 %), the highest household size comprising five to six members (16.2 and 5.2 % respectively), and the highest proportion of respondents that lived rurally (40.3 %). Despite being an island, 46.1 % of the Irish respondents lived further than 50 km from the sea, as opposed to 6.1 % of Portuguese and 16.7 % of Spanish respondents.

When the results from the questions were tested against the socio-demographic characteristics of the samples (Table 8), it was possible to find some statistical differences. Older respondents (Rho = 0.17; p = 0.00001, Spearman's correlation test), not living with parents (p = 0.0045, Kruskal-Wallis test), and living on the seaside reported a significantly higher seafood consumption frequency (p = 0.000001, Kruskal-Wallis test). A higher frequency of seafood consumption at home was reported by older respondents (Rho = 0.19, p = 0.0000014, Spearman's correlation test), not living with parents (p = 0.03, Kruskal-Wallis test), but living with children (Rho = 0.11, p = 0.007, Spearman's correlation test), and living on the seaside (p = 0.000001, Kruskal-Wallis test).

Males appear to have a significantly higher frequency of seafood consumption outside the home (p = 0.0005, Chi-squared test). This trend seems essentially driven by Portugal, the only sample for which the difference is significant (p = 0.002, Chi-squared test). Age follows the same pattern which might be explained by the fact that older people have usually better jobs and a higher economic level (Rho = 0.15, p = 0.0002, Spearman's correlation test). Students and the unemployed have lower seafood consumption outside the home (p = 0.002, Kruskal-Wallis test) which is normal due to budget limitations, together with respondents with a higher number of people living at the household (Rho = −0.098; p = 0.02 Spearman's correlation test). Respondents not living with parents (p = 0.00002, Kruskal-Wallis test) and living in cities presented higher seafood consumption outside the home (p = 0.005, Kruskal-Wallis test).

Females appear to have a higher willingness to reuse packaging (88.5 %) than males (81.3 %) (p = 0.02, Chi-squared test). There is also a slight tendency that respondents from larger households are not willing to use reusable packaging (p = 0.01, Wilcoxon signed-rank test). The older respondents are more willing to use a compostable type of packaging (p = 0.001, Two-sided Student's t-test), the result is mostly explained by the differences found among the Spanish cohort where those that responded positively were on average 11 years older than those who responded no (p = 0.006, Two-sided Student's t-test). On the contrary, respondents that are

Table 4
Percentage of respondents from each country that answered “yes” about their willingness to use the seafood packaging alternatives (%).

Seafood packaging alternative	Spain (n = 234)	Portugal (n = 228)	Ireland (n = 154)
Reuse packaging (e.g., return glass jar from canning seafood to the producer/retailer)	81.6	88.6	88.3
Use a compostable type of packaging material (e.g., flexible film made from crustacean shells) *	90.2	96.5	94.8
Use a squeeze tube type packaging instead of metal cans	44.0	48.7	37.0
Use plastic pouch packaging for canning seafood instead of metal cans	54.7	52.2	59.1
Use glass jar packaging for canning seafood instead of metal cans	93.2	95.6	90.9
Use of intelligent packaging (which extends shelf-life and maintain the quality of the food)	90.6	92.1	90.9

* Statistically significant (p < 0.05, Chi-Squared test) differences were found between countries.

Table 5
Percentage of respondents from each country that avoid seafood products with excess packaging and consider that post-harvest processing products have excess packaging (%).

		Spain (n = 234)	Portugal (n = 228)	Ireland (n = 154)
Avoid seafood products with excess packaging		49.1	56.6	44.8
Type of post-harvesting processing products	Fresh / Chilled	7.3	12.3	23.4
	Frozen	18.8	33.8	18.2
	Canned	15.0	23.2	4.5
	Pre-cooked / Ready-to-eat	37.2	39.5	30.5
	Smoked	12.0	20.2	13.6
	Dried and/or salted	5.1	4.8	3.2

students (p = 0.0003, Chi squared test), living in larger households (p = 0.046, Wilcoxon signed-rank test), living with parents (p = 0.0000002, Chi squared test), or have a higher number of children in the household (p = 0.02, Wilcoxon signed-rank test) answered that they are not willing to use a compostable type of packaging, and again the result is mostly explained by the differences found among the Spanish cohort. Finally, it was found that there is a slight trend for more educated people to accept the use of a squeeze-tube type packaging instead of metal cans (p = 0.004, Wilcoxon signed-rank test).

4. Discussion

Addressing consumer concerns by way of understanding behavioural aspects and preferences is important as this can also inform changes towards sustainable practices and promote innovation through social marketing (Domegan, 2021; Rowan and Pogue, 2021). The present study focused on the role of consumers across Portugal, Spain and Ireland in terms of understanding practices towards waste of seafood packaging that can affect environmental sustainability. Results revealed that seafood consumption frequency is relatively similar between Spain and Portugal, with the majority of respondents reporting the consumption of these products between one to three times a week, with a slightly higher frequency occurring for the Portuguese cohort. Ireland showed a different pattern where there was a general lower frequency of seafood consumption, including a small percentage of respondents (4.5 %) that never ate seafood. This result is not surprising as apparent per capita consumption of fishery and aquaculture products is highest in Portugal and Spain, with 59.9 kg and 46.0 kg per year respectively, and slightly above the EU average (24.0 kg) in the case of Ireland with 25.5 kg (EUMOFA, 2021). Data obtained for Portugal in previous studies provided a seafood consumption of four to five times a week, which is extremely high (Almeida et al., 2015a; Cardoso et al., 2013). Furthermore, Spanish citizens were associated with regular fish consumers, with 46 % of respondents saying that they consumed fish more than once a week, while Ireland was at 18 % (Zander and Feucht, 2018).

Regarding the different processing products, there is a higher consumption in the three countries for fresh/chilled seafood products. Frozen and canned seafood has the highest consumption frequency in Spain and Portugal, with the largest number of respondents answering one to three times a week for both types of products; but, in Ireland it was one or fewer times a month. Pre-cooked or ready-to-eat seafood was the least consumed in the three countries with the largest number of respondents never eating these products. Respondents that reported higher seafood consumption related to responses with higher rates of household separation of non-organic waste to recycling. This could be of relevance since most of the seafood consumption occurs at home (one to three times a week had the majority of answers from the three countries: 72.6 % in ES, 61.4 % in PT, 57.8 % in IE) and the seafood packaging waste could thus be significant in this setting. Furthermore, a higher frequency of seafood consumption at home was reported by older respondents, not living with parents, but living with children, and living on the seaside.

Respondents appear to be aligned with recycling behaviour as the largest number of respondents from Spain and Portugal said they separate all household waste to be sent to recycling whereas Ireland sends almost all. Differences in responses were noted when the question posed was specific to the type of packaging materials. There were lower rates of waste separation to recycling for Ireland for plastic waste, especially with packaging made by EPS. The reason behind these results could be related to the lack of information provided to consumers on how to deal with different waste materials or a lack of waste management capacity. Providing

Table 6
Percentage of respondents from each country that are willing to pay more for seafood products that use more sustainable packaging (e.g., package that allows to send all the packaging materials to recycling) and how much more (%).

		Spain (n = 234)	Portugal (n = 228)	Ireland (n = 154)
Pay more for seafood products that use more sustainable packaging		62.0	70.2	68.8
How much more	*10 %*	43.6	53.1	46.1
	25 %	14.1	14.9	18.2
	50 %	3.4	0.4	2.6
	> 50 %	0.9	1.8	1.9

Table 7
Summary statistics of the socio-economic characteristics of the samples (%).

		Spain sample	Portugal sample	Ireland sample
Gender	Female	64.5	67.1	66.2
	Male	34.2	32.5	29.9
	Other	0.0	0.0	1.3
	Prefer not to say	1.3	0.4	2.6
Age (years) *	≤ 20	14.1	0.0	0.0
	21–40	41.9	25.0	44.2
	41–60	38.9	64.9	46.1
	≥ 61	4.7	10.1	9.7
Education level *	Primary or below	2.1	0.0	0.0
	Lower secondary	13.7	0.9	0.6
	Higher secondary	3.4	6.1	5.2
	Additional technical training	11.1	1.8	8.4
	BSc (or similar)	23.5	27.6	30.5
	MSc (or similar)	32.5	32.9	36.4
	Bellow upper education			
	Tertiary education	–	–	–
	PhD (or similar)	13.7	30.7	18.2
Current occupation *	Student	16.7	7.9	16.2
	Employed	72.6	79.4	68.2
	Self employed	3.4	6.6	4.5
	Unemployed	4.3	2.2	2.6
	Responsible for the household	0.9	1.8	2.6
	Retired	2.1	2.2	5.8
Number of people living in the household *	1	9.4	12.3	9.1
	2	25.2	28.5	32.5
	3	31.2	27.6	16.9
	4	27.8	25.0	19.5
	5	4.3	5.3	16.2
	≥ 6	1.7	0.9	5.2
Live with parents *	No	74.8	89.9	81.8
	Yes	25.2	10.1	18.2
Number of children in the household *	0	46.2	55.7	56.5
	1	25.6	21.1	14.9
	2	23.9	18.4	14.9
	≥ 3	3.8	4.8	13.6
Type of living area *	Large town	21.8	60.5	37.7
	Rural area or village	26.5	13.6	40.3
	Small or middle-sized town	51.7	25.9	22.1
Living distance from the sea *	Inland (>50 km from the coast)	16.7	6.1	46.1
	On the seaside (<50 km from the coast)	83.3	93.9	53.9

* Statistically significant (p < 0.05, Chi-Squared test) differences were found between countries.

Table 8
Statistical test results (p values) between socio-demographic variables and answers related with seafood consumption and packaging. * Statistically significant ($p < 0.05$) differences were found; and the following statistical test was used: [1] Chi-squared test; [2] Spearman's correlation test; [3] Kruskal-Wallis test; [4] Two-sided Student's t-test, [5] Wilcoxon signed-rank test.

	Gender	Age	Education level	Occupation	N° people in the household	Live with parents	N° children in the household	Type of living area	Living distance from the sea
Seafood consumption frequency	- [1]	Rho = 0.17; $p = 0.00001$ [2] *	- [2]	- [3]	- [2]	0.0045 * [3]	- [2]	- [3]	0.000001 * [3]
Seafood consumption at home	- [1]	Rho = 0.19; $p = 0.0000014$ [2] *	- [2]	- [3]	- [2]	0.029 * [3]	Rho = 0.11; $p = 0.007$ * [2]	- [3]	0.00002 * [3]
Seafood consumption outside the home	0.0005* [1]	Rho = 0.15; $p = 0.00018$ [2] *	- [2]	0.0017 * [3]	Rho = −0.098; $p = 0.017$ * [2]	0.00002 * [3]	- [2]	0.0047 * [3]	0.0076 * [3]
Willingness to reuse packaging	0.021 * [1]	- [4]	- [5]	- [1]	0.046 [5]	- [1]	- [5]	- [1]	- [1]
Willingness to use a compostable type of packaging material	- [1]	0.001 * [4]	- [5]	0.0003 [1]	0.01 [5]	0.0000002 [1]	0.02 [5]	- [1]	- [1]
Willingness to use a squeeze tube type packaging instead of metal cans	- [1]	- [4]	0.004 * [5]	- [1]	- [5]	- [1]	- [5]	- [1]	- [1]
Willingness to use plastic pouch packaging for canned seafood	- [1]	- [4]	- [5]	- [1]	- [5]	- [1]	- [5]	- [1]	- [1]
Willingness to use glass jar packaging for canned seafood	- [1]	- [4]	- [5]	- [1]	- [5]	- [1]	- [5]	- [1]	- [1]
Willingness to use of intelligent packaging	- [1]	- [4]	- [5]	- [1]	- [5]	- [1]	- [5]	- [1]	- [1]
Avoid seafood products with excess packaging	- [1]	- [4]	- [5]	- [1]	- [5]	- [1]	- [5]	- [1]	- [1]
Pay more for seafood products that use more sustainable packaging	- [1]	- [4]	- [5]	- [1]	- [5]	- [1]	- [5]	- [1]	- [1]

recycling information is particularly important to enhance consumer knowledge of the value of packaging and to eliminate uncertainty about sorting different packaging materials (Nemat et al., 2019). From open questions, it was possible to understand that some respondents seem to be confused about what type of plastics can be recycled. Since many types of plastic exist and only a few cannot be recycled, a more efficient message could be to request consumers to separate all plastic to be sent to recycling and, in this way, avoid the risk of losing plastics from packaging that would go to undifferentiated waste treatment, for example, landfilling. Furthermore, in Ireland non-rigid plastic (e.g., plastic film, grocery bags) could only be sent to recycling waste bins since September 2021, which might explain the plastic recycling responses in Ireland to some extent were contrary to Spain and Portugal.

When we analysed the four statements relating to knowledge about recycling including where metal cans, plastic packaging from frozen, EPS boxes, and plastic bags from fresh seafood should be put or not in the recycling bin, on average, Ireland received the lowest percentage of correct answers to these statements (63.9 %) compared to Spain (68.1 %) and Portugal (78.5 %). The fact that in Ireland plastic packaging, metals, and all paper and cardboard materials should be sent to the same recycling bin (whereas in Spain and Portugal there is a yellow container for plastic and metal packaging and a blue container for paper and cardboard) can have a counter-productive effect on an individual's capacity to send packaging to recycling. There is also another difference between countries as in Ireland, in contrast to Portugal and Spain, consumers are asked to wash packaging before sending them to the recycling bin. This happens because in Ireland, since recycling materials are mixed, dirty or unsuitable items could contaminate the entire contents. This behaviour seems to be evident among Irish consumers since Irish participants reported a higher percentage of correct answers compared to the other two countries when asked if it is necessary to wash metal cans from canned seafood packaging before they are discarded (76.0 % for IE against 47.9 % for ES and 64.5 % for PT). One reason that might explain these results is that metal cans might be perceived as dirtier packaging compared to plastic due to the associated oil used to preserve canned seafood. It seems that a higher effort exists in Ireland in communicating this specific behaviour of washing dirty packaging when it is needed. In Spain and Portugal, this seems to be confusing since results for washing metal had the lowest number of correct answers from all the statements in both countries' samples. Washing packaging does not add any benefit to the recycling process since grease, traces of food, or even liquid residues do not interfere with the process, as there is a washing phase in the recycling treatment that eliminates these traces. This means that, when washing the packages at home before sending them to the recycling bin, consumers in Portugal and Spain are doubling the work and wasting water. The other three statements related to washing plastic packaging from frozen seafood, EPS boxes, and plastic bags from fresh seafood all got a higher level of correct answers from respondents from Portugal, with 72.4 %, 70.6 % and 68.9 %, and lower from Spain (57.7 %, 61.5 %, and 63.7 % respectively) and Ireland (32.5 %, 58.4 %, and 64.3 % respectively). The results suggest that improved communication using more appropriate guiding and policy information could help consumers in Spain and Portugal adjust their behaviour. This is supported by observations made by Klaiman et al. (2017) on the testing of plastic or boxboard sandwich containers, where having to clean packaging can alter preferences for packaging material and affect consumer recycling behaviour at the household.

The statement that had the highest consensus and highest number of correct answers, with 90.4 % on average, from the three countries, was if metal cans from canned seafood should be put in the recycling bin. This result is in accordance with the high level of aluminium recycling rate in Europe, which in Spain and Portugal, it can also be recovered from undifferentiated waste. Nevertheless, it demonstrates that more effort should be done for other types of packaging materials such as plastics. In general, it seems that there is a lack of information about plastic packaging recycling since some respondents intimated in open answers that they do not fully know the guidelines and believe that seafood plastic packaging cannot be sent to the recycling bin and, for example, that EPS package cannot be recycled. Also, some respondents believe that they need to wash the plastic and EPS to be recycled and, since they do not want to do it, they avoid contaminating “clean” packaging in the recycling of packages (in the case of Spain and Portugal). To overcome these misunderstandings, it would be important to communicate detailed procedures applicable to each type of material, including the different types of plastics (e.g., EPS, High-Density Polyethylene (HDPE), Low-Density Polyethylene (LDPE)), and also explain the destination of these types of materials after recycling. Specific communication for certain types of seafood products could also support the willingness of consumers to send more household waste to recycling, especially in Spain and Portugal, where seafood consumption at home is so frequent. Nevertheless, the different recycling guidelines implemented in the EU countries could impair the consistency of recycling behaviours in households at the European level.

The most consensual alternatives to using more sustainable packaging for seafood from the three countries were the use of reusable packaging (e.g., return glass jars from canning seafood to the producer/retailer), glass jar packaging for canning seafood instead of metal cans, compostable types of packaging material (e.g., flexible film made from crustacean shells), and intelligent packaging (which extends shelf-life and maintains the quality of the food). By reusing packaging, a significant reduction in the environmental impact of packaging could be feasibly achieved as a second use will allocate part of the environmental cost of the packaging. Therefore, this alternative could be promoted in case companies find it feasible (e.g. implement a return deposit scheme of glass jars for some beverage products such as water bottles). The main barrier identified by producers is a major logistical complexity, which requires the reorganisation of supply chains to ensure that packaging is available and returned through distribution and that consumers maintain their loyalty (Coelho et al., 2020). Another possibility could be the use of reusable containers from consumers when purchasing seafood at the fishmongers to avoid the use of plastic bags/heat-sealed bags. Females appear to have a higher willingness to reuse packaging than males, but not respondents from larger households that may be due to an idea that this possibility could imply extra work for the household routine which might be already complicated in households with more people.

Glass jar packaging for canning seafood could represent environmental benefits when compared to aluminium (Laso et al., 2017). In the same way, through packages with intelligent packaging consumers may also reduce seafood waste and prevent unnecessary transport and logistics from an early stage (Cammarelle et al., 2021; Salgado et al., 2021). The use of a compostable type of packaging material could improve the end-of-life of seafood packaging, which is mainly an alternative to plastic; but, it raises the point if municipalities are prepared to collect waste compost and if it is an appropriate disposal alternative. Older respondents seem to be more willing to use a compostable type of packaging, and on the contrary, respondents that are students, living in larger households, living with parents, or having a higher number of children in the household answered that they are not so willing to use compostable packaging. These results are mostly explained by the differences found among the Spanish cohort which is characterized also by younger respondents when compared with the other two samples, meaning older people could be more aware of the possibilities of composting their waste.

New design features that make packaging more sustainable could also increase the likelihood that sustainable packaging is chosen which could potentially increase the level of positive-environmental behaviour from consumers (Martinho et al., 2015). However, the use of a “squeeze-tube type packaging instead of metal cans”, commonly marketed in northern European countries, and “plastic pouch packaging for canning seafood instead of metal cans” had half or less than half of the answers so these should be considered less successful measures to improve seafood packaging from the consumers' point of view. These options imply a redesign of the packaging which may make the package less recognizable to consumers or less convenient, and the use of different types of material may reduce the appeal and attractiveness (Prendergast and Pitt, 1996). Even though, a slight trend was found for more educated people to accept the use of a squeeze-tube type packaging instead of metal cans.

Respondents appear to be concerned with packaging because around half of them intimated that they avoid seafood products due to excessive packaging (e.g., packaging that has a secondary layer of carton). This is especially the case for pre-cooked/ready-to-eat types of products since it was the type of post-harvest processed product that had a higher number of respondents who considered it to have excessive packaging. These products are less consumed in the three participating countries since the majority of respondents answered that they never eat them. The second level of products that respondents associate with excessive packaging is frozen products. In contrast, dried and/or salted products (e.g., *bacalhau* or mackerel) were the seafood products that fewer respondents from the three countries considered had excessive packaging. The majority of respondents from Portugal answered that they consumed dried and salted seafood products once or fewer times a month; but, in the case of Spain and Ireland the highest number of respondents never tried them. Fresh or chilled seafood products, the most common way of consuming seafood in these countries, were also not considered as having excessive packaging, especially by respondents from Spain and Portugal.

The majority of respondents were willing to pay more for seafood products that use more sustainable packaging (for example a 10 % increase in price was acceptable for 62.0 % of respondents in Spain, 68.8 % in Ireland 70.2 % in Portugal). This result shows the commitment of consumers in contributing to an effort that may need to be carried out in partnership with industries, presenting opportunities for innovation and more environmentally-friendly solutions (Steenson and Creedon, 2022). This result is aligned with consumer motivation to pay for more sustainable seafood products which is positively correlated to respondents' income and their environmental concerns, with a maximum premium of 10 % of the product price that consumers were happy to pay (Salladarré et al., 2016). Nevertheless, many people find difficulties in defining terms such as ‘sustainability’ in relation to something as specific as packaging and the principles in improving environmental impacts need to be translated into more clear requirements (Lewis, 2005).

Overall, the results highlight a positive attitude of consumers towards recycling and care for packaging with lower environmental impact. However, the results could also be biased by the highly-educated cohort sample which may affect the consumers' knowledge as well as their reported attitudes. This also highlights an area to improve upon for future research to reach consumers from a broader socio-demographic range and consequently obtain a more holistic perspective of environmental concerns. Other limitations of this present research should be noted. Firstly, the sample is not representative of countries' populations and results should be discussed as representing only the group of people, with their respective socio-demographic characteristics, that answered the survey. Additional research is recommended to ensure a full characterisation of the entire seafood consumer market in European countries. A second limitation is related to the data collection method adopted as the survey was processed online, representing a strong barrier for people with low motivations and/or unfamiliar with new technologies or social networks and as a result, higher educated people were more likely to answer to the survey (e.g., Cammarelle et al., 2021). Nevertheless, results can give an outlook on how much respondents are willing to contribute to reducing the environmental impact of seafood products through packaging and start a discussion on what is needed to improve the end-of-life of seafood packaging. A third issue that one should bear in mind is that answers with self-declared intentions related to more responsible behaviours or willingness to pay can be overestimated by people responding too positively when compared to reality.

Food packaging and its attributes can potentially hinder or motivate consumers to sort packaging waste correctly. The design of packaging influences recycling behaviour and, therefore, the recyclability of packaging should be considered an inherent value of the packaging, similar to other attributes such as beauty or durability (Nemat et al., 2019). Addressing consumer behavioural and attitude changes towards seafood packaging is important. In this instance the transition towards increased circularity where communication can be done by deploying appropriate marketing practices to educate and empower consumers (Domegan, 2021; Rowan and Casey, 2021). Consumers are only one of the key players in the seafood value chain and efforts are also needed from other groups of stakeholders (e.g., policymakers, dieticians, consumer organisations, retailers, fishermen, seafood processing industry) to ensure that future seafood production, processing, distribution and consumption become more sustainable. In this sense, transparent and trustful information with the help of certification labelling is recommended (Sacchettini et al., 2021). As an example, labelling based on Product Environmental Footprint (PEF) guide could be developed to deliveri information to consumers integrating topics that are still poorly described, such as the end-of-life of packaging materials (Hélias et al., 2022).

5. Conclusions

The reported rates of household waste sent to recycling of different seafood packaging types and associated materials were relatively high in the three countries analysed: Spain, Portugal, and Ireland. Respondents also appear to be willing to pay more for seafood products that use more sustainable packaging and avoid seafood products with excess packaging. A general relationship was observed between seafood consumption frequency, which mainly occurs at home, and seafood packaging separation for recycling. These results need to be linked with a sample of respondents that in general is characterized by highly-educated people and mostly employed. However, these findings can provide insights as to where to invest resources to inform on sustaining behaviour in seafood packaging waste disposal. Recycling practices at the household level could be improved upon with more appropriate information provided about specific types of seafood packaging and materials, especially for plastics that present a huge diversity of types. This may be significantly important in countries where there is high seafood consumption, especially at home, as is the case for Spain and Portugal. An increase in the quantity of packaging sent to recycling and efficiency of the waste management system can hence improve the circularity and reduce the environmental cost of seafood related to packaging and its materials.

CRediT authorship contribution statement

Cheila Almeida: Conceptualization, Methodology, Investigation, Data curation, Project administration, Writing – original draft. **Jara Laso:** Methodology, Investigation, Visualization, Writing – original draft. **David Baptista de Sousa:** Methodology, Investigation, Writing – original draft. **Ronan Cooney:** Methodology, Investigation, Writing – original draft. **Paula Quinteiro:** Methodology, Writing – original draft. **Neil Rowan:** Methodology, Writing – review & editing. **Ana Cláudia Dias:** Methodology, Writing – review & editing. **Eoghan Clifford:** Methodology, Writing – review & editing. **Rodrigo G. Reboredo:** Writing – review & editing. **María Margallo:** Writing – review & editing. **Maria Leonor Nunes:** Methodology, Writing – review & editing. **António Marques:** Methodology, Writing – review & editing, Supervision.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are most grateful to Filipe Ferreira from IPMA Informatic Systems Division for help in preparing the online survey using LimeSurvey software and Nuno Barbosa Morais from iMM for help with data analysis. The authors would like to acknowledge the promotion of the survey by the various agencies, stakeholders and groups. This work was supported by the NEPTUNUS project (EAPA_576/2018). The authors would like to acknowledge the financial support of Interreg Atlantic Area. Ana Cláudia Dias and Paula Quinteiro acknowledge FCT/MCTES for the financial support to CESAM (UIDB/50017/2020 + UIDP/50017/2020 + LA/P/0094/2020), through national funds, and to the research contracts CEECIND/02174/2017 and CEECIND/00143/2017, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160846.

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Current Opinion in
Environmental Science & Health

Empower Eco multiactor HUB: A triple helix 'academia-industry-authority' approach to creating and sharing potentially disruptive tools for addressing novel and emerging new Green Deal opportunities under a United Nations Sustainable Development Goals framework

Neil J. Rowan[1] and Orla Casey[2]

Abstract

There is a pressing drive to address climate change and environmental degradation that are global existential threats. Europe has strategically responded by unifying efforts to transform its connected communities into a modern, resource-efficient and competitive economy with a trajectory to enable net nil greenhouse gas emissions by 2050; thus, ensuring economic growth is decoupled from resource utilisation, and that no person or place is left behind. The European Green Deal is an ambitious plan to make the European economy sustainable; however, there is no reference blue-print for the safe and just transitioning to a low carbon economy. This constitutes the first description of a triple helix (academic-industry-authority) concept underpinning operation of multiactor innovation hub that can be strategically applied to enable this transition that develops green innovation and enterprises. Innovative tools for meeting the United Nations' Sustainable Development Goals are informed by appropriate technology, policy and society readiness levels from idea to final market/wider society deployment. "Empower-Eco Sustainability HUB," is a digitised "living lab" established in the Irish peatlands that converges academia, communities, social enterprises, industries, policy and decision-makers. It develops green innovation in intended environments at demo/test-beds, such as for digital, agri-food, bioeconomy and bio-based sectors, and embraces climate-proofing and COVID-19 recovery.

Addresses

[1] Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland

[2] Momentum Educate + Innovate, Orchard Court, Leitrim Village, Co. Leitrim, Ireland

Corresponding author: Rowan, Neil J (nrowan@ait.ie)

Current Opinion in Environmental Science & Health 2021, **21:**100254

This reviews comes from a themed issue on **Environmental Toxicology 2021: Disruptive Green Deal Innovations**

Edited by **Neil J. Rowan** and **Robert Pogue**

Given his role as Guest Editor Neil J. Rowan had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Damia Barceló Culleres.

For a complete overview see the Article Collection

https://doi.org/10.1016/j.coesh.2021.100254

Keywords

Just transition, New Green Deal, Sustainability, Open research, Multi-actor hub, Circularity, UN Sustainable Development Goals.

Introduction

Climate change and environmental degradation are recognised as existential threats that require urgent transnational action [1–3]. Ebi et al. [2] also stated that human health and wellbeing and the health of the biosphere are inextricably linked. Ebi et al. identified four main themes: (1) risk identification and management (including related to water, hygiene, sanitation and waste management); food production and consumption; oceans; and extreme weather events and climate change. (2) strengthening climate-resilient health systems; (3) monitoring, surveillance and evaluation; and (4) risk communication. They reported that research needs to be "transdisciplinary, multi-scalar, inclusive, equitable and broadly communicated; thus, promoting resilient and sustainable development are critical for achieving human and planetary health". The European Green Deal is an ambitious plan to make the European economy sustainable, which will inform a panoply of new policies, programmes and legislation that will propel Europe's Green Deal, Biodiversity, Farm to Fork and Circular Economy plans [4,5]; this is set against a backdrop of economic recovery, including changes to trade agreements due to BREXIT and ongoing COVID-19 pandemic [6].

Addressing pressing climate and environmental challenges will potentially create opportunities leading to sustaining or potentially new disruptive solutions [7]. For example, there is a pressing need to develop breakthrough 'circularity' solutions to replace the current 'take-make-dispose' economy that will positively have an impact on food waste regeneration, resource utilisation and climate change [8]. Adopting nonconventional approaches to

research will avoid untapped outcomes that typically can occur in silos associated with traditional research, which limit their use and benefit to stakeholders. Unlocking the value of impact from research innovation in these areas remains a significant challenge due to *inter alia* barriers, lack of trust between different actors, perceptions of the social consequences to change, economic barriers to investment, and unclear returns [9]. Gaining an appreciation of the effective use, re-use and upcycle loops will inform next-generation eco-solutions that that will meet research gaps of enterprises along with tailoring education and training [1,10]. Recovering from the ongoing COVID-19 pandemic will create opportunities directly influencing our environment, our health and our society [11]. There is also a growing interest in supporting "One Health" as a global concept that recognises that humans, animals and their shared environments are interconnected. It is increasingly apparent that it is necessary to take a One Health approach to tackle many of the health challenges we face in today's world with nexus to environmental protection, resource utilisation and sustainablity.

Developing and exploiting multiactor HUB approaches to unlock eco-innovation for new Green Deal era – lessons learnt from transnational modelling

One approach is to developing multiactor eco-innovation hubs that will drive open responsible research and innovation, such as in the areas of smart agri-food, forestry, marine, and bio-based systems [8,12]. Such a surge in interest will be informed by the outputs of transnational models and clusters that focus on drivers for change [1,13]. Multiactor HUBs will enable future solutions to improve waste mitigation and improve food security are likely to embrace open sharing of knowledge intra-regionally that can stimulate regeneration; this will be informed by life cycle assessment such as unlocking the nexus between food, energy and water to inform enterprise [14,15]. Eco-hubs will enable a structured 'holistic systems' approach that cuts across sectors and disciplines and engages multiple actors to deliver co-benefits for health, sustainability, climate and inclusion [1,12]. This is similar to the "triple-helix" concept of converging academia, industry and authorities to address complex challenges and to unlock green opportunities. For example, a circular food and resource supply can potentially become a reality where wasteful practices can stop, and circularity can be designed into all new products and services from the start [8]; however, for this to occur, a disruptive approach to channel effort will be required, including step changes in education that informs environmental literacy and behavioural change. Transnational modelling of technology core facilities and clusters for regional development, such as that described by European Interreg projects [1,13], have highlighted the need to develop multiactor hubs that link academia with industry and stakeholders. These create opportunities for sharing infrastructure and skills that underpin the needs of SMEs linked to academia that potentially lead to advances in sustaining and new disruptive technologies [7,16,17]. Loorbach et al. [18] also conceptualised that the development of such transformative initiatives can occur through growing, replicating, partnering, instrumentalising and embedding; this is supported through translocal networks that connect initiatives by sharing ideas, objects and activities across local contexts. This need also extends to econometric models for assessing government policies to support SMEs in the context of meeting ongoing and future pandemics [19]. However, there has been a significant interest in advancing supply chain disruption and risk mitigation to manage COVID-19 and to support recovery, including improving the networking of multiactors and beneficiaries [20]. Development of Green innovation from idea to market introduction, along with wider societal deployment, will also be informed technology, policy and societal readiness levels that are increasingly being used by research funding bodies [9] (Table 1).

Emerging opportunities using the peatlands

The peatlands account for ca. 3% of the earth's surface and as an important CO_2 sink where it stores approximately 1.4 trillion tonnes of carbon that is equivalent to 75% of carbon in our atmosphere [1]. Several European-funded Interreg projects, such as NWE Carbon Connects [21], North Sea CANAPE [22], NWE Care Peat [23]), have focused on restoring peatlands and biodiversity, sequestering carbon and supporting wet-peatland innovation. 'Paludiculture' crops described in the aforementioned include sphagnum moss, moor-grasses, wild rice, typha and so forth. These transnational projects, along with EC's "*Platform for Coal Regions in Transition*" initiative, support transitioning away from exploiting fossil fuels to develop more climate-resilient solutions [1,24]. Paludiculture focused-activities could be extended to supporting enterprise and research in designated 'green development zones', such as for eco-design, testing and eco-labelling of new environmental-friendly products and services [25]. For example, Bord Na Mona, a State Body that manages 80,000 ha of 'organic status' peatland in the Republic of Ireland, has aggressively adopted such a new '*Brown to Green Strategy*' that also includes controlled re-wetting of the peatlands for conservation along with forging climate solutions based on sustainable resource utilisation and green innovation. Consequently, many workers require retraining/upskilling in these areas. It is noteworthy that the global Green Technology and Sustainability market size is to potentially grow from USD 11.2 billion in 2020 to USD 36.6 billion by 2025, at a Compound Annual Growth Rate (CAGR) of 26.6% during the forecast period [26]; these include targeted technologies (such as IoT, AI and analytics, digital twin, cloud computing), and applications (such green building, carbon footprint management, weather monitoring and forecasting).

Table 1

Applying triple helix of technology, policy and society readiness levels to inform the development of a green project, a technology, a product, a process, a management practice, an intervention or an innovation from initial idea to market launch, scheme and wider societal development.

Technology Readiness Levels[a] (TRL)[a]		Policy Readiness Levels[b] (PRL)		Society Readiness Levels[c] (SFL)	
TRL 1 – Basic Research – Principles postulated and observed –no experimental proof (Discovery) **TRL 2 – Technology Concept Formulated** – concept and application defined (Concept Definition) **TRL 3 –Experimental Applied Research Concept** – first laboratory tests completed (Proof of Concept) **TRL 4 – Technology Validated in Lab** – Small scale prototype – built and tested in lab (lab validation)	Knowledge development Academia	**PRL 1 –Basic Research** – identifying issue/problem and identifying policy readiness (Discovery) **PRL 2 – Formulation** of issue/problem, proposed solutions and potential impact; expected policy readiness; concept identification relevant to stakeholders (Concept Definition) **PRL 3 – First testing** of proposed solution(s) with relevant stakeholders; modelling, consultations, feedback, development complete (Proof of Concept). **PRL 4 – Problem validated 'in lab'** through pilot testing in intended environment to substantiate proposed impact, policy readiness, feedback development (lab validation)	Knowledge development Academia	**SRL 1 – Basic Research** – identifying a problem and identifying societal readiness (Discovery) **SRL 2 – Formulation** of problem, proposed solution(s) and potential impact, expected societal readiness; identifying intended stakeholders for project (Concept Definition) **SRL 3 – Applied Research** - initial testing of proposed solution(s) with intended stakeholders (Proof of Concept) **SRL 4 – Pilot-Test Scale** - concept validated through pilot testing in relevant environment to substantiate proposed impact and societal readiness (Concept Validation)	Knowledge development Academia
TRL 5 Large-Scale Prototype tested in intended environment (test facility validation **TRL 6 – Technology demonstrated in intended environment** – close to expected performance ('open water' validation) **TRL 7 – System prototype demonstration in operational environment** – at pre-commercial scale (system demo)	Technology development Collaboration	**PRL 5 – Proposed solution(s) validated**; now by intended stakeholders in the area for application ('open water' validation) **PRL 6 – Demonstration system** in intended environment and with intended stakeholders at pre-role out scale for feedback on impact (system demo) **PRL 7 – System refinement** of scheme and/or solution(s), and possibly, retesting in intended environment with intended stakeholders to gain feedback (refinement)	Policy Development Collaboration	**SRL 5 – Large Scale Test/system -** proposed solution(s) validated; with intended stakeholders ('open water' validation) **SRL 6 – Demonstrated system** - solution(s) demonstrated in relevant environment and with intended stakeholders for feedback on policy **SRL 7 – System Refinement** – refinement of product, and/or solution(s), and if needed, retesting in intended environment with stakeholders (refinement)	Solution Development Collaboration
TRL 8 – First system complete, qualified, verified – First commercial system – manufactured issues solved (verification) **TRL 9 – Actual Full commercial system proven in operational environment** – technology available for beneficiaries (deployment)	Business development Industry	**PTL 8 – First System** - proposed solution(s), as well as plan for policy **adaptation complete, and qualified** (verification) **PRL 9 – Policy Implementation** – actual project solution(s) proven in relevant environment. Issues solved, continued monitoring, evaluation, and review of scheme/solution (deployment)	Scheme development Government	**SRL 8 – First System** – issues solved, proposed solution(s), as well as plan for societal adaption complete, and qualified (verification) **SRL 9 – Full Social System** – actual project solution(s) in intended or relevant environment (deployment)	Stakeholder development/ Society

[a] TRL – are indicators of status or maturity level of particular technology been researched and commonly used for European Commission in the context of Horizon Europe. Adapted from [9].

[b] PRL – Used to assess the level of societal adaptation to project, technology, product, process or management practice, or innovation to be integrated into policy. Adapted from [9].

[c] SRL – Used to assess the level of societal adaptation to project, technology, product, process or management practice, or innovation to be integrated into society. Adapted from [9].

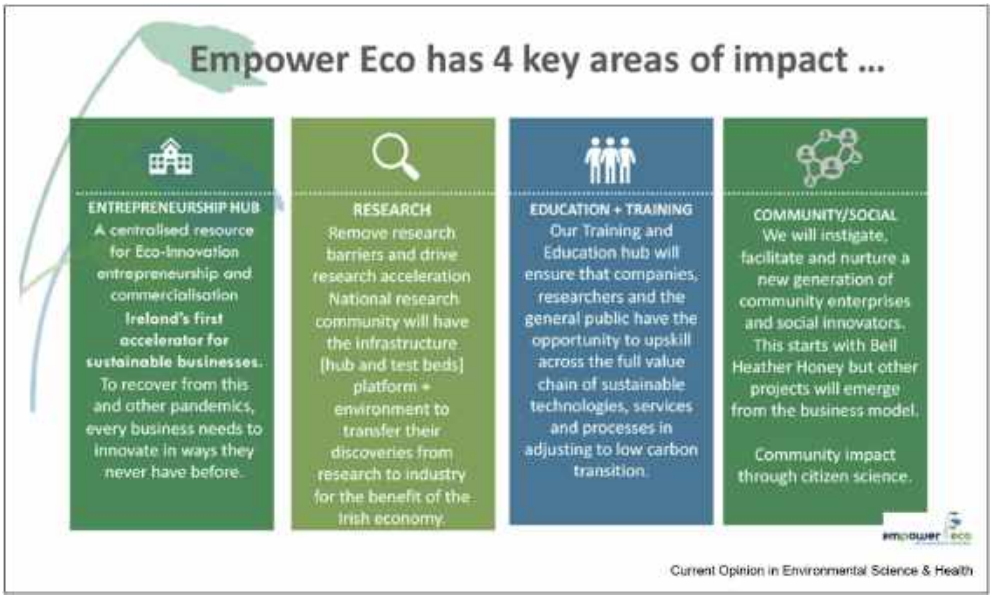
Figure 1

Empower Eco Sustainability HUB" supporting a dynamic converging community and enterprise ecosystem for transitioning to the new Green Deal era; this fosters a triple-helix concept (academia-industry-authority) to enabling and accelerating change to green innovation in the Irish midlands.

Empower Eco – Irish midlands multiactor platform to support and accelerate a just transition to low carbon economy

Empower Eco constitutes the first new Green Deal "multiactor hub" accelerator that supports and connects research, innovation, social enterprises along with enabling community transitioning to low carbon economy through engagement with broad stakeholder from a bottom-up perspective (Figure 1). It constitutes a signature multifunctional digitised building at Boora, in the heart of the Irish peatland that connects research, entrepreneurship, enterprise and the community (Figure 2). While the focus of Empower Eco is geared to support and enable other green-innovative projects funded under Just Transition [24], it is specifically developing environmental testbed activities that include the use of 6 ha of freshwater aquaculture and aeroponics, 34 ha of horticulture comprising 12 different medicinal plants and herbs, 5 ha of agroforestry. Social enterprises include deploying 200 new hives across the peatlands to promote pollination and ecosystem service management, where 'Bell Heather' honey is produced at the regional Ferbane Food Hub (Figure 1). Activities conducted across Empower Eco ecosystem meet EC Green Deal ambitions [27], including (a) supporting, investing, testing environmentally-friendly technologies

Figure 2



Empower Eco Sustainability HUB has four inter-related foundation pillars connecting entrepreneurship, research, education and training, community and social activities that will guide just transition to low carbon economy in Irish midlands with a national and international orientation.

across agri-food, horticulture, agroforestry, vertical-farming, biofuels, circular economy and digitisation; (b) supporting industry to innovate, (c) decarbonising the energy sector – specifically expanding biofuel and wind energy initiatives, (d) ensuring that buildings are more energy-efficient and (e) working with international partners to provide global environmental standards and international mobility for SME training. Herrero et al. [16] also recently highlighted the potential for such innovation to accelerate the transition to sustainable food systems. Empower Eco can also escalate this approach by way of addressing eco-design and eco-labelling that focuses on green products ranging from concept to testing at pilot scale linked to LCA [25]; this also embraces carbon emissions and energy measurements using these peatland testbeds [21–23]. In addition, this provides useful testbed data for impact climate monitoring and forecasting.

The 'living lab' concept connects the need for real-time sophisticated analysis HUB infrastructure, such as using molecular profiling (such as MinION genomic sequencing) and bioreactors for mass-balance simulated studies to inform testing and validation of in-field monitoring devices at the testbeds [28]. Early needs analysis for market research, product-market fit analysis and design for scale–up processes are addressed that includes (1) early pilot and technical validation, including 'test the tech' (beta testing/field testing); (2) experimentation and validation at prepilot scale in real-life environments, (3) providing a commercial platform, and commercialisation vehicle for research for SMEs, and emanating from connected 3rd level institutions; and (4) embracing ICT, digitisation and exploit immersive technologies for linked research, innovation and training. Empower Eco activities strongly align across several policy areas, including biodiversity (measures to protect our fragile ecosystem); from farm to fork (ways to ensure more sustainable food systems); sustainable agriculture (sustainability in agriculture and rural needs informed by common agricultural policy (CAP)); clean energy (wind and biofuels); sustainable industry (ways to ensure more sustainable, more environmentally respectful production cycles); green building and renovating; eliminating pollution (measures to cut and monitor pollution rapidly and efficiently); climate action (making Ireland and the EU climate neutral by 2050, including creating monitoring of innovation and testbeds) [9].

Empower Eco will evaluate fundamental data trends, scenario modelling, mitigation measures, risk analysis in order to implement future policies that will strengthen the environmental sustainability and competitiveness of Irish and European partnering food industries. Empower Eco will address the development of data-driven and natural capital approaches to inform bio-resources, businesses, services and value chains, which includes promoting new partnerships and innovation ecosystems between producers, processors, retailers and society. Examples of how this triple helix approach can be used as sustaining or potential disruptive green innovation for addressing United Nation's Sustainable Development Goals of this approach was also applied to

Table 2

Empower Eco activities and tools to support, enable and accelerate potential green innovation disruption under United Nations' Sustainable Development Goals (SDGS) framework.

UN Sustainable Development Goal[a]	Indicative sustaining or potentially disruptive activity under linked Empower Eco triple-helix management platform
No poverty	Food security, food systems, and sustainability along with digitisation via open knowledge and technology sharing with developing countries. Researcher mobility and exchanges creating opportunities in education.
Zero hunger	Development and future proofing sustainable agri–food processes and crops along with alternative sources for protein that includes training.
Good health and wellbeing	Adopting One Health approach to informing green innovation – there is a strong focus on community transition and social enterprise for health – this includes food for good approach that includes provision for research and innovation to pivot beyond COVID-19 [33].
Quality Education	Sharing knowledge, discoveries and growing collaborations in academia, ex-Delta Africa and Irish Research Council Coalesce Programme. Appointing Visiting Research Fellows from Developing Countries to Empower Eco projects for the dual translation of know-how that includes food for good, health and wellbeing that cross-cuts STEM with Social Science and humanities, to inform behavioural change.
Gender Equality	Empower Eco projects are strong gender equality focused with equal representation in research, innovation and entrepreneurship across projects, tasks, management that includes Project Advisory Team.
Clean Water and Sanitation	Innovative green research and enterprise to promote natural resources for water quality and mitigate waste that moves beyond end-of-pipe solutions [1,38].
Affordable and Clean Energy	Empower Eco enables and accelerates green innovations by developing affordable and clean energy that includes "Hub and spoke model" to testing and verifying technologies in intended environments using LCA, modelling and by applying technology, policy and society readiness levels [Table 1]. Carbon sink measurements on peatlands with nexus to specialist training [50,52]. Recirculating aquaculture system with 'organic status' in Irish peatlands is driven by wind turbines [1,38].
Decent work and Economic Growth	Empower Eco adopts a triple helix approach (academia-industry-authority) to inform techno-social-economic feasibility and impact that is framed upon pivoting activities to hurdle regional, national and international challenges (including COVID-19) informed by econometric and transnational modelling of key clusters for sustainable change.
Industry, Innovation, Infrastructure	Empower Eco framework, or connected ecosystem, has assembled appropriate mix of academia, industries, and enterprises to accelerate green innovation that includes sharing and access to specialist infrastructure, equipment and resources where focus is on regional transition to low carbon economy and impact. With nexus to Education
Reduced Inequalities	Empower Eco triple helix management team approach that ensures education and research for all as its' core underpinning tenet.
Sustainable Cities and Communities	Empower Eco is focused on community transitioning to low carbon economy where there was a strong reliance on fossil fuels – community groups are strongly represented in management group and Empower Eco has registered as a 'Company with Limited Guarantee' to support not-for-profit and charitable activities to support its communities.
Responsible Consumption and Production	Responsible consumption and production are core activities of Empower Eco in terms of research and enterprise – Empower Eco also develops digital twin, factory of the future, industry 4.0 concepts.
Climate Action	Empower Eco addresses climate action by developing more sustainable green innovation that are climate proofed – this includes biosensor technology for monitoring more resilient innovation [36] – this is informed by networking with international partners such as EC Horizon 2020 and Interreg programmes that includes data from transnational models. Demo and test-beds in relevant and intended environments will render big data for climate impact modelling.
Life Below Water	Empower Eco is supporting freshwater aquaculture and studies on biodiversity related to natural aquatic ecosystems in peatlands
Life On Land	Empower Eco is supporting studies on biodiversity, pollination and ecosystem service management that includes studies on key drivers affecting decline of animal pollinators (bees) [3,35]. This is co-funded by the Environmental Protection Agency.

Table 2 (*continued*)

UN Sustainable Development Goal[a]	Indicative sustaining or potentially disruptive activity under linked Empower Eco triple-helix management platform
Peace, Justice and Strong Institutions	Peace, justice and strong institutions are key founding tenets of Empower Eco that blends academia, industry, authority with communities.
Partnerships for the goals	Empower Eco supports and enable national and international partnerships aligned with UN SDGs that includes mobility and training.

[a] Alignment with https://www.un.org/sustainabledevelopment/sustainable-development-goals/.

describing potential sustaining or disruptive tools for meeting needs of the United Nations Sustainable Development Goals Framework are provided in (Table 2).

Bio-based and bio-inspired smart functional products from the peatlands

Due to its harsh and stressed environment [29], the peatlands of Europe provide a rich resource for refining new bio-inspired materials with improved functionality and sustainability for potential health and wellbeing applications, such as high value nutraceuticals, cosmetics and personal care products. This includes the development of novel bioactive compounds or ingredients for promoting health and wellbeing in humans and animals [30–33], along with testing and developing potentially disruptive green technologies [16,28,34,35]. Opportunities for harvesting immune-priming bioactives from food waste streams for potentially addressing the surge in antimicrobial resistance (AMR) that contributes to pulmonary sepsis and pneumonia should be pursued through a One Health approach [31,32]. Data-driven development solutions to optimise biomass availability and to inform the development of robust supply chains, business cases, including potential social enterprise models for sustainable and circular bio-based value chains, will be advanced [9].

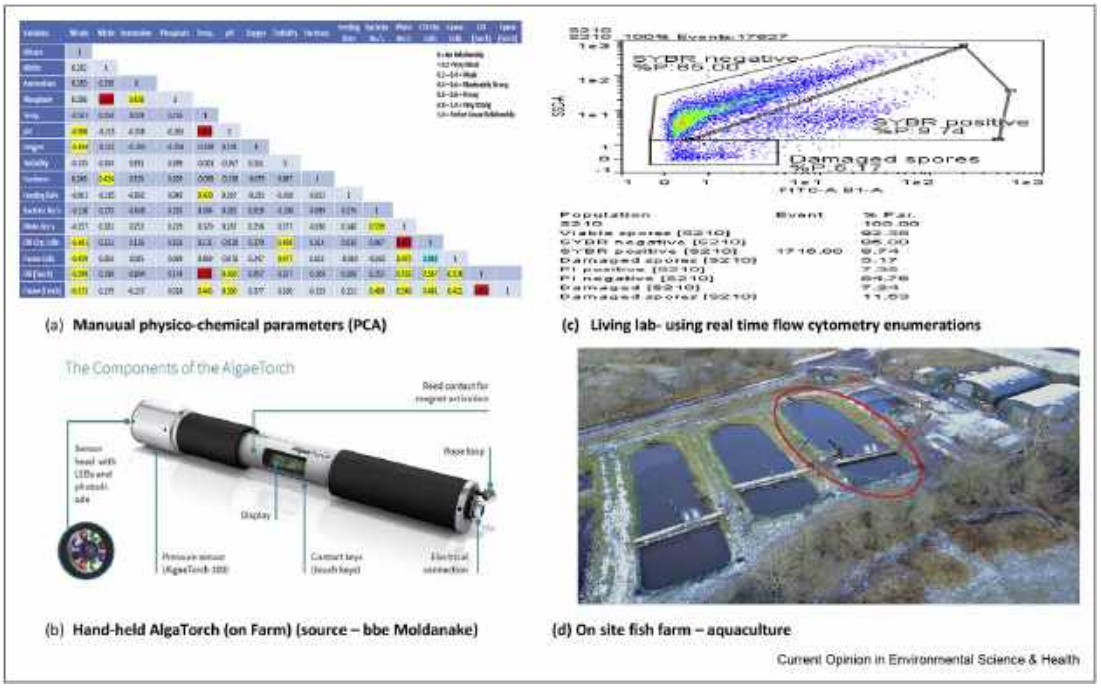
Example of sustainable innovation using "Empower Eco" HUB: freshwater aquaculture

Aquaculture is the fastest growing food production section in the world [36]. It has an average annual growth rate of 6–8% since 2000 [36] that has been positively influenced by smart fishing and the increase in the seafood and aquaculture trade. Negative connotations associated with farmed seafood, such as pollution, poor animal welfare, nonsustainable depletion of resources, disease spreading and drug use, erodes the social license to produce [37]. Freshwater aquaculture in the Irish peatlands is framed upon using naturally occurring microalgae, bacteria and duckweed in the ponds as a means of water quality and waste recycling, where trout and perch are farmed based upon organic principles and use wind turbines [38]. The process is defined to optimise 'circularity' knowledge and to mitigate waste. This sustainable system moves away from end-of-pipe effluent treatment to embrace a full recirculation system; however, unexpected operational challenges have been experienced due to large rainfalls associated with multiple storms in 2020 that caused a 'flux' in the system, causing fish fatalities. This testbed also has exploited innovative environmentally friendly diagnostic and monitoring technologies, along with connected real-time determination potential of living labs (using real-time 'in lab' evaluation) with on-site handheld microalgae torch to monitor production processes [Figure 3]. This system will be digitised to inform the full process, validation and management decision tools that include the use of machine learning along with the potential for virtual Quality of Experience training [39]. Table 3 highlights the potential for future-proofing the aquaculture industry using green innovation developed through this triple-helix management approach.

COVID-19 response and sustainability

There are increasing waves of SARS-CoV-2 infection globally that include more transmissible and pathogenic variants that exert enhanced pressure on personal and protective equipment (PPE) supply chains globally [40]. PPE shortages, such as filtering facepiece respirators or medical face masks, have been met by increased manufacture, by emergency use authorisation of nonthermal reprocessing technologies (such as VH2O2, UV) for PPE reuse and digitisation of the supply chain [41]. PPE is an important nonpharmaceutical intervention strategy that reduces the relative risk of SARS-CoV-2 infection and will continue to accompany vaccination for mitigating this COVID-19 disease and future pandemics. PPE that has single use-plastic items has generated a new waste management challenge where there are opportunities for using sustainable bio-inspired alternatives to that of using plastic constituents. These alternative bio-based solutions are to be potentially met from natural material such as food waste stream, bioplastic upcycling, and so forth [8]. For example, these biomaterials could be introduced by electrospinning to improve filtration efficacy below 300 nm cut pore size in face masks (where SARS-CoV-2 is typically 60–140 nm in size) [42,43]; however, other functional parameters must be met by original equipment manufacturers, namely pressure

Figure 3



Pressing need for end-to-end digital monitoring of big data and machine learning that connects **(a)** manual physiochemical parameters and **(b)** hand-help on-farm monitoring with **(c)** with real-time living lab analysis for **(d)** sustainable freshwater aquaculture using IMTA approach.

drop and comfort fit post nonthermal reprocessing to appropriate standards. The peatlands potentially provide a resource of alternative bioplastics to inform new PPE design and functionality for ongoing and future pandemics.

Community-focused transitioning – pressure points and challenges to overcome

Transnational modelling, and regional observational studies have highlighted key drivers to improve the transition of communities and enterprises to low carbon economies [44, 45]. These include:

Building resilient enterprises through

(a) extended supports, such as EI supports, learning from BREXIT/COVID-19 pandemic, transition vouchers; (b) SME digitisation, such as boosting awareness and take-up, simplify appreciation for innovation supports, (c) Enterprise-led networks, such as funding, lessons learned from existing clustering projects, (d) Early-movers, such as if and how to support initiatives.

Continuous pre-emptive workforce development through

(a) increased ambition, such as improved resources, higher targets, incentivise employers; (b) improve information, such as more skills audits, recognised informed skills and information sharking, (c) life-long learning, such as greater effort linked to academic institutions, and (d) better advice, such as pre-unemployment, more one-to-one coaching.

Delivering high-impact targeted funded through

(a) targeted support, including social enterprises and new business/community level engagements and mechanisms, (b) improve support, such as seed funding applications, fund local plan development, and more place-based schemes, and (c) better engagement, such as working with multiactor institutions and monitoring EU developments.

Invest and enable green enterprises and innovation

There is a need to develop and invest in enterprises informed by expert training and research, including pilot testbeds that will accelerate new products and services and dissolve technical barriers [44,46–49]. Emerging business models to roadmap initiatives supporting this include (a) transnational business modelling tailored for circularity – such as developing further successful transnational econometric and cluster models [19,47]; (b) "Invest and enable for accelerating green innovations"; (c) Enterprise HPSU Green SPRINT Programme; (d) support from local enterprise office, local government, community groups, a policy such as Just

Table 3

Climate proofing sustainable freshwater aquaculture using Irish peatlands.

Challenge Area	Detail	Technical, Commercial, Social Resolution
Environment: Climate Change	Internationally, climate change can interact with fisheries in many different ways; a decline in the industry has been attributed in part to increased water temperatures, extreme water flow events (floods and droughts), and warming of maritime and freshwater environments [14,15,47].	Harvesting the potential for peatlands to help with new innovation to transition economy beyond same [38] and designing systems for recirculating avoiding end-of-pipe treatment options [1,28]. Using big data and digitisation from aquaculture testbeds to inform climate impact modelling along with biosensors that detect real-time variations in the aquatic environment due to extreme weather events [28,36].
Environment: Waste Streams and food waste redcuction	Negative connotations associated with farmed seafood, such as pollution, poor animal welfare, nonsustainable depletion of resources, disease spreading and drug use have affected the development of aquaculture. Land-based fish farms require monitoring and treatment to ensure effluent containing nutrients and other physicochemical properties meet discharge to receiving water licence. Failure to achieve this can contribute to problematic algae blooms and fish death.	There has been an enhanced focus on valorising wastes from food production from farm to fork. For example, in the aquaculture industry, solid waste from finfish production has been identified as a potential substrate for anaerobic digestion with a secondary use as a fertiliser. However, there is a pressing need to leverage emerging 'natural 'processes to reduce operational cost and the environmental burden of food production for sustainability of the aquaculture sector. This presents opportunities for green innovation that will be informed by monitoring [37], Life Cycle Assessment, Risk modelling and cos-benefit analysis [15] and novel processes [16,34]
	Chemicals, such as antibiotics, used in the aquaculture industry, can be released into waterways. Future opportunities for Aquaculture systems to be closed or wastewater treated prior to discharge.	Extraction of bioactives, or bioinspired materials with increased functionality from aquaculture waste streams (including from microalgae) that can be used in feed ingredients to promote immune health of farmed fish, thus limiting or avoiding the use of antibiotics [32,33,51].
Disease	Aquaculture operations can spread parasites and disease into the wild. Farmed fish have an increased chance of getting parasites such as sea lice, as opposed to fish that live and breed in their natural environments. Farmed fish are exposed to diseases through the use of unprocessed fish as a food source.	Introduce precision farming techniques that monitor and control disease and prevent infection. Utilise nutritional technologies to maintain the optimum health of the farm. Investigate symbiotic feeding systems that are biodiverse and sustainable. Opportunities for reviewing green innovations from a holistic technology, policy and societal readiness from initial idea to market or system deployment [Table 2].
Cost	Treatments lead to higher costs and lower profitability	
Future need for food security, and disruptive innovation surrounding supply chain and resource utilisation	The sustained expansion of aquaculture has arisen due to the increasing global population and commensurate demand for more food. Aquaculture now accounts for ca 50% of fishery products, which is estimated to reach ca. 62% by 2030 [37]. However, issues associated with the aquaculture licensing process and the potential environmental impact caused by aquaculture effluent have hampered the expansion of the Irish Aquaculture Industry [1].	Build upon the potential demonstrated in the first radically-new concept in integrated-multitrophic aquaculture (IMTA) that uses cutaway peatland (bogs) to farm fish with an associated organic status that is powered by wind energy and utilises bacteria, microalgae, duckweed to treat rearing water. Developing IMTA systems, along with eco-innovation and monitoring/management processes, is critical for transforming the sector [38]. Use of triple-helix (academic-industry-authority) to inform new innovation linked to stakeholders and beneficiaries that also includes social change.
Feed	The feed-protein crisis has exposed the feed industry to shortages in quality protein and the need to import nonsustainably produced soy from places such as the amazon, and/or use wild-caught fish to grow fish.	Plant proteins constitute a novel avenue that has not been adequately explored in Ireland. The development of vertical farming to promote and develop high protein foods and feeds will also advance this area. It is envisaged that Irish aquaculture-farmed perch and trout benefit

(*continued on next page*)

Table 3. (*continued*)

Challenge Area	Detail	Technical, Commercial, Social Resolution
		from consuming duckweed, which along with algae and bacteria, naturally control water quality and waste [1].
Land Impact	Maintaining biodiversity, along with ensuring compliance with policies and statutory regulations, is important when considering developing and deploying land-based facilities. Aquaculture businesses locate near coastlines for easy access to clean and natural water.	Utilising bogs that support natural ecosystems. This also enables the transition from burning peat to lower carbon emissions [1]. Approx. 5% of Ireland is comprised of peat bogs, comprising potential aquaculture and other green enterprises. This will also support the community transitioning to a low carbon Bioeconomy.
COVID-19 crisis and recession	Presents uncertainly and considerable challenges and opportunities for food sustainability and security	Potential for peatlands to help with new innovation to transition economy beyond same, including agri-food and bio-based sectors informing by training [32,50,52].

Transition [24]; (e) entrepreneurship – such as Irelands New Frontiers delivered by Enterprise Ireland; mobility and specialist training for SMEs such as offered through the Hatch Blue Aquaculture Accelerator programme [50] and by National Digital Research Centre (NDRC) for accelerating start-up companies [52]. The aforementioned will also be informed by enabling innovation management through ISO 56000 series that will advance enterprises and grow capability in the green technology and service space.

Conclusions

There is a pressing need to support communities in transitioning to low carbon economies where there is an underpinning drive to empower behavioural change to meet ambitious climate and environmental challenges through creative opportunities. Empower Eco is a multitiered ecosystem approach established to support and accelerate this just transition in the Irish midland peatlands that paradoxically supported rural communities through the burning of peat or supply to products to horticulture. The controlled wetting of the peatlands presents an essential carbon sink for Ireland to meet its net zero GHS emission targets for 2050; however, this also presents considerable innovative opportunities to create social enterprises and to support businesses in forging new green innovations to help with this community transitioning and to increase employment. While lessons have been learnt from outputs of transnational sustainability modelling, life cycle assessment and cost-benefit analysis; Empower Eco constitutes the first multiactor connected ecosystem of stakeholders, spanning academia, industry, authority and communities, for informing a unified transition to low carbon economy using the Irish midland peatlands as a targeted region for regeneration. Unknowns yet to be discerned include elucidating the key drivers that inform sustainable behavioural change of citizens to low carbon economy. Also, the financial stimulus to support for enabling and accelerating green industry and businesses need to be ambitious and flexible to fast tract needs; these should also be linked to academia that can share technological core facilities and provide specialist training. For the impact of climate variance on emerging new sustainable activities, such as farm to fork, an example is the fully recirculated freshwater aquaculture process. Future studies will evaluate critically large-scale meta-data generated from these ambitious multiactor projects that will exploit advances in transnational and risk modelling, life cycle assessment and social marketing framed upon open sharing and access of knowledge. Therefore, Empower Eco describes a tool by which key green innovation and knowledge can be openly and directly transferred to decision makers and shared with stakeholders and beneficiaries. This triple-helix management approach also describes potential disruptive solutions that will inform green innovations such as required

under the UN Sustainable Development Goals (SDG) framework.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors wish to acknowledge funding from Just Transition Fund (Project No. MRTT 112_2021); Regional University Network - European Universities (Project No. 101004068) and Interreg Atlantic Area Neptunus (Project No. EAPA_576/2018).

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Journal of Cleaner Production 392 (2023) 136283

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Review

A circular economy framework for seafood waste valorisation to meet challenges and opportunities for intensive production and sustainability

Ronan Cooney [a,b], David Baptista de Sousa [c], Ana Fernández-Ríos [d], Sinead Mellett [e], Neil Rowan [e], Andrew P. Morse [f], Maria Hayes [g], Jara Laso [d], Leticia Regueiro [c], Alex HL. Wan [a,b,h], Eoghan Clifford [a,b,*]

[a] School of Engineering, University of Galway, Galway City, H91 HX31, Ireland
[b] Ryan Institute, University of Galway, Galway City, H91 TK33, Ireland
[c] ANFACO-CECOPESCA, Department of Circular Economy, Campus University 16, 36310, Vigo, Spain
[d] Department of Chemical and Biomolecular Engineering, University of Cantabria, Av. de Los Castros, s.n., 39005, Santander, Spain
[e] Bioscience Research Institute, Technological University of the Shannon, Midlands Midwest Athlone Campus, N37 HD68, Ireland
[f] School of Environmental Sciences, University of Liverpool, Liverpool, UK
[g] Department of Food BioSciences, Teagasc Food Research Centre, Ashtown, Dublin, D15 DY05, Ireland
[h] Aquaculture Nutrition Research Unit (ANRU), Carna Research Station, Ryan Institute and School of Natural Sciences, University of Galway, Carna, Connemara, Co. Galway, H91 V8Y1, Ireland

ARTICLE INFO

Handling Editor: Cecilia Maria Villas Bôas de Almeida

Keywords:
Seafood loss
Aquaculture
Nutrient recovery
Seafood supply chain
Processing by-products

ABSTRACT

There is a growing concern among societies and consumers over food security and the sustainability of food production systems. For seafood, it has been highly advocated as a healthy food source and its sustainability credentials. However, the increasing global demand for seafood and the need to supply the quantities are creating sustainability issues, e.g., the importation of plant and marine proteins for aquafeed production. Consequently, there is a necessary need to analyse the supply chain and life cycle of these systems to determine their sustainability merits and how to enhance them. The circular economy (CE) aims to reduce processing by-product underutilisation, increase the rate of reuse, and reduce pressure on natural resources and systems. For seafood, there are large quantities of biomass that are being lost through bycatch/discards, waste from aquaculture (e.g., sludge and wastewater), and by-products generated through processing (e.g., trimmings and offal). These can all be valorised for the generation of feeds, value-added products, or further food production. This review will focus on seafood by-products generated during the processing into consumer products, and the current methods that could be used to manage or treat these waste streams. The review presents a stepwise framework that outlines valorisation opportunities for seafood by-products. This framework can enable producers, operators, regulators, and investors to integrate with the principles of the CE with the consideration of achieving economic viability. The challenges of seafood loss due to climate change and emerging recycling strategies will also need to be considered and integrated into the valorisation pathways. Communication, education, and engagement with stakeholders are key to transitioning to a circular economy. Where increase awareness and acceptance will create drivers and demand for seafood by-product valorisation. Overall, the impact of such a circular production system will potentially lead to higher production efficiency, reduce demand for natural resources, and greater seafood production. All of which addresses many of the United Nation's Sustainable Development Goals by contributing towards future food security and sustainability.

1. Introduction

Human activities contribute to the significant decline in environmental quality and biodiversity. To the present date, interactions between humanity and the environment have been based on a model of extraction, processing, production, and discarding the unused products and the by-products produced along the core product as waste back into the environment (Tan and Lamers, 2021). This linear economy model is

* Corresponding author. School of Engineering, University of Galway, Galway City, H91 HX31, Ireland.
E-mail addresses: rcooney@nuigalway.ie (R. Cooney), eoghan.clifford@universityofgalway.ie (E. Clifford).

https://doi.org/10.1016/j.jclepro.2023.136283
Received 23 May 2022; Received in revised form 27 January 2023; Accepted 30 January 2023
Available online 2 February 2023

no longer compatible with the current capacities of this planet (Borrello et al., 2020; European Commission, 2020b; Laso et al., 2018; Regueiro et al., 2021). With a growing population compounded by a growing middle class with increased spending power, the global demand for food is increasing (Belton et al., 2020; Béné et al., 2015; Fernández-Ríos et al., 2021; Rohm et al., 2017). In particular, the demand for seafood and seafood products is rising also due to the advocacy as part of a healthy diet (Bohnes et al., 2020; Regueiro et al., 2021).

Seafood is a colloquial and highly broad food category. For example, in much of Europe, this often encompasses both freshwater and marine finfish species (e.g., farmed salmon, trout, carp, seabass, seabream), bivalves, (e.g., mussels, clams and oysters), decapods, (e.g., crabs, shrimp, and lobsters), cephalopods (e.g., squid and octopus), but also algae (macro and micro) and cyanobacteria. These diverse groups of seafood are considered to have a better environmental performance than other protein-rich foods, such as terrestrially farmed animals (European Commission, 2019, 2020a). Their production is either derived through farming (aquaculture) or wild caught (fisheries). Together these food production systems produced 214 million tonnes of global seafood in 2020 and over half of this production was from aquaculture (FAO, 2022). The Food Agricultural Organisation of the United Nation has estimated that the seafood trade is worth USD 151 billion and is projected that this will further grow by over 13% in production value in 2030. Much of this growth will be driven by aquaculture due to its increasing efficiency, farmed species diversification, and new production opportunities.

Like many other food production systems, seafood must employ the concepts of life cycle thinking and the circular economy (CE) to increase production efficiency and mitigate its environmental impact (Cortés et al., 2021b; Ruiz-Salmón et al., 2021). This is either through valorisation strategies or nutrient recovery technologies (de la Caba et al., 2019; Venugopal, 2021). Within the EU, policies for increased seafood consumption are being introduced to increase food and seafood circularity (European Commission, 2020b). The Circular Economy Action Plan (European Commission, 2020b) was launched by the European Union (EU) as part of the European Green Deal. The plan aims to transition the European economic bloc from a linear to a circular economy. This transition has been advocated to open new avenues for resource efficiency and places the concept of life cycle thinking as its core action (Gheewala and Silalertruksa, 2021; Ruiz-Salmón et al., 2020). This drive for circularity in value chains and processes will help to support (Raimondo et al., 2021; Regueiro et al., 2021; Zilia et al., 2021).

(i) Greater use of renewable energy,
(ii) More responsible use of resources and, crucially,
(iii) Reuse and valorisation of by-products and residue streams generated from seafood processing.

To combat the seafood nutrients, energy, and elemental loss along the supply chain, there is a need to identify value in this lost material. Thereafter, there is a need for novel designs for the valorisation and exploitation of seafood by-products, as well as the promotion of more environmentally, socially, and economically sustainable business models. Furthermore, any proposed solutions should contribute to increasing seafood circularity by maximising the potential value that can be derived. The underutilisation of by-products is regarded as a wasted opportunity. Therefore, the present review aims to identify the opportunities for CE within seafood production chains. This will be achieved by using a stepwise valorisation framework, with a particular focus on.

(i) The current protocols for seafood by-product treatment,
(ii) Opportunities for CE,
(iii) Emerging seafood loss, and
(iv) Emerging strategies for CE and opportunities.

Furthermore, this review will evaluate the barriers that prevent or limit seafood circularity and potential solutions. Overall, this review will form a catalyse in allowing researchers, industry, and policymakers in focusing key innovations to drive a CE in seafood.

2. Research methodology

The present review was carried out by searching for peer-reviewed studies relevant to the area of seafood waste, by-product valorisation, and the circular economy. Relevant literature was reviewed using academic databases (inc. Scopus, Google Scholar, Web of Science and Science Direct). The terms used for the literature search were “seafood loss”, “seafood waste”, “seafood nutrient recovery”, “aquaculture waste/loss”, “fisheries waste/loss”, “seafood circular economy” and “seafood circularity”. The inclusion criteria for the results were that the articles had to be peer-reviewed and published in the English language and must have been published in the last 10 years (2010–2022). The exclusion criteria that were applied focused on the thematic relevance of the article and that it not be an opinion article, conference article, or from the grey literature. Further refinement of the results was reached by implementing the stepwise approach to key areas of seafood waste that the article aimed to review. These areas were (i) seafood waste streams and (ii) current waste treatment protocols. The next thematic area focused on approaches which incorporate the circular economy into seafood waste management, (iii) nutrient recovery technologies, (iv) nutrient recovery strategies and management practices, (v) seafood loss, (vi) recycling strategies and (vii) bio-based resources.

Using these search terms and criteria, 142 articles derived from these searches were then broken down into the relevant thematic areas. This critical analysis of this article concludes with a discussion of the challenges with recommendations for implementation and actualisation, which will transform societies from a linear into a circular economy. This analysis will be European-centric due to the over-exploitation of aquatic environments and the economic bloc’s 88 million tonnes of food waste per annum (European Commission, 2022).

3. Current practices for seafood by-products

Within seafood production systems, large quantities of by-products are generated from both wild capture fisheries and farmed aquatic species-aquaculture. Very frequently these by-products are disposed of as waste or discharged into the aquatic environment. This brings the need to implement effective and novel treatment and utilisation protocols to reduce any environmental impact on aquatic environments or land and promote more efficient production practices that reduce biomass, energy, or nutrient losses (Caruso, 2016).

Within the seafood category, there are significant volumes of by-products being generated from the production, processing, distribution, consumption, and disposal stages (Hayes and Gallagher, 2019; Venugopal, 2021). It has been estimated that as much as 36% of seafood can be lost or wasted (FAO, 2018; Gustafsson et al., 2013). These losses are often complex and each by-product streams are unique from different seafood production systems which leads to varying composition, quantities, and quality (Fig. 1). Consequently, this results in the need for different technological requirements in its management after its produced.

In terms of by-product generation, there are a variety of sources from the seafood sector, particularly depending on the level being studied. By-product volumes, value and quality can vary from species to species, between regions, availability, and at different stages of the supply chain. For example, the use of pond culturing systems (flow through aquaculture) are extensively used for freshwater aquaculture across the world (Bohnes et al., 2019; Bohnes and Laurent, 2019; FAO, 2018). Its attractiveness to farmers is the low technology required to set up and maintain but more importantly can be easily built with limited cost in relation to aquatic farm systems. In many instances, pond systems may

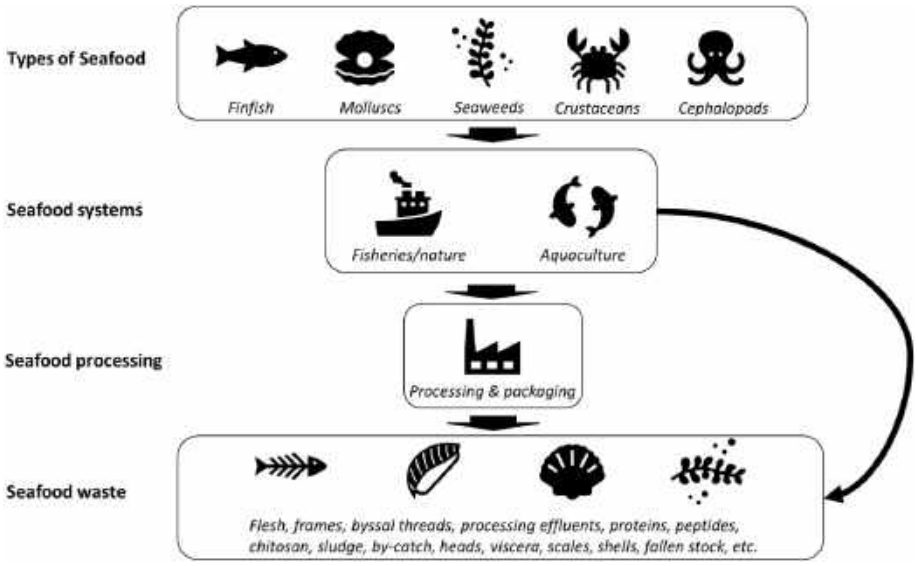

Fig. 1. An overview of the several types of seafood, their production/capture systems, and the types of by-products that are generated.

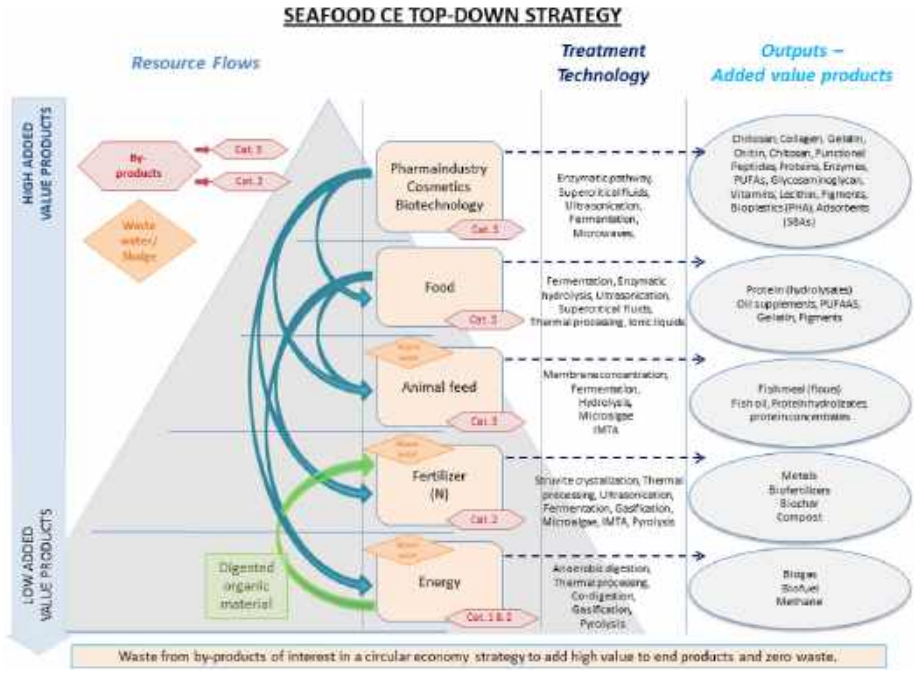

Fig. 2. A proposed framework for top-down CE strategies for seafood by-product valorisation. Animal by-products categories are presented as Cat. The triangle indicates the added value of the products and volume needed.

not allow for efficient process control, this can result in wasted and uneaten feed that can settle and accumulate over time on the pond floor. This uneaten feed can become a nutrient-rich layer that provides a substrate for microbes to convert and breakdown down biochemically (Dauda et al., 2019). This layer can also reduce process efficiencies by consuming oxygen, thus requiring larger amounts of supplementary aeration (Tahar et al., 2018). The low technology requirements of pond-based aquaculture are in contrast to the high technology requirements of recirculating aquaculture systems. These closed or semi closed aquaculture systems re-use a large proportion of the water by undergoing treatment processes. Commercial recirculating aquaculture systems as mechanical solids removal, bioreactors, heating, and cooling, and ozonation and/or ultraviolet sterilisation (Martins et al., 2010). From the solid's removal, sludge which comprises uneaten feed and biogenic wastes is captured and stored on the site for further treatment. Generally, this sludge is treated in centralised facilities such as publicly owned treatment works used for the treatment of other livestock waste as well as domestic and industrial waste (van Rijn, 2013). Other routes of food loss and waste in open aquaculture systems can be due to disease outbreaks and environmental events such as jellyfish blooms, and algal blooms which can result in mortality events (Brooks et al., 2022; Clinton et al., 2021).

Within fisheries, the main by-products are by-catches and discards. The former is classified as non-targeted caught species which can impact marine food webs, e.g., cetaceans, echinoderms, and molluscs (Bielli et al., 2020). While the latter are species that are captured which may not be of suitable grade (e.g., below harvest size), or economic value, or the fishers may not have a quota for the species. It has been estimated by

the EU that between 7 and 10 million tonnes of fish are discarded annually across the world (EC, 2022). Like bycatches, discards are returned to the sea dead which has led to a significant negative public image of fisheries (FAO, 2020). In recent years there has been a growing trend to utilise these fish for the production of fishmeal, fish oil, fertilisers, biostimulants, and even food ingredients for human consumption (Dineshbabu et al., 2013; Madende and Hayes, 2020). This trend is supported by several mitigation strategies, e.g., the EU's Common Fisheries Policy on discards ban and landing obligation. They aim to reduce the levels of bycatch and discards through modifications that include the mesh size, use of fisheye devices (FAO, 2020), implementation of circle hooks, alternate baits in longline fisheries, improvements in remote sensing, and animal tracking technologies on vessels (Komoroske and Lewison, 2015).

While the volumes of by-products from aquaculture and fisheries seem high, the major source of lost or unused material can be found in the processing stage. By-products generated during processing typically consist of biomass produced during filleting and preparation of processed seafood products direct to the end consumer. For fish by-products, there is the skin, bones, trimmings, heads, offal, shells and byssal threads from bivalve and mollusc species (Table 1). By-products should be treated on-site as food grade if required for food ingredient generation or feed grade if processed further by approved animal by-product operators. In some instances, these may be discharged to the marine environment, sent to sewage treatment plants, or disposed of in landfills (Cadavid-Rodríguez et al., 2019). For example, "stick-water" produced from the processing of fish (e.g., blood, mucus, and residue muscle proteins) can be an issue due to their quantities being generated and the requirement for sanitary disposal. However, in a number of countries, actions have been taken to reduce and valorise these by-products into food-grade and value-added food ingredients, such as collagen, chitosan, and proteins (Erasmus et al., 2021; Mathew et al.,

Table 1
Current seafood by-products and waste sources for valorisation, biorefining, or disposal.

Stage	Treatment/Process	Current waste hierarchy option	Seafood by-products	Current management strategy	Reference
Aquaculture	Use of pond systems	Disposal	Uneaten feed and sludges	Accumulation of organic by-products at the bottom of the system, where microbes act converting it to less toxic material	Dauda et al. (2019)
	Use of recirculating aquaculture systems	Disposal	Uneaten feed and sludges	Partial removal of organic by-products from aquaculture water through sedimentation and filters. Subsequent treatment in publicly owned wastewater treatment plants.	Martins et al. (2010)
Fishing	No treatment	Disposal	Bycatch	Return of the dead catch to the sea	FAO (2020)
	Fishmeal production	Valorisation	Fish bycatch	Production of fishmeal in processing plants destined for aquafeed products	Dineshbabu et al. (2013)
	Fertiliser production	Valorisation	Fish bycatch	Production of liquid or solid fertilizer, silage, or compost through fermentation or composting	
	Mitigation	Prevention	Fish bycatch	Modifications in mesh size Use of fisheye devices Implementation of circle hooks and alternate baits in longline fisheries Improvements in remote sensing and animal tracking technologies	FAO (2020) Komoroske and Lewison (2015)
Processing	No treatment	Disposal	Heads, bones, offal, and skin	Waste is eliminated through its discharge into the ocean or its disposal in landfills	Cadavid-Rodríguez et al. (2019)
	Bait	Valorisation/ biorefining	Heads, bones, offal, and skin	Valorisation of by-products for fish bait	Masilan et al. (2021)
	Silage production	Valorisation	All fish and selfish by-products	Production of silage (protein-rich liquid) through enzymatic hydrolysis of fish by-products for aquatic and terrestrial animals feeding	Islam and Peñarubia (2021)
	Fishmeal production (human consumption)	Valorisation	Heads	Production of meal in fishmeal processing plants for human consumption, such as fish mince, fish smoked sausages, or fish patties	Erasmus et al. (2021)
	Fishmeal production (animal feeds)	Valorisation	All fish by-products	Production of aquafeed for a low trophic level or warm water fish (like carp or tilapia)	Saleh et al. (2020)
	Functional food	Valorisation/ Biorefining	All fish by-products	Production of functional foods or products such as collagen, peptides, chitin, enzymes, and gelatin for human consumption	Coppola et al. (2021)
	Fertilizers production	Valorisation	Bones, heads, viscera, and wastewater (blood water)	Production of biostimulants and liquid fertilisers through degradation of viscera, biological treatment of wastewater for agricultural application	Kim et al. (2010) Kim (2011) Ching and Redzwan (2017)
	Biofuels production	Valorisation/ Biorefining	Offal and bones	Extraction of oils from fish waste and subsequent biogas or biodiesel production and by-production of glycerine for pharmaceutical, food and cosmetic applications	Karkal and Kudre (2020) Jayathilakan et al. (2012) El-Gendy et al. (2014)
	Omega-3 fatty acids extraction	Valorisation/ Biorefining	All fish by-products	Extraction of omega-3 fatty acids through anaerobic digestion for food applications (food supplements)	Mbatia et al. (2010) Nges et al. (2012)
	Collagen extraction	Valorisation/ Biorefining	Skin, scales, fins, and bones	Extraction of collagen for food, cosmetic, pharmaceutical, tissue engineering, and biomedical industries	Bhuimbar et al. (2019) Araujo et al. (2021)
	Chitin and chitosan extraction	Valorisation/ Biorefining	Shellfish shells	Extraction and purification of chitin and production of chitosan for chemical and food applications	Mathew et al. (2020)
	Protein hydrolysates extraction	Valorisation/ Biorefining	All fish and selfish by-products (especially skin and bones)	Isolation and purification of proteins for human nutrition, cosmetics, and pharmaceutical purposes	Anal et al. (2013)
Consumption	No treatment	Disposal	Bones, viscera, heads, and skin	Waste is eliminated through its disposal in organic recycling bins and later deposited in landfills	Cadavid-Rodríguez et al. (2019)

2020). These products can directly contribute to reducing the environmental impact and production costs of the primary fish product. This may provide an impact positively human health. Whereby, the production of novel, functional food ingredients potentially can reduce the risk of diseases or enhance health (Hayes, 2021).

Markets for these by-products can include low-value animal feeds or agricultural products. A common way to produce low-value feeds is to use seafood by-product-based on ensilage processes. This is used as it is a relatively simple and cheap process, taking advantage of all aspects of the by-product, i.e., protein, lipids, and bone materials (Islam and Peñarubia, 2021; Mousavi et al., 2013). Examples of higher-value alternatives for by-product use include the production of hydrolysates for human consumption, fish mince, fish smoked sausages, fish patties (Erasmus et al., 2021), and aquafeeds (Saleh et al., 2020). The use of by-products for the production of fertilisers and biostimulants has also received attention over the years as a means to increase the economic and ecological sustainability of the fishing industry (Ahuja et al., 2020), by finding avenues to derive value from bones, heads (Kim et al., 2010), viscera (Kim, 2011), and even the wastewater from processing and effluent from aquaculture farms (Ching and Redzwan, 2017; Hayes and Gallagher, 2019).

An area which has been widely studied is the valorisation of fish by-products into high-value products. Some of these studies have assessed the extraction of omega-3 fatty acids, for food supplements because of their preventive role in cardiovascular diseases (Mbatia et al., 2010; Nges et al., 2012), collagen for food, cosmetic, pharmaceutical, tissue engineering, and biomedical industries (Araujo et al., 2021; Bhuimbar et al., 2019), chitin or chitosan from shells for chemical applications (Mathew et al., 2020), and protein hydrolysates (Anal et al., 2013). Other opportunities and strategies for the extraction of oils and the subsequent production of biofuels, like biogas or biodiesel, have also been assessed (Karkal and Kudre, 2020). These valorisation processes make use of by-products generated from seafood production activities such as fishing, aquaculture, processing (e.g., offal and trimmings, Jayathilakan et al., 2012), and waste from consumers (e.g., bones) (El-Gendy et al., 2014). However, research and the uptake of seafood valorisation into commercial practices must be underpinned by the CE model. This would ensure the seafood by-products valorised for further use are sustainable through evidence-based metrics, such as life cycle assessment (LCA).

4. Incorporating the circular economy in seafood value chains

To incorporate CE principles into seafood value chains, there is a need for measurable value(s) from the utilisation of the by-products, e.g., economic, consumer perception, de-risk production portfolio, and legislation compliance. A way in which this can be facilitated is through the use and promotion of eco-design and eco-efficiency (de la Caba et al., 2019; Regueiro et al., 2021; Ruiz-Salmón et al., 2020). The former is integrating environmental attributes into the design of the value chain, while the latter is producing more from fewer resources. Opportunities exist based on a value and volume hierarchy, which can be used to valorise potential products from seafood by-products. Within the seafood industry, there are two main types of by-products that are considered waste. These by-products can be effluents from process water (i.e., sludge, aquaculture wastewater, and processing and cooking effluents) or the biological by-products resulting from the processing (e.g., crustacean, and bivalve shells, offal, fish heads, frame, and trimmings). Some whole fish material could be sourced as discarded from fisheries due to changes in the EU's Common Fisheries policy. Although the quantities are reduced in recent years due to policy changes when compared to a more static and inherent loss from seafood processing for human consumption.

Research activities have identified the potential for the valorisation of liquid effluents from seafood and processing activities, however, these are not yet as extensively applied as they could be (Alkaya and Demirer, 2016; Zilia et al., 2021). For example, it is possible to extract pigments, proteins, or flavour compounds (Tremblay et al., 2020) from processing by-products. Other avenues for the recovery of valuable products include the blood waters from the processing of fish. These waters contain substances which could be valorised into products such as antioxidant peptides, renin, and dipeptidyl peptidase (Hayes and Gallagher, 2019). Other recoverable materials from effluents include sludge or biosolids. Alternative management for sludge could generate novel resources, generating valuable elements such as carbon and different nutrients. Furthermore, as an energy resource in the form of biogas or biodiesel, sludge and recovered solids can be integrated into sustainable solutions that can help mitigate energy consumption in the sector (Gherghel et al., 2019).

With regards to organic processing by-products such as heads, skin, fins, bones, viscera, and scales are often derived into low-value commercial products such as feed, fish meal and oils (Al Khawli et al., 2019; Bruno et al., 2019a). Adhering to the principles of sustainability, these products could be important sources of new high-quality and high-value commercial compounds such as proteins, peptides, vitamins, amino acids, collagen, chitin, enzymes, gelatine, glycosaminoglycans, polyunsaturated fatty acids, minerals, etc. Furthermore, they can provide important functional and bioactive properties for food, agriculture, cosmetics, pharmaceuticals, and/or nutraceutical industries (Al Khawli et al., 2019; Ghalamara et al., 2020; Wang et al., 2019). In addition, different processes have been developed to exploit these by-products efficiently in the form of food packaging, silage, fertiliser enrichers, biofuels, etc. (Nawaz et al., 2020).

In a CE context and to achieve a zero-waste goal, a top-down classification strategy could be established according to the quality or value of the resulting products from liquid or solid by-products. This is with a view to minimising the generation of waste material, which does not result in the recovery of energy or products of interest. It is essential to identify appropriate extraction technologies that will minimise energy consumption, maximise quality and yield, guarantee the safety of the resultant product, and ensure the objectives of sustainable development. However, the valorisation of these by-products may be impacted by certain regulations. Within the EU, the European Commission Regulation (EC) 1774/2002 established the sanitary standards applicable to animal by-products not intended for human consumption (currently repealed by regulation (EC) 1069/2009). For these purposes, by-products are classified into 3 categories based on their risk to human and animal health and specify the conditions under which they can be managed (Table 2). These regulations place a limit on some of the material which can be valorised but does not unduly impact most seafood. Only designated category 3 can re-enter the food chain, typically in the form of farmed animal feed or aquafeeds.

In Fig. 2, the blue arrows represent the flows of by-products or low-value discards that are used in industries with "lower added value". These levels utilise a higher volume of material for a lower value-added product. As the material moves towards the base of the pyramid it is placed in the category below. This is because each time a lower level is used, the less value the resultant product can obtain. In the case of the green arrow, this indicates the co-product that results from energy production that could be reused in the fertilizer category, promoting recirculation of the system (i.e., digestate). The colours for wastewater/sludge (orange) and by-products (red) have no value per se. In the figure, they symbolize the types of by-products and effluents obtained during industrial processes for each of the animal by-products categories.

4.1. Pharma-industry, cosmetics, and biotechnology opportunities

There are a number of processing/biorefinery technologies that can be used to extract, concentrate, refine, and transform compounds from seafood by-products into high-value market bioactive/functional products for nutraceutical, pharma-industry, cosmetics, and biotechnology. These technologies can often include the use of supercritical extraction,

Table 2
Animal by-products (ABP) categories in seafood by-products and waste (European Union Regulation EC No. 1069/2009).

ABP Category	Risk level	By-product management	ABP material	Permitted uses
Category 1	High	Disposal only	Diseased fish, fish with notifiable diseases etc.	Incineration or as fuel in approved plants.
Category 2	Med	Not intended for animal consumption	Livestock carcasses and non-disease mortalities	Fertiliser, landfill (after sterilisation), and safe technical use.
Category 3	Low	'fit for human consumption' and derived from processing plants	Fish and shellfish processor by-products, fish trimmings.	Fertiliser, biofuel, petfood, farmed animal feeds, and aquafeeds.

A stepwise seafood by-product valorisation framework is outlined in Fig. 2. This framework is based on five levels of value for seafood by-products using a volume-value relationship (i.e., low volume, high value products at level 1 and high volume, low value products at level 5). High value applications for by-products include (Level 1) pharma-industry, cosmetics, and biotechnology, (Level 2) food. Medium value applications are valorisation as (Level 3) animal feed. Lower value applications include (Level 4) fertilisers (nitrogen, N; phosphorus, P; potassium, K) and (Level 5) energy. The nutrient recovery technologies, the value-added opportunities, and products for each of the levels of seafood by-product valorisation are presented below from high to low-value products.

membrane separation, and/or hydrolysis, inc. chemical and enzymatic. Such use allows compounds such as vitamins, flavours, essential oils, carotenoids, enzymes, amino acids, lecithin, and polyunsaturated fatty acids to be obtained from seafood by-products. The advantages of using technologies such as supercritical fluid extraction eliminates the need for extractive organic solvents that is traditionally used for extracting bioactive compounds. These solvents might not be food-safe, or environmentally friendly, and at elevated temperatures could compromise recovery rates of the compound(s) (Al Khawli et al., 2019; Haque et al., 2014; Kuvendziev et al., 2018; Uddin et al., 2011). Most studies evaluating the potential of supercritical fluids have focused on recovering lipid-soluble and antioxidative compounds. These products can often have the greatest economic value because they can improve certain technological properties in food matrices in novel foods (Al Khawli et al., 2019).

In contrast, the long-chain polysaccharides chitin and chitosan recovered from shrimp and crab shells are typically through the use of microbial proteolytic enzymes to deproteinise crustacean by-products (Wang et al., 2019). The other methodology is through the use of inorganic acids to demineralise the shells, followed by strong alkalis for deproteinisation. While the latter method produces a purer final product than the biological technique, it does however produce waste by-products, and if not removed, it can contaminate the final product.

The use of pressure membrane technology can purify recovered protein/peptide from seafood. The usefulness of this technology can avoid the need for solvents or adsorbents, as Through this technology, it is possible to obtain permeate fractions enriched in small bioactive peptides with bioactive potential as antioxidative, antimicrobial, and angiotensin-converting enzyme inhibitory activity (Chi et al., 2015; Karnjanapratum et al., 2017; Ngo et al., 2014). For example, it has been observed that cod blood can be a potential source of peptides with antioxidative properties and could be exploited as a functional food ingredient (Ghalamara et al., 2020). In addition, peptides purified from fish by-products also showed interesting antimicrobial activity (Ennaas et al., 2015; Song et al., 2012), i.e., peptides purified from cod blood and sardine cooking wastewater against *Escherichia coli* (Ghalamara et al., 2020).

The use of sludge derived from seafood was recently reviewed by Gherghel et al. (2019). The authors reported the obtention of different compounds of interest such as adsorbents obtained using microwave and pyrolysis treatments, high-quality enzymes, and proteins comparable to commercial versions using ultrasound, or bioplastics using activated sludge as raw material during bacterial fermentation. The bycatch small-spotted catshark (*Scyliorhinus canicula*) viscera have been used as a substrate to produce hyaluronic acid by *Streptococcus zooepidemicus* fermentation (Vázquez et al., 2015). Scales have also been used as a substrate to generate collagenase-like enzymes by microbial fermentation (Wang et al., 2019). Cephalopod by-products such as squid skin, it is a source of collagen for the manufacture of cosmetic products. In addition, this by-product has been investigated as a potential plasticiser in the preparation of biofilms in combination with chitosan (Wang et al., 2019).

4.2. Food opportunities

The use of seafood by-products continues to be a challenge due to food safety, their interactions with other ingredients used in the final food product, and public perception and consumer acceptance. Several products of interest can be obtained from fish by-products such as protein hydrolysates and polyunsaturated fatty acids from trimmings, heads, and frames. However, any by-products used for human consumption must be treated as food grade standards during their collection and processing, e.g., HACCP, which meets below limits of foodborne pathogens. Neglecting these standards can result in hygiene issues, spoilage, and food-borne illness due to seafood's inherent highly perishable nature.

Protein hydrolysates are perhaps the most common use of fish by-products, e.g., fish heads, frames, and offal. The generation of functional food ingredients containing bioactive peptides that can provide the consumer with a health benefit that goes beyond basic, human nutrition is a growing area of both research and commercial venture. Protein hydrolysates, concentrates and isolates are distinguished by the level and quality of protein contained in each and can command different market values based on protein content but also techno-functional and sensory attributes as well as health benefits for the consumer (Hayes et al., 2016). Hydrolysates also have applications as functional feed ingredients to improve the health of farmed aquatic species, ruminants, and companion animals (Naik et al., 2021).

Similarly, omega-3 fatty acid-rich oils from fish livers for the food/health supplement market (Al Khawli et al., 2019; Anal et al., 2013; Bhuimbar et al., 2019). Marine-derived oils (e.g., fish oil) are valuable by-products but were once treated as a low-value commodity until the recognition for their high nutritional value and now it is exploited as an omega-3 fatty acid-rich supplement. Besides this, other applications have been tested including the omega-3 fatty acid enrichment of bakery and pasta products (Nawaz et al., 2020). The traditional method of oil extraction is through cooking and separation. Although, there are other technologies such as ultrasound combined with assisted enzymatic extraction that can improve the oil extraction efficiency. Recent studies have shown that waste parts from fish (e.g., heads and frames) that were pre-treated with assisted enzymatic extraction before enzymatic hydrolysis led to a higher level of oil recovery. This included a higher percentage of polyunsaturated fatty acids level and greater oxidative stability, lower apparent viscosity, and an overall sensitivity to temperature-dependent degradation. All these attributes would lead to wider applications in food products (Al Khawli et al., 2019; Bruno et al., 2019b). While marine oils could also be co-produced along with the production of protein hydrolysates, where centrifugation or filtration technologies are typically used for recovery.

Calcium from fish bones has received attention as a natural calcium supplement for individuals that has calcium deficiency or as a health supplement (Nawaz et al., 2020; Venugopal, 2021). Studies have previously reported that calcium bioavailability is higher in tuna bones in

comparison to calcium from other sources such as milk, vegetable, and salts.

However, all previous studies suggested pre-treatment, including heating, boiling, tempering, or chemical treatment before adding it to the food matrix (Gupta et al., 2016; Nawaz et al., 2020). The reason is that the bone matrix is composed of a complex inorganic part and an organic part of collagen fibres. These fibres are difficult to break down in simple enzymatic digestion without prior softening of the bones. Likewise, by incorporating boiled fish bones from Nile tilapia (*Oreochromis niloticus*) into biscuits, it was reported that fish bone fortification may be a rich source of calcium and other minerals, along with improved fatty acids (Nawaz et al., 2020).

4.3. Animal feeds opportunities

The majority of animal by-products from fisheries and processing plants have long been used in fish meal production that is destined for animal feeds (Cho and Kim, 2011). Although from a circular business economic model perspective, the aim would be to use these by-products for greater value outputs, specifically Level 1 (pharma-industry, cosmetics and biotechnology and human food) and 2 (human food) categories (Fig. 2). However, not all by-products are suitable for CE implementation. For example, the low production yield of the seafood by-product to be economically viable, loss of quality, sporadic times of production, long distances between the by-product producer and the valorisation plant, or insufficient logistical resources to be valorised under Levels 1 and 2, the by-product could therefore be more suitable for animal feed rendering use that is lower grade. One example can be the red and vascularised fish flesh that is typically produced as waste from the fish filleting plants. It is a high-quality protein source that is often used for animal feed production or discarded without revenue being generated (Herpandi et al., 2011; Nawaz et al., 2020). This is often due to the low quantities being generated and logistical difficulty in attaining Level 1 or 2 use.

However, if it is commercially, technically, and/or logistically viable then seafood waste could undergo a series of biorefinery processes to produce functional ingredients for feed use. For instance, the use of supercritical fluid extraction makes it possible to reduce the fat content of fishmeal without affecting the quality of the protein. When operated under certain extraction conditions (10–40 MPa, 25–80 °C and with CO_2 flows of 9.5 g min^{-1}), it is possible to achieve a 90% reduction in lipid content (Al Khawli et al., 2019). Fish oil is extracted from fish viscera by pressing, microwave-assisted extraction, supercritical fluid extraction, solvent extraction, autolysis, and enzymatic hydrolysis (Wang et al., 2019). The application of supercritical fluid extraction has increased in recent years as CE and resource-efficient practices have been incorporated into commercial production practices (Al Khawli et al., 2019; Venugopal, 2021).

The high cost of fishmeal used in fish feed has prompted alternative ways of obtaining protein for feeds. A low-cost method for processing seafood by-products for feed is fish silage (Islam and Peñarubia, 2021; Mousavi et al., 2013). This process results in excellent protein products and is a valuable source of amino acids for protein biosynthesis, with high amounts of polyunsaturated fatty acids. The resulting biorefined products are widely used as feed ingredients in aquaculture for different aquaculture species. During silage processing, endogenous enzymes hydrolyse proteins and transform them into more soluble forms of nitrogen, this helps contribute to their widespread use (Ahuja et al., 2020; Herpandi et al., 2011; Mousavi et al., 2013).

As a potential solution to liquid by-product streams for aquaculture and fish processing, the cultivation of high nutritional value macroalgae (seaweeds) in integrated multitrophic aquaculture systems presents great potential. Ammonia/ammonium from protein metabolism and uneaten aquafeeds is the main aquaculture effluent. It is also often one of the most difficult nutrients to limit in flow through and open aquaculture systems, e.g., sea cages (Badiola et al., 2012; Liu et al., 2016; Song et al., 2019). In general, intensive aquaculture systems, solids are removed by sedimentation or screening, and the nitrogenous nutrient is converted to nitrate (NO_3), through nitrification in bacterial filters (Milhazes-Cunha and Otero, 2017). Therefore, current effluent treatment technology depends on bacterial systems and does not add value to the process beyond converting the toxic nutrient to a lesser form in the effluent. This is except for some aquaculture facilities possessing additional units where that host anaerobic denitrification bacteria where that convert nitrates into nitrogen gas. Although this process is technically prohibitive, e.g., the conversion process is relatively slow. Microalgae can be used for the efficient collection and recycling of nutrients in aquaculture effluents and can reduce chemical and biochemical oxygen demand concentrations and potentially toxic metals. To further enhance the economic strength of integrated multitrophic aquaculture systems, can be achieved by extracting high-value added compounds from algal biomass (fatty acids, pigments, polysaccharides, etc.) that can be valorised in premium animal feeds or as a feedstock for biobased fuels or plastics (Laurens et al., 2017; Maiolo et al., 2020; Milhazes-Cunha and Otero, 2017; Shah et al., 2018).

4.4. Fertiliser and plant biostimulant opportunities

The use of seafood by-products to produce plant fertilisers and biostimulants (i.e., enhances plant health, crop quality and stress tolerance) can potentially reduce large biomasses into commodity. This is particularly relevant if the biomass has deteriorated in shelf-life quality (i.e., rancid), or not fit for use in human or animal use. Furthermore, the recycling of elements back into food in both fisheries and aquaculture production system can mitigate the need for artificial fertilisers. For example, synthetically produced nitrogenous based fertilisers (e.g., ammonium nitrate), which uses the energy-intensive process of converting nitrogen gas in the air to ammonia using natural gas. More importantly, the generation of potassium and phosphate fertilizer from seafood by-products can also displace the need for finite mined sources, e.g., phosphorite and potash-rich rock. This could be strategically important as there is an increasing concern over the growing need for phosphorus fertilizers as a result of food demand. In addition, there is a rising scarcity of phosphate-rich mines, and more recently, global conflicts and climate impact, all of which threatens future food security (Nedelciu et al., 2020).

The benefits of using seafood by-products to produce fertiliser and biostimulant products include being a natural soil conditioner, improving soil texture, and enhancing growth performance (Radziemska et al., 2019). Different fertilisers based on fish by-products are commercially available (e.g., fish blood and bone) and some are even authorised for ecological or organic agriculture. For example, composted fish by-products with pine bark have been evaluated as organic fertilisers. The results showed that there was a positive enhancement in the nitrogen, phosphorus, potassium, sodium, calcium, and magnesium content in arable plant leaves, i.e., ice lettuce (*Lactuca sativa*) and white mustard (*Sinapis alba*) (Radziemska et al., 2019). Although the calcium: phosphorous ratio simultaneously had worsened as a result of the fertiliser regime. Similarly, the fermentation of squid feather bones inoculated with the *Lactobacillus paracasei* bacteria has been reported as a means of producing biobased fertilisers (Wang et al., 2019). In addition, as a fertiliser, it can increase the carbon storage capacity of the soil, potentially minimising greenhouse gas emissions to the atmosphere (Radziemska et al., 2019).

The recovery of phosphorus (usually as struvite) from waste sludge has been reported after anaerobic digestion, where the recovered phosphorus can be used as fertilisers. In some studies, there has been reported that aquaculture-derived sludge derived from anaerobic digestion has a higher bioavailability of nitrogen than undigested sludge (Aas and Åsgård, 2017; Del Campo et al., 2010). Although these processes involve low investment costs and can remove potentially toxic metals simultaneously, they usually require specialised equipment (e.g.,

digester vessels), high operating costs, and chemical and energy consumption (Gherghel et al., 2019). While the use of microwave technology at a laboratory scale had been shown to obtain cadmium, chromium, copper, nickel, lead and zinc after anaerobic digestion of sludge, with a total recovery of 95.3–100%. After anaerobic digestion and sludge dewatering, the production of adsorbents for metal ions (Cu^{2+} and Pb^{2+}) has been reported, with improved control of the heating process, energy savings and reduction of equipment and wastes (Gherghel et al., 2019; Madende and Hayes, 2020).

4.5. Energy generation opportunities

Different methods to obtain energy from seafood-derived sludge have been reported. For instance, biogas generation has been investigated through microwaves, ozonation, ultrasound, enzymatic treatment, and treatments with alkalis or acids. Consolidated technologies have been shown to enhance biodegradability and the capacity to obtain methane from sludge (thermal pre-treatments and high pressures), or the co-digestion of food waste with sewage sludge. Although the complexity of the reactors and the process requires a high level of technical expertise for the operation and production optimisation.

In general, biogas is produced through anaerobic digestion, which obtains a varying degree of methane (50–70%) and CO_2 (30–50%) depending on the quality of the substrate and with a minor impurity concentration, e.g., nitrogen and hydrogen sulphide. Although the process can be slow, carried out at higher than ambient temperatures, and can require large bioreactors to produce viable quantities of biogas. Anaerobic processes can remove organic matter (80–90%) (Del Campo et al., 2010; Parvathy et al., 2017). Beyond anaerobic digestion, gasification, pyrolysis, and sludge can also produce useful products such as syngas (carbon monoxide and hydrogen from gasification/pyrolysis), biochar (pyrolysis), and bio-oils. For the latter, calcined flakes have been used as a catalyst for biodiesel synthesis (Wang et al., 2019). While pyrolysis of sludge after the standard anaerobic digestion for energy production can produce biochar, which can be used for soil remediation or as fuel (Gherghel et al., 2019). The combination of anaerobic digestion and aerobic processes can also be an effective approach to reducing negative characteristics in fish processing wastewater, e.g., high biological and chemical oxygen demand, volatile solids, and typically a low pH. Other technologies such as supercritical fluid extraction have also been explored after sludge dewatering. However, they require high capital investment and maintenance costs (Gherghel et al., 2019; Parvathy et al., 2017). In the review by Pan et al. (2015), a more critical analysis of waste-to-energy supply chains was undertaken. The authors identified a series of barriers that could be categorised as technological, financial, institutional, and regulatory for the uptake of waste into energy production systems. Furthermore, evaluated successful and sustainable waste-to-energy businesses.

5. Discussion

Outlined in the previous sections of this article is the current state of the art with regards to seafood by-product materials, the processes, and technologies available and a volume to value valorisation framework. However, to increase the levels of circularity in seafood value chains, a multidisciplinary and holistic approach to its implementation is required. In order to realise this, a number of stages and steps will need to be included, expanded on and developed (Fig. 3).

- Valorisation levels in a circular economy
- Emerging waste recycling strategies
- Beyond waste to bio-based resources
- Climate impact on seafood loss in fisheries and aquaculture as well as along the value chain
- Education and outreach
- Measuring environmental performance for intensive sustainability

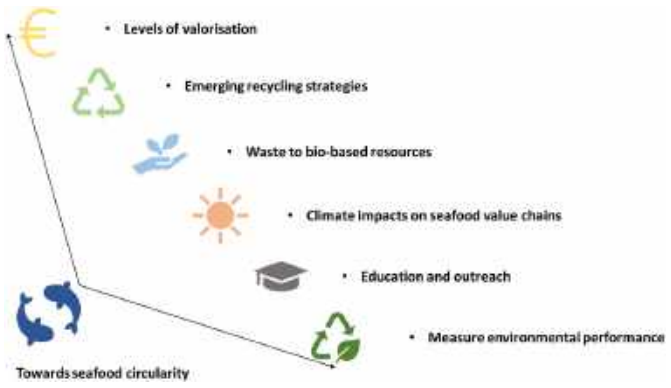

Fig. 3. The thematic areas that require implementation for increased uptake of circularity in seafood value chains.

These thematic areas address some of the key environmental, economic, and social aspects, which will impact on the successful implementation of CE in seafood value chains (Fig. 3). These areas can allow consumers, producers, and waste managers to tackle areas such as seafood loss, prepare climate adaptation measures and find value in almost all aspects of the production chain.

5.1. Valorisation levels in a circular economy

The levels for valorisation and nutrient recovery from seafood waste demonstrate the role of reuse and recovery within the CE, which subsequently adds to the sustainable seafood value chain credentials. These levels offer a decision tree framework which can allow operators, regulators, and investors to comply with the principles of CE while following options that make economic and business sense in their respective cases. For each site, the key considerations will include the volume of available material for valorisation, the distance that the material must travel and indeed, and financial costs that can be considered within this framework and allow economic and environmental considerations to develop viable valorisation strategies.

Feedback loops for by-product material in the various stages of the value chain can contribute to raw material production, in terms of feed, energy, and fertiliser (Fig. 4). By-product material from seafood processing can be valorised as feed ingredients or in higher applications such as food and biopharma products and shifted to the consumption

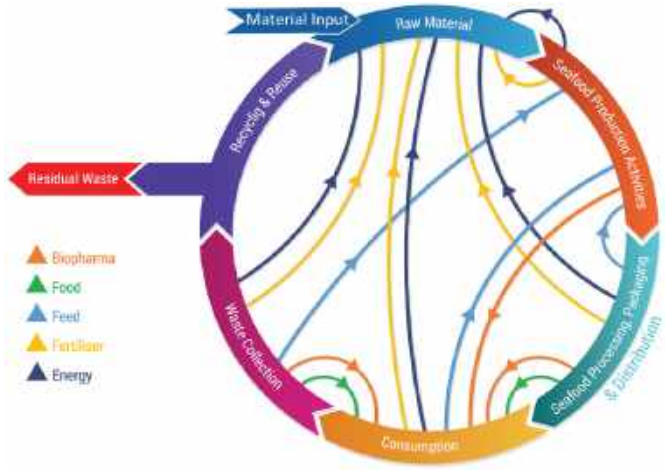

Fig. 4. Seafood circularity using the 5 levels of valorisation potential. Large arrows indicate the bulk material transfer from one step of the value chain to another, while smaller arrows demonstrate potential material transfer for regeneration along the value chain. Omitted from the diagram is the value component for each of the levels.

stage of the value chain. Opportunities for increased valorisation and recovery of lost food and material can be implemented at numerous stages in the seafood supply chain.

The CE model aims to maximise the efficiencies of resource use to reach a high level of return on the energy, time and space invested into the activity and product. The perception that the by-products from these processes are value-less is changing. However, to engage all value chain actors, there are significant knowledge gaps which need to be closed to implement sustainable CE models, which balance the needs of the business with the needs of the environment, i.e., profitability versus limiting environmental impact and sustainability. Therefore, CE practices must be implemented across the product life cycle. Extending beyond the current state of the art, where much of the focus has been on seafood production and processing.

5.2. Climate impact on seafood loss

One of the greatest threats to food and seafood security is climate change. Changes in environmental conditions can greatly impact wild fish stocks, and aquaculture productivity.

For instance, there are increasing concerns over emerging frequency and prevalence of infectious diseases, especially amongst farmed aquatic species. Brooks et al. (2022) considered the impacts of emerging infectious diseases in aquatic systems, but the case studies are mostly on tropical and crustacean-farmed species. This suggests aquatic systems elsewhere need to be evaluated against potential future climate-related impacts.

Furthermore, the increasing frequency in extreme weather events caused by climate change may have negative impacts on processing, packaging, and distribution channels, which further contribute to seafood loss. For example, Collins et al. (2020) postulated that climate change may cause an increased flood risk that could impact both shore-based facilities and their access points and routes. While increasing storm and extreme weather events (e.g., heatwaves) may also increase the time required to transport or shorten the life of seafood within and between markets. This consequently brings about a reduced useable life, quality, and value of the seafood product (Maulu et al., 2021).

5.2.1. Fisheries loss

Fisheries is one of the food production sectors that face the greatest threat of food loss from climate change. Sainsbury et al. (2021) found that fishers in the southwest region of the UK examined the trade-off of the economic rewards of continued fishing compared with the physical risk at sea when adverse weather conditions impact fishing. They looked at the socio-economic risks and potential benefits across a range of wind speeds and wave heights at sea, and how these influence the decision-making of the ship's skipper on whether to set out to fish or not. There was variability across the types of ships, gear type and other factors but in general, the utility values were seen to reduce with windspeeds above about 40 kph and wave heights >3 m. Other factors which contribute to seafood loss in the fisheries segment of seafood production can also include; discarded catch, poor chilling facilities on board the fishing vessel and damage to stock while being removed from nets (Kruijssen et al., 2020). These factors can broadly be categorised as physical, quality, nutritional and market loss (Kruijssen et al., 2020; Kumolu-Johnson and Ndimele, 2011; Love et al., 2015).

5.2.2. Aquaculture loss

The same factors for seafood loss in fisheries apply to aquaculture. Where aquaculture differs from fisheries is that the artificial conditions in which aquatic species are cultured offer little protection from natural events for example, in marine environments, storms and extreme weather events can cause cage structures to fail and in the instance of freshwater flow through systems flood events can cause considerable damage to facilities and lead to the escape of farmed stock. Food loss in aquaculture systems is something which should be mitigated given the artificial nature of the practice, which is similar to the culture of cattle and sheep, where it relies on inputs from the technosphere for production and success. Naylor et al. (2021) recognised in a comprehensive review of global aquaculture and its increasing importance over the last 20 years the impacts and thus potential losses that climate change may have on farmed seafood. They noted that losses (i.e., mortalities) from aquaculture occur mainly due to suboptimal growing temperatures, saltwater intrusion due to sea-level rise, damage to infrastructure, freshwater shortages, and droughts. They also noted climate impacts on rising costs of feed as well as climate-driven risks due to pathogens, parasites, and pests as well as harmful algal blooms.

5.2.3. Processing and distribution loss

The processing and packaging sections of seafood value chains are likely to offer the greatest opportunity and impact in recovering lost waste in the short and medium term (Cortés et al., 2021a; de la Caba et al., 2019; Laso et al., 2016; Tan and Lamers, 2021; Venugopal and Sasidharan, 2021). Seafood loss can occur in this stage of the value chain through a number of different challenges. Some of these overlap with losses that have been highlighted in other parts of the value chain. One of the ways in which loss can occur in the processing stage can be through spoilage due to inadequate equipment or equipment failure (i.e. refrigeration and conveyor systems), low levels of processing control (i.e., staff removing too little from the carcass), low use of packaging material, or low processing capacities (Kruijssen et al., 2020; Love et al., 2015; Spang et al., 2019). Another contributing factor to food loss can be the implementation of high-quality food standards. These quality standards, while they present appealing-looking products to consumers can also contribute to food and nutrient loss, by diverting damaged, but safe and healthy seafood products from the food supply (Spang et al., 2019).

From a distribution perspective, losses can occur through poor handling or stocking of the products. Damaged packaging can shorten the shelf life of the products and in cases where there is a sizeable distance to market lead to unsaleable products. Poor road infrastructure and remote landing or production sites can also contribute to seafood loss. Geopolitical events, trade barriers, and disease outbreaks can also shorten the shelf-life and availability of seafood. This can be evident by delays in the delivery of seafood products to continental Europe from Ireland and air freight of seafood from Europe to Far-East Asia and vice versa (Ahearne and Hynes, 2020; Barnes, 2020; Mahfouz et al., 2019).

Improved process control and supply chain management practices can help to reduce food loss and waste in seafood supply chains (Bruno et al., 2019a; de la Caba et al., 2019; Yan and Chen, 2015). With industry and industrial processes moving into the 4th industrial revolution (Industry 4.0), a number of advanced and smart technologies are coming online (Hassoun et al., 2022). These technologies in conjunction with wider smart systems such as energy monitoring can help to reduce the costs of cooling and refrigeration; cooling and freezing being one of the main drivers of cost, energy use, and environmental burden of seafood processing (Avadí and Acosta-Alba, 2021; Cortés et al., 2021a; Hassoun et al., 2022; Vazquez-Rowe et al., 2012). Industry 4.0 is still an emerging vision of how industrial processes and economies can increase their efficiencies. However, the EU has already begun to build capacity for Industry 5.0. It aims to complement Industry 4.0 practices by also transitioning to sustainable, human-centric, and resilient industries (European Commission, 2022).

5.2.4. Consumption loss

Some of the greatest losses and waste of seafood occur at the consumption stage. Statistics on seafood loss at the consumer level can range from 10 to 11% (Gustafsson et al., 2013) to 41–56% (Love et al., 2015) and by some estimates can be as high as 70% (Stenmarck et al., 2016). These values can vary from region to region and can reflect the cultural value that is placed on seafood within a nation's diet. Contributing factors to food loss at this stage of the supply chain can

include spoilage of the products, excess preparation (the loss of edible parts due to poor preparation), and diet culture, i.e., only eating certain parts of the seafood (Birney et al., 2017; Kruijssen et al., 2020).

Many of the recommendations which have been put forward to reduce the levels of loss and waste generated by consumers have focused on behavioural changes through educational efforts (Kruijssen et al., 2020; Love et al., 2015). These strategies aim to encourage consumers to plan their meals, control the portion size of the meals, and minimise the levels of leftovers. Other suggestions include a switch from fresh to frozen seafood products, which could lead to a decrease in levels of waste through longer shelf-life spans. Action plans are being developed by national and interregional policymakers to raise awareness on the impacts and to highlight the unsustainability of current food consumption practices. This includes the United Nations' Sustainable Development Goals, policies, and actions such as those by the EU, which have incorporated food loss and waste minimisation within many of its plans (Circular Economy, European Green Deal) and strategies (Farm to Fork, European Commission, 2018; 2020a, 200b). These actions will lead to national and interregional initiatives by member states to combat food loss and waste. However, there will also be a need to match these efforts in waste management through novel and emerging waste recycling techniques and strategies throughout seafood supply chains.

5.3. Emerging waste recycling strategies

Emerging waste recycling strategies in the seafood supply chain are essential to tackle the growing seafood waste problem. Within the EU trading bloc, the Waste Framework Directive (Commission Decision, 2011/753/EU, EU 2019) outlines some basic waste management principles with the preferred option of preventing waste. However, according to Sharma et al. (2021) and Ghosh et al. (2016), an effective waste management strategy would need to include waste minimisation, characterising the waste, and waste recycling. From a supply chain perspective, some emerging strategies are being introduced to meet sustainability goals. For example, location-based tracking technology is being introduced by the EU's commercial fisheries. This initiative will be a step towards increasing transparency and discernibility in seafood supply chains. Identifying the waste levels and the location of waste within the supply chain allows the development of an effective waste management plan, e.g., the stage, location, type, and volume of seafood waste being generated (Ghosh et al., 2016). Emerging waste recycling plans aim to develop value-added supply chains. For instance, the valorisation of abundant and available bio-wastes (de la Caba et al., 2019; Sorg et al., 2019). These plans can be facilitated through the separation of the waste into liquid/solid streams and using the best available extraction method to retain the nutritional component(s) of interest (Coppola et al., 2021; Lam et al., 2020; Rejula and Mohanty, 2018). There is also a great interest in developing sustainable and biorefinery practices to produce disruptive and high-value bioactive compounds from food waste streams. These range from the use of novel antimicrobials for eco-packaging applications, immune-stimulatory ingredients for health and wellbeing (Murphy et al., 2020; Rowan and Galanakis, 2020), and novel ingredients for fish feed from biorefining fisheries and seaweed by-products into the aquafeed ingredients (Pogue et al., 2021; Colombo et al., 2022).

Some researchers have outlined the importance of reframing what is food and what is waste as a key step in the cradle-to-cradle considering waste as part of the cycle (Martínez-Córdova et al., 2017; Soma, 2020). For example, in aquaculture, the conventional waste that is produced can take a place as a by-product alongside traditional seafood products. This approach can decrease the release of contaminants into the environment and add new revenue streams to the seafood sector (Regueiro et al., 2021).

One of the key challenges in emerging waste management strategies is consumer acceptance of recycling seafood waste and turning it into a new product. These strategies will need to balance several factors to be effective and commercially viable approaches. For example, new market opportunities, market trends, current market developments, and an end product that is competitive in the marketplace (Borrello et al., 2017). The role of the consumer in the success of the product is particularly important, as the consumer has the final say in the economic success of a product (Borrello et al., 2017; Ghosh et al., 2016). Finally, food marketing, consumer education and food labelling by retailers and producers can assist with changing consumer behaviour as a catalyst for waste management plans (Altintzoglou et al., 2021; Aschemann-Witzel et al., 2016, 2016de la Caba et al., 2019).

5.4. Beyond waste to bio-based resources

Within CE an emerging field is the circular bioeconomy (CBE). The CBE aims to utilise biomass or biological resources from all industries and economic sectors (Tan and Lamers, 2021). There are a number of definitions in use for the CBE, but it can be accepted as complementary to the CE and can help in the transition to cleaner and sustainable production (Raimondo et al., 2021; Tan and Lamers, 2021; Venugopal and Sasidharan, 2021). One of the core aims of the CBE is the production of biobased materials and products. Bio-based products are often considered to be more sustainable than their conventional counterparts. Although these claims need to be validated using life cycle thinking (Adom et al., 2014; Brunklaus and Riise, 2018; Spierling et al., 2020; Wojnowska-Baryła et al., 2020). Under a CBE framework, there are efforts to develop functional foods, feeds, and additives from seafood by-products.

Recent research and frameworks for the use of machine learning in the processing and production of feeds and food products have identified opportunities for the application of such technologies across seafood supply chains (Cooney et al., 2021; Hassoun et al., 2022; Kang et al., 2017; López-Cortés et al., 2017; Manoharan et al., 2020; Suryawanshi and Eswari, 2022). There are strong roles for machine learning, the internet of things and digitisation in the development and production of efficient, precise food products from raw or waste sources.

5.5. Measuring sustainability and environmental performance

One of the most important concepts in the use and implementation of the technologies and approaches for seafood by-product valorisation is sustainability. The sustainability of these interventions and actions needs to balance the economic and environmental needs of society and business operators. One of the approaches that are widely used to assess the environmental performance and sustainability credentials of a system, technology, process, or product is life cycle thinking, primarily in the form of life cycle assessment (LCA, Bohnes and Laurent, 2019; Ruiz-Salmón et al., 2021). Life cycle thinking uses a multimeric approach that can be used to assess the performance of a product across the three pillars of sustainability or triple bottom line: 1) environmental, 2) economic, and 3) social. These assessments can take the form of environmental LCA, social LCA (SLCA) or life cycle costing (LCC). When combined, these distinct aspects of life cycle thinking become a life cycle sustainability assessment (LCSA). LCSA has received growing attention since the early 2010s but to date has not been widely applied (Guinée, 2016). This is in part due to the ongoing development of frameworks and methodological approaches which can be used in LCSA (Lam et al., 2020). While the use of LCSA is the end goal, in the interim the use of key performance indicators (KPI) derived from LCA, LCC and SLCA can help to better inform and support decisions for the valorisation and increased utilisation of seafood by and co-products. For example, under the environmental pillar of sustainability, the use of KPIs such as carbon dioxide (kg CO2 eq. kg-1 of product), water use (m3 kg-1 of product) or energy use (MJ/kg of product) can provide very useful information on the associated environmental burden of operational decisions. Economic KPIs under an LCC approach could take the form of a payback period of the investment, internal rate of return or benefit-cost ratio (Valenti et al.,

2018). Social KPIs for example that could be applied in this context could include investment to create direct employment or indices on the seafood circularity (amount of seafood prevented from being wasted) and its contribution to nutrition (using recommended daily allowances for human nutrition or energy content if for feed) (Hallström et al., 2019; Valenti et al., 2018). The use of simple KPIs like those listed above can help to promote the sustainable use of seafood co-products under a circular economy.

Previous studies have demonstrated LCA as a suitable tool to guide and make decisions on selecting and handling strategies in food waste recycling (Lam et al., 2020; Vilariño et al., 2017; Zilia et al., 2021). The use of the water-energy-food nexus can be an effective tool in developing a waste recycling plan applied for seafood by-products. A study undertaken by Chang et al. (2016) found it was particularly useful for identifying synergies, challenges, and opportunities. In particular, the authors noted that some of the outputs which would lead to increased social justice enhanced resource efficiency and reduced environmental impact. Gephart et al. (2017) argued that there was a gap in the 'seafood' water-energy-food nexus literature. They highlighted the importance of looking at water usage in seafood production. In addition, Slorach et al. (2020) conducted a study incorporating 'health' into the water-energy-food nexus, using LCA for each sector to give a true perspective on environmental impacts. The study looked at the impact on the nexus of four treatment options: anaerobic digestion, in-vessel composting, incineration, and landfilling. The results found that anaerobic digestion was the most sustainable and in-vessel composting the least. Similarly, a study carried out by Laso et al. (2016) analysed the environmental impact of anchovy waste valorisation in contrast to incineration and landfilling. The results showed that the valorisation waste management options resulted in the least impact on the environment.

Numerous studies have outlined the benefits of using social, economic, and environmental variables including economic performance assessment tools to assist decision-makers in the waste management of seafood supply chain strategy (Alkaya and Demirer, 2016; Jacob et al., 2021; Zilia et al., 2021). Zilia et al. (2021) demonstrated that by incorporating the three pillars of economic, social, and environmental in the example of reusing sea urchin waste. They did this by presenting the frameworks under a business model canvas approach and identified social opportunities through additional jobs being created as part of CE implementation and reduced environmental burden by valorising the waste material. Vilariño et al. (2017) followed a similar approach in reviewing LCA approaches for food by-product management. A number of recent studies have suggested that the use of technological, social, and political readiness levels to supplement the use of LCA as means of developing new green innovations, including sustainable waste management (Ruiz-Salmón et al., 2020; Stead, 2019; Villanueva-Rey et al., 2018; Voyer and van Leeuwen, 2019).

According to Ghosh et al. (2016) 'undervaluing and 'underreporting' are referred to as the 'hidden costs' in food waste management. Economic benefits are key enablers of green innovation (Kelliher et al., 2020); exploring the true value of these, 'hidden costs' can enable a more accurate assessment of the economic impact of waste valorisation and can act as a facilitator for change (Ghosh et al., 2016). A good example is the use of recirculating aquaculture systems and how they can reduce water usage, increase nutrient recycling, and improve waste management (Martins et al., 2010).

5.6. Driving a seafood circular agenda through education and outreach

The linear economy has increased the distance between producers and consumers. As a result, it has affected society across all strands and social classes. In particular, how food is produced, this gap has resulted in a loss of awareness and value for the food being consume and the associated production systems. Given the increasing global demand for seafood, there is an opportunity for many seafood production systems to become "model" CE systems. Applying a multi-stakeholder approach to inform future sustainable waste management will enable an effective and inclusive strategy that will affect behavioural change. The power of the consumer as a catalyst in driving waste management in the seafood supply chain is vital. For example, the raising in public awareness on the environmental impact of single use plastics has led to a drive to prohibit their use, which resulted in the EU passing the Directive 2019/904 and bans certain types of plastics used and sold within the EU. There is a scarcity of literature on gauging socioeconomic impact, and this limits the ability of decision-makers to develop successful and long-term sustainable waste management strategies into existing business models.

Education and communication (vertical and horizontal) using the concept of a Triple Helix (academia-industry-government) ecosystem approach is required to change the behaviour to value waste as a resource in the value-added supply chain. This Triple helix approach will also support and accelerate the societal transition to a low-carbon economy using a bottom-up approach, which will inform future research and enterprise-performing activities regionally and nationally, with a global orientation. A number of emerging waste recycling strategies are reviewed from the seafood supply chain to the consumer. A holistic Triple-Helix framework approach to supporting and enabling sustainable waste recycling and management will provide strategies for by-product resource efficiency incorporating all stakeholders comprising producers, policymakers and consumers in the decision-making process and contribute to sustainable waste recycling goals. There should also be equally public-academia-industry-government driven initiatives for better seafood valorisation innovation and strategic impact. For instance, changing the misconception of fishmeal being used in aquafeeds where it is derived from the fish processing (e.g., trimming, offal, heads, and frames, Colombo et al., 2022) or hydrolysates waste by-products (partially fish protein hydrolysate and cook water, Egerton et al., 2020).

6. Conclusion

This review offers insight into the diversity of seafood by-products being generated as part of current EU and global food production practices. It also presented a stepwise framework which can allow operators to identify valorisation strategies based on a volume-value-based approach. Opportunities exist through the valorisation or reuse, which can reduce environmental impact or loss of resources, i.e., nutrients, energy, and biomass. Some of these opportunities, for example, can include nutrient recovery at the level of production in aquatic farms using polyculture, aquaponics, integrated multitrophic aquaculture, or through mechanical means such as solid waste recovery from the produced sludge. Similarly, there are many opportunities from the fisheries sector, where non-targeted species, over quotas, or poor quality and unsuitable targeted species could be exploited as a valued resource, rather than discarded back into the aquatic environment. There is now a greater need for resource efficiency use as fisheries production levels are plateauing due to over-exploitation and climate impact. However, there are significant barriers that prevent the large-scale regeneration of seafood waste into valued products. A conjunction of incentives, capital investments, policy changes, commercial, and social and consumer acceptance is needed to realise the true potential of seafood waste.

Another key area to address is that many of the technologies which had been discussed remain at the research level. To increase the technological readiness level, there needs to be greater engagement with the industry in discerning the viability and readiness with which these technologies and approaches will be received. The markets for valorised seafood by-products as they move into the higher value levels of the valorisation framework (i.e., food and biopharma). The amounts and quality of by-products that are available for valorisation will also need to be established. Eco-efficiency and eco-design will need to play strong roles in the sustainable development of seafood by-product valorisation. For example, would centralising or decentralising to valorising a

particular seafood waste be the most appropriate for commercial exploitation? To measure this environmental impact LCA would need to be employed and allow the decision-making of which valorisation option is the most sustainable. Human development has now reached a critical juncture, where the realisation of many natural resources is finite and incompatible with society's linear production chains. As the global human population increases, the regeneration of nutrients from seafood waste into new foods and products is paramount to creating a future of sustainable and responsible consumption.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by the EAPA_576/2018 NEPTUNUS project. The authors would like to acknowledge the financial support of Interreg Atlantic Area. A.H.L Wan was co-funded under the HYDROfish project (2019–2022) which was funded under the Disruptive Technologies Innovation Fund (DTIF), established under Project Ireland 2040, run by the Department of Enterprise Trade and Employment with administrative support from Enterprise Ireland. His opinions expressed are his own. The authors would also like to thank Matt Bell for his editorial assistance.

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Science of the Total Environment 748 (2020) 141362

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Review

Unlocking challenges and opportunities presented by COVID-19 pandemic for cross-cutting disruption in agri-food and green deal innovations: Quo Vadis?

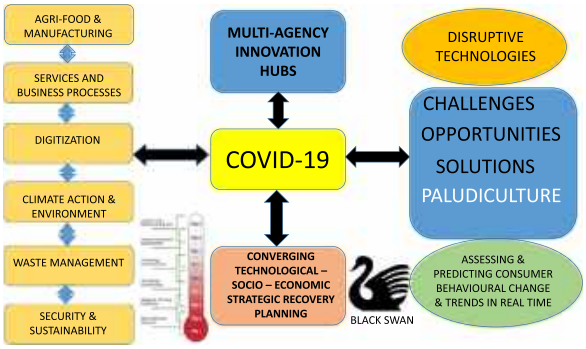
Neil J. Rowan [a,b,c,*], Charis M. Galanakis [d,e,f]

[a] Bioscience Research Institute, Athlone Institute of Technology, Ireland
[b] Centre for Disinfection, Sterilization, and Biosecurity, Athlone Institute of Technology, Ireland
[c] Empower Eco Sustainability Hub, Lough Boora, Co. Offaly, Ireland
[d] Research & Innovation Department, Galanakis Laboratories, Chania, Greece
[e] College of Science, King Saud University, Riyadh, Saudi Arabia
[f] Food Waste Recovery Group, ISEKI Food Association, Vienna, Austria

HIGHLIGHTS

- COVID-19 pandemic presents opportunities for sustainable agri-food production and to accelerate green innovation.
- Sensible yet ambitious technical-economical recovery plans are urgently needed when countries reopen.
- COVID-19 may create disruptive technologies that cross-cuts agri-food, ICT, health, and environment.
- Multi-agency converging innovation hubs have the potential to accelerate socio-economic recovery.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 June 2020
Received in revised form 27 July 2020
Accepted 28 July 2020
Available online 31 July 2020

Editor: Damia Barcelo

Keywords:
COVID-19
Disruptive technologies
Agri-food
Climate change
Green innovation
Sustainability

ABSTRACT

COVID-19 pandemic is on a trajectory to cause catastrophic global upheaval with the potential to alter geopolitical and socio-economic norms. Many countries are frantically responding with staggering financial stimulus recovery initiatives. This opinion-paper reviews challenges, opportunities, and potential solutions for the post-COVID-19 era that focuses on intensive sustaining of agri-food supply chain in tandem with meeting the high demand for new green deal innovation. For example, the development of wet peatland innovation, known as Paludiculture, can intensively sustain and blend agri-food and green innovations that will help support COVID-19 pandemic transitioning. The future looks bright for the creation of new sustainability multi-actor innovation hubs that will support, connect, and enable businesses to recover and pivot beyond the COVID-19 pandemic. The nexus between first 'Green Deal' initiative supporting 64 selected European Startups and SMEs (European Innovation Council) and 43 Irish Disruptive Technology projects are addressed in the context of cross-cutting developments and relevance to COVID-19. Candidate areas for future consideration will focus on climate action, digitization, manufacturing, and sustainable food production, security, and waste mitigation. Recommendations are also provided to facilitate community transitioning, training, enterprise, and employment to low carbon economy.

* Corresponding author at: Centre for Disinfection, Sterilization, and Biosecurity, Athlone Institute of Technology, Ireland.
E-mail address: nrowan@ait.ie (N.J. Rowan).

https://doi.org/10.1016/j.scitotenv.2020.141362
0048-9697/

Contents

1. Introduction

Coronavirus (COVID-19) has caused dramatic and unprecedented upheaval to socio-economic norms since first reported in Wuhan, China, earlier this year (Rowan and Laffey, 2020; Guan et al., 2020). COVID-19 pandemic is impossible to predict, with many countries experiencing second or successive waves of infection. Akin to a 'Black Swan' event, there is a significant gap in published research to inform companies where most have neither prepared for it nor have they planned to transition beyond it (Taleb, 2010; Reid et al., 2020). COVID-19 impacts upon health systems, governments, and businesses alike with unprecedented implications for companies worldwide. Companies are feeling the market and financial shock of the COVID-19 outbreak by factories' shutdown, labor shortages to cash flow stress, and disruptions in the supply chain (Reid et al., 2020). The global mitigation response has focused on public health strategies to curtail and reduce the spread of viral transmission through hand hygiene, social distancing, lockdown (staying-at-home, cocooning), and, community wearing of protective face masks that now occurs in 50 countries (Rowan and Laffey, 2020; Silva et al., 2020). This fact has resulted in an unprecedented technical-socioeconomic earthquake that has left many sectors seeking emergency COVID-19-related unemployment benefits (Rowan and Laffey, 2020; Guan et al., 2020). The food supply chain sector does not comprise an exception (Galanakis, 2020). Sarkis et al. (2020) noted that there is a window now for transitioning to sustainable supply chains in the aftermath of COVID-19 that includes rethinking vulnerabilities created by over-reliance on 'just-in-time' or 'business-as-usual' practices. Barcèló (2020) highlighted challenges affecting the environment and health caused by COVID-19 pandemic along with recommendations for monitoring and mitigation.

Similarly to other countries, the Republic of Ireland has been in lockdown for 3 months to flatten the curve in COVID-19 cases. On the other hand, it is in a relatively privileged position where it is adopting a phased reopening of the country that commenced 18th May 2020, with phase five intimated for 10th August 2020 (Fig. 1). While Ireland is emerging from the first phase of reopening, likely, the vast majority of foodservice and businesses will not return to regular operation for several weeks. Covid-19 adjusted employment in the Republic of Ireland has reached 28.2%, compared with traditional 5.4% for the same month last year, which is estimated to cost the Irish exchequer €6.4bn until the country reopens. COVID-19 pandemic has introduced dramatic uncertainty and unpredictability where this volatility has the potential to cause socio-economic chaos across many sectors when Ireland and other countries reopen for business (Guan et al., 2020).

While there are financial stimulus support packages (Government of Ireland, 2020), there is an unprecedented dearth in critical information (such as marketing of new consumer trends and behavioural change to offset sheltering-in-place and social distancing) in order to enabling businesses to make priority decisions on core needs for recovery post-COVID-19 (Sarkis et al., 2020). As there was almost simultaneous closure of countries due to COVID-19 mediated lockdowns (Guan et al., 2020), the Republic of Ireland will re-emerge from the COVID-19 bubble ahead of many other trading countries that will provide a useful lens to gaze through to inform recovery. This scenario will help inform real-time situational-analysis and will reveal the main challenges where different geopolitical and socio-economic landscape now potentially exists internationally. The lack of food security means that countries are dependent upon imports but may not earning sufficient foreign currency to purchase vital imported goods. The post-COVID-19 era also presents equal opportunities to address climate change and to advance green innovation matched with driving sustainable food production and security.

2. International financial recovery stimulus support initiatives for business during and post COVID-19

Europe needs to be reopened as quickly as sensible, but this will involve significant risk. In response, there has been major national, EU, and international stimulus financial aid to offset this potential socio-economic crisis potentially. On 27th May 2020, the European Commission presented an economic stimulus plan of €750 billion to help mitigate the shock from this COVID-19 pandemic and pave the way for a sustainable future. While there is a commitment to support recovery financially, there is currently a lack in specific detail underpinning a techno-socio-economic ecosystem unroadmap to guide F&D companies through and beyond COVID-19 when countries reopen. There is a recognition that the EU will still need to prioritize climate actions and a digital strategy, but also to update the new EU health program. This action is important to ensure continuity of supply chain for food and medical products, services across the EU to offset critical shortages arising for existing and future pandemics. In the Republic of Ireland, the Department of Health and the Department of Agriculture, Food and the Marine (DAFM) are collaborating as part of a holistic response to COVID-19, e.g., although remaining open for business sector (DAFM, 2020). At the time of writing, two very stark documents published in Ireland - the Irish Fiscal Advisory Council (2020) and the ESRI UK and Ireland (2020) both warned of ballooning unemployment, huge deficits, massive accumulations of public debt and painful budgetary adjustments

Phase 1 May 18th, 2020	Phase 2 8th June, 2020	Phase 3 29th June, 2020	Phase 4 20th July, 2020	Phase 5 10th August, 2020
Phased return of outdoor workers Remote working continues for all that can do so.	Limited return to onsite working subject to compliance capability Remote working continues for all that can do so.	Return to low-interaction work. Remote working continues for all that can do so.	Return to work where employees cannot remote work. Staggered hours. Remote working continues for all that can do so.	Phased return to work across **all** sectors. Remote working continues for all that can do so.
Retail that is mainly outdoor and homeware, opticians, motor, bicycle & repair, office products, electrical, IT, phone sales & repair open. All subject to social distancing.	Small retail outlets with control of numbers open. Marts open. All subject to social distancing	Open non-essential retail outlets with street level access.	Gradual easing of restrictions on higher-risk services. e.g. Barbers and hairdressers	Further easing of restrictions higher-risk services. e.g. shopping centres, tattoo, piercing.
Farmers markets, gardners and other outdoor workers return to work – social distancing requirements continue to apply . Remote working continues for all others that can do so . Return to Work Safety Protocol is the operative guide for employers and employess	Small retail outlets can reopen with a small number of staff on the basis that the retailer can control the number of individuals that staff and customers interact with at any one time.	The opening of all other non-essential retail outlets will be phased in on basis of a restriction on number of staff and customers per square metre so that social distancing can be maintained. This is to be limited to retail outlets with a street-level entrance and exit and does not include those in enclosed shopping centres due to higher risk.	Restrictions will gradually be decreased on the numbers travelling in major urban centres on public transport and in private cars. Specific measures will be introduced at ports and airports.	Enclosed shopping centres can reopen, with social distancing. A further loosening of restrictions for services involving direct physical contact for periods of time between people for which there is not a population-wide demand for later phases due to risks . Non-resident tourist travel to offshore islands can resume. Social distancing and hygiene measures are to continue for public and private transport.
Data source - https://www.gov.ie/en/news/58bc8b-taoiseach-announces-roadmap-for-reopening-society-and-business-and-u/ (accessed June 4th, 2020)				

Fig. 1. Reopening of economic, retail and commercial activities in the Republic of Ireland using a phased approach during 2020.

to the tune of up to €14bn by 2025. IBEC (2020), the group that represents Irish business, published proposed solutions to COVID-19 imposed liquidity crisis, e.g., supporting vulnerable businesses using emergency cashflow and liquidity measures from the Irish government. IBEC also denoted the importance of maintaining the operation of food and other processing facilities. This action includes specific liquidity and financing needs of farmers, fishers, and agri-food businesses and for the banks to offer flexibility to their customers at an early stage to discuss emerging cashflow issues.

3. Agri-food industry – an example of challenges ahead post COVID-19

The agri-food sector comprises a dynamic societal-technical innovation ecosystem and is one of the largest manufacturing industries (Saguy et al., 2018; Rowan, 2019). In the EU, this increasingly important sector accounts for €1098 billion turnovers and employs 4.24 million (Saguy et al., 2018). The food and drink (F&D) industry has doubled in size in the United States over the past decade, where it was estimated to be worth £6 trillion in 2015 with packaging comprising almost £1.9 trillion (Statista, 2020a). Revenue in the food market amounts to US $924,389m in 2020; the market is expected to grow annually by 1.8% (Statista, 2020a). The rate of global population growth is staggering, and there is a commensurate need to match this demand that will put increasing pressure on food production and security (Richie et al., 2018; Michelini et al., 2018). There is a pressing need to diversify the food supply chain and identify sustainable technologies across food systems that will meet changing diets, increasingly aging, ethnic and cultural population, diet-related diseases, more personalized products (Galanakis, 2020). DBEI (2018) projects that there will be a ca. 70% rise in demand for more food products and services over the next 40 years. In the years ahead, it is envisaged that organic, unprocessed, and healthy food will drive growth in domestic markets (Statista, 2020b). For example, the estimated value of shipments of the industry was US$795.4 billion in 2019, where. 15.1% of the cost of shipments generated from dairy product manufacturing.

The agri-food and beverage sector is Ireland's most important indigenous industry where the sector produced €13bn of exports to overseas markets in 2019 on foot of decades of accelerated growth where the value of exports in this sector increased by 67% (Duffy, 2020). However, at this time of writing, the impact of COVID-19 pandemic with associated international lockdown has pretty much instantaneously shut down world economies. Duffy (2020) reported that COVID-19 would also bring about a dramatic collapse in merger and acquisition activity with deals stalled, put on hold, or canceled outright. With such uncertainty and unpredictability – post-crisis, the likely immediate focus of business owners will be on managing core business and recovery. It is very challenging to forecast at this stage, for the F&D sector, the outcome and when new dawn may emerge. Duffy (2020) postulated that deals likely to be created from COVID-19 circumstances include "(a) forced mergers and acquisitions transactions as those companies unable to recover from this crisis will seek buyers, (b) corporate entitles with competitive cost structures, strong balance sheets, and case resources may seek cheaper deals including lower valuations for businesses post COVID-19, (c) consolidation of some companies of similar size to withstand market uncertainties and strengthen their financial standing by securing new customers/markets; (d) companies may seek to remedy supply chain problems, such as cash-rich retailers transitioning to procure certainty of supply for certain food products; (e) companies to see out value by adding traceability solutions to improve food security – where they will find themselves facing enhanced requirements to satisfy consumer needs for accurate and transparent information on the food they are producing". COVID-19 will be trying to understand if changes to consumer purchasing practices are permanent and how/where to adjust post-COVID-19.

4. Transitioning COVID-19 crisis by exploiting green peatland innovations (Paludiculture) - a useful nexus between agri-food, climate action, and circular economy

Europe has set out its ambitious vision to become the world's first climate-neutral continent by 2050 (A European Green Deal, 2020), while in 2019, the Irish Government published of the All of Government Climate Action Plan - to tackle Climate Breakdown. The food and environmental sectors are tightly bound. The environmental industry is nowadays facing societal changes that force companies to pay more

Table 1
European wet-peatland technologies (Paludiculture) funded projects and relevance to green deal innovation and COVID-19 pandemic recovery.

Project	Aims and approaches	Partners	Activities	Impacts
Interreg NW Europe Carbon Connects 2018–2021 €4.5 M funding https://www.nweurope.eu /projects/project-search/ cconnects-carbon-connects/	To reduce the high carbon footprint of peatland soils in Northwest Europe by introducing new bio-based business models developed for sustainable land management practices. Estimated stats offered are 50% reduction in CO_2 emissions caused by traditional practices Promote alternative approaches to wet agriculture to protect environment Low lying peatlands – can be done by raising water levels and introducing alternative crops Facilitating transformation in land use to wet agriculture – develop new business models Enhancing wetland ecosystems	Research partners Netherlands Belgium, France UK Ireland Local authorities Farmers Consumers Research Institutes	'Paludiculture or wet peatland innovation' Wet crops New business models Cattail [food/construction material/biomass] Sphagnum moss Alder carr Wild rice Blue services [water retention, cooling] Carbon credits. Online toolbox for processes leading to Carbon sink modelling Carbon and blue credit scheme for peatlands Pilot sites for testing of new business models	To reduce emissions by ca. 3200 t per year on pilot sites across ca. 80 ha Protection against environmental issues Partnerships create innovation ecosystem (living labs) with local authorities, Research Institutes, farmers, landowners Educational Programme Share best processes and knowledge
Interreg North Sea Region CANAPE €5.5 M https://northsearegion.eu /canape/measuring-greenhouse-gases-in-wetlands/	To measure greenhouse gases in wetlands. To support the restoration of 95 ha of peatland, and support the restoration of 3 peatland lakes. The project Creating a New Approach to Peatland Ecosystems responds to these issues by restoring wetland areas to reduce their CO_2 emissions and improve their capacity to store water, and by aiming to develop the markets for products produced from wetland ecosystems - a type of farming known as Paludiculture. Physical restoration of the landscape, raising water levels in drained bogs and fens, and restoring lake edges to improve their water quality. This will halt the release of CO_2 that occurs on drained peatlands, and restore the capacity of the land to act as a buffer to floods and droughts. Improving water quality will improve the recreational value of the	UK Denmark Germany Belgium The Netheralnds Pan Euroean (Nordic Baltic Wetland Group)	Paludiculture – typha, mosses and grasses Retrain Carbon sink and water store Sphagnum mosses Reed (compost, biocher] Waste wood (cooking charcoal and biocher) Greenhouse Gas Emission Site Types (GEST) Methodology. This uses the water classification and the plant types growing on the site to estimate emissions. These are based on a review of scientific literature, and uses previous studies to establish an average for each site which meets certain criteria. Deliverables Create over 60 ha of new bog habitat Create over 20 ha of new reedland habitat Trial 10 ha of new agricultural production,	Clear understanding of production methods for each product Costs and potential sale price Identify market for products Produce Carbon Pocket Guide Inform policy makers of benefit of paludiculture as alternative form of land use

	waterways and support tourism by reducing incidents of toxic algal blooms. Demonstrating sustainable use of the land, through piloting agricultural products that can be grown on wet land (known as Paludiculture), and showing that there is a viable economic alternative to draining land for agriculture.		including reed, sphagnum moss, and purple moorgrass.	
Interreg NW Europe Care-Peat €6.24 M 2019–2022 https://www.nweurope.eu/projects/project-search/care-peat-carbon-loss-reduction-from-peatlands-an-integrated-approach/	Reduce CO_2 emission and restore Carbon storage capacity in peatlands to set up and demonstrate innovative technologies for new restoration and carbon measurement techniques and involve local and regional stakeholders. Therefore the nature organisations, together with local landowners, restore peatlands of five different pilot sites ranging from 10 to 250 ha and demonstrate the (potential) carbon savings of the restoration. For each pilot site different restoration techniques are used - from manual labour to growing additional peat moss.	Scientific Research National Centre French Geological Survey Lancashire Wildlife Trust Manchester Metropolitan University National University of Ireland Galway Eurosite Natuurmonumenten University of Orleans		By 2022 – expected ca 7800 t of Carbon emissions to be retained in 5 pilot sites – equivalent greenhouse gas emission of 6072 passenger cars driven per year
Eurpean Commission – LIFE PEAT RESTOTE Project – https://life-peat-restore.eu/en/	To reduce greenhouse gas emissions by 40% until 2030 compared to 1990. Therefore conservation of peatlands must be integrated in the climate and energy policy. Especially the Baltic States as well as Poland and Germany have huge areas of peatlands, which are partly heavily degraded and which need conservation and restoration. In the project the emissions and storage of greenhouse gases, the water level as well as the wildlife (flora and fauna) will be documented, analysed and compared. This helps to prove rewetting measures and gives the chance to regulate aberrations quickly. Also the potential climate effects of the rewetting can be calculated.	Poland, Latvia, Estonia, Lithuania, Germany	The LIFE Project Peat Restore aims to rewet degraded peatlands in the partner countries Poland, Latvia, Estonia, Lithuania and Germany, covering an area of 5300 ha to restore the function as carbon sinks. National events, information materials, a photo exhibition and a documentary film will contribute to raise public awareness and to inform about the project's progress.	During the drainage of peatlands, oxygen gets into the peat, which leads to aeration and decomposition of organic matter into smallest parts. In this aerobic zone microorganisms are extremely active, so the rate of decomposition is especially high. As a result the greenhouse gases (CO_2, N_2O) are emitted into the atmosphere, what reverses the function of peatlands as a store into a source of greenhouse gases.

considerable attention to develop services, processes, and products that meet the strict legislative requirements as well as a result in a greener production, cleaner environment, and a more sustainable world. Simultaneously, companies deal with increasingly competitive markets in which innovation is regarded as a survival requirement (Galanakis, 2019). Subsequently, there is a pressing need to consider smart new solutions for ensuring the intensive sustainability of agriculture and food production processes that respond to the challenges of pandemics and climate change.

Globally, peatlands account at present for ca. 3% of the earth's surface and play a significant role in offsetting CO2 emissions through sequestration. Peatlands store 1.4 trillion tonnes of carbon-equivalent to 75% of all carbon in the atmosphere, which is twice that stored in forests. Bord Na Mona, the Irish state body that owns 80,000 ha of peatlands, are transitioning from burning peat to transitioning to a green footprint. For example, there is a strong trajectory for Bord Na Mona to develop wet peatland innovation (known as paludiculture) for new business models with academic partners and other industries under a new Empower Eco Sustainability HUB platform that is also supported by the government (Just Transition Programme, 2020). Bord Na Mona is exploiting the 'Empower Eco' platform to accelerate its transition from 'Brown to Green' during COVID-19 that will also stimulate regional development, community transitioning to low carbon economy, training, enterprise, and employment. These transitioning activities will be informed by necessary knowledge transfer from several related European-funded Interreg projects that seek to restore peatlands, including measuring greenhouse gases (GHGs), to improve biodiversity and carbon sequestration, along with developing green paludiculture innovations and blue services such as water retention (Table 1). Wet peatland crops such as cattail, sphagnum mosses, moor-grasses, Alder carr, wild rice, typha, reed, and waste wood for the food and construction industry (Carbon Connects, 2020: Restore, 2020; Care-Peat, 2020; Canape, 2020). Those mentioned above will enable carbon and blue credit scheme for the peatlands. Paludiculture will also facilitate the ambitious of advancing EU collective agriculture policy beyond 2020 (Peatlands in the EU, 2020).

This approach will also facilitate the urgent need for sustainable alternatives and innovative business models for farmers and land managers on rewetted peatlands, which will also enable significant carbon sinking. O'Neill et al. (2020) have recently reported on the development of freshwater aquaculture for high-value perch and trout in the Irish peatlands using cut-away flooded peatlands using organic principles, which is powered by a wind turbine (Fig. 2). Water quality and waste remediation are maintained in this paludiculture-based aquaculture process by using natural microalgae and duckweed. Naughton et al. (2020) also reported on the use of this freshwater aquaculture process for testing new innovations, such as the real-time relationship between the use of hand-held AlgalTorch™ on the farm and relationship with flow-cytometry to measure vital physicochemical parameters. O'Neill et al. (2019) also used this freshwater aquaculture process to demonstrate the efficacy of using microalgae as a smart green innovative tool to evaluate the ecotoxicological quality of effluent that included robustness for used during climate change, such as drought. Advances in bioinformatics and next-generation sequencing will also help with improvements in different microalgae used for this purpose as well as the determination of microbial populations in the system, including the emergence of pathogens or problematic microorganisms (Naughton et al., 2020). This is particularly relevant as less than 5% of aquatic microorganisms are culturable on conventional agar media (Rowan, 2011; Rowan et al., 2015; Fitzhenry et al., 2019). It is envisaged that there will be continued advances and potential for technology disruption in forestry and horticulture for future environmental-proofing such as the delivery of cocktail of helper microorganisms and bioactives through hydrogels from adjacent manufacturing and materials industry to respond variances in climate change and resilience. Peatlands also present an opportunity for improving pollination and ecosystem service management (Naughton et al., 2017; All Ireland Pollinator Plan, 2020). These actions include supporting new medical plant and herbs (such as Calendula, Meadowsweet, Plantain, Valerian, Marsh Mellow, Peppermint), extraction of medicinal sap from birch trees, and production of honey as a social enterprise for rural communities. These timely activities align strongly with the finding of Robroek et al. (2017), who reported that plants that makeup peat bogs adapt exceptionally well to climate change compared to diverse plant communities (such as dunes and grasslands). Peatlands lose fewer plant species as a result of climate and environmental change as they are more resilient. Robroek et al. (2017) also noted that peatlands protect ca. 10% of global CO_2 emissions and act as a sponge in our landscape for holding vast amounts of drinking water, which can be used to offset uncertainty for climate change, such as drought.

The development of peatlands presents opportunities to accelerate leading-edge knowledge and competency through innovation hubs, which would tap into abundant, diverse natural resources for enterprise development and to facilitate transition beyond COVID-19. This will inform the next generation of entrepreneurs to stimulate regional creation and environmental knowledge base with a low carbon orientation. Exploiting wet peatland innovation, known as "paludiculture"), will contribute to the new EU Farm to Fork Strategy, which is at the heart of the European Green Deal, aiming to make food systems fair, healthy, and environmentally-friendly. It will also support Europe's ambitious vision to become the world's first climate-neutral continent by 2050 (A European Green Deal, 2020). In so doing, accelerating paludiculture innovation will advance the economic diversification of territories most affected by the climate transition and the reskilling and active inclusion of their workers and jobseekers. Companies are coming under pressure to engage in sustainability and eco-friendly innovation, but lack a clear progression pathway. This new paludiculture multi-actor ecosystem platform will accelerate entrepreneurship and commercialization that will contribute to supporting the recovery from COVID-19, and possibly other future pandemics. Such a paludiculture innovation platform is being actively pursued in the Irish midlands, known as Empower Eco, which will provide sustainable transitioning-solutions for those unemployed and underemployed as a result of reliance upon burning peat as fossil fuel. It is envisaged that exploiting digitization will also advance the rate of paludiculture innovation along with creating opportunities for promoting the recovery of tourism with the support of Citizen Science. Multi-actor sustainable Hubs will also build enterprise programs by bundling added value through R & D collaboration, facilitating design and technological maturation activities, and commercial planning that should encompass financial, legal, supply chain, and market entry activities. This platform should support startup and SME funding instruments to be leveraged through elevant regional, national and European funding models, such as under Green deal and sustainable agriculture that will potentially inform technology disruption. The core tenets of multi-actor transnational innovation hubs, that connects complimentary technological core facilitities, have been successfully delineated through Interreg Atlantic Area Sharebiotech Project (2012). Such activities will also be supported and accelerated thourugh transnational modelling (Interreg Atlantic Area Neptunus Project, 2018).

5. First "Green Deal" funding from European Innovation Council to support the recovery plan for Europe – strong alignment with Paludiculture platform ambitions

On 23rd July 2020, the European Innovation Council (EIC) announced the awarding of over €307 million in funding to 64 game-changing 'green deal' startups and SMEs that will contribute to the objectives of the European Green Deal Strategy (2020), and the Recovery Plan for Europe (2020). Successful proposals ranged from innovative solutions for the automotive, aerospace, and maritime sectors to advanced materials or Internet of Things technologies (full listing of selected

projets are highlighted at European Innovation Council, 2020). Many of these EIC-funded 'Green Deal' projects align with the core tenets of Science Foundation Ireland Disruptive Technology Innovation Funded projects of 2018 and 2019 (Figs. 3 and 4). Examples of EIC 'Green Deal' selected projects that focus on pollination, bioplastics, renewable energy and GHG emission reduction, waste remediation along with specific COVID-19 interventions are noted in Table 2. Over one-third of EIC-funded companies are led by women CEOs, which is a significant increase (tripling) of the number in previous EIC funding rounds.

This paludiculture research platform aligns with The European Innovation Council (EIC), which supports visionary entrepreneurs who create transformative solutions to pressing societal and environmental challenges, supporting the Green Deal and the recovery plan for Europe (European Commision, 2020; European Innovation Council, 2020; Green Deal for Start-Ups and SMEs, 2020). These startups and SMEs are set to scale up, creating jobs and growth, and giving Europe the global leader in green technologies and solutions. Akin to the EIC's Green Deal, the paludiculture-based hub also supports companies to benefit from exclusive business acceleration services to enable rapid growth and scale (Table 3). It is noteworthy that the EIC is currently in a €3.3 billion pilot phase and is due to be fully-fledged in 2021 as part of the new Horizon Europe program (European Innovation Council, 2020). In March 2020, the Commission amended the pilot EIC 2020 Work Programme to include a €300 m budget through the EIC pilot Accelerator for funding game-changing, market-creating innovations that contribute to the goals of The European Green Deal and the United Nation's 2030 Agenda for Sustainable Development. The scheme offers startups and SMEs the option to apply for either grants or blended finance (grant or equity support). Since the start of the EIC Accelerator Pilot in autumn 2019, a total of 140 startups and SMEs active in all technology-intensive sectors (health, digital, energy, etc.) have been pre-selected for equity financing for a total of over €500 m. The amended pilot EIC Work Programme also introduced special provisions to support applications by SMEs and startups with female CEOs.

Climate change and environmental degradation are an existential threat to Europe and the world. To overcome these challenges, Europe needs a new growth strategy that transforms the Union into a modern, resource-efficient, and competitive economy where there are no net emissions of greenhouse gases by 2050 (European Innovation Council, 2020). The European Green Deal is an ambitious roadmap for making the EU's economy sustainable, which will happen by turning climate and environmental challenges into opportunities across all policy areas and making the transition just and inclusive for all. The European Green Deal provides a timely roadmap with actions that will (1) boost the efficient use of resources by moving to a clean, circular economy, (2) to restore biodiversity and cut pollution. It outlines the investments needed and financing tools available and explains how to ensure a just and inclusive transition. Those mentioned above paluciculture-focused transitioning initiative strongly aligns with A European Green Deal Action Plan (2020) including (1) climate ambition, (2) clean, affordable and secure energy, (3) industrial strategy for a clean and circular economy, (4) sustainable and smart mobility; (5) greening the ordinary agricultural policy / 'Farm to Fork Strategy, (6) preserving and protecting biodiversity, (7) towards a zero-pollution ambition for a toxic-free environment, (8) mainstreaming sustainability in EU policies, and (10) supporting the EU as a global leader in the green innovation.

6. Solutions to accelerate agri-food recovery for post-COVID-19 potential technology disruption

Several economies across the globe are braced for a short-term economic slowdown (Guan et al., 2020), where they have implemented economic stimulus measures. Reid et al. (2020) suggested that corporate leaders should consider five key steps they can take to reshape their business and plan for recovery: (i) prioritize people safety, (ii) reshape strategy for business continuity through stress-testing to evaluate short-term liquidy and risks, (iii) communicate with relevant stakeholders in a clear, transparent and timely manner, (iv) maximize the use of government support policies and packages, and finally (v) build resilience in preparation of the new reality with bold strategies based on stress tests. Companies will then have to define internal guidelines based on experience learned from the first COVID-19 wave, as well as to develop contingency to respond better to a second wave, and faster to future pandemic crises.

Virtual accelerator hubs for connecting Micro with SMEs that exploit advances in ICT through immersive technologies will become more popular for informing disruptive innovation in situ and for remote end-user applications. This tendency will enable hurdling restrictions that may come with networking and training innovators or employees in meeting rooms that may persist as a barrier to innovate for post-COVID-19 disruptors. It can be actualized through ICT and Quality of Experience (QoE) such as virtual reality (VR) and augmented reality (AR) (Braga Rodrigues et al., 2020). Rowan (2019) reported on the potential benefits of combining immersive with educational technologies for remote workforce training that has significant implications for the future provision of remote workforce training for new skills linked to education. EIC 'Green Deal' startups and SME selected projects also focus on virtual reality and training that includes provision for COVID-19 (Table 2). There is a pressing need to exploit existing or to create new multi-agency enterprise-hubs related to academia that will support and accelerate innovators and businesses (such as in agri-food) as well as full-span commercialization of products and services using the 9-stage technology readiness assessment (Mankins, 2009). This fact should include off-site pilot-data generation where there is an increasing trajectory towards sustainable innovation, the green agenda, and digitization (Table 3). This multi-agency innovation HUB concept is under strategic development in the Irish midlands region to facilitate transitioning to green innovation and for recovery post-COVID-19 era, which will facilitate resource utilization, help recovery of businesses, and accelerate technology disruption post-COVID-19 (Just Transition, 2020).

7. Definitions of disruptive technologies

Disruptive technologies (DTs) or disruptive innovations were initially defined to address market disruption in established markets, where a new product or service (a technology) is introduced (Bower and Christensen, 1995; Christensen et al., 2004). DTs arise from a global drive to discover innovations that will lead to greater competitiveness, impact and value to businesses and society (Christensen and Bower, 1996; Lauer and D'Agostin, 2013; Yongfu et al., 2017; Geels, 2018; Li et al., 2018; Sousa and Rocha, 2018). Innovations may be viewed as disruptive when they take the place of established or broadly accepted ideas arising from scientific inquiry, or in methodologies or in paradigms that disrupts knowledge (Kuhn, 1962).

In recent times, definitions of DT focus on broad factors affecting the industry and address the nexus between learning experience arising from substitutable innovations that relate explicitly to competitive pricing and performance (Rowan, 2019). The recent review by Schuelke-Leech (2018) provides an excellent insight into a diversity of DTs - where products are reduced in size (such as exploiting leading developments in nanotechnology); more lightweight and efficient (such as utilizing additive manufacturing and material science); more competitively and affordably priced (such as exploiting resource management and production including advances in innovative service and business processes); more excellent dexterity and convenience in design and functionality (using researcher creativity blended with artificial intelligence, augmented and virtual reality and so forth that includes future-proofing for needs across various platforms); and more significant performing products and services (such as exploiting physico-chemical developments combined with the use of robotics

and AI for design linked to advances in education and workforce training). For example, this concept may be applied to the food industry for the introduction and training of new technologies across the supply chain from production, distribution, and storage. Developing DTs in the agri-food domain is core to supporting and driving national strategic development plans as these generate jobs, add-value, troubleshoot, and enhance quality in changing marketplaces. These important deliverables are also strongly evident in Green Deal selected projects awarded by European Innovation Council (2020).

In the interesting work of Schuelke-Leech (2018), Beth Ann described a conceptual model to understand the orders of magnitude of DTs that may disrupt markets, businesses, institutions, and the societal norm, which constitute 'the innovation ecosystem.' Specifically, such disruptions occur at two different levels. First order-level disruption is the focus of much the business literature where it considers and addresses disrupters in innovation. Second-order includes technological disruptions that permeate through society, influencing substantial change. Second-order disruptions are more extensive than first-order disruptions. There is great desire to understand the process whereby one identify candidate technology disruptors as that is connected to innovation ecosystem (Nagji and Tuff, 2012) Schuelke-Leech (2018) noted that factors leading to the creation of DTs arise for the localized opportunity (Christensen, 2003), creativity and problem solving (Rowan, 2019), financial investments (such as from self-financing to Venture Capital and Angel investors, Rowan, 2019), networks (Rowan, 2019), broad applicability for an innovative technology (Schuelke-Leech, 2018), and supporting infrastructure and institutions (such as clustering of human capital, networking and so forth to enable a creative process to occur, Drucker, 1985).

8. How might DTs accelerate recovery post-COVID-19 and transition towards sustainable food systems?

Herrero et al. (2020) recently reviewed and identified technologies and approaches that have the potential to accelerate the transition towards a sustainable food system. While this was a pre-COVID-19 study, it is evident that many of the innovations identified as future technologies may also be led to disruption and inform recovery post-COVID-19 pandemic in terms of sustainable food production and socio-economic recovery plans. For instance, preference for

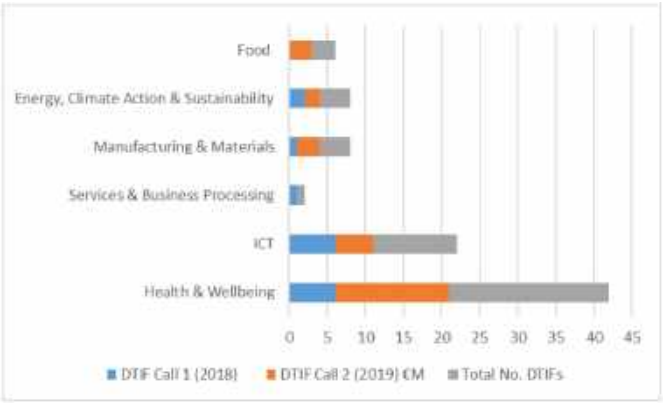


Fig. 3. Number of funded projects awarded per topic in 2018 and 2019 by the Irish Government under Disruptive Technology Innovation Fund (DTIF).

functional foods or nutraceuticals that boost immune-system and wellbeing for COVID-19 are likely to become popular (Masterson et al., 2019; Murphy et al., 2020; Galanakis, 2020). Given the gap in knowledge, there has been an increasing number of preprint publications that specifically focused on the potential benefits of nutraceuticals for used to improve the health and mental wellbeing of citizens, including a focus on those recovering post-COVID-19 pandemic. Target compounds under evaluation include vitamins, polysaccharides, natural polyphenols, bioactive lipids, and peptides (Gonzalez, 2020). Companies claim more recognition of immune-boosting ingredients (Daniells, 2020). They have seen an opportunity to develop relevant products (e.g., chocolate balls rich in β-glucan) targeting immunity during the post-lockdown era (Koe, 2020). These trends will most likely continue to drive the market within the next years (Galanakis, 2020). Subsequently, businesses will be seeking to fill the demand for nutraceuticals and functional foods to address the challenges and opportunities created by COVID-19 disease (Galanakis, 2020). These products may emerge from food processing by-products (Galanakis, 2012, 2013), seaweeds, yeast, algae, plants, and fungi or mushrooms that reduce inflammatory responses that are typically associated with cytokine storm in severe COVID-19 patients (Murphy et al., 2020). Masterson et al. (2020) also

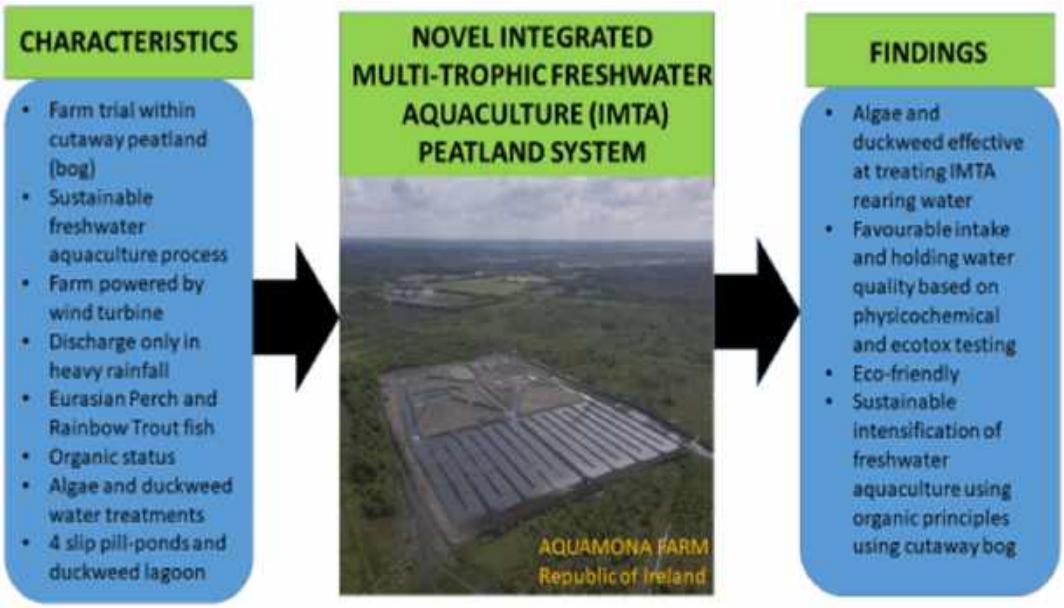


Fig. 2. Peatlands-based Freshwater Aquaculture process (adapted from O'Neill et al., 2020).

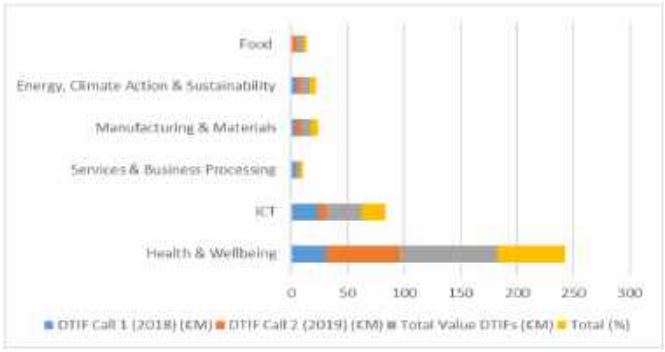


Fig. 4. Amount in Euro of funded projects awarded per topic in 2018 and 2019 by the Irish Government under Disruptive Technology Innovation Fund (DTIF). Proportional representation for each topic in overall DTIF funding is shown as percentage.

reported on the novel use of bioactives from medicinal fungi ameliorates antibiotic-resistant *Klebsiella pneumoniae*-induced pulmonary sepsis.

Future intensive sustainability of the food sector will also be influenced by pressures applied to supply chain, including uncertainties associated with the impact of global warming on crops that will include more flooding and droughts (O'Neill et al., 2019). Fisheries and seafood are viewed as desirable high protein, low carbon-intensive products with the emergence of smart aquaculture processes to meet growing consumer demands (Tahar et al., 2018a, 2018b, O'Neill et al., 2020). However, Ruiz-Salmón et al. (2020) also reported that the seafood and aquaculture sectors across European countries are embracing opportunities to mitigate key environmental pressure points (depletion of resources and climate change), social needs (changing customer attitudes and preferences) or growth in markets (services and business processes along with enhanced competition and worldwide competitiveness). Maintaining satisfactory water quality through developing sustainable innovations to meet growing populations globally will also present significant challenges and opportunities (Tahar et al., 2018c; Tiedeken et al., 2017). De-risking and policy decision making will be increasing influenced by predicitve modelling (Tahar et al., 2017). These pressing challenges are influencing the innovation ecosystem from citizens to policy-makers to adopt and foster more sustainable practices. Sharing of new knowledge across European seafood and aquaculture sector, including innovation in ecolabelling and ecodesign, will have far-reaching and cross-cutting influences to the circular economy (Ruiz-Salmón et al., 2020). Smart changes in these areas may lead to disruptive products and businesses.

Blockchain offers a security-proof approach to recording every digital transaction that can inform a broad spectrum of smart innovations from business processes to 5G networks. In the food disruption context, it has the potential to radically transform and disrupt safety and quality, waste remediation and recycling, security, and authenticity and traceability (Medical Expo, 2020). The robotics industry is estimated to be worth ca. €2.2billion by 2022 and has to potential to transform the food industry through automation, such as personalized food processing (StartUS-Insight, 2020). There has been a global push to re-address dependency on single-use plastics with a greater focus on smart packaging, including the emergence of potential for bioplastics led by large companies such as

Table 2
Examples of EIC Accelerator 'Green Dea'l Selected Projects 2020 including COVID-19.

Startup/SME	Country	Selected Project
B4plastics	Belgium	Complete biodegradable and resistent polymers that allow for circularity and a reduction in plastic consumption and microplastics pollution.
UniSieve	Switzerland	Advanced molecular separation solutions for a wide range of applications of different scales, ranging from propylene or hydrogen purification to biogas upgrading.
Vatorex AG	Switzerland	An eco-friendly solution to kill the Varroa mites and preserve the beekeeping industry.
Nanogence SA	Switerland	Innovative techniques for increasing the efficiency of cement manufacturing and the robustness of materials while achieving energy savings and reducing CO2 emissions in the process
Electrohaea GmbH	Germany	Using biological methanation technology for cost-effective large-scale energy storage and the production of multi-purpose e-fuel for use in transportation, power generation or industrial heating.
Celllugy	Denmark	Using bacteria and yeast starting from sugar or agro-industrial waste for obtaining a biomaterial able to replace plastics in several packaging applications
Brite Hellas SA	Greece	A unique transparent (80%) solar glass panel that generates clean energy. The solar glass combines a nanostructured coating material with silicon solar cell technology to de liver a product ideally suited for greenhouse applications.
Cascade	France	Game-changing disruptive solution for photosynthesis and plant development that makes growing food more sustainable by significantly reducing water, fertilizer, and pesticide use.
Outsight Sas	France	Key enabling laser technology for autonomous vehicles and drones, opening the future of emissions reduction and ecosystem monitoring
Altar	France	A disruptive platform harnessing the power of natural selection for the development of novel microorganisms fulfilling specific industrial needs.
NVP Energy	Ireland	Technology to treat municipal sewage wastewater at ambient temperatures and convert sewage pollutants into renewable biogas
N2 Applied AS	Norway	SmartNitroFarm is a ground-breaking alternative to chemical fertilisers, which reduces greenhouse gas emissions, reduces odour, stops ammonia loss and air pollution.
GlasPort Bio Ltd	Ireland	An innovative platform technology to eliminate greenhouse gas emissions from organic residues and manures that specifically inhibits methane-producing microbes and traps nitrogen.
LightSpace Technologies	Latvia	Next Generation Enhanced Augmented Reality 3D Glasses for medical education, pre-procedural planning, intra-procedural visualization, and patient rehabilitation
Hpnow APS	Denmark	Secure, safe, sustainable and affordable on-site generation of Hydrogen Peroxide
Aquila Bioscience Ltd	Ireland	COVID-19 pandemic: An innovative, safe and effective bio-decontamination technology for non-toxic removal of biological agents, including coronavirus
Reisistell AG	Switerland	Rapid diagnostic for bacterial SEPSIS and AMR urgently needed for ICU patients in COVID-19-like epidemic
Nanoscent Ltd	Isreal	Novel COVID-19 POC Screening Tool Based on Proprietary Nano-Sensors and ML Techniques

Table 3
Multi-agency ecosystem HUB activities to support and accelerate innovators, businesses post COVID-19.

Concentrated Single-Access Supports for Industry, Entrepreneurs, Disruptors	Linked Acceleration Activities
Step-Change Physical Infrastructure & Systems Supports	R&D Collaborative Facilitation
Pre-start Ups	Design Maturation Activities
Ideation & Design Thinking to inform Technology Readiness Level (TRL)	Technical Maturation Activities
Market Research and Enterprise Support	Financial Planning
– Early Needs Analysis	Legal Assistance
– Product Market Fit Analysis	Social and Digital Marketing (including informing customer behavioural change)
Early Technical Validation	Networking
– Test the Technologies	Dedicated Grant Writing/Reporting
– Experimentation/Validation in Pre-Pilot	Connection to Academic Staff/Expertise and Equipment to support commercialisation
– Scaled to Real-Life Setting	
Digitization (including AI, in situ and remote training via AR/VR)	Workforce training - placements
Conduit to State Financial Supports/Agencies	Enable Social Enterprise - Outreach Functions

Diago and Nestlé (Medical Expo, 2020). Food waste management is also the subject of many transnational research and innovation projects such as the European Commission Interreg Neptunus project that combines academic expertise with industry across the Atlantic area to address waste recycling in the fisheries and seafood area, including life cycle assessment, valorization, and ecolabeling (https://neptunus-project.eu/). There is also increasing interest in the development of 3D printers, also known as additive manufacturing, as a sustaining and potentially disruptive technology for a wide range of possibilities for the food industry. For example, 3D food bioprinters permit personalized and repeatable nutrition where it is considered to provide the correct amount of nutrients to match different lifestyles, gender, and health requirements (Brunner et al., 2018). Besides, experimental 3D Bioprinters are designed to prints living cells that have the potential to advance food supply chain needs (StartUS-Insight, 2020). However, the role of social marketing and communication to inform behavior changes and to seek feedback on attitudes, perceptions, and barriers for the uptake of this technology will be necessary (Brunner et al., 2018).

Artificial intelligence (AI) is increasingly used to develop new foods and flavors, such as Coca Cola's research into the Cherry Stripe in 2017 (Medical Expo, 2020). AI will play a prominent role in the personalization of foods and nutrition, exploiting the vast potential of digitalization. Besides, food delivery companies are beginning to concentrate on using the position of artificial intelligence (AI) for problem-solving matched with automation, such as automated guided vehicles. As an example, slow-moving pavement droids to deliver food have been tested by Just Eat, who has partnered with Starship Technologies for this exciting opportunity (StartUS-Insight, 2020). The Internet of Things (IoT) is increasingly becoming relevant for the next-generation food industry, which includes forging innovation in services and business processes. For example, Innet introduced a change suitable for all kitchen devices such as analysis of items for food refrigeration, including taking note of expiration dates with provision for suggesting recipes along with meal preparation. Food security is also an essential factor to have to the fore post-COVID-19, where the monitoring of food from the field to fork using IoT technologies presents a logical solution to this challenge. Given the necessity for food globally, disruption in products and services is likely to emerge from innovations in the delivery and online retail sector as most people remain at home to prevent infection. This infers a focus on food security (such as blockchain and the internet of things in the food supply chain), safety including smart packaging, and alternative, disruptive approaches to food sources such as protein sources. Traceability of infected workers is also very important across the entire farm to fork continuum where there have been clusters of COVID-19 cases reported in meat packaging plants, such as in Ireland (Power, 2020) and Germany (Scally, 2020). The Republic of Ireland has introduced a new COVID-19 Tracker mobile phone app for it's citizens that has ca. 200,000 daily visits.

In terms of potential global economic recovery plans post-COVID-19, and the emergence of Food DTIFs, the significant value may be placed on such things as a review of antibody testing data. For this application, it is hoped that epidemiology will show that many people were infected where this may inform a v-shaped recession with a short sharp recovery. A desirable v-shape economic recovery trend may be more likely due to a wave of online shopping and people working from home. Approximately $6.2 trillion (12.5% of retail total) is spent on food and beverage in the USA: 2015 was the year that more food was brought in than prepared in the home. COVID-19 has shocked that trend, in the US in Q2, $100bn dollars shifted from restaurants to retail space. Monopolies in food grocery services may arise, where smaller independent stores may struggle. In the UK, 7% of the population shop for groceries online, with 4% in the US. Yet, one-third of the US population bought online during the 2nd week of March, and half of them was their first time. Confidence must be provided to ensure continuity in the food supply chain to avoid friction in the food system. It is uncertain as to the state with any degree of confidence what would be the specific impact caused by the global downturn (or potential recession) in the economy as it relates to particular needs and opportunities met by emergent technologies in agri-food (including ingress from adjacent industries). Reuters (2020) described that an alternative U-shaped economic recovery might occur, that takes more than a couple of quarters as economies have suffered a faster and deeper, which Reuters feel may be the likeliest outcome. This reflects thinking that lockdown impact may last for a while after their lifting with a gradual easing of the lockdown where social distancing will continue that will continue to influence the tourist industry and so forth negatively. The occurrences of these combined risks may affect the appearance of a COVID-19-induced recession. The flexibility and adaptability of companies to meet change and adjust business models, including provision for ICT, including online delivery for supply chain, will be better placed for sustain and for potentially cause food disruption practices.

In response to COVID-19, countries may consider nationalizing their supply chains for greater control as to avoid reliance on another country that will significantly affect exports. Yet global trade feeds one-third of the world, and producing locally means buying less and the need for more land. COVID-19 may cause a contraction in the extension of the supply chain, and countries will trade with who they can trust. Question of relative advantage, will countries afford to produce things they aren't familiar with or can do, or will this be a necessity arising from potential supply chain shortage issues – for example, reprocessing Personal and Protection Equipment (Rowan and Laffey, 2020)? There is likely to be an increased demand for ICT, including areas such as robotics, blockchain, algorithms to improve processes, efficiency, and sustain or create more jobs. Industries will need to adapt in real-time – which is challenging, given very little market data available to underpin critical decisions.

9. Strategic funding initiatives to identify and accelerate DTs and relevance to COVID-19 – a case study from the Republic of Ireland.

COVID-19 has been hailed as the "big equalizer," but the reality is that we are not equally resilient as a society. It will fuel the next wave of innovators, both for economic and social impact. Given the importance of transitioning beyond COVID-19, there is likely to be increased interest internationally on providing strategic funding initiatives that merge academia and industry to identify the next disruptive technology. There is a shortage of strategic initiatives that specifically focus on DTs. Still, Enterprise-Ireland has been quick to embrace this need with its Disruptive Technology Innovation Fund (DTIF) that launched in 2018. This DTIF initiative has committed €500 m to identify and supporting DFs Project Ireland 2040 (https://dbei.gov.ie/DTIF) that will run over the period 2018 to 2027 aligned with enterprise co-funding. Relevant questions and responses are available to view on this host website. The nature of the 43 DTIF awards will also have relevance to tackling COVID-19 pressure points (Table 4). DTIF Fund is aligned with the Irish Government's Future Jobs Ireland framework with a focus on 'Embracing Innovation and Technological Change,' where there is an emphasis on creating and advancing technology disruption on a commercial footing. It is envisaged that pursuit of these strategic domains, and harnessing the potential of DTIFS emerging from these cross-cutting areas will also support national economic recovery plan for COVID-19 pandemic.

The DTIF funding initiative in Ireland is resourced to €65 m up to 2022 for projects across many thematic domains encompassing emergent preferences for advancing medical devices, ICT, artificial intelligence, blockchain, robotics, nutraceuticals, therapeutics, manufacturing and environmental and so forth. A more in-depth analysis of all funded project awards over the 2018 and 2019 period highlights the potential benefit of addressing COVID-19 often through cross-cutting contributions from these previously considered separate domains of innovation (Table 3). Analysis of the data provided shows that increased funding for this vital initiative in the priority areas Innovations in Services and Business Processing (1 project, €3.9 m (2.7%)); Food (3 projects from DTIF 2, €5.2 m (3.6%)); Energy, Climate Action and Sustainability (6 projects, €8.3 m (5.6%); Manufacturing and Materials – Advanced and Additive Manufacturing (3 projects, €8.7 m (6.0%); ICT (11 projects,€31.1 m (21.6%)), and Health and Wellbeing including Medical Devices, Diagnostics, and Therapeutics (21 projects at €86.8 m (60.3%) (Figs. 3 and 4). These domains reflect the Republic of Ireland's priority strategic areas of research and innovation to 2023. Fig. 3 describes the number of DTIF project awards in the various fields, including food for this Republic of Ireland government initiative since its' launch in 2018. However, it is appreciated that distribution of funding award to date reflects in part the presence of global Medtech and ICT industries in Ireland, in addition to the crucial partnership with leading academic institutions, Science Foundation Ireland-funded Research Centres and Enterprise-Ireland Technology Gateways that all support MNC, SMEs, startups, and entrepreneurs in a closely-knit innovation ecosystem.

The strong performance of medical technologies and ICT (Table 3) reflects in part the significant presence of global multinationals in this space connected to benchmarking universities and Institutes of Technologies across Ireland. Primary production in Ireland is represented by agriculture, fisheries, and forestry (includes food, drink, and horticulture), which pre-COVID-19 accounted for 10% of total exports worth €13bn reaching 180 markets worldwide. Approximately 80% of Ireland's agricultural land is devoted to grasslands, which makes it highly suitable for food production (Bord Bia, 2020). The innovation ecosystem is such that many of the leading food companies such as Diageo, Kerry Group, Glanbia, and so forth also are key collaborating partners in national research and innovation centers, enterprise-technology gateways, and benchmarking academic institutions that are focused sustaining and food disruption. For example, the Enterprise-Ireland funded Technology Gateways in Athlone

Table 4
COVID-19 (Coronavirus) – list of successful projects and case studies funded in the Republic of Ireland.

Topic of Research	Funding (€)
Irish Coronavirus Sequencing Consortium	378,716
NSPIRE: Facilitating Non-Invasive Ventilation (NIV) during the COVID-19 Crisis	205,667
Critical Reagent Production Addressing Supply Chain Risk for COVID-19 Diagnostics	540,263
Data Platform for Emergency Response Management (DPERM)	402,323
Prognostic indicators of critically ill patients with COVID-19: Impact on early immunology and survival	192,566
SABS-TILDA: SARS-CoV-2 specific AntiBodieS in The Irish LongituDinal Study on Aging (TILDA): an opportunity to assess COVID-19 rates and phenotypes in older adults in Ireland	199,875
Improving long-term patient recovery and reducing disability after COVID-19 critical illness using microRNA-based approaches	199,621
Defining the disease course and immune profile of COVID-19 in the immunosuppressed patient (DeCOmPRESS study)	199,908
Irish COVID-19 Vasculopathy Study (iCVS)	199,036
Screening for antiviral compounds active against SARS-CoV-2	190,036
COVID19: The North Dublin Cohort Study	190,237
Using a back-calculation model to estimate the scale of asymptomatic Covid-19 prevalence by age and determine the critical threshold of available susceptible persons within the community	199,098
Using telehealth to enhance management of vulnerable groups during the Covid-19 pandemic	96,340
Impact of Covid 19 on Individuals with Intellectual and Developmental Disabilities and Caregivers	199,965
Cupid Covid-19: paediatric emergency department attendance during covid-19	62,857
A Rapid Resource Repository for Health Professionals (RRR-HP): An online and social media individualised support intervention for return to practice, reassigned and new to practice, nursing and allied	107,015
Rapid response and learning for later: establishing high quality information networks and evaluation frameworks for the National Ambulance Service response to COVID-19	109,188
Expediting the diagnosis of COVID-19 in a clinical setting using AI enabled analysis of CT scans	62,858
Creating an evidence-based toolbox for targeted public health interventions during COVID-19: a cross-border analysis to disentangle psychological, behavioural, media and governmental responses	107,015
An investigation of psychological responses to COVID-19 in health care workers during the delay and mitigation phase of disease management: longitudinal and nested qualitative study	83,690
Altered lives in a time of crisis: Preparing for recovery from the impact of the COVID-19 pandemic on the lives of older adults	175,013
Autism specific Transition RESources: a response to the COVID-19 related restrictions (TRes)	123,370
Optimising Covid-19 social distancing communications: Identifying and addressing psychosocial determinants of social distancing during the Covid-19 pandemic	148,745
Covid-19: Estimating the burden of symptomatic disease in the community and the impact of public health measures on physical, mental and social wellbeing	199,945
Identifying mental health needs and best practice for psychological support in frontline healthcare workers during and after the COVID-19 outbreak and in future pandemics	187,254
Modelling Real Time Population Wide Impacts of COVID-19	38,310

** For further details see https://www.gov.ie/en/publication/1b4099-covid-19-list-of-successful-projects-and-case-studies/

Table 5
DTIF Projects funded by Irish Government between 2018 and 2019 – putative relationship with 2nd Order disruption for Food and COVID-19.

Title of DTIF Project and Value of Award	Priority Area of Award	Potential cross-link to Food (potential 2nd Order Disruptive Technology)	Potential COVID-19 Use
Disruptive Gene Therapy Platform, Replacing Viruses in the Treatment of Genetic Conditions [€8.4 m]	Health & Wellbeing	Not obvious, as yet	Not obvious, as yet
HOLISTICS - Holistic Human Sensing for Health, Aging and Wellness [€7.4 m]	Health & Wellbeing	Training, as Smart Wearables Industry Value - human-centric intelligent sensors and their wireless communications for products.	Yes, remote training and monitor cocooning
AuriGen Solution for Persistent Atrial Fibrillation [€5.9 m]	Health & Wellbeing	Not obvious, as yet	Not obvious, as yet
'Future of Colorectal Cancer Diagnosis and Treatment: Combining Tissue Responsive Probes, AI and Machine Learning for Medical Care [€5.7 m]	Health & Wellbeing	Not obvious, as yet	Yes, use of AI and Machine Learning
Therapeutic enzymes as a treatment for sepsis and other immune disorder diseases [€5 m]	Health & Wellbeing	Cytoflow5, had potential for informing new innovation in Food – such as nutraceuticals	Yes, potentially
Towards safe and effective off the shelf cellular therapy for cancer [€4.3 m]	Health and wellbeing	Not obvious.	Not obvious as yet
Photonics Manufacturing Pilot Line€4.1 m	ICT- Manufacturing	Pilot Line Hub will develop packaging designs tailored to fast cost-effective packaging processes and equipment and develop and next gen packaging equipment (including test) with reduced cycle-times.	Yes, smart packaging relevant for COVID-19
Microfluidic Gene Transfection Cell Analysis and Sorting Platform (€3.4 m]	Health & Wellbeing	Not obvious	Not obvious, as yet
Cooperative Energy Trading System (CENTS)	ICT	Consumers and communities will be empowered with the necessary infrastructure to generate their own electricity for artisan food production with lower carbon footprint	Yes, by stimulus to COVID-19 recovery for Micro and Small Enterprises
Nex	ICT – Internet of Things	Not obvious	Potentially
ARDENT II [€2.8 m]	Health & Wellbeing	Not obvious.	Potentially
Medical Imaging Ireland €2 m]	Health & Wellbeing	Assess impact on new nutraceuticals for lung health (Masterson et al., 2019).	Yes, lung health
ArtEngine 2.0 bridging automated, AI-Driven 3D World Creation to Market [€2 m]	ICT – AI/AR/VR	Food 3D printing using AI as tool creation of 3D models – cost of 3D content creation is prohibitive for small studios and enables co-development and adoption of AR/VR.	Yes, in Vit D supplementing products and virtual modalities
BioHealx [€1.9]	Health and Wellbeing	Not obvious	Not obvious
Sustainable Bio-Renewable Energy from Wastewater (S-BREW) for the Food & Drink wastewater sector that will reduce land-spread waste and produce high-quality renewable energy [€1.8 m]	Energy, Climate Action and Sustainability	Food waste reprocessing	Yes – economic recovery
E-BAMBI - Enhanced Biocompatibility of Additively Manufactured Biomedical Implants for Improved Clinical Outcomes [€1.9 m]	Health & Wellbeing – Medical Devices	Not obvious – but 3D printing focused	Yes, potentially
High throughput microfluidic drug screening platform [€1.9 m]	Health & Wellbeing – Diagnostics/Therapeutics	Response models for drug testing – may have cross-link to nutraceuticals (GRAS)	Yes, potentially
Future Software Systems Architectures [€1.6 m]	ICT – IoT, AI,	Future use to rapidly operationalise new software systems that are slow – with AI	Possibly, via socio-economic recovery
Irish Lasers for the Internet of the Future (iLife) [€1.6 m]	ICT – Future Networks	Not obvious, as yet	Possibly, in socio – economic recovery
Connected Medical Device Cybersecurity Transparency €1.5 m]	ICT – AI, Data Analytics - Blockchain	Possible use of AI, data analytics, blockchain for real-time platform for 2-way communication of safety-critical security information (vulnerability) across food chain	Yes, in supply chain such as PPE distribution and security
Creating Bionic Man – neural training suit for semimotor impairments[€1.5 m]	Health & Wellbeing	Not obvious	Not obvious
Advanced Environmental Decision Support System for Coastal Areas [€1.1 m]	Energy, Climate Action & Sustainability	Not obvious	Not obvious
Smart-Cardio – a paradigm shift in Cardiac Arrhythmia treatment [€1.1 m]	Health & Wellbeing – Medical Devices	Not obvious	Possibly
DEFINE- AM – Disruptive finishing using electrochemical machining for additive manufacturing €1 m]	Manufacturing & Materials	Future link to food for challenges of post- processing 3D-printed metallic parts	Not obvious
Blockchain in Technology Product Supply Chain [€1 m]	ICT - Blockchain	Food technology product supply chain	Yes, in supply chain
Developing inhaled bioengineered exosome therapeutics [€9.4 m]	Health and Wellbeing	Delivery of smart nutraceuticals via tailored aerosol delivery technology	Yes, in lung delivery of therapeutics
Qunatum computing – a software platform for multiple Qubit technologies [€7.3 m]	ICT	Possible role in financial services and logistics supporting food industry	Yes, in stimulus support to industry
Point-of-care iron stores/Ferritin testing for at risk blood donors [€7 m]	Health and Wellbeing	Not obvious	Not obvious
Data-center audio/visual intelligence on-device [€6.9 m]	ICT	Possible role between in lab and field work for audio and vision-data on devices	Yes
Pharam Latch – [€4.4]	Health & Wellbeing	Not obvious	Yes, possibly
Stroke-CIS [€4 m]	Health & Wellbeing	Not obvious	Yes, possibly
Blockchain and AI-Enabled Stratified Trial System [€3.9 m]	Innovations in Service, Business Processes	Food security – ensuring complete (GDPR) trustworthy, control and ownership of data	Yes, in supply chain and security
FreeSpace Project [€3.6 m]	ICT	Wireless connectivity with ultra-high capacity wireless laser communication technology for broad food industry – delivers combination of bandwidth, availability and distance	Yes, in communications, distribution networks and economic recovery

Table 5 (*continued*)

Title of DTIF Project and Value of Award	Priority Area of Award	Potential cross-link to Food (potential 2nd Order Disruptive Technology)	Potential COVID-19 Use
Transfer print technology for heterogeneous integration of components [€3.6 m]	Manufacturing and Materials	Possibly in food packaging	Yes
EyeVU	Health & Wellbeing	Not obvious	Not obvious
Next generation heat pump for affordable decarbonisation of heating [€2.4 m]	Energy, Climate Action, Sustainability	Possible role in food distribution and storage as zero carbon-emission, refrigerant-free, heat pump	Yes, in efficiency of food supply and security
Haemodialysis Outcomes & Patient empowerment [€2.1 m]	ICT	Possible role of AI enable software and wearable device for chronic diseases	Yes, via AI informed disease mitigation
Connected Enteral feeding healthcare system [€2 m]	Health & Wellbeing	New innovative feed delivery device design, connective and apps	Yes
TRANSPIRE – a trained AI platform for regulation [€2 m]	ICT	Combines human and AI to demystify laws and regulations making it easier to do business while protecting consumers	Yes, in innovations in business services as post disrupter
Video Intelligent Search Platform (VISP) [€1.5 m]	ICT	Not obvious	Yes, in socio-economic recovery
Optimised commercial-scale cultivation of protein-rich biomass from *Palmaria palmata* for the generation of health enhancing plant based proteinaceous ingredients.	Food	Plant-based proteinaceous ingredients as source of high quality protein and contribute to meeting the growing global demand for plant-based proteinaceous ingredients for animal and human food	Yes, economic recovery and health promoting
Beyond Food Labelling	Food	Using massively multiplexed Next Generation Sequencing to provide a crypto-anchor for food authentication and as a substitute for costly, error prone labelling and certification systems	Yes, economic recovery
HYDRO-fish: Combining targeted nutraceuticals and traceability technology for a smarter and sustainable Irish fish aquaculture industry	Food	The project entails reinforcing the supply chain of Irish salmon production	Yes, economic recovery

Institute of Technology supports ca. 300 projects per year with different startups, SMEs, MNCs nationally with cross-cutting links to the agri-food sector, including smart packaging, 3D printing, and nutraceuticals in terms of intelligent delivery systems. Drivers for informing future technology disruption in the agri-food domain will be influenced by needs arising from COVID-19 pandemic along with balancing environmental concerns for more eco-sustainable, climate-friendly products and services.

If one conducts a more in-depth review of the Irelands 43 DTIF projects, it becomes apparent that potentially 20 (46%) at a combined award value of €86.8 m (60.2%) have cross-cutting abilities to cause 2nd order disruption in the food domain (Fig. 3). This would include disruptive training using wearables via wireless communication, use of cytoflow5 for exploiting benefits of nutraceuticals, 3D printing of food, disruptive feed delivery, and future use of nutraceuticals for lung health using aerosol delivery under Health and Wellbeing domain. Disruption in the area of food security could be potentially achieved through the sole Innovative Services and Business Processes project (Table 3). Besides, disruption may be likely made in food distribution and storage through the Energy, Climate Action, and Sustainability project for reveals the use of a new zero-carbon emission, refrigerator-free, heat pump. Science Foundation Ireland has supported a Rapid COVID-19 Response Initiative that funded 26 of 350 applications to address health service readiness, medical countermeasures along with social and policy countermeasures with potential for disruption (Table 5). There is also good thematic agreement with recent Green Deal selected startup and SMEs for COVID-19 interventions (European Innovation Council, 2020).

10. Potential technology, product and business service disruptors in food beyond COVID-19 crisis

In recent times, there has been increasing interest in the augmented use of microorganisms, such as yeast, microalgae, and bacteria, in the form of protein sources. Microorganisms are commonly used in fermented products that we are very familiar with, such as yogurts and sauerkraut. This practice offers a more efficient, innovative approaches to producing the same proteins that we are already familiar with (Medical Expo, 2020). The demand for such alternative food ingredients is pushed by Millennials and Generation X, with changes in eating habits along with corresponding changes in personalized nutrition. From the disruption of introducing Greek yogurt to the emergence of new functional foods such as seaweeds (Mohamed et al., 2012), there has been increasing interest in food ingredients. Such things have informed a trend towards personalized nutrition. Considerable development in this space has been the recent partnership of Nestlé with Corbion. This combines exciting expertise of Corbion's microalgae innovation with Nestlé fermentation abilities that is renowned for its smart plant-based products. Other examples include Impossible Foods, who are making soy heme (typically found in soy plants) for plant-based burgers through microbial fermentation. Impossible food burger is made with soy leghemoglobin that mimics the taste of meat. Such innovations in food ingredients may also complement growing consumer demand for eco-sustainable food sources, which also reflects changing eating habits, diets, and the new role of personalized nutrition. Startup-Insight (2020) noted laboratory-cultured meat might provide an alternative or complementary source to actual meat where the latter requires approximately seven tons of water to produce 450 g of beef. Interestingly, the price for producing around 140 g of artificial meat dropped to €9,59 Euro in 2017 from a non-affordable initial costing base of €274.366. A useful trend to follow for disruption in food production and services is to monitor activities in the United States as more than a third of the world's top food and drink processing companies are headquartered there including Unilever, Danone, Diageo, Kirin, SABMiller, Cadbury Schweppes, Heineken and Asahi Breweries.

High protein feed for animal and human usage will prove relevant, which has been exemplified by an intensive focus on this product for intensive aquaculture production globally. Aquaculture is rapidly developing worldwide and highlights one of the fastest growth areas for the food industry (Fečkaninová et al., 2017; Liu et al., 2017; Tahar et al., 2018a; Tahar et al., 2018b; O'Neill et al., 2019). Aquacultures' pace and scale of expansion reflect a substantial increase in our worldwide population, and the commensurate demand for more safe, nutritious food (Seoane et al., 2014). Fish stocks are depleting on the oceans, and there is a countermeasure push to develop sustainable aquaculture processes to enhance disruption. Hatch-Blue is an example of accelerator SME focused on investing and progressing entrepreneurs to fast-track potential disruptive technologies for the fisheries, seafood, and aquaculture sector

(https://www.hatch.blue/) globally. Precisely, hatch-blue constitutes the first accelerator program for sustainable aquaculture that seeks out, develops, and nurtures Start-ups for disruptive innovation.

11. Conclusion

If one takes a long lens and examines how societies responded to previous catastrophic Black Swan events, (such as the Black Death the devastated the Byzantine Empire, the Cholera outbreak of the 1830s, the Spanish Flu of 1918–1920), World War II (1939–1945), such traumas unleased tremendous changes in thinking, literature, and culture, inspiration, creativity, and ambition will prevail over austerity and fuel the discovery of next-generation disruptive innovations beyond this COVID-19 pandemic. Enhanced innovation leading to the creation of new disruptive technologies in the agri-food domain will inform new exciting new products and services that will address challenges and opportunities for the intensive sustainability of the industry. Modern-day and future disruptive technologies for the agri-food sector will be influenced by the growing demand to produce more safe, nutritious foods to meet growing populations that reflects dynamic changes in eating habits such as personalized nutrition, alternative protein sources, and attitudes towards climate change and digitization. The Black Swan and anti-fragile influences, such as shock to the economy by the occurrence of COVID-19 pandemic, will create both challenges and new opportunities that include emergent or disruptive innovations in service and business processes such as home delivery. A review of the recent 43 projects funded by the Irish government under Science Foundation Ireland's Disruptive Technology Initiatives was used to highlight trends in the innovation ecosystem and the potential for both cross-cutting and future ground-breaking disruption in the agri-food, health, ICT, manufacturing and circular economy sectors with a global orientation. This trend is also reflected in the first 64 'Green Deal' selected start-ups and SME projects recently funded by the European Innovation Projects, which will help support transitioning beyond this COVID-19 pandemic and for future pandemics. Understanding where potential food technology disruptions are likely to occur will be aided by having a holistic perspective and appreciation of the complex socio-technological innovation ecosystem.

Authors' contributions

NJR and CMG conceptualized the manuscript. NJR drafted the manuscript. Both authors read, edited, and approved the final manuscript.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no competing or conflict of interests.

Acknowledgments

The authors acknowledge Pixabay for the use of a free black swan image in the graphic abstract. The authors acknowledge funding support from the Irish Health Research Board [HRB-2014-644-POR] and from H2020 MSCA RISE [ICHTHYS].

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Science of the Total Environment 866 (2023) 161455

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Challenges and future opportunities to unlock the critical supply chain of personal and protective equipment (PPE) encompassing decontamination and reuse under emergency use authorization (EUA) conditions during the COVID-19 pandemic: Through a reflective circularity and sustainability lens

Neil J. Rowan *

Department of Nursing and Healthcare, Technological University of the Shannon Midlands Midwest, Ireland
Centre for Disinfection and Sterilization, Technological University of the Shannon Midlands Midwest, Ireland
School of Medicine, Nursing and Health Sciences, University of Galway, Ireland
CURAM SFI Research Centre for Medical Devices, University of Galway, Ireland

HIGHLIGHTS

- COVID-19 pandemic caused disruption in supply chains to vital PPE
- Lack of knowledge to inform key policies and appropriate disease mitigation strategies globally
- Decontamination technologies may enable safe treatment of PPE waste for recycling and long term sustainability
- Co-circulating COVID-19 variants of concern will continue to challenge our healthcare system
- Emerging opportunities to meet harmonized pandemic responses, circular bioeconomy and green innovations

GRAPHICAL ABSTRACT

ARTICLE INFO

Editor: Damia Barcelo

Keywords:
Sustainability
PPE reuse
Medical device supply chain
COVID-19 SARS-CoV-2
Sterilization decontamination
Circularity

ABSTRACT

Severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2), and the resulting coronavirus disease (COVID-19), was declared a public health emergency of global concern by the World Health Organization (WHO) in the early months of 2020. There was a marked lack of knowledge to inform national pandemic response plans encompassing appropriate disease mitigation and preparation strategies to constrain and manage COVID-19. For example, the top 16 "most cited" papers published at the start of the pandemic on core knowledge gaps collectively constitute a staggering 29,393 citations. Albeit complex, appropriate decontamination modalities have been reported and developed for safe reuse of personal and protective equipment (PPE) under emergency use authorization (EUA) where critical supply chain shortages occur for healthcare workers (HCWs) caused by the COVID-19 pandemic. Commensurately, these similar methods may provide solutions for the safe decontamination of enormous volumes of PPE waste promoting opportunities in the circular bioeconomy that will also protect our environment, habitats and natural capital. The co-circulation of the highly transmissive mix of COVID-19 variants of concern (VoC) will continue to challenge our embattled healthcare systems globally for many years to come with an emphasis placed on maintaining effective disease mitigation strategies. This viewpoint article addresses the rationale and key developments in this important area since the onset of the COVID-19 pandemic and provides an insight into a variety of potential opportunities to unlock the long-term sustainability of single-use medical devices, including waste management.

* Department of Nursing and Healthcare, Technological University of the Shannon Midlands Midwest, Ireland.
E-mail address: nrowan@ait.ie.

http://dx.doi.org/10.1016/j.scitotenv.2023.161455
Received 23 October 2022; Received in revised form 2 January 2023; Accepted 3 January 2023
Available online 6 January 2023

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1. Introduction

Severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2), and the resulting coronavirus disease (COVID-19), was declared a public health emergency of global concern by the World Health Organization (WHO) in the early months of 2020 (Sohrabi et al., 2020). The occurrence of COVID-19 took the world by surprise (Ahmed et al., 2020; WHO, 2020). In addition to significant loss of life and morbidity, COVID-19 disrupted social daily norms that also impacted world trade (White et al., 2021). COVID-19 is also seen as a rare "black swan" event where there is no playbook to advise on best-practice solutions from prior lessons learnt, including the availability of appropriate countermeasures (Rowan and Galanakis, 2020). The aim of this viewpoint article is to critique the main developments affecting the personal and protective equipment (PPE) supply chain during the COVID-19 pandemic along with embracing emerging trends and opportunities. For example, deficiencies in our ability to maintain a supply chain of single-use PPE for frontline healthcare workers (HCWs) was underappreciated; yet, emphasis is shifting away from the potential reuse of critical PPE under conditions of extreme shortages (emergency use authorization, [EUA]) (US FDA, 2020; US FDA, 2020b) towards potentially co-creating safe decontamination and bioeconomy opportunities for recycling enormous volumes of PPE waste to protect our environment (Rubio-Romero et al., 2020; Alt et al., 2022).

A hallmark of the COVID-19 onset, in early months of 2020, was a tremendous lack of knowledge and understanding surrounding SARS-CoV-2, where the emphasis was placed on constraining and managing the spread of infection as there was no available vaccine to safeguard HCWs and citizens alike (Rowan and Laffey, 2020a; Rowan and Laffey, 2020b). Moreover, there was a marked gap in published information that addressed key needs to inform interventions and policies including viral pathogenesis, persistence, transmissiveness and efficacy of available disease prevention, control and waste management methods (Wu et al., 2020). There was a vast chasm in knowledge where the world scrambled to obtain and openly share new information in real-time, which would unlock appropriate solutions (Table 1). For example, at the time of writing, the top 16 "most cited" papers that were published in leading journals on core gaps and potential COVID-19 solutions collectively constitute a staggering 29,393 citations (Google Scholar) (Table 1). Having conducted a systematic literature review using Scopus and PubMed databases, the appearance of the key word "PPE" in journal publications increased by 71.4 % and 73.9 % respectively during the COVID-19 pandemic (Table 2). Over 7000 journal papers were published using key words "COVID-19" and "PPE". The trend showed substantial research and enterprise activities in the areas of "decontamination", "reuse" and "sterilization" generating 526 notable publications since the start of the COVID-19 pandemic (2020 to 2022 reporting period), where similar related studies had not been reported previously (Table 2). Of 516 papers of interest from the combined databases, 89 were included in this review to highlight key developments and emerging trends. The final set of complete information for this paper, represented in both Tables 1 and 2, was assembled and cross-referenced on the same day (19 October 2022). The purpose of this review is not to exhaustively digest and present copious data on the efficacy or shortcomings influencing appropriate intervention strategies (as there are many narrative and systematic reviews published on this topic cited here); but, it is to reflectively consider and articulate key findings that underpinned cues to action informing policies along with discussing commensurate opportunities that will potentially shape future needs for society, economy and our environment.

2. Observations from the early phase of COVID-19 pandemic

The World Health Organization (WHO) recommended that countries worldwide draw up a "Pandemic Plan" that would achieve clear results in managing pandemics from the early stages (Fusco et al., 2020). Each emergency in healthcare is characterized by different phases: mitigation, preparation, response and recovery (Public Health Emergency, 2012). National overarching plans provided scenarios that benefit from communication and cooperation between different multi-actors and sectors, such as healthcare management, HCWs, logistics, communication, finance and so forth (Public Health Management, 2012). In the absence of a vaccine, emphasis was placed on containing and managing the spread of SARS-CoV-2 outbreaks in the mitigation phase through the deliberate deployment of non-pharmaceutical interventions (NPIs) that addressed various modes of transmission, including contaminated contact surface and infectious aerosols (Charkaborty and Maity, 2020; Rowan and Moral, 2021). NPIs included exercising social distancing, where the WHO recommended wearing single-use N95s or FFP2 standard (or equivalent) facepiece respirators (FFRs) (WHO, 2020). A simplified holistic diagram highlighting the role of NPIs (including PPE) in breaking the chain of SARS-CoV-2 infection is illustrated in Fig. 1. Preventative and disease-controlling factors are typically focused on disrupting linkages between the infectious agent, susceptible host, mode of transmission, reservoir, mode of entry and mode of exit. Face shields and N95s or FFP2s and medical-grade face masks were also used by the general public; however, these were typically replaced by the use of improvised fabric face masks for public transport and in community settings as the months progressed and when COVID-19 vaccines were introduced (de Man et al., 2020). To protect frontline HCWs and to limit transmission, the WHO and US officials recommended wearing 100 to 300 million FFRS per month with the US having only 1 % of this in its supply chain (Lovelace, 2020).

When in sufficient supply, FFRs provide safe work environments for HCWs (Rowan and Moral, 2021). However, most countries experienced critical shortages of FFRs; therefore, it was essential to expedite alternative approaches to meet supply chain deficiencies where the US Food and Drug Administration (FDA) provided guidance for obtaining EUA to market

Table 1
Examples of "most cited" influential publications addressing critical new challenges and solutions for meeting PPE supply chain, disease mitigation and waste management during early stages of COVID-19 pandemic in 2020.

Citations	Topic	Paper title	Author(s)
10,553	Viral persistence	Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1	Van Doremalen et al. (2020)
5691	COVID-19 review	World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19)	Sohrabi et al. (2020).
4477	Biocide efficacy	Persistence of coronaviruses on inanimate surfaces and their inactivation with biocidal agents	Kampf et al. (2020)
2284	Genome sequencing	Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China	Wu et al. (2020)
1542	Environment, Prevention	COVID-19 outbreak: Migration, effects on society, global environment and prevention	Charkaborty and Maity (2020)
1276	Wastewater surveillance	First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community	Ahmed et al. (2020)
946	Air pollution & COVID-19	Association between short-term exposure to air pollution + COVID-19 infection: Evidence from China	Zhu et al. (2020)
924	Lockdown, air quality	Effect of lockdown amid COVID-19 pandemic on air quality of the megacity Delhi, India	Mahato et al., 2020
384	Disinfection + Supply Chain + solutions	Challenges and solutions for addressing critical shortage of supply chain for personal and protective equipment (PPE) arising from COVID19 pandemic – Case study from the Republic of Ireland	Rowan and Laffey (2020a)
375	Hospital waste & wastewater	Disinfection technology of hospital wastes and wastewater: Suggestions for disinfection strategy during COVID-19 pandemic in China	Wang et al. (2020)
221	Disinfecting, Sterilizing face masks	Disposable masks: Disinfection and sterilization for reuse, and non-certified manufacturing, in the face of shortages during the COVID-19 pandemic	Rubio-Romero et al. (2020).
206	PPE reuse extended use	PPE and intensive care unit healthcare worker safety in the COVID-19 era (PPE-SAFE): an international survey. Journal of Critical Care.	Tabah et al. (2020)
175	COVID-19 Interventions	An environmental and health perspective for COVID-19outbreak: Meteorology and air quality influence, sewage epidemiology indicator, hospitals disinfection, drug therapies and recommendations.	Barcelo (2022)
148	PPP waste management	Environmentally Sustainable Management of Used Personal Protective Equipment	Singh et al. (2020)
107	Face coverings, reuse, waste management	Unlocking the surge in demand for personal and protective equipment (PPE) and improvised face coverings arising from COVID-19 – Implications for efficacy, re-use and sustainable waste management	Rowan and Laffey (2020b)
84	Sterilizing face masks	Sterilization of disposable face masks via standardized dry/steam sterilization processes; an alternative in fight against mask shortages	de Man et al. (2020).

devices (equipment) for the decontamination of N95s during this period of critical use (US Food and Drug Administration, 2020). For example, Tabah et al. (2020) received 2711 responses from a worldwide survey of 1797 (67 %) physicians, 744 (27 %) nurses, and 170 (6 %) allied HCWs on the availability and use of PPE caring for COVID-19 patients in intensive care units (ICU) where at least one piece of standard PPE was unavailable for 1402 (52 %), and 817 (30 %) reported reusing single use PPE, PPE was worn for a median of 4 h. Adverse effects of PPE were associated with longer shift durations and included heat (1265, 51 %), thirst (1174, 47 %), pressure areas (1088, 44 %), headaches (686, 28 %), inability to use the bathroom (661, 27 %), and extreme exhaustion (4992, 20 %). Battista et al. (2021) also reported adverse reactions in HCWs to PPE wearing during COVID-19. Alt et al. (2022) noted that sessional use, re-use, or use of alternative PPE to that of FFRs are the limited options that face HCWs during these periods of critical shortages. The US Centres for Disease Control and Prevention also published recommendations for extended use or limited re-use, (including decontamination), as potential mitigation approaches to offset known shortages of N95s. Mtetwa et al. (2021) reported on the effective decontamination of gowns during COVID-19.

Most countries experienced several "lockdowns" in order to contain and manage COVID-19 infection where the virus evolved into different variants of concern (VoC) through the process of viral mutational change enabled by incubation in large populations of infected people including asymptomatic carriers, over the pandemic period (Rowan et al., 2021). In the early waves of COVID-19, SARS-CoV-2 was reported to exhibit a higher transmission rate (Jiang and Shi, 2020), but a lower mortality rate (2–3 %) (Shi et al., 2020) to that of outbreaks caused previously by other coronavirus (such as 40 % for Middle East respiratory syndrome) (Shi et al., 2020). Due to its enveloped morphology, the SARS-CoV-2 virus has been shown to exhibit relatively low resistance to front-line disinfection, which became its "Achilles heel". Initial emphasis was placed on trying to protect the supply chain of PPE, particularly for HCWs, where there was a focus on keeping the R_0

Table 2
Systematic Review of Scopus and PubMed databases for journal publications containing key words on personal protective equipment (PPE) use, reuse and decontamination under EUA conditions during COVID-19 pandemic over period 2010 to 2022.

Scopus			PubMed		
Key word(s) used	Encompassing COVID-19 pandemic [2010 to 2022]	Prior to COVID-19 pandemic [2010 to 2019]	Key word(s) used	Encompassing COVID-19 pandemic [2010 to 2022]	Prior to COVID-19 pandemic [2010 to 2019]
PPE	20,831	5943	PPE	46,554	12,164
PPE + COVID-19	7594	0	PPE + COVID-19	7081	0
PPE + COVID-19 + Sterilization	211	0	PPE + COVID-19 + Sterilization	388	0
PPE + COVID-19 + Decontamination	102	0	PPE + COVID-19 + Decontamination	290	0
PPE + SARS-COV-2	1500	0	PPE + SARS-COV-2	6125	0
PPE + Disinfection + SARS-CoV-2	128	0	PPE + Disinfection + SARS-CoV-2	498	0
Decontamination + N95	232	3	Decontamination + N95	243	17
PPE + Reuse	150	5	PPE + Reuse	169	8

Fig. 1. Role of key interventions strategies in breaking the chain of COVID-19 infection.

value below 1 in order to limit the number of SARS-COV-2 infected cases requiring hospital care (particularly for vulnerable patients that may need ventilator assistance in intensive care unit ICU) (Rowan and Moral, 2021).

A hallmark of the initial months of COVID-19 was the introduction of creative means of ensuring the continuity of a vital PPE supply chain including 3-D printing of face shields, bespoke production of Starmed Hoods for point-of-use in ICU (Rowan and Laffey, 2020b), use of blockchain to manage and distribute PPE to essential HCWs (Fusco et al., 2020; Nandi et al., 2021), and reuse and decontamination of single use PPE where severe shortages occurred due to this pandemic situation (Rowan and Moral, 2021). For example, blockchain has been increasingly applied to inform risk in healthcare management, as a strategic tool to strengthen efficient and effective evidence-based decision making. Fusco et al. (2020) reported on the benefits of using blockchain combined with artificial intelligence systems to affect a trace route for COVID-19 safe clinical practice. Smart use of blockchain allows for the creation of a generalizable predictive system that could contribute to the containment and management of pandemic risk nationally (Fusco et al., 2020). At the outset of COVID-19, Rowan and Laffey (2020b) reported on the trial use of bleach (sodium hypochlorite at ≤4000 ppm), along with a counter water immersion phase to remove residuals, for testing the disinfection performance of Starmed Hoods for ICU. This point-of-use chemical disinfection strategy was not put into practice due to operator concerns over the lingering unpleasant chlorine odor; also, Viscusi et al. (2009) measured the filtration performance of two FFR models submerged into a range of sodium hypochlorite solutions (0.525 %–5.25 % sodium hypochlorite) and noted some degradation in filtration performance, but not below acceptable levels.

In Ireland, as well as many countries internationally, responses and communication to the general public was coordinated by the government through the Department of Health where there was reliance on contributions from subject matter experts across various fields ranging from medical device manufacturing to clinicians/epidemiologists that were connected via a dedicated mobile WhatsApp group. This holistic approach helped to inform a rapid and flexible response to shaping new effective policies, including appropriate disease mitigation strategies. This unified approach was a stand-out abiding "response" feature from this shared COVID-19

pandemic experience as it hurdled conventional silo-based scenarios through holistic engagement where key individuals were joined by a common purpose to solve a societal challenge. This essentially unlocked the collective intellectual and creative wealth of multi-actor stakeholders to inform the government including generating and openly sharing vital information surrounding COVID-19 which informed effective guidelines and policies to constrain and manage the spread of the SARS-CoV-2 virus. Most countries had a limited number of critical-use ventilators and emphasis was also placed on the bespoke production of key equipment and safe reuse. Logistically, this was challenging given that all countries were also similarly affected by the same supply chain shortage issues for PPE; this was supported through the fast-tracking research funding nationally, and internationally from a bottom-up perspective. Addressing the safe reprocessing of vital single-use PPE for reuse applications was a complex and underappreciated task. PPE is manufactured, tested, verified and validated under very strict conditions that include an appropriate terminal sterilization step, which informs authorization by regulators, such as the FDA. As PPE items consist of varying materials where many are heat sensitive, there is a commensurate need to match this need with the use of appropriate and effective decontamination and sterilization modalities. For example, the choice of a decontamination method for the reuse of PPE must also negate any dysfunctionality post-treatment, such as retaining filtration efficacy (for FFRs), comfort fit, material compatibility and safety. Original equipment manufacturers (OEMs) of medical devices (including PPE) had not envisaged, possibly ever, that there would be a requirement to reuse PPE where vital supply was disrupted due to the pandemic. OEMs willingness to share knowledge and to provide expert advice contributed to unlocking this challenge where EUA was granted by regulators for PPE reuse using limited treatment technologies (example, VH2O2 used alone or with Ozone). At the outset of the COVID-19 pandemic, there was a gap in evidence-based publications on the persistence and survival of Coronavirus on contact surfaces (example, Kampf et al., 2020 that has reached 4477 citations) (Table 1). Based on tacit and subject matter expert knowledge of working closely with disinfection and sterilization modalities over many decades, a paper was framed on candidate appropriate technologies that could potentially address safe decontamination of PPE under EUA conditions (Rowan and Laffey, 2020a [386 citations]). The timing of this published paper also coincided with EUA US FDA on alternative strategies for PPE reuse (Rowan and Laffey, 2020b; Alt et al., 2022). Interestingly, the duration to write, review, accept and appear online in the journal in open access format was five days, which reflected the urgency in responding to critical COVID-19 knowledge gaps. At the then time of writing (3 April 2020) this paper, the global number of confirmed COVID-19 cases had reached 1,000,249 with 51,515 deaths which markedly contrasts with current epidemiology data recording 625,359,756 cases and 6,569,533 deaths (19 October 2022). Candidate technologies proposed in April 2020 for safe PPE decontamination, such as the use of vaporized hydrogen peroxide (VH2O2), mild heating and UV irradiation, remain the most popular methods used today. The potential use of VH2O2 for PPE decontamination was informed by the timely systematic review paper of McEvoy and Rowan (2019), which comprised a SWOT analysis on the use of VH2O2 compared with other technologies for terminal sterilization applications, such as physical (gamma-, x-ray, electron-beam) and gaseous (ethylene oxide) methods. This McEvoy and Rowan (2019) paper was written to provide valuable information in order to advance the field of terminal sterilization for established medical device manufacture and safe use, and not for PPE reuse.

It was also appreciated that Coronavirus was a large enveloped virus; therefore, it was likely to be susceptible to the lethal action of frontline disinfectants. Thus, moderate to high-level disinfection processes (6 $\log_{10}$ reduction in mycobacterial cells, but not bacterial endospores) could be theoretically applied for the safe reprocessing of PPE during COVID-19 without the need for full 12 D reduction in bacterial spores that is allowed for in a sterilization processes. A D value is the time taken to reduce a population of microorganisms by 1 $\log_{10}$ order using a concentration of disinfectant or steriliant (Rowan, 2019). During the early phase of COVID-19, the US FDA and US officials authorized similar decontamination modalities to address pressing PPE supply chain deficiencies under EUA for the reprocessing of PPE (encompassing Umbrella EUA for surgical masks, N95 and other respirator EUA and face shields and other barrier EUAs) during this pandemic that was restricted to a limited number of modalities, such as the use of VH2O2 (US FDA, 2022). At no time was it deemed appropriate to use physical radiation modalities (x-ray, gamma and electron-beam) or ethylene oxide gas for the reprocessing of contaminated PPE during COVID-19. Recent publications in this area corroborated this approach where Haedi et al. (2021) reported the decreased functionality of FFRs after gamma irradiation treatments. US FDA (2020) advised that "once availability of PPE (including facemasks) returns to normal (non-EUA conditions), healthcare facilities should promptly resume conventional practices".

There was a commensurate surge in interest in the use of different types of decontamination methods focused on addressing infectious aerosols and contaminated surfaces including PPE applications with some success. Viana Martins et al. (2022) reviewed 1229 studies from two databases of which only 16 studies reported on methods to "recondition PPE". However, there was frequent variance in the type and level of challenge microorganism(s) used where some researchers used SARS-VoC-2 for PPE decontamination. However, the majority of these in vitro studies did not encompass PPE functionality and material compatibility testing as part of the reported decontamination methods. Various other sophisticated decontamination methods were applied for PPE decontamination with reported successes, including the use of VH2O2 plasma vapor, ionized VHO2 (Cramer et al., 2021), plasma-generated ozone and reactive oxygen species (ROS) (Huang et al., 2022), supercritical CO_2 (Bennet et al., 2021), photoactivated methylene blue (Floreine et al., 2022). For example, plasma-ROS treated PPE (N95s) maintained acceptable mechanical and functionality properties for point-of-use decontamination applications (Huang et al., 2022). Lendvay et al. (2022) reported on the successful decontamination of N95s artificially inoculated with 2 SARS-CoV-2 viruses using novel methylene blue photochemical treatments where FFR integrity was maintained after five processing cycles. The same authors noted that one FFR model failed after five cycles using FDA-authorized VH2O2 plus ozone decontamination method that was included as a comparative control. McAvoy et al. (2021) reported on the successful use of 3D-printed frames to enable reuse, extended-use and improved fit of N95 and KN95 respirators. However, Chen et al. (2019) reported that 3D-printed polymers are less stable than injection molding fabrication when exposure to terminal sterilization by VH2O2 and electron beam technologies.

There was a concerted focus on testing less complicated, more available and affordable disinfection approaches for PPE reuse. For example, Côrtes et al. (2021) evaluated the re-use of 45 surgical masks and 69 respirators by analyzing their performance and safety before and after decontamination using oven, thermal drying, autoclave, and hydrogen peroxide plasma vapor. In addition, 14 used respirators were analyzed after work shifts before and after decontamination using reverse transcription polymerase chain reaction (RT-qPCR) and viral culturing. Oven decontamination (75 °C for 45 min) exhibited the simplest decontamination method that maintained acceptable physical and filtrations performance of treated masks and respirators for at least five processing cycles. Reprocessed respirators used in work shifts (hospital settings) were evaluated positively by users in terms of functionality and comfort, even after three decontamination cycles. Alt et al. (2022) noted that "appropriate decontamination technologies for PPE reuse under EUA FDA conditions must meet the following criteria: (1) include screening and replacement processes for these FFRs not suitable for reuse; (2) effectively inactivate SARS-CoV-2 or representative virus and other relevant bioburden on the FFR; (3) be compatible with the FFRs to avoid rendering it dysfunctional; (4) be available and practical for healthcare settings; and (5) minimize risk to operators of the decontamination equipment and end-users alike".

Clustering of decontamination and sterilization activities and services for PPE reuse under EUA pandemic conditions was centered around the use of regional hospitals, such as in Ireland that was coordinated by the Health Service Executive (HSE) in partnership with Science Foundation Ireland (SFI), academic institutions, industry and with established terminal

sterilization companies. A limited number of published studies had used actual Coronavirus strains as a bioindicator for establishing decontamination efficacy and sterility assurance levels of treated PPE during the COVID-19 pandemic. It is appreciated that the reprocessing and sterilization industries are a bastion of rigor that communicates closely with regulators to ensure compliance with standards in order to safely decontaminate and sterilize products. Thus, several non-thermal candidate laboratories pilot or commercially-available technologies used for food processing (such as gas plasma, high pressure, pulsed electric fields, photonics and so forth (Gómez-López et al., 2021)) that would not be deemed appropriate for PPE reprocessing for point-of-use healthcare applications until due to the nature of their biocidal action, material compatibility and lack of data to support regulator approval. Despite the establishment of PPE reuse methods in healthcare facilities in Ireland, there was a lack of interest in their use by HCWs. Emphasis was always placed on ensuring a continuous supply chain of new PPE items during the COVID-19 pandemic. Absent was the establishment of independent testing laboratories nationally for verification and validation of reprocessed PPE for in-house healthcare applications during COVID-19. However, a new European pandemic response hub, managed by the University of Galway, Ireland, now coordinates and supports research and enterprise in this area including provision for mitigating against future pandemics.

3. Developments during the COVID-19 pandemic

Through the passage of time, new combinational approaches were adopted to inform disease prevention and control including the introduction of effective COVID-19 vaccines (Table 3). Examples of interesting activities and innovations include the modeling of COVID-19 infection data and positive case occurrences (Zeroual et al., 2020; IHME COVID-19 Forecasting team, 2021; Rowan and Moral, 2021), and the use of machine learning and blockchain to review data from a supply chain perspective. For example, Haug et al. (2020) combined statistical modeling and machine learning techniques and estimated that wearing a mask yields a reduction of R_0 between 1.8 % and 12 %, while social distancing contributed to a reduction of approximately 20 %. Genomic, next-generation sequencing and bioinformatic breakthroughs also provided key information on countermeasures. Longitudinal modeling studies yielded new information on the efficacy of COVID-19 mitigation strategies and surveillance of transmission rates in various susceptible populations internationally, where evaluating related data over short timeframes provides limited value (Rowan and Moral, 2021). Efficacy of front-line biocides must also consider the appropriateness for meeting the emerging SARS-CoV-2 variants of concern that differed in transmissiveness and pathogenesis. There was a holistic sharing of key findings by multi-stakeholders for solutions that included the use of the Quintuple Helix Hub approach (combination of academia-industry-healthcare-government-society) to support research, development and innovation linked to education. Interestingly, there was a commensurate push for the digital transformation of adjacent industries including additive manufacturing and food systems (Rowan et al., 2022), which can potentially improve efficiencies in terms of monitoring, surveillance, sustainability, and automation. Currently, there are 706 established or new European Digital Innovation Hubs (EDIHs) where there are opportunities to align some of these appropriate EDIHs for meeting pandemic disease response, innovation, education and training (Table 3) (Rowan et al., 2022). These could also support new SMEs, start-ups, and entrepreneurs, particularly with a focus on transitioning to sustainable solutions, which may also inform new businesses and possibly, future technology disruption (Rowan and Casey, 2021). Opportunities to advance pandemic disease preparedness and responses could also be further supported through bespoke specialist training using immersive and educational technologies.

4. Impact of SARS-CoV-2 'variants of concern' of PPE usage – bracing for a winter COVID-19 surge

PPE encompassing face masks (medical grade, FFP2, N95s) remain an important intervention strategy to constrain and manage SARS-CoV-2. Despite the availability of effective vaccines and antiviral drugs such as

Table 3
Examples of new issues, activities and solutions arising for meeting PPE supply chain shortage, disease mitigation and waste management during COVID-19 pandemic.

Citations	Topic	Paper title	Author(s)
752	Symptoms (smell, taste)	Anosmia and Ageusia: Common Findings in COVID-19 Patients	Vaira et al. (2020)
326	Infection modeling	Modeling COVID-19 scenarios for the United States	IHME COVID-19 Forecasting Team
301	Diagnostics	COVID-19 diagnostics and context	Wesssleder et al. (2020)
226	Wastewater surveillance	Wastewater surveillance for population-wide Covid-19: The present and future	Daughton (2020)
212	Blockchain in supply chain bioeconomy	Redesigning Supply Chains using Blockchain-Enabled Circular Economy and COVID-19 Experiences	Nandi et al. (2021)
181	Energy & Environ footprint	The energy and environmental footprints of COVID-19 fighting measures – PPE, disinfection, supply chains	Klemeš et al. (2020)
177	Quadruple interactive HUBs	Unlocking challenges and opportunities presented by COVID-19 pandemic for cross-cutting disruption in agri-food and green deal innovations: Quo Vadis?	Rowan and Galanakis (2020)
171	Multi- actor approach	COVID-19: A Call for Physical Scientists and Engineers	Huang et al. (2020)
116	Environmental pollution	Occurrence of personal protective equipment (PPE) associated with the COVID-19 pandemic along the coast of Lima, Peru	De la Torre et al. (2021)
112	Open research datasets	COVID-19 Data Hub	Guidotti and Adria (2020)
87	3 D printed PPE	3-D Printed Protective Equipment during COVID-19 Pandemic.	Wesemann et al. (2020)
79	Blockchain in Healthcare	Blockchain in Healthcare: Insights on COVID-19	Fusco et al. (2020)
70	Sustainable PPE	COVID-19 Creating another problem? Sustainable solution for PPE disposal through LCA approach	Kumar et al., 2021
62	PPE pyrolysis waste to energy	Current plastics pollution threats due to COVID-19 and its possible mitigation techniques: a waste-to-energy conversion via Pyrolysis.	Aragaw and Mekonnen (2021)
53	Modeling of Non- pharmaceutical interventions (NPIs)	Disposable face masks and reusable face coverings as non-pharmaceutical interventions (NPIs) to prevent transmission of SARS-CoV-2 variants: Role of new sustainable NPI design innovations and predictive mathematical modeling	Rowan and Moral (2021)
33	Alternative sanitizing for Masks	Photocatalytic Rejuvenation Enabled Self-Sanitizing, Reusable, and Biodegradable Masks against COVID-19	Li et al. (2021)
30	Biodegradable Multifunctional Surgical Face masks	Biodegradable and multifunctional surgical face masks: A brief review on demands during COVID-19 pandemic, recent developments, and future perspectives	Badaahmadi et al. (2021)
21	Sustainable PPE – materials and recycling	Key ingredients and recycling strategy of personal protective equipment (PPE): Towards sustainable solution for the COVID-19 like pandemics.	Singh Siwal et al. (2021)
15	Machine learning – case mortality	Identifying mortality factors from Machine Learning using Shapley values – a case of COVID19	Smith and Alvarez, 2021

Paxlovid, COVID-19 cases caused by a mix of new VoC remain a worry given that these collectively represent one in three new SARS-CoV-2 infections in the US (Wang et al., 2022). COVID-19 cases are rising in Europe and the UK, where these VoC have taken hold (Goodman, 2022). The Omicron subvariant BA.5 causes most COVID-19 infections in the US (Smith-Schoenwalder, 2022), accounting for 88 % of new infections. Those with weakened immunity will be particularly vulnerable (Rhee et al., 2022), where recent research intimates that the last laboratory-created antibodies do not provide adequate protection against these VoC (Cao et al., 2022). These VoC descend from slightly different branches of the Omicron lineage, and this "convergent evolution" infers that several VoC can be co-circulating in our population at the same time as we enter winter. With a reduced emphasis on wearing face masks in many countries, there will be a co-challenge to meet potential surges in both COVID-19, influenza and possibly Norovirus (winter vomiting) that will place added pressure on our healthcare system and our HCWs. Interestingly, adherence to NPIs practices generally reduced the incidence of influenza and Norovirus cases over the past two winters. Real-time assessment of the large mix in VoC is becoming challenging as countries reduce surveillance (WHO, 2022). However, <10 % of the US population (14.8 million people) have received an updated COVID-19 bivalent booster vaccine, which is of concern. Thus, the use of PPE, particularly for frontline HCWs remains important; and therefore, the commensurate need to address effective sustainable clinical waste management.

Published work intimates that VoC would be similarly sensitive to disinfection and PPE as preventive measures (Rowan et al., 2021). Meister et al. (2021) reported disinfection effectiveness against SARS-CoV-2 VoC B.1.1.7 and B.1.351 using heat, soap and ethanol where treatment was carried out using a variety of artificially-seeded surfaces including face masks. While society now has effective disease mitigation strategies, we cannot become complacent which includes continued investments in vaccines and antibody therapies. Of concern is societal fatigue towards the use of NPIs (Michie et al., 2020), particularly when one considers that we will be living with COVID-19 for many years ahead. Rhee et al. (2022) surveyed COVID-19 infection control policies at 30 leading US academic hospitals in the context of the initial pandemic surge of the SARS-CoV-2 omicron variant and found that infection control practices vary substantially. The authors recommended clearer public health guidance and transparency around hospital policies that also aligns with meeting VoC responses, in order to facilitate consistent and harmonized standards.

5. Surge of PPE use and impact on waste management and our environment

The US healthcare facilities generate an estimated 1 million tons of non-infectious plastic waste every year where there are opportunities to improve recycling (Healthcare Plastics Recycling Council, 2019). Creative means of monitoring and surveillance of COVID-19 pandemic have been applied including reporting the occurrence of this virus in municipal waste that would reflect community-level transmission rates (Daughton, 2020; Barceló, 2020; Ahmed et al., 2020) (Table 1). There is also a pressing need to address appropriate clinical waste management for used PPE (Rubio-Romero et al., 2020; Rowan and Laffey, 2020b). Given the sure in single-use PPE that contains plastic globally (De la Torre et al., 2021) there is commensurate interest in effective and alternative approaches to clinical waste management. For example, Zhao et al. (2022) describe a novel pyrolysis process for used PPE that enables energy recovery through a detailed life cycle assessment approach and includes sustainability. The authors point to the environmental advantages of reducing 35.42 % of total greenhouse gas emissions from the conventional incineration and 43.50 % of total fossil fuel use from landfill processing, the optimal number, sizes, and locations of established facilities within PPE processing system in New York State where one integrated fast pyrolysis facility is used in Rockland County.

There is increasing evidence of PPE accumulation in our environment including the marine that has accelerated interest in identifying workable solutions. Waste PPE was have been reported globally that contaminates our natural capital and habitats (De la Torre et al., 2021). Consequently, there is a surge in interest in the development of alternative biodegradable materials to replace plastics in PPE including considering waste-to-energy conversion (Badaahmadi et al., 2021). It is estimated that some 44 million non-woven PPE items are used by frontline HCWs every day, resulting in some 15,000 tons of waste that are destined for landfills or incineration. Alt et al. (2022) noted that while many materials in PPE are recyclable, SARS-CoV-2 contamination significantly influences such disposal strategies, and this coupled with the natural resource consumption during manufacture may impact our natural environment. Alt et al. (2022) also evaluated and validated technologies suitable for the decontamination and re-use of contaminated N95 FFRs in response to the COVID-19 pandemic. Multiple low-temperature steam (65 to 71 °C) and vaporized hydrogen peroxide technologies were shown to be successful that inactivated feline calcivirus (FCV, $>3\ \log_{10}$) and *Mycobacterium* species ($\geq 6\ \log_{10}$) (employed as representatives on the contamination challenge) without affecting the performance of the treated PPE. This work was reported to be suitable for 10 to 20 decontamination cycles of the same PPE. This is potentially a "game-changer" as the deployment of commercial VH2O2 processes could decontaminate large amounts of PPE making it safe for circular bioeconomy applications and long-term sustainability, including reducing PPE waste destined for landfill or incineration (Aragaw and Mekonnen, 2021). Several studies have corroborated the effective use of VH2O2 technologies to decontaminate N95 face masks (Jatta et al., 2021; Deer et al., 2022). Alt et al. (2022) also reported that there are additional challenges to be considered for the reuse of PPE that includes user acceptance, traceability and stock management. Doos et al. (2022) and Kea et al. (2021) reported that the reuse of non-decontaminated PPE worn by HCWs is not an appropriate practice.

Industrial VH2O2 is a strong candidate technology for sustainable PPE waste management as it meets scalability, penetration, and compatibility with materials including innovations in sterilization chamber design and process development (McEvoy and Rowan, 2019). Not all sterilization technologies would be deemed appropriate for large-scale decontamination of PPE waste. For example, the challenges of using ethylene oxide (EO) relate to the hazardous and carcinogenic nature of the gas combined with prolonged treatment times (McEvoy and Rowan, 2019; McEvoy et al., 2021). While radiation is a relatively rapid process leaving no unwanted toxic residues, it is limited by the availability of the radiation source (cobalt), in the case of gamma and not applicable (McEvoy and Rowan, 2019). Emerging non-thermal technologies, such as cold gas plasma (Hayes et al., 2013; Qin et al., 2022), have the potential for novel point-of-use decontamination of clinical waste before transport to landfill or incineration; thus, offering new opportunities for reducing, reusing and recycling. Qin et al. (2022) reported that cold gas plasma (CAP) destroyed six major epidemic strains of SARS-CoV-2 variants of concern within 300 s, where this CAP method affects the SARS-CoV-2 spike protein rather than damaging viral RNA through an oxidative reaction. The presence of soiling on surfaces must always be considered from a cleaning perspective as the presence of organic matter can affect disinfection efficacy on contaminated surfaces including medical devices (Rowan et al., 2021).

6. Addressing future circularity and sustainability of single-use PPE

There has been a marked surge in research interest focusing on the future sustainability of medical devices, particularly, the role of using reprocessing versus single-use plastic items. The sustainability of medical devices (particularly, single-use PPE) has intensified by meeting supply chain shortage issues arising from the COVID-19 pandemic that has placed increased emphasis on reuse (where appropriate), and clinical waste management. Popular circularity and sustainability topics and activities have been captured in Table 4. In addition, use of life cycle assessment (LCA) tools to address all end-to-end stages for medical devices from design thinking to authorized commercial use will help inform resource consumption, carbon footprint, supply chain and transport energy efficiency,

Table 4
Examples of new and related research and innovation informing the future circularity and sustainability of medical devices (including PPE) arising a consequence of COVID-19 occurrence.

Topic(s)	Description of research and innovation activities	Reference
Environmental Impact Assessment LCA assessment	➢ 16 different environmental impact categories highlighting superior use of reprocessed devices over single use in 13 categories with focus on electrophysiology catheter. Also informed by LCA.	Schulte et al. (2021)
Circularity assessment	➢ Healthcare could cut emissions by half for some devices if opting for regulated, reprocessed "single-use" items	
Resource Consumption & Emissions; New job creation	➢ Avoiding use of virgin materials, remanufacturing alternators can environmental impacts of resource consumption, emissions [reducing abiotic resource use and the Global Warming impact (GWI)] – Uses LCA.	Peters (2016) Zhang et al. (2020) D'Adamo and Rosa (2016)
Use of VH2O2 for PPE waste management	➢ Development and validation of technologies for decontamination and reuse of contaminated N95 filtering facepiece respirators with provision for future sustainable waste management	Alt et al. (2022).
Rapid manufacturing; 3D printing; Biocompatability	➢ Consider material composition, toxicological end-points and improvements in compatibility testing given future sustainability and reuse opportunities for medtech	Antonini et al. (2021) Hayes et al. (2013)
Recycling challenges	➢ Recycling of complex medical device products needs extensive material flow analysis to make it economically and ecologically reasonable	Gopinath et al., 2020
	➢ The more complex a device, the more process steps, energy, and resources are needed for recycling	D'Adamo and Rosa (2016) Eze et al. (2020)
	➢ Complex devices may have specific requirements for collection and disposal after use from an infection-prevention perspective and may contain complex materials not suited to municipal waste management & recycling infrastructure (including Green technology)	Lee et al. (2017)
CE concept using R-strategies	➢ *Design smarter* [Refuse, Rethink, Reduce] ➢ *Extend lifetime* [Reuse, Repair, Refurbish, Remanufacture, Repurpose] ➢ *Circularly end-point activities*: Recycle and Recover	Potting et al. (2017)
Reuse of Polyvinyl Chloride (PVC)	➢ Recycle PVC several times (most widely used plastic in medical devices) without loss of critical properties	I'Ons (2021)
Design thinking	➢ Design medical devices smarter for circularity that includes appropriateness for reprocessing and sterilization ➢ Design from day one with disassembly and cleaning in mind, including reducing number of device components	I'Ons (2021)
Improve energy efficiency	➢ Improving energy efficiency and opting for clean energy source could reduce overall costs ➢ Additive manufacturing can reduce material waste by as much as 90 % compared to conventional manufacturing and can speed up device prototyping and testing	I'Ons (2021)
Logistical and design challenge	➢ Consider device materials in sustainable design ➢ For example, disposable surgical drapes contain polypropylene and polyethylene, each can be recycled, but used together they cannot be recycled.	La Plante (2022)
Reduce carbon footprint	➢ Recycled materials can be used without loss of technical properties or need for addition of virgin materials ➢ For example, recycled polyethylene terephthalate (PET) has 79 % lower GHG emissions that virgin PET.	La Plante (2022)
Multi-actor collaborations	➢ Work with Medical Device component and OEM manufacturers to advance circular medical device production, and to move away from traditional "take-make-waste" linear systems.	La Plante (2022) Rowan and Laffey (2020b)
AI, automation and use of novel diagnostic technologies	➢ Consider automating processes including use of artificial processes to potentially reduce resources and disinfection along with future sustainability production and waste management ➢ Role of novel diagnostic techniques in monitoring reprocessing and sterilization of devices	McEvoy et al. (2021)
Role of Supply Chain and Transport	➢ What steps are used to reduce energy, water and chemical use ➢ Reduce transport of waste from used medical devices by recycling in same region to reduce carbon footprint	La Plante (2022)
Research translation, Education, Advocacy	➢ Surveyed 'state-of-the-art' environmental sustainability research in anaesthesia and critical care where avoid, reduce, reuse, recycle and reprocess addressed ➢ Moving beyond clinical care, energy (renewables vs fossil fuel) and energy efficiency are important influencers in healthcare's ecological footprint.	McGain et al. (2020).

profitability, automation for future circularity and sustainability. Lessons can also be learnt from adjacent industries on the combined use of other sustainability tools that has influenced innovation in packaging (Ruiz-Salmón et al., 2020; Almeida et al., 2021;). There is enhanced interest among researchers in the incorporation of smart bioactive materials into medical devices that exhibit virocidal and bacteriocidal properties, which could potentially be used in the design and development of more sustainable innovations (Masterson et al., 2021); however, the appropriateness of these new materials to tolerate established high-level disinfection or sterilization processes would need to be incorporated into new product evaluation and validation including maintaining the functionality of new design innovations during testing or reuse.

Addressing such challenges is likely to have potential broader disease preventive benefits including addressing antimicrobial resistant bacterial and fungal pathogens (AMRs) that are at a crisis point globally (Garvey et al., 2022). Masterson et al. (2021) reported, for the first time, the development of a low-temperature extrusion process for the production of GRAS bioactive-polymer loaded compounds for targeting such AMR bacteria that may also be suitable for decontamination processes. There is significant scope to develop appropriate sustainability tools for evaluating the potential impact of applied decontamination technologies on the environment (Hayes et al., 2013) including elucidating cellular mechanisms of inactivation (Farrell et al., 2013). The use of life cycle assessment (LCA) confirmed that more sustainable strategies for the disposal of PPE waste are needed (Kumar et al., 2021). However, published studies have also highlighted

that data generated by medical-device-focused researchers would benefit from having an understanding of decontamination and sterilization technologies in their method (Rowan and Moral, 2021). This knowledge gap can be addressed by delivering specialist training on medical device reprocessing and terminal sterilization to academia and other stakeholders including SMEs, start-ups and entrepreneurs; thus, co-creating solutions in the adjacent circular bioeconomy or digital technologies domains (Table 3). The latter can be enabled and accelerated through sustainable business models that will de-risk investments in innovations (Rowan and Galanakis, 2020).

7. Emerging opportunities and challenges for PPE supply chain

Table 5 describes 24 observations from tackling critical PPE supply chain challenges and other adjacent COVID-19 activities and will inform the ongoing and future pandemic response plans with opportunities for promoting innovation and sustainability that will potentially benefit society, the economy and the environment. There is an emergence of opportunities to consider such as automation using robotics in medicine and healthcare including transforming reprocessing of complex medical devices (such as endoscopes) in healthcare (Allescher et al., 2022). This includes keeping

Table 5
New observations and lessons learnt from tackling critical PPE supply chain challenges during COVID-19 pandemic.

No.	Topic	Reference
1.	Efficacy in forging **multi-actor contributions to techno-, socio-economic** and environmental challenges for **real time solutions** in innovation and policies underpinning pandemic plan led by Government with subject-matter experts	Rowan and Laffey (2020a)
2.	**Global terminal sterilization technologies** have potential to scale and treat large volumes of PPE waste during pandemic for future recyclable options	Alt et al., 2022
3.	Importance of open **real-time communication** (selecting appropriate channels, messaging, timing) and **Open-Access dissemination** for sharing and reflecting upon break-through solutions (ex. COVID-19 tracker phone apps)	Zhang et al, 2022
4.	Greater need for **education, training and awareness** of the role of PPE supply chain, infection microbiology, disinfection/sterilization modalities, sustainability and circular bioeconomy in universities with industry	Rowan and Moral, (2021)
5.	**Co-creation of regional clusters** and convergence of **multi-actor innovation hubs** nationally for pandemic response plans	University of Galway (2022)
6.	Interest in **sustainable management of PPE waste**	Singh et al. (2020)
7.	**Reliance on front-line HCWs** to deliver on critical disease prevention and control service that can be prolonged and **highly stressful** where there is limited understanding on **mental health** and wellbeing including long-term wearing of medical face masks and possible use of reprocessed PPE	Tabah et al. (2020) White et al. (2021) Kea et al. (2021)
8.	Repeat waves of COVID-19 infections introduces **societal fatigue** in terms of adherence to mandatory lockdowns and restricted social movement; imposing future lockdowns will challenge society further with present-day economic pressure caused by increased cost of living, energy/commodity crisis, inflation and potential emergence of a global economic recession.	Michie et al. (2020) Rowan and Moral (2021)
9.	Reliance on single-use-plastic based PPE in healthcare globally can have a dramatic and underestimated impact on **environmental pollution** during a pandemic with urgent need to identify alternative biodegradable polymers in PPE design and appropriate clinical waste management	Kumar et al. (2021) Rowan and Galanakis (2020)
10.	SARS-CoV-2 virus mutated and changed to variants of concern that is likely to persist for many years akin to influenza, where there is requirement for regular monitoring to confirm efficacy of vaccines and boosters. Also, despite genetic adaptive changes to virion genome, **VoC are similarly sensitive to front-line non-pharmaceutical interventions (masks)** and to non-specific **chemical disinfectants and physical treatments**. Serendipitously, society was fortunate that this is a complex enveloped virus rendering it less likely to persist for long time on surfaces and readily disinfected	Rowan et al. (2021) Rhee et al. (2022) Meister et al. (2021)
11.	Creativity and ingenuity of society in **improvising and problem solving** ranging from improvised wearing of fabric-based face mask to determining indicative levels of virus in communities by monitoring of municipal wastewater, to tracking, modeling and reporting on COVID-19 globally in real-time dashboard managed by Johns Hopkins University	Rowan and Laffey (2020b). Rowan and Moral (2021)
12.	Unlike deadly Spanish flue of 1918 where rate of international transmission was comparatively slow due to absence of commercial air travel; COVID-19 **rapidly spread globally**, but society had means of disease counters measures.	Sohrabi et al. (2020).
13.	OEMs of medical devices (PPE) and contract sterilization pivoted to supporting and providing solutions for what are typically single use devices	Alt et al., 2022
14.	**Regulators** and healthcare authorities **acted rapidly** to develop appropriate pandemic plan including EUA for PPE reuse such as point-of-use in healthcare	US FDA 2020 WHO, 2020
15.	Despite suite of physical and gaseous sterilization modalities, and greater appreciation of efficacy of emerging non-thermal technologies (such as use in food industry), reprocessing of PPE for point-of-use in healthcare under EUA is likely is restricted to use of a limited number of validated modalities until such time that other modalities are appropriately developed, tested, commercially scaled and meet regulatory need with matching ISO standards	Gómez-López et al. (2021) Floreine et al. (2022) Lendvay et al. (2022)
16.	**Fast tracking funding** in research and enterprise to develop solutions, which reduces red-tape and barriers to innovation and open knowledge sharing has been seen as impactful and beneficial. Such as 3 D printing of medical devices, and bespoke production of replacement Starmed hoods for ICU.	McAvoy et al. (2021) Told et al. (2022) Rowan and Laffey (2020b)
17.	Many developed small countries including Ireland could afford maintaining single use PPE supply chains through procurement, and benefited from early freely available vaccines – however, there is a greater need in society to **share and expedite knowledge, innovation and solutions** with **developing countries** that are equally addressing co-morbidities in terms of HIV and TB	United Nations (2021)
18.	Reliance on mainstay PCR in healthcare **as sustaining innovation**; but, developments in digital technologies (real time monitoring, models, immersive technologies, blockchain) along within in situ 3D printable sterilized devices show promise as **disruptive innovation** in pandemic plan	Rowan and Moral (2021) Huang et al. (2020)
19.	Due to adopting effective face mask wearing and use of other non-pharmaceutical interventions (NPIs), there was a **marked reduction or absence** in the occurrence of influenza and winter vomiting Norovirus that challenge healthcare during winter seasons – their reemergence (including RSV) will place **added pressure on embattled healthcare system** that are already **playing catchup** from a resource and HCW fatigue perspective. It is likely that combined COVID-19 + Flu + RSV will present significant challenges.	Speare-Cole (2021) Agha and Avner (2021)
20.	**Wearing of face masks** in society (out with typically healthcare) became the **social norm** and will be used in **healthcare for the foreseeable future**	Rowan and Moral (2021)
21.	Only in the passage of time, such as through **reflection on longitudinal mixed-methods studies** including modeling will true effectiveness of interventions be understood for **harmonizing global pandemic response plan**	Rowan and Moral, 2021
21.	**Strong community engagement** including bespoke 3 D printing of face shields in local schools	Wesemann et al. (2020)
22.	Emergence of opportunities **for digital technologies** and use of machine learning during COVID-19 pandemic	Allescher et al. (2022) Taylor (2022)
23.	Surveillence of COVID-19 by use of **sewage as epidemiological indicator**	Barceló (2020)
24.	VoCs adopt and co-circulate in society, but **use of disinfectants are effective**.	Meister et al. (2021) Rhee et al. (2022)

Abbreviations: Variants of Concern (VoC); Non-pharmaceutical Interventions (NPIs); vaporized hydrogen peroxide (VH202); Emergency Use Authorization (EUA); Healthcare workers (HCWs).

pace with sustaining and disruptive practices in surgery for healthcare applications. Leading sterilization companies in partnership with universities are also developing and deploying state-of-the-art biotechnology tools such as flow-cytometry to unlock real-time microbial inactivation where such approaches may supplement conventional plate counts (McEvoy et al., 2021). Immersive (digital) technologies are also partnering with medtech companies for complex virtual training on specific technical operations. For example, Mersus Technology (Immersive) has partnered with Boston Scientific to test and apply an "Avatar Academy Program that uses computer gaming to recreate virtual laboratories and cleanrooms, allowing medtech employees to familiarize themselves remotely with a complex work environment and processes. This approach will potentially automate training where one could theoretically run six bespoke training sessions in one day that previously would have taken a month, and can be extrapolated to address the full production chain delivered in a virtual environment" (Westmeath Independent, 2020). There is a pressing interest in also ensuring the safe reprocessing of reusable medical devices in healthcare applications that are challenged by the presence of recalcitrant biofilms harboring pathogenic microorganisms where failure to effectively clean, reprocess and sterilize may lead to significant patient risk.

HCWs have been in the constant face of COVID-19 for over two years where anxiety and stress can lead to mental health issues (Peteet, 2020); thus, highlighting the importance of maintaining effective disease prevention and control measures (Vaira et al., 2020). Peteet (2020) reported growing concerns about anxiety with COVID-19 that have led to recommendations for effective staff care, and greater availability of mental health treatment. This researcher has also noted existential concerns raised by the pandemic suggesting the importance of religious resources, as seen in research into patients dealing with advanced cancer. Many Asian countries introduced tougher COVID-19 restrictions compared to other countries which caused elevated levels of stress, anxiety and isolation. A-Singapore-based "Intellect" company recently raised $20 m to develop and manage a mobile phone app that regularly checks on users' moods with connectivity to exercise and recovery sessions and to therapists for real-time interventions (Cheung and Ripley, 2022).

8. Future recommendations, research and enterprise opportunities

- Identify and invest in appropriate scalable decontamination technologies that can effectively treat large volumes of used PPE during the COVID-19 pandemic with a view to recovery, reusing and recycling that will drive a long-term sustainable waste management system.
- Harmonize pandemic response plans (disease mitigation, preparation, response and recovery phases) including infrastructure, living labs etc. that facilitate opportunities to test, verify and validate point-of-use decontamination technologies for medical devices (including 3D printing) in healthcare, which also makes provision for ongoing and future pandemics where PPE (FFRs) may require reuse under EUA conditions.
- Promoter greater engagement of OEMS, sterilization companies with academic institutions, new businesses, policymakers and civil society, such as through Quintuple Helix Hub framework to inform new green solutions, such as the expansion of Rowan and Casey (2021). An example is the efficacy of new medical devices comprising biodegradable materials from a life cycle assessment and 360 degree holistic thinking perspective that can be decontaminated without loss of functionality or biocompatibility.
- Develop and apply risk models and pathways for new businesses to help investment in entrepreneurial activities that include increased funding support for new sustainable green innovations.
- Develop further, more rapid methods in microbiology to meet the need for real-time monitoring, sustainability and diversification including "Industry 5.0" human-centric models (Rowan et al., 2022).
- Greater interface between broad users (including academia, SMEs, MNCs) and policymakers/regulators to understand policies and standards for developing innovations and processes. MNCs in medtech and connected sterilization already very effectively communicate regulators at important policy interface.
- Increased education and training for greater engagement with multi actors including social sciences, humanities and communities for feedback on pandemic response and innovations.
- Promotion of open access publications and pandemic data hubs (such as Guidotti and Adria, 2020).
- Development of regional spatial and economic strategies to support the co-creation and development of innovations for the medtech sector in partnership with academia that also addresses circular bioeconomy opportunities, and digital transformation.
- Increased multi-disciplinary role of blending Science, Technology, Engineering and Mathematics (STEM) with Arts, Humanities and Social Sciences (AHSS), and industry for solutions.
- Greater research and provision of appropriate tailored support and intervention services for HCWs suffering from stress, anxiety and mental health as a result of meeting this pandemic response for society.
- Reflect to ensure effective communication and dissemination channels, messaging and timing to inform all stakeholders in society.
- Develop appropriate sustainability tools to assist SMEs, start-ups and entrepreneurs in the creation of a new medical device that addresses the life cycle from discovery to validation and addresses technology, society and policy readiness levels. This will de-risk investment and create local employment and regional development.
- Nurture and train a talent pool of high-caliber researchers for these and future applications.
- Resource regional pandemic response hubs that address a critical supply chain for PPE linked to education and outreach, which also includes digital transformation and sustainability along with disease prevention and control.
- Effective clinical waste management balanced with environmental protection and natural capital.
- Develop and introduce immersive technologies (digital) to address important practices in disease prevention and control from a training and education perspective.
- Digital transformation of PPE supply chain from EUA perspective such as using blockchain and AI.

Consent for publication

Not applicable.

CRediT authorship contribution statement

The author solely designed and wrote this paper.

Data availability

Data will be made available on request.

Declaration of competing interest

The author declares no conflict of interest.

Acknowledgement

The author acknowledges the assistance of Gillian Kean (Momentum Consulting, Ireland) in the preparation of graphics used in this review article. The author acknowledges funding support under Phase 2 Development of CÚRAM SFI-Research Centre for Medical Devices (University of Galway) in Ireland.

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Appendix 1.

Full list of publications for Neil J Rowan that includes papers that were not used in this higher DSc submission (period covers from 1996). Red indicates publications cited below that were not included as full-length papers in thesis [44 of 142 papers (**31% unused**)].

Noting, papers included in this DSc are representative of my leading independent research contributions to field of study. It is not practical to include full length versions for all my published work in this thesis.

Section 1: Food Safety Microbiology	
1	Rowan, N.J., Anderson, J.G. and Anderton, A. (1997). Bacteriological Quality of Infant Milk Formulae Examined under a Variety of Preparation and Storage Conditions. *Journal of Food Protection*, **60** (1), 1089-1094.
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3	Rowan, N.J. and Anderson, J.G. (1998). Growth and enterotoxin production by *diarrhoeagenic Bacillus cereus* in dietary supplements prepared for hospitalized HIV patients. *Journal of Hospital Infection*, **38** (2), 139-146.
4	Rowan, N.J. and Anderson, J.G. (1997). Maltodextrin stimulates growth of *Bacillus cereus* and synthesis of diarrheal enterotoxin in infant milk formulae. Applied and Environmental Microbiology, 63 (3), https://doi.org/10.1128/aem.63.3.1182-1184.1997
5	Rowan, N.J. and Anderson, J.G. (1998). Diarrhoeal enterotoxin production by psychrotrophic *Bacillus cereus* present in reconstituted milk-based infant formulae (MIF). *Letters in Applied Microbiology*, **26** (2), 161-165).
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11	Rowan, N.J., Candlish, A.A.,G., Bubert, A., Andreson, J.G., Kramer, K. and McLauchlin, J. (2000). Virulent Rough Filaments of *Listeria monocytogenes* from Clinical and Food Samples Secreting Wild-Type Levels of Cell-Free p60 Protein. *Journal of Clinical Microbiology*, **38** (7), https://doi.org/10.1128/jcm.38.7.2643-2648.2000
12	Rowan, N.J., Kirf, D. and Tomkins, P. (2009). Studies on the susceptibility of different culture morphotypes of *Listeria monocytogenes* to uptake and survival in human polymorphonuclear leukocytes. *FEMS Immunology and Medical Microbiology*, **57** (2), 183-192.
13	Rowan, N.J. (1999). Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. *Trends in Food Science and Technology*, **10** (8), 261-270.
14	Geraghty, L., Booth, M., Rowan, N.J. and Fogarty, A. (2013). Investigations on the efficacy of routinely used phenotypic methods compared to genotypic approaches for the identification of staphylococcal species isolated from companion animals in Irish veterinary hospitals. Ir Vet J 66, 7 (2013). https://doi.org/10.1186/2046-0481-66-7
15	Rowan, N. (2004). Viable but non-culturable forms of food and waterborne bacteria: quo vadis? *Trends in Food Science and Technology*, **15** (9), 462-467.

Section 2: Non-thermal Decontamination Technology Elucidation and Development	
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112	O'Neill, E.A., Stejskal, V., Paolacci, S., Jansen, M.A.K. and Rowan, N.J. (2024). Quo vadis - Development of a novel peatland-based recirculating aquaculture multi-trophic pond

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131	O'Neill, E.A., Stejskal, V., Paolacci, S., Jansen, M.A.K. and Rowan, N.J. (2024). Quo vadis - Development of a novel peatland-based recirculating aquaculture multi-trophic pond system (RAMPS) in the Irish midlands with a global orientation. *Case Studies in Chemical and Environmental Engineering*, **9**, https://doi.org/10.1016/j.cscee.2024.100748
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16th April, 2021

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am a Professor of Anaesthesia and Intensive Care Medicine at NUI Galway, Ireland. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, data analysis and in manuscript preparation, submission and proof-editing.

Examples of these collaborative efforts are listed below.

1. NJ Rowan, JG Laffey. 2020. Challenges and solutions for addressing critical shortage of supply chain for personal and protective equipment (PPE) arising from Coronavirus disease (COVID19) pandemic–Case study from the Republic of Ireland. Science of The Total Environment 725, 138532. [IF 6.551].
2. HP Farrell, M Garvey, M Cormican, JG Laffey, NJ Rowan. 2010. Investigation of critical inter-related factors affecting the efficacy of pulsed light for inactivating clinically relevant bacterial pathogens. Journal of Applied Microbiology 108 (5), 1494-1508 79 [IF 3.066]
3. H Farrell, J Hayes, J Laffey, N Rowan. 2011. Studies on the relationship between pulsed UV light irradiation and the simultaneous occurrence of molecular and cellular damage in clinically-relevant *Candida albicans*. Journal of Microbiological Methods 84 (2), 317-326. [IF 1.81]
4. EJ Murphy, C Masterson, E Rezoagli, D O'Toole, I Major, GD Stack, J. Laffey, Rowan, N.J. 2020. β-Glucan extracts from the same edible shiitake mushroom *Lentinus edodes* produce differential in-vitro immunomodulatory and pulmonary cytoprotective effects—Implications for COVID-19. Science of the Total Environment 732, 13933 [IF 6.551]
5. D Bradley, B McNeil, JG Laffey, NJ Rowan. 2012. Studies on the pathogenesis and survival of different culture forms of *Listeria monocytogenes* to pulsed UV-light irradiation after exposure to mild-food processing stresses. Food Microbiology 30 (2), 330-339 [IF 4.155]
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OE Gaillimh,
Bóthar na hOllscoile,
Gaillimh, Eire

NUI Galway,
University Road,
Galway, Ireland

T +353 91 492566
F +353 91 494561

anaesthesia@nuigalway.ie
www.nuigalway.ie/anaesthesia/

8. CH Masterson, EJ Murphy, H Gonzalez, I Major, SD McCarthy, D O'Toole, Laffey, J, Rowan, N.J. 2020. Purified β-glucans from the Shiitake mushroom ameliorates antibiotic-resistant Klebsiella pneumoniae-induced pulmonary sepsis. Letters in Applied Microbiology 71 (4), 405-412. [IF 2.173]
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If you require any further details, please do not hesitate to contact me.

Yours Sincerely

John Laffey, Established Professor, Anaesthesia and Intensive Care Medicine, School of Medicine, NUI Galway.
Consultant Anaesthesiologist and Intensivist, Galway University Hospitals
Vice Dean for Research, College of Medicine, Nursing and Health Sciences, NUI Galway.
Director of Clinical Research, Clinical Research and Development Office, Saolta Hospitals and NUI Galway.

Memo

Abbott Diagnostics,
Lisnamuck,
Longford.
Tel: 00 353 43 3334904
Fax: 00 353 43 3331065

18th April, 2021

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am Principal Scientist (NPD) at Abbott Diagnostics Business in Ireland. Professor Rowan and I have co-authored several articles on which he was a lead or a senior author. By this I mean that he contributed to the conception, design and writing of the work, data analysis, in manuscript preparation and submission.

Examples of these collaborative efforts are listed below.

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2. H Farrell, J Hayes, J Laffey, N Rowan. 2011. Studies on the relationship between pulsed UV light irradiation and the simultaneous occurrence of molecular and cellular damage in clinically-relevant Candida albicans. Journal of Microbiological Methods 84 (2), 317-326. [IF 1.81]
3. M Garvey, H Farrell, M Cormican, N Rowan. 2010. Investigations of the relationship between use of in vitro cell culture-quantitative PCR and a mouse-based bioassay for evaluating critical factors affecting the disinfection …Journal of Microbiological Methods 80 (3), 267-273. [IF 1.81]
4. NJ Rowan, S Espie, J Harrower, H Farrell, L Marsili, JG Anderson. 2008. Evidence of lethal

Memo

and sub-lethal injury in food-borne bacterial pathogens exposed to high-intensity pulsed-plasma gas discharges. Letters in Applied Microbiology 46 (1), 80-86. [IF2.173]

5. H Farrell, M Garvey, N Rowan. 2009. Studies on the inactivation of medically important Candida species on agar surfaces using pulsed light. FEMS Yeast Research 9 (6), 956-966. [IF 3.13]

If you require any further details, please do not hesitate to contact me.

Yours Sincerely,

_____

Dr Hugh Farrell

Principal Scientist
New Product development
Abbott Diagnostics Division

16th April, 2023

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am a Senior Lecturer in the School of Engineering and Informatics at NUI Galway, Ireland. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, data analysis and in manuscript preparation, submission and proof-editing.

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National University of Ireland
Galway, University Road,
Galway, Ireland
H91 TK33
T. +353 91 524411

www.nuigalway.ie

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12. I Ruiz-Salmón, J Laso, M Margallo, P Villanueva-Rey, E Rodríguez, Cooney, R., Rowan, N., Noiret, C., Aldaco, Clifford, E. et al. 2020. Life cycle assessment of fish and seafood processed products–a review of methodologies and new challenges. Science of The Total Environment, 144094 1 citations. [IF 6.551]
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If you require any further details, please do not hesitate to contact me.

Yours Sincerely,

Dr Eoghan Clifford
Eoghan Clifford BE PhD CEng MIEI
Senior Lecturer School of Engineering,
Academic Director CÉIM Engineering programme.
NUI Galway Community and University Sustainability Partnership Executive Board.
Visiting research Fellow Athlone Institute of Technology
National University of Ireland www.nuigalway.ie
Galway, University Road,
Galway, Ireland
H91 TK33
T. +353 91 524411

Room 1035: Alice Perry Engineering Building
NUI Galway, Galway. H91HX31, Ireland.

Eoghan.clifford@nuigalway.ie

20th April, 2022

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am a Senior Lecturer in the Department of Life and Physical Science at Athlone Institute of Technology, Ireland. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, data analysis and in manuscript preparation, submission and proof-editing.

Examples of these collaborative efforts are listed below.

1. JC Hayes, M Garvey, AM Fogarty, E Clifford, NJ Rowan. 2012. Inactivation of recalcitrant protozoan oocysts and bacterial endospores in drinking water using high-intensity pulsed UV light irradiation. Water Science and Technology: Water Supply 12 (4), 513-522
2. L Geraghty, M Booth, N Rowan, A Fogarty. 2013. Investigations on the efficacy of routinely used phenotypic methods compared to genotypic approaches for the identification of staphylococcal species isolated from companion animals. rish Veterinary Journal 66 (1), 1-9
3. E McGillicuddy, I Murray, S Kavanagh, L Morrison, A Fogarty, M Cormican, Rowan, N., Morris, D. (2017). Silver nanoparticles in the environment: Sources, detection and ecotoxicology. Science of the Total Environment 575, 231-246.
4. EA O'Neill, NJ Rowan, AM Fogarty. 2019. Novel use of the alga Pseudokirchneriella subcapitata, as an early-warning indicator to identify climate change ambiguity in aquatic environments using freshwater finfish. Science of the Total Environment 692, 209-218.
5. M Broderick, A Fogarty, NJ Rowan. 2107. Development of a high-intensity, pulsed-plasma, gas-discharge technology for destruction of hazardous aqueous environment micropollutants. International Journal of Science, Environment and Technology. 1
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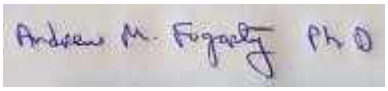
Cryptosporidium spp. and other clinically relevant microorganisms. 2011 IEEE Pulsed Power Conference, 1574-1581

If you require any further details, please do not hesitate to contact me.

Yours Sincerely,



_____

Andy Fogarty PhD,
Senuir Lecturer and Researcher,
Faculty of Science and Health,
Department of Life and Physical Science,
Phone: +353 906471861
Email: **afogarty@ait.ie**

STERIS
Applied Sterilization Technologies

16th April, 2021

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am a Senior Director Global Technologies at STERIS Advanced Sterilization Technologies. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design, data analysis and in manuscript preparation.

Examples of these collaborative efforts are listed below.

1. B McEvoy, NJ Rowan. 2019. Terminal sterilization of medical devices using vaporized hydrogen peroxide: a review of current methods and emerging opportunities. Journal of Applied Microbiology 127 (5), 1403-1420.
2. E McFadden, AL Cost Ramos, D Bradley, O Vrain, B McEvoy, N Rowan. 2016. Comparative studies on the novel sterilisation of Irish retailed infant milk formula using electron beam and pulsed light. International Journal of Science, Environment and Technology 5 (6), 4375-4377.
3. Y Chen, M Neff, B McEvoy, Z Cao, R Pezzoli, A Murphy, N Gately, Rowan, N. 2019. 3D printed polymers are less stable than injection moulded counterparts when exposed to terminal sterilization processes using novel vaporized hydrogen peroxide and electron beam. Polymer 183, 121870
4. B McEvoy, M Lynch, NJ Rowan. 2020. Opportunities for the application of real-time bacterial cell analysis using flow cytometry for the advancement of sterilization microbiology. Journal of Applied Microbiology. [IF 3.066] PMID: 33155740 DOI: 10.1111/jam.14876

If you require any further details, please do not hesitate to contact me.

Yours Sincerely,

Brian McEvoy

21th Sept, 2024

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: **Neil J Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde**

To whom it may concern,

I am a Senior Research Fellow at the Technological University of the Shannon here in Ireland. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, along with playing a key role in data analysis and in manuscript preparation, submission and proof-editing.

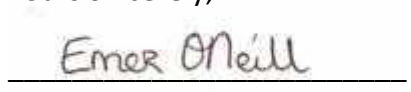
Examples of these collaborative efforts are listed below.

a. O'Neill, E.A. and Rowan, N.J. (2022). Microalgae as a natural ecological bioindicator for the simple real-time monitoring of aquaculture wastewater quality including provision for assessing impact of extremes in climate variance – A comparative case study from the Republic of Ireland. Science of the Total Environment, 802, https://doi.org/10.1016/j.scitotenv.2021.149800
b. O'Neill, E.A., Morse, A.P. and Rowan, N.J. (2022). Effects of climate and environmental variance on the performance of a novel peatland-based integrated multi-trophic aquaculture (IMTA) system: Implications and opportunities for advancing research and disruptive innovation post COVID-19 era. Science of the Total Environment, 819, https://doi.org/10.1016/j.scitotenv.2022.153073
c. O'Neill, E.A., Fehrenbach, G., Murphy, E., Alencar, S.A., Pogue, R. and Rowan, N.J. (2022). Use of next generation sequencing and bioinformatics for profiling freshwater eukaryotic microalgae in a novel peatland integrated multi-trophic aquaculture (IMTA) system: Case study from the Republic of Ireland. Science of the Total Environment, 851 (2), https://doi.org/10.1016/j.scitotenv.2022.158392
d. O'Neill, E.A., Stejskal, V., Paolacci, S., Jansen, M.A.K. and Rowan, N.J. (2024). Quo vadis - Development of a novel peatland-based recirculating aquaculture multi-trophic pond system (RAMPS) in the Irish midlands with a global orientation. Case Studies in Chemical and Environmental Engineering, 9, https://doi.org/10.1016/j.cscee.2024.100748

e. O'Neill, E.A. and Rowan, N.J. (2023). Potential disruptive effects of zoosporic parasites on peatland-based organic freshwater aquaculture: Case study from the Republic of Ireland. Science of the Total Environment, 868, https://doi.org/10.1016/j.scitotenv.2023.161495

If you require any further details, please do not hesitate to contact me.

Yours Sincerely,



Dr Emer O'Neill
Senior Research Fellow, TUS (emer.oneill@tus.ie)

1th April, 2021

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am an Emeritus Professor (granted on retiral from the former Department of Bioscience and SIPBS) and now currently a Research Fellow at the Department of Electronic and Electrical Engineering at the University of Strathclyde. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, along with playing a key role in data analysis and in manuscript preparation, submission and proof-editing.

Examples of these collaborative efforts are listed below.

1. NJ Rowan, SJ MacGregor, JG Anderson, RA Fouracre, L McIlvaney, O. Farish. (1999). Pulsed-light inactivation of food-related microorganisms. Applied and environmental microbiology 65 (3), 1312-1315 [IF 4.016].
2. JG Anderson, NJ Rowan, SJ MacGregor, RA Fouracre, O Farish. 2000. Inactivation of food-borne enteropathogenic bacteria and spoilage fungi using pulsed-light. IEEE Transactions on Plasma Science 28 (1), 83-88. [IF 1.309]
3. SJ MacGregor, NJ Rowan, L McIlvaney, JG Anderson. 1998. Light inactivation of food-related pathogenic bacteria using a pulsed power source. Letters in Applied Microbiology 27 (2), 67-70. 149 [IF 2.713]
4. NJ Rowan, SJ Macgregor, JG Anderson, RA Fouracre, O Farish.2000. Pulsed electric field inactivation of diarrhoeagenic Bacillus cereus through irreversible electroporation. Letters in Applied Microbiology 31 (2), 110-114. [IF 2.173]
5. SJ MacGregor, O Farish, R Fouracre, NJ Rowan, JG Anderson. 2000. Inactivation of pathogenic and spoilage microorganisms in a test liquid using pulsed electric fields. IEEE Transactions on Plasma Science 28 (1), 144-149 [IF 1.309]
6. NJ Rowan, JG Anderson. 1998. Effects of Above-Optimum Growth Temperature and Cell Morphology on Thermotolerance of Listeria monocytogenes Cells Suspended in Bovine Milk. Applied and Environmental Microbiology 64 (6), 2065-2071. [IF 4.016]
7. NJ Rowan, SJ MacGregor, JG Anderson, D Cameron, O Farish. 2001. Inactivation of *Mycobacterium paratuberculosis* by pulsed electric fields. Applied and Environmental Microbiology 67 (6), 2833-2836 [4.016]
8. NJ Rowan, S Espie, J Harrower, JG Anderson, L Marsili, SJ MacGregor. 2007. Pulsed-plasma gas-discharge inactivation of microbial pathogens in chilled poultry wash water. Journal of Food Protection 70 (12), 2805-2810. [IF 1.60]
9. JR Beveridge, SJ MacGregor, L Marsili, JG Anderson, NJ Rowan. 2002. Comparison of the effectiveness of biphase and monophase rectangular pulses for microbial inactivation using pulsed electric fields. IEEE Trans Plasma Sci 30 (4), 1525-1531.

10. NJ Rowan, AAG Candlish, A Bubert, JG Anderson, K Kramer, .2000. Virulent Rough Filaments of Listeria monocytogenesfrom Clinical and Food Samples Secreting Wild-Type Levels of Cell-Free p60 Protein. Journal of Clinical Microbiology 38 (7), 2643-2648. [IF 5.897]
11. NJ Rowan, JG Anderson. 1997. Maltodextrin stimulates growth of Bacillus cereus and synthesis of diarrheal enterotoxin in infant milk formulae. Applied and Environmental Microbiology 63 (3), 1182-1184. [IF 4.016]
12. NJ Rowan, JG Anderson. 1998. Diarrhoeal enterotoxin production by psychrotrophic Baccillus cereus present in reconstituted milk-based infant formulae (MIF). Letters in Applied Microbiology 26 (2), 161-165 [2173]
13. S Yaqub, JG Anderson, SJ MacGregor, NJ Rowan. 2004; Use of a fluorescent viability stain to assess lethal and sublethal injury in food-borne bacteria exposed to high-intensity pulsed electric fields. Letters in Applied Microbiology 39 (3), 246-25
14. JR Beveridge, K Wall, SJ MacGregor, JG Anderson, NJ Rowan. 2004. Pulsed electric field inactivation of spoilage microorganisms in alcoholic beverages. Proceedings of the IEEE 92 (7), 1138-1143 . [IF 10.694].
15. NJ Rowan, JG Anderson. 1998. Growth and enterotoxin production by diarrhoeagenic Bacillus cereus in dietary supplements prepared for hospitalized HIV patients. Journal of Hospital Infection 38 (2), 139-146. [IF 3.441]
16. NJ Rowan, JG Anderson, A Anderton. 1997. Bacteriological quality of infant milk formulae examined under a variety of preparation and storage conditions. Journal of Food Protection 60 (9), 1089-1094.
17. NJ Rowan, JG Anderson, AAG Candlish. Cellular morphology of rough forms of Listeria monocytogenes isolated from clinical and food samples. Letters in Applied Microbiology 31 (4), 319-322 [IF 2.173]
18. NJ Rowan, JG Anderson. 1998. Effectiveness of cleaning and disinfection procedures on the removal of enterotoxigenic Bacillus cereus from infant feeding bottles. Journal of food Protection 61 (2), 196-200 [IF 1.60]
19. NJ Rowan, JG Anderson, A Anderton. 1997. The bacteriological quality of hospital-prepared infant feeds. Journal of Hospital Infection 35 (4), 259-267 [IF 3.441]
20. NJ Rowan, JG Anderson, JE Smith. 1998. Potential infective and toxic microbiological hazards associated with the consumption of fermented foods. Microbiology of Fermented foods, 812-837. 13 citations.
21. NJ Rowan, JG Anderson, JE Smith, JA Clarke, RC McLean, NJ Kelly. 1997. Development of a computer programme for the prediction and control of mould growth in buildings using the ESP-r modelling system. Indoor and Built Environment 6 (1), 4-11. 7 citations. [IF 1.9]

If you require any further details, please do not hesitate to contact me.

Yours Sincerely,

_____

Prof John Anderson

17th April, 2021

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

To whom it may concern,

I am a Lecturer in the Department of Life Science at Sligo Institute of Technology, Ireland. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, along with playing a key role in data analysis and in manuscript preparation, submission and proof-editing.

Examples of these collaborative efforts are listed below.

1. HP Farrell, M Garvey, M Cormican, JG Laffey, NJ Rowan. 2010. Investigation of critical inter-related factors affecting the efficacy of pulsed light for inactivating clinically relevant bacterial pathogens. Journal of Applied Microbiology 108 (5), 1494-1508 79 [IF 3.066]
2. M Garvey, H Farrell, M Cormican, N Rowan. 2010. Investigations of the relationship between use of in vitro cell culture-quantitative PCR and a mouse-based bioassay for evaluating critical factors affecting the disinfection …Journal of Microbiological Methods 80 (3), 267-273. [IF 1.81]
3. J Hayes, D Kirf, M Garvey, N Rowan. 2013. Disinfection and toxicological assessments of pulsed UV and pulsed-plasma gas-discharge treated-water containing the waterborne protozoan enteroparasite Cryptosporidium parvum. Journal of Microbiological Methods. 94 (3), 325-337. [1.81]
4. M Garvey, D Rabbitt, A Stocca, N Rowan. 2015. Pulsed ultraviolet light inactivation of Pseudomonas aeruginosa and Staphylococcus aureus biofilms. Water and Environment Journal 29 (1), 36-42 [IF 1.369]
5. P Haughton, M Garvey, NJ Rowan. 2010. Emergence of Bacillus cereus as a dominant organism in irish retailed powdered infant formulae (pif). Journal of Food Safety 30 (4), 814-831 [IF 1.133]
6. JC Hayes, M Garvey, AM Fogarty, E Clifford, NJ Rowan. 2012. Inactivation of recalcitrant protozoan oocysts and bacterial endospores in drinking water using high-intensity pulsed UV light irradiation. Water Science and Technology: Water Supply 12 (4), 513-522
7. M Garvey, NJ Rowan. 2019. Pulsed UV as a potential surface sanitizer in food production processes to ensure consumer safety. Current Opinion in Food Science 26, 65-70 [IF 4.577]
8. M Garvey, N Rowan. 2015. A pulsed light system for the disinfection of flow through water in the presence of inorganic contaminants. Journal of Water and Health 13 (2), 406-412. [IF 1.349]

IT Sligo, Ash Lane, Sligo, Ireland
T +353 (0) 71 91 55222

9. M Garvey, J Hayes, E Clifford, N Rowan. 2015. Ecotoxicological assessment of pulsed ultraviolet light-treated water containing microbial species and Cryptosporidium parvum using a microbiotest test battery. Water and Environment Journal 29 (1), 27-35 [IF 1.369]
10. M Garvey, A Stocca, N Rowan. 2014. Development of a combined in vitro cell culture–Quantitative PCR assay for evaluating the disinfection performance of pulsed light for treating the waterborne enteroparasite …Experimental parasitology 144, 6-13. [IF 1.690]
11. M Garvey, E Clifford, E O'Reilly, NJ Rowan. 2013. Efficacy of Using Harmless Bacillus Endospores to Estimate the Inactivation of Cryptosporidium parvum Oocysts in Water. The Journal of Parasitology 99 (3), 448-452 [IF 1.236]
12. J Naughton, EJ Tiedeken, M Garvey, JC Stout, NJ Rowan. 2017. Pulsed light inactivation of the bumble bee trypanosome parasite Crithidia bombi. Journal of Apicultural Research 56 (2), 144-154 [IF 1 .872]
13. M Garvey, N Thokala, N Rowan. 2014. A comparative study on the pulsed UV and the low-pressure UV inactivation of a range of microbial species in water. Water Environment Research 86 (12), 2317-2324 [IF 1.269]
14. M Garvey, JP Andrade Fernandes, N Rowan. 2015. Pulsed light for the inactivation of fungal biofilms of clinically important pathogenic Candida species. Yeast 32 (7), 533-540. [IF 3.143]
15. M Garvey, E Clifford, E O'Reilly, NJ Rowan. 2012. Efficacy of using harmless Bacillus endospores as novel surrogate organisms to indicate the inactivation performance of recalcitrant cryptosporidium parvus oocysts suspended in …American Society of Parasitologists [IF 1.238]
16. M Garvey, A Stocca, N Rowan. 2016. Use of a real time PCR assay to assess the effect of pulsed light inactivation on bacterial cell membranes and associated cell viability. Water Environment Research 88 (2), 168-174 [IF 1.369]
17. M Garvey, J Hayes, E Clifford, D Kirf, N Rowan. 2013. Efficacy of measuring cellular ATP levels to determine the inactivation of pulsed UV treated Cryptosporidium parvum oocysts suspended in water. Water Science and Technology: Water Supply 13 (2), 202-213 [IF 1.050]
18. M Garvey, G Coughlan, N Murphy, N Rowan. 2016. The pulsed light inactivation of veterinary relevant microbial biofilms and the use of a RTPCR assay to detect parasite species within biofilm structures. Open Veterinary Journal 6 (1), 15-22 [IF 2.1]

If you require any further details, please do not hesitate to contact me at Garvey.mary@itsligo.ie.

Yours Sincerely,

_____
Dr Mary Garvey

Dr Mary Garvey MSc BSc
Dept of Life Science
Institute of Technology Sligo
Ash Lane
Sligo
Ireland
F91 YW50

27th September, 2024

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Professor Neil J Rowan, applicant for higher Doctorate of Science (D.Sc.) Degree on published work to the University of Strathclyde

To whom it may concern,

I am the Director for Microbiological Quality at Johnson and Johnson. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, along with playing a key role in data analysis and in manuscript preparation, submission and proof-editing.

Examples of these collaborative efforts are listed below.

Rowan, N.J., Kremer, T., McDonnell, G. (2023). A review of Spaulding's classification system for effective cleaning, disinfection and sterilization of reusable medical devices: Viewed through a modern-day lens that will inform and enable future sustainability. Science of the Total Environment, 878, https://doi.org/10.1016/j.scitotenv.2023.162976

Kremer, T., Rowan, N.J. and McDonnell, G. (2024). A proposed cleaning classification system for reusable medical devices to complement the Spaulding classification. *Journal of Hospital Infection*, **145**, 88-98.

Kremer, T.A., Felgar, J., Rowan, N.J. and McDonnell, G. (2023). Validation of the Device Feature Approach for Reusable Medical Device Cleaning Evaluations. *Biomedical Instrumentation and Technology*, https://doi.org/10.2345/0899-8205-57.4.143

Kremer, T., Murray, N., Buckley, J. and Rowan, N.J. (2023). Use of real-time immersive digital training and educational technologies to improve patient safety during the processing of reusable medical devices: Quo Vadis? *Science of the Total Environment*, **900**, https://doi.org/10.1016/j.scitotenv.2023.165673

If you require any further details, please do not hesitate to contact me.

Yours Sincerely,

Terra Kremer

UNIVERSITI MALAYA

To Whom It May Concern

24th September 2024

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

Re: Neil J. Rowan, applicant for Doctorate of Science (D.Sc.) on published work to the University of Strathclyde

I am an Associate Professor in the Institute of Biological Sciences at the Universiti Malaya, Malaysia. Prof Rowan and I have co-authored several articles on which he was a lead author. By this I mean that he contributed to the conception, design and writing of the work, data analysis and in manuscript preparation, submission and proof-editing.

1. Wan Mohtar, W.A.A.Q.I, IIham, Z., Jamaludin, A.A and **Rowan, N.J** (2021). Use of Zebrafish Embryo Assay to Evaluate Toxicity and Safety of Bioreactor-Grown Exopolysaccharides and Endopolysaccharides from European *Ganoderma applanatum* Mycelium for Future Aquaculture Applications. International Journal of Molecular Sciences, 22 (4), https://doi.org/10.3390/ijms22041675

2. Wan Mohtar, W.A.A.Q.I., Fafek, N.A., Thiran, J.P., Rahman, J.F.P., Yerima, G., Subramaniam, K. and **Rowan, N.J.** (2021). Investigations on the use of exopolysaccharide derived from mycelial extract of *Ganoderma lucidum* as functional feed ingredient for aquaculture-farmed red hybrid Tilapia (Oreochromis sp.). Future Foods, 3, https://doi.org/10.1016/j.fufo.2021.100018

3. Usuldin, S.R.A., Wan-Mohtar, W.A.A.Q.I., IIham, Z., Abdullah, N.R. and **Rowan, N**. (2021). In vivo toxicity of bioreactor-grown biomass and exopolysaccharides from Malaysian tiger milk mushroom mycelium for potential future health applications. Nature Scientific Reports, 11, https://doi.org/10.1038/s41598-021-02486-7

Your sincerely,

Official stamp:

Ts. Dr. Wan Abd Al Qadr Imad Wan Mohtar
Associate Professor (Biotechnology)
Institute of Biological Sciences, Faculty of Science
Universiti Malaya, 50603 Kuala Lumpur, Malaysia
qadyr@um.edu.my | 24/09/2024

Associate Professor Ts. Dr. Wan Abd Al Qadr Imad Wan Mohtar
Biotechnology Program, Institute of Biological Sciences, Faculty of Science
Universiti Malaya, 50603, Kuala Lumpur, Malaysia
Email: qadyr@um.edu.my

Energy Systems Research Unit
Mechanical & Aerospace Engineering
James Weir Building
75 Montrose Street
Glasgow G1 1XJ
Scotland, UK

ESRU

21 April 2021

Chair,
Higher Doctorate Committee
On behalf of Senate,
University of Strathclyde

To Whom it May Concern

Neil Rowan: DSc application, University of Strathclyde

I write in connection with Professor Rowan's DSc application. I collaborated with Neil on several research projects over the period 1996-2000 and co-authored, with him and others, publications in which he was a lead or senior author:

- Rowan N J, Johnstone C M, McLean R C, Anderson J G and Clarke J A (1999) 'Prediction of toxigenic fungal growth in buildings by using a novel modelling system', Applied and Environmental Microbiology, 65 (11), pp. 4814-4821. [IF 4.016]
- Clarke J A, Johnstone C M, Kelly N J, McLean R C, Rowan N J and Smith J E (1999) 'A technique for the prediction of the conditions leading to mould growth in buildings', Building and Environment, 34 (4), pp. 515-521. [IF 4.971]
- Rowan N J, Anderson J G, Smith J E, Clarke J A, McLean R C and Kelly N J (1997) 'Development of a computer program for the prediction and control of mould growth in buildings using the ESP-r modelling system', Indoor and Built Environment, 6 (1), pp. 4-11. [IF 1.9]

Throughout our collaboration, Neil took the lead in the bioscience aspects of the research and delivered world leading outcomes: his contributions to the conception and execution of the underlying research was substantial.

Please do not hesitate to contact me should you require any further details.

Yours sincerely,

Professor Emeritus J A Clarke

Energy Systems Research Unit
http://www.esru.strath.ac.uk
Professor J. A. Clarke BSc, PhD, DSc, D*hc*, HonFSE, CEng, FEI, FIBPSA
Email: joe@esru.strath.ac.uk
Tel: +44 (0)141 548 3986
Mob: +44 (0)7800903808