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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

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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

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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.

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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.

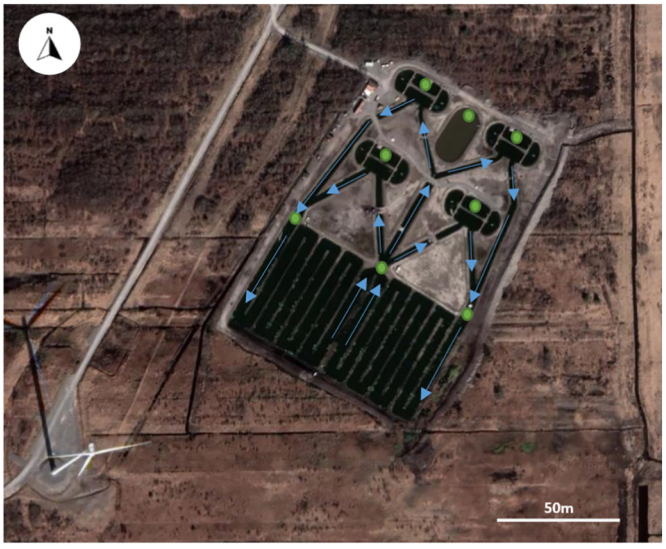

Fig. 1. Ariel view of trial fish farm. Sampling points have been indicated (green). Flow of water throughout the system has been included (blue). Image shows the four D-end culture ponds, the reservoir and the 16-channel duckweed and algae treatment lagoon. The turbine, which provides all electrical 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]

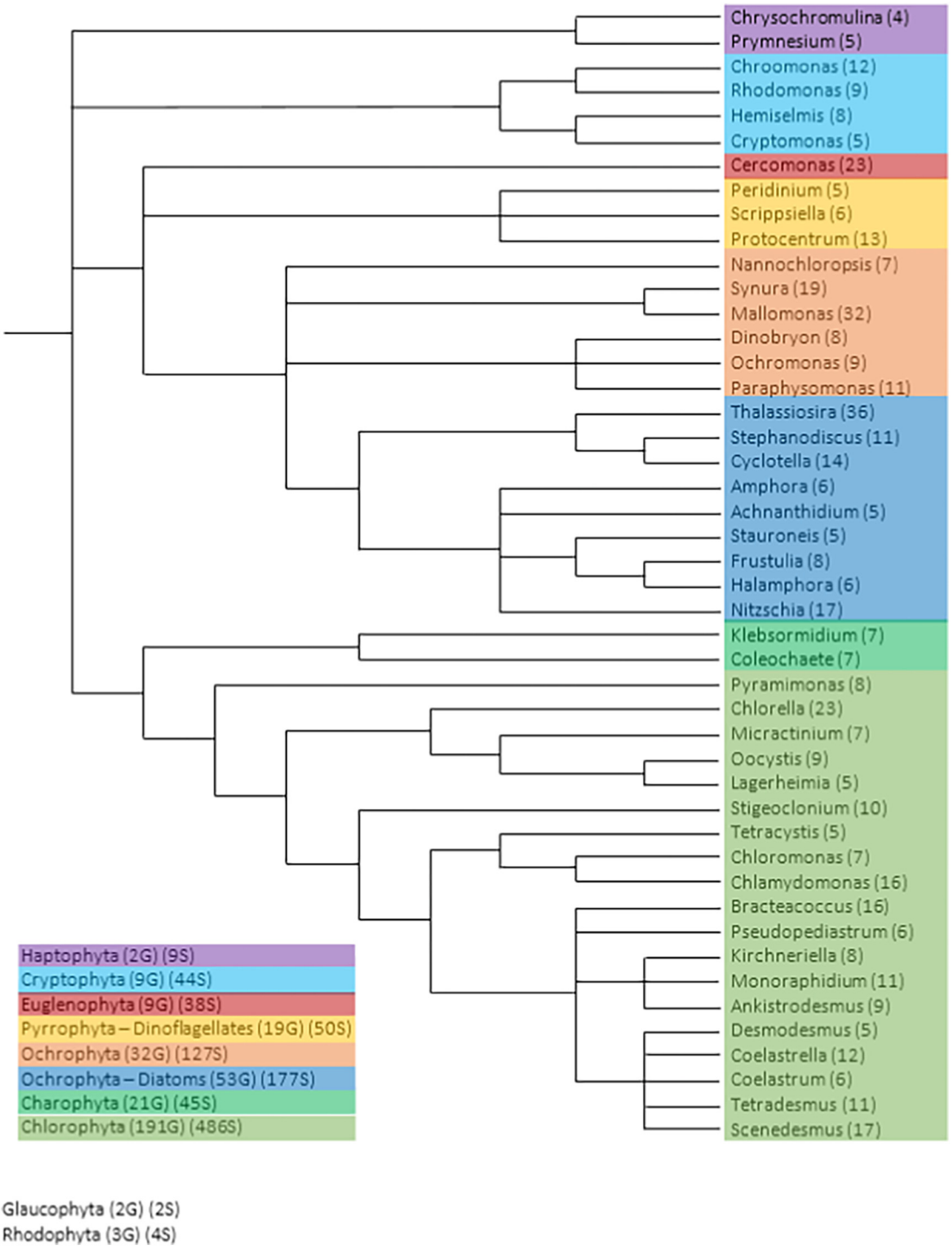

Fig. 2. Phylogenetic tree identifying genera of algae found in the novel freshwater IMTA system between December 2019 and October 2020. (n) = number of species for that specific genera, (nG) = number of genera for that specific phylum, (nS) = number of species for that specific phylum. For ease or reporting 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.

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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.

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