Efficiency of Sligo Secondary Wastewater Treatment Plants in the Removal of *Cryptosporidium* and other Human-virulent Enteropathogens

by

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Submitted to the Institute of Technology, Sligo in fulfilment of the requirement for the degree of Doctor of Philosophy

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DECLARATION

I declare that this thesis is entirely my own work, except where otherwise stated and that it has not been previously submitted to any institute or university.

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ABSTRACT

Most research on wastewater treatment efficiency compliance focuses on physiochemical and microbial indicators; however, very little emphasis has been placed so far on removal efficiencies of human-virulent enteropathogens and on determining suitable indicator organisms to predict the discharge level of human enteropathogens from municipal wastewater treatment plants to the local environment.

This project studies the occurrence of human enteropathogens, including *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, genogroup I and II noroviruses, *Enterococcus faecalis* and *Enterococcus faecium* in domestic wastewater. To investigate the capability of Sligo secondary wastewater treatment plants, (1) raw wastewater, (2) secondary sewage sludge, (3) final effluent and (4) biosolids/ final sewage sludge from four WWTPs (A-D) were investigated in the removal of human enteropathogens, over one year, using a combination of IFA, FISH and RNA-polymerase based PCR methodologies. This project also looks into the relationship between faecal indicator bacteria (*E. coli*, enterococci and *C. perfringens* spores) and human enteropathogens and the potential use for pathogen prediction, especially *Cryptosporidium*.

The results provide evidence that human-virulent enteropathogens are present throughout the wastewater processes and in end products, and can enter the aquatic environment with consequently negative implications for public health. This study also provides important scientific evidence that municipal wastewater treatment plants not only achieve pathogen removal but can also be the environmental pathogen contamination source. This research also shows that seasonal variation in concentration of human-virulent enteropathogens is highest in Spring and Summer during the calving and lambing seasons, and when tourism peaks. A suite of faecal indicator bacteria (*E. coli*, enterococci and *C. perfringens* spores) can be used as indicators for the presence of *Cryptosporidium* oocysts and *Giardia* cysts. Routine monitoring of faecal indicators in final effluents and biosolids is recommended.

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Chapter One

INTRODUCTION

1.1 General Introduction

Sewerage collecting systems receive varying types of wastewater including rainwater runoff, domestic sewage, and industrial wastewater and deliver it to municipal wastewater treatment plants (Bartlett 1981; WEF 2010). Wastewater contains a wide range of matter, such as waste from households, hospitals, schools and business units, flushed-out solids from surface runoff during heavy precipitation (termed storm-water), fats, as well as micro-organisms (i.e. native intestinal fauna and pathogenic bacteria, viruses, and protozoa) (Gerardi and Zimmerman 2004). As the runoff flows over the land or impervious surfaces (paved streets, parking lots, and building rooftops), it accumulates debris, chemicals, sediment or other pollutants that could adversely affect water quality if the runoff is discharged untreated (Report of National Research Council 2008). Due to the constant changes in the world's economic structure, rapid progression of technology (e.g. high ionic strength of wastewater resulting from high-tech industries and the usage of antibiotics), and urban housing development schemes, wastewater characteristics have changed to a more complex form (Pala and Tokat 2002; Ong *et al.* 2003; da Costa *et al.* 2006; Wang *et al.* 2008; Akiyama and Savin 2010, Honda *et al.* 2011; Fuentefria *et al.* 2011; Munir *et al.* 2011).

The level of relevant development varies from country to country. According to the Department of Economic and Social Affairs of the United Nations Secretariat, the world population is expected to reach 9 billion by 2050 and exceed 10 billion in 2100 (UN 2011). The percentage of the urban population is projected to range from 60.5% to 86% in either developed or developing countries (UN 2011). Therefore, immense volumes of wastewater are generated in all world major cities. To cope with differences in scale, wastewater treatment plant' design and capacity varies from those serving <100 population equivalent (PE) in a small village, to a plant like the Crossness Waste Water Treatment Plant (WWTP) (in London) treating

the wastewater from over 1.7 million people or a plant as big as Deer Island Sewage Treatment Plant (in Massachusetts, USA) which deals with the wastewater from a population of over 45 million in the Greater Boston area.

In urbanised European countries, such as the United Kingdom and Germany, a proportion of the population is located outside of the main cities but the generated wastewater is connected to the public sewerage system and transported to local municipal sewage treatment plants. For less urbanised countries, taking Ireland as an example, approximately 1.47 million of population live in urban areas while 0.9 millions live in rural areas in the year of 2006 (Central Statistics Office 2007). Two thirds of the wastewater generated in Ireland enters municipal sewerage networks. Municipal sewerage networks are designed with sewer pipe systems connecting wastewater from houses, business and industrial units in developed area to open-field wastewater treatment plants. Municipal WWTPs are fundamentally built to break down organic particles, remove nutrients (e.g., biodegradable phosphorus and nitrogen), and reduce the biomass load in the wastewater in urban areas to prevent pollution to the local environment (EPA 1991). A sewerage treatment network consists of collecting pipe systems and operation processes in the WWTP, such as preliminary and primary treatment facilities, biomass consumption treatment process, flocculation, sedimentation, solids settlement, and effluent clarification (Vesilind 2003). The collection and transport of wastewater to the treatment plant is via a network of sewers (i.e., sewerage system). Sewerage systems are categorised into two main systems, namely combined and separate systems. For combined sewerage systems, surface drainage from roads, paved areas, and roofs are collected in the same sewer as the foul wastewater and piped to the treatment plants (Gray 2004). Wastewater treatment plants are traditionally designed to cope with up to three times the dry weather loading amount of organic matter and to reduce biological oxygen demand (BOD), chemical oxygen demand (COD), and

three times the dry weather loading amount of organic matter and to reduce biological oxygen demand (BOD), chemical oxygen demand (COD), and total suspended solids (TSS) before discharge (Gray 2006). The infrastructures deployed in WWTPs are used to treat wastewater preventing the outgoing effluent from polluting the receiving waters, such as rivers, lakes, estuaries and bathing waters and thereby protecting drinking water supplies, aquatic ecology and recreational waters.

The incoming domestic sewage, consisting of faeces, urine, laundry and cleaning waste, food waste and toiletry waste from the catchment, represents the main proportion in the municipal wastewater (Painter 1958). After the raw wastewater (also termed influent) enters the WWTP, the coarse particles are removed by grit removal and the fat is removed by screens (Preliminary stage, Figure 1.1). In some plants, primary sedimentation is operated before the batch flow to the secondary biological treatment reservoir, especially for the system utilising membrane filtration at the secondary stage. The primary sedimentation process is operated by floating and settlement. The suspended matter with a density less than water will rise to the surface and form a scum (Gray 2004). Based on the operation processes at the secondary stage, the WWTPs can be generally categorised into extended- aerated activated sludge treatment systems or biofilm-coated filtration systems. A secondary sedimentation process is operated to settle the fine solids and to separate the microbial biomass produced in the biological treatment unit from the clarified effluent (IAWQ, 1997). After the complete treatment, the end products from the WWTPs- final effluent and sewage sludge- are discharged to the receiving water and disposed of to the licensed land, respectively (CEC 1991; US EPA 1993, 2003).

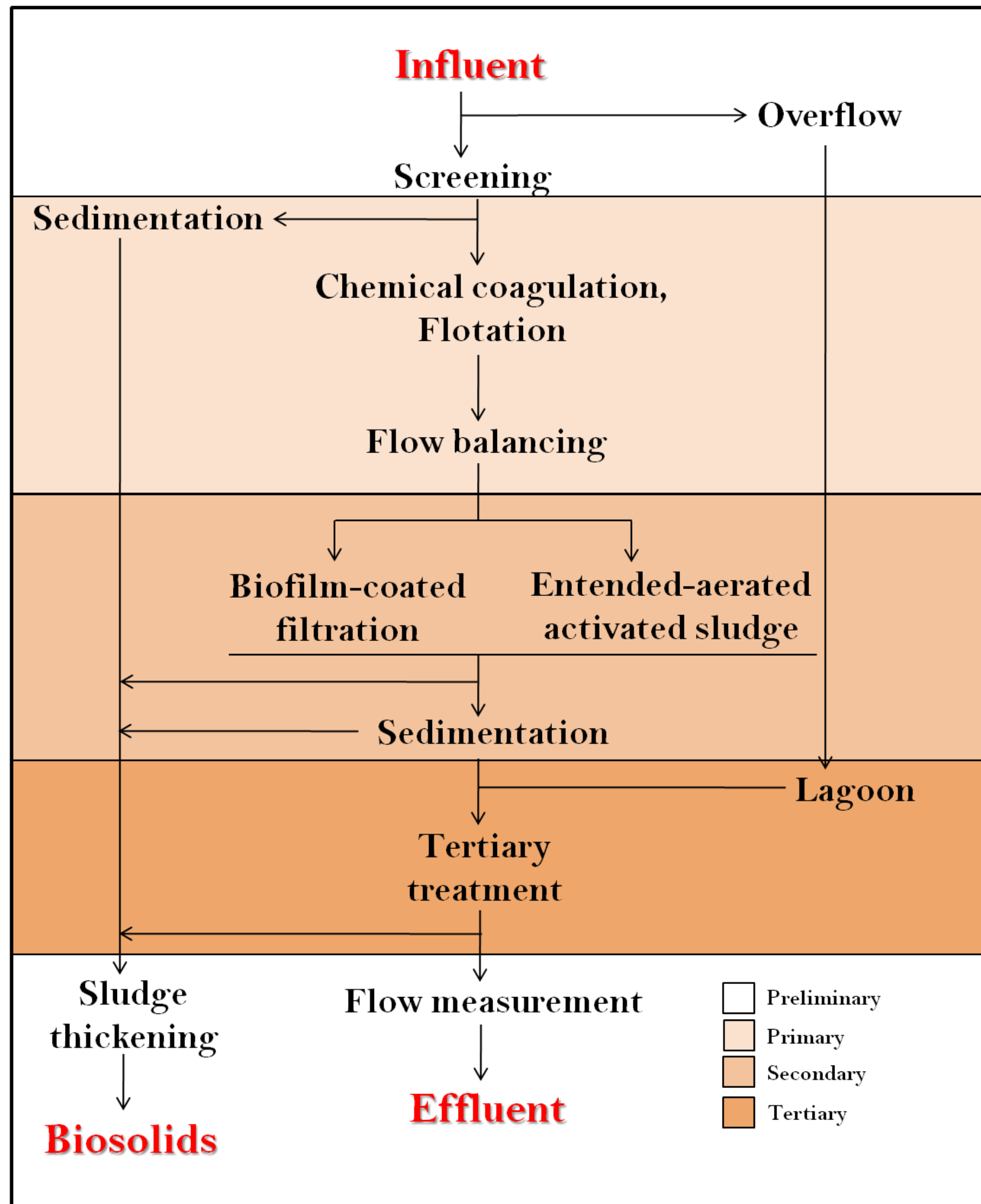

Figure 1.1. A diagram illustrating wastewater treatment process operations in a WWTP. (adapted from Gray, 2004). The wastewater treatment unit processes include four main stages: Preliminary treatment (removal and disintegration of solids and fat); Primary treatment (solids settlement); Secondary biological treatment (Oxidisation of the dissolved and colloidal organics and inactivation of the metabolism of existing pathogens from human/animal intestines and local environments; Tertiary advanced treatment facility may be equipped for the removal of BOD, nutrients, bacteria and toxic compounds). In Ireland, WWTPs are designed principally to reduce nutrient concentration in produced end-products. The resulting end-products are final effluent and biosolids.

The Urban Waste Water Treatment Regulations, 2001 (S.I. No. 254 of 2001), were introduced by the Minister for the Environment on 14th June 2001 and amended on 15th July 2004 in Statutory Instrument (Oireachtas na hEireann, 2001, 2004). The principal requirement of the 2001 Urban Waste Water Treatment Regulations is to regulate emission limit values of the discharge effluent for BOD, COD, and TSS to protect the fresh waters in Ireland. Local authorities are also requested to monitor the quality of effluent frequently to ensure the performance of the plant compliance with the emission limit (CEC 1991; EPA 1996; Oireachtas na hEireann 2001). The other main requirement of the Regulations is to schedule provision of current waste water treatment plants to fit to the size of the agglomeration and to the type of water body to which the final effluent is discharged (CEC 1991; Oireachtas na hEireann 2004). According to 2006-2007 report published by Environmental Protection Agency (EPA) in Ireland, 4% of the total of 482 monitored locations (32 WWTPs) representing a population equivalent of 5,835,495 (219,351 PE for the 32 WWTPs) in Ireland did not receive any form of treatment (Monaghan *et al.* 2009). Of the 482 WWTPs around Ireland by the end of 2007, 370 had received secondary treatment, including 112 WWTPs with tertiary nutrient reduction facilities. Compared to the national report for the years 2004-2005, 16 WWTPs arising had been refurbished with biological treatment facilities. The provision of secondary treatment with tertiary nutrient reduction has increased from 12% in 2004/2005 to 15% in 2007 (Smith *et al.* 2007; Monaghan *et al.* 2009).

Many research studies and investments have been developed to improving the wastewater treatment efficacy of chemical constituents reduction (i.e., BOD, COD, TSS, total phosphorus and nitrogen) in domestic and/or industrial WWTPs (Austermann *et al.* 1999; Bryers *et al.* 1990; Heijnen *et al.* 1991; Gorur *et al.* 1995; Daims *et al.* 2001; Liang *et al.* 2010). Chemical constituents include inorganic dissolved constituents, such as nutrients, nonmetallic constituents, metals, and gases and aggregate

organic constituents, such as microbial cell aggregate and a number of individual components that cannot be distinguished separately (Metcalf and Eddy 1991; Linek *et al.* 1997). Small microbial products (SMP), including antibiotics other pharmaceuticals, structures of cell components and products of cell metabolism, are also found in wastewater and treated effluent (Barker and Stuckey 1999; Kümmerer 2003). Raw wastewater contains known and unknown organic and inorganic constituents that are present naturally in the water supply source, present in treated drinking water, added by the water users, added from storm water in combined collection systems, formed in the collection system as a result of abiotic and biotic reactions, and added in the sewerage collection system (Graczyk *et al.* 2008). Extended-aeration activated sludge systems are well-studied in terms of bio-engineering, and are capable of reducing more than 90% of organic solids content in the wastewater before release to a watercourse (IAWQ 1997; Gray 2004). For those with nutrient removal facilities, the total carbon and nitrogen concentration can be further reduced to a total phosphorus value to less than 2 mg/L P and the total nitrogen value of less than 15 mg/L N (Metcalf and Eddy 1991; Oireachtas ne hEireann 2001) for compliance with Urban Wastewater Treatment Regulations, 2001 (S.I. 254 of 2001).

However, treatment plants are not designed to remove micro-organisms which are also delivered to the WWTPs with the sewage. The native intestinal micro-organisms, living in the human intestinal tract excreted include coliform bacteria, *E. coli* and intestinal enterococci as well as pathogenic bacteria: pathogenic *E. coli* and enterococci, *Legionella* spp., spore-forming *Clostridium* spp., *Salmonella* spp.; pathogenic protozoa: human-virulent *Cryptosporidium*, *Giardia*, helminth; fungal spores; and active human enteric viruses: enterovirus, norovirus, rotavirus in the influent can represent the microbial load in the studied human populations (Ng *et al.* 1993; da Costa *et al.* 2006; Iwai *et al.* 2009; Wen *et al.* 2009).

The microbial cells are referred to as microbial aggregate or floc, i.e., the assemblage of individual cells and micro-colonies, which are formed under specific reactor conditions at the secondary stage in WWTP (Boonaert *et al.* 1999). With the advantage of the usage of microbial aggregate naturally present in the wastewater, the issue most concerned in human public health are: How efficient does the municipal wastewater treatment plants work in the removal of the co-occurring human enteropathogens?; What is the fate of these resulting end products? Since if a treatment failure occurs, the faeces-derived microorganisms may accumulate in the wastewater or the solid wastes and contaminate the environment at its discharge (Mara *et al.* 1996).

In Ireland, as a member of the European Union, €4.7 billion was spent in the National Development Plant (NDP) 2007-2013 on wastewater/water service and infrastructure in compliance with the European Union Urban Waste Water Treatment Directive. As part of this, major investment in wastewater infrastructure has be undertaken. *The National Development Plan 2000-2006* provides for capital investment of €2.4 billion in wastewater treatment up to 2006. The *Water Services Investment Programme 2002-2004* includes all schemes needed for compliance with the EU Urban Waste Water Treatment Directive for 2005 in relation to areas with more than 2,000 population (National Report for Ireland 2002). Under the National Spatial Strategy, the city of Sligo has been identified as an urban centre of the north-west region in Republic of Ireland. Since then, Sligo city has been promoted as a Gateway city in the framework of the Sligo and Environs Development Plan 2004-2010. Benefited as commuter-belt towns in Co. Sligo, new residential areas in Collooney, Strandhill and Tubbercurry were established with secondary WWTPs in the late twentieth century. Nevertheless, these plants are apparently not capable of dealing with the dramatic increase of resulting wastewater due to urbanising

development (Sligo County Council 1985). Nowadays, a few villages in Co. Sligo, such as Rosses Point, still only have primary treatment in-plant and the new Sligo WWTP serving the city centre and environs has just come into operation in 2010. The detailed information regarding current status of WWTPs in Co. Sligo will be provided later on in this chapter (Chapter 1.2.1.3). At present, these existing secondary WWTPs are near the end of their useful lives and the upgrading of treatment infrastructures or construction of new treatment plants; therefore, are necessary not only to cope with the increasing wastewater but also to ensure an acceptable effluent quality for environmental protection (Sligo County Council 2005). Under pressure from urban-generated housing, some biological WWTPs have been commissioned or refurbished in Co. Sligo in the early 21st century. The effluent from treatment plants, sewers or drainage pipesis enforced by Environmental Protection Agency (EPA) in Ireland to ensure the local authorities control the quality of the effluents being discharged to watercourses (Oireachtas na hEireann 1992, 2008).

In terms of the risk of wastewater, it is the vector of diseases excreted by man via faeces and urine that are of primary importance to public health. Pathogens in raw or treated wastewaters are viable and potentially infectious to man and/or animals by oral ingestion (faecal-oral), via skin or by respiratory routes (WHO 1898; Shuval *et al.* 1986; Galbraith *et al.* 1987; Monpoeho *et al.* 2001; Karra and Katsivela 2007). Incidences can occur in plants (i.e. plant operator) or among residents in the plant serving catchment due to contacting faeces-contaminated surface water or food (Glaberman *et al.* 2002; Ueki *et al.* 2005; Stellacci *et al.* 2009; Laine *et al.* 2010). The majority of human diseases with diarrhoeal symptom are associated with microbially-contaminated water and food (Kosek 2003; Percival *et al.* 2003; Ishii and Sadowsky 2008). Exogeneous pathogens present in the ecosystem (i.e. surface waters, soil, air) can infect human, complete their life cycle and replicate in the host body, and they are shed from the

individuals to the municipal wastewater treatment system (Figure 1.2). At European level, water-borne and food-borne diseases, including acute gastroenteritis caused by pathogenic bacteria (i.e. Verotoxic *E. coli*, *Samonella* spp., *Clostridium* sp.), the protozoan *Cryptosporidium* and *Giardia* and viruses (i.e., norovirus and rotavirus), are challenging the policy and investments pumped into the care of human public health (Kosek 2003; HPSC 2007-2009) even though it is never an easy procedure to identify the contamination source. To do so, it is required that a well-established health care system, regularly-monitored and documented water supply and wastewater treatment, and environmental policy and governance to be in place.

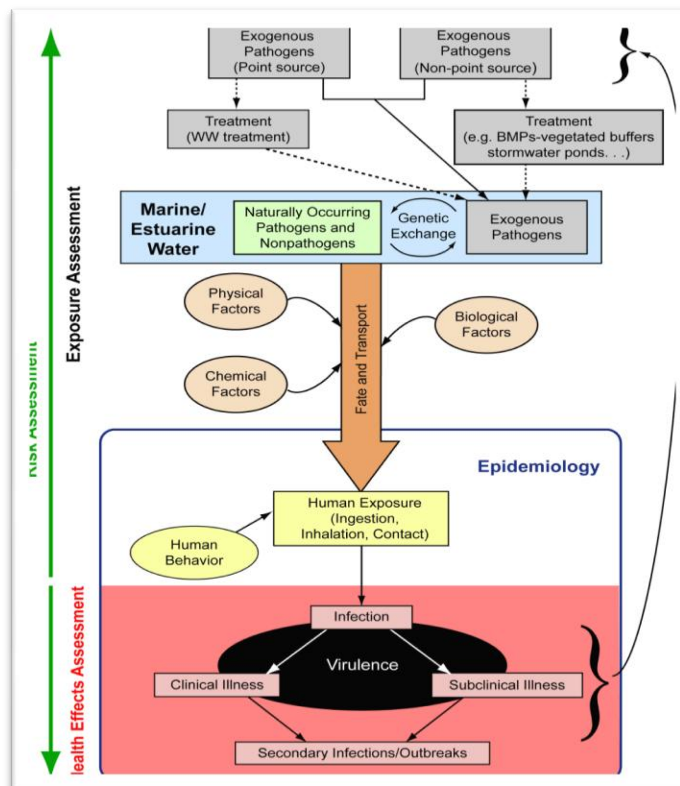

Figure 1.2. Relationship between exogeneous pathogens, WWTP, environment and human health (adapted from Stewart *et al.* 2008). Exogeneous pathogens may enter municipal WWTPs along with human sewage for the treatment process. Any residual pathogens present in the discharge effluent or biosolids from WWTPs may enter the water column to surface waters (i.e., river, lake, estuary or coastal water)(Graczyk *et al.* 2007; La Rosa *et al.* 2007; Katayama *et al.* 2008; Veronica 2008; Nordgren *et al.* 2009).

Since 1st January 2004, under the Infectious Diseases (Amendment) (No. 3) Regulations 2003 (S.I. 707 of 2003), cryptosporidiosis has been a notifiable disease in Ireland in all age groups following several outbreaks in the Midland region and County Westmeath between 2002 and 2003 (Garvey and McKeown 2002; Jennings and Rhatigan 2002; O'Tool *et al.* 2004; HPSC 2005). Although the urgent strategy on preventing raw sewage contaminating the supply of drinking water was sensed after the cryptosporidiosis outbreaks in England and Wales and Northern Ireland (Chamber *et al.* 1996; Glaberrnan *et al.* 2002), public awareness was aroused and a lesson learnt by the stakeholders and the government from the Galway cryptosporidiosis outbreak in 2007 (Irish EPA 2007, 2008; HPSC 2008). A specialist team involving the environmental investigators (HSE West Board), the local authority (Galway County Council), general practitioners, and the auditing agency (Irish EPA) were ordered to identify the contamination source and conduct risk assessment and report to the government, Irish EPA and the stakeholders. The preliminary report showed the outbreak was caused by *Cryptosporidium hominis* (HPSC 2007). Cases were clustered in areas supplied by water treatment plants which used water from Lough Corrib.

Even though no specific point source of contamination was identified, raw sewage-contaminated drinking water source in Lough Corrib was implicated as the potential source (Galway County Council 2007; HPSC 2007). In April 2008, the EPA published a Remedial Action List, a list of public water supplies which they consider required examination from source to consumer to determine whether replacements or upgrades were needed, or whether operational practices should be improved in the removal of microorganisms, including *Cryptosporidium* (EPA newsletter 2008).

Because drinking water is directly associated with infection cases, research on risk assessment of drinking water quality was conducted to evaluate health risk to Irish stakeholders (Coffeys *et al.* 2007; Pelly *et al.*

2007). Treatment failure due to inadequate infrastructure was found to be related to the occurrence of waterborne pathogens (i.e. *Cryptosporidium*) in drinking water, which resulted in outbreaks, such as cryptosporidiosis (Pelly *et al.* 2007). The work arising from this risk assessment exercise and the ongoing work on upgrading Group Water Schemes under the Rural Water Program led to a reduction in waterborne illness in Ireland in the following years. In another exposure-dependant risk assessment (Cummins *et al.* 2010), it was evident that each water supply system performed differently and a multi-barrier system including filtration and disinfection processes provided efficient *Cryptosporidium* inactivation. Besides, the annual risk of acquiring cryptosporidiosis for immunocompetent individuals by tap water consumption was below 1×10^{-4} per year, except under extreme contamination scenarios. Nevertheless, seasonal infection cases of cryptosporidiosis and other diarrhoeal diseases caused by human enteropathogens have been noticed nationwide. Solid evidence of transmission of *Cryptosporidium* in the surface water is still uncertain. WWTPs, which are designed to harbour wastewater from a whole agglomeration, can be a point contamination source. To this end, an investigation into the pathogen removal efficiency of WWTPs accompanied by a focus on the fate of the resulting end products (final effluent and sewage-derived sludge/biosolids), is important not only to identify the point sources of the pollution but also to secure a foundation for controlling and managing the spread of these pathogens to the environment.

1.2 Thesis Objectives

Even though numerous of WWTPs are designated and in place in Ireland under national and European legislation, there is a paucity of information on treatment efficiency for reducing human-virulent pathogens and their presence in potential infectious state in resulting end products. Current management systems also play an important part in ensuring the quality of wastewater treatment and waste generation in Ireland. In light of the continued threat of waterborne cryptosporidiosis in Ireland, the treatment efficiency of Irish WWTPs still remains in question. It is in this context that this study takes place. The objectives of this doctoral thesis were:

(a) To investigate the occurrence of human enteropathogens, including genogroup I and II noroviruses, *E. faecalis*, *E. faecium*, *C. parvum*, *C. hominis*, *G. duodenalis*, *E. bieneusi*, *E. hellem*, and *E. intestinalis* in domestic wastewater.

(b) To investigate the capability of Sligo secondary wastewater treatment plants (i.e. WWTP A- Strandhill, WWTP B- Collooney, WWTP C- Carney, and WWTP D- Tubbercurry WWTPs) in the removal of human enteropathogens over one year.

(c) To determine the relationship between faecal indicator bacteria (*E. coli*, enterococci and *C. perfringens* spores) and human enteropathogens and the potential use for pathogen prediction, especially *Cryptosporidium*.

1.3 Literature Review

1.3.1 Sewerage networks for wastewater treatment plants (WWTPs)

There are three types of sewerage networks: combined sewer systems, partially-separated sewer systems and separate sewer systems. Among them, the combined sewer system designed to dry out streets after rainfall or snowmelt, developed in 1855, has been used the most frequently, especially in the case of old towns established before the 1980s (Kirby and Laurson 1932; Cain 1972). The contemporary incarnation of the combined sewer was introduced in Europe in the 1840s when the cities of Hamburg and London began permitting discharge of sanitary wastewater into sewers, which were originally designed to convey only surface runoff (Burian *et al.* 1999). Starting in the 1950s, increased environmental awareness led to considerations as regards the effect of the urban drainage on the waters receiving treated sewage effluent. The combined sewer overflows were considered the main cause of degradation of the receiving waters (Mulliss *et al.* 1996; Burian *et al.* 1999; Butler and Davies 2000).

In dry weather, a combined sewer system transports an agglomeration's entire volume of wastewater to a WWTP. In wet weather, however, the resulting storm water is combined with domestic wastewater and transported to municipal WWTPs (Tibbetts 2005). Although a standard wastewater treatment plant is designed to treat up to three times the Dry Weather Flow of wastewater (DWF), problems can happen suddenly in the worst condition, e.g. heavy or continuous rainfall. In order to deal with such great amounts of diluted wastewater in wet seasons, a storm water chamber, therefore, is designed to temporarily store excess flow, which is also known as Combined Sewer Overflow (CSO) (EPA 1994). This fluctuates both in terms of the volume and the strength of wastewater. According to the National CSO Control Policy, combined sewer overflow consists of a mixture of domestic, industrial and commercial wastewater, and storm runoff (WEF 2010). Combined sewer overflow often contains high levels of

suspended solids, pathogenic microorganisms, toxic pollutants, floatables, nutrient, oxygen-demanding compounds, oil, grease, and other pollutants. The quality of CSO is variable and BOD values have been observed in excess of 7,500 mg/L (Mason 1991; Lee and Bang 2000). In addition, the entire wastewater stream is sometimes directly discharged to a watercourse as storm water if the on-site storage chamber is full or circulated back to the start of the treatment works (Mulliss *et al.* 1996; Moffa 1997). As a consequence, CSO can cause exceedances of water quality standards and poses risks to human health when discharged to receiving waters (O'Shea and Field 1992; US EPA 1994, 2001; Wojtenko *et al.* 2001).

In order to overcome fluctuations due to rainfall, separate or partially separate systems are allocated for new towns or satellite areas around old towns to separate the foul sewage and the street runoff (Burian *et al.* 1999). In contrast to one-conduit pipeline of a combined sewer system, a separate sewer system has two pipelines to carry storm water and foul sewage respectively (Figure 1.3). The foul sewage is piped to the wastewater treatment plant whilst the surface drainage (or storm water) is either bypassed to the receiving water without any treatment (storm water overflows) or is lead to the storm water tank for primary sedimentation (De Toffol 2006). In the study by De Toffol (2007), the separate sewer system with incorporated storm tank results in less pollution impact on the receiving waters but the whole system is generally more expensive than a combined sewer system (De Toffol *et al.* 2007). Wastewater enters the treatment plant via the sewerage pipelines, in the case of both separate and combined sewerage systems.

1.3.2 Characteristics of wastewater and WWTPs and the presence of human enteropathogens

WWTPs comprise physical, chemical and biological processes, depending on the requirement for the effluent standard as well as the nature of the

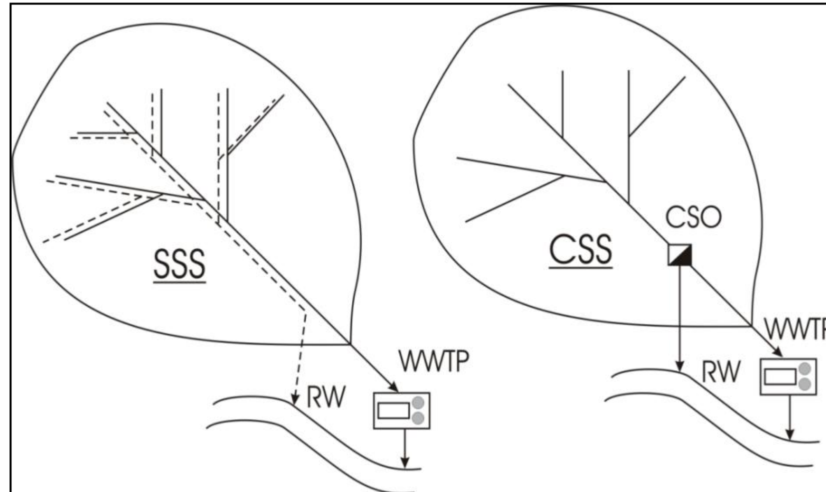

Figure 1.3. Illustration of separate sewer systems (Left) and combined sewer systems (Right) (Adapted from De Toffol, 2006). A separate sewer system (SSS) consists of two pipelines to carry urban runoff and foul sewage separately. Urban runoff is led to receiving waters (RW) with or without treatment whilst the foul sewage is led to a municipal wastewater treatment plant (WWTP) for physical and biological treatment. The treated effluent is then discharged to a receiving water body (RW). A combined sewer system (CSS) uses the same pipeline to carry storm water and foul sewage directly to WWTP. In order to reduce overloaded incoming wastewater into the plant, the excess water sometimes is discharged to RW to a receiving water body, resulting CSO-combined sewer overflow (De Toffol 2006).

wastewater. The aims of wastewater treatment fundamentally are to: (1) ensure that human-wastes are collected appropriately; (2) ensure the wastewater is effectively treated in terms of wastewater quality and the resulting bio-wastes are safely discharged to inland or coastal waters; (3) protect public health and aquatic life by reducing organic loadings and ; 4) recycle or reuse the valuable components in the end products, such as settleable solids as fertiliser and treated effluent for agricultural irrigation.

Wastewater composition is normally measured in terms of five-day biological oxygen demand (BOD_5), COD, TSS, and ammonia content. Other variables are also measured under specific circumstances, such as

total phosphorus, if the treated final effluent is to be discharged to inland water bodies or to sensitive areas (EPA 1991; Gray 2004). On average, the BOD_5 value of the influent is between 100 and 700 mg/L in Britain (Painter 1971). The compliant BOD_5 and suspended solids values of the effluent are 25 mg/L and 35 mg/L, respectively on Urban Waste Water Treatment Directive (CEC 1991). The concentration of organic matter produced per capita daily expressed in terms of BOD_5 is also known as population equivalent (PE). These variables are taken into account in the design of WWTPs (Vesilind 2003). Preliminary, primary, and biological treatment (secondary) processes are the standard operation of a modern municipal WWTP. Some advanced treatment plants are equipped with tertiary treatment infrastructures, such as constructed wetland, for further nutrient removal.

A healthy Western adult excretes approximately 135 g dry weight of faeces and 1,400 mL of urine per day, with 30% of biomass being bacteria. As they derive from the human gut, bacteria in foul wastewater are predominantly obligate anaerobes (Gray 2004). Even though the bacterial communities found in wastewater treatment plants are complex, the bacterial flora of all aerobic treatment systems are basically the same, including *Zoogloea*, *Pseudomonas*, *Chromobacter*, *Achromobacter*, *Acinetobacter, Alcaligenes*, and *Flavobacterium* (McKinny and Weichlein 1953; Fuhs and Chen 1975; Wagner *et al.* 1994; Lajoie *et al.* 2000; Oerther *et al.* 2000). In a study by Rosello-mera *et al.* (1995), 10% of the bacterial community in activated sludge floc were found to react to a fluorescein-labelled oligonucleotide probe complementary to small 16S ribosomal ribonucleic acid (16S rRNA) of *Zoogloea ramigera* 106(ATCC 19544), revealing the importance of this bacterium towastewater treatment (Wagner *et al.* 1995; Lajoie *et al.* 2000). These aerobic bacteria are able to oxidise organic compounds to carbon dioxide and water. Some bacteria can metabolise nitrogen, sulphur, methane or other compounds in wastewater

aerobically or anaerobically (Berthoux and Rudd 1977; Wagner and Loy 2002; Krul 2008; Kartal *et al.* 2010). *Escherichia coli* and the other faecal indicator bacteria (FIB) are commonly present in treatment systems but are not indigenous members of the microbial community (Berthoux and Rudd 1977; Loge *et al.* 2002).

The human gastrointestinal (GI) tract contains as many as 1014 individual bacteria, comprising over 500 different species (Possemiers *et al.* 2004; Manson *et al.* 2008) with the intestinal mucosa (microbiota) that plays a central role in host-commensal flora interactions between the immune system and the exogenous compounds or pathogens from the environment (Fuller 1989; Gibson *et al.* 1995). Human-virulent enteropathogens are agents which cause symptomatic or asymptomatic GI infection to human. Diarrhoea is the symptom that the human GI infections have in common (Casburn-Jones and Farthing 2004). Due to the human enteric component of domestic wastewater, human-infectious (or human-virulent) enteropathogens, for example, pathogenic bacteria (i.e., E. coli, *Clostridium difficile*, *Salmonella* spp., *Mycobacterium tuberculosis Campylobacter* spp.*)*, intestinal protozoa (i.e., *Cryptosporidium hominis* and *C. parvum*, *Giardia duodenalis, Acanthamoeba,* microsporidia) and enteric viruses (i.e., noroviruses, enterovirus, rotavirus) can also be found in the raw wastewater, derived from infected individuals in the community serving the plant (Robertson *et al.* 2000; Xiao *et al.* 2001; Sulaiman *et al.* 2004; van den Berg *et al.* 2005; Feng *et al.* 2009; Nordgren *et al.* 2009; Fernandes *et al.* 2011).

The plants and processes outlined below are specific to municipal secondary WWTPs, consistent with the core research of this project.

1.3.2.1 Preliminary/ primary/ treatment

Apart from faecal matter, incoming sewage contains suspended and floating debris, including paper, plastic, rags, as well as other waste process water

from industry or commercial activities. The variation of flow load is in relation to several factors, such as weather conditions and seasonal human activities. During high rainfall periods, there is a need to discharge the excess of storm water, which can increase up to 3 to 30 times the average DWF, to the WWTPs (Irish EPA 1995). Combined and semi-combined sewerage systems are designed to collect storm waters to bring with sewage to municipal WWTPs. When compared to the DWF loads, in the case of CSOs the influent mass loads to the WWTPs increased 10, 7, and 1.2 times for TSS, BOD, and nitrate (NH_3), respectively (Bertrand-Krajewski *et al.* 1995). The increased TSS in storm water was the effect caused by the flushed-out activated sludge, biomass produced from primary treatment and growing in these types of operation units (Bertrand-Krajewski *et al.* 1995; Giokas *et al.* 2002). Due to the influence of storm water on treatment efficiency, a storm overflow chamber and pipes dividing the overload flow are required for these plants (Figure 1.4, Stage I).

Cryptosporidium parvum, *C. hominis* and *G. duodenalis* are prevalent in the environment and can opportunistically infect hosts (animals/human) via their transmission stages, i.e. oocysts and cysts, respectively (Smith 1990; Robertson *et al.* 1992; Wolf 1992; McKenzie *et al.* 1994; Lucy *et al.* 2008). In the combined sewer systems, raw sewage and the overflows were found carrying detectable *Cryptosporidium* oocysts and *Giardia* cysts to urban streams, indicating that combined sewer wastewater treatment systems may significantly contribute to the load of *Cryptosporidium* and *Giardia* in ambient waters and source waters for recreational or drinking purposes (Gibson 1998; Garcia 2002; Graczyk *et al.* 2007; Robertson *et al.* 2000). Microsporidia have been associated with waterborne diseases on rare occasions, mainly happening to immunocompromised individuals (Curry and Smith 1998). Compared to the larger size of *Cryptosporidium* oocysts (3-5 µm) and *Giardia* cysts (6-12 µm), microsporidian spores are relatively small (1-5 µm), which makes them difficult to remove using a conventional

filtration system (Slifko *et al.* 2000). Nevertheless, after a one-year investigation, Fournier *et al*. (2000) pointed out that there was a low rate of water contamination by *Enterocytozoon bieneusi* (the most prevalent human-virulent microsporidia) and suggested that the risk of waterborne transmission to humans is limited.

Once the wastewater enters the treatment plant, the preliminary process containing coarse/fine screening and/or grit removal, plays a role as the first gate to remove the gross solids. A coarse screen commonly consists of steel bars spaced between 25-100 mm apart at an angle of approximate 50° to the effluent flow and positioned in an inlet chamber (Figure 1.4, Stage I). It can not only prevent the following treatment plant components from damage by the gross solids but also ensures the subsequent treatments are not impeded (Camp *et. al.* 1942). A fine screener, normally constructed of mesh materials, is introduced due to the capability to remove fine accumulated particles, in case of the fats, oils and grease, on the top of foul flows. This can reduce the loading of suspended solids to the next treatment stage. Flow velocity is another key element for effective preliminary treatment. If the velocity is too low, grit or other solid particles will settle out in the bottom of the grit chamber and the intercepted debris will be dislodged by the flow, in particular at the peak time. The suggested effluent flow rate through a screen should be maintained between 0.4-0.9 m/s (ASCE 1990; WEF 1998). Since the grit mainly comes from the surface drainage, any related problem only concerns the combined sewerage systems. It is estimated that 0.01-0.03 m^3/d of screenings may be produced in terms of a thousand PE. In the combined domestic sewer system, the quantity of grit ranges from 0.005 to 0.05 m^3 per 100 m^3 (Gray 2004).

Primary treatment is referred to as primary sedimentation. The instrumentation used in this process is sedimentation tank (Figure 1.3, Stage II). In accordance with the Urban Wastewater Directive (legislation in the Irish Statute Book under S.I. No. 419, 1994), 35 mg/L of suspended solids

is the acceptable load in the effluent to WWTPs under 2,000 PE, a ~90 % reduction over the influent. Regardless of shape (rectangular or circular), the objective of sedimentation is to slow liquid flow rate to a low point where the settleable organic matter can settle to the bottom of the tank. This can remove settleable organic matter and control the organic load remaining in the wastewater. As the sedimentation tanks are designed to operate on a continuous basis, the removal of solids must be processed continuously. In addition, scum may also accumulate on the surface of the liquid, an automatic scraper system on a continuous basis, therefore, is commonly used to remove both the settled solids and floating scum. Bacteria and viruses are not greatly reduced by primary settlement but some removal can be achieved i.e., 10% for *E. coli*, 60% for *C. perfringens*, and 33-67% for poliovirus (Berg 1966; Bonde 1977). However, oocysts and cysts of parasites can pass through primary sedimentation due to their small size and density.

1.3.2.2 Secondary treatment process

At this stage, the wastewater is treated by a variety of microorganisms with the aims: (1) to remove soluble and suspended organic substances; (2) to breakdown the existing pathogens by mechanical force; (3) to transform or remove nutrients, such as nitrogen and phosphorus. The microorganisms make up the activated sludge (also called mixed liquor suspended solids, MLSS, or activated biomass), including both aerobic and anaerobic bacteria, protozoa, rotifers, nematodes, and fungi (Curds 1975; Tomlinson and Williams 1975; Sharma 1977; Foissner and Berger 1996). Under an adequate supply of dissolved oxygen, the microorganisms can use soluble carbonaceous organic waste as food sources. Besides, some specific bacteria are capable of oxidising the nitrogen (nitrogen-fixing bacteria) or storing inorganic phosphorus (Nelson *et al.* 1978; Sedlak *et al.* 1991;

Wentzel *et al.* 1991; Wagner and Loy 2002). There is a wide range of particle sizes in the activated sludge mixed liquor ranging from individual bacterial of between 0.5 and 5.0 μm to large flocs that may be greater than 1 mm in diameter (Parker *et al.* 1971).

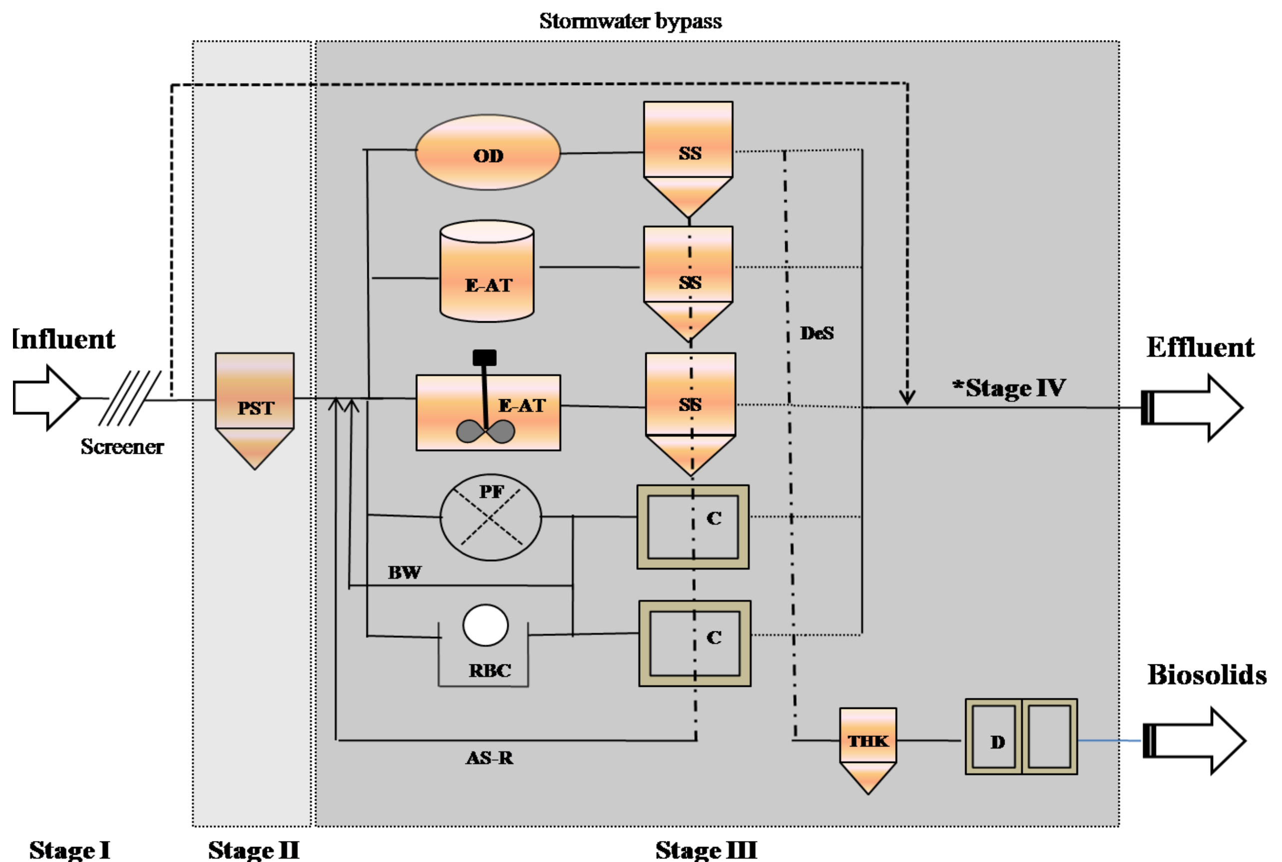

Figure 1.4. Structure of a secondary wastewater treatment plant (original figure). Influent wastewater is screened via screener and grit removal at Stage I (Preliminary). The liquid waste flows into a primary sedimentation tank (PST), if any, at Stage II and the resulting primary effluent is subjected to the biological treatment process, such as oxidation ditch (OD), extended aeration tank (E-AT), percolating filtration unit (PF) or rotating biological contactor (RBC). The backwash waste in PF and RBC system is collected and mixed with primary effluent. Part of the resulting liquid sewage sludge in Stage III returns to the biological treatment process, return activated sludge (AS-R) whilst the remaining sewage sludge (DeS, de-sludge) is taken from the bottom of the secondary settlement tank (SS)/clarifier (C), thickened (THK) and dewatered biosolids (D). The clarified final effluent wastewater is discharged to the receiving waters.

Depending on the form of the activated sludge present in the wastewater, the secondary treatment processes can be divided into two main categories: suspended growth and attached growth (biofilm) processes. In suspended growth processes, the dispersed bacteria flocs and free-living microorganisms responsible for treatment are suspended in liquid by mixing methods. The most commonly used suspended growth process for wastewater treatment is the activated sludge process consisting of two phases: aeration and sludge settlement (Metcalf and Eddy 1991). The main components of an activated sludge system include a reactor, activated sludge, aeration and mixing infrastructure, sedimentation tank, and return sludge pipeline (Figure 1.4, Stage III). Because this process involves the production of active microbial biomass, which can stabilise the waste under aerobic condition, the aeration system is essential to provide sufficient dissolved oxygen. Additionally, mixing is also crucial to maintain efficient oxygen transfer. Primary effluent is added to the reactor containing the mixed microbial population and organic matter. The reactor can be a tank, basin, ditch or a lagoon. Depending on the type of reactor, different terms are applied to specify employed biological reactors, such as aeration tank or oxidation ditch. In Ireland, most small to medium WWTPs built in the past 30 years have been based on extended aeration activated sludge systems, particularly the oxidation ditch type, which does often not include the primary sedimentation tank with the extra organic loading taken into account when designing the capacity of the secondary WWTPs (Gray 2004, pp 148-149).

The aeration has a dual function, to add air in the reactor for microbial respiration and to maintain the microbial flocs in agitated suspension and hence control the maximum contact between the surface of the floc and wastewater by mixing. Therefore, continuous aeration (if possible) is crucial to provide sufficient dissolved oxygen (0.5–2.0 mg/L) for microbial flocs and ensure adequate food source in the system.

With limited food (organic matters) and oxygen, high rates of microbial growth and respiration can be achieved, resulting in the utilisation of the organic matter present either as oxidised end-products (CO_2, NO_3, SO_4, PO_4) or the biosynthesis of new microorganisms. In wastewater, 64% of the total solids are soluble (<1 μm), whereas 34% are particulate matter including colloidal and fine particulate material (1 μm-100 μm), with 80% of the particulate fraction organic, compared with only 20% in the soluble fraction. Therefore, the major form of the organic loading to the activated sludge is in the form of colloidal material (~1 μm) or larger-sized particulate solids (Gray 2004, p 466). Aeration and mixing in the aeration tank is achieved by using either mechanical aeration (e.g. surface aerators using vertical or horizontal shafts) or by air diffusion (WEF 1988; US EPA 1999; Rao and Kumar 2006). For smaller plants, surface aerators are preferred (Chamber and Jones 1988). Surface aerators using vertical shafts or air diffusion are commonly employed in conventional and high-rate systems, while surface aerator using horizontal shafts are normally employed in extended aeration activated sludge system, for an example, oxidation ditch.

The oxidised end products can then flocculate to each other or to floc-forming bacteria to settle out. The flocculated biomass settles rapidly out of suspension to form a layer of sludge in the bottom of secondary settlement tank with the clarified effluent flowing on the top. In terms of sedimentation, size of individual floc of activated sludge and abundance of dispersed bacteria, especially filamentous bacteria, are important to separate sludge from clarified wastewater (Chudoba *et al.* 1973b; Eikelboom 1975; Eikelboom and Geurkink 2002). The maximum size of a floc is dependent on their cohesive strength and the degree of shear exerted by the turbulence created by mixing in the aeration tank (Chudoba *et al.* 1973a; Sezgin *et al.* 1978; Biggs and Lant 2000). Generally, there are two distinctive types,

microstructure (<75 μm in diameter) and macrostructure (> 75 μm) (Lau *et al.* 1984; Martins *et al.* 2003). Microstructured flocs are formed by aggregation and bioflocculation of floc-forming bacteria and are normally compact and in spherical shape. If not settled, this type of aggregated floc can easily be sheared into smaller particles in the turbulent conditions of the aeration tank (Sezgin *et al.* 1978). Under normal operating conditions, the population balance between filamentous and floc-forming organisms leads to well-settling flocs that are efficiently separated from effluent flow, producing a clarified effluent. However under certain conditions, the excessive growth of filamentous bacteria results in solids compaction and separation problems in activated sludge, called filamentous bulking (Wagner *et al.* 1994; Grady and Daigger 1999). High suspended solids concentrations in the effluent, overburden of waste sludge dewatering, and process failure caused by uncontrolled losses of biomass can also occur due to filamentous bulking (Jenkins *et al.* 2003).

The secondary-treated wastewater containing unsettled activated sludge floc then flows to a secondary settling reactor, a clarifier or a final settling tank, to achieve acceptable suspended solids concentration for discharge (Figure 1.4, Stage III). Modern sedimentation tanks are operated on a continuous-flow basis by means of classic Newton and Stokes Laws. Based on Newton's law, terminal velocity is yielded by equating the gravitational force of the particle (size-dependent) to the frictional resistance. Therefore, the rate of settlement of discrete particles varies with the particle size. Based on Stokes Law, the difference in density between the particles and fluid determines the state of particles, either suspended or settled (Metcalf and Eddy 1991). Settlement of activated sludge normally takes longer time as the flocs in secondary sedimentation tank are light and dispersed, which reduce the settling velocities (White 1976). For the activated sludge system, it is essential to return sludge solids from the settling tank or clarifier (Returned activated sludge, AS-R) to aeration tank

so that the microbial population can be maintained at the required level (Figure 1.4, Stage III).

In terms of microbial reduction, bacteria can be removed by grazing, inactivation, coaggregation, and absorption onto flocs (Curds 1968, 1971; Geldreich 1972; Bossier and Verstraete 1996; Olofsson *et al.* 1998; Soda *et al.* 1999, Malik *et al.* 2003). For instance, Drift *et al.* (1977) found the rapid adsorption of *E. coli* cells onto activated sludge floc occurred within the first hour of wastewater entering the aeration tank. Non-flocculating bacteria, such as *Acinetobacter sp.*, can coaggregate with other bacteria strains and settle (Zita and Hermansson 1997; Malik *et al.* 2003). Apart from absorption, the majority of removal mechanism of bacteria in the activated sludge process is predation by ciliate protozoans and rotifers (Curds 1968, 1971). Amoebae also play a significant role in the removal of bacteria by predation and are able to feed on flocculated particles (returned activated sludge) and freely suspended bacteria. In many studies, protozoan feeding has been reported to suppress the growth of bacteria, such as *E. coli*, *Campylobacter*, on a biofim-coated filter (Curds and Fey 1979; Pike and Carrington 1979; Curtis 2003). In studies by Scott, *Cryptosporidium* oocysts were also found to be predated on the filter by free-living ciliated protozoa in wastewater treatment systems (Scott *et al.* 2001, 2003).

Oocysts and cysts of parasites can be entrapped in the flocs and removed in the waste activated sludge. Removal rates are variable for different parasites and are dependent on the size and cell wall structure of the oocysts and cysts and other factors, such as zeta potential, which causes dispersion and clumping, and pH values of the microenvironment (Hsu 2000; Xagoraraki *et al.* 2003; Searcy *et al.* 2005). Clumping enables the pathogens to attract food, colonise, and evade host defenses. Hawkins *et al.* (2000) postulated that sedimentation can be accelerated by *Cryptosporidium* oocysts clumping with other suspended particles. In another study on three reservoirs in Biesbosch (Netherlands), storage with

long residence times (average 24 weeks) resulted in reductions of 2.3 logs for *Giardia*, 1.4–1.9 logs for *Cryptosporidium*, 2.2 logs for *Escherichia coli* and 1.7 logs for faecal streptococci (Ketelaars et al. 1995; van Breemen and Waals 1998). The removal efficiencies of spore-forming bacteria, such as *C. perfringens*, and *Cryptosporidium* oocysts and *Giardia* cysts are low due to their irreversible adsorption to sludge flocs (Wen *et al.* 2009).

Activated sludge flocs can adsorb amounts of poliovirus and enteroviruses with the assistance of human virus-binding proteins from bacterial proteins, providing the evidence that wastewater treatment processes are effective in removal of poliovirus and enteroviruses but are not be as effective in removing other viral groups, such as rotaviruses and noroviruses (Berg 1973; Safferman and Morris 1976; Farrah *et al.* 1978, 1985; Gerba *et al.* 1980; Sano *et al.* 2004; Mahjoub 2007). Viruses in the activated sludge process can be also inactivated, provided that the aeration of the wastewater continues and virus aggregation, solids attachment and settlement occurred in aeration tank and sedimentation tank (Glass and O'Brien 1980; Hejkal *et al.* 1981). Nevertheless, high residual concentrations of human enteric viruses, such as norovirus, enteroviruses and rotaviruses have been found in treated wastewater and biosolids (Lodder and de Roda Husman 2005; Ueki *et al.* 2005; da Silva *et al.* 2007).

In contrast to the suspended growth processes, the microorganisms are attached to an inert filter in attached growth processes. Percolating filter (or termed trickling filter in USA) is one of the most widely used fixed-film systems in secondary treatment process in Europe. Only the liquid section of the wastewater is subject to this process because the settleable solids may clog filters. In the first study of Jenkins in 1933, glassware was recommended as a medium in a percolating filter since the characteristics of glass didn't interfere with the methods commonly used for the analysis of nitrogen, phosphorus and potassium which have been key indicies of water quality (Jenkins 1933).

After more than 75 years of research on percolating filters, a wide range of applicable materials have been employed for packing medium, such as rock, gravel, slag, sand, redwood, and plastic or other synthetic materials. The filter consists of a graded bed (or plate) of hard material, the filter medium, about 2 meters deep (Bruce 1969; Pike 1978). The medium has interstices or voids that extend air exposure time and distribute incoming wastewater onto the surface of the bed or plate. The medium provides the base for the attachment of non-motile microorganisms, mainly bacteria and fungi, which form a biofilm (Cookes 1959; Mack *et al.* 1975; Andersson *et al.* 2008; Weber *et al.* 2009). Oxygen transfer in percolating filter is an important design criterion since BOD removal in excess of oxygen availability to biofilm can create anaerobic environment and cause odours (Logan *et al.* 1989). When the inlet stream of wastewater flows through the microbes-attached filtering medium, the present organic matter and nutrients are removed from the wastewater and form a biofilm on the surface of the filter. Basically, the wastewater is distributed over the top area of a vessel containing non-submerged packing plate and flows as a non-uniform liquid film over the attached biofilm (Williamson 1976; Randell 1992).

Size straining and particle adsorption are two main mechanisms responsible for immobilization of organic matter and microorganisms in fixed-film wastewater treatment systems and the effect is dependent on the size and morphology of the particles and grain size of porous media (Bales *et al.* 1991; Fontes *et al.* 2001; Bradford *et al.* 2004; Stevik *et al.* 2004; Marquet *et al.* 2007). Gannon *et al.* (1991) found that bacterial transport in porous media was significantly correlated to the cell size and cells with a length longer than 1μm being more efficiently removed. Based on an investigation on the transport of 14 different bacterial strains, long and rod-shaped cells were found more effectively removed through porous media (Weiss *et al.* 1995). In porous media where the pores are larger than the organic matter or microorganisms, the dominant mechanism for retention is

by adsorption (Sharna *et al.* 1985; van Loosdrecht *et al.* 1990). Percolating filters are effective in removing coliform bacteria and *Salmonella* with removal efficiencies ranging from 20 to >95% depending upon the operation (Tomlinson *et al.* 1962; Pike 1975; Feacham *et al.* 1983; Koivunen *et al.* 2003).

Once bacteria have been adsorbed onto the film, they are essentially removed and predated upon by grazing organisms. Nevertheless, percolating filtration is not very effective in the removal of parasites and and spore-forming bacteria but the mechanisms are still not well understood (Geldreich 1972; Sherman *et al.* 1975; Omura *et al.* 1989; Casson *et al.* 1990; Brush *et al.* 1999). However, intermittent sand filters, which have been used in wastewater treatment, were found efficient in removing *Cryptosporidium* oocysts from wastewaters even at high oocyst influent concentrations (Logan *et al.* 2004). Viruses and fungal spores are known to be removed by adsorption, which in the activated sludge process is more effective due the mixing in the aeration tank. In the percolating filter, there is far less chance of viruses and spores being attached to the film, especially under conditions of ponding and short-circuiting, when only a small portion of the available surface area of the medium is being used, or under high-rate loading when the retention time is short (Omura *et al.* 1989; Brusseau *et al.* 2005).

Other fixed-film systems, such rotating biological contactors, submerged filters, membrane bioreactors, reed beds, and high-rate filters, are also commonly used in secondary treatment processes either aerobically or anaerobically. The film that develops on the discs of either rotating biological contactors, submerged filters or membrane bioreactors consists of a complex and diverse microbial community including bacteria, filamentous bacteria, protozoans, and metazoans. A succession of protozoan species is seen on discs of these biofiltration reactors. This succession is similar to that observed in the activated sludge process (Kinner and Curds

1989): Bacteria colonisation →Flagellates →Amoeba →Free-swimming ciliates →Nematodes →Stalked ciliates →Rotifers. Rotating biological contactors are found to be the most efficient in the removal of bacteria even though they are the most expensive compared to other biological units (Sagy and Kott 1990; Griffin and Findlay 2000). The higher capital cost is normally uneconomic in serving populations greater than 50,000. Two main disadvantages including the higher costs compared to activated sludge systems and the larger construction area of land required makes the activated sludge systems more favourably employed at large municipal WWTPs (Jegar 1970; Parker 1999). Nevertheless, some advantages of percolating filter systems such as high tolerance on continual discharge of toxic industrial wastes and low energy consumption are reported (Table 1.1).

Similar to the activated sludge systems process, a clarifier or a final settling tank is employed to achieve acceptable discharge effluent. Excess biomass from the percolating filter tank can be settled on the bottom of the settling tank and removed periodically. Microbial growth or humus which is flushed from fixed-film reactors comprises of oxidised particles, mainly in the form of dense microbial film and also living invertebrates and associated debris. The volume of sludge produced by fixed-film reactors is much less compared with the activated sludge process (Open University 1975; Casey and O'Connor 1980). As for the treatment of the waste sludge, several methods, for example dewatering sludge in the use of chemicals (e.g. chlorine, lime treatment) or enzymes, exposure to sunlight in drying beds, ultraviolet light radiation treatment or off-site spreading, can be carried out. The water held in the sludge is either in free or bound forms and sludge of biological origin such as waste activated sludge are known to exhibit poor dewaterability (Best 1980; Sorensen *et al.* 1993; Keiding *et al.* 2001). The volumes of sludge and their solids content from primary and secondary treatment processes have been documented (Open University 1975).

Primary sludge from primary sedimentation is considered as thick liquid with moisture content of between 94-98%. Secondary sludge contains the solids washed out of the secondary treatment units, and either comprised of the wasted activated sludge or sloughed microbial film (fixed-film systems). Moreover, secondary sewage sludges are more stabilised than primary sludge with percolating filter sludge (humus) more stabilised than wasted activated sludge. Untreated sewage sludge from primary and secondary treatment units have high water contents (Table 1.2). To reduce the volume of sludge for disposal, the sludge is concentrated or thickened by gravity dewatering or thickening, heat treatment, lime stabilisation, sludge reed bed stabilisation, filter presses or composting (Bruce and Fisher 1984; Nielson 1990; CIWEM 1999; Samson and Elama 2000; Arthurson 2008). Biosolids are then produced (Figure 1.4, Stage IV).

1.3.2.3 Tertiary and advanced treatment

Ideally, the effluent resulting from the secondary biological treatment stage is expected to meet the 25:35 standard (25 mg/L of BOD_5:35 mg/L of suspended solids). In addition, the discharging effluent is expected to meet the 10:10 (10 mg/L of BOD_5:10 mg/L of suspended solids) and nutrient removal requirements for sensitive water body. However, the treatment efficiency can be affected by multiple environmental and microbial variables, such as dissolved oxygen, temperature, microbial consuming rate and microbial die-off rates. Therefore, a further treatment, tertiary treatment, to produce better quality of effluents is required. Most tertiary treatments are to designed remove suspended solids, nutrients (i.e. phosphorus and nitrogen) and also the associated BOD_5 and pathogens (Green *et al.* 1997; Gray 2004; Zhao *et al.* 2004). The most commonly used systems for tertiary treatments are: lagoons, sand filters, constructed wetlands, ozone treatment, activated carbon adsorption, ultrafiltration (pore size of 0.002 to 0.1 μm),

and reverse osmosis or disinfection of effluents to control residual nutrients and pathogens (Foster 1985; Vega *et al.* 2003; Pollice *et al.* 2004; Brusseau *et.* 2005; Lonigro *et al.* 2006; Ottoson *et al.* 2006).

For biosolids post-treatment, anaerobic digestion, alkaline stabilisation and composting are recently promoted around the world. According to UK Waste and Resources Action Programme (WRAP) report (Hogg *et al.* 2002), there has been an increase in the reuse and importance of sustainable biodegradable municipal waste around the world, in particularly in Europe due to its temperate climate and comprehensive environmental policies.

Regarding biosolids/sludge social and legal regulation in the EU 15 member states, sludge recycling is banned as impracticable in the minority of the member states (Luxemburg, Belgium, Flanders, Netherlands and Sweden) due to legislation or social opposition. In Finland, Denmark, Germany and Austria, even with notable recycling rates, the tendency is a rapid diminution, due to legal requirements and social and environmental factors. France and Sweden, by the year 2005 introduced national legislation to force post-treatment on resulting biosolids/sludge, before recycling or disposal. There are countries like Ireland, Portugal and Greece which have very low sludge yields with respect to its theoretical potential, due to the scarce development of its sanitation and wastewater treatment infrastructure (EC 1997; ADEME 1999). The leading recycling countries are France, Denmark, Great Britain and Spain, with approximate 50% land application rate.

In Ireland, industry compost standard is proposed to be applied on compost derived from biodegradable municipal waste materials, including biosolids from WWTPs (Prasad and Foster 2009). The standard includes regulations on two human pathogens: *Salmonella* (0 CFU/25 g) and *Escherichia coli* (1,000 CFU/g), which is consistent with the Animal By-Product (ABP) Regulations in Ireland (SI No. 252 of 2008).

Table 1.1. Comparison between activated sludge systems and percolating filter systems (Adapted from Gray, 2004).

	Activated sludge system	**Percolating filter system**
Capital cost	Low: average per system	High: average per system
Employed WWTPs	No PE limitation	More suitable for PE< 50,000
Area of land	Low	Large: 10 times more area required
Operating cost	High	Low
Nature of wastewater	Sensitive to toxic shocks, changes in loading, and trade wastewater; leads to bulking problem	Strong wastewater satisfactory, good tolerance to loading and toxic dischanges
Influence of weather	Works well in wet weather, slightly worse in dry weather, less affected by low winter temperatures	Works well in summer but possible ponding in winter
Technical control	High: the microbial activity can be controlled; requires labour skills and continuous operation	Little possible except process modification. Does not require continuous or skilled labour operation
Nuisance	Low odour and less fly problems. Noise may become a problem both in urban and rural areas	Moderate odour and severe fly problem in summer. Quiet during processes
Energy requirement	High: required for aeration, mixing, and maintaining sludge floc in suspension and for sewage sludge return	Low: natural ventilation and gravitation
Robustness	Maintenance on motors, not possible to operate without power supply	Very sturdy. Low maintenance is required, possible to operate without power supply
Final effluent quality	Poor nitrification but low in suspended solids except when bulking occurred	Highly nitrified, relatively high suspended solids
Secondary sludge production	Large volume, high water content, difficult to dewater, less stabilised	Small volume, less water content, highly stabilised

Table 1.2. Volumes of sludge and their solids content from primary sedimentation and secondary treatment processes (Adapted from Open University, 1975).

Type of sludge	Quantity (L/Ca./Day)	Dry solids (Kg/Ca./Day)	Moisture content (%)
Primary sludge	1.1	0.05 (4.5 %)	95.5
Percolating filter sludge			
Low-rate filter	0.23	0.014 (6.1%)	93.9
High-rate filter	0.30	0.018 (6.0%)	94
Activated sludge, surplus sludge	2.4	0.036 (1.5%)	98.5

1.3.3 Related Legislation and Regulations Associated with Wastewater Treatment in Ireland

Various laws and regulations at European Union and national levels are aimed to ensure wastewater treatment efficiency, especially relating to the reduction of organic loading in raw wastewater and to preventing environmental pollution to receiving waters. In order to protect the aquatic environment and to minimise the diffusion of pathogens by the effluent discharge and sludge disposal, relevant legislations have been introduced regionally (i.e. within EU and USA) or nationally in Ireland (WHO 1989; CEC 1991, 2006, US EPA 1992).

As an EU Member States, both wastewater treatment and waste handling in Ireland are bound to follow the policies introduced by the European Council. The quality of discharged effluent can affect the quality of receiving waters such as rivers, lakes, coastal waters, which are regulated by other water policies, such as the Bathing Waters Directive (76/160/EEC), Water Framework Directive (2000/60/EEC), and Shellfish Water Directive (2006/113/EC). The Water Framework Directive requires that at least 'good status' be achieved for all waters by individual Member State by 2015. For this purpose, Member States including Ireland had a river basin management strategy in place for each River Basin District by 2009. The integrated regulations on upstream water quality, including implementation of the Nitrates Directive (91/676/EEC) and the Urban Waste Water Treatment Directive (No. 91/271/EEC), contribute to the improved quality of downstream bathing waters and ensure public health safety.

The EU Urban Waste Water Treatment Directive makes secondary biological treatment mandatory for sewered wastewaters (CEC 1991) and was introduced as an Irish Statutory Instrument known as the Urban Waste Water Treatment Regulations in 2001 (S.I. No. 254 of 2001) and implemented in 2004 (S.I. No. 440 of 2004). According to the type of receiving waters which effluent discharged to and the size of serving

populations, the Urban Waste Water Regulations, 2001 set a minimum discharge limit to be achieved for wastewater treatment plants and a regime of monitoring by local authorities of discharge from wastewater treatment plants. Generally speaking, all legislation and regulations can be categorised to two main groups concerning (1) quality of final discharge effluent, and (2) production of biosolids and sustainable management strategy.

1.3.3.1 Regulations concerning wastewater treatment and final effluent quality in Ireland

The Urban Waste Water Regulations, 2001 composed of five Schedules, was introduced by the Department of the Environment and Local Government to give effect to the Directive of the European Parliament and of the Council of 23 October 2000 (2000/60/EC- Water Framework Directive) and to the Council Directive of May 1991 (91/271/EEC- Urban Waste Water Treatment Directive) as amended by the Commission Directive of 27 February 1998 (98/15/EC).

Sanitary authorities shall ensure that a treatment plant provided in compliance with the requirements of these Regulations is designed, constructed, operated and maintained to ensure sufficient performance under all normal local climatic conditions (Figure 1.5). Additionally, sanitary authorities should have ensured by 31 December 2005 that urban wastewater should be subjected to appropriate treatment before discharge. Wastewater from agglomerations with a PE of less than 2,000 should be discharged to freshwater and estuaries while those from agglomerations with a PE less than 10,000 should be discharged to coastal waters (S.I. No. 254/2001). More strict regulations are applied to the effluents discharging to sensitive receiving water bodies.

For collecting systems, the best technical knowledge should be applied notably regarding volume and characteristics of wastewater in

the serving area, prevention of pipe leaks, and limitation of pollution to receiving waters and human populations. Key parameters indicating treatment efficiency of WWTPs are mandatory to meet standards, such as BOD_5 (25 mg/L oxygen), COD (125 mg/L oxygen), and TSS (35 mg/L) of discharge effluent. In addition, minimum percentage of reductions on BOD_5 (70-90%), COD (75%) and TSS (90%) are also regulated (CEC 1991).

For the effluent discharging to sensitive areas, the compliance requirement also includes the value of total phosphorus (P) and total nitrogen (N). For total phosphorus, 2 mg/L P (WWTP serving PE 10,000-100,000) and 1 mg/L P(WWTP serving PE> 100,000) or 80% total phosphorus reduction are mandatory. For total nitrogen, 15 mg/L N (WWTP serving PE 10,000-100,000) and 10 mg/L N (WWTP serving PE> 100,000) or 70-80% total nitrogen reduction are mandatory. Furthermore, a minimum number of samples shall be collected at regular intervals and monitored by sanitary authorities according to the size of WWTPs (Table 1.3). The minimum annual number of samples is determined according to the size of the WWTPs as outlined in Table 1.3. The maximum number of samples which are allowed to fail the requirements, expressed in concentrations in Part 1 of the Second Schedule (S.I. No. 254/2001), is outlined in the Table 1.4. The permitted number of failures increases with additional sampling, up 25 failures for 365 samples. The Regulations do not apply to WWTPs with a population equivalent of less than 2000. For these plants, the EPA recommends that a minimum of 6 samples shall be taken.

The Irish EPA was given power by the introduction of the Waste Water Discharge (Authorisation) Regulations, 2007 (S.I. No. 687/2007) to assess discharge licence applications and grant the responsible sanitary authorities to control the treatment processing of WWTPs and the quality of resulting effluent and biosolids. Failure to meet the set out standards in the Urban Waste Water Discharge Authorisations will result in enforcement action by the EPA, including prosecution. Some WWTPs with poor

treatment efficiencies have been noted by the EPA and listed in annual quality of wastewater treatment report. The additional powers given to the EPA are believed to ensure better environmental performance from WWTPs and drive the improvement of receiving water quality. More recently the Irish government drafted a Programme for Government, which intends to take a national approach to improve quality of water services. A state-owned company, Irish Water, will take over the water investment and maintenance programmes of 34 County and City Councils to accelerate the pace of delivery of planned investment needed to upgrade the State's water and sewerage networks/systems, which are lacking at 32 agglomerations (DEHLG 2012).

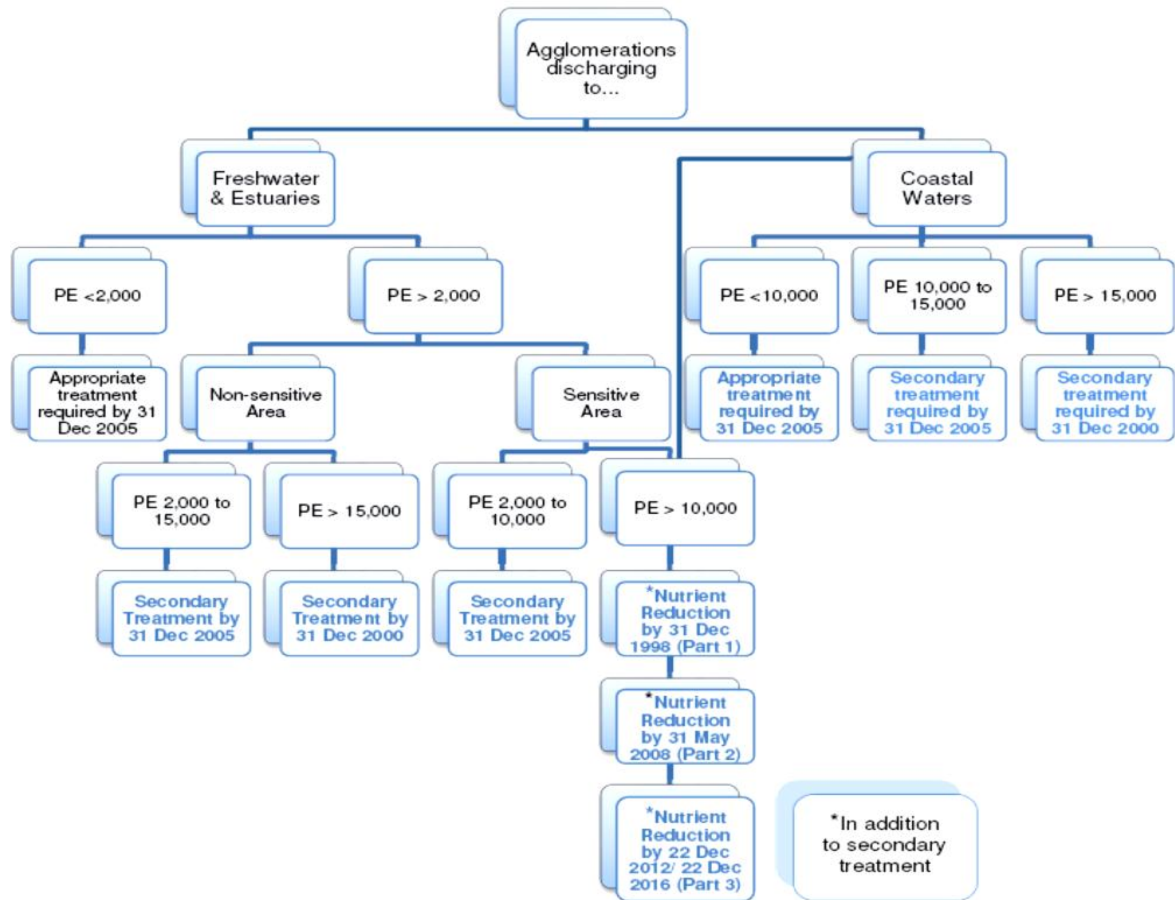

Figure 1.5. The implementation of the EU Urban Waste Water Directive (97/271/EEC) and Urban Waste Water Regulations 2001 (S.I. No. 254/2001). Specific dates and requirements are set for compliance by Member States. The type of treatment facilities and level of biological treatment required by the Directive for individual WWTP depend on the size of agglomeration (PE< 2,000, PE> 2,000, PE< 10,000, PE 10,000-15,000, PE> 15,000)and the type and status of receiving water bodies (Freshwaters and Estuary and Coastal waters; sensitive or non-sensitive). The level of treatment will depend on local circumstances and will vary from simple physical processes to physical/biological or physical/chemical processes with varying performance standards depending on the quality objectives of the receiving waters. More stringent treatment is required for agglomerations discharging to sensitive waters. The designation of ‘sensitive areas’ is a requirement of Schedule 5 of the Directive by reference to the identification criteria given in Annex II of the Directive. These criteria refer to three groups of sensitive areas: (1) freshwater bodies, estuaries and coastal waters which are eutrophic or which may become eutrophic if protective action is not taken; (2) surface waters intended for the abstraction of drinking water which contain more than 50 mg/L of nitrates; (3) areas where further treatment is required, to comply with other Council Directives.

Table 1.3. The minimum annual number of samples to be monitored and requirements by the Regulations

PE	Samples per year	Permitted No. of samples which fail to conform	Notes / Comments
500- 2,000	6	1	EPA recommended figure
2,000- 9,999	4	1	12 in first year and any year following a failed sample
9,999- 49,999	12	2	
>50,000	24	3	

Table 1.4. Maximum number samples of treated effluent allowed to fail the requirement (S.I. No. 254/2001)

Series of samples taken in any year	Maximum permitted number of samples which fail to conform
4-7	1
8-16	2
17-28	3
29-40	4
41-53	5
54-67	6
68-81	7
82-95	8
96-110	9
111-125	10
126-140	11
141-155	12
156-171	13
172-187	14
188-203	15
204-219	16
220-235	17
236-251	18
252-268	19
269-284	20
285-300	21
301-317	22
318-334	23
335-350	24
351-365	25

1.3.3.2 Regulations concerning sewage sludge handling and biosolids disposal in Ireland

A progressive increase of sewage sludge production from WWTPs, from 8 x 10^6 tonnes in 1998 to approximately over 10^7 tonnes in 2010, has been documented in the EU-15 Member States (Europa 2011). By definition, sewage sludge is residual sludge, either treated or untreated, from urban WWTPs. Sewage sludge in a treatment plant contains a high fraction of water with little percentage of solids material. After dewatering and other treatment, the remaining solids content with residual irremovable water is called biosolids (US EPA 1991). The term biosolids was introduced to reflect the beneficial characteristics of the residual solids generated from municipal WWTPs as current terminology perceive sewage sludge as a waste not a resource (Directive 2006/12/EC on waste).

European Directive and transposed Irish regulations encourage the reuse of final sludge (biosolids) from municipal WWTPs on agricultural soils, in composting, or for biogas production is (1) to encourage restitution to the biogeochemical natural cycle of recovered material, such as nutrient and metal contents; (2) to reduce ecological and economical cost to disposal in landfill or in thermo-destructive plants (i.e. incineration); (3) to fill the demand of preserving carbonaceous supply and fertility elements for soils. The European Union has introduced regulations for sewage sludge handling and disposal and use of biosolids in agriculture. The most important European guidelines are the Sewage Sludge Directive 86/278/EEC on the protection of the environment, specifically of the soil and Landfill Directive 1999/31/EC, when biosolids is used in agriculture. The Directive was transposed into Irish Statutory Instrument as the Waste Management (Use of Sewage Sludge in Agriculture) Regulations, 1998 (S.I. No. 148 of 1998) and Amendment Regulations, 2001 (S.I. No. 267 of 2001). All local authorities are now required to have local sludge management plans including structured measures in place. The Irish Regulations also

encourage the reuse of biosolids in agriculture which should be in line with this Department's Code of Good Practice for the Use of Biosolids in Agriculture (DEHLG, 1999) to ensure that the reuse of biosolids follow a few rules: (i) be compatible with good agriculture practice; (ii) not pose a risk to human, animal or plant health; (iii) maintain the integrity of the soil ecosystem; (iv) avoid water and air pollution; (v) minimise public inconvenience (DEHLG, 2008). Urban Waste Water Treatment Regulations, 2001 sets more stringent quality standards. Sludge arising from wastewater treatment shall be reused by whenever appropriate (S.I. No. 254 of 2001). All of the Directives and adopted Regulations are to meet one of the targets of Sixth Environment Action Programme to reduce final waste disposal by 20% compared with 2000 by 2010 and by 50% by 2050 (Decision No. 1600/2002/EC).

Before the Regulations were introduced in Ireland, a total of 63,369 tonnes of sewage sludge (Table 1.4) were produced during 2000 and 2001 nationally by WWTPs serving greater than 500 PE (IEPA 2003). Since sea disposal is prohibited by the Regulations, disposal of sludge to the marine environment has ceased in Ireland, with the exception of the sludge from Portrane WWTP (8,500 PE) in Co. Fingal. The EPA then advised that a sludge dewatering plant to be installed and that the practice of disposing sewage sludge to sea has then ceased (IEPA 2003). It was reported that 76,052 tonnes and 164,059 tonnes of sewage sludge were produced during 2004-2005 and 2006-2007 nationally (EPA 2007, 2009, 2012). Where final wastewater sludge (biosolids) is used in agriculture, the soil and sludge analysis for any treatment plant should be available from each local authority directly and presented in the Sludge Register (S.I. No. 267 of 2001). Agricultural application has been the most common route for sludge (biosolids) disposal (45% during 2000-2001, 63% during 2002-2003, 70% during 2004-2005, 77% during year of 2007, 62% during the year of 2009), followed by landfill on licensed private or public land until the year of 2009

(EPA, 2003, 2004, 2009, 2012). Some biosolids (up to 38% in the latest report period) were reported to be gone to composting or other unspecified application (Table 1.5).

Table 1.5. Final sewage sludge (biosolids) reuse and disposal routes and corresponding amounts (tonnes) and (percentage, %) between 2000 and 2007 (Adopted from EPA reports).

Years	Agriculture	Sea disposal	Landfill	Composting and other unspecified application	Total
2001	15,155 (45%)	0 (0%)*	18,052 (54%)	352 (1%)	33,559 (100%)
2003	26,743 (63%)	0 (0%)	14,909 (35%)	646 (2%)	42,298 (100%)
2005	45,543 (76%)	0 (0%)	10,292 (17.3%)	3,992 (6.7%)	59,827 (100%)
2006	60,019 (77%)	0 (0%)	8,536 (11%)	9,004 (12%)	77,648 (100%)
2007	60,232 (70%)	0 (0%)	4,554 (5%)	21,625 (25%)	86,411 (100%)
2009	66,194 (62%)	0 (0%)	63 (0.1%)	40,521 (38%)	106,778 (100%)

*Portrane WWTP sludge disposed to the sea was not documented.

1.3.3.3 Regulations and guidelines in respect of wastewater treatment and end products handling in other regions

In 1973, the WHO produced the publication 'Reuse of effluents: Methods of wastewater and public health safeguards', which provided guidance on how to apply the treated wastewater and excreta on agriculture and aquaculture activities. Since there is no comprehensive risk assessment on public health available, a thorough review of epidemiological studies and other new information on vector causing disease were implemented to the second normative WHO document 'Health guidelines for the use of wastewater in agriculture and aquaculture' in 1989 (WHO 1989). With the adoption of the Millennium Declaration, the United Nations (UN) established a comprehensive global framework to support poverty reduction and sustainable development, including 'Ensure environmental sustainability' and 'Develop a global partnership for development' (UN 2000). To meet the targets set by the Declaration, the WHO Guidelines was then further polished based on scientific consensus and best available evidence through broad participation (WHO 2006). The *Guidelines for the Safe Use of Wastewater, Excreta and Greywater* has been influential with respect to technical standard and also at the policy level. Many countries (e.g. USA, Australia, New Zealand, South Africa) have adopted or adapted them for their wastewater and excreta use practices and developing national policies (WHO 2006). Wastewater-related infections are communicable diseases whose causative agents (pathogenic virus, bacteria, protozoa and helminths) are released from the bodies of infected persons (or animals) in their faeces and urine. With the outcome of combined epidemiological studies and qualitative microbial risk assessment (QMRA), reduction of *E. coli* and helminth eggs counts in untreated wastewater for irrigation and associated verification requirement are recommended for health protection in the scenario of consuming raw crops (Table 1.6). Similar regulatory guidelines were adopted in Mexico, with no more 2,000 MPN/100 mL of

faecal coliforms and 5 Helminth eggs/ L for crop irrigation (Havelaar *et al.* 2001).

By employing human health and environmental risk assessments and adopting WHO guidelines, the US EPA summarises a variety of exposure pathways and evaluated two major exposure pathways through which human and environment may be affected by the surface disposal of biosolids (US EPA, 1994) in the development of the 40 Central Federal Register (CFR) Part 503 rule (Standards for the Use or Disposal of Biosolids). In addition to meeting pollutant limits and management practices, 40 CFR 503 regulates the land applied biosolids to meet either the Class A or Class B pathogen reduction criteria (Table 1.7). The criteria use a combination of technological and microbiological approaches to ensure adequate protection of human health and the environment from pathogens. Class A criteria is aimed to reduce the pathogens in biosolids to below detectable value, whereas Class B criteria is aimed to ensure that pathogen concentrations have been reduced to value that are unlikely to pose a threat to public health and the environment (McFarland 2001).

In Australia, the Environmental Protection (Scheduled Premises and Exemptions) Regulations, 1996 outlines the premises and activities that are scheduled and subject to works approcal and licensing provisions of the Environmental Protection Act, 1970 (EPA Victoria 1996). The Australian government also declared State Environmental Protection Policies (SEPP) to and compliance with the relevant SEPPs must be achieved for all activities that involve biosolids treatment and reuse (SEPP 2002, 2003). The *Guideline for Biosolids Application* is to ensure the generation of high quality biosolids (i.e. containing low pathogens) in the first instance. Frequently present pathogens in raw sewage have been included in the Guidelines, including helminths (e.g. intestinal nematodes, *Ascaris*, and tapeworm, *Taenia*), pathogenic bacteria (e.g. *Salmonella* and *Campylobacter*), protozoans (e.g. *Cryptosporidium* and *Giardia*), and

viruses (e.g. adenoviruses, hepatitis). Principally derived from the US EPA CFR503 Biosolids Rule, the Australian Guideline (EPA Victoria 2004) provides generic minimum quality assurance criteria and divided biosolids to three Grades (T1, T2 and T3) according to the detectable numbers of *E. coli*, *Salmonella*, and enteric viruses (Table 1.8).

The sanitation condition and regulatory guidelines are highly differentiated in Asian counties. In Japan, the relevant legislations concerning wastewater treatment include Basic Environment Law, Water Pollution Control Law, and Sewerage Law (JETRO 2012). The Water Pollution Control Law and Sewerage Law regulates the quality of discharge effluent from municipal WWTPs by three recommended methods: (1) Control of the pollutant concentration of wastewater (Effluent Standards); (2) Control of the pollutant concentration in water bodies (Environmental Quality Standards); (3) Control of the pollutant of wastewater in volume (Total Pollutant Load Regulations). The discharge effluent from municipal WWTPs, including industrial WWTPs, should be in compliance with parameters (e.g., temperature, pH, BOD, TSS, n-Hexane extract, Iodine consumption, phenol concentration) set in the Regulations (Okada and Peterson 2000) while there is no regulations on discharge microbiological standards. Water Pollution Control Act was introduced to Republic of China (also known as Taiwan) in 1974 and revised in 1991. As an industrialised country, the effluent quality control is regulated and the polluters are borne to be taxed based on the Article 11 of WPCA enacted in the Legislative Yuan (Taiwan Congress) on the discharge pollutants (TEPA 2007). The central sanitary authorities are responsible for collecting water pollution control fees from enterprises, sewage systems and households that discharge wastewater into surface waters based on both of the discharged waterquality and volume (TEPA 2007). The resulting biosolids is reused in a mix of composting for horticulture and co-incineration with municipal solids waste with ash reuse (McCann *et al.* 2006; He *et al.* 2007). With

regard to Peoples Republic of China, the access to official statistics on production and disposal of the produced biosolids is hindered. So far, there is no official regulation promulgated to particularly addressed to wastewater treatment, sludge treatment and disposal but a drafted regulation on urban drainage and wastewater treatment was announced by the Chinese government in December, 2011(He *et al.* 2007; Chinese State Council 2011).

Table 1.6. Revised WHO guidelines for verification monitoring in large-scale treatment systems of grey water, excreta and faecal sludge for use in agriculture (WHO, 2006)

	Helminth eggs (egg per gram of total solids or per Litre)	***E. coli* (CFU per 100 mL)**
Treated faeces and biosolids	< 1/g of total solids	< 1000/g of total solids
Grey water for use in		
Restricted irrigation	< 1/L	$< 10^{5}$ [a]. Relaxed to $< 10^{6}$.
Unrestricted irrigation of crops eaten raw	< 1/L	$< 10^{3}$. Relaxed to $<10^{4}$.

[a] These values are acceptable due to the regrowth potential of *E. coli* and other faecal coliforms in greywater.

Table 1.7. Maxmimum concentrations of pathogen permitted in biosolids (US EPA, 1993, 1999)

Classification	Indicators or pathogens	Permitted concentrations*
Class A Biosolids	Faecal coliforms	< 1 x 10^3 MPN/4 g of total solids
	Salmonella sp.	< 3 MPN/g of total solids
	Viable Helminth ova	< 1 MPN/4 g of total solids
	Enteric viruses	< 1 MPN/4 g of total solids
Class B Biosolids	Faecal coliforms	< 2 x 10^6 CFUs/g of total solids

*MPN: most probable number.

Table 1.8. Treatment grades based on microbiological criteria (EPA Victoria Australia, 2004)

Classification	Indicators or pathogens	Verified concentrations
Grade T1	*Salmonella* sp.	<1 MPN/50 g of total solids
	E. coli	<100 MPN/g of total solids
	Enteric viruses	≦1 PFU/100 g
Grade T2	*Salmonella* sp.	<10 MPN/50 g of total solids
	E. coli	<1,000 MPN/g of total solids
Grade T3	*E. coli*	<2,000,000 MPN/g of total solids

1.3.4 Current types of WWTPs in Co. Sligo and fate of the resulting end products

Wastewater treatment is essentially a separation process, consisting of concentrating and converting suspended and soluble nutrients into a settleable form (sludge) that can be separated from the liquid fraction of treated wastewater (effluent). The effluent is disposed to receiving waters whilst the accumulated sludge (biosolids) on the bottom of the operational units (i.e., primary settlement tank, secondary activated sludge unit, secondary sedimentation tank, clarifier) is transported from the wastewater treatment systems for disposal (landfill) or agriculture application with or without further sludge treatment.

Up to the time this project ended, there were 31 WWTPs in place in Co. Sligo (Table 1.9). Eight WWTPs received primary treatment (25.8 %) which was mainly comprised of primary settlement, septic tank, filtration beds and percolation area before the effluent was discharged. Approximately 3,535 people were serviced in the Environs of Ballinafad, Ballintogher, Ballybeg, Bunnanadden, Castlebaldwin, Drumcliff, Mullaghmore, and Rosses Point (Table 1.9). Twenty-two WWTPs (71%, ~18,140 population affected) have been upgraded to the secondary treatment level and eight of them received secondary treatment with nutrient reduction facilities (i.e. ferric dosing at Ballysadare, Curry, Dromore West, Geevagh, Gurteen, Monastraedan and Rockfield WWTPs or constructed wetland at Grange WWTP for phophorous/ammonia removal). Approximately 18,140 populations were affected in the Environs of Aclare, Ballinacarrow, Ballymote, Ballysadare, Carney, Cliffony, Cloonacool, Collooney, Coolaney, Culfadda, Curry, Dromore West, Easkey, Enniscrone, Geevagh, Grange, Gurteen, Monastraedan, Riverstown, Rockfield, Standhill and Tubbercurry WWTPs (Table 1.9). One tertiary WWTP, Sligo Main Drainage and WWTP, was commissioned in 2009 and mainly served Sligo town area (3.2%, ~28,000 population affected).

Table 1.9. Current treatment status of WWTPs in Co. Sligo 2012

WWTP	Treatment	Design PE	Actual PE[1]	Certificate/Licence (Authorisation no.) and contractor[2]
Aclare	Aeration tank with coarse bubble mixing and de-nitrification anoxic tank.	750	259	Sligo County Council (A0314-01). O&M contractor: EPS Ltd.
Ballinacarrow	Extended aeration package treatment (activated sludge)	250	203	Sligo County Council (A0318-01).
Ballinafad	Primary settlement, septic tank (anaerabic baffled reactor), Bord na Mona peat filtration beds (puraflo) and a percolation area.	150	192	Sligo County Council (A0311-01).
Ballintogher	Primary settlement, Bord na Mona peat filtration beds (puraflo) and peat filtration beds. Stormwater storage tank.	350	323	Sligo County Council (A0309-01)
Ballybeg	Septic tank and a percolation area	NA	28	Sligo County Council (A0315-01)
Ballymote	Extended aeration, clarifer, onsite sludge drying beds	3,000	3,547	Sligo County Council (D0094-01)
Ballysadare	Extended aeration, STM Hybrid activated sludge treatment with phosphorus removal, sludge thickening and holding chamber, Tidal Release Tank.	4,500	1,000	Sligo County Council (D0095-01). O&M contractor: EPS Ltd.
Bunnanadden	Septic tank, sludge drying bed	80	183	Sligo County Council (A0305-01).
Carney	Combined activated sludge and STM Aerotor (biofilm-coated) systems, Extended aeration	4,500	510	Application under assessment. O&M: Respond Ireland Ltd.
Castlebaldwin	Septic tank, Bord na Mona peat filtration beds and a percolation area.	100	107	Sligo County Council (A0348-01).
Cliffony	Aerated activated sludge treatment and settlement in the same tank.	450	597	Sligo County Council. Monitoring ceased from Aug 2008. Application under assessment.
Cloonacool	Aeration with coarse bubble mixing and sludge re-circulate to anoxic tank.	500	169	Sligo County Council (A0350-01). O&M contractor: EPS Ltd.
Collooney	Extended aeration, activated sludge treatment with 2 onsite sludge dewatering beds.	2,500	2,188	Sligo County Council (D0093-01).
Coolaney	Extended aeration, activated sludge. Final settlement tank.	250	883	Sligo County Council (A0350-01). O&M contractor: EPS Ltd.

[1]Actual population equivalent calculated from 2006 Census data. [2] Certificate or licence number registered with Environmental Protection Agency in 2009. NA: not applicable.

Continued Table 1.9

WWTP	Treatment	Design PE	Actual PE[1]	Certificate/Licence (Authorisation no.) and contractor[2]
Culfadda	Extended aeration, activated sludge. Final settlement tank.	150	108	Sligo County Council (A0317-01).
Curry	Extended aeration, activated sludge, filter beds for final treatment. The system is designed to remove ammonia and phosphorous from wastewater.	400	188	Sligo County Council (A0316-01).
Dromore West	Extended aeration comprising an aerator wheel. Ferric dosing to aeration tank for phosphorous removal.	2,500	306	Sligo County Council (A0313-01). O&M contractor: EPS Ltd.
Drumcliff	Septic tank, Bord na Mona peat filtration beds.	150	93	Sligo County Council (A0312-01).
Easkey	Extended aeration, activated sludge.	450	491	Sligo County Council (A0312-01). O&M contractor: EPS Ltd.
Enniscrone	Extended aeration tank comprising fine bubble diffusion.	5,000	936 (w) 4,130(s)	Sligo County Council (D0102-01). DBO: EPS Ltd.
Geevagh	Extended aeration tank comprising floor mounted membrane diffusers to release fine bubbles of air. Ferric dosing to a nutrient removal tank where the final effluent is stored.	250	216	Sligo County Council (A0347-01).
Grange	Extended aeration, activated sludge, constructed wetland for nutrient removal.	2,500	637	Sligo County Council (D0381-01).
Gurteen	Combined activated sludge and fixed-film systems. Ferric dosing to provide chemical precipitation of phosphorous from the effluent.	2,500	718	Sligo County Council (D0382-01). Application under assessment. DBO: Response Engineering Ltd.
Monastraedan	Package plant comprised of a circular inner tank (settlement) and concentric outer tank (aeration tank). Ferric dosing to provide chemical precipitation of phosphorous from the effluent.	400	111	Sligo County Council (A0310-01).

[1]Actual population equivalent calculated from 2006 Census data. [2] Certificate or licence number registered with Environmental Protection Agency in 2009. NA: not applicable.
W: winter season; S: summer season.

Continued Table 1.9

WWTP	Treatment	Design PE	Actual PE[1]	Certificate/Licence (Authorisation no.) and contractor[2]
Mullaghmore	Primary treatment comprised an inlet chamber, septic tank, and outlet chamber.	NA	1,200	Sligo County Council (D0239-01). Application under assessment.
Riverstown	Extended aeration, activated sludge.	600	475	Sligo County Council (D0383-01). Application under assessment.
Rockfield	Rotating biological contactor, settlement tank. Ferrous Sulphate dosing for phosphorous removal.	250	149	Sligo County Council (A0353). Application under assessment.
Rosses Point	Primary treatment comprised holding tank, septic tank and outlet pipeline.	NA	1,409	Sligo County Council (D0249-01). Application under assessment.
Sligo	Extended aeration with fine bubble mixing, activated sludge, sludge thickening chamber, anaerobic digestor, tertiary treatment equiped with a UV chamber for nutrient removal.	50,000	28,000	Sligo County Council (D0014). DBO: Anglian Water International.
Strandhill	Extended aeration, activated sludge, secondary settlement tank and dewatering beds.	4,500	1,887	Sligo County Council (D0107-01).
Tubbercurry	Primary settlement, Percolating biofilter, secondary settlement and dewatering beds.	5,000	2,561	Sligo County Council (D0092-01). DBO: Response Engineering Ltd.

[1]Actual population equivalent calculated from 2006 Census data. [2] Certificate or licence number registered with Environmental Protection Agency in 2009. NA: not applicable.

All the WWTPs in Sligo have been registered with the EPA and Sligo County Council had applied for licence/certificate for wastewater treatment and corresponding waste management by the Spring of 2010 (Table 1.9). The EPA under the 2007 Wastewater Discharge (Authorisation) Regulations, oversees the discharges from these facilities through the issuing of Discharge Licences and Certificates of Authorisation. Licences are required for larger discharges of >500 PE and Certificates of Authorisation are required for smaller discharges <500 PE.

As for the licence application, a detailed plant description including design capacity, upgrade record, operational facilities and the impact of resulting end products (final effluent and biosolids) to local environ is required. In order to assess the potential impact of final effluent to the receiving water and the regional river basin, three main indicators are taken into account, including the quality of the discharge effluent, the location of the discharge point and the quality and sensitivity of the receiving waters (i.e. Q value of river, Special Area of Conservation). The quality index of the discharge effluent from each WWTP and corresponding receiving waters were extracted from the official documents forwarded to the EPA by the year of 2010 (Table 1.10).

The purpose of the impact assessment is only to predict the potential influence of WWTP to the direct receiving water body but also to take the whole basin water district into account. For example, the impacts of the Collooney WWTP are directly on the Collooney River, which has been rated by the closest EPA monitoring station at Q value of 4. The Collooney River then emerges to Unshin River forming the Ballysadare River, which flows into Ballysadare Bay and in turn Sligo Bay. Although the Collooney River is not proposed as a National Heritage Area (pNHA) or a candidate of Special Area of Conservation (cSAC), the Unshin River, Ballysadare River and Ballasadare/Sligo Bay are designed by EC regulation as SAC, Special Protection Areas (SPA), and pNHA (Table 1.10).

Table 1.10. WWTP Final effluent and biosolids discharge location in Co. Sligo (from records provided by Sligo County Council, 2009)

WWTP	Effluent discharge to	Quality of discharge (Average)	
		Effluent[1]	**Receiving water[2]**
Aclare	**Eignagh River**, tributary of the **Moy River**.	pH: 7.6 SS: 98.2 mg/L BOD: 55 mg/L COD: 113.4 mg/L Total N: 33 mg/L Total P: 0.3 mg/L	**Upstream (170 m)** BOD_5: 3 mg/L Ammonia: 0.05 mg/L Ortho-P: 0.07 mg/L SS: 27.2 mg/L **Downstream (111 m)** BOD_5: 3 mg/L Ammonia: <0.01 mg/L Ortho-P: 1.15 mg/L SS: 16.8 mg/L **Q value: 4**
Ballinacarrow	**Ballinacarrow River**, discharge point is approx. 0.5 km upstream of the **Owenmore River,** which quality is monitored by EPA (used as receiving water quality). There are a number of sites of conservation importance downstream of the confluence with the Ballinacarrow River.	pH: 9.6 SS: 41.8 mg/L BOD: 78.6 mg/L COD: 126.6 mg/L Total N: 17 mg/L Total P: 0.41 mg/L	**Upstream (135 m)** BOD_5: 2 mg/L Ammonia: 0.2 mg N/L) Ortho-P: 0.03 (mg/L) SS: 7.6 mg/L **Downstream 100 m** BOD_5: 4.0 mg/L Ammonia: 1.6 mg N/L) Ortho-P: 0.04 (mg/L) SS: 10.0 (mg/L) **Q value: 4**
Ballinafad	**Ballymote groundwater body**. **Ballinafad River** (runoff from the percolation area), is approx. 0.5 km upstream of **Lough Arrow**.	NA	Ballymote ground water: **poor status** with a requirement to restore it to good status by 2015. **Balliafad River** **Upstream (100 m)** BOD_5: 3 mg/L Ammonia: 0.8 mg N/L Ortho-P: 0.08 mg/L SS: 10 mg/L **Downstream (140 m):** BOD_5: 3 mg/L Ammonia:0.01 mg N/L Ortho-P: 0.11 mg/L SS: 8 mg/L
Ballintogher	Unnamed local stream, upstream of **Lough Gill**.	pH: 7.6 SS: 48.6 mg/L BOD: 17.5 mg/L COD: 74 mg/L Total N: 17 mg/L Total P: 1.1 mg/L	**Upstream (41 m)** BOD_5: 2 mg/L Ammonia:0.49 mg N/L Ortho-P: 1.71 mg/L SS: 426 mg/L **Downstream (100 m)** BOD_5: 1 mg/L Ammonia:0.27 mg N/L Ortho-P: 0.13 mg/L SS: 25 mg/L
Ballybeg	**Groundwater** 2 km from the **Ballysadare Bay**, which is designated as an SPA, SAC and proposed NHA.	pH: 7.3 SS: 30.4 mg/L BOD: 62 mg/L COD: 95 mg/L Total N: 45 mg/L Total P: 1.24 mg/L	NA

Continued Table 1.10

WWTP	Effluent discharge to	Quality of discharge (Average)	
		Effluent[1]	**Receiving water[2]**
Ballymote	**Ballymote Stream**, tributary of the **Owenmore River.**	pH: 7.3 SS: 31 mg/L BOD: 33.6 mg/L COD: 88.7 mg/L Total N: 12.6 mg/L Total P: 3.7 mg/L	**Q value: 4**
Ballysadare	**Ballysadare Bay**; Stormwater discharge to **Ballysadare River and Estuary** (4 discharge points).	pH: 7.1 SS: 45.2 mg/L BOD: 23.2 mg/L COD: 67.8 mg/L Total N: NA Total P: NA	Ballysadare Bay is designated as an SPA, SAC and pNHA[3].
Bunnanadden	**Bunnanaden Stream,** discharge point is approx. 4 km upstream of **Cloonacleigha Lough**, which is designed as a cSAC and pNHA.	pH: 7.3 SS: 31 mg/L BOD: 33.6 mg/L COD: 88.7 mg/L Total N: 12.6 mg/L Total P: 3.7 mg/L	**Bunnanaden Stream** **Upstream (80 m)** BOD_5: 5 mg/L Ammonia:0.02 mg N/L Ortho-P: 0.19 mg/L SS: 10 mg/L **Downstream (170 m)** BOD_5: 3 mg/L Ammonia:0.01 mg N/L Ortho-P: 0.03 mg/L SS: 10 mg/L
Cliffony	**Cliffony Stream**, 50 m downstream designed as SAC and NHA.	pH: 7.3 SS: 78 mg/L BOD: 115 mg/L COD: 227 mg/L Total N: 40.6 mg/L Total P: 3.7 mg/L	**Upstream** BOD_5: <2 mg/L Ammonia:0.06 mg N/L Ortho-P: 0.009 mg/L SS: <2 mg/L **Downstream (170 m)** BOD_5: 10 mg/L Ammonia:2.2 mg N/L Ortho-P: 0.05 mg/L SS: 25.3 mg/L
Cloonacool	**River Moy**, which is a cSAC.	pH: 7.5 SS: 52.7 mg/L BOD: 33.9 mg/L COD: 68.6 mg/L Total N: 13 mg/L Total P: 0.29 mg/L	**Upstream (200 m)** BOD_5: <1 mg/L Ammonia:0.04 mg N/L Ortho-P: 0.02 mg/L SS: 32.4 mg/L **Downstream (100 m)** BOD_5: 3 mg/L Ammonia:0.01 mg N/L Ortho-P: 0.04 mg/L SS: 36.8 mg/L
Collooney	**Collooney River** and merges with the **Unshin River** forming the Ballysadare River which flows into Ballysadare Bay and in turn Sligo Bay.	pH: 7.5 SS: 13.3 mg/L BOD: 17.4 mg/L COD: 49 mg/L Total N: 9.5 mg/L Total P: 0.5 mg/L	**Q value: 4**

Continued Table 1.10

WWTP	Effluent discharge to	Quality of discharge (Average)	
		Effluent[1]	**Receiving water[2]**
Coolaney	**Owenbeg River**	pH: 7.7 SS: 2.5 mg/L BOD: 2.5 mg/L COD: 14.5 mg/L Total N: 11.2 mg/L Total P: 0.8 mg/L	**Upstream** BOD_5: <2 mg/L Ammonia:0.03 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Downstream** BOD_5: <2 mg/L Ammonia:0.03 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Q value: 4**
Culfadda	**Un-named River**, 2.7 km upstream of **Owenmore River**.	pH: 7.4 SS: 91.8 mg/L BOD: 168.5 mg/L COD: 363.5 mg/L Total N: 13 mg/L Total P: 0.6 mg/L	**Upstream (600 m)** BOD_5: 2 mg/L Ammonia:0.22 mg N/L Ortho-P: 0.87 mg/L SS: 2 mg/L **Downstream (250 m)** BOD_5: 3 mg/L Ammonia:0.19 mg N/L Ortho-P: 0.09 mg/L SS: 6 mg/L **Q value: 4**
Curry	Owengarve River	pH: 7.1 SS: 10.4 mg/L BOD: 12.4 mg/L COD: 57.2 mg/L Total N: 34 mg/L Total P: 1.4 mg/L	**Upstream (500 m)** BOD_5: 3 mg/L Ammonia:0.42 mg N/L Ortho-P: 0.49 mg/L SS: 3.2 mg/L **Downstream (100 m)** BOD_5: 2 mg/L Ammonia:0.39 mg N/L Ortho-P: 0.02 mg/L SS: 5.6 mg/L
Dromore West	Dunneill River, which is proposed as a pNHA.	pH: 7.5 SS: 13.3 mg/L BOD: 17.4 mg/L COD: 49 mg/L Total N: 9.5 mg/L Total P: 0.5 mg/L	**Upstream (1.1 km)** BOD_5: 2 mg/L Ammonia:0.1 mg N/L Ortho-P: 0.15 mg/L SS: 9 mg/L **Downstream (410 m)** BOD_5: 2 mg/L Ammonia:0.1 mg N/L Ortho-P: 0.15 mg/L SS: 1 mg/L
Drumcliff	Drumcliff River	pH: 7.1 SS: 1411 mg/L BOD: 4 mg/L COD: 18 mg/L Total N: 61 mg/L Total P: 0.3 mg/L	**Upstream (125 m)** BOD_5: 1 mg/L Ammonia:0.03 mg N/L Ortho-P: 0.01 mg/L SS: 7.2 mg/L **Downstream (100 m)** BOD_5: <1 mg/L Ammonia:0.02 mg N/L Ortho-P: 0.01 mg/L SS: 8.8 mg/L

Continued Table 1.10

WWTP	Effluent discharge to	Quality of discharge (Average)	
		Effluent[1]	**Receiving water[2]**
Easkey	Easkey River	pH: 7.4 SS: 20.5 mg/L BOD: 11.5 mg/L COD: 53.5 mg/L Total N: 12.5 mg/L Total P: 0.6 mg/L	**Upstream** BOD_5: <2 mg/L Ammonia:0.03 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Downstream** BOD_5: <2 mg/L Ammonia:0.02 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L
Enniscrone	**Kilalla Bay**, which is designed as a cSAC. Killala Bay/Moy Estuary designated as SPA and pNHA.	pH: 7.1 SS: 28 mg/L BOD: 31 mg/L COD: 99 mg/L Total N: NA Total P: NA	Kilalla Bay is rated at High Status.
Geevagh	Feorish River	pH: 7.3 SS: 57.5 mg/L BOD: 74.5 mg/L COD: 193.3 mg/L Total N: 7 mg/L Total P: 0.43 mg/L	**Upstream (81 m)** BOD_5: 3 mg/L Ammonia:0.09 mg N/L Ortho-P: 0.04 mg/L SS: 17 mg/L **Downstream (1.8 km)** BOD_5: 3 mg/L Ammonia:0.07 mg N/L Ortho-P: 0.06 mg/L SS: 5 mg/L **Q value: 4**
Grange	Grange River	pH: 7.6 SS: 137.5 mg/L BOD: 91.5 mg/L COD: 278.5 mg/L Total N: 32.4 mg/L Total P: 1.48 mg/L	**Upstream** BOD_5: <2 mg/L Ammonia:0.04 mg N/L Ortho-P: 0.02 mg/L SS: <2 mg/L **Downstream (410 m)** BOD_5: 2 mg/L Ammonia:0.03 mg N/L Ortho-P: 0.1 mg/L SS: 2 mg/L **Q value: 4-5**
Gurteen	Owenmore River	pH: 7.5 SS: 13.3 mg/L BOD: 17.4 mg/L COD: 49 mg/L Total N: 9.5 mg/L Total P: 0.5 mg/L	**Upstream** BOD_5: <2 mg/L Ammonia:0.04 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Downstream** BOD_5: <2 mg/L Ammonia:0.05 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Q value: 4-5**

Continued Table 1.10

WWTP	Effluent discharge to	Quality of discharge (Average)	
		Effluent[1]	Receiving water[2]
Monastraedan	Un-named Stream	pH: 7.7 SS: 13.7 mg/L BOD: 9.3 mg/L COD: 44.2 mg/L Total N: 3 mg/L Total P: 0.4 mg/L	**Upstream** BOD_5: 3 mg/L Ammonia:0.04 mg N/L Ortho-P: 0.13 mg/L SS: 23 mg/L **Downstream** BOD_5: 3 mg/L Ammonia:0.29 mg N/L Ortho-P: 0.12 mg/L SS: 10 mg/L
Mullaghmore	Donegal Bay	pH: 7.6 SS: 14.5 mg/L BOD: 13 mg/L COD: 35 mg/L Total N: 8.2 mg/L Total P: 0.6 mg/L	**Sea grab sample** BOD_5: 2 mg/L Ammonia:0.03 mg N/L Ortho-P: <0.02 mg/L SS: 59 mg/L
Riverstown	**Unshin River**, which is designed as Ulshin River SAC.	pH: 7.8 SS: 30.5 mg/L BOD: 42.5 mg/L COD: 54 mg/L Total N: 26.3 mg/L Total P: 1 mg/L	**Upstream** BOD_5: <2 mg/L Ammonia:0.04 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Downstream** BOD_5: <2 mg/L Ammonia:0.05 mg N/L Ortho-P: <0.02 mg/L SS: <2 mg/L **Q value: 4-5**
Rockfields	**Owenbeg River**, which is part of the Unshin River SAC.	pH: 7.5 SS: 16.7 mg/L BOD: 22.4 mg/L COD: 58 mg/L Total N: 9 mg/L Total P: 0.4 mg/L	**Upstream (100 m)** BOD_5: 3 mg/L Ammonia:0.06 mg N/L Ortho-P: 0.03 mg/L SS: 3.2 mg/L **Downstream (100 m)** BOD_5: <0.1 mg/L Ammonia:0.02 mg N/L Ortho-P: 0.56 mg/L SS: 4.8 mg/L **Q value: 4**
Rosses Point	**Sligo Bay**. The Cumeen Strand/ Drumcliff Bay cSAC comprises the entire coastal area within Sligo Bay.	pH: 7.4 SS: 36.5 mg/L BOD: 21.5 mg/L COD: 71 mg/L Total N: 19 mg/L Total P: 2 mg/L	NA
Sligo Drainage System	Garavogue Estuary	pH: 7.7 SS: 97 mg/L BOD: 92 mg/L COD: 223 mg/L Total N: 31.8 mg/L Total P: 3.9 mg/L	NA

Continued Table 1.10

WWTP	Effluent discharge to	Quality of discharge (Average)	
		Effluent[1]	Receiving water[2]
Strandhill	**Killaspug Point**, which is within Cumeen Strand/ Drumcliff Bay (Sligo Bay) SAC and within the Sligo and Drumcliff Bay NHA.	pH: 7.2 SS: 88 mg/L BOD: 61.5 mg/L COD: 150.5 mg/L Total N: 21.7 mg/L Total P: 4.9 mg/L	**NA**
Tubbercurry	**Tubbercurry River**, which is part of the River Moy SAC.	pH: 7.2 SS: 2 mg/L BOD: <2 mg/L COD: 17.5 mg/L Total N: 5 mg/L Total P: 0.04 mg/L	**Q value: 2-3**

[1]The quality of discharge effluent of WWTPs were extracted from the data enclosed in the WWDL application to the EPA by 2010; [2]The quality of receiving waters were extracted from the data enclosed in the WWDL application to the EPA by 2010; SAC: Special Area of Conversation, cSAC: Candidate of the Special Area of Conversation, NHA: National Heritage Areas, pNHA: Proposed as a National Heritage Area, SPA: Special Protection Areas. NA: Not applicable.

Table 1.11. Biosolids yields from WWTPs in Co. Sligo, 2006-2010*

Biosolids yield/year	Total amount (Tonnes)
2006	15
2007	8
2008	87
2009	72
2010	NA

* The data was extracted from EPA 2012 report*: Focus on Urban Waste Water Discharges in Ireland*.

With regard to the biosolids in Co. Sligo, a significant increase in production is documented (Table 1.11). Fifteen tonnes of biosolids (dried sludge solids) was produced in the year of 2006, followed by eight tonnes produced in the year of 2007. Ten times the amount of biosolids were produced in 2008 (87 tonnes) in Co. Sligo and the latest official figure of biosolids production (dried sludge solids) was 72 tonnes in the year of 2009 (Table 1.11).

During the period of 2008 and 2009, a total of 102,967 tonnes and 106,778 tonnes respectively of biosolids have been produced nationally by the registered WWTPs (EPA 2012). The destination routes for the sludge produced in 2009 are shown in Table 1.5. The use of biosolids in agriculture decreased from 70% of total solids arisings in 2007 to 62% in 2009. The disposal of sludge to landfill has reduced significantly from 5% to 0.01%. The other category mainly consists of composting but also includes other uses (i.e. land remediation in mine sites or biogas production). The use of biosolids in this area increased to 38% in 2009 from 25% in 2007. Where waste water sludge is used in agriculture the local authority must maintain a sludge register setting out details of the quantity of sludge produced and the quantity supplied for use in agriculture in their functional area, the composition of the sludge, the type of treatment which the sludge has undergone, the recipients of the sludge and the locations where it is to be used (S.I. No. 267 of 2001).

1.3.5 Public health and Environmental Concerns: wastewater enteropathogens, plant management, and impacts to the local population and the environment

Water quality issues throughout Europe have a lot in common, e.g. pollution from wastewater and agricultural sources (FWR 2005). However, local and regional water problems can present a quite diverse pattern, both with regard to quality and quantity. With the Urban Waste Water Treatment Directive, the EU has, for the first time in a comprehensive way, taken the nutrients dimension of water protection on board. The Directive provides for mandatory minimum design rules for sewerage system and treatment plants, which has contributed to an improvement of the quality of receiving waters. In some cases, however, scandalous delays of doing so lead to prevailing discharges of untreated or insufficiently treated wastewater (ECJ 2011).

A recent HSE and EPA joint report regarding the quality of drinking water from private wells in counties Sligo and Leitrim revealed a rate of 76% non-compliance with standards required under the Drinking Water Regulations of 2007. Coliforms, *E. coli*, Iron and Manganese were the parameters most commonly exceeded (EPA 2011). From a public health viewpoint, the presence of *E. coli*, which results from faecal contamination, is considered the most serious. The source of microbial contamination is not certain but the possibilities can include, but are not limited to, the downstream contamination by WWTPs and a deficit in animal waste and municipal biosolids management.

In accordance with the scope of this thesis, a brief overview on the occurrence of human enteropathogens, including pathogenic bacteria (i.e. antibiotic-resistant *E. coli* and enterococci), fungi (i.e. human-virulent *Encephalitozoon* and *Enterocytozoon*), enteric viruses (i.e. novoviruses) and protozoan parasites (i.e. *Cryptosporidium parvum*, *C. hominis*, and *Giardia duodenalis*) in the discharge effluent and the management of wastewater

treatment and biosolids is outlined in the following subsections.

1.3.5.1 Human pathogenic bacteria and fungi

Several studies have indicated the occurrence of human pathogenic bacteria and fungi in raw wastewater and treated effluent (Blanch *et al.* 2003; McGarvey *et al.* 2004; da Costa *et al.* 2006; Espigares *et al.* 2006; Loukiadis *et al.* 2006; Ahmed *et al.* 2007; Awais *et al.* 2007; Wilkes *et al.* 2009). Usually the isolation of pathogens from environmental samples is challenging due to the low proportion of pathogens relative to higher concentration of commensal microbes and inhibitors such as humic acid-like substances and fats. With the rapid development of molecular biological detection methodologies (e.g. polymerase chain reaction, selective culture media, immunological-based methods, biochemical test), direct/indirect detection of most commonly found bacterial and fungal pathogens can be performed to enhance disease surveillance and public health protection (Arbeit 1995; Tsai *et al.* 1993; Grimm *et al.* 1995; Stephenson 1997; Hui 2005). Moreover, local pathogen load is a crucial indicator of public health and might be estimated based on the number of pathogens present in raw wastewater of municipal WWTPs.

In Ireland, most of bacterial infectious intestinal diseases (IID) have been caused by diarrheagenic *E. coli*, *Salmonella*, invasive enterococci, *Campylobacter* and *Shigella* (HSPC reports years 2000-2010). Since the year 2001, antimicrobial-resistance enterococci (i.e. *E. faecium* and *E. faecalis*) were included in the European Antimicrobial Resistance Surveillance Network (EARS-Net). *Campylobacter* was the most prevalent pathogen clinically, with the highest notified infection cases (1,891 cases) in the year 2006 (Table 1.12). Most of the bacterial outbreaks were caused by the verotoxigenic *E. coli* (e.g. 45 outbreaks in the year of 2009; 199 notified infection cases), followed by *Salmonella* (e.g. 16 outbreaks in the year of 2009; 349 notified infection cases.

Table 1.12. Infectious intestinal diseases (outbreaks and (cases)) caused by bacterial pathogens in Ireland, 1999-2009 (HPSC annual reports years of 2000-2010)

Year[+]	Verotoxigenic *E. coli*	Invasive enterococci*		*Salmonella*	*Campylobacter*	*Shigella*	Total**
		E. faecalis	*E. faecium*				
1999	NA[++] (42)	NA	NA	(640)	NA[++]	(30)	(51)
2000	12 (52)	NA	NA	1 (428)	1 (1,288)	0 (28)	14 (1,796)
2001	11 (70)	168	85	1 (369)	1 (1,336)	1 (26)	14 (1,775)
2002	15 (88)	218	135	0 (486)	2 (1,568)	0 (36)	17 (2,142)
2003	7 (61)	242	187	8 (415)	2 (1,711)	0 (57)	17 (2,244)
2004	20 (134)	290	224	17 (349)	8 (1,803)	2 (36)	47 (2,322)
2005	30 (158)	294	265	20 (422)	7 (1,815)	3 (44)	60 (2,439)
2006	21 (167)	281	332	10 (456)	9 (1,891)	0 (43)	40 (2,557)
2007	42 (226)	301	406	22 (450)	7 (1,758)	4 (76)	75 (2,510)
2008	42 (241)	287	397	15 (333)	9 (1,808)	3 (71)	69 (2,453)
2009	45 (199)	298	392	16 (349)	2 (1,661)	3 (60)	66 (2,269)

[+]Years 1999-2002 data were extracted from national disease surveillance centre (NDSC reports 2000-2003) and years 2003-2009 data were from health protection surveillance centre (HPSC reports 2004-2010).
[++]In the year of 1999, *E. coli* O157:H7, *Salmonella*, *Campylobacter* and *Shigella* were not listed in the notifiable diseases.
*Invasive enterococci isolates of *E. faecalis* and *E faecium*, which may have antimicrobial resistance were included in EARS-Net surveillance from 2001.
**Total amounts of outbreaks and cases caused by verotoxigenic *E. coli*, *Salmonella*, *Campylobacter* and *Shigella* when data were available.

E. coli and enterococci are regarded as commensal microbes in human gastrointestinal tract. However, throughout the natural evolution cycle involving host, pathogen and the environment (Hughes 2008), verocytotoxigenic *E. coli* (including *E. coli* O157:H7) strains have been associated with several waterborne outbreaks. In Ireland, verocytotoxigenic *E. coli* (VTEC) caused the largest outbreak ever recorded in October/November 2005. VTEC are so-called because of their ability to produce one or both of two verotoxins (VT1 and VT2), VT1 being closely related to the shiga-toxin produced by *Shigella dysenteriae.* The WHO listed *E. coli* serogroups O26, O111, O103 and O145 as the four most epidemiologically important non-O157 VTEC serogroups (WHO 1998). The most renowned VTEC serotype causing illness among Irish populations is *E. coli* O157:H7, but other VTEC serogroups, for example, *E. coli* O26, O111, O103 and O145 are capable of causing the same spectrum of illnesses (HPSC 2005).

Eighteen *E. coli* O157 positive cases and one VT-2 positive case were identified in rural areas of mid-west Ireland (HPSC 2006). Half of these patients were recorded as asymptomatic. In addition, several culture-positive patients were linked to an exposure to private group water scheme in an agricultural area. It was suspected that the contamination source originated from environmental contamination of vulnerable drinking water, but was not proven (Mannix *et al.* 2007). Transmission through person-to-person was confirmed to be the main spreading route during the outbreak.

Not only the new emerging toxic strains, but the rise in antimicrobial resistance in invasive isolates of *E. coli*, *E. faecalis* and *E. faecium* is also a challenge to Irish public health (EARSS 2004; Murchan *et al.* 2006). In 2004, Ireland had the second highest proportion of vancomycin-resistant *E. faecium* (23%) in Europe, after Portugal's 42%. According to the latest report (HPSC 2010), 39.6% of the invasive *E. faecium* was found resistant to vancomycin whilst 0.3% of the invasive *E. faecalis* was vancomycin

-resistant. In addition, 95.6% of the *E. faecium* isolates were ampicillin-resistant and 8% of which were notified as multi-drug resistant strains. There is no solid answer to the significant increase in the antimicrobial-resistant strains in Europe but the obligatory presence of most antimicrobials in the gut (oral administration and bile elimination) may explain the surviving microbes by selective pressure exerted by antimicrobials (Barbosa & Levy 2000; Iversen *et al.* 2002, 2004). It is rather difficult to trace the exact transmission pathway from human end to the environment. Wastewater and the derived final effluent and biosolids from WWTPs might be favourable environments, consisting of variable mixtures of bacteria, nutrients and antimicrobial agents, for both survival and gene transfer (Lindberg *et al.* 2004; da Costa *et al.* 2006).

While micro-organisms such as *E. coli* O157 are not routinely monitored in water supplies, the presence of faecal coliforms (especially *E. coli*) indicates that the water has been recently contaminated with human and/or animal faeces (WHO 2008). Water that is known to be contaminated with faecal coliforms could therefore also be contaminated with *E. coli* O157. In 2004, two separate incidents were reported in Ireland where private wells contaminated with VTEC O157 were responsible between them for six confirmed cases of human VTEC illness. However, no VTEC infection cases have been directly linked to water supply in Ireland.

In EPA 2010 drinking water report, 95% of water supplies schemes in Ireland complied with the EU's maximum admissible concentration for faecal coliforms in drinking water. The quality of water in public water supplies and 'public' group water schemes in Ireland is high, with 98.8% and 99.1% complying with the European Union's maximum admissible concentration for faecal coliforms in drinking water of zero in 2010 (EPA 2011). Based on EPA's investigation, the concern centres on private group schemes and private wells, which serve a large proportion of households in rural communities (EPA 2004, 2011; CSO 2010). Septic tanks, slurry pits,

direct animal access to water sources, intensification of animal rearing and inappropriate spreading of animal manure have been identified as causes of pollution of group water schemes. Moreover, private group water supplies have also been found to be contaminated by adjoining septic tank systems, and also seepage has been known to occur into wells in wet seasons (EPA 2011).

1.3.5.2 Human noroviruses

In Ireland, a remarkable increase has been noticed in the number of outbreaks of viral or suspected viral infectious intestinal disease, mostly caused by norovirus and rotavirus (NDSC 2003, HPSC 2010). In a recent annual HPSC report, regional variation in all outbreaks was significant between HSE areas, with the highest rate observed in the HSE north-west area at 28.7 per 100,000 population (HPSC 2010). In addition, most of the outbreaks were attributed to norovirus.

Norovirus, formerly known as small round-like virus or Norwalk virus, is the causative agent of the well-known winter vomiting disease and classified within the family of *Caliciviridae*, with a 7.7 Kb single-stranded, positive-sense RNA genome comparative to other small round-like viruses (Green *et al.* 2000). Noroviruses are a group of genetically and antigenetically diverse RNA viruses (27-40 nm in diameter), which are the second common cause of acute non-bacterial gastroenteritis in humans worldwide, especially in children age group less than five years old (Kaplan *et al.* 1982; Cauchi *et al.* 1996; FSA 2000; NDSC 2003; Maunula & Bonsdorff 2005; Atmar & Estes 2006; Patel *et al.* 2008). The noroviral genome consists of three separate open reading frames (ORFs), encoding the non-structural proteins (ORF1), the capsid structural protein (ORF2) and a small basic structural protein (ORF3). The ORF1 (approx. 1,700 amino acids) encodes a large polyprotein which is cleaved by viral 3 C-like protease into probably 6 viral proteins, including 3 D-like RNA-dependent

RNA polymerase (RdRp polymerase), as found in other single-stranded RNA viruses (Lee *et al.* 1977; Jiang *et al.* 1993; Lambden *et al.* 1993; Belliot *et al.* 2003). ORF2 encodes the major viral capsid (~ 550 amino acids, VP1) and ORF3 is located at the 3' end of the genome and codes for a 212-amino-acids minor structural protein (VP2) of the virion (Jiang *et al.* 1990, 1993; Clarke & Lambden 2000, Green *et al.* 2001).

A standardised nomenclature was proposed to classify noroviruses into 29 genetic clusters that fall within 5 genogroups (Figure 1.6, GI-V). Among the genogroups, GI and GII are the most relevant strains to human disease, followed by GIV and GIII, which is distributed in pigs (Fankhauser *et al.* 2002; Hen *et al.* 2004; Lindell *et al.* 2005; Zheng *et al.* 2006). In A new open reading frame (ORF4) encoded by the murine norovirus sub-genomic RNA is found recently on the translation stage during virus infection (McFadden *et al.* 2011).

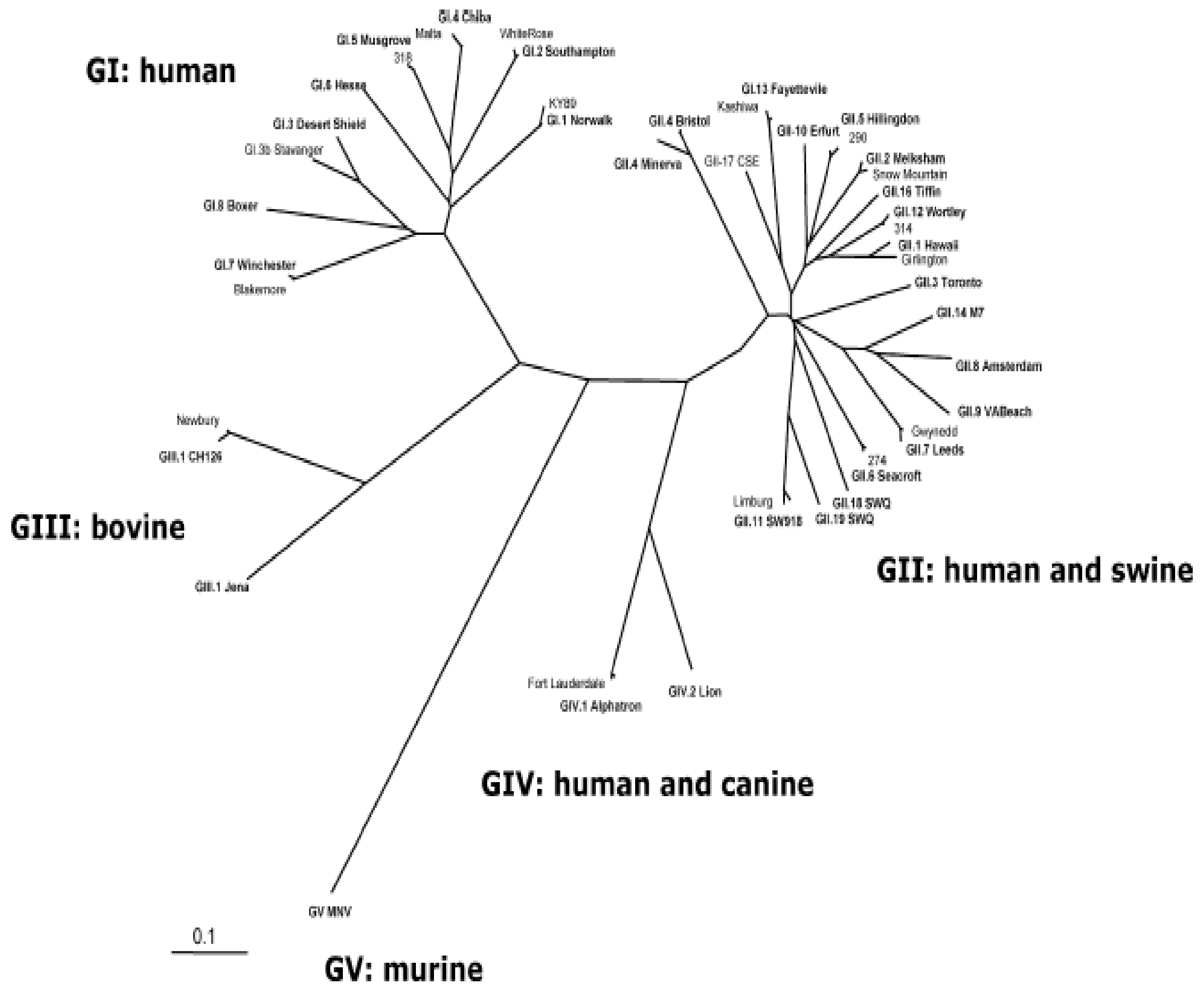

Figure 1.6. Classification of noroviruses in 5 genogroups (GI-V) by phylogenetic analysis of 32 norovirus strains in the complete capsid protein (VP1) (Adapted from Patel *et al.* 2008).

Vinje and Koopmans (1996) have shown that strains belonging to the same genogroup present more than 87% and 91% similarity to each other for GI and GII, respectively. On the basis of >85% sequence similarity in the complete VP1 genome, noroviruses can be classified further into genotypes, with at least eight genotypes belonging to GI (GI/1-GI/8) and 21 genotypes belonging to GII (Wang *et al.* 2005; Zhang *et al.* 2006). The GII/4 viruses (Lordsdale- or Grimsky-like GII/4 genotype) have predominated since the mid-1990s in the Europe, US and Oceania, causing 70–80% of all NoV outbreaks in communities, nursing homes, schools, hospitals and cruise ships associated with contaminated food or water. Since the application of molecular assays, noroviruses have been considered as the leading cause of epidemic gastroenteritis in all age groups, standing for >90% if non-bacterial and approx. 50% of all-cause epidemic gastroenteritis worldwide (Widdowson *et al.* 2005; Zheng *et al.* 2010).

The increase in norovirus outbreaks in health-care institutions in Ireland in 2002 led to the publication of guidelines by the HPSC for the diagnosis and management of outbreaks (NDSC 2003). The National Virus Reference Laboratory (NVRL) introduced reverse transcriptase–polymerase chain reaction (RT-PCR) for diagnosis of norovirus, which increased the recognition of noroviruses in patients' stool samples. In Ireland, the GII/4 genotype was also reported to be the dominant strains circulating in the years 2003 and 2004 (Waters *et al.* 2006).

In the north-west HSE region in Ireland, 33 outbreaks were recorded (one outbreak in a hospital in Sligo) and 487 people were infected before this project started (HPSC 2007). Clinical and epidemiological studies indicated person-to-person transmission of norovirus via contaminated environment, mainly contaminated food or water (Hewitt *et al.* 2007; Kelly *et al.* 2008). Based on their genetic proximity, potential zoonotic transmission or animal reservoir can be hypothesised as contaminant sources (Scipioni *et al.* 2008).

In a previous study published by NVRL, noroviruses were also identified as a major causative agent of pediatric gastroenteritis in Irish children population (≦5 years old), indicating that noroviruses were prevalent and can be the primary agent of childhood gastroenteritis (Waters *et al.* 2008). However, in most cases, the source of the contamination couldn't be identified due to the lack of library of environmentally isolated strains. The Irish IID outbreaks are dominated by human noroviruses (HPSC 2011a). In the third quarter of year 2011, 19 norovirus were confirmed and norovirus-suspected acute infectious gastroenteritis outbreaks via person-to-person or airborne transmission were reported to HPSC, standing for a majority of 74% of all general outbreaks (HPSC 2011b). Outbreaks of norovirus gastroenteritis are also associated with consumption of raw oysters (Parashar & Monroe; Doyle *et al.* 2002; Widdowson *et al.* 2005; Le Guyader *et al.* 2006; Westrell *et al.* 2010). Oysters are filter feeders and can concentrate the viruses from contaminated water, therefore are considered as an important biomonitor or disease transmission vehicle (Cheng *et al.* 2005; Comelli et al. 2008; Nenonen *et al.* 2008; Buenaventura *et al.* 2011).

Recent studies have also demonstrated that these viruses evolve over time in the VP1 sequence (recombinant variant strains), which allow evasion of immunity in the human population (Shieh *et al.* 2003; Lopman *et al.* 2004; Lindesmith *et al.* 2008; Kearney *et al.* 2007; Siegenga *et al.* 2009; Puustinen *et al.* 2012). Waters et al. (2007) also confirmed for the first time that a novel recombination event occurred within the norovirus RdRp region.

Although noroviruses are not originally considered as wastewater pathogens, more studies have recently demonstrated the occurrence of norovirus particles in raw wastewater and some cases of treated effluent and sewage-derived biosolids (Lodder et al. 1999; van der Berg et al. 2005; Katayama *et al.* 2008; Nordgren et al. 2009). Due to its low infectious dose

(~18-1000 virion) and lack of a valid vaccine (Ball *et al.* 1999; Guerrero *et al.* 2001; Teunis *et al.* 2008; Glass *et al.* 2009), the potential detectable and active noroviruses present in the discharge effluent and disposed biosolids may challenge the human population health.

1.3.5.3 Human-virulent microsporidia

Not many human-virulent fungal pathogens caused infectious intestinal diseases via the faecal-oral route (Khan *et al.* 2010). Human-virulent microsporidia (i.e. *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, *E. intestinalis*, *E. cuniculi* and *Nosema corneum*) are the most commonly detected species in the gastrointestinal tract or faeces of immuno-compromised individuals (Shadduck *et al.* 1990; Cotte *et al.* 1999; Weber *et al.* 1994; Didier 2004; Leelayoova *et al.* 2006; Espern *et al.* 2007; Willey *et al.* 2008). Microsporidia are small unicellular eukaryotic parasites that lack mitochondria, peroxisomes, Golgi membranes and eukaryotic ribosomal characteristic, with an extremely small genome size approx. 2.3 Mbp for *E. intestinalis* (Peyretaillade *et al.* 1998; Larssen 1999). Due to their prokaryotic-like feature, it indicates that microsporidia are phylogenetically very ancient protozoa with a core of gene pairs with conserved order (Vossbrinck *et al.* 1987; Larssen 1999; Corradi *et al.* 2007).

Since the first human microsporidial infection case reported in 1959 (Matsubayasi et al. 1959) and as a part of the evolving pandemic of human immunodeficiency virus (HIV) infection, microsporidia have gained attention as opportunistic pathogens of humans (Shadduck & Greeley 1989; Shadduck *et al.* 1996; Canning & Hollister 1992; Croppo *et al.* 19997; Weiss 2001; Bryan & Weber 2003; Didier 2005). Intestinal infection with *E. bieneusi* is increasingly recognized among HIV-positive patients with chronic diarrhoea where the prevalence is about 23-33% (CDC 2004). Sporadic infections can also occur in immunocompetent individuals (Nkinin *et al.* 2007; Didier *et al.* 2011; Yakoob *et al.* 2011). Symptoms of

microsporidial infection include being asymptomatic to having diarrhea, bronchitis, pneumonia, and sinusitis. Microsporidia can also cause bile duct pain and inflammation (Weber *et al.* 1994). *E. bieneusi* is associated with chronic diarrhea, unexplained weight loss, and cholangitis while *E. intestinalis* is associated with intestinal manifestations with or without disseminated symptoms (Cali *et al.* 1991; Eeftinck *et al.* 1991; Molina *et al.* 1993; Sobottka *et al.* 1998; Cotte *et al.* 1999).

The life cycle of microsporidia includes three phases: the infective phase (the spore stage), the proliferative vegetative phase (also termed merogony), and the intracellular sporogony.The 50% tissue culture infective doses ($TCID_{50}$) of *E. bieneusi*, *E. hellem*, and *E. intestinalis* have been determined as 15, 68, and 27 microsporidian spores, respectively (Johnson *et al.* 2003). The infective stage of microsporida is released into the environment in spore form. During transmission, stimulation of the spore is necessary to trigger the extrusion of the polar tubule. When digested, inoculation of infective spore content (termed sporoplasm) into a host cell can take place and then multiply intracellularly in the merogony stage. The infective offspring microsporidia are formed simultaneously as sporogony in the same infected host cell (Wittner & Weiss 1999).

Due to the electron-dense, glycoprotein-composed, and chitinous cell structure, microsporidian spores are relatively temperature insensitive, can survive in fresh and marine waters, and even after dehydration for extended periods of time (Didier *et al*. 2004; Li *et al*. 2003; Vavra and Larsson 1999) These characteristics indicate health risks due to transmission from the environment to various susceptible hosts, including humans and domestic animals such as rabbits, pigs, donkeys, and avian species. Microsporidian spores can be ingested or inhaled and then the infections are established predominantly in the small intestine (Garcia 2002; Graczyk *et al*. 2007d; Sak *et al*. 2008).

Considerable evidence to date indicates the involvement of water in the epidemiology of human microsporidiosis (Cotte *et al*. 1999; Dowd *et al*. 1998; Enriquez *et al*. 1998; Fournier *et al*. 2000; Slifko *et al*. 2000; Sparfel *et al*. 1997; Thurston-Enriquez *et al*. 2002). Microsporidian spores have been found throughout the bathing season in marine recreational waters (Graczyk *et al*. 2007c; Lucy *et al*. 2008) and in a range of lakes and rivers used for drinking water abstraction and recreation (Graczyk *et al*. 2004; Lucy *et al*. 2008). Discharge of effluents from wastewater treatment plants is an important point source of microsporidian spores in surface waters used for drinking water abstraction and recreation (Lucy *et al*. 2008); both *E. intestinalis* and *E. bieneusi* spores have been identified in such effluents (Dowd *et al*. 1998; Graczyk *et al*. 2007a). Microsporidian spores have also been detected in biosolids originating from WWTPs (Graczyk *et al*. 2007b). Microsporidian spores, range from 0.8 to 2.0 µm in size, and can pass through drinking water and wastewater treatment (Garcia 2002; Brusseau *et al.* 2005; Graczyk *et al*. 2007a,b).

1.3.5.4 Human pathogenic protozoan parasites

Some human protozoan parasites, such as *Cryptosporidium* spp. and *Giardia* spp, are known to be able to survive outside of the hosts for a long period of time in their transmission stage, oocysts and cysts, respectively (Tamburrini & Pozio 1999; Isaacs 2003).

Cryptosporidium is a protozoan enteric parasite, which infects epithelial cells of gastrointestinal tract of humans and animals, causing acute watery and profuse diarrhoea (also known as cryptosporidiosis) worldwide. To date, there are 30 valid named species of *Cryptosporidium* of which seven have been reported from human cases (Caccio 2005; Xiao *et al.* 2004). The first human cases of cryptosporidiosis were reported in the 1970s (Meisel *et al.* 1976; Nime *et al.* 1976). Tzipori et al. (1980) reported cryptosporidiosis in an immunocompetent adult, who suffered severe, water

but self limiting diarrhoea and vomiting. The AIDS epidemic has played an important role in the recognition and clinical management of *Cryptosporidium* because all of the oocyst-forming protozoa are important AIDS associated pathogens (Peterson 1993; Pape *et al.* 1994; Weber et al. 1994; Goodgame 1996). The sporulated oocyst is the exogenous stage, which is excreted from the body of infected host in the faeces. The oocyst wall of *Cryptosporidium parvum* is a trilaminar structure with an average thickness of 49 mm (Harris and Petry 1999). These thick-walled oocysts maintain viability of four internal sporozoites under adverse environmental conditions. The endogenous life cycle begins with the ingestion of a viable oocyst containing four sporozoites by a host (Figure 1.6).

Cryptosporidiosis is transmitted via the fecal-oral route by the oocyst stage. *Cryptosporidium hominis* is restricted to humans, while *C. parvum* is not only found in mammals but is also zoonotic (Caccio *et al.* 2005; Hunter and Thompson 2005; Thompson *et al.* 2008). Cryptosporidiosis has an incubation period of 5-7 days. During the large drinking water –related outbreak in Milwaukee, Wisconsin, the average incubation period was estimated from 3 to 7 days.

Three *Cryptosporidium* species are considered important human-virulent enteropathogens, namely *Cryptosporidium hominis*, *Cryptosporidium parvum* and *Cryptosporidium meleagridis* (McLauchlin *et al.* 2000; Morgan-Ryan *et al.* 2002; Peng *et al.* 1997; Xiao *et al.* 2001). *Cryptosporidium* has been identified in Irish river basins (Graczyk *et al.* 2004; Lucy *et al.* 2008).

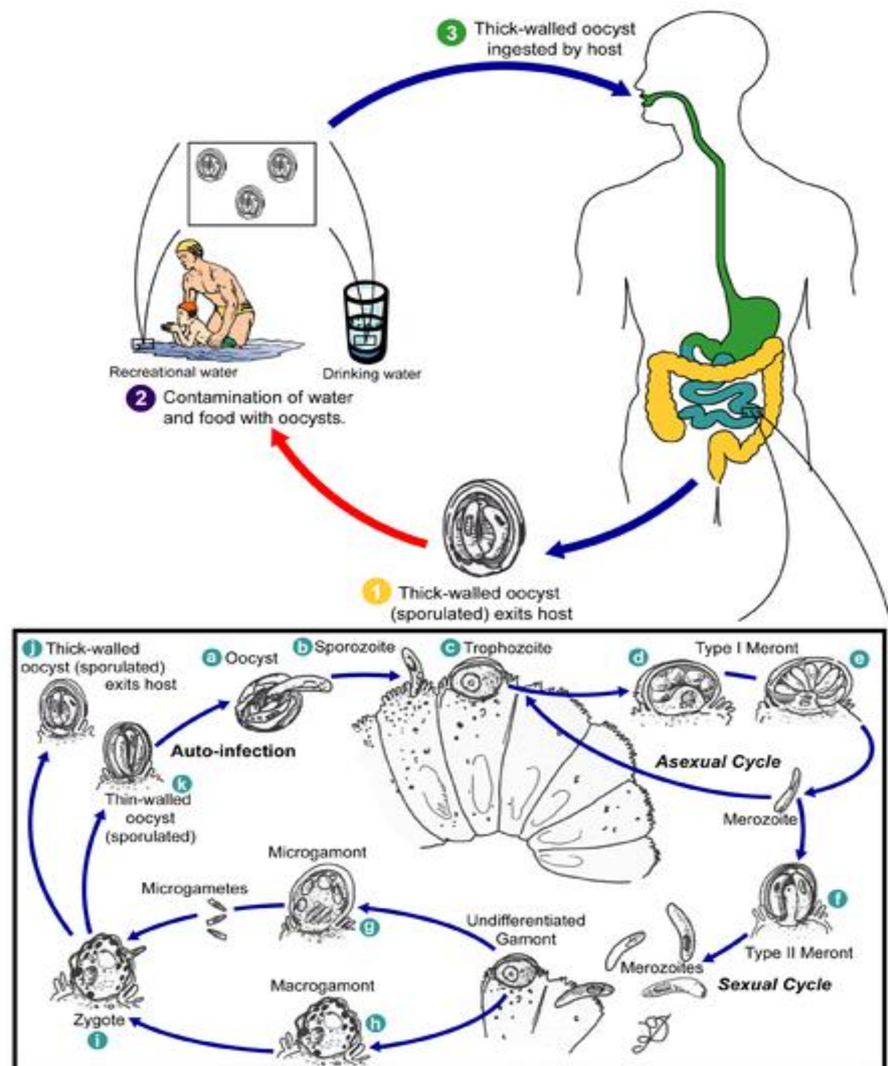

Figure 1.6. Life cycle and transmission route of *Cryptosporidium* (Adapted from CDC).

Giardiasis is one of the most common pathogenic intestinal protozoan infections globally. *Giardia duodenalis* is the most frequently identified etiologic agent in outbreaks associated with the ingestion of surface water, often due to ineffective filtration or pretreatment (Wolfe 1992). Cysts are ingested; these resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in the feces (diagnostic stages). The cysts are hardy and can survive several months in cold water. Infection occurs by the ingestion of cysts in contaminated water, food, or by

the fecal-oral route (hands or fomites). In the small intestine, excystation releases trophozoites (each cyst produces two trophozoites). Trophozoites multiply by longitudinal binary fission, remaining in the lumen of the proximal small intestine where they can be free or attached to the mucosa by a ventral sucking disc. Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in nondiarrhoeal faeces . Because the cysts are infectious when passed in the stool or shortly afterward, person-to-person transmission is possible (www.CDC.gov). *G. duodenalis* has been identified in Irish river basins (Graczyk *et al.* 2004; Lucy *et al.* 2008).

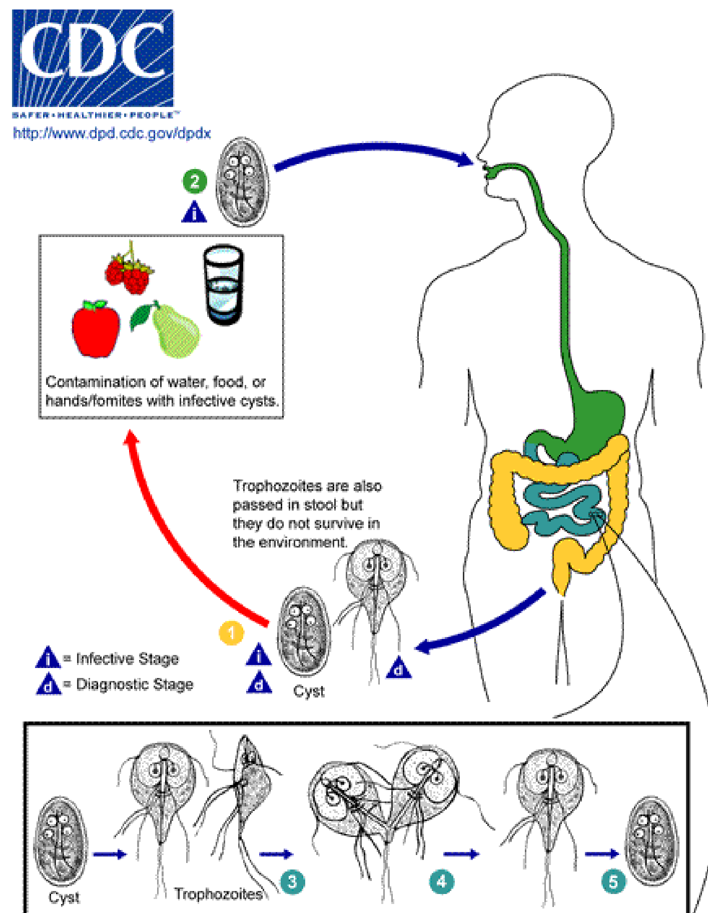

Figure 1.7. Life cycle and transmission route of *Giardia duodenalis* (Adapted from CDC)

Chapter Two
MATERIALS AND METHODS

2.1 Characteristics of Secondary WWTPs in the Project

In accordance with Sligo and Environs Development Plans 2004-2010, the Sligo sub-region Development Strategy, 2001-2021 established a framework to manage the growth of the Sligo Sub-region, including the towns of Strandhill, Carney, and Collooney included in this study, which was under pressure from urban-generated housing, owing to its proximity to Sligo City and its environs. Four municipal secondary WWTPs in Co. Sligo, including Strandhill WWTP (plant A), Collooney WWTP (plant B), Carney WWTP (plant C), and Tubbercurry WWTP (plant D) were investigated for this research thesis from 2008 to 2010: in the months of March, April, July, October in 2008 and monthly from January 2009 to February 2010. The geographical locations of the areas which individual plants are serving are shown in Figure 2.1. These four plants serve local settlements ranging from 1,100 to 2,500 PE with wastewater originating from houses, schools, hospitals, health care centres, and local business sectors (Table 2.1). All plants are designed as separated sewerage systems receiving only domestic wastewater, except for plant B, which accepts a combination of domestic sewage and proportional urban run-off in wet conditions (combined sewer).

2.1.1 Strandhill WWTP (WWTP A)

Strandhill WWTP (54°16’43”N, 08°36’20”W) is located at a coastal town-Strandhill, approximately 8 km from Sligo city. Strandhill is traditionally a seaside town, attracting tourism and recreational visitors. A business park has developed on the outskirts of Strandhill town and a regional airport is operated with daily links to Dublin (until August 2011) and to some cities in England. Strandhill WWTP was commissioned in 1993 and designed to cater for a population equivalent of 1,500. Based on an occupancy rate of 2.84 persons per house and the average number of 728.72 housing units in the area, the present day theoretical loading is 2,069 PE (Sligo County Council 2005). The Strandhill sewerage pipe system comprises of

approximately 6.4 km of fully separated sewerage pipelines. Strandhill WWTP includes an inlet chamber with coarse screen, storm overflow chamber, oxidation ditch (2 surface aerators), clarifier, sludge return sump, six sludge drying beds and a plant house (plants electrical switch boards and staff welfare facilities). When the influent enters the plant, it passes through a screen which removes any coarse materials. It then flows through a measurement flume to the oxidation ditch. The oxidation ditch incorporates two surface aerators, and has a design retention period of 24 hours. After the biological treatment process, the effluent flows onto the clarifier which promotes sludge settlement, and allows the final effluent to spill out over a weir. The final effluent is then transported to the outfall in Sligo Bay via the outlet pipe. The settled sludge in the clarifier is partially pumped back to the oxidation ditch to maintain the ML/SS ratio in the oxidation ditch and the remaining is collected and transferred onto the sludge dewatering beds to produce biosolids. There is a care-taker employed by county council in the operation and maintenance of the plant.

2.1.2 Collooney WWTP (WWTP B)

Collooney WWTP (54°11’11”N, 08°29’10”W) is located in a commuter-belt, stable residential town and was designed to cater for a population equivalent of 1,058. Based on an occupancy rate of 2.84 persons per house and the average number of 1,144 housing units in the area, the present day theoretical loading is 3,249 PE (Sligo County Council 2005). The Collooney sewerage pipe system is a semi-combined system taking part of the storm water and combined with the inflowing domestic wastewater to the plant. Collooney WWTP includes an inlet chamber, extended aeration tank, clarifier, sludge return sump, and two sludge drying beds. When the influent enters the plant, it passes the inlet chamber without screening and enters the rectangular aeration tank with one aerator operating at the centre of the tank. The extended aeration tank has a design retention time of 12

hours. After the biological treatment process, the effluent flows onto the rectangular clarifier for sludge settlement. The final effluent then flows through a pipeline to the Collooney River. The settled sludge in the clarifier is partially pumped back to the aeration tank to maintain the ML/SS ratio and the remaining is collected and transferred onto the sludge dewatering beds to produce biosolids. There is a care-taker employed by county council in the operation and maintenance of the plant.

2.1.3 Carney WWTP (WWTP C)

Carney WWTP (54°20'11.3"N, 08°31'44.3"W) is located in a stable residential town. The plant was commissioned in 2004 and designed for a population equivalent of 2,500. The average daily flow is 500 m^3/day with peak hourly flow in dry weather at 62.5 m^3/h. The sewerage pipe system in Carney is a fully separated pipeline system. At secondary treatment process, the plant is designed with a combined process, which uses a combination of the submerged fixed film process (with biofilm-coated STM-aerotor, 75% submerged) and the activated sludge system to treat approx. 138.96 m^3 of aerobic volume and 111.23 m^3 of anoxic volume of all tank unit in a 7.5 meter in length, 7.5 meter in width and 4.5 meter water-high circular aeration tank. The STM-aerotor operates at slow speeds driven by a low-powered motor and can provide consistent simultaneous nitrification/ denitrification, as well as biological phosphorous removal in a single basin (EPA export, 2011). Less waste sludge is produced with better dewaterbility. After biological treatment process, the effluent is transported to a circular clarifier tank with horizontal streaming, including sludge scraper, sludge hopper and inclination for sedimentation and simultaneous partial sludge stabilization on the bottom of the tank. The effluent is discharged to Carney River. The maximum return sludge ratio to the secondary biological tank is 83%. There is a care-taker employed by the private contractor for plant maintenance and operation and all the functioning data are recorded

automatically by meters.

2.1.4 Tubbercurry WWTP (WWTP D)

Tubbercurry WWTP (54°02'54"N, 08°43'28"W) was commissioned since 1970 and originally designed for a population equivalent of 1,400. With the growth of the town size, the loading was found considerably to exceed the designed value and the county council has upgraded the capacity of the plant to a load of 5,000 PE in 2009. The refurbished Tubbercurry WWTP includes an inlet chamber, fine screen screening and grit removal, storm overflow chamber, pumping station with two dry weather pumps and two storm pumps, ferric sulphate storage tank and bund, primary clarifier, final clarifier, sludge sump, Imhoff tank, two bio-film-coated percolating filtration systems, humus tank, sludge dewatering beds. The incoming raw wastewater is stored in a reservoir (inlet chamber) and discharged by gravity sewer to the pumping station sump. The inlet chamber to the sump is fitted with a manual cleaned coarse bar screen. The influent is pumped via pipe and injected with ferric sulphate to a primary clarifier for sedimentation. The tank is equipped with a rotating half-bridge sludge scraper. The clarified effluent is discharged over a peripheral notched weir into a peripheral collector channel and distributed onto plastic biofilm-coated media. The primary settled effluent flows to the secondary percolating filtration system. The secondary filtered effluent flows to secondary clarifier for sedimentation. The clarified effluent flows through rectangular humus tank (hopper-bottom with a floor slope of 30° to the horizontal) and discharged to the Tubbercurry River. The resulting sludge is primary sludge and transported to the onsite dewatering beds (8 beds) every 6 months. The plant is monitored by local county council.

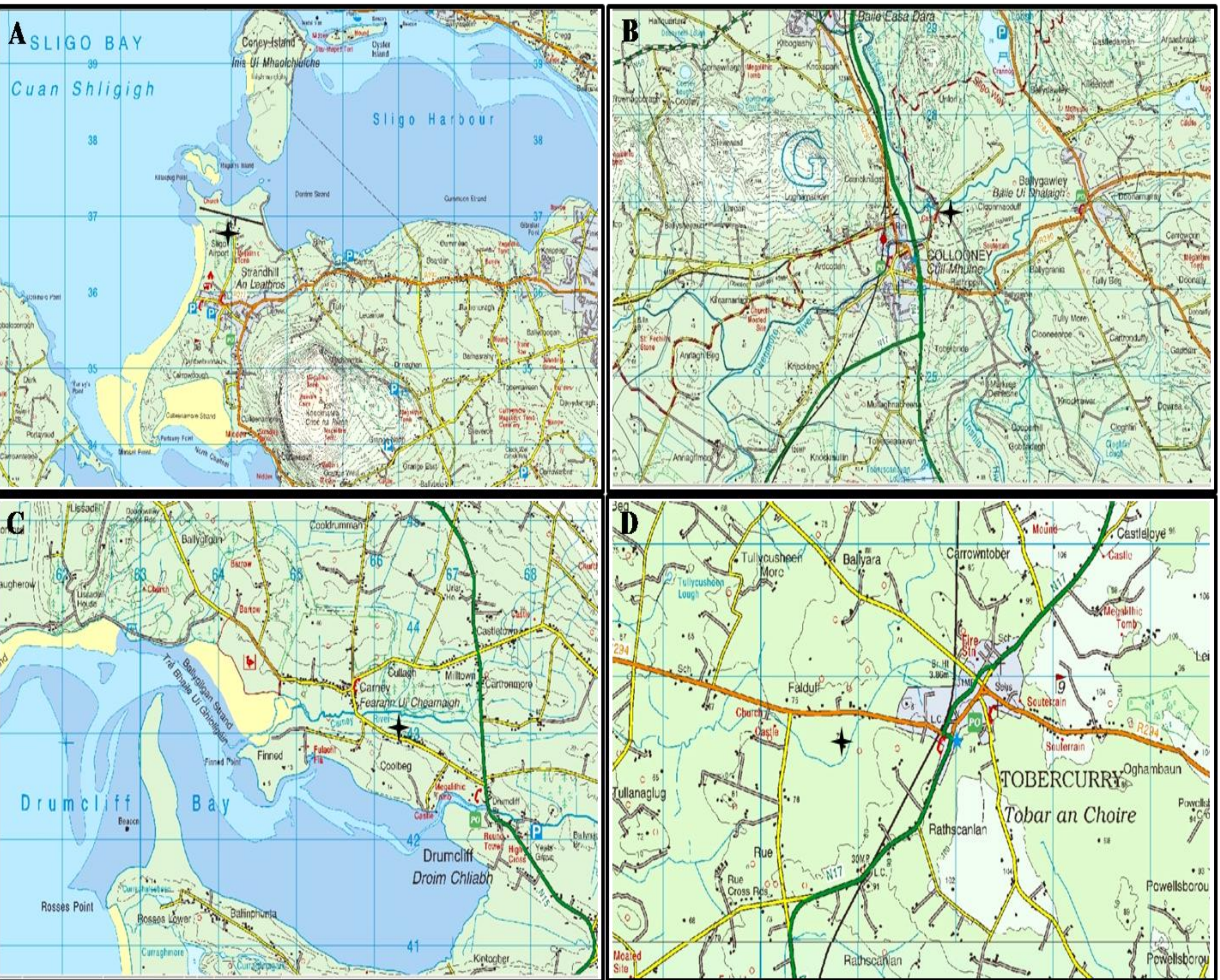

Fig 2.1. Plants A-D on maps of Ireland's North-West. Plant A is in Strandhill, plant B is in Collooney, plant C is in Carney, and plant D is in Tubbercurry. The symbol indicates the location of WWTPs A-D in the area.

Table 2.1 Characteristics of the four WWTPs in this project (Original table)

Type	Strandhill WWTP (plant A)	Collooney WWTP (plant B)	Carney WWTP (plant C)	Tubbercurry WWTP (plant D)
PE	1,500	1,058	2,500	5,000
Actual agglomeration	2,069	3,249	2,000	2,154
System	Extended-aerated activated sludge	Extended-aerated activated sludge	Extended-aerated activated sludge	Biofilm-coated filtration
Preliminary/ primary process	Coarse screen, grit removal	None	Coarse/ fine screen, grit removal, primary sedimentation	Coarse screen, grit removal, and two Imhoff tanks for primary sedimentation
Secondary process	Oxidation with oxygen (oxidation ditch), and sedimentation (clarifier)	Activated sludge, extended aeration (aeration tank), and settlementation (clarifier)	STM-Aerator, activated sludge, (aeration tank), and settlementation (settling tank)	Biofilm-coated filter, percolation, and settlementation (clarifier)
Chemical treatment	None	Polyelectrolite for coagulation	None	Ferric sulphate
Final waste product	Biosolids	Biosolids	Liquid sewage sludge	Biosolids
Yield annually	4.12 Tonnes (0.75% dried solids)	0.76 Tonnes (0.75% dried solids)	1.82 Tonnes (2.50% dried solids)	8 Tonnes (0.75% dried solids)
Waste application	Agriculture	Agriculture	Return to aeration tank	Agriculture
Effluent discharge	Sea	Collooney River	Carney River	Tubbercurry River
Management	Sligo County Council	Sligo County Council	Private contractor- Response Group	Sligo County Council

2.2 Sample Collection and Physiochemical Parameters

Grab liquid wastewater samples (i.e., influent, activated sludge, and mixed liquor) and treated end-products (i.e., final effluent and biosolids), originating from plants A, B, and D were collected during the study period. All wastewater samples were obtained using a long-handled 1-L capacity sampler and transferred to 1-L sterilised polyethene bottles. Approximately 100 gram of biosolids was collected by trowel from 10-cm in depth of the dewatering beds at plants A, B, and D, while 1-L of biological-treated liquid sewage sludge was obtained from plant C discharge valve at the bottom of the clarifier. All samples were collected in triplicate and transported to the environmental laboratory (Business Innovation Centre, IT Sligo) and stored at 4℃. Physiochemical parameters, such as temperature, pH, conductivity, dissolved oxygen (DO), and total dissolved solids of wastewater were measure on-site using portable meters (Sens-Ion, Hach Company, Colorado, USA). The liquid samples were shaken vigorously and were ready for microbiological analysis. Twenty grams of the biosolids was suspended in 1-L MilliQ water by vortexing and transferred to 1-L sterilised polyethene bottles and ready for tests.

2.3 Analysis for Faecal Indicator Bacteria

All the liquid wastewater samples (i.e. influent, activated sludge, effluent) were mixed vigorously and ten milliliter (mL) of the samples was transferred to a 15-mL sterilised conical plastic centrifuge tube, labeled as sub-samples, in triplicate. Twenty grams of biosolids sample was diluted 1:50 in MilliQ water (in triplicate) and vortexed. The sub-samples were mixed vigorously and 1-mL of the mixture was subjected to ten-fold serial dilution in Ringer’s solution (Oxoid, UK) to 10^{-4}. For *E. coli* and intestinal enterococci, two hundred microliters of the sub-samples was aseptically spread onto chromogenic coliform/*E. coli* agar and Slanetz and Bartley agar, and cultivated at 37℃ for 24 hours and 44℃ for 24-36 hours,

respectively. In accordance with the manufacturer's direction, pinky purple colonies on chromogenic coliform/*E. coli* agar (CM1046, Oxoid, UK) and burgundy red colonies on Slanetz and Bartley agar (CM0377, Oxoid, UK) were enumerated as positive for *E. coli* and enterococci, respectively. For *C. perfringens* spores, sub-samples (10 mL) for were heat shocked at 75°C for 20 min and then cooled on ice (Haudschild et al. 1974). Two hundred microliter of the heat shock-processed serial diluant was aseptically spread onto Perfringens agar (CM0587, Oxoid, UK) with supplementary D-cycloserine (SR0088, Oxoid, UK). Non-supplemented Perfringens agar was overlaid onto the base and settled at room temperature. The plates were incubated anaerobically in gas jars at 35± 2°C for 18- 24 h (Haudschild *et al.* 1974). Black colonies were enumerated as positive.

2.4 *Enterococcus faecalis* and *Enterococcus faecium* Identification

For *E. faecalis* and *E. faecium* detection, 10% of the positive colonies on Slanetz and Bartley agar were transferred individually using sterilised plastic needle to microcentrifuge tubes containing 100 µL of DNase and RNase-free water (Promega). To release the DNA from enterococci isolates, the mixture underwent 3 freeze-and-thaw cycles: heating in a dry heating block at 99℃ for 10 min then cooling in an ice box at 0℃ for 5 min (Hsu *et al.* 2006). The suspension was centrifuged (13,500 x g, 10 min) then the supernatant was transferred to new sterilised microcentrifuge tubes. 200 µL of the wastewater samples were subjected to total DNA extraction using DNA stool extraction kit (Qiagen, USA). Aliquots of the extracted DNA samples was subjected to identification first using enterococcal genus primers (Deasy *et al.* 2000), followed by a *sod*A gene targeted species-specific primers for *E. faecalis* and *E. faecium* (Jackson *et al.* 2004). The sequences of the oligonucleotide primers are listed in Table 2.2. Enterococcal cultures of *E. faecalis* (NCTC775) and *E. faecium* (ATCC19434) were used in this study as positive controls. Total genomic

DNA extracted from both culture of *E. faecalis* and *E. faecium* were used as positive controls in this study. Briefly, a DNA solution was added to a microcentrifuge tube containing 12.5 µL of 2X PCR buffer (Promega), 0.5µL of 1.0 µmol of primers (Integrated DNA Technologies (IDT), Inc., USA) and 7.5 µL of nuclease-free water (Promega). The reaction was carried out using a modified protocol (Deasy *et al.* 2000). Initially, the DNAs were denatured at 95℃ for 4 min, followed by 30 cycles of 95℃ for 30 sec, 50℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 7 min. A nested PCR was prepared with 1:10 diluted first-run PCR products and the sequences were amplified with species-specific primers targeting *E. faecalis* and *E. faecium* respectively. The reaction was carried out using a modified protocol (Jackson *et al.* 2004). Following an initial denaturation at 95℃ for 4 min, products were amplified by 30 cycles (35 cycles for *E. faecium*) of 95℃ for 30 sec, 53℃ for 1 min (*E. faecalis*) or 48℃ for 1 min (*E. faecium*), 72℃ for 1 min, followed by a final extension at 72℃ for 7 min. Five µL of product was mixed with 1µL of loading buffer (Promega) and electrophoresed on a 2% 1X Tris-acetate-EDTA agarose gel. The products were visualised by ethidium bromide staining and UV trans-illumination (Figure 2.2). Each fragment size was compared with DNA molecular weight marker (100 bp, Promega). Fecal samples from infected patients were used as positive controls. DNase-free water was used as negative control.

2.5 Norovirus Identification

The combination of US EPA Manual: Optimizing Molecular Methods to Detect Human Caliciviruses in Environmental Samples (Vinje 2008) and the protocol established by Katayama *et al.* (2002) was adapted for norovirus concentration. One liter of each wastewater sample was centrifuged at 7,280 x g for 15 min. The supernatant was transferred to a new tube whereas the pellet was resuspended in 10 mL of PBS and the

mixture was centrifuged (2000 x g, 15 min). The pellet was resuspended in 2 mL of PBS and stored at -20 ℃ (Vinje 2008). The two supernatants were combined and subsequently filtered through a glass fiber prefilter (AP15, Millipore, Ireland). The filtrate was adjusted with $MgCl_2$ to a final concentration at 0.05 M (Lukasik *et al.* 2000; Hsu *et al.* 2009) and then filtered through nitrocellulose membranes (HA series, 0.45 µm pore size, Millipore, Ireland) for adsorption using a glass filter holder unit (Millipore, USA) with a stainless steel screen. The stainless steel screen was cleaned and sterilised before use. The filter membrane was gently stirred in 10 mL of 1X PBS buffer (phosphorus buffer saline, pH 7.0) for 15 min (Hsu *et al.* 2009). The solution was mixed well and the aliquot was transferred to sterilised 1.5 ml microcentrifuge tubes and stored at -80℃ before use. Processed faecal samples from norovirus-positive patients were utilised for positive control.

The concentrated samples, biosolids pellet and norovirus-positive faecal samples were 1:10 diluted in nuclease-free water and transferred to silica column provided in the Viral Nucleic Acid Extraction Kit III (Geneaid, Taiwan), following extraction instructions provided by the manufacturer. All primers were listed in Table 2.2. The extracted RNA was then subjected to one-step reserve transcription-polymerase chain reaction (RT-PCR) with 5X reaction buffer, Enzymix containing DNA and RNA *Taq* polymerase (Invitrogen, USA), 100 nmol of primers JV12/JV13 (IDT, Inc., USA) and nuclease-free water (Promega). The reaction was carried out as follows: 1 cycle of 50℃ for 30 min and 95℃ for 15 min; 45 cycles of 94℃ for 30 sec, 37℃ for 1 min, 72℃ for 1 min; 1 cycle of 72℃ for 10 min (Green *et al.* 1998). The parallel semi-nested PCRs were conducted subsequently for norovirus genogroup I and norovirus genogroup II. Briefly, The PCR was carried out in a new microcentrifuge tube containing 1µL of each RT-PCR product, 2.5 µL of 10X PCR buffer (Invitrogen, USA), 0.4 µL of dNTPs (10 mM of each dNTP), 0.25 µL of Platinum *Taq* DNA

Polymerase (5 U/µL, Invitrogen, USA), 0.5 µL of primers JV13/GI (for norovirus genogroup I) and JV12/NoroII-R (for norovirus genogroup II) and 19.85 µL of nuclease-free water. The reaction condition was modified from Boxman's protocol (Boxman *et al.* 2006). Briefly, an initial denaturation step was conducted at 94℃ for 5 min, followed by 40 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec, and a final extension at 72℃ for 10 min. Five-µL of the product was mixed with 1µL of loading buffer (Promega) and the electrophoresis was carried out on a 3% 1X Tris-acetate-EDTA agarose gel. The products were visualised by ethidium bromide staining and UV trans-illumination (Figure 2.3). The fragment size was compared with DNA molecular weight marker (100 bp, Promega). Fecal samples from infected patients were used as positive controls. DNase-free water was used as negative control.

Table 2.2. Primers (*E. faecium*, *E. faecalis*, noroviruses genogroups I and II) and probes (*Cryptosporidium*, *Giardia duodenalis*, *E. bieneusi*, *E. hellem* and *E. intestinalis*) used in this study

Microorganisms	Primers/ probes	Sequence (sense)	Target
Enterococci	E1	5'-TCAACCGGGGAGGGT-3' (+)	16S rRNA
	E2	5'-ATTACTAGCGATTCCGG-3' (-)	
E. faecalis	FL1	5'-ACTTATGTGACTAACTTAACC-3' (+)	SodA gene, genomic DNA
	FL2	5'-TAATGGTGAATCTTGGTTTGG-3' (-)	
E. faecium	FM1	5'-GAAAAAACAATAGAAGAATTAT-3' (+)	SodA gene, genomic DNA
	FM2	5'-TGCTTTTTTGAATTCTTCTTTA-3' (-)	
Noroviruses			
Genogroup I& II	JV12	5'-ATACCACTATGATGCAGATTA-3' (+)	RNA-dependent RNA polymerase
	JV13	5'-TCATCATCACCATAGAAAGAG-3' (-)	
Genogroup I	GI	5'-TCNGAAATGGATGTTGG-3' (+)	RNA-dependent RNA polymerase
	JV13	5'-TCATCATCACCATAGAAAGAG-3' (-)	
Genogroup II	JV12	5'-ATACCACTATGATGCAGATTA-3' (+)	RNA-dependent RNA polymerase
	NoroII-R	5'-AGCCAGTGGGCGATGGAATTC-3' (-)	
Cryptosporidium	CRY-1	(HEX)5'-CGGTTATCCATGTAAGTAAAG-3'	18S rRNA
Giardia duodenalis	Giar4	(HEX)5'-CGGCGGGGGGCCAACTAC-3'	18S rRNA
	Giar6	(HEX)5'-CGGGGCTGCCGCGGCGCG-3'	18S rRNA
Enterocytozoon bieneusi	BIEN-1	(TET)5'-AUCAACGAAUGACUUGA-3'	16S rRNA
Encephalitozoon hellem	HEL878F	(6-FAM)5'-ACTCTCACACTCACTTCAG-3'	16S rRNA
Encephalitozoon intestinalis	INT-1	(HEX)5'-GTTCTCCTGCCCGCTTCAG-3'	16S rRNA

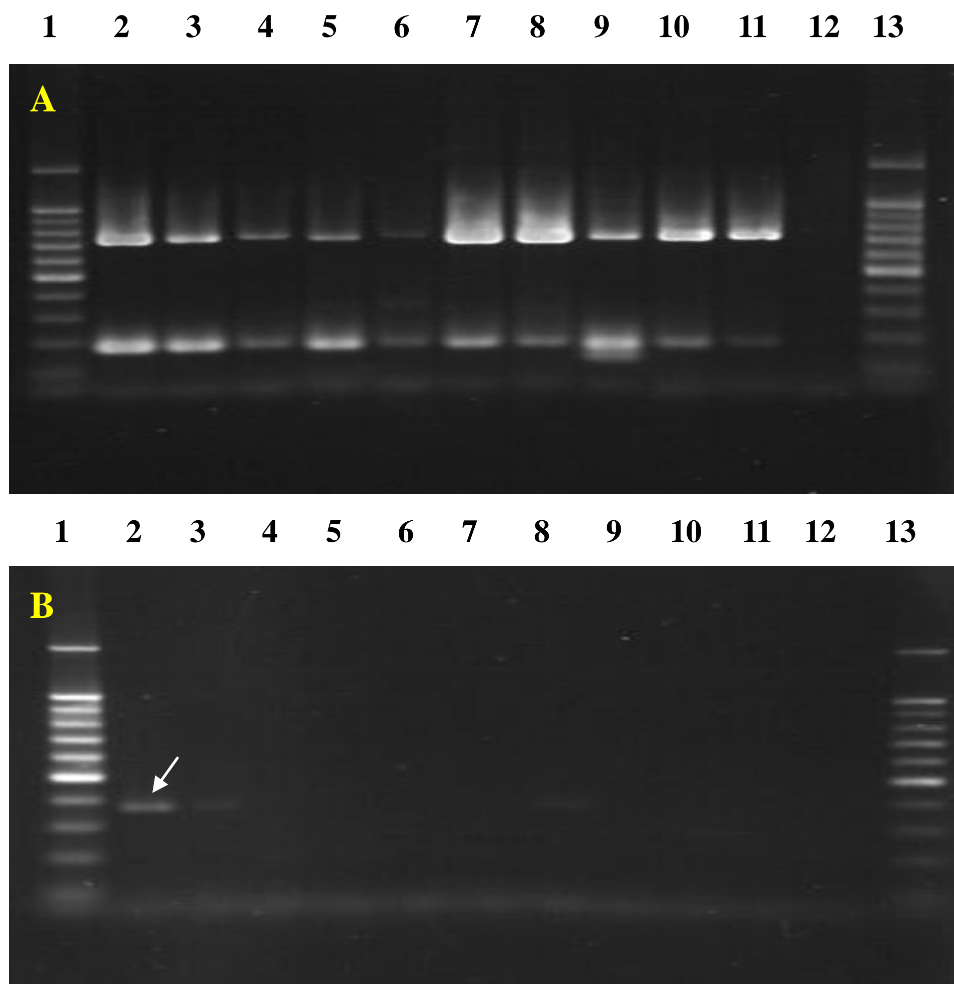

Figure 2.2. Genus PCR of enterococci isolated from Collooney WWTP and *Enterococcus faecalis*-specific PCR of isolates from Collooney WWTP. (A) Genus-specific bands are indicated by the arrow, approx. 707 bp. Positive controls are presented as isolates from influent (Lane 2-6), effluent (Lane 7-9), and biosolids (Lane 10 and 11) respectively. Lane 12, Negative control. Lane 1 and 13, DNA ladder (100 bp). (B) The first PCR products were diluted1:10 (Lane 5-7) and 1: 100 (Lane 2-4, Lane 8-11) respectively and were subjected to a nested PCR amplification. Species-specific bands were indicated by the arrow, approx. 360 bp. Positive controls are presented as isolates from influent (Lane 2-3) and effluent (Lane 8) respectively. Lane 12, Negative control. Lane 1 and 13, DNA ladder (100 bp).

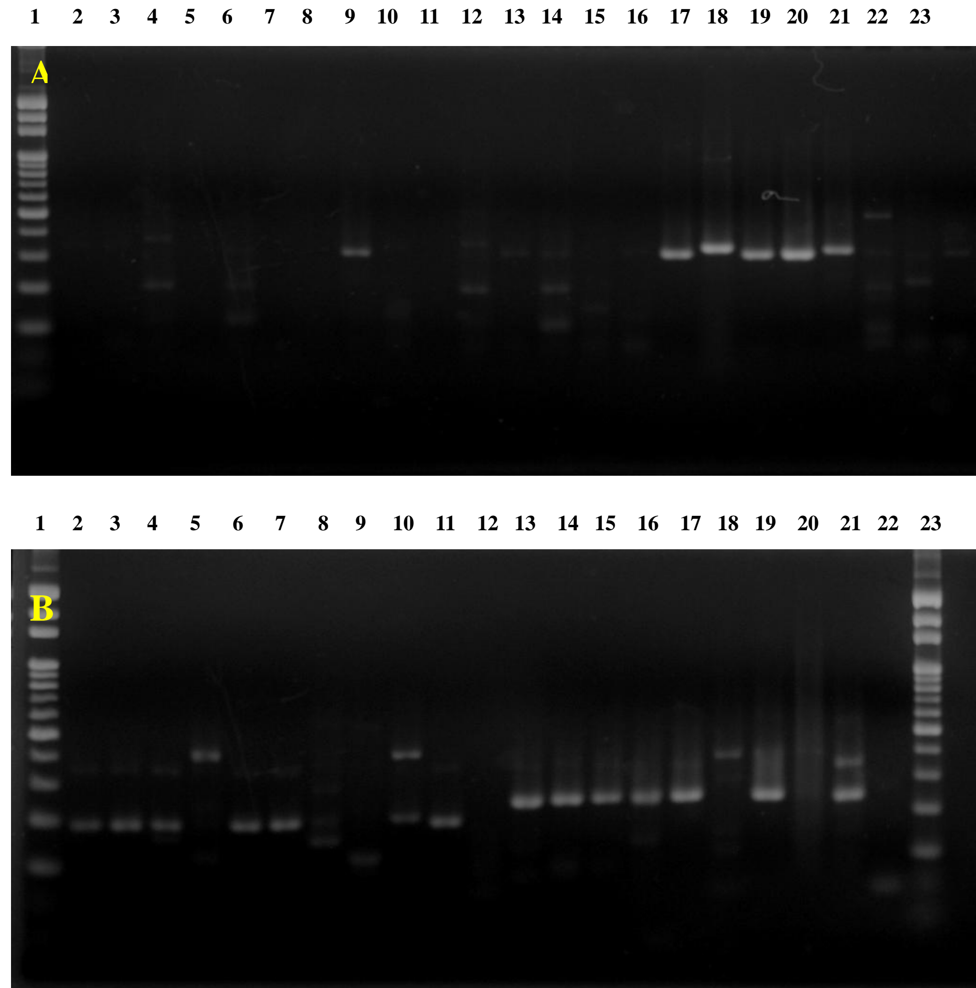

Figure 2.3. RT-PCR results of extracted noroviral RNA from infleunt, effluent, biosolids samples from Collooney WWTP (April, Jull, October 2008; January-March 2009) and positive controls from patients. (A) RT-PCR for noroviruses genogroup I and II with JV12/JV13 primer, positive band 327 bp. Samples shown here included influent (Lane 2, 5, 8, 11, 14, 21), effluent (Lane 3, 6, 9, 12, 15, 22), and biosolids (4, 7, 10, 13, 16, 23). Lane 1, DNA ladder (100 bp). Lane 17-20, positive controls. RT-PCR positive samples were subjected to genogroup-specific semi-nested PCR (B). Lane 2-11 for genogroup I (JV13/GI, 187 bp) and Lane 13-22 for genogroup II (JV12/NoroII-R, 286 bp). Positive controls (Lane 2-5 and 13-16) are stool No. 168, 196, 203, and 216, respectively. Wastewater samples included influent (Lane 10 and 21), effluent (Lane 7, 8, 18, 19), and biosolids (Lane 6, 9, 11, 17, 20, 22). Lane 12, Negative control.

2.6 *Cryptosporidium* and *Giardia* Identification

All liquid samples were shaken vigorously and transferred to 1-L-capacity Imhoff settlement cones. For the dewatered sewage sludge samples, 20 g of the dewatered cake was added to a sterile food processor containing 1-L MilliQ water and homogenized. After homogenization, the liquid was transferred to 1-L conical plastic Imhoff cones. All samples were left overnight at 4℃ for gravity sedimentation (Ash and Orihel 1987; Graczyk *et al.* 2007). Fifty-ml of the top sediment layer was transferred using a 50-ml glass pipette to a plastic 50-ml conical centrifuge tube and centrifuged (3,000 x g, 5 min). The supernatant was discarded and the pellet transferred to a 1.5-ml microcentrifuge tube and 75 % ethanol was added up to 1 ml. The samples were stored at 4℃ (Graczyk *et al.* 2007). Positive samples were provided by Johns Hopkins Bloomberg School of Public Health. Sterile PBS was used as negative control.

The ethanol was washed from the pellet by centrifugation (8,000 rpm, 5 min) twice using an equal volume of sterile phosphate-buffered saline (PBS) and the resulting pellet was subjected to sugar-phenol flotation (Ash *et al.* 1987). The resulting pellet was divided equally for combined multiplex fluorescence *in situ* hybridization (FISH) and a direct immunofluorescence assay (IFA) to target *C. parvum*, *C. hominis*, and *G. duodenalis* (Lemos *et al.* 2005). All probes were listed in Table 2.2. Briefly, an oligonucleotide probe (CRY-1) and two probes (i.e., Giar4 and Giar6), were each 5' labelled with hexachlorinated 6-carboxyfluorescein (HEX), and used to hybridize with the 18S rRNA of *C. parvum*/*C. hominis* and *G. duodenalis* for 1 hr at 57℃ (Deere *et al.* 1989; Dorsch and Veal 2001; Smith *et al.* 2004). The processed samples were placed into three lysine-coated immunofluorescent wells on slides (Carlow Ltd. U.S.A). The slides were stored at room temperature to dry and covered to avoid direct light.

FISH-treated *Cryptosporidium* oocysts and *Giardia* cysts were confirmed by IFA applied on the same slides. The cell wall was targeted by

fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Meri*fluor,* Meridian Diagnostic, Inc., Cincinnati, OH, USA). The slides were air-dried. *Cryptosporidium* oocysts and *Giardia* cysts were enumerated using an epifluorescence microscope (Eclipse E600, Nikon, Japan), dry X60 objective, and BP450-490 exciter filter (B-2A, Nikon, Japan). For oocysts and cysts, only those that were both IFA and FISH positive were assigned as potentially viable.

2.7 Microsporidian Spore Identification

All liquid samples were shaken vigorously and transferred to 1-L capacity plastic Imhoff settlement cones and were left overnight for gravity sedimentation (Ash and Orihel 1987; Graczyk *et al.* 2007). Fifty milliliters of the top sediment layer was centrifuged (3,000 x g, 5 min). The supernatant was discarded and the pellet was transferred to a 1.5-mL microcentrifuge tube, preserved in 75% ethanol, and stored at 4°C. The ethanol was washed from the pellet by centrifugation (8,000 x g, 5 min) twice using equal volume of sterile PBS and the pellet was subjected to sugar-phenol flotation. After sugar-phenol purification, 500 μL of the treated supernatant was transferred to a 1.5-mL microcentrifuge tube. The pellet was washed by centrifugation (5,000 x g, 5 min) using 1 x PBS (Graczyk *et al.* 2007).

The resulting pellet was assayed in 1.5-ml microcentrifuge tubes by multiplex fluorescent *in situ* hybridization (FISH) for identification of *E. bieneusi*, *E. hellem*, and *E. intestinalis* spores (Graczyk *et al.* 2004; Slodkowicz-Kowalska *et al.* 2006). Briefly, species-specific, fluorochrome-labelled probes, i.e. HEL878F, INT-1, and BIEN-1 (Table 2.2), were used for detection of *E. hellem*, *E. intestinalis* and *E. bieneusi* spores, respectively (Dorsh *et al.* 2001; Graczyk *et al.* 2004). The probes, which hybridize the 16S ribosomal RNA of specific microsporidian spore species, were added to purified samples and incubated at 57°C for 3 hours (Graczyk

et al. 2004). The processed samples were placed onto the wells of immunofluorescent slides and the slides were stored at room temperature to dry. Microsporidian spores were identified and enumerated without knowledge of sample identity using an epifluorescent microscope (Eclipse E600, Nikon, Japan), at 100X objective magnification, and BP450-490 exciter filter (B-2A, Nikon, Japan). Positive samples were provided by Johns Hopkins Bloomberg School of Public Health. Sterile PBS was used as negative control.

2.8 Statistical Analysis

Multi-dimensional scaling (MDS) analysis with 100 restarts and minimum accepted stress of 0.01 based on the Bray-Curtis coefficient of similarity was performed to reveal if the composition and concentration of faecal indicator bacteria and pathogens were dependent on such factors as the 'plant', 'treatment stage', and 'season'. Negative samples were removed from the analysis. The data were fourth-root transformed to reduce the variance. Analysis of similarity (ANOSIM) was applied to test if the clustering of samples visually detected on a two dimensional MDS plot was statistically significant (Clarke and Warwick 2001). As most of the quantitative variables (i.e. temperature, pH, DO, TSS) measured in this research project were non-normally distributed, median and quartile values were used to describe the central tendency and variation for those variables, respectively. Wilcoxon matched pairs tests were applied to compare the concentration of faecal indicator bacteria and pathogens and environmental parameters among seasons. Correlations between microbial concentration and environmental parameters were estimated using Spearman rank coefficient γ_s (Zar 1999). MDS and ANOSIM were performed using PRIMER 6 software (PRIMER-E Ltd, 2006, Plymouth, UK). All statistical tests were considered significant at $P < 0.05$.

As it is generally accepted that faecal indicator bacterial concentration

in sewage samples follow a log_{10} normal probability, the mean concentration is then calculated as log10 (χ+1), where χ = concentration of the indicator bacteria (CFU 100 mL^{-1}). Correlation between log_{10}–transformed indicator bacteria concentration and (oo)cysts and spores concentration was conducted using Spearman rank coefficient γ_s. Pathogen detection frequency was determined and the removal efficiency was calculated as a percentage of the difference in pathogen concentration in the effluent v.s. influent. Detection frequency of individual pathogens was presented corresponding to the factors associated with plants A-D, i.e. season (spring, summer, autumn, winter) and treatment stage (influent, activated sludge, effluent and biosolids). PERMANOVA (Anderson 2005) was used to test the significant differences among different factors of the two-way crossed design. The factors (i.e. plant, season, treatment stage) were considered as fixed. Results were considered at P(PERM) < 0.01 and P(MC) < 0.01. P(PERM) is permutation P-value and P(MC) is Monte-Carlo asymptotic P-value (Anderson 2005). Wilcoxon matched pairs tests were applied to compare the detection frequencies in different plants, treatment stages and seasons. The differences among independent factors (i.e. treatment plants, seasons) were calculated with the STATISTICA 6.0 (StatSoft Inc., 2002, Tulsa, USA). When multiple comparisons were performed with the Wilcoxon test, the resultant P-values were adjusted using the Bonferroni correction (Rice 1989).

Chapter Three

CRYPTOSPORIDIUM OOCYSTS AND GIARDIA CYSTS

This chapter originated from one peer-reviewed journal paper entitled 'Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during secondary wastewater treatment' (Journal of Parasitology Research, Appendix 1). This was a preliminary study on wastewater samples from four Sligo WWTPs, carried out in Johns Hopkins Bloomberg School of Public Health (JHSPH), USA. Subsequently, the research was extended to a one-year study on the loadings and fate of these protozoan pathogens, throughout the wastewater treatment process, in these plants. Biosolids loadings were also analysed and these are dealt with in Chapter 5. Datasets are based on protocols conducted in JHSPH (Professor Thaddeus Graczyk's laboratory) as part of a PhD training programme. The core methodologies (i.e. fluorescent in situ hybridisation (FISH) and immunofluorescent antibody assay (IFA)) were transferred to the Biomolecular Environmental and Public Health research group at the Institute of Technology, Sligo via this training course.

3.1 Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during secondary wastewater treatments

Introduction

Cryptosporidium parvum, *Cryptosporidium hominis*, and *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) are obligate protozoan parasites and causal agents of cryptosporidiosis and giardiasis, respectively, in both HIV-infected and immunocompetent individuals (Wolfe 1992; DuPont *et al*. 1995; Caccio *et al*. 2003). These pathogens are prevalent in the environment and can be opportunistically spread via their transmissive stages, i.e., oocysts and cysts, respectively (Graczyk *et al*. 2008a, b).

The end-products of municipal wastewater treatment plants (WWTPs), i.e., final effluent and stabilized sludge (biosolids), have been reported to contain the aforementioned pathogens (Ahmad *et al*. 1997; Lonigro *et al*. 2006; Graczyk *et al*. 2007) as they are common fecal constituents in raw sewage delivered via sewerage network systems to municipal WWTPs (Robertson *et al*. 2000, 2006). Conventional secondary sewage treatment systems are not designed to completely remove these pathogens (Robertson *et al*. 2000; Caccio *et al*. 2003). In Ireland, raw sewage may be transported separate to urban run-off or alternatively the sewerage network may be combined with urban run-off. Primary treatment involves the settlement of preliminary treated wastewater in a sedimentation tank whereas in secondary treatment the wastewater is aerobically broken down during sludge activation in an aeration tank, oxidation ditch, or by a percolating filter system (Gray 2004). Following this treatment, the wastewater is removed to a clarifier where the solids settle by gravity. Raw liquid sludge, with more than 94% of water, is regularly removed from the bottom of either a primary sedimentation tank (in the case of primary sludge) or from a clarifier (in the case of secondary sludge) and the sewage sludge is

transported to a sludge drying bed for thickening and dewatering (Gray 2004). The effluent is released to surface or ground water.

The end-products of municipal WWTPs are used in many countries (Graczyk *et al*. 2008b). The biologically treated effluent is discharged to rivers, lakes, groundwaters, or coastal waters, or it may be applied in agricultural irrigations (Pollice *et al*. 2004; Lonigro *et al*. 2006; Al-Saed 2007). Untreated sewage sludge may be mixed with soil as fertilizer or spread directly on land. An estimated 45,543 tonnes of sewage sludge was spread on Irish farmland in 2005 (Irish Environmental Protection Agency, IEPA 2007). Various international studies have identified high levels of *Cryptosporidium* oocysts and *Giardia* cysts in both treated biosolids and in untreated sewage sludge (Charles *et al*. 2003; Rimhanen-Finne *et al*. 2004; Brianesco and Bonadonna 2005; Montemayor *et al*. 2005; Graczyk *et al*. 2007, 2008b) that negatively impact on public health. This presents a risk factor as residual human-virulent pathogens can be transmitted via surface run-off into waters used for drinking water abstraction or for recreational purposes (Rimhanen-Finne *et al*. 2004; Gale 2005; Hutchison *et al*. 2005). Once the public makes direct contact with oocysts or cysts via consumption of inadequately treated or contaminated waters or crops, then cryptosporidiosis or giardiasis outbreaks can take place (MacKenzie *et al*. 1994; Caccio *et al*. 2003; Gale 2005). Treated municipal WWTP effluents released to surface waters used for drinking water abstraction and recreational purposes also present public health risk factors (Lucy *et al*. 2008). This health hazard is long-lasting as *Cryptosporidium* oocysts can remain viable for almost a year in the environment (Tamburini and Pozio 1999) and in animal liquid waste (Hutchison *et al*. 2005). Several studies have detected *Cryptosporidium* species (Chalmers *et al*. 1997; Lowery *et al*. 2001; Smith and Grimason 2003; Graczyk *et al*. 2004; Lucy *et al*. 2008), *G. duodenalis* (Graczyk *et al*. 2004; Lucy *et al*. 2008), and microsporidia, i.e., *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* (Graczyk *et al*.

2004; Lucy *et al*. 2008) in Irish river basins. Many studies have indicated that these pathogens are still viable, even when present at low concentrations in sludge (Graczyk *et al*. 2007; Montemayor *et al*. 2005). Their low ID50, of less than ten oocysts or cysts, and resistance to environmental stressors reveal a health risk for both the public and for workers in wastewater treatment plants (DuPont *et al*. 2005; Hutchison *et al*. 2005). In Ireland, very little published data are available on the efficiency of municipal wastewater treatment systems in pathogen removal. Historically, the treatment efficiency and management of sewage sludge end-products in Ireland has not been well-documented. Since the outbreaks of cryptosporidiosis in Galway, Ireland, in 2005 and 2007 (Pelly *et al*. 2007), public awareness related to biosolids management has dramatically increased. The aims of this study were to investigate survival and fate of human pathogens, *C. parvum*, *C. hominis*, and *G. duodenalis* at four Irish municipal WWTPs. The removal efficiency at each wastewater processing stage was evaluated and the survival of these pathogens through the sewage treatment was determined based on their viable transmissive stages present in the discharged effluent. The pathogen removal efficiency of the different types of biological treatment applied in these plants was also compared. The pathogen burden in the corresponding biosolids, while mentioned in the chapter will be dealt with separately in Chapter 5.

Materials and methods

Wastewater and sewage sludge samples were collected from four municipal wastewater treatment plants: Plant A (54°16′43″N, 08°36′20″W); Plant B (54°11′11″N, 08°29′10″W); Plant C (54°20′11.3″N, 08°31′44.3″W); and Plant D (54°16′44″N, 08°36′90″W) in north-western Ireland (Figure 1) between April 2007 and January 2008. The population served by these plants ranged from 1,050 to 2,500 people (Table 1), and wastewater originated from domestic houses, schools, and local businesses. The raw

sewage reached each plant via separated sewerage systems, except for Plant B. Preliminary treatment for raw sewage at Plants A, C, and D were coarse screening and grit removal whereas no screening was available at Plant B. The influent at Plant D was stored at a large open chamber prior to the secondary treatment. The following secondary wastewater treatments were applied: (1) sludge activation in an oxidation ditch (Plant A); (2) sludge activation in extended aeration tanks (Plants B and C); and 3) treatment by biofilm-coated percolating filter (Plant D; Table 1). The wastewater in each WWTP was subsequently settled in a secondary settlement tank, i.e., clarifier, separating gravitationally final effluent and sewage sludge. Plants A, B, and D were equipped with sludge drying beds. Wastewater samples were collected at the influent and effluent stages of each WWTP. Secondary wastewater samples were also obtained from the sludge activation processes at Plants A (i.e., oxidation ditch), B, and C (i.e., aeration tanks) and from the percolating filter tank at Plant D.

All wastewater samples were obtained using a long handled 1-L-capacity sampler and transferred to 1-L sterilized polyethene bottles. All samples were collected in triplicate, delivered to the laboratory in a cooler, and processed within 2 h. Physicochemical parameters of the wastewater, i.e., temperature, pH, conductivity, dissolved oxygen (DO), and total dissolved solids (TDS) were measured on-site using a portable Hach (Sens-Ion) meter (Table 1). All liquid samples were shaken vigorously and transferred to 1-L-capacity Imhoff settlement cones. All samples were left overnight at 4°C for gravity sedimentation (Graczyk *et al*. 2007). Fifty milliliters of the top sediment layer was transferred using a 50-ml glass pipette to a plastic 50-ml conical centrifuge tube and centrifuged (3,000 x g, 5 min). The supernatant was discarded and the pellet transferred to a 1.5-ml microcentrifuge tube and 75% ethanol was added up to 1 ml. The samples were stored at 4°C (Graczyk *et al*. 2007). Positive samples were provided by Johns Hopkins Bloomberg School of Public Health. Sterile PBS was

used as negative control.

The ethanol was washed from the pellet by centrifugation (8,000 x g, 5 min) twice using equal volume of sterile phosphate-buffered saline (PBS) and the pellet was subjected to sugar–phenol flotation (Graczyk *et al*. 2007). The resulting pellet was divided equally for combined multiplex fluorescence in situ hybridization (FISH) and a direct immunofluorescence assay (IFA) to target *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts (Lemos *et al*. 2005). Briefly, an oligonucleotide probe (CRY-1) and two probes (i.e., Giar-4 and Giar-6), were each 5′-labeled with hexachlorinated 6-carboxyfluorescein (HEX), and used to hybridize with the 18S rRNA of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts for 1 h at 57°C (Deere *et al*. 1989; Dorsch and Veal 2001; Smith *et al*. 2004). The processed samples were placed into three lysine-coated immunofluorescent wells on slides. The slides were stored at room temperature to dry and covered to avoid direct light. *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts were enumerated using an Olympus epifluorescence microscope, dry X60 objective, and BP450-490 exciter filter. For oocysts and cysts, only those that were both IFA and FISH-positive were assigned as viable.

Variable distribution values were analysed using ranked plots. The correlation between the pathogen presence and the physicochemical factors was carried out using Statistica 6.0 software. Level of significance was indicated by $P < 0.05$.

Figure 3.1. Location of four wastewater treatment plants (i.e., A, B, C, and D) in north-western Ireland for this PhD study. The effluent from Plant A was discharged to Sligo bay. The effluents from Plants B, C, and D were released to local rivers.

Table 3.1. Physicochemical characteristics of municipal wastewater samples (April 2008) from four wastewater treatment plants in north-western Ireland in this study.

Plant	Secondary Treatment System	Population Equivalent	Source	Temp (°C)	pH	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)	TDS* (mg/L)
A	Oxidation ditch	1,914	Influent	10	8.6	670	4.3	341.5
			Secondary treatment	9.8	8.5	606.2	6.1	302.1
			Effluent	9.8	8.1	607	2.2	302.7
B	Extended aeration tank	1,058	Influent	9	7.4	790	6	391.2
			Secondary treatment	8.5	6.8	660	5.6	330.3
			Effluent	8.2	7	690	7.7	339.1
C	Extended aeration tank	2,500	Influent	6	7.6	444	3.84	223
			Secondary treatment	7.1	7.7	477	8.87	241
			Effluent	6.3	7.5	475	11.1	239.3
			Sewage sludge	7	7.8	427	3.41	213.4
D	Percolating filter	2,154	Influent	8.5	6.7	514	6.7	242
			Secondary treatment	8	7.2	430	8.4	212
			Effluent	8.2	7.1	462	8.5	231

* Total Dissolved Solids

Results

Temperature of collected samples ranged from 6 to 10°C (Table 1). The pH value of the influent was 8.6 at Plant A and 7.4, 7.6, and 6.7 at Plant B, C, and D, respectively (Table 3.1). The hardness of supply waters in these WWTPs were categorized into moderately soft at Plants A and C, slightly hard at Plant B, and hard at Plant D (Table 1). Samples from Plant B had the highest conductivity and TDS values in the influent and showed the greatest TDS reduction throughout the wastewater treatment process (Table 1). Dissolved oxygen showed an increasing trend through the treatment process, in general reaching the highest level in the final effluent, except at Plant A, where the DO of the final effluent was only 2.2 mg/L (Table 3.1).

Viable *G. duodenalis* cysts vs. nonviable and non-*G. duodenalis* cysts were clearly differentiated by colour as a result of FISH and mAb-labeling. Nonviable cysts were represented by: (1) shells with apparently structurally damaged walls; and (2) intact cells with a very small amount of internal structures with diffused appearance. In comparison, viable intact cysts were filled out completely with cytoplasm without the gap between the internal structures and the wall. Viable oocysts labeled by FISH and mAb were predominantly intact, revealing a small gap between the oocyst wall and internal structures and, in most of them, the sporozoites were visible. In comparison, dead oocysts, i.e., oocyst shells, frequently had discernable damage to their walls. Rarely, FISH-positive viable oocysts had noticeable ruptures in their walls which were clearly revealed by mAb-staining.

The concentration of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts at different stage of wastewater treatments is shown in Table 3.2. The prevalence of both pathogens in the raw sewage samples was 100%, except the July sample from Plant D (0%). The concentration of *C. parvum* and *C. hominis* oocysts was considerably higher than the concentration of *Giardia* cysts at all WWTPs throughout the treatment processes ($t=2.43$, $p< 0.05$). Plant B had the highest concentration of

oocysts and cysts in April wastewater influent (317 oocysts/L; 99 cysts/L). In other months, Plant A had the highest concentration of oocysts and cysts in wastewater influents, especially in July samples i.e., 592 oocysts/L and 320 cysts/L, respectively (Table 3.2). Among July samples, Plants C and D had relatively low concentration of *Cryptosporidium* oocysts (Plant C: 11 oocysts/L; Plant D: 1 oocyst/L) and *Giardia* cysts (Plant C: 7 oocysts/L; Plant D: 0 oocysts/L) when compared to Plants A and B.

Table 3.2. Concentration (cells/L) of *Cryptosporidium parvum* and *C. hominis* oocysts, and *Giardia duodenalis* cysts during secondary wastewater treatment processes at four wastewater treatment plants in north-western Ireland

	Cryptosporidium(Oocysts/L)			***Giardia(Cysts/L)***		
	Raw sewage	Sewage sludge	Effluent	Raw sewage	Sewage sludge	Effluent
Apr- A	193	125	19	64	35	0
Apr- B	317	149	19	99	69	13
Apr- C	93	28	0	19	12	0
Apr- D	101	5	20	43	3	11
Jul- A	592	128	4	320	56	1
Jul- B	280	8	8	123	4	3
Jul- C	11	0	4	7	2	0
Jul- D	1	2	4	0	6	1
Oct- A	304	128	56	104	48	0
Oct- B	88	232	64	72	120	1
Oct- C	152	184	3	56	40	0
Oct- D	168	8	0	32	0	0
Jan- A	190	83	0	50	7	0
Jan- B	173	133	30	43	40	10
Jan- C	183	84	3	77	37	1
Jan- D	60	233	2	40	153	0

Removal efficiency of secondary treatment and final treatment are displayed in Table 3.3. In April, Plant C had the greatest removal efficiency of both *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts (i.e., 100 %) with Plant A being the second best at 90.3% for oocysts and 100% cysts (Table 3.3). In July, Plant C had the greatest removal efficiency of cysts (100%), whilst Plant B had the greatest removal efficiency of *C. parvum* and *C. hominis* oocysts (98%). Negative removal capability of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts was observed at Plant D (Table 3.3). In October, all plants had greater than 80% removal efficiency of *C. parvum* and *C. hominis* and *G. duodenalis*, except one occasion of low removal rate of *C. parvum* and *C. hominis* at Plant B. In terms of the overall efficiency of the wastewater treatment, over 80% of the viable *C. parvum* and *C. hominis* oocysts and 85% of *G. duodenalis* cysts were eliminated, except at Plant D, which had negative removal efficiency for the oocysts and cysts (Table 3.3).

Table 3.3. Removal of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during the wastewater treatment processes at four wastewater treatment plants in north-western Ireland (April 2008- Jan 2009)

Months-WWTPs	**Removal (%) *C. parvum* and *C. hominis***		**Removal (%) *G. duodenalis***	
	Secondary treatment	**Final effluent**	**Secondary treatment**	**Final effluent**
Apr- Plant A	35.1	90.3	45.8	100
Apr- Plant B	53.1	94.1	29.8	86.5
Apr- Plant C	70	100	35.8	100
Apr- Plant D	94.8	80.3	93.7	74.9
Jul- Plant A	78.4	78.4	82.5	99.7
Jul- Plant B	97.1	98	96.7	97.6
Jul- Plant C	100	63.6	71.4	100
Jul- Plant D	-100	-300	-600	-100
Oct- Plant A	57.9	81.6	53.8	100
Oct- Plant B	-164	27.3	-66.7	98.6
Oct- Plant C	-21.1	98	28.6	100
Oct- Plant D	95.2	100	100	100
Jan- Plant A	56.2	100	86.6	100
Jan- Plant B	23.1	82.7	7.6	76.9
Jan- Plant C	54.2	98.4	52.2	98.7
Jan- Plant D	-288.8	97.2	-283.2	100

Discussion

Municipal WWTPs are not designed to completely remove human waterborne pathogens and therefore they are recognized as reservoirs of such microbiological contaminants occurring in surface waters (Robertson *et al*. 2000; Caccio *et al*. 2003). The present study demonstrated however the high effectiveness of four secondary wastewater treatment systems in the reduction of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts from inflowing raw sewage to the final wastewater effluent. These pathogen reduction efficiencies of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts throughout the wastewater treatment processes observed in the present study are in agreement with removal rates obtained in other studies (Ahmad *et al*. 1997; Robertson *et al*. 2000, 2006; Suwa and Suzuki 2001; Caccio *et al*. 2003; Brianesco and Bonadonna 2005).

The variation in the level of pathogens reduction in secondary-treated wastewater samples can be related to the different operational treatment processes utilized at these WWTPs. The aeration tanks at Plant C yielded considerably higher pathogen reduction rates than the oxidation ditch at Plant A and the aeration tank at Plant B (Table 2). The physical agitation of the aerator systems at these two plants resulted in increased DO levels (Table 1) and may have also caused the mechanical breakdown of the oocysts and cysts (Graczyk *et al*. 2007, 2008b). In addition, the concentration of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts from the oxidation ditch system at Plant A were far greater than those from the aeration tank systems at Plants B and C (Table 2).

The main difference between these 2 aeration tanks at Plants B and C is maintenance and design. Plant C is equipped with latest design of bubble diffusion aeration tank whilst Plant B is equipped with traditional single-aerator based treatment.

The concentration of both oocysts and cysts in WWTP influents were considerably lower at Plants C and D even though the population served by

these WWTPs were considerably higher (Table 1). This phenomenon may reflect a lower incidence of cryptosporidiosis and giardiasis in local populations. It could also be related to the fact that the influent wastewater at Plant D was stored in a large open chamber prior to treatment, which caused settlement of *C. parvum* and *C. hominis* oocysts, and *G. duodenalis* cysts to the bottom of the chamber thus eliminating them from further treatment processes.

The results of the present study showed that, in the first place, treatment plant operatives are exposed to these human enteropathogens during their working day (Cole *et al*. 2000). For the wider community, the provision of efficiently and safely treated effluents and the subsequent stabilization of sewage sludge into pathogen-free biosolids is an important public health issue in Ireland and other countries. This is because both cryptosporidiosis outbreaks (Pelly *et al*. 2007) and enteric pathogen surveys (Graczyk *et al.* 2004, Lucy *et al*. 2008) have revealed *Cryptosporidium* contamination in the aquatic environment in Ireland (Skerrett and Holland 2000), in watersheds where land-spreading and wastewater discharge occurs (Council of the European Community, CEC 1986, Council of the European Community, CEC 1991). This presents a risk factor when these waters are used for drinking water abstraction or for recreational activities (Dorn *et al*. 1985, Ingallinella *et al*. 2002). The European Union (EU) urban wastewater directive aims to protect the environment from the adverse effects of wastewater discharges; however, unfortunately, the relevant effluent quality requirements do not legislate for the presence of pathogens (Council of the European Community, CEC 1986).

All the wastewater treatment processes investigated in the present study appear to be highly effective in removing human pathogens from wastewater stream; however, because of the fact that detected pathogens were viable, the introduction of a regular monitoring program is required to ascertain whether this pathogen removal efficacy is affected by various

factors, particularly seasonal environmental changes and volume of raw sewage entering the WWTP. For example, Plant A was located in a coastal tourist town, with a seasonally increasing population of tourists potentially introducing *Cryptosporidium* and *Giardia* into this community. Known seasonal peaks during spring lambing and calving may also increase the pathogen concentration (Zintl *et al*. 2006); this may impact on pathogen loadings via combined sewerage systems in the event of flooding. This spring peak has been explored in the Irish EPA project (2008-EH-MS-3-S3) Cryptosporidiosis: human, animal and environmental interface, with human results published (Zintl *et al.* 2010) and the project soon due for release. Seasonal effects will be discussed in more depth in Chapters 6 and 7.

Microbial source tracking, which is applied to reveal source of *C. parvum* oocysts and *G. duodenalis* cysts may allow the policy-makers and the operatives to understand the degree of stabilization and sanitization treatments present through the different stages in wastewater treatment processes (Veronica 2008). This study into the survival and fate of the human-virulent pathogens, *C. parvum* and *C. hominis*, and *G. duodenalis*, demonstrated the importance of monitoring these pathogens both within individual wastewater treatment plant processes and particularly in researching their end-products, which are released back into the environment.

Chapter Four

MICROSPORIDIAN SPORES

This chapter consists of one peer-reviewed journal paper published in *Parasitology Research* entitled 'Municipal wastewater treatment plants as removal systems and environmental sources of human-virulent microsporidian spores.' (Appendix II)

4.1 Municipal wastewater treatment plants as removal systems and environmental sources of human-virulent microsporidian spores

Introduction

Microsporidia are widespread obligate intracellular parasites representing more than 1,000 species in 100 genera. Among the 15 species infecting humans, *Enterocytozoon* and *Encephalitozoon* species, especially *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, are the most commonly detected in transplant recipients or in human immunodeficiency virus-infected persons (Didier 2005; Weber *et al*. 1994). Sporadic infections can also occur in immunocompetent individuals (Nkinin *et al*. 2007). Anthropozoonotic potential has been documented for *E. bieneusi*, *Encephalitozoon hellem* (i.e., humans and birds), *Encephalitozoon cuniculi*, and *E. intestinalis* (i.e., humans and other mammals) based on phylogenetic analysis on the internal transcribed spacer (Dengjel *et al*. 2001; Mathis *et al*. 2005; Sulaiman *et al*. 2003). The 50% tissue culture infective doses (TCID50) of *E. bieneusi*, *E. hellem*, and *E. intestinalis* have been determined as 15, 68, and 27 microsporidian spores, respectively (Johnson *et al*. 2003).

Due to the electron-dense, glycoprotein-composed, and chitinous cell structure, microsporidian spores are relatively temperature insensitive, can survive in fresh and marine waters, and even after dehydration for extended periods of time (Didier *et al*. 2004; Li *et al*. 2003; Vavra and Larsson 1999). These characteristics indicate health risks due to transmission from the environment to various susceptible hosts, including humans and domestic animals such as rabbits, pigs, donkeys, and avian species. Microsporidian spores can be ingested or inhaled and then the infections are established predominantly in the small intestine (Garcia 2002; Graczyk *et al*. 2007d; Sak *et al*. 2008).

Considerable evidence to date indicates the involvement of water in the epidemiology of human microsporidiosis (Cotte *et al*. 1999; Dowd *et al*. 1998; Enriquez *et al*. 1998; Fournier *et al*. 2000; Slifko *et al*. 2000; Sparfel *et al*. 1997; Thurston-Enriquez *et al*. 2002). Microsporidian spores have been found throughout the bathing season in marine recreational waters (Graczyk *et al*. 2007c; Lucy *et al*. 2008) and in a range of lakes and rivers used for drinking water abstraction and recreation (Graczyk *et al*. 2004; Lucy *et al*. 2008). Discharge of effluents from wastewater treatment plants is an important point source of microsporidian spores in surface waters used for drinking water abstraction and recreation (Lucy *et al*. 2008); both *E. intestinalis* and *E. bieneusi* spores have been identified in such effluents (Dowd *et al*. 1998; Graczyk *et al*. 2007a). Microsporidian spores, range from 0.8 to 2.0 µm in size, and can pass through drinking water and wastewater treatments together with other human parasites such as *Cryptosporidium* oocysts and *Giardia* cysts (Garcia 2002; Graczyk *et al*. 2007a, 2009).

Legislative instruments such as the European Union Urban Wastewater Treatment Directive provide legal limits for physical and chemical parameters such as biochemical oxygen demand, pH, oxygen saturation, electro-conductivity, and total suspended and dissolved solids (Bontoux 1998; CEC 1991). However, microbiological indicators, such as *Escherichia coli* and intestinal enterococci are not included in this legislation. Furthermore, human waterborne pathogens such as microsporidian spores are also not bound to the directive. In a previous study, up to one-third of viable *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts survived secondary wastewater treatment (Cheng *et al*. 2009). In this study, the presence of microsporidian spores of human-virulent species during secondary wastewater treatment processes in derived biosolids and final effluents at four municipal wastewater treatment plants

were investigated in order to assess the reduction of pathogen load through the wastewater treatments. We also measured the level of intestinal enterococci to investigate their relationship with the load of microsporidian spores.

Materials and methods

Wastewater samples were collected from four municipal wastewater treatment plants; Plant A (54°16′43″N, 08°36′20″W), plant B (54°11′11″N, 08°29′10″W), plant C (54°20′11.3″N, 08°31′44.3″W), and plant D (54°16′44″N, 08°36′90″W) in north western Ireland (Table 4.1). These four plants serve local settlements ranging from 1,100 to 2,500 population equivalent with wastewater originating from houses, schools, hospitals, and local business sectors. Plant A was located in a popular tourist coastal town with beach amenities. The raw sewage entered each plant via combined sewerage systems. Preliminary and primary treatments were applied prior to the secondary biological treatment at plants A, C, and D, while at plant B, the raw sewage was delivered directly to an aeration tank without any screening or grit removal (Table 4.1). At plant D, raw sewage was stored in a large open chamber for approximately 2 h and lead to Imhoff tanks for primary sedimentation before entering the percolating system. In addition, all solids at plant D were settled as primary sludge for removal to a sludge drying bed. The secondary wastewater treatments were applied as follows: (a) sludge activation in an oxidation ditch (plant A); (b) sludge activation in extended aeration tanks (plants B and C); and (c) treatment by percolating filter (plant D). After the activated-sludge

treatment, the final effluent was discharged to adjacent surface waters, i.e., river (plant B, C, and D) and sea (plant A). Due to the engineering design at plant C, liquid sewage sludge was collected directly for disposal from a secondary settling tank (Table 4.1).

Grab sewage samples (i.e., raw sewage, activated sludge, and mixed liquor) and treated end-products (i.e., final effluent), originating from plants A, B, C, and D were collected in April and July 2008. All wastewater samples were obtained using a long-handled 1-L capacity sampler and transferred to 1-L sterilized polyethene bottles. Sewage sludge cake (approximately 100 g) was collected by trowel from 10 cm in depth of the dewatering beds at plants A, B, and D, whereas 1 L of biological treated liquid sewage sludge was obtained from plant C discharge valve at the bottom of the clarifier. All samples were collected in triplicate. Physiochemical parameters, such as temperature, pH, conductivity, dissolved oxygen, and total dissolved solids of wastewater were measured on-site using portable meters (Sens-Ion, Hach Company, Colorado, USA).

The liquid samples were shaken vigorously and transferred to 1-L capacity plastic Imhoff settlement cones. Twenty grams of the dewatered cake was suspended in 1-L MilliQ water by vortexing and transferred to 1-L Imhoff cones. All samples were left at 4°C overnight for gravity sedimentation (Ash and Orihel 1987; Graczyk *et al*. 2007a). Fifty milliliters of the top sediment layer was centrifuged (3,000 x g, 5 min). The supernatant was discarded and the pellet was transferred to a 1.5-ml microcentrifuge tube, preserved in 75% ethanol, and stored at 4°C. The ethanol was washed from the pellet by centrifugation (8,000 x g, 5 min) twice using equal volume of sterile PBS and the pellet was subjected to sugar-phenol flotation. After sugar-phenol purification, 500 µl of the treated supernatant was transferred to a 1.5-ml microcentrifuge tube. The pellet was washed by centrifugation (5,000 x g, 5 min) using 1 × PBS (Graczyk *et al*. 2007a).

The resulting pellet was assayed in 1.5-ml microcentrifuge tubes by multiplex fluorescent in situ hybridization (FISH) for identification of *E. bieneusi*, *E. hellem*, and *E. intestinalis* spores (Graczyk *et al*. 2004; Slodkowicz-Kowalska *et al*. 2006). Briefly, species-specific, fluorochrome-

labelled probes, i.e., HEL878F, INT-1, and BIEN-1, were used for detection of *E. hellem*, *E. intestinalis*, and *E. bieneusi* spores, respectively (Dorsch and Veal 2001; Graczyk *et al*. 2004). The probes, which hybridize the 16S ribosomal RNA of specific microsporidian spore species, were added to purified samples and incubated at 57°C for 3 h (Graczyk *et al*. 2004). The processed samples were placed onto the wells of immunofluorescent slides and the slides were stored at room temperature to dry. Microsporidian spores were identified and enumerated without knowledge of sample identity using an epifluorescent microscope at ×100 objective magnification, and BP450–490 exciter filter.

A 1-ml aliquot of the raw samples was subjected to serial dilution for the detection of enterococci using Slanetz and Bartley medium (Oxoid, UK). Two hundred microliters of the diluant was pipetted onto the agar and spread aseptically. The total counts of pink to red colonies were calculated according to standard methods for the examination of water and wastewater.

Multi-dimensional scaling (MDS) analysis with 100 restarts and minimum accepted stress of 0.01 based on the Bray–Curtis coefficient of similarity was performed to reveal if the composition and concentration of microsporidian spores were dependent on such factors as the "plant", "treatment stage", and "season". Six spore-negative samples were removed from the analysis. The data were fourth-root transformed to reduce the variance. Analysis of similarity (ANOSIM) was applied to test if the clustering of samples visually detected on a two dimensional MDS plot was statistically significant (Clarke and Warwick 2001). As most of the quantitative variables measured in this study were non-normally distributed, median and quartile values were used to describe the central tendency and variation for those variables, respectively.

Wilcoxon matched pairs tests were applied to compare the concentration of microsporidian spores and environmental parameters among seasons. Correlation between spore concentration and water

temperature was estimated using Spearman rank coefficient r_s (Zar 1999). Pathogen removal efficiency was calculated as a percent of the difference in pathogen concentration in the effluent vs influent. Negative values were obtained for the cases in which the pathogen concentration was higher in the effluents as compared to the influents. All descriptive statistics, correlation coefficients, and Wilcoxon tests were calculated with the STATISTICA 6.0 (StatSoft, Inc, 2002, Tulsa, USA). MDS and ANOSIM were performed using PRIMER 6 software (PRIMER-E Ltd, 2006, Plymouth, UK). All statistical tests were considered significant at $P<0.05$.

Results

Microsporidian abundance was clustered in relation to season with two divergent clusters (ANOSIM, P=0.03) corresponding to the sampling periods of April and July (Figure 4.1). There was a significantly higher concentration of all microsporidian spore species in the samples collected in July when compared to April (Tables 4.2 and 4.3). For wastewater samples, among the three microsporidian species, *E. bieneusi* was the predominant species at all four plants in July, with the highest concentration at plant A (up to 497 spores/L), followed by plant C (up to 184 spores/L) and plant B (up to 173 spores/L). Plant A had the highest concentration of all microsporidian spores (up to 1,014 spores/L), including 240 and 277 spores/L for *E. hellem* and *E. intestinalis*, respectively. Plants with the next highest concentrations were plants C and B. There were no spores detected at plant D at any of the stages except in the biosolids (Table 4.2) No statistically significant relationship was found between the microsporidian abundance and the other trained factors: i.e., the four different plants (ANOSIM, P=0.885; Figure 4.1a) and the various treatment stages (ANOSIM, P=0.158; Figure 4.1b).

Of all the environmental parameters, only the temperature differed significantly (P=0.0004, Wilcoxon tests) between April and July (Table

4.3). In addition, there was a significant positive correlation between the concentration of *E. bieneusi*, *E. hellem*, *E. intestinalis* and temperature (r_s= 0.37, r_s=0.55, and r_s=0.54, respectively, P<0.05). A significant correlation was also found between levels of intestinal enterococci and *E. bieneusi*, in July (r_s=0.72, P<0.05) but not in April.

Table 4.1. Characteristics of the WWTPs

WWTPs	Location	Served PE	Preliminary and primary process	Secondary process	Resulting sewage sludge[a]
Plant A	54°16′43″N 08°36′20″W	1,950	Grit removal and coarse screening	Oxidation with oxygen and settlementation	4.12 Tonnes; 0.75% dried solids
Plant B	54°11′11″N 08°29′10″W	1,060	None	Activated sludge, extended aeration, and settlementation (clarifier)	0.76 Tonnes; 0.75% dried solids
Plant C	54°20′11.3″N 08°31′44.3″W	4,000	Grit removal, screening, and sedimentation	Activated sludge, extended aeration, and settlementation (settling tank)	1.82 Tonnes; 0.75% dried solids
Plant D	54°02′54″N 08°43′28″W	2,500	Grit removal, screening, and two Imhoff tanks for primary sedimentation	Percolating filtration system and settlementation (clarifer)	8 Tonnes; 0.75% dried solids

WWTPs wastewater treatment plants, PE population equivalent
[a] Yearly produced volume obtained from the local authority

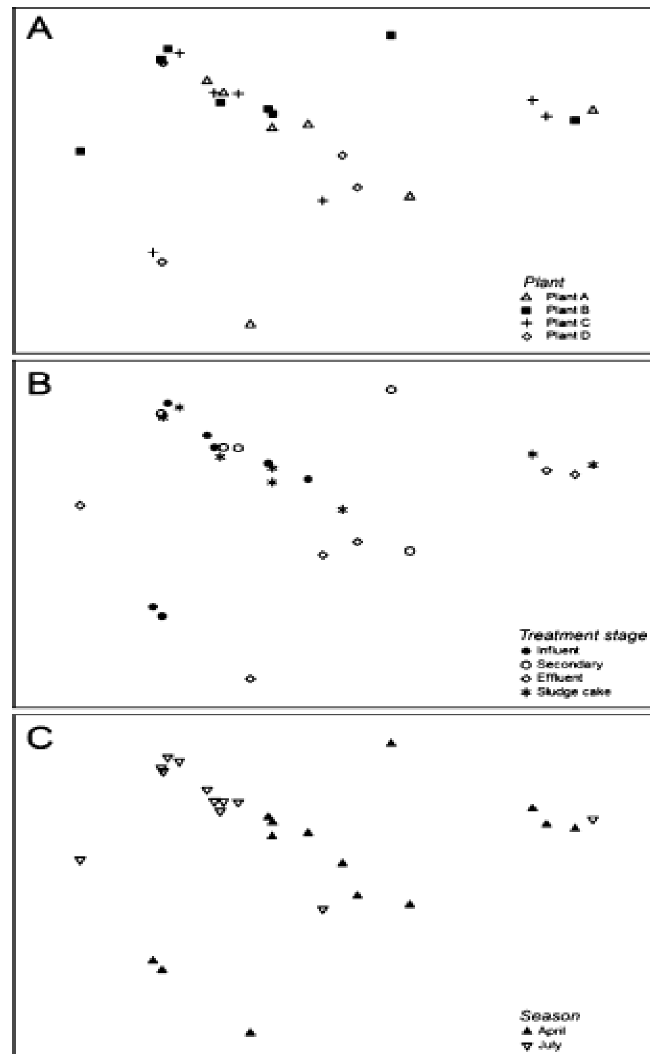

Figure 4.1. MDS ordination of the microsporidian assemblages in relation to the factors ''plant'' (a), ''treatment stage'' (b), and ''season'' (c). The stress value is 0.09, indicating on high quality of this 2D ordination.

Table 4.2. Potentially viable human-virulent microsporidian spores and enterococci during wastewater treatment processes

			No. of spores/colonies[a]							
			E. bieneusi		*E. hellem*		*E. intestinalis*		Enterococci	
Sampling	**Plants**	**Treatment**	**Median**	**Quartiles**	**Median**	**Quartiles**	**Median**	**Quartiles**	**Median**	**Quartiles**
April	A	Influent	12	(1; 16)	2	(1; 7)	12	(8; 12)	2,650	(2,280; 2,900)
		AcSS	2	(0; 11)	3	(2; 6)	0	(0; 0)	4,250	(4,200; 4,670)
		Effluent	0	(0; 0)	20	(13; 27)	0	(0; 0)	0	(0; 0)
	B	Influent	46	(37; 89)	8	(7; 9)	22	(12; 31)	883	(650; 980)
		AcSS	22	(12; 32)	0	(0; 0)	3	(3; 3)	2,350	(1,980; 2,500)
		Effluent	4	(2; 4)	0	(0; 0)	0	(0; 0)	0	(0; 0)
	C	Influent	0	(0; 0)	5	(5; 6)	3	(1; 7)	5,460	(4,900; 5,940)
		AcSS	0	(0; 0)	0	(0; 0)	0	(0; 0)	1,850	(1,760; 2,670)
		Effluent	6	(3; 9)	0	(0; 0)	0	(0; 0)	0	(0; 0)
	D	Influent	0	(0; 0)	9	(6; 9)	1	(1; 3)	0	(0; 0)
		AcSS	0	(0; 0)	0	(0; 0)	0	(0; 0)	0	(0; 0)
		Effluent	13	(10; 15)	15	(14; 19)	0	(0; 0)	0	(0; 0)

Continued Table 4.2.

			No. of spores/colonies[a]							
			E. bieneusi		***E. hellem***		***E. intestinalis***		**Enterococci**	
Sampling	**Plants**	**Treatment**	**Median**	**Quartiles**	**Median**	**Quartiles**	**Median**	**Quartiles**	**Median**	**Quartiles**
July	A	Influent	470	(400; 620)	180	(140; 400)	280	(250; 300)	8,250	(7,880; 8670)
		AcSS	120	(100; 130)	540	(480; 1,090)	390	(310; 500)	3,400	(3,000; 3480)
		Effluent	0	(0; 0)	40	(30; 50)	220	(100; 260)	16	(16; 20)
	B	Influent	180	(150; 190)	30	(30; 40)	70	(50; 80)	2,450	(2,200; 2,560)
		AcSS	120	(120; 140)	10	(0; 10)	60	(40; 70)	1,950	(1,900; 2,740)
		Effluent	20	(10; 20)	130	(100; 180)	0	(0; 0)	0	(0; 0)
	C	Influent	200	(130; 200)	130	(90; 230)	30	(20; 70)	2,000	(1,720; 2,100)
		AcSS	120	(120; 130)	80	(80; 100)	10	(0; 40)	1,720	(1,500; 1,900)
		Effluent	0	(0; 0)	0	(0; 0)	0	(0; 0)	20	(18; 20)
	D	Influent	0	(0; 0)	0	(0; 0)	0	(0; 0)	0	(0; 0)
		AcSS	0	(0; 1)	0	(0; 0)	0	(0; 0)	10	(10; 10)
		Effluent	0	(0; 0)	0	(0; 0)	0	(0; 0)	0	(0; 0)

[a]For microsporidia: spore/L; for Enterococci: 10^3 CFUs/ml

In terms of pathogen removal efficacy, 100% of the enterococci were removed at all of the four plants in both April and July (Figure 4.2). More efficient microsporidian spore removal was observed in July than in April (Figures 4.1 and 4.2); no spores were detected at plant D. In July, plant A had the less efficient removal (i.e., 32%) of *E. intestinalis* spores with almost 100% removal at other plants in April and July. In some samples collected in April, pathogen removal efficacy attained negative values for *E. bieneusi*, e.g., at plants C and D (−100%) and for *E. hellem* at plants A and D (−90% and −50%, respectively). In terms of microsporidian spore removal efficiency, only plant B had negative values in July (*E. hellem*).

Table 4.3. Median values and quartiles (in parentheses) of the microsporidian abundance and environmental parameters in April and July

Parameter	April	July	*P* value for Wilcoxon test
E. bieneusi	7.1 (0.0; 27.1)	93.4 (0.0; 150.0)	0.014
E. hellem	4.4 (0.0; 9.0)	51.7 (0.0; 143.4)	0.008
E. intestinalis	0 (0.0; 7.0)	46.7 (0.0; 211.7)	0.005
Temperature, °C	11.7 (10.7; 12.0)	16.5 (15.8; 17.1)	0.0004
Ph	7.5 (7.05; 7.7)	7.6 (7.1; 7.8)	0.842
Dissolved oxygen, mg/L	5.3 (4.3; 7.8)	4.3 (3.0; 7.5)	0.438
Conductivity, µS/cm	588 (512.0; 649.0)	675.6 (485.2; 740.5)	0.379
Turbidity, NTU	150 (55.6; 536.0)	170.2 (67.3; 494.0)	0.535

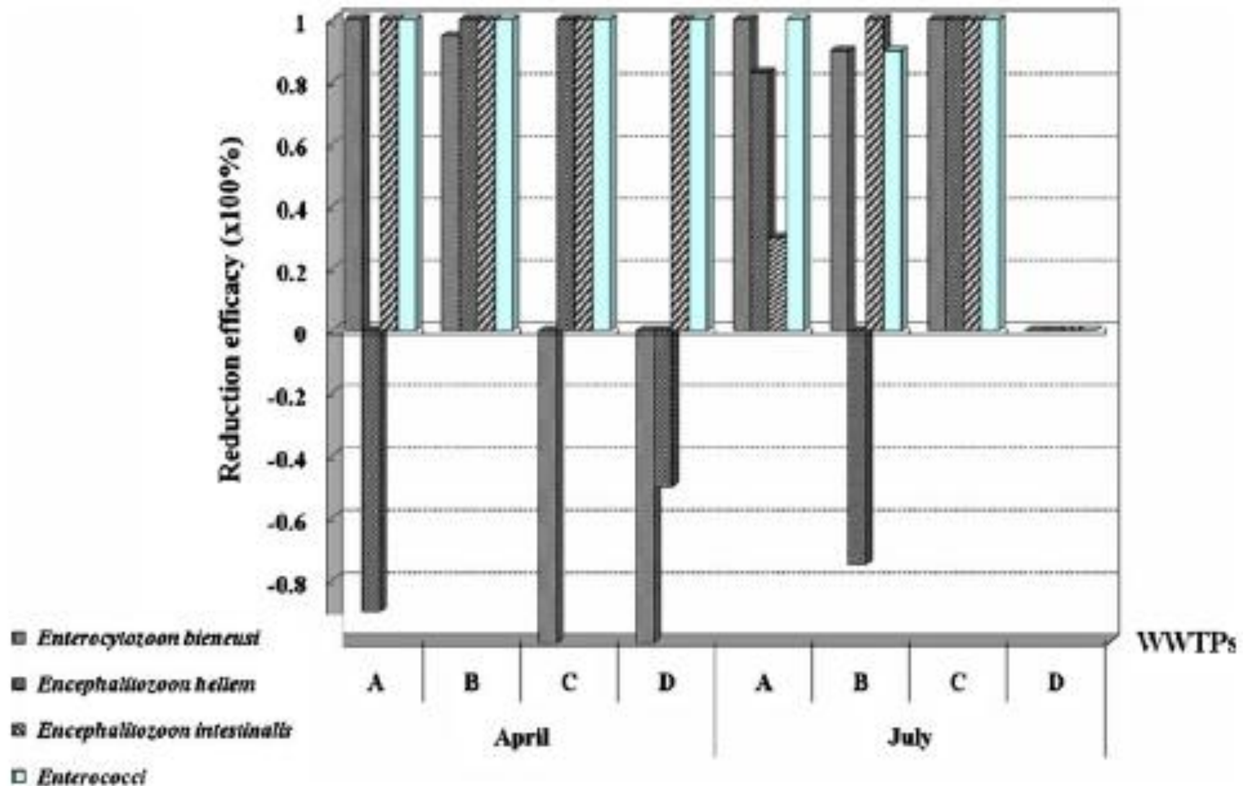

Figure 4.2. Reduction efficacy of the wastewater treatment plants (a-d) on three different species of microsporidia in April and July 2008

Discussion

Very little research has been carried out on the efficacy of secondary wastewater treatment systems in the removal of human-virulent species of microsporidian spores (Dowd *et al*. 1998; Graczyk *et al*. 2007b, 2009). This study reports high concentrations of microsporidian spores in inflowing wastewaters and in the wastewater processing end-products, i.e., biosolids and final effluents. Relatively high concentrations of such human pathogens as *Cryptosporidium* oocysts and *Giardia* cysts, have been reported in municipal wastewater treatment studies (Caccio *et al*. 2003; Cheng *et al*. 2009; Rimhanen-Finne *et al*. 2004; Robertson *et al*. 2006), indicating that

WWTPs can act as pathogen reservoirs. This study is the first report of a significant seasonal increase in microsporidian spore concentrations in municipal wastewaters. Significantly higher microsporidian spores released from the community may indicate that more cases of microsporidiosis occurred in local areas at this time.

This study demonstrated that the spore removal efficacy of four secondary wastewater treatment systems varied. Although *E. bieneusi* was found at higher concentration than *E. hellem* and *E. intestinalis*, the removal of the former species at all four plants was the most efficient. The removal efficacy of *E. hellem* spores varied among plants from 100−90% (Figure 4.2). These negative removal efficacy values for *E. hellem* spores indicate that some spores were likely delivered to the plants during the treatment processes by visiting avian wildlife, as birds were abundant at wastewater operations. Wildlife contribution of pathogens to the wastewater during the processing has been reported previously (Graczyk *et al*. 2009). Increased levels of *E. hellem* in the biosolids might also be due to fecal contribution of birds as sludge treatment and storage takes place in open drying beds. It is noted that there is limitation of sampling volume and frequency as well as applied methodology.

In the present study, approximately 20% of *E. bieneusi* and 40% of *E. hellem* spores went through into the next treatment stage and were still potentially viable in the secondary activated sludge during extended aeration. Compared with a parallel investigation at the same treatment plants on *C. parvum*, *C. hominis*, and *G. duodenalis*, the microsporidian loads in treated effluents were statistically higher than in the influents. In particular, plants A and B, where the effluents had 4 oocysts/L and 8 oocysts/L of *Cryptosporidium*, respectively, while the microsporidian spores were found at much higher concentration: 40 spores/L for *E. hellem* and 220 spores/L for *E. intestinalis* (plant A) and 130 spores/L of *E. intestinalis* (plant B) (six, this study). The surplus microsporidian spores in

the final effluents may be due either to wildfowl inputs from visiting wildlife or alternatively are due to the accumulation of colloid-associated spores in the clarifier. According to previous studies, *Columba livia* (feral pigeon) and other free-ranging birds have been considered as potential carriers of spores of some microsporidia, such as *E. bieneusi* and *E. hellem*, which are also virulent to humans (Dengjel *et al*. 2001; Graczyk *et al*. 2007d). In addition, an increase in these pathogens was previously observed in constructed wetlands associated with wastewater treatment plants (Graczyk *et al*. 2009). In this study, the same phenomenon was observed in the final clarifier with elevated numbers of microsporidian spores in the final effluents. From a public health point of view, bioaerosols that arise from the secondary activated sludge tank and the clarifier may contain microsporidian spores (Graczyk *et al*. 2007b). Possible aerosolization of microsporidian spores could be responsible for infections among wastewater treatment plant personnel. Moreover, the negative removal efficacy of wastewater treatment plants can substantially contribute to contamination of human-virulent microsporidian spores to surface waters used for recreation and drinking water abstraction (Lucy *et al*. 2008).

In Europe, the management of bathing water quality has been entered into force in accordance with the bathing water directive 2006/7/EC, which implemented historically reliable fecal bacterial indicators, i.e., *E. coli* and enterococci, for predicting microbiological health risks in order to achieve a high level of protection of human health (CEC 2006). However, no standards are specified in the EU Urban Wastewater Directive for allowable discharging concentration or removal percentage for either microbial indicators or pathogens. In the present study, intestinal enterococci showed a significant correlation with concentration of human-virulent *E. bieneusi* spores in wastewater samples. This is the first known published study on the relationship between enterococci and microsporidian spores in wastewater. As enterococci levels are relatively easy and cost-effective to

assess and are commonly used for assessment of pathogens, they may have potential as indicators for the possible presence of human-virulent microsporidia in wastewaters (Ulrich *et al*. 2005). Intestinal enterococci have definite advantages as indicators; high resistance to harsh environmental conditions, limited host range, and rare replication outside their hosts (Wheeler *et al*. 2002). On this basis, we suggest that an enterococcibased maximum discharge allowance should be taken into consideration on implementation of microbiological index to the Urban Wastewater Directive.

Results obtained for plant D for both April and July demonstrate that primary sedimentation can enhance removal of microsporidian spores from wastewaters by settling out the spores with primary solids. Other techniques, such as chlorine and UV light treatments, have been suggested to eliminate microsporidian spores that bypassed treatment processes and are found in final effluents (John *et al*. 2003; Johnson *et al*. 2003). Discharges of final effluents to surface waters used for recreation or drinking water abstraction can result in human exposure or direct contact with pathogens present in the effluents (Cheng *et al*. 2009; Lucy *et al*. 2008). As plant A discharged effluents offshore, close to a tourist seaside amenity with seasonally increasing population, spores of *E. intestinalis* discharged with the effluent are of a particular public health concern. It has been demonstrated that immunocompetent people can acquire chronic diarrhea after coming in contact with *E. intestinalis* contaminated recreational waters during holiday seasons (Wichro *et al*. 2005). In conclusion, the present study showed seasonal variation in concentration of microsporidian spores in incoming wastewater that was correlated with seasonal differences in water temperature. We also demonstrated seasonal increase in levels of microsporidian spores in final effluents and biosolids released to the environment. This emphasizes high public health risks, as in Ireland demand for water recreation and drinking water abstractions are highest during the summer months.

Chapter Five

PATHOGEN BURDEN IN THE CORRESPONDING BIOSOLIDS AT WWTPS AND WASTE MANAGEMENT

5.1 Pathogen Burden in the corresponding biosolids and waste management

Introduction

Characteristics of sewage sludge depend on the wastewater treatment processes and sludge treatment. Generally speaking, sewage sludge is composed of nutrients, non-essential trace metals, organic micro pollutants and microorganisms (Kulling 2001). Treated sewage sludge, also known as biosolids, has been used as a soil amendment for agricultural purposes since the onset of municipal wastewater treatment worldwide (EC 1986; EPA Victoria 2004; Metcalf and Eddy 2003; US EPA 1993). Biosolids is the final solid form of product from wastewater treatment; the term is often used interchangeably with sewage sludge but in this study three of the plants were considered to produce biosolids while untreated sewage sludge was analysed from WWTPs Although the macronutrients in biosolids serve as good source of nutrients to crops and the micro constituents provide beneficial soil conditioning properties, the presence of pathogenic microorganisms (i.e. human-virulent bacteria, fungi, viruses, protozoa) in biosolids may pose risks to human, farm animals, and plant health (US EPA 1993). In addition, groundwater and surface water contamination, pathogens and odour are some of the problems associated with the use of biosolids on cropland (US EPA 1994).

Some pathogens are found in biosolids and they may affect quality of agricultural produce after their land application (Estein 1998; Eamens *et al.* 2006; LeBlanc *et al.* 2008). Eamens *et al.* (2006) studied the prevalence of *E. coli, Clostridium perfringens* and *Salmonella* spp. in soil amended with anaerobically digested biosolids and found that bacterial numbers were above detection limits for 10 to 17 months. Previous work by Crute *et al.* (2005) found that *E. coli* and *Enterococci* were detectable

for up to 6 months in biosolids-amended soil spread to agricultural land. Internationally, there have also only been limited studies on pathogens in amended with biosolids. Lang *et al.* (2007) measured the survival of *E. coli* in agricultural soil amended with treated biosolids in a wet temperate environment in the United Kingdom. They determined that *E. coli* in the conventional biosolids treatment reached detection limits by 3 months whereas enhanced-treated biosolids were not a source of *E. coli.*

There is a gap in the scientific data as to the presence and survival of human enteropathogens in dewatered biosolids cake (Gerba and Smith 2005; Lang *et al.* 2007; Sidhu and Toze 2009; Singh *et al.* 2011). Human enteropathogens, such as *Cryptosporidium parvum*, *C. hominis*, *Giardia duodenalis* and human-virulent microsporidia, may present a serious health risk to consumers of agricultural products (Gale 2005; Gerba and Smith 2005; Hays 1977; Haas *et al.* 1999; Nasser *et al.* 2003; Sidhu and Toze 2009) even though the risk may seem minor where biosolids have been used on field crops, processing crops or crops not used directly for human consumption (Epstein 1998).

In terms of public health, the management of final sewage sludge and resulting biosolids has epidemiological implications (IEPA 2000; Gale 2005; Veronica 2008). Implementation of sewage sludge management has been introduced and implemented in the EU legislation (CEC 1991) but research is needed into the safe practice of sewage sludge storage, biosolids treatment, and land-spreading to prevent contamination of watercourses, which can result in further epidemics (Pelly *et al*. 2007). *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores have also been detected in biosolids originating from secondary wastewater treatment (Graczyk *et al*. 2007b; Lucy *et al.* 2008). In addition, viable oocysts and cysts present in biosolids resulting from wastewater treatment pose potential risks to the public and wildlife once biosolids are released to the environment as fertilizer for spreading on agricultural land (Cheng

et al. 2009).

This study was conducted to investigate the pathogen burden in the dewatered biosolids and secondary sewage sludge in four secondary wastewater treatment plants in North Western Ireland. Pathogen removal efficiency of the dewatering bed was also within the scope of this study.

Materials and methods

Biosolids (Plants A, B and D) and final sewage sludge (Plant C) samples were collected from four municipal wastewater treatment plants; Plant A (54°16′43″N, 08°36′20″W), Plant B (54°11′11″N, 08°29′10″W), Plant C (54°20′11.3″N, 08°31′44.3″W), and Plant D (54°16′44″N, 08°36′90″W) (Figure 5.1). As previously noted, these four plants serve local settlements in north western Ireland, ranging from 1,100 to 2,500 population equivalent.

Plant A was located in a popular tourist coastal town with beach amenities. The raw sewage entered each plant via combined sewerage systems. Preliminary and primary treatments were applied prior to the secondary biological treatment at Plants A, C, and D, while at Plant B, the raw sewage was delivered directly to an aeration tank without any screening or grit removal. At Plant D, raw sewage was stored in a large open chamber for approximately 2 h and lead to Imhoff tanks for primary sedimentation before entering the percolating system. In addition, all solids at Plant D were settled as primary sludge for removal to a sludge drying bed. The secondary wastewater treatments were applied as follows: (a) sludge activation in an oxidation ditch (Plant A); (b) sludge activation in extended aeration tanks (Plants B and C); and (c) treatment by percolating filter (Plant D). After the activated-sludge treatment, the final effluent was discharged to adjacent surface waters, i.e., river (Plant B, C, and D) and sea (Plant A). Due to the engineering design at Plant C, liquid sewage sludge was collected directly for disposal from a secondary settling tank.

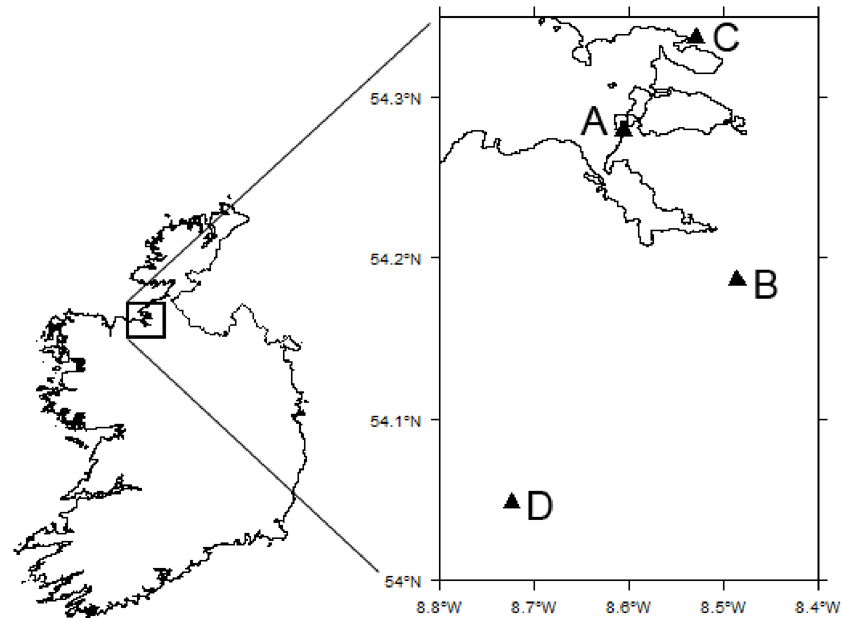

Figure 5.1. Locations of WWTPs A-D.

Sampling was carried out in April 2008 (Spring), July 2008 (Summer), October 2008 (Autumn) and January 2009 (Winter). Sewage sludge cake (approximately 100 g) was collected by trowel from 10 cm in depth of the dewatering beds at plants A, B, and D, whereas 1 L of biological treated liquid sewage sludge was obtained from plant C discharge valve at the bottom of the clarifier. All samples were collected in triplicate. At Plant B, a monthly sampling regime was implemented in a marked area of the sludge bed, to assess long-term changes in pathogen burden between July 2008 and January 2009.

Twenty grams of the dewatered cake was suspended in 1-L MilliQ water by vortexing and transferred to 1-L Imhoff cones. All samples were left at 4°C overnight for gravity sedimentation (Ash and Orihel 1987; Graczyk *et al*. 2007a). Fifty milliliters of the top sediment layer was centrifuged (3,000 x g, 5 min). The supernatant was discarded and the pellet was transferred to a 1.5-ml microcentrifuge tube, preserved in 75% ethanol, and stored at 4°C. The ethanol was washed from the pellet by centrifugation (8,000 x g, 5 min) twice using equal volume of sterile

PBS and the pellet was subjected to sugar-phenol flotation. After sugar-phenol purification, 500 µl of the treated supernatant was transferred to a 1.5-ml microcentrifuge tube. The pellet was washed by centrifugation (5,000 x g, 5 min) using 1 × PBS (Graczyk *et al*. 2007a).

The resulting pellet was assayed in 1.5-ml microcentrifuge tubes by multiplex fluorescent in situ hybridization (FISH) for identification of *C. parvum* and *C. hominis*, *G. duodenalis*, *E. bieneusi*, *E. hellem*, and *E. intestinalis* spores (Graczyk *et al*. 2004; Slodkowicz-Kowalska *et al*. 2006). Positive controls for each pathogen were provided by Johns Hopkins Bloomberg School of Public Health. Sterile PBS was used as negative control.

Briefly, species-specific, fluorochrome- labelled probes, i.e., CRY-1, Giar-4, Giar-6, HEL878F, INT-1, and BIEN-1, were used for detection of *C. parvum* and *C. hominis* oocysts, *G. duodenalis* cysts, *E. hellem*, *E. intestinalis*, and *E. bieneusi* spores, respectively (Deere *et al.* 1989; Dorsch and Veal 2001; Graczyk *et al*. 2004; Smith *et al.* 2004). The probes, which hybridize with the 18 S ribosomal RNA of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts and 16S ribosomal RNA of specific microsporidian spore species respectively, were added to purified samples and incubated at 57°C for 1 h for *C. parvum*, *C. hominis* and *G. deodenalis* and 3 h for microsporidia (Graczyk *et al*. 2004). The processed samples were placed onto the wells of immunofluorescent slides and the slides were stored at room temperature to dry. *Cryptosporidium* oocysts and *Giardia* cysts were enumerated without sample identity using an epifluorescent microscope at x 60 objective magnification, and BP450-490 exciter filter. Microsporidian spores were identified and enumerated without knowledge of sample identity using an epifluorescent microscope at x100 objective magnification, and BP450–490 exciter filter.

Multi-dimensional scaling (MDS) analysis with 100 restarts and minimum accepted stress of 0.01 based on the Bray–Curtis coefficient of

similarity was performed to reveal if the composition and concentration of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in biosolids (Plants A, B and D) and sewage sludge (Plant C) were dependent on such factors as the "plant", and "season". Negative samples were removed from the analysis. The data were fourth-root transformed to reduce the variance. Analysis of similarity (ANOSIM) was applied to test if the clustering of samples visually detected on a two dimensional MDS plot was statistically significant (Clarke and Warwick 2001). As most of the quantitative variables measured in this study were non-normally distributed, median and quartile values were used to describe the central tendency and variation for those variables, respectively.

Student's T tests were applied to compare the concentration of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in biosolids and period of dewatering treatment. Pathogen removal efficiency was calculated as a percent of the difference in the initial pathogen concentration in biosolids vs treated biosolids. Negative values were obtained for the cases in which the pathogen concentration was higher in the treated biosolids as compared to the initial pathogen load. All descriptive statistics, correlation coefficients, and Wilcoxon tests were calculated with the STATISTICA 6.0 (StatSoft, Inc, 2002, Tulsa, USA). MDS and ANOSIM were performed using PRIMER 6 software (PRIMER-E Ltd, 2006, Plymouth, UK). All statistical tests were considered significant at $P<0.05$. Analysis at Plant C is different due to the design of wastewater treatment processing.

Results

Concentration of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in Biosolids was shown in Table 5.1. Spore concentration in biosolids was higher in July than in other sampling seasons, especially at plants B and D. Plant B had the highest concentration of *E. bieneusi* spores (19,000 spores/Kg) and *E. intestinalis*

(16,000 spores/Kg). The highest microsporidian biosolids loading observed in this study was for *E. hellem* at plant D in July, with a median concentration of 32,000 spores/Kg.

Generally, median concentration of *Cryptosporidium* oocysts and *Giardia* cysts in biosolids was higher in July and January than in April and October. *Giardia* cysts was below detectable limit in Plant B biosolids in October. Plant D biosolids, which originated from primary sewage sludge, had significant higher abundance of *Cryptosporidium* oocysts and *Giardia* cysts than Plant B ($t=3.61$, $p<0.05$). Median concentration of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in sewage sludge from Plant C was shown in Table 5.2. *Cryptosporidium* oocysts were the most frequently detected protozoa at Plant C. The highest concentration of *Cryptosporidium* oocysts (48 oocysts/L) was found in April sewage sludge samples followed by January sewage sludge samples (14 oocysts/L). In terms of abundance, higher load of microsporidian spores were detected in January compared to other sampling seasons (Table 5.2). Significantly higher abundance of *E. hellem* spores were detected in January compared to April and July samples ($t=20.4$, $p< 0.05$).

In terms of long-term pathogen removal efficacy of dewatering bed in Plant B, 100% of *Giardia* cysts and *E. bieneusi* spores were inactivated by October and January, respectively (Table 5.1, Figure 5.2). More efficient *Cryptosporidium* oocyst removal was observed in July (94%) than in October (14.3%) and January (-96%). In July, negative efficient removal of microsporidian spores was observed, i.e. -82.4% for *E. intestinalis* spores; -96.3% for *E. hellem* and *E. intestinalis* (Figure 5.2). In January, pathogen removal efficacy attained negative values for *Cryptosporidium* oocysts and *Giardia* cysts, with -96% and -100% removal efficacy respectively. However, 91.7% of *E. hellem* and 86.4% of *E. intestinalis* were reduced in the same month.

Table 5.1. Loads of human-virulent *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in the resulting biosolids

Sampling	Plants	No. of spores/colonies (median, quartiles)[a]				
		Cryptosporidium	*G. duodenalis*	*E. bieneusi*	*E. hellem*	*E. intestinalis*
April	A	2,400 (1,200; 2,400)	600 (500; 600)	1,550 (600; 3,500)	500 (450; 750)	600 (400; 600)
	B	3,082 (3,000; 3,200)	1,130 (1,000; 1,250)	3,350 (2,250; 4,450)	350 (300; 450)	600 (500; 600)
	D	5,060 (4,500; 5,100)	2,330 (2,000; 2,500)	3,950 (3,450; 4,450)	500 (400; 600)	0 (0; 0)
July	A	9,400 (9,400; 9,500)	3,925 (3,900; 3,950)	3,500 (3,000; 4,000)	0 (0; 950)	1,500 (1,500; 5,000)
	B	175 (150; 250)	100 (100; 100)	19,000 (19,000; 22,000)	9,500 (7,500; 14,000)	16,000 (12,000; 17,000)
	D	9,800 (9,500; 9,800)	4,000 (2,500; 4,000)	6,000 (5,000; 7,000)	32,000 (29,000; 34,000)	11,000 (9,500; 14,000)
October	A	4,325 (4,000; 4,500)	1,300 (1,000; 1,750)	500 (500; 500)	1,100 (0; 1,100)	1,250 (1,200; 1,250)
	B	150 (150; 175)	0 (0; 0)	8,300 (8,000; 8,300)	12,000 (9,500; 12,500)	6,100 (6,100; 6,100)
	D	3,630 (3,100; 3,630)	1,550 (1,500; 1,550)	15,700 (15,500; 16,000)	4,580 (4,500; 4,580)	8,300 (8,000; 8,300)
January	A	4,000 (4,000; 4,000)	330 (300; 350)	0 (0; 0)	160 (160; 160)	3,000 (2,500; 3,000)
	B	3,750 (3,500; 3,750)	1,000 (0; 1,000)	0 (0; 50)	1,000 (250; 1,000)	830 (800; 830)
	D	13,080 (12,500; 13,500)	3,170 (2,500; 3,250)	800 (400; 800)	6,800 (6,500; 7,000)	3,400 (3,000; 3,400)

[a] For *C. parvum* and *C. hominis*: oocysts/Kg; *G. duodenalis*: cysts/Kg; microsporidia: spores/Kg

Table 5.2. Loads of human-virulent *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in the final sewage sludge at Plant C (L^{-1}).

Months	*Cryptosporidium*	*Giardia*	*E. bieneusi*	*E. hellem*	*E. intestinalis*
April	48 (0; 48)	8 (0; 8)	50 (50; 50)	0 (0; 0)	0 (0; 0)
July	12 (12; 12)	12 (12; 12)	0 (0; 0)	5 (0; 5)	0 (0; 0)
October	9 (9; 9)	0 (0; 0)	0 (0; 0)	8 (8; 8)	0 (0; 0)
January	14 (0; 14)	0 (0; 0)	5 (5; 5)	120 (90; 120)	216 (210; 216)

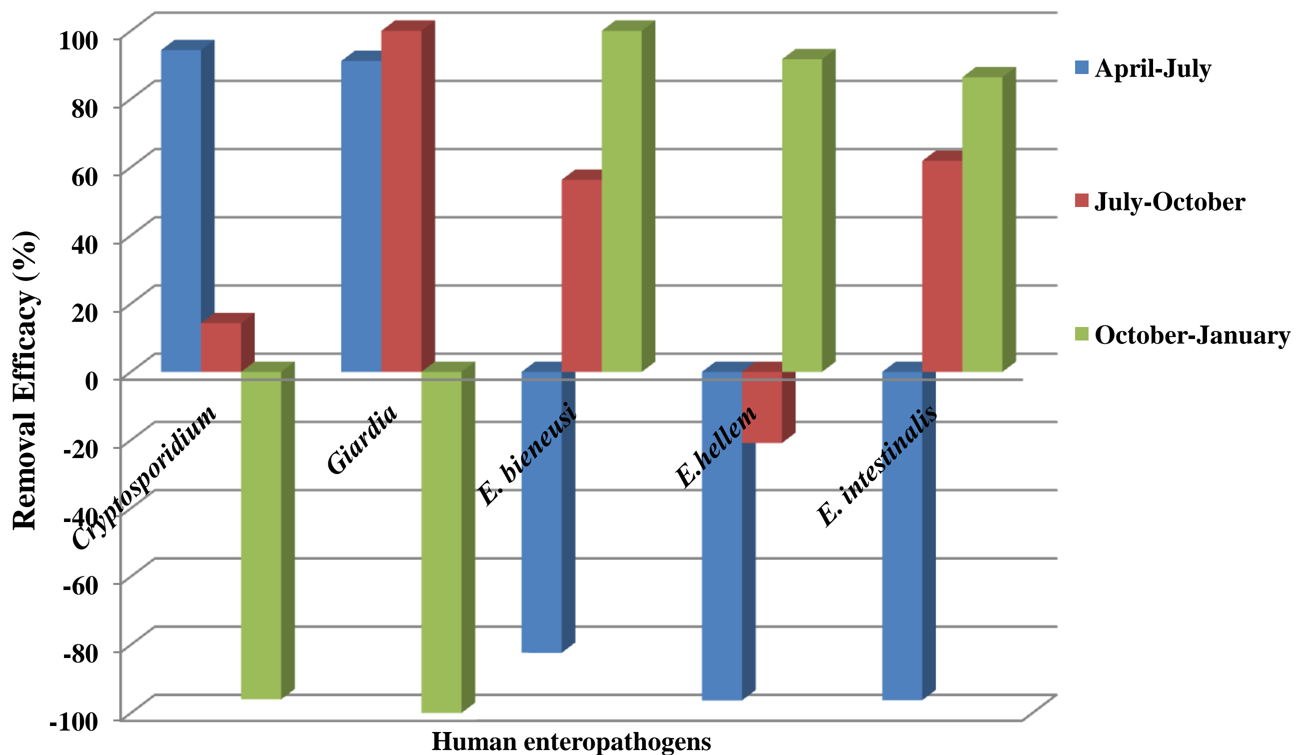

Figure 5.2. Removal efficacy of dewatering bed in Plant B between April 2008 and January 2009.

Cryptosporidium oocysts, *Giardia* cysts and microsporidian abundance was clustered in relation to season with three divergent clusters (ANOSIM, P=0.03) corresponding to the sampling periods (Figure 5.3). There was a significantly higher concentration of all microsporidian spore species in the samples collected in July when compared to April and October (Tables 2, Figure 5.2). Among the three microsporidian species, *E. bieneusi* was the predominant species at all four plants in April and July, with the highest concentration at Plant B (19,000 spores/Kg, July biosolids), followed by Plant D (6,000 spores/L, July biosolids). Plant D had the highest concentration of all microsporidian spores (up to 49,000 spores/Kg), including 6,000, 32,000 and 11,000 spores/Kg for *E. bieneusi*, *E. hellem* and *E. intestinalis*, respectively. It was noted that the concentration of microsporidian spores in Plant D influent and effluent samples were low while the concentration of microsporidian spores in Plant D biosolids was considerably high. It may due to the fact that biosolids collected was from the previous batches of raw wastewater.

The Plant with the next highest concentrations was Plants B, with total microsporidian load of 44,500 spores/Kg biosolids. No statistically significant relationship was found between the microsporidian abundance and the other trained factors: i.e., the four different plants (ANOSIM, P=0.79).

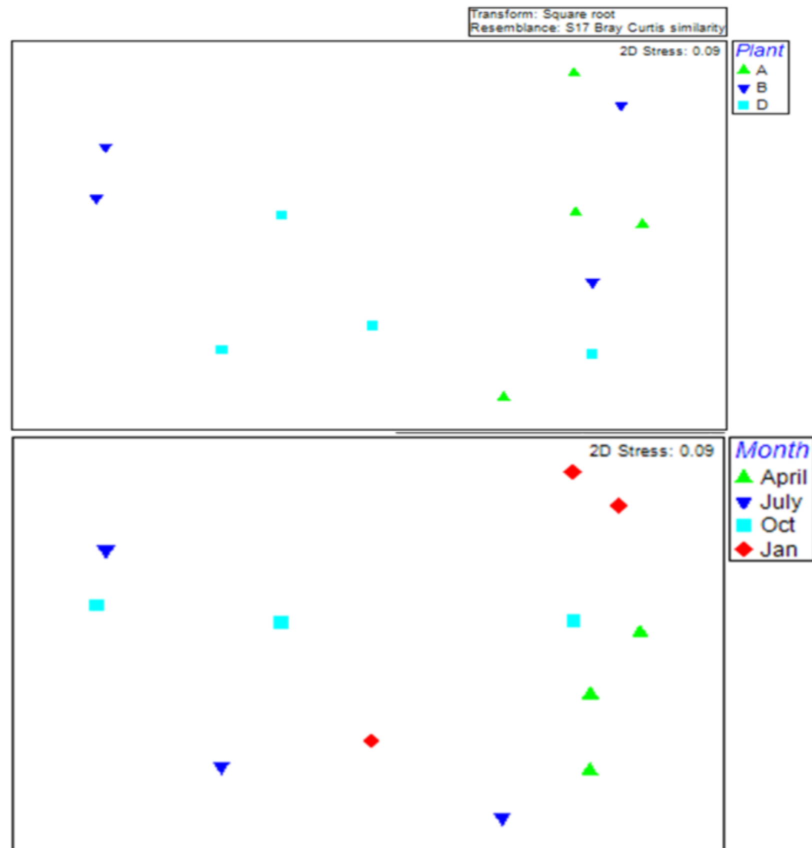

Figure 5.3.

MDS ordination of the human enteropathogens assemblages in relation to the factors 'plant' (top) and 'month' (bottom). The stress value is 0.09, indicating the high quality of this 2D ordination.

Discussion

Although the final effluent at Plants A, B and D had very low pathogen loadings (data shown in Chapter 3 and Chapter 4), the corresponding biosolids had considerably higher concentrations of human-virulent pathogens (Table 5.1). This is important from the public health standpoint because transmission stages of these pathogens were viable. The highest pathogen concentration in biosolids was observed at Plant D, where the liquid form of sewage sludge was collected at the primary stage and was not exposed to any secondary treatment process, which could reduce pathogen levels. As all the dewatering beds in this study were exposed to the environment, the possibility also exists that wildlife visiting these beds may increase the load of *C. parvum* and *C. hominis* oocysts, *G. duodenalis* cysts, and microsporidian spores (Wolfe 1992; Graczyk *et al*. 2008a).

This study reports high loadings of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in the treated biosolids. Relatively high concentrations of such human pathogens as *Cryptosporidium* oocysts and *Giardia* cysts, have been reported in municipal wastewater treatment studies (Caccio *et al*. 2003; Rimhanen-Finne *et al*. 2004; Robertson *et al*. 2006), indicating that WWTPs can act as pathogen reservoirs.

The occasional negative removal efficacy values for *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores indicate that some protozoa were likely delivered to the plants during the treatment processes by visiting avian wildlife, as birds were abundant at wastewater operations. Increased levels of these pathogens in the biosolids might also be due to fecal contribution of birds as sludge treatment and storage takes place in open drying beds. The increase of *Cryptosporidium* oocysts and *Giardia* cysts in January may due to unaware addition of new batch of sewage sludge. It is noted that there is limitation of sampling volume and frequency as well as detection methods applied in this study.

In conclusion, the present study showed efficacy variation in pathogen loads between plants and seasons. We also demonstrated seasonal increase in levels of *Cryptosporidium* oocysts and *Giardia* cysts in January biosolids, which may be utilised for agriculture in the approaching farming season. 106,778 tonnes of biosolids have been produced nationally by the registered WWTPs in Ireland (IEPA 2012). This may end up in local watercourses, following spread of biosolids on agricultural lands, providing a pathogen source via drinking water abstraction and recreational activities (Beach 2008; Lucy *et al.* 2008).

Chapter Six

PREVALENCE OF HUMAN-VIRULENT NOROVIRUSES AND *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* AND REMOVAL EFFICIENCY OF WWTPS

6.1 General Introduction

This chapter originated from one peer-reviewed journal paper ('Municipal wastewater treatment plants as pathogen removal systems and as a contamination source of noroviruses and *Enterococcus faecalis',* Journal of Water and Health, Appendix III) and explores the prevalence of human-virulent noroviruses and *Enterococcus faecalis* and *E. faecium* and the related removal efficiency of WWTPs. Faecal-origin pathogens, such as norovoruses and *Enterococcus faecium* and *Enterococcus faecalis* can be delivered to WWTPs via shedding from faeces of infected hosts. If end products of WWTPs are not well-managed, it can pose risks to human health, e.g. diarrhoeal diseases.

Noroviruses are considered as a common cause of human infectious gastroenteritis in all age groups (especially in the age of 2-8) in restaurants and institution such as nursing homes and hospitals (Vinje *et al.* 1996; Verbelen *et al.* 2004). These viruses belong to the genus Norovirus within the family Caliciviridae and are divided into five genogroups (GI, GII, GIII, GIV and GV). Among the genogroup, GI and GII are the most frequently detected ones in human origin, followed by GIV and GIII, which is detected in pig (Zheng *et al.* 2006). During 2006, 300 gastrointestinal/infectious intestinal disease (IID) outbreaks were notified in Ireland; with approximately 5,100 people became ill. The IID outbreaks are in continuous trend in Ireland, which are dominated by noroviruses. In the case of the north-west HSE region, 33 outbreaks were reported (one outbreak occurred in a hospital in Sligo) and 487 people were infected (NDSC 2006). One-hundred-and-seventy-seven norovirus-related outbreaks were reported in 2006, with approximately 4,200 people ill.

Clinical and epidemiological studies indicated that person-to-person transmission of norovirus via contaminated environment is involved in the majority of the infection cases. In some cases, the contamination source originated couldn't be identified due to the lack of library of environmental

isolated strains and surveillance. Additionally, it is difficult to assess the number of patients suffering from NoVs in an epidemiological surveillance, mainly because people do not, necessarily, visit the hospital with diarrhoea. Based on their genetic proximity, potential zoonotic transmission or animal reservoir can be hypothesised as contaminant sources (Scipioni *et al.* 2008). Sedmak *et al.* (2003) and Borchardt *et. al.* (2003) surveyed the prevalence of enteroviruses and bacterial diarrhoea in sewerage systems and the comparison between clinical isolates and wastewater isolates was also assessed in local communities. It should be possible to investigate the transmission point of NoV in the human environment from the occurrence data of NoVs in the end products of and annual notified infection cases in communities.

This chapter investigates this hypothesis by studying the occurrence frequency of noroviruses, *Enterococcus faecalis* and *Enterococcus faecium* in influent, final effluent and biosolids from 4 WWTPs in north western Ireland (WWTPs A-D). The seasonal and spatial variation of the plant treatment efficiencies in pathogen removal is also within the scope of this Chapter.

6.2 Municipal WWTPs as pathogen removal systems and as a contamination source of norovirus and *Enterococcus faecalis* circulating in human populations

Introduction

Diarrhoeal disease remains a major leading cause of morbidity and mortality with 2.16 million deaths per year globally (Kosek *et al.* 2003; Mathers *et al.* 2004). In developed countries, improvement in water and sanitation is one of the key factors leading to the decreasing trend in diarrhoeal disease. However, according to national disease surveillance report in 2003, 4.5 percent of the Irish population was still affected by gastroenteritis every month. In a recent Irish Health Protection Surveillance Centre (HPSC) 2009 annual report, infectious intestinal disease (IID) outbreaks accounted for 56.4% of all outbreaks, with 6% decrease compared to the national statistics in 2008 (HPSC 2010).

Regional variation in all IID outbreaks was significant between Health Service Executive (HSE) areas, with the highest rate observed in the HSE north-west area at 28.7 per 100,000 population. In addition, most of the outbreaks were attributed to norovirus (HPSC 2010). According to a study on invasive enterococcal bacteraemia surveillance undertaken in the same year, 289 *Enterococcus faecalis* isolates and 397 *Enterococcus faecium* isolates were identified in the acute hospitals, with 0.7% (*E. faecalis*) and 39.1% (*E. faecium*) showing resistance to Vancomycin (EARS-Net 2009). Enterococci are one of the predominant bacterial flora in human/animal gastrointestinal tract. Nowadays, enterococci play dual roles as both commensal organisms and human-virulent pathogens, representing the second most leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial bacteraemia (Moellering 1992). Norovirus, formerly known as small roundlike virus or Norwalk virus, is

the causative agent of the well-known winter vomiting disease and classified within the family of *Caliciviridae*, with 7.5 Kb single-stranded, positive-sense RNA genome comparative to other small round-like viruses. Noroviruses genome encodes three large open reading frames (ORFs), including the ORF1 encoding replicase polyprotein (e.g. RNA-dependant RNA polymerase) and the ORFs 2 and 3 encoding major (e.g. capsid protein) and minor structural proteins, respectively (Lindesmith *et al.* 2008). Noroviruses can be grouped into five genetically differentiable genogroups (genogroups I-V), with noroviruses genogroups I and II accounting for the majority of viral gastroenteritis in human populations internationally (Caul 1996; Lopman *et al.* 2004). An international research study demonstrated that 1~3% of people were expected to become infected with noroviruses each year (Food Standards Agency 2000). In Ireland, norovirus are the commonest cause of outbreak of acute gastroenteritis standing for 48.1% of all IID outbreaks (HPSC 2010). The majority of infection cases are of norovirus genogroup II/genotype 4 (Waters *et al.* 2006; HPSC 2010).

The modes of transmission of enterococcal infection and norovirus infection are recognised as being via consumption of faeces-contaminated food (e.g. raw oyster or shellfish), drinking or bathing waters, or through person-to-person contact (Green *et al.* 2001; Koopmans *et al.* 2003). The infected individuals release the pathogens in the excreta, which is transported to wastewater treatment systems. Wastewater treatment plants (WWTPs) comprise physical, chemical and biological processes, depending on the requirement for the effluent standard as well as the nature of the wastewater. The aims of wastewater treatment fundamentally are to ensure the human-wastes are collected appropriately, to ensure the wastewater is effectively treated and the resulting bio-hazardous products are safely discharged to inland or coastal waters, and to possibly recycle or reuse the

valuable components in the end products. By the nature of the domestic wastewater, human-virulent enteropathogens can also be found in the raw wastewater derived from the infected individuals in the plant serving communities.

The aims of this study were to investigate the occurrence of noroviruses, and also *Enterococcus faecalis* and *Enterococcus faecium* in human sewage samples (raw and treated end-products) from four secondary wastewater treatment plants in north western Ireland and to observe any seasonal and spatial variation. Second-PCR was conducted in this study not only to overcome the sensitivity limitation but also to differentiate norovirus genogroup I and genogroup II. Furthermore, this study was undertaken to evaluate the treatment efficiency of these municipal secondary WWTPs on pathogen removal (presence/absence in the treated effluent and biosolids) and to assess the potential impact of the discharge wastes from the plants to human public health.

Materials and methods

Wastewater treatment plants and source of wastewater samples

Four secondary wastewater treatment plants (plants A-D) in north western Ireland were investigated in the months of April, July and October in 2008 and monthly between January 2009 and February 2010 in this study (Figure 6.1). Plant A is located in a seaside tourist town (serving 1,950 population equivalents), where a flux of travellers visit during holidays, especially in summer period. Plants B, C, and D are situated in stable residential areas and serve up to 4000 population equivalents each. Regional hospitals and health care centres are located in the areas where plants A, B, and D serve. All plants are designed as separated sewerage systems receiving only domestic wastewater, except for plant B, which accepts a combination of domestic sewage and proportional urban run-off in wet conditions (combined sewer). The characteristics of the WWTPs were mentioned in a

previous study (Cheng *et al.* 2011). Briefly, the raw wastewater (except at WWTP B) went through grit removal and coarse screening before reaching the secondary treatment stage. The subsequent secondary wastewater treatments were applied: sludge activation in an oxidation ditch (plant A); sludge activation in extended aeration tanks (plants B and C); and treatment by biofilm-coated percolating filter (plant D). The wastewater in each WWTP was then settled in a secondary settlement tank, i.e., clarifier, separating gravitationally final effluent and sewage sludge. The resulting sewage sludge (mixture of the sludge on the bottom of secondary treatment facility and the settlement tank) was taken out of the tank and spread onto the on-site drying beds, where the dewatered biosolids samples were collected. Due to different plant design, sewage sludge at plant C was obtained via discharge valve from the secondary settling tank. The biosolids obtained from plants A, B, and D contained 0.75% dried solids while the sewage sludge at plant C contained 2.5% dried solids.

All wastewater samples (influent and effluent) were collected using a long-handled 1 L sampler and transferred to 1 L sterilised polyethene bottles. Biosolids (approx. 100 g) were collected by trowel from 10-cm depth in the drying beds at plants A, B, and D whereas 1 L of liquid sewage sludge was obtained through the plant C discharge valve. All samples were collected in triplicate, and delivered to the laboratory in a cooler (Cheng *et al.* 2009).

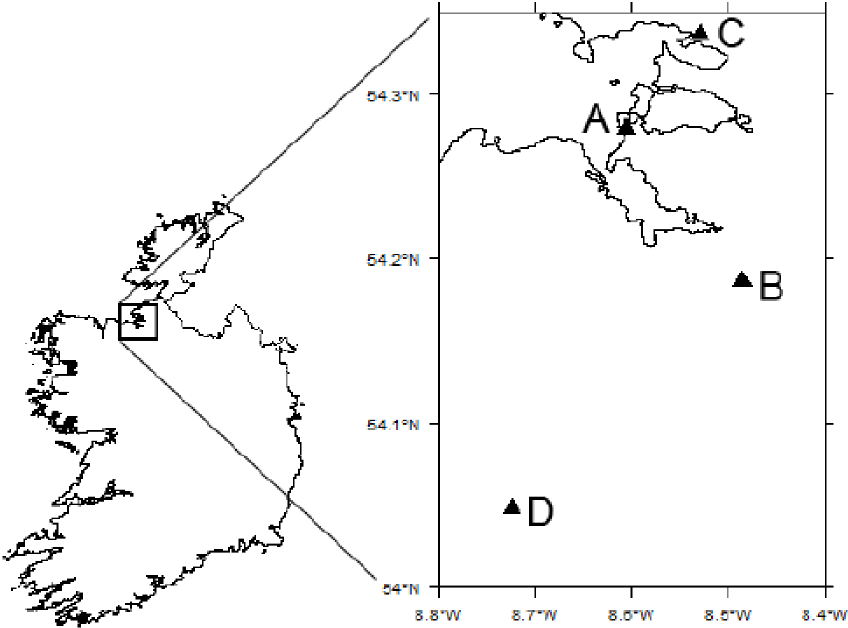

Figure 6.1. Location of plants A-D in north-western Ireland (this study).

Enumeration of enterococci and detection of E. faecalis and E. faecium

All the liquid wastewater samples were mixed vigorously and ten milliliter (mL) of the samples were transferred to a 15 mL sterilised conical plastic centrifuge tube, labelled as subsamples, in triplicate. Twenty grams of biosolids sample were diluted 1:50 in phosphate-buffered saline (in triplicate) and vortexed. The sub-samples were mixed vigorously and 1 mL of the mixture was subjected to ten-fold serial dilution in Ringer's solution (Oxoid, UK) to 10^{-4}. Two hundred microliters of the sub-samples were aseptically spread onto Slanetz and Bartley agar (Oxoid, UK) and cultivated at 44℃ for 24-36 hours. In accordance with the manufacturer's direction, burgundy red colonies on Slanetz and Bartley agar were enumerated as positive.

For *E. faecalis* and *E. faecium* detection, 10% of the positive colonies on Slanetz and Bartley agar were transferred individually using sterilised plastic needle to microcentrifuge tubes containing 100 µL of DNase and RNase-free water (Promega). To release the DNA from enterococci isolates, the mixture underwent 3 freeze-and-thaw cycles: heating in a dry heating block at 99℃ for 10 min then cooling in an ice box at 0℃ for 5 min (Hsu *et al.* 2006). The suspension was centrifuged (13,500 x g, 10 min) then the supernatant was transferred to new sterilised microcentrifuge tubes. Two hundred µL of the wastewater samples were subjected to total DNA extraction using DNA stool extraction kit (Qiagen, USA). Aliquots of the extracted DNA samples were subjected to identification first using enterococcal genus primers (Deasy *et al.* 2000), followed by a *sod*A gene targeted species-specific primers for *E. faecalis* and *E. faecium* (Jackson *et al.* 2004). The sequences of the oligonucleotide primers are listed in Table 6.1. Enterococcal cultures of *E. faecalis* (NCTC775) and *E. faecium* (ATCC19434) were used in this study as positive controls. Total genomic DNA extracted from both cultures of *E. faecalis* and *E. faecium* were used as positive controls in this study. Briefly, a DNA solution was added to a

microcentrifuge tube containing 12.5 µL of 2X PCR buffer (Promega), 0.5 µL of 1.0 µmol of primers (Integrated DNA Technologies (IDT), Inc., USA) and 7.5 µL of nuclease-free water (Promega). The reaction was carried out using a modified protocol (Deasy *et al.* 2000). Initially, the DNAs were denatured at 95℃ for 4 min, followed by 30 cycles of 95℃ for 30 sec, 50℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 7 min. A nested PCR was prepared with 1:10 diluted first-run PCR products and the sequences were amplified with species-specific primers targeting *E. faecalis* and *E. faecium* respectively. The reaction was carried out using a modified protocol (Jackson *et al.* 2004). Following an initial denaturation at 95℃ for 4 min, products were amplified by 30 cycles (35 cycles for *E. faecium*) of 95℃ for 30 sec, 53℃ for 1 min (*E. faecalis*) or 48℃ for 1 min (*E. faecium*), 72ºC for 1 min, followed by a final extension at 72ºC for 7 min. Five µL of product was mixed with 1 µL of loading buffer (Promega) and electrophoresed on a 2% 1X Tris-acetate-EDTA agarose gel. The products were visualised by ethidium bromide staining and UV trans-illumination. Each fragment size was compared with DNA molecular weight marker (100 bp, Promega).

Pre-treatment, RNA extraction, detection for noroviruses

The combination of US EPA Manual "Optimizing Molecular Methods to Detect Human Caliciviruses in Environmental Samples" (Vinje 2008) and the protocol established by Katayama *et al.* (2002) was adapted for norovirus concentration. One litre of each wastewater sample was centrifuged at 7,280 x g for 15 min. The supernatant was transferred to a new tube whereas the pellet was resuspended in 10 mL of PBS and the mixture was centrifuged (2000 x g, 15 min). The pellet was resuspended in2 mL of PBS and stored at -20 ℃ (Vinje 2008). The two supernatants were combined and subsequently filtered through a glass fiber prefilter (AP15, Millipore, Ireland). The filtrate was adjusted with $MgCl_2$ to a final

concentration at 0.05 M (Lukasik *et al.* 2000; Hsu *et al.* 2009) and then filtered through nitrocellulose membranes (HA series, 0.45 μm pore size, Millipore, Ireland) for adsorption using a glass filter holder unit (Millipore, USA) with a stainless steel screen. The stainless steel screen was cleaned and sterilised before use. The filter membrane was gently stirred in 10 mL of 1X PBS buffer (phosphorus buffer saline, pH 7.0) for 15 min (Hsu *et al.* 2009). The solution was mixed well and the aliquot was transferred to sterilised 1.5 ml microcentrifuge tubes and stored at -80℃ before use. Processed faecal samples from norovirus-positive patients were utilised for positive control.

The concentrated samples, biosolids pellet and norovirus-positive faeces samples were 1:10 diluted in nuclease-free water and transferred to silica column provided in the Viral Nucleic Acid Extraction Kit III (Geneaid, Taiwan), following extraction instructions provided by the manufacturer. The extracted RNA was then subjected to one-step reserve transcription polymerase chain reaction (RT-PCR) with 5X reaction buffer, Enzymix containing DNA and RNA *Taq* polymerase (Invitrogen, USA), 100 nmol of primers JV12/JV13 (IDT, Inc., USA) and nuclease-free water (Promega). The reaction was carried out as follows: 1 cycle of 50℃ for 30 min and 95℃ for 15 min; 45 cycles of 94℃ for 30 sec, 37℃ for 1 min, 72℃ for 1 min; 1 cycle of 72℃ for 10 min (Green *et al.* 1998). The parallel semi-nested PCRs were conducted subsequently for norovirus genogroup I and norovirus genogroup II. Briefly, The PCR was carried out in a new microcentrifuge tube containing 1 μL of each RT-PCR product, 2.5 μL of 10X PCR buffer (Invitrogen, USA), 0.4 μL of dNTPs (10 mM of each dNTP), 0.25 μL of Platinum *Taq* DNA Polymerase (5 U/μL, Invitrogen, USA), 0.5 μL of primers JV13/GI (for norovirus genogroup I) and JV12/NoroII-R (for norovirus genogroup II) and 19.85 μL of nuclease-free water. The reaction condition was modified from Boxman's protocol (Boxman *et al.* 2006). Briefly, an initial denaturation step was conducted at

94℃ for 5 min, followed by 40 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec, and a final extension at 72ºC for 10 min. Five µL of the product was mixed with 1 µL of loading buffer (Promega) and the electrophoresis was carried out on a 3% 1X Tris-acetate-EDTA (TAE) agarose gel. The products were visualised by ethidium bromide staining and UV trans-illumination. The fragment size was compared with DNA molecular weight marker (100 bp, Promega). In addition, the amplified gene segments were compared to the size of amplicons obtained from norovirus positive patients' stool control samples on TAE gels.

Statistical analysis

The detection frequency of individual pathogens was presented corresponding to the factors associated with plants A-D, i.e. season (spring, summer, autumn, winter) and treatment stage (influent, effluent and biosolids). PERMANOVA (Anderson 2005) was used to test the significant differences among different factors of the two-way crossed design. The factors (i.e. plant, season, treatment stage) were considered as fixed. Results were considered at P(PERM)< 0.01 and P(MC)< 0.01. P(PERM) is permutation P-value and P(MC) is Monte-Carlo asymptotic P-value (Anderson 2005). Wilcoxon matched pairs tests were applied to compare the detection frequencies in different plants, treatment stages and seasons. The differences among independent factors (i.e., treatment plants, seasons) were calculated with the STATISTICA 6.0 (StatSoft Inc., 2002, Tulsa, USA). When multiple comparisons were performed with the Wilcoxon test, the resultant P-values were adjusted using the Bonferroni correction (Rice 1989).

Table 6.1. Primers used in this study for detection of *E. faecalis*, *E. faecium* and noroviruses genogroups I and II.

Microorganism	Primers	Sequence (Sense)	Target	Product size	Reference
Enterococci	E1	5'-TCAACCGGGGAGGGT-3' (+)	16S rRNA	707 bp	Deasy *et al*. 2000
	E2	5'-ATTACTAGCGATTCCGG-3' (-)			
E. faecalis	FL1	5'-ACTTATGTGACTAACTTAACC-3' (+)	*Sod*A gene, genomic DNA	360 bp	Jackson *et al*. 2004
	FL2	5'-TAATGGTGAATCTTGGTTTGG-3' (-)			
E. faecium	FM1	5'-GAAAAAACAATAGAAGAATTAT-3' (+)	*Sod*A gene, genomic DNA	215 bp	Jackson *et al*. 2004
	FM2	5'-TGCTTTTTTGAATTCTTCTTTA-3' (-)			
Noroviruses					
GI/GII	JV12	5'-ATACCACTATGATGCAGATTA-3' (+)5'-	RNA-dependent	327 bp	Vinje and Koopmans
	JV13	TCATCATCACCATAGAAAGAG-3' (-)	RNA polymerase		1996
Norovirus genogroup I	GI	5'-TCNGAAATGGATGTTGG-3' (+)	RNA-dependent	187 bp	Vinje and Koopmans
	JV13	5'-TCATCATCACCATAGAAAGAG-3' (-)	RNA polymerase		1996; Greens *et al*. 1998
Norovirus genogroup II	JV12	5'-ATACCACTATGATGCAGATTA-3' (+)	RNA-dependent	286 bp	Vinje and Koopmans
	NoroII-R	5'-AGCCAGTGGGCGATGGAATTC-3' (-)	RNA polymerase		1996; Greens *et al*. 1998

Results and Discussion

The detection frequencies of noroviruses (genogroups I and II), *E. faecalis*, and *E. faecium* at four plants are shown in Table 6.2. The results were obtained by the interpretation of the amplicons (both wastewater samples and controls) amplified with species-specific primers. *E. faecalis* was detected in all of the influent samples from plants A and B, where 52.9% and 58.8% of the samples were found positive for *E. faecium*. While 58.8% and 47.1% of the influent samples from plant D were detected positive for *E. faecalis* and *E. faecium*, respectively. It was noted that *E. faecalis* and *E. faecium* were absent during the whole investigation period in plant C influents, except one *E. faecalis*-positive sample in April 2008. Norovirus genogroup II was more frequently detected in the influents at plants A and B (88.2%), followed by 64.7% at plant D. Overall, plant C effluent and final biosolids samples had significantly lower pathogen detection frequencies when compared to that at plant B ($P = 0.02$), but not plant A ($P = 0.04$) and plant D ($P = 0.04$) (Wilcoxon tests with Bonferroni-corrected significance level of 0.017).

It was also observed that the prevalence of the pathogens differed significantly by seasons at all of the plants (P(PERM)< 0.001; P(MC)< 0.001). Compared to plants A, B, and D, plant C had the least pathogen load in spring (P=0.002), summer (P=0.002), and autumn (P=0.006) (Wilcoxon tests, with Bonferroni-corrected significance level of 0.017). In winter months, noroviruses were frequently present in the influent samples at all plants (median: 80% for norovirus genogroup I; 100% for norovirus genogroup II). In the same period of investigation, the effluent samples from plants C and D were negative for all pathogens, except for norovirus genogroup II. It was also noted that all winter effluent samples from plant A were positive for norovirus genogroup II. The prevalence of individual pathogens differed significantly between *E. faecalis* and *E. faecium* (P=0.005, Wilcoxon test) and norovirus genogroups I and II (P=0.002,

Table 6.2. Detection rates (%) of *Enterococcus faecalis*, *Enterococcus faecium* and Norovirus genotype I and genotype II in wastewater samples at plants A-D (year 2008-2010)

Samples	Overall			Spring			Summer			Autumn			Winter		
	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids
Plant A															
Enterococcus faecalis	100.0	58.8	29.4	100.0	50.0	25.0	100.0	100.0	75.0	100.0	50.0	0.0	100.0	40.0	20.0
Enterococcus faecium	52.9	11.8	5.9	100.0	0.0	25.0	100.0	25.0	0.0	25.0	25.0	0.0	0.0	0.0	0.0
Norovirus genogroup I	58.8	17.6	11.8	25.0	0.0	25.0	75.0	25.0	25.0	50.0	0.0	0.0	80.0	40.0	0.0
Norovirus genogroup II	88.2	70.6	76.5	50.0	50.0	75.0	100.0	50.0	75.0	100.0	75.0	75.0	100.0	100.0	80.0
Plant B															
Enterococcus faecalis	100.0	70.6	47.1	100.0	50.0	75.0	100.0	100.0	100.0	100.0	75.0	25.0	100.0	60.0	0.0
Enterococcus faecium	58.8	17.6	5.9	100.0	0.0	0.0	100.0	50.0	25.0	25.0	25.0	0.0	20.0	0.0	0.0
Norovirus genogroup I	58.8	17.6	11.8	75.0	0.0	0.0	25.0	0.0	0.0	50.0	50.0	50.0	80.0	20.0	0.0
Norovirus genogroup II	88.2	52.9	47.1	100.0	25.0	25.0	75.0	75.0	25.0	75.0	50.0	50.0	100.0	60.0	80.0
Plant C															
Enterococcus faecalis	5.9	0.0	*0.0	25.0	0.0	*0.0	0.0	0.0	*0.0	0.0	0.0	*0.0	0.0	0.0	*0.0
Enterococcus faecium	0.0	0.0	*0.0	0.0	0.0	*0.0	0.0	0.0	*0.0	0.0	0.0	*0.0	0.0	0.0	*0.0
Norovirus genogroup I	41.2	0.0	*0.0	25.0	0.0	*0.0	0.0	0.0	*0.0	50.0	0.0	*0.0	80.0	0.0	*0.0
Norovirus genogroup II	47.1	5.9	*41.2	25.0	0.0	50.0	0.0	0.0	*0.0	50.0	0.0	*25.0	100.0	20.0	*80.0

Continued Table 6.2

Samples	Overall			Spring			Summer			Autumn			Winter		
	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids	Influent	Effluent	Biosolids
Plant D															
Enterococcus faecalis	58.8	17.6	41.2	50.0	25.0	25.0	100.0	50.0	100.0	75.0	0.0	50.0	20.0	0.0	0.0
Enterococcus faecium	47.1	5.9	11.8	25.0	25.0	25.0	75.0	0.0	25.0	75.0	0.0	0.0	20.0	0.0	0.0
Norovirus genogroup I	35.3	5.9	23.5	25.0	0.0	0.0	25.0	0.0	25.0	50.0	25.0	25.0	40.0	0.0	40.0
Norovirus genogroup II	64.7	23.5	47.1	50.0	25.0	25.0	50.0	0.0	25.0	75.0	25.0	75.0	80.0	40.0	60.0

*Samples were taken bottle of secondary settlement tank as final sewage sludge.

Wilcoxon tests). As more *E. faecalis* than *E. faecium* was frequently detected in the treated effluent samples, it may demonstrate that the former may be either naturally present at higher concentration in the influent or more resistant to treatment processes (either activated sludge systems or the biofilm system) in municipal secondary wastewater treatment plants. This explanation can also be applied on the observed pattern of norovirus genogroup II. The introduction of this genogroup into the local aquatic environment following the wastewater treatment process coincides with the observed clinical prevalence in the previous study (Waters *et al.* 2006). Even though the fecal-oral route has been known as the transmission route leading to acute gastroenteritis, very little research has focussed on the complete environmental infection cycle starting from municipal wastewater treatment plants. Nevertheless, Borchardt *et al*. study (2003) has associated the viral and bacterial diarrhoea occurring in studied populations living in Marshfield, Wisconsin with the density of septic systems in the same epidemiologic study area. According to the multivariate analysis, viral and bacterial diarrhoea cases were independently associated with the number of septic systems in the studied residential area (Borchardt *et al.* 2003), indicating wastewater treatment systems as a risk factor for enteric infections.

For the biosolids at plants A, B, and D, samples indicating the least effective treatment occurred in the summer season when 75-100% of the biosolids were *E. faecalis*-positive, followed by norovirus genogroup II (25-75%) and norovirus genogroup I and *E. faecium* (0-25%). In terms of the treatment efficiency based on the overall data, plant D (biofilm-coated percolating system) was found to be more efficient than plants A and B (extended-aerated activated sludge system), although the observed statistical significance was contributed mostly by the scenarios of poorly treated effluents and biosolids produced in spring and summer seasons at plants A and B, where the resulted wastes may pose a health risk to local

inhabitants, if they are spread on land in the vicinity (Gale 2005). For example, on average 70.6% and 76.5% of plant A effluents (to local watercourses) and biosolids (to the land) contained norovirus genogroup II. For primary biosolids produced at plant D, since pathogen levels were higher, there may have been a higher risk to the catchment where the wastes are spread.

This study was conducted in a coastal area in the north-west of Ireland, which accommodates thousands of holiday visitors annually especially in spring and summer. Seasonal variation of pathogen detection frequency in the incoming human sewage at plants A and B demonstrated that tourist influx may be the key factor to the insufficient treatment as well as a potential source of new pathogens. Wastewater treatment plants are traditionally designed to cope with up to 3 times the dry weather loading amount of organic matter and to reduce BOD, suspended solids and nutrients to a compliant level before discharge (Gray 2006; CEC 1991). However, wastewater treatment plants are not originally designed for pathogen removal and the active human-virulent pathogen accumulated in the biosolids or partially suspended in the effluents may facilitate the environmental circulation of opportunistic pathogens causing infectious diarrhoea, such as *Cryptosporidium hominis*, *C. parvum* and noroviruses if the stakeholders consume the contaminated shellfish, crops, or bathing waters (Gale 2005; Graczyk *et al.* 2007; Nenonen *et al.* 2008). Although relevant regulations, i.e. the EU shellfish water directive (CEC 2006), the WHO guidelines for the reuse of wastewater in agriculture and aquaculture (WHO 1989), The EU bathing water directive (CEC 2006) and Water Framework Directive are in place to prevent risks to human health, no pathogen emission limit is documented into legal force (CEC 1991) to ensure consistent treatment efficiency at municipal wastewater treatment systems, which are considered as pathogen accumulation reservoirs (Graczyk *et al.* 2009; Cheng *et al.* 2011).

The management of biosolids has epidemiological and public health implication (Gale 2005; US EPA 1999; Veronica 2008). In the previous studies, abundant potentially human-infectious *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores were reported in the wastes from municipal wastewater treatment plants (Rimhanen-Finne *et al.* 2004; Cheng *et al.* 2011). In the Irish 2007 national record (Irish EPA 2009), 86,411 tonne of final biosolids were produced. For the studied areas, 4.12, 0.76, 1.82, and 8 tonnes of biosolids were from plants A-D respectively. Apart from those from plant C, the dewatered biosolids contained active viral particles deposited onto farmland, which may facilitate norovirus circulation. In this study, we also revealed that investigated municipal wastewater treatment systems were insufficient to inactivate norovirus particles and *Enterococcus faecalis* in discharged effluents. For reasons of public health, it is extremely important to provide facilities with tertiary and advanced treatments which are capable of pathogen inactivation, such as the add-on membrane filtration compartment, slow sand filtration or well-managed constructed wetland (Liu *et al.* 2010; Simmons *et al.* 2011; Heistad *et al.* 2009; Vega *et al.* 2003) before effluents reach receiving watercourses. If possible, disinfection of treatment facilities in summer is recommended for preventive maintenance.

The notified infection cases (years 2007-2010) caused by noroviruses, *E. faecalis*, and *E. faecium* nation-wide and in the HSE north western area are shown in Table 6.3. The incident data were provided by different sources (i.e. Computerised Infectious Disease Reporting system, Health Service Executive, and EARS-Net). Among enterococcal bacteraemia notification, more infections were caused by *E. faecium* than *E. faecalis*, with 406 bacteraemia cases caused by the former notified in 2008 and slightly less cases in the following years. One hundred-and-sixty-three and 115 Norovirus outbreaks occurred in 2008 and 2009 respectively, resulting in 1,777 and 1,638 infection cases nationwide. Ten percent of the infection

cases in 2008 and 96 cases were notified in HSE north western area. Fournorovirus infection cases were reported in plant A and B serving areas and one case was reported in plant D serving area during the investigation period (personal communication, A. Murray, HSE). Over 40% of plant C influent samples were norovirus-positive but no effluents contained detectable viral particles before discharge. This may be a factor in the absence of norovirus infection cases notified in the area serving plant C. The stability of the noroviruses, combined with regular test on the influents and the discharge wastes (effluent and biosolids), may provide epidemiological information (Iwai *et al.* 2009). Moreover, the association between the quality of sewage-derived waste products and infectious gastroenteritis cases should be carried out and evaluated in other regions internationally.

Table 6.3. Notified infection cases caused by verotoxigenic *Escherichia coli*, *Enterococcus faecalis, E. faecium* and norovirus in Ireland between 2007-2010*

Pathogen	2007	2008	2009	2010
Escherichia coli				
Notified verotoxigenic *E. coli*** cases in Ireland	167	226	241	225
Notified verotoxigenic *E. coli*** cases in HSE North Western Area	69	16	26	39
Enterococcus bacteraemia				
Enterococcus faecali	281	301	289	298
Enterococcus faecium	332	406	397	392
Norovirus outbreaks	111	163	115	NA***
Notified infection cases in Ireland	1317	1777	1638	1931
Notified infection cases in HSE North Western area	36	173	96	66

*Outbreaks include family and general outbreaks. 2007-2009 national data were extracted from Health Protection Surveillance Centre annual reports. 2010 data were taken from national Communicable Infectious Diseases Reporting (CIDR) system was provisional. Notified verotoxigenic *E. coli* and enterococci infection cases were taken from EARSS system and 2010 Norovirus cases in HSE North Western area were taken from CIDR system.
**National Health Protection Survellance Centre only notify EHEC cases directly to HPSC through EARSS system.
*** 2010 national Norovirus outbreaks data was not available in CIDR system.

Conclusions

1. Human noroviruses, *E. faecalis*, and *E. faecium* were frequently detected in plants A, B and D serving agglomerations. The connection with infection cases notified in the agglomerations, especially in those where health care centres are located, demonstrated that raw wastewater can provide epidemiological information to health protection agencies.
2. Norovirus genogroup II and *E. faecalis* were found more frequently discharged to fresh waters than norovirus genogroup I and *E. faecium*, despite the treatment process (activated sludge system and biofilm percolating system). The biofilm-coated percolating system may present better treatment for norovirus particles in suspension but may however, accumulate potentially active viral particles in the produced primary sludge.
3. In this study, we suggest that local authorised waste management bodies re-evaluate their current wastewater treatment systems in order to adjust to modern human activities, such as tourism or seasonal migration to regular holiday destinations (e.g. holiday homes in other regions or countries)and to effectively provide pathogen inactivation systems rather than acting as pathogen reservoirs.

Chapter Seven

POTENTIAL MICROBIAL INDICATORS FOR *CRYPTOSPORIDIUM* AND OTHER PATHOGENS

7.1 General Introduction

This chapter originated from one peer-reviewed journal paper entitled 'Determining potential indicator of *Cryptosporidium* oocysts throughout the wastewater treatment process' (Appendix IV).

Most research on wastewater treatment efficiency compliance focuses on physicochemical and microbial indicators; however, very little emphasis has been placed so far on determining suitable indicator organisms to predict the discharge level of pathogens from treatment plants. In this study, raw wastewater, activated sludge, and the resulting final effluents and biosolids in four municipal wastewater treatment plants (WWTPs A, B, C and D) were seasonally investigated for human-virulent water-borne pathogens *Cryptosporidium parvum*/*hominis* and *Giardia duodenalis*, and microsporidia (e.g. *Encephalitozoon hellem*, *E. intestinalis*, and *Enterocytozoon bieneusi*) between 2008 and 2009. A suite of potential microbial indicators for human-virulent protozoa and microsporidia was also determined. A combination of multiple fluorescent in situ hybridization and immunofluorescent antibody assays were applied to detect *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores. *Escherichia coli*, enterococci and *Clostridium perfringens* spores were cultivated in selected media.

7.2 Determining potential indicators of *Cryptosporidium* oocysts throughout the wastewater treatment process

Introduction

Secondary biological treatment processes, for example, activated-sludge treatment, percolating filtration systems, rotating biological contactors, and submerged fixed film systems have been used at municipal wastewater treatment plants (WWTPs) world-wide for removal of nutrients. In order to meet the EU Urban Wastewater Treatment Directive standard, the environmental agencies and local authorities throughout the EU-15 Member States have been requested to monitor the residual nutrient in the discharged final effluent and biosolids on a regular basis since 1998 (CEC 1991; EEA 2005). However, there is very little emphasis on restraining the discharge of waterborne pathogens from the wastewater treatment facilities (Blumenthal *et al.* 2000; EEA 2005).

Evidence shows that the Irish national burden of gastroenteritis caused by pathogenic water-borne microorganisms, for instance pathogenic *Escherichia coli*, *Clostridium perfringens*, and human-virulent protozoa: *Cryptosporidium parvum*, *C. hominis* and *Giardia duodenalis*, has been increasing in the new millennium. Since 2004, when cryptosporidiosis was added to the list of notifiable infectious diseases, cases of acute infectious gastroenteritis have increased in scale (Garvey & McKeown 2008). *Cryptosporidium* and *Giardia* are human-virulent protozoan parasite species, forming resistant and long-lasting transition stages in the environment, termed oocysts and cysts respectively (Caccio *et al.* 2006). Microsporidian spores have also been found in a range of lakes and rivers used for drinking water abstraction and recreation (Lucy *et al.* 2008). Similar to *Cryptosporidium* oocysts and *Giardia* cysts, microsporidian spores (ranging from 0.8 to 2.0 µm in size) can resist

drinking water and wastewater treatment processes and survive in the surface water for a period of time (Fournier *et al.* 2000; Didier *et al.* 2004).

Studies utilising bacterial surrogates, i.e. *E. coli*, enterococci and *C. perfringens*, have been evaluated at laboratory scale, acting as potential indicators of the presence of *Cryptosporidium* and *Giardia* in soil, fresh waters, drinking water, and treated effluent from wastewater treatment plants (Robertson *et al.* 1992; Costan-Longares *et al.* 2008; Wilkes *et al.* 2009). A preliminary report from our research group indicated the evidence of human-virulent *Cryptosporidium* and *Giardia* prevailing in the local human populations (Cheng *et al.* 2009). The objective of this study was to quantitatively determine and compare the concentration of *Cryptosporidium* oocysts, *Giardia* cysts, microsporidian spores and faecal indicator bacteria, i.e. *E. coli*, enterococci, and *C. perfringens* spores throughout the wastewater treatment process and in the corresponding final effluent and biosolids seasonally between 2008 and 2009. Evidence-dependent regression analysis was carried out to create a potential *Cryptosporidium* predictive model.

Materials and methods

Characteristics of WWTPs and source of wastewater samples

This study focused on four secondary wastewater treatment plants (WWTPs A–D) located in north-western Ireland, serving up to 4,000 population equivalents. Wastewater samples (raw sewage, secondary activated sludge, final effluent) and biosolids were collected in April, July, October in 2008 and January and February in 2009. WWTP A is located in a seaside tourist town, in which a flux of travellers, particularly surfers, take regular holiday visits, especially in the summer period. WWTPs B, C, and D are situated in stable residential areas. All plants are designed as separate sewerage systems receiving only domestic wastewater, except Plant B, which accepts a combination of domestic sewage and proportional surface run-off in wet

conditions. The specific treatment processes used in each of the four wastewater treatment plants are described in Table 7.1. Briefly, the raw wastewater (except at WWTP B) underwent grit removal and coarse screening before reaching the secondary treatment stage. Subsequent
secondary wastewater treatments (where secondary wastewater samples were collected for this study) were applied: sludge activation in an oxidation ditch (WWTP A); sludge activation in extended aeration tanks (WWTPs B and C); and treatment by biofilm-coated percolating filter (WWTP D). The wastewater in each WWTP was then settled in a secondary settlement tank, i.e. clarifier, separating gravitationally final effluent and sewage sludge. The resulting sewage sludge was taken out of the tank and spread onto the on-site drying beds, where the dewatered biosolids samples were collected. Sewage sludge at WWTP C was obtained via a discharge valve from the secondary settling tank. All wastewater samples were collected using a long-handled 1-L sampler and transferred to 1-L sterilised polyethylene bottles. Sewage sludge cake (biosolids, approx. 100 g) was collected, by trowel, from 10-cm depth in the drying beds at WWTPs A, B, and D, while 1 L of liquid sewage sludge was obtained through the WWTP C discharge valve. All samples were collected in triplicate, and delivered to the laboratory in a cooler box (Cheng *et al.* 2009).

Enumeration of faecal indicator bacteria

Fifty millilitres (mL) of the sub-samples was mixed vigorously and 1-mL of the mixture was subjected to a ten-fold serial dilution in Ringer's solution (Oxoid, UK) to 10^{-4}. Sub-samples for *C. perfringens* spores (10 mL) were heat shocked at 75°C for 20 min and then cooled on ice (Hauschild *et al.* 1874). For *E. coli* and enterococci, 0.2-mL of the sub-samples was aseptically spread onto chromogenic *E. coli*/coliform agar (Oxoid, UK) and Slanetz and Bartley agar (Oxoid, UK) and cultivated at 35 ± 2°C and 44°C,

respectively. In accordance with the manufacturer's direction, purple colonies present on Chromogenic *E. coli*/coliform agar and burgundy red colonies on Slanetz and Bartley agar were enumerated as positive. For *C. perfringens* spores, 0.2-mL of the heat shock-processed serial diluent was aseptically spread onto Perfringens agar with supplement (Oxoid, UK). Non-supplemented Perfringens agar was overlaid onto the base and settled at room temperature. The plates were incubated anaerobically at 35 ±2°C for 18–24 h (Hauschild *et al.* 1974). Black colonies were enumerated as positive.

Pre-treatment and purification

All liquid wastewater samples were mixed vigorously and transferred to 1-L capacity Imhoff settlement cones. Twenty grams of the dewatered biosolids was rehydrated in 1 L of MilliQ water in a sterile food processor and homogenized. The liquid was then transferred to 1-L imhoff cones for gravity sedimentation. Fifty millilitres of the top sediment layer was transferred using a 50-mL glass pipette to a plastic 50-mL conical centrifuge tube and centrifuged at 3,000 g for 5 min. The supernatant was removed and the pellet was transferred and reserved in 75% ethanol in a 1.5-mL microcentrifuge tube (Graczyk *et al.* 2004). The samples were stored at 4°C. The ethanol was washed from the pellet by centrifugation (8,000 g, 5 min) twice and the pellet was subjected to sugar–phenol flotation (Ash & Orihel 1987).

Fluorescent in situ hybridization and immunofluorescent antibody assay

For *Cryptosporidium* oocysts and *Giardia* cysts, aliquots of the purified samples were treated in equal volumes of acetone for 15 min and washed in 1× phosphate-buffered saline (PBS). The Hex-fluorochrome-labelled probes (100 μmol) were added subsequently and the whole mixture was

incubated at 57°C for 1 h. After centrifugation (8,000 g, 4 min), the pellets were re-suspended in 20 µL of MilliQ water and transferred into three lysine-coated immunofluorescent wells on slides. The slides were stored at room temperature to dry and the hybridisation-processed samples were then subjected to a direct immunofluorescent antibody assay (Graczyk *et al.* 2004; Cheng *et al.* 2009).

For microsporidian spores, the resulting pellet was assayed in 1.5-mL microcentrifuge tubes by multiplex fluorescent in situ hybridisation for identification of *E. bieneusi*, *E. hellem*, and *E. intestinalis* spores. Briefly, species-specific, fluorochrome-labelled probes, i.e. HEL878F, INT-1, and BIEN-1, were used for detection of *E. hellem*, *E. intestinalis* and *E. bieneusi* spores, respectively. The probes, which hybridise the 16S ribosomal RNA of specific microsporidian spore species, were added to purified samples and incubated at 57°C for 3 h (Graczyk *et al.* 2004). The processed samples (20 µL in MilliQ water) were placed onto the wells of immunofluorescent slides and the slides were stored at room temperature to dry. *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores were identified and enumerated without knowledge of sample identity using an epifluorescent microscope, at 100× objective magnification, and BP450-490 exciter filter.

Statistical analysis

As it is generally accepted that faecal indicator bacterial concentration in sewage samples follow a $\log_{10}$ normal probability, the mean concentration is then calculated as $\log_{10}$ (x+1), where x=concentration of the indicator bacteria (CFU 100 mL^{-1}). Correlation between Log_{10}-transformed indicator bacteria concentration and (oo)cysts and spore concentration was conducted using Spearman rank coefficient r_s. Pathogen detection frequency was determined and the removal efficiency was calculated as a percent of the difference in pathogen concentration in the effluent vs. influent. Negative

Table 7.1. Characteristics of four wastewater treatment plants (WWTPs A-D), 2009

Plants	Population equivalent	Sewerage system	Primary treatment	Secondary treatment	Receiving water body	Operation
Plant A 54°16043″ N 08°36020″W	1,950	Separate	Grit removal and course screening	Oxidation ditch, sedimentation, onsite dewatering	Sea	Local authority
Plant B 54°11011″ N 08°29010″W	1,060	Semi-combined	None	Extended aerated activated sludge system, onsite dewatering	River	Local authority
Plant C 54°20011.3″ N 08°31044.3″W	4,000	Separate	Grit removal, course screening, and sedimentation	Extended aerated activated sludge, settlement	River	Private sector
Plant D 54°02054″ N 08°43028″W	2,500	Separate	Grit removal and primary sedimentation	Percolating settlement, onsite dewatering	River	Local authority

values were obtained for the cases in which the pathogen concentration was higher in the effluents as compared to the influents. All descriptive statistics, Spearman rank correlation coefficients and Wilcoxon tests were calculated using STATISTICA 6.0 (StatSoft, Inc, 2002, Tulsa, USA).

Results and discussion

Presence and abundance of faecal indicator bacteria

The geometric mean concentrations, 95% intervals and range concentration of faecal indicator bacteria, expressed as Log10, are shown in Figure 7.1. The geometric means (GM) have shown WWTPs A and B had averagely higher microbiological concentration, with 4.3 Log unit 100 mL^{-1} (WWTP A) and 4.2 Log unit 100 mL^{-1} (WWTP B) of *E. coli*, 2.8 Log unit 100 mL^{-1} (WWTP A) and 2.3 Log unit 100 mL^{-1} (WWTP B) of enterococci, and 3 Log unit 100 mL^{-1} of *C. perfringens* spores at both plants. WWTP C had the lowest GM concentration of all indicator bacteria. However, higher concentrations of *E. coli* (3.5 Log unit 100 mL^{-1}) were observed in raw wastewater samples in February 2009, compared to WWTPs A, B, and D (mean: 3.1 unit 100 mL^{-1} of *E. coli*). The results also highlighted the wide variability of faecal indicator bacteria concentration in sewage samples by site, treatment stage and season (95% intervals and range concentration, Figure 7.1). For example, *E. coli* had the highest concentration of 6.6 Log unit 100 mL^{-1} in WWTP B raw wastewater, in summer 2008, followed by concentration of *C. perfringens* spores (4.1 Log unit 100 mL^{-1}) and enterococci (3.3 Log unit 100 mL^{-1}). In the same season, WWTP B had the highest amount of indicator bacteria, followed by WWTP A (*E. coli*: 6.4 Log unit 100 mL^{-1}; enterococci: 4.0 Log unit 100 mL^{-1}; *C. perfringens* spores: 3.6 Log unit 100 mL^{-1}), and WWTP D had the least faecal indicator bacteria with 3.7 Log unit 100 mL^{-1} of *C. perfringens* spores and no enterococci detected. In most scenarios, over 92% of the indicator bacteria were removed during treatments whilst *E. coli* and *C. perfringens* spores

were occasionally detected at high concentration in the discharging effluents (e.g. *E. coli*: 5.8 Log unit 100 mL^{-1} at WWTP D in October 2008; *C. perfringens* spores: >3.9 Log unit 100 mL^{-1} at WWTP B in both summer and autumn seasons). The order of the indicator bacteria concentration was: *E. coli*> *C. perfringens* spores> enterococci. However, *C. perfringens* spores were frequently detected in greater concentrations than *E. coli* at WWTP D.

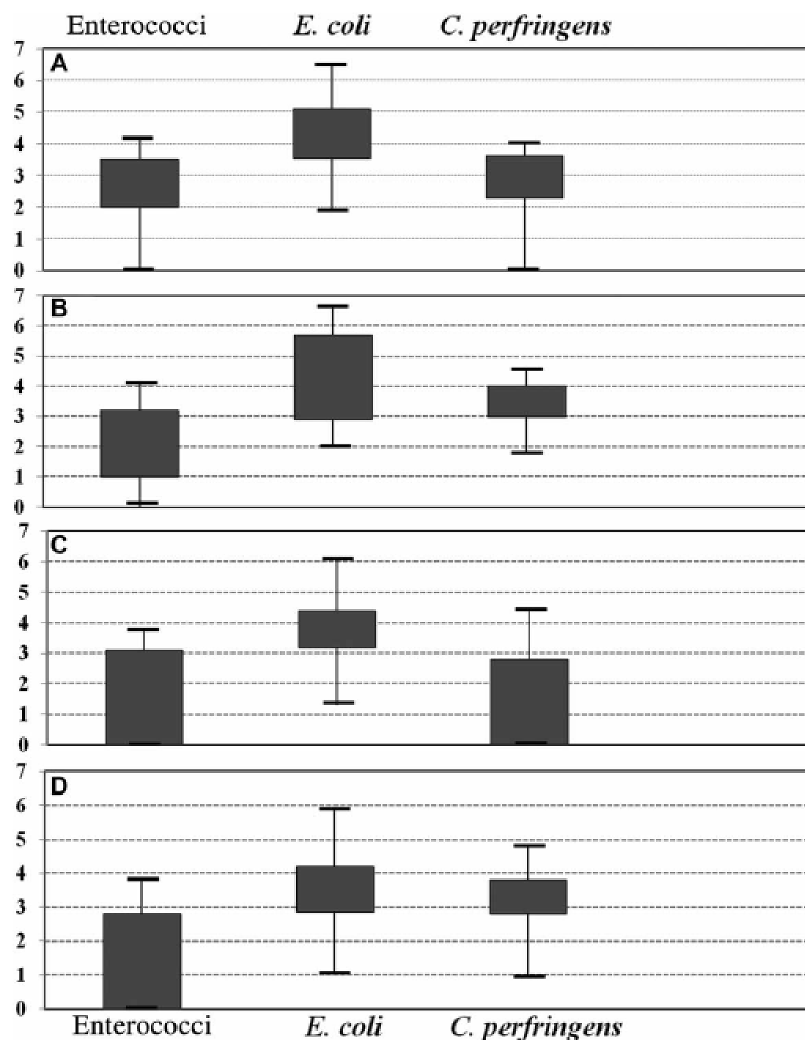

Figure 7.1. Geometric mean, 95% intervals (box) and range (line) of Log10 faecal indicator bacteria concentration in all wastewater samples at Plants A–D, 2008–2009.

Presence and abundance of human-virulent Cryptosporidium oocysts, Giardia cysts, and microsporidian spores

The geometric mean concentrations, 95% intervals and range concentration of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores are shown in Figure 7.2. *Cryptosporidium* oocysts were found in higher concentration than other pathogens at all plants with GM ranging from 20 to 180 oocysts/L among WWTPs, followed by *E. intestinalis* spores (GM= 6–83 oocysts/L) and *Giardia* cysts (GM=6–130 oocysts/L). All of the raw sewage samples were shown positive for *Cryptosporidium*, with the highest abundance at WWTP B (>590 oocysts/L) in the summer season. WWTP C had the least pathogen concentration, with averagely 20 *Cryptosporidium* oocysts/L, 6 *Giardia* cysts/L, and 34 microsporidian spores detected. On average, 76% of *Cryptosporidium* oocysts and 90% of *Giardia* cysts in wastewaters were removed after treatment. Among the four plants, WWTP C had the highest removal efficiency of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores at 84, 98, and 99.5%, respectively. *Cryptosporidium* was dominant over all other pathogens, i.e. *Giardia* cysts, which were found in a higher concentration than *Cryptosporidium* oocysts and *E. bieneusi* spores in the previous Irish study (Graczyk *et al.* 2007), and spores of *E. hellem*, *E. bieneusi* and *E. intestinalis* (in this study). Seasonal variation of pathogen detection frequency with high abundance in the incoming human sewage, especially at WWTPs A and B. WWTP A is a tourist town, demonstrating that seasonal-dependant sudden traveller influx may be the key factor for the insufficient treatment as well as a potential source of new pathogens. WWTP B is overloaded due to the expansion of town size. Even though the highest abundance of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores were detected in summer at WWTPs A and B, it was found that an averagely high abundance of *Cryptosporidium* oocysts (GM=195 oocysts/L), *Giardia* cysts (GM=81 cysts/L) and *E. intestinalis* spores (GM=73 spores/L) were present in winter

samples at all of the four plants, intermitting medium background levels of pathogens in the studied human populations.

The detection frequencies among individual pathogens were varied (Table 7.2). *Cryptosporidium* was detected in over 93% of the samples in July (summer), October (autumn) 2008 and January and February (winter) in 2009, with the exception of April (spring) samples at 69% detection frequency. *Giardia* had a higher detected frequency in the summer (87.5%) and winter (>81%) seasons whilst *E. hellem* was detected more in autumn (>93%). All in all, seasonal variation was noticed in the pathogen detection frequencies, especially between the spring and winter seasons (r_s=0.89, p<0.05), indicating higher *Cryptosporidium* and *Giardia* detection frequency in winter. The finding in this study presents different *Cryptosporidium* occurrence trends in comparison to the pronounced spring peak in other studies in the Republic of Ireland (Chalmers *et al.* 1997; Zintl *et al.* 2006). However, Zintl *et al.*(2008) later pointed out that a second small *Cryptosporidium* peak appeared in late autumn, the year after the large cryptosporidiosis outbreak in Co. Galway, Ireland (Zintl *et al.* 2008) although this was not observed in a later EPA study (de Waal et al. 2013, *In prep*). The variation among pathogen abundance was noticed at the four WWTPs from plant to plant (within seasons) as well as in different seasons. In many cases, the pathogens were most frequently detected in the highest abundance at WWTP B. However, a seasonally-dependent high pathogen load was determined at WWTP A in both summer and autumn seasons (Wilkes *et al.* 2009). WWTP C had the least pathogen load in all seasons. It was obvious that the pathogen presence scenario differed from plant to plant, with WWTP A versus D (t-value=2.75, df=8, p=0.02); WWTP B versus C (t-value=2.64, df=8, p=0.03) and D (t-value=3.41, df=8, p=0.01). It is noted that plant C is under-loaded during investigation.

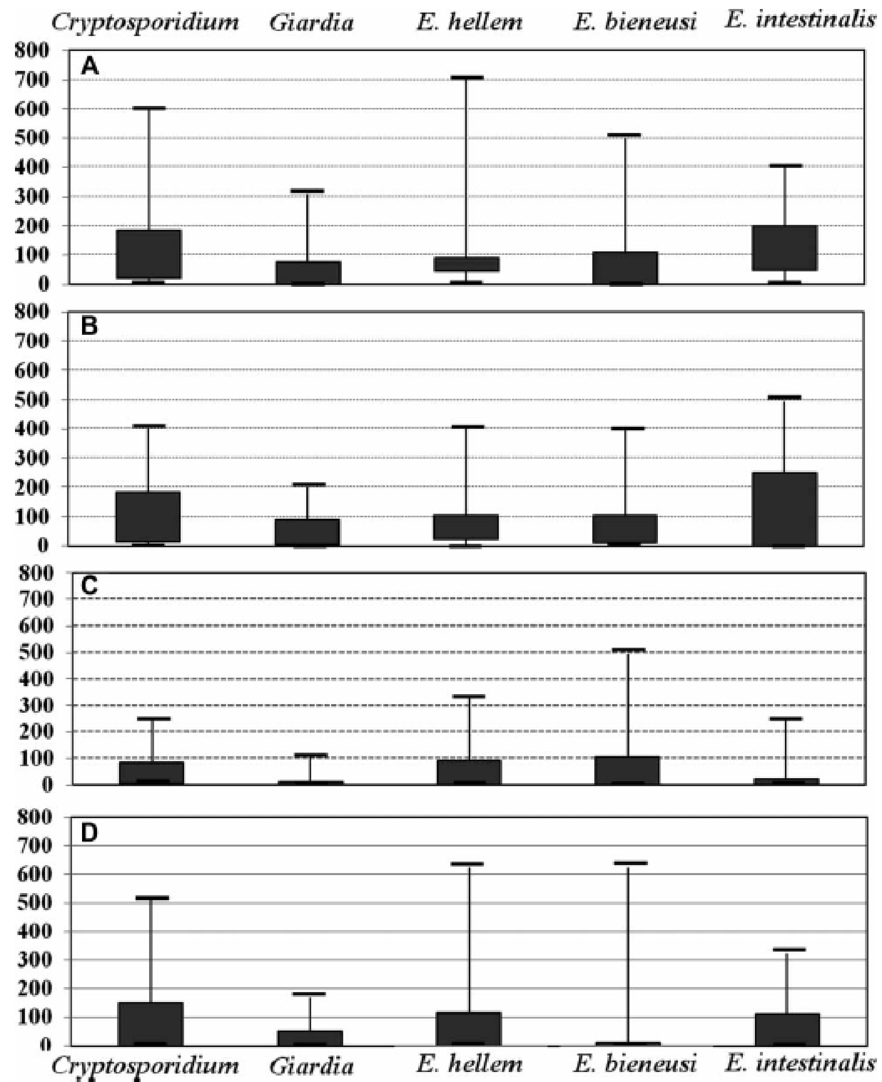

Figure 7.2. Geometric mean, 95% intervals (box) and range (line) of pathogen concentration in all wastewater samples at Plants A–D, 2008–2009.

Relationship between the abundance of faecal indicator bacteria and abundance of pathogens

Spearman rank correlation analyses were conducted using all non-categorical microbial data and pathogen abundance during the study period (Table 7.3). Significant correlations ($\alpha<0.05$) were found between enterococci and *E. coli* (r_s=0.6072). Correlations between *C. perfringens* spores and other faecal indicator bacteria were weak, ranging from 0.21 to 0.29. All faecal indicator bacteria had a significant correlation with *Cryptosporidiu*m (r_s=0.40–0.48), *Giardia* (r_s=0.44–0.50), and microsporidian spores (r_s=0.43–0.57). There was no significant correlation between spores of *C. perfringens* and *E. bieneusi* and *E. intestinalis*. It was noted that *E. coli* had the strongest correlation (r_s=0.48–0.57) with the detected pathogens. Furthermore, the combination of *E. coli*, enterococci, and *C. perfringens* spores contributed to the significantly positive correlation with the occurrence of *Cryptosporidium* oocysts, *Giardia* cysts and *E. hellem* spores. Microsporidia are included in the frequently detected pathogens in the Irish disease control and prevention list. Therefore, the source of high detected concentration of microsporidian spores in the studied domestic wastewater is still unknown. Nevertheless, a few studies linked microsporidian spores detected in urban wastewaters to domestic dogs and livestock and avian visiting to the treatment plants (Graczyk *et al.* 2007, 2009).

A strong positive correlation was found between abundance of *Giardia* cysts and abundance of *Cryptosporidium* oocysts (r_s=0.89, $p<0.05$). Instead of detecting both of the parasites, it is time-effective and economic to detect one of them. As *Giardia* cysts have a larger size than *Cryptosporidium* oocysts and since *Giardia* cysts are commonly found in a significant relationship with *Cryptosporidium* in the same environment (Bajer 2008), we suggest that *Giardia* cysts can be used as a potential indicator to predict the occurrence of human-virulent *Cryptosporidium* oocysts.

Table 7.2. Presence, absence and detection frequency of *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores

	Spring			Summer			Autumn			Winter		
	Presence (N)	Absence (N)	Detection (%)	Presence (N)	Absence (N)	Detection (%)	Presence (N)	Absence (N)	Detection (%)	Presence (N)	Absence (N)	Detection (%)
Cryptosporidium	22	10	68.8	15	1	93.8	15	1	93.8	15	1	93.8
Giardia	17	15	53.1	14	2	87.5	10	6	62.5	13	3	81.3
E. bieneusi	23	9	71.2	10	6	62.5	12	4	75.0	15	1	93.8
E. intestinalis	16	16	50.0	12	4	75.0	15	1	93.8	10	6	62.5
E. hellem	16	16	50.0	10	6	62.5	10	6	62.5	13	3	81.3

Table 3. Spearman rank order correlations. Marked coefficients are significant at p< 0.01

	Enterococci	*E. coli*	*C. perfringens*	*Cryptosporidium*	*Giardia*	*E. hellem*	*E. bieneusi*	*E. intestinalis*
Enterococci	1.0000	**0.6072**	0.2134	**0.4781**	**0.4437**	**0.4516**	**0.4930**	**0.4308**
E. coli	**0.6072**	1.0000	0.2926	**0.4809**	**0.5054**	**0.5695**	**0.5310**	**0.4742**
C. perfringens	0.2134	0.2926	1.0000	**0.4020**	**0.4669**	**0.4570**	0.3450	0.3602
Cryptosporidium	**0.4781**	**0.4809**	**0.4020**	1.0000	**0.8919**	**0.5458**	**0.4150**	**0.5331**
Giardia	**0.4437**	**0.5054**	**0.4669**	**0.8919**	1.0000	**0.5613**	**0.4185**	**0.5240**
E. hellem	**0.4516**	**0.5695**	**0.4570**	**0.5458**	**0.5613**	1.0000	**0.5498**	**0.7865**
E. bieneusi	**0.4930**	**0.5310**	0.3451	**0.4150**	**0.4185**	**0.5498**	1.0000	**0.5475**
E. intestinalis	**0.4308**	**0.4743**	**0.3602**	**0.5331**	**0.5240**	**0.7865**	**0.5475**	1.0000

Multiple linear regression was analysed using the abundance of *Cryptosporidium* oocysts and *Giardia* cysts in all samples, resulting in the overall predictive regression model: *Cryptosporidium* oocysts=(3 ± 0.9) ± (1.2± 0.8) *Giardia* cysts (Table 7.4). Since the pathogen presence scenario differed from plant to plant, the predictive regression models specific for WWTPs A–D were also determined (Table 7.4).

Table 7.4. The *Cryptosporidium* regression predictive models (at WWTPs A-D and overall)

Plants	Regression predictive models	*p* value
Plant A	*Cryptosporidium* oocysts = (44.6 ± 1.8) + (11.7 ± 0.1) *Giardia* cysts	$R^2 = 0.91$, $p < 0.05$
Plant B	*Cryptosporidium* oocysts = (1.82 ± 0.23) *Giardia* cysts	$R^2 = 0.78$, $p < 0.05$
Plant C	*Cryptosporidium* oocysts = (1.78 ± 0.17) *Giardia* cysts	$R^2 = 0.84$, $p < 0.05$
Plant D	*Cryptosporidium* oocysts = (2.45 ± 0.27) *Giardia* cysts	$R^2 = 0.81$, $p < 0.05$
Overall	*Cryptosporidium* oocysts = (3 ± 0.9) + (1.2 ± 0.8) *Giardia* cysts	$R^2 = 0.74$, $p < 0.05$

Conclusions

A suite of faecal indicator bacteria can be used as microbial indicators for the presence of *Cryptosporidium* oocysts, *Giardia* cysts and *E. hellem* spores. Spatial variation of the bacteria abundance and pathogen loads in the studied areas may be caused by sudden traveller influx in tourism seasons, especially at WWTP A in summer and autumn. A background abundance of *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores were also noticed at WWTPs A and B. The overall predictive regression model: *Cryptosporidium* oocysts= (3 ± 0.9) + (1.2 ± 0.8) *Giardia* cysts (R^2=0.74, p<0.05).

Chapter Eight

FINAL DISCUSSION

8.1 Prevalence of Human-virulent Pathogens in the Plants serving Regions in Co. Sligo and Related Implications

Waterborne diseases occur worldwide and outbreaks caused by contamination of community water systems have the potential to cause disease in large number of consumers. National statistics on outbreaks linked to contaminated water have been available in the USA since 1920 (Craun 1986). Since 1971, the Centers for Disease Control (CDC), the US Environmental Protection Agency (US EPA) and the Council of State and Territorial Epidemiologists have maintained a collaborative surveillance system for collecting data pertaining to the occurrence and causes of outbreaks of waterborne disease (Barwick *et al.* 2000; Lee *et al.* 2002; CDC 2007). In Europe, 277 outbreaks associated with drinking water and recreational water were reported from 16 European countries during 1986-1996 (Kramer *et al.* 2001). In Ireland, several risk assessment projects on *Cryptosporidium* have been carried out, for example the NUIG project on 'Enhancing human health through improving water quality' (Workpackage 5: the impact of water supply, weather, agriculture and environment on cryptosporidiosis in the west of Ireland) and two projects in UCD ('Cryptosporidiosis Network Ireland' and 'Towards developing a microbial risk assessment/forecast model for Cryptosporidiosis'). Nevertheless, the most likely root cause of water contamination, microbial pathogens present in human faeces-originated discharging effluent, is not routinely assessed and analyzed. This highlights a gap in understanding the fate of pathogens in wastewater, during wastewater processing and their subsequent common release to the aquatic environment.

It is crucial to develop robust and rapid detection methods, providing efficient microbial examination in wastewater, final effluent and biosolids samples. Therefore, technique transfer (i.e. IFA and FISH methodologies on wastewater sample detection) from Johns Hopkins Bloomberg School of

Public Health, Baltimore was a prerequisite for the methodologies utilised in this project. Based upon collected microbial data, it was clearly demonstrated that potentially viable/active human-virulent pathogens (i.e. *Cryptosporidium parvum*, *C. hominis*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Enterococcus faecium, Enterococcus faecalis* and norovirus genotype I and genotype II) are transferred to municipal wastewater treatment plants via sewerage networks. It was also observed that the prevalence of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in inflowing wastewater differed significantly at all WWTPs. The prevalence of individual pathogens differed significantly from season to season. For example, higher concentration of *Cryptosporidium* oocysts, *Giardia* cysts microsporidian spores were monitored in summer compared to spring, autumn and winter samples (Outlined in Chapter 3 and Chapter 4). This may link to seasonal tourism, agricultural activities (calving, lambing) and spreading of slurry. Prevalence of noroviruses (both genotypes I and II) and *Enterococcus faecium* and *E. faecalis* differed significantly by seasons at all plants (Outlined in Chapter 6). In winter months, noroviruses were frequently detected in the influent samples at all monitored WWTPs.

Concurrently, this project provided information on the presence/levels of human-virulent *Cryptosporidium parvum*, *C. hominis*, *G. duodenalis*, *E. bieneusi*, *E. intestinalis*, *E. hellem*, *E. faecium*, *E. faecalis* and noroviruses in studied communities by looking at their occurrence in wastewater. The studies presented in Chapter 7 showed that there was connection between frequencies of microbial occurrence and notified infection cases in the agglomerations. The study demonstrates that raw wastewater can provide epidemiological information to health protection agencies and this information is of use to Public Health scientists as it provides parallel information to analysis gained from human stool samples (clinical samples).

8.2 Comparison of Removal Efficiency of Human-Virulent Pathogens among secondary WWTPs in Co. Sligo

The overarching aim of this project is to evaluate how efficient secondary WWTPs were in the removal of human enteric pathogens. It is outlined in Chapter 3 and Chapter 4 that WWTP C (combined biofilm-coated STM-aerotor and activated sludge system, Chapter 2.1.3) had the highest removal efficiency in the removal of *Cryptosporidium* oocysts (averagely 90% removal rate), *Giardia* cysts (averagely 99.7% removal rate) and microsporidian spores (averagely 99%). It was noted that *E. faecalis* and *E. faecium* were absent during the whole investigation period in WWTP C. Overall, WWTP C provided the most efficient treatment in the removal of human-virulent pathogens. Negative removal capability of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores was observed frequently at WWTP D (biofilm-coated percolating filtration system, Chapter 2.1.3). Generally speaking, studied activated sludge systems were found to be more effective processes to remove *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian sporesthan the percolating filtration system. In Stadterman's study (Stardtman *et al.* 1995), it was found that oocysts were poorly removed without a settling blanket (activated sludge biofloc), which facilitates mechanisms of sedimentation. In the same study, 48% more oocysts were removed in the activated sludge system in 2.8 hours than in the percolating filtration system (Stardtman *et al.* 1995). The biofilm-coated percolating system was however found to provide better treatment for norovirus particles in this study as outlined in Chapter 6.

System design, maintenance and management are critical control measures in process capability (i.e. removal efficiency of WWTPs). WWTP C was commissioned in 2004 and equipped with the state-of-art treatment system, whilst WWTP D was commissioned in 1970 and upgraded with capital funds from Sligo County Council in 2009. WWTP C is operated and managed by private subcontracted company whilst WWTP D is operated

and managed by the local county council. According to one United Nations Environment Programme (UNEP) publication (Corcoran *et al.* 2010), countries must adopt a multi-sectoral approach to wastewater management as a matter of urgency, incorporating principles of ecosystem based management from the watersheds into the sea, connecting sectors that will reap immediate benefits from better wastewater management. This is in line with the EU water framework directive and the targets for good water quality that must be achieved in Ireland and other member states by 2015. Developing and maintaining better wastewater treatment processes would improve overall water quality (particularly nutrients and suspended solids) as well as reducing pathogen loads to the aquatic environment. Successful and sustainable management of wastewater requires a cocktail of innovative approaches that engage the public and private sector at local, national and trans-boundary scales. Planning processes should provide an enabling environment for innovation, including at the community level. Innovative financing of appropriate wastewater infrastructure should incorporate design, construction, operation, maintenance, upgrading and/or decommissioning. Financing should take account of the fact that there are important livelihood opportunities in improving wastewater treatment processes (Corcoran *et al.* 2010).

8.3 Potential Use of Faecal Indicator Bacteria on Human-virulent Pathogen Prediction

Studies utilising faecal indicator bacteria, i.e. *E. coli*, enterococci and *C. perfringens*, have been evaluated at laboratory scale, acting as potential indicators of the presence of human-virulent pathogens (Robertson *et al.* 1992; Ferguson *et al.* 1996; Costan-Longares *et al.* 2008; Wen *et al.* 2009; Wilkes *et al.* 2009). The studies in Chapter 7 demonstrated the all faecal indicator bacteria (i.e. *E. coli*, enterococci and *C. perfringens*) had a significant correlation with *Cryptosporidium* (rs=0.40-0.48), *Giardia*

(rs=0.44-0.50) and microsporidian spores (rs=0.43-0.57). A strong positive correlation was found between abundance of *Giardia* cysts and abundance of *Cryptosporidium* oocysts.

8.4 Limitations of this study

Some limitations were noted during the investigation. First of all, the influent, activated sludge and effluent samples were collected on the same day when visiting the plants. At maximum one hour was spent at plants during sample collection. Influent samples were taken from inlet chamber (Plants A, B and D) and influent bottle (Plant C). Activated sludge samples were taken from aeration tank (Plants A-C) and percolating tank (Plant D). This brings into question whether activated sludge samples can be considered as the same wastewater batch as the influent and the effluent as the wastewater process takes at least 24 hours to process.

Second, some results obtained demonstrated a zero target pathogen result present in the samples. Due to the limitation of both the sampling and the laboratory methods applied, notably percentage loss from each recovery stage (during sampling and in the laboratory), then it must be assumed that the ‘Zero target pathogen in the sample’ reported in fact means that the target pathogen is not detectable by applied methodology and not necessarily that is absent from the sample in question.

This study also demonstrates the value of wastewater testing for future epidemiological studies. During the data collection, it was noted that healthcare database may under-estimate the cases of cryptosporidiosis and giardiasis in the community. In order to ensure that all cases of cryptosporidiosis and giardiasis are captured in healthcare database, it is crucial to encourage and urge general practitioners to report observed cases (either confirmed or suspected) to the health protection surveillance centre.

Chapter Nine

CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

This research project was funded under the broad EPA research theme: **Health Risks from Exposure to Contaminated Soil and Waters** and designed to investigate adverse health impacts of human enteropathogens (i.e. *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Giardia duodenalis*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Enterococcus faecium*, *Enterococcus faecalis* and noroviruses) in wastewater and sludge end products. This thesis is the first comprehensive study focused on treatment efficiencies of regional secondary WWTPs in the removal of human-virulent enteropathogens in the Republic of Ireland. This research provides scientific information on 1) comparison of human enteropathogen removal between WWTPs during different seasons of the year, 2) seasonal variation of pathogen loads existing in WWTPs serving local population and indication of public health in studied communities, 3) identifying microbial risks of discharging end products (i.e. final effluent and biosolids) from WWTPs to local population, 4) the potential use of faecal indicator bacteria to establish *Cryptosporidium* prediction model.

Robust and innovative molecular biological technologies (i.e. combination of multiple fluorochrome-based immunofluorescent antibody staining, rRNA-based fluorescent in situ hybridiation, RNA-polymerase based PCR methods) were successfully transferred from collaborative laboratories (i.e. Johns Hopkins Institute of Public Health, Baltimore and Tatung University, Taipei) to the Centre for Biomolecular Environmental and Public Health research group in the Institute of Technology, Sligo. These methodologies were used to qualitatively and quantitatively assess the load, origin, and viability of human-virulent enteropathogens, including bacterial, protozoan, viral pathogens, throughout the secondary wastewater treatment process and in end products at WWTPs A-D in Co. Sligo. WWTP

C operated by the private sector and equipped with the state-of-art facilities (combination of activated sludge and filtration treatments) provided the most effective pathogen removal during wastewater treatment processes. This research project also showed seasonal variation in concentration of human-virulent enteropathogens is correlated with seasonal factors, such as calving and lambing seasons, tourism, and wildfowl input from visiting wildlife.

During the investigation period, new management of biosolids and WWTP waste was introduced in Co. Sligo, including the development of regional sludge processing hub and requirement of nutrient removal and management plans before the treated biosolids applied to land. Additional, a tertiary WWTP equipped with anaerobic digester was launched in Sligo City. Plants A and D were also upgraded and improved to provide timely treatment. Plant D was upgraded with two additional primary settlement tanks.

This intensive investigation of treatment efficiencies of WWTPs in Co. Sligo highlights that municipal WWTPs act as pathogen reservoirs. It is also worth mentioning that even though WWTPs remove the microbial loads in wastewater during treatment processes, not all pathogens are removed and surviving human-virulent enteropathogens in the discharging end products from WWTPs are therefore an environmental pathogen contamination source. This emphasizes high public health risks to local populations due to potential contamination of surface waters used for drinking water abstraction and recreation. When the correlation between faecal indicator bacteria and human-virulent enteropathogens are compared, this study demonstrates that a suite of faecal indicator bacteria can be used as microbial indicators for the presence of *Cryptosporidium* oocysts, *Giardia* cysts and *E. hellem* spores. The selection of the indicator depends on availability of resources, skilled personnel and budget constraints.

9.2 Recommendations

In light of rapid global change, wastewater management should be planned against future scenarios, e.g. increased population size and elevated rainfall due to climate change predictions and not just according to the current demographic situation. It also has to take economic parameters (budget constraints) and environmental variables into account. It is vital to carry out routine surveillance on treatment efficiencies of municipal WWTPs in the removal of human-virulent enteropathogens. Additionally, routine surveillance of pathogen loads in the discharging end products (i.e. final effluents) and receiving waters is recommended to provide accurate risk assessment of discharging treated wastewater to local environments. Control strategy of wastewater treatment (i.e. process capability, control indicators for microbial abundances and nutrients discharge) should be in place for each WWTP to prevent potential causes of wastewater treatment failure made in Ireland. In order to achieve continuous improvement of municipal wastewater treatment, Wastewater Management-focused Education Programmes (for wastewater treatment operators, management team and stakeholders) are also recommended to local county councils, private companies and to the Irish Water state agency.

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APPENDIX I

Parasitol Res
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ORIGINAL PAPER

Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during secondary wastewater treatments

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Abstract This study investigates the fate of *Cryptosporidium parvum* and *C. hominis* oocysts and *Giardia duodenalis* cysts at four Irish municipal wastewater treatment plants (i.e., Plant A, B, C, and D) that utilize sludge activation or biofilm-coated percolating filter systems for secondary wastewater treatment. The fate of these pathogens through the sewage treatment processes was determined based on their viable transmissive stages, i.e., oocysts for *Cryptosporidium* and cysts for *Giardia*. Analysis of final effluent indicated that over 97% of viable oocysts and cysts were eliminated, except at Plant C, which achieved only 64% of oocyst removal. A significant correlation between the removal of oocysts and cysts was found at Plants A, B, and D (R=0.98, P<0.05). All sewage sludge samples were positive for *C. parvum* and *C. hominis*, and *G. duodenalis*, with maximum concentrations of 20 oocysts and eight cysts per gram in primary sludge indicating the need for further sludge sanitization treatments. This study provides evidence that *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts are present throughout the wastewater processes and in end-products, and can enter the aquatic environment with consequent negative implications for public health.

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Introduction

Cryptosporidium parvum, *Cryptosporidium hominis*, and *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) are obligate protozoan parasites and causal agents of cryptosporidiosis and giardiasis, respectively, in both HIV-infected and immunocompetent individuals (Wolfe 1992; DuPont et al. 1995; Caccio et al. 2003). These pathogens are prevalent in the environment and can be opportunistically spread via their transmissive stages, i.e., oocysts and cysts, respectively (Graczyk et al. 2008a, b).

The end-products of municipal wastewater treatment plants (WWTPs), i.e., final effluent and stabilized sludge (biosolids), have been reported to contain the aforementioned pathogens (Ahmad et al. 1997; Lonigro et al. 2006; Graczyk et al. 2007) as they are common fecal constituents in raw sewage delivered via sewerage network systems to

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municipal WWTPs (Robertson et al. 2000, 2006). Conventional secondary sewage treatment systems are not designed to completely remove these pathogens (Robertson et al. 2000; Caccio et al. 2003). In Ireland, raw sewage may be transported separate to urban run-off or alternatively the sewerage network may be combined with urban run-off. Primary treatment involves the settlement of preliminary treated wastewater in a sedimentation tank whereas in secondary treatment the wastewater is aerobically broken down during sludge activation in an aeration tank, oxidation ditch, or by a percolating filter system (Gray 2004). Following this treatment, the wastewater is removed to a clarifier where the solids settle by gravity. Raw liquid sludge, with more than 94% of water, is regularly removed from the bottom of either a primary sedimentation tank (in the case of primary sludge) or from a clarifier (in the case of secondary sludge) and the sewage sludge is transported to a sludge drying bed for thickening and dewatering (Gray 2004). The effluent is released to surface or ground water.

The end-products of municipal WWTPs are used in many countries (Graczyk et al. 2008b). The biologically treated effluent is discharged to rivers, lakes, groundwaters, or coastal waters, or it may be applied in agricultural irrigations (Pollice et al. 2004; Lonigro et al. 2006; Al-Saed 2007). Untreated sewage sludge may be mixed with soil as fertilizer or spread directly on land. An estimated 45,543 tonnes of sewage sludge was spread on Irish farmland in 2005 (Irish Environmental Protection Agency, IEPA 2007). Various international studies have identified high levels of *Cryptosporidium* oocysts and *Giardia* cysts in both treated biosolids and in untreated sewage sludge (Charles et al. 2003; Rimhanen-Finne et al. 2004; Brianesco and Bonadonna 2005; Montemayor et al. 2005; Graczyk et al. 2007, 2008b) that negatively impact on public health. This presents a risk factor as residual human-virulent pathogens can be transmitted via surface run-off into waters used for drinking water abstraction or for recreational purposes (Rimhanen-Finne et al. 2004; Gale 2005; Hutchison et al. 2005). Once the public makes direct contact with oocysts or cysts via consumption of inadequately treated or contaminated waters or crops, then cryptosporidiosis or giardiasis outbreaks can take place (MacKenzie et al. 1994; Caccio et al. 2003; Gale 2005). Treated municipal WWTP effluents released to surface waters used for drinking water abstraction and recreational purposes also present public health risk factors (Lucy et al. 2008). This health hazard is long-lasting as *Cryptosporidium* oocysts can remain viable for almost a year in the environment (Tamburini and Pozio 1999) and in animal liquid waste (Hutchison et al. 2005). Several studies have detected *Cryptosporidium* species (Chalmers et al. 1997; Lowery et al. 2001; Smith and Grimason 2003; Graczyk et al. 2004; Lucy et al. 2008), *G. duodenalis* (Graczyk et al. 2004; Lucy et al. 2008), and microsporidia, i.e., *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* (Graczyk et al. 2004; Lucy et al. 2008) in Irish river basins. Many studies have indicated that these pathogens are still viable, even when present at low concentrations in sludge (Graczyk et al. 2007; Montemayor et al. 2005). Their low ID_{50}, of less than ten oocysts or cysts, and resistance to environmental stressors reveal a health risk for both the public and for workers in wastewater treatment plants (DuPont et al. 2005; Hutchison et al. 2005). In Ireland, very little published data are available on the efficiency of municipal wastewater treatment systems in pathogen removal. Historically, the treatment efficiency and management of sewage sludge end-products in Ireland has not been well-documented. Since the outbreaks of cryptosporidiosis in Galway, Ireland, in 2005 and 2007 (Pelly et al. 2007), public awareness related to biosolid management has dramatically increased.

The aims of this study were to investigate survival and fate of human pathogens, *C. parvum*, *C. hominis*, and *G. duodenalis* at four Irish municipal WWTPs. The removal efficiency at each wastewater processing stage was evaluated and the survival of these pathogens through the sewage treatment was determined based on their viable transmissive stages present in the discharged effluent and corresponding sewage sludge. The pathogen removal efficiency of the different types of biological treatment applied in these plants was also compared.

Materials and methods

Wastewater and sewage sludge samples were collected from four municipal wastewater treatment plants: Plant A (54°16′43″N, 08°36′20″W); Plant B (54°11′11″N, 08°29′10″W); Plant C (54°20′11.3″N, 08°31′44.3″W); and Plant D (54°16′44″N, 08°36′90″W) in north-western Ireland (Fig. 1). The population served by these plants ranged from 1,050 to 2,500 people (Table 1), and wastewater originated from domestic houses, schools, and local businesses. The raw sewage reached each plant via combined sewerage systems. Preliminary treatment for raw sewage at Plants A, C, and D were coarse screening and grit removal whereas no screening was available at Plant B. The influent at Plant D was stored at a large open chamber prior to the secondary treatment. In Plant D, all solids were settled as primary sludge for removal to a sludge drying bed. The following secondary wastewater treatments were applied: (1) sludge activation in an oxidation ditch (Plant A); (2) sludge activation in extended aeration tanks (Plants B and C); and 3) treatment by biofilm-coated percolating filter (Plant D; Table 1). The wastewater in each WWTP was subsequently settled in a secondary settlement tank, i.e., clarifier, separating gravita-

Fig. 1 Location of four wastewater treatment plants (i.e., *A*, *B*, *C*, and *D*) in north-western Ireland. The effluent from Plant A was discharged to Sligo bay. The effluents from Plants B, C, and D were released to local rivers

tionally final effluent and sewage sludge. Plants A, B, and D were equipped with sludge drying beds, from which the sludge was removed. Sewage sludge at Plant C was collected directly for disposal from a secondary settling tank.

Wastewater samples were collected at the influent and effluent stages of each WWTP. Secondary wastewater samples were also obtained from the sludge activation processes at Plants A (i.e., oxidation ditch), B, and C (i.e., aeration tanks) and from the percolating filter tank at Plant D. Sewage sludge samples were taken from the drying beds at Plants A, B, and D and from the settling tank discharge valve at Plant C.

All wastewater samples were obtained using a long-handled 1-L-capacity sampler and transferred to 1-L sterilized polyethene bottles. Sewage sludge cake (approximately 100 g) was collected by trowel from 10 cm in depth of the drying beds at Plants A, B, and D, whereas 1 L of liquid sewage sludge was obtained from the Plant C discharge valve. All samples were collected in triplicate, delivered to the laboratory in a cooler, and processed within 2 h. Physicochemical parameters of the wastewater, i.e.,

Table 1 Physicochemical characteristics of municipal wastewater samples from four wastewater treatment plants in north-western Ireland

Plant	Secondary treatment system	Population equivalent	Source	Temp (^{o}C)	pH	Conductivity (μS/cm)	Dissolved oxygen (mg/L)	TDS (mg/L)
A	Oxidation ditch	1,914	Influent	10	8.6	670	4.3	341.5
			Secondary treatment	9.8	8.5	606.2	6.1	302.1
			Effluent	9.8	8.1	607	2.2	302.7
B	Extended aeration tank	1,058	Influent	9	7.4	790	6	391.2
			Secondary treatment	8.5	6.8	660	5.6	330.3
			Effluent	8.2	7	690	7.7	339.1
C	Extended aeration tank	2,500	Influent	6	7.6	444	3.84	223
			Secondary treatment	7.1	7.7	477	8.87	241
			Effluent	6.3	7.5	475	11.1	239.3
			Sewage sludge	7	7.8	427	3.41	213.4
D	Percolating filter	2,154	Influent	8.5	6.7	514	6.7	242
			Secondary treatment	8	7.2	430	8.4	212
			Effluent	8.2	7.1	462	8.5	231

TDS total dissolved solids

temperature, pH, conductivity, dissolved oxygen (DO), and total dissolved solids (TDS) were measured on-site using a portable Hach (Sens-Ion) meter (Table 1).

All liquid samples were shaken vigorously and transferred to 1-L-capacity Imhoff settlement cones. For the dewatered sewage sludge samples, 20 g of the dewatered cake was added to a sterile food processor containing 1-L MilliQ water and homogenized. After homogenization, the liquid was transferred to 1-L conical plastic Imhoff cones. All samples were left overnight at 4°C for gravity sedimentation (Graczyk et al. 2007). Fifty milliliters of the top sediment layer was transferred using a 50-ml glass pipette to a plastic 50-ml conical centrifuge tube and centrifuged (3,000 rpm, 5 min). The supernatant was discarded and the pellet transferred to a 1.5-ml microcentrifuge tube and 75% ethanol was added up to 1 ml. The samples were stored at 4°C (Graczyk et al. 2007).

The ethanol was washed from the pellet by centrifugation (8,000 rpm, 5 min) twice using equal volume of sterile phosphate-buffered saline (PBS) and the pellet was subjected to sugar–phenol flotation (Graczyk et al. 2007). The resulting pellet was divided equally for combined multiplex fluorescence in situ hybridization (FISH) and a direct immunofluorescence assay (IFA) to target *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts (Lemos et al., 2005). Briefly, an oligonucleotide probe (CRY-1) and two probes (i.e., Giar-4 and Giar-6), were each 5′-labeled with hexachlorinated 6-carboxyfluorescein (HEX), and used to hybridize with the 18S rRNA of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts for 1 h at 57°C (Deere et al. 1989; Dorsch and Veal 2001; Smith et al. 2004). The processed samples were placed into three lysine-coated immunofluorescent wells on slides. The slides were stored at room temperature to dry and covered to avoid direct light. *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts were enumerated using an Olympus epifluorescent microscope, dry X60 objective, and BP450-490 exciter filter. For oocysts and cysts, only those that were both IFA and FISH-positive were assigned as viable.

Variable distribution values were analyzed using ranked plots. The correlation between the pathogen presence and the physicochemical factors was carried out using Statistica 6.0 software. Level of significance was indicated by $P<0.05$.

Results

Temperature of collected samples ranged from 6 to 10°C (Table 1). The pH value of the influent was 8.6 at Plant A and 7.4, 7.6, and 6.7 at Plant B, C, and D, respectively (Table 1). The hardness of supply waters in these WWTPs were categorized into moderately soft at Plants A and C, slightly hard at Plant B, and hard at Plant D (Table 1). Samples from Plant B had the highest conductivity and TDS values in the influent and showed the greatest TDS reduction throughout the wastewater treatment process (Table 1). Dissolved oxygen showed an increasing trend through the treatment process, in general reaching the highest level in the final effluent, except at Plant A, where the DO of the final effluent was only 2.2 mg/L (Table 1).

Viable *G. duodenalis* cysts vs. nonviable and non-*G. duodenalis* cysts were clearly differentiated by color as a result of FISH and mAb-labeling. Nonviable cysts were represented by: (1) shells with apparently structurally damaged walls; and (2) intact cells with a very small amount of internal structures with diffused appearance. In comparison, viable intact cysts were filled out completely with cytoplasm without the gap between the internal structures and the wall. Viable oocysts labeled by FISH and mAb were predominantly intact, revealing a small gap between the oocyst wall and internal structures and, in most of them, the sporozoites were visible. In comparison, dead oocysts, i.e., oocyst shells, frequently had discernable damage to their walls. Rarely, FISH-positive viable oocysts had noticeable ruptures in their walls which was clearly revealed by mAb-staining.

The removal of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts during the wastewater treatments is shown in Table 2 and Fig. 2. The prevalence of both pathogens in the WWTP samples was 100% and the concentration of *C. parvum* and *C. hominis* oocysts was considerably higher than the concentration of cysts at all WWTPs (Table 2, Fig. 2). Plant A had the highest concentration of oocysts and cysts in wastewater influents, i.e., 592 oocysts/L and 320 cysts/L, respectively (Table 2, Fig. 2). In addition, the concentrations of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts in the influent were higher at Plants A and B than at Plants C and D (Table 2, Fig. 2).

Plant B had the greatest removal efficiency of both oocysts and cysts (i.e., on average 96.7%) with Plant A being the second best at 82.5% for both oocysts and cysts (Table 3). At Plant C, 100% of *C. parvum* and *C. hominis* oocysts were removed, while only 71.4% removal efficiency was observed for *G. duodenalis* cysts at that plant (Table 3). In terms of the overall efficiency of the wastewater treatment, over 97% of the viable *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts were eliminated, except at Plant C, which had only 64% removal efficiency for the oocysts (Table 3). A significant correlation between the removal of oocysts and cysts was found at Plants A, B, and D ($R=0.98$; $P<0.05$).

Sewage sludge at Plants A and D had higher concentrations of both oocysts and cysts than the other two plants, i.e., B and C (Table 2, Fig. 2). Nineteen viable oocysts per

Table 2 Concentration (cells/L) of *Cryptosporidium parvum* and *C. hominis* oocysts, and *Giardia duodenalis* cysts during secondary wastewater treatment processes at four wastewater treatment plants in north-western Ireland

Wastewater treatment plant	Sample source	Oocysts (mean±SD)	Cysts (mean±SD)
A	Influent	592±22.6	320±22.6
	Secondary treatment	128±45	56±34
	Effluent	4±2	1±1.5
	Sewage sludge cake[a]	19±3.2	8±1.7
B	Influent	280±33.9	123±9.2
	Secondary treatment	8±4.9	4±2.6
	Effluent	8±3.6	3±1.5
	Sewage sludge cake[a]	7±2.8	4±2
C	Influent	11±4.5	7±4.1
	Secondary treatment	0	2±1
	Effluent	4±1.5	0
	Sewage sludge[a]	4±1.5	4±3
D	Influent	1±1	0
	Secondary treatment	2±2.8	6±1.1
	Effluent	4±2	1±1.1
	Sewage sludge cake[a]	20±2.4	9±1.8

[a] Cells/g at Plants A, B, and C; and cells/L at Plant D

gram and eight cysts per gram were detected in the sludge at Plant A; seven oocysts per gram and four cysts per gram were detected at Plant B (Table 2, Fig. 2). At Plant C, only four oocysts and cysts were identified. The highest pathogen concentration was observed in the primary sewage sludge at Plant D, with 20 oocysts per gram and nine cysts per gram (Table 2, Fig. 2).

Discussion

Municipal WWTPs are not designed to completely remove human waterborne pathogens and therefore they are recognized as reservoirs of such microbiological contaminants occurring in surface waters (Robertson et al. 2000; Caccio et al. 2003). The present study demonstrated however the high effectiveness of four secondary wastewater treatment systems in the reduction of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts from inflowing raw sewage to the final wastewater effluent. Notably, over 98% of pathogen removal was achieved for final effluent in two of four WWTPs in the present study (i.e., Plant A and B); these had the highest concentration of both pathogen species in the influent sewage (Table 3). The final effluents of these plants contained maximum only eight oocysts and three cysts per liter (Table 2). These pathogen reduction efficiencies of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts throughout the wastewater treatment processes observed in the present study are in agreement with removal rates obtained in other studies (Ahmad et al. 1997; Robertson et al. 2000, 2006; Suwa and Suzuki 2001; Caccio et al. 2003; Brianesco and Bonadonna 2005).

The variation in the level of pathogens reduction in secondary-treated wastewater samples can be related to the different operational treatment processes utilized at these WWTPs. The aeration tanks at Plant A and C yielded considerably higher pathogen reduction rates than the oxidation ditch at Plant B (Table 2). The physical agitation of the aerator systems at these two plants resulted in increased DO levels (Table 1) and may have also caused the mechanical breakdown of the oocysts and cysts (Graczyk et al. 2007, 2008b). In addition, the concentration of *C. parvum* and *C. hominis* oocysts and *G. duodenalis* cysts from the oxidation ditch system at Plant A were far greater than those from the aeration tank systems at Plants B and C (Table 2).

The concentration of both oocysts and cysts in WWTP influents were considerably lower at Plants C and D even though the population served by these WWTPs were considerably higher (Table 1). This phenomenon may reflect a lower incidence of cryptosporidiosis and giardiasis in local populations. It could also be related to the fact that the influent wastewater at Plant D was stored in a large open chamber prior to treatment, which caused settlement of *C. parvum* and *C. hominis* oocysts, and *G. duodenalis* cysts to the bottom of the chamber thus eliminating them from further treatment processes.

Although the final effluent at Plant A had very low pathogen loadings, the corresponding end-products, i.e., sewage sludge, had considerably high concentrations of both *C. parvum* and *C. hominis* oocysts, and *G. duodenalis* cysts (Table 2). This is important from the public health standpoint because transmissive stages of these pathogens were viable. The low dissolved oxygen content of the final

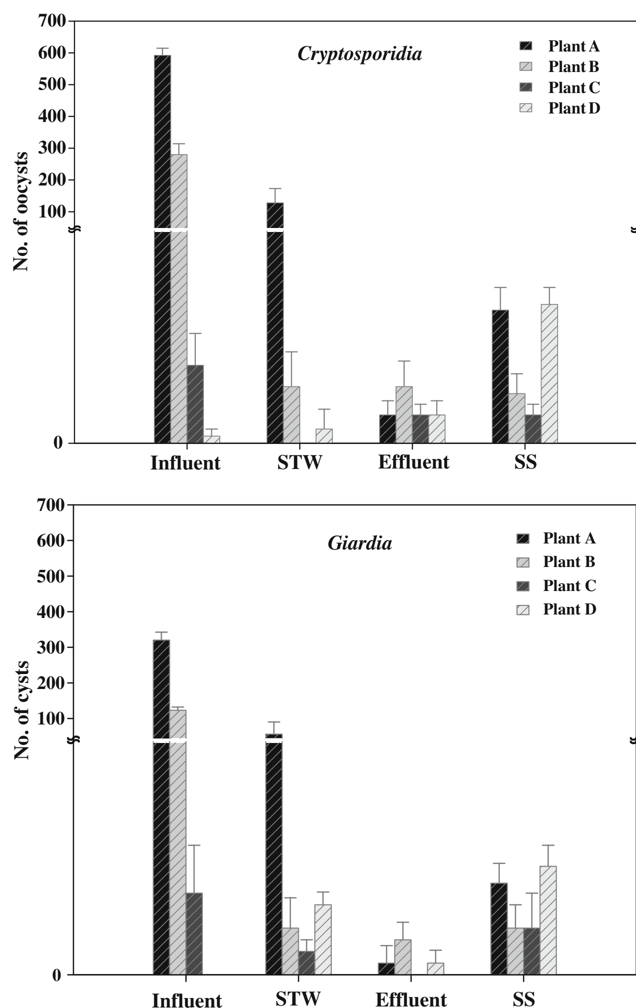

Fig. 2 Removal of *Cryptosporidium parvum* and *C. hominis* (oocysts/L) and *Giardia duodenalis* (cysts/L) during the wastewater treatment at four wastewater treatment plants (i.e., *A*, *B*, *C*, and *D*) in north-western Ireland. *STW* Secondary-treated wastewater, *SS* sewage sludge

effluent at this WWTP (Table 1) was also an indicator that the secondary treatment process had not completed the oxidation of wastewater and therefore was not fully efficient in inactivation of microbiological contaminants. The highest pathogen concentration in sewage sludge were observed at Plant D, where the sludge was collected at the primary stage and was not exposed to any secondary treatment process, which could reduce pathogen levels. As all the sewage sludge beds in this study were exposed to the environment, the possibility also exists that wildlife visiting these beds may increase the load of *C. parvum* oocysts and *G. duodenalis* cysts (Wolfe 1992; Graczyk et al. 2008a).

In terms of public health, the management of sewage sludge both during and following wastewater treatment processes has epidemiological implications (Irish Environmental Protection Agency, IEPA, 2000; Gale 2005; Veronica 2008). The results of the present study showed that, in the first place, treatment plant operatives are exposed to these human enteropathogens during their working day (Cole et al. 2000). For the wider community, the provision of efficiently and safely treated effluents and the subsequent stabilization of sewage sludge into pathogen-free biosolids is an important public health issue in Ireland and other countries. This is because both cryptosporidiosis outbreaks (Pelly et al. 2007) and enteric pathogen surveys (Lucy et al. 2008) have revealed *Cryptosporidium* contamination in the aquatic environment in Ireland (Skerrett and Holland 2000), in watersheds where land-spreading and wastewater discharge occurs (Council of the European Community, CEC, 1986, Council of the European Community, CEC, 1991). This presents a risk factor when these waters are used for drinking water abstraction or for recreational activities (Dorn et al. 1985, Ingallinella et al. 2002). The European Union (EU) urban wastewater directive aims to protect the environment from the adverse effects of wastewater discharges; however, unfortunately, the relevant effluent quality requirements do not legislate for the presence of pathogens (Council of the European Community, CEC, 1986). Implementation of sewage sludge management has been introduced and implemented in the EU legislation (Council of the European Community, CEC, 1991) but research is needed into the safe practice of sewage sludge storage, treatment, and land-spreading to prevent contamination of watercourses, which can result in further epidemics (Pelly et al. 2007).

All the wastewater treatment processes investigated in the present study appear to be highly effective in removing human pathogens from wastewater stream; however, because of the fact that detected pathogens were viable,

Table 3 Removal of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during the wastewater treatment processes at three wastewater treatment plants in north-western Ireland

Wastewater treatment plant	Removal (%) *C. parvum* and *C. hominis*		Removal (%) *G. duodenalis*	
	Secondary treatment	Final effluent	Secondary treatment	Final effluent
Plant A	78.4	99.3	82.5	99.7
Plant B	97.1	97.5	96.7	98
Plant C	100	64	71.4	100

the introduction of a regular monitoring program is required to ascertain whether this pathogen removal efficacy is affected by various factors, particularly seasonal environmental changes and volume of raw sewage entering the WWTP. For example, Plant A was located in a coastal tourist town, with a seasonally increasing population of tourists potentially introducing *Cryptosporidium* and *Giardia* into this community. Known seasonal peaks during spring lambing and calving may also increase the pathogen concentration (Zintl et al. 2006); this may impact on pathogen loadings via combined sewerage systems. Research is also required to establish whether further treatment options could render the sewage sludge into pathogen-free biosolids, which can be safely disposed into the environment. Microbial source tracking, which is applied to reveal source of *C. parvum* oocysts and *G. duodenalis* cysts may allow the policy-makers and the operatives to understand the degree of stabilization and sanitization treatments present through the different stages in wastewater treatment processes (Veronica 2008). This study into the survival and fate of the human-virulent pathogens, *C. parvum* and *C. hominis*, and *G. duodenalis*, demonstrated the importance of monitoring these pathogens both within individual wastewater treatment plant processes and particularly in researching their end-products, which are released back into the environment.

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APPENDIX II

Parasitol Res
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ORIGINAL PAPER

Municipal wastewater treatment plants as removal systems and environmental sources of human-virulent microsporidian spores

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Abstract Municipal wastewater treatment plants play a vital role in reducing the microbial load of sewage before the end-products are discharged to surface waters (final effluent) or local environments (biosolids). This study was to investigate the presence of human-virulent microsporidian spores (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Encephalitozoon hellem*) and enterococci during treatment processes at four Irish municipal secondary wastewater treatment plants (plants A–D). Microsporidian abundance was significantly related to seasonal increase in water temperature. Plant A had the least efficient removal of *E. intestinalis* spores (32%) in wastewater, with almost 100% removal at other plants both in April and July. Some negative removal efficiencies were obtained for *E. bieneusi* (at plants C and D, −100%) and for *E. hellem* (at plants A and D, −90% and −50%). In addition, a positive correlation was found between the levels of enterococci and *E. bieneusi* in July (r_s=0.72, P<0.05). In terms of the dewatered biosolids, a median concentration as high as 32,000 spores/Kg of *E. hellem* was observed at plant D in July. Plant C sewage sludge contained the lowest microsporidian loadings (*E. bieneusi*; 450 spores/L and 1,000 spores/L in April and July, respectively). This study highlights the seasonal variation in concentrations of microsporidian spores in the incoming sewage. Spores in final effluents and dewatered biosolids can be the source of human-virulent microsporidian contamination to the local environment. This emphasizes a considerably high public health risk when sewage-derived biosolids are spread during summer months. This study also suggested enterococci as a potential indicator of the presence

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of microsporidian spores in wastewater, especially for *E. bieneusi*.

Introduction

Microsporidia are widespread obligate intracellular parasites representing more than 1,000 species in 100 genera. Among the 15 species infecting humans, *Enterocytozoon* and *Encephalitozoon* species, especially *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, are the most commonly detected in transplant recipients or in human immunodeficiency virus-infected persons (Didier 2005; Weber et al. 1994). Sporadic infections can also occur in immunocompetent individuals (Nkinin et al. 2007). Anthropozoonotic potential has been documented for *E. bieneusi*, *Encephalitozoon hellem* (i.e., humans and birds), *Encephalitozoon cuniculi*, and *E. intestinalis* (i.e., humans and other mammals) based on phylogenetic analysis on the internal transcribed spacer (Dengjel et al. 2001; Mathis et al. 2005; Sulaiman et al. 2003). The 50% tissue culture infective doses ($TCID_{50}$) of *E. bieneusi*, *E. hellem*, and *E. intestinalis* have been determined as 15, 68, and 27 microsporidian spores, respectively (Johnson et al. 2003).

Due to the electron-dense, glycoprotein-composed, and chitinous cell structure, microsporidian spores are relatively temperature insensitive, can survive in fresh and marine waters, and even after dehydration for extended periods of time (Didier et al. 2004; Li et al. 2003; Vavra and Larsson 1999). These characteristics indicate health risks due to transmission from the environment to various susceptible hosts, including humans and domestic animals such as rabbits, pigs, donkeys, and avian species. Microsporidian spores can be ingested or inhaled and then the infections are established predominantly in the small intestine (Garcia 2002; Graczyk et al. 2007d; Sak et al. 2008).

Considerable evidence to date indicates the involvement of water in the epidemiology of human microsporidiosis (Cotte et al. 1999; Dowd et al. 1998; Enriquez et al. 1998; Fournier et al. 2000; Slifko et al. 2000; Sparfel et al. 1997; Thurston-Enriquez et al. 2002). Microsporidian spores have been found throughout the bathing season in marine recreational waters (Graczyk et al. 2007c; Lucy et al. 2008) and in a range of lakes and rivers used for drinking water abstraction and recreation (Graczyk et al. 2004; Lucy et al. 2008). Discharge of effluents from wastewater treatment plants is an important point source of microsporidian spores in surface waters used for drinking water abstraction and recreation (Lucy et al. 2008); both *E. intestinalis* and *E. bieneusi* spores have been identified in such effluents (Dowd et al. 1998; Graczyk et al. 2007a). Microsporidian spores have also been detected in biosolids originating from secondary wastewater treatment (Graczyk et al. 2007b). Microsporidian spores, range from 0.8 to 2.0 μm in size, and can pass through drinking water and wastewater treatments together with other human parasites such as *Cryptosporidium* oocysts and *Giardia* cysts (Garcia 2002; Graczyk et al. 2007a, 2009).

Legislative instruments such as the European Union Urban Wastewater Treatment Directive provide legal limits for physical and chemical parameters such as biochemical oxygen demand, pH, oxygen saturation, electro-conductivity, and total suspended and dissolved solids (Bontoux 1998; CEC 1991). However, microbiological indicators, such as *Escherichia coli* and intestinal enterococci are not included in this legislation. Furthermore, human waterborne pathogens such as microsporidian spores are also not bound to the directive. In a previous study, up to one-third of viable *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts survived secondary wastewater treatment (Cheng et al. 2009). In addition, viable oocysts and cysts present in the wastewater treatment resulting biosolids pose potential risks to the public and wildlife once biosolids are released to the environment as fertilizer for spreading on agricultural land (Cheng et al. 2009).

In this study, the presence of microsporidian spores of human-virulent species during secondary wastewater treatment processes in derived biosolids and final effluents at four municipal wastewater treatment plants were investigated in order to assess the reduction of pathogen load through the wastewater treatments. We also measured the level of intestinal enterococci to investigate their relationship with the load of microsporidian spores.

Materials and methods

Wastewater and sewage sludge samples were collected from four municipal wastewater treatment plants; Plant A (54°16′43″N, 08°36′20″W), plant B (54°11′11″N, 08°29′10″W), plant C (54°20′11.3″N, 08°31′44.3″W), and plant D (54°16′44″N, 08°36′90″W) in northwestern Ireland (Table 1). These four plants serve local settlements ranging from 1,100 to 2,500 population equivalent with wastewater originating from houses, schools, hospitals, and local business sectors. Plant A was located in a popular tourist coastal town with beach amenities. The raw sewage entered each plant via combined sewerage systems. Preliminary and primary treatments were applied prior to the secondary biological treatment at plants A, C, and D, while at plant B, the raw sewage was delivered directly to an aeration tank without any screening or grit removal (Table 1). At plant D, raw sewage was stored in a large open chamber for approximately 2 h and lead to Imhoff tanks for primary sedimentation before entering the percolating system. In addition, all solids at plant

Table 1 Characteristics of the WWTPs

WWTPs	Location	Served PE	Preliminary and primary process	Secondary process	Resulting sewage sludge[a]
Plant A	54°16′43″N, 08°36′20″W	1,950	Grit removal and screening	Oxidation with oxygen and settlementation	4.12 Tonnes; 0.75% dried solids
Plant B	54°11′11″N, 08°29′10″W	1,100	None	Activated sludge, extended aeration, and settlementation (clarifier)	0.76 Tonnes; 0.75% dried solids
Plant C	54°20′11.3″N, 09°31′44.3″W	2,500	Grit removal, screening, and sedimentation	Activated sludge, extended aeration, and settlementation (settling tank)	1.82 Tonnes; 2.50% dried solids
Plant D	54°16′44″N, 08°36′90″W	2,150	Grit removal, screening, and two Imhoff tanks for primary sedimentation	Percolating filteration system and settlementation (clarifier)	8 Tonnes; 0.75% dried solids

WWTPs wastewater treatment plants, *PE* population equivalent

[a] Yearly produced volume obtained from the local authority

D were settled as primary sludge for removal to a sludge drying bed. The secondary wastewater treatments were applied as follows: (a) sludge activation in an oxidation ditch (plant A); (b) sludge activation in extended aeration tanks (plants B and C); and (c) treatment by percolating filter (plant D). After the activated-sludge treatment, the final effluent was discharged to adjacent surface waters, i.e., river (plant B, C, and D) and sea (plant A), while the settled biosolids were deposited onto sludge dewatering beds. Plants A, B, and D were equipped with sludge dewatering beds, from which sludge was removed. Due to the engineering design at plant C, liquid sewage sludge was collected directly for disposal from a secondary settling tank (Table 1).

Grab sewage samples (i.e., raw sewage, activated sludge, and mixed liquor) and treated end-products (i.e., final effluent and sludge), originating from plants A, B, C, and D were collected in April and July 2008. All wastewater samples were obtained using a long-handled 1-L capacity sampler and transferred to 1-L sterilized polyethene bottles. Sewage sludge cake (approximately 100 g) was collected by trowel from 10 cm in depth of the dewatering beds at plants A, B, and D, whereas 1 L of biological-treated liquid sewage sludge was obtained from plant C discharge valve at the bottom of the clarifier. All samples were collected in triplicate. Physiochemical parameters, such as temperature, pH, conductivity, dissolved oxygen, and total dissolved solids of wastewater were measured on-site using portable meters (Sens-Ion, Hach Company, Colorado, USA).

The liquid samples were shaken vigorously and transferred to 1-L capacity plastic Imhoff settlement cones. Twenty grams of the dewatered cake was suspended in 1-L MilliQ water by vortexing and transferred to 1-L Imhoff cones. All samples were left at 4°C overnight for gravity sedimentation (Ash and Orihel 1987; Graczyk et al. 2007a). Fifty milliliters of the top sediment layer was centrifuged (3,000 rpm, 5 min). The supernatant was discarded and the pellet was transferred to a 1.5-ml microcentrifuge tube, preserved in 75% ethanol, and stored at 4°C. The ethanol was washed from the pellet by centrifugation (8,000 rpm, 5 min) twice using equal volume of sterile PBS and the pellet was subjected to sugar-phenol flotation. After sugar-phenol purification, 500 μl of the treated supernatant was transferred to a 1.5-ml microcentrifuge tube. The pellet was washed by centrifugation (5,000 rpm, 5 min) using 1 × PBS (Graczyk et al. 2007a).

The resulting pellet was assayed in 1.5-ml microcentrifuge tubes by multiplex fluorescent in situ hybridization (FISH) for identification of *E. bieneusi*, *E. hellem*, and *E. intestinalis* spores (Graczyk et al. 2004; Slodkowicz-Kowalska et al. 2006). Briefly, species-specific, fluorochrome-labelled probes, i.e., HEL878F, INT-1, and BIEN-1, were used for detection of *E. hellem*, *E. intestinalis*, and *E. bieneusi* spores, respectively (Dorsch and Veal 2001; Graczyk et al. 2004). The probes, which hybridize the 16S ribosomal RNA of specific microsporidian spore species, were added to purified samples and incubated at 57°C for 3 h (Graczyk et al. 2004). The processed samples were placed onto the wells of immunofluorescent slides and the slides were stored at room temperature to dry. Microsporidian spores were identified and enumerated without knowledge of sample identity using an epifluorescent microscope at ×100 objective magnification, and BP450–490 exciter filter.

A 1-ml aliquot of the raw samples was subjected to serial dilution for the detection of enterococci using Slanetz and Bartley medium (Oxoid, UK). Two hundred microliters of the diluant was pipetted onto the agar and spread aseptically. The total counts of pink to red colonies were calculated according to standard methods for the examination of water and wastewater.

Multi-dimensional scaling (MDS) analysis with 100 restarts and minimum accepted stress of 0.01 based on the

Bray–Curtis coefficient of similarity was performed to reveal if the composition and concentration of microsporidian spores were dependent on such factors as the "plant", "treatment stage", and "season". Six spore-negative samples were removed from the analysis. The data were fourth-root transformed to reduce the variance. Analysis of similarity (ANOSIM) was applied to test if the clustering of samples visually detected on a two dimensional MDS plot was statistically significant (Clarke and Warwick 2001). As most of the quantitative variables measured in this study were non-normally distributed, median and quartile values were used to describe the central tendency and variation for those variables, respectively. Wilcoxon matched pairs tests were applied to compare the concentration of microsporidian spores and environmental parameters among seasons. Correlation between spore concentration and water temperature was estimated using Spearman rank coefficient r_s (Zar 1999). Pathogen removal efficiency was calculated as a percent of the difference in pathogen concentration in the effluent vs influent. Negative values were obtained for the cases in which the pathogen concentration was higher in the effluents as compared to the influents. All descriptive statistics, correlation coefficients, and Wilcoxon tests were calculated with the STATISTICA 6.0 (StatSoft, Inc, 2002, Tulsa, USA). MDS and ANOSIM were performed using PRIMER 6 software (PRIMER-E Ltd, 2006, Plymouth, UK). All statistical tests were considered significant at $P<0.05$.

Results

Microsporidian abundance was clustered in relation to season with two divergent clusters (ANOSIM, $P=0.03$) corresponding to the sampling periods of April and July (Fig. 1). There was a significantly higher concentration of all microsporidian spore species in the samples collected in July when compared to April (Tables 2 and 3). For wastewater samples, among the three microsporidian species, *E. bieneusi* was the predominant species at all four plants in July, with the highest concentration at plant A (up to 497 spores/L), followed by plant C (up to 184 spores/L) and plant B (up to 173 spores/L). Plant A had the highest concentration of all microsporidian spores (up to 1,014 spores/L), including 240 and 277 spores/L for *E. hellem* and *E. intestinalis*, respectively. Plants with the next highest concentrations were plants C and B. There were no spores detected at plant D at any of the stages except in the biosolids (Table 2). No statistically significant relationship was found between the microsporidian abundance and the other trained factors: i.e., the four different plants (ANOSIM, $P=0.885$; Fig. 1a) and the various treatment stages (ANOSIM, $P=0.158$; Fig. 1b).

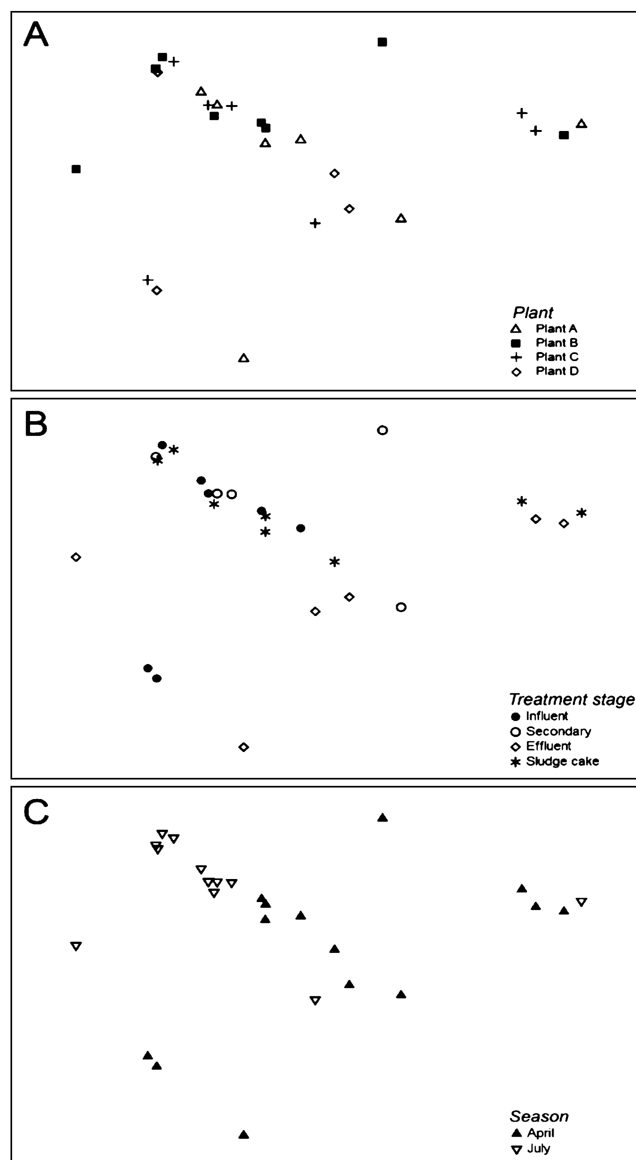

Fig. 1 MDS ordination of the microsporidian assemblages in relation to the factors "plant" (**a**), "treatment stage", (**b**) and "season" (**c**). The stress value is 0.09, indicating on high quality of this 2D ordination

Of all the environmental parameters, only the temperature differed significantly ($P=0.0004$, Wilcoxon tests) between April and July (Table 3). In addition, there was a significant positive correlation between the concentration of *E. bieneusi*, *E. hellem*, *E. intestinalis* and temperature ($r_s=0.37$, $r_s=0.55$, and $r_s=0.54$, respectively, $P<0.05$). A significant correlation was also found between levels of intestinal enterococci and *E. bieneusi*, in July ($r_s=0.72$, $P<0.05$) but not in April.

In terms of pathogen removal efficacy, 100% of the enterococci were removed at all of the four plants in both April and July (Fig. 2). More efficient microsporidian spore removal was observed in July than in April (Figs. 1 and 2); no spores were detected at plant D. In July, plant A had the

Table 2 Potentially viable human-virulent microsporidian spores and enterococci during secondary wastewater treatment processes

			No. of spores/colonies[a]							
			E. bieneusi		*E. hellem*		*E. intestinalis*		Enterococci	
Sampling	Plants	Treatment	Median	Quartiles	Median	Quartiles	Median	Quartiles	Median	Quartiles
April	A	Influent	12	(1; 16)	2	(1; 7)	12	(8; 12)	2,650	(2,280; 2,900)
		AcSS	2	(0; 11)	3	(2; 6)	0	(0; 0)	4,250	(4,200; 4,670)
		Effluent	0	(0; 0)	20	(13; 27)	0	(0; 0)	0	(0; 0)
	B	Influent	46	(37; 89)	8	(7; 9)	22	(12; 31)	883	(650; 980)
		AcSS	22	(12; 32)	0	(0; 0)	3	(3; 3)	2,350	(1,980; 2,500)
		Effluent	4	(2; 4)	0	(0; 0)	0	(0; 0)	0	(0; 0)
	C	Influent	0	(0; 0)	5	(5; 6)	3	(1; 7)	5,460	(4,900; 5,940)
		AcSS	0	(0; 0)	0	(0; 0)	0	(0; 0)	1,850	(1,760; 2,670)
		Effluent	6	(3; 9)	0	(0; 0)	0	(0; 0)	0	(0; 0)
	D	Influent	0	(0; 0)	9	(6; 9)	1	(1; 3)	0	(0; 0)
		AcSS	0	(0; 0)	0	(0; 0)	0	(0; 0)	0	(0; 0)
		Effluent	13	(10; 15)	15	(14; 19)	0	(0; 0)	0	(0; 0)
July	A	Influent	470	(400; 620)	180	(140; 400)	280	(250; 300)	8,250	(7,880; 8,670)
		AcSS	120	(100; 130)	540	(480; 1,090)	390	(310; 500)	3,400	(3,000; 3,480)
		Effluent	0	(0; 0)	40	(30; 50)	220	(100; 260)	16	(16; 20)
	B	Influent	180	(150; 190)	30	(30; 40)	70	(50; 80)	2,450	(2,200; 2,560)
		AcSS	120	(120; 140)	10	(0; 10)	60	(40; 70)	1,950	(1,900; 2,740)
		Effluent	20	(10; 20)	130	(100; 180)	0	(0; 0)	0	(0; 0)
	C	Influent	200	(130; 200)	130	(90; 230)	30	(20; 70)	2,000	(1,720; 2,100)
		AcSS	120	(120; 130)	80	(80; 100)	10	(0; 40)	1,720	(1,500; 1,900)
		Effluent	0	(0; 0)	0	(0; 0)[a]	0	(0; 0)	20	(18; 20)
	D	Influent	0	(0; 0)	0	(0; 0)	0	(0; 0)	0	(0; 0)
		AcSS	0	(0; 1)	0	(0; 0)	0	(0; 0)	10	(10; 10)
		Effluent	0	(0; 0)	0	(0; 0)	0	(0; 0)	0	(0; 0)

[a] For microsporidia: spores/L; for Enterococci: 10^3 CFUs/ml

less efficient removal (i.e., 32%) of *E. intestinalis* spores with almost 100% removal at other plants in April and July. In some samples collected in April, pathogen removal efficacy attained negative values for *E. bieneusi*, e.g., at plants C and D (−100%) and for *E. hellem* at plants A and D (−90% and −50%, respectively). In terms of microsporidian spore removal efficiency, only plant B had negative values in July (*E. hellem*).

For the biosolids, viable *E. bieneusi* spores were not only the most frequently detected but in general had the highest concentration at all plants in April and July (Table 4). Spore concentration in biosolids was higher in July than in April, especially at plants B and D. Plant B had the highest concentration of *E. bieneusi* spores (19,000 spores/Kg) and *E. intestinalis* (16,000 spores/Kg). The highest microsporidian biosolids loading observed in this study was for *E. hellem* at

Table 3 Median values and quartiles (in parentheses) of the microsporidian abundance and environmental parameters in April and July

Parameter	April	July	*P* value for Wilcoxon test
E. bieneusi	7.1 (0.0; 27.1)	93.4 (0.0; 150.0)	0.014
E. hellem	4.4 (0.0; 9.0)	51.7 (0.0; 143.4)	0.008
E. intestinalis	0 (0.0; 7.0)	46.7 (0.0; 211.7)	0.005
Temperature, °C	11.7 (10.7; 12.0)	16.5 (15.8; 17.1)	0.0004
pH	7.5 (7.05; 7.70)	7.6 (7.10; 7.80)	0.842
Dissolved oxygen, mg/L	5.3 (4.3; 7.8)	4.3 (3.0; 7.5)	0.438
Conductivity, µS/cm	588 (512.0; 649.0)	675.6 (485.2; 740.5)	0.379
Turbidity, NTU	150 (55.6; 536.0)	170.2 (67.3; 494.0)	0.535

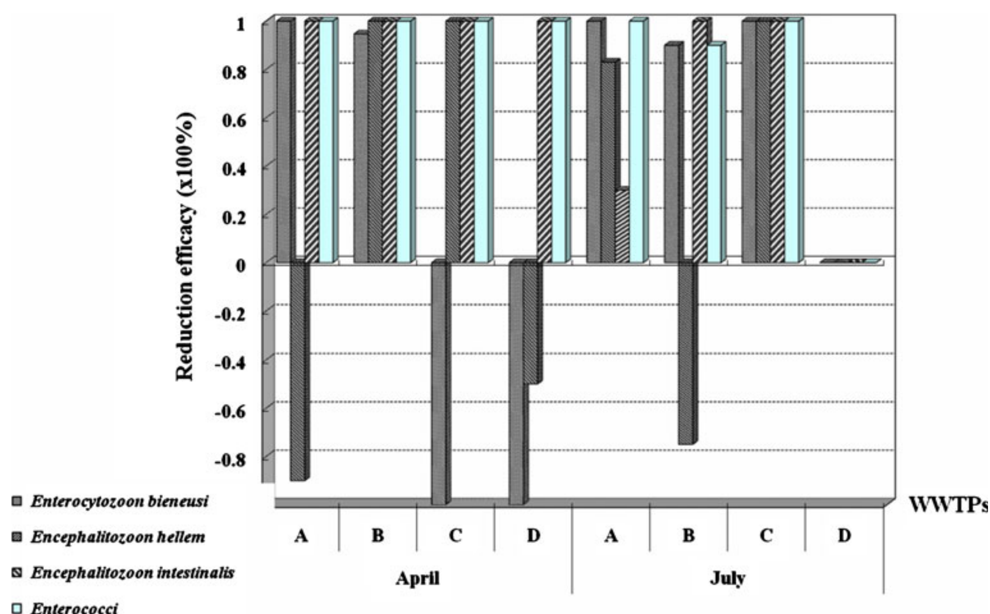

Fig. 2 Reduction efficacy of the wastewater treatment plants (**a**–**d**) on three different species of microsporidia in April and July 2008

plant D in July, with a median concentration of 32,000 spores/Kg. Plant C sewage sludge contained the lowest microsporidian loadings with *E. bieneusi* concentrations of 450 spores/L in April, and 1,000 spores/L in July.

Discussion

Very little research has been carried out on the efficacy of secondary wastewater treatment systems in the removal of human-virulent species of microsporidian spores (Dowd et al. 1998; Graczyk et al. 2007b, 2009). This study reports high concentrations of microsporidian spores in inflowing wastewaters and in the wastewater processing end-products, i.e., biosolids and final effluents. Relatively high concentrations of such human pathogens as *Cryptosporidium* oocysts and *Giardia* cysts, have been reported in municipal wastewater treatment studies (Caccio et al. 2003; Cheng et al. 2009; Rimhanen-Finne et al. 2004; Robertson et al. 2006), indicating that WWTPs can act as pathogen reservoirs. This study is the first report of a significant seasonal increase in microsporidian spore concentrations in municipal wastewaters. Significantly higher microsporidian spores released from the community may indicate that more cases of microsporidiosis occurred in local areas at this time.

This study demonstrated that the spore removal efficacy of four secondary wastewater treatment systems varied. Although *E. bieneusi* was found at higher concentration than *E. hellem* and *E. intestinalis*, the removal of the former species at all four plants was the most efficient. The removal efficacy of *E. hellem* spores varied among plants from 100−90% (Fig. 2). These negative removal efficacy values for *E. hellem* spores indicate that some spores were

Table 4 Loads of human-virulent microsporidia and enterococci in the resulting biosolids

Sampling	Plants	No. of spores/colonies (median, quartiles)[a]			
		E. bieneusi	*E. hellem*	*E. intestinalis*	Enterococci
April	A	1,550 (600; 3,500)	500 (450; 750)	600 (400; 600)	0 (0; 0)
	B	3,350 (2,250; 4,450)	350 (300; 450)	600 (500; 600)	100 (100; 150)
	C	450 (250; 600)[b]	400 (250; 450)[b]	0 (0; 0)	0 (0; 0)
	D	3,950 (3,450; 4,450)	500 (400; 600)	0 (0; 0)	320 (300; 320)
July	A	3,500 (3,000; 4,000)	0 (0; 950)	1,500 (1,500; 5,000)	122 (115; 124)
	B	19,000 (19,000; 22,000)	9,500 (7,500; 14,000)	16,000 (12,000; 17,000)	0 (0; 0)
	C	1,000 (0; 1,500)[b]	0 (0; 0)	0 (0; 0)	0 (0; 0)
	D	6,000 (5,000; 7,000)	32,000 (29,000; 34,000)	11,000 (9,500; 14,000)	0 (0; 0)

[a] For microsporidia: spores/Kg; for enterococci: CFU/ml

[b] Unit: spores/L

likely delivered to the plants during the treatment processes by visiting avian wildlife, as birds were abundant at wastewater operations. Wildlife contribution of pathogens to the wastewater during the processing has been reported previously (Graczyk et al. 2009). Increased levels of *E. hellem* in the biosolids might also be due to fecal contribution of birds as sludge treatment and storage takes place in open drying beds.

In the present study, approximately 20% of *E. bieneusi* and 40% of *E. hellem* spores went through into the next treatment stage and were still potentially viable in the secondary activated sludge during extended aeration. Compared with a parallel investigation at the same treatment plants on *C. parvum*, *C. hominis*, and *G. duodenalis*, the microsporidian loads in treated effluents were statistically higher than in the influents. In particular, plants A and B, where the effluents had 4 oocysts/L and 8 oocysts/L of *Cryptosporidium*, respectively, while the microsporidian spores were found at much higher concentration: 40 spores/L for *E. hellem* and 220 spores/L for *E. intestinalis* (plant A) and 130 spores/L of *E. intestinalis* (plant B) (six, this study). The surplus microsporidian spores in the final effluents may be due either to wildfowl inputs from visiting wildlife or alternatively are due to the accumulation of colloid-associated spores in the clarifier. According to previous studies, *Columba livia* (feral pigeon) and other free-ranging birds have been considered as potential carriers of spores of some microsporidia, such as *E. bieneusi* and *E. hellem*, which are also virulent to humans (Dengjel et al. 2001, Graczyk et al. 2007d). In addition, an increase in these pathogens was previously observed in constructed wetlands associated with wastewater treatment plants (Graczyk et al. 2009). In this study, the same phenomenon was observed in the final clarifier with elevated numbers of microsporidian spores in the final effluents. From a public health point of view, bioaerosols that arise from the secondary activated sludge tank and the clarifier may contain microsporidian spores (Graczyk et al. 2007b). Possible aerosolization of microsporidian spores could be responsible for infections among wastewater treatment plant personnel. Moreover, the negative removal efficacy of wastewater treatment plants can substantially contribute to contamination of human-virulent microsporidian spores to surface waters used for recreation and drinking water abstraction (Lucy et al. 2008).

In Europe, the management of bathing water quality has been entered into force in accordance with the bathing water directive 2006/7/EC, which implemented historically reliable fecal bacterial indicators, i.e., *E. coli* and enterococci, for predicting microbiological health risks in order to achieve a high level of protection of human health (CEC 2006). However, no standards are specified in the EU Urban Wastewater Directive for allowable discharging concentration or removal percentage for either microbial indicators or pathogens. In the present study, intestinal enterococci showed a significant correlation with concentration of human-virulent *E. bieneusi* spores in wastewater samples. This is the first known published study on the relationship between enterococci and microsporidian spores in wastewater. As enterococci levels are relatively easy and cost-effective to assess and are commonly used for assessment of pathogens, they may have potential as indicators for the possible presence of human-virulent microsporidia in wastewaters (Ulrich et al. 2005). Intestinal enterococci have definite advantages as indicators; high resistance to harsh environmental conditions, limited host range, and rare replication outside their hosts (Wheeler et al. 2002). On this basis, we suggest that an enterococci-based maximum discharge allowance should be taken into consideration on implementation of microbiological index to the Urban Wastewater Directive.

Results obtained for plant D for both April and July demonstrate that primary sedimentation can enhance removal of microsporidian spores from wastewaters by settling out the spores with primary solids. Other techniques, such as chlorine and UV light treatments, have been suggested to eliminate microsporidian spores that bypassed treatment processes and are found in final effluents (John et al. 2003; Johnson et al. 2003). Discharges of final effluents to surface waters used for recreation or drinking water abstraction can result in human exposure or direct contact with pathogens present in the effluents (Cheng et al. 2009; Lucy et al. 2008). As plant A discharged effluents offshore, close to a tourist seaside amenity with seasonally increasing population, spores of *E. intestinalis* discharged with the effluent are of a particular public health concern. It has been demonstrated that immunocompetent people can acquire chronic diarrhea after coming in contact with *E. intestinalis*-contaminated recreational waters during holiday seasons (Wichro et al. 2005).

The management of biosolids both during and following wastewater treatment processes has epidemiological and public health implications (Gale 2005; US EPA 1999; Veronica 2008). In many countries, biosolids are often spread on agricultural lands; approximately 86,411 tonnes of biosolids were deposited onto Irish farmland in 2007 (Irish EPA 2007), and therefore may result in introduction of microsporidian spores to watersheds and river basin districts used for drinking water abstraction and recreation (Graczyk et al. 2004; Lucy et al. 2008). Agricultural spreading of biosolids takes place during summer months and as demonstrated by the present study, it coincides with high levels of microsporidian spores in biosolids. Further investigations, such as an assay on the disinfection of human-virulent spores, are needed for a proper sanitization of sewage sludge in order to produce safe biosolids. For

instance, ultrasonic treatment and quicklime stabilization demonstrated inactivation and disintegration of microsporidian spores (Graczyk et al. 2007b). Finally, guidelines for accepted levels of human-virulent microsporidian spores in biosolids are urgently needed.

In conclusion, the present study showed seasonal variation in concentration of microsporidian spores in incoming wastewater that was correlated with seasonal differences in water temperature. We also demonstrated seasonal increase in levels of microsporidian spores in final effluents and biosolids released to the environment. This emphasizes high public health risks, as in Ireland demand for water recreation and drinking water abstractions are highest during the summer months.

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APPENDIX III

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Municipal wastewater treatment plants as pathogen removal systems and as a contamination source of noroviruses and *Enterococcus faecalis*

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ABSTRACT

Municipal wastewater treatment plants play a crucial role in reducing the microbial and pathogen load of human wastes before the end-products are discharged to surface waters (final effluent) or land spread (biosolids). This study investigated the occurrence frequency of noroviruses, *Enterococcus faecalis* and *Enterococcus faecium* in influent, final effluent and biosolids from four secondary wastewater treatment plants in northwestern Ireland (plants A–D) and observed the seasonal and spatial variation of the plant treatment efficiencies in the pathogen removals. It was noted that norovirus genogroup II was more resistant to the treatment processes than the norovirus genogroup I and other active viral particles, especially those in the discharge effluents. The percolating biofilm system at plant D resulted in better effluent quality than in the extended aerated activated sludge systems (plants A and B); primary biosolids produced at plant D may pose a higher health risk to the locals. The spread of norovirus genogroup II into the environment, irrespective of the wastewater treatment process, coincides with its national clinical predominance over norovirus genogroup I. This study provides important evidence that municipal wastewater treatment plants not only achieve pathogen removal but can also be the source of environmental pathogen contamination.

Key words | biosolids, *Enterococcus faecalis*, *Enterococcus faecium*, noroviruses, wastewater

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INTRODUCTION

Diarrhoeal disease remains a major leading cause of morbidity and mortality with 2.16 million deaths per year globally (Kosek *et al.* 2003; Mathers *et al.* 2004). In developed countries, improvement in water and sanitation is one of the key factors leading to the decreasing trend in diarrhoeal disease. However, according to national disease surveillance report in 2003, 4.5% of the Irish population were still affected by gastroenteritis every month (NDSC 2003). In a recent Irish Health Protection Surveillance Centre annual report (HPSC 2010), infectious intestinal disease outbreaks accounted for 56.4% of all outbreaks, with 6% decrease compared to the national statistics in 2008 (HPSC 2010).

Regional variation in all infectious intestinal disease outbreaks was significant between Health Service Executive (HSE) areas, with the highest rate observed in the HSE northwest area at 28.7 per 100,000 population. Most of the outbreaks were attributed to norovirus (HPSC 2010). According to a study on invasive enterococcal bacteraemia surveillance undertaken in the same year, 289 *Enterococcus faecalis* isolates and 397 *Enterococcus faecium* isolates were identified in acute hospitals, with 0.7% (*E. faecalis*) and

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39.1% (*E. faecium*) showing resistance to vancomycin (EARS-Net 2009). Enterococci are among the predominant bacterial flora in the human/animal gastrointestinal tract. Nowadays, enterococci play dual roles as both commensal organisms and human-virulent pathogens, representing the second leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial bacteraemia (Moellering 1992). Norovirus, formerly known as small round-like virus or Norwalk virus, is the causative agent of the well-known winter vomiting disease and classified within the family of *Caliciviridae*, with 7.5 kb single-stranded, positive-sense RNA genome comparative to other small round-like viruses. The noroviruses genome encodes three large open reading frames (ORFs), including the ORF1 encoding replicase polyprotein (e.g. RNA-dependant RNA polymerase) and the ORFs 2 and 3 encoding major (e.g. capsid protein) and minor structural proteins, respectively (Lindesmith *et al.* 2008). Noroviruses can be grouped into five genetically differentiable genogroups (genogroups I–V), with noroviruses genogroups I and II accounting for the majority of viral gastroenteritis in human populations internationally (Caul 1996; Lopman *et al.* 2004). An international research study demonstrated that 1–3% of people were expected to become infected with noroviruses each year (Food Standards Agency 2000). In Ireland, noroviruses are the commonest cause of outbreaks of acute gastroenteritis standing for 48.1% of all infectious intestinal disease outbreaks (HPSC 2010). The majority of cases of norovirus infection are of genogroup II/genotype 4 (Waters *et al.* 2006; HPSC 2010).

The modes of transmission of enterococcal infection and norovirus infection are recognised as being via consumption of faeces-contaminated food (e.g. raw oyster or shellfish), drinking or bathing waters or through person-to-person contact (Green *et al.* 2001; Koopmans *et al.* 2003). The infected individuals release the pathogens in excreta, which is transported to wastewater treatment systems. Wastewater treatment plants (WWTPs) operations comprise physical, chemical and biological processes, depending on the requirement for the effluent standard as well as the nature of the wastewater. The fundamental aims of wastewater treatment are to ensure the human wastes are collected appropriately, to ensure the wastewater is effectively treated and the resulting biohazardous products are safely discharged to inland or coastal waters, and to possibly recycle or reuse the valuable components in the end products. By the nature of domestic wastewater, human-virulent enteropathogens can also be found in the raw wastewater derived from the infected individuals in the communities that the plant serves. The aims of this study were to investigate the occurrence of noroviruses and *Enterococcus faecalis* and *E. faecium* in human sewage samples (raw and treated end-products) from four secondary WWTPs in northwestern Ireland and to observe any seasonal and spatial variation. Semi-nested polymerase chain reaction (PCR) was conducted to overcome the sensitivity limitation and to differentiate norovirus genogroup I and genogroup II. Furthermore, this study was undertaken to evaluate the treatment efficiency for pathogen removal (presence/absence in the treated effluent and biosolids) and to assess the potential impact of the discharge wastes from the plants to human public health at the study municipal secondary WWTPs.

MATERIALS AND METHODS

Wastewater treatment plants and source of wastewater samples

Four secondary wastewater treatment plants (plants A–D) in northwestern Ireland were investigated in April, July and October 2008 and monthly between January 2009 and February 2010 (Figure 1). Plant A is located in a seaside tourist town (serving 1,950 population equivalents), where an influx of travellers visits during holiday seasons (April, July, August, October), especially in summer. Plants B, C and D are situated in stable residential areas and serve up to 4,000 population equivalents each. Regional hospitals and health care centres are located in the areas that plants A, B and D serve. All plants are designed as separated sewerage systems receiving only domestic wastewater, except for plant B, which accepts a combination of domestic sewage and proportional urban run-off in wet conditions (combined sewer). The characteristics of the WWTPs were described in a previous study (Cheng *et al.* 2011). Briefly,

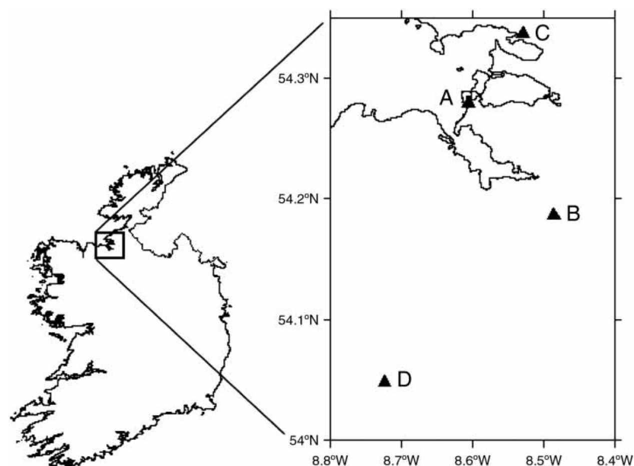

Figure 1 | Location of plants A–D in northwestern Ireland.

the raw wastewater (except at WWTP B) went through grit removal and coarse screening before reaching the secondary treatment stage. The subsequent secondary wastewater treatments were applied: sludge activation in an oxidation ditch (plant A); sludge activation in extended aeration tanks (plants B and C) and treatment by biofilm-coated percolating filter (plant D). The wastewater in each WWTP was then settled in a secondary settlement tank, i.e. clarifier, gravitationally separating final effluent and sewage sludge. The resulting sewage sludge (mixture of the sludge on the bottom of secondary treatment facility and the settlement tank) was taken out of the tank and spread onto the on-site drying beds, where the dewatered biosolids samples were collected. Due to different plant design, sewage sludge at plant C was obtained via a discharge valve from the secondary settling tank. The biosolids obtained from plants A, B and D contained 0.75% dried solids while the sewage sludge at plant C contained 2.5% dried solids.

All wastewater samples (influent and effluent) were collected using a long-handled 1 L sampler and transferred to 1 L sterilised polyethylene bottles. Biosolids (approx. 100 g) were collected by trowel from 10 cm depth in the drying beds at plants A, B and D, and 1 L of liquid sewage sludge was obtained through the plant C discharge valve. All samples were collected in triplicate and delivered to the laboratory in a cooler box (Cheng *et al.* 2009).

Enumeration of enterococci and detection of *E. faecalis* and *E. faecium*

All the liquid wastewater samples were mixed vigorously and triplicate 10 mL sub-samples were transferred to 15 mL sterilised conical plastic centrifuge tubes. Twenty grams of biosolids sample were diluted 1:50 in phosphate-buffered saline (in triplicate) and vortexed. The sub-samples were mixed vigorously and 1 mL of the mixture was subjected to 10-fold serial dilution in Ringer's solution (Oxoid, UK) to 10^{-4}. Two hundred microlitres of the sub-samples were aseptically spread onto Slanetz and Bartley agar (Oxoid, UK) and cultivated at 44 °C for 24–36 h. Burgundy red colonies on Slanetz and Bartley agar were enumerated as positive, in accordance with the manufacturer's instructions.

For *E. faecalis* and *E. faecium* detection, 10% of the positive colonies on Slanetz and Bartley agar were transferred individually by sterilised plastic needle to microcentrifuge tubes containing 100 µL of DNase and RNase-free water (Promega). To release the DNA from enterococci isolates, the mixture was freeze–thawed three times with heating in a dry heating block at 99 °C for 10 min then cooling in an ice box at 0 °C for 5 min (Hsu *et al.* 2006). The suspension was centrifuged (13,500 × g, 10 min) then the supernatant was transferred to new sterilised microcentrifuge tubes. Total DNA was extracted using DNA stool extraction kit (Qiagen, USA) from 200 µL aliquots of the wastewater samples. Aliquots of the extracted DNA samples were subjected to identification first using enterococcal genus primers (Deasy *et al.* 2000), followed by a *sod*A gene targeted species-specific primers for *E. faecalis* and *E. faecium* (Jackson *et al.* 2004). The sequences of the oligonucleotide primers are listed in Table 1. Total genomic DNA was extracted from cultures of *E. faecalis* (NCTC775) and *E. faecium* (ATCC19434) to use as positive controls in this study. Briefly, a DNA solution was added to a microcentrifuge tube containing 12.5 µL of 2X PCR buffer (Promega), 0.5 µL of 1.0 µmol of primers (Integrated DNA Technologies (IDT), Inc., USA) and 7.5 µL of nuclease-free water (Promega). The reaction was carried out using a modified protocol (Deasy *et al.* 2000). Initially, the DNAs were denatured at 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 sec, 50 °C for 1 min, 72 °C for 1 min and final

Table 1 | Primers used in this study

Microorganism	Primers	Sequence (Sense)	Target	Product size	Reference
Enterococci	E1	5′-TCAACCGGGGAGGGT-3′ (+)	16S rRNA	707 bp	Deasy *et al.* (2000)
	E2	5′-ATTACTAGCGATTCCGG-3′ (−)	16S rRNA		
E. faecalis	FL1	5′-ACTTATGTGACTAACTTAACC-3′ (+)	SodA gene, genomic DNA	360 bp	Jackson *et al.* (2004)
	FL2	5′-TAATGGTGAATCTTGGTTTGG-3′ (−)	SodA gene, genomic DNA		
E. faecium	FM1	5′-GAAAAAACAATAGAAGAATTAT-3′ (+)	SodA gene, genomic DNA	215 bp	Jackson *et al.* (2004)
	FM2	5′-TGCTTTTTTGAATTCTTCTTTA-3′ (−)	SodA gene, genomic DNA		
Noroviruses (GI/GII)	JV12 JV13	5′-ATACCACTATGATGCAGATTA-3′ (+) 5′-TCATCATCACCATAGAAAGAG-3′ (−)	RNA-dependent RNA polymerase RNA-dependent RNA polymerase	327 bp	Vinje & Koopmans (1996)
Norovirus genotype I	GI JV13	5′-TCNGAAATGGATGTTGG-3′ (+) 5′-TCATCATCACCATAGAAAGAG-3′ (−)	RNA-dependent RNA polymerase RNA-dependent RNA polymerase	187 bp	Vinje & Koopmans (1996); Greens *et al.* (1998)
Norovirus genotype II	JV12 NoroII-R	5′-ATACCACTATGATGCAGATTA-3′ (+) 5′-AGCCAGTGGGCGATGGAATTC-3′ (−)	RNA-dependent RNA polymerase RNA-dependent RNA polymerase	286 bp	Vinje & Koopmans (1996); Greens *et al.* (1998)

extension at 72 °C for 7 min. A nested PCR was prepared with 1:10 diluted first-run PCR products and the sequences were amplified with species-specific primers targeting *E. faecalis* and *E. faecium* respectively. The reaction was carried out using a modified protocol (Jackson *et al.* 2004). Following an initial denaturation at 95 °C for 4 min, products were amplified by 30 cycles (35 cycles for *E. faecium*) of 95 °C for 30 sec, 53 °C for 1 min (*E. faecalis*) or 48 °C for 1 min (*E. faecium*), 72 °C for 1 min, followed by final extension at 72 °C for 7 min. Five microlitres of product was mixed with 1 μL of loading buffer (Promega) and electrophoresed on a 2% 1X Tris-acetate-EDTA agarose gel. The products were visualised by ethidium bromide staining and UV trans-illumination. Each fragment size was compared with a DNA molecular weight marker (100 bp, Promega).

Pre-treatment, RNA extraction, detection for noroviruses

The combination of USEPA Manual 'Optimizing Molecular Methods to Detect Human Caliciviruses in Environmental Samples' (Vinje 2008) and the protocol established by Katayama *et al.* (2002) was adapted for norovirus concentration. One litre of each wastewater sample was centrifuged at 7,280 × g for 15 min. The supernatant was transferred to a new tube, and the pellet was resuspended in 10 mL of PBS and the mixture was centrifuged (2,000 × g, 15 min). The pellet was resuspended in 2 mL of PBS and stored at −20 °C (Vinje 2008). The two supernatants were combined and subsequently filtered through a glass fibre prefilter (AP15, Millipore, Ireland). The filtrate was adjusted with $MgCl_2$ to a final concentration at 0.05 M (Lukasik *et al.* 2000; Hsu *et al.* 2009) and then filtered through nitrocellulose membranes (HA series, 0.45 μm pore size, Millipore, Ireland) for adsorption using a glass filter holder unit (Millipore, USA) with a stainless steel screen. The stainless steel screen was cleaned and sterilised before use. The filter membrane was gently stirred in 10 mL of 1X PBS buffer (pH 7.0) for 15 min (Hsu *et al.* 2009). The solution was mixed well and aliquots were transferred to sterilised 1.5 ml microcentrifuge tubes and stored at −80 °C before use. Processed faecal samples from norovirus-positive patients were used for positive controls.

The concentrated samples, biosolids pellet and norovirus-positive faeces samples were 1:10 diluted in nuclease-free water and transferred to silica column provided in the Viral Nucleic Acid Extraction Kit III (Geneaid, Taiwan), following the manufacturer's extraction instructions. The extracted RNA was then subjected to one-step reverse transcription-polymerase chain reaction (RT-PCR) with 5X reaction buffer, Enzymix containing DNA and RNA *Taq* polymerase (Invitrogen, USA), 100 nmol of primers JV12/JV13 (IDT, Inc., USA) and nuclease-free water (Promega). The reaction was carried out as follows: 1 cycle of 50 °C for 30 min and 95 °C for 15 min; 45 cycles of 94 °C for 30 sec, 37 °C for 1 min, 72 °C for 1 min; 1 cycle of 72 °C for 10 min (Green *et al.* 1998). The parallel semi-nested PCRs were conducted subsequently for norovirus genogroup I and norovirus genogroup II. Briefly, the PCR was carried out in a new microcentrifuge tube containing 1 μL of each RT-PCR product, 2.5 μL of 10X PCR buffer (Invitrogen, USA), 0.4 μL of dNTPs (10 mM of each dNTP), 0.25 μL of Platinum *Taq* DNA Polymerase (5 U/μL, Invitrogen, USA), 0.5 μL of primers JV13/GI (for norovirus genogroup I) and JV12/NoroII-R (for norovirus genogroup II) and 19.85 μL of nuclease-free water. The reaction condition was modified from Boxman's protocol (Boxman *et al.* 2006). Briefly, an initial denaturation step was conducted at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 10 min. Five microlitres of the product was mixed with 1 μL of loading buffer (Promega) and the electrophoresis was carried out on a 3% 1X Tris-acetate-EDTA agarose gel. The products were visualised by ethidium bromide staining and UV trans-illumination. The fragment size was compared with DNA molecular weight marker (100 bp, Promega). In addition, the amplified gene segments were compared to the size of amplicons obtained from norovirus-positive patients' stool control samples on TAE gels.

Statistical analysis

The detection frequency of individual pathogens was presented corresponding to the factors associated with plants A–D, i.e. season (spring, summer, autumn, winter) and treatment stage (influent, effluent, biosolids). PERMANOVA (Anderson 2005) was used to test the significant differences among different factors of the two-way crossed design. The factors (i.e. plant, season, treatment stage) were considered as fixed. Results were considered at P(PERM) <0.01 and P(MC) <0.01. P(PERM) is permutation P-value and P(MC) is Monte-Carlo asymptotic P-value (Anderson & Robinson 2003). Wilcoxon matched pairs tests were applied to compare the detection frequencies in different plants, treatment stages and seasons. The differences among independent factors (i.e. treatment plants, seasons) were calculated with STATISTICA 6.0 (StatSoft Inc., 2002, Tulsa, USA). When multiple comparisons were performed with the Wilcoxon test, the resultant P-values were adjusted using the Bonferroni correction (Rice 1989).

RESULTS AND DISCUSSION

The detection frequencies of noroviruses (genogroups I and II), *E. faecalis* and *E. faecium* at four plants are shown in Table 2. The results were obtained by the interpretation of the amplicons (both wastewater samples and controls) amplified with species-specific primers. *E. faecalis* was detected in all of the influent samples from plants A and B, where 52.9 and 58.8%, respectively, of the samples were found positive for *E. faecium*. At plant D, 58.8 and 47.1% of the influent samples were positive for *E. faecalis* and *E. faecium*, respectively. *E. faecalis* and *E. faecium* were absent during the whole investigation period in plant C influents, except one *E. faecalis*-positive sample in April 2008. Norovirus genogroup II was more frequently detected in the influents at plants A and B (88.2%), followed by 64.7% at plant D. Overall, plant C effluent and final biosolids samples had significantly lower pathogen detection frequencies when compared to that at plant B ($P = 0.02$), but not plant A ($P = 0.04$) and plant D ($P = 0.04$) (Wilcoxon tests with Bonferroni-corrected significance level of 0.017).

It was also observed that the prevalence of the pathogens differed significantly by seasons at all of the plants (P(PERM) <0.001; P(MC) <0.001). Compared to plants A, B and D, plant C had the lowest pathogen load in spring ($P = 0.002$), summer ($P = 0.002$) and autumn ($P = 0.006$) (Wilcoxon tests, with Bonferroni-corrected significance level of 0.017). In winter months, noroviruses were frequently present in the influent samples at all plants

Table 2 | Detection rates of *Enterococcus faecalis*, *E. faecium* and norovirus genotype I and genotype II in wastewater samples at plants A–D (year 2008–2010)

	Overall			**Spring**			**Summer**			**Autumn**			**Winter**		
Samples	**Influent (%)**	**Effluent (%)**	**Biosolids (%)**	**Influent (%)**	**Effluent (%)**	**Biosolids (%)**	**Influent (%)**	**Effluent (%)**	**Biosolids (%)**	**Influent (%)**	**Effluent (%)**	**Biosolids (%)**	**Influent (%)**	**Effluent (%)**	**Biosolids (%)**
Plant A															
E. faecalis	100.0	58.8	29.4	100.0	50.0	25.0	100.0	100.0	75.0	100.0	50.0	0.0	100.0	40.0	20.0
E. faecium	52.9	11.8	5.9	100.0	0.0	25.0	100.0	25.0	0.0	25.0	25.0	0.0	0.0	0.0	0.0
Norovirus genotype I	58.8	17.6	11.8	25.0	0.0	25.0	75.0	25.0	25.0	50.0	0.0	0.0	80.0	40.0	0.0
Norovirus genotype II	88.2	70.6	76.5	50.0	50.0	75.0	100.0	50.0	75.0	100.0	75.0	75.0	100.0	100.0	80.0
Plant B															
E. faecalis	100.0	70.6	47.1	100.0	50.0	75.0	100.0	100.0	100.0	100.0	75.0	25.0	100.0	60.0	0.0
E. faecium	58.8	17.6	5.9	100.0	0.0	0.0	100.0	50.0	25.0	25.0	25.0	0.0	20.0	0.0	0.0
Norovirus genotype I	58.8	17.6	11.8	75.0	0.0	0.0	25.0	0.0	0.0	50.0	50.0	50.0	80.0	20.0	0.0
Norovirus genotype II	88.2	52.9	47.1	100.0	25.0	25.0	75.0	75.0	25.0	75.0	50.0	50.0	100.0	60.0	80.0
Plant C															
E. faecalis	5.9	0	0.0[a]	25.0	0.0	0.0[a]	0.0	0.0	0.0[a]	0.0	0.0	0.0[a]	0.0	0.0	0.0[a]
E. faecium	0	0	0.0	0.0	0.0	0.0[a]	0.0	0.0	0.0[a]	0.0	0.0	0.0[a]	0.0	0.0	0.0[a]
Norovirus genotype I	41.2	0	0.0	25.0	0.0	0.0[a]	0.0	0.0	0.0[a]	50.0	0.0	0.0[a]	80.0	0.0	0.0[a]
Norovirus genotype II	47.1	5.9	41.2	25.0	0.0	50.0[a]	0.0	0.0	0.0[a]	50.0	0.0	25.0[a]	100.0	20.0	80.0[a]
Plant D															
E. faecalis	58.8	17.6	41.2	50.0	25.0	25.0	100.0	50.0	100.0	75.0	0.0	50.0	20.0	0.0	0.0
E. faecium	47.1	5.9	11.8	25.0	25.0	25.0	75.0	0.0	25.0	75.0	0.0	0.0	20.0	0.0	0.0
Norovirus genotype I	35.3	5.9	23.5	25.0	0.0	0.0	25.0	0.0	25.0	50.0	25.0	25.0	40.0	0.0	40.0
Norovirus genotype II	64.7	23.5	47.1	50.0	25.0	25.0	50.0	0.0	25.0	75.0	25.0	75.0	80.0	40.0	60.0

[a]Plant C produced liquid sewage sludge on the bottom of settlement tank, where the samples were collected.

(median: 80% for norovirus genogroup I; 100% for norovirus genogroup II). In the same period, the effluent samples from plants C and D were negative for all pathogens, except for norovirus genogroup II. It was also noted that all winter effluent samples from plant A were positive for norovirus genogroup II. The prevalence of individual pathogens differed significantly between *E. faecalis* and *E. faecium* ($P = 0.005$, Wilcoxon test) and norovirus genogroups I and II ($P = 0.002$, Wilcoxon tests). As more *E. faecalis* than *E. faecium* was frequently detected in the treated effluent samples, it may demonstrate that the former may be either naturally present at higher concentration in the influent or more resistant to treatment processes (either activated sludge systems or the biofilm system) in municipal secondary wastewater treatment plants. This explanation can also be applied to the observed pattern of norovirus genogroup II. The introduction of this genogroup into the local aquatic environment following the wastewater treatment process coincides with the observed clinical prevalence in a previous study (Waters *et al.* 2006). Even though the faecal-oral route has been known as the transmission route leading to acute gastroenteritis, very little research has focused on the complete environmental infection cycle starting from municipal wastewater treatment plants. Nevertheless, Borchardt *et al.* (2003) associated the viral and bacterial diarrhoea occurring in studied populations living in Marshfield, Wisconsin with the density of septic systems in the same epidemiological study area. According to the multivariate analysis, viral and bacterial diarrhoea cases were independently associated with the number of septic systems in the studied residential area (Borchardt *et al.* 2003), indicating wastewater treatment systems as a risk factor for enteric infections.

For the biosolids at plants A, B and D, samples indicating the least effective treatment occurred in the summer season when 75–100% of the biosolids were *E. faecalis*-positive, followed by norovirus genogroup II (25–75%) and norovirus genogroup I and *E. faecium* (0–25%). In terms of the treatment efficiency based on the overall data, plant D (biofilm-coated percolating system) was found to be more efficient than plants A and B (extended-aerated activated sludge system), although the observed statistical significance was contributed mostly by the scenarios of poorly treated effluents and biosolids produced in spring and summer seasons at plants A and B, where the resultant wastes may pose a health risk to local inhabitants, if they are spread on land in the vicinity (Gale 2005). For example, on average 70.6 and 76.5% of plant A effluents (to local watercourses) and biosolids (to the land) contained norovirus genogroup II. For primary biosolids produced at plant D, since pathogen levels were higher, there may have been a higher risk to the catchment where the wastes are spread.

This study was conducted in coastal area in the northwest of Ireland, which accommodates thousands of holiday visitors annually especially in spring and summer. Seasonal variation of pathogen detection frequency in the incoming human sewage at plants A and B demonstrated that tourist influx may be the key factor to the insufficient treatment as well as a potential source of new pathogens. Wastewater treatment plants are traditionally designed to cope with up to three times the dry weather loading amount of organic matter and to reduce biochemical oxygen demand, suspended solids and nutrients to a compliant level before discharge (CEC 1991; Gray 2004). However, wastewater treatment plants are not originally designed for pathogen removal and the active human-virulent pathogen accumulated in the biosolids or partially suspended in the effluents may facilitate the environmental circulation of opportunistic pathogens causing infectious diarrhoea, such as *Cryptosporidium hominis*, *C. parvum* and noroviruses following consumption of contaminated shellfish, crops or bathing waters (Gale 2005; Graczyk *et al.* 2007; Nenonen *et al.* 2008). Relevant regulations, i.e. EU shellfish water directive (CEC 2006a), WHO guidelines for the reuse of wastewater in agriculture and aquaculture (WHO 1989), EU bathing water directive (CEC 2006b), are in place to prevent risks to human health. However, no pathogen emission limit is documented into legal force (CEC 1991) to ensure consistent treatment efficiency at municipal wastewater treatment systems, which are considered as pathogen accumulation reservoirs (Graczyk *et al.* 2009; Cheng *et al.* 2011).

The management of biosolids has epidemiological and public health implications (USEPA 1999; Gale 2005; Veronica 2008). In previous studies, abundant potentially human-infectious *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores were reported in the wastes

from municipal wastewater treatment plants (Rimhanen-Finne *et al.* 2004; Cheng *et al.* 2011). In the Irish 2007 national record (Monaghan *et al.* 2009), 86,411 tonnes of final biosolids were produced. For the studied areas, 4.12, 0.76, 1.82, and 8 tonnes of biosolids were from plants A–D respectively. Apart from those from plant C, the dewatered biosolids contained active viral particles deposited onto farmland, which may facilitate norovirus circulation. In this study, we also revealed that investigated municipal wastewater treatment systems were insufficient to inactivate norovirus particles and *E. faecalis* in discharged effluents. For reasons of public health, it is extremely important to provide facilities which are capable of pathogen inactivation, such as the add-on membrane filtration compartment, slow sand filtration or well-managed constructed wetland (Vega *et al.* 2003; Heistad *et al.* 2009; Liu *et al.* 2010; Simmons *et al.* 2011) before effluents reach receiving watercourses.

The notified infection cases (2007–2010) caused by noroviruses, *E. faecalis* and *E. faecium* nationwide and in the HSE northwestern area are shown in Table 3. The incident data were provided by different sources (i.e. Computerised Infectious Disease Reporting system, Health Service Executive, and EARS-Net). Among enterococcal bacteraemia notification, more infections were caused by *E. faecium* than *E. faecalis*, with 406 bacteraemia cases caused by the former notified in 2008 and slightly fewer cases in the following years. In 2008 and 2009, respectively, 163 and 115 norovirus outbreaks occurred resulting in 1,777 and 1,638 infection cases nationwide. In 2008 and 2009, respectively, 178 and 96 infection cases were notified in the HSE northwestern area. Four norovirus infection cases were reported in plant A and B serving areas and one case was reported in plant D serving area during the investigation period (personal communication, Mrs Amanda Murray, HSE west). Over 40% of plant C influent samples were norovirus-positive but no effluents contained detectable viral particles before discharge, except January 2010 samples which were positive for norovirus genogroup II. This may be a factor in the absence of norovirus infection cases notified in the area serving plant C. The stability of the noroviruses, combined with regular testing of the influents and the discharge wastes (effluent and biosolids), may provide epidemiological information (Iwai *et al.* 2009). Moreover, the association between the quality of sewage-derived waste products and infectious gastroenteritis cases should be carried out and evaluated in other regions internationally.

Table 3 | Notified infection cases caused by verotoxigenic *Escherichia coli*, *Enterococcus faecalis, E. faecium* and norovirus in Ireland between 2007 and 2010[a]

Pathogen	2007	2008	2009	2010
Escherichia coli				
Notified verotoxigenic *E. coli*[b] cases in Ireland	167	226	241	225
Notified verotoxigenic *E. coli*[b] cases in HSE Northwestern area	69	16	26	39
Enterococcus bacteraemia				
Enterococcus faecali	281	301	289	298
Enterococcus faecium	332	406	397	392
Norovirus outbreaks	111	163	115	NA[c]
Notified infection cases in Ireland	1317	1777	1638	1931
Notified infection cases in HSE Northwestern area	36	173	96	66

[a]Outbreaks include family and general outbreaks. 2007–2009 national data were extracted from Health Protection Surveillance Centre annual reports. 2010 data were taken from the national Communicable Infectious Diseases Reporting (CIDR) system was provisional. Notified verotoxigenic *E. coli* and enterococci infection cases were taken from EARSS system and 2010 norovirus cases in HSE Northwestern area were taken from CIDR system.

[b]National Health Protection Survellance Centre only notify EHEC cases directly to HPSC through EARSS system.

[c]2010 national norovirus outbreaks data were not available in the CIDR system.

CONCLUSIONS

1. Human noroviruses, *E. faecalis*, and *E. faecium* were frequently detected in plants A, B and D serving conurbations. The connection with infection cases notified in the conurbations, especially in those where health care centres are located, demonstrated that raw wastewater can provide epidemiological information to health protection agencies.
2. Norovirus genogroup II and *E. faecalis* were found more frequently discharged to fresh waters than norovirus genogroup I and *E. faecium*, irrespective of the treatment process (activated sludge system and biofilm percolating system). The biofilm-coated percolating system may present better treatment for norovirus particles in suspension but may, however, accumulate potentially active viral particles in the produced primary sludge.

3. We suggest that local authorised waste management bodies re-evaluate their current wastewater treatment systems in order to adjust to modern human activities, such as tourism or seasonal migration to regular holiday destinations (e.g. holiday homes in other regions or countries) and to provide effective pathogen inactivation systems to mitigate against acting as pathogen reservoirs.

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APPENDIX IV

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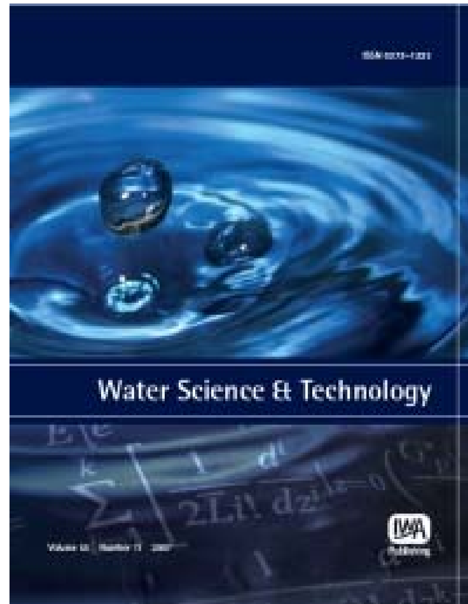

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Determining potential indicators of *Cryptosporidium* oocysts throughout the wastewater treatment process

H.-W. A. Cheng, M. A. Broaders, F. E. Lucy, S. E. Mastitsky and T. K. Graczyk

ABSTRACT

Most research on wastewater treatment efficiency compliance focuses on physicochemical and microbial indicators; however, very little emphasis has been placed so far on determining suitable indicator organisms to predict the discharge level of pathogens from treatment plants. In this study, raw wastewater, activated sludge, and the resulting final effluents and biosolids in four municipal wastewater treatment plants (WWTPs A, B, C and D) were seasonally investigated for human-virulent water-borne pathogens *Cryptosporidium parvum*/*hominis* and *Giardia duodenalis*, and microsporidia (e.g. *Encephalitozoon hellem*, *E. intestinalis*, and *Enterocytozoon bieneusi*) between 2008 and 2009. A suite of potential microbial indicators for human-virulent protozoa and microsporidia was also determined. A combination of multiple fluorescent *in situ* hybridization and immunofluorescent antibody assays were applied to detect *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores. *Escherichia coli*, enterococci and *Clostridium perfringens* spores were cultivated in selective media. Positive correlations were found between the abundance of enterococci and *E. coli* and abundance of *Cryptosporidium* oocysts ($r_s > 0.47$, $p < 0.01$) and *Giardia* cysts ($r_s > 0.44$, $p < 0.01$) at WWTPs A–D. *Clostridium perfringens* spores were positively correlated to *Cryptosporidium* oocysts ($r_s = 0.40$, $p < 0.01$) and *Giardia* cysts ($r_s = 0.46$, $p < 0.01$). There was a strong positive correlation between abundance of *Giardia* cysts and that of *Cryptosporidium* oocysts ($r_s > 0.89$, $p < 0.01$). To sum up, a suite of faecal indicator bacteria can be used as indicators for the presence of *Cryptosporidium* oocysts and *Giardia* cysts in these activated-sludge systems (WWTPs A, B and C). Overall, *Giardia duodenalis* was noted to be the best *Cryptosporidium* indicator for human health in the community-based influent wastewater and throughout the treatment process.

Key words | *Cryptosporidium* oocysts, faecal indicator bacteria, *Giardia* cysts, microsporidian spores, multiple linear regression predictive model, wastewater treatment process

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INTRODUCTION

Secondary biological treatment processes, for example, activated-sludge treatment, percolating filtration systems, rotating biological contactors, and submerged fixed film systems have been used at municipal wastewater treatment plants (WWTPs) world-wide for removal of nutrients. In order to meet the EU Urban Wastewater Treatment Directive standard, the environmental agencies and local authorities throughout the EU-15 Member States have been requested to monitor the residual nutrient in the discharged final effluent and biosolids on a regular basis since 1998 (CEC 1991; EEA 2005). However, there is very little emphasis on restraining the discharge of waterborne pathogens from the wastewater treatment facilities (Blumenthal *et al*. 2000; EEA 2005).

Evidence shows that the Irish national burden of gastroenteritis caused by pathogenic water-borne microorganisms, for instance pathogenic *Escherichia coli*, *Clostridium perfringens*, and human-virulent protozoa: *Cryptosporidium parvum*, *C. hominis* and *Giardia duodenalis*, has been increasing in the new millennium. Since 2004, when cryptosporidiosis was added to the list of notifiable infectious diseases, cases of acute infectious gastroenteritis have

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increased in scale (Garvey & McKeown 2008). *Cryptosporidium* and *Giardia* are human-virulent protozoan parasite species, forming resistant and long-lasting transition stages in the environment, termed oocysts and cysts respectively (Caccio *et al.* 2006). Microsporidian spores have also been found in a range of lakes and rivers used for drinking water abstraction and recreation (Lucy *et al.* 2008). Similar to *Cryptosporidium* oocysts and *Giardia* cysts, microsporidian spores (ranging from 0.8 to 2.0 μm in size) can break through the barriers during drinking water and wastewater treatments and survive in the surface water for a period of time (Fournier *et al.* 2000; Didier *et al.* 2004).

Studies utilising bacterial surrogates, i.e. *E. coli*, enterococci and *C. perfringens*, have been evaluated at laboratory scale, acting as potential indicators of the presence of *Cryptosporidium* and *Giardia* in soil, fresh waters, drinking water, and treated effluent from wastewater treatment plants (Robertson *et al.* 1992; Costan-Longares *et al.* 2008; Wilkes *et al.* 2009). A preliminary report from our research group indicated the evidence of human-virulent *Cryptosporidium* and *Giardia* prevailing in the local human populations (Cheng *et al.* 2009). The objective of this study was to quantitatively determine and compare the concentration of *Cryptosporidium* oocysts, *Giardia* cysts, microsporidian spores and faecal indicator bacteria, i.e. *E. coli*, enterococci, and *C. perfringens* spores throughout the wastewater treatment process and in the corresponding final effluent and biosolids seasonally between 2008 and 2009. Evidence-dependent regression analysis was carried out to create a potential *Cryptosporidium* predictive model.

MATERIALS AND METHODS

Characteristics of WWTPs and source of wastewater samples

This study focused on four secondary wastewater treatment plants (WWTPs A–D) located in north-western Ireland, serving up to 4,000 population equivalents. Wastewater samples (raw sewage, secondary activated sludge, final effluent) and biosolids were collected in April, July, October in 2008 and January and February in 2009. WWTP A is located in a seaside tourist town, in which a flux of travellers, particularly surfers, take regular holiday visits, especially in the summer period. WWTPs B, C, and D are situated in stable residential areas. All plants are designed as separate sewerage systems receiving only domestic wastewater, except Plant B, which accepts a combination of domestic sewage and proportional surface run-off in wet conditions. The specific treatment processes used in each of the four wastewater treatment plants are described in Table 1. Briefly, the raw wastewater (except at WWTP B) underwent grit removal and coarse screening before reaching the secondary treatment stage. Subsequent secondary wastewater treatments (where secondary wastewater samples were collected for this study) were applied: sludge activation in an oxidation ditch (WWTP A); sludge activation in extended aeration tanks (WWTPs B and C); and treatment by biofilm-coated percolating filter (WWTP D). The wastewater in each WWTP was then settled in a secondary settlement tank, i.e. clarifier, separating gravitationally final effluent and sewage sludge. The resulting sewage sludge was taken

Table 1 | Characteristics of four wastewater treatment plants (WWTPs A–D)

Plants	Population equivalent	Sewerage system	Primary treatment	Secondary treatment	Receiving water body	Operation
Plant A 54°16′43″ N 08°36′20″ W	1,950	Separate	Grit removal and coarse screening	Oxidation ditch, sedimentation, onsite dewatering	Sea	Local authority
Plant B 54°11′11″ N 08°29′10″ W	1,060	Semi-combined	None	Extended aerated activated sludge system, onsite dewatering	River	Local authority
Plant C 54°20′11.3″ N 08°31′44.3″ W	4,000	Separate	Grit removal, coarse screening, and sedimentation	Extended aerated activated sludge, settlement	River	Private sector
Plant D 54°02′54″ N 08°43′28″ W	2,500	Separate	Grit removal and primary sedimentation	Percolating, settlement, onsite dewatering	River	Local authority

out of the tank and spread onto the on-site drying beds, where the dewatered biosolids samples were collected. Sewage sludge at WWTP C was obtained via a discharge valve from the secondary settling tank. All wastewater samples were collected using a long-handled 1-L sampler and transferred to 1-L sterilised polyethylene bottles. Sewage sludge cake (approx. 100 g) was collected, by trowel, from 10-cm depth in the drying beds at WWTPs A, B, and D, while 1 L of liquid sewage sludge was obtained through the WWTP C discharge valve. All samples were collected in triplicate, and delivered to the laboratory in a cooler box (Cheng *et al.* 2009).

Enumeration of faecal indicator bacteria

Fifty millilitres (mL) of the sub-samples was mixed vigorously and 1-mL of the mixture was subjected to a ten-fold serial dilution in Ringer's solution (Oxoid, UK) to 10^{-4}. Sub-samples for *C. perfringens* spores (10 mL) were heat shocked at 75 °C for 20 min and then cooled on ice (Hauschild *et al.* 1974). For *E. coli* and enterococci, 0.2-mL of the sub-samples was aseptically spread onto chromogenic *E. coli*/coliform agar (Oxoid, UK) and Slanetz and Bartley agar (Oxoid, UK) and cultivated at 35 ± 2 °C and 44 °C, respectively. In accordance with the manufacturer's direction, purple colonies present on Chromogenic *E. coli*/ coliform agar and burgundy red colonies on Slanetz and Bartley agar were enumerated as positive. For *C. perfringens* spores, 0.2-mL of the heat shock-processed serial diluent was aseptically spread onto Perfringens agar with supplement (Oxoid, UK). Non-supplemented Perfringens agar was overlaid onto the base and settled at room temperature. The plates were incubated anaerobically at 35 ± 2 °C for 18–24 h (Hauschild *et al.* 1974). Black colonies were enumerated as positive.

Pre-treatment and purification

All liquid wastewater samples were mixed vigorously and transferred to 1-L capacity Imhoff settlement cones. Twenty grams of the dewatered biosolids was rehydrated in 1 L of MilliQ water in a sterile food processor and homogenized. The liquid was then transferred to 1-L imhoff cones for gravity sedimentation. Fifty millilitres of the top sediment layer was transferred using a 50-mL glass pipette to a plastic 50-mL conical centrifuge tube and centrifuged at 3,000 g for 5 min. The supernatant was removed and the pellet was transferred and reserved in 75% ethanol in a 1.5-mL microcentrifuge tube (Graczyk *et al.* 2004). The samples were stored at 4 °C. The ethanol was washed from the pellet by centrifugation (8,000 *g*, 5 min) twice and the pellet was subjected to sugar–phenol flotation (Ash & Orihel 1987).

Fluorescent *in situ* hybridization and immunofluorescent antibody assay

For *Cryptosporidium* oocysts and *Giardia* cysts, aliquots of the purified samples were treated in equal volumes of acetone for 15 min and washed in 1× phosphate-buffered saline (PBS). The Hex-fluorochrome-labelled probes (100 μmol) were added subsequently and the whole mixture was incubated at 57 °C for 1 h. After centrifugation (8,000 *g*, 4 min), the pellets were re-suspended in 20 μL of MilliQ water and transferred into three lysine-coated immunofluorescent wells on slides. The slides were stored at room temperature to dry and the hybridisation-processed samples were then subjected to a direct immunofluorescent antibody assay (Graczyk *et al.* 2004; Cheng *et al.* 2009).

For microsporidian spores, the resulting pellet was assayed in 1.5-mL microcentrifuge tubes by multiplex fluorescent *in situ* hybridisation for identification of *E. bieneusi*, *E. hellem*, and *E. intestinalis* spores. Briefly, species-specific, fluorochrome-labelled probes, i.e. HEL878F, INT-1, and BIEN-1, were used for detection of *E. hellem*, *E. intestinalis* and *E. bieneusi* spores, respectively. The probes, which hybridise the 16S ribosomal RNA of specific microsporidian spore species, were added to purified samples and incubated at 57 °C for 3 h (Graczyk *et al.* 2004). The processed samples (20 μL in MilliQ water) were placed onto the wells of immunofluorescent slides and the slides were stored at room temperature to dry.

Cryptosporidium oocysts, *Giardia* cysts and microsporidian spores were identified and enumerated without knowledge of sample identity using an epifluorescent microscope, at 100× objective magnification, and BP450-490 exciter filter.

Statistical analysis

As it is generally accepted that faecal indicator bacterial concentration in sewage samples follow a $\log_{10}$ normal probability, the mean concentration is then calculated as $\log_{10}(x+1)$, where x = concentration of the indicator bacteria (CFU 100 mL^{-1}). Correlation between Log_{10}-transformed indicator bacteria concentration and (oo)cysts and spore concentration was conducted using Spearman rank coefficient r_s. Pathogen detection frequency was determined and the removal efficiency was calculated as a percent of the difference in pathogen concentration in the effluent vs.

influent. Negative values were obtained for the cases in which the pathogen concentration was higher in the effluents as compared to the influents. All descriptive statistics, Spearman rank correlation coefficients and Wilcoxon tests were calculated using STATISTICA 6.0 (StatSoft, Inc, 2002, Tulsa, USA).

RESULTS AND DISCUSSION

Presence and abundance of faecal indicator bacteria

The geometric mean concentrations, 95% intervals and range concentration of faecal indicator bacteria, expressed as Log_{10}, are shown in Figure 1. The geometric means (GM) have shown WWTPs A and B had averagely higher microbiological concentration, with 4.3 Log unit 100 mL^{-1} (WWTP A) and 4.2 Log unit 100 mL^{-1} (WWTP B) of *E. coli*, 2.8 Log unit 100 mL^{-1} (WWTP A) and 2.3 Log unit 100 mL^{-1} (WWTP B) of enterococci, and 3 Log unit 100 mL^{-1} of *C. perfringens* spores at both plants. WWTP C had the lowest GM concentration of all indicator bacteria. However, higher concentrations of *E. coli* (3.5 Log unit 100 mL^{-1}) were observed in raw wastewater samples in February 2009, compared to WWTPs A, B, and D (mean: 3.1 unit 100 mL^{-1} of *E. coli*). The results also highlighted the wide variability of faecal indicator bacteria concentration in sewage samples by site, treatment stage and season (95% intervals and

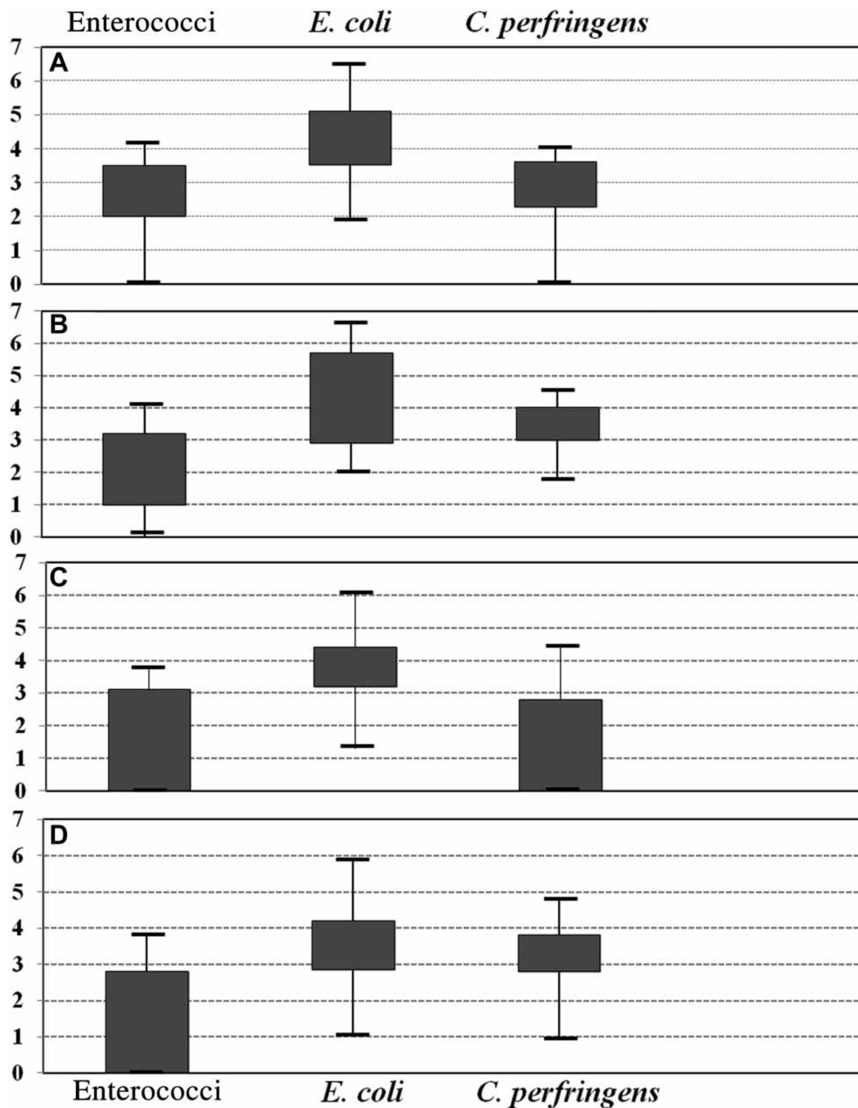

Figure 1 | Geometric mean, 95% intervals (box) and range (line) of Log_{10} faecal indicator bacteria concentration in all wastewater samples at Plants A–D, 2008–2009.

range concentration, Figure 1). For example, *E. coli* had the highest concentration of 6.6 Log unit 100 mL^{-1} in WWTP B raw wastewater, in summer 2008, followed by concentration of *C. perfringens* spores (4.1 Log unit 100 mL^{-1}) and enterococci (3.3 Log unit 100 mL^{-1}). In the same season, WWTP B had the highest amount of indicator bacteria, followed by WWTP A (*E. coli*: 6.4 Log unit 100 mL^{-1}; enterococci: 4.0 Log unit 100 mL^{-1}; *C. perfringens* spores: 3.6 Log unit 100 mL^{-1}), and WWTP D had the least faecal indicator bacteria with 3.7 Log unit 100 mL^{-1} of *C. perfringens* spores and no enterococci detected. In most scenarios, over 92% of the indicator bacteria were removed during treatments whilst *E. coli* and *C. perfringens* spores were occasionally detected at high concentration in the discharging effluents (e.g. *E. coli*: 5.8 Log unit 100 mL^{-1} at WWTP D in October 2008; *C. perfringens* spores: >3.9 Log unit 100 mL^{-1} at WWTP B in both summer and autumn seasons). The order of the indicator bacteria concentration was: *E. coli* > *C. perfringens* spores > enterococci. However, *C. perfringens* spores were frequently detected in greater concentrations than *E. coli* at WWTP D.

Presence and abundance of human-virulent *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores

The geometric mean concentrations, 95% intervals and range concentration of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores are shown in Figure 2. *Cryptosporidium* oocysts were found in higher

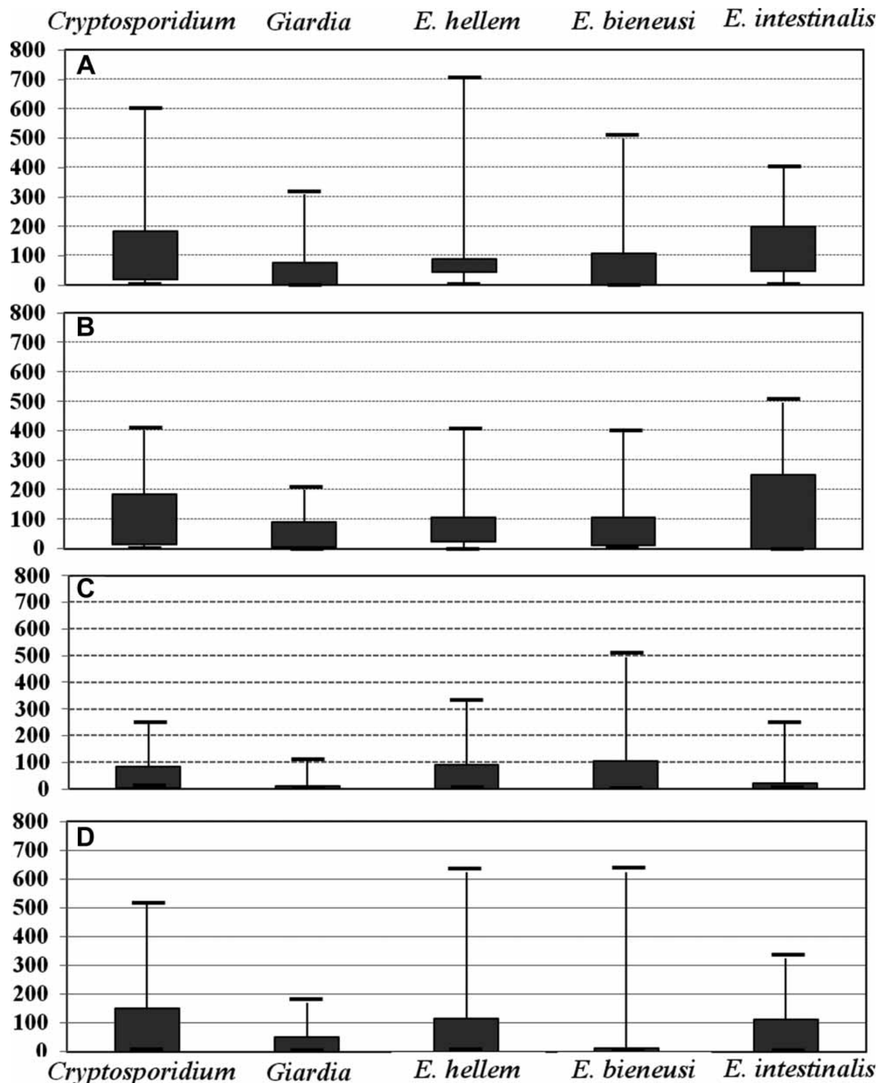

Figure 2 | Geometric mean, 95% intervals (box) and range (line) of pathogen concentration in all wastewater samples at Plants A–D, 2008–2009.

concentration than other pathogens at all plants with GM ranging from 20 to 180 oocysts/L among WWTPs, followed by *E. intestinalis* spores (GM = 6–83 oocysts/L) and *Giardia* cysts (GM = 6–130 oocysts/L). All of the raw sewage samples were shown positive for *Cryptosporidium*, with the highest abundance at WWTP B (>590 oocysts/L) in the summer season. WWTP C had the least pathogen concentration, with averagely 20 *Cryptosporidium* oocysts/L, 6 *Giardia* cysts/L, and 34 microsporidian spores detected. Averagely, 76% of *Cryptosporidium* oocysts and 90% of *Giardia* cysts in wastewaters were removed after treatment. Among the four plants, WWTP C had the highest removal efficiency of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores at 84, 98, and 99.5%, respectively. *Cryptosporidium* was dominant over all other pathogens, i.e. *Giardia* cysts, which were found in a higher concentration than *Cryptosporidium* oocysts and *E. bieneusi* spores in the previous Irish study (Graczyk *et al.* 2007), and spores of *E. hellem*, *E. bieneusi* and *E. intestinalis* (in this study). Seasonal variation of pathogen detection frequency with high abundance in the incoming human sewage, especially at WWTPs A and B which are tourist town, demonstrated that season-dependent sudden traveller influx may be the key factor for the insufficient treatment as well as a potential source of new pathogens. Even though the highest abundance of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores were detected in summer at WWTPs A and B, it was found that an averagely high abundance of *Cryptosporidium* oocysts (GM = 195 oocysts/L), *Giardia* cysts (GM = 81 cysts/L) and *E. intestinalis* spores (GM = 73 spores/L) were present in winter samples at all of the four plants, intermitting medium background levels of pathogens in the studied human populations.

The detection frequencies among individual pathogens were varied (Table 2). *Cryptosporidium* was detected in over 93% of the samples in July (summer), October (autumn) 2008 and January and February (winter) in 2009, with the exception of April (spring) samples at 69% detection frequency. *Giardia* had a higher detected frequency in the summer (87.5%) and winter (>81%) seasons whilst *E. hellem* was detected more in autumn (>93%). All in all, seasonal variation was noticed in the pathogen detection frequencies, especially between the spring and winter seasons ($r_s = 0.89$, $p < 0.05$), indicating higher *Cryptosporidium* and *Giardia* detection frequency in winter. The finding in this study presents different *Cryptosporidium* occurrence trends in comparison to the pronounced spring peak in other studies in the Republic of Ireland (Chalmers *et al.* 1997; Zintl *et al.* 2006). However, Zintl *et al.* later pointed out

Table 2 | Presence, absence and detection frequency of *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores

	Spring			Summer			Autumn			Winter		
	Presence (N)	Absence (N)	Detection (%)	Presence (N)	Absence (N)	Detection (%)	Presence (N)	Absence (N)	Detection (%)	Presence (N)	Absence (N)	Detection (%)
Cryptosporidium	22	10	68.8	15	1	93.8	15	1	93.8	15	1	93.8
Giardia	17	15	53.1	14	2	87.5	10	6	62.5	13	3	81.3
E. bieneusi	23	9	71.2	10	6	62.5	12	4	75.0	15	1	93.8
E. intestinalis	16	16	50.0	12	4	75.0	15	1	93.8	10	6	62.5
E. hellem	16	16	50.0	10	6	62.5	10	6	62.5	13	3	81.3

Table 3 | Spearman rank order correlations. Marked coefficients are significant at $p < 0.01$

	Enterococci	*E. coli*	*C. perfringens*	*Cryptosporidium*	*Giardia*	*E. hellem*	*E. bieneusi*	*E. intestinalis*
Enterococci	1.0000	**0.6072**	0.2134	**0.4781**	**0.4437**	**0.4516**	**0.4930**	**0.4308**
E. coli	**0.6072**	1.0000	0.2926	**0.4809**	**0.5054**	**0.5695**	**0.5310**	**0.4742**
C. perfringens	0.2134	0.2926	1.0000	**0.4020**	**0.4669**	**0.4570**	0.3450	0.3602
Cryptosporidium	**0.4781**	**0.4809**	**0.4020**	1.0000	**0.8919**	**0.5458**	**0.4150**	**0.5331**
Giardia	**0.4437**	**0.5054**	**0.4669**	**0.8919**	1.0000	**0.5613**	**0.4185**	**0.5240**
E. hellem	**0.4516**	**0.5695**	**0.4570**	**0.5458**	**0.5613**	1.0000	**0.5498**	**0.7865**
E. bieneusi	**0.4930**	**0.5310**	0.3451	**0.4150**	**0.4185**	**0.5498**	1.0000	**0.5475**
E. intestinalis	**0.4308**	**0.4743**	**0.3602**	**0.5331**	**0.5240**	**0.7865**	**0.5475**	1.0000

that a second small *Cryptosporidium* peak appeared in late autumn, the year after the large cryptosporidiosis outbreak in Co. Galway, Ireland (Zintl *et al.* 2008).

The variation among pathogen abundance was noticed at the four WWTPs from plant to plant (within seasons) as well as in different seasons. In many cases, the pathogens were most frequently detected in the highest abundance at WWTP B. However, a seasonally-dependent high pathogen load was determined at WWTP A in both summer and autumn seasons (Wilkes *et al.* 2009). WWTP C had the least pathogen load in all seasons. It was obvious that the pathogen presence scenario differed from plant to plant, with WWTP A versus D (t-value $= 2.75$, $df = 8$, $p = 0.02$); WWTP B versus C (t-value $= 2.64$, $df = 8$, $p = 0.03$) and D (t-value $= 3.41$, $df = 8$, $p = 0.01$).

Relationship between the abundance of faecal indicator bacteria and abundance of pathogens

Spearman rank correlation analyses were conducted using all non-categorical microbial data and pathogen abundance during the study period (Table 3). Significant correlations ($\alpha < 0.05$) were found between enterococci and *E. coli* ($r_s = 0.6072$). Correlations between *C. perfringens* spores and other faecal indicator bacteria were weak, ranging from 0.21 to 0.29. All faecal indicator bacteria had a significant correlation with *Cryptosporidium* ($r_s = 0.40$–0.48), *Giardia* ($r_s = 0.44$–0.50), and microsporidian spores ($r_s = 0.43$–0.57). There was no significant correlation between spores of *C. perfringens* and *E. bieneusi* and *E. intestinalis*. It was noted that *E. coli* had the strongest correlation ($r_s = 0.48$–0.57) with the detected pathogens. Furthermore, the combination of *E. coli*, enterococci, and *C. perfringens* spores contributed to the significantly positive correlation with the occurrence of *Cryptosporidium* oocysts, *Giardia* cysts and *E. hellem* spores. Microsporidia is included in the frequently detected pathogens in the Irish disease control and prevention list. Therefore, the source of high detected concentration of microsporidian spores in the studied domestic wastewater is still unknown. Nevertheless, a few studies linked microsporidian spores detected in urban wastewaters to domestic dogs and livestock and avian visiting to the treatment plants (Graczyk *et al.* 2007, 2009).

A strong positive correlation was found between abundance of *Giardia* cysts and abundance of *Cryptosporidium* oocysts ($r_s = 0.89$, $p < 0.05$). Instead of detecting both of the parasites, it is time-effective and economic to detect one of them. As *Giardia* cysts have a larger size than *Cryptosporidium* oocysts and *Giardia* cysts are commonly found in a significant relationship with *Cryptosporidium* in the same environment (Bajer 2008), we suggest that *Giardia* cysts can be used as a potential indicator to predict the occurrence of human-virulent *Cryptosporidium* oocysts. Multiple linear regression was analysed using the abundance of *Cryptosporidium* oocysts and *Giardia* cysts in all samples, resulting in the overall predictive regression model: *Cryptosporidium* oocysts $= (3 \pm 0.9) \pm (1.2 \pm 0.8)$ *Giardia* cysts (Table 4). Since the pathogen presence scenario differed from plant

Table 4 | The *Cryptosporidium* regression predictive models (at WWTPs A–D and overall)

Plants	Regression predictive models	p value
Plant A	*Cryptosporidium* oocysts $= (44.6 \pm 1.8) + (11.7 \pm 0.1)$ *Giardia* cysts	$R^2 = 0.91$, $p < 0.05$
Plant B	*Cryptosporidium* oocysts $= (1.82 \pm 0.23)$ *Giardia* cysts	$R^2 = 0.78$, $p < 0.05$
Plant C	*Cryptosporidium* oocysts $= (1.78 \pm 0.17)$ *Giardia* cysts	$R^2 = 0.84$, $p < 0.05$
Plant D	*Cryptosporidium* oocysts $= (2.45 \pm 0.27)$ *Giardia* cysts	$R^2 = 0.81$, $p < 0.05$
Overall	*Cryptosporidium* oocysts $= (3 \pm 0.9) + (1.2 \pm 0.8)$ *Giardia* cysts	$R^2 = 0.74$, $p < 0.05$

to plant, the predictive regression models specific for WWTPs A–D were also determined (Table 4).

CONCLUSIONS

A suite of faecal indicator bacteria can be used as microbial indicators for the presence of *Cryptosporidium* oocysts, *Giardia* cysts and *E. hellem* spores. Spatial variation of the bacteria abundance and pathogen loads in the studied areas may be caused by sudden traveller influx in tourism seasons, especially at WWTP A in summer and autumn. A background abundance of *Cryptosporidium* oocysts, *Giardia* cysts, and microsporidian spores were also noticed at WWTPs A and B. The overall predictive regression model: *Cryptosporidium* oocysts $= (3 \pm 0.9) + (1.2 \pm 0.8)$ *Giardia* cysts ($R^2 = 0.74$, $p < 0.05$).

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