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,

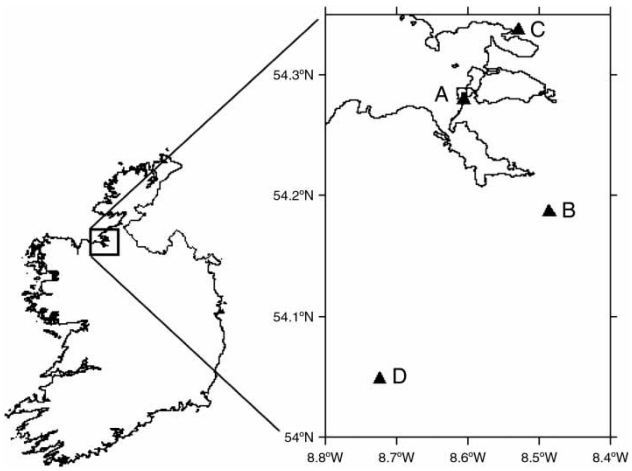

Figure 1 | Location of plants A–D in northwestern Ireland.

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 Surveillance 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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