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

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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

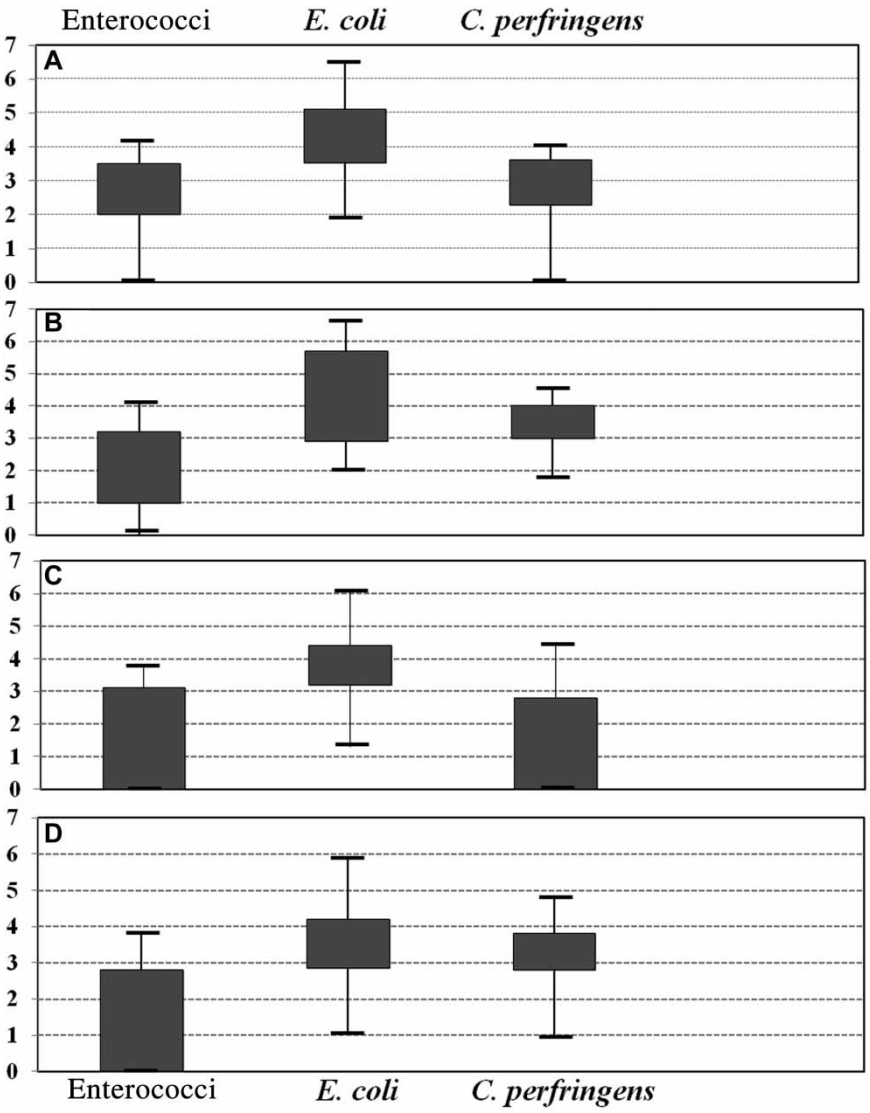

Figure 1 | Geometric mean, 95% intervals (box) and range (line) of Log_{10} faecal indicator bacteria concentration in all wastewater samples at Plants A–D, 2008–2009.

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

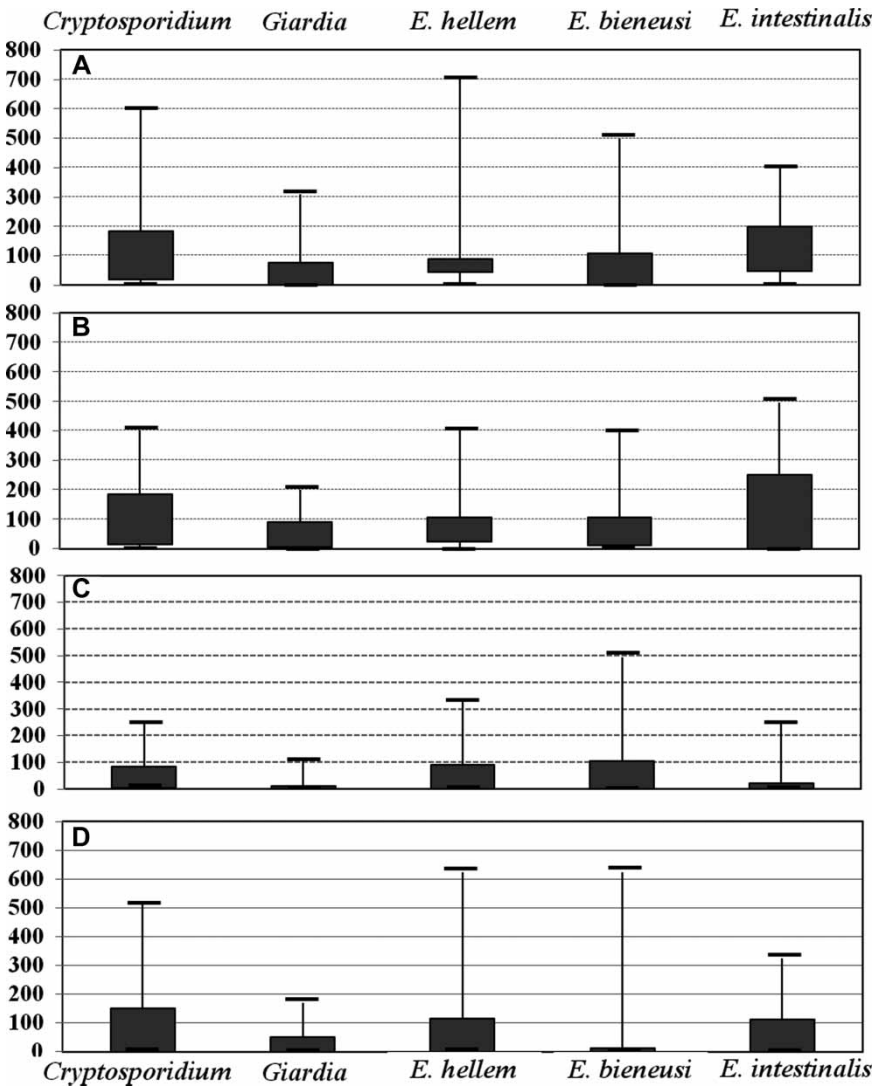

Figure 2 | Geometric mean, 95% intervals (box) and range (line) of pathogen concentration in all wastewater samples at Plants A–D, 2008–2009.

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 ± 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 = $(3 \pm 0.9) + (1.2 \pm 0.8)$ *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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