

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

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Received: 8 January 2009 / Accepted: 3 April 2009
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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 transmissible 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 transmissible 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 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 polyethylene 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 (°C)	pH	Conductivity (μS/cm)	Dissolved oxygen (mg/L)	TDS (mg/L)
<i>A</i>	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
<i>B</i>	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
<i>C</i>	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
<i>D</i>	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 transmissible stages of these pathogens were viable. The low dissolved oxygen content of the final

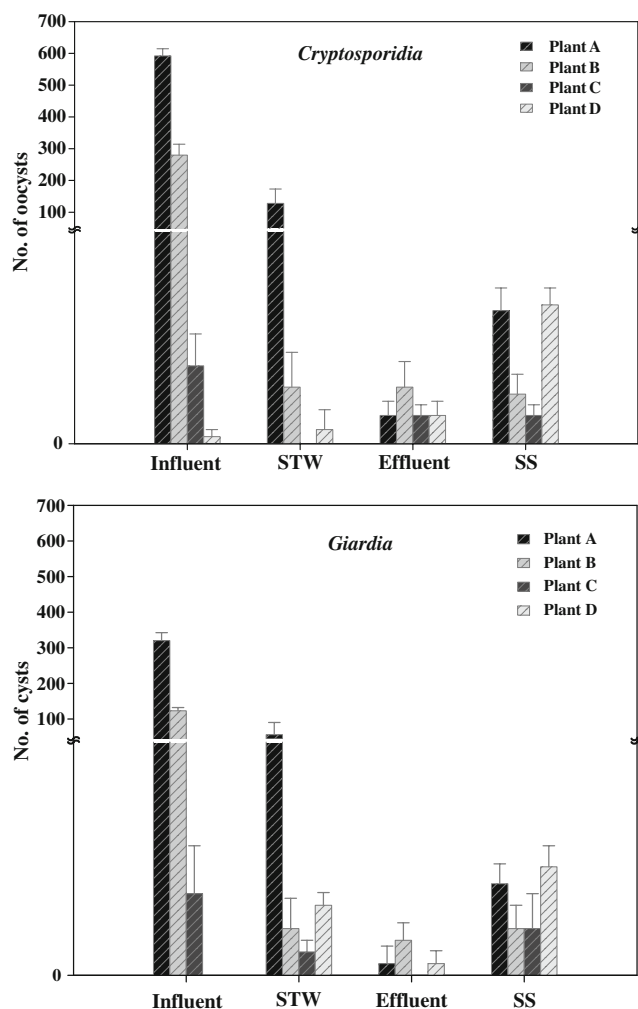


Fig. 2 Removal of *Cryptosporidium parvum* and *C. hominis* (oocysts/L) and *Giardia duodenalis* (cysts/L) during the wastewater treatment at four wastewater treatment plants (i.e., A, B, C, and D) in north-western Ireland. STW Secondary-treated wastewater, SS sewage sludge

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 (%) <i>C. parvum</i> and <i>C. hominis</i>		Removal (%) <i>G. duodenalis</i>	
	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.

Acknowledgments The study was supported by the Irish EPA Strive PhD grant 2007-PhD-EH-3, Fulbright Senior Specialist Fellowship (grant no. 2225 Graczyk), Johns Hopkins Center in Urban Environmental Health (grant no. P30 ES03819), School of Science Institute of Technology, Sligo, Ireland, and the U.S. Environmental Protection Agency Science to Achieve Results (STAR) Program (grant no. RD83300201). The views expressed herein have not been subjected to the U.S. EPA review and therefore do not necessarily reflect the views of the agency, and no official endorsement should be inferred.

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