

# Human zoonotic enteropathogens in a constructed free-surface flow wetland

Thaddeus K. Graczyk · Frances E. Lucy ·  
Yessika Mashinsky · R. C. Andrew Thompson ·  
Ozgur Koru · Alexandre J. daSilva

Received: 6 January 2009 / Accepted: 8 March 2009  
© Springer-Verlag 2009

**Abstract** Effluents from a small-scale free-surface flow constructed wetland, used for polishing of secondary treated wastewater, contained significantly higher concentrations of potentially viable *Giardia duodenalis* cysts and *Enterocytozoon bieneusi* spores than did wetland influents consisting of secondary treated wastewater. Zoonotic Assemblage A of *G. duodenalis* cysts was identified in wetland inflows, while Assemblage A and two nonhuman infective Assemblages (i.e., C, and E) were present in wetland effluents. *E. bieneusi* spores represented genotype K based on DNA sequencing analysis of internal transcribed spacer. The study demonstrated that: (1) free-surface flow small-scale constructed wetlands may not provide sufficient remediation for human zoonotic protozoa and fungi present in secondary treated wastewater; (2) dogs and livestock can

substantially contribute human-pathogenic protozoan and fungal microorganisms to engineered vegetated wetland systems; and (3) large volumes of wetland effluents can contribute to contamination of surface waters used for recreation and drinking water abstraction and therefore represent a serious public health threat.

## Introduction

Demand for high-quality drinking and recreational waters rises exponentially due to global demographic growth in the human population, reinforcing an urgent need for microbiologically safe reclaimed waters (Graczyk and Lucy 2007). Wastewater discharges are worldwide risk factors

---

T. K. Graczyk (✉) · Y. Mashinsky  
Department of Environmental Health Sciences,  
Division of Environmental Health Engineering,  
Johns Hopkins Bloomberg School of Public Health,  
Baltimore, MD 21205, USA  
e-mail: tgraczyk@jhsph.edu

T. K. Graczyk  
Department of Molecular Microbiology and Immunology,  
Johns Hopkins Bloomberg School of Public Health,  
Baltimore, MD 21205, USA

T. K. Graczyk  
Johns Hopkins Center for Water and Health,  
Johns Hopkins Bloomberg School of Public Health,  
Baltimore, MD 21205, USA

F. E. Lucy  
Department of Environmental Science,  
School of Science, Institute of Technology,  
Sligo, Ireland

T. K. Graczyk · F. E. Lucy  
Centre for Biomolecular Environmental and Public Health  
Research, School of Science, Institute of Technology,  
Sligo, Ireland

F. E. Lucy  
Environmental Services Ireland, Lough Allen,  
Carrick on Shannon, Co. Leitrim, Ireland

R. C. Andrew Thompson  
WHO Collaborating Centre for the Molecular Epidemiology of  
Parasitic Infections, School of Veterinary and Biomedical  
Sciences, Murdoch University,  
Murdoch, Western Australia 6150, Australia

O. Koru · A. J. daSilva  
Division of Parasitic Diseases, National Center for Zoonotic,  
Vector-borne, and Enteric Diseases, Centers for Disease Control  
and Prevention, Public Health Service,  
U.S. Department of Health and Public Services,  
Atlanta, GA 30341, USA

for the introduction of human pathogens into surface waters used as drinking and recreational resources. *Cryptosporidium parvum*, *Giardia duodenalis*, and human-virulent microsporidia, (i.e., *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, and *Enterocytozoon bieneusi*) are waterborne enteric pathogens inflicting morbidity in healthy people and mortality (e.g., *Cryptosporidium* and microspora) in highly immunodeficient individuals (Weber and Bryan 1994; Graczyk et al. 1997; Savioli et al. 2006). Their transmissive stages, i.e., oocysts, cysts, and spores, respectively, are resistant to environmental stressors and are therefore long-lasting and relatively ubiquitous in the environment (Wolfe 1992; Graczyk et al. 1997; Matchis et al. 2005). These pathogens are category B biodefense agents on the National Institute of Health list, and microsporidian spores are on Contaminant Candidate List of the US Environmental Protection Agency because spore identification, removal, and inactivation in drinking water are technologically challenging (Nwachuku and Gerba 2004). Surface water is not routinely monitored for these pathogens, despite considerable evidence demonstrating environmental contamination derived from wastewater discharges (Graczyk and Lucy 2007). Environmentally, the aforementioned parasites have a broad zoonotic reservoir (Graczyk et al. 1997; Matchis et al. 2005; Savioli et al. 2006).

Constructed wetlands of either vertical or horizontal flow are increasingly being used worldwide for secondary or tertiary treatment of municipal sewage (Davison et al. 2005). In wetlands, human-pathogenic microorganisms are removed and biodegraded by sedimentation (Karim et al. 2004; Dai and Boll 2006), filtration and attachment to plant roots (Gerba et al. 1999), UV radiation (Quinonez-Diaz et al. 2001), and protozoan predation (Stott et al. 2001). It is thought that the performance of engineered wetlands in removing microorganisms is superior to that of conventional activated sludge plants (Ulrich et al. 2005). Reed-bed systems usually discharge processed wastewater to surface waters that are frequently used for recreation or drinking water abstraction (Davison et al. 2005). It is commonly assumed that human enteropathogens identified in waters receiving effluents from sewage treatment operations through engineered wetlands originate from the wastewater (Thurston et al. 2001). However, it is possible that constructed wetlands can act as endemic sites supporting both propagation and transmission of the pathogens entering the wetlands with wastewater (Graczyk et al. 2007a). Sizing reed-bed systems for a residence time of 5 days has become a standard practice (Davison et al. 2005), leaving plenty of time for propagation and spreading of wastewater-derived pathogens in wetland habitats via a wide variety of wildlife (Graczyk and Lucy 2007; Graczyk et al. 2007a).

Because *Cryptosporidium*, *Giardia*, and microsporidia can infect a variety of nonhuman hosts, identification of

human-infective species represents a challenge. Another challenge is determination of viability of the aforementioned pathogens as they may be nonviable and thus not of epidemiological importance. Both challenges are addressed by fluorescence in situ hybridization (FISH) technique. FISH employs fluorescently labeled oligonucleotide probes targeted to species-specific sequences of 18S rRNA, and therefore, identification of pathogens is species-specific (Graczyk et al. 2007a). As rRNA has a short half-life and is only present in numerous copies in viable organisms, FISH allows for differentiation between potentially viable and nonviable pathogens (Vesey et al. 1998; Hester et al. 2000; Dorsch and Veal 2001).

The purpose of the present study was to quantitatively, qualitatively, and genetically characterize potentially viable *C. parvum* oocysts, *G. duodenalis* cysts, and human-infective microsporidian spores entering and leaving a small-scale engineered horizontal free-surface flow wetland used for tertiary treatment of municipal sewage.

## Materials and methods

Two grab samples (2 L) of secondary treated wastewater entering a constructed small scale free-surface flow horizontal wetland and three 2-L samples of wetland effluents (i.e., tertiary treated wastewater) were collected at an urban wastewater treatment plant, Keadue (54°03.18' N; 08° 09.12' W), Ireland. The plant utilized primary treatment by coarse screening, secondary treatment, i.e., sludge activation and sedimentation, and then the effluent was polished using a reed-bed wetland filtration system, with effluent discharging directly to Lough Meelagh, a lake used for recreational activities. Secondary (i.e., wetland influent) and tertiary (i.e., wetland effluent) treated wastewater samples were transported to the laboratory in a cooler and processed by gravity sedimentation (Graczyk et al. 2007a). The resulting pellet was divided evenly into five aliquots. The first aliquot was processed for *C. parvum* and *G. duodenalis* using multiplexed FISH combined with direct immunofluorescent antibody (IFA), and the second for human-infective microsporidia (i.e., *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi*) by multiplexed FISH (Graczyk et al. 2007a). FISH-reactive pathogen cells were enumerated (Graczyk et al. 2007a). The third aliquot was assayed by polymerase chain reaction (PCR) for *Cryptosporidium* oocysts and then by nested PCR for *C. parvum* and *Cryptosporidium hominis* (Downey and Graczyk 2007). The fourth aliquot was assayed by PCR using primers based on 18SrRNA gene for detection of *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi*, and by sequencing for genotyping of *E. bieneusi* spores (de Groote et al. 1995; Visvesvara et al. 1995; daSilva et al.

1996, 1997). DNA from these samples were extracted using the FastDNA method as described elsewhere (daSilva et al. 1999) Samples positive for microsporidia with the 18SrRNA PCR were subjected to PCR amplification with primers designed to amplify the internal transcribed spacers (ITS) as described elsewhere (Katzwinkel-Wladarsch et al. 1996). ITS amplicons were sequenced on both strands with the BigDye® V3.1 chemistry on an ABI Prism® 3130xl genetic analyzer. Genotypes were assigned based on similarities obtained with ITS sequences through BLAST searches and alignments with sequences deposited in the GenBank® database. The fifth aliquot was used for molecular characterization of *Giardia* isolates using a nested PCR for amplification at the 18S rDNA locus as previously described (Leonhard et al. 2007).

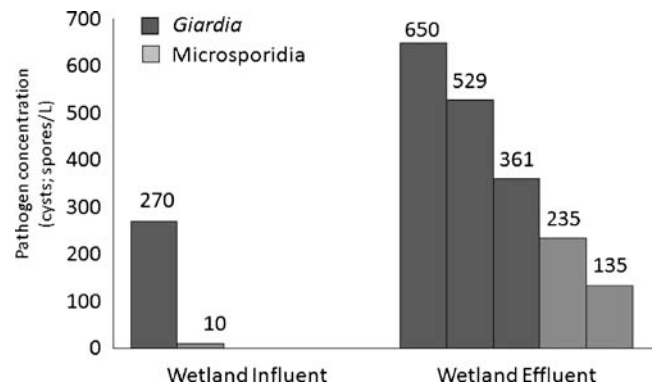
Rainfall data for 2 months preceding sample collection and for the month of sample collection were obtained electronically from the local weather station.

Statistical analysis was carried out with Statistix 9.0 (Analytical Software, St. Paul, MN, USA). Variables were tested by Wilk–Shapiro/Ranking Plots to determine whether their distribution conformed to a normal distribution, and if not, nonparametric tests were used. Differences in spore concentration were assessed by Wilcoxon signed rank test, and differences in fraction of pathogens entering and leaving the wetland by chi-square test ( $\chi^2$ ). Statistical significance was considered to be  $P < 0.05$ , and all  $P$  values for the Wilcoxon signed rank test were two-tailed.

## Results

Multiplexed FISH assay combined with IFA identified only *G. duodenalis* cysts. The cysts were present in a single wetland influent sample and in all three wetland effluent samples. The concentration of *G. duodenalis* in the wetland influent was 270 cysts/L; and 650, 529, and 361 cysts/L (mean, 513 cysts/L) in three wetland effluent samples (Fig. 1). Genetic analysis revealed that the cysts in the wetland influent sample represented Assemblage A; whereas Assemblages A, C, and E were detected in three wetland outfall samples.

Of all four microsporidian species tested, only *E. bienersi* DNA was amplified with the 18SrRNA PCR and identified by multiplexed FISH method. The spores were present in a single wetland influent sample at a concentration of 10 spores/L and in two of three wetland effluent samples at concentration of 135 and 236 per liter (mean, 185 spores/L; Fig. 1). The genotypes of *E. bienersi* spores in all three spore-positive samples were the same and represented ITS sequences that were identical to genotype K (also identified as genotypes PtEb IX, PtEb IV, and PtEb III). This genotype has been isolated from humans, cattle,



**Fig. 1** Concentration of potentially viable *G. duodenalis* cysts and *E. bienersi* spores identified by the fluorescence in situ hybridization method in influent and effluent samples collected from constructed, small-scale, free-surface flow wetland used for tertiary treatment of municipal wastewater

cats, and dogs and is represented in GenBank entries AF267141, AF242478, AY371277, AY331009, DQ836343, and DQ885579.

Neither *C. parvum* nor *C. hominis* oocysts were detected by FISH, PCR, or nested PCR in the influent or effluent samples.

All pathogens cells detected by the FISH assays were potentially viable.

The mean concentration of combined transmissible stages (i.e., *G. duodenalis* cysts and *E. bienersi* spores; mean, 382/L) identified in wetland effluent samples was statistically significantly higher than the mean of pathogen number in the wetland influent samples (mean, 185/L; Wilcoxon signed rank test;  $t=4.6$ ,  $P < 0.03$ ). Also, the total number of pathogens identified in the wetland effluent (1,911 cysts and spores) was statistically higher than that detected in the wetland influent samples (280 cysts and spores; chi-square test;  $\chi^2=4.21$ ,  $P < 0.03$ ).

Unusually high rainfall (i.e., 123.5 mm) was reported for the month of sample collection as compared for 2 months preceding sampling (i.e., 65.4 and 70.9 mm, respectively).

## Discussion

The results of the present study demonstrate that: (1) the concentration of *G. duodenalis* cysts and *E. bienersi* spores in wetland effluents was significantly higher when compared to the influent; (2) in addition to *G. duodenalis* Assemblage A cysts detected in both wetland influent and effluent samples, other human nonvirulent Assemblages C and E were identified in wetland effluent samples; and (3) all human enteropathogens discharged to Lake Meelagh were potentially viable. Thus, it is most likely the secondary treated wastewater contained more human-infective enteropathogens

than the tertiary treated wastewater (Fig. 1). The presence of a higher pathogen concentration in tertiary treated, wetland-polished wastewater than in secondary treated wastewater can be explained by the facts that *G. duodenalis* and *E. bieneusi* were: (1) propagated in the wetland by dogs and livestock; or (2) contributed to the wetland by visiting wildlife. This conclusion is supported by the genetically different cyst isolates found in wetland effluent vs. influent samples. There are five morphologically distinct species representing *Giardia* genus: *G. duodenalis* (syn. *Giardia lamblia*, *Giardia intestinalis*; mammals), *Giardia muris* (rodents), *Giardia ardea* and *Giardia psittaci* (birds), and *Giardia agilis* (amphibians) (Thompson 2004; Thompson and Monis 2004). Molecular epidemiology revealed genetic diversity in *G. duodenalis*; mammalian isolates with zoonotic potential belong to one out of two assemblages (Thompson 2004; Thompson and Monis 2004). These assemblages are distributed worldwide and are referred in Europe as “Polish” and “Belgian,” in North America as groups 1 and 2, and in Australia as assemblages A and B (Thompson 2004; Thompson and Monis 2004). Assemblage A most commonly infects humans and also can also be found in other mammals. Assemblages C and E infect animals and not humans (Thompson 2004; Thompson and Monis 2004), and finding these assemblages in wetland effluent clearly demonstrates the involvement of dogs and livestock.

Wildlife that inhabit or visit constructed wetlands were previously demonstrated to significantly contribute fecal coliforms (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) to wetlands (Thurston et al. 2001). It has been suggested that wildlife plays an important role in the elevation of total and fecal coliform counts in wetland effluents due to their fecal deposition (Thurston et al. 2001) and the spontaneous multiplication of wildlife-derived coliforms in wetlands during warm summer months (Geldreich 1996). We conclude that, in addition to fecal coliform bacteria, dogs and livestock can also substantially contribute human-pathogenic protozoan and fungal microorganisms to these constructed wetland systems.

The results of the present study are in contrast to some existing data. Horizontal subsurface flow-engineered wetland has been shown to remove *Cryptosporidium* and *Giardia* from wastewater with efficiencies of 64.2% and 87.8% (Thurston et al. 2001); 100% and 100% (Quinonez-Diaz et al. 2001), 99.9% (Karpiscek et al. 2001), 44.3% and 98.6% (Nokes et al. 2003), 1.5–2.5 and 1.5–2.5 log removal (Ulrich et al. 2005), and 98.9% and 97.69% (Reinoso et al. 2008), respectively. Removal efficiency of *Cryptosporidium* and *Giardia* was lower for a free-surface flow wetland, 47.8 and 63.1%, (Reinoso et al. 2008), respectively. However, none of the aforementioned studies genetically characterized the pathogenic protozoa, leaving some doubt whether these contaminants truly originated

from the wastewater or from wildlife. Also in terms of public health, none of the aforementioned studies assessed the viability or infectivity of pathogens in wetland effluents to indicate their epidemiological importance. These significant omissions in sewage treatment wetland research were due to the lack of available molecular technology in the past (Graczyk and Lucy 2007).

There are several possibilities in the present study why the levels of *Giardia* cysts and microsporidian spores in the effluent were high and considerably higher than in the influent samples. This wetland was a free-surface flow system located in a densely wooded area, without implemented means to prevent animal access. Robust wetland vegetation (i.e., common reed (*Phragmites australis*) and tall trees around the wetland reduced exposure to sunlight and prevented heating and full exposure to UV light. Intensive precipitation diluted microorganism concentration and potentially caused: (1) inflow of runoff water to the wetland from wetland banks inhabited by rodents; or (2) surface runoff from other sources. Potential malfunctioning caused by clogged inlet pipe(s) could cause temporal hydraulic short circuits that bypass part of the wetland filtration area and consequently result in reduction or collapse of removal performances. These factors have been showed to extend survival of human pathogens in wetlands and to significantly lower wetland performance in microorganism removal (Quinonez-Diaz et al. 2001; Ulrich et al. 2005). Irrespective of the causative mechanism, we conclude that free-surface flow small-scale constructed wetlands may not always provide sufficient remediation for human enteropathogens present in primary or secondary treated wastewater, although such systems are excellent in absorbing, removal, and storage of nitrogen and phosphorus from the wastewater (Kadlec 2005; Zhang et al. 2008). We further conclude that the large volume of effluents discharged by free-surface flow wetlands can contribute to contamination of surface waters and thereby represent a serious public health threat. As an example, one of this study’s participants contracted gastric symptoms after brief contact with Lough Meelagh water in the vicinity of wetland effluent discharges.

Performance of constructed wetlands in removing microorganisms was previously concluded to be superior to that of conventional activated sludge processes (Ulrich et al. 2005). However, this is not supported by new data. The previous study demonstrated a significant reduction (i.e., on average 88%) of *C. parvum* oocysts, *G. duodenalis* cysts, and *E. intestinalis* and *E. bieneusi* spores associated with sewage sludge activation at small-scale wastewater plants (Graczyk et al. 2007a).

The lack of detection of nonviable pathogens in the present study indicates that, when the pathogen walls become permeable to compounds and microorganisms

present in large quantities in wastewater, they undergo fast biodegradation. Such a phenomenon was observed previously for human-pathogenic protozoa during the sewage sludge activation process (Graczyk et al. 2007b, 2008). Loss of pathogen viability in constructed wetland was attributed to the lytic action of bacteria and bacteriophages, oxidation reactions, adsorption, and exposure to plant and microbial toxins (Thurston et al. 2001).

Improving reclaimed water quality by lowering fecal coliforms is not a sound solution for human protozoan parasites (Graczyk and Lucy 2007), as multiple studies have shown the inadequacy of standard fecal coliforms (i.e., *E. coli*, enterococci, fecal and total coliforms) as indicators for contamination of wastewater with pathogenic protozoa (Rimhanen-Finne et al. 2004). This reinforces a need for better water quality indicators or, alternatively, for testing constructed wetlands which discharge to drinking or recreational water reservoirs for *Cryptosporidium*, *Giardia*, and human-virulent microsporidia. Source-tracking of fecal coliform indicators does not offer a satisfactory solution to the safety of reclaimed waters (Graczyk and Lucy 2007); however, *Clostridium perfringens* spores offer some hope as an alternative water quality indicator for wetland-polished wastewaters (Karpiscak et al. 2001; Ulrich et al. 2005). The provision of safe and high microbiological quality reclaimed waters through constructed wetland systems should be an outcome of partnership between wastewater engineering, environmental health and epidemiological sciences, and research-sponsoring institutions and reinforced by the relevant regulatory agencies.

**Acknowledgments** The study was supported by the 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 US Environmental Protection Agency Science to Achieve Results (STAR) Program (grant no. RD83300201). The views expressed herein have not been subjected to the US EPA review and therefore do not necessarily reflect the views of the agency, and no official endorsement should be inferred. We acknowledge Roscommon County Council for access and samples from sewage treatment plant.

## References

- Dai X, Boll J (2006) Settling velocity of *Cryptosporidium parvum* and *Giardia lamblia*. *Water Res* 40:1321–1325
- daSilva AJ, Slemenda SB, Visvesvara GS, Schwartz DA, Wilcox CM, Wallace S, Pieniazek NJ (1997) Detection of *Septata intestinalis* (Microsporidia) Cali et al. 1993 using polymerase chain reaction primers targeting the small subunit rRNA coding region. *Mol Diagn* 2:47–52
- daSilva AJ, Schwartz DA, Visvesvara GS, de Moura H, Slemenda SB, Pieniazek NJ (1996) Sensitive PCR diagnosis of infections by *Enterocytozoon bienersi* (Microsporidia) using primers based on the region coding for small subunit rRNA. *J Clin Microbiol* 34:986–987
- daSilva AJ, Bornay-Llinares FJ, Moura INS, Slemenda SB, Tuttle TL, Pieniazek NJ (1999) Fast and reliable extraction of protozoan parasite DNA from fecal specimens. *Mol Diagn* 4:57–63
- Davison L, Headley T, Pratt K (2005) Secondary treatment by reed-bed—eight year experience in northeastern New South Wales. *Water Sci Technol* 51:129–138
- de Groote MA, Visvesvara GS, Wilson ML, Pieniazek NJ, Slemenda SB, daSilva AJ, Leitch GJ, Bryan RT, Reeves R (1995) Polymerase chain reaction and culture confirmation of disseminated *Encephalitozoon cuniculi* in patient with AIDS: successful therapy with albendazole. *J Infect Dis* 171:1375–1378
- Dorsch MR, Veal DA (2001) Oligonucleotide probes for specific detection of *Giardia lamblia* cysts by fluorescent *in situ* hybridization. *J Appl Microbiol* 90:836–842
- Downey AS, Graczyk TK (2007) Maximizing recovery and detection of *Cryptosporidium parvum* oocysts from spiked Eastern oysters (*Crassostrea virginica*). *Appl Environ Microbiol* 73:6910–6915
- Geldreich EE (1996) Microbial quality of water supply in distribution systems. Lewis, New York
- Gerba CP, Thurston JA, Falabi JA, Watt PM, Karpiscak MM (1999) Optimization of artificial wetland design for the removal of indicator microorganisms and pathogenic protozoa. *Water Sci Technol* 40:363–368
- Graczyk TK, Lucy FE (2007) Quality of reclaimed waters; a public health need for source-tracking of wastewater-derived protozoan enteropathogens in engineered wetlands. *Trans Royal Soc Trop Med Hyg* 101:532–533
- Graczyk TK, Fayer R, Cranfield MR (1997) Zoonotic potential of *Cryptosporidium parvum*: implications for waterborne cryptosporidiosis. *Parasitol Today* 13:348–351
- Graczyk TK, Lucy FE, Tamang L, Mirafior A (2007a) Human enteropathogen load in activated sewage sludge and corresponding sewage sludge-end products. *Appl Environ Microbiol* 73:2013–2015
- Graczyk TK, Kacprzak M, Neczaj E, Tamang L, Graczyk H, Lucy FE, Girouard AS (2007b) Human-virulent microsporidian spores in solid waste landfill leachate and sewage sludge samples and comparative analysis of effects of various sanitization treatments on their inactivation. *Parasitol Res* 100:569–575
- Graczyk TK, Kacprzak M, Neczaj E, Tamang L, Graczyk H, Lucy FE, Girouard AS (2008) Occurrence of *Cryptosporidium* and *Giardia* in sewage sludge and solid waste landfill leachate and quantitative comparative analysis of sanitization treatments on pathogen inactivation. *Environ Res* 106:27–33
- Hester FD, Linquist HDA, Bobst AM, Schaffer FW (2000) Fluorescent *in situ* detection of *Encephalitozoon hellem* spores with a 6-carboxyfluorescein-labeled ribosomal RNA targeted oligonucleotide probe. *J Eukaryot Microbiol* 47:299–308
- Kadlec RH (2005) Phosphorus removal in emergent free surface wetlands. *J Environ Sci Health A Toxic Hazard Subst Environ Eng* 40:1293–1306
- Karim MR, Manshadi FD, Karpiscak MM, Gerba CP (2004) The persistence and removal of enteric pathogens in constructed wetlands. *Water Res* 38:1831–1837
- Karpiscak MM, Sanchez LR, Freitas RJ, Gerba CP (2001) Removal of bacterial indicators and pathogens from dairy wastewater by a multi-component treatment system. *Water Sci Technol* 44:183–190
- Katzwinkler-Wladarsch S, Lieb M, Helse W, Loscher T, Rinder H (1996) Direct amplification and species determination of microsporidian DNA from stool specimens. *Trop Med Int Health* 1:373–378
- Leonhard S, Pfister K, Beelitz P, Wielinga C, Thompson RCA (2007) The molecular characterisation of *Giardia* from dogs in Southern Germany. *Vet Parasitol* 150:33–38
- Matchis A, Weber R, Deplazes P (2005) Zoonotic potential of microsporidia. *Clin Microbiol Rev* 18:423–445

- Nokes RL, Gerba CP, Karpiscak MM (2003) Microbial water quality improvement by small scale on-site subsurface wetland treatment. *J Environ Sci Health A Toxic Hazard Subst Environ Eng* 38:1849–55
- Nwachuku N, Gerba CP (2004) Emerging waterborne pathogens: Can we kill them all? *Curr Opin Biotechnol* 15:175–180
- Quinonez-Diaz MJ, Karpiscak MM, Ellman ED, Gerba CP (2001) Removal of pathogenic and indicator microorganisms by a constructed wetland receiving untreated domestic wastewater. *J Environ Sci Health A Toxic Hazard Subst Environ Eng* 36:1311–1320
- Reinoso R, Torres LA, Becares E (2008) Efficiency of natural systems for removal of bacteria and pathogenic parasites from wastewater. *Sci Total Environ* 395:80–86
- Rimhanen-Finne R, Vourinen A, Marmo S, Malmberg S, Hanninen ML (2004) Comparative analysis of *Cryptosporidium* and *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. *Lett Appl Microbiol* 38:301–305
- Savioli L, Smith H, Thompson A (2006) *Giardia* and *Cryptosporidium* join the “Neglected Disease Initiative”. *Trends Parasitol* 22:203–208
- Stott R, May E, Matsushita E, Warren A (2001) Protozoan predation as a mechanism for the removal of *Cryptosporidium* oocysts from wastewaters in constructed wetlands. *Water Sci Technol* 44:191–8
- Thompson RCA (2004) The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. *Vet Parasitol* 126:15–35
- Thompson RCA, Monis PT (2004) Variation in *Giardia*: Implications for taxonomy and epidemiology. *Adv Parasitol* 58:69–137
- Thurston JA, Gerba CP, Foster KE, Karpiscak MM (2001) Fate of indicator microorganisms, *Giardia* and *Cryptosporidium* in subsurface flow constructed wetlands. *Water Res* 35:1547–1551
- Ulrich H, Klaus D, Irmgard F, Annette H, Juan LP, Regine S (2005) Microbiological investigations for sanitary assessment of wastewater treated in constructed wetlands. *Water Res* 39:4849–4858
- Vesey G, Ashbolt N, Fricker EJ, Deere D, William KL, Veal DA, Dorsch M (1998) The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labeling of viable *Cryptosporidium parvum* oocysts. *J Appl Microbiol* 85:429–440
- Visvesvara GS, daSilva AJ, Croppo GP, Pieniazek NJ, Leitch GJ, Ferguson D, Moura H, Wallace S, Slemenda SB, Tyrrell I (1995) *In vitro* culture and serologic and molecular identification of *Septata intestinalis* isolated from urine of a patient with AIDS. *J Clin Microbiol* 33:930–936
- Weber R, Bryan RT (1994) Microsporidial infections in immunodeficient and immunocompetent patients. *Clin Inf Dis* 19:517–521
- Wolfe MS (1992) Giardiasis. *Clin Microbiol Rev* 5:93–100
- Zhang Z, Rengel Z, Meney K (2008) Interactive effects of nitrogen and phosphorus loadings on nutrient removal from simulated wastewater using *Schoenoplectus validus* in wetland microcosm. *Chemosphere* 72:1823–182