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Improving the Efficacy of Wastewater-Polishing Reed Beds

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IMPROVING THE EFFICACY OF WASTEWATER-POLISHING REED BEDS

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1.0 Background

Microbiological Contaminants in Treated Wastewater

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 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*, *E. hellem, E. cuniculi*, and *Enterocytozoon bieneusi*) are waterborne enteropathogens inflicting considerable morbidity in healthy people and mortality (e.g., *Cryptosporidium* and microspora) in immunodeficient individuals (Savioli, et al., 2006; Weber and Bryan, 1994). 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 (Graczyk, et al., 1997; Matchis, et al., 2005; Wolfe,1992). These pathogens are category B biodefense agents on the NIH list. Microsporidian spores are on Contaminant Candidate List of the U.S. EPA (Nwachcuku and Gerba, 2004) because spore identification, removal, and inactivation in drinking water are technologically challenging. Surface water is not routinely monitored for these pathogens, despite evidence demonstrating environmental contamination derived from wastewater discharges (Graczyk and Lucy, 2007). Environmentally, all these pathogens have a broad zoonotic reservoir (Graczyk et al., 1997; Matchis et al., 2005; Savioli, et al., 2006).

Constructed Wetland Concept

Constructed wetlands of either vertical or horizontal flow are increasingly used worldwide for secondary or tertiary treatment of municipal wastewater due to minimum electric requirements and low maintenance costs (Davidson et al., 2005; Reinoso et al., 2008). The wetland concept has become an attractive wastewater treatment alternative to conventional tertiary treatment processes for: a) municipal wastewater; b) on-site domestic wastewater treatment; and c) concentrated animal feeding operations (CAFO) (Karpiscak et al., 2001). In wetlands, human-pathogenic microorganisms are physically removed and biodegraded by sedimentation (Dai and Boll, 2006; Karim et al., 2004), filtration and evapotranspiration-driven attachment to plant roots (Gerba et al., 1999; Dorsch and Veal, 2001; Weaver et al., 2003), natural die-off (Nokes et al., 2003), UV radiation, straining and sorption by the biofilm (Quinonez-Diaz et al., 2001), and protozoan predation (Stott et al., 2001). It is thought that performance of wetlands in removing human pathogens is superior to that of secondary wastewater treatment, i.e., conventional sewage sludge activation (Ulrich et al., 2005). Horizontal wetlands usually discharge to surface waters that are frequently used for recreation or drinking water production (Davidson et al., 2005).

Constructed Wetland Operation

In general, wastewater can be injected under the wetland surface for plug flow hydraulics (Weaver et al., 2003), or be delivered to the wetland surface for free-surface flow. Because the wastewater resides in wetlands for certain time, these areas can act as endemic sites supporting both propagation and transmission of human zoonotic pathogens (Graczyk et al., 2007). Sizing

reed-bed systems for a residence time of 5 days has become a standard practice (Davidson et al., 2005; Quinonez-Diaz et al., 2001; Thurston et al., 2001), 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., 2007). In addition, any temporal or permanent malfunctioning caused by clogged inlet pipes can cause hydraulic short circuits that bypass part of the filtration area in wetlands.

Aims of the Project

The purposes of the project were to: a) determine species of microbiological contaminants entering, residing, and leaving constructed horizontal wetlands used for tertiary treatment of municipal wastewater; and b) determine removal efficacy of *Cryptosporidium* oocysts, *G. duodenalis* cysts, and human-virulent microsporidian spore species by wetlands from secondary-treated wastewater.

2.0 Methodology

Selection of Wetlands

Samples originated from four constructed horizontal wetlands, i.e., Wetland A (53°40'41"N, 08°34'24"W), Wetland B (53°03'12"N, 08°08'57"W), Wetland C (54°04'07"N, 08°12'12"W), and Wetland D (53°41'11"N, 08°45'17"W). All wetlands received unchlorinated secondary treated municipal wastewater after sewage sludge activation and secondary sedimentation. All wetlands were small scale wetlands discharging to surface waters. All wetlands were multispecies systems with both emergent and submerged plants; overwhelmed by the Common reed, *P. australis.* The inflow, outflow and vegetation densities were similar at all wetlands, and the influent and effluent flow rates were relatively constant.

Sample Collection

Two grab samples (2 L) of both wetland influents and effluents were collected in addition to two samples from the wetland longitudinal transect in regular intervals. Samples were collected from August through December 2009 (Table, 1, 2, 3, 4, and 5). Samples were transported to the laboratory in a cooler and processed by gravity sedimentation (Graczyk et al., 2007). Briefly, samples were vortexed, transferred to 1-L-capacity Imhoff settlement cones and left overnight at 4°C. Fifty ml of the top sediment layer were transferred to a plastic 50-ml tube and centrifuged (3,000*g*, 10 min). The supernatant was discarded and the pellet transferred to a 1.5-ml tube and preserved with 75 % ethanol. The recovery efficacy of human waterborne pathogens from wastewater matrices was determined previously to be approximately 77% (Graczyk et al., 2007).

Sample Processing

The ethanol was washed using phosphate buffered saline (PBS) (pH 7.4), centrifugation (5,000*g*, 10 min) and the pellet purified by sugar floatation; 2.5M sucrose solution with a specific gravity of 1.34 was used (Kahle and Thurston-Enriquez, 2007). The resulting pellet was divided evenly into two aliquots. The first was processed for *C. parvum* and *G. duodenalis* by multiplexed fluorescence *in situ* hybridization (FISH) in combination with IFA, and the second

for human-virulent microsporidia (i.e., *E. intestinalis*, *E. hellem, E. cuniculi*, and *E. bieneusi*) by multiplexed FISH (Graczyk, 2007).

3.0 Results

Table 1. Results for Samples Collected in August 2009 and Processed by the Fluorescence *In Situ* Hybridization Assay.

Wetland	Sample ID	*Cryptosporidium parvum* oocysts/L	*Giardia duodenalis* cysts/L	Microsporidian (*Encephalitozoon bieneusi*) spores/L
A	Influent	12	2	2
	Wetland transect 1	3	2	4
	Wetland transect 2	0	1	2
	Final effluent	5	0	0
B	Influent	0	0	3
	Wetland transect 1	3	4	0
	Wetland transect 2	1	2	0
	Final effluent	9	1	2
C	Influent	4	0	1
	Wetland transect 1	2	3	0
	Wetland transect 2	0	1	0
	Final effluent	8	0	0
D	Influent	1	1	0
	Wetland transect 1	0	1	0
	Wetland transect 2	0	0	0
	Final effluent	2	3	3

Table 2. Results for Samples Collected in September 2009 and Processed by the Fluorescence *In Situ* Hybridization Assay.

Wetland	Sample ID	*Cryptosporidium parvum* oocysts/L	*Giardia duodenalis* cysts/L	Microsporidian (*Encephalitozoon bieneusi*) spores/L
A	Influent	1	2	2
	Wetland transect 1	3	0	3
	Wetland transect 2	2	0	2
	Final effluent	4	2	1
B	Influent	0	0	3
	Wetland transect 1	3	4	2
	Wetland transect 2	1	7	5
	Final effluent	2	6	0
C	Influent	0	0	1
	Wetland transect 1	2	3	0
	Wetland transect 2	1	2	0
	Final effluent	7	0	0
D	Influent	1	1	0
	Wetland transect 1	0	1	3
	Wetland transect 2	0	4	2
	Final effluent	4	6	1

Table 3. Results for Samples Collected in October 2009 and Processed by the Fluorescence *In Situ* Hybridization Assay.

Wetland	Sample ID	*Cryptosporidium parvum* oocysts/L	*Giardia duodenalis* cysts/L	Microsporidian (*Encephalitozoon bieneusi*) spores/L
A	Influent	1	3	3
	Wetland transect 1	0	0	0
	Wetland transect 2	2	0	0
	Final effluent	0	0	1
B	Influent	4	0	1
	Wetland transect 1	1	0	4
	Wetland transect 2	1		5
	Final effluent	3	0	0
C	Influent	0	0	0
	Wetland transect 1	2	3	0
	Wetland transect 2	0	0	0
	Final effluent	2	2	0
D	Influent	1	1	0
	Wetland transect 1	0	1	3
	Wetland transect 2	3	4	2
	Final effluent	4	6	1

Table 4. Results for Samples Collected in November 2009 and Processed by the Fluorescence *In Situ* Hybridization Assay.

Wetland	Sample ID	*Cryptosporidium parvum* oocysts/L	*Giardia duodenalis* cysts/L	Microsporidian (*Encephalitozoon bieneusi*) spores/L
A	Influent	7	4	7
	Wetland transect 1	3	0	3
	Wetland transect 2	2	0	2
	Final effluent	0	0	0
B	Influent	0	0	3
	Wetland transect 1	3	2	2
	Wetland transect 2	1	5	3
	Final effluent	0	6	0
C	Influent	0	0	1
	Wetland transect 1	2	3	0
	Wetland transect 2	1	2	2
	Final effluent	3	0	0
D	Influent	1	1	0
	Wetland transect 1	0	1	3
	Wetland transect 2	0	2	2
	Final effluent	3	4	1

Table 5. Results for Samples Collected in December 2009 and Processed by the Fluorescence *In Situ* Hybridization Assay.

Wetland	Sample ID	*Cryptosporidium parvum* oocysts/L	*Giardia duodenalis* cysts/L	Microsporidian (*Encephalitozoon bieneusi*) spores/L
A	Influent	7	6	2
	Wetland transect 1	3	0	5
	Wetland transect 2	2	0	2
	Final effluent	1	0	0
B	Influent	0	0	3
	Wetland transect 1	3	4	2
	Wetland transect 2	1	5	2
	Final effluent	1	6	0
C	Influent	0	0	1
	Wetland transect 1	2	3	0
	Wetland transect 2	1	2	0
	Final effluent	3	0	0
D	Influent	1	1	0
	Wetland transect 1	0	1	3
	Wetland transect 2	0	4	1
	Final effluent	4	3	1

Overall, three species of human protozoan enteropathogens were detected, i.e., *C. parvum, G. duodenalis, and E. bieneusi*. Most pathogens detected by the FISH assays were viable; a fraction of non-viable cells represented less than 2%. Viable *G. duodenalsi* cysts vs. nonviable cysts were clearly differentiated by color as a result of FISH and mAb labeling. Nonviable cysts were represented by: a) shells with apparently structurally damaged walls; and b) intact cells with a very small amount of internal structures with diffused appearance. In comparison, potentially viable intact cysts were filled out completely with cytoplasm without the gap between the internal structures and the wall. *Cryptosporidium parvum* oocysts labeled by FISH and mAb were predominantly intact, revealed 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.

Analysis of samples collected in August demonstrated that in 5 of 12 (42%) sample sets, the number of pathogens found in the final effluent was lower than in the influent; and in 6 of 12 (50%) samples sets the number of pathogens the final effluent was higher than in the influent.

Analysis of samples collected in September demonstrated that in 3 of 12 (25%) sample sets, the number of pathogens identified in the final effluent was lower than in the influent; and in 7 of 12 (58%) sample sets, the number of pathogens in the final effluent was higher than in the influent. Analysis of samples collected in October demonstrated that in 6 of 12 (50%) sample sets, the number of pathogens identified in the final effluent was lower than in the influent; and in 5 of 12 (42%) sample sets, the number of pathogen in the final effluent was higher than in the influent. Analysis of samples collected in November demonstrated that in 6 of 12 (50%) sample sets, the number of pathogens identified in the final effluent was lower than in the influent; and in 5 of 12 (42%) sample sets, the number of pathogens in the final effluent was higher than in the influent. Analysis of samples collected in December demonstrated that in 5 of 12 (42%) sample sets, the number of pathogens identified in the final effluent was lower than in the influent; and in 6 of 12 (50%) sample sets, the number of pathogens in the final effluent was higher than in the influent.

Overall, 42% of sample sets showed decrease of pathogens in the final effluent; 48% of sample sets showed increase of pathogens in the final effluent; and 10% of sample set showed even number of pathogens in influent as comared to the final effluent.

4.0 Conclusions

The project demonstrated that: a) composition of human pathogen species in secondary treated wastewater entering constructed wetlands and in tertiary treated wastewater is highly complex and dynamic; b) small-scale constructed wetlands may not provide sufficient remediation for human zoonotic protozoan pathogens; and c) most of the pathogens discharged by wetlands to surface waters were viable thus potentially capable of causing human infections.

Presence of pathogens at higher concentrations in wetland-polished wastewater than in influents may be explained by the fact that these pathogens were: a) propagated in the wetlands by residing wildlife; b) contributed to the wetland water by visiting wildlife; or c) originated from other sources, e.g., surface runoff from wetland banks utilized by rodents as habitats. Aquatic birds and mammalian wildlife that inhabit wetlands can disseminate human-virulent species of *Cryptosporidium*, *Giardia*, and microsporidia, i.e., *E. hellem* and *E. bieneusi* (Graczyk et al., 2007; Graczyk et al., 2008; Slodkowicz-Kowalska et al., 2007; Sulaiman et al., 2003). It has been estimated that a single visitation of an average size waterfowl flock can introduce into surface water reservoirs approximately: a) 9.3×10^6 *C. parvum* oocysts; b) 1.1×10^7 *G. duodenalis* cysts; and c) 9.1×10^8 *E. hellem* spores (Graczyk et al., 2008)

Wildlife that inhabits or visit constructed wetlands has previously been demonstrated to significantly contribute fecal coliforms (e.g., *Escherichia coli* and *Klebsiella pneumonia*) to wetlands (Thurston et al., 2001). It has been suggested that wildlife plays an important role in the elevation of total and fecal coliform levels in wetland effluents due to their fecal deposition (Thurston et al., 2001), and the spontaneous multiplication of wildlife-derived coliforms in wetlands during summer months (Geldreich,1996). The pathogens identified in the present study cannot multiply in the environment without their hosts.

There are several possibilities why the levels of *Cryptosporidium* oocysts, *Giardia* cysts and microsporidian spores in the wetland outfalls were higher than in the influents. All wetlands operated without implemented means to prevent animal access. Vegetation density in constructed wetlands has been shown not to influence the removal rates of *Cryptosporidium* oocysts and *Giardia* cysts (Nokes et al., 2003.). However, robust vegetation (i.e., *P. australis*) and tall trees around the wetland reduced exposure to sunlight, and prevented heating and full exposure to UV light. In all wetlands, precipitation potentially caused: a) inflow of runoff water to the wetland from wetland banks inhabited by rodents; and b) 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 consequently resulting in reduction or collapse of removal performances (Quinonez-Diaz et al., 2005). Concentration of human pathogens in wetland samples may also show diurnal fluctuation. Irrespective of the causative mechanism, we conclude that small-scale constructed wetlands may not 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).

The minimal levels of non-viable pathogens in the present study indicate that the pathogen walls become permeable to compounds and microorganisms present in large quantities in wastewater and they undergo fast biodegradation. Such a phenomenon was observed previously for human-pathogenic microorganisms in wastewater matrices (Graczyk et al., 2007; Graczyk et al., 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).

Because *Cryptosporidium*, *Giardia*, and microsporidia can infect a variety of non-human hosts, identification of human-pathogenic species represents a challenge. Another challenge is determination of viability of these pathogens as they may be non-viable and thus, not of epidemiological importance. Both challenges are addressed by the fluorescence *in situ* hybridization (FISH) technique used in the present study. 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., 2007). As rRNA has a short half-life and is only present in numerous copies in viable organisms, FISH allows for differentiation between viable and non-viable pathogens (Dorsch et al., 2000; Vesey et al., 1998).

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West Palm Beach, City of

Georgia
Atlanta Department of Watershed Management
Augusta, City of
Clayton County Water Authority
Cobb County Water System
Columbus Water Works
Fulton County
Gwinnett County Department of Public Utilities
Savannah, City of

Hawaii
Honolulu, City & County of

Idaho
Boise, City of

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Decatur, Sanitary District of
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Kankakee River Metropolitan Agency
Metropolitan Water Reclamation District of Greater Chicago
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Maryland
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Massachusetts
Boston Water & Sewer Commission
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Michigan
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Saginaw, City of
Wayne County Department of Environment
Wyoming, City of

Minnesota
Rochester, City of
Western Lake Superior Sanitary District

Missouri
Independence, City of
Kansas City Missouri Water Services Department
Little Blue Valley Sewer District
Metropolitan St. Louis Sewer District

Nebraska
Lincoln Wastewater & Solid Waste System

Nevada
Henderson, City of
Las Vegas, City of
Reno, City of

New Jersey
Bergen County Utilities Authority
Ocean County Utilities Authority

New York
New York City Department of Environmental Protection

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Ohio
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Columbus, City of
Metropolitan Sewer District of Greater Cincinnati
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Northeast Ohio Regional Sewer District
Summit, County of

Oklahoma
Oklahoma City Water & Wastewater Utility Department
Tulsa, City of

Oregon
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Clean Water Services
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Gresham, City of
Portland, City of Bureau of Environmental Services
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Prince William County Service Authority
Richmond, City of
Rivanna Water & Sewer Authority

Washington
Everett, City of
King County Department of Natural Resources
Seattle Public Utilities
Sunnyside, Port of
Yakima, City of

Wisconsin
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Kenosha Water Utility
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STORMWATER UTILITY

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Colorado
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