Applied and Environmental Microbiology, Mar. 2007, p. 2013–2015 Vol. 73, No. 6
0099-2240/07/$08.00+0 doi:10.1128/AEM.02412-06

Human Enteropathogen Load in Activated Sewage Sludge and Corresponding Sewage Sludge End Products▽

Thaddeus K. Graczyk,[1,2]* Frances E. Lucy,[3] Leena Tamang,[1] and Allen Miraflor[4]

Department of Environmental Health Sciences, Division of Environmental Health Engineering, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205[1]; Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205[2]; School of Science, Institute of Technology, Sligo, Ireland[3]; and Johns Hopkins University, Homewood Campus, Baltimore, Maryland 21218[4]

Received 13 October 2006/Accepted 21 January 2007

This study demonstrated a significant reduction in the concentrations of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts, *Giardia lamblia* cysts, and spores of human-virulent microsporidia in dewatered and biologically stabilized sewage sludge cake end products compared to those of the respective pathogens in the corresponding samples collected during the sludge activation process.

Cryptosporidium parvum, *Cryptosporidium hominis*, *Giardia lamblia*, and human-virulent microsporidia (i.e., *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, and *Enterocytozoon bieneusi*) are enteric pathogens that inflict considerable morbidity in healthy people and can cause mortality (e.g., *Cryptosporidium*) in individuals with various immunodeficiencies (6, 18, 21). Their transmissive stages, i.e., oocysts, cysts, and spores, are highly resistant to environmental stressors, and therefore, they are long lasting and ubiquitous in the environment (3, 6, 12). It is believed that spreading sewage sludge on agricultural lands, which has increased during recent years due to economic and environmental reasons (13), facilitates the circulation of *Cryptosporidium* and *Giardia* in the environment and contaminates potable waters via surface runoff (15), thus posing a public health threat. However, other studies provided evidences that the sludge activation process significantly reduced (i.e., up to 99.8%) the level of *Giardia* compared to that of the raw sewage (2, 20). The purpose of the present study was to quantitatively determine and compare the concentrations of viable *Cryptosporidium parvum* and *C. hominis* oocysts, *G. lamblia* cysts, and spores of *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *E. bieneusi* in sewage sludge during the activation secondary treatment process and in the corresponding sewage sludge end products.

The samples originated from urban wastewater treatment plants: Collooney (54°11′37″N, 08°28′93″W), Strandhill (54°16′89″N, 08°35′95″W), Grange (54°23′53″N, 08°31′79″W), and Keadue (54°03′18″N, 08°09′12″W), Ireland. All plants utilized primary treatment by coarse screening. The Collooney, Strandhill, and Grange plants employed secondary treatment, i.e., sludge activation and sedimentation. The Keadue plant uses a rotating filter and settlement tank. The sewage effluent is then polished using a reedbed, wetland filtration system, with effluent discharging directly to Lake Meelagh. The activated sludge samples (2 liters) were collected directly from the top wastewater layer during the activation process. The corresponding dewatered biologically stabilized sewage sludge cake samples (1 kg) were collected from the top layer of the dewatering beds. At the Keadue wastewater plant, a dewatered biologically stabilized sewage sludge sample was collected as described above in addition to a sample of final wetland filtration effluent (2 liters). Dewatered sludge samples were homogenized with water to a volume of 2 liters. All collected samples were vigorously homogenized, transferred to conical plastic flasks of 2.5-liter capacity, and left overnight at 4°C for gravity sedimentation (1, 5, 16). Fifty milliliters of the top sediment layer was transferred to a plastic 50-ml-capacity tube and centrifuged (3,000 × *g*, 5 min), the supernatant was decanted, and the resulting pellet was transferred to a 1.5-ml-capacity plastic tube. The solution was mixed with an equal volume of 75% ethanol and stored at 4°C. Alcohol was washed from the pellets by centrifugation (10,000 × *g*, 10 min) two times using sterile phosphate-buffered saline, and the resulting pellet was subjected to sugar-phenol flotation (1). The pellet was evenly divided into two aliquots. One aliquot was processed for *C. parvum*, *C. hominis*, and *G. lamblia* by multiplexed combined fluorescent in situ hybridization and a direct immunofluorescence assay and the other for human-virulent microsporidia by multiplexed fluorescent in situ hybridization (7–11, 14, 17).

There was a significant reduction of pathogen concentration in dewatered and biologically stabilized sewage sludge cake compared to the concentrations of the respective pathogens in the corresponding sludge samples collected during the activation process (Table 1). *Giardia lamblia* was found at a significantly higher concentration in activated sewage sludge at Collooney, Strandhill, and Grange (mean, 843 cysts/liter) than *Cryptosporidium* (mean, 338 oocysts/liter) or microsporidia (mean, 166 spores/liter) ($F = 5.2$, $P < 0.05$ [Kruskal-Wallis analysis of variance]). However, the highest concentrations of *G. lamblia* (i.e., 1,540 cysts/liter) and *E. bieneusi* (371 spores/liter) were found in the final effluent from the wetland filtration system at Keadue (Table 1). The highest concentration of *Cryptosporidium* was 650 oocysts/liter in the activated sewage sludge, and the lowest was 43 oocysts/liter (Table 1). *Enterocytozoon bieneusi* was found in activated sewage sludge samples

* Corresponding author. Mailing address: Department of Environmental Health Sciences, Division of Environmental Health Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205. Phone: (410) 614-4984. Fax: (410) 955-0105. E-mail: tgraczyk@jhsph.edu.

▽ Published ahead of print on 2 February 2007.

TABLE 1. Effect of the sewage sludge activation process on the removal of viable human enteropathogens[a]

Wastewater plant	Pathogen species	Pathogen concn in:		Removal efficiency (%)
		AcSS	SSC	
Collooney	*Cryptosporidium parvum*/*C. hominis*	320	110	65.6
	Giardia lamblia	1,100	43	96.1
	Enterocytozoon bieneusi	245	13	94.7
	Encephalitozoon intestinalis	47	5	89.4
Strandhill	*Cryptosporidium parvum*/*C. hominis*	43	5	88.4
	Giardia lamblia	540	3	99.4
	Enterocytozoon bieneusi	31	5	83.9
Grange	*Cryptosporidium parvum*/*C. hominis*	650	64	90.2
	Giardia lamblia	890	114	87.2
	Enterocytozoon bieneusi	247	33	86.7
	Encephalitozoon intestinalis	54	3	94.4
Keadue	*Cryptosporidium parvum*/*C. hominis*	0*	0	
	Giardia lamblia	1,540*	5	
	Enterocytozoon bieneusi	371*	0	

[a] The concentrations of enteropathogens in activated sewage sludge (in cells/liter) during the activation process and in the corresponding sewage sludge cake samples (in cells/kg) were compared. AcSS, activated sewage sludge; SSC, sewage sludge cake; *, final effluent from wetland filtration treatment.

from all wastewater treatment plants, and the mean concentration (i.e., 224 spores/liter) was significantly higher than the concentration of *E. intestinalis* (i.e., 51 spores/liter) ($F = 13.7$, $P < 0.02$ [Kruskal-Wallis analysis of variance]) (Table 1). The overwhelming majority of oocysts, cysts, and spores identified in sewage sludge-derived samples were viable; the fraction of nonviable pathogens constituted no more than 1%.

The present study demonstrated a significant reduction of human enteropathogen load associated with the sewage sludge activation process. The concentration of human pathogens in dewatered and biologically stabilized sewage sludge cake was on average 88% lower than in the same wastewater-derived sludge during its activation process. This pattern was uniform throughout the wastewater treatment plants that utilized sludge activation treatment and for all pathogen groups.

The low level of nonviable enteropathogens indicates that when the pathogen walls become permeable to compounds and the large quantities of microorganisms present in wastewater and sewage sludge, they undergo fast biodegradation. In addition to this, the dewatering and biological stabilization of sewage sludge extend direct exposure of potential human pathogens to environmental stressors such as UV, desiccation, and temperature fluctuations, which, in addition to activation, impact their viability and survival.

Although the present study demonstrated high efficiency of pathogen removal and their relatively low concentration in sewage sludge end products, the detected pathogens were viable. Thus, given the massive amounts of sewage sludge end products utilized by agriculture or deposited in landfills (4, 15, 17, 19), the environmental robustness of these enteropathogens (3, 6, 10, 18), and their high virulence to humans, it should be considered that the environmental contamination derived from such activities presents a serious public health threat.

For the sewage sludge end products to be used without restriction on agricultural lands growing ready-to-eat crops, the pathogen load must be reduced to a level which does not pose an unacceptable public health risk (4, 19). Current risk assessment modeling for cryptosporidiosis and giardiasis acquired through consumption of root crops grown on land where treated sewage sludge end products have legally been applied predicts 50 and 1 disease case per year, respectively (4). Another study predicted up to 25% annual increases in the cryptosporidiosis rate in a community consuming products from agricultural lands where sewage sludge end products were legally applied (19).

This study was supported by the Fulbright Senior Specialist Fellowship (grant no. 2225 to T. K. Graczyk), the Johns Hopkins Center in Urban Environmental Health (grant no. P30 ES03819), the Procter & Gamble Foundation, the Johns Hopkins Center for a Livable Future, and the School of Science, Institute of Technology, Sligo, Ireland.

We acknowledge the Sligo County Council and Roscommon County Council for access to and samples from sewage treatment plants.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1987. Parasites: a guide to laboratory procedures and identification. ASCP Press, Chicago, IL.
2. **Cacciò, S. M., M. De Giacomo, F. A. Aulicino, and E. Pozio.** 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. **69:**3393–3398.
3. **deRegnier, D. P., L. Cole, D. G. Schupp, and S. L. Erlandsen.** 1989. Viability of *Giardia* cysts suspended in lake, river, and tap water. Appl. Environ. Microbiol. **55:**1223–1229.
4. **Gale, P.** 2005. Land application of treated sewage sludge: quantifying pathogen risk from consumption of crops. J. Appl. Microbiol. **98:**380–396.
5. **Graczyk, T. K., R. Owens, and M. R. Cranfield.** 1996. Diagnosis of subclinical cryptosporidiosis in captive snakes based on stomach lavage and cloacal samples. Vet. Parasitol. **67:**143–151.
6. **Graczyk, T. K., R. Fayer, and M. R. Cranfield.** 1997. Zoonotic potential of *Cryptosporidium parvum*: implications for waterborne cryptosporidiosis. Parasitol. Today **13:**348–351.
7. **Graczyk, T. K., J. Bosco-Nizeyi, A. J. DaSilva, I. N. S. Moura, N. J. Pieniazek, M. R. Cranfield, and H. D. A. Lindquist.** 2002. A single genotype of *Encephalitozoon intestinalis* infects free-ranging gorillas and people sharing their habitats in Uganda. Parasitol. Res. **88:**926–931.
8. **Graczyk, T. K., B. H. Grimes, R. Knight, A. J. DaSilva, N. J. Pieniazek, and D. A. Veal.** 2003. Combined FISH and mAb detection of *Cryptosporidium parvum* and *Giardia lamblia* carried by synanthropic flies. Am. J. Trop. Med. Hyg. **68:**228–232.
9. **Graczyk, T. K., D. B. Conn, F. Lucy, D. Minchin, L. Tamang, L. N. S. Moura, and A. J. DaSilva.** 2004. Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage, Ireland. Parasitol. Res. **93:**389–391.
10. **Graczyk, T. K., A. S. Girouard, L. Tamang, S. P. Nappier, and K. J. Schwab.** 2006. Recovery, bioaccumulation, and inactivation of human waterborne pathogens by the Chesapeake Bay nonnative oyster, *Crassostrea ariakensis*. Appl. Environ. Microbiol. **72:**3390–3395.
11. **Lemos, V., T. K. Graczyk, M. Aves, M. L. Lobo, C. S. Neto, M. C. Sousa, F. Antunes, and O. Matos.** 2005. Identification and determination of the viability of *Giardia lamblia* cysts, *Cryptosporidium parvum* and *C. hominis* oocysts in human fecal samples and water supply samples by fluorescent *in situ* hybridization and monoclonal antibodies. Parasitol. Res. **98:**48–53.
12. **Mathis, A., R. Weber, and P. Deplazes.** 2005. Zoonotic potential of microsporidia. Clin. Microbiol. Rev. **18:**423–445.
13. **Rimhanen-Finne, R., A. Vourinen, S. Marmo, S. Malmberg, and M. L. Hanninen.** 2004. Comparative analysis of *Cryptosporidium* and *Giardia* and indicator bacteria during sewage sludge hygienization in various composting processes. Lett. Appl. Microbiol. **38:**301–305.
14. **Slodkowicz-Kowalska, A., T. K. Graczyk, L. Tamang, S. Jedrzejewski, A. Nowosad, P. Zduniak, P. Solarczyk, A. S. Girouard, and A. C. Majewska.** 2006. Microsporidian species known to infect humans are present in aquatic birds: implications for transmission via water? Appl. Environ. Microbiol. **72:**4540–4544.
15. **Straub, T. M., I. L. Pepper, and C. P. Gerba.** 1993. Hazard from pathogenic microorganisms and land-disposed sewage sludge. Rev. Environ. Contam. Toxicol. **132:**55–91.

16. **Suwa, M., and Y. Suzuki.** 2001. Occurrence of *Cryptosporidium* in Japan and counter-measure in wastewater treatment plants. Water Sci. Technol. **43:** 183–186.
17. **Szostakowska, B., W. Kruminis-Lozowska, M. Racewicz, R. Knigh, L. Tamang, P. Myjak, and T. K. Graczyk.** 2004. *Cryptosporidium parvum* and *Giardia lamblia* recovered from flies on a cattle farm and in a landfill. Appl. Environ. Microbiol. **70:**3742–3744.
18. **Weber, R., and R. T. Bryan.** 1994. Microsporidial infections in immunodeficient and immunocompetent patients. Clin. Infect. Dis. **19:**517–521.
19. **Westrell, T., C. Schonning, T. A. Stenstrom, and N. J. Ashbolt.** 2004. QMRA (quantitative microbial risk assessment) and HACCP (hazard analysis and critical control points) for management of pathogens in wastewater and sewage sludge treatment and reuse. Water Sci. Technol. **50:**23–30.
20. **Wiandt, S., A. M. Grimason, B. Balez, and J. Bontoux.** 2000. Efficiency of wastewater treatment plants at removing *Giardia* sp. cysts in Southern France. Schriftenr. Ver. Wasser Boden Lufthyg. **105:**35–42.
21. **Wolfe, M. S.** 1992. Giardiasis. Clin. Microbiol. Rev. **5:**93–100.