MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH
Part A—Toxic/Hazardous Substances & Environmental Engineering
Vol. A38, No. 7, pp. 1259–1268, 2003

Removal of *Encephalitozoon intestinalis*, Calicivirus, and Coliphages by Conventional Drinking Water Treatment

Charles P. Gerba,[1,*] Kelley R. Riley,[1] Nena Nwachuku,[2] Hodon Ryu,[3] and Morteza Abbaszadegan[3]

[1]Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, Arizona, USA
[2]Office of Water, Office of Science and Technology, Environmental Protection Agency, Washington, DC, USA
[3]Department of Civil and Environmental Engineering, Arizona State University, Tempe, Arizona, USA

ABSTRACT

The removal of the Microsporidia, *Encephalitozoon intestinalis*, feline calicivirus and coliphages MS-2, PRD-1, and Fr were evaluated during conventional drinking water treatment in a pilot plant. The treatment consisted of coagulation, sedimentation, and mixed media filtration. Fr coliphage was removed the most (3.21 log), followed by feline calicivirus (3.05 log), *E. coli* (2.67 log), *E. intestinalis* (2.47 log), MS-2 (2.51 log), and PRD-1 (1.85 log). With the exception of PRD-1 the greatest removal of the viruses occurred during the flocculation step of the water treatment process.

Key Words: Encephalitozoon intestinalis; Microsporidia; Calicivirus; PRD-1; Fr; MS-2; Conventional water treatment; Filtration; Flocculation.

*Correspondence: Charles P. Gerba, Department of Soil, Water, and Environmental Science, University of Arizona, 1117 E. Lowell Street, Building 90, Room 217, Tucson, AZ, 85721, USA; E-mail: gerba@ag.arizona.edu.

DOI: 10.1081/ESE-120021124
1093-4529 (Print); 1532-4117 (Online)

INTRODUCTION

The Environmental Protection Agency (EPA) is required under the Safe Drinking Water Act (amended in 1996) to publish a list of unregulated contaminants that are known or expected to occur in public water systems that may pose a risk in drinking water. In 1998, the first of these lists was produced and is referred to as the Drinking Water Contaminant Candidate List, or CCL. The Drinking Water CCL includes 50 chemical and 10 microbiological contaminants. To assess whether current treatment rules were adequate to control the waterborne pathogens on this list, procedures for evaluating the treatability of these organisms were needed. The CCL included the Microsporidia and caliciviruses. Microsporidia are a group of protozoan parasites which primarily cause illness in AIDS patients[1] although they have been a cause of gastroenteritis in normal, healthy individuals visiting developing countries. Epidemiological evidence has indicated their potential transmission by drinking water[2] and recreational waters.[3] They produce an environmentally resistant spore 1–3 μm in diameter. Feline calicivirus, which is similar in structure to human calicivirus (i.e., Norwalk virus), does not infect humans[4] and can be grown in the laboratory to high titers. Human caliciviruses can not currently be grown in the laboratory. Norwalk virus is the calicivirus most commonly associated with waterborne disease.[5] There are a large number of caliciviruses which infect man and animals.

A conventional treatment plant involving coagulation, sedimentation, and filtration was used to evaluate the removal of the microsporidium *Encephalitozoon intestinalis* and a feline calicivirus. These microorganisms were selected because of the lack of data on their removal by conventional treatment and due to safety considerations. *Encephalitozoon intestinalis* removal was assessed with inactivated spores since the spores could be detected microscopically and eliminated a potential health risk during the operation of the pilot plant. For comparative purposes, the removal of coliphages MS-2, PRD-1, and Fr were also included in this phase of the study. All of these coliphages have different isoelectric points (Table 1) which could potentially affect their degree of removal. Finally, *Escherichia coli* removal was also assessed since it is similar in size to *E. intestinalis* and a commonly used indicator of water quality.

MATERIALS AND METHODS

Viruses

MS-2 coliphage (ATCC #15597-B1) was propagated by inoculating a one-liter flask containing 200 mL tryptic soy broth (TSB) with 2 mL of the host bacteria,

Table 1. Isoelectric points for bacteriophages.

Bacteriophage	Isoelectric point
MS-2	3.5
PRD-1	4.2
Fr	8.9–9.0

E. coli (ATCC #15597). The culture flask was placed in a shaking incubator maintained at 37°C. When the bacterial density reached approximately 1×10^8 colony forming units (cfu)/mL (previously determined), an aliquot of the virus stock was added. The culture was shaken continuously for 4 h and 0.02 g of lysozyme and 6 mL of sterile 0.2 M ethylenediaminetetraacetic acid (EDTA) were added to the culture to lyse any intact host cells and incubated for an additional 30 min. The suspension was centrifuged at $3300 \times g$ for 20 min and then passed through a 0.45 μm pore size sterile filter to remove cellular debris. The stock was titered by the agar overlay method[6] and stored at 4°C until needed.

PRD-1 and Fr bacteriophage were propagated in the same manner except *Salmonella typhimurium* (LT2) and *E. coli* ATCC #19853 were used as the host bacteria, respectively.

Feline calicivirus (FCV) was propagated and assayed in feline kidney cells (CRFK cells). CRFK cells were grown in 162 cm^2 tissue culture flasks until a monolayer was formed, which usually took within 5–7 days. The cell culture media was decanted and the cell monolayer was inoculated with approximately 1×10^6 $TCID_{50}$ (Tissue Culture Infective Dose) of FCV followed by incubation at 37°C for 30 min. After incubation, fresh cell culture media (Eagles minimum Essential Media) containing 10% FBS (fetal bovine serum) was placed on the cell monolayer and incubated at 37°C until complete destruction of the cell monolayer occurred, usually within 3 days. The flasks were frozen and thawed two times. The cell culture media containing the virus was pooled into a container for storage. FCV was assayed by the $TCID_{50}$ method.[7,8]

Spores of *Encephalitozoon intestinalis* (ATCC #50603) were obtained from American Type Culture Collection and grown in RK-13 cells. After addition of the spores to RK-13 cell monolayers, flasks were supported on Eagles minimum Essential Media (MEM) with 8% fetal bovine serum. To initiate the propagation of large numbers of fresh spores, loose spores in the cell culture supernatant were concentrated and re-inoculated upon media changes. This was performed by collecting cell culture media and centrifuging in a 50 mL centrifuge tube for 20 min at $1050 \times g$. The supernatant from this concentrate was aspirated down to 5 mL, and the pellet resuspended and added back to the infected monolayer from which it was collected with the fresh media. This procedure was followed for four weeks, after which the media containing the spores was collected, changed, and replaced with fresh media. The support media continued to be replaced twice a week with fresh MEM containing 8% FBS. Collected media contained newly-formed *E. intestinalis* spores from infected cells and was stored at 4°C until purified. After four weeks of infection, the cell monolayer in the infected flasks was detached using trypsin-EDTA as described above and split into several 162 cm^2 cell culture flasks to increase the number of flasks producing microsporidia. After several weeks of infection, the cell monolayer began to detach *en masse*, and required supplementation with uninfected stock cells. When cells began to show such deleterious effects of microsporidial infection, the flask was supplemented with 1×10^6 uninfected cells. These fresh cells were able to reestablish the cell monolayer and support the growth of microsporidia.

To purify spores from cell culture media, the spores and cell debris were concentrated by centrifugation at $1500 \times g$ for 15 min in 220 mL conical centrifuge

bottles. The supernatant was aspirated by pipet and the pellet re-suspended and transferred to 50 mL centrifuge tubes. The 220 mL bottle was also rinsed with 0.01 M phosphate buffer saline (PBS)(pH 7) and this rinse was also added to the 50 mL centrifuge tube. The 50 mL centrifuge tubes were centrifuged again at $1050 \times g$ for 15 min, and the supernatant aspirated to 5 mL. At this point, phosphate buffer and Percoll (Sigma-Aldrich, St. Louis, MO) were added to achieve a mixture of 50% Percoll mixture. This mixture was centrifuged at $2000 \times g$ for 30 min. The cell debris formed a layer on top and microsporidial spores formed a loose pellet at the bottom of the tubes. The Percoll mixture and cell debris were carefully aspirated to 5 mL, with care taken to not disturb the spore pellet. The tubes were then refilled with phosphate buffer and centrifuged at $1050 \times g$ for 15 min. The spores were resuspended in PBS and stored at 4°C not more than 14 days before use in experiments.

Spores were fixed in 5% formalin prior to use. Samples for *E. intestinalis* assay were serially diluted (with the exception of the filter effluent sample) with 0.01 M PBS. Samples were filtered through 13 mm diameter 0.2 μm Isopore membrane filters (Millipore, Bedford, MA) to collect the spores for staining with antibody. The filters were treated with 1 mL of bovine serum albumin (BSA) prior to filtering the sample. After plugging the bottom of the syringe filter housing with parafilm, 200 μL of diluted antibody was placed on the filter. A labeled monoclonal antibody to *E. intestinalis* (Waterborne Inc., Tulane LA) was diluted 1:20 with 0.01 M PBS. The filter housing was covered with aluminum foil and incubated at room temperature for 1 h. After incubation, the filters were rinsed twice by passing approximately 3 mL of 0.01 M PBS through the filter. The filters were removed from the housing and mounted on a slide with 1,4 diazobicyclo[2.2.2] octane (DABCO). The coverslip was sealed with clear fingernail polish. The filters were enumerated by fluorescent microscopy using an Olympus BH-2 microscope (Olympus America Inc., Melville, NY).

Escherichia coli (ATCC #25922) was prepared by inoculating 100 mL of tryptic soy broth (TSB) and incubating overnight at 37°C. The membrane filter technique using mEndo medium was used to enumerate *E. coli* by Standard Method 9222.[9] The samples were incubated at 37°C for 24 h before counting the colonies. Samples were placed at 4°C immediately after collection and assayed within 24 h.

Pilot Design and Operation

A pilot plant located at the City of Chandler Water Treatment Plant (Chandler, AZ) was used to evaluate the removal of the selected CCL microorganisms through conventional treatment, including coagulation, flocculation, sedimentation, and multimedia filtration with granular activated carbon (GAC), sand, and gravel. A schematic of the pilot plant is shown in Fig. 1.

Water treatment occurs through a series of physical and chemical processes, which are interrelated. They generally include flocculation, sedimentation, filtration, and disinfection. The pilot plant included the first three stages. In flocculation, a dose of coagulant is added and the water is gently agitated. This gentle mixing encourages the particles to stick together, thereby increasing particle size, which allows it to be more easily separated from the water column. Sedimentation allows a certain amount of time for the particles to settle out via gravity. Additionally, there is an

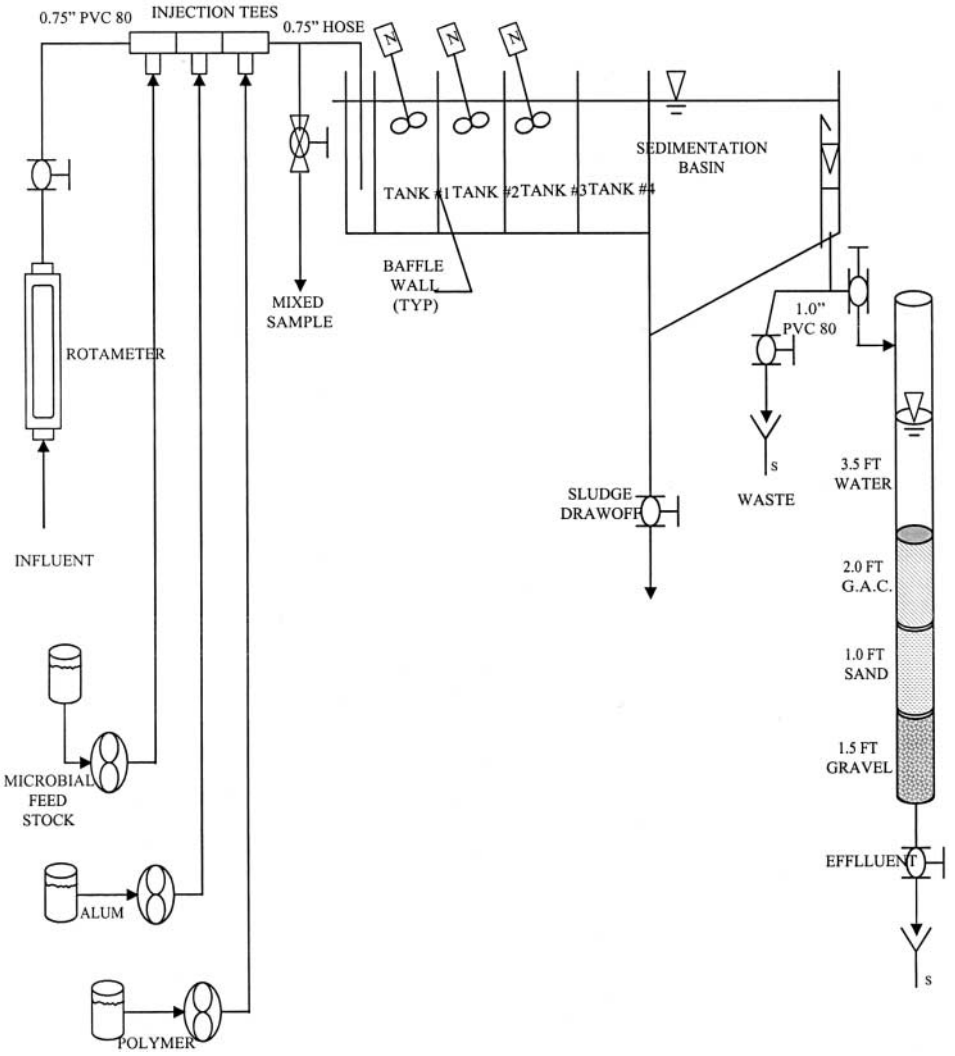

Figure 1. Schematic for the city of Chandler pilot plant.

upflow velocity created where the water flows out over a weir. Filtration is the removal of particles by water flowing down through granular media.

Central Arizona Project (CAP) raw water was fed into the pilot plant at a flow rate of 0.4–0.5 gallon/min (1.5–1.8 L/min). Three injection ports installed on the inflow water pipe were used for the addition of alum, polymer, and microorganisms (Fig. 1). For the three experimental runs, the alum (Rhodia, Shelton, CT) feed concentrations ranged between 20–23 mg/L and the polymer (CATFLOC-L equivalent; Sweetwater Tech., Temecula, CA) feed concentration ranged between 1.8–2.3 mg/L. The chemical concentrations used for the pilot study were based on the concentrations used for the full scale plant at the time of the experiment. The

Table 2. Pilot plant tank measurements.

Tank	Dimensions (inch)	Volume (gal)	Mixing (rpm)
#1	12″L × 12″ W × 7.5″D	4.5	35
#2	11.5″ L × 12″ W × 7.5″D	4.5	25
#3	11.5″ L × 12″ W × 7.25″D	4.5	15
#4	11.5″ L × 12″ W × 7.25″D	4.5	None
Sedimentation basin	48″ L × 12″ W × 15–22″D	46	None

water then entered a series of four mixing tanks, with rapid mixing in the first tank, followed by gradually declining speeds of mixing for each subsequent tank. The paddle speed for Tank 1 was 35 rpm, the paddle speed for Tank 2 was 25 rpm, and the paddle speed for Tank 3 was 15 rpm. There was no mixing in Tank 4. Floc formation was observed increasing in size within each series of tanks after approximately 15 min. The flocculated water was allowed to settle in the sedimentation basin. The upper portion of the water in the sedimentation basin entered the pipe leading to the multimedia filter, which had a layer of gravel, a layer of sand, and a layer of granular activated carbon. The tanks' dimensions are listed in Table 2. The depths listed in the table are the depths of water in each tank.

Hydraulics

In a water treatment process, the water flow characteristics are intimately coupled with the process chemistry and reactions. Since it generally takes a certain amount of time to achieve the required chemical change, the process is designed to allow time for this to take place. This occurs by sizing a tank to allow sufficient contact time, assuming the flow rate is known. This is known as hydraulic residence time (HRT).

As with many large scale processes, general rules of thumb have been established over time, which tend to yield good results. The first three tanks were the flocculation basins, which typically have an HRT of approximately 37 min. For this study, the HRT was approximately 27 min for the three mixing tanks. The sedimentation basin had an HRT of approximately 1 h and 39 min (including the no mixing tank #4). Typical residence times in sedimentation basins in full-scale plants range from 2–3 h.

The filter in the pilot plant contained the same media as in full-scale facilities but was contained in a $3\frac{1}{2}$ in diameter clear acrylic pipe. Residence time is not a critical parameter for proper filter operation. The surface loading of the filter is more important, which was approximately 10 gpm/ft^2. The recommended value is in the range of 5–10 gpm/ft^2.

Microbial Seeding Protocol and Collection

Three challenges were conducted at the pilot plant with *Escherichia coli* (ATCC #25922), MS-2 and PRD-1, *E. intestinalis* and feline calicivirus on May 30, July 12,

and September 12, 2000. One challenge included Fr coliphage (September 12, 2000). Approximately 100 mL of 1×10^8 cfu/mL of stock *E. coli*, 50 mL of 1×10^{11} pfu/mL of stock MS-2, 50 mL of 1×10^{12} pfu/mL of stock PRD-1, 100 mL of 1×10^6 spores/mL of stock *E. intestinalis*, and 300 mL of 1×10^6 $TCID_{50}$/mL of stock feline calicivirus were added to a 3.78 L plastic cubitainer. Approximately 100 mL of 1×10^8 pfu/mL of stock Fr was also added during the third pilot run. Raw water was added to the cubitainer for a total volume of 3 L. This was done to ensure there would be enough microbial seed to last the duration of the pilot plant run.

Filter effluent turbidity was checked prior to the start of the pilot run to ensure the turbidity was 1 NTU. A sample was collected from the seed cubitainer to verify the concentration of the microorganisms added. A sample was collected from the influent port before entering the first mixing tank approximately 10 min after starting the microbial feed. The microbial seed was allowed to feed into the pilot plant for about 7 min before the coagulant and polymer were added. The post-sedimentation sample was collected after 45 min of seeding the pilot and the filter effluent was collected after 50 min. Sterile 1 L polypropylene bottles were used for sample collection. A separate sample was collected for feline calicivirus and 1 mL of FBS was added to the sample for preservation of the virus.

RESULTS

The average log removal of *E. coli* by the pilot plant for the three experiments was 2.67. Following sedimentation, the log removal averaged 1.23. An additional average removal of 1.44 log was measured following filtration. Therefore, the greatest removal of the bacteria occurred by the filtration process.

E. intestinalis was consistently removed through the process with an average 2.47 log removed for the three pilot plant tests conducted (Table 3). Following sedimentation, the log removal averaged 0.78. An average additional removal of 1.69 was measured following filtration. Therefore, the greatest removal for the protozoa occurred during filtration.

Of the three coliphages studied, Fr was removed to the greatest degree (3.21 log) and PRD-1 was removed the least (1.85 log) (Table 3). Feline calicivirus removal was similar to Fr (FCV > 3.05 log vs. Fr 3.21 log). The greatest removal of the viruses occurred during sedimentation, except for PRD-1 which showed greater removal during filtration.

Discussion

The Guidance Manual[10] assumes that conventional treatment plants meeting minimum performance criteria are achieving at least a 2.5-log removal of *Giardia* cysts and at least a 2-log removal of viruses. The literature indicates that well operated plants can achieve up to a 3-log reduction of *Giardia* cysts and viruses.[10,11] Thus, it was felt that limiting the credit to 2.5-log for *Giardia* and 2-log for viruses provided a margin of safety.

Table 3. Average removal of challenge microorganisms in pilot plant study.

	Concentration of test organism/mL				
Organism	Influent	After sedimentation	Log removal	After filtration	Total log removal
E. coli	2.4E + 04	1.4E + 03 ± 1130	1.23	51 ± 52	2.67
E. intestinalis	2.6E + 03	4.3E + 02 ± 247	0.78	8.9 ± 10.5	2.47
MS-2	5.5E + 08	8.1E + 06 ± 6.9E + 06	1.83	1.7E + 06 ± 1.3E + 06	2.51
PRD-1	8.5E + 07	1.4E + 07 ± 1.5E + 07	0.78	1.2E + 06 ± 9.1E + 05	1.85
Fr	2.9E + 08	3.1E + 06	1.97	1.8E + 05	3.21
Feline calicivirus	3.1E + 03	9.9 ± 12	2.49	> 2.79 ± 0.18	3.05

Several different coliphages with differing isoelectric points and *E. coli* were used as controls. Conventional treatment was most effective in the removal of the larger *E. coli* (2.67 log) and *E. intestinalis* (2.47 log), which are similar in size. Interestingly, the feline caliciviruses were removed on average to a greater degree (3.05 log) than any of the microorganisms tested. This could be due to a greater degree of removal by the treatment processes or a greater degree of inactivation. Feline calicivirus removal was similar to that reported for rotavirus.[12] Coliphage MS-2 removal (2.51 log) was similar to *E. intestinalis* (2.47 log). PRD-1 was removed the least (1.85 log) of all the study organisms. This may be a reflection of its resistance to removal or greater survival in water. PRD-1 is known to survive better in water than MS-2.[13] MS-2 removal was similar to that reported for poliovirus type 1.[12] Fr, with the highest isoelectric point of the viruses, was removed to the greatest degree. In general, viruses were removed to the greatest degree by flocculation and the larger microorganisms, *E. intestinalis* and *E. coli* were primarily removed by filtration. PRD-1 was the virus least removed by flocculation. Since PRD-1 was removed the least of any microorganism studied it could be considered a worst case example of removal for a microorganism by conventional treatment before disinfection.

In summary, *E. intestinalis* removal was in the range required for *Giardia* and feline calicivirus was greater than the 2-log removal required for viruses. Thus, current EPA guidance for conventional surface water treatment appears to be adequate for the control of these organisms in drinking water supplies.

ACKNOWLEDGMENTS

This work was supported by the Office of Water, United States Environmental Protection Agency under contract 68-C-99232. The options expressed here are those of the authors and not necessarily those of the Environmental Protection Agency.

REFERENCES

1. Weber, R.; Bryan, R.T.; Schwartz, D.A.; Owen, R.L. Human microsporidial infections. Clin. Microbiol. Rev. **1994**, *7*, 426–461.
2. Cotte, L.; Rabodonirina, M.; Chapuis, F.; Bailly, F.; Bissuel, F.; Raynal, C.; Gelas, P.; Persat, F.; Piens, M.A.; Trepo, C. Waterborne outbreak of intestinal microsporidiosis in persons with and without human immunodeficiency virus infection. J. Infect. Dis. **1999**, *180*, 2003–2008.
3. Hutin, Y.J.; Sombardier, M.N.; Liguory, O.; Sarfati, C.; Derouin, F.; Modai, J.; Modina, J.M. Risk factors for intestinal microsporidiosis in patients with human immunodeficiency virus infection: a case-control study. J. Infect. Dis. **1998**, *178*, 904–907.
4. Green, K.Y.; Ando, T.; Balayan, M.S.; Berke, T.; Clarke, I.N.; Estes, M.K.; Matson, D.O.; Nakata, S.; Neill, J.D.; Studdert, J.; Thiel, H.J. Taxonomy of the caliciviruses. J. Infec. Dis. **2000**, *18*, 5222–5330.
5. Hunter, P.R. Drinking water and waterborne disease. In *Waterborne Disease*; John Wiley: Chichester, UK, 1997.

6. Adams, M.H. In *Bacteriophages*; Interscience Publishers: New York, NY, 1959.
7. Dahling, D.R.; Wright, B.A. Optimization of suspended cell method and comparison with cell monolayer technique for virus assays. J. Virological Meth. **1988**, *20*, 169–179.
8. Grabow, W.O.K.; Puttegill, D.L.; Bosch, A. Propagation of adenovirus types 40 and 41 in the PLC/PRF/5 primary liver carcinoma cell line. J. Virological Meth. **1992**, *37*, 201–208.
9. American Public Health Association (APHA). Part 9000 – Microbiological Examination, In *Standard Methods for the Examination of Water and Wastewater*, 20th Ed.; Clesceri, L.S., Greenberg, A.E., Eaton, A.D., Ed.; Washington, D.C., 1998.
10. United State Environmental Protection Agency. Office of Drinking Water, Criteria and Standards Division. Guidance Manual for Compliance With The Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Sources. Washington, DC, 1997.
11. Robeck, G.G.; Clarke, N.A.; Dostal, K.A. Effectiveness of water treatment processess in virus removal. J. Am. Water Works Assoc. **1962**, *54*, 1275–1290.
12. Rao, V.C.; Symons, J.M.; Ling, A.; Wang, P.; Metcalf, T.G.; Hoff, J.C.; Melnick, J.L. Removal of hepatitis A virus and rotavirus by drinking water treatment. J. Am. Water Works Assoc. **1988**, *80*, 59–67.
13. Yahya, M.T.; Galsomies, L.; Gerba, C.P.; Bales, R.C. Survival of bacteriophages MS-2 and PRD-1 in groundwater. Water Sci. Tech. **1993**, *27*, 409–411.

Received August 29, 2002