APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2007, p. 4218–4225 Vol. 73, No. 13
0099-2240/07/$08.00+0 doi:10.1128/AEM.02713-06

Multistate Evaluation of an Ultrafiltration-Based Procedure for Simultaneous Recovery of Enteric Microbes in 100-Liter Tap Water Samples[▽]

Vincent R. Hill,[1]* Amy M. Kahler,[1,2] Narayanan Jothikumar,[1] Trisha B. Johnson,[1,2] Donghyun Hahn,[1,2] and Theresa L. Cromeans[1,2]

Centers for Disease Control and Prevention, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Division of Parasitic Diseases, Atlanta, Georgia,[1] and Atlanta Research and Education Foundation, Atlanta, Georgia[2]

Received 20 November 2006/Accepted 27 April 2007

Ultrafiltration (UF) is increasingly being recognized as a potentially effective procedure for concentrating and recovering microbes from large volumes of water and treated wastewater. Because of their very small pore sizes, UF membranes are capable of simultaneously concentrating viruses, bacteria, and parasites based on size exclusion. In this study, a UF-based water sampling procedure was used to simultaneously recover representatives of these three microbial classes seeded into 100-liter samples of tap water collected from eight cities covering six hydrologic areas of the United States. The UF-based procedure included hollow-fiber UF as the primary step for concentrating microbes and then used membrane filtration for bacterial culture assays, immunomagnetic separation for parasite recovery and quantification, and centrifugal UF for secondary concentration of viruses. Water samples were tested for nine water quality parameters to investigate whether water quality data correlated with measured recovery efficiencies and molecular detection levels. Average total method recovery efficiencies were 71, 97, 120, 110, and 91% for ϕX174 bacteriophage, MS2 bacteriophage, *Enterococcus faecalis*, *Clostridium perfringens* spores, and *Cryptosporidium parvum* oocysts, respectively. Real-time PCR and reverse transcription-PCR (RT-PCR) for seeded microbes and controls indicated that tap water quality could affect the analytical performance of molecular amplification assays, although no specific water quality parameter was found to correlate with reduced PCR or RT-PCR performance.

Ultrafiltration (UF) is a technique that has been used in various approaches since the 1970s for concentrating microbes, especially viruses, in water (4). Because UF membranes have pore sizes small enough to remove molecules having molecular masses on the order of 10,000 to 100,000 Da, these filter membranes are also capable of simultaneously concentrating diverse waterborne microbes, including viruses, bacteria, and parasites. The capability of hollow-fiber UF for simultaneous recovery of diverse microbes raises the potential for employing hollow-fiber UF techniques for time-efficient and cost-effective monitoring of water quality with regard to a wide range of microbes.

Morales-Morales et al. (15) reported using hollow-fiber UF for simultaneously recovering *Escherichia coli*, *Cryptosporidium parvum* oocysts, T1 bacteriophage, and PP7 bacteriophage from 10-liter surface water samples. Their UF procedure used a reusable UF cartridge, calf serum pretreatment of the UF, and UF elution using a surfactant solution. Hill et al. (12) reported a UF procedure using reusable commercial dialysis filters, filter backflushing using a surfactant solution, and a chemical dispersant (sodium polyphosphate) to reduce potential microbial adsorption to the UF fibers. The research of Morales-Morales et al. (15) and Hill et al. (12) demonstrated that hollow-fiber UF could be effective for simultaneously recovering viruses, bacteria, and parasites from water, but the UF conditions used by these research groups were different and the techniques were investigated using relatively small volumes of water (10 liters). In the present study, we report a new UF-based protocol that incorporates elements of the procedures reported by Morales-Morales et al. (15) and Hill et al. (12) for large-volume (100-liter) water samples.

When developing a microbial concentration technique for large-volume water sampling, it is important to demonstrate that commonly used analytical techniques can be used effectively to assay the final concentrates produced by the method. It has been shown in previous studies that the performance effectiveness of immunological techniques (e.g., immunomagnetic separation–immunofluorescent-antibody staining [IMS-IFA staining]) (14, 24) and molecular assays (e.g., PCR and reverse transcription-PCR [RT-PCR]) (1, 11) can be negatively affected by various water quality conditions (e.g., pH, iron content, or organic compounds), although relatively few studies of analytical method inhibition have focused on drinking water. Because detection of *Cryptosporidium* and *Giardia* is important for water quality monitoring, understanding the effects of water quality on available IMS techniques for these microbes, such as those incorporated into USEPA Method 1623 (22), is critical. Because assay inhibition is an issue in using molecular amplification techniques for water testing, it is important to evaluate the magnitude of potential inhibition, using controls such as internal standards or external control procedures (11, 18).

The water sample processing protocol described by the

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, National Center for Infectious Diseases, Division of Parasitic Diseases, 4770 Buford Highway, Mail Stop F-36, Atlanta, GA 30341-3724. Phone: (770) 488-4432. Fax: (770) 488-4253. E-mail: vhill@cdc.gov.

▽ Published ahead of print on 4 May 2007.

present study was investigated to evaluate the following issues: (i) the effectiveness of a hollow-fiber UF procedure for recovering viruses, bacteria, and parasites in 100-liter tap water samples; (ii) the effectiveness of secondary sample processing techniques performed in conjunction with primary UF concentration; (iii) the effect of tap water quality on total method performance; and (iv) the effect of tap water quality on microbe detection using real-time PCR and RT-PCR. To investigate these issues, a suite of enteric microbes was used to encompass diverse microbial characteristics. This suite included the ϕX174 and MS2 bacteriophages, *Enterococcus faecalis*, *Clostridium perfringens* spores, *C. parvum* oocysts, and *Giardia intestinalis* cysts. ϕX174 and MS2 are well-characterized surrogates for pathogenic human enteric viruses and have different surface charge properties that may help in evaluating the performance of water sampling methods (19). *E. faecalis* is an established microbial water quality parameter that has previously been studied for recovery by UF methods (12). *C. perfringens* has also been studied as a microbial water quality parameter because its spores are resistant to environmental degradation. *C. parvum* and *G. intestinalis* are important human pathogenic parasites of concern for drinking water quality.

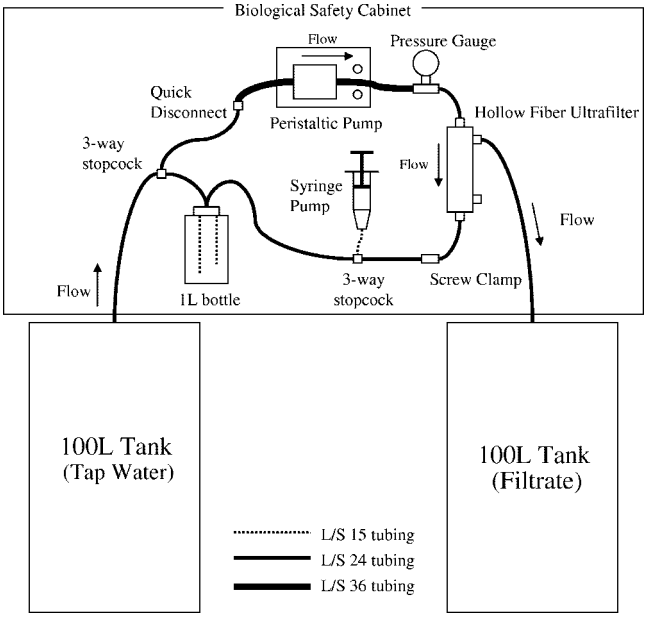

FIG. 1. Schematic of hollow-fiber UF procedure, as performed in a biological safety cabinet.

MATERIALS AND METHODS

Water samples. Tap water samples were obtained from eight cities in the United States located in six different U.S. Geological Survey (USGS) hydrologic regions. One-hundred-liter samples were collected from the laboratories or offices of collaborating facilities in 10- and 20-liter Cubitainer containers. Sodium thiosulfate was added to each container to achieve a final concentration of 40 mg/ml. The containers comprising each 100-liter sample were shipped to the CDC by priority overnight shipping in coolers. Although it was not feasible to chill these large-volume water samples during shipment, the sample containers were stored in open coolers at 4°C upon receipt by the CDC. No more than 2 to 3 days elapsed between sample receipt and use in an experiment. The night before an experiment, each sample Cubitainer was stored at room temperature to allow the water samples to warm slowly. Using this procedure, the average water temperature for experiments was 18 ± 4°C. For each experiment, tap water from Cubitainer containers was poured into a sterile 30-gallon high-density polyethylene tank that had been calibrated to 100 liters, using 10-liter gradations.

Water quality testing. All water samples were characterized on the day of an experiment, using the following water quality parameters: pH, turbidity, specific conductance (SC), alkalinity, total iron, total organic carbon (TOC), dissolved organic carbon (DOC), UV absorbance (at 254 nm), and heterotrophic bacterial plate count (HPC). Water quality measurements for the 100-liter tap water samples were performed immediately prior to UF. Water quality measurements for the UF concentrates were generally performed immediately following UF. Sample pH was measured with a Fisher Scientific Accumet Research AR25 pH/mV/°C/ISE meter. Turbidity was measured using a Hach model 2100N laboratory turbidimeter (Hach Company). SC and temperature were measured with an Oakton CON 100 conductivity/°C meter. Alkalinity was determined using a Hach alkalinity test kit (AL-DT digital titrator). Total iron was measured using the Hach FerroVer iron (total) reagent and a Hach DR/2400 portable spectrophotometer. TOC and DOC were both measured using a Hach low-range TOC reagent set and a Hach DR/2400 portable spectrophotometer. To process a sample for DOC determination, an Ahlstrom 0.7-μm prebaked borosilicate microfiber disc filter (Environmental Express) was conditioned by filtering 300 ml deionized (DI) water. Sixty milliliters of the sample was then passed through the filter, and the last 10 ml was retained for analysis. UV absorbance at 254 nm was measured with a Genesys 2 spectrophotometer (Thermo Electron Corporation). HPC was enumerated by membrane filtration using R2A agar according to the protocol in *Standard Methods for the Examination of Water and Wastewater* (7).

Microorganisms and microbial assays. A suite of the following five microbes was used in this study: ϕX174 bacteriophage, MS2 bacteriophage, *E. faecalis*, *Clostridium perfringens* spores, and *C. parvum* oocysts. In addition, EasySeed (BTF Pty. Ltd., Australia) was used to seed UF concentrates with *Giardia intestinalis* cysts to evaluate the effectiveness of IMS for this microbe under controlled conditions.

MS2 (ATCC 15597-B1) and ϕX174 (ATCC 13706-B1) stocks were produced using tryptic soy broth and respective antibiotics and host bacteria as described by the American Type Culture Collection (ATCC; Manassas, VA). Frozen MS2 stocks (1.5×10^6 PFU/ml) and ϕX174 stocks (1.2×10^9 PFU/ml) were diluted using 10-fold dilutions and vigorous vortex mixing in a diluent containing 0.01 M phosphate-buffered saline (PBS; Dulbecco's modification, pH 7.40), 0.01% (wt/vol) Tween 80 (Fisher), and 0.001% (wt/vol) Y-30 antifoam emulsion (Sigma) in order to disperse viral particles. MS2 and ϕX174 were seeded into the 100-liter water samples at concentrations of 72,000 ± 24,000 and 92,000 ± 31,000 PFU, respectively (measured seed concentrations for 19 experiments). MS2 and ϕX174 were enumerated by plaque assay per USEPA Method 1602 (21). Frozen stocks of *E. faecalis* (ATCC 19433), produced per ATCC guidance and having a concentration of 8.3×10^8 CFU/ml, were diluted as described for the viruses and seeded into 100-liter samples at a concentration of 8,400 ± 740 CFU. *E. faecalis* was assayed by membrane filtration according to the protocol in *Standard Methods for the Examination of Water and Wastewater* (7). *C. perfringens* spores were purchased as "BioBalls" from BTF Pty. Ltd. (Australia). One 10,000-CFU BioBall was added to each 100-liter water sample. BioBalls were prepared for use in experiments according to the manufacturer's guidelines, including vigorous shaking (600 oscillations/min) in 10 ml of sample water containing 0.001% Y-30 antifoam emulsion for 30 min, using a Pall laboratory shaker. *C. perfringens* spores were enumerated by membrane filtration using mCP agar (6). *C. parvum* oocysts were obtained from the laboratory of Michael Arrowood (CDC) and from Waterborne, Inc. (New Orleans, LA). Oocysts produced by the CDC were processed according to the method of Arrowood and Donaldson (3) and were used for experiments with tap water from sites 1 to 5. Oocyst stocks (6.7×10^7 [CDC] and 1.2×10^8 oocysts/ml [Waterborne]) were less than 4 months old when used for experiments and did not contain appreciable oocyst aggregates. *C. parvum* oocyst stocks were diluted in PBS-Tween 80 diluent and seeded into 100-liter water samples at an average concentration of 590,000 ± 84,000 oocysts. This seeding level was chosen to enable direct assay of UF concentrates by IFA microscopy for *Cryptosporidium* without the need for additional concentration (e.g., using centrifugation) or IMS.

The same microbial stock dilutions used to seed a 100-liter sample were also used to seed a 1-liter control sample of experimental tap water, which was used to quantify the microbial seeding concentrations for each experiment. The control sample was kept at room temperature during the UF experiment and was assayed when UF concentrates, secondary concentrates, and IMS samples were assayed.

UF setup. The filtration unit configuration is shown in Fig. 1. New high-

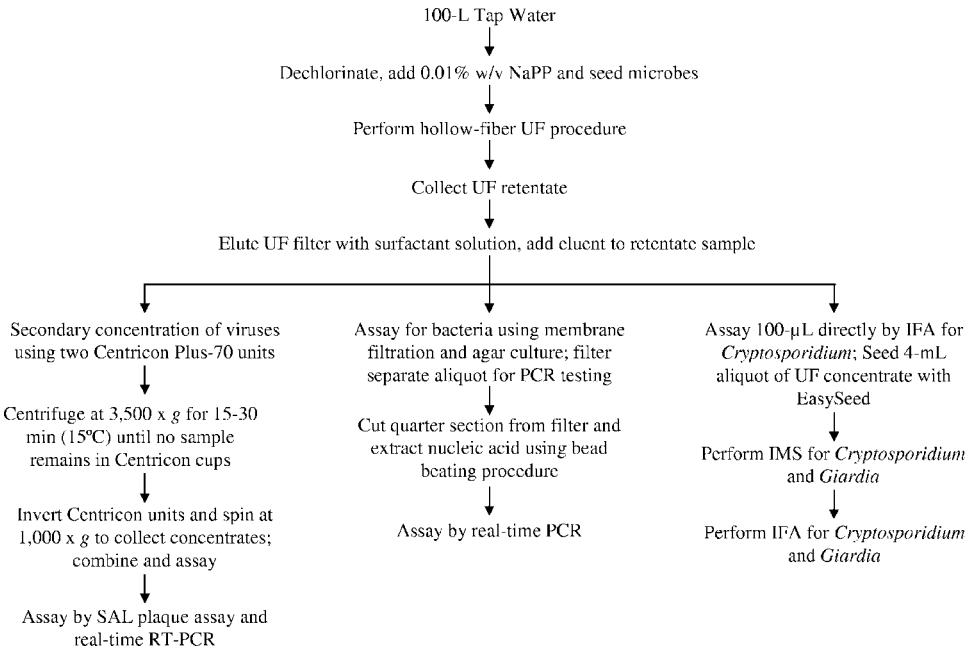

FIG. 2. Flow diagram showing procedure for primary concentration of all microbe classes by hollow-fiber UF, followed by secondary concentration and analysis of specific microbes.

performance, platinum-cured L/S 36, L/S 24, and L/S 15 silicon tubing (Masterflex; Cole-Parmer Instrument Co.) was used for each filtration experiment. All tubing connectors and clamps were autoclaved, and the brass fitting of the pressure gauge was sanitized with 3% hydrogen peroxide and 10% bleach solution (0.6% sodium hypochlorite) and then washed thoroughly with DI water prior to use in the filtration setup. The UFs and tubing were discarded after each experiment. The hollow-fiber UFs used were single-use Fresenius F200NR polysulfone dialysis filters with a molecular mass cutoff of ~30,000 Da (30 kDa), a surface area of 2.0 m^2, and a fiber inner diameter of 200 μm (Fresenius Medical Care, Lexington, MA). A Cole-Parmer model 7550-30 peristaltic pump was used for all experiments.

Hollow-fiber UF blocking. UFs were blocked by recirculating 500 ml of filter-sterilized 5% calf serum (e.g., Invitrogen no. 16170-078) through the UFs for 5 min with the filtrate port closed. The calf serum was then allowed to contact the UF fibers overnight at ambient temperature on a rotisserie in a hybridization oven. Prior to use in an experiment, the calf serum was flushed from the UF by using 1 liter of DI water.

Hollow-fiber UF procedure. Prior to beginning the UF procedure, 0.01% (wt/vol) sodium polyphosphate (NaPP; Sigma-Aldrich) was added to the 100-liter water sample and mixed for 30 s using a Stir-Pak laboratory mixer (Cole-Parmer) (Fig. 2). The peristaltic pump was set to pump water at 2,900 ml/min. Generally, the system was operated at 13 ± 2 lb/in^2 (90 ± 14 kPa) to achieve a filtrate rate of approximately 1,200 ml/min (and corresponding cross-flow rate within the hollow-fiber UF of 1,700 ml/min). Filtration was performed until ~200 ml of concentrated sample remained in the loop shown in Fig. 1 (i.e., the hold-up volume of the tubing, UF, and remaining volume in the 1-liter bottle). At this point, the effluent tubing from the 1-liter bottle was disconnected from the bottle, and the peristaltic pump was used to force as much of the retentate as possible into the 1-liter bottle. The 1-liter bottle was then set aside, and a bottle containing 500 ml of a surfactant solution (0.01% Tween 80, 0.01% NaPP, 0.001% Y-30 antifoam emulsion) was connected in its place. This eluent was recirculated through the UF (with an open filtrate port and no screw clamp for backpressure) until the system started to draw air. The inlet tubing was immediately pulled from the eluent bottle to flush the remaining eluent from the system. The total volume of eluent (150 to 200 ml) was collected and added to the retentate sample to produce the final "UF concentrate." The average UF concentrate volume achieved using this protocol was 420 ± 50 ml.

Secondary sample processing procedures. Two 30-kDa Centricon Plus-70 units (Millipore, Billerica, MA) were used according to the manufacturer's instructions to concentrate 110 to 140 ml of the UF concentrate for each experiment, depending on the sample volume available (Fig. 2). The average final Centricon concentrate volume achieved was 2.1 ± 1.2 ml. The concentrates were assayed for culturable bacteriophages as well as for MS2 and an inhibitor control by real-time RT-PCR. Another 100- to 140-ml aliquot of the UF concentrate was filtered through a 47-mm, 0.2-μm Supor membrane filter (Pall, Ann Arbor, MI) to capture *C. perfringens* and *C. parvum* for subsequent detection by real-time PCR.

IMS was performed for *Cryptosporidium* and *Giardia* to investigate potential water quality effects on this procedure. Five milliliters of UF concentrate was processed directly by IMS (i.e., no centrifugation), using an Aureon Crypto kit (Aureon Biosystems, Austria) and an Aureon Giardia kit to determine *C. parvum* recoveries and the potential background presence of *Giardia* in the samples. IMS-processed samples were examined by the IFA procedure, using an Easy-Stain kit (BTF, Australia). One vial of EasySeed (BTF, Australia), containing 100 *G. intestinalis* cysts, was added to a second (4-ml) aliquot of UF concentrate as a controlled side experiment to evaluate water quality effects on IMS performance for *Giardia* recovery.

Nucleic acid extraction. Real-time PCR was performed on UF concentrates by filtering ~120 ml (representing roughly 30% of the volume of each UF concentrate) through a 0.2-μm membrane filter and performing a bead-beating procedure, using a one-quarter section from the filter. Real-time RT-PCR was performed on Centricon concentrates for MS2 and a hepatitis A virus (HAV) inhibitor control. Nucleic acid extraction from membrane filters and Centricon concentrates was performed using a modification of a previously reported noncommercial lysis buffer (2). To make this lysis buffer, 120 g of guanidine thiocyanate (Boehringer Mannheim, Indianapolis, IN) was added to 100 ml of TE buffer (5 mM Tris, pH 8.0, 0.5 mM EDTA, pH 8.0) prepared with nuclease-free water (Ambion, Austin, TX). After the guanidine thiocyanate was dissolved, 0.5 g sodium pyrophosphate (Alfa Aesar, Ward Hill, MA), 11 ml of 5 M sodium chloride (Ambion, Austin, TX), and 11 ml of 3 M sodium acetate, pH 5.5 (Ambion, Austin, TX), were added. Carrier nucleic acid [2.2 ml from a 2-mg/ml stock solution of poly(A) (Sigma, St. Louis, MO)] was added to a final concentration of 20 μg/ml.

To extract nucleic acids from membrane filters, the filters were cut aseptically, using a scalpel, into four one-quarter sections. One of these one-quarter sections was inserted into a 2-ml screw-cap polypropylene tube (National Scientific Supply) containing 100 mg of 106-μm glass beads, 100 mg of 425- to 600-μm glass beads, and 500 μl of diluent buffer (Dulbecco's PBS with 0.01% Tween 80 and 0.001% antifoam A). The tube was shaken for 3 min at a high-speed setting in a bead beater (Biospec, Bartlesville, OK). After the bead-beating step, the sample was centrifuged for 30 s at 14,000 × *g*, and the supernatant was transferred to a 1.6-ml microcentrifuge tube containing 500 μl lysis buffer. The sample was

TABLE 1. Primers and probes used in the present study

Target analyte	Primer direction	Primer sequence (5′–3′)	TaqMan probe sequence (5′-FAM–3′-BHQ)[e]
Cryptosporidium spp.[a]	Forward	ATGACGGGTAACGGGGAAT	CGCGCCTGCTGCCTTCCTTAGATG
	Reverse	CCAATTACAAAACCAAAAAGTCC	
C. perfringens[b]	Forward	CACAAGTAGCGGAGCATGTG	AACCTTACCTACACTTGACATCCCTTGC
	Reverse	CCCCGAAGGGATTTCCTCGATT	
S. enterica serovar Typhimurium[c]	Forward	GCCTTTCTCCATCGTCCTGA	TGCGATCCGAAAGTGGCG
	Reverse	TGGTGTTATCTGCCTGACC	
MS2[d]	Forward	TGCCATTTTTAATGTCTTTAG	AGACGCTACCATGGCTATCGC
	Reverse	TGGAATTCCGGCTACCTAC	

[a] Primer positions for the forward primer (100 to 118), reverse primer (258 to 223), and probe (161 to 185) were based on GenBank accession no. AY458612. The PCR product length is 159 bp.
[b] Primer positions for the forward primer (852 to 870), reverse primer (952 to 931), and probe (894 to 921) were based on GenBank accession no. DQ298091. The PCR product length is 101 bp.
[c] Primer positions for the forward primer (304 to 323), reverse primer (389 to 371), and probe (329 to 346) were based on GenBank accession no. EF113938. The PCR product length is 86 bp.
[d] Primer positions for the forward primer (47 to 67), reverse primer (110 to 92), and probe (70 to 90) were based on GenBank accession no. V00642. The RT-PCR product length is 64 bp.
[e] FAM, 6-carboxyfluorescein; BHQ, black hole quencher.

vortexed with the lysis buffer for 30 s and then passed through a silica spin column (QIAGEN, Valencia, CA) by centrifugation at 14,000 × *g* for 30 s. The column was loaded twice to process the entire sample volume (~1 ml, including 500 μl lysis buffer and 500 μl sample). The column was washed once with 500 μl of 100% ethanol (and centrifuged at 14,000 × *g* for 1 min) and twice with 75% ethanol (and spun at 14,000 × *g* for 1 min). The column was centrifuged again to remove any excess ethanol. The column was then transferred to a clean microcentrifuge tube, and nucleic acid was eluted by adding 200 μl TE buffer and centrifuging the column for 1 min at 14,000 × *g*. Further purification of the eluted nucleic acid sample was performed using a Microcon YM-100 (Amicon Inc., Beverly, MA) microconcentrator by adding the sample (200 μl) to the Microcon YM-100 reservoir containing 200 μl TE buffer. The mixture (400 μl) was filtered by centrifugation at 14,000 × *g* for 1 min. The filter was then inverted and inserted into a clean microcentrifuge tube to recover the nucleic acid by centrifugation at 4,500 × *g* for 30 s. Approximately 160 μl DNA was collected (samples with less volume were adjusted to a 160-μl final volume with TE buffer).

To extract nucleic acids from Centricon concentrates (for MS2 analysis), 1 ml of lysis buffer was amended with 5 μl of 10% sodium dodecyl sulfate. This amended lysis buffer was added to 500-μl Centricon concentrate samples at a ratio of 1:1. If less than 500 μl was available, nuclease-free water was added to the sample to increase the effective volume to 500 μl. The sample was vortexed with the lysis buffer for 30 s, centrifuged at 14,000 × *g* for 30 s, transferred to a silica spin column (QIAGEN, Valencia, CA), and processed as described previously for membrane filters.

Real-time PCR, RT-PCR, and inhibitor controls. Four *Salmonella enterica* serovar Typhimurium BioBalls (30 CFU each, for a total of 120 CFU) were seeded into each 120-ml UF concentrate that was filtered to evaluate the recovery of *C. perfringens* spores and *C. parvum* oocysts by real-time PCR. The *Salmonella* seeding allowed for controlled evaluation of the effects of water quality on the extraction and PCR detection of vegetative bacteria in UF concentrates. The relative difference in *Salmonella* crossing-point (CP) values between sample collection sites was used as an indication that water quality was associated with PCR inhibition and/or poor nucleic acid extraction performance. The effect of water quality on real-time RT-PCR was evaluated using HAV RNA as an external control. HAV RNA was extracted from a stock of HAV strain HM-175, clone 24A (8), using the lysis buffer and silica column described previously. For each Centricon concentrate tested, an aliquot of the Centricon nucleic acid extract sample was seeded with HAV RNA (at a concentration reflecting 10,000 PFU/reaction), as was an aliquot of molecular-grade water. The difference in the CP values for the seeded reagent-grade water sample and the Centricon sample was used as an indication of the magnitude of RT-PCR inhibition associated with a given tap water sample. This approach to inhibition evaluation has been used by other researchers (11). A relative difference of 3.3 CP values was used as a minimum value for indicating PCR or RT-PCR inhibition, based on the ideal slope for a real-time PCR standard curve of 3.3 CP values per 10-fold difference in target amounts present in a reaction mix.

Amplification of DNA and RNA targets was performed using an iCycler iQ4 real-time PCR detection system (Bio-Rad, Hercules, CA). Five different assays (for *C. perfringens*, *Cryptosporidium*, *Salmonella*, MS2, and HAV) were performed in a 96-well plate format. Table 1 provides sequence data for primers and TaqMan probes used for the assays. HAV RT-PCR was performed according to the method of Jothikumar et al. (13). For each assay, a TaqMan probe was used at a final concentration of 100 nM, and the primers were used at a final concentration of 250 nM (each). Real-time PCR amplifications were performed under the following conditions: reverse transcriptase reaction for 30 min at 50°C, followed by denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s. Each 50-μl reaction mix contained 25 μl of 2× master mix (QuantiTect probe PCR kit [QIAGEN, Valencia, CA] or QuantiTect probe RT-PCR kit [QIAGEN, Valencia, CA]) and different volumes of sample depending on the target analyte (10 μl of DNA for *C. parvum* and *C. perfringens*, 20 μl of DNA for *Salmonella*, 10 μl of RNA for MS2, and 20 μl of RNA for inhibitor control testing using HAV). Each water sample was tested once using these molecular assays. Aside from *C. perfringens* testing of samples from sites 1 to 4, molecular testing of nonseeded water samples was not performed.

Data analysis and statistics. Recovery efficiencies were calculated by dividing the number of each microbe recovered after a procedure (concentration times sample volume) by the experimentally determined number of each microbe present in the sample prior to performing the procedure (concentration times sample volume) and then multiplying the result by 100. In general, data results have been averaged, with standard deviations indicated by a "±" symbol. Coefficients of variation (COV) were calculated by dividing standard deviations by their respective data averages. For statistical tests using molecular data, a CP value of 43 was assumed for negative results because the highest observable CP value for a positive result was 42. Correlation tests were performed using the Pearson product moment correlation. Scientifically meaningful correlations were limited to those correlations with Pearson's r values of ≥ 0.6 or ≤ -0.6, at a significance level of ≤ 0.05.

RESULTS

Water quality results. A total of 26 100-liter samples were collected from eight U.S. cities located in the USGS hydrologic regions indicated in Table 2. To protect the identities of the water systems and facilities providing water samples for this study, the identities of the cities are not reported in this paper. However, a wide range of water quality tests were performed in order to characterize the chemical and biological quality of the water samples used in the study. The average pHs of the tap water used in this study ranged from 7.28 at site 6 to 9.30 at site 1. The pHs of the UF concentrates were usually lower than the pHs of nonconcentrated tap water and ranged from 6.38 (site 6) to 7.47 (site 8). Turbidity of the nonconcentrated tap water also varied widely, with a low of 0.108 nephelometric turbidity

TABLE 2. Water quality data for 100-liter tap water samples collected from eight U.S. cities[c]

Site (USGS region)	Water source-treatment[a]	*n*	pH	Turbidity (NTU)	SC[b] (μS/cm at 25°C)	Alkalinity (mg/liter as $CaCO_3$)	Total iron (mg/liter)	TOC (mg/liter as C)	DOC (mg/liter as C)	A_{254}	HPC (CFU/ml)
1 (3)	SW-FC	2	9.21, 9.39	0.121, 0.221	119, 147	17, 19	0.03, 0.06	1.7, 2.2	2.4, 2.9	0.072, 0.084	360
2 (17)	SW-FC	3	8.38 ± 0.14	0.152 ± 0.019	102 ± 0.764	21 ± 0.58	0.02 ± 0.02	1.5 ± 0.78	1.7 ± 0.29	0.074 ± 0.005	240 ± 130
3 (10)	SW-MC	4	7.98 ± 0.14	0.108 ± 0.024	333 ± 71.8	46 ± 11	0.02 ± 0.01	4.1 ± 2.6	2.5 ± 1.9	0.096 ± 0.013	4,600 ± 7,600
4 (5)	SW-FC	3	8.07 ± 0.23	0.142 ± 0.060	670 ± 11.5	48 ± 3.6	<0.02	5.0 ± 0.75	4.5 ± 1.0	0.108 ± 0.008	1,800 ± 2,400
5 (13)	GW-FC	3	7.51 ± 0.08	0.201 ± 0.048	983 ± 54.9	160 ± 7.4	0.02 ± 0.01	11 ± 4.5	9.6 ± 2.7	0.121 ± 0.010	28,000 ± 40,000
6 (2)	SW-FC	4	7.28 ± 0.02	1.12 ± 0.349	139 ± 2.39	14 ± 0.96	0.02 ± 0.01	3.7 ± 0.69	3.6 ± 1.1	0.090 ± 0.019	3,600 ± 5,100
7 (3)	GW-FC	4	8.94 ± 0.23	1.46 ± 0.903	400 ± 32.0	51 ± 5.4	0.56 ± 0.31	11 ± 1.7	11 ± 2.1	0.263 ± 0.016	92,000 ± 168,000
8 (2)	SW-MC	3	8.20 ± 0.09	1.63 ± 0.517	467 ± 6.66	97 ± 5.3	<0.02	8.3 ± 4.3	5.1 ± 4.7	0.139 ± 0.041	385,000 ± 120,000

[a] SW, surface water source; GW, groundwater source; FC, utility performs chlorination to produce disinfectant residual; MC, utility performs chloramination to achieve disinfectant residual.

[b] SC was measured after sodium thiosulfate was added to water samples, potentially increasing SC values by approximately 80 μS/cm at 25°C. Thus, these data do not reflect the true SC of the tap water sampled but do reflect the SC of the tap water used in the experiments.

[c] For site 1, each data point is reported ($n = 2$ for all parameters, except $n = 1$ for HPC); for sites 2 to 8, averages and standard deviations are reported.

units (NTU) at site 3 and a high of 1.63 NTU at site 8. Turbidities were increased by a median of 110× (concentration factor) following UF (data not shown). Alkalinity results indicated that most of the tap waters had moderate to low buffering capacities, with the exception of site 5, which had an average alkalinity of 160 mg/liter as $CaCO_3$. Total iron concentrations in all nonconcentrated tap water samples were consistently low to nondetectable but were increased by a median of 100× during UF (ranging from a final concentration of 0.60 mg/liter Fe [site 4] to 59 mg/liter Fe [site 7]). TOC concentrations in tap water samples were also quite low and were found to be comprised primarily of DOC. TOC and DOC concentrations in UF concentrates were 40 to 96 mg/liter as C, levels that were 11 to 12 times higher than those in nonconcentrated tap water (data not shown). UV absorbance measurements were included in the study to enable calculations of specific UV absorbance (SUVA; ratio of UV absorbance at 254 nm [expressed per meter of path length] to DOC), a parameter suggested by previous researchers as being indicative of humic acid content and the potential presence of PCR inhibitors (1). Several sites had average SUVA values above 4 (the suggested lower limit for indicating that measured DOC is comprised primarily of humic compounds), including site 2 (SUVA = 4.4), site 3 (SUVA = 7.6), and site 8 (SUVA = 5.2) (data not shown). HPC concentrations varied greatly, with average HPC concentrations ranging from 240 CFU/ml at site 2 to 385,000 CFU/ml at site 8.

Microbial recovery. Measured microbial recovery efficiencies for the hollow-fiber UF procedure were 86% (ϕX174) to 100% and higher (MS2, *E. faecalis*, and *C. perfringens* spores) when recovery data for all eight cities were averaged together (Table 3). The cross-site UF recovery data were consistent for ϕX174, MS2, and *C. parvum*, with COV of 15, 18, and 11%, respectively. No study site was associated with UF recovery efficiencies that were significantly different from the average recovery efficiencies for all cities combined. In addition, water quality parameters were not generally observed to be associated with measured UF microbial recovery efficiencies. The only microbe for which statistically significant correlations were observed between water quality parameters and UF recovery efficiency was *E. faecalis*, with significant correlations observed for conductivity ($r = -0.84$; $P = 0.0047$), alkalinity ($r = -0.81$; $P = 0.0082$), TOC ($r = -0.85$; $P = 0.0034$), and $\log_{10}$-converted HPC ($r = -0.67$; $P = 0.047$).

Two Centricon Plus-70 units were used to process approximately 30% of each UF concentrate (i.e., ~120 ml of ~400-ml UF concentrates). Average ϕX174 recovery efficiencies ranged from 62% (site 6) to >100% (sites 1 and 5) (Table 3). Average MS2 recovery efficiencies ranged from 61% (site 6) to >100% (site 5). Although sites 5 and 6 did have very different water

TABLE 3. Average recovery efficiencies for total method procedure and constituent methods comprising the total procedure (UF, Centricon procedure, and IMS) for the different enteric microbes seeded into 100-liter tap water samples

Site	*n*	Avg recovery efficiency (%)[a]											
		ϕX174			MS2			*E. faecalis* (total)	*C. perfringens* spores (total)	*C. parvum* oocysts			*G. intestinalis* cysts (IMS)
		UF	Centricon	Total	UF	Centricon	Total			UF	IMS	Total	
1	2	97	110	100	ND	ND	ND	ND	ND	81	110	86	63
2	2	80	92	74	140	78	110	ND	ND	84	100	93	41
3	3	79	82	67	100	82	88	ND	ND	83	110	91	56
4	2	76	78	59	110	86	97	ND	ND	88	120	93	69
5	2	75	110	78	100	120	120	67	64	85	91	76	69
6	3	100	62	63	140	61	86	170	120	92	95	88	65
7	3	86	72	63	120	65	77	100	170	98	94	91	65
8	2	87	85	71	110	89	100	100	34	92	110	98	66
Cross-site avg		86	86	71	120	82	97	120	110	88	100	91	64
Cross-site SD		13	23	21	22	26	26	51	81	10	12	8.8	8.0

[a] ND, no data.

qualities (e.g., turbidity, conductivity, alkalinity, and TOC/DOC), their respective values for these parameters were not substantially different enough from data from other sites to enable an association of these water quality parameters with observed differences in the microbial recovery performance of the Centricon procedure.

IMS was performed for the separation of *C. parvum* oocysts and *G. intestinalis* cysts from the UF concentrate matrix. The IMS procedure was found to be more effective for recovering *Cryptosporidium* oocysts than *Giardia* cysts, but the procedure consistently achieved the same levels of performance for all the tap water samples tested (with the possible exception of site 2 for *Giardia* recovery) (Table 3). The cross-site average for *Cryptosporidium* IMS was 100% ± 12% (with a COV of 12%). The cross-site average for IMS recovery of the *Giardia* oocysts in the EasySeed control was 64% ± 8.0% (with a COV of 12%).

Average total method recovery efficiencies (incorporating UF only [for bacteria], UF plus Centricon units [for viruses], and UF plus IMS-IFA [for *C. parvum*]) were >50% for all microbes at each study site, with the exception of *C. perfringens* spores in tap water from site 8 (34%). The average cross-site total method recovery efficiencies were 71% ± 21% (ϕX174), 97% ± 26% (MS2), 120% ± 51% (*E. faecalis*), 110% ± 81% (*C. perfringens* spores), and 91% ± 8.8% (*C. parvum* oocysts). No water quality parameter was significantly associated with total method recovery efficiency when UF and a secondary concentration technique were incorporated.

Molecular analysis of UF-concentrated samples. A wide variation in real-time PCR and RT-PCR CP values was observed for microbes seeded into 100-liter tap water samples (i.e., MS2, *C. perfringens* spores, and *C. parvum*) (Table 4). The lowest CP values for MS2 were obtained with site 2 water samples (average CP value = 35.6); the highest CP values were obtained with site 5 and 7 water samples (for which one negative result was obtained for each site). *C. perfringens* spores were investigated as a water-seeding study parameter for sites 5 to 8; for these sites, average CP values ranged from 30.6 (site 8) to 36.1 (site 5). *C. parvum* oocysts were detected with average CP values of 25.0 (site 4) to 30.4 (site 5).

Data for the *Salmonella* extraction/inhibitor control indicated that water samples from sites 2 and 5 were associated with the poorest molecular detection performance, likely due to PCR inhibition and/or reduced nucleic acid extraction efficiency. However, no significant correlations were observed between *Salmonella* detection (i.e., CP values) and any of the water quality parameters studied. Tap water samples from sites 5 and 6 were associated with the greatest RT-PCR inhibition (~9 and ~7 CP value differences, respectively) for the HAV inhibitor control compared to the CP value (28.0) obtained when this assay was performed using the same HAV RNA amount in molecular-grade water. Tap water samples from all sites, except site 7, exhibited some level of RT-PCR inhibition (defined as a delay in amplification detection of at least 3 CP values). As observed for the *Salmonella* control, no significant correlations were observed between HAV external control detection (i.e., CP values) and any of the water quality parameters studied.

TABLE 4. Average real-time PCR and RT-PCR CP results for seeded microbes, extraction control, and inhibitor control

Site	CP value[a]				
	MS2	*C. perfringens* spores[b]	*C. parvum* oocysts	*Salmonella* serovar Typhimurium inhibitor control	HAV inhibitor control
1	35.7	NEG	25.4	36.9	31.3
2	35.6	NEG	30.3	41.8	31.9
3	38.0	NEG	27.2	38.1	31.2
4	36.9	NEG	25.0	37.8	31.2
5	40†	36.1	30.4	43[c]	37†
6	37†	34.5	26.6	38.5	35.2
7	40†	32.7	25.5	37.6	28.4
8	37.8	30.6	25.4	36.4	31.3
Cross-site avg	38	33.8	27.0	39	32
Cross-site SD	2.9	2.84	2.30	2.4	3.6

[a] †, one sample tested was negative; a CP value of 43 was assumed for data averaging and comparison.
[b] NEG, PCR tests for *C. perfringens* spores were negative for these tap water samples in which no *C. perfringens* spores were seeded.
[c] Both samples tested were negative; a CP value of 43 was assumed for data averaging and comparison.

DISCUSSION

UF is an emerging technique for accomplishing simultaneous recovery of viruses, bacteria, and parasites in relatively large-volume water samples (~100 liters). Research studies focused on developing hollow-fiber UF water sampling methods have shown that this technique can be effective for recovering diverse waterborne microbes (12, 15–17). These studies have shown that a number of procedures can be effective for minimizing attachment of microbes to UF membranes and for detachment of those microbes which do become adhered to UF membranes. Several of these procedures (calf serum blocking of UF membranes and NaPP amendment of water samples to minimize adsorption, elution of UF membranes with surfactants) have been incorporated into the UF method reported in the present study. This is the first report of a UF method that utilizes these three techniques. In addition, this is the first study of centrifugal UF (i.e., with Centricon Plus-70 microconcentrators) as a secondary concentration procedure for viruses in UF concentrates.

A total of 26 100-liter tap water samples, collected from eight U.S. cities representing six different hydrologic regions, surface water and groundwater sources, and chlorination and chloramination residual disinfection processes, were included in this study. As demonstrated by the water quality data summarized in Table 2, these tap water samples differed greatly in a number of important water quality parameters, including turbidity, SC, and organic carbon content. These water quality data represent a unique data set demonstrating the robustness of the UF procedure as well as providing insights into the water quality characteristics of UF concentrates. As anticipated, UF-concentrated tap water was found to contain higher concentrations of particulate and dissolved materials than did nonconcentrated tap water, as reflected by the calculated concentration factors for turbidity (median increase of 110×), total iron (100×), TOC (11×), DOC (12×), and UV absor-

bance (5×). Information regarding UF concentrate water quality is important because UF methods can capture more water constituents than microfiltration techniques do and therefore may increase the likelihood of reduced performance for downstream sample processing techniques (such as IMS) or analytical techniques (such as real-time PCR).

In general, high seeding levels (i.e., higher than likely background microbial concentration levels) were used to compensate for any low-level background presence of study microbes and to allow for direct determination of recovery efficiencies for the UF procedure (i.e., without incorporating secondary concentration techniques to enable microbial detection). The microbial recovery efficiency data in the present study show that the UF procedure can effectively concentrate and recover viruses, bacteria, and parasites. Microbial recovery efficiencies measured for the hollow-fiber UF procedure varied from a cross-site average of 86% ± 13% for ϕX174 to one of >100% for MS2, *E. faecalis*, and *C. perfringens* spores. These results are similar to recovery efficiencies (80 to 90%) for these microbes reported by Hill et al. for a different UF procedure (no calf serum blocking, with microbe desorption accomplished using surfactant backflushing instead of elution) developed using 10-liter tap water samples (12). In two early studies of hollow-fiber UF, Bicknell et al. and Dziewulski and Belfort reported recovery efficiencies of 76 to >100% and 80 to 100%, respectively, for poliovirus seeded into 100-liter tap water samples (5, 9). While no recent research studies have reported microbial recovery data for hollow-fiber UF techniques applied to large-volume finished water or tap water samples, the data from the present study are similar to virus recovery data (70 to 80% recovery efficiencies) reported by Olszewski et al. for 100-liter groundwater samples (16).

Recovery data of >120% reported for several microbes suggest that microbial aggregation in seed stocks could have contributed to these recovery efficiencies despite efforts to disperse the microbial stocks by using dilution in a PBS diluent containing Tween 80 and vigorous vortexing. In particular, the highly variable *C. perfringens* data may have been associated with the use of the *C. perfringens* "BioBalls" to seed the water samples. The BioBalls were produced by freeze-drying, which could have increased spore aggregation, although the vendor's guidance was followed for hydrating and disaggregating the BioBalls. However, when the recovery efficiency data for the full suite of seed microbes are considered, they consistently indicate that the UF protocol, with and without the Centricon procedure and IMS procedure, achieved effective microbial recovery performances for a wide range of tap water samples. The recovery efficiencies for the Centricon procedure indicate that this technique can be effective for recovering viruses from tap water concentrates. However, the approach used in this study required two Centricon Plus-70 units, which were used to process approximately 30% of the hollow-fiber UF concentrate volume. To process a greater proportion of the UF concentrate volume using the Centricon technique, either additional Centricon units would be needed (which would increase the cost of the procedure) or the two Centricon units would need to be double loaded. This is the subject of additional work by our research group.

The other secondary sample processing technique evaluated in conjunction with hollow-fiber UF was IMS. Although previous research indicated that IMS efficiencies can be affected by water quality parameters such as pH (14) and iron content (24), no specific water quality parameter was found to correlate with IMS efficiency data in this study. IMS was found to provide consistently high recovery performances for *C. parvum* (90 to 100%). These recovery efficiencies are similar to the IMS efficiencies (78 to 84%) reported by Fontaine and Guillot for IMS recovery of *C. parvum* oocysts seeded into 100-liter tap water samples, although these researchers used a different IMS kit than that used in the present study (10). IMS performance data in the present study were not as good for the *G. intestinalis* EasySeed control as those for *C. parvum*, but *G. intestinalis* IMS recovery efficiencies were consistently in the range of 50 to 70% (with the exception of those for water from site 2). The EasySeed *Giardia* IMS results reported in the present study for recovery from 100-liter tap water concentrates were slightly lower than the 75% ± 14% average recovery efficiency reported by Warnecke et al. for EasySeed *Giardia* added to 10-liter finished-water concentrates (23).

The real-time PCR and RT-PCR data indicate that the quality of 100-liter tap water samples can affect the performance of molecular methods for detecting waterborne microbes. However, statistical analysis of water quality data did not identify any water quality parameter that was significantly associated with inhibition of PCR or RT-PCR. *Cryptosporidium* detections at CP values of 25 to 27 suggest that the sampling and detection method described in this study can be effective for detection of lower *Cryptosporidium* levels than those used in this study. The *C. perfringens* PCR data demonstrate that this sampling and analytical protocol can be effective for consistently detecting 10,000 CFU of *C. perfringens* spores in 100 liters of tap water. The *Salmonella* PCR data show that consistent detection of 120 CFU of *Salmonella* was achieved when these bacteria were captured on a 47-mm membrane filter following UF.

A single bead beating-based nucleic acid extraction technique was used to extract and purify DNA and RNA from study microbes in liquid UF concentrates and from study microbes collected on membrane filters. The real-time PCR and RT-PCR data indicate that this nucleic acid extraction technique was effective, although RT-PCR inhibition (3 CP values and higher) was apparent for Centricon concentrates associated with seven of eight sites (based on HAV inhibitor control results), and PCR inhibition was consistently observed for samples associated with site 5. The volume concentration factor for the Centricon samples was >10,000× (100 liters to 2.1 ml), so it was not unexpected that RT-PCR inhibitors would be present in Centricon concentrates. However, these data do warrant additional research into improving the nucleic acid extraction procedure to achieve higher-purity DNA and RNA.

This study has shown that a UF-based procedure can be highly effective for simultaneously concentrating viruses, bacteria, and parasites in 100-liter tap water samples. Additional research is needed to compare this technique with established methods for sampling water for human pathogenic viruses (such as the EPA's virus adsorption-elution technique [20]) and parasites (EPA method 1623 [22]). In addition, the results for the Centricon sample processing technique suggest that this technique could be investigated further as a method for

simultaneously concentrating viruses, bacteria, and parasites in UF concentrates, which, if effective, would simplify the secondary sample processing performed in conjunction with the hollow-fiber UF procedure.

ACKNOWLEDGMENTS

This study was supported in part by funds provided by the American Water Works Association Research Foundation (AwwaRF) for AwwaRF project 3108.

We thank Michael Arrowood (CDC) for providing *C. parvum* oocyst stocks for this project and Jacquelin Roberts (CDC) for performing statistical analyses. We also thank the staff from the collaborating facilities for this project who collected and shipped tap water samples to CDC.

Use of trade names and commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the U.S. Department of Health and Human Services.

REFERENCES

1. **Abbaszadegan, M., P. Stewart, and M. LeChevallier.** 1999. A strategy for detection of viruses in groundwater by PCR. Appl. Environ. Microbiol. **65:**444–449.
2. **American Water Works Association Research Foundation.** 2004. Development of molecular methods to detect infectious viruses in water (no. 2591 90995F). American Public Health Association, Washington, DC.
3. **Arrowood, M. J., and K. Donaldson.** 1996. Improved purification methods for calf-derived Cryptosporidium parvum oocysts using discontinuous sucrose and cesium chloride gradients. J. Eukaryot. Microbiol. **43:**S89.
4. **Belfort, G., Y. Rotem, and E. Katzenelson.** 1974. Virus concentration using hollow fiber membranes. Water Res. **9:**79–85.
5. **Bicknell, E. A., D. M. Dziewulski, L. S. Sturman, and G. Belfort.** 1985. Concentration of seeded and naturally-occurring enteroviruses from waters of varying quality by hollow fiber ultrafiltration. Water Sci. Technol. **17:**47–62.
6. **Bisson, J. W., and V. J. Cabelli.** 1979. Membrane filter enumeration method for *Clostridium perfringens*. Appl. Environ. Microbiol. **37:**55–66.
7. **Clesceri, L. S., A. E. Greenberg, and A. D. Eaton (ed.).** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.
8. **Cromeans, T., H. A. Fields, and M. D. Sobsey.** 1989. Replication kinetics and cytopathic effect of hepatitis A virus. J. Gen. Virol. **70:**2051–2062.
9. **Dziewulski, D. M., and G. Belfort.** 1983. Virus concentration from water using high-rate tangential-flow hollow fiber ultrafiltration. Water Sci. Technol. **15:**75–89.
10. **Fontaine, M., and E. Guillot.** 2003. An immunomagnetic separation-real-time PCR method for quantification of Cryptosporidium parvum in water samples. J. Microbiol. Methods **54:**29–36.
11. **Guy, R. A., P. Payment, U. J. Krull, and P. A. Horgen.** 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. **69:**5178–5185.
12. **Hill, V., A. Polaczyk, D. Hahn, N. Jothikumar, T. Cromeans, J. Roberts, and J. Amburgey.** 2005. Development of a rapid method for simultaneously recovering microbes in drinking water using ultrafiltration with sodium polyphosphate and surfactants. Appl. Environ. Microbiol. **71:**6878–6884.
13. **Jothikumar, N., T. L. Cromeans, M. D. Sobsey, and B. H. Robertson.** 2005. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl. Environ. Microbiol. **71:**3359–3363.
14. **Kuhn, R. C., C. M. Rock, and K. H. Oshima.** 2002. Effects of pH and magnetic material on immunomagnetic separation of *Cryptosporidium* oocysts from concentrated water samples. Appl. Environ. Microbiol. **68:**2066–2070.
15. **Morales-Morales, H. A., G. Vidal, J. Olszewski, C. M. Rock, D. Dasgupta, K. H. Oshima, and G. B. Smith.** 2003. Optimization of a reusable hollow-fiber ultrafilter for simultaneous concentration of enteric bacteria, protozoa, and viruses from water. Appl. Environ. Microbiol. **69:**4098–4102.
16. **Olszewski, J., L. Winona, and K. H. Oshima.** 2005. Comparison of 2 ultrafiltration systems for the concentration of seeded viruses from environmental waters. Can. J. Microbiol. **51:**295–303.
17. **Oshima, K. H., T. T. Evans-Strickfaden, A. K. Highsmith, and E. W. Ades.** 1995. The removal of phages T1 and PP7, and poliovirus from fluids with hollow-fiber ultrafilters with molecular weight cut-offs of 50,000, 13,000, and 6000. Can. J. Microbiol. **41:**316–322.
18. **Parshionikar, S. U., J. Cashdollar, and G. S. Fout.** 2004. Development of homologous viral internal controls for use in RT-PCR assays of waterborne enteric viruses. J. Virol. Methods **121:**39–48.
19. **Polaczyk, A., J. Roberts, and V. Hill.** 2007. Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. J. Microbiol. Methods **68:**260–266.
20. **USEPA.** 2001. USEPA manual of methods for virology. EPA 600/4-84/013 (N14). USEPA Office of Research and Development, Washington, DC.
21. **USEPA.** 2001. Method 1602: male-specific (F^+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA 821-R-01-029. USEPA Office of Water, Washington, DC.
22. **USEPA.** 2001. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA-821-R-01-025. USEPA Office of Water, Washington, DC.
23. **Warnecke, M., C. Weir, and G. Vesey.** 2003. Evaluation of an internal positive control for Cryptosporidium and Giardia testing in water samples. Lett. Appl. Microbiol. **37:**244–248.
24. **Yakub, G. P., and K. L. Stadterman-Knauer.** 2000. Evaluation of immunomagnetic separation for recovery of *Cryptosporidium parvum* and *Giardia duodenalis* from high-iron matrices. Appl. Environ. Microbiol. **66:**3628–3631.