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Tangential-Flow Ultrafiltration with Integrated Inhibition Detection for Recovery of Surrogates and Human Pathogens from Large-Volume Source Water and Finished Drinking Water[▽]

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Tangential-flow ultrafiltration was optimized for the recovery of *Escherichia coli*, *Enterococcus faecalis*, *Clostridium perfringens* spores, bacteriophages MS2 and PRD1, murine norovirus, and poliovirus seeded into 100-liter surface water (SW) and drinking water (DW) samples. SW and DW collected from two drinking water treatment plants were then evaluated for human enteric viruses.

More than 140 microorganisms are known to be associated with waterborne diseases, including bacteria (*Campylobacter*, *Escherichia coli*, and *Vibrio cholerae*), protozoa (*Cryptosporidium* and *Giardia*), and viruses (norovirus [NoV], adenovirus [AdV], hepatitis A virus, and enterovirus [EV]) (35). Even with the advancement of drinking water (DW) treatment processes (e.g., membrane filtration and alternative disinfectants, such as ozone or UV radiation) and more stringent regulatory requirements, waterborne disease outbreaks (WBDOs) associated with drinking water still occur in the United States (25, 40). On average, between 1991 and 2002, 17 WBDOs associated with drinking water were reported annually in the United States, with the dominant etiologic agents being *Cryptosporidium*, *Giardia*, norovirus, and *E. coli* O157:H7 (7). Since 1971, approximately half of all reported WBDOs causing acute gastrointestinal illness were due to unidentified microorganisms, and symptoms were most often consistent with a viral etiology (35).

Recently, tangential-flow, hollow-fiber ultrafiltration (UF) has been used to investigate microbial contamination of drinking water (17, 33). By applying UF to concentrate microorganisms from water, limitations related to the direct analysis of small-volume grab samples—in particular, problems associated with detection sensitivity due to low concentrations of microbes—may be overcome. UF has previously been limited by suboptimal elution of the retained microorganisms (10). However, the application of membranes with ultra-low protein binding properties (e.g., polyethersulfone and cellulose acetate) in combination with nonionic surfactants and chemical dispersants has improved the recovery of microorganisms (19, 23). For this study, the use of UF to concentrate viral pathogens related to WBDOs was of particular interest. Human noroviruses (HuNoV) are estimated to cause more than 80% of all nonbacterial outbreaks of gastroenteritis in the United States and Europe (11, 27). Members of the *Caliciviridae* family, HuNoVs are 27- to 30-nm, icosahedral, nonenveloped human enteric viruses that cause acute gastroenteritis (14). Because of their nonenveloped structure, HuNoVs are relatively resistant to chemical inactivation (i.e., chlorination) and environmental degradation. However, the presence of infectious HuNoV has been difficult to study in environmental waters and finished drinking water supplies due to the lack of an *in vitro* cell culture system or small animal model (8). Thus, viral surrogates—bacteriophages (e.g., MS2), attenuated vaccine strains of poliovirus (PV), and feline calicivirus—have been utilized for studying the physicochemical properties of HuNoVs (13, 16). More recently, murine norovirus 1 (MNV-1) has been suggested as an effective surrogate for the study of HuNoV infectivity in the environment (3, 4, 31). MNV-1 is located within the *Norovirus* genus (genogroup V) of the *Caliciviridae* family and is morphologically and genetically similar to HuNoVs, unlike the aforementioned viral surrogates. In addition, MNV-1 is the only NoV that is amenable to routine growth in cell culture, thus making it ideal for the study of infectious HuNoVs (39).

Molecular assays, including real-time PCR and real-time reverse transcription-PCR (RT-PCR), are increasingly used to detect human enteric viruses, especially HuNoVs, in environmental water samples (1, 3). Real-time PCR and real-time RT-PCR are attractive for the assessment of viruses in environmental samples due to the rapid detection capabilities and high sensitivity and specificity of these assays. However, because of these characteristics, the inclusion of appropriate positive and negative controls, including detection of sample inhibition, is imperative. The presence of inhibitors (i.e., humic acids, bacterial debris, complex polysaccharides, and metal ions) in real-time PCR assays increases the difficulty of amplifying target nucleic acids (NA) and potentially leads to the reporting of false negatives (38). Detecting inhibition is of particular importance in environmental samples with low levels of viral contamination as this minimal viral load may be obscured during analysis. Mechanisms to limit or control molecular inhibitors include optimization of nucleic acid extraction and purification techniques and the use of internal standards

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for the detection of inhibition during nucleic acid amplification (24, 37).

The goal of the present study was to concentrate and recover viral surrogates (MNV-1, bacteriophages PRD1 and MS2, and PV) and endogenous human enteric viruses, including EV, human adenovirus (HuAdV), and human polyomavirus (HuPyV), from environmental waters (i.e., surface waters [SW] and finished DW) by utilizing tangential-flow, hollow-fiber UF and real-time PCR with detection of sample inhibition. To date, no studies investigating the recovery of infectious enteric viruses by UF have utilized MNV-1 or PRD1 as a model viral surrogate for HuNoVs and HuAdVs, respectively. Moreover, few studies have reported using tangential-flow UF for the recovery and molecular detection of viral surrogates and endogenous viruses from large-volume finished drinking water samples and their source waters (29, 32). Additionally, studies using molecular detection of viruses in UF concentrates have not controlled for potential false negatives by systematically evaluating sample inhibition.

Water. Water samples were collected from four different sites and included surface water (SW) ($n = 11$) and dechlorinated drinking water (DW) ($n = 12$). For seeding experiments, 100-liter DW samples ($n = 6$) were collected from a laboratory at Johns Hopkins Bloomberg School of Public Health in Baltimore, MD, and 100-liter SW samples ($n = 5$) were collected from a stream in Harford County, MD. Additional 100-liter water samples—not seeded with microorganisms—were collected from two drinking water treatment plants (DWTPs), one in California and the other in Maryland, and included both the source (surface) water ($n = 6$) and DW ($n = 6$). Water quality parameters for nonseeded samples were collected using onsite monitoring instruments and reported by the respective DWTPs. For seeded samples, water quality parameters were collected using an Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA), a Hach DPD free chlorine kit and DR/890 colorimeter (Hach, Loveland, CO), and a Hach 2100N turbidimeter (Hach). Laboratory DW and DWTP finished DW samples were dechlorinated by adding 100 ml of 200 mM sodium thiosulfate (Sigma) to achieve a final concentration of 0.2 mM sodium thiosulfate per liter. Dechlorinated samples were tested for free chlorine using a Hach DPD free chlorine kit and DR/890 colorimeter (Hach) to ensure that no residual chlorine remained. After dechlorination, the chemical dispersant sodium polyphosphate (NaPP) (Sigma) was added to each sample to a final concentration of 0.01% (19).

Microorganism preparation and assays. The following microbes were used in this study: *Enterococcus faecalis*, *E. coli* CN-13, *Clostridium perfringens* spores, PRD1 bacteriophage, MS2 bacteriophage, MNV-1, and PV. Stocks of *E. faecalis* (ATCC 29212) were generated by inoculating tryptic soy broth (TSB) (Invitrogen, Carlsbad, CA) with 5 μl of frozen stock and incubating overnight at 37°C with mixing. *E. faecalis* stocks were enumerated with an Enterolert Quanti-tray system (IDEXX Laboratories, Westbrook, ME) to determine the most probable number (MPN). *E. coli* CN-13 (ATCC 700609) stocks were produced as described for *E. faecalis* with the addition of 1% nalidixic acid solution (Sigma, St. Louis, MO) to the TSB. *E. coli* CN-13 stocks were enumerated with a Colilert Quanti-tray system (IDEXX Laboratories) to determine the MPN.

C. perfringens (ATCC 13124) spores were generated in modified Duncan-Strong sporulation broth by incubation at 42°C, anaerobically, for 48 h (9). *C. perfringens* cultures were pelleted and resuspended in Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen). *C. perfringens* spores were isolated from vegetative cells by heating for 30 min at 65°C and enumerated as described previously (2).

MS2 (ATCC 16696-B1) and PRD1 bacteriophages were generated using the double-agar-layer method with *E. coli* F_{amp} (ATCC 700891) and *Salmonella typhimurium* LT2 (ATCC 19585) bacterial hosts, respectively. Bacteriophages were extracted from cell lysates with an equal volume of chloroform (Sigma), centrifuged at 4,000 × *g* for 30 min at 4°C, sterile filtered, aliquoted, and stored at −80°C.

MNV-1 and PV stocks were generated in monolayers of RAW 264.7 cells (ATCC TIB-71) and BGMK cells, respectively, as previously described (3). MNV-1 and PV stock titers were determined by plaque assay as described in Bae and Schwab (3), with modifications for MNV-1. Briefly, 6-well tissue culture plates were seeded with RAW 264.7 cells at a concentration of 2×10^6 viable cells per well and incubated for 24 h at 37°C and 5% CO_2. Viral stock dilutions were prepared in complete Dulbecco's modified Eagle's medium (DMEM) with 2% low-endotoxin fetal bovine serum (FBS; Invitrogen) and 2.5 μg/ml Fungizone antimycotic (Invitrogen), and 200 μl was inoculated into each well. The plates were incubated at 37°C and 5% CO_2 for 1 h with continuous rocking, followed by removal of the inoculum and the application of 2 ml of prepared overlay medium (3). The plates were incubated at 37°C and 5% CO_2 for 24 h, and then each well was stained with an additional 2 ml of overlay medium supplemented with 1% 3.3 g/liter stock solution of neutral red (Invitrogen) for visualization of plaques.

Seeding experiments. Water samples were seeded with microbial surrogates to evaluate the recovery efficiency (RE) of UF. The total number of PFU or CFU added was determined for each experiment from the stock titer for each microorganism using the culture assays described in the present study. After the addition of microorganisms, water samples were held for 30 min prior to UF to allow microorganisms to acclimate. Surface water samples ($n = 5$) were assayed for the presence of bacterial indicators (total coliforms, *E. coli*, and fecal enterococci) before seeding. All SW ($n = 5$) and DW ($n = 6$) samples were assayed for study microorganisms after seeding and in the UF concentrates, using culture assays described in the present study. To minimize microbial aggregation, all dilutions of microbial stocks were prepared in a diluent containing 0.01 M DPBS, pH 7.40 (Gibco), 0.01% (wt/vol) Tween 80 (Sigma), and 0.001% antifoam A (Sigma) (34).

Ultrafiltration setup and procedure. Ultrafiltration was conducted as previously reported, with modifications (34). High-performance, platinum-cured LS/36 and LS/24 silicon tubing (Masterflex; Cole-Parmer Instrument Co, Vernon Hills, IL) was used. Following each experiment, the tubing was disinfected in 10% hypochlorous acid, rinsed with deionized water, and autoclaved at 15 lb/in^2, 121°C, for 15 min. All polypropylene NS4 quick-disconnect couplings (Colder Products Company, St. Paul, MN), screw clamps, brass fittings, rubber stoppers (Fisher Scientific), and polypropylene tanks (120 liters) (Nalgene Nunc, Rochester, NY) were autoclaved before

TABLE 1. Virus primers and probes

Microorganism	GenBank accession no.	Primer or probe	Primer/probe final concn (nM)	Probe label[a]	Sequence[b]	Product size (bp)	Product region[c]	Reference(s)
Murine norovirus	AY228235	MNVKS1	400		5′ AGGTCATGCGAGATCAGCTT 3′	159	ORF1	3
		MNVKS2	400		5′ CCAAGCTCTCACAAGCCTTC 3′			
		MNVKS3	200	FAM	5′ CAGTCTGCGACGCCATTGAGAA 3′			
Human norovirus GI	M87661	COG1F	1,000		5′ CGYTGGATGCGNTTYCATGA 3′	85	ORF1-ORF2 junction	22
		COG1R	1,000		5′ CTTAGACGCCATCATCATTYAC 3′			
		RING 1A	100	FAM	5′ AGATYGCGATCYCCTGTCCA 3′			
		RING 1B	100	FAM	5′ AGATCGCGGTCTCCTGTCCA 3′			
Human norovirus GII	AF145896	COG2F	1,000		5′ CARGARBCNATGTTYAGRTGGA TGAG 3′	88	ORF1-ORF2 junction	22
		COG2R	1,000		5′ TCGACGCCATCTTCATTCACA 3′			
		RING2-TP	200	FAM	5′ TGGGAGGGCGATCGCAATCT 3′			
Human adenovirus (Type A-F)	AC_000008	JTVXF	400		5′ GGACGCCTCGGAGTACCTGAG 3′	96	Hexon region	21
		JTVXR	400		5′ ACIGTGGGGTTTCTGAACTTGTT 3′			
		JTVXP	150	FAM	5′ CTGGTGCAGTTCGCCCGTGCCA 3′			
Human polyomavirus (JC and BK)		SM2	500		5′ AGTCTTTAGGGTCTTCTACCTTT 3′	173 (JC), 176 (BK)	Partial T antigen	28
		P6	500		5′ GGTGCCAACCTATGGAACAG 3′			
		KGJ3	400	FAM	5′ TCATCACTGGCAAACAT 3′			
Human enteroviruses	AJ293918	EV1R	700		5′ TGTCACCATAAGCAGCCA 3′	143	5′ UTR	15
		EV1F	700		5′ CCCTGAATGCGGCTAAT 3′			
		EV probe	120	FAM	5′ ACGGACACCCAAAGTAGTCG GTTC 3′			
Hepatitis G virus	U44402	HepG-F	400		5′ CGGCCAAAAGGTGGTGGATG 3′	185	5′ UTR	24, 36
		HepG-R	400		5′ CGACGAGCCTGACGTCGGG 3′			
		HepG probe	200	FAM	5′ AGGTCCCTCTGGCGCTTGTGG CGAG 3′			

[a] The FAM (6-carboxyfluorescein) quencher is BHQ-1 (Black Hole Quencher). The FAM quencher is a minor groove binder nonfluorescent quencher (MGBNFQ).
[b] Mixed bases in degenerate primers and probes are as follows: Y = C, T; R = A, G; B = C, T, G; N = A, C, T, G.
[c] ORF, open reading frame; UTR, untranslated region.

use. Disposable Baxter Exceltra Plus 210 dialysis filters (Baxter International, Deerfield, IL) composed of a cellulose triacetate filter with a molecular mass cutoff of ~70 kDa and surface area of 2.1 m^2 were utilized during UF. New filters were used for each experiment. A Cole-Parmer model 7524-40 peristaltic pump and Masterflex model 77800-52 pump heads were used for all experiments. Before filtration, ultrafilters were blocked with 0.1% NaPP, and filtration was conducted as described in Polaczyk et al. (34).

UF concentrate sample processing. Using a Centricon Plus-70 (Millipore) centrifugal filtration device with a molecular mass cutoff of 100 kDa, 70 ml of UF concentrates were further concentrated, following the manufacturer's protocol. Before secondary concentration, SW UF concentrate samples were preclarified by centrifugation at 5,000 × *g* for 5 min. Supernatant was removed and applied to the Centricon filter unit. The pellet was archived and processed separately during total viral NA extraction as described below. For the molecular analysis of viral surrogates and endogenous viruses, total viral NA was extracted from secondary concentrates and SW pellets archived from the clarified 70-ml UF concentrates. Two hundred-microliter amounts of the secondary concentrates and pellets were processed using QIAamp MinElute virus spin kits (Qiagen, Valencia, CA), following the manufacturer's protocol. Total viral NA was eluted from the Qiagen spin column by a double elution using, for each elution, 50 μl diethyl pyrocarbonate (DEPC)-treated water supplemented with 0.01% 500 U/μl RNase inhibitor (Applied Biosystems, Foster City, CA). Eluted NA was aliquoted and archived at −80°C until analysis.

Real-time PCR and RT-PCR. Amplification of viral DNA and RNA targets was performed using an ABI Prism 7300 sequence detection system (Applied Biosystems). In seeding experiments, an assay for MNV-1 was performed in a 96-well plate format as reported in Bae and Schwab (3). In nonseeding experiments, five different assays (pan-EV, HuNoV GI, HuNoV GII, HuAdV, and HuPyV) were performed separately, using 96-well plate formats (15, 21, 22, 28). The sequences, concentrations, and sources of the primers and probes used in this present study are shown in Table 1. All assays were validated using positive controls, i.e., viral RNA or DNA extracted from cell lysate stocks or, in the case of HuNoV, clarified 10% stool in DPBS, and negative controls consisting of nontarget NA and DEPC-treated water (Quality Biologicals, Gaithersburg, MD).

For viral RNA amplification, each 25-μl reaction mixture contained 12.5 μl of 2× master mix (QuantiTect probe RT-PCR kit [Qiagen],) 5 U RNase inhibitor (Applied Biosystems), custom primers (Invitrogen), and dually labeled TaqMan probes (Biosearch Technologies, Novato, CA), 5 μl of prepared sample, and DEPC-treated water for the remaining volume. Real-time RT-PCR amplification for four assays (MNV-1, HuNoV GI and GII, and pan-EV) was performed under the following conditions: reverse transcription for 30 min at 50°C and denaturation for 15 min at 95°C, followed by

TABLE 2. Recovery efficiency of microorganisms from 100-liter water samples during laboratory tangential-flow UF optimization by culture-based assays

Sample type (no. of samples)	Seed level	Avg % recovery (CoV)[a] of:						
		E. coli CN-13	*E. faecalis*	*C. perfringens*	MS2	PRD1	Murine norovirus 1	Poliovirus
SW (4)	High	68.3 (66.8)	56.4 (46.8)	ND	48.2 (51.3)	42.6 (74.4)	ND[d]	40.2
SW (1)	Low[b]	70.5	182.9	30.1	83.7	80.4	74.0	15.7
DW (3)	High	51.2 (9.5)	ND	78.7 (15.7)	58.2 (17.13)	ND	41.5 (82.4)	ND
DW (3)	Low[b]	47.9 (22.8)	52.9 (15.0)[c]	64.1 (13.0)	38.1 (32.1)	57.4 (28.3)[c]	ND[d]	ND

[a] CoV, coefficient of variation; ND, not done. DW was dechlorinated.
[b] Seeded at a low concentration (1 to 10 PFU or CFU per 100 ml).
[c] $n = 2$ samples.
[d] Recovery was assessed by real-time RT-PCR.

40 cycles of denaturation at 95°C for 15 s and primer annealing/extension at 60°C for 60 s. For viral DNA amplification, each 25-μl reaction mixture contained 10 μl of 2× master mix (QuantiTect probe PCR kit [Qiagen]), custom primers (Invitrogen), and dually labeled TaqMan probes (Biosearch Technologies), 5 μl of prepared sample, and DEPC-treated water for the remaining volume. Real-time PCR amplification for HuAdV and HuPyV were performed under the conditions described in Jothikumar et al. and McQuaig et al., respectively (21, 28). Dilutions of sample NA extracts were prepared in DEPC-treated water. All PCR analyses utilized a positive control for each target and DEPC-treated water as the negative control with each thermocycler run to ensure reagent and cycling efficiency.

Detection of sample inhibition. An internal standard for the identification of inhibition in real-time PCR and RT-PCR assays was prepared using hepatitis G virus (HGV) Armored RNA (Asuragen, Austin, TX). RNA was extracted from 140 μl HGV Armored RNA using a QIAamp viral RNA mini spin kit (Qiagen) following the manufacturer's spin protocol. The extracted RNA was then amplified by real-time RT-PCR. Primers and probe sequences and concentrations for the HGV assay are reported in Table 1, and amplification was performed as described previously, with modifications (24, 36). For each extracted water sample, inhibition was evaluated in a separate real-time RT-PCR assay. Each 25-μl reaction mixture was prepared as described above for viral RNA, with the inclusion of 2 μl of a 100-fold dilution of internal standard HGV RNA. Real-time RT-PCR amplification for HGV was performed under the same conditions described in the present study for MNV-1, HuNoV, and pan-EV. Each batch of samples assayed for inhibition included a negative control of HGV master mix containing no HGV RNA and at least 3 positive-control reaction mixtures containing only HGV RNA and no sample. For controls, 5 μl of DEPC-treated water was added to bring the reaction mixture volume to 25 μl.

Recovery and volume calculations and statistical analysis. For seeded samples, the percent recovery efficiency (RE) of microbial surrogates was calculated as the number of microbes recovered after UF divided by the number of microbes seeded, multiplied by 100. Coefficients of variation were calculated by dividing the standard deviations by their corresponding mean percent recoveries. Back volume values were calculated for molecular data in order to estimate the volume of preconcentrated sample analyzed by individual NA assays.

Microbial recovery in seeded DW and SW. The recovery efficiencies of microbial surrogates were assessed via standard culture methods. The average % RE results by culture methods for vegetative bacteria (*E. coli* and *E. faecalis*), viral surrogates (MS2, PRD1, MNV-1, and PV), and bacterial spores (*C. perfringens*) in SW and DW are shown in Table 2. Elution of SW samples resulted in lower % RE values for viruses and viral surrogates. These lower % RE values were apparent when the UF retentate was analyzed before and after the elution step (data not shown). Therefore, the elution step was only performed on DW samples and a subset of SW samples ($n = 7$).

Molecular analysis and detection of inhibition. The total viral NA extracted from the secondary concentrates and pellets of each seeded 100-liter UF concentrate were analyzed for MNV-1. The cycle threshold (C_T) values for MNV-1 real-time RT-PCR analysis, along with the estimated original sample volume analyzed, indicate consistent recovery of MNV-1 for source water type and seed level (Table 3). Total viral NA extracts from nonseeded 100-liter-UF concentrates were analyzed for HuAdV (types A to F), EV (pan-EV), HuPyV (JC and BK), and HuNoV (GI and GII). No human enteric viruses

TABLE 3. Real-time RT-PCR analysis of MNV-1 in seeded DW and SW sample total NA extracts[a]

Sample type	C_T value	Original sample vol analyzed (ml)
DW (high seed)	26.76	5.36
	25.39	4.48
	25.23	6.82
DW (low seed)	33.53	16.14
	35.42	4.91
	34.47	6.28
SW (high seed)	31.60	1.06
	29.55	0.93
	Undetermined	1.93
SW (low seed)	35.38	10.8
SW pellets (high seed)	34.52	Not applicable
	32.21	
	Undetermined	

[a] MNV-1, murine norovirus 1; DW, drinking water; SW, surface water; NA, nucleic acid.

TABLE 4. Back volume calculations to determine average final sample volume in tangential-flow UF concentrates, secondary concentrates, and total viral NA extracts

Sample type	No. of samples	Total sample vol (liters)	Avg vol (range) of concentrate (ml)	Total sample vol/ avg vol of concentrate (ml)	Avg total vol of:			
					Secondary concentrate[a] (ml)	Sample/ml of secondary concentrate (liters)	Viral NA extract (μl)[b]	Sample in 5 μl of NA extract (ml)
SW	11	100	314 (82–512)	459	4.82	13.9	91.7	74.8
DW	12	100	310 (100–510)	430	0.87	53.0	91.0	547.1

[a] Total secondary concentrate from 70 ml of UF concentrate.
[b] Total viral NA extract from 200 μl of secondary concentrate.

were detected in total viral NA extracts from nonseeded environmental UF concentrates. Negative and positive controls were negative and positive for all assays, respectively.

Inhibition was evaluated in seeded and nonseeded viral NA extracts from DW, SW, and pellets using the HGV RNA internal standard. Amounts of 5 μl of viral NA extracts were analyzed undiluted and/or at 10-fold and 100-fold dilutions to determine the level of inhibition. Sample inhibition had occurred if the sample's HGV RNA C_T value deviated from the average C_T value of the positive-control HGV RNA by one C_T value. Inhibition was detected in 22 of 29 samples analyzed. Analysis for target viral NA was then determined based on the dilutions where no inhibition occurred. For example, if total inhibition (i.e., a C_T value output of "undetermined") was identified in the undiluted sample but not at the 10^{-1} or 10^{-2} dilution, then analysis for target NA would be done at the 10^{-1} and 10^{-2} dilutions but not undiluted. If partial or no inhibition occurred, then undiluted and 10^{-1}-diluted portions were analyzed.

Back volume calculations. To determine the volume of the initial sample analyzed, back calculations from the total viral NA extracts were completed for each DW and SW sample. Table 4 shows the average volumes per sample processing step as they relate to the initial sample volume. SW and DW samples were grouped separately for clarity. The average sample volumes analyzed in a 5-μl real-time PCR or real-time RT-PCR reaction mixture were 75 ml and 547 ml for SW and DW, respectively. These volumes decreased by 10- or 100-fold as the sample was diluted to overcome the effects of inhibition.

This study describes an optimized tangential-flow, hollow-fiber UF method for the recovery of intact bacterial, protozoan, and viral surrogates from 100-liter SW and DW samples, with a focus on viruses. The recovery of traditional viral surrogates (MS2 and PV) in the present study was similar to the results reported previously in studies using tangential-flow, hollow-fiber UF methods for the concentration of microorganisms in 100-liter DW samples (20, 32, 34). The additional viral surrogates used in the present study, including PRD1 and MNV-1, have not been used in published studies involving UF of 100-liter environmental water samples. The bacteriophage PRD1 was chosen here as a suitable surrogate for the study of HuAdVs (12). Similar to HuAdVs, PRD1 is a 63-nm, icosahedral virus with double-stranded DNA, making it an appropriate model. MNV-1 has been utilized as a surrogate for HuNoV in studies evaluating resistance to heat and chemical (i.e., chlorine and monochloramine) inactivation, persistence in the environment, and bioaccumulation in bivalves (3, 6, 26, 31). However, MNV-1 has not been used in the evaluation of UF methods for the concentration of large-volume environmental water samples. This is the first study to utilize MNV-1 to optimize and evaluate a UF method.

Secondary concentration of the UF concentrates with the Centricon Plus-70 100-kDa filter units was used as an intermediate step for the extraction of total viral NA (18). Preclarification before secondary concentration of SW UF concentrates was demonstrated as a way to analyze the entire (i.e., pellet and supernatant) sample for viral surrogates and endogenous human enteric viruses.

Real-time RT-PCR inhibition ($\geq$1 C_T) was detected in NA extracts from seeded SW (including pellets) and DW samples that were undiluted (12 out of 16) and 10-fold diluted (7 out of 16), based on the HGV inhibition control analysis. Irrespective of this inhibition, the MNV-1 real-time RT-PCR data demonstrated that the optimized sampling and analytical protocol can be used for the consistent detection of MNV-1 in 100 liters of DW, while detection in SW was variable. As expected, similar levels of inhibition were also detected in nonseeded SW and DW samples. Additional research involving the application of commercially available NA extraction kits to UF secondary concentrates should be done to address issues of molecular inhibition. Published research has also suggested the elimination of molecular inhibition by utilizing Sephadex G-100 columns as a final NA purification step before real-time PCR analysis (5).

Since inhibition in real-time PCR and RT-PCR assays appears to be inevitable in environmental samples, the detection of inhibition is imperative for proper quality assurance/quality control during analysis and reporting. A simple system for the detection of sample inhibition utilizing commercially available RNA, similar to previously published research, was optimized in this study (24). The lack of detection methods for inhibition in environmental water samples is problematic, and providing a standardized method, as described in the present study, may assist in more widespread application.

Back volume calculations for each sample are also reported in this study. These calculations were done to demonstrate the estimated original sample volume that was analyzed during molecular analyses. These back calculations have often not been reported in previously published studies involving concentration from large volumes of water combined with real-time PCR (18, 30, 34). Not unexpectedly (due to greater turbidity) nearly a 10-fold difference in the original sample volumes analyzed was found between SW and DW. The ability to relate a C_T value output to a certain volume of water is

important when reporting the levels of human enteric viruses potentially present in the original 100-liter samples.

Conclusions. This study describes the application of UF to 100-liter SW samples and the incorporation of PRD1 or MNV-1 as a viral surrogate in the development of UF concentration methods for large volumes of water. By applying this UF method to DWTP source and finished waters, water utilities and regulators may have a better understanding of the microbial threats entering DWTPs. A methodology combining concentration from large-volume water samples by UF with real-time PCR detection allows for a more representative assessment of microbial water quality. The ability to analyze 100-liter samples of water within a 300-ml concentrate provides a more complete picture of true water quality than does a smaller-volume grab sample. In addition, the present study reports a standardized system for the detection of inhibition in environmental water samples that may be easily transferred to laboratories, using real-time PCR and RT-PCR for the detection of human enteric viruses.

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