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Concentration of Enteroviruses, Adenoviruses, and Noroviruses from Drinking Water by Use of Glass Wool Filters[▽]

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Available filtration methods to concentrate waterborne viruses are either too costly for studies requiring large numbers of samples, limited to small sample volumes, or not very portable for routine field applications. Sodocalcic glass wool filtration is a cost-effective and easy-to-use method to retain viruses, but its efficiency and reliability are not adequately understood. This study evaluated glass wool filter performance to concentrate the four viruses on the U.S. Environmental Protection Agency contaminant candidate list, i.e., coxsackievirus, echovirus, norovirus, and adenovirus, as well as poliovirus. Total virus numbers recovered were measured by quantitative reverse transcription-PCR (qRT-PCR); infectious polioviruses were quantified by integrated cell culture (ICC)–qRT-PCR. Recovery efficiencies averaged 70% for poliovirus, 14% for coxsackievirus B5, 19% for echovirus 18, 21% for adenovirus 41, and 29% for norovirus. Virus strain and water matrix affected recovery, with significant interaction between the two variables. Optimal recovery was obtained at pH 6.5. No evidence was found that water volume, filtration rate, and number of viruses seeded influenced recovery. The method was successful in detecting indigenous viruses in municipal wells in Wisconsin. Long-term continuous filtration retained viruses sufficiently for their detection for up to 16 days after seeding for qRT-PCR and up to 30 days for ICC–qRT-PCR. Glass wool filtration is suitable for large-volume samples (1,000 liters) collected at high filtration rates (4 liters min^{-1}), and its low cost makes it advantageous for studies requiring large numbers of samples.

Waterborne viruses are an important cause of disease, being responsible for 14% of outbreaks (9 of 64 cases) and 38% of illnesses (1,153 of 3,008 cases) associated with drinking water in the United States from 1999 to 2002 (21, 49). During the same period, noroviruses were responsible for 6% (8 of 66 cases) of outbreaks and 17% (348 of 2,093 cases) of illnesses associated with recreational water. If waterborne illnesses of unknown etiology during the period 1999–2002 are included in the above statistics, as these are believed to be of viral origin, up to 56% and 28% of illness cases associated with drinking water and recreational water, respectively, may be attributed to viruses.

To detect and quantify waterborne viruses from environmental samples, the first step in the protocol usually requires concentration from a large water volume. Several concentration methods have been developed and applied successfully in the past two decades (see reviews by Wyn-Jones and Sellwood [48] and Grabow [13]). These include adsorption onto (and subsequent elution from) electropositive cartridges and membranes (3, 25, 27, 29, 33, 35), gauze pads and glass powder (2, 9, 34), electronegative membranes, and microporous materials (1, 8, 12, 16, 20, 27) and concentration by ultrafiltration (15, 17, 36, 37) and ultracentrifugation (26). Adsorption onto electropositive cartridges, for example, the CUNO 1-MDS Virosorb filter, is currently the most popular method.

Sodocalcic glass wool offers a promising alternative as an adsorptive material for virus concentration. Glass wool, held together by a binding agent and coated with mineral oil, presents both hydrophobic and electropositive sites on its surface. When a virus suspension flows through the pore space of the packed material, the fiber surface is able to attract and retain negatively charged virus particles at near-neutral pH (7). The fibers are inexpensive and require no water conditioning outside of pH adjustment in some circumstances (30, 48). Glass wool has been used in virus monitoring studies involving wastewater (10), drinking water (14, 41, 46), groundwater (6, 30, 31, 43), river water (18, 41), and reservoirs (6, 43). However, only a handful of studies have attempted to quantify how effective glass wool is for concentrating viruses (7, 44, 45), and these examined only enteroviruses and rotavirus. Investigators using glass wool for quantitative virus monitoring have implicitly assumed 100% recovery (18, 31, 41) or an average of 40% (42). Such assumptions contribute additional uncertainty when virus data derived from glass wool concentration are used in exposure and risk assessment analyses (40, 42).

The objective of the present study was to validate the glass wool method for concentrating the four virus groups on the contaminant candidate list (CCL) of the U.S. Environmental Protection Agency (EPA), namely, coxsackievirus, echovirus, adenovirus, and norovirus (39). The validation was motivated by the need to collect more than 2,000 water samples to target these viruses as part of an ongoing epidemiological study on groundwaterborne disease transmission. If the standard elec-

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tropositive cartridge filter were used, concentration of such a large number of samples would be cost prohibitive. The validation focused on groundwater matrices at pH levels typically found in municipal drinking water, and as it was necessary in the epidemiological investigation to collect as large a water sample in as short a time as possible, virus recovery tests were conducted at filtration rates of 2 liters per minute or greater.

MATERIALS AND METHODS

Glass wool filter preparation. The method for constructing glass wool filters was derived from procedures described by Vilaginès et al. (45), the UK Environment Agency (7), and W. O. K. Grabow (personal communication). Oiled sodocalcic glass wool (Bourre 725QN; Saint Gobain, Isover-Orgel, France) was rinsed for 15 min with 18-mohm reverse osmosis (RO) water, washed for 15 min with 1 M HCl, rinsed again with RO water, washed with 1 M NaOH for 15 min, and finally rinsed with RO water until the pH was 7.0. Washed glass wool was stored in phosphate-buffered saline (PBS) at 4°C.

Glass wool was packed into columns to a density of 0.5 g cm^{-3} dry weight by use of a metal plunger. The following three column sizes were used in these experiments, depending on the filtration rate: 16-mm-diameter by 6.6-cm polyethylene tubes for a filtration rate of 0.5 liters min^{-1}, 3.8-cm-diameter by 10.2-cm polyvinyl chloride (PVC) threaded pipes with caps for a filtration rate of 2 liters min^{-1}, and 5.1-cm-diameter by 10.2-cm PVC threaded pipes with caps for a filtration rate of 4 liters min^{-1}. Packed columns were flushed with PBS (pH 7.0) prior to use.

Virus stocks. The following six viruses were used to evaluate the recovery capabilities of glass wool filters: poliovirus Sabin type 3, coxsackievirus B5, echovirus 18, adenovirus 41, norovirus GI, and norovirus GII. The Sabin type 3 poliovirus is an attenuated vaccine strain. Coxsackievirus B5, echovirus 18, adenovirus 41, norovirus GI, and norovirus GII had previously been isolated from patients and serotyped by the Wisconsin State Laboratory of Hygiene. Concentrated stocks of poliovirus Sabin type 3, coxsackievirus B5, echovirus 18, and adenovirus 41 were obtained by cell culture, and after cytopathic effects were complete, the cultures were freeze-thawed three times, followed by removal of cell debris at 900 × *g* for 10 min. Noroviruses GI and GII were extracted from stool specimens by vortex mixing the specimens with PBS and 1,1,2-trichlorotrifluorethane (Freon; Sigma T-5271), centrifuging the mixture, and retrieving the aqueous phase. All virus preparations were stored at −80°C.

Water. Recovery experiments were conducted with the following three water matrices. The first matrix was tap water from Marshfield, WI, which is groundwater treated at a conventional municipal treatment plant that includes sand filtration and chlorination at pH 8.0. The water was dechlorinated with sodium thiosulfate and its pH adjusted to 7.0 with HCl before glass wool filtration. The second and third matrices were groundwater from two drilled wells near Marshfield, WI, with one drawing from a glacial till aquifer, pH 7.2 (well 1), and the other drawing from a Precambrian granite aquifer, pH 6.8 (well 2). These water matrices did not require dechlorination or pH adjustment prior to glass wool filtration. None of these groundwater sources is under the influence of surface water. In addition, as a test of field capability, glass wool filtration of untreated municipal drinking water from groundwater sources was performed in 15 Wisconsin communities. When necessary, the water pH was lowered to neutral by continuously injecting 1N HCl with a high-pressure precision peristaltic pump (Cole-Parmer model K-07520-50; pump head model K-77250-62).

Seeding experiments. Viruses were seeded "live" into 10-, 20-, or ~1,500-liter volumes of the water matrix to be tested at concentrations ranging from 8.5×10^0 to 2.7×10^7 genomic copies $liter^{-1}$. Virus stock solutions were diluted with a small volume of sterile water on the day of the recovery trial and then mixed into the entire volume of water to be filtered. The seeded water was pumped by peristaltic pump from carboys or large plastic garbage cans through a glass wool filter. All tubing and containers had previously been sterilized with 0.5% chlorine for at least 30 min.

The effect of pH on glass wool concentration of viruses was evaluated by adjusting the pH of dechlorinated tap water to end values of between 6.0 and 9.0 (poliovirus recovery) and between 6.0 and 7.5 (adenovirus recovery). Two or four trials of 20-liter volumes were conducted at each pH. Because it is known that virus concentration with electropositive filters is very inefficient at pHs of ≥8, investigating the pH range from 6 to 7.5 was of primary interest, while tests at pH 8 and 9 were performed separately as an additional check.

Besides grab samples, the capability of glass wool filters to adsorb and retain viruses during long-term continuous sampling was also evaluated. Two trials were conducted, each with four glass wool filters connected in parallel to a single manifold fed water from a faucet at well 2. Effluent from the filters passed through an activated carbon filter to trap all viruses released from the glass wool during the experiments. At the start of each trial, three filters were seeded with poliovirus Sabin type 3 (3.4×10^8 genomic copies in trial 1 and 3.6×10^8 genomic copies in trial 2) by injecting 1 ml virus stock diluted into 30 ml of water directly into the glass wool filter by use of a syringe. The fourth filter remained unseeded. Water was then flushed continuously at 200 ml min^{-1} through all four filters, and periodically over the course of 10 days (trial 1) or 30 days (trial 2), one of the seeded filters was removed and tested for the amount of poliovirus retained. At the end of the trials, the fourth unseeded filter was returned to the laboratory, seeded with the same quantity of poliovirus as its three companion filters, and flushed with 300 ml well 2 water to evaluate the effect of long-term water flushing on recovery efficiency.

Filter elution and flocculation. Viruses were eluted by saturating the filter with 3% beef extract (wt/vol) containing 0.5 M glycine (pH 9.5). The elution buffer was kept in contact with the glass wool for 15 min before an additional volume was syringed through the filter and finally evacuated with air. The eluent was adjusted to pH 7.0 to 7.5 with 1 N HCl and then flocculated with polyethylene glycol 8000 (8% [wt/vol]) and NaCl (final concentration, 0.2 M). This mixture was stirred for 1 h at 4°C, incubated overnight at 4°C, and centrifuged at 4,200 × *g* for 45 min at 4°C. The pellet was resuspended in 2 ml of sterile 0.15 M Na_2HPO_4 solution (pH 7.0). This final concentrated sample volume (FCSV) was stored at −80°C.

Virus quantification. Viral nucleic acids were extracted from 140 μl of FCSV with a QIAamp DNA blood mini kit and buffer AVL (Qiagen, Valencia, CA) to yield a viral nucleic acid suspension of 50 μl.

Two-step reverse transcription-PCR (RT-PCR) was performed to quantify enteroviruses (poliovirus Sabin type 3, coxsackievirus B5, and echovirus 18). Extracted RNA (8.6 μl) was mixed with 8.6 μl of nuclease-free water and 0.7 μl (0.007 μg μl^{-1}) of random hexamers (Promega, Madison, WI). The mixture was heated for 4 min at 99°C and then supplemented with 32.1 μl RT master mix containing the indicated final concentrations of the following components: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, a 70 μM concentration of each deoxynucleoside triphosphate (Promega), 30 U of RNasin (Promega), and 100 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies, Rockville, MD). The reaction mix was incubated at 25°C for 15 min, 42°C for 60 min, 99°C for 5 min, and then 4°C until PCR amplification. The RT mixture and reaction conditions for noroviruses were the same, except that the initial 8.6 μl of extracted RNA was added to 8.05 μl of nuclease-free water and 1.25 μl of reverse gene-specific primer (final concentration, 250 nM).

Quantitative PCR (qPCR) was performed on a LightCycler 1.2 machine (Roche Diagnostics, Mannheim, Germany), using PCR mixes prepared with a LightCycler DNA master hybridization probe kit (Roche Diagnostics), with fluorescence generated by TaqMan probes (TIB Molbiol, Berlin, Germany). The sources of the PCR primers and hybridization probes and their final concentrations used in the present study were as follows: enteroviruses (5), 300 nM forward primer, 900 nM reverse primer, and 100 nM probe; adenoviruses (4), 500 nM primers and 100 nM probe; and noroviruses (19), 250 nM primers and 100 nM probe. Reactions were not multiplexed. All reaction mixtures contained 4 mM $MgCl_2$. Amplification reactions for enteroviruses and adenoviruses started with a hot start polymerase activation step for 10 min at 95°C, followed by 45 cycles of 15 s at 94°C and 1 min at 60°C. For noroviruses, thermal conditions were 10 min at 95°C, followed by 45 cycles of 15 s at 94°C, 20 s at 55°C, and 15 s at 72°C (19).

RT-PCR controls for each batch of reactions included an extraction negative control (unseeded FCSV), negative controls for the RT and PCR cocktails, and a positive control of known low viral concentration seeded into an FCSV matrix. This positive control also served as the LightCycler reference control, validating the use of the standard curves. qRT-PCR inhibition was evaluated by seeding 800 copies of hepatitis G virus (HGV) armored RNA (Asuragen Inc., Austin, TX) into the RT reaction mixtures for 10 samples from the three water sources (3, 4, and 3 samples from tap water, well 1, and well 2, respectively). The 10 samples had glass wool-filtered volumes of 20 liters. qRT-PCR was performed as described above, using HGV primers provided by the manufacturer and a laboratory-designed probe. Inhibition was considered absent when the crossing point of the HGV-seeded samples was less than one cycle higher than that for the inhibition reference control (crossing point = 32). PCR inhibitors were not detected in the three water sources. HGV is used in our laboratory as an inhibition control to avoid completely the increased contamination potential that would result if an aliquot of every FCSV were seeded with every target virus to test for virus-specific inhibition. The assumption is that HGV emulates the PCR inhibition level of the other viruses.

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TABLE 1. Recovery of viruses by glass wool filtration[a]

Virus group	Virus strain	Water type	No. of trials	Vol of water filtered (liters)	Filtration rate (liters min^{-1})	Concn range seeded (genomic copies $liter^{-1}$)	Mean recovery (%)	Coefficient of variation (%)
Enterovirus	Poliovirus	Tap	12	10	2	3.5×10^5–3.8×10^5	98	24
	Poliovirus	Well 1	7	20–1,597	2, 4	2.7×10^4–2.7×10^7	56	81
	Poliovirus	Well 2	6	20–1,439	2, 4	3.0×10^4–2.7×10^7	31	45
	Coxsackie B5	Well 1	6	20	2	3.2×10^6–6.6×10^6	12	33
	Coxsackie B5	Well 2	6	20	2	3.2×10^6–6.6×10^6	15	62
	Echovirus 18	Well 1	6	20	2	2.8×10^6–3.4×10^6	15	76
	Echovirus 18	Well 2	6	20	2	2.8×10^6–3.4×10^6	24	79
Adenovirus	41	Tap	16	10–1,500	0.5–4	8.5×10^0–1.7×10^3	28	51
	41	Well 1	8	20	2	8.1×10^2–1.6×10^3	22	72
	41	Well 2	8	20	2	8.1×10^2–1.6×10^3	8	47
Norovirus	GI	Well 1	2	20	2	1.1×10^7	33	2
	GI	Well 2	2	20	2	1.1×10^7	45	1
	GII	Tap	3	20	2	1.3×10^3	30	91
	GII	Well 1	10	10–20	2	1.9×10^5–5.0×10^6	32	45
	GII	Well 2	6	20	2	1.9×10^5–5.0×10^6	16	21

[a] Each row represents the summary recovery for a specific virus strain and water type, averaged over the volume filtered, filtration rate, and concentration range of the seeded virus.

Standard curves were established by treating stocks of each virus type with Benzonase (Novagen, Madison, WI) for 30 min at 37°C, followed by incubation for 2 days at 4°C, leaving only the nucleic acid contained within intact capsid-protected virions and removing extraneous viral nucleic acid that would have inflated the estimate of genomic copy number. Viral RNA or DNA mass was measured fluorometrically using RiboGreen (Molecular Probes, Eugene, OR) or PicoGreen (Molecular Probes) and a CytoFluor series 4000 fluorimeter (Applied Biosystems, Framingham, MA) and then converted to genomic copies based on the nucleic acid molecular weight of that virus (32). Intact viruses were serially diluted, and each dilution was seeded into separate 0.14-ml volumes of negative FCSV and extracted using a QIAamp DNA blood mini extraction kit (Qiagen). Therefore, the standard curves represent the entire quantitation process and include any matrix effects from the elution and flocculation procedures. Crossing points were calculated automatically by the LightCycler instrument by the second-derivative-maximum method and plotted against the decimal logarithm of viral RNA or DNA concentration.

Infectious enterovirus numbers were measured for long-term continuous filtration trials. A negative-strand/positive-strand RNA hybrid, a marker of infectious replicating virus, was quantified by qRT-PCR following the methods of Cromeans et al. (4). A 0.1-ml volume of FCSV was inoculated into each of six flasks containing Buffalo green monkey kidney monolayers. One flask was harvested at selected time points from 4 h to 44 h postinoculation, and the quantity of negative/positive-strand hybrids was determined for each time point. The following exponential growth model was fit to the increase in hybrids over time: $N_T = N_0 e^{rt}$, where N_T is the number of hybrids at time T, r is the specific growth rate, and t is time. N_0, the number of infectious genomes present at time zero in the FCSV, was obtained by solving for the y intercept of the exponential phase of the growth curve after natural log transformation.

Controls for native viruses and glass wool effect on lowering PCR inhibition. For each set of trials in which a particular water matrix was tested, two unseeded water samples of the same volume as the seeded sample were filtered through two glass wool filters. One filter was processed as the negative control to check for native viruses, which, if present, would have inflated the recovery estimates. No native viruses were detected at any time in the three water sources used for recovery experiments. The second filter was eluted and flocculated, and the resulting FCSV was seeded with the same quantity and type of virus as the corresponding water sample. Viral nucleic acid was extracted from the seeded FCSV and enumerated for virus genomic copies following the qRT-PCR or qPCR method described above. The number of genomic copies in the seeded FCSV was used as the denominator for calculating virus percent recovery. Ensuring that viruses were in the same eluate matrix in determining the numerator and denominator of the percent recovery calculation was necessary because it was discovered that passing the eluent through the glass wool fibers reduced the level of PCR inhibition introduced by the beef extract and water sample. If viruses were seeded into FCSV medium not previously passed through glass wool, the PCR was slightly inhibited; the resulting lower concentration value would decrease the denominator and yield a falsely elevated percent recovery (data not shown).

Recovery calculation and statistical analysis. Percent recovery was calculated as the genomic copy number of the virus recovered after filtration of the water sample divided by the genomic copy number of the virus seeded into the FCSV of the unseeded water sample multiplied by 100. Linear mixed-effect models (23) were used to evaluate the association between percent recovery (the dependent variable) and the following five independent variables: virus type, water matrix, water volume filtered, virus amount seeded, and filtration rate. Since the distribution of recovery percentages was skewed, natural log transformation was applied prior to fitting of models. All models included a random "day" effect, defined as the variation observed in the same filtration experiment performed on different days, using one to five replicates each day, to account for any day-to-day variation in experimental results. All analyses were conducted using SAS release 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

Glass wool filters were effective at concentrating all the CCL viruses and poliovirus, although the levels of recovery differed among virus types (Table 1). Considering each individual filtration trial across the three water matrices, recovery efficiencies ranged from 17% to 155% for poliovirus ($n = 25$), 5% to 32% for coxsackievirus B5 ($n = 12$), 4% to 60% for echovirus 18 ($n = 12$), 4% to 58% for adenovirus 41 ($n = 32$), and 7% to 60% for noroviruses ($n = 23$). Poliovirus in tap water had the highest recovery efficiency, whereas adenovirus 41 in well 2 water had the lowest.

The initial step in the statistical analysis was to fit a model containing the random day effect and fixed effects for virus type, water matrix, water volume filtered (natural log transformed), virus amount seeded ($\log_{10}$ transformed), and filtration rate. After controlling for day-to-day variability, the following three variables were found not to be associated with virus recovery: water volume filtered ($P = 0.44$), quantity of virus seeded ($P = 0.62$), and filtration rate ($P = 0.51$). Only the effects for virus type ($P = 0.009$) and water matrix ($P = 0.013$) attained statistical significance. Subsequently, a model with the random day effect and fixed effects for virus type, water matrix, and their interaction was fit. Both virus type and water matrix were found to be highly significant variables in explaining differences in virus recovery (Table 2). The interaction term was statistically significant ($P = 0.003$), and hence, comparisons of

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TABLE 2. Results of mixed-model analysis of effects of virus type and water matrix on virus recovery

Effect	Numerator df[a]	Denominator df[a]	*F* value	*P* value
Virus type	5	71	10.02	<0.0001
Water matrix	2	71	4.61	0.0131
Virus type and water matrix	7	71	3.44	0.0032

[a] df, degrees of freedom.

virus types were performed within categories of water matrix and vice versa. For example, norovirus GII and poliovirus had statistically significant different recovery rates only for tap water, and adenovirus and norovirus GI had such differences only for well 2 water, while the difference in recovery between adenovirus and poliovirus was significant for all three water matrices. As an example of the effect of water matrix, tap and well 1 waters had statistically different recovery rates only for poliovirus, while tap and well 2 waters were significantly different with respect to adenovirus and poliovirus recovery. Well 1 and well 2 waters differed in recovery for only adenovirus and norovirus GII. This interaction is the reason that recovery efficiencies presented in Table 1 are subdivided by both virus type and water matrix.

The effect of pH was evaluated by controlling for a single water matrix, tap water, and adjusting the pH between 6.0 and 9.0 for poliovirus recovery and between 6.0 and 7.5 for adenovirus 41 recovery. Poliovirus recovery was maximal at a pH of 6.5 and declined with increasing pH, reaching a value close to zero at pH 9.0 (Fig. 1). A similar trend was observed for adenovirus 41, with maximum recovery at a pH between 6.0 and 6.5 and substantially diminished recovery at pH 7.5 (Fig. 2).

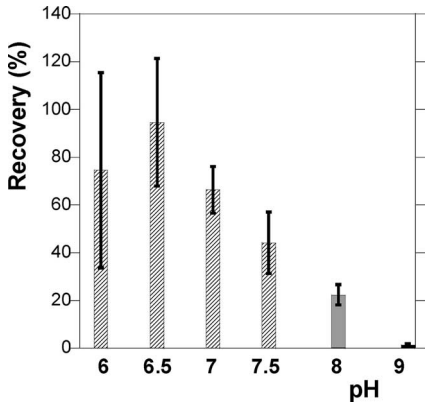

FIG. 1. Effect of pH on efficiency of glass wool concentration of poliovirus in tap water. Recovery within the pH range of 6.0 to 7.5 was evaluated in two experiments, each with two filtration trials per pH level. Each histogram (hatched bars) represents the combined results of experiments 1 and 2 (four replicates). Experiment 1 was performed by seeding 6.39×10^7 genomic copies in 20 liters; experiment 2 was done with 1.14×10^8 genomic copies seeded in 20 liters. Recovery at pH 8.0 and 9.0 was evaluated in two additional separate experiments of four replicates each, i.e., experiment 3 (gray bar), performed at pH 8 with 3.13×10^6 genomic copies seeded in 10 liters, and experiment 4 (black bar), performed at pH 9 with 7.64×10^6 genomic copies seeded in 10 liters. Error bars represent 1 standard deviation.

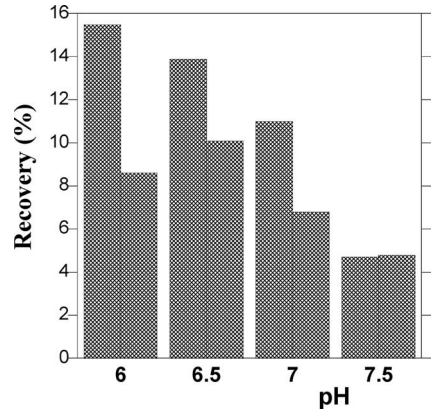

FIG. 2. Effect of pH on efficiency of glass wool concentration of adenovirus 41 in tap water (5.25×10^3 genomic copies were seeded into 20 liters). Two filtration trials were conducted at each pH value.

To test the operation of glass wool filtration under field conditions, drinking water was sampled from the distribution systems of 15 Wisconsin communities. No difficulties unique to glass wool filtration were encountered, and the goal of collecting samples larger than 1,000 liters at filtration rates of 2 to 4 liters min^{-1} was achieved (Table 3). The filter size used in these field trials was a 3.7-cm inner diameter by a 10.2-cm length, packed with 90 g (washed weight) of glass wool. The glass wool filters were judged effective in the field, as evidenced by the concentration of enteroviruses and adenoviruses from community water systems (Table 3). Ideas for improving field sampling with glass wool filters arose during this part of the study and were incorporated into the final standard operating procedure. The improvements included using a high-pressure precision-motor peristaltic pump to deliver HCl for pH adjustment, placing the entire apparatus (pump, acid bottle, glass wool filter, and totalizing flow meter) in an enclosed sealed box with only the inlet and outlet tubing and power cord showing, using easy on-off fittings, and attaching warning and safety signs to the sampling box so it could be left unattended as the sample was collected.

In testing the possible effect of long-term continuous sampling on virus recovery, where poliovirus was seeded on day 0 and then the filters were flushed continuously with virus-free water for the rest of the testing period, viruses were still detectable by qRT-PCR after 10 days of water flushing in trial 1 and after 16 days in trial 2 (Table 4). Infectious viruses were recovered at higher efficiencies than were total viruses, and they remained detectable even after 30 days of water flushing (Table 4). To check for possible water flushing effects on the efficiency of glass wool during long-term continuous sampling, two unseeded glass wool filters were flushed with virus-free water from well 2 for 10 or 30 days before being spiked with poliovirus. Recovery efficiencies were 17% total genomic copies after 10 days of water flushing and 9% after 30 days of water flushing.

DISCUSSION

Glass wool filtration proved to be an effective means of concentrating the four enteric viruses on the U.S. EPA's CCL

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TABLE 3. Enterovirus and adenovirus levels in drinking water distribution systems of 15 nonchlorinating Wisconsin municipalities sampled with glass wool filters

Municipality	Vol of water sampled (liters)	Ambient water pH	Adjusted pH[b]	Filtration rate (liters min^{-1})	No. of enterovirus genomic copies $liter^{-1}$[a]	No. of adenovirus genomic copies $liter^{-1}$[a]
1	1,245	7.60	NA	4.0	19.2	ND
2	1,257	8.00	6.98	4.1	13.8	ND
3	1,185	7.07	NA	3.9	7.6	ND
4	1,476	7.17	NA	3.8	12.2	ND
5	1,821	7.27	NA	5.5	5.9	ND
6	1,514	7.37	7.00	4.6	0.2	ND
7	1,401	7.36	6.90	4.8	ND	ND
8	1,647	6.94	NA	5.4	0.8	ND
9	1,401	7.18	6.74	4.9	ND	ND
10	1,514	7.78	6.98	5.0	ND	ND
11	1,620	7.79	7.02	4.3	ND	ND
12	1,658	7.47	6.84	4.2	ND	0.07
13	1,139	7.38	6.92	3.3	ND	0.02
14	1,370	7.32	7.06	3.7	0.4	0.01
15	1,431	7.30	6.91	4.7	ND	ND

[a] Every sample was evaluated for PCR inhibition by seeding of an aliquot of the FCSV with HGV (see Materials and Methods), and if necessary, inhibition was corrected by diluting the nucleic acid extract 1:5 or 1:10 with nuclease-free water. ND, not detectable.
[b] NA, not applicable.

from water. How effective glass wool is depends on the type of virus, water pH, and water matrix. The water matrix effect was not likely a result of confounding by PCR inhibition because, at least as tested by HGV seeding, inhibition was not detected in samples from the three water sources. There was an interaction between virus type and water matrix with an effect on virus recovery, meaning that, ideally, like for other virus concentration methods, in a field study the recovery efficiency should be checked for each water matrix and virus type of interest. Glass wool filters are simple to construct from inexpensive materials and simple to use in field settings. After concentration, the virus suspension eluted from the filter is compatible with quantification by PCR and cell culture. As far as we know, the present study provides the most comprehensive evaluation of glass wool filter performance to date, using a variety of environmental and sampling conditions and virus types.

TABLE 4. Effectiveness of long-term continuous glass wool filtration for poliovirus recovery

Trial	Continuous time of filter operation (days)	Total virus genomes recovered		Infectious virus genomes recovered	
		No. of genomic copies	% Recovered	No. of genomic copies	% Recovered
1[a]	3	6.6×10^7	19.4	3.4×10^5	17.0
	8	1.5×10^7	4.4	4.4×10^5	22.0
	10	1.5×10^7	4.5	3.0×10^5	15.0
2[b]	7	6.6×10^7	18.3	6.2×10^5	34.4
	16	2.6×10^5	0.07	2.2×10^4	1.2
	30	ND[c]	ND	6.0×10^3	0.3

[a] In trial 1, a total amount of 3.4×10^8 poliovirus genomic copies was seeded on day 0, of which 2.0×10^6 were from infectious virions (0.6%). Unchlorinated tap water was then continuously passed through the filter for the specified number of days, with no viral seed. Both trials 1 and 2 were performed with well 2 water.
[b] In trial 2, a total amount of 3.6×10^8 poliovirus genomic copies was seeded on day 0, of which 3.6×10^6 were from infectious virions (1%). Unchlorinated tap water was then continuously passed through the filter for the specified number of days, with no viral seed.
[c] ND, not detectable.

Virus recovery measured in the present study compares favorably with values observed in previous glass wool validation studies. The average poliovirus recovery rate across the three water matrices was 70%, within the ranges of 62% to 77% and 60% to 83% reported by Vilaginès et al. (44, 45), and the 70% to 91% range noted in the UK Environment Agency study (7). These three past studies adopted working parameters different from those used here, such as filtration rate, water source, and filter dimensions, making direct comparison of recovery efficiencies equivocal. It is also important that the virus enumeration techniques used were not the same; specifically, Vilaginès et al. (45) and the UK Environment Agency (7) used a plaque assay. The observation that recovery seemed independent of the amount of virus seeded in the present study is consistent with results reported by Vilaginès et al. (45) for poliovirus. The average recovery rates of coxsackievirus and echovirus observed in our study were 14% and 19%, respectively, which are much lower than the recovery rates for the same viruses reported by Vilaginès et al. (45). The average recovery rates of norovirus and adenovirus were 29% and 21%, respectively. No previous recovery studies of norovirus and adenovirus with glass wool are available.

A variety of other filtration methods to concentrate viruses from water have been studied, and their performance can be compared to that of glass wool. Recoveries of 51% and 4% to 24% were observed for poliovirus, using electronegative cellulose acetate/nitrate and glass borosilicate filters, respectively (8). Haramoto et al. (16) obtained values ranging from 37% to 100% recovery of poliovirus by using various electronegative nitrocellulose filters and of 19% for electronegative glass filters, with significantly higher values if the filters were coated with cations. Electropositive filters showed average poliovirus recovery rates of 73% for MK filters, 90% for 1MDS filters (25), and 69% for Zetapor glass filters (16). Sobsey and Glass (35) recovered 60% and 56% of seeded poliovirus with electronegative Filterite and electropositive AMF Cuno Zeta Plus 50S filters, respectively. A positively charged filter developed

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by Li et al. (22) recovered 89% to 96% of poliovirus, as well as 50% to 95% of coxsackievirus B3 and 92% of echovirus 7. Coxsackievirus B3 was also recovered at efficiencies of 33% and 96% with MK and 1MDS filters, respectively (25). Ultrafiltration is another method for the recovery of viruses from environmental waters. Ultrafiltration systems were observed to recover ranges of 82% to 90% (hollow fiber) and 43% to 95% (tangential flow) of poliovirus 2 in groundwater when the retentate was recirculated (28, 47). Hill et al. (17) used ultrafiltration and different water amendments to recover echovirus 1, with efficiencies between 49% and 97%.

Glass wool recovery efficiency was significantly affected by water pH, similar to the pH dependence of other virus concentration techniques relying on electronegative and electropositive media (48). In the present study, virus recovery decreased substantially at pHs of >7.5, although in contrast, Vilaginès et al. (45) did not observe significant variations in virus recovery by using glass wool over the pH range of 7.1 to 8.2. According to the U.S. EPA protocol for using 1MDS filters, the water pH must be adjusted downward only when it is above 8.0 (38). Glass wool, thus appears to have a narrower range of acceptable ambient pH values. Our current guideline for glass wool filtration is to adjust the pH to 7.0 if the ambient pH is ≥7.5. It is important that compared to that of poliovirus, the isoelectric points of the other common enteroviruses are lower (11), which means that they would be more strongly electronegative than poliovirus at near-neutral pH and should attach as well or better to positively charged surfaces. Because waterborne viruses present different isoelectric points and adsorption-desorption behaviors (11), it may be advisable to optimize the pH adjustment to the specific virus types and waters to be tested.

For sampling in the field from wellheads and drinking water distribution systems, the glass wool filters showed no operational difficulties. The filters never clogged, broke, or leaked and could be left unattended for weeks. If necessary for highly turbid water, a prefilter could easily be installed and eluted along with the glass wool filter. Large volumes and high filtration rates did not pose any problems; the 2- to 4-liters min^{-1} filtration rate used in the present study exceeded the previously reported maximum filtration rates of 1.7 liters min^{-1} (44, 45) and 0.27 liters min^{-1} (7). Another advantage was that passing water samples through glass wool appeared to diminish PCR inhibition, an observation also noted by van Heerden et al. (41).

Glass wool was found to have sufficient adsorptive capacity and strength to be used in long-term continuous sampling. The continuous sampling experiments simulated the extremes along the temporal continuum of virus occurrence, where virus is present in the water source on only the first day of sampling and needs to be retained for the remainder of the sampling period or where no virus is present until the last sampling day, after the filter has been flushed for an extended period of time. Indeed, poliovirus seeded on the first day was still detectable by qRT-PCR after 16 days and still detectable by cell culture after 30 days. Glass wool appears to be more effective in retaining infectious intact virus than naked viral RNA or partially degraded virions that have lost their infectivity. This is possible if the factors affecting adsorptive strength, such as charge, isoelectric point, size, and hydrophobicity, are more favorable for infectious intact virus. How much of the virus loss was due to desorption versus decay is unknown. After being flushed with water for 30 days, the glass wool filter had a poliovirus recovery efficiency of 9%, suggesting that the filter did not work as well at the end of the sampling period as at the beginning, but still it exhibited some adsorptive capacity. Under more realistic conditions in the environment, viruses are probably present constantly in the water or appear intermittently over the course of sampling and are present at much lower concentrations than the seeded concentration used in these experiments. The effects of these temporal patterns and virus concentrations on the effectiveness of long-term continuous sampling with glass wool filters should be evaluated further.

The primary limitation of the glass wool method in our hands was that recovery efficiencies were highly variable. For example, poliovirus recovery rates for the three water matrices tested had coefficients of variation ranging from 24% to 81% (Table 1), whereas Vilaginès et al. (45) reported coefficients of variation of between 8% and 40%. The reasons for the variation are unknown. Perhaps the filter construction and operating protocols need further standardization. Variability in recovery is not specific to glass wool filtration and has been observed for other adsorption-elution methods (16, 48) as well as for ultrafiltration methods (28). In considering the entire molecular biology-based analytical process for detecting viruses in water, the volume of water filtered and the presence of PCR inhibitors in the water likely have a more significant impact on the overall method detection limit than does the recovery efficiency of the virus concentration method (24).

The primary benefit of the glass wool method is its low cost. If 1MDS filters were used for the 2,000 water samples required for the epidemiological study mentioned in the introduction, the direct cost without including institutional overhead would be $340,000. A glass wool filter has a one-time PVC housing cost of $3.65 (the housings are sanitized and reused), and the expendable glass wool cost is $0.75 per filter. In our laboratory, the glass wool washing step has been semiautomated, and packing it into the housing is performed with a modified handpress. After washing the filters, one person can assemble 40 glass wool filters in about 4 h. Considering supplies and all labor, we estimate the cost of 2,000 glass wool filters to be $20,000, a savings of $320,000. Ultrafiltration has a one-time equipment cost of $6,000 to $16,000, and the reusable filters (hollow fiber or tangential flow) cost $250 to $1,500 (28). Glass wool filters give virus recovery efficiencies that are comparable to those of other concentration methods at a fraction of the cost. It is now affordable to take large numbers of samples for viruses, the very kind of sampling program that is necessary to better understand the fate and distribution of human pathogenic viruses in the environment.

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