Comparison of filters for concentrating microbial indicators and pathogens in lake-water samples

Running Title: Filters for concentrating microorganisms in lake water

Donna S. Francy,1# Erin A. Stelzer,1 Amie M.G. Brady,1 Carrie Huitger,1 Rebecca N. Bushon,1 Hon S. Ip,2 Michael W. Ware,3 Eric N. Villegas,3 Vicente Gallardo,4 and H.D. Alan Lindquist4

U.S. Geological Survey, Ohio Water Science Center, Columbus, Ohio1 and National Wildlife Health Center, Madison, Wis.2; U.S. Environmental Protection Agency, National Exposure Research Laboratory3 and National Homeland Security Research Center4, Cincinnati, OH

#Corresponding author at U.S. Geological Survey, 6480 Doubletree Ave., Columbus, OH 43229, USA. Tel: +1 614 430 7769; fax: +1 614 430 7777. E-mail address: dsfrancy@usgs.gov
Abstract

Bacterial indicators are used to indicate increased health risk from pathogens and to make beach closure and advisory decisions; however, beaches are seldom monitored for the pathogens themselves. Studies of sources and types of pathogens at beaches are needed to improve estimates of swimming-associated health risks. It would be advantageous and cost effective, especially for studies on a regional scale, to use a method that can simultaneously filter and concentrate all classes of pathogens from the large volumes of water needed to detect pathogens. In seven recovery experiments, stock cultures of viruses and protozoa were seeded into 10-L lake-water samples, and concentrations of naturally-occurring bacterial indicators were used to determine recoveries. For the five filtration methods tested, the highest median recoveries were as follows: glass wool for adenovirus (4.7%); NanoCeram for enterovirus (14.5%) and MS2 coliphage (84%); continuous flow centrifugation (CFC) +Virocap for Escherichia coli (68.3%) and Cryptosporidium (54%); automatic ultrafiltration (UF) for norovirus GII (2.4%); and dead-end UF for Enterococcus faecalis (80.5%), avian influenza virus (0.02%), and Giardia (57%). In evaluating filter performance in terms of both recovery and variability, the automatic UF resulted in the highest recovery while maintaining low variability for all nine microorganisms. The automatic UF was used to demonstrate that filtration can be scaled up to field deployment and the collection of 200-L lake water samples.

[Introduction]

To protect beachgoers from illnesses associated with fecal contamination from sewage and other sources, officials generally rely on quantification of bacterial indicators to make decisions about beach closures and advisories. Concentrations above established standards indicate an
unacceptable human health risk from the possible exposure to human or animal waste and
pathogenic microorganisms. In 2007 and 2008, it was reported that E. coli O157:H7, Shigella,
Cryptosporidium, and norovirus caused outbreaks of illness in the United States as a result of
recreational exposure to contaminated waters (1). In recreational epidemiological studies,
diarrhea and respiratory ailments are commonly reported health outcomes, and it is believed
that these may be associated with a variety of unidentified enteric viruses (2).

There is no current legal requirement to examine recreational waters for pathogenic
microorganisms. There is wide recognition, however, that bacterial indicators may not be
adequate indicators of all types of pathogens. A few studies describe concentrations of
pathogens in recreational waters, but these were generally small scale, local investigations (2,
3, 4, 5, 6). The exception was a 15-mo study of pathogens and indicators at 25 freshwater
recreational and water supply sites in New Zealand (7), the results of which were used to better
understand pathogen presence and sources of fecal contamination in recreational waters,
develop a quantitative microbial risk assessment for campylobacteriosis, and derive new
national freshwater recreation guidelines for New Zealand. Studies of sources and types of
potential pathogens present at beaches on a regional scale in other areas can provide similar
benefits. These types of studies can also provide data on the relationships of bacteria indicator
concentrations and environmental and water-quality parameters that are used to provide
estimates of swimming-associated health risks to pathogen concentrations.

Because pathogens are typically found in low numbers in environmental waters, it is necessary
to concentrate relatively large sample volumes. It would be advantageous and cost effective,
especially for studies on a regional scale, to use a method that can simultaneously target all classes of pathogens (viruses, bacteria, and protozoa), provided the method can provide acceptable and consistent recoveries. We evaluated two types of filtration approaches—virus adsorption-elution (VIRADEL) and ultrafiltration (UF). The VIRADEL filtration method is primarily used for recovering human enteric viruses from water matrices and concentrates viruses by charge interactions (8), with only limited testing of other microorganisms (9, 10). Ultrafiltration is a physical removal process, and has been shown to effectively concentrate viruses, bacteria, and protozoa simultaneously (11).

This study was done to test and evaluate five filtration methods for recovery of seeded viruses and protozoa and naturally-occurring bacterial indicators in lake-water samples. One method was applied in a field setting. Although only lake-water samples were tested during this study, the results may be applicable to other types of water samples.

Materials and methods

Sites and sampling methods

The study included seven recovery experiments with 10-L lake water samples collected during June–Nov, 2010–11 at one inland lake (site 1) and six Great Lakes beaches (Fig. 1). Field deployment studies (described below) were done during Aug–Sept 2010 at two sites in Ohio and at five sites in Wisconsin (Fig. 1). Site names, latitude and longitudes, study dates, and water-quality data are presented in supplemental materials (Table S1).

For recovery experiments, 10 L of lake water was collected into sterile carboys for seeding, and one 3-L sample was collected for direct processing without filtration. Samples were collected
by immersing carboys or bottles below the water surface at the center of the swimming area where water depths were 0.5 to 1.0 m. Lake samples were kept on ice and transported to the USGS Ohio Water Microbiology Laboratory in Columbus, OH (USGS Lab) for further processing. Two types of recovery experiments were designed—single and variability experiments. Single experiments were when multiple carboys were collected, and one carboy was subsequently seeded for each filter method. For variability experiments, multiple carboys were collected, and triplicate filtrations for each filter method were run on the same day. Typically, 2 to 3 d were required to conduct all triplicate filtrations for a variability experiment. For all experiments, unseeded controls were collected, filtered, and analyzed within 24 h of collection. The unseeded controls added a duplicate (single studies) or quadruplicate (variability studies) sample for some organisms.

In the USGS Lab, duplicate measurements of turbidity were made with a portable turbidimeter (Hach Company, Loveland, CO) and pH was measured by established USGS methods (12), both from the 3-L sample. If the pH of the sample was greater than 7.0, it was adjusted to pH 6.5–7.0 by adding 0.5 N HCl to the 10-L sample before seeding and filtration. Reusable equipment was washed and sterilized as described elsewhere (13). To measure any potential for contamination, one equipment blank was processed for each filtration method. Equipment blanks were 10 L of unseeded dechlorinated tap water that were filtered and processed in the same manner as regular samples.
Microorganisms and seeding

Stock cultures of MS2 coliphage (an F-specific coliphage), enteric viruses (adenovirus, enterovirus, norovirus GII, and avian influenza virus), and protozoan pathogens (Cryptosporidium parvum and Giardia lamblia) were used to seed 10-L water samples. Enterococci and Escherichia coli were not seeded so that recovery of these naturally-occurring bacterial indicators (often abundant in lake-water samples) could be determined. Wide ranges of seed amounts for coliphage and enteric viruses and of concentrations of naturally-occurring bacterial indicators were included (Table 1). Concentrations for seeding were established to ensure each microorganism was recovered after filtration. Seed amounts were representative of moderate to worst-case scenarios in natural settings.

MS2 coliphage (ATCC 15597-B1) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained per ATCC instructions. Mahoney strain Poliovirus (belongs to the enterovirus group and will hereafter be referred to as “enterovirus”) and adenovirus serotype 41 were made from pure culture inoculated cell lines, propagated and provided by other researchers (U.S. Environmental Protection Agency, Cincinnati, OH; The University of North Carolina at Chapel Hill, Chapel Hill, NC). Norovirus GII stock culture was made by treating a norovirus GII positive stool sample (obtained from the Ohio Department of Health) with Vertrel XF® (Miller-Stephenson Chemical Company, Inc, Danbury, CT), a hydrocarbon degreasing compound. Avian influenza virus (AIV) strain A/turkey/Minnesota/3689-1551/1981 (H5N2) was propagated by inoculation into the allantoic sac of day eight specific pathogen-free embryonated chicken eggs and incubated at 37.2°C and 50% relative humidity for three days at the USGS National Wildlife Health Center (NWHC), Madison, WI. The titer of the AIV recovered on June 27, 2017 by guest http://aem.asm.org/ Downloaded from
from the allantoic fluid following incubation was quantified by inoculating serial dilutions, and the resultant 50% egg infectious dose (EID_{50}) was calculated (14). Enterovirus, adenovirus, and norovirus GII were diluted in phosphate buffered saline (Hardy Diagnostics, Santa Maria, CA), MS2 coliphage stocks were diluted in tryptic soy broth (TSB), and AIV was diluted in Viral Transport Medium (VTM) (15) for seeding.

Parasitic cysts and oocysts were propagated and flow sorted at the U.S. Environmental Protection Agency (USEPA), National Exposure Research Laboratory (NERL), Cincinnati, OH. C. parvum oocysts (Iowa strain) were propagated and purified as previously described using sucrose and cesium chloride floatation (16). G. lamblia cysts (H3 strain) were propagated in Mongolian gerbils and purified using sucrose floatation followed by Percoll sedimentation (17,18). Oocysts and cysts were used within 2 mo and 3 wk of purification, respectively. The parasite seeds of 100 or 500 cysts and oocysts were prepared and verified as previously described for C. parvum, except the FACS Aria II (BD Biosciences, San Jose, CA) and Aqua-glo (Waterborne Inc., New Orleans, LA) were used for detection and sorting. Prepared seeds were stored and shipped overnight at 4°C to the USGS Lab and used within ten days of preparation (19). Shipment temperature conditions and spike stability were verified with the Thermocron® i-button tracking system (Maxim, Inc., Sunnyvale, CA) and trip controls (20). Stock tubes of protozoan oocysts and cysts were rinsed three times with 1 mL of 0.001% Tween 20 for seeding 10-L water samples.
Filtration and post-filtration processing

Each 10-L carboy was filtered with a different filtration method, described in detail in supplemental materials (Figs S1-S5). General steps are shown in Fig. 2 and described below.

Three VIRADEL methods and two UF methods were tested. For most experiments, permeates (from the waste stream) from each filtration were analyzed for *E. coli* and F-specific coliphage to determine if these target organisms were lost as a result of crossing the filter medium and entering the waste stream. These microorganisms were chosen as representatives of filter compromise for bacteria and viruses.

VIRADEL filtration methods

The VIRADEL filtration method concentrates microorganisms through charge interactions by use of an electropositive filter. These filters were designed specifically to concentrate negatively-charged viruses. This study included the following three VIRADEL filters:

- Glass-wool fiber filter (special order from the USDA Agricultural Research Station, Marshfield, WI). For this method, the pH of the sample must be adjusted to pH 6.5–7.0 before filtration (21).

- NanoCeram® filter (Argonide, Inc., Sanford, FL), 2 μm average nominal pore size

- Continuous flow centrifugation (CFC) with ViroCap® capsule filter (CFC+ViroCap) (Scientific Methods, Granger, IN). The ViroCap contains a NanoCeram cartridge filter incorporated into a 12.7 cm disposable capsule.

The 10-L sample was pumped through the filter and microorganisms were eluted and concentrated for glass-wool filtration (21, 22) (Fig. S1) and NanoCeram filtration (23) (Fig. S2).
Aliquots for bacteria, coliphage, and protozoa analyses were removed from the filter eluate for glass-wool filtered samples collected at sites 1–4 (original procedure) and from all NanoCeram samples. For glass wool filtered samples collected at sites 5–7, aliquots for bacteria, coliphage and protozoa analyses were removed from the final concentrate (modified procedure) so that larger proportions were analyzed for these microorganisms (9). For CFC+Virocap filtration, the 10-L samples were pumped through the CFC bowl and ViroCap filter in sequence and were eluted and concentrated from each (8) (Fig S3). Aliquots for bacteria and protozoa were removed directly from the CFC bowl eluate for samples collected at sites 1–4 (original procedure). For samples collected at sites 5–7, the CFC bowl eluate was further concentrated by centrifugation and the resultant CFC bowl eluate concentrate was used for bacteria and protozoan analyses. For all CFC+Virocap samples, coliphage analyses were done from the filter eluates. For all VIRADEL methods, enteric virus analyses were done from the final concentrates (Fig. 2). Comparisons between the original and modified procedures for the glass-wool and CFC+ViroCap filters showed no consistent differences in recoveries of affected microorganisms (data not shown).

Ultrafiltration methods

Ultrafiltration (UF) methods rely on size-exclusion and have pore sizes rated by molecular weight cutoffs which enable concentration by sieving, instead of adsorption or sedimentation (24). The ultrafilters used in this study were Rexbrane Membrane High-Flux, REXEED-25S (Asahi Kasei Kuraray Medical Co., Ltd., Japan) with a molecular weight cutoff of 29,000-Da, surface area of 2.5 m², and fiber inner diameter of 185 μm. Two UF methods were included in this study:
An automatic tangential flow UF sampler (automatic UF) provided by USEPA, National Homeland Security Research Center (Teledyne Isco, Lincoln, NE)

Dead-end UF

The 10-L sample was pumped through and eluted from the automatic UF sampling device (25) (Fig. S4) and the dead-end UF (26) (Fig. S5). A computer controlled system automates the process of concentrating microorganisms in the automatic method. The dead-end UF differs from automatic UF in that dead-end UF involves a single pass of water that is not recirculated. For both UF methods, the filter eluate was centrifuged and the resultant pellet was used for bacteria and protozoa analyses and the supernatant for coliphage analysis (Fig. 2). Enteric virus analyses were done from the final concentrates.

Bacterial indicator and coliphage quantification

Enterococci and E. coli were enumerated by use of standard membrane filtration methods on mEI agar (27) and modified mTEC (28), respectively. F-specific coliphage were enumerated by use of the single-agar layer procedure (29). This method detects any F-specific coliphage that is able to infect the host bacterium (E. coli F<sub>amp</sub>) and produce a circular lysis zone (plaque); MS2 is one strain of F-specific coliphage and was used to seed water samples in recovery experiments.

Protozoan quantification

Cryptosporidium and Giardia were isolated and enumerated using USEPA Method 1623 with heat dissociation (30, 31). Processed samples were shipped overnight at 4°C from the USGS Lab to the USEPA. One IMS reaction was performed per sample. In highly turbid samples, an additional 10 mL deionized water rinse was added after the first IMS purification. The slides...
were stained with EasyStain™ G&C (BTF Pty Lmt., North Ryde, NSW, Australia) following the manufacturer’s protocol except steps 3, 6, and 7 were omitted.

**Enteric virus quantification**

Viral RNA and DNA were extracted from 400 μL of the final concentrates using the QIAamp DNA Mini Extraction kit (Qiagen, Valencia, CA) according to manufacturer’s instructions, except the AL general lysis buffer was substituted for the AVL viral lysis buffer with the addition of carrier RNA (Qiagen, Valencia, CA). Of the 100 μL of extracted concentrate, 5 μL was analyzed by use of quantitative polymerase chain reaction (qPCR) for adenovirus (32) or quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for enterovirus (33) and norovirus GII (34).

PCR inhibition was determined using matrix spikes by seeding the sample with an extracted positive control virus in a duplicate qPCR or qRT-PCR reaction. The concentration of target virus in the sample was then compared to the concentration of target virus in the clean matrix control that was seeded with the same extracted positive control virus. Sample extracts were considered inhibited and were diluted if the seeded test sample was >2 Ct cycles higher than the seeded clean matrix control.

The standard curves for molecular detection of adenovirus, enterovirus, and norovirus GII were created using virus stocks treated with Benzonase™ (Novagen, Madison, WI), as described previously (21) except that the treated stocks were incubated overnight at 37° C as recommended by Novagen, instead of 30 minutes at 37°C and 2 days at 4°C. Treated stocks were extracted and the amount of virus RNA or DNA was measured by using RiboGreen™ or PicoGreen™ (Molecular Probes, Eugene, OR) using a spectrophotometer, and the number of
genomic copies (gc) was calculated. After quantification, viral stocks were serially diluted using a 2% beef extract solution. Each standard point was extracted in duplicate and then tested by qPCR or qRT-PCR in duplicate on every plate.

Samples for AIV were quantified as described elsewhere (35). Briefly, avian influenza viral RNA was recovered from a 50 μl aliquot of the eluate using the Ambion MagMax AI/ND viral RNA kit according to manufacturer’s instructions. Viral RNA was eluted in a total of 50 μl and 8 μl of the RNA was quantified in a qRT-PCR test to the matrix gene of the AIV genome. A standard curve was generated in each experiment using RNA extracted in a similar manner from AIV stocks of known concentration and the curve used to calculate the amount of AIV recovered. The qRT-PCR assay is capable of quantification of between 2.5-2.5x10^7 EID₅₀ units of virus per reaction.

Sample inhibition was detected by spiking with a heterologous AIV RNA (H7) to the recovered eluate in parallel qRT-PCR assays (35).

**Field deployment experiments**

Eight samples were collected during field deployment experiments at seven sites (Fig 1, Table S1). Sources of fecal contamination at sites 2 and 4 in Cleveland, OH, include urban storm water runoff and large populations of birds. An adjacent stream with combined sewer overflows and a wastewater pump station additionally affects water quality at site 4. The Wisconsin beaches are located in Manitowoc County and are potentially impacted by agricultural sources and storm-water runoff. Water quality at sites 8 and 11, located within the city of Manitowoc, is affected by the Manitowoc River. Site 12 is located in the small
community of Two Rivers, and potential sources include storm sewers and the Twin River. Site 10 is 11 miles north of Manitowoc in a recreational and agricultural area.

For field experiments, ambient concentrations of microorganisms were determined and samples were not seeded. The automatic UF method (24) was selected because a portable unit was available at the time and this method provided acceptable recoveries in early experiments. From the UF sampling unit, approximately 9 m of sterile inlet tubing was attached to the middle of a bar anchored to the lake bottom where water depths were 0.6–0.9 m. For some samples, a 100-mesh Alsco pre-filter (Alsco Industrial Products, Lithia Springs, GA) with 150 μm openings was attached in-line outside the sampling unit to filter algae and other large particles and prevent filter clogging (supplemental materials, Fig. S6.A). In substitution of or in addition to the pre-filter for some samples, a 14-cm long tubing end connector with irregular holes (approximately 2 mm in diameter) was attached to the end of the inlet tubing out in the lake (Fig. S6.B). As described above for recovery experiments, one 3-L sample was collected for direct processing of bacterial indicators and coliphage and for measurements of turbidity.

Recovery calculations and statistical analysis

For recovery and field deployment experiments, concentrations of each microorganism were determined by applicable analytical methods in the initial seed or in the grab sample for unseeded microorganisms (enterococci and E. coli) and from post-filtration processed samples specific to the target organism and filter.

The data from recovery experiments were analyzed to determine percent recoveries of each organism using different filtration methods. The recoveries, as calculated, do not distinguish
between the effects of the matrix from the effects from filtration, processing, and analysis.

Recoveries for seeded microorganisms were not adjusted for concentrations found in unseeded lake-water samples; in most cases, these concentrations were negligible as compared to seeded amounts. Percent recoveries were calculated for each result, as follows:

(1) \[ \text{Percent recovery (of each organism)} = \frac{\text{no. recovered in post-filtration sample}}{\text{no. in seed or in unseeded grab sample}} \times 100 \]

Median percent recoveries were calculated by organism and by filter. A non-parametric analysis of variance (ANOVA), the rank transform test, and the Tukey-Kramer multiple comparison test were used to compare median recoveries among all filter types for each organism.

A ranking system was developed to gain insight into which filter methods provided the highest recoveries of microorganisms in lake water while still maintaining low variability. The median percent recoveries were used to rank five filters for recovering each organism from 1 to 5, with the highest median recovery receiving the lowest rank (R). A variability rank score (RCV) was then calculated for each organism and filter method as follows:

(2) \[ \text{RCV} = R \times CV \]

where CV is the median coefficient of variation for the three variability recovery studies for each organism and filter.

The RCV was then ranked from lowest to highest (R'), with the lowest value being the filter with the lowest RCV.
An average RCV was calculated for each filter for all microorganisms, giving equal weight to each type of organism (bacteria, viruses, and protozoa) as follows:

$$\text{Average RCV} = \left( \frac{\text{RCV}_{E. coli} + \text{RCV}_{E. faecalis}}{2} \right) + \left( \frac{\text{RCV}_{MS2} + \text{RCV}_{adenovirus} + \text{RCV}_{norovirus GII} + \text{RCV}_{enterovirus} + \text{RCV}_{AIV}}{5} \right) + \left( \frac{\text{RCV}_{Cryptosporidium} + \text{RCV}_{Giardia}}{2} \right) / 3$$

Results

Because turbidity was expected to affect recoveries, the samples collected represented a wide range of turbidities. Turbidities ranged from <5 to 280 NTRU in recovery and field deployment experiments (Table S1) with an average of 58 NTRU (SD= 87). Because pH adjustment to pH 6-7.0 is required for glass-wool filtration, it was measured and adjusted for all filtered samples. Initial sample pH values ranged from 7.6 to 8.8 (Table S1) with an average of pH 8.4 (SD=0.2).

Recoveries in 10-L seeded lake-water samples.

Median percent recoveries of microorganisms in lake-water samples are presented in Table 1 with information on seed amounts and numbers of trials. Ranges for the numbers of trials were included because fewer protozoan analyses were done using the NanoCeram, and because unseeded controls for enterococci and E. coli were added in the sample counts for glass-wool filtration and automatic UF. Boxplots show the distributions of recoveries for each microorganism (Fig. 3); boxplots for AIV recoveries were not included because recoveries were very low. Results from Tukey-Kramer multiple comparison tests for median recoveries among filtration methods are presented as letters on the plots.

Recoveries for E. coli and enterococci were determined using concentrations from unseeded, naturally-occurring bacteria in samples directly processed by membrane filtration (Table 1).
Median recoveries of *E. coli* (Fig. 3A) and enterococci (Fig. 3B) for the CFC+ViroCap and UF methods were statistically higher than those for the glass wool and NanoCeram filtration methods. Concentrations of *E. coli* in permeates (waste stream) were below the limit of detection (<20 CFU/10L) for all filters except for the glass wool (data not shown). For the glass wool method, however, the average *E. coli* concentration in the permeates was 1,300 CFU/10 L (SD=1,800) (data not shown).

The analytical method for coliphage detects F-specific coliphages that are able to infect the host bacterium. In contrast, MS2 coliphage is one strain of F-specific coliphage and was used to seed water samples in recovery experiments. Percent recoveries of MS2 coliphage, therefore, were determined in seeded lake-water samples, and concentrations of F-specific coliphage were determined in unseeded lake-water samples. F-specific coliphage were detected in 53% of unseeded lake-water samples; however, concentrations in unseeded samples were negligible (<4 to 170 PFU/10 L, data not shown) compared to seed concentrations of MS2 coliphage (Table 1). Median recoveries of MS2 coliphage were >20% for all methods except for the glass-wool filtration, which was statistically lower than all the other methods (Fig. 3C).

Concentrations of MS2 coliphage in the permeates from the UF filters were all below detection (<100 PFU/10L, data not shown), and small amounts of MS2 coliphage were found in the permeates from the NanoCeram and CFC+ViroCap (averages of 2,300 and 900 PFU/10 L, respectively, data not shown). From the glass wool, however, the average concentration in the permeates was 420,000 PFU/10L (SD=320,000, data not shown), indicating poor trapping efficiency for coliphage.
Median recoveries of enteric viruses ranged from 0% to 14.5% (Table 1). For adenovirus in lake water, the highest median recovery was with the glass wool (4.7%), but this was not statistically higher than the automatic UF method (Fig. 3D). For norovirus GII, the highest median recovery was for the automatic UF (2.4%), but this was not statistically higher than any other method (Fig. 3E). For enterovirus, the highest median recovery was for the NanoCeram (14.5%), but this was not statistically higher than any other method except for the dead-end UF (Fig. 3F). In unseeded lake-water samples, norovirus GII and enterovirus were not detected. Adenovirus was detected in 47% of unseeded lake-water samples in concentrations ranging from 3 to 170 gc/10L (data not shown), much lower than seed amounts (Table 1). Avian influenza virus was not detected in any unseeded lake-water samples. Recoveries of seeded AIV were very low (Table 1), with the highest median recovery for the dead-end UF (0.02%).

Aliquots of 100 or 500 Cryptosporidium oocysts and Giardia cysts were seeded into lake-water samples. Fifteen unseeded lake-water samples were analyzed and background averages were generally low; eight of those had <1 cysts or oocysts per 10L and four had either 1 oocyst or cyst per 10L. The remaining three samples included results from glass wool and (or) automatic UF methods in two samples, with concentrations ranging from 1 to 52 oocysts or cysts per 10 L. Only one sample had results with >16 oocysts and cysts per 10 L, and these results were from a glass wool filtered sample where considerable particulate clumping was observed. These results were considered an aberration due to particulate clumping and the fact that a small percentage of the sample was analyzed, both of which lead to in higher variability. Consequently, seed values were not adjusted for background concentrations and recoveries may be slightly inflated. Median recoveries of Cryptosporidium and Giardia were >20% for the
CFC+ViroCap and UF methods. Median recoveries were statistically higher for the CFC+Virocap for Cryptosporidium and the dead-end UF for Giardia than those for the glass wool and NanoCeram (Fig. 3G, 3H).

**Recovery and variability ranks for microorganisms**

In order to identify which filter performed best with lake-water samples, median recoveries were ranked from 1-5 for each organism (R), with 1 being the filter with the highest recovery (Table 2). Variability rank scores (RCV) and variability-weighted ranks (R') based on the RCVs were calculated. The mean coefficient of variation, used as the variability measure for these calculations, was determined for each microorganism and filter method in the three lake-water variability experiments. Different filters were ranked highest for different microorganisms (Table 2). When variability was considered along with recovery (R'), the #1 rankings remained the same for all microorganisms as determined based on recovery alone (R); however, the #2 rankings changed for enterovirus, AIV, Cryptosporidium, and Giardia. For recovery of all microorganisms, the automatic UF resulted in the lowest average RCV and R' (Table 3).

**Field deployment of automatic ultrafiltration**

To evaluate the practicality and efficiency in using a filtration method at recreational beaches, eight samples were collected during field deployment experiments at seven sites (Fig. 1, Table 4) using the automatic UF method. None of the samples were seeded with microorganisms. Ambient concentrations found by use of direct plating ranged from 54 to 5,100 CFU/L for bacterial indicators and <10 to 30 PFU/L for F-specific coliphage (Table 4). Results for enteric virus analyses were inconclusive because all filtered samples showed inhibition due to matrix interference (data not shown). By use of automatic UF, two samples were positive for
Cryptosporidium (0.06 oocysts/L) and all were below detection for Giardia. Sample turbidities ranged from 2.9 to 280 NTRU (Table 4).

The automatic UF filtration method was relatively easy to deploy in the field. Although we attempted to filter approximately 150–200 L, we were not able to filter the full volume in all samples due to filter clogging or system problems (Table 4). Pre-filters were used in four of the early experiments; however, in later experiments, we learned that filtration worked more efficiently with attachment of a tubing end connector (Fig. S6.B). With inclusion of the tubing end connector, filtration times at sites 13 and 14 (the last two sites sampled) were 113 and 174 min for ≥ 200 L at a maximum 2-3 L/min. Median recoveries of E. coli and enterococci in field deployment experiments (56.2% and 35.7%, respectively) were slightly lower than those found for automatic UF in 10-L recovery experiments (64.6 and 45.8%, respectively). The median recovery of coliphage in field deployment experiments (14.0%) was substantially lower than the median in 10-L recovery experiments (67.7%).

Discussion

This study was done to identify the filtration method(s) that could be used efficiently and simultaneously to sample for all classes of pathogens (bacteria, viruses, and protozoa) in recreational lake-water studies. Even though the three virus adsorption-elution (VIRADEL) methods were designed specifically to concentrate viruses through charge interactions, we evaluated the use of VIRADEL for all microorganisms. Automatic and dead-end ultrafiltration (UF) methods were designed to concentrate all types of microorganisms. Stock cultures of MS2 coliphage, adenovirus, enterovirus, norovirus GII, avian influenza virus (AIV), C. parvum, and G.
lamblia were used to seed 10-L lake-water samples. *E. faecalis* and *E. coli* were not seeded to determine recoveries of naturally-occurring bacterial indicators.

There are a few studies that present data on recoveries of microorganisms in surface water that can be used as a comparison to recoveries of microorganisms in the present study. In the present study using the automatic UF, median recoveries of naturally-occurring *E. coli* and enterococci and seeded MS2 coliphage and *Cryptosporidium* in lake-water samples were 64.6, 45.8, 67.7%, and 35.3%, respectively. Gibson and Schwab (36) reported recoveries of 68, 56.4, and 51.3% for *E. coli*, enterococci, and coliphage in seeded stream-water samples of by use of tangential-flow UF. Using a similar UF method, Morales-Morales et al. (37) found an average recovery in seeded lake-water samples for *E. coli* of 91.6% and for *Cryptosporidium* of 31.6%.

Using dead-end ultrafiltration, Mull and Hill (38), recovered *E. coli* (81%), enterococci (85%), MS2 coliphage (66%), and *Cryptosporidium* (49%) from surface water samples. In the present study using dead-end ultrafiltration, median recoveries were considerably lower for *E. coli* (62.1%), but only slightly lower for enterococci (80.5%), MS2 coliphage (58.7%), and *Cryptosporidium* (41.0%). Bennett et al. (8) found an average recovery for MS2 coliphage of 53% in surface water using the CFC+ViroCap filter; in the present study, median recovery of seeded MS2 coliphage by use of CFC+ViroCap filtration was 20.5%. Deboosere et al. (39) found that glass wool filtration could achieve an average recovery of 1% of H1N1 influenza virus—a higher recovery than they achieved using NanoCeram. In the present study, median recoveries of AIV were much lower—the median was 0% and the highest recovery of an individual sample was 0.009% (data not shown). Deboosere et al. (39) noted that the recovery was much more variable in lake water (0.01% - 7.89%) and that 50-L samples had poor recovery due to
interfering substances. These may be additional factors that resulted in poor AIV recovery in the current study. Millen et al. (9) used glass-wool filtration to determine seeded enterovirus and Cryptosporidium recoveries in tap water amended with different amounts of silt loam soil and to determine AIV recoveries in seeded surface-water samples. Enterovirus and AIV were quantified by qRT-PCR. Average recoveries of enterovirus, Cryptosporidium, and AIV ranged from 38-81%, 28-53%, and 7.8-42.9%, respectively, much higher than those in the current study (medians of 10.5%, 10.7%, and 0%). However, Millen et al. (9) calculated recoveries by setting the denominator equal to the seed concentration in a negative eluate (after filtration), therefore, eliminating results from matrix differences. In the current study, recoveries were calculated by setting the denominator equal to the concentration in the seed before filtration. This enabled us to investigate how recoveries were affected by both the filtration method and the water matrix. To our knowledge, there are no other published studies of virus recovery from surface-water samples using qPCR or qRT-PCR.

For E. coli, enterococci, and protozoa, median recoveries in lake-water samples were >22% for the CFC+Virocap, automatic UF, and dead-end UF and were statistically higher than for the glass wool and NanoCeram. This is not surprising, in that the glass-wool and NanoCeram filters were not originally designed for recovering bacteria and protozoa. Indeed, appreciable concentrations of E. coli were noted in the permeates (waste stream) from the glass wool, but not from the other filters. For MS2 coliphage, median recoveries were >20% for all filters except for the glass wool. Similar to E. coli, lower recoveries of MS2 coliphage were attributed to poor trapping efficiency by glass wool filtration as shown by high concentrations in the permeates.
Variability-weighted ranks ($R'$) were calculated to combine both recovery and variability (from multiple lake-water recovery measurements) into one value. Using this approach, the best filtration method would have a relatively high recovery while maintaining low variability. The five filters were ranked best ($R'=1$), as follows: the glass wool for adenovirus, the NanoCeram for MS2 coliphage and enterovirus, the CFC+ViroCap for *E. coli* and *Cryptosporidium*, the automatic UF for norovirus GII, and the dead-end UF for *E. faecalis*, AIV, and *Giardia*. The glass-wool method performed consistently well in recovering the three targeted enteric viruses from lake-water samples, and ranked 1st for adenovirus, 2nd for norovirus G11, and a close third for enterovirus. Although the glass-wool ranked low for the other microorganisms, it should not be discounted from including in lake-water pathogen studies when viruses are target microorganisms. Among the five filters, the automatic UF resulted in the best average $R'$ for all nine organisms.

Finally, we showed that the automatic UF method could be used efficiently for sampling 100–200 L volumes of lake water for pathogens. During field sampling at seven Great Lakes beaches, the issue of filter clogging from high turbidity or algae was identified and reduced. The automatic UF method worked more efficiently with attachment of a tubing end connector for removing large particles (Fig. S5B). To improve field filtration efficiency in future work, a PVC “end connector” (40-cm long with 10 0.254-mm slotted openings) (Fig. S5.C) was designed to replace the shorter tubing end connector (14-cm in length) used in the present study (Fig. S5.B).
In addition to the automatic UF, other filtration methods have the potential to be practical and efficient for sampling large volumes of lake water. A dead-end concentrator was designed for sampling recreational waters (40) and is commercially available (IntelliSense Design, Inc., Tampa, FL). A manual UF system (41), that operates on the same principle as the automatic UF but without a computer controlled system, worked well in recovering pathogens from 100-L samples during field studies at Great Lakes and inland lake beaches (data not shown). The glass-wool filtration system, contained in simple plastic tub, was used successfully to collect large volume samples at beaches in Wisconsin (Steve Corsi, USGS, written commun., 2011). In future studies, work is needed to identify appropriate sample volumes for detecting pathogens and to test the PVC end connector with 200-L sample volumes, if larger volumes are needed. The volume identified would need to be a compromise between sampling as much water as practical while reducing inhibition found in samples analyzed for viruses by qPCR and qRT-PCR.

To our knowledge, this was the first comprehensive evaluation for multi-pathogen sampling in recreational waters that included an evaluation of different filtration methods. Many previous studies were done with tap water or a relatively clean water matrix, whereas the present study was done with a variety of lake-water samples. After comparison of filtration methods with 10-L samples, we were able to scale up the methods to 150–200 L and successfully deploy the technique to collect lake-water samples from multiple public beaches in two states. The use of one or two filters to effectively concentrate all types of microorganisms makes filtration efficient for regional studies of public health risk at beaches.
Acknowledgements

We thank Vincent R. Hill and Bonnie Mull (Centers for Disease Control and Prevention) for help with the dead-end UF, Fu-Chi Hsu (Scientific Methods Inc.) for help with the CFC+ViroCap, Mark A. Borchardt and Susan K. Spencer (USDA ARS Laboratory) for help with glass wool filtration, and Brian Morris (Pegasus Technical Services) for help with the automated UF device. We also thank David O. Erisman for technical support. We thank Elizabeth Bohuski and other members of the National Wildlife Health Center Diagnostic Virology Laboratory for their capable assistance in AIV analysis.

Support for this study was provided by the U.S. Geological Survey, Coastal Marine Program, and by the U.S. Environmental Protection Agency through its Office of Research and Development.

This publication has been reviewed by the U.S. Environmental Protection Agency but does not necessarily reflect Agency views. No official endorsement by the U.S. Environmental Protection Agency should be inferred. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

References


## TABLE 1  
Seed amounts and median percent recoveries of microorganisms in lake-water samples with the results from the filtration method yielding the highest recovery for each organism shaded

<table>
<thead>
<tr>
<th>Seed amounts (per 10 L)</th>
<th>Median recoveries (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glass wool</td>
</tr>
<tr>
<td>Number of trials</td>
<td>14–20</td>
</tr>
<tr>
<td>E. coli (CFU)</td>
<td>4,000 (4,000)*</td>
</tr>
<tr>
<td>Enterococci (CFU)</td>
<td>5,000 (6,000)*</td>
</tr>
<tr>
<td>MS2 coliphage (PFU)</td>
<td>460,000 (310,000)</td>
</tr>
<tr>
<td>Adenovirus (gc)</td>
<td>500,000 (340,000)</td>
</tr>
<tr>
<td>Norovirus GII (gc)</td>
<td>46,000 (81,000)</td>
</tr>
<tr>
<td>Enterovirus (gc)</td>
<td>8.4E+6 (1.5E+7)</td>
</tr>
<tr>
<td>Avian influenza virus (gc)</td>
<td>1.0E+9 (9.3E+8)</td>
</tr>
<tr>
<td>Cryptosporidium (oocysts)</td>
<td>100 (0)*</td>
</tr>
<tr>
<td>Giardia (cysts)</td>
<td>100 (0)*</td>
</tr>
</tbody>
</table>

* Naturally-occurring concentrations of microorganisms were used to determine recoveries.

b 500 oocysts and cysts were used for one glass wool and one NanoCeram filtration

c Genomic copies
<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>E. faecalis</th>
<th>MS2 coliphage</th>
<th>Adenovirus</th>
<th>Norovirus GII</th>
<th>Enterovirus</th>
<th>Avian influenza virus</th>
<th>Cryptosporidium</th>
<th>Giardia</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLASS WOOL</td>
<td>R</td>
<td>RCV</td>
<td>R'</td>
<td>R</td>
<td>RCV</td>
<td>R'</td>
<td>R</td>
<td>RCV</td>
<td>R</td>
</tr>
<tr>
<td>R</td>
<td>4</td>
<td>2.32</td>
<td>4</td>
<td>4</td>
<td>4.88</td>
<td>5</td>
<td>5</td>
<td>1.4</td>
<td>4</td>
</tr>
<tr>
<td>RCV</td>
<td>1</td>
<td>0.29</td>
<td>1</td>
<td>2</td>
<td>1.28</td>
<td>2</td>
<td>3</td>
<td>1.2</td>
<td>4</td>
</tr>
<tr>
<td>R'</td>
<td>2</td>
<td>1.28</td>
<td>2</td>
<td>3</td>
<td>1.4</td>
<td>4</td>
<td>5</td>
<td>6.70</td>
<td>5</td>
</tr>
<tr>
<td>NANO CERAM</td>
<td>5</td>
<td>2.8</td>
<td>5</td>
<td>5</td>
<td>4.05</td>
<td>4</td>
<td>1</td>
<td>0.67</td>
<td>3</td>
</tr>
<tr>
<td>R</td>
<td>1</td>
<td>0.67</td>
<td>1</td>
<td>3</td>
<td>2.32</td>
<td>5</td>
<td>5</td>
<td>5.15</td>
<td>5</td>
</tr>
<tr>
<td>RCV</td>
<td>2</td>
<td>1.14</td>
<td>2</td>
<td>4</td>
<td>1.68</td>
<td>3</td>
<td>1.4</td>
<td>4.35</td>
<td>5</td>
</tr>
<tr>
<td>R'</td>
<td>3</td>
<td>1.68</td>
<td>3</td>
<td>2</td>
<td>0.43</td>
<td>1</td>
<td>1.68</td>
<td>5.85</td>
<td>5</td>
</tr>
<tr>
<td>AUTO UF</td>
<td>2</td>
<td>0.94</td>
<td>2</td>
<td>2</td>
<td>1.28</td>
<td>2</td>
<td>2</td>
<td>0.68</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>1</td>
<td>0.68</td>
<td>1</td>
<td>3</td>
<td>0.45</td>
<td>2</td>
<td>1</td>
<td>0.47</td>
<td>3</td>
</tr>
<tr>
<td>RCV</td>
<td>2</td>
<td>1.76</td>
<td>3</td>
<td>3</td>
<td>0.57</td>
<td>2</td>
<td>3</td>
<td>0.57</td>
<td>2</td>
</tr>
<tr>
<td>R'</td>
<td>3</td>
<td>0.57</td>
<td>2</td>
<td>3</td>
<td>0.57</td>
<td>2</td>
<td>3</td>
<td>0.57</td>
<td>2</td>
</tr>
<tr>
<td>DEAD END UF</td>
<td>3</td>
<td>0.96</td>
<td>3</td>
<td>3</td>
<td>0.36</td>
<td>4</td>
<td>4</td>
<td>0.63</td>
<td>4</td>
</tr>
<tr>
<td>R</td>
<td>1</td>
<td>0.36</td>
<td>4</td>
<td>3</td>
<td>0.54</td>
<td>3</td>
<td>3</td>
<td>1.14</td>
<td>3</td>
</tr>
<tr>
<td>RCV</td>
<td>2</td>
<td>1.14</td>
<td>3</td>
<td>5</td>
<td>3.45</td>
<td>3</td>
<td>5</td>
<td>4.2</td>
<td>5</td>
</tr>
<tr>
<td>R'</td>
<td>3</td>
<td>3.45</td>
<td>3</td>
<td>5</td>
<td>4.2</td>
<td>5</td>
<td>1</td>
<td>0.63</td>
<td>4</td>
</tr>
</tbody>
</table>

R is the rank of the median percent recoveries in all lake-water samples, with the highest median recovery receiving the lowest rank.
RCV is the variability rank score, calculated by multiplying the rank (R) times the median coefficient of variation for the three multiple lake-water samples.
R' is the rank of the RCVs, with the lowest value being the filter with the lowest RCV.
<table>
<thead>
<tr>
<th>Filter</th>
<th>Avg RCV</th>
<th>Avg R'</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUTO UF</td>
<td>0.79</td>
<td>2.00</td>
</tr>
<tr>
<td>DEAD END UF</td>
<td>1.33</td>
<td>2.56</td>
</tr>
<tr>
<td>VIROCAP</td>
<td>1.79</td>
<td>2.89</td>
</tr>
<tr>
<td>GLASS WOOL</td>
<td>2.84</td>
<td>3.67</td>
</tr>
<tr>
<td>NANOCERAM</td>
<td>3.58</td>
<td>3.89</td>
</tr>
</tbody>
</table>
### TABLE 4

Recoveries of bacterial indicators and F-specific coliphage in unseeded lake-water samples using automatic ultrafiltration during field deployment studies at seven sites in Ohio and Wisconsin, 2010

<table>
<thead>
<tr>
<th>Site number</th>
<th>Ambient concentrations, per L*</th>
<th>Filter information</th>
<th>Recovery after filtration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient concentrations</td>
<td>Filter type</td>
<td>Volume filtered</td>
</tr>
<tr>
<td></td>
<td>E. coli (CFU)</td>
<td>Enterococci (CFU)</td>
<td>F-specific coliphage (PFU)</td>
</tr>
<tr>
<td>2</td>
<td>2,300</td>
<td>1,500</td>
<td>&lt;10</td>
</tr>
<tr>
<td>4</td>
<td>460</td>
<td>160</td>
<td>&lt;10</td>
</tr>
<tr>
<td>10</td>
<td>5,100</td>
<td>2,700</td>
<td>&lt;10</td>
</tr>
<tr>
<td>11</td>
<td>2,100</td>
<td>1,200</td>
<td>&lt;10</td>
</tr>
<tr>
<td>11</td>
<td>4,500</td>
<td>4,200</td>
<td>&lt;10</td>
</tr>
<tr>
<td>12</td>
<td>160</td>
<td>110</td>
<td>10</td>
</tr>
<tr>
<td>13</td>
<td>1,800</td>
<td>930</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>140</td>
<td>54</td>
<td>10</td>
</tr>
</tbody>
</table>

*a* Determined by direct plating of the lake-water sample for bacterial indicators or filtered samples for protozoan pathogens

*b* Not readable because of algae and debris

*c* Pre-filter is a 100 mesh, 150 mm pre-filter; end is an end connector (tubing with irregular holes)

*d* Not applicable because coliphage were not present in the direct plating sample
For CFC+ViroCap, a CFC bowl eluate and ViroCap filter eluate were produced in sequence.
Glass Wool NanoCeram CFC + ViroCap Automatic UF Dead-End UF Method

Percent Recovery

B C A AC AC

(13)

(13)

(13)(13)

(14)

on June 27, 2017 by guest http://aem.asm.org/ Downloaded from
Glass Wool NanoCeram CFC + ViroCap Automatic UF Dead-End UF
Method

H.

Glass Wool  NanoCeram  CFC + ViroCap Method  Automatic UF  Dead-End UF

(14) (8) (13) (13) (13)