EFFICIENT AND PREDICTABLE RECOVERY OF VIRUSES AND *CRYPTOSPORIDIUM PARVUM* OOCYSTS FROM WATER BY ULTRAFILTRATION SYSTEMS

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ABSTRACT

Current methods to concentrate viruses and *Cryptosporidium parvum* oocysts from large volumes of water are prone to inconsistent results and are costly and complex. In addition, the recovery of viruses and oocysts requires the use of two different concentration methods. Ultrafiltration utilizes size selection rather than adsorption/elution to concentrate any organisms larger than the pore size of the ultrafilter. This approach in the concentration of pathogens from water may offer greater flexibility in developing methods that can provide more consistent recoveries among different viruses and widely varying water conditions. This study involved the characterization and optimization experiments were done using two small-scale (2L) and two large-scale ultrafiltration (100L) systems (hollow fiber and tangential flow) with virus suspended in reagent grade water, tap, ground, and surface waters. Recovery experiments were done with three viruses: bacteriophage PP7 and T1 and poliovirus as well as a protozoan parasite (*C. parvum* oocysts) to compare, characterize and optimize the recovery with two ultrafiltration systems. Pretreatment of the ultrafilters with blocking agents (fetal bovine serum or other proteinaeous solutions) and the use of elution agents can serve to prevent viral adsorption to the filter surface or to elute bound virus and keep viral agents suspended in the retentate. Blocking the membrane also improved *C. parvum* recovery. The use of a blocking and an elution step efficiently concentrated (>60% recovery) viruses and *C. parvum* oocysts from widely varying water qualities including surface water. Both ultrafiltration systems appear to be able to recover viruses efficiently; however, the hollow fiber systems may provide slightly better and more consistent results in the 2 and 100L volumes tested. These results indicate that the hollow fiber ultrafiltration system can efficiently and reproducibly recover viruses and *C. parvum* from small- and large-scale systems and from widely varying water qualities, and that both ultrafiltration systems can provide efficient recovery of viruses from water.

Keywords: ultrafiltration, waterborne virus, poliovirus, enterovirus, *Cryptosporidium parvum*

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INTRODUCTION

Human pathogens can be introduced into surface and groundwater through complex and highly variable process involving wastewater and agricultural runoff contaminated with human and animal fecal matter. These pathogens can then pose health risks to humans through exposure via recreational water, drinking water, and contaminated agricultural products. The availability of highly sensitive and reproducible methods for detecting waterborne microbial pathogens is important in order to determine the extent of microbial contamination, the types of pathogens involved, and the correlation between the isolation of microbial agents and environmental factors. This information can then be used to help identify indicators of systems at risk for these contaminants. Such information will lead to a better understanding of the health risks and better methods to prevent and/or reduce the level of waterborne microbial pathogens, and to reduce the impact on public health.

Concentration of Viruses by Filtration

The concentration and detection of viruses from environmental samples are particularly problematic because of the variability of recovery among the different viruses, variable recovery due to water quality, and the cost and time involved with these methods. The current standard method for the concentration of viruses utilizes filter membranes with pore sizes that are larger than viral particles (American Public Health Association 1995; Farrah and Bitton 1978; Goyal and Gerba 1982; Goyal et al. 1980; Sobsey 1976) and relies on the adsorption of viruses to the filter surface and then the elution of the adsorbed particles into a much smaller volume. Viruses have been shown to have variable adsorption efficiencies to membrane filters based on differences in their surface characteristics (Guttman-Bass and Armon 1983; Rose et al. 198;

Shields and Farrah 1983, Shields et al. 1986; Sobsey and Glass 1984; Sobsey and Hickey 1985, Sobsey et al. 1981). Properties of viral particles such as size, shape, composition of the outer most layer and stability can affect the adsorption of viral particles. The adsorption of viral particles is also affected by water characteristics including pH, salt, levels of organics and volume of water filtered (Gerba et al. 1978; Guttman-Bass and Catalano-Sherman 1985; Mix 1974; Shields et al. 1986; Sobsey 1976). As water quality deteriorates, the efficiency of virus recovery generally declines (Melnick et al. 1984; Shields et al. 1986). Recovery using adsorption/elution methods with an electropositive membrane is quite variable depending on the volume filtered and the source of water (Lucena et al. 1995; Rose et al. 1984; Shields et al. 1986).

An alternative to the adsorption and elution method for concentrating viral particles is the use of ultrafilters that utilize size exclusion to concentrate viruses into smaller volumes. Several studies with ultrafilters have been done to recover human viruses (Belford et al. 1975a and b; Berman et al. 1980; Bicknell et al. 1985; Jansons and Bucens, 1985; Gilgen et al. 1997; Juliano and Sobsey 1998; Soule et al. 2000). However, these studies did not examine viral recovery when combinations of different viruses, water qualities and ultrafiltration systems were used. Ultrafiltration has also been used as a second step concentration procedure (after adsorption/elution) for the recovery of hepatitis A virus and poliovirus from small volumes (Divizia et al. 1989), and to concentrate planktonic viruses and bacteriophage from marine environments (Paul et al. 1991; Suttle et al. 1991). Results from the concentration of marine viruses have shown promise. However, recoveries of low concentrations of human viral pathogens were not investigated. Concentration of marine bacteriophage has also been achieved via ultrafiltration after prefiltration through 0.2 and 0.1 μm filters for <0.5 L volumes

(Wommack et al. 1995). A previous study with the hollow fiber ultrafilter described the efficient recovery of phages T1 and PP7, and poliovirus when a high concentration of virus was suspended in 500 ml of fluids containing proteinaceous material (Oshima et al. 1995).

Downstream Processes to Further Concentrate Viral Particles

A number of methods have been used to further concentrate viral particles after the initial filtration step. These have included organic flocculation (acidifying the eluent and recovering the precipitate by centrifugation and suspending the pellet in a small volume (American Public Health Association 1995), polyethylene glycol hydroextraction (American Public Health Association 1995), small-scale microfiltration (Logan 1980; Sobsey et al. 1980) and ultrafiltration has also been used (Divizia 1989). The efficiency of these steps with different viruses and water qualities has not been reported in much detail.

Concentration of *Cryptosporidium parvum* Oocysts From Water

Cryptosporidium is an enteric protozoan parasite that infects a wide range of hosts, including humans. The potential for oocysts contaminating drinking water is a concern because of their small size (4-7 um) and resistance to chlorine (Venczel et al. 1997). Waterborne outbreaks of *Cryptosporidium* have been on the increase in recent years (Mayer and Palmer 1996).

To protect against *Cryptosporidium* outbreaks, water treatment facilities need to monitor their source and finished water. Large volumes of water (10-100L of raw and up to 1000L of finished water) should be analyzed to ensure sensitive detection (Rochelle et al., 1999). One of the main difficulties with detection of *Cryptosporidium* is the lack of a reliable method that can

efficiently and reproducibly concentrate oocysts from large volumes of water, especially those of a higher turbidity (Awad-El-Kariem et al. 1994; Bukhari et al. 1998; Sartory et al. 1998). The updated EPA Method 1622 and 1623 utilizes an absolute pore-size filter cartridge (1 μm microfilter) and purification of the oocysts by immunomagnetic separation (IMS) (USEPA 1999a and b). The absolute pore filters can be expensive since these filters are designed for single use and variations in oocyst recoveries have been observed (Campbell and Smith 1997; Connell et al. 2000; Hsu and Huang 2000).

Ultrafiltration offers some of the same advantages over microfiltration in the concentration of oocysts as with viral particles. The cross-flow recirculation maintains oocysts in suspension and retains oocysts based on size exclusion. This circulation pattern, as mentioned previously also serves to reduce fowling of the filter and makes it possible to filter turbid waters.

The objectives of this study were to:

1) characterize and optimize the efficiency of virus recovery using two different ultrafiltration systems and to determine if ultrafiltration could efficiently concentrate viruses over a wide range of water conditions; recovery was initially tested in small-scale (2L) spiked samples and then tested with 100L volumes

2) determine the effectiveness of ultrafiltration as a second step concentration system for viruses

3) characterize and optimize the efficiency of *Cryptosporidium parvum* oocyst recovery using the hollow fiber ultrafiltration system. Recovery was initially tested in small-scale (2L) spiked samples and then tested with 10L volumes.

4) develop and test polymerase chain reaction-based detection systems for both enteroviruses and *C. parvum* that have been adapted to ultrafiltration.

To date, a systematic approach (different filters, multiple target organisms, water qualities, blocking agents) to determine the effectiveness of ultrafiltration as a first-step and possibly second-step virus concentration procedure has not been done. The results of the present study have led to optimized methods to recover viruses from small 2L and large 100L volumes and *Cryptosporidium* oocysts from 2L and 10L and to a better understanding of factors affecting the performance of ultrafiltration to recover these organisms.

MATERIALS AND METHODS

Filters

Polyacrylonitrile (PAN) 50,000 MWCO (pencil module, AHP-0013; small pilot module, AHP-1010; pilot AHP-2013) hollow fiber ultrafilter (Microza; Pall Corp. Glen Cove, NY) and polyethersulfone 10,000 MWCO (Ultrasette, OS010C70; Centramate, FS013C10; Centrasette, OS010C05 Pall-Filtron, Ann Arbor, MI) were used to concentrate viruses from water (Table 1).

The hollow fiber ultrafilter consists of hollow tubes of filter material bundled together. The flow of the filtered water was from the inside to the outside of the fiber. The fiber diameter was 0.8 mm.

The tangential ultrafilter consists of two sheets of membrane material with the feed going between the two sheets. Back-pressure was used to control the rate of filtration. During filtration, a portion of the feed was forced through the filter membrane while the remaining water flowed across the surface of the membrane and back to the central reservoir.

TABLE 1. Filter surface area of the ultrafilters used in the concentration of viruses from 2L and 100L water samples.

	2L	10L	100L
Hollow Fiber	pencil module AHP-0013 (0.017m^2)	small pilot module AHP-1010 (0.2m^2)	pilot module AHP 2013 (1m^2)
Tangential Flow	Ultrasette OS010C70 (0.085m^2) Centramate FS013C10 (0.09m^2)	none	Centrasette OS010C05 (0.92m^2)

Viruses

Escherichia coli (ATCC 11303) was used as the host strain for growth and assay of phage T1 (ATCC 11303-B1) (Table 2). *Pseudomonas aeruginosa* (ATCC 15692-B2) was used

for growth and assay of phage PP7 (ATCC 15692-B2). The plaque assay was conducted as described previously (Oshima et al. 1994).

The Sabin 2 vaccine strain was used as the challenge virus for experiments with poliovirus. The virus was grown in HeLa cells and the plaque assay conducted as described previously (Oshima et al. 1995).

TABLE 2. Physical characteristics and host of model viruses and *Cryptosporidium* oocysts used in this study.

Virus	Size	Host	Enveloped	Nucleic Acid
T1	50 nm head 150 nm tail	E.coli	No	dsDNA
PP7	25 nm	P. aeruginosa	No	ssRNA
Poliovirus (Sabin 2 strain)	25 nm	HeLa cells	No	ss RNA
Cry. oocysts	5-6 um	NA	NA	dsDNA, rRNA, mRNA

Stability Tests

The stability of phage T1 and PP7 and poliovirus suspended in sterile ultrapure water (UPW) and PBS at room temperature over a 24-hour period was evaluated. Three replicate experiments were done with each virus. The viruses were suspended in 1000 ml of sterile UPW or UPW buffered with PBS at a concentration of ~1000 PFU/ml. The viral suspension was stirred for 10 minutes and a sample was assayed to determine the initial viral concentration. After 1-, 3-. and 24-hours, the suspension was assayed to determine the virus concentration by the plaque assay method.

Filtration Procedure for the Small-scale (2L) Ultrafiltration Experiments

Hollow Fiber

The hollow fiber ultrafilter module was fitted into a filtration system (PS24001; Asahi Chemical Industry Co., Tokyo, Japan) containing a gear type pump and valves to control transmembrane pressure and flow rate (Figure 1). Before and after each experiment, the ultrafilter module was sanitized by circulating a solution containing 100 mg/L free sodium hypochlorite for 30 minutes. The free sodium hypochlorite concentration was determined at the end of sanitation by measuring the absorbance at 530 nm by the DPD method (PR/2010; Hach, Loveland, CO.) The ultrafilter module was flushed with sterile ultrapure water (UPW) until the residual concentration of free sodium hypochlorite was <0.04 mg/L.

Viruses were suspended in 2L volumes of water buffered with phosphate buffered saline (PBS, pH 7.2, 1.54 mM KH_2PO_4, 154 mM NaCl, 6.05 mM Na_2P0_4) with a virus concentration of ~1000 PFU/ml. A 10X solution of PBS was added to the 2L sample prior to the addition of virus. Viruses were resuspended in UPW, tap, ground or surface water buffered with PBS. The virus solution was stirred for 10 minutes then recirculated throughout the ultrafiltration unit by cross-flow circulation for 5 minutes.

The 2L samples were processed at a transmembrane pressure of 0.8 bar (1 bar = 100 kPa). Each experiment was designed such that the retentate was recirculated back to a central reservoir (Figure 1). Filtration was terminated when only the hold-up volume (volume of fluid contained in the filter apparatus) remained. For each experiment, the virus concentration was determined for the initial virus suspension, retentate and overall “bulk” permeate by plaque assay.

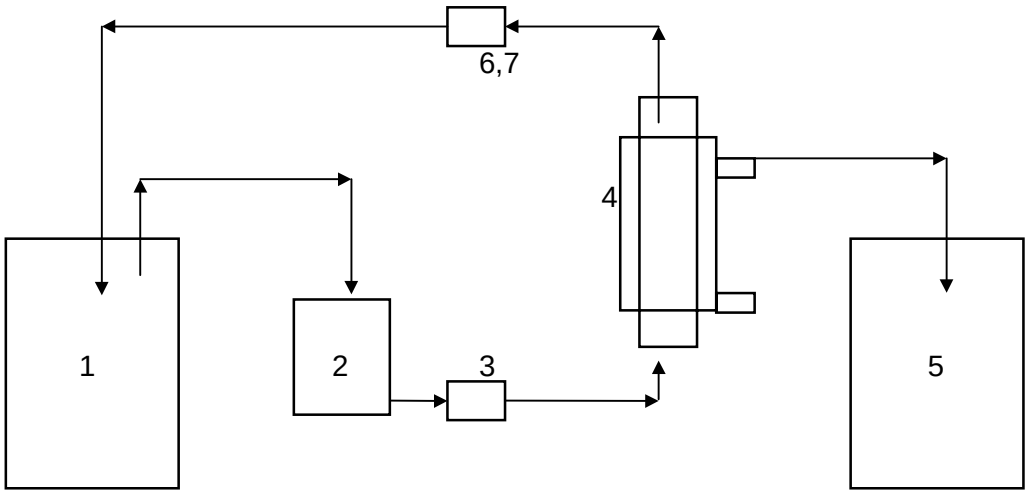

Figure 1. Diagram of filtration scheme for the hollow fiber and tangential flow filtration systems: 1, retentate reservoir; 2, peristaltic pump; 3, pressure gauge; 4, hollow fiber module; 5, permeate reservoir; 6, pressure gauge at module outlet; 7, valve to control module outlet pressure.

Tangential Flow Ultrafiltration System

The membrane cassette was assembled as recommended by the manufacturer, connected to a peristaltic pump (model 7520-25, Cole Parmer, Chicago IL) with the appropriate valves and gauges to control transmembrane pressure. The system was sanitized with 1L of a 0.1-0.5 N sodium hydroxide solution. The input pressure was set at 2.1 bar and the sodium hydroxide solution was filtered for one hour through the entire system. The sodium hydroxide solution was then removed by flushing ~3,500 ml of sterile UPW through the system. The pH (~7.0) of the permeate was measured to ensure the removal of the sanitant. A 2L viral suspension was prepared in the same manner as the hollow fiber system (buffered with PBS [pH 7.2]). In some cases, a blocking solution was used prior to the introduction of the 2L virus suspension (see below). The 2L sample was processed with an inlet pressure of 1.70-2.40 bar and the retentate pressure at 0.70 bar. The experiment was terminated when only the hold-up volume remained

(~50 ml). After completion of each experiment the system was sanitized again. For each experiment, the total infectious virus in the initial suspension, the retentate and permeate was determined by plaque assay and the percent recovered was estimated using the equation below.

Pretreatments of Environmental Water Samples Prior to Ultrafiltration

The recovery of phages T1 and PP7 suspended in different types of water was evaluated for both filtration systems. Tap water was dechlorinated with 0.05 mg/L of sodium thiosulfate (final concentration) prior to being seeded with viruses. The chlorine level was monitored using the same method as the sanitation procedure. Ground water was collected from a well located within the NMSU campus and surface water was collected from the Rio Grande at Mesilla, NM. In 2L tests, most surface samples were prefiltered using a 11 μm filter (Whatman No 1, Maidstone, England) for the hollow fiber and tangential flow system prior to the addition of viruses. Surface water experiments with the tangential flow system were not prefiltered.

Blocking and Elution Methods

Hollow Fiber System

For some experiments, the membrane was blocked with proteinaceous agents prior to filtration of the virus suspension (Table 3). This was done with a 100-ml suspension of blocking solution. This solution was allowed to flow across the membrane (cross-flow) for 1 hour (no permeate flow) or blocked overnight with agitation at room temperature. All blocking solutions were prefiltered through a 47-mm nylon 66 membrane filter (0.2-μm; NT, Pall Corp.) prior to use. Unbound blocking material was removed by flushing the system with 500 ml of UPW (single pass) prior to filtration.

In other experiments, elution of the viral particles from particulates or the filter was done after the concentration step by the addition of 0.05 M glycine (final concentration) at pH 7.0 or 9.0 to the retentate (retentate volume was usually 30-50 ml for the hollow fiber and ~50 ml for the tangential flow system). The retentate (eluent added) was then passed through the membrane for 15 or 30 min. in the cross-flow mode only (no permeate flow). A sample of the retentate was then taken for analysis by plaque assay. Elution was also tested with the addition of 10% fetal bovine serum (FBS; final concentration) directly to the retentate followed by elution in the cross-flow configuration for 30 minutes. The amount of virus recovered was then determined by plaque assay (Oshima et al. 1994 and 1995).

TABLE 3. Agents used to reduce viral adsorption to the filter or to resuspend bound virus.

Blocking Agents	Added to Virus Suspension	Elution
2 and 4% nutrient broth (Becton Dickenson, Cockeyeville Md)	0.5% FBS in 2 L (FBS, Gibco-BRL, Grand Island NY)	10% FBS
5% beef extract (Sigma, St. Louis Mo)	0.05% FBS in 2 L	0.05M glycine pH 9.0 (IBI, New Haven Cn)
1 and 5% FBS		
5% calf serum (Gibco-BRL)	0.5% FBS added to retentate in last 500 ml	0.05M glycine pH 7.0
5% BSA (Sigma, St. Louis Mo)		

Tangential Flow System

For some experiments, a 5% solution of FBS (100ml) was circulated through the filter (permeate closed) for 1 hour to pre-block the membrane prior to the addition of the viral suspension. The filter was then flushed with 1L UPW to remove any unbound FBS. For some surface water experiments no prefiltration was done. In the other set of experiments when the retentate volume reached 500 ml, FBS was added to a final concentration of 0.5% as was done with the hollow fiber system. Filtration was then continued until only the hold-up volume remained. Like the hollow fiber system, for some experiments, glycine was added to the retentate (glycine 0.05M, pH 7.0) and recirculated for 30 minutes. In other experiments, the retentate was removed and a glycine solution (0.05M pH 7.0, 100 ml) was recirculated through the cassette for five minutes. After five minutes, the eluent was added to the retentate and the virus concentration in this solution was determined.

Large-scale Ultrafiltration Experiments

For the large-scale hollow fiber and tangential flow ultrafiltration system's (100L) a peristaltic pump (Cole Palmer model 7549-32) was used with tubing (Cole Palmer Phar Med 6485-89). Stainless steel filter housing was used for the centramate (FS013K10, Pall-Filtron) and the centrasette (FS014K05, Pall-Filtron). In the 100L systems, the sanitation, blocking, and elution steps were similar to that of the small-scale system.

Hollow Fiber

After each use, the filter system was sanitized with 200 ppm of free chlorine and 0.5M sodium hydroxide solution in 3L of RO water. The unit was recirculated in the same manner as

the small-scale system and the free sodium hypochlorite level was tested by the DPD method. The system was then flushed with reverse osmosis (RO) water until the pH was ~ 7 and the free sodium hypochlorite level was less than 0.04 mg/L. When surface water was used, additional cleaning consisting of 200 ml of 10% sodium dodecyl sulfate (SDS) added to the filter module and incubated at 37^{o}C overnight was used. The following day, the SDS was flushed out with RO water until the pH was ~ 7.0. The filter module was cleaned until the clean water flux was 7.8-8.0L/minute. If this flux was not achieved, additional cleaning was done with either 0.5M NaOH or 10% acetic acid recirculated through the filter module for one hour followed by flushing with RO water to remove the sanitant. When not in use, the filter module was placed in the refrigerator for storage in 200 ml of NaOH or 10% SDS.

When the proper clean water flux was obtained, the filter module was blocked with 3L of 5% calf serum by cross-flow recirculation of the blocking solution for one hour (flow parallel with the filter membrane (no permeate flow). The filter was then incubated with the blocking agent added to the module (200 ml) overnight (with all the openings to the modules capped) at RT with agitation. The following day the blocking solution was then recirculated for one hour through the module and then the system flushed with 40L of RO water to remove any unbound blocking agent.

Two 50L carboys of water sample were inoculated with virus (to a final concentration of ~1000 plaque forming units (PFU)/ml) and mixed thoroughly. For tap and well water samples, the 100 L of water was then poured into a 100L tank and recirculated through the pilot ultrafiltration system for five minutes before taking an input sample (10 ml). For surface water, a 10-ml sample of the initial 100L virus suspension was taken before the virus suspension was prefiltered through a 75, 53 and 38 μm 12-inch diameter stainless steel sieves (VWR, Denver

Colorado) in sequential order to remove some of the larger particulates. Following prefiltration, the 100L virus suspension was then filtered at an input pressure of 30 psi and an outlet of 15-18 psi. When filtration was completed, the retentate volume remaining was measured (~2.5L) and a 10 ml sample was taken.

For elution, 0.05 M glycine at pH 7.0 (final concentration) was added to the retentate sample to produce a 3L retentate sample containing 0.05M glycine. The retentate with glycine added was then recirculated for 15-30 minutes in the cross-flow mode only. A 10-ml sample was then taken to determine the effectiveness of the elution process. For surface water experiments, after the elution step, the retentate solution was removed and a fresh elution solution (250 ml) was poured into the filter module and the ends capped and the filter agitated for 15 minutes (10 ml collected for virus assay). After agitation, the elution solution was added to the 3L that was previously collected.

Ten-fold serial dilutions of the input, retentate, retentate after the addition of glycine, elution off the filter, and permeate samples were done and the dilutions were tested for virus concentration via the plaque assay as previously described (Oshima et al. 1994, 1995).

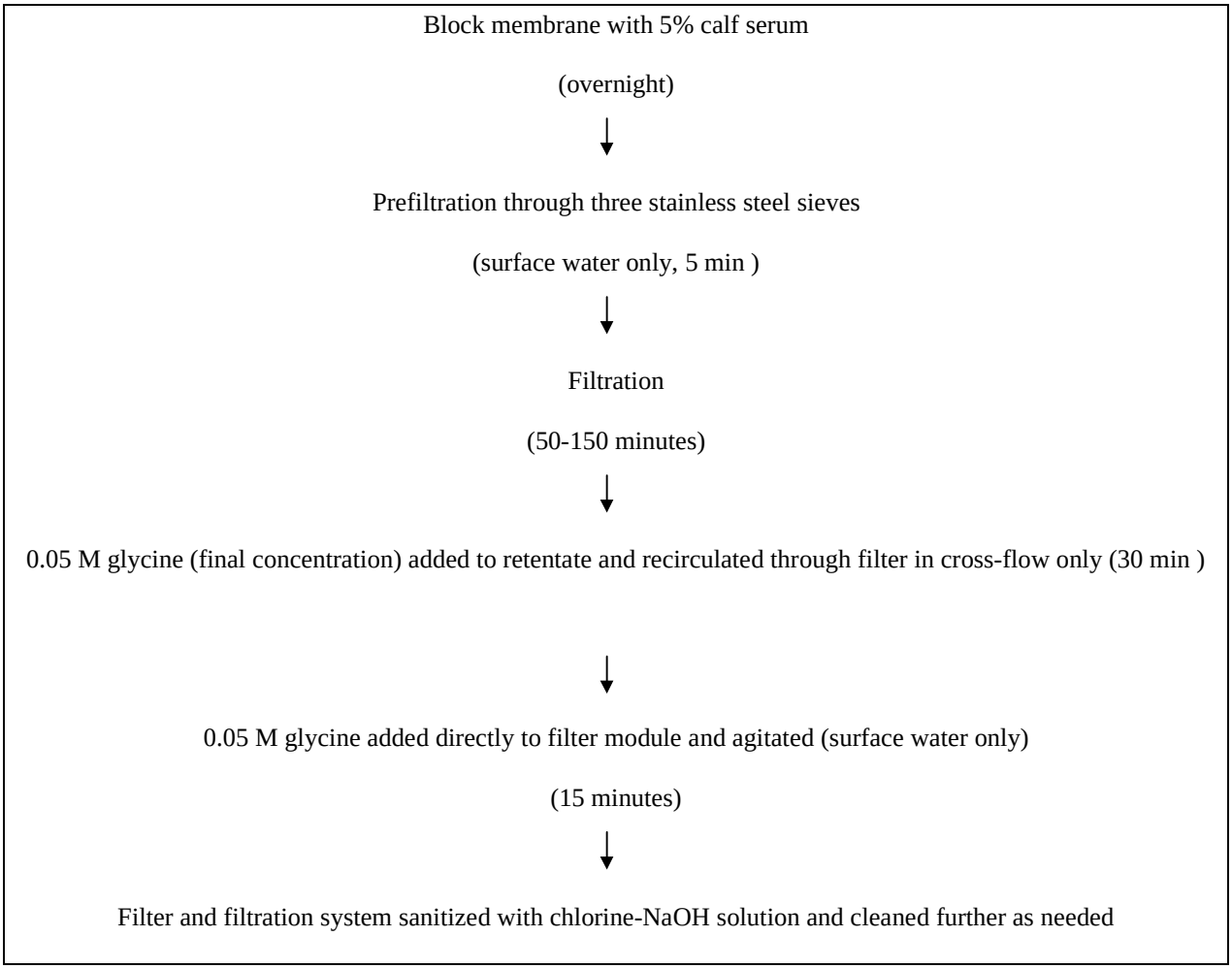

FIGURE 2. Optimized filtration scheme for 100L for the hollow fiber ultrafiltration system.

Tangential Flow Ultrafiltration

After each use, the filter system was sanitized with 200 ppm of free chlorine and 0.5M sodium hydroxide solution in 3L of RO water. The unit was recirculated in the same manner as the small-scale system and the free sodium hypochlorite level was tested by the DPD method. The system was then flushed with RO water until the pH was ~ 7 and the free sodium hypochlorite level was less than 0.04 mg/L. When surface water was used, additional cleaning was done periodically with 10% acetic acid recirculated through the filter module for one hour

followed by flushing with RO water to remove the sanitant. When not in use, the filter module was placed in the refrigerator for storage in 200 ml of NaOH.

When the proper clean water flux was obtained, the filter module was blocked with 3L of 10% calf serum by cross-flow recirculation of the blocking solution for two hours (flow parallel with the filter membrane (no permeate flow), and then the system was flushed with 10L of RO water to remove any unbound blocking agent.

Two 50L carboys of water sample were inoculated with virus (to a final concentration of ~1000 pfu/ml) and mixed thoroughly. For tap and well water samples, the 100L of water was then poured into a 100L tank and recirculated through the pilot ultrafiltration system for five minutes before taking an input sample (10 ml). For surface water, a 10-ml sample of the initial 100L virus suspension was taken before the virus suspension was prefiltered through a 75, 53 and 38 um 12-inch diameter stainless steel sieves (VWR, Denver Colorado) in sequential order to remove some of the larger particulates. Following prefiltration, the 100L virus suspension was then filtered at an input pressure of 30 psi and an outlet of 15-18 psi. When filtration was completed the retentate volume remaining was measured (~2.5 L) and a 10-ml sample was taken.

For elution, 0.05 M glycine at pH 7.0 (final concentration) was added to the retentate sample. The retentate with glycine added was then recirculated for 30 minutes in the cross-flow mode only. A 10-ml sample was then taken to determine the effectiveness of the elution process. For surface water experiments, after the elution step, the retentate solution was removed and a fresh elution solution 1.5L was recirculated through the system for 15 minutes. The elution solution was added to the retentate sample.

Ten-fold serial dilutions of the input, retentate, retentate after the addition of glycine, elution off the filter and permeate samples were done and the dilutions tested for virus concentration via the plaque assay as previously described (Oshima et al. 1994, 1995).

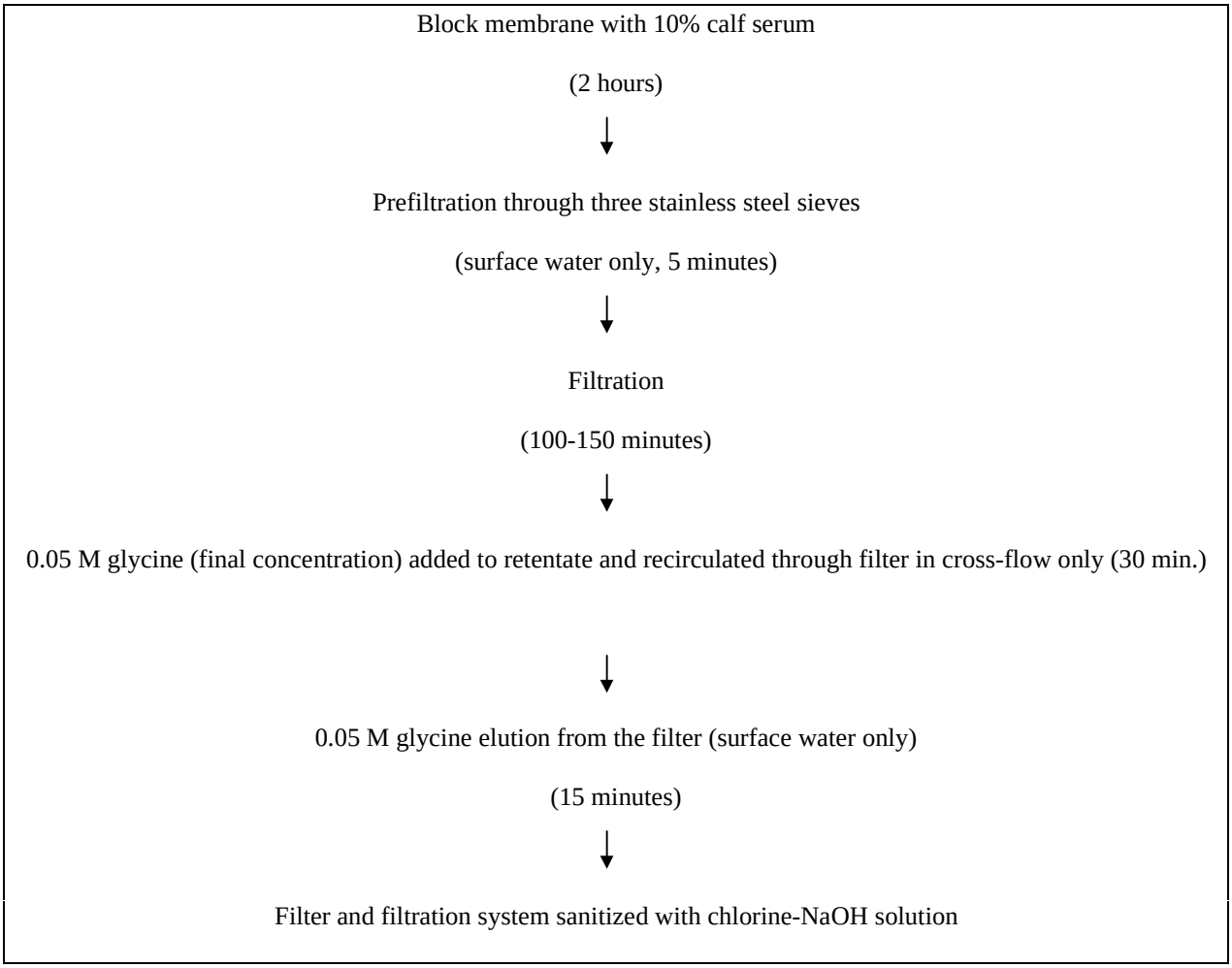

FIGURE 3. Optimized filtration scheme for 100L for the tangential flow ultrafiltration system.

Calculations

Plaque assays were done to determine the percentage of virus recovered from each experiment. The following equations were used to calculate the efficiency of viral recovery. The following equations were used.

Virus recovery from the retentate with no elution:

$$(1)\ \%\ \text{recovery in the retentate prior to any elution step(s)} = \frac{\text{Total PFU of virus in the retentate}}{\text{Initial total PFU of virus in 2L or 100L}} \times 100$$

For the retentate containing 0.05M glycine and after 15-30 min recirculation of the concentrate:

$$(2)\ \%\ \text{recovery from elution} = \frac{\text{retentate/eluant recirculation PFU/ml x volume}}{\text{Total input PFU/ml x 2 L or 100 L}} \times 100\%$$

For the elution of the virus off the filter (for large-scale surface water experiments only):

$$(3)\ \%\ \text{recovery off of the filter} = \frac{\text{elution off the filter PFU/ml x 200 ml}}{\text{Total input PFU/ml x 2L or 100L}} \times 100\%$$

For total recovery (for 100 L surface water samples only):

$$(4)\ \%\ \text{total recovery} = (2) + (3)$$

The data was usually reported as the mean and standard deviation of three replicate experiments.

Cryptosporidium

Purified *C. parvum* oocysts (human/mouse strain AZ-1) were purchased from Parasitology Research Labs., LLC (Neosho, MO). Oocysts were density gradient purified and resuspended in an antibiotic solution for overnight shipment to our laboratory. Oocysts were enumerated via fluorescent antibody (FA) prior to use and were used within three months of receipt.

Small-scale filter setup involved silconized tygon (96420-15, Cole-Parmer, Vernon Hills, IL, USA) tubing extending from the 2L retentate beaker through a peristaltic pump (pump-head 7518, Cole-Parmer, Vernon Hills, IL, USA) to a pressure gauge. A second piece of tubing

connected the pressure gauge to the inlet of the filter module. Tubing also connected the outlet of the module to a screw-down pressure regulator and back to the retentate beaker where the sample was concentrated. Another tube was connected from the permeate port to the permeate beaker (Figure 1).

Filter preparation involved blocking with 5% FBS in the same manner as with the virus challenges. Unbound FBS was removed by flushing the module with 10L of sterile deionized water. After each experiment, the filter module was drained of all excess water and filled with 10% sodium dodecyl sulfate (SDS), and incubated at 37^{o}C for 24 hours. After treatment with SDS the filter was rinsed by flushing 12.0L of sterile deionized water once through the module outlet and permeate port. The filter was re-blocked before each experiment. As a control, unblocked filters were challenged with oocysts suspended in sterile deionized water to determine if the use of FBS improved oocyst recovery. In these experiments, the filters were sanitized by recirculating a 5% chlorine bleach solution through the filter to prevent microbial growth during storage as outlined in previous viral protocols. Excess bleach was removed prior to each subsequent challenge by flushing 12L of sterile deionized water through the filter. On three randomly selected challenges, the filter was treated with SDS, blocked with FBS, and re-challenged with sterile deionized water to determine if oocysts were being carried over from the previous challenge.

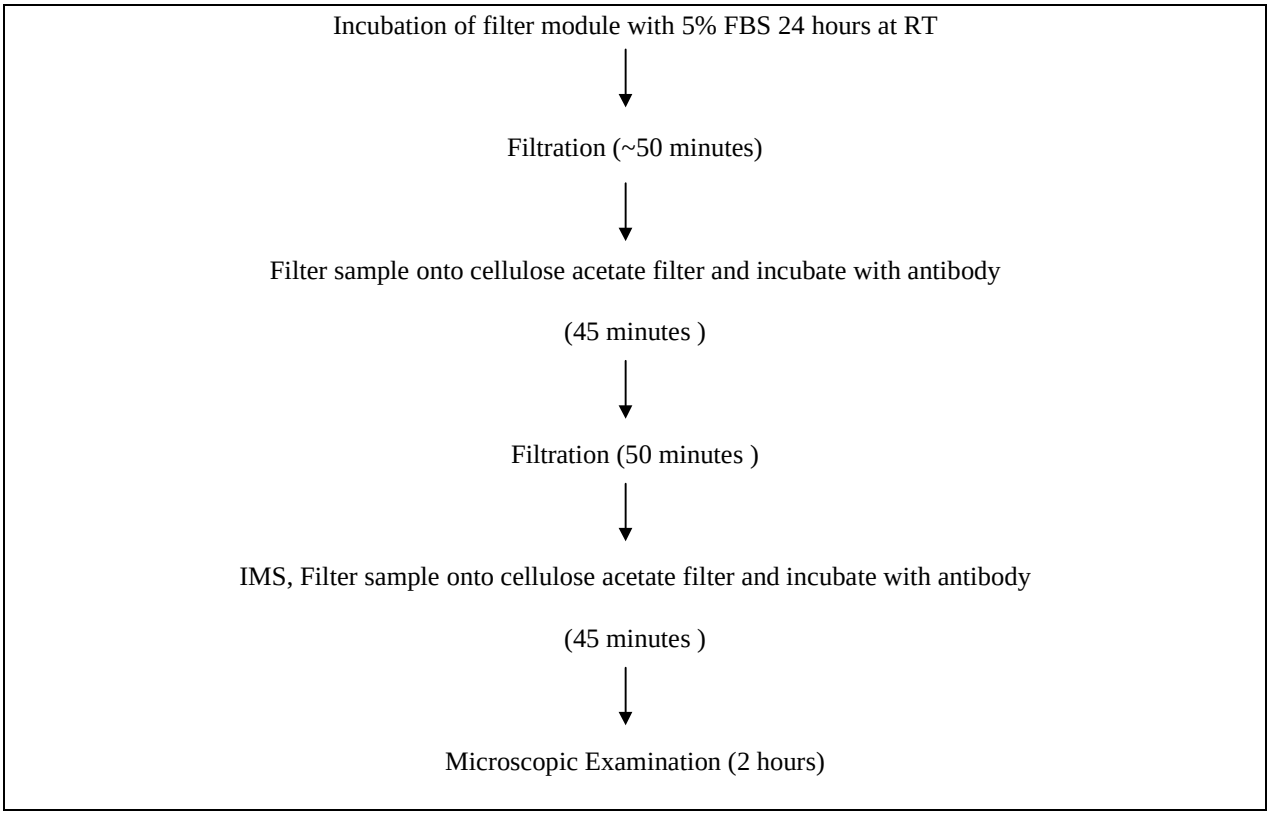

FIGURE 4. Optimized procedure for concentrating Cryptosporidium oocysts from 10L of water.

Water Samples

Water samples were collected from the following sources: Las Cruces tap water, Las Cruces well water (New Mexico State University Fisheries and Wildlife Lab), the Arkansas River below Pueblo Dam (Pueblo, CO), the Fountain River (southern Colorado) the Rio Grande (Las Cruces, NM) and the San Juan river (near San Juan, New Mexico). Water samples were kept at 4C until time of use. A 125-ml sample was analyzed for turbidity at the time of use.

Filtration

A 2L sample was spiked with 550 to 210,000 oocysts, together with 0.05% FBS to block the beaker and tubing. The water was allowed to circulate through the filtration system in the

cross-flow mode for five minutes (permeate port closed) to further mix the sample. An initial sample of 200 ml was taken and initial oocyst concentrations determined via fluorescent antibody test. During filtration the pressure was maintained at 8psi. Filtration was continued until 25-40 ml of sample remained in the retentate beaker, at which time the pump was shut off and the entire retentate volumes collected. To remove additional oocysts from the system, 20 ml of sterile deionized water was circulated in the cross-flow mode for 5 minutes. The samples were then pooled, mixed and the entire retentate (50-95ml) and permeate volume (~1750 ml) analyzed by FA. Due to the amount of suspended particles in the Arkansas River sample, the retentate volume (50-95 ml) was divided in half and both fractions were then individually analyzed to reduce the background during FA analysis. Due to the high turbidity of the Rio Grande sample, the retentate was diluted 1:10 to decrease interference by suspended particles and then 1 ml of the diluted sample was analyzed.

Fluorescent Antibody (FA) Staining

Three, 0.8 μm, 13-mm cellulose acetate (Sartorious Corp., Filter Div., Hayward CA, USA) filters were moistened with 1X phosphate-buffered saline (PBS), pH 7.4, and placed into separate 13-mm stainless steel syringe filter holder (Fisher Scientific, Pittsburgh, PA) attached to a 30-cc syringe clamped to a ring stand. The output of the filter holder was connected to a 250-ml vacuum flask. One ml of a 1% bovine serum albumin (BSA) solution (1% BSA in sterile deionized water) (Sigma, St. Louis, MO USA) was added to the syringe and a vacuum of 2-4 inches Hg was applied to the syringe to pull the BSA through the filter. The sample (1-90 ml depending on the sample type) was then added to the syringe and the vacuum was again applied until the entire sample was filtered. The system was then rinsed with 10 ml of 1X PBS. The

filter holder was removed, and 200 µl of antibody (Crypto-glo, Waterborne, New Orleans, LA, USA) diluted 1:20 in 1% BSA was pipetted into the filter holder. Both ends of the filter holder were covered with aluminum foil and the sample was incubated for 40 minutes at room temperature. Following incubation, the excess antibody was rinsed from the filters with 15 ml of 1X PBS via vacuum. The filter was then removed and placed on a glass microscope slide. Five ml of 1X PBS was placed on the filter and a glass cover slip was placed over the top. The slides were viewed immediately using epifluorescence. The entire surface of the filter was scanned under 400X magnification following the method outlined in EPA method 1622. The recovery of the oocysts in the retentate was calculated by the following equation:

$$(5)\quad \%\ \text{recovery} = \frac{\text{Total number of oocysts in the retentate}}{\text{Total number of oocysts in the 1.8L suspension}} \times 100$$

Large-scale Ultrafiltration of *C. parvum* oocysts

The optimized procedure for large-scale (10L) samples was the same as the 2L samples except for the use of a larger filter module (2.2 sq ft membrane surface area) and a larger hold-up volume in the retentate (300-400 ml). In addition, a high through put pump head (Cole Parmer 77250-62) was used on the peristaltic pump.

Downstream Processes After the Initial Ultrafiltration Step

Viruses

Viruses were further concentrated using a second ultrafiltration step with a small-scale tangential flow ultrafiltration system (ultrasette). Concentration procedure was the same as the 2L experiments previously described. The retentate from the first step (100L initial volume) was concentrated using a tangential filtration system down to ~3L. The retentate was stored at 20C until ready for use. For surface water samples, the retentate (with 0.05M glycine added and 0.1% Tween 80, Sigma Chemical Co.) was then centrifuged at 6,000 x g to remove the particulates. The virus concentration was determined by plaque assay before and after centrifugation.

The supernatant was then filtered through an ultrasette filter that was blocked as previously described. The retentate was then recirculated for five minutes and the viral concentration in the retentate determined by plaque assay. The final retentate volume was 80-150 ml.

Purification of *Cryptosporidium* Oocysts by IMS

Once the retentate was obtained, the sample was centrifuged at 3,000 x g. The pellet was resuspended in 10 ml of reagent grade water and the IMS (Dynal, Oslo, Norway) was used as per manufacturers procedure. If the sample contained more than a 0.5 ml packed pellet, only a 0.5 ml packed pellet was used and the recovery of oocysts back calculated from the percent of the packed pellet that was sampled. The recovered oocysts were then counted as described above using IFA.

RESULTS

Recovery of Viruses

Stability Tests

The stability of phages T1 and PP7 and poliovirus in UPW and UPW buffered with PBS was determined by suspending the viruses in these fluids. More uniform stability among the three viruses was observed when the virus suspensions were buffered with PBS (Table 4). However, phage T1 was not as stable as phage PP7 and poliovirus. In all filtration experiments, viral suspensions were buffered with PBS to maximize viral stability among the three viruses during the concentration process (a one to three hour process).

TABLE 4. Stability of bacteriophage T1, PP7 and poliovirus in ultrapure water (UPW) and phosphate buffered saline (PBS, pH 7.0) for a 24-hour period at room temperature.

			Percent Recovery [a,b]		
	Suspension Fluid	Initial Suspension	1 hour	3 hours	24 hours
T1	UPW	100	89 (25)	28 (20)	0.2 (0.4)
	PBS	100	68 (16)	34 (16)	4 (4)
PP7	UPW	100	56 (12)	5 (3)	0 (0)
	PBS	100	90 (12)	73 (15)	57 (3)
Polio	UPW	100	81 (3)	57 (21)	48 (15)
	PBS	100	84 (19)	65 (8)	32 (29)

[a] Each data point is the mean of three experiments
[b] Standard deviation inside parenthesis

Recovery of Virus from Reagent Water Using Ultrafiltration

Hollow Fiber - In all recovery experiments, a 2L virus suspension was concentrated down to 30-50 ml (hold-up volume of the system). The concentration of virus in the initial suspension (2 L) was compared to the amount of virus recovered in the retentate.

Without treatments to prevent viral adsorption to the filter or the use of elution steps to resuspend virus bound to the filter, low recovery of all three model viruses was observed. Pretreatment of the ultrafilter by blocking the membrane surface with proteinaceous materials appears to reduce the ability of viral particles to bind to the filter during concentration. Several pretreatment regimes were tested (nutrient broth, beef extract, bovine serum albumen or FBS) to block viral adsorption to the filter (Table 5).

Results from the hollow fiber ultrafiltration system indicated that all the blocking agents (except 1% beef extract) had a positive effect on the efficiency of virus recovery compared to filters that were not pretreated (Table 5). As a group, the most efficient recovery among the three viruses was obtained using a 1% FBS solution as a blocking agent. Similar results were observed when a lower concentration of virus was used (~10 PFU/ml) with phages T1 and PP7 and the hollow fiber blocked with 2% nutrient broth (results not shown).

TABLE 5. Comparison of various blocking agents to enhance recovery of viruses from reagent grade water with the hollow fiber, 50 000 MWCO polyacrylonitrile ultrafilter.

Blocking Agents	% Virus Recovery[a] Phage T1 (S.D.)[c]	PP7 (S.D.)[c]	Poliovirus (S.D)[c]	Time min.[b] Phage	Polio
None[d]	2 (1)	5 (5)	ND[e]	12,	ND
None[f] PBS	22 (25)	38 (30)	4 (5)	10	15
2% Nutrient Broth	58 (7)	91 (12)	52 (25)	13	15
4% Nutrient Broth	69 (39)	100 (48)	28 (18)	15	19
5% BSA	40 (22)	98 (7)	57 (42)	45	70
1% Beef Extract	12 (20)	29 (46)	ND[f]	11	ND
5% Beef Extract	47 (32)	99 (41)	43 (18)	15	13
1% FBS	**47 (10)**	**94 (20)**	**98 (7)**	25	28

[a] Average virus recovery for three replicate experiments
[b] Time to concentrate 2L suspension of virus to the holdup volume
[c] Standard deviation
[d] Virus suspended in ultrapure water
[e] Not determined
[f] Viruses suspended in reagent water buffered with PBS pH 7.0

Tangential Flow – Based on the results from the hollow fiber system, a smaller array of blocking agents was tested with the tangential flow system (MWCO of 10,000 Da) (Table 6). The results indicate that recovery also improved with the addition of a blocking step for the tangential flow system. Both ultrafiltration systems appear to have similar efficiencies in terms of virus recovery. Like the hollow fiber system, the highest recovery (among the three viruses as a group) for the tangential flow system was also observed when 1% FBS was used as a blocking agent (Table 6).

TABLE 6. Comparison of various blocking agents to enhance recovery of viruses with a tangential flow, 10 000 MWCO polyethersulfone ultrafilter.

Blocking Agents[c]	% Virus Recovery[a] Phage T1 (S.D.)[d]	PP7 (S.D.)[d]	Poliovirus (S.D)[d]	Time min.[b] phage, polio
2% Nutrient Broth	57 (9)	65 (14)	53 (7)	25, 35
4% Nutrient Broth	68 (21)	68 (9)	19 (7)	55, 63
5% Beef Extract	40 (13)	91 (9)	15 (2)	31, 36
1% FBS	**52 (18)**	**87 (12)**	**74 (11)**	18, 35

[a] Average virus recovery for three replicate experiments
[b] Time to concentrate 2L suspension of virus to the hold-up volume
[c] Viruses suspended in 2L of reagent water buffered with PBS
[d] Standard deviation

Recovery of Virus from Environmental Water (Tap, Ground, Surface)

Hollow Fiber - Based on results with reagent water, pretreatment of the ultrafilter with 1% FBS produced the highest and most consistent recoveries for PP7 and poliovirus (Tables 4 and 5). However, recoveries with 1% FBS were not as efficient when viruses were suspended in other types of water. Increasing the FBS concentration in the blocking solution to 5% and the duration of the blocking step to overnight improved recovery from groundwater. However, lower recoveries were observed when this method was used with surface water (Table 7). Prefiltration (11 μm) did not appear to improve recoveries from surface water although the flow rate did improve and was used for most of the surface water experiments (Table 7).

TABLE 7. Recovery of phages T1 and PP7 from 2000 ml of tap, ground and surface water (buffered with PBS) using a hollow fiber 50 000 MWCO polyacrylonitrile ultrafilter that was pretreated with FBS prior to filtration.

Blocking Agent	Water Type	% virus recovery [a] Phage T1 (S.D.)[b]	Phage PP7 (S.D.)[b]	Time [c]
1% FBS[d]	Tap	46 (24)	78 (18)	32
1% FBS[d]	Ground	12 (2)	37 (8)	37
1% FBS, 10% serum elution [e]	Ground	44 (17)	55 (20)	37
5% FBS [f]	Ground	72 (9)	68 (12)	54
5% FBS [f]	Surface	6 (3)	32 (10)	74
5% FBS prefiltered[g]	Surface	17 (8)	13 (7)	63
5% FBS prefiltered[h]	Surface	35 (22)	51 (20)	57

[a] Average virus recovery for three replicate experiments
[b] Standard deviation
[c] Time to concentrate 2L suspension of virus to the holdup volume
[d] Membrane blocked with a 1% solution of FBS prior to concentration
[e] After the concentration step, the virus was eluted off of sediments and filter with 10% FBS added to the retentate
[f] Membrane blocked with a 5% solution of FBS prior to concentration
[g] Raw water was prefiltered through an 11μm filter before the addition of virus
[h] Raw water was prefiltered through a 0.2 μm filter before the addition of virus

The use of FBS as an elution agent was examined closely. The addition of FBS (10%) to the retentate and recirculating the retentate utilizing cross-flow (no backpressure) for 30 minutes did not improve recovery to the desired levels when groundwater was used (Table 8). In another set of experiments, 0.5% FBS was added to the buffered groundwater in the original 2L virus suspension. Recovery was quite variable depending on the virus and filtration proceeded at a much slower rate. The addition of 0.5% FBS to the retentate after it was concentrated to 500 ml produced improved recoveries particularly when the final retentate was recirculated for an additional 15-30 minutes (Table 8).

TABLE 8. Recovery of phages T1 and PP7 and poliovirus from different waters using the hollow fiber 50 000 MWCO polyacrylonitrile ultrafilter. Combination blocking and elution procedure was used to improve virus recovery.

Treatment	Water Type	Mean % Virus Recovery[a] (+/- standard deviation)			Time[b] (min)
			Immediate	w recirc.[c]	
1% FBS block, 10% FBS elution[c,d]	Ground	T1	44 (17)	ND	37
		PP7	55 (20)	ND	37
		Polio	ND	ND	
0.5% FBS added last	Ground	T1	28 (7)	**57 (22)**	58
		PP7	38 (6)	**61 (13)**	58 500 ml
		Polio	71 (16)	**90 (7)**	44
5% FBS Block with elution 0.05M glycine	Ground	T1	24 (16)	**87 (3)**	102
		PP7	41 (33)	**88 (23)**	102
		Polio	21 (25)	**90 (10)**	87
Elution with 0.05M glycine no block	Ground	T1	2 (1)	14 (9)	37
		PP7	2 (0)	23 (11)	37
0.5% FBS added to 2L	Surface	T1	38 (12)	ND	127
		PP7	78 (21)	ND	127
		Polio	21 (11)	ND	121
0.5% FBS added last 500ml	Surface	T1	7 (4)	**27[e] (8)**	64
		PP7	12 (6)	**51[e] (4)**	64
		Polio	15 (16)	**81[e] (3)**	55
0.05% FBS 500 ml[e]	Surface	T1	14	ND	69
		PP7	7	ND	69
5% FBS block with elution 0.05M glycine pH 9.0	Surface	T1	33 (33)	**61 (11)**	59
		PP7	41 (23)	**85 (2)**	59
		Polio	10 (9)	**82 (12)**	106

Groundwater was 0.1 NTU, surface water was 15-40 NTU

Bold numbers represent conditions that produced the most efficient viral recoveries.

[a] Each data point is the mean of three replicate experiments

[b] Time to complete filtration from 2L

[c] 30 min recirculation of retentate prior to virus assay

[d] Membrane blocked with a 1% solution of FBS prior to concentration and after the concentration step FBS was added to a final concentration of 10% in the retentate and recirculated for 30 min as an eluent

[e] 15 min recirculation of retentate prior to virus assay

[f] One experiment

In other experiments, glycine (0.05M final retentate concentration at pH 7.0 or 9.0) was added to the retentate as an elution agent with filters that were also pretreated with 5% FBS as a blocking agent. These results produced the most efficient recovery (>60%) among the three viruses in surface and groundwater compared to any other process examined (Table 8). Recovery of virus was not as efficient when only the glycine elution step was used (without a blocking agent) when tested with virus suspended in groundwater (Table 8).

Tangential Flow - Blocking agents were also used to improve recoveries of phages T1 and PP7 and poliovirus using the tangential flow ultrafiltration system (Tables 6 and 9). In contrast to the hollow fiber system, several differences were noted when the same methods were applied to the tangential flow system. The recirculation of the retentate for 30 minutes resulted in a decrease in virus recovery and the use of 0.05M glycine as an eluent added to the retentate directly did not appear to enhance recovery when coupled with the use of 5% FBS as a pretreatment to block the membrane before use. Recoveries were actually lower when this was done with surface water (Table 9). However, the use of 0.05M glycine recirculated for five minutes (retentate collected and removed prior to glycine elution) did improve the recovery of poliovirus (Table 9). The use of 0.5% FBS added to the retentate when the retentate volume reached 500 ml did not appear to recover the phages from surface water as well as with the hollow fiber system (Tables 8 and 9). Prefiltration was not needed to concentrate 2L of surface water to efficiently recover phages T1 and PP7 (Table 9).

Table 9. Recovery of phages T1 and PP7 from different waters using a 10 000 MWCO polyethersulfone tangential flow ultrafiltration system.

Treatment	Water Type		Mean % Virus Recovery[a] (+/- standard deviation) Immediate	With Recirculation.[c]	Time[b] (min)
0.5% FBS added to last 500 ml	Ground	T1	65 (22)	21 (16)	13
		PP7	81 (16)	43 (10)	13
0.5% FBS added to last 500 ml	Surface	T1	24 (13)	18 (18)	15
		PP7	51 (14)	41 (19)	15
5% FBS block with glycine elution 0.05M	Ground	T1	**63 (27)**	61 (19)	17
		PP7	**77 (35)**	72 (20)	17
		polio	**43 (10)**	ND	18
5% FBS block with glycine elution 0.05M	Surface	T1	**53 (2)**	36 (15)	22
		PP7	**92 (17)**	55 (22)	22
		polio	**52 (13)**	ND	21
5% FBS	Surface	T1[d]	**51 (13)**	ND	20
		PP7[d]	**81 (25)**	ND	20
		polio[d,e]	ND	**77 (10)**	26

Bold numbers represent conditions that produced the most efficient viral recoveries

[a] Each data point is the mean of three replicate experiments

[b] Time to complete filtration from 2L

[c] 30 min recirculation of retentate prior to virus assay; in some cases refers to the recirculation of the elution agent

[d] No prefiltration

[e] Virus bound to the membrane was eluted with the recirculation of 100 ml, 0.05% FBS in 0.05M glycine pH. 7.0 for five minutes. This was then combined with the retentate to determine the % recovery.

ND = not done

Large-scale Ultrafiltration

Once the small 2L ultrafiltration systems were tested and optimized, large-scale 100L volumes where then tested. The knowledge from the 2L experiments was used as the starting point for the large-scale experiments.

For large-scale ultrafiltration, the same ultrafiltration systems were used except the modules contained more surface area (Table 1) and larger peristaltic pumps were used. In addition, stainless steel sieves were used after spiking 100L of Rio Grande water to prefilter the water sample prior to filtration through either the hollow fiber or tangential ultrafilter. At least 96% of spiked virus (phages T1, PP7 and poliovirus) was recovered from the prefiltration process (Table 10).

TABLE 10. Virus recovery after prefiltration of 100L of surface water through 75, 53 and 38 μm sieves.

Virus	Mean % recovery[a] (S.D.)
T1	98 (1)
PP7	97 (2)
poliovirus	96 (2)

[a]Mean recovery and standard deviation from 3 replicate experiments.

Hollow Fiber Ultrafiltration - Like the small-scale system, virus recovery was tested with three model viruses and with ground and surface water. Recoveries were efficient (> 70%) and consistent among all three viruses in ground and surface water. Filtration was competed within 100 minutes (Table 11).

Results indicate that viral recoveries similar to the small-scale hollow fiber systems were achievable from 100L (Table 12). Procedural differences between the small-and large-scale systems were few. One difference was the addition of a filter elution step to elute viruses remaining on the filter surface when surface water was concentrated.

TABLE 11. Optimal recovery of phages T1 and PP7 with a hollow fiber 50,000 MWCO polyethersulfone ultrafilter from 100L water samples.

Blocking Agent	Water Type	% virus recovery Phage T1 (S.D.)[a]	Phage PP7 (S.D.)[a]	Polio	Time (min.)[b] phage, polio
5% calf serum with 0.05M glycine elution	Ground 100L	5 (2)[c] **71[d] (10)**	4 (2)[c] **70 (15)**	ND **82 (5)**	47 56
5% calf serum with 0.05M glycine elution	Surface 100L	ND **70[d] (9)**	ND **86 (4)**	ND **69(18)[e]**	98 92

Final recovery in 100L are in bold. Most efficient concentration methods are in bold.
[a] Standard deviation
[b] Time to complete filtration of 100L
[c] Top row are % recoveries without the elution step
[d] Second row recoveries after elution of bound viruses with 0.05M glycine
[e] Results from 4 replicate experiments

TABLE 12. Comparison of the recovery of viruses from 2L and 100L virus suspensions with the hollow fiber ultrafiltration system.

Water Type	Virus	2L recovery [a] (mean and SD)	100 L recovery a (mean and SD)
Ground water	T1	87 (3)	71 (11)
	PP7	88 (23)	70 (15)
	Poliovirus	90 (10)	82 (5)
Surface water	Ti	61 (11)	70 (9)
	PP7	82 (2)	86 (4)
	Poliovirus	82 (12)	69 (18)[b]

[a] Average recovery and standard deviation from 3 replicate experiments
[b] Four replicate experiments

In order to determine the effectiveness of the sanitation process of the large-scale hollow fiber ultrafiltration system, experiments were done to determine if the model viruses could be detected following sanitation when a virus challenge was done with either ground or surface water. When a 100L of RO water was filtered with no virus added, in all the experiments no viruses were detected in the concentrate except for one poliovirus experiment that followed a concentration experiment with surface water. However, in this experiment virus was detected in the initial 100L pool indicating that the tank may not have been properly disinfected since virus was detected directly from the 100L of RO before it had been filtered with tap water (Table 13).

TABLE 13. Viral carry-over after 100L experiments in the large-scale hollow fiber system. 100 L of RO water was concentrated with no spiked virus and otherwise processed in the same manner as a normal experiment.

Virus	Groundwater	Surface water
T1 phage	not detected	not detected
PP7 phage	not detected	not detected
poliovirus	not detected	not detected in 2/3 2.0×10^2 in one replicate

Tangential Flow Ultrafiltration - Recovery with the tangential flow ultrafiltration system in 100L was also similar to the 2 L system (Table 9). Much like the 2L volumes, the use of an elution step did not improve recovery as much as with the hollow fiber ultrafilter. Still, mean recovery in ground water ranged from 57-95%. Filtration was completed within 150 minutes. Results between the 2 and 100L volumes were not as consistent as with the hollow fiber system (Tables 14 and 15).

Table 14. Optimal recovery of phages T1 and PP7 with a 10,000 MWCO tangential flow ultrafilter from 100L water samples.

Blocking Agent	Water Type	% virus recovery [a] Phage T1 (S.D.)[a]	Phage PP7 (S.D.)[a]	Polio	Time (min.) [b]
5% calf serum with	Ground	53 (9) [c]	69 (5) [c]	92 (15)	120
0.05M glycine elution	100L	**57[d] (11)**	**74 (7)**	**95 (15)**	
5% calf serum with	Surface	114 (25)	99 (8)	52 (15)	150
0.05M glycine elution	100L	**123 (25) [e]**	**104 (10)**	**56 (6)**	

Final recovery in 100L are in bold.
[a] Mean of three replicate experiments
[b] Time to complete filtration of 100L
[c] Standard deviation
[d] Top row are % recoveries without the elution step. [b] Time to complete filtration of 100L
[e] Second row recoveries after elution of bound viruses with 0.05M glycine

TABLE 15. Comparison of the recovery of viruses from 2 L and 100 L virus suspensions with the tangential flow ultrafiltration system.

Water Type	Virus	2L recovery [a] (mean and SD)	100 L recovery a (mean and SD)
Ground water	T1	63 (27)	57 (11)
	PP7	77 (35)	74 (7)
	Poliovirus	43 (10)	95 (15)
Surface water	Ti	51 (13)	123 (25)
	PP7	92 (17)	104 (10)
	Poliovirus	77 (10) [b]	56 (6)

[a] Average recovery and standard deviation from three replicate experiments
[b] A additional elution step with 0.05 M glycine (recirculation for five minutes) off the filter was done after the retentate was removed.

Comparison Between the Hollow Fiber and Tangential Flow Ultrafiltration Systems for the Recovery of Viruses

Viral recoveries were similar between the hollow fiber and tangential flow ultrafiltration systems. However, recoveries were more consistent among the three viruses and the different water types with the hollow fiber than the tangential flow system (Table 16). The time to filter 100L was slightly faster with the hollow fiber system.

TABLE 16. Comparison of viral recoveries (%) from 100L of environmental waters using a 50,000 MWCO hollow fiber and 10,000 MWCO tangential flow ultrafilters.

Water	Virus	Hollow Fiber % Recovery[a]	Tangential % Recovery [a]
Ground	T1	71 (11)	57 (11)
	PP7	70 (15)	74 (7)
	Poliovirus	82 (5)	95 (15)
Surface	T1	70 (9)	123 (25)
	PP7	86 (4)	104 (10)
	Poliovirus	69 (18)	57 (6)

[a] Mean and standard deviation of three replicate experiments

Recovery of Virus after Further Steps to Concentrate Viral Samples

Experiments were also done to assess methods to further concentrate viral samples after the initial ultrafiltration process. These experiments included centrifugation (surface water samples only) to pellet particulates from the retentate samples and a second small-scale ultrafiltration process to further concentrate the retentate. Results with centrifugation step indicate that viruses were efficiently maintained in the supernatant during the centrifugation process (Table 17).

Preliminary experiments from ground and surface water indicated that efficient recoveries were possible from a second ultrafiltration step to further concentrate the samples (>50%) although the recoveries were not as efficient as in the initial large-scale ultrafiltration process (Table 18). Filtration was completed in <1 hour.

TABLE 17. Viral recoveries (%) from 3L of concentrated surface water (100L) following centrifugation at 3000 rpm for 20 minutes to remove sediments.

Treatment	Virus	Recovery %[a] (S.D.)[b]
None	T1	70 (45)
	PP7	68 (32)
	Poliovirus	82 (48)
0.1% between 80	T1	80 (7)
	PP7	92 (11)
	Poliovirus	68 (16)

[a] Mean of three replicate experiments
[b] Standard deviation

TABLE 18. Comparison of viral recoveries (%) by a second-step concentration from 3L of concentrated environmental waters through a 10,000 MWCO polyethersulfone screen channel ultrafilter.

Water[a]	Virus	Recovery % w/o recirculation[a, b] (S.D.)[c]	Recovery % w/ recirculation[a, b] (S.D.)[c]	Time (min)[d]
Ground	T1	32 (4)	54 (9)	35
	PP7	39 (6)	52 (12)	
	poliovirus	45 (8)	51 (12)	
Surface	T1	99 (23)	72 (22)	55
	PP7	79 (11)	53 (14)	
	poliovirus	37 (14)	50 (10)	

[a] Ground and surface water samples (100L) were first concentrated through a field-scale tangential flow system concentrated and resuspended in a 3L volume before the second concentration step through the small scale tangential ultrafilter
[b] Mean of three replicate experiments
[c] Standard deviation
[d]Time to complete filtration

Recovery of *Cryptosporidium* Oocysts from Water in a Small-Scale System

The hollow fiber ultrafilter without SDS/FBS treatment recovered an average of 37.2% (47.8% with five minute recirculation) during the first four challenges from deionized water. A gradual decrease in oocysts recovery was noted during each subsequent challenge, resulting in the average recovery to decrease to 29.3% over the subsequent four challenges (Table 19). Treatment of the membrane with SDS to remove bound particles, followed by the addition of a 5% FBS blocking solution dramatically improved the recovery and also decreased the variation between samples (Table 19). On average, the permeate flow of an unblocked filter was 165 ml/min (S.D. 5.6 ml/min), compared to a blocked membrane where the flow slowed to an average of 40 ml/min (S.D. 7.7 ml/min). The addition of 0.05% FBS to the 2L sample produced a small increase in oocyst recovery (75.3% S.D. 6.3, n=3) over samples that contained no additional FBS (62% S.D. 8.5, n=3). Further increases in the concentration of FBS resulted in even lower permeate flow rates and oocysts could not be counted by FA because of premature clogging of the cellulose acetate filter for FA analysis (data not shown).

Three experiments were performed to determine if oocysts could be carried over from one experiment to the next. No oocysts were detected by FA in these experiments.

Environmental samples had a turbidity from 0.11 to 30.9 NTU. When low-turbidity samples (deionized, well, and tap) were used, the entire retentate could be filtered through a single 13-mm filter (0.8 μm) for analysis. The increased amount of suspended particles found in the Arkansas River sample resulted in the need to divide the retentate into two fractions to reduce the interference of the suspended particles on visualization of oocysts. Rio Grande samples required a 1:10 dilution, followed by the filtration of 1 ml of the 10 ml dilution through a 13 mm, 0.8-μm filter disk . The average recovery from tap, well, Arkansas River and the Rio Grande were 64.8, 75.8, 76.6 and 81.2%, respectively (Table 19). No oocysts were detected in the permeate samples.

TABLE 19. Recovery efficiency (%) of *Cryptosporidium* oocysts from 2L of deionized, tap, ground, and surface water using a 50,000 MWCO hollow-fiber ultrafilter.

No. of replicates	Water type	Retentate volume (S.D.)	Turbidity (NTU)	% Retentate analyzed	Oocyst seed density (S.D.)	% recovery efficiency (S.D.)
4	Deionized[a]	31.3 (2.1)	0.0	100	7933 (748)	47.8 (3.1)
4	Deionized[b]	56.0 (8.5)	0.0	100	12275 (5602)	29.3 (13.7)
3	Deionized[c]	36.6 (4.7)	0.0	100	8389 (1247)	48.1 (0.7)
3	Deionized[d]	38.2 (4.9)	0.0	100	0 (0)	Not Detected
3	Tap[e]	84.0 (8.2)	0.1	100	613 (45)	64.8 (9.9)
3	Well[e]	86.3 (5.4)	0.3	100	887 (465)	75.8 (9.4)
3	Arkansas[e]	84.6 (9.3)	1.4	100	866 (225)	76.6 (6.2)
3	Rio Grande[e]	71.0 (6.4)	30.9	0.1	201000 (12328)	81.0 (11.4)

[a]First four replicates of an unblocked membrane, with bleach sanitation
[b]Subsequent four replicates of an unblocked membrane (a)
[c]Membrane treated with SDS after use, without FBS block
[d]Determination of oocyst carry over between experiments
[e]Membrane treated with SDS and 5% FBS

Recovery from 10L of ground and surface water shows efficient and reproducible recoveries of oocysts (Table 20). Detection of naturally occurring oocysts was possible for a number of surface water samples (Table 21). No carry-over of oocysts was detected between experiments (Table 20). Filter modules were sanitized using standard methods and 10 of DI water was concentrated and the presence of oocysts determined by IFA. Three replicate experiments were done for water sample.

TABLE 20. Recovery efficiency (%) of *Cryptosporidium* oocysts from 10L of tap, ground, and surface water using a 50,000 MWCO hollow-fiber ultrafilter.

No. of replicates	Water type	Turbidity (NTU)	Oocyst seed density	% recovery efficiency (S.D.)
3	Tap	0.1	7693	88 IFA[a] (7)
4	Rio Grande	6.1	48607	82 IMS[b] (6)
3	Rio Grande[c]	3.6	13379	66 IMS (10)
1	Arkansas River	12.0	312	70 IFA/73 IMS
1	Arkansas River	12.0	13846	98 IFA/95 IMS
3	Arkansas River	2	177	54 IMS (3)
3	Carry over control[d]	ND	0	Not Detected
3	Fountain river	45	10293	68 IMS (21)
3	San Juan River	6	983	68 IMS (3)

[a]IFA, immunofluorecent antibody was used without IMS to quantify the number of oocysts

[b]IMS, immunomagnetic separation was used to prior to quantifying by IFA

TABLE 21. Recovery of naturally occurring *Cryptosporidium* oocysts from 10L of surface water using a 50,000 MWCO ultrafilter.

Water type	Retentate volume (S.D.)	Turbidity (NTU)	% Retentate analyzed	Number of oocysts/10L[a]
Arkansas River	175	12.0	100	18
Fountain River	330	45.0	100	195.
Rio Grande	250	52.0	100	21
Rio Grande	330	3.6	100	not detected
Fountain River	280	4.5	100	33
Rio Grande	330	16.2	100	21
Fountain River	285	226.0	100	12
Rio Grande	300	106.0	100	130
San Juan River	300	NA	100	6

[a]Results from three replicate tests

DISCUSSION

At the appropriate MWCO, ultrafiltration has the capacity to concentrate viruses and larger waterborne pathogens by size exclusion. Thus the simultaneous concentration of viral particles and *C. parvum* oocysts during filtration was achievable because the filter pore size is smaller than the pathogens targeted for recovery. This mechanism is in contrast to microfiltration where the pores are larger than the viral particle and adsorption of the viral particle to the filter must occur for there to be efficient concentration of the viral particles.

Microfiltration relies on the adsorption and elution of viral particles off microfilters as the mechanism for the concentration of viruses from water. This has resulted in variable recoveries among viruses because of surface chemistry differences between different viral particles and variation in water quality affecting the efficiency of the adsorption and elution process. The efficiency of virus recovery by microfiltration tends to be more difficult from surface water or water with higher turbidity (Sobsey and Glass 1984).

When ultrafilters of the appropriate MWCO are selected, little to no virus should be detected in the permeate regardless of the type of water or ultrafiltration system tested (less than 1% of the original inoculum was detected in the permeate of the ultrafiltration systems used in this study) (data not shown). There are however, other factors that can lower recovery efficiency of the ultrafiltration process such as the adsorption of viruses and adherence of oocysts to the filter or to particulates in the water. In this study, steps were examined that were intended to minimize these causes for the lower recovery of viral particles and oocysts during ultrafiltration.

Three viruses and *C. parvum* oocysts were used to examine the efficiency and consistency of recovery with the two ultrafiltration systems. In addition, several types of water (reagent, tap, ground, and surface) were also tested. These filtration systems were selected

because they represent two common configurations of ultrafiltration systems and both are available with additional filter modules or cassettes that can handle large volumes of water (tables 21 and 22).

TABLE 22. Available membrane modules for the hollow fiber module (50,000 MWCO polyacrylonitrile) characteristics.

Process Scale	Membrane Area (ft^2)	Hold-up Volume (in the filter) Feed Side	Permeate Side
Lab (pencil)	0.18	9 ml	9 ml
Small	2.2	90 ml	120 ml
Pilot	11.0	300 ml	500 ml
Production	51.0	1,200 ml	2,700 ml

TABLE 23. Tangential flow cassettes (10,000 MWCO polyethersulfone).

Process Scale	Membrane Area (ft^2)	Hold-up Volume (in the filter) Feed Side	Permeate Side
Pilot	1	35 ml	NA[a]
Production	5-25	90-360 ml	NA[a]

[a] Data not available.

Initial experiments were with small volumes (2L) to allow for experiments to be done conveniently and with less expense than if large systems were used. Viruses were spiked at ~1000 PFU/ml in order to get a accurate determination of the virus concentration in the initial 2L suspension. Oocysts were spiked at various concentrations to determine if there was a relationship between oocyst concentration and recovery efficiency. An accurate assessment of the initial virus and oocyst concentration is important in determining the virus recovery in the retentate.

Virus Recovery

Three viruses with different shapes and diameters were selected to examine recovery from widely varying viral particles. The results indicated that T1 recoveries were generally lower than either phage PP7 or poliovirus. Phage T1 is a tailed phage and thus may be more susceptible to shear forces that could damage its ability to adsorb to cells and initiate the infectious process. Stability experiments also indicated that phage T1 was not as stable as phage PP7 and poliovirus. Because of the size, shape, and receptor characteristics, phage PP7 and polioviruses may be more indicative of viral recoveries of pathogenic human viruses. Thus, optimal recoveries of these viruses are probably more useful than phage T1. However, phage T1 may be a good model virus for a conservative estimate of virus recovery.

Ultrafiltration to Concentrate Viruses from 2L

Small-scale results indicated that virus recoveries were low with both ultrafiltration systems unless measures were taken to either prevent viral adsorption to the filter membrane or to elute bound viruses. Although blocking the membranes with a proteinaceous agent before

filtration resulted in lower flux, this approach has advantages compared to traditional elution methods because less proteinaceous agent is needed, and once the sample processing has begun, there are fewer steps involved than by adsorption/elution. This can be an advantage from the standpoint of recovery efficiency, expense, minimal use of blocking agent in the retentate, and for more efficient detection by PCR because lower levels of inhibitory substances are introduced during the concentration process.

As water quality deteriorates, such as with surface water, additional steps were needed to prevent virus from binding to particulates in the water or to the filter surface. Adsorption of viruses to particulates during filtration may increase since particulates will also be concentrated during the filtration process.

Results indicate that both ultrafiltration systems show promise for the efficient recovery of viruses from water. Performance characteristics were quite similar between the two, although slightly different elution steps were needed. The use of a 30 minutes elution of the retentate improves virus recovery for the hollow fiber system and should be incorporated into the filtration procedure while little to no improvement was noted for the tangential flow system when the same conditions were used (tables 8 and 9).

For the hollow fiber system, the most efficient and consistent viral recovery was obtained using a 5% FBS block coupled with a 30 minutes, 0.05M glycine elution of the retentate (recoveries of phage PP7 and poliovirus of >80%). The addition of 0.5% FBS at 500 ml may also be effective, but our experiments appeared to produce recoveries that were not as high (recoveries of >50% for phage PP7 and poliovirus).

For the tangential flow system, the use of a 5% FBS block with no recirculation of the retentate produced the most efficient recoveries (recoveries of phage PP7 and poliovirus of

>43%) or the addition of 100ml, 0.05M glycine elution step for the membrane after the retentate was removed (poliovirus 77%). Under optimal conditions, both ultrafiltration systems produced similar recovery for phage PP7 and poliovirus. However, more consistent results among the viruses were observed with the hollow fiber system (tables 8 and 9).

These results indicate the feasibility of using ultrafiltration to concentrate viruses from small volumes of environmental water. With the appropriate filtration conditions, the use of these systems appears to have the flexibility to allow efficient recoveries from a wide range of water qualities. In addition, both ultrafiltration systems can be sanitized and reused. Each of the filters were reused more than 30 times with little to no observable change in recovery performance. Small volume concentration could be done as a first-step concentration procedure or as a second concentration step after elution of viruses from microfilters in large volume applications.

Ultrafiltration to Concentrate Viruses From 100L

Large-scale (100L) volumes were tested with both the tangential flow and hollow fiber ultrafiltration systems. Similar recoveries were observed between the 2L and 100L volumes with both ultrafiltration systems. However, slightly higher and more consistent recoveries were observed with the hollow fiber system (Table 15).

In both ultrafiltration systems, 100L could be filtered in less than 2 hours. Each system can be sanitized and reused such that the cost of the filter per use will be similar or lower than the cost of a single use disposable microfilter. For surface water, the use of stainless steel sieves removed large particles that could get trapped within the filter housing while allowing for little to no loss of viral particles. The use of sieves allowed prefiltration to be done very rapidly.

The initial cost of either filtration system will be higher than the cost of the current microfiltration based systems for either virus or oocyst recovery. However, the reusable nature of either ultrafilter reduces the cost of the filters such that the cost per filtration will be lower than the current methods. Pump systems will also be more expensive, but after the initial cost, these systems will be functional for a longer period of time. The ability to concentrate viruses and *Cryptosporidium* oocysts and possibly bacterial agents simultaneously by ultrafiltration will further reduce the cost by allowing a single process to do what currently requires three different methods.

Ultrafiltration also appears to be feasible as a second-step concentration procedure for viruses. Viral recovery was similar as when it was used as a first-step concentration procedure although further experimentation is needed to optimize this process.

Cryptosporidium parvum Recovery

Incubation of the hollow fiber ultrafiltration module after each experiment with 10% SDS, followed by a thorough flushing of the membrane with deionized water appeared to remove bound oocysts as well as proteinaceous materials that may have accumulated on the membrane surface. The fact that there was no carry-over of oocysts after filtration demonstrates that the SDS was able to eliminate any oocysts build-up and that the filter can be reused without affecting the performance (Table 20). The laboratory-scale ultrafiltration module has been used more than 40 times and no difference in oocyst recovery or flow has been observed.

The use of an unblocked membrane resulted in a decrease in oocyst recovery after repeated uses. This was likely due to the enhanced adhesion of oocysts to the membrane surface because of inadequate cleaning between uses. Oocysts have been demonstrated to have the ability to adhere to glass and plastic (Swabby-Cahill et al. 1998).

When a 5.0% solution of FBS was used as a blocking agent, improved oocyst recoveries were observed. This was probably due to the formation of a proteinaceous layer on the membrane surface that reduced the ability of the oocysts to adhere to the filter and helped to keep oocysts in suspension during filtration.

Efficient and consistent recoveries were also obtained from a wide range of water qualities (up to 106 NTU). The use of ultrafiltration to concentrate oocysts followed by IMS is feasible and recoveries appeared to be equal to or better than that reported for method 1623 (Bukhari et al. 1998; Connell et al. 2000) using similar methods as was used in concentrating viral particles.

These results indicate that it is feasible to use ultrafiltration to concentrate viruses and oocysts simultaneously in the initial ultrafiltration step. The use of ultrafiltration could therefore replace the current separate microfiltration methods used for viral agents and *Cryptosporidium parvum* oocysts with a single method that can be used for both viral and parasitic organisms.

Development of PCR Methods for the Detection of Viruses and *Cryptosporidium* Oocysts

Methods have been developed to adapt a ELISA-based detection PCR amplified DNA products from enteroviruses and *Cryptosporidium* oocysts using RT-PCR (enteroviruses) and PCR (*Cryptosporidium*). These systems have been optimized to very low sensitivity when stock viruses and oocysts (five oocysts per PCR reaction) are used.

Preliminary experiments have indicated low sensitivity of the PAN enterovirus PCR when environmental samples were used. Experiments are ongoing to optimize the assay for enteroviruses and *Cryptosporidium* oocysts concentrated from environmental samples.

The use of an ELISA-based detection system for PCR product is advantageous because of the potential for high through put coupled with high sensitivity. These systems also require equipment that is less costly and more readily available than other methods to detect PCR product that are commercially available.

The characterization and optimization of PCR based detection of viruses and *Cryptosporidium* oocysts is important because the utility of ultrafiltration for PCR assays has not been adequately addressed for pathogens isolated from environmental water samples. In addition, PCR-based detection is rapid and will specifically identify *Cryptosporidium parvum* whereas the antibody used in the IFA also binds to other *Cryptosporidium* species that may not cause disease in humans. PCR based detection may not be as subjective as detection by IFA. However, one potential weakness of PCR methodologies is the difficulty in determining viability of the oocysts. Recently this has been overcome by amplifying oocysts in cell culture prior to detection by PCR to establish the viability of the isolated oocysts (Di Giovanni et al. 1999).

Experimentation is continuing on the optimization of PCR-based detection from environmental samples. Due to the preliminary nature and the desire to publish this data, a detailed description of the PCR results will be available at a later date. Developing this approach will make it more feasible to process a greater number of PCR samples with equipment that is readily available and more economical than other PCR detection methods.

Future Work

Additional work will focus on optimizing the hollow fiber system for a two-step ultrafiltration procedure for viruses and other reconcentration methods. Work will focus on optimizing the integration of the PCR-based detection of viruses and oocysts from the final concentrate. Additional water samples will also be tested for virus and *Cryptosporidium parvum* recovery as well as PCR- based detection of viruses and oocysts in environmental samples.

CONCLUSIONS

Virus Recovery

1) Results indicate that the recovery of the model viruses from small-scale (2 L) samples produced similar results when expanded to a 100L field-scale system for both ultrafiltration systems. This suggests that it is appropriate to use small-scale experiments to predict performance from a large-scale system.

2) Both ultrafiltration systems appear to be reusable many times after sanitation.

3) Both ultrafiltration systems were able to filter 100L of surface water (as high as 50 NTU) in 2.5 hours with minimal prefiltration.

4) The most efficient recoveries were produced when the filters were blocked with 5% FBS or calf serum (overnight best) and after the filtration process 0.05M glycine (final concentration) is added to the retentate and the retentate is recirculated through the ultrafilter for 30 minutes for the hollow fiber and for the tangential ultrafilter. For surface water samples, after the retentate/eluant is recirculated, a fresh solution of 0.05 glycine is added to the filter module and agitated for 15 minutes and the eluent is added to the retentate. Recoveries of 69-86% were obtained for the three model viruses from 100L of ground or surface water with the hollow fiber ultrafiltration system and 57- 100% from the tangential flow system from 100L.

5) The hollow fiber ultrafiltration system appeared to provide slightly more consistent recoveries between the three viruses than the tangential flow ultrafilter, although both systems appear to be feasible for concentrating viruses from field-scale volumes.

***Cryptosporidium* Oocysts**

1) Recoveries of *Cryptosporidium* oocysts appears to be efficient and similar in 2L and 10L hollow fiber ultrafiltration systems. In 10L of surface water recoveries were from 54-88%.

2) Filters can be sanitized between uses to remove oocysts by overnight incubation of the filter modules in 5% SDS at 37^{o}C.

3) Viruses and oocysts can be concentrated together in the hollow fiber ultrafiltration system, taking the place of what has been two separate processes.

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