Journal of Microbiological Methods 160 (2019) 143–153

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

The use of hollow fiber dialysis filters operated in axial flow mode for recovery of microorganisms in large volume water samples with high loadings of particulate matter

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A B S T R A C T

Ultrafiltration concentration of microorganisms in large volume water samples containing high levels of particulate matter was evaluated in a proof of concept study. The organisms tested were *Bacillus atrophaeus* subspecies *globigii* spores and MS2 bacteriophage. To produce the large volume samples, fresh water sediment of a known particle size was added to 51 l of tap water. Five different concentrations of particulate matter were studied: 0, 50, 100, 150 and 750 mg solids/l. The concentration procedure used a dialysis filter as the ultrafilter configured for axial flow, either with or without recirculation. The target number of organisms spiked was 1×10^5 of either spores or bacteriophage per 51 l. After concentration, the filters were dissected to retrieve the fibers which were then washed using surfactant solution which was then analyzed for the target organisms. Two washes of the filter fibers were carried out sequentially. For axial flow with recirculation, the first wash produced statistically greater recovery of *B. globigii* spores (26–40% of spike) compared to the second wash (8–13% of spike). Total recovery (the sum of the recoveries for the first and second washes) ranged from 35 to 53%. Recovery increased as the solids level increased from 0 to 150 mg solids/l. Recovery at the 100 and 150 mg solids/L loadings was statistically higher at the $P < .05$ level than recovery at 0 mg/L solids. At 150 mg solids/L, axial flow without recirculation (dead end) yielded lower recovery than axial flow with recirculation, however the difference was not significant at the $P < .05$ level. Recovery of *B. globigii* at 750 mg solids/L averaged 38% using dead end axial flow. The average recovery of MS2 bacteriophage was 45% at a solids concentration of 150 mg/L using axial flow with recirculation. PhiX174 and Phi8 were also studied, however these bacteriophage appeared to be inactivated in the matrix of concentrated wash water. One hundred liters of water containing 750 mg solids/L was concentrated using dead end axial flow, and only minimal problems with filter clogging were observed. Results described herein suggest axial flow ultrafiltration is an effective concentration method for microorganisms in water containing high levels of particulate matter.

1. Introduction

In the aftermath of the terrorist events of 2001, the U.S. government initiated a multi-faceted effort to strengthen homeland security. One facet of this effort addressed vulnerabilities in the nation's drinking water systems to intentional contamination of a biological, chemical or radiological nature. In turn this led to research, development and testing of methods to improve detection of highly pathogenic microorganisms in water samples. The collection of larger samples of drinking water, i.e., 40 to 100 l (L) and then the subsequent concentration of the microorganisms in these larger samples were recommended to improve the capability to detect biological agents such as *Bacillus anthracis* (*B. anthracis*) spores (EPA, 2003; Lindquist et al., 2006). A larger sample size increased the probability of capturing target organisms present at low levels, and subsequent concentration of the microorganisms theoretically allowed the analysis of all the microorganisms in the original large volume water sample.

Much of the ensuing research and development focused on hollow fiber ultrafiltration (HFUF) as a primary concentration method. Prior to this a similar use of hollow fiber ultrafiltration was for monitoring source water for naturally occurring protozoa i.e., *Giardia* and *Cryptosporidium*. Although not included in the USEPA method 1623 for the analysis of these organisms, HFUF in a tangential flow configuration was seen to be an effective concentration method for these organisms (Kuhn and Oshima, 2001).

Fig. 1 shows one common way to set up a tangential flow HFUF system. In this process the water to be concentrated enters the inlet of the filter where it is then distributed to the interior of individual hollow fibers. The fiber walls are porous walls and allow passage of molecules that are smaller than a certain molecular weight. As water goes along the inside of the fibers, the water and other small molecules will either cross the fiber wall i.e., the filter membrane, or else will continue to flow along the inside of the fiber until exiting the other end where it then is recirculated back into the system. It is hypothesized that the flow of this latter fraction of water through the fibers helps to keep microorganisms in suspension and thus aids in their subsequent

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https://doi.org/10.1016/j.mimet.2019.04.005
Received 12 March 2019; Received in revised form 5 April 2019; Accepted 5 April 2019
Available online 08 April 2019

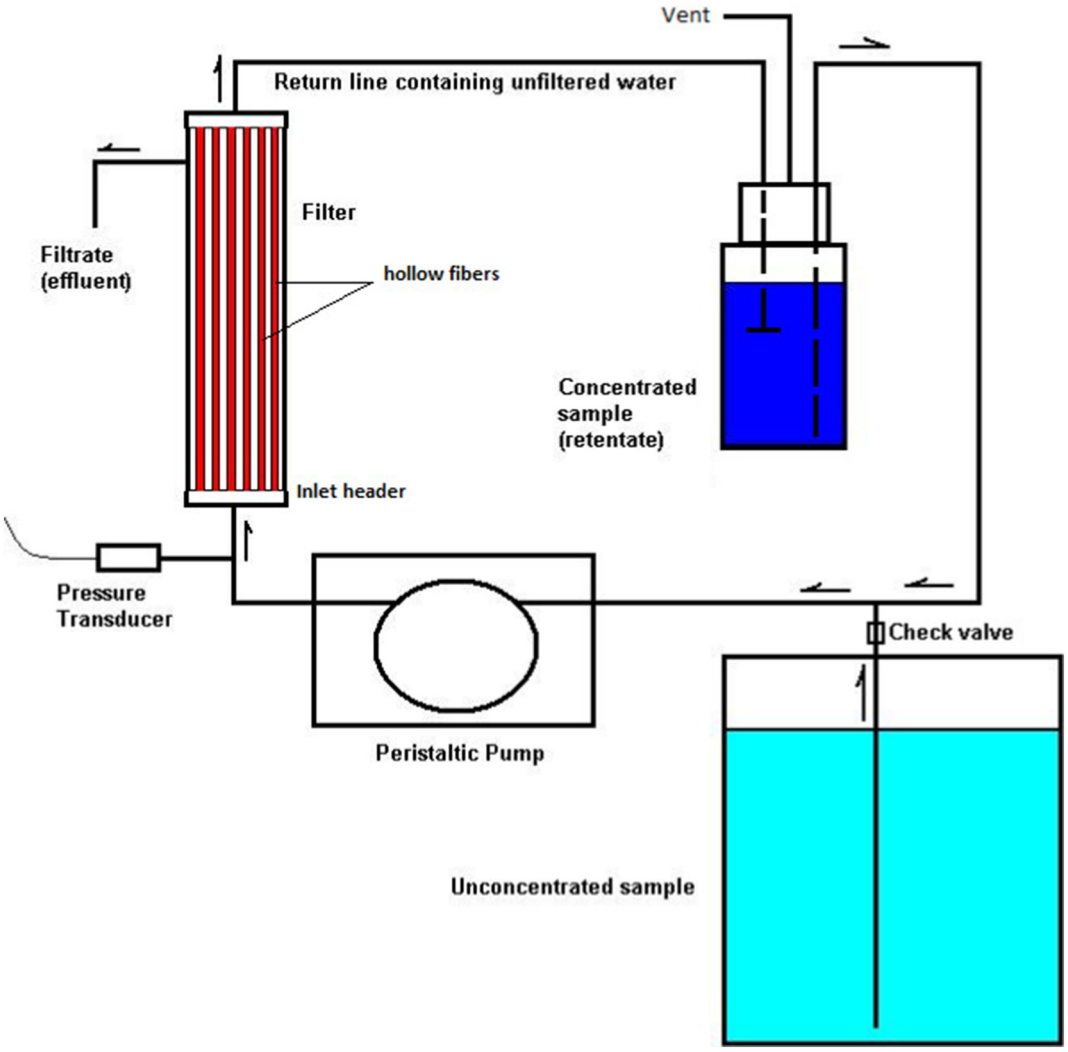

Fig. 1. Tangential Flow HFUF Process.

recovery (Lindquist et al., 2007). Over the course of the process, the majority of the water exits the system as filtrate, and the filter and the small fraction of water that remain will contain the large molecules, microorganisms, and other particulate matter in the original large volume sample. The filter is typically eluted with a surfactant solution to capture the target organisms from the fibers, and the resulting concentrated water sample, also known as the retentate, can then be processed for downstream microbiological analysis.

Building on the earlier work with tangential flow HFUF for *Giardia* and C*ryptosporidium*, both the United States Centers for Disease Control and Prevention (CDC) and the United States Environmental Protection Agency (EPA) developed tangential flow hollow fiber ultrafiltration procedures for concentrating biothreat agents in response to intentional contamination of drinking water systems. In both efforts single-use dialysis filters, also known as hemodialyzers, were used since they were relatively inexpensive and provided a large (1.5–2.5 m^2) surface area for filtration and had an effective pore size that enabled capture of protozoa, bacteria and viruses (Hill et al., 2005; Holowecky et al., 2009). Sakai (2000) provides an informative background on the physical and chemical characteristics of this type of filter. The CDC developed a protocol for use in laboratories for the concentration of large volume drinking water samples (Polaczyk et al., 2008; Hill et al., 2005). In addition, the US EPA in conjunction with Idaho National Laboratory (INL) developed the Water Sample Concentrator (WSC), a semi-automated portable device for concentrating large volume samples in the field (Carpenter et al., 2016; Humrighouse et al., 2015). While differing in certain details, the two tangential flow HFUF methods yielded comparable recovery of the organisms studied, which included viruses, vegetative bacteria, bacterial endospores and protozoa (EPA, CDC, 2011).

In addition to detection of biothreat agents in drinking water, situations can arise where detection of such organisms in turbid water is a desired capability. There have been a number of events where buildings had been contaminated with biothreat agents, most notably the buildings impacted by the intentional mailing of letters with *B. anthracis* spores (Schmitt and Zacchia, 2012). In addition, residences were contaminated by *B. anthracis* spores which had originated from imported animal skins (Guh et al., 2010). During the subsequent cleanups at these sites, wash water was generated as part of remediation activities. This wash water had the potential for containing low levels of *B. anthracis* spores, but there were no reliable methods for concentrating and detecting low levels of microorganisms in large volumes of water laden with particulate matter. This limited disposal options for such wash water. For example, for the U.S. Capitol building cleanup, during the decontamination of personnel in personal protective equipment, approximately 14,000 gal of wash water were generated and subsequently required steam sterilization prior to disposal because of the potential of it containing *B. anthracis* (EPA, 2002).

Another event where the detection of microorganisms in wash water proved challenging occurred during a full scale clean up exercise at an

INL test site. In this exercise an aerosol made up of *Bacillus atrophaeus* subspecies *globigii* spores (*B. globigii*), a surrogate for *B. anthracis*, was released to evaluate decontamination procedures for buildings and other wide areas, (EPA, 2013). Activities during this exercise produced large volumes of wash water that likely contained some level of *B. globigii* spores. Attempts were made to concentrate the microorganisms in the wash water using the WSC ultrafiltration device. However, efforts were largely unsuccessful due to particulate matter clogging the filter during concentration. It was concluded that current methods for concentration of such matrices were inadequate, and an effort was initiated to address this challenge.

Our early trials to concentrate turbid water using tangential flow HFUF resulted in varied success, depending on the particular water processed. When the water originated from washing laboratory floors and other surfaces, problems with clogging were typically encountered as was seen at the INL test site mentioned previously. However, when the water source was a surface water such as the Ohio River or one of its tributaries, clogging was not a major hinderance for concentration. It was concluded that in the concentration of turbid water using tangential flow HFUF, clogging was most problematic at the entrance to the fibers, i.e., at the inlet header shown in Fig. 1. Clogging of the membrane pores along the fiber wall did not appear to be an issue with the volumes of water processed (50–100 l). Furthermore, it appeared that fine particulate matter, e.g., the suspended material found in surface waters such as the Ohio River, was small enough to not clog the inlet header. Studying a similar type of water, Mull and Hill (2012) concentrated 72 l of lake water with a turbidity of 92 Nephelometric Turbidity Units (NTU) using dead end HFUF. Concentration was successful even though dead end filtration is single pass (no recirculation) and thus more susceptible to clogging. However, it was seen from our initial work that larger particles could not enter the fibers' openings, thus resulting in the filter clogging. Since the particle size distribution is not always known prior to collecting a large volume of turbid water for concentration, it was concluded that a reliable method for concentrating turbid water would be one capable of processing water without clogging, regardless of particle size.

Our Initial efforts to develop a reliable concentration process included modifying tangential flow HFUF by periodically backwashing the filter to dislodge the material accumulating at the inlet header of the filter. This backwashed material was collected and added to the final retentate sample (Gallardo, 2014). Although this was more effective than conventional tangential flow HFUF, it still was not consistently effective and clogging still proved problematic in wash waters with high solids content. A strainer using a 100 mesh (150 μm) screen at the inlet was another modification investigated but was not found to be effective to prevent clogging for the wash water studied. Magaña et al. (2013) processed vegetable wash water through a series of sieves (75, 53 and 35 μm) prior to successfully concentrating water and only observed small losses of the target organism from the sieving process: unsieved recovery, 101.25%; sieved recovery, 96.4%. However, the particle size distribution of the particulate matter was not reported.

These early difficulties prompted trials with axial flow HFUF where the filtrate and retentate flow paths are switched. In axial flow HFUF, shown in Fig. 2, water enters the side port and either permeates the fiber from the outside and flows along the inside of the fiber and exits at the end port as filtrate, or else flows out the side port at the opposite end and is recirculated back into the system. In this configuration the particulate matter that would normally clog the filter at the inlet header during tangential flow HFUF instead collects along the outside of the fibers for the entire length of the filter. This configuration provides a much larger volume for particle accumulation before clogging becomes a hinderance. The disadvantage in using axial flow HFUF is that elution of the filter fibers involves more steps including dissection of the filter to remove the fibers for subsequent washing, i.e., elution, and then decanting of the eluent prior to analysis. Although the elution process is more cumbersome, the results presented in this article suggest that axial flow HFUF is a reliable method for concentrating turbid water regardless of the size of particulate matter in the water. Applicable waters for this method include municipal waste water, storm water, surface water, irrigation water, and wash water originating from various types of activities such as cleanup of biologically contaminated areas, and fruit and vegetable washing.

The objective of this study was to evaluate axial flow HFUF in the recovery of target organisms in large volumes of turbid water that would be similar to water encountered in the cleanup of a biologically contaminated area such as the *B. anthracis* cleanups mentioned above. Henceforth this type of water will be referred to as wash water. The target size of the large volume sample was 51 l, which falls within the range of the recommended volume (40–100 l) mentioned earlier. The target organisms studied were *B. globigii* spores, and a number of bacteriophage: MS2, PhiX174 and Phi8. The bacteriophage served as surrogates for pathogenic viruses.

It was desired to test the process using water that would be challenging to tangential flow HFUF (i.e., likely to cause problems with clogging), thus water with a significant percentage (e.g., 10–20%) of particles with a diameter similar to the fiber diameter (0.180 mm) was preferred. Given that the target volume of water for each concentration test was 51 l and that the water needed to be consistent from test to test, it was seen as impractical to collect and store the water needed for all the tests by generating run off water from activities such as floor washing. Instead a source of clean river sediment was located that had a particle size distribution which would likely be challenging for the HFUF process. This sediment would then be added to tap water to generate large volume samples for testing. In general, the wash water that is relevant to this study originates from water flowing over surfaces such as floors and parking lots and picking up particulate matter as it travels, eventually collecting in a tank or other type of reservoir where a fraction of the particulate matter settles to the bottom. Since river sediment originates in an analogous way, it was seen as a realistic and practical source for the particulate matter used in this study. Future studies would involve adding surfactants and/or disinfectants since these would be likely components in some wash waters.

For axial flow HFUF, the filters were operated with recirculation, similar to conventional tangential flow HFUF where a fraction of the water is also recirculated back into the system. In addition for another series of tests, filters were operated in dead end mode. It was anticipated that axial flow with recirculation would yield higher recovery, but dead end flow is simpler to set up and operate and thus also of interest. For most of the recovery studies, the solids content in the wash water ranged from 0 to 150 mg/l. Additional testing was done with wash water with a solids content of 750 mg/l. Turbidity was not found to be a useful parameter since much of the particulate matter would settle out during measurement with a turbidimeter. Instead solids content was calculated from the mass of solids added to a given volume of water.

2. Materials and methods

2.1. Sediment

The sediment for this study had been previously collected from the Ottawa River in Toledo, Ohio and characterized for particle size distribution and organic content. The organic content was 3.4% (*w*/w) and the particle size distribution of the sediment is given in Table 1.

2.2. Preparation of large volume wash water samples

Sediment was weighed and added to 51 l of dechlorinated tap water in a 30 gal polypropylene tank (catalog # 9224, US Plastics, Lima, OH) that had been washed and sterilized via autoclaving. Laboratory tap water was used and originated from the Richard Miller Treatment Plant, Cincinnati, Ohio. The amount of sediment added was the mass

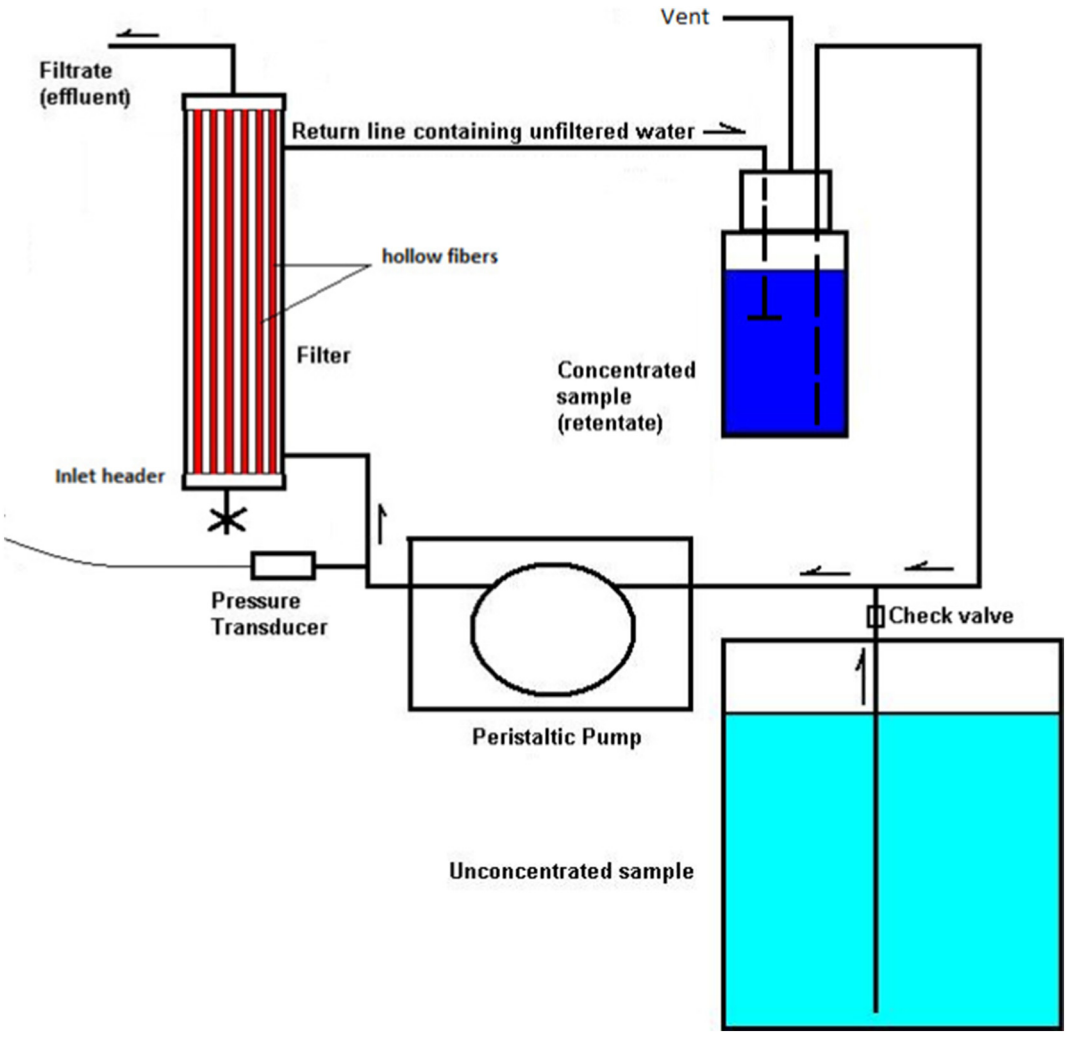

Fig. 2. HFUF Modified for Axial Flow.

that would yield, on a dry weight basis, the desired concentrations of particulate matter: 50, 100, 150, or 750 mg solids/l. Prior to addition to the water, the sediment was mixed in a small volume of water in a weigh boat to break up the sediment into smaller clumps in order to facilitate suspension of the sediment in the large sample. The suspension of wash water was mixed using a magnetic stirrer (model 6795–611 Corning, Corning NY) operating at 90% of full speed and a stir bar (catalog # 16–800-530, Fisher Scientific, Pittsburgh, PA) for approximately 10–30 min prior to concentration. Mixing continued in the concentration process that followed. Ten ml of a sterile 10% sodium thiosulfate solution was added to quench the chlorine residual in the 51 l of tap water. The prepared large volume of wash water was then spiked with a known volume of a diluted stock suspension of *B. globigii* spores or bacteriophage. The diluted stock suspension was analyzed according to the procedure described below to calculate the number of target organisms spiked into the large volume.

2.3. Microorganisms

2.3.1. Spore preparation

The strain of *B. globigii* spores used in this study was originally obtained from J. Wright, U.S. Army, Dugway Proving Grounds, UT. Spores were prepared following the procedure outlined by Humrighouse et al. (2015). Briefly, spores were prepared in a generic sporulation media and purified using gradient separation with RenoCal-76 (Bracco Diagnostics, Princeton, NJ). The spores were then washed three times by centrifugation using cold sterile Type 1 water (Milli-Q, Burlington, MA) which produced purified spore preparations at a level of approximately 1×10^9 colony forming units (CFU)/ml and were then stored in 40% (*v*/v) ethanol at 5 °C until use. Microscopic examination using phase-contrast microscopy of the purified spore preparations exhibited < 0.1% vegetative cells. A dilution series of the spore suspension was performed in diluent (0.01 M PBS (Sigma P4417), 0.01% Tween 80 (Sigma P1754), 0.001% Antifoam A (Sigma A5758)) and plated out to determine which dilution to use to achieve a spike containing the

Table 1
Particle size distribution of sediment used to generate turbid water.

Particle size (mm)	9.5	4.75	2	0.85	0.425	0.25	0.106	0.075	0.074	0.005	0.001
Mass % of particles[a]	100	99	98.4	96.2	94.5	89.7	74.9	69	67.8	50.5	17.3

[a] Mass % of particles smaller than the associated particle size, e.g., 99% of the particles were smaller than 4.75 mm

desired number of spores: ~1 × 10^5 spores. Two trials also were done where the target spike was ~1 × 10^3 spores.

2.4. Bacteriophage

2.4.1. MS2 and PhiX174

A phage culture technique was adopted from McMinn et al. (2017) and Rhodes et al. (2011) using the double ager layer method (EPA, 2001). A titer was performed in 1× phosphate buffered saline (PBS) pH 7 (P3744, Sigma-Aldrich, St. Louis, MO) of the frozen stocks to determine the dilution needed to generate ~1 × 10^5 spike of MS2 (ATCC 15597-B1) or PhiX174 (φX174) (ATCC 13706-B1). Briefly, one day prior to analysis, 5 ml of tryptic soy broth (TSB) (Fisher Scientific, Waltham, MA) containing 50 µL antibiotics (streptomycin/ampicillin (15 mg/l) or nalidixic acid (100 mg/l)) was inoculated with the bacterial host *E. coli* F_{amp} (ATCC 700891) or *E. coli* CN-13 (ATCC 700609), for MS2 and φX174 respectively, and incubated over night at 36 ± 1.0 °C. For the analysis, 1 ml of the bacteriophage control spike or the concentrated sample was added to 5 ml of 0.7% tryptic soy agar (TSA) (BD Scientific, Sparks, MD) containing the specific antibiotic and a 0.1 ml mid-log culture of the corresponding *E. coli* host. This solution was poured over a 1.5% TSA plate containing the antibiotics at the concentrations mentioned. After the top agar solidified the plates were inverted and incubated at 37 °C for approximately 18–24 h and the plaques were then counted.

2.4.2. Phi 8

Phi 8 (φ8) and *Pseudomonas syringae* LM2489 were obtained from Leonard Mindich of the Public Health Research Institute, 225 Warren Street, Newark, NJ 07103–3535 (Mindich et al., 1999) and were assayed using the double ager layer method (EPA, 2001). A titer was performed from the frozen stock in Tryptone broth with $MgCl_2$. The host culture of LM2489 was grown in Tryptone broth with $MgCl_2$ and incubated at 26 ± 2 °C for 24 h. For the analysis, 1 ml of the bacteriophage control spike or the concentrated sample was added to 3 ml of 0.7% tryptone agar with $MgCl_2$ and a 0.5 ml mid-log culture of the LM4289 host. This solution was poured over a 1.5% tryptone agar with $MgCl_2$ plate. After the top agar solidified the plates were inverted and incubated at 37 °C for approximately 18–24 h and the plaques were then counted.

2.5. Ultrafiltration

For axial flow with recirculation, the WSC device that was developed for tangential flow HFUF was used. To allow for axial flow, the underlying process was modified by switching the inlet and outlet ports: water to be concentrated entered the side port of the filter, filtrate exited through the opposite end port, and unfiltered retentate exited the other side port. These changes are shown in Fig. 2. In addition, barbed Hansen connectors (catalog # MP-773, Molded Products, Harlan, IA) were used for the side ports. Other than these alterations, the procedure for the WSC as outlined in EPA, CDC (2011) was followed. The cited procedure is described briefly below. A REXEED 25S hemodialyzer filter (Asahi Kasei Kuraray Medical Co. Ltd., Tokyo, Japan) was used for ultrafiltration and a Masterflex 77411–00 peristaltic pump drive and Easy-Load 77601–00 pump head (Cole Parmer, Vernon Hills, IL) using Norprene tubing with a 3/8" inner diameter and 5/8" outer diameter (AAL00029, Saint-Gobain, La Défense, Courbevoie, France) were used to drive the water through the process with the filtrate flowrate averaging about ~1,500 ml/min. Inlet pressure was measured via a Pendotech PRESS-S-000 pressure sensor (Cole Parmer). The process was controlled by a computer program that had been developed for the WSC and written with Labview software (National Instruments, Austin, TX). Fig. 3 is a screen shot of the software and shows, conceptually, how the WSC is configured. Pretreatment (i.e., blocking) of the filter consisted of recirculating approximately 1 l of a sterile aqueous solution of 0.055% (*v*/v) Tween 80, 0.1% (*w*/*v*) sodium polyphosphate, and 0.001% (v/v) Antifoam A through the system for 3 min. The purpose of blocking is to lessen the tendency for microorganisms to adhere to filter surfaces. After recirculation of the blocking solution, all but 250 ml was removed from the system and sample concentration would begin with a fraction of the water exiting the system as filtrate and the remaining faction being recirculated back to the inlet of the filter. The sample was mixed during the process using the same stirring equipment and conditions mentioned previously. The target pressure at the inlet of the filter was 30 psig. The software would adjust the pump speed to maintain the target pressure. After sample concentration, approximately 600 ml of a sterile eluting solution containing 0.001% (v/v) Tween 80 was recirculated for 3 min through the filter and was then discharged from the system as filtrate. An additional volume of eluting solution then flushed the fluid from the filter and into the retentate container. The volume of retentate produced after this step was ~250 ml and would be used to wash the fibers in a subsequent step. The WSC was used for the experiments reported here due to the automated control and parameter recording that the device offered. A manual concentration process could be set up by modifying the procedure in Lindquist et al. (2007) for axial flow and should yield similar performance.

For the set up using dead end axial flow, the main difference was that there was no re-circulation, and a manual system rather than the WSC was used and is shown in Fig. 4. The same pump, pump head, pump tubing and pressure transducer used in the WSC was also used in the dead end system. The water crossed the fiber wall from the outside to the inside and flowed out the opposite end port. The other filter ports were closed off. A 500 ml volume of the pretreatment solution mentioned above was recirculated through the dialysate volume of the filter (i.e., the volume outside the fibers and inside the cartridge) for 3 min. The pump then proceeded to draw up the large volume sample into the filter, and concentration commenced. The target pressure was 25 psig ± 5 and was maintained by manually adjusting the pump speed. Pump speed was 20–25% of full scale (650 RPM). After the large volume sample had been concentrated, the pump would continue to run until no more filtrate exited the filter. No eluting solution would be drawn up into the filter, and there was no retentate fluid except the residual water that remained in the filter.

Blank concentration runs were conducted with 51 l of unspiked tap water as well as 51 l of unspiked turbid water containing 150 mg solids/l. The retentate was processed and analyzed as described previously. No target organisms were detected.

2.6. Fiber recovery

After concentration the particulate matter was easily visible inside the filter cartridge. Fig. 5 shows an example of a filter after concentration. Attempts to flush the particulate matter out of the filter proved unsuccessful and recovery of target organisms was very low. Greater amounts of particulate matter could have been retrieved if greater flushing volumes were used, but this would have led to a larger final sample volume. In order to increase recovery, dissection of the filter was carried out, and the fibers were retrieved for further processing.

To retrieve the fibers, the filter was capped at both the side and end ports using the caps that came with the filter and held horizontally with a clamp connected to a ring stand in a biosafety cabinet. A picture of this set up is in Fig. 5. Using standard safety precautions for a biosafety level 2 laboratory, the filter was processed in the following manner. A 130 watt hot knife (catalog # 60313 Chicago Electric, Carol Stream, IL), was used to cut through the filter casing at points A and B along the dashed lines shown in Fig. 5. A sterile plastic Stomacher bag (catalog # 14–258-207, Fisher Scientific) was held open beneath the area of cutting in order to catch any water that would drip out. The fibers were left intact. After the cartridge walls had been cut, a sterile scalpel was used to cut the fibers at point A. The fibers were then pulled out while still

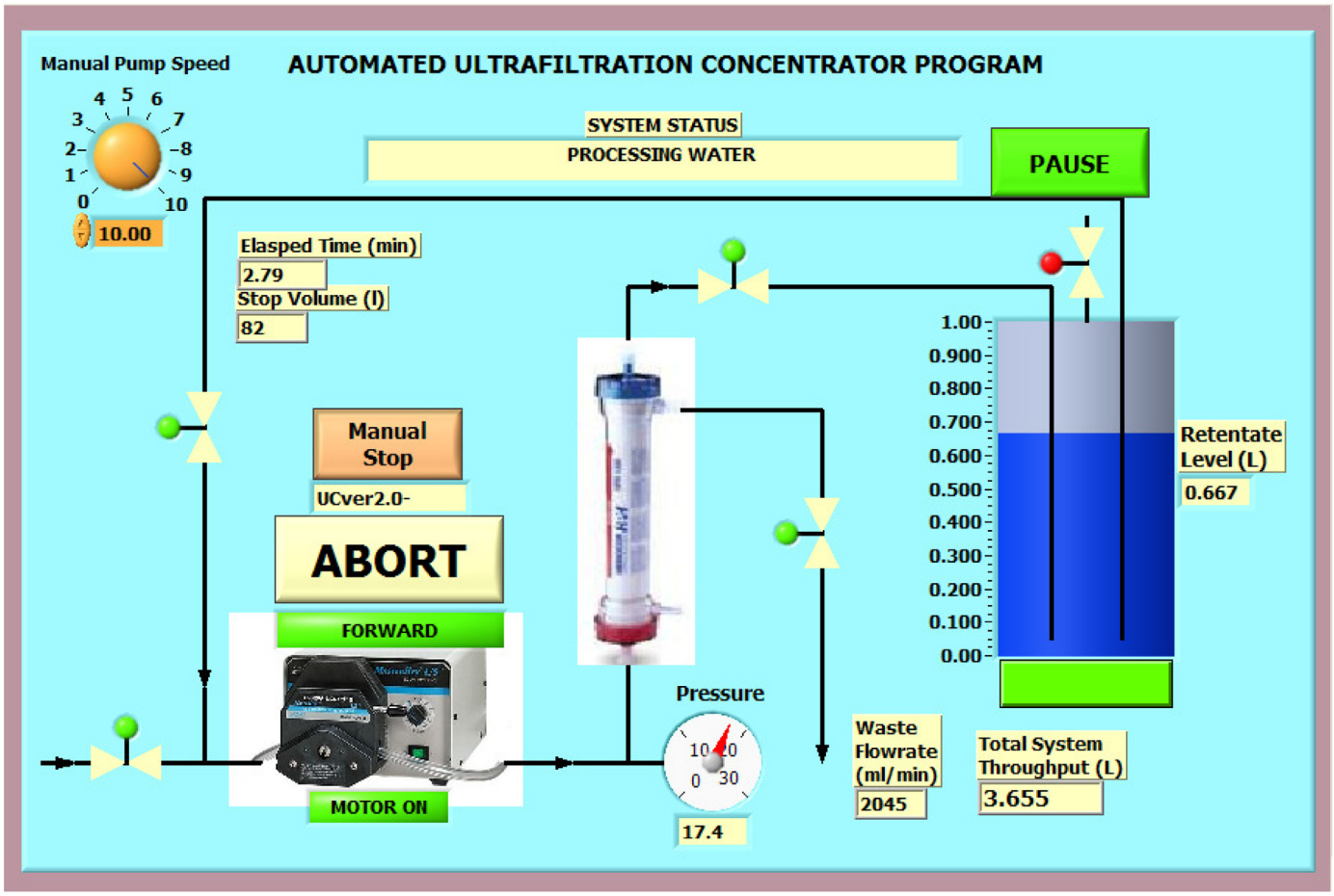

Fig. 3. Screen Shot of Software Used to Control the Automated Water Sample Concentrator.

being connected at point B and placed in the Stomacher bag. Once in the bag the fibers were cut at point B and the other end port discarded. To this bag a volume of eluting solution was added. For axial flow with recirculation, the eluting solution was the entire retentate volume collected at the end of the previous step. For axial flow in dead end mode, the eluting solution was 250 ml of the 0.001% Tween 80 solution mentioned previously. The bag was then sealed and Stomached for 1 min at high intensity using a Stomacher 3500 laboratory blender (Seward Ltd., West Sussex, UK). Using a motorized pipettor, the liquid was then decanted as the fibers inside the bag were manually squeezed from the outside of the bag to release as much water as practically possible. The decanted liquid, i.e., the retentate, was placed in a sterile sample bottle and the volume recorded. A second wash was carried out with fresh eluting solution, and the stomaching and liquid retrieval steps were repeated. The sum of the volumes of the recovered water from the first and second washes was the total volume of the final retentate. In the earlier experiments, the first wash and second wash were processed and analyzed separately. After the relative amount of recovery from both the washes had been evaluated, the two washes were combined and analyzed as one sample in the later experiments.

2.7. Microbial analysis

For *B. globigii* analysis a 25 ml volume of the retentate was transferred to a sterile 50 ml centrifuge tube and heat treated in a water bath at 75 °C for 1 h to inactivate vegetative bacteria. The sample was allowed to cool at room temperature, and serial dilutions were then carried out. Replicates of 1 ml aliquots of the retentate sample were spread plated over 5 tryptic soy agar plates (B21283X, Fisher Scientific), 0.2 ml per individual plate. The plates were incubated at 37 °C for 24 h after which colony forming units (CFUs) of *B. globigii* were counted. The CFUs on each of the 5 plates were added together and divided by 1 ml to calculate the concentration of *B. globigii* in the retentate. The average concentration of the two replicates was the value used for the concentration of the target organisms in the retentate. Separate aliquots of the same diluted *B. globigii* stock added to the large volume wash water sample were similarly plated and incubated. The *B. globigii* colonies growing on the latter plates were compared to the colonies from the plated retentate samples. Using both color and colony morphology, the *B. globigii* colonies from the retentate samples were easily identified amidst background microbiological growth. The heat treatment step described earlier likely aided the identification process by reducing the amount of background growth. Culture and analysis of matrix blanks (wash water with no added *B. globigii*) showed no colonies resembling *B. globigii* amidst the background growth.

In earlier related research, environmental samples containing *B. globigii* spores were plated out on TSA and incubated as described above. The resulting colonies that were identified, via color and morphology, as *B. globigii* were picked and analyzed using real time PCR. This latter analysis confirmed that the picked colonies were *B. globigii* (EPA, 2013).

For MS2, Phi8 and PhiX174, a 25 ml retentate sample was likewise transferred to a sterile 50 ml centrifuge tube but not heat treated. Plating commenced and followed the same phage culture technique mentioned previously for enumerating the amount of bacteriophage in the spike.

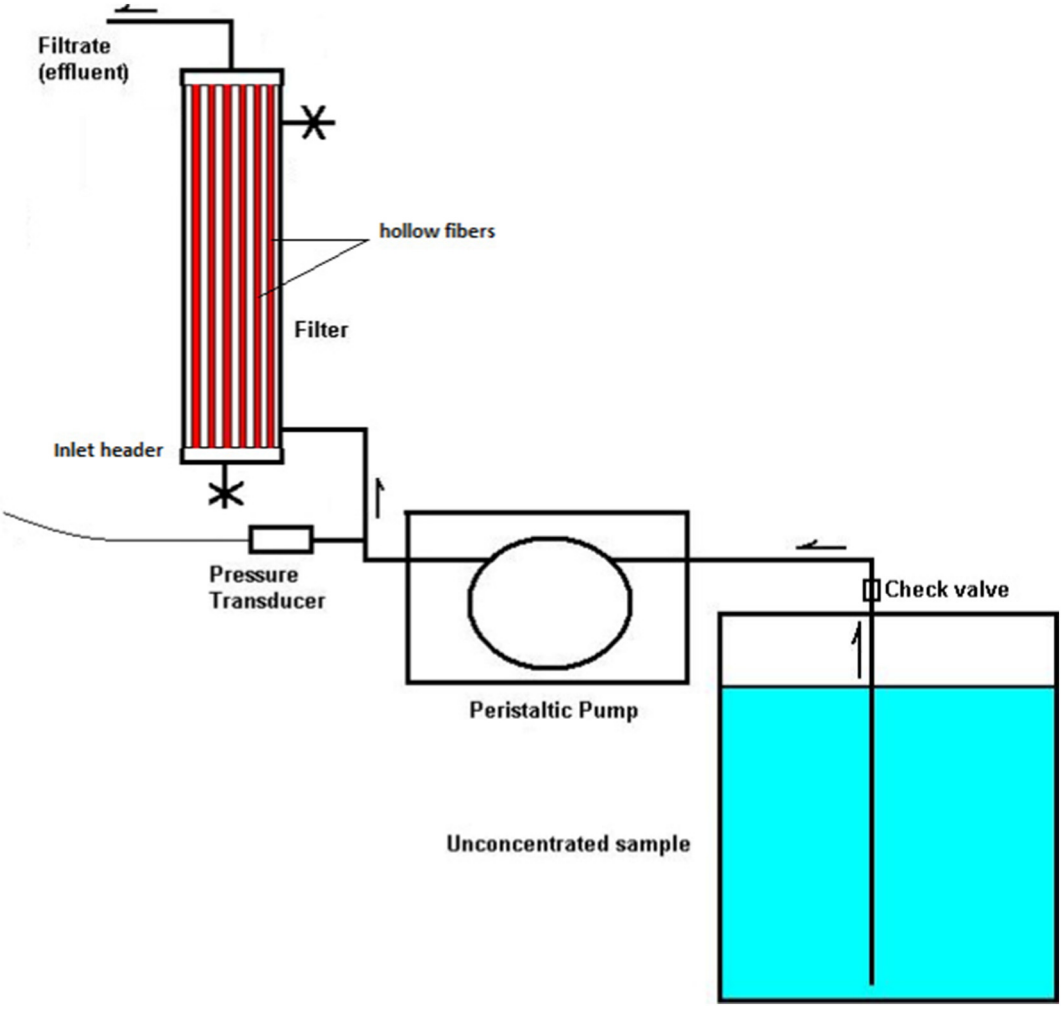

Fig. 4. Dead End Ultrafiltration Configured for Axial Flow.

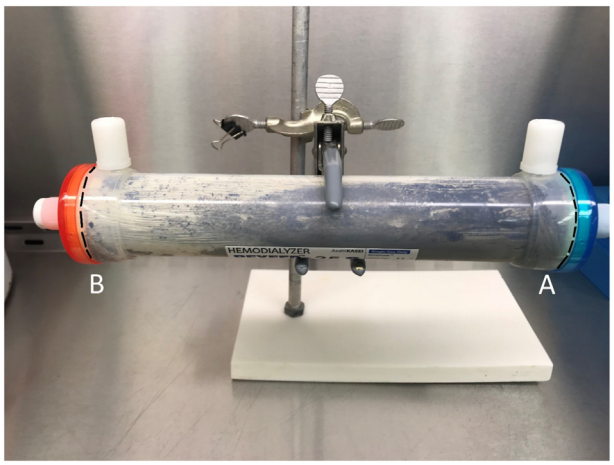

Fig. 5. Hollow Fiber Dialysis Filter Used in the Axial Flow Configuration. Ready for Dissection.

2.8. Calculation of percent recovery and statistical analysis

The percent recovery for each experiment was calculated by first obtaining the number of organisms added to the large volume sample: aliquots of the spike were analyzed as described above, and the resulting CFUs or plaque forming units (PFUs) per ml were then multiplied by the volume of the spike to determine the total number of organisms added. The amount of recovered organisms were similarly calculated from the concentration of the target organisms measured in the retentate multiplied by the total volume of retentate. The amount of recovered organisms divided by the amount of spiked organisms multiplied by 100 yielded percent recovery.

Data were statistically analyzed and graphed using Prism 6 (GraphPad Software, USA). When 3 or more sets of recovery data were compared, statistical significance was determined using two-way analysis of variance (ANOVA) with a Tukey post-hoc adjustment of *P*-values to account for multiple comparisons. When two sets of data were compared, a non-parametric *t*-test was used for statistical significance.

2.9. Strainer to prevent clogging

To test the filter's capacity for solids accumulation (but not organism recovery), 100 l of wash water containing 750 mg/l solids was concentrated via dead end axial flow. To prevent clogging of the inlet tubing by large particles (i.e., > 1 mm diameter), a simple strainer was made from a nylon screen with 1.1 × 1.4 mm openings and wrapped around a plastic pipe fitting with 3/4“ nominal pipe threads (NPT); the other end of the fitting was a compression fitting with a short segment of a 3/8” outside diameter tube on which inlet tubing was attached.

Table 2
Percent Recovery of B. globigii spores. Axial Flow with Recirculation. Target spike: 100,000 spores/51 l.

Trial	Tap water (0 mg solids/l)			50 mg solids/l			100 mg solids/l			150 mg solids/l		
	1st wash	2nd wash	Total	1st wash	2nd wash	Total	1st wash	2nd wash	Total	1st wash	2nd wash	Total
1	32.0	7.8	39.8	27.3	10.8	38.1	42.5	9.0	51.5	34.4	14.9	49.3
2	18.9	13.2	32.1	30.5	10.5	40.9	35.4	8.7	44.1	39.4	11.9	51.3
3	27.2	5.9	33.1	31.8	5.6	37.4	37.6	4.9	42.5	45.56	12.07	57.63
Avg.	26.1	8.9	35.0	29.9	9.0	38.8	38.5	7.5	46.0	39.8	13.0	52.7
Std. Dev.	6.7	3.8	4.2	2.3	2.9	1.9	3.6	2.3	4.8	5.6	1.7	4.4

3. Results and discussion

3.1. High spike results, bacillus spores. axial flow with recirculation

Table 2 shows percent recovery using axial flow with recirculation where the target spike amount was 100,000 *B. globigii* spores in 51 l of water. Results for the first wash, second wash, and combined washes (total recovery) are presented in separate columns of Table 2. The level of particulate matter in the unconcentrated water samples ranged from 0 to 150 mg solids/l. The filtrate flow rate did not appear to be influenced by solids loading and averaged about 1500 ml/min for this set of experiments.

Results consistently showed that higher recovery of target organisms occurred during the first wash compared to the second wash (3–5 times greater than the second wash), and the difference in recovery for the two washes was significant ($P < .05$) using a non-parametric *t*-test. The average percent recovery of the first wash ranged from 26 to 40% for the first wash and from 7.5 to 13% for the second wash. It is conjectured that additional washes would have increased total recovery, however each additional wash would have also increased the final retentate volume and would have thus been counter-productive to the purpose of sample concentration.

Recovery data for the experiments shown in Table 2 are also plotted in in Fig. 6. Recovery of the organisms from dechlorinated tap water (Tap Water Recovery) was unexpectedly less than the recovery of organisms from the wash water containing the two highest solids content studied, 100 and 150 mg/l. Using a 2-way ANOVA Tukey multiple comparison test to evaluate the data, the difference was statistically significant at a $P < .05$ level. The recovery from 50 mg/l wash water was also higher compared to tap water but was not statistically significant. Prior to this set of studies, it was assumed that the particulate matter would cause lower recovery compared to tap water, however the data suggest the opposite. One hypothesis for the higher recovery is that the target organisms adhere to particulate matter and are subsequently captured during the elution step and become part of the final retentate sample. If particles with adhered spores are included in the aliquots plated out and incubated, this could increase recovery if the adhered spores have access to the media so that they germinate and form visible colonies. Rhodes et al. (2016) also saw higher recovery of certain viruses in river water compared to tap water. In addition, greater recovery at the higher solids levels may be partially the result of longer contact times between the surfactant and adhered organisms on associated solid surfaces. In general, the purpose of the surfactant is to extract attached organism from solid surfaces (i.e., from the outside of the filter fibers or from particulate matter) and into the bulk sample water. For both types of concentrated samples (tap water and wash water) the surfactant is in contact with the fibers for approximately the same amount of time (from filter dissection to decanting in the procedure described earlier.) For concentrated tap water samples, once the eluting solution is decanted from the stomacher bag containing the filter fibers, the surfactant is no longer in contact with any solid surfaces that may have target organisms still attached. However, for the concentrated wash water samples, although the surfactant is no longer in contact with the fibers after the decanting step, all the particulate matter that was decanted is still in contact with the surfactant in the remaining steps of the procedure. This includes the time that the retentate sample is held prior to heat treatment of a 25 ml aliquot; the time of heat treatment, i.e., 60 min; and the time between heat treatment and plating out. This affords the surfactant greater opportunity to extract adhered spores into the bulk water sample. In addition, the higher temperature (75 °C) of the one hour heat treatment and the vortexing prior to plating likely increase extraction efficiency of the surfactant. From a practical standpoint, the lower recovery in tap water samples may not be relevant since other concentration methods, i.e., methods not using axial flow, would likely be used for tap water samples. These latter methods have shown to yield high recovery of a wide variety of microorganisms including bacterial endospores and are simpler to use (EPA, CDC, 2011; Smith and Hill, 2009).

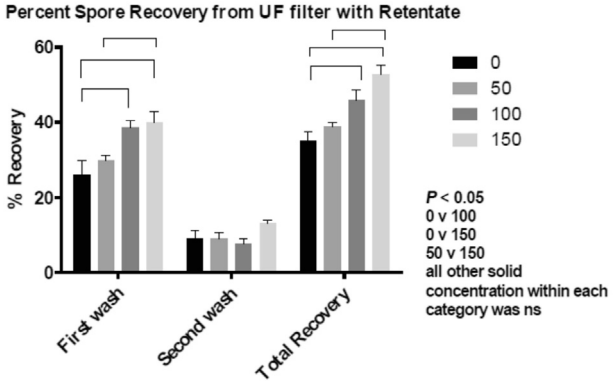

Fig. 6. Percent Recovery of B. globigii spores. Axial Flow with Recirculation. Target Spike =. 100,000 spores/51 l.

3.2. Low spike results, bacillus spores, axial flow with recirculation

Limited studies were conducted with axial flow with recirculation where the target spike was 1×10^3 *B. globigii* spores. For one trial, a 10 ml sample of the first wash was membrane filtered and plated (five, 2 ml aliquots, 1 aliquot per membrane filter) and a 25 ml sample of the second wash was membraned filtered and plated (five, 5 ml aliquots, 1 aliquot per membrane filter). No *B. globigii* colonies were visible after incubation. Assuming 30% of the organisms could be recovered during the first wash, 2 CFUs per plate were expected. After incubation there was significant background growth of unidentified microorganisms, and this may have negatively affected growth and detection of the target organisms.

For the other trial, the volume filtered per membrane filter was lowered with the goal of reducing background growth. In addition the two washes were combined. Five, 2 ml aliquots and five, 1 ml aliquots were membraned filtered (1 aliquot per filter). After incubation, although there was similar overgrowth as seen in the first trial, one of the plates that received a 1 ml aliquot showed 2 *B. globigii* CFUs. Theoretically this amounts to a recovery of 7.5%, although there is uncertainty in this value since no other plates revealed any target colonies, and there was still considerable background growth. It was

Table 3
Percent Recovery of B. globigii spores, Axial Flow, Dead End and with Recirculation. Target spike: 100,000 spores/51 l.

Trial	Dead-end axial flow 150 mg solids/l			Axial flow with recirculation 150 mg solids/l		
	1st wash	2nd wash	Total	1st wash	2nd wash	Total
1	36.1	10.5	46.5	34.4	14.9	49.3
2	30.6	13.6	44.3	39.4	11.9	51.3
3	31.7	8.9	40.6	45.56	12.07	57.63
Avg.	32.8	11.0	43.8	39.8	13.0	52.7
Std. Dev.	2.9	2.4	3.0	5.6	1.7	4.4

concluded that in order to see low levels of target organisms in complex matrices such as the one studied here, secondary concentration methods and molecular techniques should be evaluated. Background growth for the higher spike tests was not an issue since only 0.2 ml were spread plated per TSA plate, and thus contained less background organisms compared to the lower spike tests which involved membrane filtration of much larger aliquots (1 to 5 ml) per filter.

3.3. Dead-end axial flow recovery

Table 3 shows the percent recovery of organisms using dead end ultrafiltration with axial flow at 150 mg solids/l. Dead end ultrafiltration has the advantage of being simpler to set up compared to axial flow with recirculation. For comparison purposes, results for axial flow with recirculation at the same solids level were included in the table. Recovery in the dead end mode averaged 44%. This is lower than the average percent recovery from axial flow with recirculation (53%) although the difference was not significant at the $P < .05$ level using a non-parametric *t*-test. In conventional tangential flow ultrafiltration where the water to be concentrated enters the end of the fibers, there is the phenomenon of wall scouring that occurs as a large percentage of the water goes along the inside of the fiber and does not cross the fiber membrane but is recirculated (Lindquist et al., 2007) and aids in keeping microorganisms in suspension. However, with axial flow the flow dynamics are different, and recirculation may not offer similar advantages. In addition, in the procedures reported here, elution entails dissecting the filter cartridge, removing the fibers then stomaching the fibers in a bag containing eluting solution. This is an inherently different method of elution compared to conventional hollow fiber ultrafiltration where eluting solution flushes the interior of the fibers and then exits the end of the fibers for final sample collection. Thus it is not unexpected that the eluting dynamics for the two modes would perform differently regarding recovery.

3.4. Recovery in severely turbid water

Table 4 shows the results of concentrating *B. globigii* in water with 750 mg solids/l using dead end filtration with axial flow. Average recovery of *B. globigii* spores was 38.3%, slightly lower than the recovery of dead end filtration at a solids loading of 150 mg/l: 44%, (also shown in Table 4) but not statistically different at the $P < 0.05$ level using a non-parametric t-test. Operationally, the high loading of particulate matter did pose some challenges not encountered at the lower loadings. Clogging, especially on the inlet side of the pump, i.e., vacuum side, was more common since there was a greater mass of large diameter particles at the higher loading. A check valve between the pump and the large volume sample was a common place for clogging and had to be removed in order to complete the concentration of the target volume of wash water. Clogging of the filter itself was not highly problematic. Although not used in the recovery studies, larger tubing upstream of the pump (e.g., 0.375″ inner diameter) would have decreased problems with clogging. Also, if a coarse strainer with ~1.5 mm openings was attached to the entrance of the inlet tubing this would have reduced clogging by preventing large particles from entering the system. Straining out such particles could result in a loss of the target organism, but since the vast majority of the particulate matter studied was smaller than 1 mm, loss of organisms would likely be minimal. In general, since the finer solid material has more surface area per mass, it is anticipated that most of the organisms would adhere to these smaller diameter particles.

Table 4
Percent recovery of B. globigii spores, 750 mg solids/l, and 150 mg solids/l dead end axial flow target spike: 100,000 spores/51 l.

Trial	750 mg solids/l	150 mg solids/l
	% Recovery	% Recovery
1	33.8	46.5
2	37.7	44.3
3	43.4	40.6
Avg.	38.3	43.8
Std. Dev.	4.9	3.0

3.5. Upper limit for particulate loading

The hollow fiber filters used in these studies provided 2.5 m^2 of filter area which allows for the concentration of microorganisms in large volumes of water. For finished drinking water thousands of liters of water can be processed before clogging could become an issue. However, for turbid water such as the type used in this study, the volume of water that can be processed is not limited by this aforementioned surface area but by the available volume for macroscopic particulate matter (e.g., soil particles, debris) to accumulate inside the filter housing. (The microscopic pores themselves that are along the fibers walls have not been observed to clog.) Theoretically, when the filters are operated in axial flow, this available volume is the dialysate volume of the filter, i.e., the space outside of the fibers and inside the filter cartridge housing. As a concentration run proceeds, this volume becomes more packed with particulate matter and will eventually reach a point where no more water can be processed. For the filters used in this study, the dialysate volume was 95 ml. The practical amount of particulate matter that could accumulate in the filter would be a percentage of this dialysate volume.

To further test the capacity of the filter, 100 l (twice as much as normally processed) of wash water containing 750 mg solids/l were concentrated. Recovery of microorganisms was not studied. A strainer (described in the methods and materials section) was used to prevent clogging on the suction side of the pump. Although resistance to flow increased over time and thus necessitated lowering the pump speed to maintain a pressure of 25 psig, the 100 l volume was processed in about 60 min, and all but ~1 g was collected in the filter. This latter amount was made up mostly of large particles that could not pass through the strainer.

After 100 l of wash water had been concentrated, the retentate in the filter contained, on a dry weight basis, 74 g of solid material. Since recovery was not measured, no further steps were carried out on this particular filter. However, if the filter was later dissected and the elution procedure carried out, the resulting solids concentration of the final retentate would have been approximately 140,000 mg/l (14% solids). Whether target organisms could be detected and quantified in this matrix would depend on the specific analytical methods used, but this level of solids shows the promise of this procedure to concentrate water heavily laden with particulate matter. After the 100 l sample was concentrated, the filtrate flow rate was 1000 ml/min, and this indicates that the filter still had some capacity left for more particulate matter.

Table 5
Recovery of Bacteriophage, target spike: 100,000 phage/51 l, 150 mg solids/l. Axial Flow with Recirculation.

Trial	MS2	phi 6	phi 174	B. globigii Axial Flow w/ Recirc.
	% Recovery			% Recovery
1	57.5	did not survive matrix		49.3
2	40.2			51.3
3	36.8			57.6
Avg.	44.9			52.7
Std. Dev.	11.1			4.33

4. Virus recovery. axial flow with recirculation

Table 5 shows the recovery results for the bacteriophage, MS2 in 150 mg solids/l wash water. For this set of experiments the eluent from the first wash and second wash were combined into one sample and analyzed, and the amount of total recovery reported. Total recovery from both the first and second washes ranged from 36.8 to 57.5%, with an average of 44.9% recovery. This is comparable to the average recovery of *B. globigii* under the same solids loading (also shown), although the variability for MS2 recovery was higher. Higher variability for virus recovery compared to *Bacillus* spore recovery has been observed in previous related work (EPA, CDC, 2011).

PhiX174 and Phi8 were also studied, however these bacteriophage appeared to be inactivated in the matrix of concentrated wash water. Retentate samples were first produced from 51 l of wash water containing 150 mg solids/l, and then spiked with approximately 1×10^5 phage particles. After plating and incubating no plaques were visible on any of the plates, which suggests that both of the bacteriophage were inactivated in the matrix of concentrated turbid water. Since bacteriophage were inactivated when spiked into the retentate samples, no experiments were carried out in which these organisms were spiked into the large volume samples of turbid water and then concentrated. The particular reason for the apparent inactivation is not known.

5. Conclusions

The results of a proof of concept study for a method to concentrate highly turbid water were presented and shown to yield recovery of 38 to 53% percent of target organisms. Parameters such as inlet pressure, volume and concentration of eluting solution used, and stomaching time/intensity were not variables in this study, but it is possible that recovery efficiency could be improved by optimizing these parameters. The wash water studied was chosen as a challenging test for the concentration process, and it appears that the concentration method, at the solids levels studied, was not hindered by wash water containing particles of a size that normally would clog conventional HFUF. Results for axial flow with and without recirculation at the same level of solids show that the recovery of *B. globigii* organisms was not significantly different at the $P < 0.05$ level.

The limited testing done at the low spike levels suggest that culture analysis of the final retentate, by either direct spread plating or membrane filter plating was not adequate for detecting low levels of microorganisms in the matrix studied. Since the concentration process described herein was developed for detecting low levels of biothreat agents in wash water, secondary concentration methods and molecular analytical methods should be developed to detect such agents at low levels in complex water matrices.

In addition to wash water from biothreat cleanups, water types applicable for this concentration method include municipal waste water, storm water, surface water, irrigation water, and water produced from fruit and vegetable washing. The practical limit of the volume of water that can be concentrated by a single filter will depend on the mass of solids loading but appears to be > 74 g of solids per filter on a dry weight basis. As the solids level increases, the water will begin to exhibit characteristics of sludge, and as this point is approached, the concentration method presented here will become less practical.

Acknowledgements and disclaimer

The authors gratefully acknowledge Mr. Brian McMinn and Dr. Sangdon Lee for their critical review of this manuscript and Dr. Helen Buse and Mr. Larry Wymer for their assistance in conducting the statistical analysis of the data. The U.S. Environmental Protection Agency (EPA) through its Office of Research and Development managed and funded the research described herein under Contract EP-C-15-010 to Pegasus Technical Services, Inc. It has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Any mention of trade names, products, or services does not imply an endorsement by the U.S. Government or EPA. The EPA does not endorse any commercial products, services, or enterprises.

Impact statement

The paper describes the results of a proof of concept study of a method to concentrate microorganisms in large volumes of highly turbid water. It is not as subject to clogging as other methods are. The method has shown to have a very high capacity for wash water containing high levels particulate matter, e.g., 0.075% solids. The method has potential for enabling the analysis of low levels of biothreat agents in wash water generated from the cleanup of a site containing such agents. In addition the method shows promise for investigating irrigation water and vegetable wash water as sources for food borne outbreaks.

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