MANUAL
of
AQUATIC VIRAL ECOLOGY

MAVE Chapter 12, 2010, 110–117

Filtration-based methods for the collection of viral concentrates from large water samples

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Abstract

Ecological investigations rely on data describing the biomass, diversity, and composition of living things. In the case of microbial communities, these data are primarily gathered using microscopy and molecular genetic approaches. The diminutive size of viruses means that obtaining genetic material sufficient for molecular approaches for examining the diversity and composition of aquatic viral assemblages can be challenging. Moreover, in procedures for the isolation and cultivation of novel viruses from natural waters, high-density viral inocula provide the best chance for success. To address the need for samples containing a high-density of viruses, investigators have used tangential-flow filtration (TFF) to concentrate viruses from large-volume (>20 L) water samples. This report outlines procedures for the preparation of viral concentrates from large volume water samples using TFF and discusses the effect of concentration procedures on viral recovery and downstream molecular genetic analyses.

Introduction

Two procedures, direct enumeration and concentration of viruses, form the core methodological basis of most ecological investigations of natural viral assemblages. Indeed, nearly all of the methods outlined in this volume contain enumeration or viral concentration as a component procedure. Because these procedures are especially critical, aquatic viral ecologists have continually sought improvements in sample throughput, precision, and yield. As a consequence, today there are dozens of technical approaches to viral enumeration and concentration. This plethora of techniques can be confusing to a new investigator, and in too few cases, has thorough consideration been given to methods comparison. This report will briefly review approaches for the concentration of viruses from large (>1 L to hundreds of liters) water samples, outline an example method, and provide guidance for the assessment of concentration methods geared to a particular analytical outcome.

Early methods for viral concentration from water samples relied on adsorption of viruses to a solid matrix such as a fiberglass (Sobsey et al. 1977), membrane filter (Farrah et al. 1976; Katayama et al. 2002), or diatomaceous earth (Farrah et al. 1991) followed by elution of viruses into a small volume of buffer. Whereas the details of these methods vary widely, they essentially rely on increasing the concentration of cations and decreasing the pH of the water sample, which encourages positively charged viral particles to adsorb to negatively charged surfaces. Because of the extensive water conditioning required for negatively charged membranes, electropositive filters have also been employed and have shown more consistent recovery of polioviruses (Sobsey and Glass 1980). Subsequently,

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Acknowledgments

Publication costs for the Manual of Aquatic Viral Ecology were provided by the Gordon and Betty Moore Foundation. This document is based on work partially supported by the U.S. National Science Foundation (NSF) to the Scientific Committee for Oceanographic Research under Grant OCE-0608600. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF.

The authors would like to thank those who have spent countless hours watching water move from bottle to bottle and back to the sea, in particular: Kui Wang, Sharath Srinivasiah, Kurt Williamson, Shellie Bench, Shannon Williamson, Matt Simon, and Tim Mills. Work contributing to this manuscript was supported through National Science Foundation grants (MCB-0132070 and OCE-0221825) to K.E.W. and an NSF predoctoral fellowship to D.M.W.

ISBN 978-0-9845591-0-7, DOI 10.4319/mave.2010.978-0-9845591-0-7.110

Suggested citation format: Wommack, K. E., T. Sime-Ngando, D. M. Winget, S. Jamindar, and R. R. Helton. 2010. Filtration-based methods for the collection of viral concentrates from large water samples, p. 110–117. *In* S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

adsorbed viruses are eluted (removed) from the solid matrix into a small volume of a positively charged buffer such as 50 mM glycine or dilute beef extract. Despite the speed and efficiency of absorption-elution methods for the concentration of viruses, those researchers focused on the ecology of autochthonous aquatic viral assemblages have not adopted these methods. The primary reason is that because viruses can differ in biophysical characteristics, not all viruses are concentrated with equal efficiency through adsorption-elution. Indeed, all adsorption-elution methods for viral concentration have focused on the detection of specific pathogenic viruses within freshwater, and to a lesser extent, seawater samples. Moreover, differences in the characteristics of a given water sample can influence viral recovery, and eluant buffers such as beef extract can be incompatible with downstream analyses such as molecular genetic assays and microscopy (Williamson et al. 2003).

Aquatic viral ecology studies conducted over the past two decades have avoided the inherent limitations of adsorption-elution techniques through the use of two distinct approaches for the concentration of viruses from water samples. Ultrafiltration has been primary among these and is the focus of this review; however, direct collection of viruses by ultracentrifugation has also been used (Short and Short 2008; Steward et al. 2000). The primary technical challenge in concentrating sub-micron particles from large aqueous samples is the prevention of filter clogging. While a number of approaches have been developed to avoid clogging of ultrafiltration membranes, the most widely adopted has been tangential-flow filtration (TFF).

In TFF, the process fluid (in our case, a water sample) flows along a parallel tangent to the filter surface. Application of hydrodynamic pressure to the process flow (usually through restriction of the flow at one end of the filter) causes water and particles smaller than the pore size of the ultrafiltration membrane to flow through the membrane (Fig. 1). The water flowing across the membrane is known as the retentate, while the water flowing through the filter is the permeate. The speed of filtration is controlled by varying the amount of back pressure on the retentate flow (Fig. 1). Although back pressure can be controlled by both the retentate flow rate and the amount of restriction on the retentate flow; in practice, the flow rate is held constant while the restriction to the flow is used to control the overall filtration rate. Higher flow rates will more effectively prevent filter clogging as will more modest levels of back pressure. Manufacturers of TFF systems provide operational limits in terms of back pressure and often recommend optimal flow rates to prevent filter clogging. Thus, TFF represents a balance between the prevention of filter clogging (high retentate flow and low back pressure) and the speed of filtration (high permeate flow and high back pressure). In the special case of concentrating viruses from natural water samples, it is unlikely that an investigator will really challenge the operational limits of most TFF systems with sheet-type filter membranes; however, hollow fiber-based systems can be less tolerant to excessive back pressure. Nevertheless, the prudent investigator will carefully monitor TFF operating conditions and check filter integrity. Poor operating procedures or inappropriate cleaning and storage procedures can ruin the integrity of the filter. Such loss of integrity will allow viruses to pass through the filter resulting in a reduction in viral concentration efficiency.

There are at least three manufacturers that supply TFF systems suitable for the concentration of viruses from natural water samples. Millipore Corporation (www.millipore.com) is the largest supplier of TFF technologies to the research market and manufactures TFF platforms in two reusable cartridge formats including spiral-wound (e.g., Helicon and Prep-Scale) and flat plate (e.g., Pellicon). These systems are designed in various sizes to allow for scaling process volumes from ~100 mL up to thousands of liters. Smaller TFF systems in a flat-plate format are supplied by Sartorius Stedim Biotech

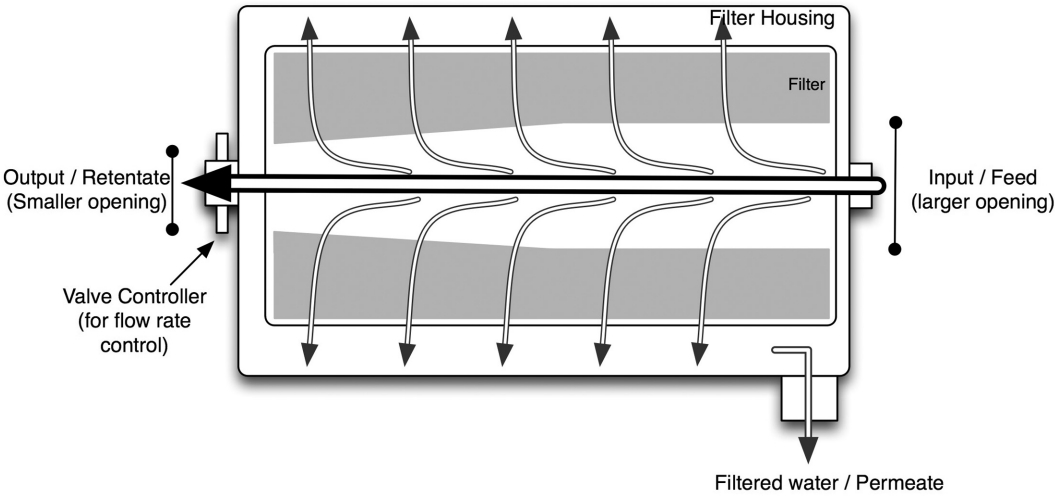

Fig. 1. Schematic diagram of tangential flow filtration cartridge

(www.sartorius-stedim.com; Vivaflow) and Pall Corporation (www.pall.com; Ultrasette) and are rated for initial filtration volumes of up to 5 L and can be expanded by parallel connection of multiple filters.

In most cases, initial concentration of viruses from large (10 to hundreds of liters) water samples by TFF in the field is followed by laboratory procedures designed to further concentrate viruses into a small volume (e.g., <10 mL). Initial TFF-based concentration of viruses within large water samples is preceded by microfiltration to remove bacterial cells and protists. Most TFF filtration housings can withstand inclusion of particles up to 100 µm in size; however, most investigators choose to remove all particles and cells larger than 0.22 µm prior to TFF so as to ensure the purity of viral concentrates. This report will outline and evaluate the filtration steps needed for the preparation of samples (viral concentrates) containing a density of viral particles concentrated from large volumes of natural water samples.

Materials and procedures

I. List of materials
   A. Large-scale concentration.
      i. Tubing: silicone, various sizes
      ii. Tubing: PharMed for pump heads only
      iii. Connectors to tubing and filters
      iv. Diaphragm pump (Jabsco Industrial Diaphragm pump #31801, 12 L per min capacity)
      v. Two 50-L plastic carboys
      vi. 25-L carboy
      vii. 25-µm wound polypropylene sediment filter (pool filter) and housing
      viii. Large peristaltic pump with 2 pump heads
      ix. 0.22-µm TFF filter
      x. 30- to 50-kD TFF filter
      xi. 0.22-µm syringe filters
      xii. 60-mL syringes
      xiii. 50-mL conical tubes
   B. Small-scale concentration.
      i. Tubing: silicone, various sizes
      ii. Tubing: PharMed for pump heads only
      iii. Connectors to tubing and filters
      iv. Small, reversible peristaltic pump with 1 pump head
      v. Two 2-L polycarbonate bottles
      vi. 500-mL polycarbonate bottle
      vii. 30- to 50-kD compact spiral filter
   C. Postconcentration.
      i. Ultracentrifuge
      ii. Swing bucket rotor
      iii. Polyallomer tubes
      iv. Balance
      v. Waste container
      vi. Final collection tubes of needed size
      vii. 200-µL pipettman and tips

II. List of reagents
   A. Sterile 60% glycerol

III. General protocol for the concentration of virioplankton from a large volume (>20 L) water sample

   Note: All filters should be cleaned and rinsed according to manufacturer's recommendations before use in any application during the concentration procedures. (Fig. 2A and B)

   A. Prefiltration.
      i. Prefilter ambient water sample with a 25-µm wound polypropylene sediment filter before any concentration (Fig. 2A). Use a diaphragm pump to filter raw water into a 50-L plastic carboy.
      ii. Rinse the 50-L carboy three times with a few liters of the prefiltered water before filling with final sample.
   B. Tangential flow microfiltration to remove particles and cells >0.22 µm (Fig. 2B).
      i. Once a 50-L carboy is full of 25 µm prefiltered ambient water, attach the 0.22-µm TFF filter feed and retentate tubing. Run the feed tubing through one of the large peristaltic pump heads (Caution: check for the correct flow direction before making connections!). Attach the permeate tubing to a 25-L carboy to contain the viral concentrate. The permeate valve on the 0.22-µm TFF filter should be in the OFF position.
      ii. If the peristaltic pump head has an occlusion setting, set it to maximal occlusion to help prime the 0.22-µm TFF filter and remove all air. Increase the pump speed to 20%. Once the tubing and filter are completely filled with water, adjust the occlusion knob to a looser setting to prevent excessive tubing wear. Slowly increase the pump speed to 45%. Once the system is running smoothly, partially open the permeate valve on the 0.22-µm TFF filter to the second tick mark (~20° open) and collect the >0.22 µm permeate into a 25-L carboy.
      iii. Cells and particulates between 25 µm and 0.22 µm will concentrate within the 50-L carboy as TFF through the 0.22-µm filter proceeds.
   C. Concentration of virioplankton.
      i. Large-scale (Fig. 2B)
         a. When the 25-L carboy is more than half full with <0.22 µm permeate, prepare the 30-kD TFF filter for viral concentration. Slow the large peristaltic pump speed to 10% (Do not stop the pump as this encourages adherence of viruses to filter matrix). Attach the feed and retentate tubing to the appropriate ports on the 30-kD TFF. Make sure that the backpressure knob on the 30-kD TFF filter is completely open (counter-clockwise). Direct the permeate

tubing from the 30-kD TFF filter into a clean carboy to collect ultrafiltrate (UF; virus-free water). Carefully work the feed tubing into the second pump head of the large peristaltic pump.

b. Increase the large peristaltic pump speed to 20%. Again, tighten the occlusion knob on the pump head to prime the 30-kD TFF filter and remove all air. Once the filter and lines are fully primed loosen the pump head occlusion knob. Slowly increase the pump speed to 45%. Slowly close the backpressure knob on the 30-kD TFF filter until permeate begins to flow. Monitor the level of <0.22 µm water in the 25-L carboy. The level should be maintained at half full until the prefiltered ambient water is nearly gone. Collect 1 L UF in a 2-L bottle for rinsing of the 30-kD TFF filter.

c. When ~5 L of 25 µm filtered ambient water remains in the large carboy, release the pump head and remove the 0.22-µm TFF filter feed tubing from the large peristaltic pump. Continue to run the 30-kD TFF filter until ~1 L of <0.22 µm water remains in the 25 L carboy. Avoid entry of air into the feed line as the level of retentate water nears this minimum. Stop the large peristaltic pump and drain the 30-kD TFF filter into the retentate carboy. Slowly prime the 30-kD TFF filter from a 1-L stock of UF and then recirculate the UF at 30% pump speed for 5 min. Be careful to avoid air bubbles. Stop the pump, release the pump head, and drain all tubing into the 2-L UF bottle. (Note: Recirculation of UF after primary viral concentration has been reported by the manufacturer to substantially improve recovery of retained molecules and viruses [Millipore 2003]).

d. Pool the recirculated UF with the ~1 L of retentate from the primary viral concentration through the 30-kD TFF filter. The volume of the ~2 L viral concentrate can be further reduced by using a small-scale TFF ultrafilter. To avoid excessive degradation of viruses, it is advisable to store the first stage VC at 4°C and perform a second stage small-scale concentration a soon as possible (i.e., within no more than 1 d).

ii. Small-scale

The steps involved in the concentration of viruses from smaller water samples (i.e., <2 L) are similar to those for large samples. However, the TFF filters used for concentration of viruses from small-scale samples are usually 10-fold smaller in filter area. The smaller size of these filters, and the tubing connected to them, results in a coordinately smaller minimum hold-up volume. In our experience, small-scale TFF filtration results in viral concentrates of ca. 250 mL in volume. Small-scale concentration of viruses from ambient water samples will require prefiltration to remove cells and particulates larger than 0.22 µm in size. If small-scale TFF filtration is used as a second step viral concentration following a large-scale (50 L to 2 L) procedure no prefiltration is required. After this two step process, theoretical viral concentration ratios of 200 fold (50 L to 0.25 L) can be achieved. Actual viral concentration ratio will depend on overall filtration efficiency. Oftentimes, large- and small-scale TFF concentration of viruses is performed in the field, and the final 250 mL concentrate is frozen (preferably snap frozen in LN2) for transport back to the lab. Some investigators have reported better viral preservation by adding glycerol to a final concentration of 10% prior to snap freezing (Glass and Williamson pers. comm.).

iii. Postconcentration procedures

Oftentimes, it is desirable to reduce the volume of viral concentrates below the ca. 250 mL minimum hold-up volume of most small-scale TFF apparatus. In particular, fingerprinting of viral assemblages by pulsed-field gel electrophoresis (Steward 2001; Wommack et al. 1999) or preparation of viral concentrates for metagenomic sequencing (Bench et al. 2007; Breitbart et al. 2002) requires viral particle densities of $\geq 10^9$ mL^{-1}.

By and large, investigators have produced high-density viral concentrates using either spin filters, which rely on centrifugal force to push water through a 30- to 50-kD molecular sieve, or an ultracentrifuge to pellet viruses followed by resuspension in a smaller volume of UF or buffer. Disposable spin filters are provided by a number of manufacturers and require only a benchtop swinging bucket rotor for filtration (Bench et al. 2007). Pelleting of viruses can only be done in an ultracentrifuge at centrifugal forces exceeding 100,000*g*, a requirement that can be cost prohibitive or unavailable at smaller research facilities (Wommack et al. 1992). Recently, Colombet and coworkers (2007) adapted polyethylene glycol (PEG) precipitation of viruses, for postconcentration of viruses within 1 L viral concentrates derived from 20-L water samples. The PEG protocol showed a greater than 2-fold increase in the recovery efficiency of virus particles as compared with the ultracentrifugation

procedure. The lower cost of this procedure is welcomed; however, we have found that postconcentration procedures can have a dramatic effect on the quality of PFGE virioplankton fingerprints. In contrast to the reported improvement in fingerprints after PEG precipitation (Colombet et al. 2007), we found that postconcentration of viral concentrates by ultrafiltration consistently produces the best resolved PFGE fingerprints (Fig. 3). The source of loss in PFGE band clarity and sharpness for samples processed by PEG precipitation or spin filtration is not known and did not appear to be attributable to sample loading as the PEG and Centricon samples were loaded with 2-fold less and 3-fold more viruses, respectively, than the ultracentrifuged sample.

Assessment

Because each processing step can result in the loss of viruses it is important to optimize filtration procedures for maximal viral recovery. Essentially, there are three major factors that can have a substantial effect on viral recovery: i) filter material; ii) flow rates; and iii) the physical means by which the process fluid travels through the filtration apparatus. An experiment to assess prefiltration and TFF methods is illustrative of the combined impact of these factors on viral recovery as assessed through viral direct counts by epfluorescence microscopy (Fig. 4). At least 60 L estuarine water samples (~30‰ salinity) collected at the entrance to the Delaware Bay near Lewes, DE (38°48′N; 75°07′W) were subjected to five methods for removal of larger particles and cells: gravity filtration through a 30-µm Nitex screen; pump-driven filtration through a 25-µm polypropylene sediment filter; vacuum filtration through a

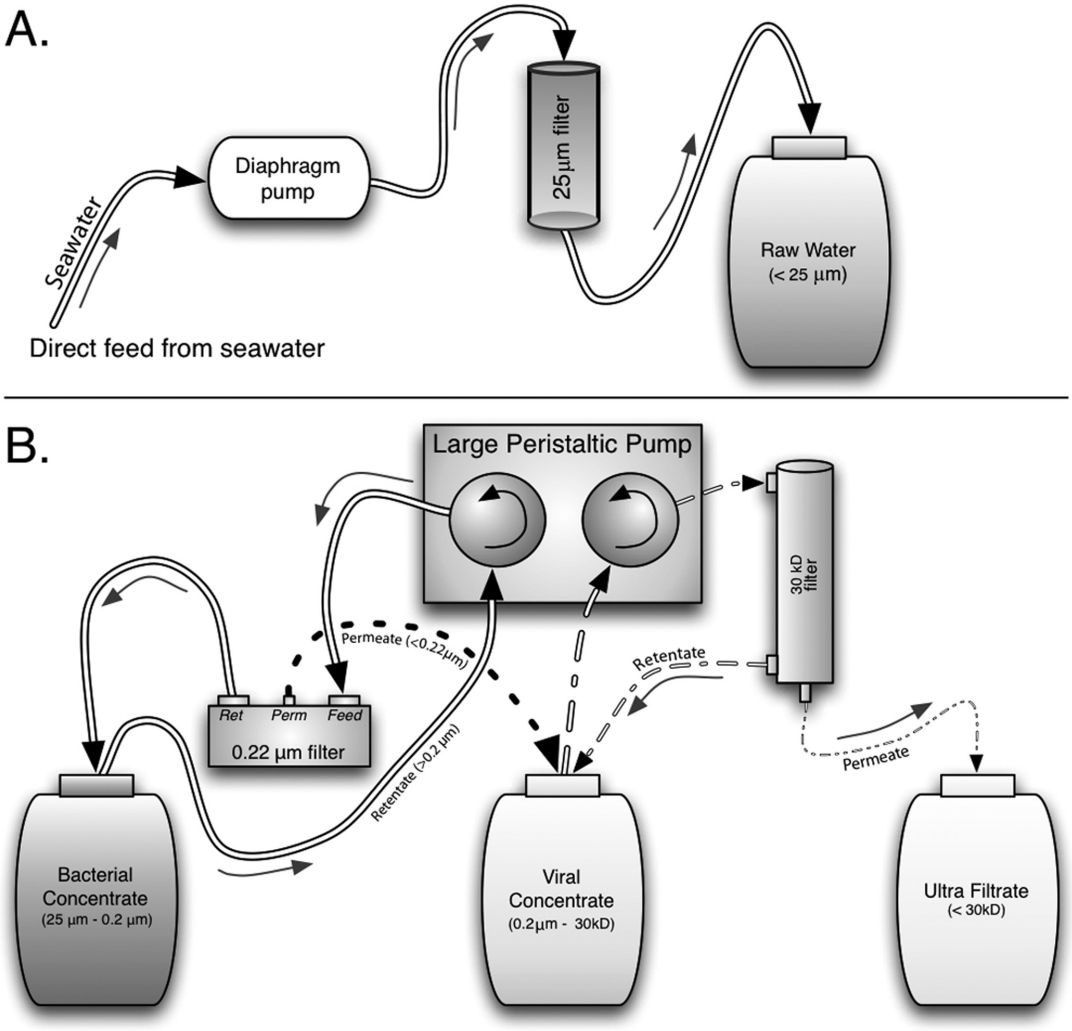

Fig. 2. Schematic diagrams of prefiltration (panel A) and TFF procedures (panel B) for the concentration of viruses from large volume water samples. Abbreviations are as follows: Ret, retentate; Perm, permeate; kD, kilodalton.

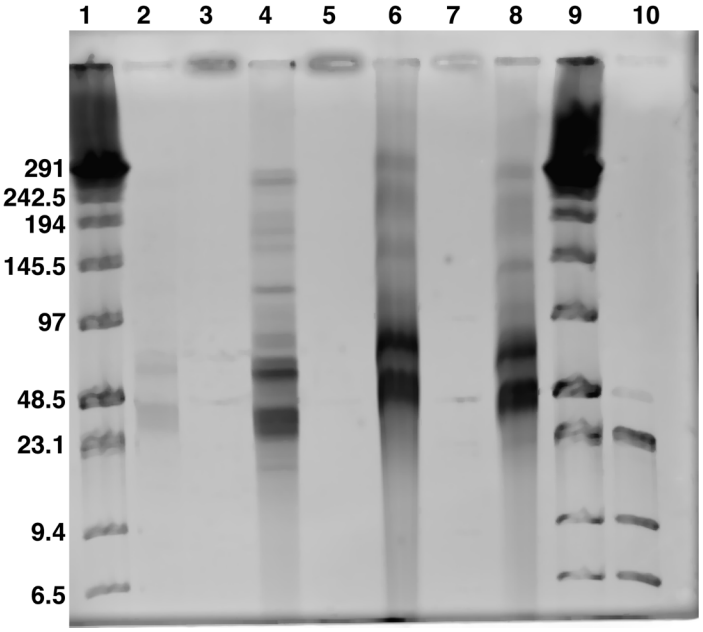

Fig. 3. Effect of final viral concentration procedures on pulsed-field gel electrophoretic analysis of Chesapeake Bay virioplankton assemblages. Lanes are as follows: 1, 9, and 10) molecular weight markers (band size in kilobases of DNA are shown on the left); 2) viral concentrate without additional concentration procedure (0.1×10^9 viruses); 4) ultracentrifugation pelleting and resuspension of viral concentrate (1.6×10^9 viruses); 6) polyethylene glycol precipitation and resuspension of viral concentrate (0.8×10^9 viruses); 8) ultrafiltration of viral concentrate using Centricon Plus 70 (Millipore) (5×10^9 viruses). All initial viral concentrates were 0.22 µm filtered before final concentration procedures or loading on PFGE gel.

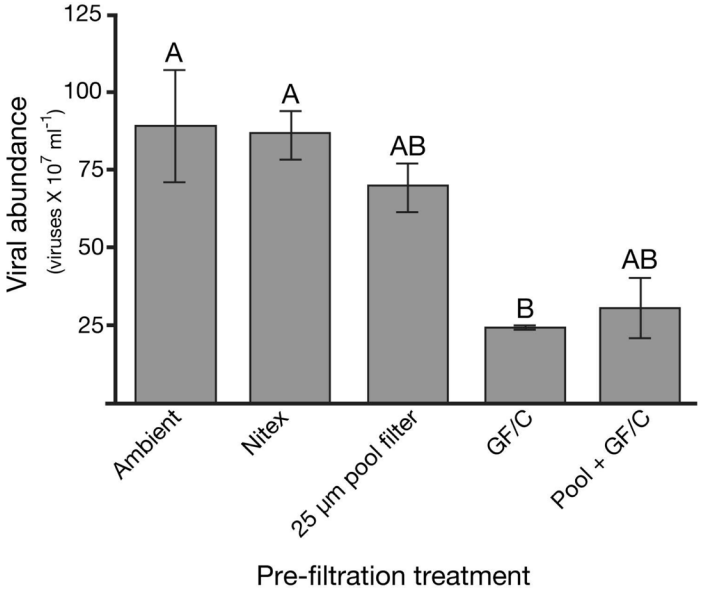

Fig. 4. Effect of prefiltration procedures on recovery of viral particles from seawater. Treatments are as follows: Ambient, seawater without prefiltration; Nitex, 30-µm Nitex screen; Pool, pump-driven filtration through a 25-µm wound polypropylene sediment filter; GF/C, glass fiber filter C nominal pore size (0.7 µm); Pool + GF/C, GF/C filtration following pool filter. Letters above bars denote significance groupings by Tukey post hoc tests ($P < 0.05$). Error bars are standard deviations of replicate measurements.

glass fiber (GF/C) impact filter, and filtration through the 25-µm sediment filter followed by GF/C filtration. Ambient viral and bacterial abundances were typical of mid-Atlantic coastal waters at ca. 8×10^7 and 3.4×10^6 mL^{-1}, respectively. Among these methods, GF/C filtration had the worst viral recovery at 27% and was significantly lower than ambient water and 30 µm Nitex-filtered water (one-way ANOVA, Tukey post hoc, $P < 0.05$) whereas all other methods produced no significant differences in viral abundances. It is important to remember that the influence of prefiltration procedures on viral recovery seen in this experiment is highly correlated to the physiochemical conditions of the modestly estuarine waters at the entrance to the Delaware Bay. Oligotrophic pelagic waters or those containing greater levels of particulate matter would likely show a different outcome in the same experiment.

Subsequently, seawater samples prefiltered through the 25-µm sediment pool filter were subjected to four treatments for the removal of cells and small particulates: 1 and 2) vacuum-driven impact filtration (a.k.a., dead-end filtration) through 0.22-µm or 0.45-µm filters; 3 and 4) TFF through 0.22-µm or 0.45-µm TFF filters. In the case of TFF through 0.22-µm and 0.45-µm TFF filters, three cross-filter flow rates were tested as measured by the ratio of retentate to permeate flow. In this scenario, the high flow rate treatment was a retentate to permeate flow ratio of 4 to 1 (i.e., in a given amount of time, 4-fold more water flowed through the retentate than the permeate). Whereas statistically significant differences in viral recovery were not found among these filtration procedures, clear trends were apparent (Fig. 5). In general medium cross-filter flow rates (a 2.5:1 retentate to permeate flow ratio) resulted in better viral recovery for TFF procedures. Impact filtration and TFF performed similarly; however, because limited sample volumes were tested, the influence of impact filter clogging on viral recovery was not assessed. A key factor in deciding whether to use impact or TFF procedures for removal of cellular material is the degree to which filter clogging will influence viral recovery for a given volume of sample water. In productive coastal waters that support high levels of bacterial abundance, impact filtration is less advisable as significant clogging can occur for relatively low sample volumes (e.g., ~5 L). Moreover, impact filtration through 0.45-µm filters tended to pass more bacterial cells into the filtrate as compared with impact filtration through 0.2-µm filters or tangential flow filtration with either 0.45- or 0.22-µm filters.

Assessment of final viral concentration by TFF using a 30-kD spiral cartridge (Helicon, Millipore) showed no significant differences in viral abundance resulting from TFF at each of three

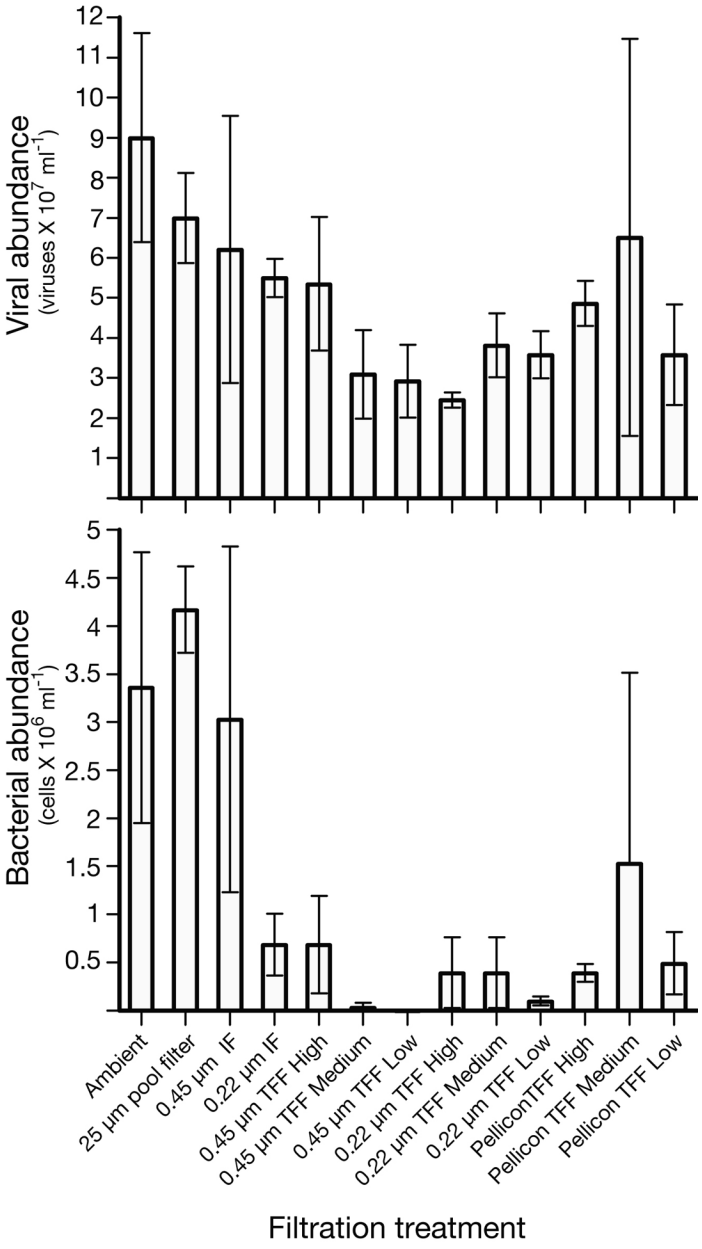

Fig. 5. Effects of prefiltration on viral recovery (upper panel) and bacterial cell removal (lower panel). Category designations are as follows: Ambient, initial unprocessed water samples; 25-µm pool filter, filtration of ambient water through wound polypropylene sediment filter; IF, impact filtration; TFF, tangential-flow filtration; High, Medium, and Low, 4:1, 2.5:1, and 1:1 retentate to permeate flow ratio, respectively; Pellicon, 0.1-m^2, 0.22-µm TFF filter. Error bars are standard deviations of replicate measurement.

cross-flow rates (one-way ANOVA, $P > 0.05$; data not shown), although concentration efficiency tended to be highest for the high flow rate TFF procedure. Thus, among the three filtration steps used in preparation of viral concentrates from large volume water samples, the greatest potential for loss of viruses occurs with the removal of cells and particulates of ≥0.22 µm.

Discussion

Tangential-flow filtration procedures create high-density viral concentrates that are clear of contaminating cells and particles larger than 0.22 µm. These concentrates can directly feed a number of downstream analyses common to viral ecological investigations such as isolation of new viral-host systems (Suttle and Chan 1995; Wang and Chen 2008) and assessing the impact of increased viral predation on host physiology (Suttle et al. 1990); detection of gene targets by PCR (Wang and Chen 2004; Zhong et al. 2002); characterization of whole virioplankton assemblages by randomly amplified polymorphic DNA-PCR (Winget and Wommack 2008) or PFGE profiling (Wommack et al. 1999). In the case of PCR procedures, many investigators have chosen to treat viral concentrates with nucleases to remove free DNA and ensure that PCR amplicons are derived from only capsid-enclosed viral genomic DNA (Winget and Wommack 2008). This consideration is also critical in the creation of viral metagenome libraries where procedures such as CsCl purification and nuclease digestion have been used prior to high-throughput sequencing (Breitbart et al. 2002).

The relative scale of viral concentration necessary for each procedure can differ. PCR-based procedures may require only modest levels of viral concentration (10 to 50 times concentration), whereas PFGE profiling or metagenomic analyses may require viral densities in excess of 100-fold that of ambient viral abundance. Thus, it is important to match the filtration apparatus to the required degree of viral concentration and initial sample volume. The end-point consideration for the investigator is the surface area of TFF filters. Filter surface area is directly proportional to filtration rate, thus, larger filters can process larger volumes of process fluid in a fixed amount of time. One caveat, however, is that minimum hold-up volumes will also increase with larger TFF filters ensuring that a second, smaller scale TFF system will be needed to further boost viral density within concentrates.

While viral concentration methods have been essential to advancing our understanding of viruses in aquatic environments, these methods also present significant limitations. The required separation of viruses and cells means that viruses with capsid sizes exceeding 220 nm are lost from endpoint viral concentrates. For example, DNA polymerase sequences from phycoviruses are commonly detected in whole microbial communities from the Chesapeake Bay, but are absent from viral concentrates prepared from Bay waters (Chen pers. comm.). As a consequence of prefiltration, extant genetic diversity of large viruses, such as those within the Phycodnaviridae and nucleo-cytoplasmic large DNA viruses (NCLDVs), has been under-sampled within virioplankton metagenome libraries (Monier et al. 2008).

Among the aquatic viral ecology research community, it is widely believed that TFF filtration methods lack the inherent and possibly systematic biases in viral concentration that

might result from adsorption-elution methods (Suttle et al. 1991). Thus, TFF viral concentration procedures operate on the assumption that all viruses are concentrated with equal efficiency. To our knowledge, however, this assumption has not been thoroughly tested nor compared with alternate procedures such as adsorption-elution. Although it is a tedious and difficult experiment, certainly more extensive testing for systematic loss of virus groups during TFF is warranted. A secondary alternative to such an extensive test would be a quick and inexpensive means to monitor concentration efficiency. At present, few investigators report the efficiency of their viral concentration procedures. As shown in Figs. 4 and 5, even slight alterations in filtration media or filtration conditions can dramatically affect the efficiency of viral concentration. Ironically, simply increasing initial water sample volume 5-fold (from 50 to 250 L) lowered concentration efficiency by ~10-fold (50% to 5%) for Chesapeake Bay water samples (data not shown) and eliminated possible gains in the concentration of viruses from processing a larger sample volume.

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