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Tangential flow diafiltration: an improved technique for estimation of virioplankton production

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ABSTRACT: Accurate estimates of viral production in natural environments are critical for assessing the impacts of viral lysis on bacterial mortality and dissolved organic matter release. Here, viral production was estimated using a tangential flow diafiltration (TFD) dilution method, which reduced viral abundance to about 25% of ambient while maintaining near ambient levels of bacterial abundance. In subsequent incubations, the rate of virus-like particle increase was measured and used to calculate viral production. TFD viral production estimates were compared to those from simultaneous incubations using a vacuum diafiltration procedure. At 4 stations in the Chesapeake and Delaware Bays, viral production averaged $4.8 \pm 1.7 \times 10^{10}$ and $5.9 \pm 4.4 \times 10^{10}$ viruses l^{-1} d^{-1} as assessed by the TFD and vacuum methods, respectively. The TFD procedure improved upon the vacuum-based method by recovering significantly more of the bacterial community and requiring less sample processing time. Optimization tests of the TFD procedure found that a 0.22 µm pore size filter with a flushing rate of 40 ml min^{-1}, and a flushing volume 4-fold the initial sample volume gave the best combination of bacterial recovery, viral dilution, and processing time. Based on TFD viral production estimates, viral lysis was responsible for the loss of 14 to 93% of the bacterial standing stock and the release of 22 to 47 µg C l^{-1} d^{-1} in the Chesapeake and Delaware Bays. These results indicate that viral lysis is a significant factor for microbial mortality and dissolved organic matter cycling within these estuaries.

KEY WORDS: Viral production · Tangential flow diafiltration · Viral-mediated mortality · Dilution method

INTRODUCTION

Marine viruses were initially discovered to be highly abundant members of the marine environment about 15 yr ago (Bergh et al. 1989). Since then, many studies have focused on marine cyanophage host specificity (Suttle & Chan 1993, Waterbury & Valois 1993, Lu et al. 2001), viral diversity (Cottrell & Suttle 1991, Wommack et al. 1999, Wang & Chen 2004), and the potential impacts of viral infection on phyto- and bacterioplankton community composition (Thingstad & Lignell 1997, van Hannen et al. 1999, Fuhrman & Schwalbach 2003, Winter et al. 2004b). By comparison, fewer investigations on the potential influence of viruses and viral infection on the cycling of dissolved organic matter (DOM) through the marine food web have been conducted. In addition to causing bacterial and phytoplankton death, viral lysis diverts microbial cell biomass into the DOM pool and away from higher trophic levels (Proctor & Fuhrman 1991, Bratbak et al. 1992, Fuhrman 1992, 1999, Wilhelm & Suttle 1999, Wommack & Colwell 2000). Accurate estimates of *in situ* viral production are critical for quantifying the effects of viral lysis on DOM cycling and host population mortality.

Viral production (VP), the amount of viruses produced within a given time period and volume, can be inferred from the rate of increase in viral abundance over time. Using an assumed or average burst size for a typical viral infection, VP rates can be used to calculate viral-mediated bacterioplankton lysis, i.e. viral-mediated mortality (VMM) (Steward et al. 1996, Noble & Fuhrman 2000, Wilhelm et al. 2002), and to estimate the

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amount of C, N, and P released into a system by viral cell lysis (Fuhrman 1992, Steward et al. 1996, Gobler et al. 1997, Weinbauer & Höfle 1998, Wilhelm et al. 2002). Approaches to estimation of VP have been numerous and include electron microscopic observation of the frequency of visibly infected cells (FVIC) (Proctor et al. 1993, Steward et al. 1996, Binder 1999, Guixa-Boixereu et al. 1999, Hwang & Cho 2002, Middelboe et al. 2002, Choi et al. 2003, Weinbauer et al. 2003a), contact rates of viruses and bacteria (Murray & Jackson 1992, Suttle & Chan 1994), incorporation of ^{3}H-thymidine or ^{32}P into viral DNA (Steward et al. 1992a, 1996, Fuhrman & Noble 1995, Kepner et al. 1998), viral decay rates (Heldal & Bratbak 1991, Bratbak et al. 1992, Guixa-Boixereu et al. 1999, Tuomi & Kuuppo 1999), fluorescently labeled viruses (FLVs) as tracers of viral decay and VP (Noble & Fuhrman 2000, Helton et al. 2005, this issue), and dilution methods (Wilhelm et al. 2002, Winter et al. 2004a, Helton et al. 2005). The assumptions, advantages, and disadvantages of each of these approaches to measuring VP are summarized in Table 1. For many of these methods, 1 or more of the assumptions or conversion factors upon which the assessment of VP is based remains untested or poorly constrained.

Recently, Wilhelm et al. (2002) introduced a dilution approach in which vacuum filtration through a 0.22 µm impact filter reduced viral abundance prior to incubation. This method allows for direct observation of VP from a natural microbial consortia and requires only 1 correction factor, the reciprocal of the percent loss of bacteria, to estimate VP (Weinbauer et al. 2002, Wilhelm et al. 2002, Poorvin et al. 2004). Thus, it bypasses many of the assumptions and conversion factors necessary for other VP techniques. Removal of viruses by dilution prior to incubation lowers the occurrence of new viral infections during incubation and reduces the background level of viruses to ease enumeration throughout the experiment. However, a critical limitation to dilution-based estimates of VP is the loss of bacteria during filtration. In our experience, >80% of bacteria are typically lost during vacuum diafiltration using 0.22 µm impact filters. To improve bacterial recovery and viral dilution, a new technique employing tangential flow diafiltration was developed and tested using water samples from the Chesapeake and Delaware Bays. Loss of viruses, recovery of bacteria, and estimates of VP obtained from both vacuum and tangential flow diafiltration methods (Wilhelm et al. 2002) were compared.

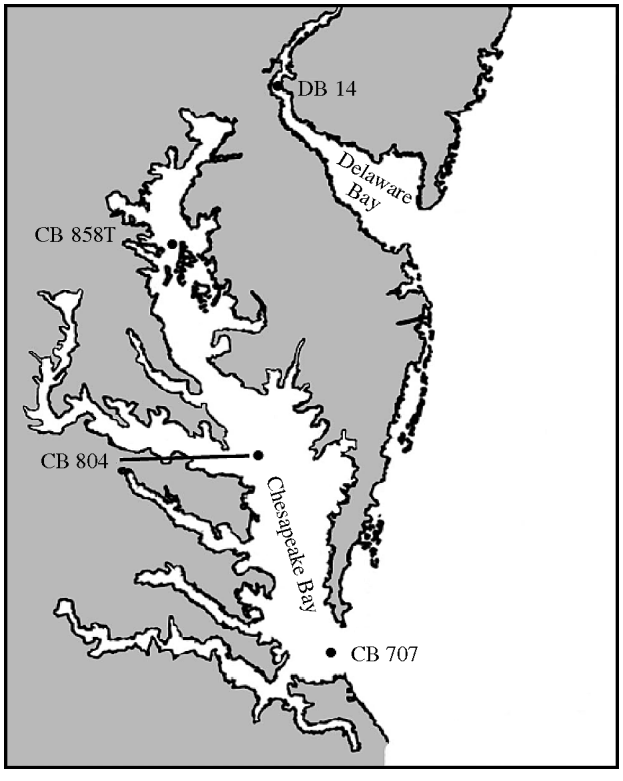

Fig. 1. Diagram of sampling station locations in the Chesapeake and Delaware Bays

MATERIALS AND METHODS

Sampling. Water samples for VP experiments were collected along the main stem and tributaries of the Chesapeake Bay at Stns CB 858T, CB 804 and CB 707 (Fig. 1) in August and September 2002; March, April, June, July, and August 2003; and July 2004. Samples were also gathered in the Delaware Bay at Stn DB 14 during August 2002. For all samples, surface water from approximately 1 m depth was collected in 10 l Niskin bottles using a CTD (Seabird 911 Plus) and then transferred to a 1 or 2 l acid-cleaned and triple-rinsed polycarbonate bottle. Sub-samples of 5 or 15 ml of ambient water were collected, preserved with addition of formalin to a final concentration of 1%, and stored at 4°C on the August 2002, September 2002, and March 2003 cruises until analysis for viral and bacterial abundance via epifluorescence microscopy. All other water samples were frozen in liquid nitrogen within 12 h of collection and stored at –70°C. The remaining ambient water was filtered through 50 µm Spectra mesh and kept in the dark at ambient water temperature in an on-board flow table until diafiltration (1 to 2 h).

The rate of VP in water samples was assumed to be equal to the rate of appearance of new viral particles after removal of the majority of the natural viral community via dilution with virus-free seawater (Wilhelm et al. 2002). Two methods of viral removal were performed in this study.

Vacuum diafiltration. The first closely followed the dilution procedure reported by Wilhelm et al. (2002).

Table 1. Summary of assumptions, advantages, and disadvantages of viral production (VP) techniques. FLV: fluorescently labeled virus

VP method	Assumption	Advantages	Disadvantages	Source
Frequency of visibly infected cells	•All cells containing viruses will lyse •Latent period equals host generation time •All viruses visible in cell for same time period and percentage of latent period •Bacterial infection and lysis occur continuously •Burst size is constant or within a stated range •Infected and uninfected bacteria grow and are grazed at the same rate	•No incubation required to calculate VP •Only transmission electron microscopy sample preparation •Average burst size can be directly calculated for each sample	•Calculations of VP dependent on conversion factors •Does not directly observe changes in viral abundance over time •Large numbers of cells must be examined for accurate estimates •Selective loss of infected cells may occur during sample centrifugation •No distinction can be made between lytic and lysogenic VP •Viruses visible for varying portions of latent period •VP estimation can vary with sample preparation used	Proctor et al. (1993), Fuhrman & Noble (1995), Binder (1999), Noble & Steward (2001), Weinbauer et al. (2002)
Contact rates	•All cells equally sensitive to viral infection •All cells lyse after viral infection •All virus to host adsorption rates similar to that of Phage T4 •Every virus–host contact results in infection •Burst size and host cell size are constant or within a stated range	•Rapid and easy to calculate VP	•Calculations of VP dependent on conversion factors •No direct observation of changes in viral abundance over time •No distinction can be made between lytic and lysogenic VP	Murray & Jackson (1992), Waterbury & Valois (1993), Suttle & Chan (1994)
Radioisotope incorporation	•Bacteriophages dominate free virus populations •Environment is in a steady-state condition •Constant conversion factor for incorporation rate of isotope into DNA to VP •All isotope incorporation into viral sized fraction is viral in origin •Genome size of replicating viruses is constant or within a given range •No de novo nucleotide or DNA synthesis	•Directly measures VP rates •Easily reproducible with low error between replicates •Measures flux of bacterial to viral biomass	•Requires working with radioactive materials •Magnitude of intracellular isotope dilution can vary between samples •RNA viruses not included in assessment •Calculations of VP dependent on conversion factors used •No distinction can be made between lytic and lysogenic VP •Small, isotope-labeled bacteria or DOM may contaminate samples •Viral abundance losses can occur during filtration •Viral isotope incorporation rate can be below background level	Steward et al. (1992a,b), Fuhrman & Noble (1995), Steward et al. (1996), Kepner et al. (1998), Noble & Steward (2001)
Viral decay	•Chemical inhibitor halts all viral production •Viral burst size is constant or within a stated range •Viral decay equals VP •Chemical inhibitor does not affect viral degradation rates	•Directly observes changes in viral abundance over time •No sample filtration necessary •Easy to perform	•Only abiotic viral decay sources assessed •Resulting viral decay curves can be difficult to interpret •No distinction can be made between lytic and lysogenic VP	Heldal & Bratbak (1991), Guixa-Boixereu et al. (1999)
FLV tracer	•Burst size is constant or within a stated range •FLVs act the same as unlabeled viruses and are non-infective •FLV addition does not alter or affect viral or bacterial communities	•Directly observes changes in viral abundance over time •VP and viral decay can be measured simultaneously •Easy and inexpensive to perform	•FLV tracers dim over time •Lengthy tracer preparation necessary •Requires extensive sample enumerations •Sample incubation must be performed in dark •No distinction can be made between lytic and lysogenic VP •Can overestimate VP estimates for eutrophic environments	Noble & Fuhrman (2000), Noble & Steward (2001), Helton et al. (2005)
Dilution method	•All VP from bacteria infected at the initiation of the incubation •Filtration does not alter or affect the system •No new infections occur during incubation	•Directly observes changes in viral abundance over time •Lowered initial viral abundance eases enumeration •Easy and relatively inexpensive to perform •Only conversion factor for bacterial loss needed	•Sample manipulation during diafiltration unavoidable •Portion of ambient bacterial community always lost during diafiltration •Requires extensive sample enumerations •No distinction can be made between production of virulent and temperate viruses •Sample incubation must be performed in dark	Wilhelm et al. (2002), Winter et al. (2004a), Helton et al. (2005), Present study

Briefly, 300 ml water samples were vacuum filtered through a 47 mm, 0.2 µm pore size Supor 200 filter (Pall Corporation). Continuous flushing with a 25 ml transfer pipette was employed to keep bacteria suspended in the filter reservoir during diafiltration (Weinbauer et al. 2002). As seawater was removed via filtration, virus-free seawater (30 kDa filtered) from the same location was added to the filter reservoir until 3 vol (900 ml total) had been flushed through the filter. This flushing volume was expected to remove about 90% of the ambient viral population. After completion of diafiltration, 100 ml of the remaining viral-reduced water sample was immediately aliquoted into each of 3 polycarbonate bottles (250 ml) and incubated in the dark at *in situ* temperatures in an on-board flow table. Immediately after aliquoting, 5 or 10 ml sub-samples (t_0 samples) of each of the 3 replicates were taken, preserved, and stored as above. Sub-sampling was repeated at 3 h intervals for 12 h. Samples from this method are hereafter referred to as 'vacuum' samples.

Tangential flow diafiltration. The second method of viral removal employed tangential flow diafiltration (TFD) instead of vacuum diafiltration. In the TFD method, water is drawn into the feed line using a peristaltic pump, flows over a 0.22 µm pore size filter (PelliconXL, Millipore), and out the retentate line (Fig. 2). Reducing the diameter of the retentate line with a clamp creates back pressure inside the filter cartridge and forces water and particles smaller than the filter's pore size through the filter and out the permeate line. A similar procedure, using 30 to 100 kDa filters, is routinely employed for collection and concentration of viruses from ambient water samples (Chen et al. 1996, Wommack et al. 1999, Wang & Chen 2004, Winter et al. 2004b). Throughout the process, the retentate line is directed into the sample container to return particles >0.22 µm (i.e. bacteria) to the sample. As in vacuum diafiltration, 300 ml of 50 µm pre-filtered water was poured into a 500 ml square polycarbonate bottle. After the feed line was primed with a peristaltic pump, the combined permeate plus retentate flow rate was adjusted to 40 ± 2 ml min^{-1} with a back pressure of 8 to 10 psi by changing the pump speed and retentate line diameter. The permeate line was then removed from the sample bottle to filter out water and particles <0.22 µm in size from the sample. Permeate volume was replaced by the addition of virus-free, ultra-filtered seawater to the sample. Three vol (900 ml total) of virus-free seawater were used for diafiltration in all experiments except as noted for specific optimization experiments. After completion of viral dilution, the filter was back-flushed according to manufacturer recommendations to recover water remaining in the filter. The viral-reduced sample was aliquoted, incubated, and sub-sampled as described previously for the vacuum diafiltration method. Samples from this method are hereafter referred to as 'TFD' samples.

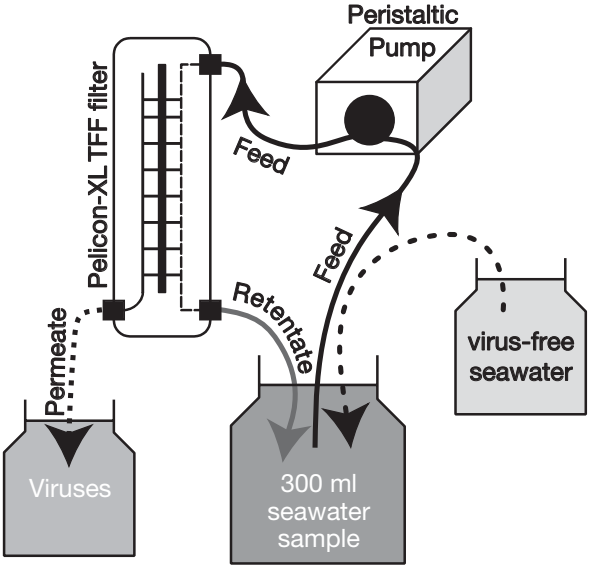

Fig. 2. Tangential flow diafiltration (TFD) procedure. Sample water is pumped through the feed line (solid arrow) into the TFD filter using a peristaltic pump. As the water passes through the 0.22 µm filter, back pressure forces viruses and seawater through the filter into the permeate (dotted arrow). Remaining water and bacteria flow back into the sample container through the retentate line (gray arrow). The volume of water lost through the permeate is replaced by the addition of virus-free water (dashed arrow)

TFD method optimizations. To determine the effects of dilution volume and flow rate on bacterial recovery and viral loss, a 0.22 µm pore size TFD filter was assessed at 3-fold (900 ml) and 4-fold (1200 ml) flushing volumes at each of 3 flow rates—low (30 ml min^{-1}), medium (40 ml min^{-1}), and high (50 ml min^{-1}). A 0.45 µm pore size filter was subjected to the same set of conditions to determine the effects of filter pore size on bacterial recovery and viral loss. Samples of 5 to 10 ml of ambient water were collected prior to and immediately after dilution was completed and were stored as described above. Each set of conditions was replicated twice at different stations and on different sampling dates. The results of the 2 experiments were averaged to determine which combination of factors produced the greatest bacterial recovery and viral dilution.

Viral and bacterial abundance. In all samples, viral and bacterial abundances were assessed via epifluorescence microscopy after staining with SYBR Gold (Chen et al. 2001). Briefly, 0.5 to 3 ml of sample was filtered onto a 25 mm, 0.02 µm Anodisc filter (Whatman) supported by a 0.2 µm Supor filter (Pall Corp.) and an extra thick glass fiber filter (Pall Corp.) and stained with 2.5× SYBR Gold for 15 min in the dark. Anodisc filters were then mounted onto glass slides using

immersion oil and topped with an anti-fade solution (50% glycerol, 50% PBS, 0.1% anti-fade solution; Noble & Fuhrman 1998) and an 18 mm round coverslip. For each filter, at least 200 virus-like particles (VLPs) and 10 microscopic fields were viewed under FITC excitation at 1000× magnification on a Zeiss Axioskop 2 (100× Plan-NEOFLUAR oil objective). Twelve-bit TIFF digital images of each field were captured using an ORCA-ER (Hamamatsu) camera. VLPs and bacteria were differentiated based on differences in size and fluorescence intensity. Both were enumerated from the digital images using FoVeaPro (Reindeer Software) plug-ins for Adobe Photoshop (Helton et al. 2005). For Stns DB 14 and CB 858T (August 2002) 0, 6, and 12 h sub-samples were analyzed within 12 d of sample collection, while the 3 and 9 h sub-samples were enumerated after 121 d. Stn CB 707 and CB 804 experiments (March 2003) were entirely enumerated within 25 d of collection.

Calculations of viral production and statistical analyses. For both the vacuum and TFD methods, the same calculations were used for bacterial recovery, viral loss, and VP. Bacterial recovery efficiency was calculated as the bacterial abundance at initiation of the experimental incubation divided by the bacterial abundance in ambient water, multiplied by 100 (Wilhelm et al. 2002). Similarly, percent viral loss or dilution, L, was calculated as:

$$L = [1 - (V_{t_0}/V_a)] \times 100$$

where V_{t_0} is viral abundance at the initiation of the experimental incubation, and V_a is viral abundance in ambient water. VP was estimated from the slope of a first-order regression line of viral abundance versus incubation time (Wilhelm et al. 2002). The slopes of the 3 replicates were averaged and corrected for bacterial loss by multiplying by the ratio of bacterial abundance in ambient water to bacterial abundance in experimental incubations at the initiation of the experiment (t_0). VMM was calculated as the VP estimate divided by the burst size, assumed to be 50 as in Wilhelm et al. (2002). The percentage of bacterial production (BP) consumed by viral lysis was computed as the BP in cells l^{-1} d^{-1} divided by VMM. All BP rates were assessed using a modified ^{3}H-leucine incorporation method (Kirchman et al. 1985, Smith & Azam 1992). The percentage of bacterial standing stock lysed per day was calculated as VMM divided by ambient bacterial abundance.

All linear regressions were performed using Prism version 4.0 (Software Mackiev). Student's *t*-tests were employed to assess differences in bacterial recovery, viral dilution, and VP rates between the vacuum and TFD diafiltration methods and significant differences between ambient and initial abundances of bacteria and viruses. Multi-way and 1-way ANOVAs were performed to detect significant differences in bacterial recovery and viral dilution among the various optimization parameters tested. Tukey's post hoc tests were used to identify exact relationships when significant differences were found. The significance level for all tests was set at $p < 0.05$, and all statistical tests were performed using SPSS 11 software (SPSS).

RESULTS

Dilution method comparisons

At 2 stations in August 2002 and 2 stations in March 2003, the vacuum and TFD methods for VP were performed simultaneously. Although each diafiltration was started simultaneously, completion of the vacuum technique took about 1 h longer than the 90 min needed for completion of TFD. As a result, TFD incubations started and ended approximately 1 h earlier than the vacuum incubations. No significant differences were found in either viral or bacterial abundance after 50 µm pre-filtration (data not shown). Bacterial abundances in estuarine water samples ranged from 2.2×10^9 to 1.0×10^{10} cells l^{-1} (Table 2). Bacterial recovery using vacuum diafiltration averaged 15% of ambient bacterial abundance and ranged from 7 to

Table 2. Bacterial abundance, viral production (VP), and recovery efficiencies. All numbers are mean ± SE. *Significant differences between tangential flow diafiltration (TFD) and vacuum methods. Student's *t*-test, $p < 0.05$

Stn	Date	Ambient bacterial abundance (bacteria l^{-1})	Method	VP (10^{10} viruses l^{-1} d^{-1})	Ambient bacteria recovered (%)	Ambient viruses removed (%)
CB 858T	Aug 2002	1.0×10^{10}	Vacuum	6.7 ± 6.8	25 ± 1.7*	62 ± 11
			TFD	6.6 ± 0.85	68 ± 1.6*	57 ± 6.2
DB 14	Aug 2002	4.3×10^9	Vacuum	–3.9 ± 1.6	19 ± 2.6*	74 ± 2.5
			TFD	4.7 ± 2.8	53 ± 4.6*	79 ± 3.5
CB 707	Mar 2003	2.3×10^9	Vacuum	25 ± 10	9.8 ± 4.0*	92 ± 2.2
			TFD	–2.2 ± 1.2	32 ± 3.2*	84 ± 0.53
CB 804	Mar 2003	2.2×10^9	Vacuum	–3.7 ± 1.8*	6.5 ± 1.2*	97 ± 0.20*
			TFD	10 ± 3.5*	66 ± 9.6*	73 ± 5.6*

25%. For the TFD method, bacterial recovery averaged 55% and varied from 32 to 68% (Table 2). For all sampling stations, bacterial recovery efficiency by the TFD method was significantly higher than that of the vacuum procedure ($p < 0.05$). Bacterial abundances at the initiation of TFD experimental incubations were always significantly higher than those of vacuum incubations (Fig. 3, $p < 0.05$). At 3 of the 4 stations, the loss of bacteria using both methods was significant (Fig. 3, $p < 0.05$). At the fourth station (CB 804), ambient and initial incubation bacterial abundances were statistically identical using the TFD method, whereas ambient bacterial abundance exceeded initial incubation abundance using the vacuum method ($p < 0.05$).

Vacuum diafiltration removed an average of 83% of ambient viruses and ranged from 62 to 97% viral dilution efficiency. TFD removed an average of 75% of ambient viruses and varied from 57 to 84% viral dilution efficiency. Despite the lower average viral loss rate of TFD, at only Stn CB 804 did vacuum diafiltration remove a greater percentage of ambient viruses than TFD (Table 2, $p < 0.05$).

VP estimates based on the entire 12 h incubation for both TFD and vacuum methods ranged from a net loss of viruses (e.g. -3.7×10^{10} viruses l^{-1} d^{-1} at Stn CB 804) to a high VP estimate of 25×10^{10} viruses l^{-1} d^{-1} at Stn CB 707 (Table 2). In experiments conducted at Stns DB 14, CB 707, and CB 804 a net loss of viruses was recorded from one of the incubation methods, leading to a significant or nearly significant difference in resulting VP estimates from the 2 methods (DB 14: $p = 0.055$; CB 804: $p = 0.026$; CB 707: $p = 0.059$). For the station where both methods yielded a positive VP estimate, mean values were not significantly different (CB 858T: $p = 0.995$). Comparisons of VP estimates performed prior to correction for bacterial recovery efficiency indicated that the TFD and vacuum-based VP methods differed significantly at Stns CB 707 and CB 804 (Fig. 4a, $p < 0.05$). No significant correlation or regression was found between VP estimates and either the percentage of ambient bacteria recovered or the percentage of ambient viruses removed (data not shown). Correlation and regression analysis of VP estimates and ambient or t_0 bacterial and viral abundances or virus to bacteria ratios (VBR) were not significant either (data not shown).

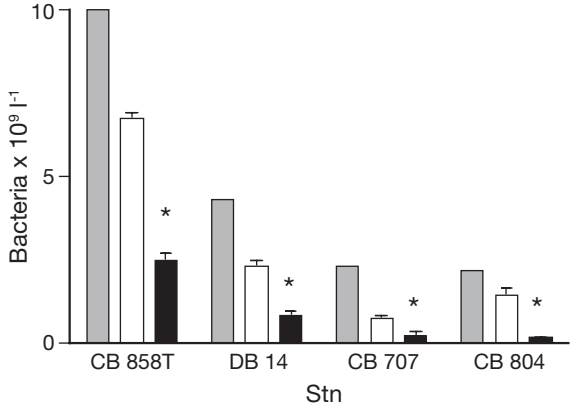

Fig. 3. Comparison of TFD and vacuum diafiltration methods. Bacterial abundance per liter in ambient water samples and water samples taken immediately after manipulation by either the TFD or vacuum dilution method (grey bars: ambient water; open bars: TFD method; black bars: vacuum method counts; *: significant differences between TFD and vacuum methods; error bars: +SE)

TFD method optimizations

To determine the optimal conditions for TFD, combinations of 3 different flow rates (low, medium, and high), 2 filter sizes (0.22 and 0.45 µm), and 2 flushing volumes of virus-free seawater (3- and 4-fold sample

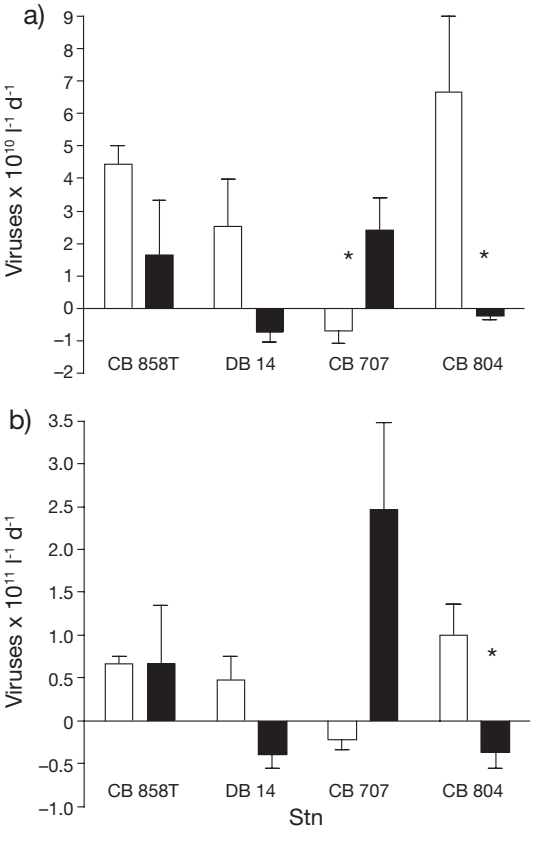

Fig. 4. Comparison of average viral production estimates between TFD and vacuum diafiltration methods. Viral production estimates (a) prior to correction for bacterial recovery efficiency and (b) after correction for bacterial recovery efficiency (open bars: TFD-based viral production estimates; black bars: vacuum-based viral production estimates; *: significant differences between estimates; error bars: +SE)

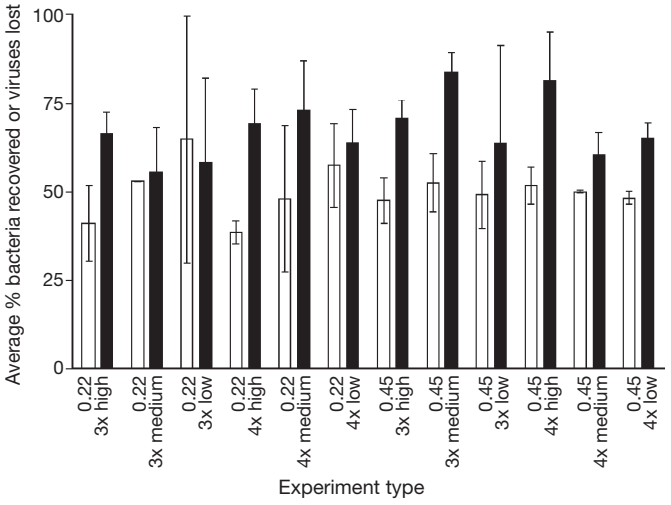

Fig. 5. Comparison of the percentages of ambient bacteria recovered and ambient viruses diluted out by various TFD method conditions (0.22: use of a 0.22 µm pore size TFD filter; 0.45: use of a 0.45 µm pore size TFD filter; 3× and 4×: 3- and 4-fold, respectively, of the original sample volume of virus-free water used to replace water lost to permeate; high: a flow rate of 50 ml min^{-1}; medium: 40 ml min^{-1}; low: 30 ml min^{-1}; open bars: % of ambient bacteria recovered; black bars: % of ambient viruses diluted out by diafiltration; error bars: ± SE)

volume) were tested on water samples collected from March 2003 through July 2004 (Fig. 5). Replicates of experimental conditions were performed at different stations on separate cruises to account for effects of changing environmental conditions such as increased turbidity. None of the parameters tested had a significant effect on either bacterial recovery or viral loss efficiency ($p > 0.05$). Average viral loss and bacterial recovery rates were similar for the 0.45 and 0.22 µm pore size filters: 71% viral loss, 50% bacterial recovery and 68% viral loss, 44% bacterial recovery, respectively.

Average bacterial recovery and viral loss rates for the 3- and 4-fold flushing volumes were as follows: 45% bacterial recovery, 70% viral loss and 49% bacterial recovery, 69% viral loss, respectively. In 25 subsequent experiments, the 4-fold flushing volume averaged 68% viral dilution and 55% bacterial recovery (data not shown). The low flow rate of 30 ml min^{-1} required 45 min longer to complete than the other flow rates and averaged 46% bacterial recovery and 68% viral loss. The highest flow rate of 50 ml min^{-1} had the lowest average bacterial recovery efficiency at 45%, but the highest mean viral dilution at 72%. The medium flow rate of 40 ml min^{-1} averaged 51 and 68% for bacterial recovery and viral dilution rates, respectively.

Examination of TFD VP experiments revealed internal fluctuations of VP at various time points. For instance, an increasing rate of VP followed by a decreasing rate was seen at 3 h intervals at Stn CB 858T. These variations resulted in different VP estimates based on the end time point chosen for the regression (data not shown). However, the VP estimate significantly varied with the time interval (6, 9, or 12 h) used in the calculation only at Stn CB 858T ($p < 0.05$).

Biogeochemical impacts of viral lysis

From TFD-based VP estimates, several biologically important parameters were calculated for each station (Table 3). Because experiments that indicate a net loss of viruses (negative VP rate) are counter-intuitive when applied to calculations of viral-mediated bacterial mortality, the experiment conducted at Stn CB 707 was excluded from further analyses. VMM ranged from a low of 9.4×10^8 to a high of 2.0×10^9 bacteria l^{-1} d^{-1}. The percentage of BP lost to viral lysis (84 to 207%) was generally higher in the late summer, and corresponded to a loss of between 14 and 93% of the bacterial standing stock d^{-1}. Assuming that 23.3 fg C per bacterial cell is released into the DOM pool upon cell lysis (Wilhelm et al. 1998, 2002), the amount of carbon released based on TFD VP estimates varied from 22 to 47 µg C l^{-1} d^{-1} (Table 3). Carbon release calculated for Stn CB 804 in March was almost double the average C release for the August stations.

DISCUSSION

Assessment of TFD method

Termed the 'viral-shunt' (Wilhelm & Suttle 1999), the loss of microbial biomass to viral lysis is among the most poorly understood processes within the microbial loop (Wilhelm & Suttle 1999, Wommack & Colwell 2000). Accurate determination of viral-mediated

Table 3. Viral and bacterial parameters calculated from viral production (VP) estimates. VMM: virally mediated mortality, VP/burst size of 50; % bacterial production (BP) consumed by viral lysis: BP/VMM; viral turnover time: VP/viral abundance × 24 h d^{-1}; carbon released: VMM × 23.3 fg C $bacteria^{-1}$ (1×10^{-9})

Stn	Date	VMM (10^9 bacteria l^{-1} d^{-1})	BP consumed by viral lysis (%)	Viral turnover time (d^{-1})	Carbon released (µg C l^{-1} d^{-1})
CB 858T	Aug 2002	1.5	207	1.1	34
DB 14	Aug 2002	0.9	105	0.51	22
CB 804	Mar 2003	2.0	84	0.49	47

Table 4. Comparison between recent studies employing different methodologies to estimate viral production (VP) and related parameters in a variety of habitats. VTT: viral turnover time; VMM: virally mediated bacterial mortality; BP: bacterial production; TFD: tangential flow diafiltration; FLV: fluorescently labeled virus

VP method	Location	VP (viruses $\times 10^9$ l^{-1} d^{-1})	VTT (d^{-1})	VMM (% of BP lost to viral lysis)	VMM (% of bacterial community lost to viral lysis d^{-1})	C released (µg l^{-1} d^{-1})	Source
TFD dilution	Chesapeake & Delaware Bays	47–100		84–207	14–93	22–47	Present study
Vacuum dilution	Coastal British Columbia	53–430	5.1–28		18–137	6.7–62	Wilhelm et al. (2002)
	Peruvian upwelling	97–380			5–36		Poorvin et al. (2004)
Modified vacuum dilution	North Sea	0.07–12					Winter et al. (2004a)
	Coastal California—nearshore	2.8–28	0.48–1.5		24–50	1.1–12	Noble & Fuhrman (2000)
FLV tracer	Coastal California—offshore	3.7	0.45		26		Noble & Fuhrman (2000)
Viral decay rates	Gulf of Mexico			6–52	2–39	0.12–5.2	Wilhelm et al. (1998)
	Antarctic Ocean			50–400	10–110		Guixa-Boixereu et al. (2002)
	Aarhus Bay, Denmark	168			72	111	Bratbak et al. (1992)
	Lake Kuhworte, Danube River	10–31		15–30			Mathias et al. (1995)
^{3}H-thymidine incorporation	Coastal California	25–43	1.7–2.2		29–67		Fuhrman & Noble (1995)
	Coastal California—nearshore	12–230	0.25–1.8		1–740		Steward et al. (1992a)
	Coastal California—offshore	0.6–1.4	0.03–0.11		1–32		Steward et al. (1992a)
	Bering and Chukchi Seas	0.51–4.2	0.07–0.83	2–12			Steward et al. (1996)
	Masan Bay, Korea	1.3–34	0.007–0.56	2–20			Choi et al. (2003)
Frequency of visibly infected cells	East Sea	0.04–1.4	0.04–1.1	10–19			Hwang & Cho (2002)
	Bering and Chukchi Seas	0.02–14	0.1–2.6	2–36			Steward et al. (1996)
	North Water	0.11–1.3	0.13–0.25	6–28			Middelboe et al. (2002)
	Adriatic Sea—mesotrophic	0.00003–0.0029	0.028–0.26		3.5–24		Weinbauer et al. (1993)
	Adriatic Sea—eutrophic	0.0017–0.017	0.078–0.5		7.0–64		Weinbauer et al. (1993)
	Mediterranean Sea	0.0001–0.09			3–15		Weinbauer et al. (2003a)
	Baltic Sea	0.03–1.8			12–51		Weinbauer et al. (2003a)
	Solar salterns, Spain	10–90			10–20		Guixa-Boixereu et al. (1996)
	Lake Plußsee, Germany	1.5–19	7.7–98			0.36–8.1	Weinbauer & Hofle (1998)
	Lake Pavin, France	0.93–3.1			14–19		Bettarel et al. (2004)
	Lake Aydat, France	6.3–16			7–11		Bettarel et al. (2004)

bacterioplankton mortality is critical for constraining the flux of C through the viral-shunt and relies on 2 key estimates: average burst size and VP rate. Reported calculations of VMM have used either an assumed average burst size or direct observation of the average number of viruses within infected cells through electron microscopy (Weinbauer et al. 1993, Winter et al. 2004a). An assumed burst size of 50 viruses per burst has been used in several studies (Heldal & Bratbak 1991, Steward et al. 1996, Tuomi & Kuuppo 1999, Noble & Fuhrman 2000, Wilhelm et al. 2002). Based on the large variability inherent to burst size estimates from electron microscopy (Steward et al. 1996, Noble & Steward 2001), and the larger burst sizes observed in locations with higher bacterial activity (Weinbauer & Peduzzi 1995, Bettarel et al. 2004, Winter et al. 2004a), such as the Chesapeake and Delaware Bays, we felt the choice of an assumed burst size of 50 was also valid and appropriate for the estuarine waters studied here.

Dilution-based VP estimates for Chesapeake and Delaware Bays varied from $-2.2 \pm 1.2 \times 10^{10}$ to $10 \pm 3.5 \times 10^{10}$ viruses l^{-1} d^{-1} using TFD and from $-3.9 \pm 1.6 \times 10^{10}$ to $25 \pm 10 \times 10^{10}$ viruses l^{-1} d^{-1} using vacuum diafiltration. These observations are within the range of previously reported VP estimates for near-shore coastal waters, e.g. between 2.8×10^9 and 43×10^{10} viruses l^{-1} d^{-1} (Table 4; Steward et al. 1992b, Guixa-Boixereu et al. 1996, Noble & Fuhrman 2000, Wilhelm et al. 2002, Poorvin et al. 2004), indicating that both methods produce reasonable estimates of VP. Most importantly, TFD demonstrated consistently higher bacterial recovery than vacuum diafiltration. Assuming equal viability of bacteria recovered by each procedure, TFD-based VP estimates are more accurate, because a larger proportion of the natural bacterial community is present during the experiment and a smaller conversion factor is needed to account for bacterial loss.

The TFD method, like other VP methods, relies on 3 basic assumptions (Table 1): (1) all viruses produced during the incubation period are from the lysis of bacteria already infected at the start of the

experiment; (2) no new infections occur during the incubation time period; and (3) filtration does not affect community diversity or composition. The reduction of free viruses in water samples from 16 to 46% of ambient levels through TFD and the short incubation time greatly reduces the possibility of new viral infection during dilution experiments (Wilhelm et al. 2002). Steady increases in viral abundance throughout experimental incubations were not always observed in this study. Instead, 2 or more increases in viral abundance followed by decreases were observed in this study and in subsequent experiments using TFD. Explanations for this behavior of viral abundance within experimental incubations include the release of viruses with different latent periods during the experiments or absorption, new infection, and cell death from viruses released in early lysis events. The complication of new infection–lysis events within the experimental incubation can be overcome by calculating VP rates using varying incubation times (Noble & Fuhrman 2000). With this approach we found that significant changes in VP estimates occurred once when the incubation time was cut from 12 h to 6 or 9 h. A shortened incubation time with more frequent sampling could resolve this dilemma of VP from new infection events.

The dilution approaches used in this study allow the estimation of net VP. Thus, virulent and temperate VP are indistinguishable from one another with this method. However, both contribute to VP in natural environments, and, assuming the incidence of lysogenic induction is not altered by diafiltration, it is accurately included in the dilution approach's estimation of net VP. Implementation of the TFD protocol is technically easier and less time-consuming than vacuum diafiltration for the highly productive and turbid waters of the Chesapeake and Delaware Bays. Despite attempts to alleviate the problem, filter clogging always resulted in greater manipulation of samples during vacuum diafiltration than during TFD. Excessive manipulation increases the risk of sample contamination, loss of bacterial viability from additional exposure to air and filtration hardware, and possibly induction of temperate bacteriophages. Lengthened exposure to temperatures and light levels different from ambient conditions may also induce changes in the bacterial community that could affect subsequent VP estimates. Therefore, TFD with less sample manipulation is again the preferred method.

Both diafiltration methods showed lower actual viral dilution and bacterial recovery efficiency than theoretically possible. However, subsequent VP estimates were independent of bacterial recovery or viral dilution efficiency at the start of an experiment (data not shown). Thus, the percentage of bacterial recovery or viral dilution did not affect the VP estimation. Interestingly, in only 1 of the experiments did VP estimates based on the different protocols agree on whether the viral population was declining or increasing (Table 2).

Several possible explanations exist for the observed discrepancies in VP trends between the 2 methodologies. First, the difference in starting time of the experiments may have captured diurnal changes in VP. However, the 1 to 1.5 h delay in starting incubations of vacuum-diafiltered water was probably not long enough to introduce diurnal changes in the balance between VP and decay (Winter et al. 2004a). A difference in the composition, activity, or viability of the bacterial community remaining after each diafiltration procedure may also have caused the observed differences. For example, discrepancies in VP estimates between the 2 methods could result from differences in the number or types of infected bacteria lost during diafiltration. Vacuum diafiltration, with its lower bacterial recovery efficiency and longer handling times, may be more susceptible to these effects than TFD. Finally, inconsistent filter and slide preparation within an experiment could lead to erroneous VLP enumeration (Noble & Fuhrman 1998, Chen et al. 2001). This would affect VP estimates and could cause the observed discrepancies between the diafiltration methods. Enumeration of replicate filters from a given sample would resolve this problem.

Although negative VP estimates occur at the stations with the highest viral abundance, no significant relationship was found between VP and ambient or t_0 viral or bacterial abundance or VBR for either method or the combined data set. Therefore, negative VP estimates were not due to changing viral or host abundances or VBRs in ambient water samples or incubations. The lack of correlation between VP estimates and viral abundance, bacterial abundance, or VBR agrees with previous findings (Choi et al. 2003). However, this disagrees with other observed correlations between VP and bacterial abundance (Steward et al. 1992b, Weinbauer et al. 2003b), indicating that other environmental factors, such as temperature, light, or ambient nutrient levels, may influence VP. The negative VP estimates observed in this study may be due to variations in abiotic factors, such as DOM and nutrient levels, between sample locations or between sample filtration processes. The vacuum method is more likely to result in artificially altered DOM concentrations in incubations because of the increased potential for cell lysis when using impact filters during diafiltration.

Experimentation with the filter size, flow rate, and flushing volume used for TFD showed that the bacterial recovery and viral dilution efficiencies of this

method varied little over the range of conditions tested. Ultimately, the 0.22 µm filter and larger, 4-fold flushing volume were chosen as optimal conditions for presumably more representative recovery of the natural bacterial community. The medium flow rate (40 ml min^{-1}) was selected as the most advantageous for its faster processing time and higher bacterial recovery.

Since these experiments were performed, the storage of seawater samples at 4°C with the addition of an aldehyde preservative has been shown to cause significant declines in viral abundance, as assessed via epifluorescence microscopy (Brussaard 2004, Wen et al. 2004). In our study, sample degradation in the TFD and vacuum experiments occurred. Some t_1 (3 h) and t_3 (9 h) time point samples were enumerated approximately 5 mo after the other time points. The t_0 time points for all VP experiments involved were recounted on the same day as the t_1 and t_3 samples. Average loss of VLPs per day due to sample degradation for each experiment was calculated as the difference in the t_0 counts divided by the time in days that elapsed between assessment dates. Subsequently, average loss rate in VLPs per day was used to correct VLP counts in the t_1 and t_3 water samples. Removal of the t_1 and t_3 counts from these experiments marginally increased VP estimates for 3 experiments and made the VP rate of the vacuum-based experiment at Stn DB 14 more negative. None of the changes in VP estimates due to the exclusion of these data points significantly altered VP estimates ($p > 0.05$).

Corrections to all viral abundances for the VP experiments were also made using the decay models presented in Wen et al. (2004). VP estimates were then recalculated based on revised viral abundances and compared to original VP estimates. The VP estimates for 5 experiments increased after re-calculation, while 3 decreased. However, for 7 out of 8 experiments no statistically significant differences were found between the original and re-calculated VP estimates ($p > 0.05$). For all experiments, results of comparisons of VP estimates between TFD and vacuum diafiltration methods remained unchanged ($p > 0.05$). Thus, the VP values presented here have not been corrected for viral degradation except at the time points mentioned above. Both the TFD and vacuum diafiltration samples for each of these experiments were subjected to the same preservation conditions and demonstrated similar degradation rates. Therefore, assessments of differences in VP estimates were not affected by storage conditions. Determinations of bacterial recovery or viral loss with each method were not affected by storage conditions, as these statistics were based on the ambient water and t_0 counts made shortly after sample collection.

Bacterioplankton mortality and biogeochemical impacts of viral lysis

VP estimates from this study and other coastal environments (Bratbak et al. 1992, Steward et al. 1992b, Fuhrman & Noble 1995, Guixa-Boixereu et al. 1996, Noble & Fuhrman 2000, Wilhelm et al. 2002, Poorvin et al. 2004) are higher than VP rates in more oligotrophic environments and freshwater lakes (Table 4; Weinbauer et al. 1993, 2003a, Mathias et al. 1995, Steward et al. 1996, Noble & Fuhrman 2000, Hwang & Cho 2002, Middelboe et al. 2002, Bettarel et al. 2004, Winter et al. 2004a). These observed differences in production rates may be due to the variety of methods used. However, it appears that none of the methods consistently produces higher or lower estimates. Further measurement of VP and related variables at a variety of locations using consistent methodology and sample preservation is needed to identify potential correlation between the nutrient and DOM status of an environment and VP.

Compared to the findings of this study, similar proportions of bacterial standing stock were lost to viral infection off the coast of British Columbia (Wilhelm et al. 2002), in Tampa Bay (Jiang & Paul 1994), and in Antarctic waters (Guixa-Boixereu et al. 2002). Levels of VMM found in this study were high compared to a variety of other environments (Table 4; Weinbauer et al. 1993, Guixa-Boixereu et al. 1996, Steward et al. 1996, Wilhelm et al. 1998, Noble & Fuhrman 2000, Hwang & Cho 2002, Middelboe et al. 2002, Choi et al. 2003, Bettarel et al. 2004). Despite falling within the range of previously reported values, observations of >100% of BP and standing stock lost to viral lysis appear unrealistic for the Chesapeake and Delaware Bay systems, which typically demonstrate high rates of BP. High VMM, in excess of bacterial standing stock, has been observed in other studies (Weinbauer et al. 1995, 2002, Guixa-Boixereu et al. 2002, Wilhelm et al. 2002), but low rates of VMM have also been reported for eutrophic lakes (Bettarel et al. 2004). Thus, VMM may also vary with location and ambient conditions. VP estimates used to calculate VMM represent instantaneous snapshots of community dynamics that, when averaged with other observations taken over a longer time period, might yield numbers more closely correlated to BP. Furthermore, VMM estimates in this study are based on an assumed viral burst size of 50 viruses released per cell lysed. Doubling the burst size used in the calculations to 100 would decrease all but 1 of the percentages of BP consumed to values <100% (Table 3). Future work is needed to determine an accurate average bacteriophage burst size for the Chesapeake Bay.

Dissolved organic carbon (DOC) release estimates from the Chesapeake and Delaware Bays exceed or

fall within those reported in previous VP studies from a range of marine ecosystems (Table 4). Bratbak et al. (1992) estimated a higher rate of DOC release than in this study; however, these authors concede that such high DOC flux was an overestimation. Our increased estimates are probably due to the high level of VMM observed in this study, which is used to calculate DOC release. Paradoxically, the March experiment at Stn CB 804 showed the highest levels of VMM and C release, but the lowest level of BP lost to lysis (Table 3). Together, these results indicate that the impacts of viruses on bacterial populations and BP may vary with changes in environmental conditions occurring over annual cycles, geographic location, and depth. As VP varies over space and time, viral lysis may have varying impacts on the quantity of C and other cell constituents added to the DOM pool.

In conclusion, the TFD method yields a marked improvement in bacterial recovery efficiency over the previously described vacuum diafiltration method for estimation of VP by dilution. As a result, the magnitude of a key conversion factor, the inverse of bacterial recovery, is reduced and the resultant VP estimates are closer to actual production values. Compared to the vacuum method, the shorter processing time and reduced sample manipulation in the TFD method decrease the possibility of the diafiltration process altering VP estimates. The large amounts of C released and BP lost to viral lysis and the high rates of VP observed in the Chesapeake and Delaware Bays in this study offer tantalizing insights into the influence of viruses on bacterioplankton and DOM production in estuaries. Further investigations are necessary to determine if VP and associated parameters of burst size, VMM, and percent BP lost to lysis fluctuate seasonally or vary spatially with changing DOM concentrations and abiotic factors.

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