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An Inexpensive, Accurate, and Precise Wet-Mount Method for Enumerating Aquatic Viruses

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Viruses affect biogeochemical cycling, microbial mortality, gene flow, and metabolic functions in diverse environments through infection and lysis of microorganisms. Fundamental to quantitatively investigating these roles is the determination of viral abundance in both field and laboratory samples. One current, widely used method to accomplish this with aquatic samples is the "filter mount" method, in which samples are filtered onto costly 0.02-μm-pore-size ceramic filters for enumeration of viruses by epifluorescence microscopy. Here we describe a cost-effective (ca. 500-fold-lower materials cost) alternative virus enumeration method in which fluorescently stained samples are wet mounted directly onto slides, after optional chemical flocculation of viruses in samples with viral concentrations of $<5 \times 10^7$ viruses ml^{-1}. The concentration of viruses in the sample is then determined from the ratio of viruses to a known concentration of added microsphere beads via epifluorescence microscopy. Virus concentrations obtained by using this wet-mount method, with and without chemical flocculation, were significantly correlated with, and had precision equivalent to, those obtained by the filter mount method across concentrations ranging from 2.17×10^6 to 1.37×10^8 viruses ml^{-1} when tested by using cultivated viral isolates and natural samples from marine and freshwater environments. In summary, the wet-mount method is significantly less expensive than the filter mount method and is appropriate for rapid, precise, and accurate enumeration of aquatic viruses over a wide range of viral concentrations ($\geq 1 \times 10^6$ viruses ml^{-1}) encountered in field and laboratory samples.

Viruses are the most abundant biological entities in aquatic systems, and their infection of microorganisms has substantial influences on microbial ecology, biogeochemical cycling, and gene transfer in aquatic environments (reviewed in references 1 and 2). An accurate method to quantify aquatic viruses is thus essential for use in field and laboratory studies to investigate the roles of viruses in aquatic environments. Enumeration of viruses in aquatic samples has previously been accomplished by using transmission electron microscopy (TEM) (3), epifluorescence microscopy (reviewed in reference 4), and flow cytometry (reviewed in reference 5).

While each of the above-mentioned methods requires the use of relatively expensive laboratory equipment, the per-sample cost of the widely used epifluorescence microscopy method has recently increased dramatically. This method involves filtering the sample onto 0.02-μm-pore-size ceramic filters, staining viruses on the filters by using one of several available nucleic acid dyes, mounting the filter onto a slide, and visually enumerating the deposited viruses by epifluorescence microscopy (reviewed in reference 4). However, the filters used for this "filter mount" method have risen in cost to ca. $10 each in the United States (with increased costs in some other countries), creating a significant financial burden for researchers pursuing studies of environmental viruses. To address this, we have developed a new, less costly "wet-mount" epifluorescence microscopy method to enumerate aquatic viruses, in which fluorescently stained samples are wet mounted directly onto a slide, with quantification of viral concentrations based on the relative abundance of viruses and silica beads in the sample.

MATERIALS AND METHODS

Comparison of the wet-mount and filter mount methods for virus enumeration. The wet-mount method was tested by comparing viral concentrations obtained with the wet-mount and filter mount methods in triplicate samples collected from a variety of marine and freshwater environments as well as in cultivated viral lysates (described in Table S1 in the supplemental material). Briefly, field samples included those from a 6-depth profile (5 to 300 m) from the Eastern Tropical North Pacific Ocean (using whole seawater samples); 8 surface ocean locations throughout the Pacific, Atlantic, and Southern Oceans chosen for their range of chlorophyll concentrations (collected on the *Tara* Oceans Expedition [6], using 0.2-μm-filtered samples); and a freshwater location in South Carolina. All field samples were preserved with glutaraldehyde (0.5% final concentration), flash frozen in liquid nitrogen, and stored at −80°C until analysis. Lysate samples included the *Synechococcus* virus S-WHM1 (7), two dilutions of the *Synechococcus* virus S-SM1 (8), and the *Prochlorococcus* virus P-HM2 (9). Triplicate independent 1-ml samples were processed by using each of the filter mount and wet-mount methods, as described below. Statistical comparison of viral concentrations obtained by using each method was then performed by using two-tailed *t* tests and Pearson correlation (SigmaPlot v12.5; Systat Software Inc.).

Filter mount sample preparation and analysis. The filter mount method was performed according to methods described previously by

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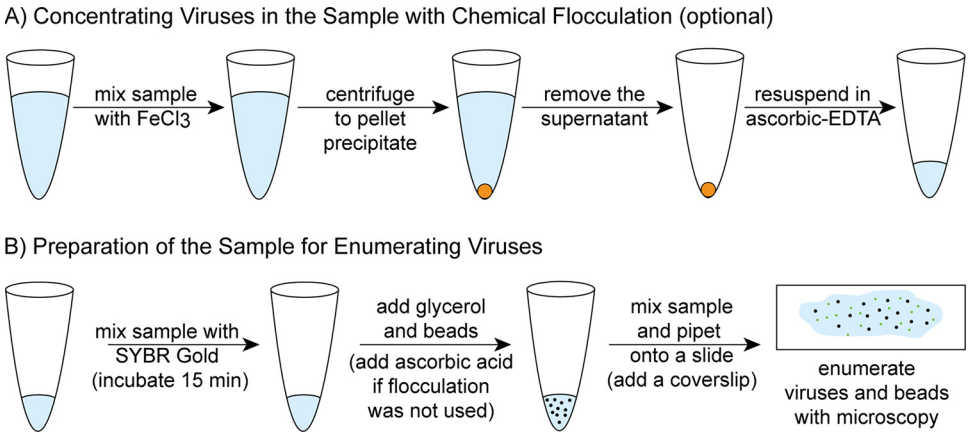


FIG 1 Overview of the wet-mount method for enumeration of aquatic viruses.

Suttle and Fuhrman (4). Briefly, samples were filtered onto 0.02-μm-pore-size ceramic filters (Whatman Anodisc), stained with SYBR gold (Invitrogen) for 15 min, and mounted onto a glass slide with an antifade solution (Acros Organics). Viruses were viewed under blue excitation using a Nikon TS100 inversion microscope or a Zeiss Axio Imager epifluorescence microscope at a ×1,000 magnification. The viral concentration was determined by using the average number of fluorescent viruses within a given area of the microscope reticle in 20 fields of view and the total volume of sample filtered through a measured area on the filter.

Wet-mount sample preparation and analysis. The wet-mount method for enumerating viruses involves an optional virus concentration step followed by combining a known volume of stained sample with a known volume and concentration of silica beads for relative enumeration of viruses and beads to calculate the virus concentration in the sample (Fig. 1). The reagents for assessing viral concentrations using the wet-mount method are fully described in Table 1, and the protocol is as follows.

1. If the virus concentration is expected to be $<5 \times 10^7$ viruses ml^{-1}, concentrate the viruses by chemical flocculation, as follows. Add 1 μl iron chloride solution to a 1-ml sample in a microcentrifuge tube, mix the sample by inversion, and centrifuge the mixture for 20 min at ~14,000 × *g*. Remove the supernatant and resuspend the pellet in 10 μl ascorbate-EDTA buffer. (If a lower concentration factor is desired, the pellet may be resuspended in a larger volume of ascorbate-EDTA buffer. In this case, increase the amounts of SYBR gold, glycerol, and silica beads accordingly in subsequent steps.)
2. Add 2 μl SYBR gold working stock to the concentrated sample, vortex the mixture, and incubate the mixture for 15 min in the dark. If the sample was not concentrated with chemical flocculation (i.e., the concentration is expected to exceed 5×10^7 viruses ml^{-1}), mix 10 μl unconcentrated sample with 2 μl SYBR gold working stock in a microcentrifuge tube.
3. Add 5 μl glycerol, vortex the mixture, and add 2 μl working bead solution (thoroughly vortex the working bead solution before addition to the sample to ensure accurate pipetting of the beads). If the sample was not concentrated with chemical flocculation, add 1 μl ascorbic acid antifade solution as well.
4. Mix the prepared sample thoroughly by pipetting up and down and then immediately pipette 10 μl onto a glass microscope slide and place a coverslip over the sample (both the glass slide and coverslip should be cleaned with isopropanol). Avoid trapping air under the coverslip.
5. Using an epifluorescence microscope, count the number of viruses in a given area within the microscope reticle under blue (~495-nm) excitation at a ×1,000 magnification. Within the same field of view, count the beads under white light. Continue counting fields of view until at least 100 each of viruses and beads have been counted.
6. The virus concentration is calculated as $c_{virus} = n_{virus}/n_{beads} \times v_{beads}/v_{sample} \times c_{beads}$, where c_{virus} is the virus concentration in the sample (viruses ml^{-1}), n_{virus} is the total number of viruses counted, n_{beads} is the total number of beads counted, v_{beads} is the volume of working bead solution added (μl), v_{sample} is the sample volume

TABLE 1 Reagent preparation for the wet-mount virus enumeration protocol

Reagent	Prepn method
SYBR gold working stock	Dilute SYBR gold (Invitrogen) (10,000× stock) into PBS to prepare a 1,000× solution
Ascorbic acid antifade solution	Dissolve ascorbic acid into PBS to create a 10% (wt/vol) solution[a]
Working bead solution	Thoroughly vortex the stock bead solution (2.34-μm silica spheres) (catalog no. SS04N/4186; Bangs Laboratories), dilute it 10-fold into PBS to obtain a concn of ~10^8 beads ml^{-1}, and store it at 4°C
Iron chloride solution	Dissolve $FeCl_3 \cdot 6H_2O$ into ultrapure water to form a solution of 10 g Fe liter^{-1}; the solution has expired if a cloudy precipitate forms[b]
Ascorbate-EDTA buffer	Combine equal parts of 0.4 M Mg_2EDTA and 0.8 M ascorbic acid and adjust with 10 N NaOH to reach a pH of 6–7; prepare fresh within 48 h of use[c]

[a] See reference 11.
[b] See reference 10.
[c] An alternative ascorbate-EDTA buffer can be made with $MgCl_2$ and Na_2EDTA if Mg_2EDTA is unavailable (10).

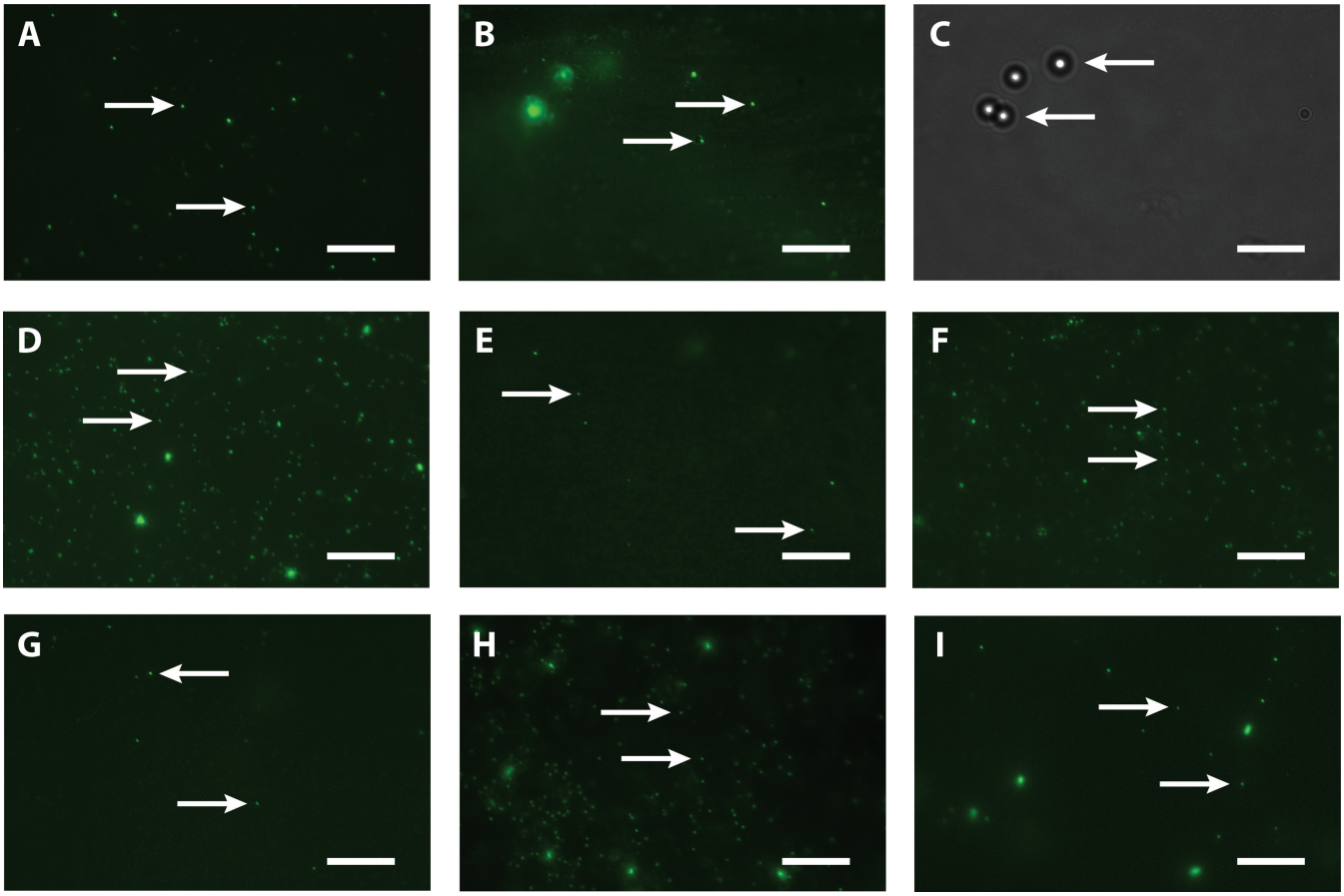
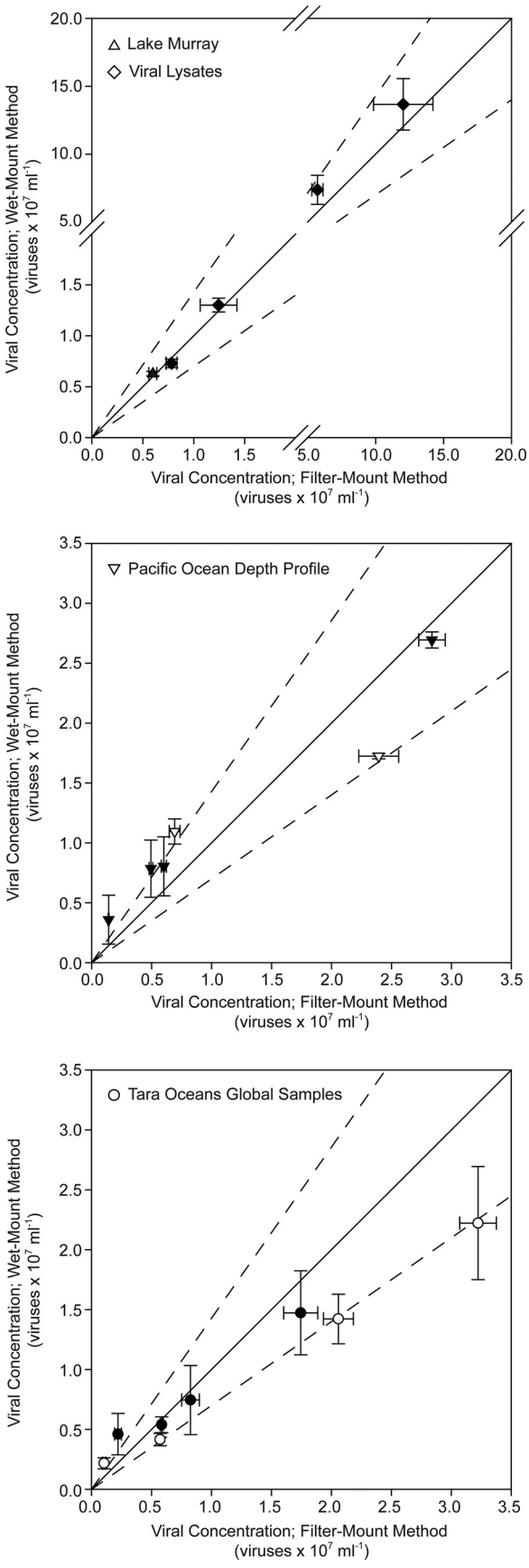


FIG 2 Images of samples prepared by use of the filter mount and wet-mount virus enumeration methods. Shown are epifluorescence images of purified S-SM1 lysate obtained by using the filter mount (A) and wet-mount (B) methods, seawater from 30 m in the Pacific Ocean depth profile by using the filter mount (D) and wet-mount (E) methods, freshwater from Lake Murray by using the filter mount (F) and wet-mount (G) methods, and unpurified S-SM1 lysate with *Synechococcus* cells by using the filter mount (H) and wet-mount (I) methods. These epifluorescence images include arrows pointing to two of the viruses in each image. Under white light (C), beads are visible in the same field of view as for the wet-mount sample (B), with arrows pointing to two of the beads in the image. Bar, 10 μm.

(μl) (the volume prior to concentration if chemical flocculation is used), and c_{beads} is the bead concentration in the working bead solution (beads ml^{-1}).

A full, illustrated protocol describing this method is also available online for convenience (http://eebweb.arizona.edu/faculty/mbsulli/protocols/). For analysis of samples with $<5 \times 10^7$ viruses ml^{-1}, viruses must be concentrated with chemical flocculation by using a method adapted from methods described previously by John et al. (10), to obtain a sufficient concentration of viruses for analysis (Fig. 1A). Samples in this study with viral concentrations below that threshold were first concentrated 100-fold with this chemical flocculation method (step 1 in the list of procedures above) before being stained with SYBR gold for 15 min and then combined with silica beads and glycerol (added to create a more viscous solution and reduce clumping of beads) (steps 2 and 3 in the list of procedures above) (Fig. 1B). The silica bead size (2.34 μm) was selected due to the ease of visually counting the beads under white light. Due to the relatively large size of the beads (compared to the size of viruses), the bead solution must be vortexed thoroughly prior to the addition of beads to the sample to ensure the addition of an accurate concentration of beads. These concentrated samples did not require the addition of an antifade solution since they were resuspended in a buffer containing ascorbic acid, which reduces fading of the fluorescent signal (11). Samples were then pipetted directly onto an isopropanol-cleaned glass microscope slide and covered with a cleaned glass coverslip (step 4 in the list of procedures above). Viruses and beads were enumerated in multiple fields of view on a Nikon TS100 inversion microscope or a Zeiss Axio Imager epifluorescence microscope at a ×1,000 magnification until at least 100 each of viruses and beads were enumerated to calculate the virus concentration (steps 5 and 6 in the list of procedures above). For each field of view, the total number of fluorescent viruses was determined under blue excitation, after which the total number of beads within the same field of view was determined under white light (Fig. 2).

For analysis of samples with $>5 \times 10^7$ viruses ml^{-1}, chemical flocculation of viruses prior to wet-mount sample preparation was not necessary to obtain a sufficient concentration of viruses for enumeration. These samples (S-SM1 lysates) were prepared by staining 10 μl of sample with SYBR gold, followed by the addition of an ascorbic acid solution (to act as an antifade solution), glycerol, and silica beads (steps 2 and 3 in the list of procedures above) (Fig. 1B). These samples were then wet mounted onto slides and enumerated exactly as described above for the samples that had been concentrated with chemical flocculation. To compare the wet-mount method with the filter mount method for these samples with high viral concentrations, they were diluted 10-fold in phosphate-buffered saline (PBS) prior to filtering 1 ml of sample for the filter mount method, as the undiluted sample would have resulted in an excessive viral density on the filter, preventing analysis. We also note that while *p*-phenylenedi-



amine is a popular antifade chemical (4), it reacted with glutaraldehyde to form a precipitate in these wet-mount samples and thus should not be used in the wet-mount method with fixed samples.

The minimum number of beads and viruses enumerated per sample is justified as follows. Counting statistics (also known as shot noise) dictates that the error in the quantity of viruses or beads counted is given by $1/\sqrt{n}$ (12), where n is the number of objects enumerated, and therefore, the total error in viral abundance is $\sigma_{total} = \sqrt{1/n_{virus} + 1/n_{beads}}$, where n_{virus} and n_{beads} are the total numbers of viruses and beads counted, respectively. When at least 100 each of viruses and beads are enumerated, the maximum error is 14%.

Storage conditions for samples prepared by using the wet-mount method. Storage conditions were assessed by using two different lysates concentrated 100× using the flocculation method described above. To assess storage after samples were mounted onto slides, triplicate samples (S-SM1 lysate) were prepared and analyzed by using the full protocol described above, with slides being stored vertically at −20°C immediately after enumeration, and viruses and beads were recounted 7 days later. Additional triplicate samples (S-WHM1 lysates) were prepared through step 3 in the list of procedures above, with 10 μl of the prepared sample being analyzed immediately and the remaining sample (~10 μl) being stored in a microcentrifuge tube at −20°C until analysis 7 days later.

RESULTS AND DISCUSSION

The wet-mount method resulted in fluorescently stained viruses with an intensity similar to those of the filter-mount method (Fig. 2). While there was typically a lower density of viruses in the images derived from samples prepared by using the wet-mount method, this is favorable because viruses are enumerated in larger fields of view with the wet-mount than with the filter mount method. However, images depicting a greater density of viruses and cells can be obtained with more concentrated samples (Fig. 2I). Viral concentrations obtained by using the wet-mount method were strongly correlated (Pearson correlation coefficient of 0.986; $P < 0.001$) with those obtained by using the filter mount method for all sample types tested, including viral lysates and samples from a variety of oceanic and freshwater regions (Fig. 3). There was no significant difference in viral concentrations obtained by the use of these methods for the majority of samples (13 of 19 samples; two-tailed t tests) (see Table S1 in the supplemental material). For the remaining samples with significantly different viral concentrations, neither method consistently resulted in higher or lower viral concentrations, nor were these differences restricted to a specific range of viral concentrations (i.e., high versus low) or sample type (i.e., freshwater versus marine sample, natural sample versus lysate, or low versus high chlorophyll concentration), indicating stochastic variability inherent to analyses of samples (Fig. 3; see also Table S1 in the supplemental material). Furthermore, we consider the low magnitude of the differences in

FIG 3 Viral concentrations in natural samples and lysates obtained by using the filter mount and wet-mount enumeration methods. Error bars are standard deviations of the means of data from triplicate samples. Closed symbols represent samples in which there was no significant difference in virus concentrations obtained by using the filter mount and wet-mount methods ($P > 0.05$ by two-tailed t tests), while open symbols represent samples in which there was a significant difference (see Table S1 in the supplemental material). Average viral concentrations for all samples obtained by using each method were strongly and positively correlated (Pearson correlation coefficient, 0.986; $P < 0.001$). The solid lines represent a 1:1 relationship, and dashed lines represent an interval of 70% agreement between methods around the 1:1 relationship to facilitate visual comparison of results.

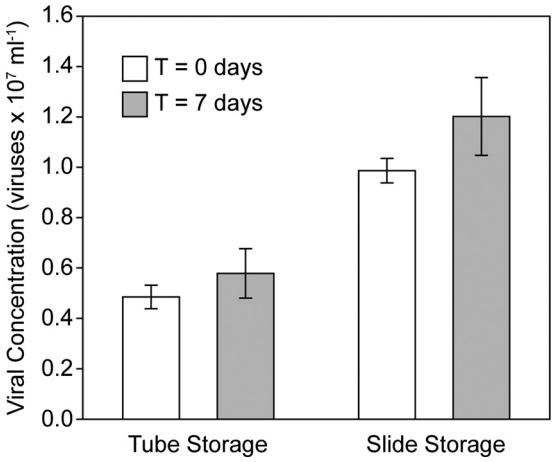


FIG 4 Storage of samples prepared by using the wet-mount method. Concentrations of viruses in triplicate samples (S-SM1 lysate for tube storage and S-WHM1 lysate for slide storage) prepared according to the wet-mount protocol and stored at −20°C in microcentrifuge tubes (tube storage) or wet mounted onto slides (slide storage) are shown. Viruses were enumerated immediately after preparation (time [T] = 0 days) and after 7 days of storage (T = 7 days). Error bars are standard deviations of the means for triplicate samples.

average viral concentrations for the few significantly different samples to be acceptable for studies of aquatic viruses.

It is important to note that there is no available standard used in aquatic virus enumeration methods. Previous studies comparing aquatic viral concentrations determined by using different methods (i.e., TEM, filter mount, and flow cytometry) have shown discrepancies between methods, with one method usually resulting in consistently higher viral concentrations (13–17). However, we observed no such consistent differences in our comparison of the wet-mount and filter mount methods. Furthermore, the comparison in this study showed that most of the samples had at least 70% agreement between virus concentrations obtained by use of the wet-mount method and those obtained by use of the filter mount method (Fig. 3), which is similar to data from previously reported comparisons of methods used to enumerate viruses (16, 17). The wet-mount method also had high precision; standard deviations of the means for triplicate samples were 2 to 18% (average, 7% ± 4%) of the mean virus concentration and were not significantly different from those obtained by using the filter mount method (P = 0.531 by two-tailed t test). Thus, the wet-mount method and the filter mount method can be used with equal confidence.

When enumerating viruses, it is sometimes advantageous to store prepared samples for enumeration at a later date. For example, samples prepared with the filter mount method can be stored at −20°C for at least 4 months, with no significant change in viral concentrations (4). For wet-mount samples, we tested storage of prepared samples both in microcentrifuge tubes and on slides at −20°C (Fig. 4). While the calculated virus concentration was higher after storage of the prepared samples under both storage conditions, these differences were not significant (P = 0.210 and P = 0.083, respectively, by two-tailed t tests). Thus, samples prepared by use of the wet-mount method can be stored frozen either before or after mounting the sample onto slides, with no significant change in the calculated virus concentration.

The wet-mount method had one major drawback compared to the filter mount method, which was the inability to efficiently enumerate samples with viral concentrations of $<1 \times 10^6$ viruses ml^{-1}. Attempted analysis of samples with lower viral concentrations (i.e., samples below 300 m in the Pacific Ocean depth profile) using the wet-mount method resulted in ≤1 virus per field of view, even after maximum concentration (100-fold) with chemical flocculation. Thus, the wet-mount method is not recommended for samples with viral concentrations of $<1 \times 10^6$ viruses ml^{-1} because the low density of viruses on the slide significantly extends the time for analysis of a sample. Although this limitation prevented analysis of the deep-sea samples (>300 m) in the Pacific Ocean depth profile in this study, many deep-sea samples have viral concentrations above this limit (e.g., see reference 18), and thus, the wet-mount method should be useful for a wide range of environmental samples.

The available methods to enumerate aquatic viruses each have benefits and limitations that are worth considering when planning research projects. For example, TEM-based analyses of aquatic samples can generate information about the morphological characteristics of viruses (e.g., see reference 21) in addition to viral abundance (e.g., see reference 3) but can potentially underestimate the number of viruses because they may be obscured by debris in the sample (16). Fluorescence-based methods for viral enumeration (i.e., epifluorescence microscopy and flow cytometry) are significantly faster than TEM but can potentially falsely include gene transfer agents or cell debris as viruses (reviewed in reference 19) while excluding single-stranded DNA (ssDNA) viruses that have very faint fluorescence (20). One additional advantage of fluorescence-based methods is the ability to enumerate both viruses and bacteria (if present) by using the same prepared sample (e.g., see reference 16). However, the wet-mount method presented here has not yet been evaluated for accuracy in counting of bacterial cells. Among the available epifluorescence-based methods, the filter mount method also provides an opportunity to obtain images with a high density of viruses and cells, while the flow cytometry method does not. The viral density in images obtained by use of the wet-mount method is generally much lower than that for filter mount samples, although the density of viruses and cells increases when more concentrated samples are used. While each of these variables is important when evaluating potential virus enumeration methods for a given project, we offer the wet-mount method as a cost-effective alternative to the widely used filter mount epifluorescence method.

A significant advantage of the wet-mount method over the filter mount method is the lack of a requirement for costly 0.02-μm-pore-size ceramic filters. Currently, these filters are available from only one supplier and are expensive (~$10 each). Instead, the wet-mount method uses microsphere silica beads that can be purchased from several suppliers at a ~500-fold-lower cost ($0.02 for 20 μl of a 10^8-bead ml^{-1} working solution per sample, calculated based on $150 for 15 ml of a 10^9-bead ml^{-1} stock solution). Even after accounting for the cost of other reagents and slides, the per-sample materials cost for the wet-mount method is much lower (~$0.10 per sample). Thus, the wet-mount method is recommended as an equivalently accurate and precise but cheaper alternative for enumerating viruses in field and laboratory samples with viral concentrations of $>1 \times 10^6$ viruses ml^{-1}.

Conclusion. Enumeration of viruses in field and laboratory samples is an important tool for investigating the numerous in-

fluences of viruses in aquatic environmental systems. However, the high cost of enumerating viruses in aquatic samples using the established filter mount epifluorescence microscopy method can be a significant burden in conducting aquatic virus research. In this study, we present a new, less expensive wet-mount method for aquatic virus enumeration that can be used with accuracy and precision equivalent to those of the filter mount method for a variety of environmental and laboratory samples.

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REFERENCES

1. **Suttle CA.** 2005. Viruses in the sea. Nature **437:**356–361. http://dx.doi.org/10.1038/nature04160.
2. **Breitbart M.** 2012. Marine viruses: truth or dare. Annu Rev Mar Sci **4:**425–448. http://dx.doi.org/10.1146/annurev-marine-120709-142805.
3. **Bergh O, Borsheim K, Bratbak G, Heldal M.** 1989. High abundance of viruses found in aquatic environments. Nature **340:**467–468. http://dx.doi.org/10.1038/340467a0.
4. **Suttle CA, Fuhrman J.** 2010. Enumeration of virus particles in aquatic or sediment samples by epifluorescence microscopy, p 145–153. *In* Wilhelm SW, Weinbauer MG, Suttle CA (ed), Manual of aquatic viral ecology. ASLO, Waco, TX.
5. **Brussaard C, Payet J, Winter C, Weinbauer M.** 2010. Quantification of aquatic viruses by flow cytometry, p 102–109. *In* Wilhelm SW, Weinbauer MG, Suttle CA (ed), Manual of aquatic viral ecology. ASLO, Waco, TX.
6. **Karsenti E, Acinas SG, Bork P, Bowler C, De Vargas C, Raes J, Sullivan MB, Arendt D, Benzoni F, Claverie J-M, Follows M, Gorsky G, Hingamp P, Iudicone D, Jaillon O, Kandels-Lewis S, Krzic U, Not F, Ogata H, Pesant S, Reynaud EG, Sardet C, Sieracki ME, Speich S, Velayoudon D, Weissenbach J, Wincker P.** 2011. A holistic approach to marine eco-systems biology. PLoS Biol **9:**e1001177. http://dx.doi.org/10.1371/journal.pbio.1001177.
7. **Millard A, Clokie MRJ, Shub DA, Mann NH.** 2004. Genetic organization of the *psbAD* region in phages infecting marine *Synechococcus* strains. Proc Natl Acad Sci U S A **101:**11007–11012. http://dx.doi.org/10.1073/pnas.0401478101.
8. **Sullivan MB, Waterbury JB, Chisholm SW.** 2003. Cyanophages infecting the oceanic cyanobacterium *Prochlorococcus*. Nature **424:**1047–1051. http://dx.doi.org/10.1038/nature01929.
9. **Sullivan MB, Huang KH, Ignacio-Espinoza JC, Berlin AM, Kelly L, Weigele PR, DeFrancesco AS, Kern SE, Thompson LR, Young S, Yandava C, Fu R, Krastins B, Chase M, Sarracino D, Osburne MS, Henn MR, Chisholm SW.** 2010. Genomic analysis of oceanic cyanobacterial myoviruses compared with T4-like myoviruses from diverse hosts and environments. Environ Microbiol **12:**3035–3056. http://dx.doi.org/10.1111/j.1462-2920.2010.02280.x.
10. **John SG, Mendez CB, Deng L, Poulos B, Kauffman AKM, Kern S, Brum J, Polz MF, Boyle EA, Sullivan MB.** 2011. A simple and efficient method for concentration of ocean viruses by chemical flocculation. Environ Microbiol Rep **3:**195–202. http://dx.doi.org/10.1111/j.1758-2229.2010.00208.x.
11. **Patel A, Noble RT, Steele JA, Schwalbach MS, Hewson I, Fuhrman JA.** 2007. Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR green I. Nat Protoc **2:**269–276. http://dx.doi.org/10.1038/nprot.2007.6.
12. **John SG, Adkins JF.** 2010. Analysis of dissolved iron isotopes in seawater. Mar Chem **119:**65–76. http://dx.doi.org/10.1016/j.marchem.2010.01.001.
13. **Bettarel Y, Sime-Ngando T, Amblard C, Laveran H.** 2000. A comparison of methods for counting viruses in aquatic systems. Appl Environ Microbiol **66:**2283–2289. http://dx.doi.org/10.1128/AEM.66.6.2283-2289.2000.
14. **Hennes KP, Suttle CA.** 1995. Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol Oceanogr **40:**1050–1055. http://dx.doi.org/10.4319/lo.1995.40.6.1050.
15. **Weinbauer MG, Suttle CA.** 1997. Comparison of epifluorescence and transmission electron microscopy for counting viruses in natural marine waters. Aquat Microb Ecol **13:**225–232. http://dx.doi.org/10.3354/ame013225.
16. **Noble R, Fuhrman J.** 1998. Use of SYBR green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat Microb Ecol **14:**113–118. http://dx.doi.org/10.3354/ame014113.
17. **Marie D, Brussaard C, Thyrhaug R, Bratbak G, Vaulot D.** 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl Environ Microbiol **65:**45–52.
18. **Parada V, Sintes E, van Aken HM, Weinbauer MG, Herndl GJ.** 2007. Viral abundance, decay, and diversity in the meso- and bathypelagic waters of the North Atlantic. Appl Environ Microbiol **73:**4429–4438. http://dx.doi.org/10.1128/AEM.00029-07.
19. **Wommack KE, Colwell RR.** 2000. Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev **64:**69–114. http://dx.doi.org/10.1128/MMBR.64.1.69-114.2000.
20. **Holmfeldt K, Odić D, Sullivan MB, Middelboe M, Riemann L.** 2012. Cultivated single-stranded DNA phages that infect marine Bacteroidetes prove difficult to detect with DNA-binding stains. Appl Environ Microbiol **78:**892–894. http://dx.doi.org/10.1128/AEM.06580-11.
21. **Brum JR, Schenck RO, Sullivan MB.** 2013. Global morphological analysis of marine viruses shows minimal regional variation and dominance of non-tailed viruses. ISME J **7:**1738–1751. http://dx.doi.org/10.1038/ismej.2013.67.