# Enumeration of virus particles in aquatic or sediment samples by epifluorescence microscopy

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## Abstract

Microbes and microbial processes are crucial and quantitatively important players in aquatic environments, and viruses as major agents of microbial mortality and nutrient cycling are a key component of aquatic systems. These roles have led to the need to routinely quantify viral abundance as the part of many investigations. Electron microscopy was first used to demonstrate high viral abundances in aquatic samples; by the mid-1990s, the greater accuracy and higher precision of estimates of viral abundance made by epifluorescence microscopy (EFM) were evident. Initially, DAPI (6-diamidino-2-phenylindole) was the stain used to enumerate virus particles in natural samples, but this dye was soon superseded by a new generation of much brighter fluorochromes. This article outlines detailed protocols for enumerating virus particles in aquatic or sediment samples using SYBR Green, SYBR Gold, and Yo-Pro-1. Each of these stains has advantages and disadvantages, but for natural water samples they produce indistinguishable estimates of viral abundance when the appropriate protocols are carefully followed.

Evidence for high abundances of viruses in aquatic systems date back more than 40 years (Anderson et al. 1967; Torrella and Morita 1979); however, it was not until 20 years ago that

more quantitative estimates of abundance were made by transmission electron microscopy (TEM) (Bergh et al. 1989; Børsheim et al. 1990; Proctor and Fuhrman 1990). Methods using TEM are time consuming, requiring specialized training and access to expensive equipment with limited availability. Moreover, TEM-based methods are subject to a large number of artifacts that can lead to relatively inaccurate and low-precision estimates of viral abundance (Hennes and Suttle 1995; Weinbauer and Suttle 1997). As a consequence, efforts were made to develop accurate, rapid, and affordable techniques of viral enumeration based on epifluorescence microscopy (EFM). Today, EFM is routinely used to estimate virus abundances in aquatic samples and sediments.

It must be emphasized that estimates of viral abundance by TEM or EFM are operationally defined by the technique used, that is, virus-like particles (TEM) or small pinpricks of light (EFM). The techniques provide no information on infectivity or the biological nature of the particles. Although the large majority of observed particles are likely infectious viruses, they may also include defective viruses, gene transfer agents, viruses damaged by solar radiation, or other unknown particles. As well, there are RNA and single-stranded DNA viruses that are difficult, or in some cases impossible, to see using the methods presented below. These limitations should always be kept in mind when following these methods.

EFM-based methods to estimate viral abundance were adapted from approaches in which fluorescent dyes with high binding coefficients for nucleic acids are used to stain heterotrophic prokaryotes, which are then captured on filters and enumerated using EFM (Daley and Hobbie 1975; Porter and Feig 1980). Initially, DAPI (4′,6-diamidino-2-phenylindole) was used for estimating viral abundance in natural samples (Hara et al. 1991, 1996; Proctor and Fuhrman 1992; Ricciardi-Rigault et al. 2000; Suttle 1993; Suttle et al. 1990), and although estimates were accurate and precise relative to TEM (Hara et al. 1991; Weinbauer and Suttle 1997), the small amount of DNA in viruses, relatively low fluorescence yield of DAPI, and rapid fading made counting difficult (Bettarel et al. 2000). The advent of nucleic-acid stains with much higher fluorescent yields circumvented this limitation, and counts of viruses in natural waters and sediments can now routinely be made in any laboratory with access to an epifluorescence microscope and the appropriate filter sets.

A new generation of very bright, cyanine-based nucleic-acid stains, Yo-Pro-1, SYBR Green 1, and SYBR Gold, which are excited by blue light (491 to 495 nm) and emit green or yellow light (509 to 537 nm), has superseded the use of DAPI. Studies with Yo-Pro-1 (Hennes and Suttle 1995) and SYBR Green 1 (Noble and Fuhrman 1998) on natural water samples yield highly reproducible estimates of viral abundance. Moreover, estimates of viral abundance made by epifluorescence microscopy have much greater precision and are routinely higher than estimates made by TEM (Chen et al. 2001; Hennes and Suttle 1995). Yo-Pro has a high fluorescence yield, a strong binding coefficient to DNA, and stable fluorescence; however, samples need to be stained for 48 h and cannot be preserved with aldehyde-based fixatives. Treatment with microwave radiation has been recommended as a means to overcome these limitations (Bettarel et al. 2000; Xenopoulos and Bird 1997) and has been used in a number of studies (Bird et al. 2001; Juniper et al. 1998). In a study by Bettarel et al. (2000), samples prepared in this manner had a higher coefficient of variation than the original method and give slightly lower estimates of viral abundance, although the difference was not significant given the small sample size examined.

A widely adopted alternative to Yo-Pro-1 is SYBR Green 1 (Noble 2001; Noble and Fuhrman 1998; Patel et al. 2007) or SYBR Gold (Chen et al. 2001; Shibata et al. 2006). The primary advantages of SYBR dyes over Yo-Pro-1 are a much shorter staining time (<30 min), lower cost, and less sensitivity to aldehyde fixatives. Yo-Pro-1 has the advantages of extremely bright and long-lasting fluorescence and does not require the use of an antifade reagent. When bound to DNA, SYBR Green 1 has less stable fluorescence than Yo-Pro-1 (Suzuki et al. 1997) or SYBR Gold (Chen et al. 2001), which has caused concerns as to the reproducibility and accuracy of viral abundance estimates made using SYBR Green (Bettarel et al. 2000; Chen et al. 2001). However, it is clear that if procedures are carefully followed, SYBR and Yo-Pro stains yield stable brightly fluorescent viruses and estimates of viral abundance that are indistinguishable (Wen et al. 2004).

There are several constraints to using EFM to enumerate viruses in natural samples. High humic content can result in an unacceptable level of background fluorescence. As well, other microorganisms and some detrital particles are also stained. Viruses can generally be distinguished from bacteria and detritus by their staining characteristics and shape, although some very small bacteria may be counted as viruses. Because there are typically more than 10 times as many viruses as bacteria, however, even if all bacteria in a natural sample were counted as viruses, the introduced error would typically be relatively small (Hennes and Suttle 1995). It is much more likely that bacterial abundances will be overestimated by counting some viruses as bacteria.

Another potential pitfall, which went unrecognized for several years, is that stainable viral particles decline very quickly in aldehyde-based fixatives, although loss rates decrease markedly with time (Wen et al. 2004). For example, Wen et al. found that ~16% of the particles were lost within the first hour, whereas about half the counts remained after 2 days. Many earlier EFM counts that used SYBR staining were done on fixed samples that were refrigerated for days or weeks before the slides were made, and are likely significant underestimates of actual abundances. As aldehyde-based fixatives are not compatible with Yo-Pro staining, slides have to be made on unfixed samples as soon as possible after collection; hence, decay in preserved samples is not an issue. Accurate estimates of viral abundance require that slides are made immediately after sample collection or that the samples are fixed and then flash-frozen in liquid nitrogen.

Estimates of viral abundance made using Yo-Pro-I, SYBR Green I, and SYBR Gold give comparable estimates of viral abundance, and detailed procedures for sample preparation, staining, and counting have been published for aquatic (Hennes and Suttle 1995; Noble 2001; Noble and Fuhrman 1998; Ortmann and Suttle 2009; Patel et al. 2007; Shibata et al. 2006; Wen et al. 2004) and sediment (Danovaro et al. 2001; Fischer et al. 2005; Hewson et al. 2001; Maranger and Bird 1996; Ortmann and Suttle 2009) samples. Despite the brighter and more stable fluorescence of Yo-Pro, SYBR stains are most widely used because of the much shorter staining times and lower cost. The fluorescence of SYBR Gold–stained samples is reported to be more stable than that of samples stained with SYBR Green (Shibata et al. 2006), and as the procedures are the same and SYBR Gold is less expensive, it a good choice for most samples. However, SYBR Gold emits at a longer wavelength (537 versus 520 nm), and the gold color can make it difficult to count viruses in samples with a high humic content or a lot of detritus, which is often autofluorescent in a similar wavelength.

This article draws on previous publications, in particular Patel et al. (2007) and Ortmann and Suttle (2009), and the authors' experience to provide detailed protocols for using epifluorescence microscopy to determine the abundance of viral particles in aqueous and sediment samples.

## Materials and procedures

*Equipment—*
- Epifluorescence microscope equipped with the following:
  - ≥100 W Hg-vapor or 150 W xenon lamp (xenon lamps are richer in the blue spectrum and have longer lifetimes and lower lifetime cost)
  - 100× fluorescence objective (phase contrast objectives typically reduce brightness)
  - blue excitation filter (wide bandpass preferred for maximum brightness, e.g., 450–480 or 460–500 nm; excitation peaks 497, 495, and 491 nm for SYBR Green 1, SYBR Gold, and Yo-Pro-1, respectively)
  - dichroic mirror (beam splitter), typically 500 or 510 nm
  - long-pass sharp cutoff filter (typically 515 nm; emission peaks 520, 537, and 509 nm for SYBR Green 1, SYBR Gold, and Yo-Pro-1, respectively; a long-pass filter allows maximum brightness)
  - ocular reticule divided into 100 grid squares
  - stage micrometer
- Filtration unit, to hold 25-mm-diameter filters
- Vacuum pump
- Vacuum flask
- Pipettes suitable for dispensing 1 µL to 2 mL
- Event counter
- Filter forceps
- Sonicating bath (sediment samples only)
- Squeeze bottle (or similar) containing 0.2-µm filtered MilliQ water that will be used to wet the underlay filter.

*Reagents and solutions—*
- (SYBR only) SYBR Green or SYBR Gold nucleic-acid gel stain, 10,000× concentrate in anhydrous DMSO (Invitrogen)
- (Yo-Pro only) Yo-Pro-1, 1 mM stock solution in a 1:4 solution of dimethyl sulfoxide and water (Invitrogen)
- (SYBR only) Antifade solution: *p*-phenylenediamine dihydrochloride or 1,4-phenylenediamine dihydrochloride (se Sigma P-1519 and not P-6001 [Patel et al. 2007] and store in a tightly capped container away from light)
- Spectrophotometric-grade glycerol
- Phosphate-buffered saline (PBS): 0.05 M $Na_2HPO_4$, 0.85% NaCl (wt/vol), pH 7.5)
- (Sediment samples only) Pyrophosphate (10 mM)
- 0.02-µm filter autoclaved MilliQ $H_2O$
- Tris EDTA (TE) buffer, pH 8 (for acidic samples)
- (Yo-Pro only) Aqueous NaCl solution (0.3% wt/vol)
- (SYBR only) 25% EM-grade glutaraldehyde, kept at 4°C
- Ethanol
- DF- or FF-grade immersion oil (refractive index 1/4, 1.516; Olympus).

  Cautions: Nucleic acid stains, phenylenediamine dihydrochloride and aldehyde-based fixatives are toxic; great care should be taken to avoid inhalation or contact with skin.

*Disposable supplies—*
- Anodisc $Al_2O_3$ filters, 0.02 µm pore size, 25 mm diameter, with support ring (Whatman)

  Note: At the time of writing, GE Healthcare, which recently purchased Whatman, is considering discontinuing production of the Anodisc membrane. Currently, there are no other membranes known that are suitable for EFM counts of virus particles. For polycarbonate filters, the background fluorescence is too high (even if stained black) and the porosity is too low to filter an adequate volume of water.
- Nitrocellulose membrane filters, 0.45 µm pore size, 25 mm diameter
- Precleaned glass microscope slides, 25 × 75 mm
- Glass coverslips (25 × 25 mm) of proper thickness (each microscope objective has an optimal coverslip thickness—the cover glass is part of the optics; for example, an Olympus infinity-corrected 100× UVPlanApo is imprinted "∞/0.17," which indicates a 0.17-mm-thick cover glass, also known in the US as #1 1/2)
- 2.0 mL sterile polypropylene microcentrifuge tubes
- 2.0 mL screw-cap cryovials
- 10 µL, 200 µL, and 1 mL sterilized pipette tips
- Polypropylene centrifuge tubes (15 or 50 mL)
- Petri plates
- Kimwipes (Kimberly-Clark) or other lint-free paper wipes
- 9-cm-diameter paper filters (Whatman #1)
- Additional supplies for long-term storage of samples for SYBR staining:
  - Liquid nitrogen
  - Nylon stockings.

*Preparation of reagents—*Reagents must be made in freshly prepared deionized 0.02-µm filtered water to prevent virus particles being introduced into the samples and causing high blanks.

Stock stain solution: Stains should always be handled in low light to prevent photodegradation. Because the stains are sensitive to repeated freezing and thawing, the stains should be aliquoted as stock solutions in small volumes. For SYBR stains, make a secondary stock by diluting the concentrated dye supplied by the manufacturer 10-fold with 0.02-µm filtered deionized water ($dH_2O$) and dispense into polypropylene screw-cap microcentrifuge tubes. For Yo-Pro-1, dilute to 50 µM in an aqueous solution of 2 mM NaCN to prevent any microbial growth during the 48-h staining period. Because the fluorescence of the dyes is very pH sensitive, it can be helpful to dilute the stain in TE buffer (pH 8) if processing strongly acid or basic samples (Chan, pers. comm.). The diluted stains should be stored at –20°C, and ideally should be used within a week; the dye should be checked before use to make sure that it has not precipitated or adsorbed to the walls of the storage tube. Adsorption of the stain to the tube walls is minimized when stored in polypropylene. Each filter requires 2 µL SYBR stain; hence, 40 µL dispensed into each tube provides

enough stain for 20 filters. For Yo-Pro, freeze 800-μL aliquots for enough dye to stain 10 filters.

Glycerol/PBS solution: Prepare a solution of 50% glycerol and 50% PBS. The solution should be shaken or vortexed to ensure complete mixing, 0.02 μm filtered, and stored as a liquid at –20°C. Alternatively, for long-term storage, 990 μL of the filtered mixture can be dispensed into microfuge tubes and frozen until ready for use.

Antifade (SYBR only): Prepare a 10% stock solution of the antifade reagent by diluting 1 g phenylenediamine (PDA) in 10 mL of 0.02-μm filtered autoclaved $dH_2O$. The PDA should go completely into solution, producing a colorless liquid. If the stock solution is tea-colored or darker, it has oxidized and should not be used. Dispense 500-μL aliquots of the working solution into microcentrifuge tubes and store at –20°C to minimize freezing and thawing. The frozen reagent should be white; if it has a brownish tint it should not be used. It is possible to use other antifade reagents such as 0.5% (wt/vol) ascorbic acid in 50% (vol/vol) glycerol/PBS or SlowFade (Invitrogen), but they may provide less protection against fading (Noble and Fuhrman 1998). In contrast, DABCO (1,4-diazabicyclo[2.2.2]octane) in TE/glycerol is reported to be a superior antifade to PDA (Ortmann, pers. comm.). Immediately before preparing the slides, make a 0.1% working solution of the antifade by adding the 10% phenylenediamine solution to the glycerol-PBS mixture. Estimate 50 μL of reagent per slide.

*Sample collection and preparation—*

Aqueous samples: Collect the samples in sterile containers that are rinsed three times with the sample water. Polypropylene centrifuge tubes or bottles work well as sample containers. The range of viral abundances suitable for enumeration is ~$10^5$ to $10^7$ mL$^{-1}$; hence, dilution may be necessary for very productive natural samples or cultures. If necessary, dilute the sample with 0.02-μm filtered water, ideally prepared from the same or very similar water from which the sample was obtained.

Sediment samples: Undisturbed sediment samples should be collected with a piston corer. The sediment–water interface can be sampled with a wide-bore serological pipette to minimize disruption to the sediment surface. Samples from deeper within the core can be obtained by carefully pushing the core up from the bottom of the core barrel and slicing the sediment at the desired depth. The sediment should be sampled from the center of the core, leaving a well-defined area of sediment around the periphery to ensure the sample is not contaminated with sediment smeared along the side of the core barrel. Remove a 0.5-cm$^3$ subsample of sediment from the center of the core slice and transfer it into 4 mL of 0.02-μm filtered seawater and 1.0 mL pyrophosphate (10 mM final concentration). Sonicate the mixture for 3 min and centrifuge at 800*g* for 1 min (Ortmann and Suttle 2009). The supernatant can then be diluted and the slides prepared as outlined below. For different types of sediments and soils, the amount of pyrophosphate and length of sonication may need to be

tested and optimized. Potassium citrate has been reported to be superior to sodium pyrophosphate for extracting phage from soils (Williamson et al. 2003) and may be an alternative for aquatic sediments, as well.

Preservation of samples (SYBR only): If slides cannot be made in the field on freshly collected samples, preservation will be necessary. Many earlier estimates of viral abundance have been made on preserved samples; however, several studies have shown rapid and significant decay of viral particles in aldehyde-fixed samples (Brussaard 2004; Danovaro et al. 2001; Wen et al. 2004), although there is evidence that the decay is lessened when larger-volume samples are preserved (Patel et al. 2007). Samples preserved in the field should be flash-frozen in liquid nitrogen. Even freezing with dry ice (frozen $CO_2$) or immediately freezing in a –87°C freezer is not adequate for preservation, and to our knowledge, freezing in a dry ice/ethanol slurry (–78°C) has not been tested. Samples to be preserved should be fixed in 0.5% (final concentration) EM-grade glutaraldehyde and flash-frozen in liquid $N_2$. For relatively productive samples with virus abundances ≥$10^6$ mL$^{-1}$, add 30 μL of 25% EM-grade glutaraldehyde into prelabeled 2-mL cryovials, add 1470 μL of the sample to be counted, and mix. These volumes can be halved for samples with virus abundances ≥$10^7$ mL$^{-1}$ and increased to 4 mL in 5-mL cryovials for very oligotrophic samples. Allow the samples to stand for at least 15 min but no longer than 30 min, and then freeze immediately in liquid $N_2$. To facilitate easy retrieval, vials can be placed in women's nylon stockings before freezing. (We find black sheer ones the most aesthetically pleasing.) Once frozen, the vials can be transferred to –80°C for long-term storage, until the slides can be prepared. For counting, the frozen samples are thawed in a 37°C water bath and immediately stained with SYBR Green I or SYBR Gold, as outlined below.

In the field, if it is impossible to make slides or preserve in liquid N, then it probably is best to collect the samples in as large volumes as is practical and maintain them under in situ conditions for as short a time as is possible until slides can be made.

*Filtration and staining of sample—*

1. Prepare slide labels with critical information such as the date, sample location, and volume filtered to keep track of the samples once they have been filtered and stained. The labels should be affixed when each slide is prepared.

2. For every set of four samples to be stained, use a permanent pen to mark the bottom of a plastic Petri plate into four labeled sections.

3. (SYBR only) For each filter that is to be prepared, add a 78-μL drop of 0.02-μm filtered $dH_2O$ on each section of the marked Petri plates. Note that the efficacy of the stain is pH dependent; hence, if samples are from an acidic environment, diluting the stain in pH 8 TE buffer rather than $dH_2O$ has been found to result in more stable fluorescence (Chan, pers. comm.).

4. (SYBR only) Thaw a 40-µL vial of the stock SYBR solution and add 2 µL stock solution to each drop (78 µL) of sterile dH$_2$O or buffer. Mix the stain by gently pipetting up and down. If a large number of filters are to be processed, it is easier to prepare a working solution of the stain and transfer 80-µL drops onto the plates. Place the Petri plates in the dark so that the stain is not bleached.

5. (SYBR only) Prepare the antifade solution in a clean, sterilized 2-mL microcentrifuge tube. About 40 µL of antifade solution will be required for each filter. Dilute the 10% (wt/vol) stock of *p*-phenylenediamine 1:100 using glycerol/PBS as the diluent. For example, add 1 µL stock *p*-phenylenediamine per 99 µL of 1:1 glycerol:PBS solution. Keep the solution on ice and protect it from light.

6. (Yo-Pro only) For each filter that is to be prepared, add a 80-µL drop of thawed Yo-Pro working solution on each section of the marked Petri plates. Place a 9-cm-diameter filter paper soaked with 3 mL aqueous NaCl solution (0.3% wt/vol) in the lid of the Petri plates to prevent evaporation of the stain.

7. Connect a filtration unit for 25-mm filters to a vacuum source, ensuring the vacuum is ≤13 kPa. A stronger vacuum will likely crack the filter.

8. Place a 0.45-µm nitrocellulose backing filter on each filter support, and overlay it with a thin layer of dH$_2$O. If the filter does not wet evenly (if there are areas or spots that remain white), replace the filter with another one. Backing filters can be reused if they are smooth, are wet evenly with water, and have no holes.

9. Carefully pick up a 0.02-µm Anodisc filter by its plastic ring and lay it over the wet backing filter, with the plastic ring facing upward. The Anodiscs are ceramic and break easily; make sure it is not cracked and that air is not trapped between the filters. If necessary, pull excess water through the filters using the vacuum, but make sure the filters remain wet.

10. (SYBR only) If the sample has been preserved and frozen as described above, thaw it in a 37°C water bath. For a sample that has just been collected, fix it with 0.5% glutaraldehyde for 15–30 min at 4°C before preparing slides. In addition, prepare duplicate control samples by fixing 1 mL of the 0.02-µm filtered water that was used to dilute the SYBR stain. For some samples, fixation may improve the fluorescence of the particles and make counting easier. Also, some virus particles are prone to breakage if not fixed (Chan, pers. comm.).

11. (Yo-Pro only) Because divalent cations interfere with the binding of the stain, seawater samples should be diluted to <7 psu with 0.02-µm filtered dH$_2$O before filtration.

12. It is a good idea to make test slides (including a control with no sample added) to be sure an appropriate volume is filtered, that the procedure is working, and that the filters and reagents do not have viruses on or in them (some batches of Anodiscs have been covered with bacteria and

viruses). For most lake and coastal seawater samples, which have viral abundances of ~$10^7$ mL$^{-1}$, 0.8–1.0 mL sample is added to the surface of the Anodisc filter while the vacuum is off. A filtration tower is not needed, as surface tension will hold the water on the surface of the filter. Make sure the entire volume is within the plastic ring, or the sample will be pulled under the edge of the filter. Turn on the vacuum and suck the sample through the filter.

13. For oligotrophic or very deep ocean samples, it may be necessary to filter 4 mL or more. If the volume to be filtered is slightly greater than 1 mL, the additional volume can be added while the sample is filtering, being careful to ensure the entire filter surface is continuously covered with liquid during filtration. For larger volumes, a sterile filtration tower can be used; if the inside diameter of the tower is less than the filter diameter inside the plastic ring, it must be taken into consideration when calculating viral abundance (*see* below). Filter towers must be cleaned between samples. Rinse the towers with 0.02-µm filtered dH$_2$O followed by ethanol. Dry with lint-free paper (e.g., Kimwipe).

14. Once the sample is filtered, remove the Anodisc with the vacuum still on. There should not be any liquid on the surface of the filter. Touch only the plastic ring, so as not to crack the membrane. To assist in lifting the filter, a 10-µL pipette tip can be cut at a slight angle and slid under the filter edge. It also helps to place the Anodisc filter about a millimeter off center on the 0.45 underfilter, to provide an edge you can grasp with forceps. Allow the filter to air-dry (typically a minute or less), until the surface is visibly dry.

15. Place the Anodisc, sample side up, on a drop of stain in the Petri dish.

16. (SYBR only) Allow the filter to stain for 15 min in the dark.

17. (Yo-Pro only) Allow the filter to stain for 48 h in the dark, at room temperature.

18. Add a drop of dH$_2$O on the backing filter, lay the stained Anodisc on top, and use the vacuum to remove any remaining fluid. Do not use the filter if there is visible water on top of the Anodisc when it is done staining, as it is likely cracked.

19. (Yo-Pro samples and samples with high background fluorescence) Some samples (e.g., sediments, vent fluid, and humic waters) may require the filters to be rinsed to reduce background fluorescence. If so, while the vacuum is still on and the filter is damp, rinse the filter twice with 1 mL of 0.02-µm filtered dH$_2$O. For some samples, such as those with high humic content, or from vent environments, samples can be rinsed with TE buffer to reduce background fluorescence (Chan and Winget, pers. comm.).

20. Remove the Anodisc while the vacuum is on. Place the Anodisc, sample-side up, on a 9-cm filter paper or Kimwipe in the dark, and allow the filter to dry until it appears opaque. The filter paper can be placed inside the lid of a Petri plate, and a foil-lined Petri-plate bottom can be used

as a lid. It usually takes about 5 min for the filter to dry, but it can be longer when humidity is high. The process can be accelerated by laying the filter on a glass slide that is heated to 35–37°C on a heating block, often very helpful when humidity is high.

21. (SYBR only) Place 12–15 µL antifade solution on a labeled glass slide and lay the dry Anodisc on top. Add ~20 µL antifade on top of the Anodisc and cover with a coverslip. If the slides are to be frozen, add a little more antifade to compensate for sublimation.

22. (Yo-Pro only) Place 12–15 µL spectrophotometric-grade glycerol on a labeled glass slide and lay the dry Anodisc on top. Add ~20 µL glycerol on top of the Anodisc and cover with a coverslip.

23. Remove any air bubbles that are trapped under the coverslip by gently pressing on the surface.

24. The slides can be counted immediately or stored frozen at –20°C for at least 4 months with no decrease in estimates of viral abundance. Slides can be individually wrapped in a Kimwipe and placed in small batches in foil packets before freezing. This allows a few slides to be thawed at a time. Once thawed, the slides should be counted immediately.

*Determining abundance—*

1. Count the viruses at 1000× magnification using a 100× oil-emersion objective. Make sure that the area of the filter covered by the 10 × 10 ocular reticule has been determined using a stage micrometer.

2. Begin by checking the test filters to ensure that the reagents or filters were not contaminated and the filtered volumes were appropriate. Viral and bacterial particles will appear green (SYBR Green and Yo-Pro) or yellow (SYBR Gold) when excited with blue light (Fig. 1).

3. Check each slide before counting to make sure that the filter is evenly stained and that the viruses are on a single plane of focus and not suspended in the mounting medium and are evenly distributed across the filter. Using the ocular reticule, select an appropriate number of grid squares so that there are 10–100 stained viruses in each field. Viruses can generally be distinguished from cells by their staining characteristics. Viruses appear as bright pinpricks of light, whereas cells generally have discernable size (Fig. 1). If the 95% confidence intervals (*see* step 7) overlap for three transects of 20 random fields containing at least 200 viruses, it indicates that 20 fields is adequate to compensate for the variation in viral abundance among fields.

4. Estimate the abundance of viruses by counting at least 20 random fields. Keep a tally of the number of particles in each field so that the variation in abundance of particles among fields can be determined. These data can be used to determine whether the distribution of particles across the filter is random. (The data should follow a Poisson distribution where the mean equals variance, although 20 fields should resemble a normal distribution.) Particles touching two edges of the grid (e.g., left side and top) should be

counted, whereas particles touching the other two edges (e.g., right side and bottom) should not be counted.

5. For each sample, record the number of particles counted in each field, the number of fields counted, the area of the field, and the volume of sample filtered.

6. The abundance of viruses mL$^{-1}$ ($V_t$) in the sample = $V_c \div F_c \times A_t \div A_f \div S$, where $V_c$ = total number of viruses counted, $F_c$ = total number of fields counted, $A_t$ = surface area of the filter (µm$^2$) (*see* note below), $A_f$ = area of each field (µm$^2$), and $S$ = volume of sample filtered (mL). (Note that for a 25-mm Anodisc filter, the diameter of the filter inside the plastic ring is 19 mm, which corresponds to an area of 283,528,737 µm$^2$. If a filtration tower is used that has a diameter less than that of the surface of the filter, it will be necessary to correct for the smaller filtration surface area. This is most accurately done by using the microscope to determine the maximum width of the filter across which virus particles can be observed [Patel et al. 2007].)

7. The total number of particles counted will determine the size of the 95% confidence intervals on the estimates of viral abundance. By assuming a Poisson distribution, the 95% confidence intervals can be estimated using the following equations (Suttle 1993):

$$\text{Upper } 95\% = V_c + 1.96 \times \sqrt{(V_c + 1.5)} + 2.42$$

$$\text{Lower } 95\% = V_c - 1.96 \times \sqrt{(V_c + 0.5)} + 1.42$$

## Assessment and discussion

The importance of microbes and microbial processes in aquatic environments, coupled with the major role of viruses as agents of microbial mortality and nutrient cycling, has led to the need to quantify viral abundance as part of many investigations. The collection of these data have been greatly facilitated by the development of epifluorescence microscopy as a relatively inexpensive and quick method to quantify viral particles in natural waters and cultures. There have been many modifications and improvements to the method since the first data were published using DAPI-stained samples (Hara et al. 1991; Proctor and Fuhrman 1992; Suttle et al. 1990). However, the development of these methods has also identified challenges that can affect the accuracy of results. These are briefly summarized below.

*Sample preservation—*A significant issue that has been problematic for many years is the preservation of samples for later analysis. One of the first studies that used epifluorescence microscopy to estimate viral abundance in natural waters (Hara et al. 1991) preserved samples in 1% (vol/vol) formalin for 1–2 weeks before analysis. Similarly, many studies that used SYBR stains analyzed aldehyde-preserved samples on the premise that the abundance of viruses would remain stable over time. The same issue did not arise with Yo-Pro staining, as aldehyde fixatives are not compatible with the method. Numerous studies have now shown that the abundance of stainable viral particles decreases rapidly once fixed (Brussaard
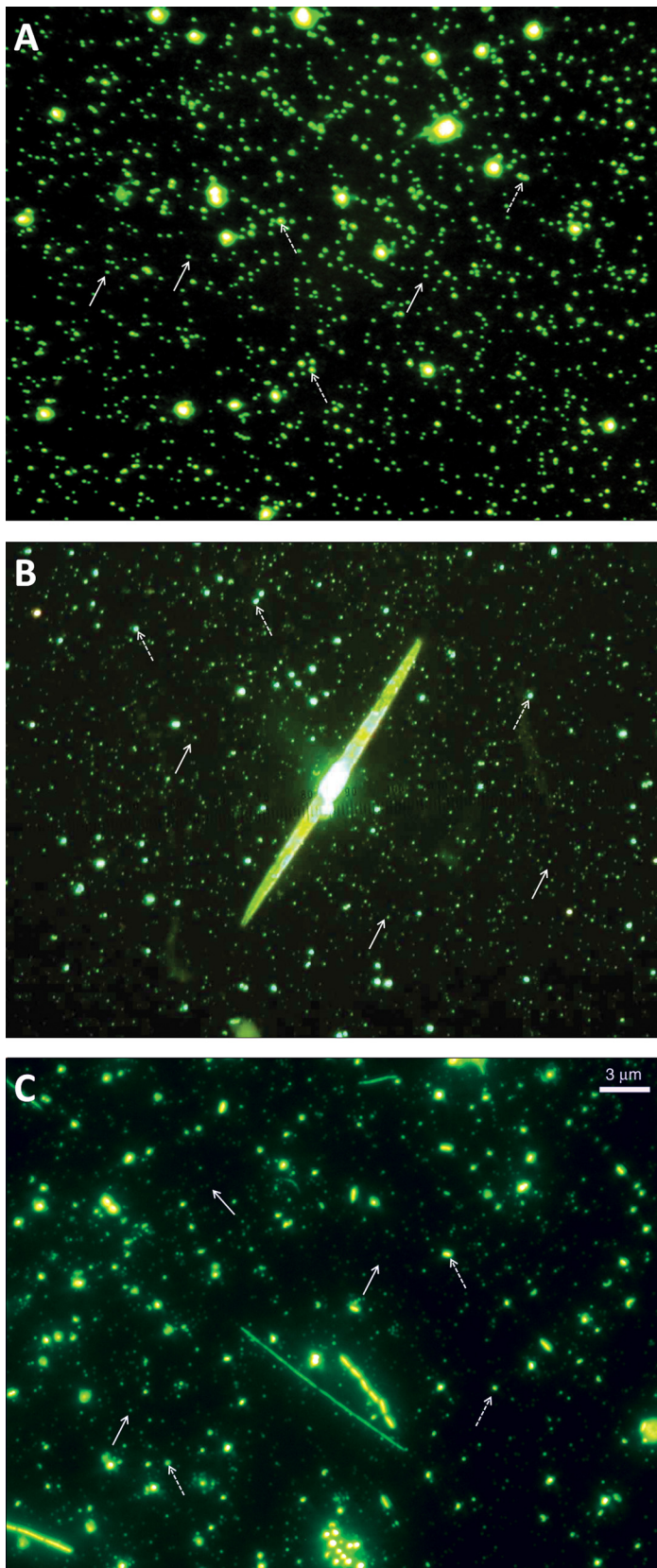
**Fig. 1.** Epifluorescence micrographs of natural water samples. Viruses are indicated by solid arrows and bacteria by broken arrows. (A), Yo-Pro 1–stained slide of a water sample from the Arctic Ocean (courtesy of J. P. Payet). (B), SYBR Green I–stained water sample from the coastal waters of Southern California. The large object in the center is a pennate diatom. (C) SYBR Gold–stained sample of a water sample from the Chesapeake Bay (courtesy of F. Chen and K. Wang). The slides emphasize the differences that occur between samples and different staining protocols.

2004; Danovaro et al. 2001; Shibata et al. 2006; Wen et al. 2004). Consequently, if accurate estimates of viral abundance are to be obtained, slides must be prepared immediately following sample collection, or the samples must be fixed and frozen in liquid nitrogen, as outlined above.

*Distinguishing between stained viruses and other fluorescent particles*—Even if some bacteria are counted as viruses, this should be of relatively minor consequence, because the error introduced would generally be relatively small even if all the bacteria were counted as viruses (Hennes and Suttle 1995). The typical high-precision viral abundance estimates obtained within laboratories (generally <10%) gives the illusion of accuracy. However, an interlaboratory comparison made on the same samples by researchers who routinely estimate viral abundance by epifluorescence microscopy yielded estimates that varied by as much as threefold (unpubl. data, SCOR Working Group on Marine Viral Ecology). Yet the reproducibility within labs for individual samples was high. This indicates that small differences in methodology (e.g., the person counting, staining times, etc.) or equipment (e.g., light source, filter sets, etc.) can have a significant impact on estimates of viral abundance made by epifluorescence microscopy. The reasons behind these discrepancies remain to be resolved, but care must be taken when comparing absolute estimates of viral abundance made by different investigators.

*Lack of a suitable standard*—In part, reproducibility of estimates among laboratories is exacerbated by the lack of a suitable standard that can be used to calibrate estimates of viral abundance. Even though it is relatively easy to stain and generate high-precision estimates of abundance for stock cultures of viruses, the accuracy of these estimates is typically unknown. Moreover, in natural samples, there is a much wider spectrum of fluorescent signatures than is found within cultures of viruses, making an appropriate standard difficult to define. Nonetheless, it is generally accepted that the most accurate estimates of viral abundance for natural samples are made by epifluorescence microscopy. However, until standards that are widely accepted by the community are available, the accuracy of individual estimates of viral abundance will remain unknown.

*All virus particles are not equally stained*—Another source of error is that all viruses are not stained equally, and may not be visible by epifluorescence microscopy. Current evidence suggests that the majority of viruses in the ocean are double-stranded DNA with genome sizes >20 kbp (Steward et al. 2000; Wommack et al. 1999), which should be easily stained and

resolved by EFM. Nevertheless, it is becoming increasingly apparent that RNA viruses and single-stranded (ss)DNA viruses are also widely distributed and likely relatively abundant members of the viroplankton (Angly et al. 2006; Culley et al. 2006; Lang et al. 2009). However, the small genome sizes of RNA and ssDNA viruses, and the weaker fluorescence yield of the dyes when bound to these nucleic acids, makes it unlikely that these viruses would typically be visible by EFM.

*Infectivity remains unknown*—EFM provides high precision and relatively accurate estimates of the abundance of virus particles in natural samples; however, the proportion of particles that are infectious is unknown, as is the identity of the hosts that they infect. It is reasonable to assume that the majority of virus particles are infectious based on estimates of contact rates with host cells (Suttle and Chen 1992) and photoreactivation (Wilhelm et al. 1998).

In this contribution we have attempted to bring together the state of methodologies for using EFM to determine the abundance of viruses in aquatic and sediment samples. If protocols are carefully adhered to, EFM can give highly reproducible and relatively accurate estimates of the abundance of viral particles in water samples, sediments, and cultures. Care must be taken to understand the limitations of the methods, and challenges remain to develop standards that can be used to obtain absolute and accurate estimates of viral abundance. However, the methods are suitable for obtaining routine estimates of viral abundance in a wide range of natural samples.

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