Limnol. Oceanogr., 40(6), 1995, 1050–1055

Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy

Kilian P. Hennes[1] *and Curtis A. Suttle*[2]
Marine Science Institute, University of Texas at Austin, POB 1267, Port Aransas 78373-1267

Abstract

Epifluorescent microscopy was used to determine the abundance of viruses in samples from marine and freshwater environments and in laboratory cultures that were filtered onto 0.02-μm pore-size filters and stained with a cyanine-based dye (Yo-Pro-1). Estimates of viral abundance based on Yo-Pro-stained samples were 1.2–7.1 times greater than estimates obtained with transmission electron microscopy (TEM). Moreover, the precision of the Yo-Pro-based method was much greater than that for TEM (C.V. 7% vs. 20%, respectively). DNase treatment of samples did not result in lower numbers of particles that could be stained by Yo-Pro, suggesting that the fluorescence was not the result of nucleic acids associated with the surface of particles. These results indicate that the concentration of viruses in natural waters may be higher than previously recognized and imply that the TEM-based method significantly underestimates virus abundance. Virus abundances ranged from $10^7 -> 10^8$ ml^{-1} in surface waters along a transect in the western Gulf of Mexico to 10^9 ml^{-1} in water overlying a submerged cyanobacterial mat. High counting efficiency, ease of preparation, modest equipment requirements, and the possibility of preparing specimens for long-term storage, make the Yo-Pro-based method ideal for routine environmental analysis.

Viruses are an abundant and biologically active component of the surface waters of marine and freshwaters. Central to many investigations on viruses in aquatic ecosystems has been the enumeration of virus particles. Typically, viruses are sedimented from the samples directly onto electron-microscopy grids by ultracentrifugation, negative stained, and counted by transmission electron microscopy (TEM) (Bergh et al. 1989; Børsheim et al. 1990; Wommack et al. 1992), although in some studies viruses have been concentrated by ultrafiltration rather than centrifugation prior to enumeration by TEM (Proctor and Fuhrman 1990; Paul et al. 1991). TEM methods are time consuming, require expensive equipment, and cannot be done at sea. An alternative approach is to stain the viruses with DAPI and enumerate them by epifluorescence microscopy (Suttle et al. 1990; Hara et al. 1991; Proctor and Fuhrman 1992). The DAPI method has the advantage that the equipment is much less expensive than required for TEM but has the disadvantage that viruses stained in this manner are close to the limit of visual detection by epifluorescence microscopy. Consequently, TEM-based methods have been used most frequently for enumerating the total concentration of viruses in natural water samples. Further discussion on the advantages and limitations of these methods can be found elsewhere (Suttle 1993).

The problems with these approaches motivated us to develop a method that is simple, accurate, and suitable for routine environmental analysis. Viruses are filtered onto 0.02-μm pore-size filters, stained with a fluorochrome that is specific for nucleic acids (Hirons et al. 1994), and enumerated by epifluorescence microscopy. Results with this method are consistent with TEM significantly underestimating the abundance of viruses in natural waters.

Materials and methods

Sample collection—Triplicate water samples (200 ml) were collected in polyethylene bottles from marine and freshwater environments that ranged from oligotrophic to hypereutrophic and which varied in detrital and humic acid content. These included a submerged cyanobacterial mat, a freshwater marsh, a humic ditch, the Port Aransas municipal water supply, and a 6,000-liter tank containing natural seawater; other samples were collected near Austin, Texas (mesotrophic Lake Austin and Barton Springs spillway). Duplicate samples were also collected from the surface along a transect in the western Gulf of Mexico (from 27°49′N, 97°01′W to 27°49′N, 96°59′W), and from the boat harbor and pier at the University of Texas Marine Science Institute (MSI; 27°50′N, 97°02′W).

Virus isolates—The cyanophages S-BBS1 (Siphoviridae), S-PWP1 (Podoviridae), and S-PWM2 (Myoviridae) and bacteriophages PWH3a-P1 and LB1VL-P1b (Myoviridae and Podoviridae) were isolated from the coastal waters of Texas (Suttle and Chen 1992; Suttle and Chan

[1] Present address: Limnological Institute, University of Constance, POB 5560, D-78434 Constance, Germany.
[2] Corresponding author.

Acknowledgments

The assistance and helpful discussions of Amy M. Chan and Feng Chen are appreciated. We also thank Anthony F. Amos for providing the salinity data and the exact locations of the sampling stations, Jan Culbertson from Texas Parks and Wildlife for allowing participation on the cruise of the RV *Longhorn*, and Troy A. Roepke for collecting water samples from Austin. Molecular Probes, Inc., provided stain for evaluation.

This research was supported in part by grants from the Office of Naval Research to CAS (N00014-92-J-1676) and the German Academic Exchange Service to K.P.H. (515 402 783 3).

Contribution 941 from the Marine Science Institute, University of Texas at Austin.

1993). The algal virus MpV-SP1 (Phycodnaviridae) was isolated from seawater collected off the coast of southern California (Cottrell and Suttle 1991). The viruses were amplified using their respective hosts: *Synechococcus* strains BBC1, SNC1, DC2 (WH 7803); heterotrophic bacterial strains PWH3a and LB1; *Micromonas pusilla,* strain Plymouth 27 (UTEX 991). Following lysis the cultures were filtered through 0.2-μm pore-size (0.45 μm for MPV-SP1) Durapore membranes (Millipore) and diluted in media up to 10,000-fold prior to staining.

Transmission electron microscopy—Samples were fixed with glutaraldehyde at a final concentration of 1% and harvested by ultracentrifugation (3 h at 155,000 × *g*) directly onto carbon and Formvar-coated electron microscopy grids (Børsheim et al. 1990). More than 200 virus particles were counted in 20 randomly chosen fields using TEM (Philips EM301 at 80 kV and 34,000 × magnification). A taper correction factor was applied to the estimates of virus concentration, and the precision of the estimates was calculated as outlined by Suttle (1993).

Epifluorescence microscopy—Epifluorescence microscopy was used to view viruses stained either with the cyanine-based nucleic acid stain, Yo-Pro-1 {4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethylene]-1-(3′-trimethylammoniumpropyl)-quinolinium diiodide} (Molecular Probes), or DAPI (4′6-diamidino-2-phenylindole), which specifically stains double-stranded DNA.

For the Yo-Pro-stained samples a stock solution of Yo-Pro-1 supplied by Molecular Probes (1 mM Yo-Pro-1 in a 1 : 4 solution of dimethyl sulfoxide and water) was diluted to 50 μM in an aqueous solution of 2 mM NaCN. Immediately before sample collection, a series of 80-μl drops of this solution were dispensed on the bottom of a 10-cm-diameter plastic Petri dish, in the lid of which a filter paper soaked with 3 ml of an aqueous NaCl solution (0.3% wt/vol) were placed to prevent evaporation of the staining solution. Unfixed samples (100 μl) were diluted with 700 μl of deionized-distilled water and placed on the surface of a 0.02-μm pore-size Al_2O_3 Anodisc 25 membrane filter (Whatman); care was taken to hold the samples within the plastic support ring of the filter by surface tension. It is important that the samples not be fixed in aldehydes which interfere with binding of the stain. Dilution of the unfixed samples is necessary as divalent cations also interfere with binding of Yo-Pro-1.

Each sample was gently filtered (15 kPa) with a pre-moistened 0.45-μm pore-size cellulose nitrate membrane as a backing filter. While still moist, the Anodisc membranes with the filtered samples were laid (sample side up) on drops of the staining solution and incubated in the covered Petri dish for 2 d in the dark at room temperature. The filters were then washed twice by filtering 800 μl of deionized-distilled water through the membrane. The damp membranes were transferred to glass slides, immediately covered with a drop of spectrophotometry-grade glycerol and a cover slip, and stored at −20°C until processed. For each sample, >200 viruses in 20 randomly selected fields were counted at 1,000× with an epifluorescence microscope (Olympus IMT-2) equipped with an acridine orange filter set (excitation <490 nm, dichroic filter 500 nm, barrier filter >515 nm).

For DNase-treated samples, 250 Kunitz units of DNase was added per milliliter of sample and incubated for 30 min (Suttle 1993) before dilution of the sample with deionized-distilled water. This concentration of DNase was adequate to digest several μg ml^{-1} of DNA in seawater (~1,000-fold more dissolved DNA than is typically found in seawater; Jiang and Paul 1995) in <10 min (change in absorbance at 260 nm for 35 μg ml^{-1} of DNA in seawater = 0.04 min^{-1}; data not shown).

Bacteria and viruses were also enumerated following staining with DAPI. Virus stock solutions from culture lysates of marine bacteria were incubated for 30 min at room temperature in 1 μg ml^{-1} final concentration of DAPI and transferred to glass slides and counted as outlined by Suttle (1993). Bacteria were stained, filtered onto 0.2-μm pore-size, black polycarbonate filters (Poretics), and counted (Porter and Feig 1980).

Chlorophyll a and salinity—For chlorophyll *a* determinations, 100 ml of sample was filtered onto 0.45-μm pore-size nitrocellulose filters, extracted in 90% acetone overnight at −20°C, and measured fluorometrically (Parsons et al. 1984). Results were corrected for pheopigment concentrations. Salinity was measured by conductivity.

Results and discussion

The two most important results of these studies are that epifluorescence microscopy of samples stained with the cyanine-based stain Yo-Pro-1 can be used to enumerate viruses in aqueous samples and that the TEM-based method seems to underestimate viral abundance in many instances. Given its simplicity, high precision, and modest equipment requirements, the Yo-Pro protocol should be ideal for routine determinations of virus abundance in natural water samples.

Virus staining—Yo-Pro-1 fluoresces green (510 nm) when bound to DNA or RNA and excited with blue light (Hirons et al. 1994), while the unbound dye has very low background fluorescence. We tested the stain on a variety of viral taxa (Podoviridae, Syphoviridae, Myoviridae, and Phycodnaviridae), as well as on natural virus communities from fresh and marine waters. The preparation of each sample was rapid (<5 min); however, the samples had to be incubated for 2 d to ensure adequate staining. The stained viruses were brilliant green, relatively stable while illuminated (~ 5 min) and could easily be seen with epifluorescence microscopy (Fig. 1). In contrast, DAPI-stained viruses were less stable and fluoresced much less brightly. Detritus particles fluoresced yellow and could be clearly distinguished from viruses. The method was not tested for RNA viruses, but Yo-Pro stains both RNA and DNA so there is no reason to anticipate difficulties.

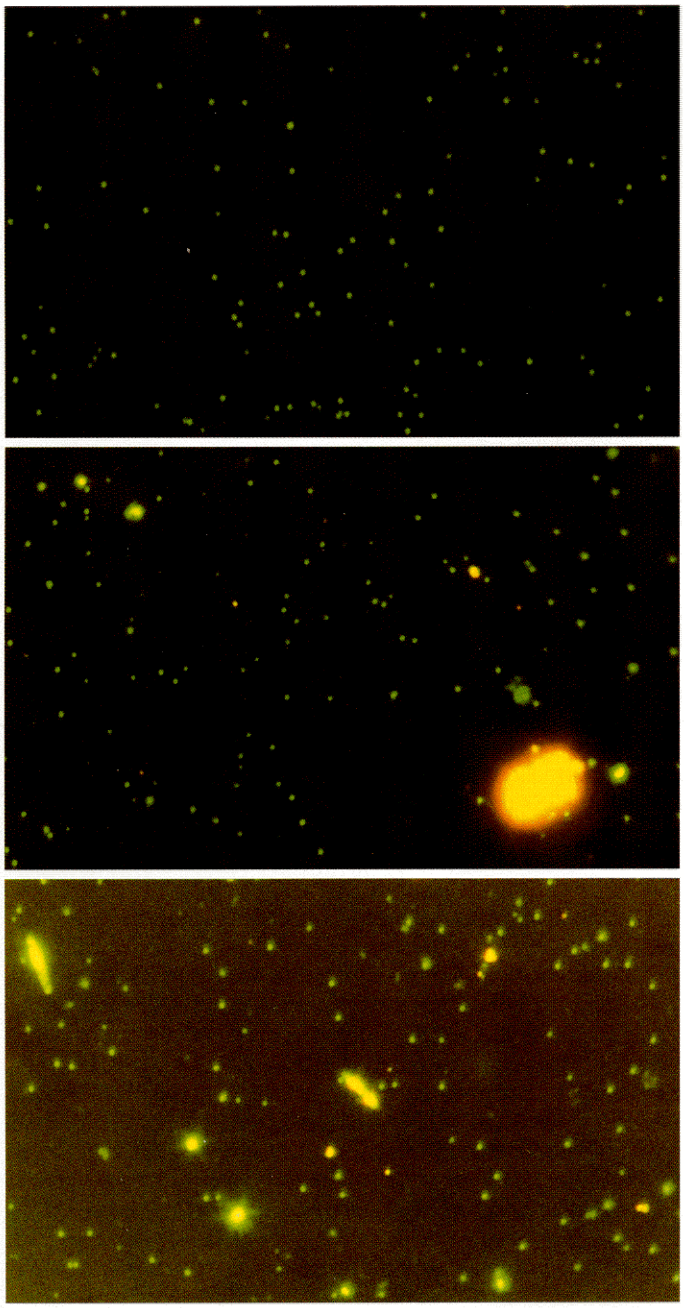

Fig. 1. Virus samples filtered onto 0.02-μm pore-size membranes, stained with Yo-Pro-1 and photographed at a magnification of 1,000× (Kodachrome 400 ASA film, 30-s exposure). Top—marine bacteriophage (PWH3a-P1). Center and bottom—natural seawater samples. Detritus particles are yellow, while bacteria are larger in size, typically irregular in shape, and appear more yellow than the viruses.

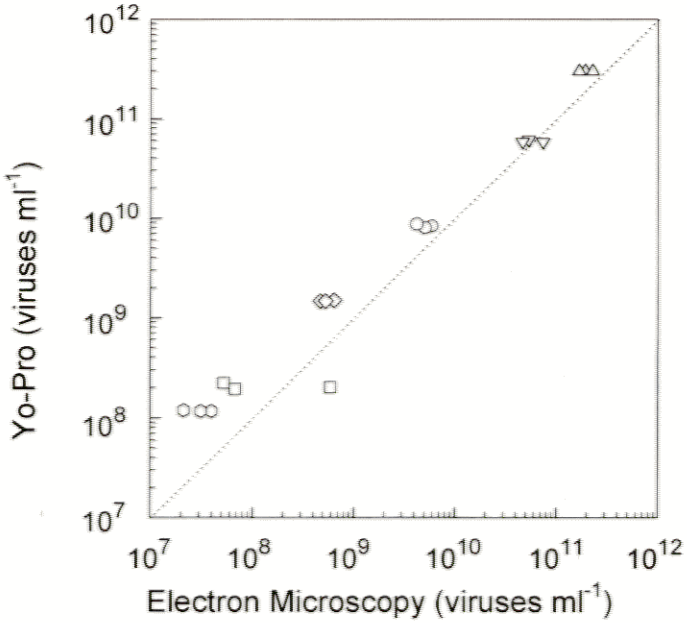

Fig. 2. Counts of virus particles using Yo-Pro-1 and transmission electron microscopy for a variety of marine virus isolates: cyanophages S-BBS1 (◇), S-PWP1 (○), and S-PWM2 (○); phycovirus MPV-SP1 (□); and bacteriophages PWH3a-P1 (△) and LB1VL-P1b (▽). Dashed line indicates a relationship of 1 : 1.

Virus isolates—The Yo-Pro and TEM methods were used to estimate the concentration of marine bacteriophages, cyanophages, and a phycovirus in culture lysate. Estimates of viral abundances using these methods were well correlated ($r = 0.98$, $P < 0.01$, $n = 18$, Fig. 2), although the Yo-Pro method consistently yielded higher values for virus concentrations $\lesssim 10^{10}$ ml^{-1}, implying that the TEM method underestimated the true concentration of viruses. The precision of the Yo-Pro protocol was also much greater than for the TEM method (Fig. 2); the average C.V. (C.V., SD × 100/mean) of triplicate samples was 3% for the Yo-Pro method vs. 39% for the TEM method. Consequently, TEM-based counts could not be used as an absolute standard against which the Yo-Pro staining method could be evaluated.

The relative difference between the methods was greatest when the abundance of viruses was the least. For example, both methods yielded similar estimates at concentrations of ~10^{11} ml^{-1}, whereas, when the concentration of viruses estimated with the Yo-Pro method was ~10^8 ml^{-1},the estimate based on TEM counts was only about half as much. The error was least for samples in which the concentration of viruses was greatest because these samples needed to be diluted 10–10,000-fold before being processed for counting by TEM. As viruses must be counted at a much higher magnification by TEM (30,000×) than by epifluorescence microscopy (1,000×), the virus concentration must be ~30 times higher on the surface of the electron-microscopy grid than on the filter used for the Yo-Pro method. Therefore, particulate material which interferes with the TEM counting method is also more concentrated on the grid surface. When the high-virus-concentration culture lysates are diluted to allow counting by TEM, the abundance of interfering particulate material is also diluted, which results in improved accuracy for the TEM method.

Table 1. Abundance of viruses in aquatic microbial communities determined by transmission electron microscopy (TEM) and by epifluorescent microscopy of Yo-Pro-stained samples (Yo-Pro). The percent coefficient of variation (%C.V.) of triplicate samples is shown in parentheses.

Location	1994	Salinity (‰)	Yo-Pro counts (10^7 ml^{-1})	TEM counts (10^7 ml^{-1})	TEM/ Yo-Pro
Cyanobacterial mat	11 Jan	37.0	96.1(15)	44.3(47)	0.46
Boat harbor	5 Jan	29.5	14.9(8)	3.7(16)	0.25
MSI pier	5 Jan	31.3	10.1(2)	1.4(29)	0.14
MSI pier (1200 hours)	10 Mar	26.3	28.3(7)	6.8(27)	0.24
MSI pier (1400 hours)	10 Mar	25.8	23.7(8)	7.3(18)	0.31
MSI pier (1630 hours)	10 Mar	28.6	26.1(5)	8.6(7)	0.33
MSI pier (1900 hours)	10 Mar	30.9	22.5(4)	9.6(7)	0.43
Marsh A	11 Jan	13.3	70.0(4)	22.7(15)	0.32
Marsh B	11 Jan	6.3	57.4(6)	*	
Ditch	11 Jan	0.7	†	14.6(14)	
Seawater tank	15 Mar	0.2	16.1(9)	10.2(6)	0.64
Lake Austin	15 Mar	0.2	14.2(4)	6.1(5)	0.43
Barton Springs	15 Mar	0.3	0.53(11)	0.39(49)	0.73
Port Aransas water supply	15 Mar	0.5	0.18(7)	0.16(18)	0.86

* Could not be counted because of high concentration of detritus.
† Could not be counted because of high fluorescence of humic materials.

Natural samples—The Yo-Pro and TEM methods were also used to estimate the abundance of viruses in natural water samples that ranged widely in salinity (0.2–37‰) and productivity (oligotrophic Gulf of Mexico to a eutrophic pond) (Table 1). It was not possible to use both methods for all samples. Background fluorescence prevented use of the Yo-Pro method for samples with a high humic content, and high concentrations of particulate material interfered with the TEM protocol. As was the case for the virus stocks, counts made with the Yo-Pro method were consistently higher than those made by TEM, and the average C.V. for triplicate samples was lower (7% vs. 20%, respectively). Estimates of virus abundance using TEM were only 14–86% (avg, 43%) of those obtained with the Yo-Pro method (Table 1) although the results for the two methods were correlated ($r = 0.87$, $P < 0.01$, $n = 36$). The estimates were most comparable when the concentration of suspended matter was lowest.

Unfortunately, as the degree of underestimation depends on the characteristics of the sample, a single factor cannot be applied to correct estimates of virus concentration made with TEM. For example, estimates made with TEM will be less accurate for productive environments because particle concentrations will be higher than in oligotrophic habitats. Viruses can also be lost when uranyl acetate is wicked off the grids subsequent to staining or the grids are rinsed to prevent formation of salt crystals. For example, the error was significantly larger (t-test, $P < 0.05$) for marine samples (2.2–7.1-fold) than for freshwater samples (1.2–2.3-fold), presumably because the grids for the marine samples were rinsed to remove salts (Table 1). Nonetheless, even for freshwater samples, estimates of virus abundance made with TEM were only 66%, on average, of those based on the Yo-Pro method.

Other difficulties include correcting for the fact that virus particles will not sediment in parallel paths (Suttle 1993) and that small viruses may not be recovered with 100% efficiency during ultracentrifugation (Børsheim et al. 1990; Hara et al. 1991). As well, it is often not possible to count all areas of the grid because the viruses and stain are not uniformly distributed across the grid surface, making it very difficult to get accurate estimates of virus abundance. The above difficulties are eliminated or substantially reduced with the Yo-Pro staining protocol.

An alternate explanation for the different estimates obtained with epifluorescence microscopy and TEM is that fluorescent particles other than viruses were counted. Potential sources of error include autofluorescent particles, very small bacteria, nucleic acids associated with particles, or other particles which stain in a similar manner to nucleic acids. Such particles would have to be extremely small and stain brightly to be confused with viruses.

Particles that could be confused with stained viruses were not present in the stain or in unstained samples. However, in some samples, small dimly fluorescent coccoid bacteria were present that were difficult to distinguish from viruses. Therefore, we counted DAPI-stained bacteria as well as Yo-Pro-stained viruses in natural water samples (Table 2). Even if all the bacteria that were visible by DAPI staining were also counted as viruses, the bacteria would not have contributed significantly to our estimates of virus abundance. In all cases the bacterial abundance was at least 4 times less than the standard deviation of virus counts for triplicate samples. As well, the yellow fluorescence of detritus, and the distinctive shape (e.g. rods, bacilli, or filaments) and much brighter fluorescence of most bacteria, generally distinguished these particles from viruses. Finally, viral-size particles which stained in a similar manner to viruses could result in

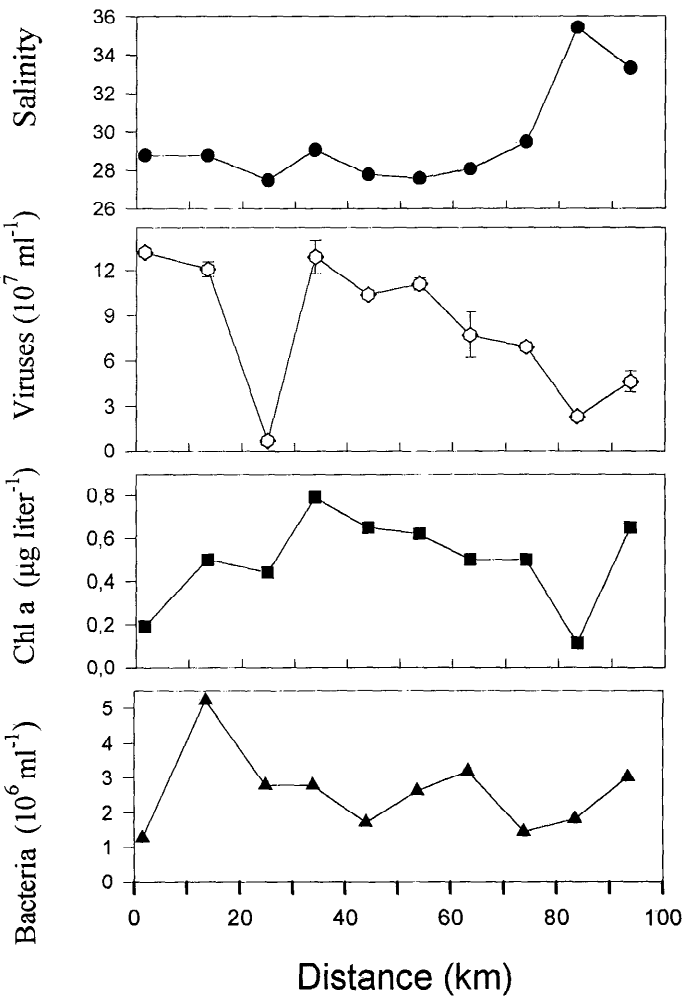

Fig. 3. Salinity, abundance of Yo-Pro-stained viruses and DAPI-stained bacteria, and chlorophyll *a* concentration along a transect offshore from Port Aransas into the western Gulf of Mexico. Error bars on the estimates of virus abundance respresent the standard deviation of duplicate samples; where they are not shown they are less than the width of the symbols.

overestimates of viral abundance. Theoretically, these particles could include free ribosomes, mitochondria, or extremely small particles that bind nucleic acids. It seems extremely unlikely that free mitochondria or ribosomes could persist and be abundant in seawater.

Table 2. Counts of viruses (Yo-Pro) and bacteria (DAPI) in natural water samples.

	Viruses*	Bacteria*
	(10^7 ml^{-1})	
MSI pier		
1200 hours	28.3±1.9	0.35±0.01
1400 hours	23.7±1.9	0.43±0.01
1630 hours	26.1±1.4	0.15±0.02
1900 hours	22.5±1.0	0.06±0.01

* Average and standard deviation of triplicate samples.

Table 3. Counts of viruses in DNase-treated samples.

	Yo-Pro counts* (10^7 viruses ml^{-1})		
Location	DNase-treated samples	Untreated samples	Treated/ untreated
Aquarium	14.6±0.9	14.7±0.7	1.00
Boat harbor	17.2±1.2	15.3±0.2	1.12
Seawater tank	36.3±1.4	34.7±1.3	1.04
MSI pier	18.9±0.8	18.8±1.1	1.00

* Average and standard deviation of duplicate samples.

We tested for the presence of unprotected DNA that may have been associated with particles by incubating samples in the presence of DNase. Estimates of virus abundance in Yo-Pro-stained samples to which DNase was added were not significantly different from samples that were not treated with DNase before filtration (paired *t*-test, $P > 0.05$; C.V. = 4%) (Table 3), indicating that DNase-sensitive DNA associated with particles was not responsible for the discrepancy between counts of viruses made by TEM and epifluorescence microscopy.

Unfortunately, there is no test that can absolutely eliminate the possibility that viral-size particles other than viruses are stained by Yo-Pro. However, such particles would have to be extremely abundant, of relatively uniform size, and enriched with nucleic acids or another unknown substance that reacts with Yo-Pro in a similar way to nucleic acids. At present, we are unaware of any particles other than viruses that fit these criteria. Overall, there is good evidence that the discrepancy between TEM and Yo-Pro estimates of virus abundance stems from the TEM protocol underestimating virus concentrations.

The stability of Yo-Pro-stained viruses stored at −20°C was tested by recounting triplicate slides 1 d, 6 d, 2 months, and 4 months after preparation. There was no significant change in viral numbers after 4 months (paired *t*-test, $P > 0.05$) and coefficients of variation remained within the range of those for most of the natural water samples (Tables 1 and 4).

The Yo-Pro method was used to determine the concentrations of viruses along a transect in the western Gulf of Mexico. Virus abundances ranged from $>10^8$ ml^{-1} at the stations closest to shore to $\sim 4 \times 10^7$ ml^{-1} at the most offshore station, although the lowest abundance ($\sim 10^7$ ml^{-1}) occurred relatively nearshore (Fig. 3). These estimates are considerably greater than others reported for

Table 4. Effect of storage of prepared slides on estimates of virus concentration. The percent coefficient of variation (%C.V.) of triplicate samples is shown in parentheses.

	Virus concn* (10^7 ml^{-1})	
Date of count	MSI pier	Boat harbor
7 Jan 94	11.1(2)	14.8(7)
13 Jan 94	11.5(7)	14.2(4)
22 Mar 94	10.9(3)	14.6(2)
9 May 94	11.1(3)	15.1(2)

* Triplicate slides stored at −20°C.

the southeastern Gulf of Mexico (Boehme et al. 1993) and for most coastal marine environments (*see* Børsheim 1993). The concentrations of chlorophyll and bacteria varied within a relatively narrow range (from ~ 0.01 to 0.8 μg liter^{-1}, and 1–5 $\times$ 10^6 ml^{-1}, respectively) and were not correlated with viral abundance ($r < 0.32$). Others have also found that bacteria and virus concentrations are not necessarily highly correlated (Cochlan et al. 1993; Paul et al. 1993), although when waters of wide-ranging trophic status are compared, more productive environments tend to have higher virus abundances (Paul et al. 1991; Weinbauer et al. 1993).

Our results indicate that the concentration of viruses in marine and freshwaters is probably underestimated by the TEM method; hence, the abundance of viruses in natural waters is likely several-fold higher than indicated by previous studies. In addition, the Yo-Pro procedure can be performed on ship or in remote locations without need for expensive equipment. Similar to the TEM method, the Yo-Pro protocol does not provide any information on the infectivity of the virus particles that are counted nor what the potential hosts might be. Nonetheless, the method should be suitable for many laboratory and field applications as well as for routine environmental analysis.

References

BERGH, Ø., K. Y. BØRSHEIM, G. BRATBAK, AND M. HELDAL. 1989. High abundance of viruses found in aquatic environments. Nature **340:** 467–468.

BOEHME, J., AND OTHERS. 1993. Viruses, bacterioplankton, and phytoplankton in the southeastern Gulf of Mexico: Distribution and contribution to oceanic DNA pools. Mar. Ecol. Prog. Ser. **97:** 1–10.

BØRSHEIM, K. Y. 1993. Native marine bacteriophages. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. **102:** 141–159.

———, G. BRATBAK, AND H. HELDAL. 1990. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. Appl. Environ. Microbiol. **56:** 352–366.

COCHLAN, W. P., J. WIKNER, G. F. STEWART, D. C. SMITH, AND F. AZAM. 1993. Spatial distribution of viruses, bacteria and chlorophyll *a* in neritic, oceanic and estuarine environments. Mar. Ecol. Prog. Ser. **92:** 77–87.

COTTRELL, M. T., AND C. A. SUTTLE. 1991. Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter, *Micromonas pusilla.* Mar. Ecol. Prog. Ser. **78:** 1–9.

HARA, S., K. TERAUCHI, AND I. KOIKE. 1991. Abundance of viruses in marine waters: Assessment by epifluorescence and transmission electron microscopy. Appl. Environ. Microbiol. **57:** 2731–2734.

HIRONS, G. T., J. J. FAWCETT, AND H. A. CRISSMAN. 1994. TOTO and YOYO: New very bright fluorochromes for DNA content analyses by flow cytometry. Cytometry **15:** 129–140.

JIANG, S. C., AND J. H. PAUL. 1995. Viral contribution to dissolved DNA in the marine environment as determined by differential centrifugation and kingdom probing. Appl. Environ. Microbiol. **61:** 317–325.

PAUL, J. H., S. C. JIANG, AND J. B. ROSE. 1991. Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. Appl. Environ. Microbiol. **57:** 2197–2204.

———, J. B. ROSE, S. C. JIANG, C. A. KELLOGG, AND L. DICKSON. 1993. Distribution of viral abundance in the reef environment of Key Largo, Florida. Appl. Environ. Microbiol. **59:** 718–724.

PARSONS, T. R., Y. MAITA, AND C. M. LALLI. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon.

PORTER, K. G., AND Y. S. FEIG. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:** 943–948.

PROCTOR, L. M., AND J. A. FUHRMAN. 1990. Viral mortality of marine bacteria and cyanobacteria. Nature **343:** 60–62.

———, AND ———. 1992. Mortality of marine bacteria in response to enrichments of the virus size fraction from seawater. Mar. Ecol. Prog. Ser. **87:** 283–293.

SUTTLE, C. A. 1993. Enumeration and isolation of viruses, p. 121–134. *In* P. F. Kemp et al. [eds.], Handbook of methods in aquatic microbial ecology. Lewis.

———, AND A. M. CHAN. 1993. Marine cyanophages infecting oceanic and coastal strains of *Synechococcus*: Abundance, morphology, cross-infectivity and growth characteristics. Mar. Ecol. Prog. Ser. **92:** 99–109.

———, ———, AND M. T. COTTRELL. 1990. Infection of phytoplankton by viruses and reduction of primary productivity. Nature **347:** 467–469.

———, AND F. CHEN. 1992. Mechanisms and rates of decay of marine viruses in seawater. Appl. Environ. Microbiol. **58:** 3721–3729.

WEINBAUER, M. G., D. FUKS, AND P. PEDUZZI. 1993. Distribution of viruses and dissolved DNA along a coastal trophic gradient in the northern Adriatic Sea. Appl. Environ. Microbiol. **59:** 4047–4082.

WOMMACK, K. E., R. T. HILL, M. KESSEL, E. COHEN, AND R. R. COLWELL. 1992. Distribution of viruses in the Chesapeake Bay. Appl. Environ. Microbiol. **58:** 2965–2970.

Submitted: 29 March 1994
Accepted: 11 April 1995
Amended: 5 June 1995