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Comparison of epifluorescence and transmission electron microscopy for counting viruses in natural marine waters

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ABSTRACT: Transmission electron microscopy (TEM) and epifluorescence microscopy of DAPI and Yo-Pro-1 stained samples were used to estimate viral abundance in natural communities along a transect from the oligotrophic central Gulf of Mexico to the productive near-shore waters at Port Aransas, Texas (USA). Estimates of viral abundance based on TEM averaged only 66% (range 26 to 108%) of those made using epifluorescence microscopy and the cyanine-based dye, Yo-Pro-1. DAPI staining provided estimates that were much closer and averaged 86% (range 72 to 109%) of those made using Yo-Pro. However, all 3 methods provided similar estimates at viral abundances $<10^6$ ml^{-1}. The precision of the Yo-Pro and DAPI methods (coefficient of variation 8 and 11%, respectively) was much greater than for the TEM method (25%). Experiments with cultures indicated that grazing by flagellates was unlikely to be a significant source of viral-size particles that could interfere with the DAPI or Yo-Pro method. Estimates of viral abundance made using the Yo-Pro method ranged from 0.3×10^6 to 79×10^6 ml^{-1} in surface water along the transect. Across the investigated environments viral and bacterial abundances were well correlated ($r = 0.929$), although the slope of the relationship was significantly greater than 1, indicating that viral abundance increased more rapidly than that of bacteria. These results extend previous observations by showing that epifluorescence microscopy is suitable for counting viruses in very oligotrophic waters, that DAPI and Yo-Pro stained samples provide similar estimates of viral abundance and that grazing by flagellates is not a significant source of particles that could interfere with the epifluorescence method. The study supports the use of epifluorescence microscopy over TEM for obtaining accurate estimates of viral abundances in natural waters.

KEY WORDS: Virus enumeration · Gulf of Mexico · Yo-Pro · DAPI · Virus abundance

INTRODUCTION

Viruses are an important and dynamic component of aquatic microbial food webs, and because they are responsible for significant mortality in microbial communities (e.g. Suttle 1994) they potentially affect nutrient flow and community composition. Estimates of high viral abundances ($<10^5$ to $>10^8$ particles ml^{-1}) in surface waters were originally obtained by concentrating natural viral communities by ultracentrifugation (Bergh et al. 1989, Børsheim et al. 1990) or ultrafiltration (Proctor & Fuhrman 1990) and quantifying virus-like particles by transmission electron microscopy (TEM). The high cost and limited availability of TEM led a number of investigators (Suttle et al. 1990, Hara et al. 1991, 1996, Proctor & Fuhrman 1992, Fuhrman et al. 1993, Suttle 1993) to use DAPI (4',6-diamidino-2-phenylindole) to stain viruses so that they could be enumerated by epifluorescence microscopy, modifying methods used to count bacteria (Porter & Feig 1980). Recently, a cyanine-based dye, Yo-Pro-1 {4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethyledene]-1-(3'-trimethylammoniumpropyl)-quinoliniumdiiodide} (Molecular Probes) was substituted for DAPI (Hennes & Suttle 1995) and results from this study indicate that the TEM

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method can significantly underestimate viral abundance in some natural waters.

There are a number of reasons why it is important to have accurate estimates of viral abundance in marine waters. One reason is to estimate viral production rates. Viral populations are dynamic, and as most viruses are produced via lysis of host cells, one way to infer the effect of viruses on microbial populations is to use direct counts of viral particles to determine the rate at which viruses are produced (e.g. Heldal & Bratbak 1991, Weinbauer & Suttle 1996). Secondly, accurate estimates of viral abundance can be important in determining the contribution of viruses to specific pools. For example, marine viruses are typically smaller than 0.2 µm (Børsheim 1993) and are included in measurements of dissolved DNA. Dissolved DNA includes DNase digestible and non-digestible fractions, with the latter consisting of viruses and an uncharacterized component that together constitute about 50 to 90% of the total dissolved DNA (Beebee 1991, Paul et al. 1991, Maruyama et al. 1993, Jiang & Paul 1995). Data suggest that viral DNA can be either a minor (Paul et al. 1991, Boehme et al. 1993, Jiang & Paul 1995) or a major (Weinbauer et al. 1993, 1995) contributor to the pool of dissolved DNA. One difficulty is that estimates of viral DNA can depend upon determinations of viral abundance. For instance, based on TEM counts, Jiang & Paul (1995) calculated that viruses accounted for only 17 to 30% of the DNase insensitive dissolved DNA. However, if the TEM method underestimated viral abundance, as has been suggested (Hennes & Suttle 1995), then the contribution of viruses to the dissolved DNA pool would also be underestimated. Another reason that accurate estimates of viral abundance are required is to determine contact rates between viruses and bacteria; contact rates are proportional to the abundance of each (Murray & Jackson 1992). Consequently, calculations such as the proportion of contacts that result in infection require accurate estimates of viral abundance.

One of the concerns with using epifluorescence microscopy to enumerate viral particles is the possibility that DAPI or Yo-Pro may stain non-viral particles of similar size. This seems unlikely as the particles would have to be extremely numerous, of virus-size, and not recognizable using TEM. One potential source of interference are membrane bound particles that are produced by flagellates grazing on bacteria and which are resistant to enzyme digestion (Nagata & Kirchman 1992). It is possible that these particles include DNA that can be stained with Yo-Pro and DAPI and visualized by epifluorescence microscopy.

The purpose of this investigation was to further examine the use of nucleic acid stains and epifluorescence microscopy to quantify viruses in seawater. Currently, quantitative data are not available comparing estimates of viral abundance made with DAPI and Yo-Pro stained samples, as well as TEM. Our goals in this study were 4-fold. First, we wished to determine if repeatable estimates of viral abundance could be made using DAPI stained natural samples. In order to do this we used a microscope equipped with better optics than previously employed. Second, we wanted to quantitatively compare the 3 approaches of counting viruses (DAPI, Yo-Pro, TEM) and to determine if previous observations that estimates of viral abundance in coastal waters were lower using TEM were confirmed. Third, we wished to extend observations of viral abundance into more oligotrophic environments than had previously been examined using the 3 methods. Finally, we wanted to determine if grazing of protists on bacteria produced particles that interfere with estimates of viral abundance made by epifluorescence microscopy.

MATERIAL AND METHODS

Sample collection. Water samples were collected from the pier of the Marine Science Institute, Port Aransas, Texas, USA (27° 50' N, 97° 02' W), as well as along a transect from the central to the western Gulf of Mexico (Stn A: 26° 14' N, 95° 11' W; Stn B: 25° 19' N, 94° 07' W; Stn C: 25° 41' N, 94° 26' W; Stn E: 27° 00' N, 96° 14' W; Stn F: 27° 32' N, 96° 45' W). Samples were pumped from the surface (0.5 m) or collected from the deep chlorophyll maximum (Stn A, 98 m; Stn E, 72 m) using Niskin bottles mounted on a rosette equipped with a Sea-Bird CTD (model SBE 9 plus).

Enumeration of viruses and bacteria. For the TEM method, viruses were preserved in glutaraldehyde (2% final concentration) and enumerated using the protocol of Bergh et al. (1989) as described in Suttle (1993). Briefly, viruses were collected quantitatively onto Formvar-coated, 400-mesh electron-microscope grids by centrifugation in a swinging-bucket rotor (SW 40; 25 000 rpm, 77100 × g, for 3 h). This will pellet particles of 110 S with 100% efficiency. The grids were stained for 30 s with 1% uranyl acetate and rinsed 3 times with deionized-distilled water. Typically, for the TEM method viruses from ca 10 ml of sample were pelleted onto electron-microscope grids; however, volumes were increased up to 50 ml for the oligotrophic offshore waters. This was accomplished by pelleting viruses from a 10 ml aliquot, removing the supernatant, and pelleting the next 10 ml aliquot. This procedure was repeated until the viruses from up to 50 ml of sample were collected onto the grids. Regardless of the method used at least 200 viral particles were counted per replicate sample.

Viruses were also enumerated using epifluorescence microscopy following staining with Yo-Pro (Hennes & Suttle 1995) or DAPI (Suttle 1993), as described in the cited references. For the Yo-Pro method, viruses in 0.1 to 2.4 ml of unfiltered sample were gently filtered (15 kPa vacuum) onto 0.02 µm pore-size Anodisc filters (Whatman) using a 10 place filtration unit (Hoeffer). While still damp the filters were placed on 80 µl drops of Yo-Pro-1 for 2 d. Since fixation with aldehydes interferes with binding of Yo-Pro, viruses were filtered and stained immediately after collection. Subsequent to staining the filters were rinsed twice by filtering 800 µl of deionized-distilled water through the membrane. The damp membranes were transferred to glass slides, covered with a drop of glycerol and a cover slip. Once stained in this manner the samples are stable for several months. The slides were counted using an Olympus BX 40 microscope equipped with a UPlanApo 100× objective and a wide blue (WB) filter set.

For the DAPI protocol, unfiltered samples were preserved with glutaraldehyde (2% final concentration), and 1 part DAPI solution (15 µg ml^{-1} in McIlvaine's buffer) was added to 15 parts of sample (1 µg ml^{-1} final DAPI concentration) and incubated in the dark for 30 min. The stained samples were collected onto Anodisc filters (Fuhrman et al. 1993, Suttle 1993) and while still damp placed on a glass slide and then counted using a wide UV (WU) filter set as described above. Samples for bacteria were also stained with DAPI as described in Turley (1993) and filtered onto 0.2 µm pore-size polycarbonate filters (Poretics), to compare with estimates of bacterial abundance obtained on the 0.02 µm pore-size Anodisc filters. Filter blanks to check for contamination of fluorescent or staining particles in the preservative, stains and filtration apparatus were prepared for all protocols and were found to be insignificant.

Flagellate cultures. Six isolates of marine flagellates were used for experiments. *Paraphysomonas imperforata* (strain VS1) and *Pseudobodo parvulus* (ATCC 50091, formerly *Bodo parvulus*; Garza & Suttle 1995) were kindly provided by David Caron. The bodonid strains E1 and E4 (both *Bodo* sp.), as well as the strains E9 and B3 which were tentatively identified as *Bodo* sp., were isolated by Juan González. Flagellates were maintained in exponential growth by adding a 10% inoculum of an exponentially growing culture into autoclaved seawater which had been filtered through a 30 000 MW cutoff ultrafiltration cartridge (Suttle et al. 1991). Bacteria which served as food for the flagellates were also transferred during inoculation. Samples for the enumeration of bacteria, flagellates and virus-size particles were collected from the incubations and preserved in glutaraldehyde (final concentration: 2%). Flagellates were stained with Lugol's iodine and counted with a hemocytometer. Bacteria and virus-size particles were stained with DAPI and filtered onto 0.02 µm pore-size filters as described above. The abundance of virus-size particles was also determined at the end of the experiments using the Yo-Pro method. Viruses and non-viral particles <0.2 µm were also enumerated using TEM.

RESULTS AND DISCUSSION

Epifluorescence microscopy of DAPI and Yo-Pro stained samples and transmission electron microscopy were used to estimate viral abundance in seawater ranging from the oligotrophic central Gulf of Mexico to highly productive waters along the Texas coast. Estimates of viral abundance made on DAPI and Yo-Pro stained samples were similar, whereas the TEM-based method consistently resulted in lower estimates, when viral abundance exceeded ca 10^6 ml^{-1}. Moreover, flagellate grazing was not a significant source of virus-size particles that could be stained with DAPI or Yo-Pro. These results are examined in detail below.

Comparison of methods for counting viral numbers

Estimates of viral abundance in DAPI stained samples were 77 to 109% (mean 86%, n = 14) of those obtained with Yo-Pro (Table 1). Although the difference was generally small, it was significant (2-tailed paired *t*-test; $p < 0.05$). In contrast, TEM-based estimates were frequently much less than those made with Yo-Pro (35 to 103%, mean 63%, n = 12). The precision of the DAPI protocol was slightly less than for the Yo-Pro method, but greater than for TEM; the average coefficient of variation (SD × 100/mean) of triplicate samples for the DAPI, Yo-Pro and TEM methods was 11, 8 and 25%, respectively. The difference in results for the DAPI and Yo-Pro samples may result from the fact that DAPI only stains double-stranded DNA, whereas Yo-Pro stains double-stranded and single-stranded DNA, as well as RNA. Thus, the presence of single-stranded DNA or RNA viruses might have contributed to the small difference between the 2 methods. In addition, the lower brightness and faster fading made DAPI stained samples more difficult to count. Based on our experience, DAPI stained viruses are near the limit of detection by epifluorescence microscopy. We found a noticeable difference in our ability to visualize viruses by DAPI staining when we switched from an older Olympus IMT-2 inverted microscope to an optically superior Olympus BX-40 microscope equipped with a UPlanApo 100× objective.

Table 1. Abundance of viruses in natural marine water determined by transmission electron microscopy (TEM) and by epifluorescence microscopy of DAPI and Yo-Pro stained samples. The percent coefficient of variation of triplicate samples is shown in parenthesis. MSI: Marine Science Institute, Port Aransas, Texas, USA; DCM: deep chlorophyll maximum; ND: not determined

Location	Date	DAPI	Yo-Pro (10^6 ml^{-1}) (%CV)	TEM	DAPI/Yo-Pro	TEM/Yo-Pro
MSI pier	26 Nov 1994	29.8 (12)	36.6 (9)	ND	0.81	ND
MSI pier	9 Jun 1995	20.3 (11)	28.3 (5)	10.4 (8)	0.72	0.37
Stn A	21 Jun 1995	0.3 (10)	0.3 (5)	0.3 (29)	0.92	0.81
Stn A_{DCM}	21 Jun 1995	0.8 (9)	1.0 (4)	ND	0.84	ND
Stn B 04:00 h	22 Jun 1995	0.4 (14)	0.4 (20)	0.4 (24)	0.83	1.03
Stn B 08:00 h	22 Jun 1995	0.3 (10)	0.4 (6)	0.4 (27)	0.77	0.95
Stn C 04:00 h	24 Jun 1995	0.3 (20)	0.3 (9)	0.3 (44)	1.05	0.96
Stn C 08:00 h	24 Jun 1995	0.2 (5)	0.3 (6)	0.3 (19)	0.80	1.08
Stn E 04:00 h	26 Jun 1995	4.8 (11)	5.3 (5)	2.1 (37)	0.91	0.39
Stn E 08:00 h	26 Jun 1995	5.1 (7)	4.7 (2)	1.2 (27)	1.09	0.26
Stn E_{DCM}	27 Jun 1995	5.9 (9)	7.1 (5)	3.0 (25)	0.83	0.42
Stn F 04:00 h	28 Jun 1995	6.4 (16)	7.9 (8)	5.7 (19)	0.81	0.71
Stn F 08:00 h	28 Jun 1995	6.6 (6)	8.2 (8)	4.8 (24)	0.80	0.59
MSI pier	20 Jun 1995	68.9 (9)	79.2 (13)	27.9 (15)	0.87	0.35

The fluorescence of Yo-Pro stained viruses is much greater and more stable than for DAPI stained viruses; however, Yo-Pro has a disadvantage in that samples cannot be preserved with aldehydes and must be filtered and stained immediately after collection (Hennes & Suttle 1995, Suttle 1996). Furthermore, staining requires 2 d for Yo-Pro but only 30 min for DAPI. Since estimates of viral abundance were similar using the Yo-Pro and DAPI methods (Table 1), the DAPI protocol may be particularly useful when the time scale of sampling is short or when estimates of viral abundance must be obtained quickly.

The DAPI, Yo-Pro and TEM methods produced similar results at viral abundances $<10^6$ ml^{-1}, but at higher viral densities the TEM-based estimates were lower (Table 1). These data are consistent with those of Hennes & Suttle (1995) and show that the difference between estimates obtained using epifluorescence microscopy and TEM increases with viral abundance (Fig. 1). Similarly, data from Hara et al. (1991) show that at densities $>10^6$ ml^{-1} TEM counts of viruses were only about 80% of those made using DAPI. In contrast, Proctor & Fuhrman (1992) did not find a significant difference between TEM and DAPI estimates of viral abundance in natural seawater samples and culture lysates. In part, this may have been because the scatter in the relationship was too large to detect a significant difference, and because they may have lost viruses by using 0.1 and 0.08 µm pore-size filters; the majority of marine viruses have a head size smaller than 60 nm (Børsheim 1993). Nonetheless, if a 1:1 line is drawn through the data of Proctor & Fuhrman, in 10 of 12 samples the estimate of viral abundance is greater with the DAPI method than by TEM. Overall, there is good evidence that when viral abundance exceeds ca $>10^6$ ml^{-1}, estimates obtained using epifluorescence microscopy are usually higher than those obtained by TEM. This is probably because particulate material in the samples interferes with the TEM method when viral abundance is high. As viruses have to be concentrated 10- to 1000-fold more to be counted by TEM than by epifluorescence microscopy, this results in a high concentration of particulate material (e.g. cells, cell debris, and detritus) on the surface of the electron microscopy grids which interferes with the estimation of viral abundance (Hennes & Suttle 1995, Suttle 1996).

Very small, dimly fluorescent coccoid bacteria could be confused with viruses. Therefore, we counted bacteria on both 0.02 µm and 0.2 µm pore-size filters. Estimates of bacterial abundance counted on 0.02 µm pore-size filters were 86 to 123% (average 106%, n = 9)

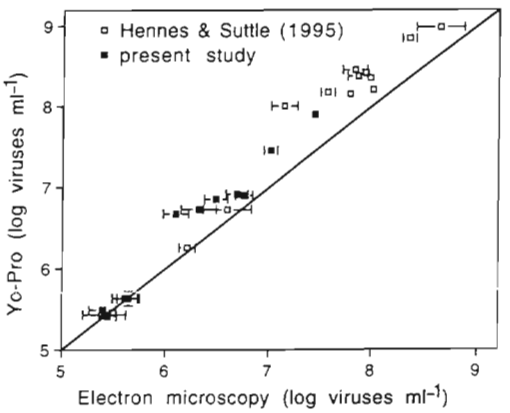

Fig. 1. Counts of viral particles using Yo-Pro and transmission electron microscopy in natural waters. Data from Hennes & Suttle (1995) and this study. Solid line: relationship of 1:1. Standard deviation (x and y error) is calculated from triplicate samples. Regression equation: $y = 1.78x - 5.57$. Where error bars are not shown the SD was smaller than the width of the symbols

of those obtained on 0.2 µm pore-size filters (Table 2). The coefficient of variation was 9% on both types of filters. At bacterial densities $<10^6$ cells ml^{-1}, counts on 0.02 µm pore-size filters were significantly higher (average 17%; 2-tailed paired *t*-test; $p < 0.05$) than the numbers obtained from the 0.2 µm pore-size filters. At higher bacterial densities the counts were similar on both filter types. It is known that a fraction of the bacterial community can pass a 0.2 µm pore-size filter (Stockner et al. 1990). Since the smallest bacteria occur in oligotrophic water where abundance is also the least, this may be the reason for the difference between filters at bacterial densities $<10^6$ ml^{-1}. Moreover, the TEM counts of bacteria at oligotrophic stations were more similar to counts made on 0.02 µm pore-size filters than on 0.2 µm pore-size filters (Table 2). Hara et al. (1996) have also recently compared counts of DAPI stained bacteria on 0.02 and 0.2 µm pore-size Anodisc and polycarbonate filters, respectively. They also found higher estimates of bacterial abundance on the 0.02 µm pore-size membranes. However, as they did not find significant differences in estimates of bacterial abundance made using 0.015 and 0.2 µm pore-size polycarbonate filters, or between 0.02 and 0.20 µm pore-size Anodisc membranes, they interpreted the higher counts on the Anodisc membranes to be the result of the flatter surface of the Anodiscs.

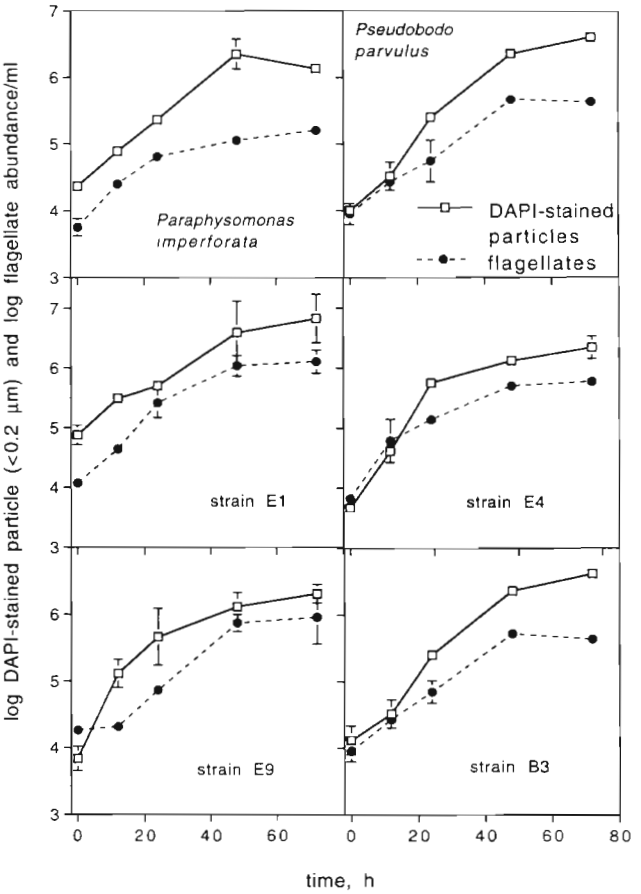

Fig. 2. Abundance of flagellates and DAPI-stained <0.2 µm particles in flagellate cultures. Data are expressed as means ± standard deviations of duplicate cultures. Where error bars are not shown the SD was smaller than the width of the symbols

Virus-size particles produced during flagellate grazing

During grazing by flagellates, colloids or liposome-like structures (Koike et al. 1990, Nagata & Kirchman 1992, Tranvik 1994) and dissolved DNA (Turk et al. 1992) are produced. Thus, it is possible that DNA bound to colloids or trapped into liposome-like structures interferes with the DAPI and Yo-Pro methods for counting viruses. In stock cultures of flagellates, virus-size particles that could be stained with DAPI were undetectable. During exponential growth the numbers of DAPI stained particles increased along with flagellate abundance (Fig. 2). TEM showed that viruses were present in all of the incubations, however, DAPI counts of virus-size particles at the end of the experiments

Table 2. Comparison of bacterial numbers counted on 0.02 and 0.2 µm pore-size filters and determined by TEM. The percent coefficient of variation of triplicate samples is shown in parenthesis. ND: not determined

Location	Date	Bacterial abundance (10^6 ml^{-1})			0.02 µm/0.2 µm	0.02/TEM
		0.02 µm	0.2 µm	TEM		
MSI pier	26 Nov 1994	3.2 (2)	3.0 (10)	ND	1.08	ND
MSI pier	12 Dec 1994	1.6 (5)	1.7 (8)	ND	0.94	ND
MSI pier	9 Jun 1995	6.9 (8)	7.3 (11)	5.9 (12)	0.95	1.17
Stn A	21 Jun 1995	0.5 (6)	0.4 (5)	0.5 (23)	1.23	0.98
Stn B	22 Jun 1995	0.4 (11)	0.3 (5)	0.4 (13)	1.15	0.96
Stn C	24 Jun 1995	0.6 (7)	0.5 (20)	0.6 (21)	1.13	0.97
Stn E	26 Jun 1995	1.5 (7)	1.7 (7)	1.2 (9)	0.86	1.17
Stn F	28 Jun 1995	2.3 (6)	2.1 (9)	1.9 (17)	1.11	1.24
MSI pier	20 Jul 1995	4.6 (12)	4.5 (7)	4.0 (17)	1.06	1.18

Table 3. Final abundances of viruses and non-viral particles <0.2 µm counted by TEM, and of DAPI-positive particles <0.2 µm. Yo-Pro counts of particles <0.2 µm were not significantly different from DAPI counts (2-tailed paired *t*-test; $p > 0.05$; data not shown). Data calculated from duplicate incubations; standard deviations given in parentheses

Flagellate culture	DAPI particles <0.2 µm (10^6 ml^{-1})	TEM counts of viruses (10^6 ml^{-1})	TEM counts of non-viral particles <0.2 µm (10^6 ml^{-1})	TEM counts of non-viral particles/TEM counts of viruses
Paraphysomonas imperforata	1.4 (0.33)	0.7 (0.09)	0.09 (0.01)	0.12
Pseudobodus parvulus	2.9 (1.47)	0.7 (0.27)	0.05 (0.01)	0.07
Bodonid strain E1	8.2 (6.73)	1.3 (0.55)	0.48 (0.21)	0.37
Bodonid strain E4	2.3 (0.98)	0.6 (0.05)	0.07 (0.01)	0.11
Bodonid strain E9	2.1 (0.66)	1.4 (0.34)	0.32 (0.03)	0.23
Bodonid strain B3	4.2 (0.65)	0.9 (0.13)	0.08 (0.01)	0.09

were between 1.5 and 6.2 (mean 3.8, n = 6) times higher than TEM counts of viruses (Table 3). The finding that estimates of viral abundance using TEM were considerably lower than those obtained by DAPI staining is likely a consequence of interference by particulate matter with the TEM method (see above and Hennes & Suttle 1995). The abundance of particulate matter in the samples was high, about 10^5 flagellates ml^{-1} (Fig. 2) and about 10^7 bacteria ml^{-1} (data not shown). The abundance of non-viral particles in the <0.2 µm fraction was also determined by TEM and ranged from 7.1 to 36.9% (average 16.7%) of the TEM counts of viruses (Table 3). This indicates that the majority of the particles <0.2 µm in the flagellate incubations were viruses. The increase in viruses during the incubations (Fig. 2) likely resulted from higher encounter rates between viruses and bacteria during exponential bacterial growth (data not shown).

On average only 13.7% (SD 7.7, n = 6) of the virus-size particles visible in the cultures by TEM were not recognizable as viruses (Table 3). Even if all of these particles were stained by DAPI (which is unlikely), it suggests that less than 15% of the DAPI stained, virus-size particles were non-viral. Therefore, even in exponentially growing flagellate cultures with cell densities $>10^6$ ml^{-1}, only about 0.2×10^6 to 1.1×10^6 particles ml^{-1} could be attributed to non-viral particles <0.2 µm. An even more conservative approach would be to assume that the TEM-based estimates of viral abundance are accurate, and that the difference between the TEM- and DAPI-based counts results from DAPI stained, non-viral particles. Even using these assumptions, the number of DAPI stained particles that were produced (ca 10^6 ml^{-1}) could not account for the observed differences between estimates of viral abundance made using DAPI and TEM in coastal water. In nature, where heterotrophic flagellate abundances are typically 3 orders of magnitude lower, the abundance of such particles would presumably also be much lower.

Viruses stained with DAPI are close to the visual detection limit of epifluorescence microscopy and can only be seen because their DNA is densely packed (Proctor & Fuhrman 1992). Moreover, natural viral communities in which the DNA is uncoiled by heating and re-natured by cooling are no longer discernible as DAPI-positive particles (Suttle et al. 1990). This implies that the DNA in virus-size DAPI-positive particles is densely packed, consistent with such particles being viruses. Given that flagellate grazing does not appear to produce sufficient DAPI-positive virus-size particles to interfere with estimates of viral abundance, and that other processes are not likely to produce tightly packed virus-size DNA, it is reasonable to conclude that estimates of viral abundance obtained by epifluorescence microscopy are accurate.

Viral and bacterial abundances in natural water

Viral abundances in offshore stations determined by TEM are typically in the range of 10^5 to 10^6 ml^{-1}, although occasionally abundances $<10^5$ ml^{-1} are reported (Bergh et al. 1989, Hara et al. 1991, 1996, Boehme et al. 1993, Cochlan et al. 1993). Boehme et al. (1993) found viral abundances in surface waters of the Gulf of Mexico ranging from 3.8 to 8.5×10^5 ml^{-1}. In our study viral abundances were also determined to be about 10^5 ml^{-1} at the most offshore stations using both TEM and epifluorescence microscopy. Given that both methods yield similar estimates in oligotrophic waters (Table 1) it is not surprising that results among studies are comparable. Nearer shore, viral concentrations were about 10^6 ml^{-1} in shelf waters and about 10^7 ml^{-1} in waters collected from the pier of the Marine Science Institute. Numerous other studies have also found similar estimates of viral abundance in coastal waters using TEM (e.g. Bergh et al. 1989, Proctor & Fuhrman 1990, Hara et al. 1991). As estimates of viral abundance by TEM and epifluorescence microscopy are generally within the same order of magnitude (Table 1; Proctor & Fuhrman 1992, Hennes & Suttle 1995), it is reasonable that results should be similar among

studies. However, it is also true that estimates of viral abundance in coastal waters made by TEM are probably underestimates by several fold.

Unlike a previous study (Hennes & Suttle 1995) which found no correlation between bacterial and viral abundances at several of the same stations examined in the present investigation, we found a good relationship between the two (Fig. 3). This is likely because the previous study was restricted to a short transect on the Texas shelf which did not include areas of low viral and bacterial abundances. In the present study viral abundance increased more rapidly than that of bacteria. One explanation for this observation is that bacteria are larger in more productive waters and therefore produce more viruses. This is supported by findings from a cruise in the Gulf of Mexico that burst sizes ranged from 10 to 28 at oligotrophic stations and from 21 to 64 at the productive coastal stations (unpubl. data). Also, burst sizes were higher at eutrophic than at mesotrophic stations in the northern Adriatic Sea (Weinbauer et al. 1993). A second possibility is that viral production is higher at eutrophic than oligotrophic stations. We found that the frequency of visibly infected bacteria was lower at oligotrophic stations (0.1 to 0.3%) than at more productive stations in the Gulf of Mexico (0.7 to 1.6%; unpubl. data). This is consistent with other observations that viral production and the frequency of visibly infected bacteria are generally higher in more eutrophic environments (Steward et al. 1992, 1996, Weinbauer et al. 1993). Therefore, higher burst sizes and higher infection frequencies may be the reason why viral abundance increased more rapidly than that of bacteria along a transect from oligotrophic to productive coastal waters of the Gulf of Mexico. In contrast to our study, Maranger & Bird (1995) found that bacterial and viral abundances increased approximately proportionally (slope = 0.93); the slope of the regression in their study was only 52% of that we obtained. One explanation for the different slopes is that viral abundances in the study of Maranger & Bird were determined by TEM, which might have underestimated viral abundances in productive environments. In addition their data were derived from a wider range of environments than were sampled in the present study.

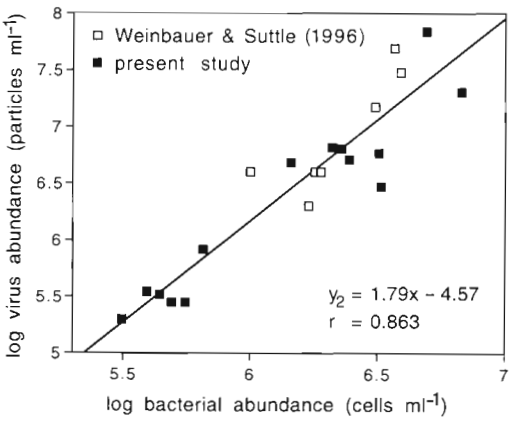

Fig. 3. Relationship between bacterial and viral abundances for marine environments ranging from oligotrophic oceanic to productive coastal waters. Data are from Weinbauer & Suttle (1996) and this study. Viral and bacterial abundances were obtained from direct counts on 0.02 µm pore-size filters using DAPI staining

Counting of Yo-Pro or DAPI stained viruses by epifluorescence microscopy provided fast and inexpensive alternatives to the TEM method and also resulted in more precise and accurate estimates of viral abundance in marine waters. Hennes & Suttle (1995) estimated that in productive coastal waters only about 43% of viruses are counted by TEM, while in the present study we estimated that about 66% were counted. It is significant that given adequate equipment, DAPI can also be used to quantify viruses in natural waters. Moreover, we could find no evidence that grazing on bacteria by flagellates produced particles that could interfere with estimates of viral abundance determined by epifluorescence microscopy. Consequently, epifluorescence microscopy should provide a superior alternative to TEM for estimating viral abundance in natural waters.

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