MICROBIAL ECOLOGY

Microb Ecol (2002) 43:168–173
DOI: 10.1007/s00248-001-1021-9

A Dilution Technique For The Direct Measurement Of Viral Production: A Comparison In Stratified and Tidally Mixed Coastal Waters

S.W. Wilhelm,[1] S.M. Brigden,[2] C.A. Suttle[2]

[1] Department of Microbiology, The University of Tennessee, Knoxville, TN, USA
[2] Departments of Earth and Ocean Sciences, Botany and Microbiology and Immunology, The University of British Columbia, Vancouver, BC, Canada

Received 10 April 2001; Accepted 12 September 2001; Online Publication: 23 January 2002

ABSTRACT

The abundance of heterotrophic bacteria and viruses, as well as rates of viral production and virus-mediated mortality, were measured in Discovery Passage and the Strait of Georgia (British Columbia, Canada) along a gradient of tidal mixing ranging from well mixed to stratified. The abundances of bacteria and viruses were approximately 10^6 and 10^7 mL^{-1}, respectively, independent of mixing regime. Viral production estimates, monitored by a dilution technique, demonstrated that new viruses were produced at rates of 10^6 to 10^7 mL^{-1} h^{-1} across the different mixing regimes. Using an estimated burst size of 50 viruses per lytic event, ca. 19 to 27% of the standing stock of bacteria at the stratified stations and 46 to 137% at the deep-mixed stations were removed by viruses. The results suggest that mixing of stratified waters during tidal exchange enhances virus-mediated bacterial lysis. Consequently, viral lysis recycled a greater proportion of the organic carbon required for bacterial growth under non-steady-state compared to steady-state conditions.

Introduction

Primary productivity in the inlets and channels along the western coast of British Columbia (Canada) is greatly affected by tidally driven mixing [17, 19]. Biological fronts develop at the interface between well-mixed and stratified waters. These frontal boundaries are characterized by high rates of primary productivity and phytoplankton biomass. For example, rates of primary productivity at a tidal front near Saanich Inlet have been shown to range from 2.8 to 6.3 g C m^{-2}, while rates within the stratified waters of the inlet were 1.1 to 2.2 g C m^{-2} [18].

Over the past decade, viruses have been shown to be ubiquitous in marine [4, 5, 27, 30] and freshwater [8, 13, 24, 28] environments. Because viruses are known to be subject to a number of loss factors [11, 23, 31], their persistence at high abundance indicates that removal and replacement rates are relatively balanced. This also implies that viruses play an integral role in the destruction of planktonic organisms, as these obligate pathogens destroy bacteria and phytoplankton to proliferate.

Correspondence to: Curtis A. Suttle; E-mail: csuttle@eos.ubc.ca

One way to infer the production rate of viruses in aquatic systems is from the decay rates of viral particles [7, 25] or infectivity [23, 25, 31] and to assume that decay is balanced by production. However, studies of viral destruction rates can produce results that are difficult to interpret [1, 7, 26]. Furthermore, natural viral communities differ in their sensitivity to damaging solar radiation [6, 15, 26, 31], implying that decay rates of isolates are not always good proxies for natural viral communities. Another technique that has been used to infer viral production rates is monitoring the incorporation of ^{32}P into the DNA of the viral size fraction [21]. This technique, however, is susceptible to error associated with the breakthrough of labeled bacterial DNA into the viral fraction and the availability of appropriate conversion factors to convert radioactivity into viral production. A third technique used to estimate viral production involves the addition of fluorescently labeled viral tracers [16]. In this technique, viral isolates are stained with a fluorescent dye and added to a water sample. The rate of change of abundance of fluorescent viruses as well as the change in total virus abundance is then used to estimate a production rate. Although this technique also provides estimates of viral production rates, it requires significant preparation to produce the fluorescent viral tracers and can only be used to measure production rates in the dark.

Ideally, viral production should be measured as directly as possible, without conversion factors and using the native microbial population. To accommodate these considerations we used a dilution technique to investigate rates of viral production along a gradient of tidal mixing that ranged from homogeneously mixed to stratified. The technique reduces the background of free viruses through the addition of virus-free seawater, allowing rates of viral production to be readily monitored from small changes in viral concentration. As the viruses produced are from cells infected prior to the beginning of the experiment, altered contact rates between viruses and bacteria because of changes in virus or host abundances will not affect cell-specific viral production rates.

Our results indicated that strong tidal mixing resulted in high viral abundances and production rates despite relatively low growth rates of bacteria. As well, viral-mediated lysis of bacteria appeared to be important in maintaining the cycling of organic carbon supplying bacterial growth. The results have implications for the regulation of bacterial productivity and regeneration of nutrients from viral lysis in areas of strong tidal mixing.

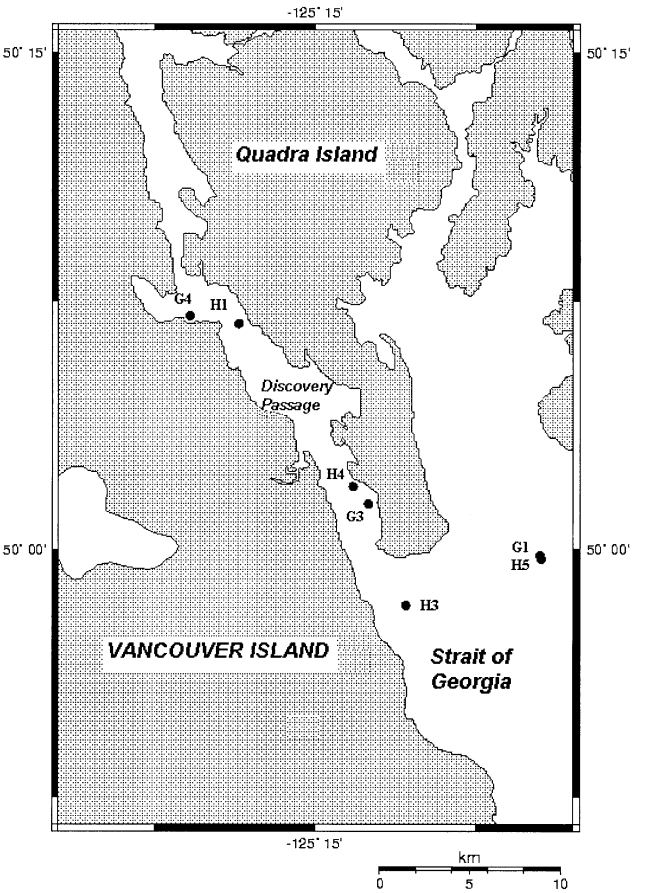

Fig. 1. Location of the sampling sites in and near Discovery Passage in the northern Strait of Georgia, British Columbia, Canada.

Materials and Methods

Study Site, Characteristics, and Sample Collection

Discovery Passage is a channel between Vancouver Island and Quadra Island (Fig. 1) through which the northern portion of the Strait of Georgia flushes. At its narrowest point (Seymour Narrows, the narrowest part of Discovery Passage near our northern station HI), tidal flows can exceed 20 km h^{-1}, resulting in complete mixing of the water column. As stratified water moves northward from the Strait of Georgia into Discovery Passage during the ebb tide, the current speed increases and the water column becomes progressively well mixed, before becoming completely mixed as it passes through Seymour Narrows. On the flood tide, the completely mixed water flows southwards, back through Seymour Narrows. Consequently, the water that flows southward into Discovery Passage during the flood tide has been mixed for a longer time than the water flowing northward on the ebb tide. Stations G1, G3, and G4 were sampled on 25 June 1997 as water from the Strait of Georgia moved northward into Discovery Passage on the ebb tide. Stations H1, H3, H4, and H5 were sampled on 26 June 1997, as water moved southward out of the passage and into the Strait on the flood tide. At each station,

salinity, temperature, and *in situ* chlorophyll fluorescence were determined using a CTD and fluorometer. The instruments were attached to a rosette frame that held an array of 10-L Niskin bottles for sampling at discrete depths.

Abundance of Viral Particles and Bacteria

Viral particles were quantified by epifluorescence microscopy following YO-PRO-1 staining (Molecular Probes) [9]. Samples were not prefiltered or treated with DNase. The high viral abundance required that 100-µl samples were diluted with 700 µl of ultrafiltered (<30,000 Da) seawater. The virus-free ultrafiltrate was prepared from near surface (ca. 2-m depth) water using an S10Y30 ultrafiltration cartridge (Amicon) as previously described [2]. Viruses were collected on 0.02-µm pore-size filters (Anodisc 25, Whatman) immediately after sampling, stained for 48 h, and enumerated with an Olympus BX60 epifluorescence microscope. Bacteria were enumerated in acridine-orange-treated water samples (2 ml) after collection on 0.2-µm pore-size black polycarbonate filters (Millipore GTBP) [10], The model of Murray and Jackson [14] was used to calculate virus–host contact rates ($cell^{-1}$ d^{-1}) as previously described [22]. The percentage of bacterial cells lysed d^{-1} was determined from the rate of virus-mediated bacterial destruction (the rate of viral production divided by an assumed burst size of 50 viruses produced per lytic event) divided by the total bacterial abundance. Contact success (the proportion of virus–host contacts that result in an infection) was determined from the estimated abundance of cells lysed per day *in situ* corrected for the total number of contacts [22, 25]. This assumes that the number of cells lysed is equal to the number of successful contacts.

Estimates of Viral Production

The rate of viral production in surface water samples was determined from the production rate of new viral particles after the dilution of the *in situ* viral community. Surface water samples (300 mL) were collected and gently vacuum filtered (<258 mm Hg) through 47-mm diameter, 0.2-µm pore-size polycarbonate filters (Millipore). During this process, the sample was mixed by drawing water into and injecting it from a transfer pipette, while volume was maintained (>50 mL, final volume = 300 mL) by adding virus-free, ultrafiltered seawater (<30,000 Da). This resulted in viruses being diluted to ca. 10–20% of the initial abundance. Samples (50 ml) of this virus-reduced retentate were placed in 250-ml polycarbonate flasks and incubated in the dark at *in situ* temperatures. Subsamples (ca. 1 mL) for viral enumeration were collected every 3 h and stained with YO-PRO-1 as described (without dilution). This time frame was chosen to exclude viruses produced as a result of infections occurring during incubation. Viral production rates were determined from first-order regressions of viral abundance vs time for triplicate incubations. Mean estimates and standard deviations for viral production rates were determined from the slope of abundance vs time for independent replicates. Viral turnover rates were estimated by dividing viral production rates by the viral abundance in surface-water samples after correcting for the dilution of the bacterial hosts between the samples and the natural seawater community. The correction for lost bacteria is necessary to account for the loss of potentially infected cells during the diafiltration. Viral production will be proportional to cell abundance (i.e., cell-specific rates should be constant for a sample); therefore, viral production rates must be corrected for lost cells.

Results

Station Characteristics

Stations were separated into stratified, partially stratified, and well-mixed (Table 1) based on vertical profiles of temperature and salinity (data from G Stations shown in Fig. 2; data from H Stations not shown). Stations G1 and H5 (Fig. 1) were stratified and located outside of Discovery Passage; Stations G3, H3, and H4 were partially stratified and located at the southern end of Discovery Passage; Stations G4 and H1 were near Seymour Narrows and were well mixed.

Bacterial and Viral Abundance And Production

Bacterial abundance *in situ* ranged from 0.6 to 2.1×10^6 mL^{-1} and was reduced to ca. 11 to 43% of ambient concentrations subsequent to rinsing with virus-free water (Table 2). No trend was seen between station characteristics and bacterial abundance.

Table 1. Maximum depth at each station and surface values of chlorophyll, temperature and salinity

Station	Mixing	Depth (m)	Temperature (°C)	Salinity (psu)	Chl (µg L^{-1})
Gl	Stratified	248	16.8	20.3	5.5
G3	Partially stratified	77	15.4	22.5	5.9
G4	Well mixed	90	11.6	26.4	1.2
H1	Well mixed	136	10.9	27.3	2.0
H3	Partially stratified	275	14.7	23.5	7.2
H4	Well mixed	77	11.7	26.2	4.9
H5	Stratified	240	15.4	21.4	8.4

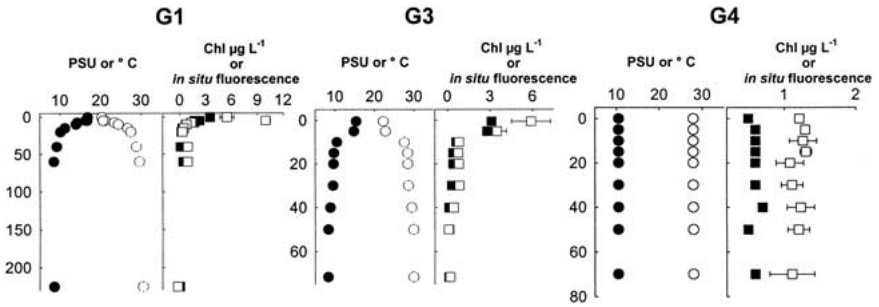

Fig. 2. Temperature (●), salinity (○) extractable chlorophyll (■) and *in situ* fluorometry (□) for Stations G1, G3, and G4.

Viral abundance among the stations ranged from 4.2 to 10.7×10^7 mL^{-1} (Table 3). There was no correlation between abundance and the degree of stratification. As well, estimated contacts between bacteria and viruses were frequent with each bacterium being contacted by ca. 43–111 viral particles each day. Viral abundance in the diluted samples increased linearly with time while there was no net change in bacterial abundance (e.g., Fig. 3). Calculated viral turnover rates were lower during the ebb tide than the flood tide and ranged from ca. 5.0 to almost 30 d^{-1}. Assuming a burst size of 50, the viral production rates inferred high rates of microbial mortality with estimated lysis rates removing between 18 and 137% of the bacteria per hour, clearly indicating the microbial communities were not in steady state. At stations sampled during the ebb tide (G stations), only 0.2 to 0.3% of the contacts were calculated to result in cell lysis. In contrast, during the flood tide (H stations), 0.5 to 1.5% of the contacts were calculated to cause lysis.

Discussion

Two important conclusions can be drawn from the data presented. First, we established that a simple dilution approach can be used to directly measure rates of viral production and turnover in coastal seawater samples. Second, viral production rates implied microbial mortality rates that were higher than could be sustained in a steady-state system.

Table 2. Bacterial abundance in samples pre- and post-dilution

	Bacteria ($\times 10^5$ ml^{-1})		
	In situ	After dilution	Cells remaining (%)
G1	21.9	2.8 (0.4)	12.8
G3	20.0	2.3 (0.3)	11.6
G4	20.5	2.4 (0.5)	11.7
H1	6.3	2.7 (0.2)	43.0
H3	21.2	3.9 (0.5)	18.5
H4	4.9	0.6 (0.3)	12.7
H5	19.4	2.8 (0.3)	14.3

The dilution technique provided rapid and reproducible estimates of viral production. Although it requires direct enumeration of virus particles, it does not suffer from some of the potential problems associated with other techniques (see Introduction). Measured rates of viral production were consistent with high rates of virus-mediated mortality of the microbial communities in Discovery Passage. Viral production rates increased with the length of time since deep mixing and were markedly higher in samples collected during flood tide (H stations) than ebb tide (G stations). These results suggest that high viral production rates may be a characteristic of non-steady-state systems. In all cases the viral production rates were supported by the calculated contact rates. Contact success rates were low relative to those determined for the Gulf of Mexico [26] and suggest there is a mismatch between the bacterial and viral communities or that the diversity of the bacterial community is high so that most viruses collide with the wrong hosts. This could physically be achieved by spatial separation of viruses and hosts in the stratified waters of the Strait that is overcome by deep vertical mixing in the Pass (an idea that is supported by the increased contact efficiencies after mixing).

Carbon and Nutrient Recycling by Viral Lysis

Estimates for carbon (or other elements) released by viral lysis can be made by applying elemental estimates per cell (cell quotas) to our estimates of cell lysis [12, 29]. At stations within the Passage or those occupied during the flood tide, virus-mediated bacterial lysis would have released a significant amount of organic material to bacteria (Table 3). In unproductive environments, it has been suggested that bacterial growth efficiencies are commonly 10 to 25% [3]. If one was to assume a slow growth rate of marine bacteria in this region (ca. 0.2 d^{-1}) and that the

Table 3. Viral production, abundance, and turnover, and the percentage of bacterial stock destroyed by viral lysis in experiments

STA	Viral production in dilution samples (10^6ml^{-1} h^{-1}, ±SD)	*In situ* viral abundance ($\times 10^7$ ml^{-1})	Viral turnover (d^{-1})	*In situ* contacts (viruses cell^{-1} d^{-1})	% Cells lysed (h^{-1})	OC released (ug L^{-1} d^{-1})	% contact causing lysis
Gl	3.3 (0.5)	7.7	7.2	79.9	23.2	11.8	0.3
G3	3.1 (0.8)	9.0	7.0	93.6	26.8	12.5	0.3
G4	2.2 (0.7)	9.6	5.1	99.8	18.5	8.8	0.2
HI	6.2 (1.5)	4.2	8.4	43.4	45.9	6.7	1.1
H3	17.9 (0.7)	8.4	27.8	86.9	90.4	44.7	1.0
H4	4.3 (1.1)	8.9	9.8	92.6	137	61.9	1.5
H5	7.9 (0.7)	10.7	12.5	111.1	56.9	25.7	0.5

[a] Estimates of organic carbon (OC) release are based on the % cells lyse, the *in situ* bacterial abudance (Table 2), and a cellular carbon quota of 23.3 fg C cell^{-1} [11]. Viral turnover rates were determined from the rate of production in the diluted samples, and corrected for the loss of bacteria in the dilution (multiplied by *in situ* abundance/diluted abundance). Contact success (% contacts causing cell lysis) was determined by taking the number of successful contacts (one per cell lysed d^{-1}) and dividing it by the total contacts.

carbon released as the result of viral lysis was available for bacterial production, then at Station H1 and H3 viral release of C could have supplied all of the bacterial demand. In the steady-state environment of the Strait of Georgia, bacterioplankton can obtain required DOM from components released primary producers. However, in the non-steady-state tidally mixed regime of Discovery Passage, where primary producers are mixed well below the critical depth for photosynthesis, DOM released during the viral lysis of bacterioplankton would have supplied most of the bacterial requirements. The results demonstrate that viral activity can bridge an uncoupling of supply and demand of bacterial carbon demand in non-steady-state systems.

Nitrogen, phosphorus, and iron are not respired by bacteria and therefore are more efficiently recycled than organic carbon. Bacteria generally contain these elements at static stoichiometric ratios with carbon (e.g., bacterial C:N is about 4:1 [20]). Assimilation of organic substrates from the lysis of bacteria with growth efficiencies on the order of 25% implies that nitrogen, phosphorus, and iron in excess of bacterial requirements must be remobilized. This suggests that viral lysis contributes to the release of organic nutrients that, after conversion to inorganic forms by bacterial activity, can help fuel the high rates of primary production that are associated with frontal boundaries [18].

This study demonstrates that tidally driven mixing can stimulate high rates of viral production and virus-induced mortality in natural microbial communities. The results suggest that virus-mediated bacterial mortality is different in environments subject to turbulent mixing relative to those in steady state. The mechanisms causing these higher rates of viral production following turbulent mixing need further investigation.

Acknowledgments

We gratefully acknowledge the assistance of the captain and crew of the CCGS *Vector*. A. Ramanarine, S. Short, E. Spinelli, and W. Mah assisted in collecting samples. M.G.

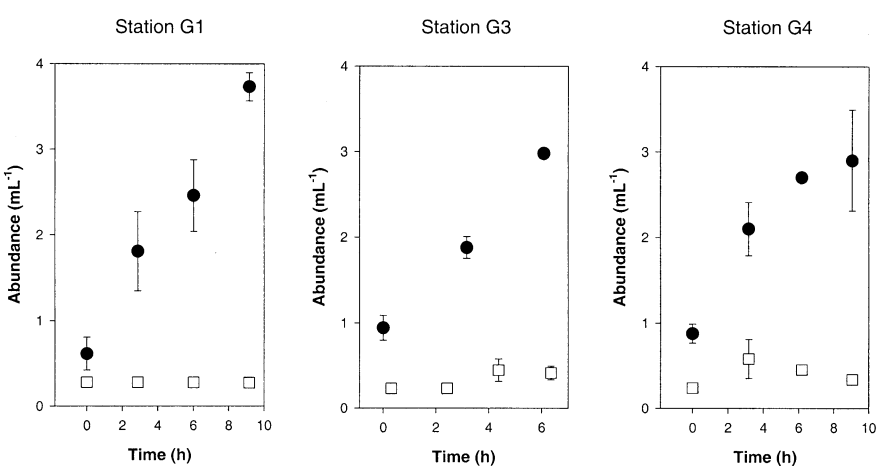

Fig. 3. Changes in viral (● × 10^7) and bacterial abundance (□ × 10^6) in incubations from Stations G1, G3 and G4. Samples were diluted with virus-free water (as described in text) at $T = 0$. The rates of viral production at each station were determined from first-order regressions of viral abundances in each independent incubation.

Weinbauer improved the manuscript with his comments. Supported by grants from the Natural Sciences and Engineering Research Council (Canada) to CAS and the National Science Foundation (OCE-9977040) to SWW.

References

1. Bratbak G, Heldal M, Thingstad TF, Riemann B, Haslund OH (1992) Incorporation of viruses into the budget of microbial C-transfer. A first approach. Mar Ecol Prog Ser 83:273–280
2. Chen F, Suttle CA, Short SM (1996) Genetic diversity in marine algal virus communities as revealed by sequence analysis of DNA polymerase genes. Appl Environ Microbiol 62:2869–2874
3. del Giorgio PA, Cole JJ, Cimbleris A (1997) Respiration rates in bacteria exceed phytoplankton production in unproductive aquatic systems. Nature 385:148
4. Fuhrman JA, Suttle CA (1993) Viruses in marine planktonic systems. Oceanogr 6:51–63
5. Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548
6. Garza DR, Suttle CA (1998) The effect of cyanophages on the mortality of *Synechococcus* spp. and selection for UV resistant viral communities. Microb Ecol 36:281–292
7. Heldal M, Bratbak G (1991) Production and decay of viruses in aquatic environments. Mar Ecol Prog Ser 72:205–212
8. Hennes KP, Simon M (1995) Significance of bacteriophages for controlling bacterioplankton growth in a Mesotrophic lake. Appl Environ Microbiol 61:333–340
9. Hennes KP, Suttle CA (1995) Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol Oceanogr 40:1050–1055
10. Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225–1228
11. Kapuscinski RB, Mitchell R (1980) Processes controlling virus inactivation in coastal waters. Water Res 14:363–371
12. Lee SH, Fuhrman JA (1987) Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl Environ Microbiol 53:1298–1303
13. Maranger R, Bird DF (1995) Viral abundances in aquatic systems: a comparison between marine and fresh waters. Mar Ecol Prog Ser 121:217–226
14. Murray AG, Jackson GA (1992) Viral dynamics: a model of the effects of size, shape, motion and abundance of single-celled planktonic organisms and other particles. Mar Ecol Prog Ser 89:103–116
15. Noble RT, Fuhrman JA (1997) Virus decay and its causes in coastal waters. Appl Environ Microbiol 63:77–83
16. Noble RT, Fuhrman JA (2000) Rapid virus production and removal as measured with fluorescently labeled viruses as tracers. Appl Environ Microbiol 66:3790–3797
17. Parsons TR, Stronach R, Borstad G, Louttit G, Perry RJ (1981) Biological fronts in the Strait of Georgia, British Columbia and their relation to recent measurements of primary productivity. Mar Ecol Prog Ser 6:237–242
18. Parsons TR, Perry RJ, Nutbrown ED, Hsieh W, Lalli CM (1983) Frontal zone analysis at the Mouth of Saanish Inlet, British Columbia, Canada. Mar Biol 73:1–5
19. Parsons TR, Dovey HM, Cochlan WP, Perry RI, Crean PB (1984) Frontal zone analysis at the mouth of a fjord, Jervis Inlet, British Columbia. Sarsia 69:133–137. 1984
20. Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic marine bacteria. Mar Ecol Prog Ser 51:201–213
21. Steward GF, Wikner J, Smith DC, Cochlan WP, Azam F (1992) Estimation of virus production in the sea: 1. Method development. Mar Microb Food Webs 6:57–78
22. Suttle CA, Chan AM (1994) Dynamics and distribution of cyanophages and their effect on marine *Synechococcus* spp. Appl Environ Microbiol 60:3167–3174
23. Suttle CA, Chen F (1992) Mechanisms and rates of decay of marine viruses in seawater. Appl Environ Microbiol 58:3721–3729
24. Weinbauer MG, Höfle MG (1998) Significance of viral lysis and flagellate grazing as controlling factors of bacterioplankton production in an eutrophic lake. Appl Environ Microbiol 64:431–438
25. Wilhelm SW, Weinbauer MG, Suttle CA, Jeffrey WH (1998) The role of sunlight in the removal and repair of viruses in the sea. Limnol Oceanogr 43:586–592
26. Wilhelm SW, Weinbauer MG, Suttle CA, Pledger RJ, Mitchell DL (1998) Measurements of DNA damage and photoreactivation imply that most viruses in marine surface waters are infective. Aquat Microb Ecol 14:215–222
27. Wilhelm SW, Suttle CA (1999) Viruses and nutrient cycles in the sea. BioScience 49:781–788
28. Wilhelm SW, Smith REH (2000) Bacterial carbon production in Lake Erie is influenced by viruses and solar radiation. Can J Fish Aquat Sci 57:317–326
29. Wilhelm SW, Suttle CA (2000) Viruses as regulators of nutrient cycles in the sea. In: CR Bell, M Brylinsk, P Johnson-Green (eds) Proceedings of the Eighth International Symposium on Microbial Ecology, Atlantic Canada Society for Microbial Ecology, Halifax, pp 551–556
30. Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev 64:69–114
31. Wommack KE, Hill RT, Muller TA, Colwell RR (1996) Effects of sunlight on bacteriophage viability and structure. Appl Environ Microbiol 62:1336–1341