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FINAL TECHNICAL REPORT

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INSTITUTION: University of Texas at Austin, Marine Science Ins...

GRANT TITLE: Development and use of Fluorescent Probes for Detection, Enumeration and Identification of Naturally-occurring Marine Viruses

AWARD PERIOD: 1 October 1989 to 31 May 1992

OBJECTIVES: 1) To isolate representative marine virus systems; 2) to develop taxon-specific probes for marine viruses; 3) to determine spatial and temporal distributions of specific viral taxa; 4) to establish a library of natural marine virus communities and a culture collection of marine viruses and their hosts; 5) to characterize the viruses that we isolate.

APPROACH: Natural virus communities were concentrated from seawater using ultrafiltration and screened for the presence of viruses that infect marine bacteria, cyanobacteria and eukaryotic phytoplankton. The isolated host-viral systems were used as models with which to determine spatial and temporal distributions of viruses and virus turnover times. As well, we have begun to examine the nucleic acids from some of the virus isolates which should ultimately lead to the development of probes specific to groups of viruses.

ACCOMPLISHMENTS AND SIGNIFICANCE: We have isolated pathogens that infect a number of phytoplankton including a prasinophyte (*Micromonas pusilla*), pennate (*Navicula* sp.) and centric (of uncertain taxonomy) diatoms and numerous cyanobacteria of the genus *Synechococcus* (*Nature* 343:60-62; *Appl. Environ. Microbiol.* 57:721-726). These represent some of the major taxa of primary producers in the sea. As well, we have isolated a number of bacteriophage, including several which infect bioluminescent bacteria. These virus-host systems are providing excellent models for addressing basic questions such as determining host-virus dynamics in the sea and potential of viruses to regulate the abundance and community structure of planktonic organisms in the sea.

To determine if viruses might affect primary productivity, material in the 0.002 to 0.2 μm size range was concentrated from seawater using ultrafiltration and added to natural phytoplankton communities. Within minutes photosynthetic rates were reduced by up to 80 % and over the longer term *in vivo* chlorophyll fluorescence decreased. Although the reduction in photosynthesis cannot be definitively attributed to viruses, the causative agent was filterable through a 0.2 μm filter and was sensitive to autoclaving, consistent with a viral-mediated process (*Nature* 343:60-62; *Mar. Ecol. Prog. Ser.* in press).

A couple of these systems were chosen as models to examine spatial and temporal distributions of marine viruses. One of the great surprises were the extremely high abundances of cyanophages infecting the genus *Synechococcus* in seawater. Both in nearshore seawater and in transects along the western Gulf of Mexico we routinely measured concentrations of infectious cyanophages in excess of 10^5 ml^{-1} (two submitted

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manuscripts). In contrast, viruses infecting the eukaryotic phytoplankter *Micromonas pusilla*, although widely occurring were much less abundant, with titers ranging from thousands per ml to one per 30 ml. Although the viruses appeared identical they were genetically and phenotypically distinct based on DNA restriction fragment analysis and molecular weights of the major proteins. This was true of viruses isolated from the same water sample (*Mar. Ecol. Prog. Ser.* 78:1-9). These results indicate the high genetic diversity and dynamic nature of virus populations in the sea.

Ultrafiltration has also been used to create a library of natural virus communities. Viruses are concentrated from seawater obtained from different locations and times, and then preserved at $-80°$ and $4°$ C. The library is a source for new virus isolates or can be screened to determine if a specific virus is present at a given location and time. It is anticipated that the library will be a very valuable asset as probes become available for more types of viruses. Currently, we have over 50 virus communities preserved.

Considerable effort has also been spent characterizing the viruses. The cyanophages infecting *Synechococcus* belong to three different families of viruses (Siphoviridae, Myoviridae, Podoviridae). The viruses vary widely in host specificity, some infecting both red and green strains of *Synechococcus*, whereas others were specific to a single strain of eleven *Synechococcus* strains tested (two submitted manuscripts). Unlike the cyanophages, the viruses infecting *M. pusilla* are large untailed polyhedrons. They contain about 200 kb of methylated double-stranded DNA. They appear to belong to the recently recognized virus family, Phycodnaviridae. As part of our objective to develop virus-specific probes, we have identified DNA in *Micromonas-pusilla* viruses (MPV) that hybridizes to a DNA polymerase gene probe and have begun to sequence this region. As well, we have developed a plaque-assay for MPV which will make this a better system for studying the molecular biology of marine algal viruses.

We have documented loss rates of marine viruses from seawater and the mechanisms responsible for their decay. In sunlight decay rates were 0.4-0.8 h^{-1}. Even when UV-B was blocked decay rates were as high as 0.17 h^{-1}. Loss rates for marine viruses in the absence of solar radiation were much lower (0.009 to 0.028 h^{-1}). In the absence of sunlight viruses did not decay or decayed very slowly in 0.2-μm-filtered, ultracentrifuged or autoclaved seawater, but continued to decay in natural seawater in which bacteria were poisoned with KCN⁻. As well, decay rates were reduced in 1.0-μm-filtered seawater even though bacterial numbers were not. Our calculation suggest that solar radiation can be the most important mechanism causing loss of viral infectivity to a depth of 100 m in oceanic regions.

SIGNIFICANCE: 1. The ubiquitous distribution of cyanophages and MPV suggests that other marine viruses may be equally widespread. 2. A DNA polymerase gene in MPV may permit development of an algal-virus probe, and allow genetic relatedness to be inferred among algal viruses. 3. The plaque assay should make MPV a useful tool for studying the molecular biology of marine algal viruses. 4. Viral decay data indicates that in clear oceanic waters the decay rate due to sunlight, averaged over 200 m, could be greater than that attributable to all other processes combined. This should cause a strong diel signal in viral infectivity. In addition, as sunlight destroys infectivity but not viral particles, a large proportion of the viruses in seawater are probably not infective.

PRIMARY PUBLICATIONS:

1. Suttle, C.A., A.M. Chan & M.T. Cottrell. 1990. Infection of viruses by phytoplankton and reduction of primary productivity. *Nature* 347:467-469.

2. Suttle, C.A., A.M. Chan & M.T. Cottrell. 1991. Use of ultrafiltration to isolate viruses from seawater which are pathogens to marine phytoplankton. *Applied and Environmental Microbiology*, 57:721-726.

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5. Suttle, C.A. & F. Chen. 1992. Mechanisms and rates of decay of marine viruses in seawater. *Appl. Environ. Microbiol.* (in press).

6. Suttle, C.A. (1992). Inhibition of photosynthesis in phytoplankton by the submicron fraction concentrated from seawater. *Mar. Ecol. Prog. Ser.* (in press).

BOOK CHAPTERS AND SHORT COMMUNICATIONS:

1. Suttle, C.A. (in press). Enumeration and isolation of viruses. *in:* Current Methods in Aquatic Microbial Ecology, (*eds.* P.F. Kemp, B.F. *et al.*), Lewis Publ.

2. Suttle, C.A., F. Chen & A.M. Chan. (in press). Marine viruses: Decay rates, diversity and ecological implications. pp. xxx-yyy in International Marine Biotechnology Conference "IMBC-91": Short Communications of the Invited Lectures ecology, C.C. Nash II (*ed.*), Dev. Microbiol. Ser., W. Brown Co.

3. Suttle, C.A., A.M. Chan. F. Chen & D.R. Garza (in press). Cyanophages and sunlight: a paradox. in: *Proceed. Inter. Symp. Microb. Ecol.*

ABSTRACTS:

1. Suttle, C.A., A.M. Chan and M.T. Cottrell. 1990. Viruses infect marine phytoplankton and reduce primary productivity by up to 80%. EOS 71:162.

2. Suttle, C.A. 1991. Response of phytoplankton communities to addition of material in the 30,000 to 0.2 μm size fraction concentrated by ultrafiltration. Abs. Am. Soc. Limnol. Oceanogr.

3. Suttle, C.A. 1991. Abundance, diversity and nature of virus communities in the sea. Internat. Phycol. Congr., Workshop on Phycovirology, Aug. 1991.

4. Suttle, C.A., M.T. Cottrell & A.M. Chan. 1992. Viruses infecting marine phytoplankton. *6th Intern. Symp. Mar. Ecol.*, Barcelona, Sept. 1992.

5. Suttle, C.A. & F. Chen. 1992. Bacteriophage decay rates and processes responsible for virus decay in seawater. Abs. Am. Soc. Limnol. Oceanogr., Feb. 1992.

6. Gonzalez, J.M., C.A. Suttle, E.B. Sherr & B.F. Sherr. 1992. Grazing on viruses by marine phagotrophic protists. Abs. Am. Soc. Limnol. Oceanogr., Feb. 1992.

7. Cottrell, M.T. & C.A. Suttle. 1992. Wide spread occurrence and clonal variation in viruses which lyse the marine microalga *Micromonas pusilla*. Abs. Am. Soc. Limnol.

Oceanogr., Feb. 1992.

8. Chan, A.M. & C.A. Suttle. 1992. Host specificity, occurrence and morphology of cyanophages infecting marine *Synechococcus*. Abs. Am. Soc. Limnol. Oceanogr., Feb. 1992.

9. Suttle, C.A. 1991. Viruses, viral dynamics and productivity. Internat. Mar. Biotech. Conf., Oct. 1991.

MANUSCRIPTS SUBMITTED:

1. Suttle, C.A. & A.M. Chan. High concentrations of cyanophages in seawater infecting marine *Synechococcus* spp. (submitted).

2. Suttle, C.A. & A.M. Chan. Marine cyanophages infecting oceanic and coastal strains of *Synechococcus*: Abundance, morphology, cross-infectivity and growth characteristics (submitted).

3. Gonzalez, J.M., C.A. Suttle, E.B. Sherr & B.F. Sherr. Virus ingestion by marine protists. (submitted).

4. Gonzalez, J.M. & C.A. Suttle. Protist grazing on viral particles: ingestion and digestion. (submitted).

APPENDIX

PUBLICATIONS RESULTING FROM THIS AWARD

Infection of phytoplankton by viruses and reduction of primary productivity

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NATURAL marine waters contain roughly 10^6 to 10^9 virus particles per ml, yet their role in aquatic ecosystems and the organisms that they infect remain largely unknown. Electron microscopy has been used to study interactions between viruses and their hosts, focusing mainly on pathogens to prokaryotic organisms[1-5]. Here we demonstrate that viral pathogens infect a variety of important marine primary producers, including diatoms, cryptophytes, prasinophytes and chroococcoid cyanobacteria. Also, addition to sea water of particles in the 0.002–0.2 μm size range, concentrated from sea water by ultrafiltration, reduced primary productivity ([^{14}C]bicarbonate incorporation) by as much as 78%. These results indicate that, in addition to grazing and nutrient limitation, infection by viruses could be a factor regulating phytoplankton community structure and primary productivity in the oceans.

Relatively large volumes of water (20–100 litres) were prefiltered through glass-fibre and membrane filters (142-mm diameter; 0.2-μm pore size) to remove zooplankton, phytoplankton and most bacteria. The remaining particulate matter in the filtrate was concentrated to 10–30 ml using Amicon hollow-fibre or spiral cartridge filters (molecular weight cutoff, 100,000 and 30,000, respectively). Negative-staining electron microscopy[6] confirms that these methods yield diverse assemblages of virus-like particles essentially free of bacteria, clays and other particulates of non-viral appearance. We used the fluorochrome 4'-6-diamidino-2-phenylindole (DAPI) to estimate the concentration of particulates in the concentrates that were associated with double-stranded DNA. Correcting for the concentrating factor of the cartridges, the number of DAPI-positive particles smaller than 0.2 μm (presumed to be viruses) in the sea water ranged from 10^6 to 10^7 ml^{-1} (Table 1). These concentrations are comparable to electron microscopy estimates[2-5].

Isolation and screening of viruses infecting phytoplankton was achieved initially in liquid culture. Phytoplankton were grown exponentially (for several transfers) in batch culture using microwave-sterilized f/2-enriched[7] natural sea water. To test for lytic viruses, growth rates and yields of unperturbed cultures were compared with cultures to which aliquots of natural viral communities, concentrated by ultrafiltration, had been added. Growth rates of the phytoplankton were monitored in vivo by chlorophyll fluorescence (an approximate measure of phytoplankton chlorophyll and hence biomass). When the addition of viral concentrate resulted in lower growth rates or cell lysis, the pathogen was purified by inoculating a small amount of supernatant from a centrifuged infected culture into an uninfected culture. The process was repeated many times to dilute non-replicating viruses from the original virus addition and to ensure that the effect could be propagated. The nature of the pathogen was confirmed by repeating these experiments using 0.2 μm-filtered culture lysate as inoculum. Samples of culture lysate were also preserved for electron microscopy. Using this method we were successful in isolating several viruses that infect eukaryotic phytoplankton of diverse taxonomy, including a pennate and a centric diatom (both very small and of uncertain taxonomy), a cryptophyte (*Rhodomonas* sp.) and a prasinophyte (*Micromonas pusilla*). (We were unable to propagate the *Rhodomonas* virus, but lysis of the experimental cultures and the observation of viral-like particles in electron micrographs indicated that viruses probably were responsible for the treatment effect.) In all cases addition of the viruses to cultures of
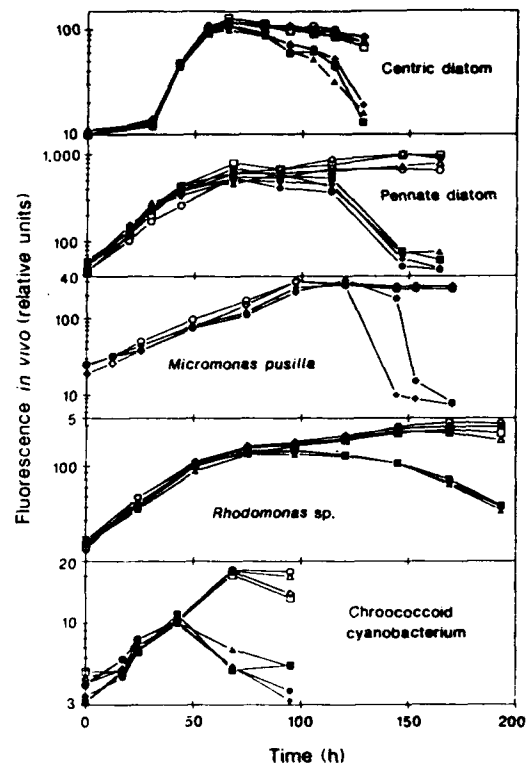

FIG. 1 Effect of adding naturally occurring marine viruses to cultures of their phytoplankton hosts. In vivo fluorescence gives a rough measure of chlorophyll a and hence of relative phytoplankton biomass. Each symbol represents a different replicate culture: open symbols, controls; closed symbols, cultures to which viruses have been added.

their respective hosts caused dramatic decreases in fluorescence as the cultures approached stationary phase (Fig. 1). As far as we are aware, the only virus previously known to infect a marine eukaryotic phytoplankton was found in British Columbia coastal waters and, interestingly, was also a pathogen of *Micromonas pusilla*[8,9]. Viruses have also been isolated that infect freshwater *Chlorella* spp. that are endosymbiotic in *Paramecium* and *Hydra*[10,11]. We have also used ultrafiltration to isolate a phage that infects a chroococcoid cyanobacterium. As the alga and phage could withstand the temperatures required to pour top agar, this phage was further purified by combining centrifuged culture lysate with algae, embedding the mixture in a soft top layer of agar medium and screening for plaque formation[12].

As it was clear that particles in the 0.002–0.2 μm size range (assuming a molecular weight of 30,000 corresponds to a particle diameter of about 0.002 μm) contained pathogens that infected a variety of phytoplankton, we investigated whether this fraction could affect rates of primary productivity. The 0.002–0.2 μm fraction was concentrated from sea water and added back at a range of dilutions to 50-ml seawater samples from the same location. Samples were labelled with 5 μCi of [^{14}C]bicarbonate and incubated under fluorescent lights at an irradiance of 120 μmol m^{-2} s^{-1} for 4 h. Primary productivity, estimated from the radioactivity incorporated into particulate material, revealed that the rates of photosynthesis were as low as 22% of the controls without concentrate (Fig. 2). The inhibitor was also sensitive to autoclaving. We have repeated this experiment five times: on four occasions the results were similar to those described, with primary productivity being reduced to an average 44% (s.e. 21%), but in one instance there was no effect on photosynthesis when concentrate was added.

These data do not prove that viruses were responsible for the decrease in carbon-fixation rate: proteins of relatively high molecular weight would probably also be concentrated by our
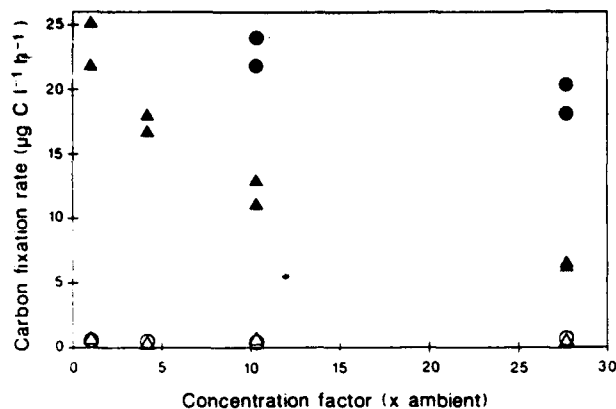
FIG. 2 Effect on phytoplankton photosynthetic rates of adding sea water from the 0.002-0.2 μm size fraction concentrated by ultrafiltration. Closed symbols represent samples that were incubated in the light and to which either autoclaved (circles) or non-autoclaved (triangles) concentrates were added. Open symbols represent samples that were incubated in the dark and to which either autoclaved (circles) or non-autoclaved (triangles) concentrates were added. On the basis of DAPI counts in the concentrate and the concentrating efficiency of the spiral cartridge, the ambient concentration of DAPI-positive particles in the size range 0.002-0.2 μm fraction was 2.1×10^7 ml^{-1}.

TABLE 1 Concentration of DAPI-positive particles in 0.2-μm filtered sea water

Location	Water type	DAPI-positive particles per ml	n
Laguna Madre	Hypersaline	3.3×10^7 (1.90×10^7)	6
MSI Pier/Harbour	Estuarine to clear coastal	1.4×10^7 (0.75×10^7)	6
Gulf of Mexico	Oligotrophic	2.0×10^6 (1.11×10^6)	3

Epifluorescence microscopy was used to obtain means and standard deviations of DAPI-positive particles in 0.2-μm filtered sea water in which the remaining particulate material was concentrated by ultrafiltration (n, number of sampling dates). The data are from several sampling dates between March and September 1989. Samples from Laguna Madre, a large (1,660 km^2) hypersaline lagoon separated from the Gulf of Mexico by a barrier island were collected at 27°28.6' N, 97°19.2' W. The Marine Science Institute (MSI) Pier and Harbour are situated adjacent to Aransas Pass on the north end of a barrier island (27°50.2' N, 97°00.5' W). Gulf of Mexico samples were obtained 70 km offshore from Aransas Pass. Estimates are from direct epifluorescent microscope counts of known volumes of DAPI-stained[18] samples collected by ultrafiltration of sea water, which was prefiltered through glass-fibre and 0.2-μm membrane filters. Electron microscopy revealed that the concentrates were virtually free of bacteria or other particulate material that might interfere with the DAPI-counting method. Unstained samples did not contain fluorescent material. The DAPI-positive material was destroyed by heating to 95 °C for 20 min. Samples were also treated for 30 min with protease-free DNase (250 units ml^{-1}) to remove dissolved free DNA and DNA not protected by a protein coat. The method was tested by digesting a sample to which DNA had been added with DNase and monitoring the change in absorbance at 260 nm. The accuracy of this method in enumerating viruses was tested using known titres of two double-stranded DNA marine bacteriophages that we have isolated. Estimates of the efficiency of recovery were made by adding known numbers of marine bacteriophage to 20 litres of ultrafiltered (particle size < 0.002 μm) sea water and subjecting it to our prefiltration and concentration procedures, then determining the concentration of DAPI-positive particles and plaque-forming units recovered. These results were compared with controls without bacteriophage. The overall concentration efficiencies of the spiral and hollow-fibre cartridges were 35 and 44% respectively.

procedure, are heat labile and can have antibiotic activity. Whatever the causative agents, they were able to inhibit the principal primary producers in our system. If host-specific viruses were responsible, they evidently occur in close spatial and temporal proximity to the phytoplankton they infect.

It is significant that the phytoplankton for which we have isolated viral pathogens are representative of groups of algae that are important primary producers. Chroococcoid cyanobacteria are the most abundant primary producers in the oceanic environment and account for a substantial proportion of the total primary productivity in the nutrient-depleted waters that make up much of the world's oceans[13]. Micromonas pusilla is a small flagellate (~1 μm in diameter) which is found throughout the world in both coastal and oceanic habitats and which commonly exceeds 10^6 cells per litre[14]. Centric diatoms are the principal primary producers in upwelling and coastal areas, the most productive regions of the world's oceans. By contrast, pennate diatoms are major benthic primary producers and also constitute a significant component of marine fouling communities. Rhodomonas and other cryptomonads are a common element of coastal phytoplankton communities but remain poorly studied.

Virus-host isolates provide model systems by which to study the interactions of indigenous marine viruses with phytoplankton. Such systems will help us in determining the effect of viral infection on the primary productivity and structure of natural phytoplankton communities. As viruses can infect a variety of marine phytoplankton, they may affect pathways of nutrient and energy flow in the ocean, restrict or terminate phytoplankton blooms, maintain phytoplankton species diversity and serve as vectors for the transfer of genetic material between phytoplankton. The presence of algal viruses in sea water may also explain previous reports of lysis of natural phytoplankton communities[15] and difficulties in balancing rates of primary productivity with loss rates for phytoplankton and sinks for organic carbon[16,17]. □

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Use of Ultrafiltration To Isolate Viruses from Seawater Which Are Pathogens of Marine Phytoplankton†

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Viruses may be major structuring elements of phytoplankton communities and hence important regulators of nutrient and energy fluxes in aquatic environments. In order to ascertain whether viruses are potentially important in dictating phytoplankton community structure, it is essential to determine the extent to which representative phytoplankton taxa are susceptible to viral infection. We used a spiral ultrafiltration cartridge (30,000-molecular-weight cutoff) to concentrate viruses from seawater at efficiencies approaching 100%. Natural virus communities were concentrated from stations in the Gulf of Mexico, a barrier island pass, and a hypersaline lagoon (Laguna Madre) and added to cultures of potential phytoplankton hosts. By following changes in in vivo fluorescence over time, it was possible to isolate several viruses that were pathogens to a variety of marine phytoplankton, including a prasinophyte (*Micromonas pusilla*), a pennate diatom (likely a *Navicula* sp.), a centric diatom (of unknown taxa), and a chroococcoid cyanobacterium (a *Synechococcus* sp.). As well, we observed changes in fluorescence in cultures of a cryptophyte (a *Rhodomonas* sp.) and a chlorophyte (*Nannochloropsis oculata*) which were consistent with the presence of viral pathogens. Although pathogens were isolated from all stations, all the pathogens were not isolated from every station. Filterability studies on the viruses infecting *M. pusilla* and the *Navicula* sp. showed that the viruses were consistently infective after filtration through polycarbonate and glass-fiber filters but were affected by most other filter types. Establishment of phytoplankton-pathogen systems will be important in elucidating the effect that viruses have on primary producers in aquatic systems.

Despite estimates of 10^6 to 10^9 free viral particles per ml in coastal and oligotrophic oceanic waters (3, 19, 23, 27), very little is known about indigenous marine viruses. The existence of planktonic marine bacteriophage has been known for some time (e.g., see references 25, 26), and more recent studies (e.g., see references 8, 10, 11, 17) have continued to isolate and describe such viruses. Viruses infecting marine cyanobacteria and eukaryotic algae have been virtually ignored, yet numerous studies suggest that they are important. Most work has been confined to freshwater organisms. Safferman and Morris (21) isolated a cyanophage from freshwater which infected 11 strains of filamentous cyanobacteria of a total of 78 host organisms tested. The potential significance of such viruses was demonstrated when artificially induced cyanobacteria blooms contained in 112-liter ponds were eliminated by the addition of viruses (22). Many other studies have reported infection of freshwater cyanobacteria by viruses (e.g., see references 5, 18, 24), but complimentary studies have generally not been conducted for marine systems, even though cyanobacteria are important primary producers in the sea. Eukaryotic algae are also susceptible to viruses. Electron microscopy studies have documented viruslike particles in at least 27 genera and numerous classes of eukaryotic algae (6, 7). Recently, Sieburth et al. (23) published micrographs illustrating the presence of viruslike particles in the brown-tide chrysophyte *Aureococcus anophagefferens*. The most substantial work on eukaryotic algal viruses has been done on those infecting freshwater *Chlorella* spp. which are endosymbiotic in *Paramecium* and *Hydra* spp. (reviewed in references 15 and 30). Of viruses infecting eukaryotic marine phytoplankton, the virus infecting *Micromonas pusilla* has been best characterized experimentally (13, 14, 31). Samples of seawater from the region where the virus was isolated indicated 10^4 to 10^7 U of a lytic agent specific for *M. pusilla* per liter. Recently, it was demonstrated that indigenous marine viruses infect marine eukaryotic and prokaryotic phytoplankton of diverse taxonomy and may cause a reduction in primary productivity in short-term incubations (27).

In this paper we describe a method for isolating viruses which are pathogens to marine phytoplankton. Such virus-host isolates will provide excellent model systems with which to study interactions between indigenous marine viruses and phytoplankton.

MATERIALS AND METHODS

Study site. Water for the studies was collected from three locations in Texas coastal waters during 1989 and 1990 (Fig. 1). Station 1 was located in Laguna Madre, a hypersaline lagoon which is separated from the Gulf of Mexico by a barrier island. There were two sampling sites at station 2. One was from the pier at the Marine Science Institute and the other was in the nearby small-boat harbor. Depending on tide, rainfall, wind, and season, the water off of the pier can vary from low salinity (22‰) and estuarine conditions (3.0 to 10.0 µg of chlorophyll $a \cdot$ liter^{-1}) to high salinity (37‰) and oligotrophic conditions (0.1 to 1.0 µg of chlorophyll $a \cdot$ liter^{-1}). Station 3 was approximately 70 km off the Texas coast in the Gulf of Mexico.

Virus concentration. Zooplankton, phytoplankton, and most bacteria were removed by gentle filtration of 20 to 100 liters of seawater. The seawater was dispensed in aliquots into a 20-liter stainless steel vessel, pressure filtered (<130 mm Hg [17,329 Pa]) through 142-mm-diameter glass-fiber (MFS GC50; nominal pore size, 1.2 µm) low-protein-binding
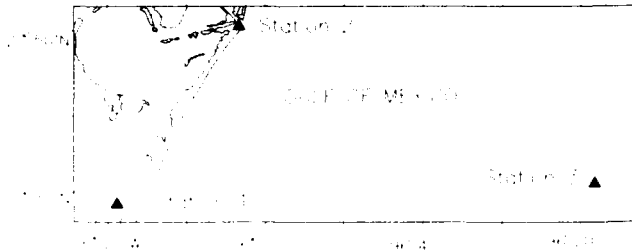
FIG. 1. The northwestern Gulf of Mexico, showing the locations of the sampling stations.

Millipore GVWP membrane filters (0.22-μm pore size), connected in series, and held in place by stainless steel filter holders. The filtrates were concentrated to final volumes of 23 to 130 ml by using a peristaltic pump and spiral-cartridge ultrafiltration (30,000 molecular weight [MW]; Amicon S1Y30). Filtrate was pumped through the cartridge at a flow rate of 850 ml · min^{-1} and at a back pressure of 1,000 mm Hg (133,300 Pa). The cartridge was cleaned after use by flushing with 2 liters of 0.1 N NaOH heated to 40°C. It was then stored refrigerated in 0.01 N NaOH, per the manufacturer's recommendation. Prior to reuse the cartridge was flushed with 7 liters of deionized distilled water.

Viral concentrates were stored in the dark at 4°C until use. Aliquots were also cryopreserved in liquid nitrogen and kept at −80°C for long-term storage.

Concentration efficiency. The efficiency with which viruses were concentrated was determined by adding known concentrations of specific double-stranded DNA viruses to ultrafiltered virus-free seawater (as determined by plaque assays) and determining the number of viruses at each stage of the concentration procedure. For these experiments, we used two bacteriophages (PWH3a-P1 and LMG1-P4) that we have isolated from seawater. Duplicate plaque assays were done on each of three independent samples taken at each step of the procedure. As well, epifluorescence microscopy and DAPI (4′,6-diamidino-2-phenylindole) staining was used to make direct counts of the number of viruses after concentration. DAPI is specific for double-stranded DNA; hence, only double-stranded DNA viruses can be enumerated by this method. The DAPI was dissolved in McIlvaine's buffer (pH 4.4) to achieve a final concentration of 5 μg · ml^{-1} (4). Subsamples (20 μl) of the viral concentrates were dispensed into autoclaved polypropylene microcentrifuge tubes. The subsamples were treated with DNase I (Sigma D-4527) to digest any dissolved double-stranded DNA in seawater that might be stained by DAPI and interfere with counting of viruses. A 5-μl drop of 0.15 M NaCl containing 1.5 Kunitz units of DNase was placed on the inside of each microcentrifuge tube; the tubes were then briefly centrifuged (15,600 × g for 5 s), vortexed, recentrifuged, and allowed to incubate for 30 min at room temperature. The method was tested by adding dissolved double-stranded DNA to seawater and following the change in A_{260} after the addition of DNase. After the DNase treatment, 5 μl of the DAPI solution was added in the same manner and incubated on ice for 20 min. Subsequently, a 9-μl drop was placed on a clean glass slide, covered with a 18-mm^2 coverslip, and observed by using an Olympus epifluorescence microscope equipped with a mercury vapor lamp. The excitation and emission filters had peak transparencies of 334 to 365 and >420 nm, respectively. A minimum of 20 random fields containing not

less than 200 fluorescent particles was counted. Viruses tended to adsorb to the glass, so care was taken to enumerate the DAPI-positive particles on both the coverslip and slide surface, which were in different planes of focus. As well, some viruses did not attach to the surfaces and were difficult to count because of Brownian motion. The problem was reduced by counting smaller volumes of samples (3 to 5 μl).

Virus isolation. The phytoplankton which were screened as potential hosts for viruses were obtained from two sources. *Synechococcus* sp. strain BBCY1, a pennate diatom (likely *Navicula* sp. strain PWPD1), and a centric diatom (PWCD1) were isolated from waters adjacent to the Marine Science Institute, whereas *M. pusilla* UTEX LB 991, *Nannochloropsis oculata* UTEX LB 2164, and *Rhodomonas* sp. strain UTEX LB 2163 were obtained from the algal culture collection at the University of Texas at Austin.

With the exception of the *Synechococcus* sp., phytoplankton to be screened for susceptibility to viruses were grown exponentially either in batch culture, by using microwave-sterilized f/2-enriched (12) ultrafiltrate produced during concentration of the viruses, or in artificial seawater (ESAW [9]). Growth rates of the phytoplankton were monitored nondestructively by following in vivo fluorescence of the cultures by using a Turner Designs fluorometer. This made it possible to monitor growth rates in hundreds of tubes on a daily basis. Near the top of the exponential growth phase 1 to 2% inocula of the phytoplankton were transferred to 5 or 40 ml of fresh media in borosilicate culture tubes (13 by 100 or 25 by 150 mm) with polypropylene caps. Once exponential growth was established, 2 to 8% inocula of viral concentrate were added to replicate tubes (usually in quadruplicate) and growth rates and yields of phytoplankton in these tubes were compared with those in cultures which received no addition. Viral concentrates collected at the same sampling station were pooled. This was done to give some information on the distribution of viral pathogens without resulting in a prohibitive number of experiments. When the addition of viral concentrate resulted in a decrease in fluorescence relative to that of control cultures, the pathogen was purified by transferring a 2.5 to 5% inoculum from an infected culture into an exponentially growing culture that had never been exposed to the suspected pathogen. The process was repeated numerous times to dilute nonreplicating viruses from the original viral concentrate.

The virus infecting the *Synechococcus* sp. was isolated by plaque assay. For phytoplankton, which grow well on solid medium and can withstand the temperature required to pour top agar, plaque assays are preferable to the fluorometric assay because subsequent plaque purification is possible. Mid-log-phase, 40-ml cultures of the *Synechococcus* sp. were harvested by centrifugation at 18°C for 15 min at 10,000 × g. The pellet was resuspended in 500 μl of ESAW to which 100 μl of a natural virus community concentrated from seawater was added. The viruses were adsorbed to the host cells for 60 min at room temperature with occasional agitation, added to 2.5 ml of molten top agar (ESAW plus 0.4% purified agar heated to 44°C), gently vortexed, and poured evenly over hardened agar in a petri plate (ESAW plus 1.0% purified agar). The plates were incubated under continuous light at 25°C and 18 μmol of quanta · m^{-2} · s^{-1} and monitored daily for plaque formation.

Membrane filtration. For three of the pathogens which could be successfully propagated, filtration was used to remove bacteria and any remaining phytoplankton from culture lysates before addition to exponentially growing

TABLE 1. Titers of viruses PWH3a-P1 and LMG1-P4 and coefficients of variation during experiments to test the recovery efficiency of viruses by using a 30,000-MW cutoff spiral ultrafiltration cartridge

Virus	Titer (PFU/ml) (coefficient of variation [%]) for the following concn step[a]:					Efficiency (%)	
	Calculated prefiltration (10^6)	Assayed prefiltration (10^6)	Assayed postfiltration (10^6)	Assayed ultrafiltration (10^9)	Assayed ultrafiltrate (10^3)	Calculated prefiltration titer	Assayed prefiltration titer
PWH3a-P1	6.4	5.7 (7.6)	6.3 (11.8)	2.7 (5.3)	3.0	88.7	99.6
LMG1-P4	4.2	3.5 (5.8)	3.6 (12.1)	1.5 (4.1)	3.0	75.1	90.1

[a] Titers for each virus were determined at each step of the procedure by plaque assay. The concentration factor (initial volume/final volume) was 475.6. The calculated titers are the concentration of PFU in the prefiltration reservoir, assuming no loss of infectivity as a result of addition of viruses to the reservoir.

cultures of their hosts both as an initial purification step and to provide further evidence that the infectious agents were viral. For the pathogens infecting the diatoms, subsamples were drawn from an infected culture and pressure filtered using a 3-ml-capacity syringe through a variety of sterile 25-mm-diameter filter types. Each filter was rinsed three times with 1 ml of microwave-sterilized ESAW and 1 ml of lysate before the filtrate was collected. The filtrate was screened for the presence of the lytic agent in borosilicate culture tubes (13 by 100 mm) by adding 2.5% inocula to duplicate exponentially growing cultures of the host and monitoring in vivo fluorescence.

M. pusilla was grown in larger volume cultures; hence, more lysate was available for filtration experiments. Generally, 40 ml of culture lysate was vacuum filtered through 47-mm-diameter filters by using a Millipore Sterifil unit. The first 10 ml of culture filtrate was discarded, and the remainder was used for screening. For the Acrodisc filter, which was only 25 mm in diameter, 10 ml was syringe-filtered and the first 1 ml was discarded as a rinse. Screening was done by adding a 1% inoculum of the filtrate to exponentially growing cultures of M. pusilla growing in culture tubes (25 by 150 mm).

Electron microscopy. Negative-staining electron microscopy (16) was used to confirm that ultrafiltration was effective for concentrating viruses from seawater and that the pathogens causing lysis were viral. Samples were preserved in 1% glutaraldehyde before application to carbon-coated 400-mesh copper grids. The grids were rinsed in several drops of deionized distilled water to remove salt water, stained with 1% (wt/vol) uranyl acetate, and observed by using a JEOL JEM-100CX transmission electron microscope.

RESULTS AND DISCUSSION

Concentration efficiency. The only significant loss of viral titer in these experiments occurred after LMG1-P4 was added to the ultrafiltered seawater in the stainless steel reservoir used for the pressure filtration (Table 1). The titer of LMG1-P4 decreased by 17%, as determined from the number of plaque-forming units (PFU). The decrease in titer was not unexpected because this bacteriophage and its host were isolated from hypersaline Laguna Madre, and decreases in titer had been observed when this virus was added to less-saline water, as was done in these experiments. Further decreases in PFU during the filtration and concentration procedures were less than 10%. The concentrations of PFU detected in the ultrafiltrates were only about 0.1% of those retained by the ultrafiltration cartridge (Table 1).

Counts of DAPI-stained viruses also indicated that overall, concentration efficiencies of the viruses were in excess of 80%. Recovery efficiency of viruses was determined on the basis of the concentration of DAPI-positive particles in samples which were ultrafiltered by using a 30,000-MW cutoff spiral cartridge. Postaddition and prefiltration titers were determined as PFU. The calculated prefiltration titer (the concentration of PFU added to the prefiltration reservoir) was 10.6 × 10^6 PFU ml^{-1}, whereas the assayed prefiltration titer was somewhat lower (9.2 × 10^6 PFU ml^{-1}). After ultrafiltration, the concentration of DAPI-positive particles was 4.1 × 10^9 ml^{-1}, yielding overall concentration efficiencies of 81.3 and 94.1% when determined from the calculated and assayed prefiltration titers, respectively.

In these experiments the ultrafiltration procedure was tested under well-defined conditions by adding known titers of viruses to ultrafiltered seawater, concentrating them, and then determining the titer in the concentrate. Clearly, the efficiency of recovery may be lower when viruses are concentrated directly from natural seawater because of adsorption of viruses to particulate material that is removed during prefiltration. Consequently, for quantitative recovery we recommend doing concentration experiments in parallel and adding an internal standard of a representative virus to one of the experiments. This will allow the absolute recovery efficiency to be determined. As well, it is important to determine titer at each step of the concentration procedure so that the source of any losses can be identified. For example, lower recovery efficiencies previously reported (27) stemmed from a loss in titer that occurred when viruses were added to ultrafiltrate and not from the concentration procedures directly.

We have also used a 100,000-MW hollow-fiber (Amicon H1P100-43) ultrafiltration cartridge to concentrate viruses from seawater, and we obtained efficiencies similar to those reported here. However, we changed to a 30,000-MW cutoff because we were concerned that some small viruses might not be retained by the larger-MW cutoff. We also switched from a hollow-fiber to a spiral cartridge because the greater strength (which allows for more back pressure) and surface area of the membrane reduced filtration time by about 75%. As well, the membrane of the spiral cartridge is less adsorbent to proteins, although adsorption of viruses to either type of cartridge was not observed in our experiments.

Virus isolation. Ultrafiltration combined with an in vivo fluorescence assay, which allowed one person to monitor several hundred cultures on a daily basis, proved to be a sensitive and easy method for screening water samples for viruses which infect marine phytoplankton. Although ultrafiltration has previously been used to concentrate specific types of viruses (e.g., see references 1 and 2) and natural viral communities from water (19), concentrates produced in this manner had not been screened for the presence of viruses which infect phytoplankton. Addition of the concentrates to cultures of phytoplankton either had no effect on the growth rates and yields of the phytoplankton cultures
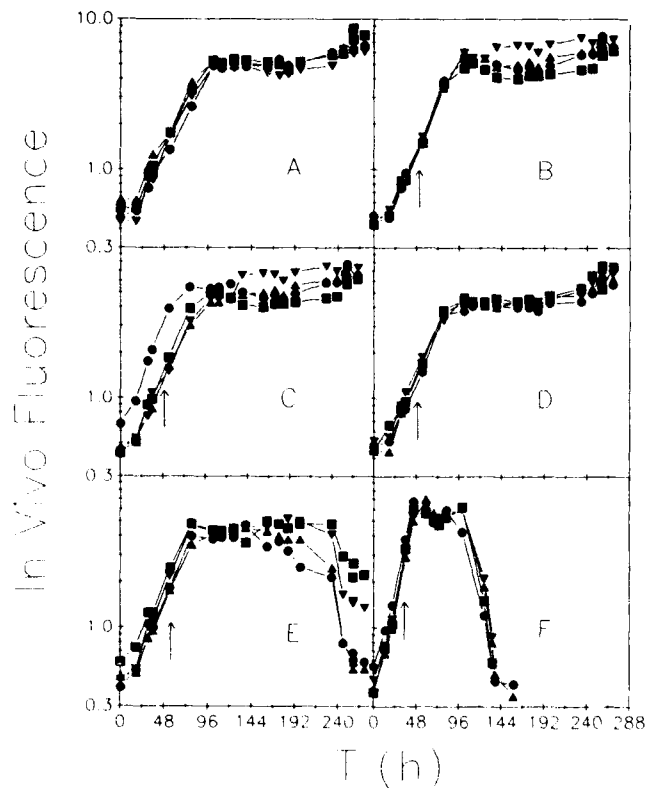
FIG. 2. An example showing how the fluorescence assay was used to screen natural virus communities for the presence of viral pathogens which infect the *Navicula* sp. Different symbols represent fluorescence measurements of quadruplicate cultures. The arrows indicate where viruses were added. (A) Control (no virus added); (B) viruses from station 1 (Laguna Madre); (C) viruses from station 2 (Marine Science Institute boat basin); (D) viruses from station 2 (Marine Science Institute pier); (E) viruses from station 3 (Gulf of Mexico); (F) culture lysate taken from one of the replicates shown in E and added as a 2% inoculum to each of the cultures illustrated.

relative to that of controls or resulted in a substantial drop in fluorescence as the cultures approached stationary phase (Fig. 2). Generally, changes in fluorescence caused by the presence of pathogens were easily recognized and highly reproducible among replicate treatments. When aliquots from lysed cultures were transferred to exponentially growing cultures which had not been exposed to viral concentrate, the observed effect was generally propagated. By using this method, we were able to document six phytoplankton isolates of diverse taxonomy that were susceptible to pathogens present in seawater. They included a pennate diatom (likely a *Navicula* sp.), a centric diatom (of uncertain taxonomy), a prasinophyte (*M. pusilla*), a cryptophyte (a *Rhodomonas* sp.), a eustigmatophyte (*N. oculata*) and a cyanobacterium (a *Synechococcus* sp.). Some of the algal pathogens have been difficult to culture. We were unable to propagate the pathogen infecting the *Rhodomonas* sp., although electron microscopy revealed viruslike particles in the medium from lysed cultures. As well, addition of culture lysate to exponentially growing cultures of *N. oculata* and the diatoms does not always cause a significant decrease in fluorescence relative to controls.

None of the phytoplankton were affected by pathogens from all three stations (Table 2). The centric diatom re-

TABLE 2. Results of screening experiments of phytoplankton against concentrated natural virus communities collected from several locations

Isolate	Origin of virus communities[a]		
	Stn 1	Stn 2	Stn 3
Micromonas pusilla UTEX LB 991	+	+	−
Nannochloropsis oculata UTEX LB 2164	−	+	−
Rhodomonas sp. strain UTEX LB 2163	+	−	−
Synechococcus sp. strain BBCY1	+	+	−
Navicula sp. strain PWPD1	−	−	+
Centric diatom (PWCD1)	−	+	+

[a] Stn, Station.

sponded to the addition of virus communities from estuarine (station 2) and offshore (station 3) waters, whereas the *Navicula* sp. was sensitive only to pathogens from the Gulf of Mexico. The *Synechococcus* sp. and *M. pusilla* were not sensitive to viral concentrates collected offshore but did respond to material concentrated in hypersaline Laguna Madre (station 1) and from estuarine waters adjacent to the Marine Science Institute. As well, we were able to isolate viruses infecting *M. pusilla* from water collected from Peconic Bay Estuary, Long Island, N.Y. and from two locations in the Pacific (coastal waters of southern California and Strait of Georgia, British Columbia, Canada). The *Rhodomonas* sp. and *N. oculata* only responded to viruses from water collected at station 1 and station 2, respectively.

Membrane filtration. Concentration of viruses from seawater initially requires prefiltration to remove zooplankton, phytoplankton, bacteria, and other particulate material larger than viruses. In addition, filtration of culture lysates or eluted plaques is often desirable as an initial purification step. Hence, determining the effects of filter type on the infectivity of algal viruses is important. Infectivity of the viruses infecting *M. pusilla* and the *Navicula* sp. was dependent on the type of filter used and was not strictly dictated by pore size (Table 3). Viruses remained infective after filtration through glass-fiber filters with nominal pore sizes ranging from 0.7 to 2.0 μm. The effect of filtration through cellulose-based filters produced less predictable results, and several filters interfered with the virus infecting the *Navicula* sp. The PBCV-1 virus, which infects certain *Chlorella* species, is also disrupted by filtration through 0.22- and 0.45-μm-pore-size Millipore filters (29) but can be filtered through 0.4-μm-pore-size polycarbonate filters (28). The fact that the 0.22-μm-pore-size polyvinylidene difluoride filter (Millipore GVWP) affected the infectivity of the *Navicula* sp. pathogen indicates that this filter was not ideal for prefiltering seawater prior to concentration by ultrafiltration. Unfortunately, the 0.45-μm-pore-size Millipore HVLP filter did not exclude many of the bacteria. As well, filtration through a modified polysulfone filter (0.2-μm pore size; Supor) prevented infection of both the *Navicula* sp. and *M. pusilla*. In contrast, viruses which were filtered through 0.2-μm-pore-size polycarbonate filters were infectious. Only one filtration experiment was done with the centric diatom virus, and it remained infectious after filtration through a 0.45-μm-pore-size Millipore HVLP filter (data not shown). Filtration studies have not been done for the viruses infecting the *Synechococcus* sp., the *Rhodomonas* sp., or *N. oculata*.

Electron microscopy. Electron microscopy demonstrated

TABLE 3. Infectivity of viruses after filtration through membranes of various types and rated pore sizes on *M. pusilla* and the *Navicula* sp.

Filter[a]	RPS (μm)[b]	Membrane type[c]	Host[d]	
			PD	Mp
Gelman Acrodisc	0.2	Polysulfone	±	+
Gelman Supor	0.2	Modified polysulfone	−	−
Gelman Supor	0.45	Modified polysulfone	−	ND
Millipore GVWP	0.22	PVD	−	+
Millipore HVLP	0.45	PVC	+	ND
Gelman GN-6	0.45	Mixed cellulose	−	+
Millipore GSWP	0.22	Mixed cellulose	±	ND
Millipore HAWP	0.45	Mixed cellulose	±	ND
MFS A045A025A	0.45	Cellulose nitrate	−	+
MSI Acetate Plus	0.22	Cellulose acetate	−	ND
Poretics PCTE	0.2	Polycarbonate	+	+
Poretics PCTE	0.2	Polycarbonate (high porosity)	+	ND
MFS GF75	0.7	Glass fiber	+	+
Gelman AE	1.0	Glass fiber	+	+
MFS GC50	1.2	Glass fiber	+	+
MFS GB100R	2.0	Glass fiber	+	+

[a] MFS, Micro Filtration Systems; MSI, Micro Separations, Inc.
[b] RPS, Rated pore size.
[c] PVD, Polyvinylidine difluoride.
[d] The infectivity after filtration is indicated as follows: +, infective; ±, variable; −, no longer infective. ND, Not determined; PD, the *Navicula* sp.; *Mp, M. pusilla.*

that ultrafiltration of prefiltered seawater produced a concentrated, diverse viral assemblage that was virtually free of bacteria. Preliminary characterization indicates that the virus infecting *M. pusilla* is a relatively large polyhedron about 100 nm in diameter and appears morphologically similar to the *M. pusilla* virus previously described (13, 14). The cyanophage infecting the *Synechococcus* sp. has an isometric head 65 to 70 nm in diameter and a lightly staining, long, flexible tail. It is similar in morphology to members of the genus *Cyanostylovirus* which also infect *Synechococcus* spp. (20). Although we were unable to propagate the pathogen infecting the *Rhodomonas* sp., examination of the culture lysate revealed numerous tailless viruslike particles approximately 100 nm in diameter.

Ecological implications. The results presented here indicate that pathogens exist in seawater which infect a number of phytoplankton taxa representing some of the major primary producers in aquatic ecosystems. This implies that viruses have the potential to be major structuring elements of phytoplankton communities and thereby may affect nutrient and energy fluxes in aquatic ecosystems. It is only through the isolation of specific phytoplankton-pathogen systems that we will be able to gather the data necessary to predict the impact of naturally occurring marine viruses on primary production in aquatic ecosystems.

viruses were subsequently isolated. The comments of two anonymous referees and F. Chen improved the manuscript.

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Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter, *Micromonas pusilla*

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ABSTRACT: Seven clonal isolates of viruses which cause lysis of the eukaryotic, naked, photosynthetic flagellate *Micromonas pusilla* (Prasinophyceae) were isolated from the coastal waters of New York, Texas, California and British Columbia, as well as the oligotrophic waters of the central Gulf of Mexico. The viruses are large polyhedrons (ca 115 nm dia.) lacking tails, and are morphologically similar to a previously described virus (MPV) which infected *M. pusilla*. Restriction fragment analysis of the DNA from these clones using *EcoRI* revealed unique banding patterns, demonstrating that each of the clones (including 3 that were isolated from the same water sample) were genetically different. Summation of the 17 to 26 visible fragments from the restriction digests, for each of the clones, yielded estimated genome sizes of 77 to 110 kilobase pairs. In contrast, only 4 different types of viruses could be recognized based on the molecular weights of the major proteins. In field samples the concentrations of viruses causing lysis of *M. pusilla* were found to be spatially and temporally variable, ranging from < 20 to 4.6×10^6 infective units l^{-1}. Our results demonstrate that this genetically diverse but morphologically similar group of viruses is widespread in nature. As viral infections propagate rapidly when host density is high, the presence of these viruses may place constraints on the maximum density that *M. pusilla* could reach in nature. If viruses infecting other phytoplankton taxa are similarly widespread then they are probably a major factor maintaining diversity in phytoplankton communities, and are also likely important players in nutrient and energy cycling.

INTRODUCTION

Recently, a number of studies (Bergh et al. 1989, Proctor & Fuhrman 1990, Suttle et al. 1990) have reported concentrations of viral particles in marine waters in excess of 10^7 ml^{-1} in a variety of marine habitats. Yet, little is known about this abundant and apparently diverse natural virus community or its role in aquatic ecosystems. Nonetheless, observations from transmission electron microscopy (TEM) (e.g. Pienarr 1976, Johnson & Sieburth 1982, Sieburth et al. 1988, Proctor & Fuhrman 1990) and from culture studies (Spencer 1963, Hidaka 1977, Moebus 1980, Suttle et al. 1990) have indicated that heterotrophic bacteria, cyanobacteria and eukaryotic algae are infected by viruses in seawater.

Virtually nothing is known about the geographic distribution or the genetic diversity of viruses which infect phytoplankton in the sea. In order to explore these unknowns we have isolated from a variety of geographic locations viruses which infect a common algal host. The eukaryotic, naked photosynthetic flagellate *Micromonas pusilla* (Prasinophyceae) was chosen as the experimental organism for a number of reasons. First, a virus which lyses this alga had been isolated from waters adjacent to the British Columbia coast (Mayer & Taylor 1979, Waters & Chan 1982). Second, *M. pusilla* has been reported to occur in the central (Hoepffner & Haas 1990) and western (Furuya & Marumo 1983) Pacific Ocean, the eastern Atlantic Ocean (Manton & Parke 1960), the fjords of Norway (Throndsen 1976), the Caribbean Sea (Throndsen 1973), the western Baltic Sea (Jochem 1990), and the Strait of Georgia (Cochlan et al. 1990). Third, we initially isolated viruses infecting this

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widely distributed host from Texas and New York coastal waters which suggested that the viruses might be widely distributed as well.

This is the first detailed study comparing viral clones that infect a widely distributed and abundant component of marine phytoplankton communities. We isolated 7 viral clones from 5 widely separated regions of the Atlantic and Pacific Oceans, as well as the Gulf of Mexico, and compared them with respect to their morphology, DNA restriction fragment banding patterns, genome sizes, and molecular weights of major proteins. We also obtained estimates of the concentration of viruses which lyse *Micromonas pusilla* in the water samples from which the viral clones were obtained. Our results demonstrate that viruses which lyse *M. pusilla* are geographically widespread and genetically diverse.

MATERIALS AND METHODS

Algal cultures. *Micromonas pusilla* (UTEX 991, Plymouth 27) was obtained from the Culture Collection of Algae at the University of Texas at Austin (Starr & Zeikus 1987). Algal cultures were grown in ESAW, an enriched artificial seawater medium (Harrison et al. 1980) modified by the addition of 5 mM Tris-HCl (pH = 7.7) and 10 nM Na_2SeO_3. *M. pusilla* was found to have an obligate Se requirement. Cultures were grown in borosilicate containers (25 × 150 mm screw-cap test tubes, 2 l Erlenmeyer flasks capped with beakers, or 9 l carboys plugged with cotton bungs) at 18 °C under continuous light (Philipps F96712/CW/VHO) at 100 μmol m^{-2} s^{-1}. Culture biomass was monitored via in vivo chlorophyll fluorescence using a Turner Designs fluorometer or by cell counts of Lugol's-fixed (Parsons et al. 1984) samples using a hemocytometer.

Study sites. Water from a number of geographically distant locations was screened for the presence of viruses infecting *Micromonas pusilla*. The study sites included productive coastal waters on the eastern, western, and southern coasts of North America and the oligotrophic waters of the Gulf of Mexico. Dates and locations of sampling are given in Table 1.

The Peconic Bay estuary (PB) is located at the eastern-most end of Long Island, New York, USA. Peconic Bay is vertically well mixed with little or no seasonal stratification. Phytoplankton primary productivity ranges from 162 to 213 g C m^{-2} yr^{-1} (Bruno et al. 1980), similar to other estuaries of the northwest Atlantic coast. The largest source of freshwater input is nonpoint sources (Hardy 1976) and the salinity near the head of the estuary ranges seasonally between 23.5 and 29.5 ppt (Bruno et al. 1980).

Both the Marine Science Institute pier lab (PL) and boat basin are located on the south side of the Aransas

Pass located at the northern end of Mustang Island, Texas, USA. The Pass allows the exchange of water between the Gulf of Mexico and a lagoon between the mainland and the series of barrier islands which include Mustang Island. Depending on tide, wind, and rainfall, the water at this location can be representative of oligotrophic offshore water or productive estuarine water.

The Strait of Georgia (SG) is a partially enclosed basin which lies between the mainland of British Columbia and the southern half of Vancouver Island, Canada. The salinity in the Strait is diluted considerably by freshwater input from the Fraser River. Near the location where these samples were taken, temperature and salinity ranged between ca 7 to 17 °C and 12 to 29 ppt respectively. The chlorophyll concentration of the mixed depth averages several μg l^{-1} (Stockner et al. 1979).

Scripps pier (SP) is located in La Jolla, California, USA, and juts out past the surf zone into the Pacific Ocean. Salinities in the region are generally high (> 33 ppt) and representative of conditions farther out in the California Bight, whereas temperature and chlorophyll values are variable and strongly influenced by upwelling events (Wyllie & Lynn 1971, Owen 1974).

The samples from the central Gulf of Mexico were collected along a transect that intersected a warm-core eddy which had broken away from the Gulf of Mexico Loop Current. Chlorophyll concentrations at 20 m reached a maximum of 0.05 μg l^{-1} along the transect and a deep chlorophyll maximum with chlorophyll concentrations as high as 0.34 μg l^{-1} was present at each station along the transect (Biggs 1991).

Virus isolation. Generally, 1 l surface water samples were collected at each location described above and transported back to the laboratory where they were filtered and screened for viruses as outlined below. Water samples from the oligotrophic Gulf of Mexico were collected from Niskin-bottle samples taken aboard the RV 'Gyre' on Cruise 90G-15 during the period 13 to 15 October 1990. Water samples (250 ml) were collected from Stns 3, 5, 7, 9, 11, 13, and 15 at depth intervals of ca 20 m between 40 and 105 m below the surface. The cruise track and station locations were chosen to sample warm-core eddy Q in the Gulf of Mexico (Biggs 1991).

Water samples from which viruses were isolated were filtered under vacuum (< 30 cm Hg) either through 47 mm diameter, 0.2 μm pore-size (Nuclepore) polycarbonate membrane filters (oceanic samples) or 47 mm diameter, 0.45 μm pore-size (Millipore) polyvinylidene difluoride (PVD) Durapore filters (coastal samples). Viruses infecting *Micromonas pusilla* were previously shown to remain infective after filtration through polycarbonate and PVD membranes (Suttle et

al. 1991). Samples from the cruise were transported back to the laboratory in polyethylene screw cap bottles at 4 °C.

The titer of viruses which lyse *Micromonas pusilla* in the filtered water samples was estimated using 3 or 4 ten-fold dilution steps in ESAW. Known volumes (0.1 to 1.0 ml) of each dilution were added to exponentially growing cultures of *M. pusilla*. Five replicate cultures were used to assay each dilution step and the viral titer was estimated using the most probable number (MPN) method (Taylor 1962). The detection limit of the method was 0.02 viruses ml^{-1}. Repeated titering of a stock viral solution indicated that the concentration of infective viruses can be estimated in a reproducible manner with this method (coefficient of variation = 45 %).

Viral clones were isolated by adding 0.2 infective units of a titered seawater sample to 20 *Micromonas pusilla* cultures. The probability that a culture received 1 virus was 0.1637 and therefore would be expected to occur with a frequency of 3.274 out of 20 cultures. The fact that no more than 4 of the 20 cultures lysed during any of the cloning experiments suggests that we were successful in obtaining lysates initiated by a single virus (data not shown). The probability that a culture received 2 or more viruses was 0.0176 and therefore would be expected to occur with a frequency of 0.352 out of 20 cultures.

Virus amplification and purification. Each *Micromonas pusilla* virus (MPV) clone was amplified by adding 40 ml of lysate into an exponentially growing 8 l culture of *M. pusilla*. After about 3 d the lysed culture was prefiltered through either a 142 mm diameter 0.45 µm pore-size Durapore filter or a 0.2 µm pore-size polycarbonate QR Nuclepore cartridge (2.3 m^2 of filtration area). The viruses were collected from the filtered lysate by ultrafiltration through either a 100 000 MW-cutoff hollow-fiber or 30 000 MW-cutoff spiral Amicon cartridge filter, and the retentate (160 ml) centrifuged at 7700 rpm (7087 × *g*) for 20 min in an SS34 rotor (Sorvall) to pellet the small amount of bacteria and cellular debris not retained by the prefilter. The viruses were then pelleted from the supernatant by either centrifuging at 9200 rpm (10 117 × *g*) for 90 min in an SS34 rotor or at 17 000 rpm (38 400 × *g*) for 30 min in an AH-629 rotor (Sorvall). The glassy pellet was resuspended in 50 mM Tris-HCl (pH = 7.8). Pure MPV DNA could be extracted from samples processed in this manner. The restriction fragment banding pattern of DNA isolated from viruses purified with this protocol was the same as that obtained from viruses which had been further purified on a sucrose gradient. In order to isolate the viral proteins it was necessary to further purify the viral material on 10 to 40 % w/v sucrose gradients made up in 50 mM Tris-HCl (pH = 7.8). The samples

(1 to 3 ml) were centrifuged at 17 000 rpm for 15 min in an AH-629 rotor and a single band containing infective viruses recovered from the gradient. The band material was diluted about 10-fold with 50 mM Tris-HCl (pH = 7.8) and the virus was once again collected by ultracentrifugation to remove most of the sucrose.

Isolation of viral DNA and restriction enzyme analysis. The procedure of Maniatis et al. (1982) was used for the extraction of the DNA. Samples (0.5 to 1.0 ml) were incubated for 15 min at 68 °C in the presence of sodium dodecylsulfate to disrupt the viral proteins and then extracted with phenol, phenol/chloroform (1:1), and chloroform. The final aqueous phase was adjusted to 0.1 M sodium chloride and the DNA precipitated by the addition of 2 volumes of ice-cold 95 % ethanol and incubation at −20 °C for 2 h.

Viral DNA was digested with *Eco*RI restriction enzymes (Promega) following the suppliers' procedure. The DNA fragments were separated on 0.5 % agarose gels in Tris-borate EDTA (TBE) buffer (Maniatis et al. 1982) and *Hind* III-digested lambda DNA was used to establish the relationship between distance migrated and fragment size in base pairs.

Sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Viruses purified on sucrose gradients were disintegrated by boiling for 10 min in sample buffer (Laemmli 1970). The peptides were separated by electrophoresis on a 12 % polyacrylamide gel at 200 V and silver stained (Wray et al. 1981). SDS-Page standards (Bio-Rad), 10 000 to 100 000 MW, were used for molecular weight calibration.

Transmission electron microscopy. Lysates were prepared for TEM by centrifuging 0.22 µm Durapore-filtered lysates at 20 000 rpm (50 400 × *g*) for 30 min in a SW40 rotor (Beckman). Pellets were resuspended in 100 µl of ESAW and the viral particles transferred to carbon-coated copper grids by floating the grids on drops of the virus suspension for 20 min. The grids were then stained (2 s) with 2 % aqueous Uranyl Acetate and photographed at a magnification of 72 000 × on an Hitachi HU11-E transmission electron microscope. Particle diameters were estimated by measuring the images on the negatives and dividing by the magnification.

RESULTS

Lysis of cultures by virus and in vivo fluorescence assay

All of the viral clones caused lysis of *Micromonas pusilla*. Cultures to which viruses were added at a virus to host ratio of 1 showed a decrease in growth rate after about 24 h relative to control cultures to which viruses were not added. Cell lysis occurred in infected cultures
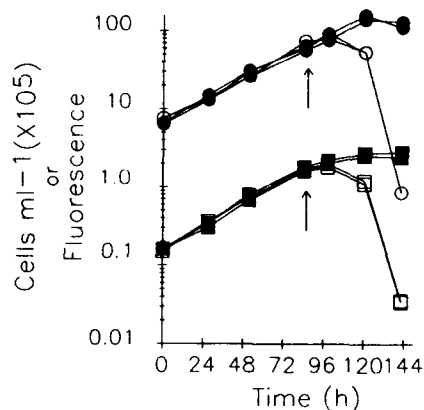
Fig. 1. *Micromonas pusilla*. Growth curves determined from in vivo chlorophyll fluorescence (■,) and cell counts (●,). Arrow indicates where one of the virus clones (MPV-PL1) was added to two of the cultures (open symbols) at a virus to host ratio of 1.0. Control cultures to which viruses were not added are shown as filled symbols

after about 36 h (Fig. 1), and was apparent whether growth was monitored by cell counts or in vivo fluorescence. Screening cultures for lysis by in vivo fluorescence allowed us to routinely monitor the large number of cultures required to conduct these experiments.

Virus distribution

We isolated viruses which lyse *Micromonas pusilla* from a wide range of environments representative of estuarine, coastal and oceanic habitats. Water samples obtained from Peconic Bay, Gulf of Mexico, Pier Lab in the Aransas Pass, Scripps Pier, Strait of Georgia, and

the UT Boat Basin contained concentrations of viruses which lyse *M. pusilla* ranging from not detectable to 4600 ml^{-1} (Table 1). The highest concentrations occurred in the small boat harbor at the University of Texas Marine Science Institute (UT Boat Basin) and the lowest in the oligotrophic waters of the central Gulf of Mexico. Virus concentrations were also temporally variable with concentrations of 3 and 4600 ml^{-1} estimated in the UT Boat Basin at different times.

We assayed 34 water samples taken from 7 stations at ca 43 n mile intervals along a transect across a warm-core eddy in the Gulf of Mexico and were able to detect virus in only 1 water sample. The detection limit in these assays was 0.02 ml^{-1}. The thermal structure along the transect showed that we sampled water from outside the eddy, as well as water inside the eddy and the frontal region between the two. We sampled water above, below, and within the deep chlorophyll maximum which was present at all the stations (Biggs 1991). Interestingly, the 1 water sample from which we were able to isolate a virus which lyses *Micromonas pusilla* was taken from the deep chlorophyll maximum in the area of the front.

Characteristics of viruses

Transmission electron microscopy of positively stained preparations of the 7 viral clones revealed particles with maximum diameters ranging from 104 to 118 nm with an average of 113 nm. No morphological differences were apparent between clones. The viruses appeared to be polyhedral and tailless (Fig. 2).

The structural proteins of the 7 viral clones were

Table 1. Concentrations of viruses lysing *Micromonas pusilla* in water samples collected from different locations. For the Gulf of Mexico stations, discrete samples were taken at each of the depths indicated

| Location		Depth (m)	Date of collection	Titer[a] (MPN ml^{-1})
Peconic Bay	(40° 00' N, 72° 30' W)	Surface	25 Jun 1990	90
Gulf of Mexico				
Stn 3	(27° 20' N, 92° 00' W)	40, 60, 75, 90, 105	13 Oct 1990	Not detectable
Stn 5	(27° 00' N, 91° 19' W)	40, 60, 75, 90, 105	14 Oct 1990	Not detectable
Stn 7	(26° 40' N, 90° 40' W)	20, 40, 60, 75, 90, 105	14 Oct 1990	Not detectable
Stn 9	(26° 20' N, 89° 59' W)	40, 60	14 Oct 1990	Not detectable
Stn 9	(26° 20' N, 89° 59' W)	76	14 Oct 1990	0.1
Stn 9	(26° 20' N, 89° 59' W)	90, 105	14 Oct 1990	Not detectable
Stn 11	(25° 59' N, 89° 20' W)	20, 60, 75, 90, 105	14 Oct 1990	Not detectable
Stn 13	(25° 40' N, 88° 40' W)	40, 60, 75, 90, 105	15 Oct 1990	Not detectable
Stn 15	(25° 19' N, 88° 00' W)	60, 75, 90, 110	15 Oct 1990	Not detectable
Strait of Georgia	(49° 18' N, 124° 11' W)	Surface	Sep 1990	9
Pier Lab	(27° 50' N, 97° 04' W)	Surface	31 Oct 1990	10
Scripps Pier	(32° 45' N, 117° 15' W)	0.5	31 Oct 1990	9
UT Boat Basin	(27° 50' N, 97° 04' W)	Surface	24 May 1990	3
UT Boat Basin	(27° 50' N, 97° 04' W)	Surface	10 Mar 1991	4600

[a] Detection limit = 0.02 ml^{-1}
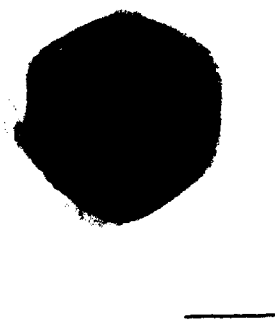
Fig. 2. MPV-PB5 positively stained with uranyl acetate and observed by transmission electron microscopy. Scale bar = 50 nm
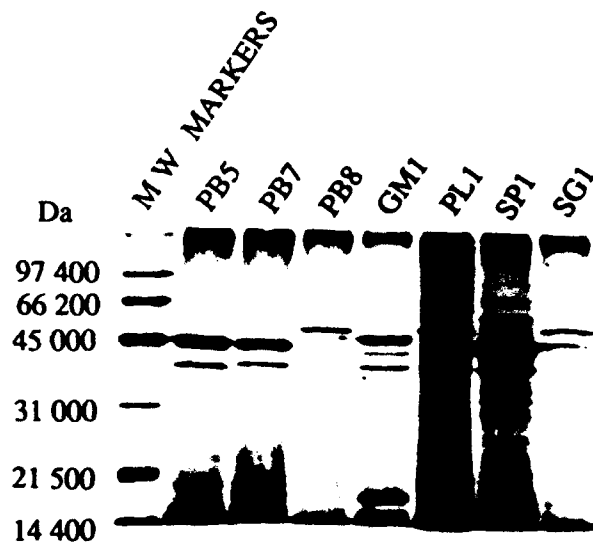

Fig. 3. Silver-stained polyacrylamide gel of proteins from the 7 viral clones infecting *Micromonas pusilla*. PB5, PB7, and PB8 were isolated from Peconic Bay, New York. Clones GM1 and PL1 were isolated from the central Gulf of Mexico and coastal waters of Texas respectively. SP1 was isolated from the waters off Southern California and SG1 was isolated from waters near British Columbia. Molecular weights of the standards are given in daltons (Da)

analyzed by SDS polyacrylamide gel electrophoresis. All 7 clones contained at least 3 major proteins which appeared as distinct bands (Fig. 3). The viruses probably contain more than 3 proteins since more bands are visible when a larger sample is loaded into the gel. For example, clone SP1 produces lysates with titers about 10 times higher than the other clones (data not shown) and clearly contains more than 3 proteins. In addition, peptides of less than 21500 daltons (21.5 kDa) are visible in preparations with especially low background (i.e. GM1), but may be hidden in preparations which had high background even after 1 purification on a sucrose density gradient (i.e. PL1).

The molecular weights of the major proteins varied among clones (Fig. 3). Based upon the molecular weights of the major proteins the 7 clones could be separated into 4 types (Table 2). Type I includes 2 clones from Peconic Bay (PB5, PB7) and the clone isolated from Southern California (SP1). The major proteins from these clones have molecular weights of 48.7, 47.9, and 44.6 kDa. Type II includes the third clone from Peconic Bay (P38) whose major proteins have molecular weight of 54.4, 48.7, and 44.6 kDa. Note that Type I and Type II share peptides of 48.7 and 44.6 kDa. Type III includes the clone isolated from the central Gulf of Mexico (GM1) whose major proteins have molecular weights of 51.8, 47.9, and 44.6 kDa; again note that Type III shares the 44.6 kDa peptide with Types I and II. Type IV includes the clones isolated from Aransas Pass (PL1) and the Strait of Georgia (SG1) whose major proteins have molecular weights of 55.8, 50.7, and 45.1 kDa. These proteins are not shared by any other clones (Table 2).

EcoRI restriction endonuclease digests of the DNA of

Table 2. Estimates of the molecular weights of the major proteins, size of the genomes and number of restriction fragments from an *EcoRI* digest of the viral genomes for each of the viral clones examined. Based on the protein banding patterns the virus clones could be classified into 4 types

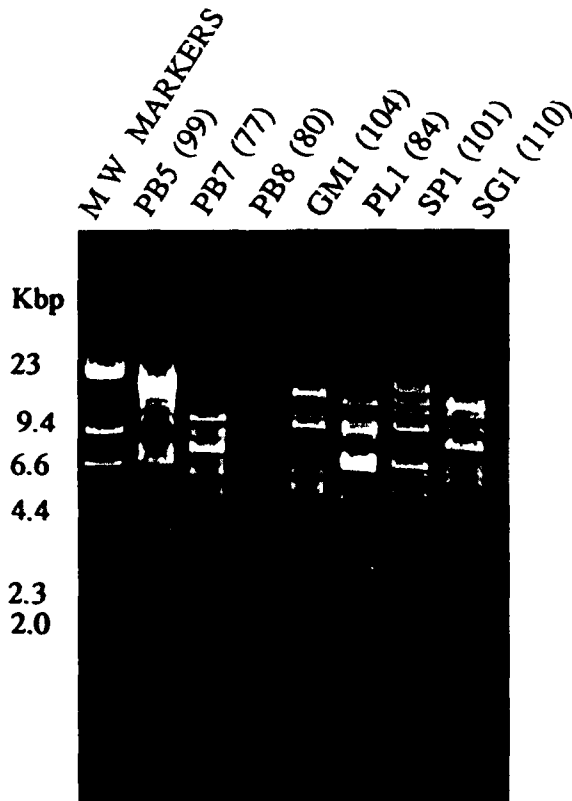| Clone	Major protein (kDa)	Type	Genome size (kbp)	Fragments
MPV-PB5	48.7 47.9 44.6	I	99	19
MPV-PB7	48.7 47.9 44.6	I	77	21
MPV-PB8	54.4 48.7 44.6	II	80	17
MPV-GM1	51.8 47.9 44.6	III	104	26
MPV-PL1	55.8 50.7 45.1	IV	84	24
MPV-SP1	48.7 47.9 44.6	I	101	23
MPV-SG1	55.8 50.7 45.1	IV	110	25

Fig. 4. Agarose gel electrophoresis of *EcoRI* restriction enzyme digests of DNA from 7 clones of viruses which lyse *Micromonas pusilla*. Clone designation of the isolates are as in Fig. 3. Estimated genome sizes from summing the restriction fragments are shown in parentheses

the 7 clones revealed 17 to 26 visible bands (Fig. 4). Each of the clones had a unique restriction fragment banding pattern including the 3 clones isolated from a single 1 l water sample collected from Peconic Bay (PB5, PB7, PB8). Repeated amplification of the same viral clone demonstrated that restriction fragment banding patterns were conserved within a clone (data not shown). Summation of the *EcoRI* fragments yielded estimates of the genome sizes of the individual clones from 77 and 110 kbp (Table 2). It should be noted that because of co-migrating restriction fragments genome sizes may be underestimated when based on the summation of restriction fragment sizes.

DISCUSSION

Several important findings emerged from our study. First, viruses infecting *Micromonas pusilla* are geographically wide-spread and occur in the coastal waters of the Pacific and Atlantic Oceans, as well as the Gulf of Mexico. Second, virus concentrations were different between geographic locations, and were different temporally at the same location. Third, no 2 virus clones were identical based upon *EcoRI* restriction fragment banding patterns. This included clones that were isolated from the same water sample. Fourth, based on the molecular weight of the viral proteins the 7 clones fell into 4 phenotypes. However, 2 of the 4 phenotypes were isolated from the same water sample. Fifth, no morphological differences were observed among clones. These observations are discussed in further detail below.

We cannot distinguish our viruses from the virus isolated by Mayer & Taylor (1979). The virus they isolated was large (ca 130 nm), hexagonal in profile and lacked a distinctive tail-like or other external structure (Mayer 1979). Their isolate of this virus was capable of lysing strains of *Micromonas pusilla* isolated from the English Channel, the coastal waters off Baja California, the coastal waters of British Columbia, and the NE Pacific at Weather Ship P. It would not lyse any other algal taxa they tested (Mayer 1978). They did not conduct any biochemical investigations of the virus which is no longer in culture (F. J. R. Taylor pers. comm.). Therefore, we are calling these viruses *Micromonas pusilla* virus (MPV) as well. On the other hand, the viruses we have isolated are easily distinguishable from the *Chlorella* viruses which are the only other well-characterized viruses infecting a eukaryotic alga. The latter are polyhedrons of about 175 nm in diameter, containing more than 50 structural proteins and having a genome of 333 kbp (Van Etten et al. 1991).

Comparisons among clones

Restriction enzyme analysis of the DNA from the 7 clonal isolates showed that the genome of each clone is different from the others, although the 7 clones do share some phenotypic characteristics. They all lyse *Micromonas pusilla* and are tailless polyhedra with a diameter of about 115 nm. The molecular weights of the major viral proteins vary among clones, but are not as variable as the restriction fragment patterns. In addition, clones which share major proteins of equal molecular weight have different restriction fragment banding patterns. The differences in restriction fragment banding patterns between clones may be due to differences in nucleotide sequences of their genomes. Our current interpretation of this result is that each of the 7 viral clones is a different strain of virus which lyses *M. pusilla*, but lacking more detailed information about the genomes, we cannot say at this time how closely related these clones might be. We are currently examining the genomes more closely. The existence of conserved regions among the genomes would lend support to our tentative conclusion based on the

phenotypic similarity among these clones that they represent a group of closely related viruses.

The diversity of genotypes among clones of MPV, indicated by the restriction endonuclease analysis, is not unique to this eukaryotic algal virus. It is possible to isolate from nature many clones of viruses which infect an exosymbiotic *Chlorella*-like alga. Restriction enzyme analysis of these clones shows that each has a different restriction fragment banding pattern (Van Etten et al. 1985, Schuster et al. 1986, Zhang et al. 1988). It has also been demonstrated that some bacteriophages also exist in nature as populations with high clonal diversity. For example, it is possible to isolate from nature phages which are φX174-like with respect to having the same sized single stranded circular genome, icosahedral particle morphology, similar particle density, and the same number of proteins with approximately the same molecular weights (Godson 1974). Godson (1974) isolated 4 classes (called the G-phages) of these φX174-like phages and showed that while the genomes of phages G4, G6, G13, and G14 will form DNA heteroduplexes with φX174 DNA, the resulting heteroduplexes contain regions of at least 24 % base sequence mismatch and appeared as single stranded loops when viewed with TEM. A similar examination of T7-related phages using restriction enzyme analysis led Studier (1980) to the conclusion that during the evolution of the T7-related phages a highly successful morphology and genetic functional organization is conserved while the sequence of nucleotides in the DNA is plastic and can vary widely.

Studier's conclusion seems reasonable, but many questions remain unanswered. For example, what is the source of the genetic diversity observed in presumably closely related viral clones? Point mutations are the accepted mechanism by which base sequences of genes are modified, but DNA/DNA interactions such as homologous or illegitimate recombination which move homologous or nonhomologous sequences between viruses may be the prime accelerators of the evolution of bacteria and their viruses (Reanney & Ackermann 1982). Studies of the lambdoid phages using heteroduplexing reveal that the DNA of phages like lambda and φ80 have regions of near-perfect homology interspersed with regions of total nonhomology. Examination of the nucleotid sequence of the genomes of phages T3 and T7 reveals regions of high similarity abutting regions of much lower similarity (Beck et al. 1989). It has been suggested that recombinations among ancestors of T3 and T7 gave rise to this pattern of sequence similarity and dissimilarity (Beck et al. 1989).

It would be interesting to examine the similarities and differences among the genomes of these algal viruses and to determine the potential for recombination between these viruses under controlled laboratory conditions to assess whether recombination might be a mechanism by which they evolve. The probability that a single *Micromonas pusilla* cell would encounter 2 viral particles at the same time is certainly low given the titers of viruses we observed. Co-infection would seem to be a possible but inefficient mechanism by which recombination might take place between these viruses in the sea. On the other hand, if MPV can exist as a provirus within *M. pusilla*, there may be a number of *M. pusilla* cells in the sea which already contain a copy of the MPV genome. Recombination between a provirus and the genome of a free viral particle could be an efficient mechanism for recombination between different MPV genomes in the sea.

Ecological implications

The results of this study greatly extend previous observations of the presence of viral pathogens of phytoplankton in the sea, and the ecological implications posed by these results are significant. Viruses infecting *Micromonas pusilla* are widespread in nature. In fact, every coastal seawater sample that we have screened has contained viruses which cause lysis of *M. pusilla*. This must pose strict controls on the densities that can be maintained in *M. pusilla* populations. For example, Wiggins & Alexander (1985) found that rapid propagation of viruses infecting *Escherichia coli* occurred when host density exceeded about 10^4 ml^{-1}. At significantly lower densities the probability of a virus encountering a suitable host dictates that the infection propagates at a much slower rate. Hence, it would appear to be very unlikely that densities of *M. pusilla* could significantly exceed about 10^4 ml^{-1} in the presence of the viral pathogen. Interestingly, this is approximately the same maximum density that has been reported for *M. pusilla* populations in field samples (Cochlan et al. 1990). However, virus-resistant strains of *M. pusilla* may exist in nature which are not represented in culture collections and which would not be affected by the presence of the lytic virus. If host-specific viral pathogens do exist for each phytoplankton species then viral infection is the only mechanism that is required to maintain diversity in phytoplankton communities (sensu Hutchinson 1961). This is not unreasonable as viral pathogens infecting a variety of ecologically important phytoplankton taxa have been demonstrated to occur in seawater (Suttle et al. 1990). This leads to another significant ecological question in terms of the structure of phytoplankton communities, namely 'What are the mechanisms that allow phytoplankton to escape viral pathogens and form blooms?'

The phenotypic and genotypic diversity observed

among viruses infecting *Micromonas pusilla* (even within the same water sample) also poses interesting ecological and evolutionary questions. The phenotypic differences among clones may be under selective pressures. The nature of the selective agents is unknown, although obvious candidates would be phenotypic and physiological differences among the hosts. However, one cannot exclude the possibility of other factors being important (e.g. loss processes). Alternatively, the different phenotypes may all have equivalent fitness so that the differences between them are selectively neutral.

This study clearly demonstrates that, while widespread, viruses infecting *Micromonas pusilla* have spatially variable concentrations. In coastal waters concentrations were typically a few to less than 100 ml[1]; however, in one instance a titer of 4600 ml[1] was observed. In contrast, in oligotrophic oceanic waters only one of 36 samples had a titer as high as 0.1 ml[1]. In the other 35 samples MPV was not detected. Clearly, some mechanism is operating to regulate the density of viruses which lyse *M. pusilla* in nature. Intuitively, as the virus is apparently highly host-specific (Mayer 1978) the density of *M. pusilla* must be important. We do not have estimates of host density from the samples from which viruses were isolated, but others (Throndsen 1973, 1976, Furuya & Marumo 1983, Cochlan et al. 1990, Hoepffner & Haas 1990, Jochem 1990, Harrison et al. 1991) have observed that *M. pusilla* is relatively abundant in coastal waters and particularly in subsurface chlorophyll maxima at coastal and offshore stations. This is consistent with our observations of higher viral titers in coastal waters. Furthermore, the sample from which MPV-GM1 was isolated was taken from the subsurface chlorophyll maximum near the western front of the warm-core eddy. Not surprisingly, these results suggest that populations of viruses which infect *M. pusilla* are found in environments where the alga has been shown to be abundant.

Concentrations of viruses infecting *Micromonas pusilla* also varied widely at a single location. This was demonstrated by the ca 1000-fold difference in viral titre between 2 water samples collected from the same location on separate occasions (Table 2). Such large changes in the abundance of viruses infecting *M. pusilla* indicates that loss and production processes of the viruses are not necessarily tightly coupled. Nonetheless, it remains to be determined what the important factors are which control the distribution and abundance of these viruses in the sea.

If the cosmopolitan distribution and variability in titre of viruses infecting *Micromonas pusilla* are typical of viruses which infect other phytoplankton then such viruses are likely important players in nutrient and energy cycles in aquatic ecosystems.

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Virus ecology

Sir — Suttle et al.[1] suggest that in addition to grazing and limitation of nutrients, infection of phytoplankton by marine viruses could be a factor regulating their production. The evidence for this hypothesis is the huge amounts of virus particles found in marine waters, and the finding that up to 20-fold concentrations of such can reduce the photosynthesis of marine phytoplankton under experimental conditions[1]. Although this hypothesis is attractive, we think that one important aspect of this phenomenon has not been considered. When a new, highly virulent virus type is introduced into a population of initially susceptible individuals, an enormous selection pressure for virus-resistant variants is generated. A well-known example of this phenomenon is the artificial introduction of myxoma virus into Australia to help control the rabbit pest problem[2]. In this giant field experiment, genetic resistance to myxomatosis increased so that the mortality rate after infection with a particular strain of virus deceased from 90 to 25 per cent within a few years.

This phenomenon is not restricted to virus–animal systems. Shortly after his discovery of bacteriophages[3], d'Herelle suggested that bacterial viruses might serve a useful therapeutic purpose; but he was never able to prove his idea experimentally[1]. The suggestion that exogenous marine viruses should continuously be able to kill a large proportion of a phytoplankton community seems to us as unlikely as the idea that bacteriophages should eliminate pathogenic or other bacteria within a given ecological niche. Therefore, if marine viruses are involved in the control of primary plankton production, this probably occurs only if a new virulent virus strain emerges, and the effects on primary production would most likely only be transient, owing to the rapid evolution and reproduction of resistant phytoplankton strains.

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SUTTLE ET AL. REPLY — Olofsson and Kjelleberg doubt that viruses could reduce primary productivity because of selection for virus-resistant variants. This point is an important aspect of viral ecology and we agree that resistant phytoplankton would arise if put under strong selective pressure by a viral pathogen.

The greatest impact of viruses on photosynthetic rates probably occurs in specific situations, such as during the demise of large monospecific phytoplankton blooms. Resistant variants would not prevent a transitory decrease in productivity as the infection propagated, but would presumably protect the host species from local extinction. In non-bloom situations, viruses probably remove a portion of the primary productivity, as lytic viruses occur in sea water and must be produced at the expense of hosts. As viral infections propagate rapidly when host density becomes high[5], viruses may also indirectly affect primary productivity by modifying community structure. In other situations viruses could stimulate primary productivity by causing cell lysis and enhancing the rates of nutrient recycling, analogous to the effect of grazers in some planktonic communities[6]. Observations that aquatic viruses infect particular hosts over wide geographic areas[7,8], that a significant portion of marine cyanobacteria are infected with viruses[9], and that titres of viral pathogens to specific hosts are highly variable (our unpublished results) emphasize the dynamic interactions of host-virus systems in the sea. But the role of host resistance is likely to be limited, because marine virus and phytoplankton communities are generally diverse with only a small proportion of the total numbers made up of any given pathogen or host. So the low probability of a virus encountering a suitable host minimizes selection for host resistance relative to other factors.

We would also like to point out that we did not conclude that viruses caused the observed decreases in photosynthetic rates.

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Mechanisms and Rates of Decay of Marine Viruses in Seawater[1]

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ABSTRACT

Loss rates and loss processes for viruses in coastal seawater from the Gulf of Mexico were estimated using three different marine bacteriophages. Decay rates in the absence of sunlight ranged from 0.009 to 0.028 h^{-1}, with different viruses decaying at different rates. In part, decay was attributed to adsorption by heat-labile particles, as viruses did not decay or decayed very slowly in 0.2-μm-filtered, autoclaved or ultracentrifuged seawater, but continued to decay in cyanide-treated seawater. Cyanide did cause decay rates to decrease, however, indicating that biological processes were also involved. As decay rates were often greatly reduced in 0.8- or 1.0 μm-filtered seawater, whereas bacteria numbers were not, it suggested that most bacteria were not responsible for the decay. Decay rates were also reduced in 3-μm-filtered or cycloheximide-treated seawater, but not in 8-μm-filtered water, implying that flagellates consumed viruses. Viruses added to flagellate cultures decayed at 0.15 h^{-1}, corresponding to 3.3 viruses ingested flagellate^{-1} h^{-1}. Infectivity was very sensitive to solar radiation and in full sunlight decay rates were 0.4-0.8 h^{-1}. Even when UV-B was blocked, rates were as high as 0.17 h^{-1}. Calculations suggest that in clear oceanic waters exposed to full sunlight that most of the viral decay, averaged over a depth of 200 m, would be attributable to solar radiation. In coastal waters, when decay rates were averaged over 24 h and 10 m depth, loss rates of infectivity attributable to sunlight were similar to those resulting from all other processes combined. Consequently, there should be a strong diel signal in the concentration of infectious viruses. In addition, as sunlight destroys infectivity but does not remove virus particles, a large proportion of the viruses in seawater are probably not infective.

INTRODUCTION

High concentrations of viral particles are now recognized as being a ubiquitous component of surface seawaters (1, 5, 20, 23, 32, 35). Furthermore, a relatively large proportion of bacteria and cyanobacteria in surface waters contain virus-like particles, suggesting that infection by viruses may be a significant loss process for microbes in the sea (10, 23). Yet, despite their abundance and potential importance to nutrient and energy cycling, little is known about the processes responsible for regulating virus concentrations in the sea.

Observations of high concentrations of viruses in the sea are recent; nevertheless, interest in the fate of viruses in marine systems, is not. In fact, a great deal of effort has centered on the "survival" of viruses in seawater (*e.g.* 3, 17, 18), but these studies have largely focussed on human pathogens and coliphages. Data from these model systems suggest that viral infectivity decays significantly over periods of days and that the decay is primarily biologically mediated, although chemical effects and effects from solar radiation have also been implicated (*e.g.* 3, 18). In addition, colloids and particulate material have been shown to adsorb and re-release coliphages in seawater (4). Most recently, it was reported that the disappearance of natural communities of viral particles from seawater was extremely rapid, with measured decay rates of up to $1.1\ h^{-1}$ (10). The agents responsible for the disappearance were not identified, but unlike other investigations high decay rates persisted when particulate material was removed by centrifugation or when cyanide was added to the seawater.

Ultimately, a number of mechanisms are likely responsible for the natural removal of viruses from seawater. In this study we examined the processes responsible for the decay of infectivity of marine bacteriophages in seawater. Infectivity rather than the abundance of viral particles was followed, as infection is the process by which most viral-mediated effects occur. Our use of indigenous marine bacteriophages rather than exotic viruses is an important distinction between our studies and those of others, given the recent interest in the role of viruses in marine ecosystems. We also extend the observations of previous work by examining the effect of solar radiation and micr flagellate grazing on decreases in viral infectivity, as well as, determining which size fractions are associated with the decay of viruses in seawater. The relative importance of these processes are considered using a simple model.

MATERIALS AND METHODS

Marine bacteriophages and their hosts. The viruses (LMG1-P4, PWH3a-P1 and LB1VL-P1b) and bacteria (LMG1, PWH3a and LB1) used for these studies were isolated from the coastal waters of Texas. LMG1-P4 and its host (LMG1) were isolated from a large, typically hypersaline lagoon (Laguna Madre), whereas, PWH3a, LB1 and the viruses infecting them were isolated from seawater collected from the Marine Science Institute (MSI) pier at Port Aransas, Texas. The taxonomic status of LMG1 and PWH3a are currently unknown. They were isolated as colony-forming units from seawater spread on 1 % agar plates made up with natural seawater enriched with 0.05 % peptone and 0.05 % casamino acids. LMG1 is red and was isolated from Laguna Madre; PWH3a forms milky colonies and was isolated from pier water. LB1 is a bioluminescent bacterium tentatively identified as *Photobacterium leiognathi*. It was also isolated from pier water using the same agar-solidified medium described above, supplemented with 0.05 % yeast extract and 3 ml l^{-1} of glycerol.

The viruses LMG1-P4 and PWH3a-P1 were isolated from natural virus communities concentrated by ultrafiltration from water collected from Laguna Madre and the MSI pier (33). One ml of exponentially-growing host cells were combined with 20 or 40 μl aliquots of the virus communities and allowed to adsorb for 30 min. These mixtures were combined with 2.5 ml of nutrient-enriched 0.6 % molten agar, poured over a 1 % agar underlay and screened for plaque formation. The virus infecting *P. leiognathi* was isolated from a culture in which a 2.5 ml culture of the bacterium and 25 ml of the enriched seawater medium was added to 100 ml of seawater. After 48 h, aliquots of the culture were plaque-assayed and viruses detected. Clonal isolates of all the viruses were obtained by standard plaque purification procedures (16).

Characterization of the viruses. Each of the viruses were characterized using electron microscopy. Samples from amplified stocks were spotted onto 400 mesh carbon-coated copper grids and allowed to adsorb for 30 minutes. The adsorbed viruses were rinsed through several drops of deionized-distilled water to remove salts, stained with 1 % w/v uranyl acetate and observed using transmission electron microscopy.

Evidence for the nucleic-acid composition of the viruses was obtained by staining with the DNA-specific fluorochrome, DAPI (4',6 diamidino-2-phenylindole) and examining them using epifluorescent microscopy. Further details on procedures that were used to isolate, purify and visualize the viruses are described elsewhere (33, 31).

1   **Plaque assays.** The concentration of viruses in amplified stocks and seawater samples to which viruses

2   had been added was determined by assaying for the number of plaque-forming units (PFUs). A colony from a

3   plate of host cells was transferred to sterile medium in the late afternoon and the cells shaken overnight. In the

4   morning a 5-10 % inoculum was transferred to fresh medium and 4-5 h later the exponentially-growing culture

5   was suitable for plaque assays. This protocol ensured that the number of non-viable host cells to which phage

6   could attach was extremely small. Aliquots (20-500 μl) of the virus sample to be assayed were combined with

7   500 μl from the culture of host cells and adsorbed for 30 min (LMG1 and PWH3a). A shorter adsorption time

8   (10 min) was found to be optimal for *P. leiognathi*. The culture containing the adsorbed virus was then

9   combined with 2.5 ml of molten agar (44-46 $^\circ$C) to make a final concentration of 0.5 % and immediately

10   poured over a 1 % agar underlay. Plaques would begin to appear 12-16 h later on plates that were incubated at

11   23-25 $^\circ$C. Triplicate plaque assays were done on each culture for every time point. In the event that it was

12   necessary to dilute the concentration of viruses before assaying, the dilutions were always done in triplicate and

13   independently of each other using autoclaved, artificial (9) or ultrafiltered (33) seawater.

14   **Virus decay experiments.** The seawater for the decay experiments was collected from the MSI pier

15   during incoming tides when the seawater was relatively clear and representative of water farther offshore

16   (salinity typically 30-35 ppt). Experiments were initiated when treatments to the seawater were completed. The

17   decay of infectivity of each of the marine bacteriophages was assayed in whole seawater and seawater that had

18   been treated by autoclaving (121 $^\circ$C for 20 min), centrifugation in swinging-bucket rotors (16,000 or 121,000 x g

19   for 20 and 300 min, respectively), or gentle filtration (<120 mmHg) through polycarbonate filters (Poretics;

20   0.2-, 0.8-, 1.0-, 3.0- or 8.0-μm pore-sizes). A tracer level (*ca.* 10^3 PFUs ml^{-1}) of each of the viruses was

21   introduced into duplicate treated or untreated seawater samples contained in polycarbonate Erlenmeyer flasks

22   or borosilicate glass bottles, incubated at 23-25 $^\circ$C and the decay of infectious units over time followed.

23   As well, decay rates were measured in samples in which biological activity was stopped by the addition

24   of 2 mM NaCN$^-$. The effect of the cyanide on microbial activity was determined by measuring thymidine

25   incorporation. Tritiated thymidine (methyl-^{3}H, 3.7 x 10^5 Bq) was added to poisoned and non-poisoned

26   seawater subsamples (35 ml each) at 0, 22, 42, 114, and 159 h after the start of the experiment, incubated for

27   60 min, and filtered through 0.2 μm pore-size polycarbonate membranes (Poretics), overlaid on glass-fiber

5

1   filters. The radioactivity trapped on the filters was determined by liquid scintillation counting.

2   For the grazing experiments 5×10^7 PFUs ml^{-1} of PWH3a-P1 was introduced into cultures containing

3   6×10^5 cells ml^{-1} of a marine phagotrophic nanoflagellate (strain E4), and into culture filtrate in which grazers

4   and bacteria were removed by filtration through 0.8 and 0.2 μm pore-size filters, respectively. The density of

5   viruses approximated concentrations in the field, while the abundance of flagellates ensured measurable decay

6   rates over the duration of the experiment. In another experiment, cycloheximide (200 mg l^{-1}), a inhibitor of

7   protein synthesis in eukaryotes, was added to natural seawater to which 1.7×10^4 PFUs ml^{-1} of PWH3a-P1 had

8   been added. The viral decay rates were compared to rates in 0.2-μm-filtered seawater and seawater to which

9   no cycloheximide was added.

10   The effect of solar radiation on the decay of viruses was investigated by adding *ca.* 2×10^3 PFUs ml^{-1}

11   of each virus into ten 19-cm wide by 7-cm deep glass dishes containing 1.5 l of natural seawater. We had

12   previously determined that each of the viruses could not cross infect any of the other hosts. The dishes were

13   placed in a trough of flowing water to maintain constant *in situ* seawater temperatures and dark controls were

14   compared to treatments that were incubated for 4.5 h in full sunlight, or in sunlight that was reduced by 80 or

15   53 % using neutral density screening. In addition, 6mm-thick glass covers were used to exclude wavelengths

16   < 320 nm from two full-sunlight treatments. The percentage of light transmitted by the glass at given

17   wavelengths was 450 nm, 88 %; 400 nm, 86 %; 370 nm, 78 %; 360, 71 %; 350, 58 %; 340, 36 %; 330, 12.3 %; 320,

18   1.5 %; 310, < 0.3 %. All treatments were duplicated. Samples for virus titer were taken at time zero and at

19   1.5 h intervals, thereafter. Irradiance was measured using a Licor Li-1000 quanta data logger and cosine

20   collector measuring photosynthetically-active radiation (PAR).

21   The decay rate constants were calculated by fitting a least-squares linear regression to a plot of

22   *ln* PFUs ml^{-1} versus time. The slope of the line is the decay constant (h^{-1}) and the reciprocal is the turnover

23   time of the virus population.

24

25   **RESULTS**

26

27   **Characterization of the viruses.** Two of the bacteriophages used in these studies were very similar in

28   size and appearance (Fig. 1a & b). LMG1-P4 and PWH3a-P1 are approximately 78 and 83 nm in diameter, and

have rigid tails about 97 and 104 nm in length, respectively. In contrast, LB1VL-P1b is smaller (50 nm in diameter) and has a very short tail (Fig. 1c). The DAPI-stained viruses could be visualized using epifluorescent microscopy, suggesting that they contained double-stranded DNA.

Decay rates in seawater in the absence of solar radiation. Viruses generally did not decay or decayed very slowly in seawater that was autoclaved or filtered through 0.2 μm polycarbonate membrane filters (Fig. 2). In untreated natural seawater (NSW) the disappearance of PFUs was typically linear ($r^2 > 0.95$) when plotted against time on a semi-logarithmic axis (Table 1, Fig. 2 & 3). The range of measured decay rates in NSW was 0.008 to 0.023 h^{-1} for PWH3a-P1 and 0.023 to 0.028 h^{-1} for LB1VL-P1b (Tables 1-3). This corresponds to turnover times for the viral populations that ranged between 1.5 and 5.2 d. Experiments run with the same virus on the same day resulted in very similar decay rates (Table 1); however, in paired experiments the infectivity of LB1VL-P1b decayed at about twice the rate of PWH3a-P1 (t-test, $p < 0.0001$, n = 8). When particles > 3 μm were removed both LB1VL-P1b and PWH3a-P1 decayed more slowly (paired t-test, $p < 0.001$, n = 4), but there was no difference between 8.0-μm filtered and unfiltered seawater (Tab ?, Fig. 2, paired t-test, $p = 0.256$, n = 6). Likewise, removal of particles by centrifugation at 16,000 xg for 20 min resulted in reduced decay rates that were similar to 0.2-μm filtered seawater; however, there was no detectable decay in viruses that were added to ultracentrifuged seawater (Table 3). Non-living particles were partially responsible for the decay, as decay continued in cyanide-treated seawater (Table 3) even though there was no detectable microbial activity over the 7 days of the experiment (data not shown). Furthermore, when particles > 0.8 or > 1.0 μm were removed decay rates were substantially reduced (Table 2, Fig. 3, paired t-test, $p < 0.001$, n = 4); yet, bacterial abundances remained high. Nonetheless, biological processes were also involved as decay rates were reduced by the addition of cyanide.

As a significant amount of decay was at attributable to material in the 3.0-8.0 μm size fraction, and as decay rates were reduced by the addition of cycloheximide (Table 3, t-test, $p < 0.03$, n = 4), it suggested that protozoan grazers might be responsible for some of the observed decay. Addition of PWH3a-P1 to cultures of a phagotrophic marine nanoflagellate resulted in accelerated decay rates (Fig. 4) relative to viruses that were added to filtrate from a 0.2- or 0.8-μm filtered culture.

Effect of solar radiation on virus decay rates. The average surface irradiance during the decay

1	experiment was 1872 μmol quanta m^{-2} s^{-1} but ranged between 1288 and 2228 μmol quanta m^{-2} s^{-1} during the

experiment was 1872 μmol quanta m^{-2} s^{-1} but ranged between 1288 and 2228 μmol quanta m^{-2} s^{-1} during the

15 min periods over which the irradiance was integrated. The decay rates of the viruses were very sensitive to

solar radiation. In fact as little as 20 % of the surface irradiance resulted in decay rates for PWH3a-P1 that

were 3.2 to 8.9 times those measured in natural seawater not exposed to solar radiation; for LB1VL-P1b the

range was about 3.9 to 4.8 (Fig. 5, Tables 1-3). Similar decay rates were obtained if the viruses were added to

ultrafiltered seawater and exposed to sunlight (data not shown); hence, the presence of phytoplankton, bacteria

or other particulate material was not required for sunlight to cause decay of infectivity. The decay rates were

proportional to the amount of radiation received (Fig. 6). For example, eliminating 80 % of the incident

irradiance resulted in decay rates that were 20 % of those in full sunlight. Similar experiments were done on

three other occasions with comparable results. When wavelengths < 320 nm (UV-B) were eliminated, decay

rates were still very high relative to the dark controls and were similar to the treatment in which 80 % of the

sunlight was removed (Fig. 5). The loss of infectivity was not reversable by overnight incubation in the dark.

Given that the decay of infectivity was directly related to the amount of radiation received (Fig. 6), it

was possible to approximate the light-related decay of viruses in seawater from the attenuation coefficient (k)

for damaging radiation. Hence, the decay rate at any depth (z) due to solar radiation (d_r) can be approximated

from $d_{r0} * e^{-kz}$ where, d_{r0} is the decay rate at the surface. Using a range of attenuation coefficients for

biologically-damaging radiation in seawater (0.15-5.0 m^{-1}), and the average decay rate of infectivity at the

surface for the three sunlight-exposed viruses (0.5 h^{-1}), we calculated the decay rate of infectivity at different

depths for an average surface irradiance of 1872 μmol quanta m^{-2} s^{-1} (Fig. 7).

DISCUSSION

This study provides the first data of which we are aware on the decay of infectivity of natural marine

viruses in seawater. Our results indicate that a number of factors contribute to the decay of viral infectivity in

seawater including solar radiation, living and non-living particulates and grazing by protozoa.

Characterization of viruses. The marine bacteriophages used in our studies are tailed icosahedrons.

They are morphologically similar to other bacteriophages which have been isolated from seawater (8, 11, 12),

and resemble many of the viruses that are observed in electron micrographs of marine viral communities (1, 23,

35). It is reasonable to expect that the mechanisms and rates of decay of these viruses are representative of

1   those experienced by the rest of the marine bacteriophage community.

2   **Effect of solar radiation on viral decay.** Sunlight greatly accelerated the decay of viral infectivity. This

3   was not unexpected as viruses are very sensitive to UV irradiation. More surprising was that decay rates were 2

4   to 10 times faster than dark controls when wavelengths < 320 nm were removed, or when 80 % of the incident

5   irradiance was excluded (Fig. 5). Biologically-damaging effects of UV-A (320-400 nm) and even longer

6   wavelengths have been documented (21). Unlike the effects of shorter wavelength UV, the damage is not

7   inflicted by direct interaction of the radiation with DNA and is thought to be the result of nonDNA

8   photosensitizers and reactive species of oxygen (*e.g.* 22). For example, thymine dimerization and single-strand

9   breaks in DNA occur when intact bacteriophage T4 is irradiated at 365-nm (36); whereas, isolated phage DNA

10   from X174 was unaffected by wavelengths > 320 nm (19).

11   The sensitivity of marine viruses to solar radiation has major implications in terms of the persistence of

12   viral infectivity in seawater. Significant amounts of UV-B (290-320 nm) and UV-A (320-400 nm), which directly

13   and indirectly damage DNA, penetrate to considerable depth in seawater. For example, estimated attenuation

14   coefficients for 310 nm radiation range from 0.15 m^{-1} in clear oceanic water to 0.86 m^{-1} in moderately

15   productive, high-DOM coastal waters (27). Hence, 22 % and 2 %, respectively, of the surface irradiance would

16   remain at 10 m. At the bacteriophage-damaging wavelength of 365 nm (36) the attenuation coefficient is even

17   lower (k = .040 for clear ocean water) and continues to decrease at longer wavelengths of UV-A (28).

18   Radiation will effect decay rates to significant depths over a wide range of attenuation coefficients

19   (Fig. 7). Even at a coefficient of 1 m^{-1}, the decay rate of viruses at 3 m resulting from radiation (0.025 h^{-1}),

20   would be similar to the highest decay rates that we measured in the absence of sunlight (Tables 1-3). If decay

21   rate is integrated $((d_0/-k)(e^{-kz}-1))$ to determine the average decay rate in the water column, then not until

22   30 m is the mean decay attributable to solar radiation equal to the average rate measured in the absence of

23   sunlight (0.017 h^{-1}). For coastal waters with a higher attenuation coefficient (2.0 m^{-1}), the depth where they

24   would be equal is 15 m. In clear oceanic waters (k = 0.15 m^{-1}), only at depths exceeding 200 m would the

25   average decay rate over the water column resulting from radiation be exceeded by the average decay rate

26   measured in the absence of sunlight (0.017 h^{-1}). In offshore waters the relative importance of solar radiation to

27   decay is probably even more important than indicated here, as loss rates resulting from other processes (*e.g.*

9

1　attachment to particulate material) are probably greatly reduced. The significance of solar radiation is

2　supported by data documenting that the survival of repair-deficient mutants of *Escherichia coli* was affected by

3　sunlight at depths of 10 m in Antarctic waters (13).

4　　　As the rate of decay of infectivity is proportional to the radiation received (Fig. 6), the average decay

5　rate at the surface as a result of radiation can be estimated from the rate of incident radiation averaged over

6　24 h. For example, on the date of the sunlight-decay experiments (Fig. 5) the average quantum flux (PAR) at

7　Port Aransas, over 24 h, was 521 μmol quanta m^{-2} s^{-1}. Using the regression equation relating decay rate to

8　irradiance (Fig. 6; y = 0.2646x + 0.01938) the average daily decay rate at the surface caused by radiation would

9　be 0.16 h^{-1}, approximately 10-fold greater than the average loss of viral infectivity in the absence of sunlight

10　(Table 1-3). In the upper 60 m of clear oceanic water (k = 0.15) the estimated daily decay attributable to

11　radiation (0.018 h^{-1}) exceeds the average rate measured without sunlight. In coastal water (k = 1.0 m^{-1})

12　sunlight would be responsible for most of the total daily decay in the upper 10 m. Clearly, solar radiation is a

13　major factor responsible for the decay of viral infectivity in marine waters.

14　　　An important consequence of decay caused by radiation is that the viruses will not be removed.

15　Therefore, in sunlight-exposed surface waters a large proportion of the viruses are probably not infective.

16　Consequently, estimates of abundance made using electron microscopy probably greatly overestimate the

17　number of infective viruses. A caveat is that photoreactivation and cell-mediated UV reactivation (2, for brief

18　review) can repair radiation-damaged phage DNA and restore viral infectivity. The significance of these

19　processes in nature remains to be evaluated.

20　　　**Viral decay in the absence of solar radiation.** Decay rates in the absence of sunlight ranged from 0.008

21　to 0.028 h^{-1}, corresponding to turnover times of between 1.5 and 5.2 d (Tables 1-3). The kinetics were first

22　order and well described by linear regression of logarithmically-transformed data, suggesting that most of the

23　decay was attributable to a single rate process. The viruses should have served as good tracers, as they were

24　added at low concentrations (*ca.* 10^3 ml^{-1}) relative to the number of viral particles typically present (*ca.*

25　10^7 ml^{-1}). The decay rates that we report are similar to those observed by others (3, 4, 26) for enteroviruses in

26　non-polluted seawater. In contrast, recently reported disappearance rates for viruses in seawater, determined

27　using electron microscopy, were 9 to 39 times more rapid (0.26-1.1 h^{-1}) than the highest rates we observed (10).

1   These observations are difficult to reconcile as decreases in infectivity should provide more conservative

2   estimates of viral decay rates than disappearance of viral particles. As well, Heldal and Bratbak (10) found that

3   centrifugation of seawater did not reduce virus disappearance rates, whereas, in our studies and those of others

4   decay rates decreased in seawater that was centrifuged to the same (our studies) or a lesser extent (3).

5   In our studies, the 0.8- to 8.0-μm size fraction was associated with much of the viral decay (Table 2,

6   Figs. 2 & 3). Decreased decay rates associated with the removal of particles has generally been attributed to

7   removal of bacteria (e.g. 18, 26). Yet, decay rates were significantly reduced by removal of particles > 0.8 or

8   1.0 μm (Table 2, Figs. 2-4) even though bacterial numbers were not. Nevertheless, bacteria or other organisms

9   were involved in viral decay as rates were reduced in the presence of cyanide.

10   Considerable decay was also associated with the 3.0- to 8.0-μm size fraction. Moreover, as decay rates

11   were reduced by the addition of cycloheximide (Table 3), and marine flagellates have been observed to

12   consume viruses (Gonzalez, Suttle and Sherr unpubl. data), it suggested that some of the decay might result

13   from grazing. Decay rates in the presence of flagellates were accelerated, however, the consumption rate was

14   relatively modest (3.3 viruses flagellate^{-1} h^{-1}) when the viruses were added at a natural concentration

15   (5 x 10^7 ml^{-1}). Consequently, even if flagellates were present at 10^4 ml^{-1} they could only consume about 0.1 %

16   of the virus community h^{-1} and could not account for the decay attributable to the > 1-μm size fraction.

17   The element responsible for decay in the absence of sunlight was heat labile and could be greatly

18   reduced by filtration or centrifugation, but was only partially attributable to bacteria or protists. Lycke et al.

19   (15) found that viriocidal material in seawater was destroyed by heating to as little as 45 °C. They interpreted

20   their results as evidence for non-living organic material being responsible for the decay of viral infectivity, but

21   offered no explanation as to its nature. We suggest that viruses irreversibly bind to heat-labile, loosely-

22   associated aggregates, and in the process loose their infectivity. This hypothesis is supported by observations

23   that fluorescently-stained viruses rapidly adsorb onto microscopic particulates in seawater (Gonzalez and Suttle,

24   unpubl. data). The origin of the particles is unknown, but their appearance and the variable efficiency with

25   which they can be removed by filtration suggests that they are loosely-associated microscopic aggregates.

26   Although viruses reversibly bind to a number of organic and inorganic materials (4, 25) we were unable to

27   recover a significant number of infectious units using a number of standard protocols for desorption, or by using

1   gentle sonication to break-up aggregates (data not shown). Enteroviruses can also strongly adsorb to natural

2   particles in estuarine waters and only be desorbed with low efficiency (14). Our results suggest that attachment

3   to particles removes both infective and non-infective viruses from seawater, as once adsorbed the viruses will be

4   lost by sedimentation or potentially via zooplankton grazing. Studies have observed large numbers of viral

5   particles affiliated with material collected from sediment traps (24) and viruses embedded in slime associated

6   with a collapsing diatom bloom (6).

7   **Implications.** Our study demonstrated that solar radiation is likely the primary mechanism causing

8   decay of viral infectivity in surface waters. Even when averaged over 24 h and integrated over the upper 30 m of

9   the water column, the estimated destruction of infectivity by solar radiation in clear ocean water (0.033 h^{-1})

10   exceeded the greatest loss rates that we measured for coastal waters in the absence of sunlight (Tables 1-3). In

11   addition, as infectivity is probably destroyed much more quickly than viruses are physically removed, it implies

12   that a large proportion of the viruses that are counted using electron microscopy are non-infective.

13   Abundance and infectivity of viruses in seawater are controlled by several processes. The most

14   important of these are production of viruses via cell lysis, destruction of infectivity by solar radiation, removal of

15   viruses through adhesion to particulates, and perhaps digestion by bacterial enzymes (Fig. 8). The relative

16   importance of solar radiation will vary as a function of insolation and water transparency. In near surface and

17   shallow coastal waters sunlight can destroy the infectivity of most of the viral community in a few hours. As

18   well, unlike adsorption to particles or grazing by protists, sunlight will not remove viruses. Hence, viral

19   production needs only to balance the removal of viral particles to maintain a constant number. Average loss

20   rates of infectivity in our studies in the absence of sunlight averaged 0.41 d^{-1} (range, 0.22-0.67; Tables 1-3).

21   This implies similar production rates, assuming that these decay rates reflect the removal of free viral particles.

22   In order to support this production, and assuming an average burst size for marine bacteria of 50 viruses (10),

23   the loss rate from the virus community required to support this production, via infection of bacteria, would be

24   approximately 0.02 of the viral production rate (range, 0.004 to 0.013 d^{-1}). For typical viral and bacterial

25   abundances of 10^7 and 10^6 ml^{-1}, respectively, this would require about 4-13 % of the bacteria to be infected

26   daily, assuming that lysogenic cells represent a minor source of viral progeny. The proportion of the bacterial

27   mortality that this represents depends strongly on bacterial growth rates, being highest at the slowest rates.

1   These values seem reasonable given that 0.7-16 % of marine bacteria have been estimated to contain mature

2   phage (10, 23, 24). The viral production and removal rates are similar to the decay rates that would be

3   attributable to sunlight in coastal waters with an attenuation coefficient of (1.0 m^{-1}). The average daily decay

4   rate of viral infectivity for a 10 m water column would be 0.38 d^{-1}. Hence, almost all of the daily viral

5   production would be made noninfective during a sunny day. This suggests that there is a strong diel signal in

6   the number of infectious viruses.

7   The effect of solar radiation on viral infectivity may also explain a paradox. It is puzzling that despite

8   the great abundance of viruses in seawater and the nι .nerous host-viral systems that have been isolated, the

9   concentration of infectious viruses for a given host (except cyanobacteria) is generally low, ranging from below

10   detection limits, or a fraction of an infectious unit ml^{-1}, to 10^4 infectious viruses ml^{-1} (e.g. 7, 29, 30, 34). The

11   possible explanations for this observation are that 1) the host-pathogen systems that have been isolated are not

12   representative of those occurring most abundantly in nature, 2) the viruses are extremely host specific and there

13   are 10^3 to 10^4 different hosts in each ml of seawater or, 3) a large proportion of the viruses in seawater are not

14   infective. Given the potentially strong impact of solar radiation on viral infectivity it seems that the latter

15   hypothesis is likely correct.

16

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TABLE 1. Decay rates and turnover times of PWH3a-P1 and LB1VL-P1b in natural seawater.

Date	PWH3a-P1				LB1VL-P1b			
	k (h^{-1})	TT^{b} (d)	r^2	n	k (h^{-1})	TT^{b} (d)	r^2	n
22 May 1991	0.012	3.4	0.994	5	0.023	1.8	0.983	5
22 May 1991	0.014	2.9	0.982	5	0.023	1.8	0.992	5
24 May 1991	0.011	3.7	0.988	4	0.028	1.5	0.974	4
24 May 1991	0.013	3.2	0.983	4	0.025	1.6	0.932	4

TABLE 2. Decay rates (h^{-1}) of marine viruses in filtered and unfiltered natural seawater.[a]

| Date | Virus | Filtration treatment | | | | |
		0.2-μm	0.8-μm	3.0-μm	8.0-μm	Unfiltered
24 May 91	PWH3a-P1	0.000 (0.0001)	-	0.005 (0.0000)	0.011 (0.0000)	0.012 (0.0005)
24 May 91	LB1VL-P1b	0.004 (0.0004)	-	0.020 (0.0012)	0.028 (0.0001)	0.027 (0.0014)
25 July 91	PWH3a-P1	0.011 (0.0009)	0.011 (0.0015)	-	-	0.023 (0.0014)
30 July 91	PWH3a-P1	0.004 (0.0001)	0.007 (0.0007)	-	-	0.015 (0.0005)
27 Jan 92	PWH3a-P1	0.003 (0.0001)	0.002 (0.0004)[b]	-	0.012 (0.0008)	0.008 (0.0002)

[a] Rates determined from a minimum of 4 time points; standard deviation of duplicate determinations in parentheses.

[b] Filtered through a 1.0 μm pore-size filter.

18

TABLE 3. Effect of selective inhibitors and centrifugation on decay rates (h^{-1}) of PWH3a-P1 in treated and untreated natural seawater.[a]

Date	Treatment					
	0.2-μm filtered	Cycloheximide[b]	NaCN[c]	Centrifuged[d]	Ultracentrifuged[e]	untreated
30 July 1991	0.004 (0.0001)	0.011 (0.0007)	-	0.005 (0.0008)	0.000 (0.0007)	0.015 (0.0005)
19 June 1992	0.001 (0.0001)	-	0.005 (0.0003)	-	-	0.012 (0.0003)

[a] Rates determined from a minimum of 4 time points; standard deviation of duplicate determinations in parentheses.

[b] 2 mM, final concentration

[c] 200 mg l^{-1}, final concentration

[d] Centrifuged at 16,000 x g for 20 min

[e] Centrifuged at 121,000 x g for 300 min

FIGURE LEGENDS

2   FIG. 1. Electron micrographs of the bacteriophages used in the decay experiments. a) LMG1-P4; b) PWH3a-

3   P1; c) LB1VL-P1b. Scale bar is equivalent to 50 nm.

4   FIG. 2. Decay of infectivity of the marine viruses LB1VL-P1b (A) and PWH3a-P1 (B & C) in untreated

5   natural seawater samples (o) and seawater samples that were autoclaved (◊), or filtered through 0.2-

6   (△), 3.0- (□) or 8.0-μm (▽) pore-size filters. Where error bars are not shown 1 SD was less than the

7   width of the symbols.

8   FIG. 3. Titers of PWH3a-P1 (upper panel) and changes in bacteria numbers (lower panel) in natural seawater

9   (NSW, open symbols) and in seawater that was filtered through 1.0-μm pore-size polycarbonate filters

10   (closed symbols). Where error bars are not shown 1 SD was less than the width of the symbols.

11   FIG. 4. Titers of PWH3a-P1 in cultures of a marine phagotrophic nanoflagellate (6 x 10^5 cells ml^{-1}) and in 0.2-

12   and 0.8-μm filtered culture filtrate.

13   FIG. 5. Effect of solar radiation on the titers of three different marine viruses (LB1VL-P1b, LMG1-P4,

14   PWH3a-P1) on 29 May 1991. The treatments were as follows: (●) dark controls; (●) No UV-B; (▲)

15   20 %; (■) 53 %; and (▽) 100 % of incident irradiance, respectively.

16   FIG. 6. Effect of solar radiation on the decay rates of LMG1-P4 (y = 0.3746x + 0.0019; r^2 = 1.000), LB1VL-

17   P1b (y = 0.2363x + 0.0332; r^2 = 0.992), and PWH3a-P1 (y = 0.1830x + 0.0230; r^2 = 0.987). Where

18   error bars are not shown 1 SD was less than the width of the symbols. The regression equation

19   describing all of the bacteriophage data is y = 0.2646x + 0.0194; r^2 = 0.918

20   Fig. 7. The effect of solar radiation on the decay rate of marine viruses using attenuation coefficients (k)

21   ranging from clear oceanic water (k = 0.15) to turbid coastal waters (k = 5). The surface decay rate is

22   based on the averaged decay rate of LB1VL-P1b, LMG1-P4, and PWH3a-P1 during a 4.5 h incubation,

23   during which the average irradiance was 1872 μmol quanta m^{-2} s^{-1} (PAR).

24   FIG. 8. A simple model depicting the estimated daily rate constants for the decay and production of marine

25   viruses, averaged over a 10 m coastal water column, for a daily averaged surface irradiance of

26   45.052 mol quanta m^{-2} and an attenuation coefficient of 1.0 m^{-1}. Arrows represent loss and

27   production of viral particles, except for the hatched arrow which represents the destruction of viral

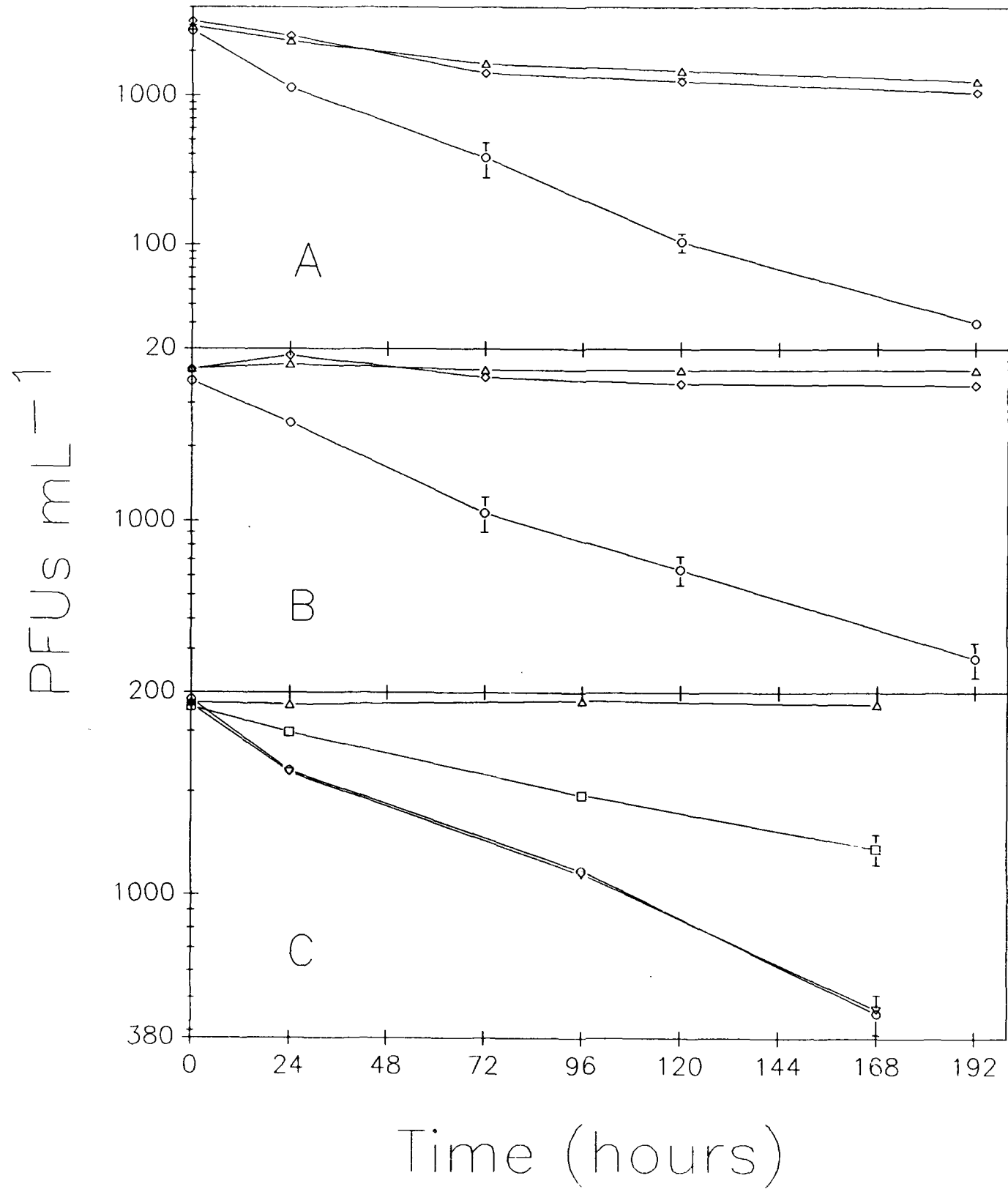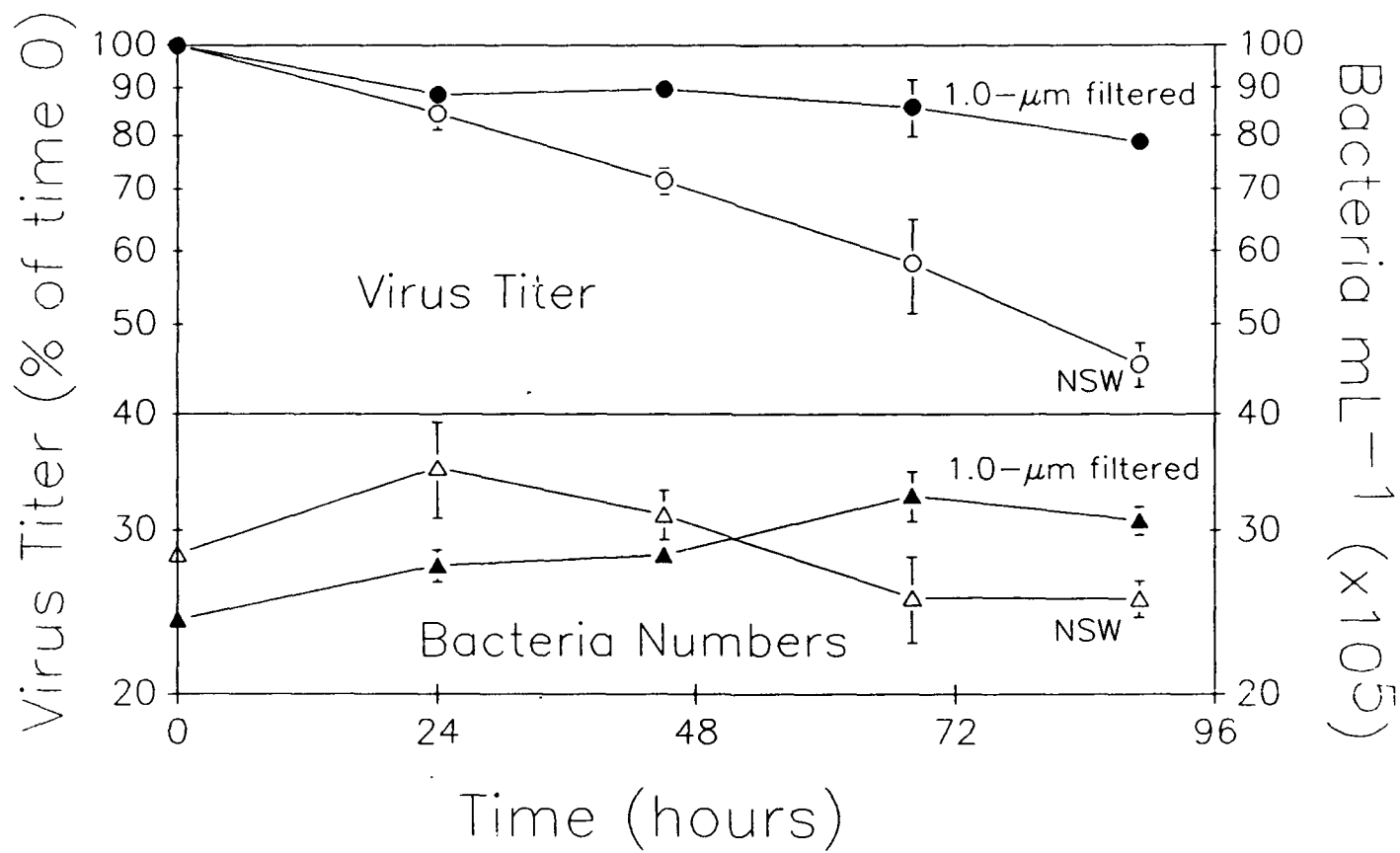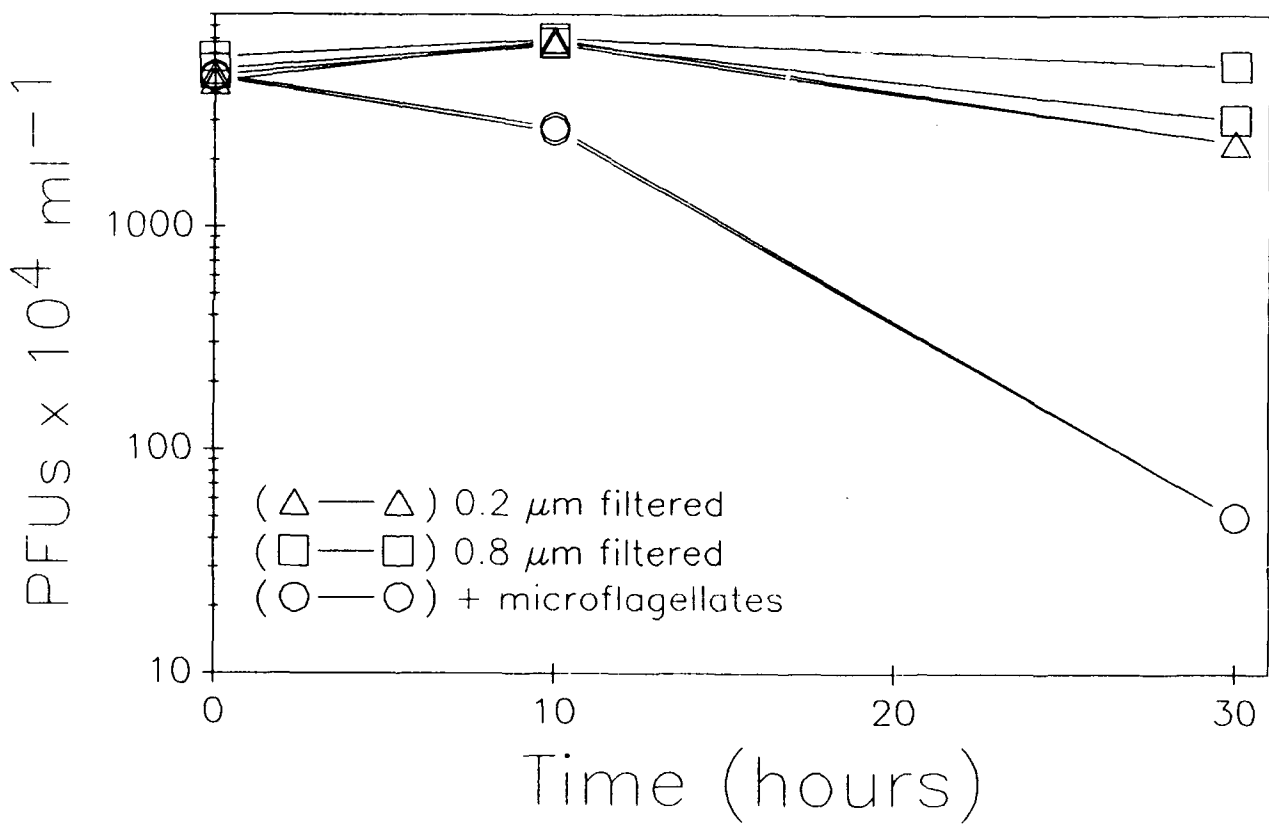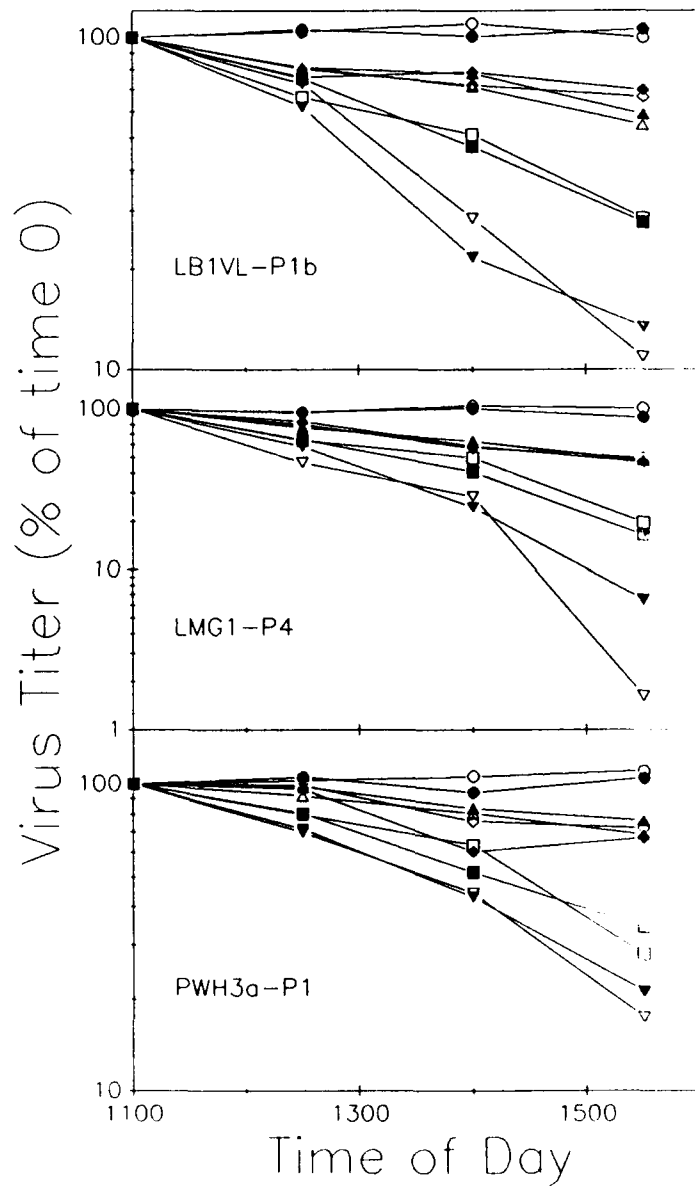28   infectivity resulting from solar radiation.

FIG 3

FIG 5

SOLAR RADIATION 0.38 d^{-1}

FREE VIRUSES

ADSORPTION 0.41 d^{-1}

INFECTION 0.008 d^{-1}

INGESTION 0.002 d^{-1}

LYSIS 0.41 d^{-1}

MICRO-AGGREGATES

NANO-FLAGELLATES

BACTERIA

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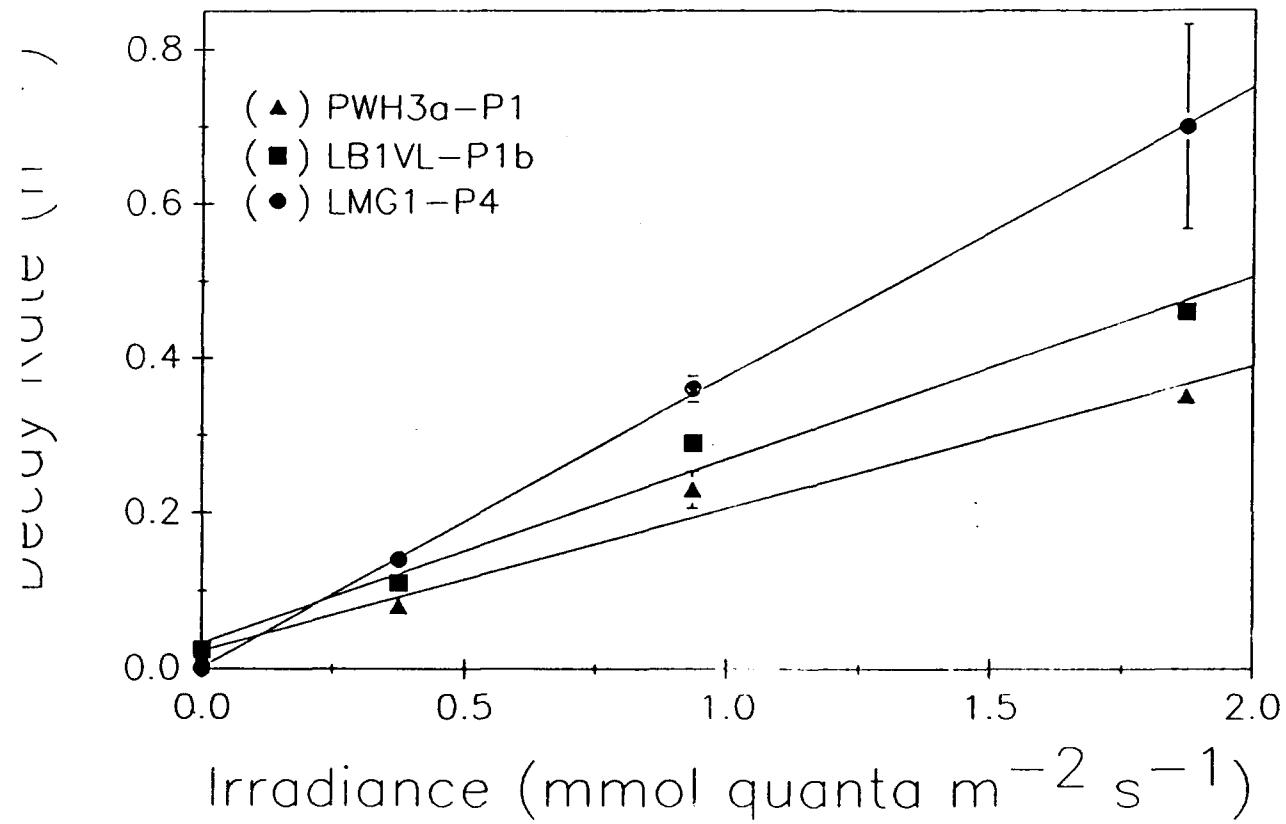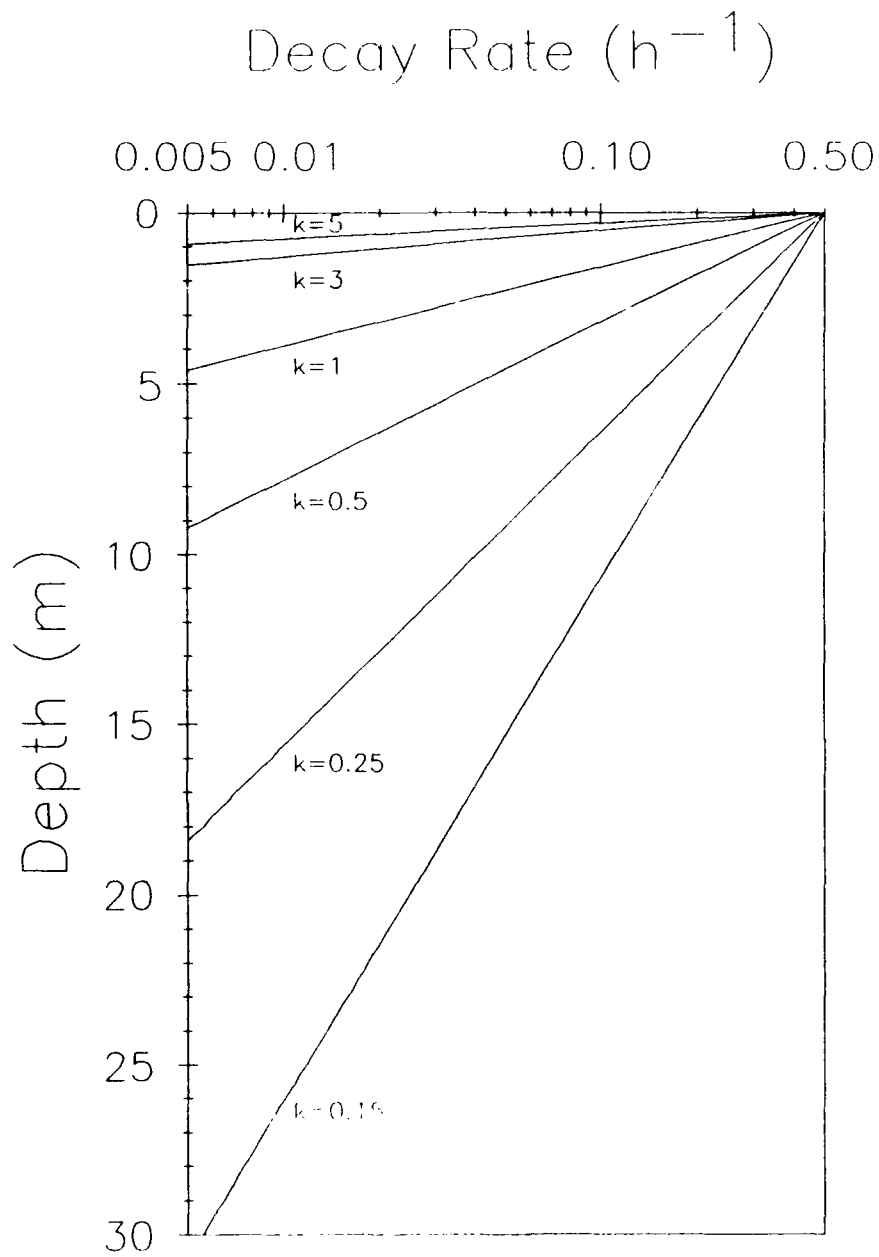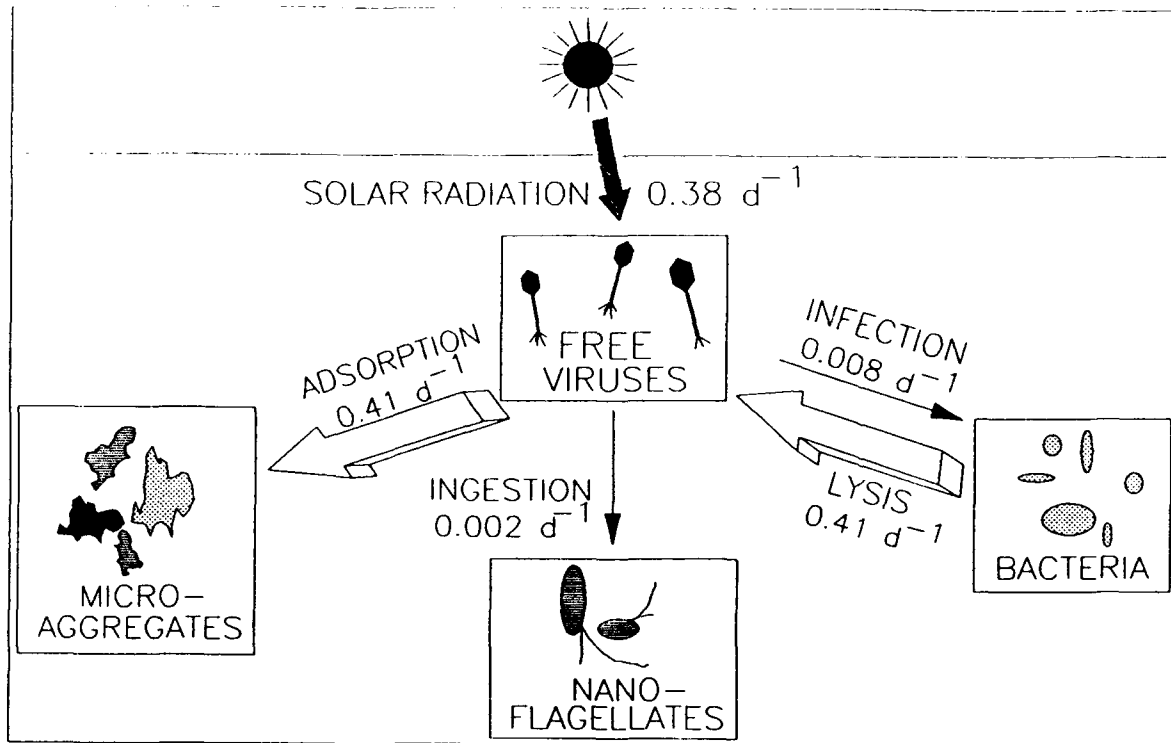MARINE ECOLOGY PROGRESS SERIES
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Inhibition of photosynthesis in phytoplankton by the submicron size fraction concentrated from seawater

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ABSTRACT: Ultrafiltration was used to concentrate the 2–200 nm size fraction from seawater samples 100- to 1000-fold. Electron microscopy indicated that these concentrates were heavily enriched with virus-like particles. When aliquots from these concentrates were added to natural seawater samples, primary productivity (^{14}C-bicarbonate uptake) was either little affected or suppressed within minutes by up to 78%. In some instances as little as a 20% increase in the concentration of the 2–200 nm size fraction in seawater reduced relative carbon fixation rates by nearly 50%. Autoclaving the concentrates before addition reduced or eliminated the bioactive effect. Larger additions caused greater inhibition, although a point was reached where further additions of concentrate did not result in greater photosynthetic suppression, relative to samples to which autoclaved concentrate was added. Microautoradiography indicated that there were fewer photosynthetically-active cells in seawater samples to which untreated concentrates were added, when compared to samples receiving autoclaved concentrate. Phytoplankton >3 µm were affected the most. This indicated that specific organisms were affected as opposed to photosynthesis being reduced throughout the community. In vivo chlorophyll fluorescence paralleled changes in carbon incorporation, and increased most slowly when the greatest additions of concentrate were made. Nonetheless, growth of phytoplankton recovered several days after the concentrates were added, suggesting either that some cells were unaffected by the addition or that resistance to the bioactive agent(s) developed over time. The data provide further evidence for the presence of a strongly bioactive component that inhibits primary productivity, which is associated with the virus-rich 2–200 nm size fraction of seawater.

INTRODUCTION

It is now well established that high concentrations of virus-like particles are a ubiquitous feature of marine waters (Torrella & Morita 1979, Bergh et al. 1989, Proctor & Fuhrman 1990, Hara et al. 1991, Paul et al. 1991) and apparently of freshwater systems as well (Klut & Stockner 1990). These natural viral communities can be extremely dynamic (Bratbak et al. 1990, Heldal & Bratbak 1991), and data suggest that they may cause significant mortality in natural marine bacterial and cyanobacterial communities (Proctor & Fuhrman 1990, Heldal & Bratbak 1991). Yet, despite the abundance of virus-like particles in seawater, much remains to be learned about the impact of viruses on planktonic communities.

Based on observations that large numbers of virus-like particles could be recovered from seawater using ultrafiltration (Proctor et al. 1988, Proctor & Fuhrman 1990) we used similar methodology in a successful attempt to isolate from seawater pathogens that infected marine phytoplankton (Suttle et al. 1990, 1991a). As pathogens infecting a number of important groups of phytoplankton were present in the ultrafiltration retentate (2–200 nm size fraction), an aliquot of this relatively high molecular weight concentrate was added to a seawater sample to determine if photosynthesis (uptake of ^{14}C-bicarbonate) was inhibited. Surprisingly, a 6 % (v/v) addition of concentrate to a natural seawater sample reduced the rate of ^{14}C incorporation by 46 %. Based on this observation a series of experiments was initiated to examine the relationship between the volume of concentrate added to seawater and the degree of inhibition of photosynthesis. These results demonstrated that addition of this bioactive material to natural seawater samples could reduce rates of primary productivity.

These observations coupled with the recent interest in 'dissolved' organic carbon in the sea (e.g. Benner et al.

1992, Martin & Fitzwater 1992, Ogawa & Ogura 1992) and observations of high concentrations of viruses in coastal and offshore waters, prompted further investigation into the effects of material concentrated from the 2-200 nm size fraction of seawater on photosynthetic rates of the phytoplankton. This paper presents additional data on the bioactive nature of material concentrated from seawater in the 2-200 nm size fraction.

MATERIALS AND METHODS

Concentration of the 2-200 nm size fraction from seawater. Surface seawater samples (50 to 100 l) were collected either from the pier or the entrance to the small-boat harbor at the University of Texas Marine Science Institute (USA), and dispensed into acid-washed and rinsed polyethylene carboys. Aliquots of this water were gently poured into a 20 l stainless-steel vessel and pressure filtered (< 130 mm Hg) through 142 mm diameter glass-fiber (MFS GC50, 1.2 μm nominal pore size) and low protein-binding Millipore GVWP 'Durapore' membrane filters (0.22 μm pore size) to remove zooplankton, phytoplankton and most bacteria. Filters were connected in series and held in place by stainless-steel filter holders. A peristaltic pump and spiral-wound ultrafiltration cartridge (30 000 MW, Amicon S1Y30) was used to concentrate the 2-200 nm size fraction ca 100- to 1000-fold (assuming 30 000 MW is equivalent to 2 nm), based on the final volume of the concentrate. Further details of the method are described in Suttle et al. (1991a).

Microscopy. Virus-like particles (VLPs) in the concentrates were observed using a JOEL JEM-1000X transmission electron microscope (TEM). Carbon-coated 400 mesh copper grids were floated on a drop of concentrate for 20 min, rinsed through a series of distilled water drops to remove the salt and negative-stained for 10 s with 1 % w/v uranyl acetate.

The abundance of viral particles in the concentrates were estimated by staining samples with a fluorochrome (DAPI, 4,6 diamidino-2-phenylindole) specific for double-stranded DNA (dsDNA), and enumerating the stained particles with an epifluorescence microscope. This method likely underestimates virus numbers as non-dsDNA viruses and some small dsDNA viruses cannot be visualized using DAPI (Suttle unpubl.). The microscopy methods are described in detail elsewhere (Suttle ~~1991~~. 1992.

Photosynthetic rates. The 2-200 nm size fraction was concentrated from seawater as described above and split into 2 portions, one of which was autoclaved at 121 °C for 20 min. These concentrates were added back at a range of dilutions to 40 to 60 ml of seawater collected at the same location from which the viral size fraction was concentrated. The samples were labelled with 5 μCi of ^{14}C-bicarbonate and incubated either in darkness or under daylight fluorescent bulbs at an irradiance of 120 μmol m^{-2} s^{-1}. Each experiment included autoclaved and untreated concentrates added at a range of dilutions, as well as controls receiving no addition. Treatments and controls were in duplicate. Immediately following the addition of the label and at 4 and 8 h afterwards aliquots of the samples were filtered onto 0.2 μm pore size Poretics polycarbonate filters underlain with glass-fiber filters (Suttle et al. 1991b). Filters were placed in open scintillation vials in an airtight container and fumed overnight in the presence of an open bottle of concentrated HCl. The container was then opened in a fume hood, the bottle of HCl capped, and the moisture in the vials allowed to evaporate. Scintillation fluor was added to the vials and the radioactivity retained by the filters determined using a Beckman LS 5801 scintillation counter.

Fluorescence measurements. In vivo chlorophyll fluorescence measurements were initiated in conjunction with one of the primary productivity experiments. After the seawater was filtered through 120 μm pore size Nitex screening to remove the large zooplankton, 100 ml was dispensed into each of four 125 ml borosilicate flasks. Nothing was added to 2 of the flasks and 10 ml of concentrate collected the previous day was added to the others. This was equivalent to a final concentration factor of 86.6. Fluorescence was monitored using a Turner Designs (Model 10) fluorometer at predetermined time intervals for 7 d following the addition of the concentrate. As well, at the end of the concurrent primary productivity experiment, samples for in vivo fluorescence were collected from each of the treatment and control samples.

Microautoradiography. Microautoradiography was used to determine the number of photosynthetically-active cells in treated and untreated seawater samples. No attempt was made to use autoradiography to quantify the radioactivity incorporated by individual cells. Samples for microautoradiography were collected from seawater receiving no addition (control), or a 7.4 % (v/v) inoculum (concentration factor: 69) of autoclaved or unautoclaved material concentrated from the viral size fraction. Incubations were carried out as outlined above for the photosynthetic rate experiments except that 32 μCi of ^{14}C-bicarbonate was added to each of the bottles from which subsamples for microautoradiography were obtained. At the end of the 8 h incubation, 5 ml samples were filtered under gentle vacuum onto the shiny sides of 25 mm diameter, 0.2 μm pore size filters. Immediately after filtration the polycarbonate filters and underlays were transferred into a light-tight slide box. Shortly afterwards the filters were placed sample side down onto the surface of a glass slide which had been coated with autoradiographic

emulsion. After drying at 4°C overnight the filters were carefully peeled away from the dried emulsion in total darkness, and the slides exposed for 4 wk at 4°C before development. The relatively high level of radioactivity used and the long exposure times for the autoradiograms minimized the possibility that photosynthetically-active cells would not be detected.

At 1000× magnification a minimum of 20 random fields were examined and 200 labelled cells of each size class counted. The 3 size classes (<3, 3–10 and >10 µm) were based on the maximum cell dimension.

RESULTS

Rates of inorganic C uptake

Ultrafiltration of seawater which was prefiltered through glass-fiber and 0.22 µm pore size membrane filters resulted in concentration of the viral size fraction. The number of viruses in these concentrates was ca 10^9 ml^{-1} based on counts of DAPI-positive particles. TEM revealed that this material was very enriched with a diverse community of virus-like particles and was virtually free of bacteria or other recognizable particulate material (Fig. 1).

Addition of these concentrates to seawater samples typically resulted in inhibition of rates of photosynthesis,

relative to samples which received either autoclaved concentrates or no addition (i.e. concentration factor 1) (Figs. 2A, B & D), although occasionally there was little or no inhibition of photosynthetic rates in response to the addition of concentrate (Fig. 2C). Autoclaving removed the effect of the inhibitory material, however, in some samples the largest additions caused reduced photosynthetic rates relative to the no-addition controls (Figs. 2A & D). The decreases in photosynthetic rates occurred within minutes of the addition of concentrate and remained unchanged over a 2 h incubation (Fig. 3). Addition of ultrafiltrate to seawater samples did not affect photosynthetic rates (data not shown), indicating that the biological activity was not the result of a water soluble toxin resulting from the concentration procedure. As well, the pH of the retentate was the same as that of the seawater from which it was concentrated.

Fig. 1. Electron micrograph of a coastal natural marine viral community concentrated from seawater using ultrafiltration. Notice the great morphological diversity of the virus-like particles. Scale bar = 100 nm
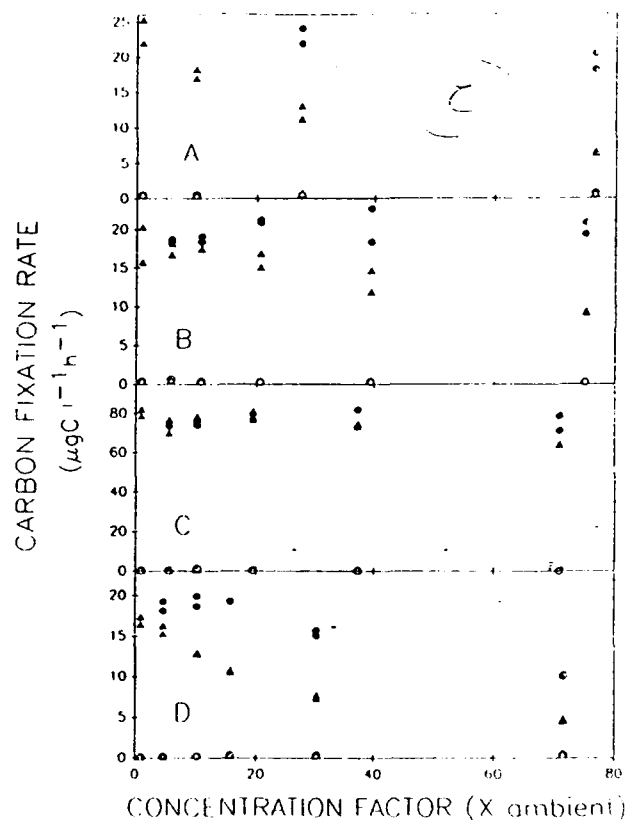

Fig. 2. Results of experiments conducted on (A) 14 and (B) 26 September 1989, and (C) 15 March and (D) 5 April 1990. High molecular weight material concentrated from seawater by ultrafiltration was added to seawater and rates of primary productivity measured during 4 h incubations. Closed symbols represent duplicate samples that were incubated in the light and to which either autoclaved (●) or non-autoclaved (▲) concentrates were added. Open symbols represent samples that were incubated in the dark and to which either autoclaved (○) or non-autoclaved (△) concentrates were added. The concentration factors assume that the >30 000 MW fraction was concentrated with 100% efficiency
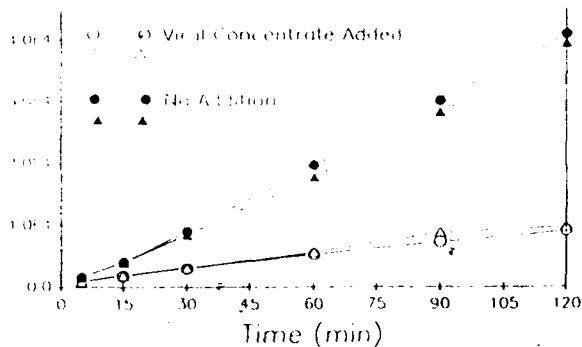
Fig 3 Time course of accumulation of ^{14}C-bicarbonate into phytoplankton in natural seawater and in natural seawater to which high molecular weight concentrate (2–200 nm size fraction) had been added. Different symbols indicate data collected from duplicate treatments

claved concentrate (t-test, p = 0.014). However, cells in the <3 µm size class appeared to be inhibited by the addition of autoclaved concentrate relative to cells which received no addition, although the difference was not significant (t-test, p = 0.052). The results were even more striking for larger cells. In the 3–10 µm size fraction the number of photosynthetically-active cells was reduced by about 90 % in seawater samples exposed to concentrate, relative to samples receiving autoclaved concentrate (t-test, p = 0.0002). Similar results were observed for the >10 µm size fraction, although the number of photosynthetically-active phytoplankton was only reduced by about 70 % (t-test, p = 0.023).

In vivo chlorophyll fluorescence

Moreover, the degree of photosynthetic suppression in response to the addition of concentrate was similar whether measured over 4 or 8 h (Fig. 4). As well, photosynthesis was never completely inhibited. A point was reached where further addition of concentrate did not result in greater photosynthetic suppression, relative to samples to which autoclaved concentrate had been added (Fig. 4).

Photosynthetically-active cells were easily recognized using microautoradiography (Fig. 5). These data were collected in conjunction with the experiments of 5 April 1990 (i.e. Fig. 2D & 4D). In all 3 size classes the concentration of photosynthetically-active cells was lower in seawater samples exposed to concentrate than in samples receiving autoclaved concentrate (Fig. 6). In the <3 µm size fraction about 60 % as many cells were associated with exposed silver grains in samples inoculated with concentrate as compared to those samples that received auto-

Temporal changes in fluorescence were consistent with phytoplankton growing at a lower rate in seawater samples to which concentrate had been added (Fig. 7). Values of in vivo fluorescence remained similar in control and treated seawater samples for 4.5 h following the addition of concentrate. Subsequently, the values diverged so that by 23 h the fluorescence in the samples to which concentrate was added was less than half that of samples which received no addition. After about 36 h the fluorescence began to increase in the seawater samples to which concentrate had been added, eventually surpassing that in the control cultures. This indicated that some cells were capable of growth in the presence of the concentrate.

The in vivo chlorophyll fluorescence of seawater samples was affected by the volume of concentrate added in much the same way as rates of photosynthesis were affected (Fig. 8). After 9 h of incuba-
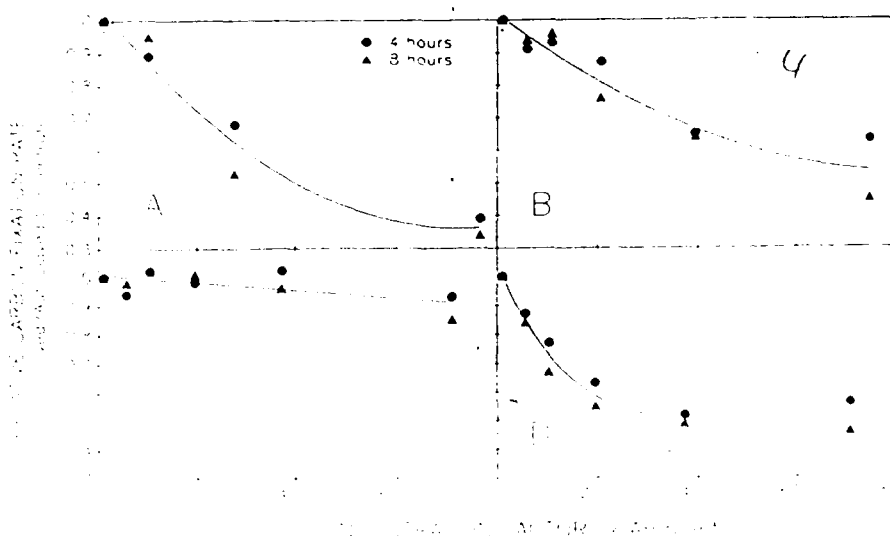

Fig 4 Changes in photosynthetic rates in seawater to which 30 000 MW ultrafiltration retentate has been added, relative to seawater to which autoclaved retentate was added, after (●) 4 h and (▲) 8 h Curves were fitted by non-linear, least-squares regression analysis Dates of experiments as in Fig 2
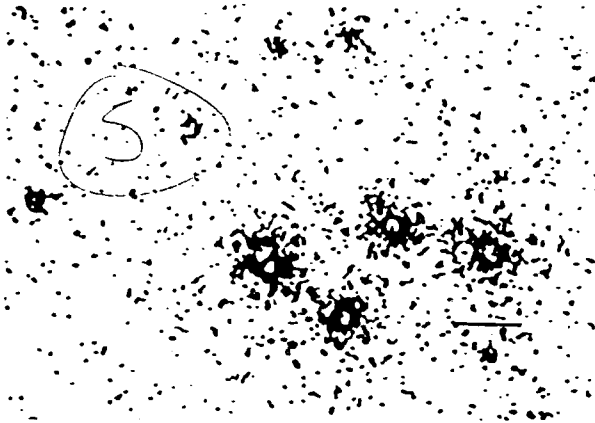
Fig. 5. Microautoradiograms showing 5 photosynthetically-active cells in the <3 μm size fraction (primarily *Synechococcus* spp.). Scale bar = 3 μm

Fig. 6. Concentration of radioactively-labelled phytoplankton cells in natural seawater and natural seawater to which either autoclaved or untreated concentrate had been added. Experiments were in duplicate (Rep A and Rep B). The <3 μm cells consisted of *Synechococcus* spp. while the larger cells were made up of diatoms and larger flagellates

tion samples exposed to untreated concentrate had lower levels of fluorescence than samples to which autoclaved concentrate had been added. Just as in the productivity experiments, a point was reached where further increases in the amount of concentrate added did not result in further decreases in fluorescence. Increases in the concentrate beyond a factor of about 10 times ambient, resulted in fluorescence values that were about 60 % of those to which the same quantity of autoclaved concentrate had been added.
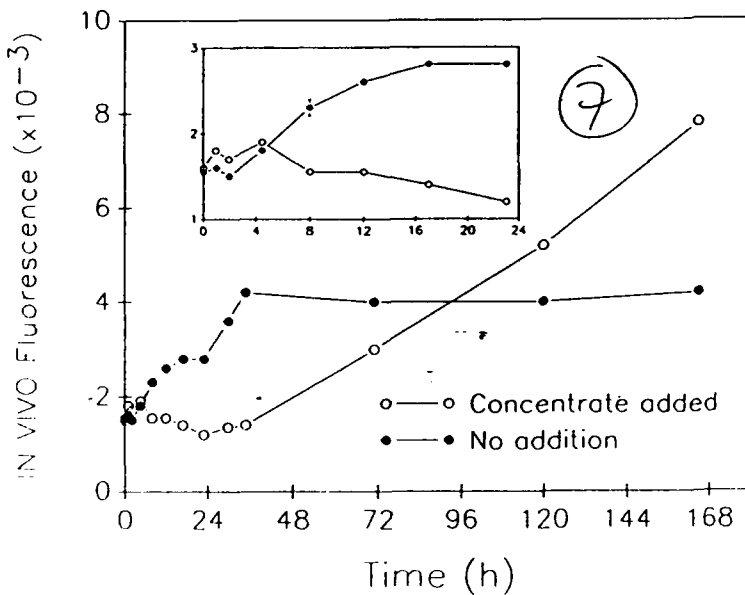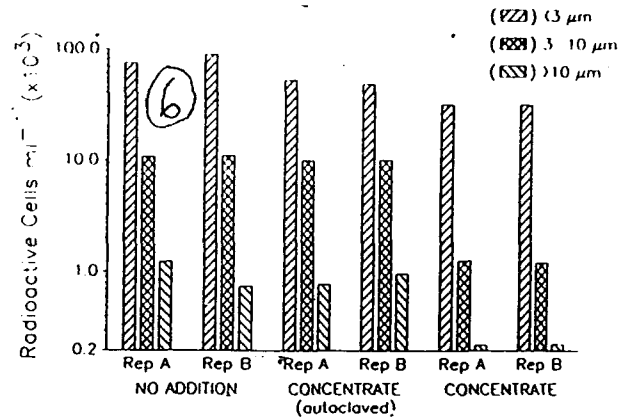

Fig. 7. *In vivo* chlorophyll fluorescence in natural seawater and seawater which was amended by adding a 9 % inoculum of 30 000 MW concentrate. Each point is the mean value from duplicate cultures. SD were less than the width of the symbols

DISCUSSION

Photosynthetic rates

These data clearly demonstrate that one or more substances can be concentrated from the 2–200 nm size fraction of seawater that strongly inhibit rates of photosynthesis in natural communities of marine phytoplankton. Bacterial growth rates have also been observed to decrease when virus-enriched material, concentrated from seawater by ultrafiltration, has been added back to seawater samples (Proctor et al. 1988). The degree to which photosynthesis was affected by the addition of the concentrates varied between seawater samples. At times little or no inhibition was observed, whereas on other occasions rates were substantially reduced (Figs. 2 & 4). There were at least 2 bioactive components to the substance. One component was heat labile and often caused strong reductions in photosynthetic rates when added at relatively modest enrichments. The other component remained bioactive after autoclaving, and was most effective at higher concentration factors. Consequently, even though rates of photosynthesis continued to decrease at concentration factors above 40 (Fig. 2), these decreases occurred whether or not the concentrate had been autoclaved, indicating that the effect was due to non-heat-labile components. The fluorescence data corroborated the productivity data
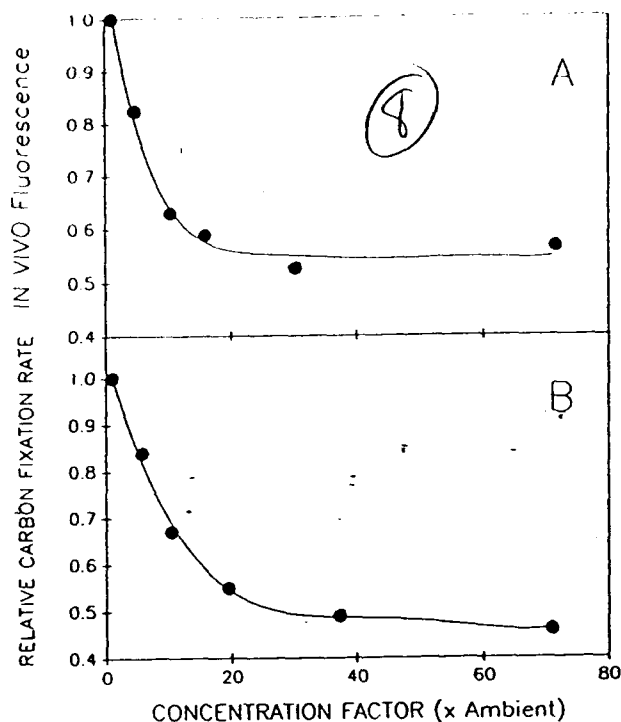
Fig. 8. (A) *In vivo* chlorophyll fluorescence and (B) carbon-fixation rate in natural seawater to which concentrate had been added, relative to seawater to which autoclaved concentrate was added. Data were collected ca 8 h after the addition of the concentrate

and showed that after a several hour lag in both treatments, growth rates were higher in samples to which concentrate was not added (Fig. 7).

There are 2 interpretations to the observation that photosynthetic rates decreased after addition of the concentrates. One is that carbon fixation was inhibited throughout the phytoplankton community. The other is that photosynthesis was suppressed in certain members of the community while other components were unaffected. Two aspects of the data suggest that the decreases in productivity resulted from selective inhibition of a subset of the phytoplankton community. First, the relative decrease in photosynthetic rates was less at higher concentration factors, suggesting that some cells were unaffected by the bioactive material. If the entire community were affected one would expect photosynthetic rates to continue to decline as the concentration factor increased. Second, after ca 36 h, fluorescence began to increase in the samples to which concentrate had been added, suggesting that a subset of the phytoplankton community was growing.

Autoradiography showed that cells in the >3 and >10 µm size fractions (diatoms and flagellates) were very sensitive to the addition of the concentrate; whereas, cells in the <3 µm size fraction (largely

Synechococcus spp.) were much less so (Fig. 6). As the addition of untreated concentrate resulted in fewer cells with incorporated isotope, relative to samples to which autoclaved concentrate was added, it indicated that only certain cells were affected by the addition. If the entire assemblage were affected there should have been no difference in the concentration of photosynthetically-active cells (i.e. those associated with exposed silver grains) in seawater samples receiving autoclaved or untreated concentrate (although phytoplankton in the treatment samples would photosynthesize at a reduced rate). Given the strong grain development observed (Fig. 5), even cells with reduced photosynthetic rates should have been detected. Furthermore, in hundreds of experiments where >30 000 MW concentrates were added to unialgal cultures of phytoplankton (e.g. Suttle et al. 1990, 1991a), growth rates were not reduced relative to controls receiving no addition, unless a pathogen for the particular alga being screened was present. Thus primary productivity, fluorescence, and microautoradiography data were all consistent with the hypothesis that carbon fixation was only suppressed in a subset of the phytoplankton community.

It is worth emphasizing that modest increases in the concentration factor caused substantial decreases in photosynthetic rates (Figs. 2 & 3). For example, as little as a 20-fold increase in the concentration factor caused nearly a 50 % decrease in relative carbon fixation rates in some instances (Figs. 4A & 4D). This assumes that the >30 000 MW fraction was concentrated with 100 % efficiency. If concentration efficiencies were lower this would indicate that the retentate was even more potent. It is conceivable that the bioactive substance was formed as part of the ultrafiltration procedure. Although this possibility cannot be easily dismissed there is no evidence of which I am aware indicating that bioactive substances can be formed when seawater is ultrafiltered.

Relative changes in fluorescence were similar to those observed for photosynthetic rates (Fig. 8). This was because growth rates were lower in seawater samples to which concentrate was added; therefore, fluorescence also increased more slowly. It was not because fluorescence decreased when concentrates were added (e.g. Fig. 7).

Nature of the bioactive agents

Transmission electron microscopy indicated that the material concentrated from 0.2 µm filtered seawater was highly enriched in virus-like particles, with no other recognizable particulate material being visible (Fig. 1). This confirms previous reports that both

natural viral communities (Proctor & Fuhrman 1990, Suttle et al. 1990, 1991■, Paul et al. 1991) and specific types of viruses (e.g. Belfort et al. 1974, 1976) can be concentrated efficiently from seawater by ultra-filtration.

The heat-labile nature of the bioactive material also suggests that a biologically-mediated process was responsible for the decreases in photosynthetic rate. There are few candidates other than viruses that are strongly bioactive, fall in the 2–200 nm size range and are heat labile. The small molecular size of antibiotics (e.g. Austin 1989) and marine humics (900 to 1200 MW; Harvey & Boran 1985) makes it unlikely that either is the active agent; as well, humic acids are refractory by definition and should be relatively stable during auto-claving. Nonetheless, the possibility remains that free proteins or perhaps certain large heat-sensitive humic or fulvic acids or colloids could be responsible for sup-pressing primary productivity.

As photosynthetic rates were affected in a subset of the phytoplankton community it suggests that taxon-specific pathogens might be involved. The reason that the number of photosynthetically-active cells in the <3 μm size class was not as greatly affected may be that cyanophage-infected *Synechococcus* spp. photo-assimilate carbon until the point of cell lysis, many hours later (MacKenzie & Hazelkorn 1972). If viral pathogens were not the responsible agent it would imply that the bioactive substance specifically inhibits eukaryotes.

The fact that carbon assimilation was reduced within minutes of adding the concentrates to seawater and continued at the same reduced rate for hours after-wards (Fig. 4) does not refute the possibility that viruses caused the reduction of photosynthetic rates. Intuitively, one would expect viral infection to cause a gradual reduction in primary production as more cells became infected and as cellular processes began to be affected; however, this is not the case for all unicellular algae. Although data are limited, in some *Chlorella*-like algae carbon fixation is suppressed almost imme-diately following infection (Van Etten et al. 1983); whereas in *Micromonas pusilla* carbon fixation rates do not decrease until about 4 h post infection (Waters & Chan 1982). It remains to be seen which, if either, will turn out to be typical for marine phytoplankton.

The fact that the bioactive component could not always be eliminated entirely by autoclaving implies that viruses were not the only agent responsible for reducing primary productivity. It was not possible to completely remove the inhibitory material by centrifu-gation of a concentrate at 114 000 × *g* for 150 min (data not shown). Therefore, some of the bioactive substance had a sedimentation coefficient of <98 *S*, less than that of even the smallest viral particles.

Initial observations of decreased productivity of marine bacteria (Proctor et al. 1988) and phytoplank-ton (Suttle et al. 1990) have been associated with the addition of concentrated natural marine viral commu-nities to seawater. Even though viruses are essentially the only particles visible in these concentrates using TEM, other less heat-labile substances which also have the potential to inhibit primary productivity must be present, as well. The present challengers are to deter-mine how much of the photosynthetic suppression is the result of viral infection and how much is attribut-able to other high molecular weight bioactive sub-stances. Ultimately, the goal must be determine the degree to which primary productivity is affected by these substances when they are present at ambient concentrations.

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Enumeration and Isolation of Viruses

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Paul - looks good. Only a few minor changes.

Curtis

I. INTRODUCTION

The methods described here are designed to be used for the enumeration and isolation of viral pathogens that infect aquatic bacteria, cyanobacteria and phytoplankton. However, many of the procedures are similar to virological methods that have been in place for many years in other scientific and medical disciplines. This contribution brings together in a convenient forum some of the more suitable methods.

Basically, four methods are currently used for enumerating viruses in aquatic environments. These are plaque assays, most-probable-number assays (MPNs), transmission electron microscopy (TEM) and epifluorescence microscopy. The methods tell us different things. Plaque assays and MPNs are for quantifying the abundance of infectious units which cause lysis of a particular host. Obviously these assays require the host of interest to be culturable. TEM is typically used for enumerating the number of viral-like particles (VLPs), either in whole water or in culture medium, while epifluorescence microscopy has been used for quantifying viral-sized particles containing double-stranded DNA. The procedure used depends on the question being addressed and the accuracy and sensitivity required. The advantages and disadvantages associated with each of the methods are addressed in the following protocols.

Successful isolation of a virus depends on bringing host and pathogen together so that infection of the host and amplification of the virus occurs. Unless viruses infecting the host are abundant the chances of isolating one directly by plaque or MPN assays are slim; hence, the probability of finding a virus increases with the volume of water screened. Also, keep in mind that host density is the major factor dictating the rate of viral propagation. Screening a relatively large volume of water can be accomplished either by growing the host to high density in the water to be assayed, or by concentrating the virus community using ultrafiltration and adding some of the concentrate to an exponentially growing culture of the host. Additional information on recovering viruses from the natural environment can be found in Berg.[1]

II. MATERIALS REQUIRED

A. Enumeration of viruses

i. Plaque assays

a. Equipment

Autoclave
Microwave oven
25 mm polysulfone filter funnel (Gelman)
1 ml and 5 ml adjustable pipettes
Constant-temperature dry bath or water bath
Centrifuge *Microcentrifuge*
Culture facilities for bacteria and/or algae
Vortex mixer

b. Supplies

Polypropylene or polycarbonate sampling bottles
25 mm, 0.22 and 0.45 μm pore-size polyvinylidine difluoride (PVD) "Durapore" membrane filters (Millipore)
25 mm, 0.2 and 0.4 μm pore-size polycarbonate membrane filters (Nuclepore or Poretics)
Petri plates
Pipet tips
250 ml screw-cap borosilicate-glass media bottle or Erlenmeyer flask
1.5 ml microfuge tubes
13 x 100 mm disposable glass culture tubes

Purified agar (e.g. Fisher Scientific)
Liquid culture medium

c. Solutions

Bottom agar: In a 1-l container add 5 g of agar to 500 ml (1 % w/v) of an
appropriate growth medium. Partially melt the agar by microwaving (e.g.
3 min at 700 W) and autoclave for 20 min at 121 °C. Allow the medium to
cool to about 60 °C and under sterile conditions pour 15-20 ml into each
of 20-30 plates. Leave the petri plate lids slightly ajar while the
plates are cooling to prevent condensation accumulating on the lids.
Once the agar has solidified, stack the plates and invert them to
prevent condensation dripping on the surface of the agar. These plates
can be used about 12 h after pouring if the surface of the agar is not
wet. The plates can be kept at room temperature for a week or more if
they are sealed in a plastic bag.

Top agar: Prepare 0.6 % (w/v) top agar by adding 0.6 g of agar to 100 ml of
medium in a 250 ml screw-cap Erlenmeyer flask or media bottle and
sterilize, with the lid off, as described above. If sealed after
sterilization the medium can be stored at room temperature for many
months and remelted in a microwave oven before use. Repeated melting
may result in precipitates forming. For each water sample that is to be
assayed pour 2-2.5 ml of molten top agar into each of three 7-ml
13 x 100 mm disposable glass culture tubes and keep at 45-47° C. For
each experiment also include 3 tubes of agar that will be used for
controls.

Host cells: For microalgae such as *Synechococcus* or *Chlorella*, cells are
grown in liquid culture to about 10^7 cells ml^{-1}. While still in
exponential growth the cultures are harvested by gentle centrifugation
and resuspended in liquid medium at a concentration of about $10^9 ml^{-1}$. For
bacteria grown in nutrient-rich broth it is generally not necessary to
concentrate the cells.

ii. Most-Probable-Number (MPN) assays

a. Equipment

5 ml adjustable pipette
Fluorometer with filter set for chlorophyll *a* determination (420_{ex}, $>640_{em}$;
e.g. Corning CS 5-60 and CS 2-64)
Culture facilities for phytoplankton
25 mm polysulfone filter funnel (Gelman)

b. Supplies

1 L polypropylene or polycarbonate container
25 mm, 1.0 μm pore-size polycarbonate membrane filters (Nuclepore or
Poretics)
7 and 50 ml glass culture tubes (13 x 100 and 25 x 150 mm, respectively)
with polypropylene screw caps
Pipet tips
Liquid culture of host organism in exponential growth (10^7 cells ml^{-1}).

iii. Transmission Electron Microscopy (TEM)

a. Equipment

Ultracentrifuge with swinging-bucket rotor
Transmission electron microscope
Tweezers for handling EM grids
Centrifuge support platforms for EM grids

b. Supplies

200 to 400 mesh copper TEM grids coated with carbon and Formvar
Ultracentrifuge tubes
Parafilm
TEM grids and grid platforms: The grid platforms must be strong but not so
   dense that the safety of the rotor is compromised. The simplest
   approach is to mold perfectly balanced platforms out of epoxy resin or
   machine them out of hard plastic such as Plexiglass[2] (also see Cochlan et
   al., submitted ms.). The platforms can be easily removed if a threaded
   hole is included in the platform into which a small rod can be inserted.
   Preferably, the grids should be held in place within grid-size shallow
   depressions on the surface of each platform. A small notch beside each
   depression will facilitate removal of the grids.

For this technique, carbon-coated Formvar films on copper grids are
probably the best compromise between strength and electron transparency.
These are commercially available (e.g. Ted Pella, Inc.), but expensive,
or they can be prepared if one has access to a carbon evaporator.[3] A
problem with support films is that uneven charge on the surface can
affect the distribution of VLPs, thereby resulting in inaccurate
estimates of virus density. Consequently, the surface of the films
should be made evenly hydrophylic by UV irradiation, by exposure to a
strong electrical field in a reduced atmosphere (glow discharge) or by
chemical treatment.[3] A simple treatment is to float the grids for 1 min
on a drop of 4000 MW poly-l-lysine and wick away the excess solution
immediately before using the grids.

c. Solutions

25 % (v/v) EM-grade glutaraldehyde
1 % (w/v) uranyl acetate solution
Uranyl-acetate Stain: Add 0.25 g of uranyl acetate ($C_4H_6O_6U$) to 25 ml of
   distilled water and gently stir for about 30 min to make up a 1 % stain
   solution. Be careful when handling uranyl acetate as it is toxic and
   radioactive. Store it at 4 °C in a tightly-capped dark bottle as it is
   light sensitive and prone to oxidation. The stain can be stored for
   several weeks, but as precipitate may form it should be centrifuged or
   filtered prior to use. This is nasty stuff so always dispose of
   unwanted stain as toxic waste.

iii. Epifluorescence microscopy of DAPI-positive particles

a. Equipment

Epifluorescent microscope with a 100 watt Hg bulb, 334-365 nm excitation
   and > 420 nm emission filter sets, and a ocular quadricule divided into
   100 grid squares.
20 μl and 2 ml adjustable pipettes
Microcentrifuge for 250 μl centrifuge tubes

b. Supplies

25 mm polysulfone filter funnel (Gelman)
Polypropylene or polycarbonate bottles for sampling
0.03 and 0.2 μm pore-size, 25 mm, polycarbonate membrane filters (Poretics)
250 μl centrifuge tubes — 0.45μm pore-size, 25mm, nitrocellulose membrane filters
Microscope slides
22 x 22 mm cover slips
Low-fluorescence immersion oil

c. Solutions

5 μg ml⁻¹ solution of DAPI (4′, 6-diamidino-2-phenylindole) (Sigma D 1388).

Make up a 5 μg ml⁻¹ solution of DAPI in McIlvaine's buffer (pH = 4.4). To
make the buffer dissolve 3.561 g of $Na_2HPO_4 \cdot 2H_2O$ in 100 ml of distilled
water (Solution A) and 2.101 g of citric acid·H_2O in 100 ml of distilled
water (Solution B) and combine 8.82 ml of Solution A with 11.18 ml of
Solution B. Store at 4 °C in the dark as DAPI is very light sensitive.
Irgalan black (Ciba-Geigy). Make the 0.03 μm pore-size filters non-
fluorescent, by soaking them for several hours at 90 °C in a solution of
2 g Irgalan black dissolved in 1 l of 2 % acetic acid. After staining
rinse the filters in filtered distilled water. For larger orders
stained 0.03 μm pore-size filters are currently available from Poretics
Corp. (Livermore California, USA).
0.2 M solution of $Na_2HPO_4 \cdot 2H_2O$ (M.W. 178.05)
0.1 M solution of Citric acid·H_2O (M.W. 210.14)
0.015 M solution of NaCl (M.W. 58.44)
Acetic acid ($C_2H_4O_2$; M.W. 60.05)
Formaldehyde or 25 % EM-grade glutaraldehyde
DNase (DNase I, Sigma D 4527) Solution: The proportion of DAPI-positive
particles that are DNase-sensitive is usually small, however, the use of
DNase will often make counting easier by reducing background
fluorescence. To make a stock solution of DNase I dissolve 10,000
Kunitz units in 1.0 ml of ice cold 0.15 M NaCl. Aliquot 50 μl
subsamples into 250 μl microfuge tubes and freeze at -80 °C. This
should keep for many months, but it should be assayed periodically for
activity per the manufacturer's instructions.

B. Isolation of viruses

*i. Equipment (AP = amplification procedure, CP = concentration
procedure)*

Fluorometer (Turner Designs) to monitor phytoplankton growth or
  spectrophotometer to measure bacteria growth (AP & CP)
Filtration apparatus with plastic or stainless-steel filter supports for
  47 mm diameter filters (AP)
1 and 5 ml adjustable pipettes (AP & CP)
Lighted incubator or other system for culturing phytoplankton and bacteria
  (AP & CP)
Pump for vacuum and pressure filtration (AP & CP)
Two 142 mm stainless-steel filter holders (CP)
Stainless-steel reservoir for pressure filtration (CP)
Peristaltic pump (several liters per min) for ultrafiltration (CP)
30,000 MW-cutoff spiral ultrafiltration cartridge (Amicon S1Y30) (CP)

*ii. Supplies (AP = amplification procedure, CP = concentration
procedure)*

0.2 and 1.0 μm pore-size, 47 mm, polycarbonate membrane filters (Nuclepore,
  Poretics) (AP)
Culture flasks for phytoplankton and bacteria (AP)
Nutrients for enriched sample water and media (AP & CP)
13 x 100 mm borosilicate culture tubes (AP & CP)
142 mm-diameter glass-fiber filters (MFS CC50, 1.2 μm nominal pore-size)
  (CP)
142 mm-diameter, low protein-binding "Durapore" membrane filters
  (Millipore, 0.22 or 0.45 μm pore-size) (CP)

III. PROCEDURES

A. Enumeration of viruses

i. Plaque assays

Plaque assays are routinely used to estimate titres of viruses that cause
lysis of bacteria, cyanobacteria and algae that can be grown on solid media.[46]

The basis of the method is that each infective unit will form a clearing (plaque) on a lawn of host cells. Lawns are typically made by mixing host cells and viruses in molten agar and quickly and evenly pouring this mixture over a bottom layer made with a higher percentage of agar. The number of plaque-forming units (PFUs) in a given volume of water can be estimated using this method. One must be cautious, however, as a number of aquatic bacteria will form plaques on lawns of bacteria and algae.[7,8] Filtration or pretreatment of the water sample with chloroform are the methods traditionally used to distinguish viral and bacterial pathogens. However, as some algal viruses may be 0.4 μm or larger in diameter,[9] and some are chloroform sensitive, these approaches may be selective.

Advantages of the plaque-assay method include the following: 1) It is relatively sensitive with a practical detection limit of about 5 PFUs ml^{-1}. However, in natural seawater samples viruses infecting specific hosts are often present at even lower concentrations. 2) Accurate results can be achieved with a modest amount of effort. Plaque counts can even be done by image analysis. 3) Pathogens can be easily purified by cloning individual plaques picked from a plate. 4) Only infective viruses are enumerated.

Disadvantages include that 1) the host must be culturable on solid medium; 2) different pathogens cannot easily be distinguished; 3) natural bacteria can interfere with lawn-formation, particularly of slower-growing hosts; 4) filtration may remove some viruses.

1. Sample collection and filtration: Ideally, samples should be collected immediately before plaque assaying. Use a clean container that has relatively low protein binding, such as a polycarbonate or polypropylene bottle. Although prefiltration of the sample selects against some viruses it is often necessary in order to prevent bacteria overgrowing the lawn. Filtration through a 0.45-μm PVD or 0.4-μm polycarbonate filter is often adequate, although in some instances 0.2-μm pore-size filters may be required. Also, many marine viruses show little decrease in titer for several weeks if samples are prefiltered and stored in the dark at 4°C (unpubl. data).

2. In a sterile microfuge tube combine 500 μl of cell concentrate with 500 μl of the sample or control water to be assayed. Mix gently and allow adsorption of the viruses to the host cells. If virus titers are high, decrease the volume of sample added or do a dilution series using sterile medium, prior to adsorption. A 30-45 min incubation should be adequate if the adsorption kinetics are unknown.

[handwritten annotations: "quickly by vortexing to prevent multiple infection of single cell"; "Mix agent 10 µl and yellow"]

3. Following adsorption add the sample to a tube containing 2-2.5 ml of molten top agar, and quickly pour the contents onto the bottom agar.

[handwritten: "Vortex"]

4. After 30 min invert the plates and incubate them under appropriate conditions. The plaques are counted after an even lawn appears.

ii. Most-probable-number Assays (MPNs)

MPNs are usually done to determine the concentration of lytic viruses that infect hosts which cannot be grown on solid medium but which can be grown in liquid.[10] This is particularly a problem with delicate phytoplankton. The assay is simple but labor intensive. It is also very sensitive; a detection limit of 0.01 viruses ml^{-1} is not unreasonable. However, MPNs lack the accuracy and precision of a plaque assay. Traditionally, MPN assays have been done using a series of 10-fold dilutions with 3-10 replicates at each dilution. The replicates in which no growth or growth followed by cell lysis occurs are assumed to contain at least one infective virus. By comparing the number of replicates that contain viruses to a MPN table, the number of infective units can be estimated. With the wide availability of computers and other technologies such as microplate readers, it is not necessary to be constrained by a specific number of replicates or order-of-magnitude dilution regimes. The first step is to determine the detection limit desired. For

[handwritten right margin: "from the top and side of the tube and"]

example, to achieve a detection limit of 1 virus ml^{-1}, several ml of water needs to be screened. In lieu of information on viral titer it is necessary to do a broad dilution series. A series of five 10-fold dilutions starting with 1 ml of sample should span the range of concentrations typically found, although larger volumes can be screened if desired. Changes in phytoplankton biomass are monitored by measuring *in vivo* chlorophyll fluorescence, which allows hundreds of cultures to be non-destructively monitored on a daily basis. The culture tubes specified fit directly into a fluorometer. The MPN, standard error and confidence interval are calculated using a program written in basic.[11]

1. Transfer 50 ml of a exponentially growing batch culture of the host to be screened into 450 ml of fresh medium. As soon as exponential growth of the culture begins, transfer 4 ml into each of fifty-five 7 ml culture tubes. Immediately add 1 ml to each tube from the dilution series outlined below (see steps 2d-f).

2. Collect sample water in a clean polypropylene or polycarbonate container and prepare the dilution series as follows:
   a) Filter 25 ml of sample water through a 1 μm pore-size polycarbonate filter into a filter flask that has been pre-rinsed with filtrate.
   b) Into each of four 50 ml culture (dilution) tubes place 18 ml of medium. Into the first dilution tube add 2 ml of 1 μm-filtered sample water. Mix well.
   c) Take 2 ml from the first dilution tube (10^{-1}) and add it to the second (10^{-2}). Complete the dilution series for the 10^{-3} and 10^{-4} dilutions.
   d) Add 1 ml from the undiluted sample to each of 10 replicate 7 ml culture tubes, each containing 4 ml of exponentially growing host-cell culture (from step 1).
   e) For each of the 4 dilutions, add 1 ml to each of 10 replicate 7 ml culture tubes.
   f) Reserve the 5 remaining tubes as controls.

3. Gently mix each tube and measure the fluorescence on a daily basis. Cultures which have not cleared after 7 days in stationary phase are assumed not to contain a virus.

4. For each dilution, record the number of tubes that have cleared and use these data to calculate the MPN for the concentration of viruses in the sample. The results are generally expressed as the number of tubes from each dilution in which lysis occurs (*e.g.* 10, 10, 3, 0, 0). The MPN can be determined from published tables of values[12] or preferably using a computer program which also provides confidence intervals and standard errors for the MPN estimates.[11]

iii. Transmission Electron Microscopy (TEM)

There are a variety of protocols for determining the concentration of viral-like particles (VLPs) in aqueous solutions using TEM.[13,14] Currently, only two of these have been adapted for use in enumerating viruses in natural aquatic communities. Both methods require that the viruses be concentrated before they can be enumerated because of the high magnifications used in electron microscopy. The viruses can be concentrated using ultrafiltration and subsamples of the concentrate spotted onto EM grids[15] or the viruses can be pelleted directly onto grids by ultracentrifugation.[2,16] The grids are then stained and viewed using TEM.

Both protocols share several disadvantages. 1) They require access to a transmission electron microscope, an expensive facility that is generally only available at large research centers. 2) There are no definitive criteria for what constitutes a VLP in a natural viral community. Although many viruses are easy to recognize because of the presence of phage-like tails, many non-tailed particles are difficult to categorize. 3) Particulates and aggregates can interfere with counting by obscuring large areas of the EM grid. 4) At

the present time *no information can be obtained* on whether the VLPs are infective and if so whether the host is a bacterium, phytoplankton or other organism. As well, if ultrafiltration is used the concentration efficiency must be determined using a tracer. Inert 50 nm polystyrene beads (Polyscience) can be easily enumerated in concentrates using epifluorescence microscopy and can be used as internal standards for determining the concentration of VLPs on EM grids. Alternatively, concentration efficiencies can be ascertained using virus tracers and plaque assays. If viruses are being concentrated from seawater there is the added difficulty that the salts must be removed before the sample can be spotted and dried onto a grid. Proctor and Fuhrman[13] accomplished this by pipetting a 2 μl sample onto a formvar-coated grid and floating this on a drop of distilled water to dialyse the salts. Care must be taken to ensure that there are no holes in the film through which viruses could diffuse. If an ultracentrifuge and swinging-bucket rotor are available pelleting of the viruses is simpler and more rapid.

The significant advantages of using TEM to count viruses are that information is obtained on the morphological diversity and abundance of the total virus community, and because infectivity is not an issue unfiltered samples can be preserved immediately after collection, thereby eliminating problems of virus decay and amplification.

1. Sampling and fixation: Collect duplicate water samples and if possible centrifuge them immediately without fixatives or preservatives, which can cause artifacts such as clumping. However, keep in mind that in unpreserved samples continuing biological process may influence the concentration of VLPs. If necessary the samples can be stored for several weeks in 1-2 % EM grade glutaraldehyde, in the dark, at 4°C. Viruses can be dispersed from aggregates in preserved samples by adding 10 μg ml^{-1} of a surfactant such as polyoxyethylene sorbitan monooleate (*i.e.* Tween 80) to the sample prior to centrifugation (Cochlan, pers. comm.), similar to the method used by Yoon and Rosson[17] for dispersing bacteria attached to particulate material.

2. Ultracentrifugation: Place, carbon-coated side up, duplicate hydrophylic grids onto each platform and carefully fill and balance each centrifuge tube. A variety of swinging-bucket rotors can be used, although those holding *ca.* 100 mm centrifuge tubes are suitable for most water samples. In order to pellet the smallest viruses, centrifuge the samples until particles of 80S are sedimented with 100 % efficiency. For samples in distilled water at 20 C° this can be calculated using the following formula:

(1) $T = (1/s) * (\ln[r_{max}/r_{min}]/[w^2 * 60])$,

where: T = time in minutes; s = sedimentation coefficient in seconds (*e.g.* a 80S particle has a sedimentation coefficient of 80 x 10^{-13} s); r_{min} = distance (cm) from the center of the rotor to the top of the sample in the centrifuge tube; r_{max} = distance (cm) from the center of the rotor to the surface of the EM grid; w (angular velocity in radians) = 0.10472 * rpm.

Example for a Beckman SW 40 rotor at 30,000 rpm with a full centrifuge tube and a 0.5 cm-high grid platform:

$$\frac{1}{80 \times 10^{-13}} * \frac{\ln (15.38 / 6.67)}{(0.10472 * 30000)^2 * 60} = 176.4 \text{ min}$$

Sedimentation times will vary as a function of temperature and salinity because of the associated changes in the density and viscosity of water. Increase centrifugation times about 12 % if pelleting viruses out of 3.5 % salt versus distilled water. Lower temperatures have a more dramatic effect on sedimentation times because of the associated changes in the viscosity of water. Increasing sedimentation times by 25 % for each 5 °C decrease in temperature below 20 °C will adequately compensate for the increased viscosity. Decreases in the viscosity of water at temperatures above 20 °C

are much less pronounced.

3. Staining: VLPs are most easily enumerated if they are positively stained. In negatively-stained preparations the stain deposition is often uneven making it difficult to count all of the VLPs. Some experimentation may be necessary to achieve optimum results for a given sample, although the following protocol is generally adequate.

   a) After centrifugation remove the grids, wick off the excess water with a piece of filter paper, and remove the remaining salts by floating the grids, sample-side down, through several drops of distilled water. Wick off the excess water at each step but make sure the surface of the grid is never dry.

   b) To stain, float the grid face-down on a drop of 1 % uranyl acetate for about 30 s. Wick off the excess stain, rinse through two or three drops of distilled water, briefly allow the grids to dry on filter paper and view with TEM. One advantage of using uranyl acetate over other stains is that the grids can be stored in a desiccator for several weeks without appreciable loss of detail. Hayat and Miller[3] present an authoritative review on staining and visualization of viruses by TEM.

4. Enumeration: Establish objective criteria for recognizing VLPs and scan each grid to make sure that the VLPs are distributed randomly and that larger particles will not interfere with counting. VLPs can be counted directly off the phosphorescent screen but for better resolution should be counted from photographic negatives. Matthews and Buthala[18] obtained a precision of ± 6 % at the 95 % confidence interval by counting a minimum of 100 VLPs from 5 random fields at a magnification of 10,000 x. For most applications, counting a minimum of 20 fields containing at least 200 VLPs represents a reasonable compromise between accuracy and effort. Assuming a Poisson distribution this yields upper and lower 95 % confidence limits of 174 and 230. As increases in accuracy are a function of the square root of the number counted it is necessary to count much larger numbers of viruses to get any appreciable increase in accuracy. If desired, 95 % confidence intervals can be estimated from the following formulae:

(3)   Lower = $n + 1.96 * \sqrt{(n + 1.5)} + 2.42$

(4)   Upper = $n - 1.96 * \sqrt{(n + 0.5)} + 1.42$

where n is the number of viruses counted.

   In order to determine the VLP concentration first calculate a taper correction factor (S_f), because the particles do not sediment in parallel paths. This can be approximated as follows:[18]

(5) $S_f = (0.5\ r_{max} - (0.5\ r_{min}^2 / r_{max})) / (r_{max} - r_{min})$

Use the following formula to convert the number of VLPs in a field to a concentration:

(6)   VLPs ml^{-1} = $P_f / (A_f * H * S_f)$

where: P_f = number of particles counted per field; A_f = area of grid represented in the microscope field or by the photographic negative (cm^2); H = height of sample (cm) (i.e. $r_{max} - r_{min}$).

   iv. *Epifluorescence microscopy of DAPI-positive particles*

   The protocol has the advantages that DAPI-positive is an objective criterion, a modest amount of equipment is required compared to TEM, and the method is amenable to use at sea. The most notable disadvantages are that 1) the viruses must be concentrated on a filter or by ultrafiltration before being counted; 2) no information is obtained on the composition of the viral community or the organisms that the viruses infect; 3) DAPI is specific for double-stranded DNA (dsDNA), so only dsDNA viruses are DAPI-positive (DAPI

binds to dsRNA as well, but the binding constant is an order of magnitude less than that of DNA:[19] 4) some small dsDNA viruses are difficult to visualize using DAPI; 5) samples must be prefiltered to remove bacteria which can also remove a large proportion of the viruses; 6) small bacteria cannot be distinguished from large viruses; 7) individual viruses cannot be distinguished from small clumps of viruses (although we have not found clumping of viruses to be a problem).

Viruses can be concentrated and enumerated in a variety of ways. The simplest way is by filtration onto stained 25 mm polycarbonate filters (e.g. Hara et al. 1991). Alternatively, viruses can be concentrated to $> 10^8$ ml^{-1} using ultrafiltration or vortex flow filtration and enumerated directly on glass slides.[20-22] A variety of ultrafiltration systems can be used to concentrate viruses from tens or hundreds of liters of water (see virus-isolation procedures), but as this generally takes several hours it is not practical for routine virus counts. Small-volume centrifugation concentrators potentially provide an alternative, but more research is required to increase the efficiency of virus recovery from natural water samples.

a. Filtration method

1. Filter a freshly collected water sample through a 0.2 μm pore-size polycarbonate filter.

2. To a 2 ml subsample add 50 μl of stock DNase solution (500 Kunitz units) and incubate for 30 min at room temperature. After DNase treatment the sample can be fixed in 1 % glutaraldehyde or formalin.

3. Add 0.2 ml of the DAPI stock solution (1 μg ml^{-1} final concentration) and incubate in the dark for 30 min. The fluorescence is less if higher concentrations of DAPI are used.[23]

4. Filter the entire sample through a 25 mm, 0.03 μm pore-size, irgalan-black stained polycarbonate filter (vacuum 200 mmHg); this filtration may take > 30 min. Do not rinse the filter as the DAPI binding is reversible. The 0.03 μm filter should be laid over a pre-wetted 0.45 μm pore-size nitrocellulose filter for even filtration. These filters are very fragile and must be handled with care. Turn the vacuum off as soon as the filter drys. If the filter is too wet or dry the slide will be poor.

5. Lay the filter over a small drop of low-fluorescence immersion oil on a microscope slide. Place three tiny drops of oil on a cover slip and gently lay this over the filter, trapping the oil between the cover slip and filter. The filter should be of even color, with no wrinkles, dry spots, or emulsion of water and oil.

6. At 1000 x magnification count 20-100 DAPI-positive particles in each of 20 random fields. Calculate the number of viruses per ml (N_v) from:

(7) $N_v = P_f * (A_s/A_f) * (1/V_s) * ([V_s + V_e + V_g + V_D]/V_s)$ ~~* 1000~~ /ml

where: P_f = number of fluorescent particles per field; A_s = filtration area of filter (μm^2); A_f = area of field (μm^2); V_s = Volume of sample (μl); V_e = Volume of DNase added (μl); V_g = Volume of glutaraldehyde or formalin added (μl); V_D = Volume of DAPI added (μl).

b. Concentration method

In order to count DAPI-stained viruses directly on glass slides the abundance must be about 10^{10} ml^{-1}. The method is particularly well suited for counting viruses in laboratory experiments where further concentration is frequently not required. However, 10^{10} ml^{-1} is considerably greater than in most natural water samples; hence, the viruses must be concentrated before

they can be counted.

The efficiency of concentration should be checked using an internal standard such as 50 nm fluorescent beads, labelled with fluorescein isothiocyanate (FITC, excitation/emission = 458/540 nm), or by adding a trace addition of a marine bacteriophage to one of a pair of duplicate samples and titering the replicates by plaque assay before and after concentration.

1. Concentrate the viruses from a natural water sample as is outlined below in the virus isolation procedures. Transfer 20 μl of the concentrate to a microfuge tube.

2. Dilute 10 μl of stock DNase solution with 90 μl of ice cold 0.15 M NaCl, and for each 20 μl sample place 5 μl of DNase I (5 Kunitz units) on the inside of each microfuge tube.

3. Briefly centrifuge the sample (ca. 15,000 x g for 3 s), vortex, re-centrifuge and allow to incubate for 30 min at room temperature. DNase will dissolve free DNA that could interfere with the counting method, but will not interfere with DNA protected by a protein coat. If required the samples can be fixed at this point with 1 % formalin or glutaraldehyde. Do not add excess glutaraldehyde as it imparts background fluorescence and makes viruses difficult to count.

4. To stain, add 5 μl of DAPI stock solution (1 μg ml^{-1} final concentration), centrifuge and vortex, as above, and incubate in the dark at 4° C for at least 30 min.

5. Pipet 3-5 μl on a clean glass slide and cover with a clean 22 x 22 mm cover slip. At 1000 x magnification count 20-100 DAPI-positive particles in each of 20 random fields. Use the appropriate number of grid squares in the quadricule. The viruses will tend to adsorb to the surfaces of the cover slip and slide, so be careful to count the DAPI-positive particles in both planes of focus. Calculate the concentration of viruses per ml (N_v) using the following formula:

$$(8) \quad N_v = P_f * (A_c/A_f) * (1/[V_c * C_f]) * ([V_a + V_c + V_g + V_D]/V_a) * 1000,$$

where: P_f = number of fluorescent particles per field; A_c = area of cover slip (μm^2); A_f = area of field (μm^2); V_c = Volume of sample under the cover slip (μl); C_f = concentration factor of the sample; V_a = Volume of sample to which DNase, fixative and DAPI added (μl); V_c = Volume of DNase added (μl); V_g = Volume of glutaraldehyde or formalin added (μl); V_D = Volume of DAPI added (μl).

B. Isolation of viruses

   i. Amplification method

This protocol is extremely simple, requires little equipment and allows relatively large volumes of water to be assayed for the presence of lytic viruses. The major disadvantages are that separate amplifications must be done for each host that is screened and the culture volumes can be large.

1. Filter several liters of sample water through a 0.2 or 1.0 μm pore-size polycarbonate filter to remove the natural bacteria or phytoplankton community, depending on whether one is screening for bacteriophages or algal viruses. *numbered?*

2. Enrich seawater with inorganic (e.g. ESNW, Harrison et al. 1980) or organic (0.05 % Bacto-Peptone and .05 % Casamino Acids, Difco) nutrients as appropriate and a 10 % inoculum of an exponentially growing culture of the potential host added.

3. Measure *in-vivo* fluorescence or optical density (600 nm) to monitor the growth of the phytoplankton or bacteria, respectively. Within a few days after growth of the host has ceased the water is screened for the presence of viruses.

4. For each enriched water sample to be screened add a 10 % inoculum from an exponentially-growing host-cell culture into each of ten 13 x 100 mm borosilicate culture tubes (with polypropylene caps) containing fresh medium (0.4 ml of culture to 3.6 ml of medium).

5. When exponential growth is observed add 1 ml of 0.2- or 1.0-μm-filtered water from the enriched sample into 5 of the tubes. As a control add 1.0-μm-filtered water from a stationary culture to the other 5 tubes.

6. If a decrease in fluorescence or optical density is observed relative to that of the control cultures, repeat the experiment by transferring 5 % inocula from a control and a potentially infected culture into ten additional tubes containing exponentially growing cultures that had never been exposed to the suspected pathogen. Repeat the process numerous times to dilute non-replicating viruses from the original concentrate.

For hosts that will grow on solid substrate, screening of the enriched water sample can be done by plaque assay as described in the above section on enumeration.

ii. Concentration method

The other approach of increasing the probability of host-virus encounter is to increase the concentration of the natural virus community by ultrafiltration, and add aliquots of the concentrate to potential hosts. Using this method a large number of different bacteria and phytoplankton can be screened expeditiously. As well, the concentrates serve as a library of natural virus communities that can be screened for other pathogens. The disadvantages of the approach are that *prefiltration* removes viruses[22] and that ultrafiltration is somewhat expensive and time consuming.

1. Dispense 20-100 l of water into a pressure vessel. Stainless steel pressure vessels can often be obtained from soft drink wholesalers for a modest deposit.

2. Pressure filter the water (<130 mmHg) through 142 mm-diameter glass-fiber and Durapore membrane filters, connected in series and held in place by stainless-steel filter holders. The 0.45 μm pore-size filters let bacteria through and make ultrafiltration slower, but the 0.22 μm pore-size likely excludes many of the large algal viruses. The 0.22 μm pore size is suitable for smaller viruses.

3. Concentrate the filtrates 100-1000 fold using a a 30,000 MW ultrafiltration cartridge (Amicon). Flow rates at a back pressure of 1000 mmHg are about 850 ml min⁻¹. The cartridge is cleaned after use by flushing with 2 l of 0.1 N NaOH heated to 45°C and is then stored refrigerated in 0.01 N NaOH, as per the manufacturer's recommendation. Prior to reuse flush the cartridge with 7 l of deionized-distilled water.

4. The concentrate is screened in the same manner as is used for the enriched water samples. Inocula from a concentrate are introduced into exponentially growing cultures of phytoplankton which are monitored fluorometrically. If there is evidence of a pathogen the effect is propagated. Alternatively, the concentrate can be screened by plaque assay if the host will grow on solid medium.

iii. Cloning of the virus

If the pathogen can be propagated the first step is to demonstrate that it

is viral, as a number of bacteria are predatory on other bacteria and phytoplankton.[7,8,24] Test if the infective agent can be removed by filtration, whether it is sensitive to antibiotics and whether it is host specific. Finally, use TEM to confirm the presence of VLPs.

Cloning of the virus is also accomplished by a modification of the above methods. If the host is amenable to growing on agar the simplest way to clone the virus is through plaque purification. Serially-diluted culture lysate from a virus amplification is plaque assayed, and a single well-separated plaque removed from the lawn. The plaque is eluted in medium overnight and the eluent used for another dilution series and plaque assay. This procedure should be repeated several more times to be sure that the virus has been cloned.

For hosts that will not grow on solid medium cloning must be accomplished by amplifying a single infectious unit in a liquid culture. The first step is to determine the titer of a culture lysate by an MPN assay. Based on these results a dilution series is set up and 0.2 of an infectious unit is added into each of 20 exponentially-growing cultures of the host. The probability of a culture receiving a single virus is 0.164 and therefore would be expected to occur with a frequency of 3.27 out of 20 cultures. The probability of a culture receiving 2 or more viruses is < 0.02.[10] Hence, by repeating this procedure twice one can be very confident that the virus has been cloned.

IV. ACKNOWLEDGEMENTS

Please add

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Marine Viruses:

Decay Rates, Diversity and Ecological Implications

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Running Title: Virus decay rates, titer and host range

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Numerous investigators (1, 4, 14) have confirmed the observation of Proctor et al. (10) that concentrations of viruses in seawater are typically in the range of 10^5-10^8 mL^{-1}. These virus communities are extremely diverse and include pathogens which infect bacteria, cyanobacteria and eukaryotic phytoplankton. The viruses range in morphology from large polyhedrons, such as the virus which infects the photosynthetic flagellate *Micromonas pusilla*, to long-tailed cyanophages, to bacteriophages with T-type morphologies (Fig. 1). Despite their great abundance and diversity little is known about aquatic viruses or their role in marine ecosystems. Although they are likely of great ecological importance and harbor many potentially useful attributes much research will be required before these secrets are unlocked. The data presented in this paper are relevant to understanding the ecological significance of marine viruses to planktonic communities.

Decay rates: Before the dynamics of viral populations in aquatic systems can be understood, the loss rates and the processes responsible for these losses must be known. We have done a number of experiments using marine bacteriophages to quantify loss rates of viruses in seawater and to identify the factors responsible for the decay of infectivity. We chose decay of infectivity rather than disappearance of viral particles, as infection is the mechanism through which most viral-mediated process occur. These experiments were conducted by adding *ca.* 5 x 10^4 infective units mL^{-1} of a given virus and following the loss of infective units over time. These concentrations were chosen to trace the decay of the natural viral community, but still be high enough so that the decay process could be followed for some time. The data were fit to exponential regressions to calculate a decay constant (d^{-1}). Each experiment was repeated on several occasions. As well, different treatments were carried out in an effort to identify the major factors responsible for the decay. Data

for two bacteriophages is shown in Table 1. The decay constants for viruses added to seawater but not exposed to sunlight were approximately 0.37 and 0.60 d^{-1}, corresponding to turnover times of 2.7 and 1.7 days, respectively. When the seawater was exposed to full sunlight the rates were 20-fold higher; however, eliminating UV radiation < 310 nm resulted in much lower decay rates. The addition of heterotrophic microflagellate grazers (6 x 10^5 ml^{-1}) to a higher density of viruses (5 x 10^7) also resulted in much more rapid decay, supporting recent observations of grazing by microflagellates on viruses (3).

Geographical and temporal distribution: Given that the decay of indigenous viruses is relatively rapid in seawater, it is of interest to know the distribution of viruses which infect particular hosts. If they are geographically and temporally widespread it implies that production must be relatively constant. Table 2 shows estimated titers of two marine bacteriophages in natural viral communities concentrated by ultrafiltration (15). Viruses infecting PWH3a were frequently undetectable, and when detected they were always present at relatively low concentrations. In contrast, viruses infecting LMG1 were always present and on one date in southern Laguna Madre reached titers of > 10^5 L^{-1} during a dense dinoflagellate bloom. Clearly, the titers of viruses infecting these hosts are variable, but even at the highest concentrations there were only a few hundred infectious units mL^{-1}. These data support previous findings that viruses infecting specific marine bacteria generally do not exceed a few hundred infectious units mL^{-1} (11, 12), or are below the detection limit of conventional techniques (5, 7). Yet, they remain geographically extremely wide spread (8). Similarly, viruses infecting the marine prasinophyte, *M. pusilla*, are found ubiquitously in marine coastal waters, but typically at concentrations of < 100 infective units mL^{-1} (2).

Host-specificity and genetic diversity: Little data are available on host range or genetic diversity of viruses infecting marine bacteria or phytoplankton. Table 3 provides data showing the host range of 6 virus clones which infect marine *Synechococcus* spp. The host range differs amongst cyanophages. For example, UTSYN1 and UTSYN2 were specific for one of 9 strains of *Synechococcus* tested, whereas, UTSYN5 infected 4 of 9 strains. This implies that there is considerable genetic variation amongst the cyanophages. This would be consistent with observations made on viruses infecting *M. pusilla*. Although the viruses are host specific (6) and morphologically indistinguishable from each other, restriction fragment analysis of the DNA and electrophoresis of the major proteins indicates that the viruses are genetically and phenotypically diverse (2).

Summary and ecological implications: There are several important ecological implications that can be drawn from these and other studies. First, the relatively rapid decay rates of viruses in seawater, combined with the widespread occurrence but low concentrations of pathogens infecting specific hosts, implies that viruses are being continuously produced and lost. As viruses are obligate pathogens that can only be produced at the expense of host metabolism, and as they generally cause lysis of the host at the time of production, they must have important effects on aquatic nutrient cycling. Second, the bacteriophage, cyanophage and algal virus data so far collected indicate that viruses infecting particular hosts are widely, if not ubiquitously, distributed in the sea. Nonetheless, the concentration of viruses infectious for a specific host are generally present at concentrations of a few hundred per mL, or less. As well, it is evident that virus concentrations in seawater, whether determined from counts of viral-like (1, 9) or DAPI-stained (4, 14) particles, are in the range of 10^6-10^7 mL^{-1}. If the virus-host systems that have been

isolated are good models, and if most of the viruses in seawater are infective, then in each mL of seawater there are 10^4-10^5 viruses which infect different hosts! This diversity is conceivable given the great deal of genetic diversity found among morphologically-identical virus clones that infect the same host (2, 13). In addition, as rapid viral propagation only occurs when host densities are high (16), this is the only mechanism that is required to maintain the high diversity that is observed in planktonic communities.

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Figure Legend

Figure 1: Viruses which infect a) the photosynthetic flagellate *M. pusilla*, b) the

cyanobacterium *Synechococcus* strain BBC1, and c) a heterotrophic

bacterium LMG1. The scale bar is 100 nm.

TABLE 1. Decay rates (d^{-1}) of marine bacteriophages in seawater. The standard deviation and number of individual experiments are shown in parentheses.

Treatment	Virus	
	PWH3a-P1	LB1VL-P1b
Natural seawater	0.37 (0.090, 8)	0.60 (0.048, 4)
Full sunlight	8.46 (0.311, 2)	11.08 (0.368, 2)
50 % full sunlight	5.53 (0.562, 2)	6.86 (0.281, 2)
20 % full sunlight	1.81 (0.355, 2)	2.58 (0.356, 2)
Full sunlight (uv blocked)	2.09 (0.273, 2)	1.50 (0.003, 2)
Microflagellate grazers	3.81 (0.011, 2)	(not determined)

TABLE 2: Titers of viruses infecting the marine bacteria PWH3a and LMG1 (expressed in plaque-forming units L^{-1}).

Location	Date	PWH3a	LMG1
Laguna Madre	15 Jul 1989	< 1.0	23.3
	18 Aug 1989	< 0.9	1224
	19 Dec 1989	< 0.7	11.8
Stn 1	15 Dec 1990	46.6	57200
Stn 2	15 Dec 1990	68.4	312000
Stn 3	15 Dec 1990	101.2	397000
MSI Boat Basin	20 Aug 1989	8.0	122
	24 Aug 1989	1.9	19.4
MSI Pier Lab	21 Jun 1989	3.2	(not determined)
	2 Oct 1990	< 1.3	(not determined)
	1 Nov 1990	< 1.0	(not determined)

TABLE 3: Host specificity of six cyanophage clones which infect *Synechococcus* spp.

| *Synechococcus* | Cyanophage Clone | | | | | |
Strain	UTSYN1	UTSYN2	UTSYN3	UTSYN4	UTSYN5	UTSYN6
DC2[1]	-	-	+	+	+	+
SYN48[1]	-	-	-	-	+	+
SNOOPYC2[1]	-	-	-	-	+	-
BBC1	+	-	-	-	-	-
BBC2	-	+	-	-	-	-
SNOOPYC1	-	-	-	-	+	-
838BG	-	-	+	+	-	-
UTEX1634	-	-	-	-	-	-
UTEX2380	-	-	-	-	-	-

[1] These are phycoerythrin-containing red strains.
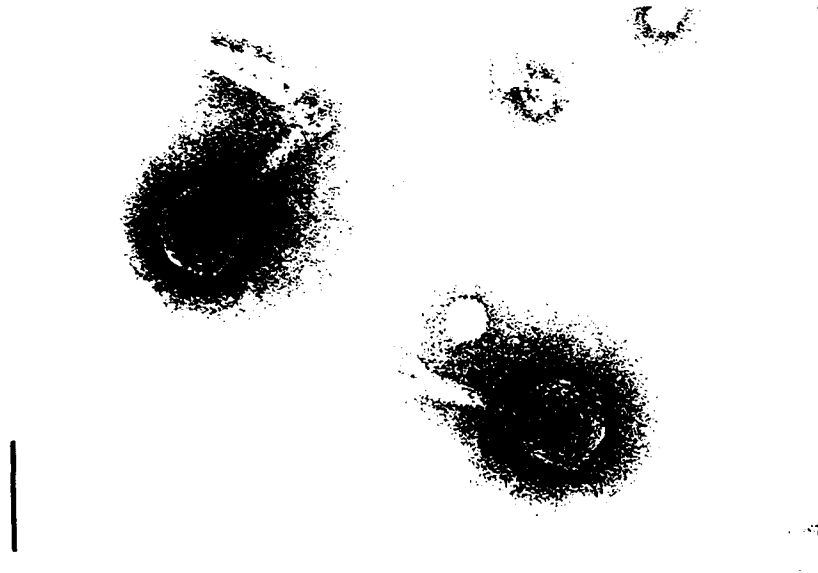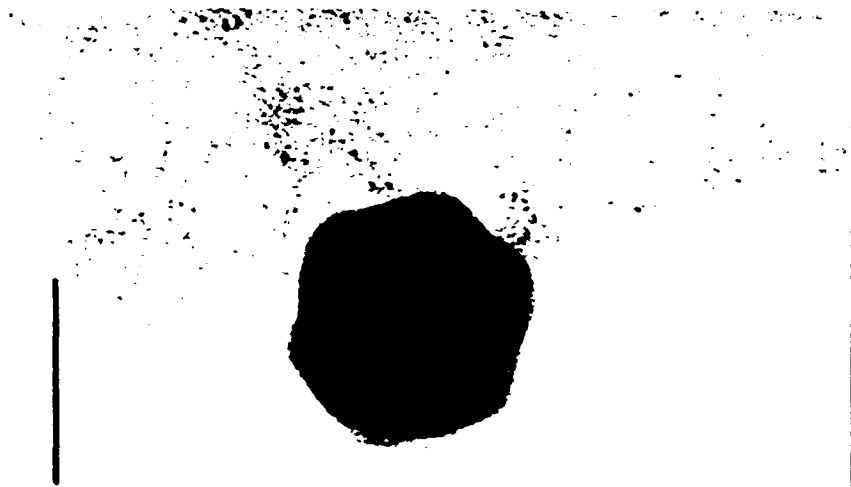
Cyanophages and Sunlight: A Paradox.

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Summary: We have investigated viruses that infect marine cyanobacteria in terms of their occurrence, diversity and abundance in seawater.   Cyanophages infecting *Synechococcus* spp. comprise a diverse group of viruses that routinely occur in excess of 10^5 ml^{-1}. Abundances were highest at stations closest to shore and in near-surface waters along a transect in the Gulf of Mexico. The infectivity of marine phages was very sensitive to solar radiation and viruses in natural seawater exposed to sunlight decayed much more rapidly than those in the dark. Infectivity decayed much more rapidly than viral particles, suggesting that most viruses in surface waters should be noninfective. This poses the paradox: "Why are the concentrations of infective cyanophages so great?"

1. Introduction

In recent years observations using electron microscopy have indicated the presence of an abundant and diverse community of viruses in seawater (1, 4, 11). This has provided impetus for elucidating the role of viruses in structuring marine communities and as regulators of nutrient and energy cycles in the sea.   Although the presence of cyanophages in freshwater systems has been recognized for many years (6) it has only recently been demonstrated that a significant proportion of the *Synechococcus* cells in marine systems are infected with viruses (4) and that cyanophages can be readily isolated from seawater (5). As *Synechococcus* spp. are among the most important primary producers in open ocean environments (3) defining the role of cyanophages is of critical importance. As a first step in this direction we have examined the abundance, diversity and aspects of the life cycle of some marine cyanophages.   Essential to understanding the importance of cyanophages and other viruses to marine ecosystems is quantifying the fate of viral infectivity in seawater. As damaging radiation penetrates to considerable depth (8) we have also examined the effect of sunlight on viral decay.

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2. Cyanophage Diversity, Host Range and Life-cycle

The cyanophages that we have isolated include representatives from each of the genera that have been proposed based on freshwater cyanophages (5). We have isolated *Cyanopodovirus* spp. and *Cyanostylovirus* sp. that infect green strains of marine *Synechococcus*, and *Cyanomyovirus* spp. that infect both red and green *Synechococcus* strains (Fig. 1). There was considerable variation among the viruses in terms of host specificity. Some viruses infected a single strain of *Synechococcus*, whereas, another infected 4 different strains of the 9 tested. The growth cycle of the viruses seems to be similar to their freshwater counterparts. S-BBP1, which infects a green *Synechococcus* strain from Texas coastal waters had relatively slow adsorption kinetics (3.94×10^{-9} ml min^{-1}) similar to those of other cyanophages (7). Data from a one-step growth experiment indicated that cell lysis began about 9 h following infection and was complete by 17 h post infection. Photosynthetic rates were similar to those in non-infected controls until near the onset of cell lysis. Approximately 250 progeny viruses were produced per infected host cell when lysis occurred.
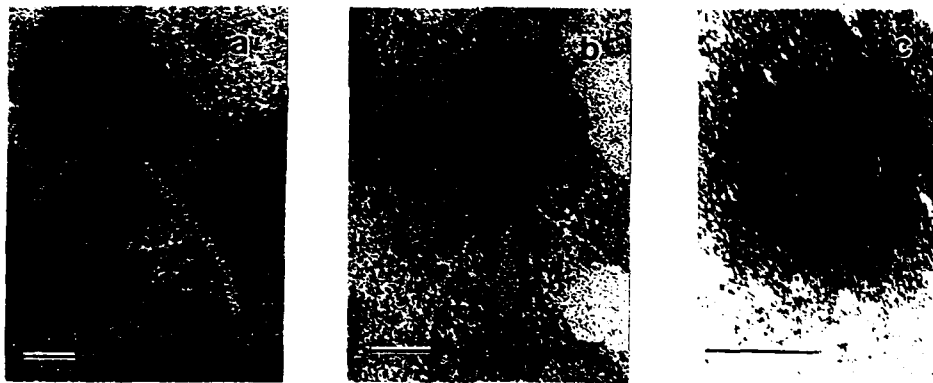
Fig. 1. Representative cyanophages infecting marine *Synechococcus* spp. a) *Cyanostylovirus* S-BBS1, b) *Cyanomyovirus* S-PWM4 and c) *Cyanopodovirus* S-BBP1. Scale bar is equivalent to 50 nm.

3. Cyanophage Concentrations in Seawater

We routinely determined the concentration of cyanophages in seawater collected off of the pier of the Marine Science Institute, in hypersaline Laguna Madre and along transects in the western Gulf of Mexico. Cyanophages were detected, and were frequently very abundant, in every seawater sample that we screened. They occurred over the entire

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range of temperature (12-30.5 °C) and salinity (18-70 ppt) that was assayed. The concentration of viruses was titered by adding aliquots of seawater from a dilution series into 96-well microtiter plates, each well of which contained an exponentially-growing culture of *Synechococcus*. The wells were examined for 7-10 days for evidence of cell lysis. A similar protocol has been used to titer the concentration of cyanophages in seawater off of the coast of the northeastern United States (Waterbury, pers. comm.). The concentration of cyanophages infecting a particular host was estimated from the number of wells in which lysis was observed, and analyzing the results by computer (2). Cyanophage concentrations were very dependent on the host which was screened. In seawater collected from the Institute pier over a 28 month period the cyanophages infecting *Synechococcus* strain DC2 varied from about 100 to > 10^5 ml^{-1}. In contrast, maximum titers observed for some of the other strains were 10-fold less. The lowest titers were associated with cooler water temperatures and lower salinities.

Along transects in the Gulf of Mexico the highest cyanophage titers were observed nearshore in a region of lower salinity (Fig. 2). Farther offshore the highest titers occurred within 30 m of the surface. This pattern was similar to the abundance of *Synechococcus* spp. which ranged from < 100 ml^{-1} at 97 m offshore to > 10^4 ml^{-1} in shallower depths near shore.
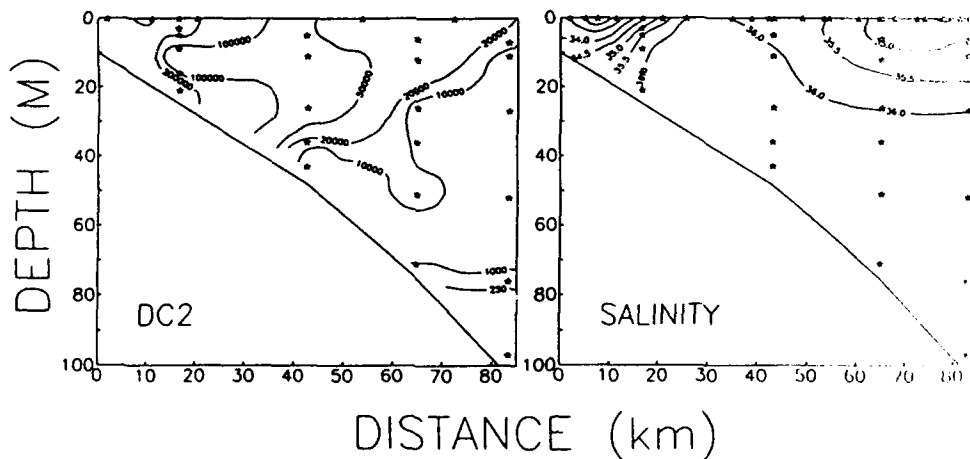

Fig. 2. Salinity and concentrations of cyanophages (viruses ml^{-1}) infecting *Synechococcus* strain DC2 along a transect in the western Gulf of Mexico.

4. Effect of Sunlight on Viral Decay

Marine bacteriophages are extremely sensitive to solar radiation and decay at rates up to 28 times faster than viruses incubated in the dark,

suggesting that sunlight is one of the major mechanisms causing viral decay in natural waters (10). In order to determine if infectivity decays more rapidly than viral particles, we incubated marine viruses in sterile seawater, in UV-transparent bags suspended *in situ*. Infectivity was much more sensitive to sunlight than were viral particles (Fig 3). At the end of the 12 h diurnal incubation the number of infective viruses in the surface incubation had decreased from 9.51×10^6 to $33 \ ml^{-1}$ (over 4 orders of magnitude); whereas, the number of viral particles only decreased by about 57 %. Significant decreases in viral infectivity still occurred at a few % of surface irradiance, even though there was no concomitant decrease in the number of viral particles. Similar results have been obtained using marine virus, PWH3a (Myoviridae). These results strongly suggest that a large portion of the viral particles in near-surface seawater may be non-infective.
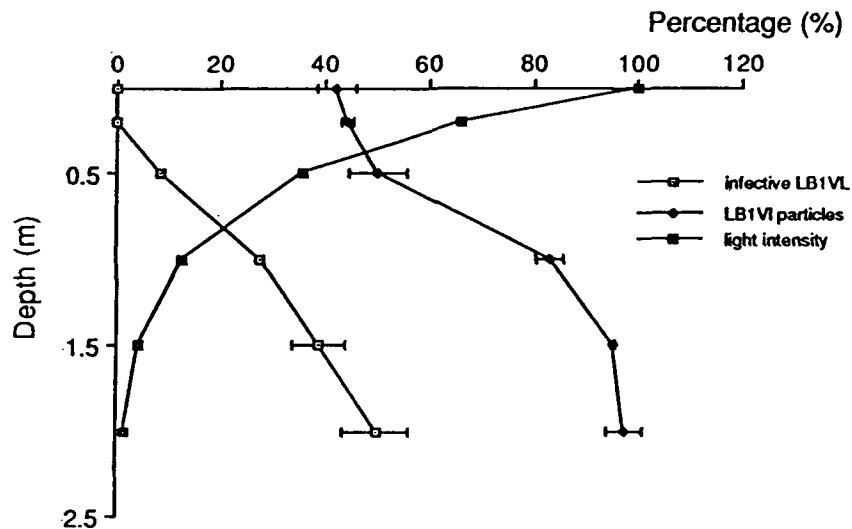
Fig. 3. Light penetration and decay of viral particles and infectivity for the marine bacteriophage LB1VL (Podoviridae), in turbid coastal seawater.

This presents a paradox. Marine phages are very sensitive to radiation; yet, high concentrations of infective cyanophages often occur in surface waters. As cyanophages belong to the same three virus families as most bacteriophages, it is unlikely that they are more resistant to radiation. One hypothesis is that because cyanobacteria are routinely exposed to damaging radiation in order to photosynthesize, they may have very efficient DNA repair mechanisms. As the host DNA-repair machinery cannot distinguish between its own DNA and the phage DNA it would repair the viral DNA, ensuring its own demise.

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Viruses Infect Marine Phytoplankton and Reduce Primary Productivity by up to 80%.

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We have isolated viruses that infect a variety of marine phytoplankton including a diatom, cryptophyte, prasinophyte and a chroococcoid cyanobacterium. Data will be presented showing that addition of viruses to cultures of their hosts results in subsequent death of the host. Electron micrographs of some of the viruses that we have in culture, that infect eukaryotic algae, indicate the viruses are large polyhedrons. In addition, we have been using ultrafiltration methodology to concentrate natural virus communities from seawater. Addition of these virus concentrates back to seawater reduces primary productivity (^{14}C-bicarbonate incorporation) by up to 80%. In contrast, addition of concentrates inactivated by autoclaving has no affect on primary productivity relative to controls to which nothing has been added. These data indicate that natural communities of marine viruses may be very important in controlling primary productivity and regulating phytoplankton community composition in marine environments.

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RESPONSE OF PHYTOPLANKTON COMMUNITIES TO ADDITION OF MATERIAL IN THE 30,000 MW TO 0.2 μm SIZE FRACTION, CONCENTRATED BY ULTRAFILTRATION

Rates of bicarbonate incorporation in natural phytoplankton communities were reduced when exposed to material concentrated by ultrafiltration, in the 30,000 MW to 0.2 μm size fraction. The decrease in photosynthetic rates were rapid, ard coincided with sharp decreases in *in-vivo* fluorescence that were especially pronounced in dark-incubated samples. Autoradiographic data suggest that all components of the phytoplankton were not equally affected by the addition of concentrate. The biological effect of the concentrate was generally reduced by autoclaving but not eliminated entirely. As well, some but not all of the biologically-active material could be pelleted by centrifugation at 70,000 g for 150 min. Counts of viral-like particles by electron microscopy and of DAPI-positive particles by epifluorescence microscopy indicate that there are about 10^9 to 10^{10} viruses mL^{-1} in the concentrate.

Abundance, Diversity and Nature of Virus Communities in the Sea

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Virus concentrations in coastal seawater from the coast of Texas were on the order of 10^7 viruses per mL^{-1}. Electron microscopy revealed that this community consisted of a variety of virus types including tailed, untailed and filamentous viruses. Bioassays indicated that some of these viruses were pathogens to representatives from a number ecologically important phytoplankton taxa including a prasinophyte, pennate and centric diatoms, a cyanobacterium and a cryptophyte. As well, when the size fraction containing the viruses was concentrated by ultrafiltration, and added to seawater it was associated with up to a 78 % decrease in the rate of carbon fixation. Addition of this highly bioactive size fraction to seawater also caused a decrease in chlorophyll fluorescence. Investigations using autoradiography suggested that the smallest cells were the most affected by this concentrated material. Investigations using representative marine viruses indicated that they decay very quickly in seawater as the result of sunlight and biological processes. As well, titres of specific types of viruses are highly variable in nature, and morphologically similar viruses contain a great deal of genetic variability.

VIRUSES INFECTING PHYTOPLANKTON.

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We have isolated a number of pathogens that infect prokaryotic and eukaryotic phytoplankton. Two of the systems that we have studied in detail are viruses which infect the picophytoplankton *Synechococcus* spp. and *Micromonas pusilla*. A genetically diverse group of viruses which infect *M. pusilla* were found ubiquitously in coastal seawater, including in samples from the Atlantic and Pacific oceans, and the Gulf of Mexico. Similarly, cyanophages that infect coastal and oceanic strains of *Synechococcus* were found in 30 of 31 marine virus communities. Both types of viruses occurred at concentrations up to $>10^3$ infectious units per ml. Host specificities among the cyanophages were variable. Some viruses infected a single strain of *Synechococcus*, whereas, another infected 4 different strains of the 9 tested. In cyanobacteria photosynthesis was not affected until the final stage of the lytic cycle, which was approximately 10 h post infection. The burst size was about 250 infective viruses. Decay rates for infectivity of bacteriophages in seawater in the absence of solar radiation were between 0.2 and 0.6 d^{-1}. In the presence of sunlight decay rates were as high as 16.8 d^{-1}, but were reduced 4 to 6 fold when UV-B radiation was blocked. Bacteria probably have little effect on viral decay rates.

Suttle, Curtis A. and Feng Chen, Marine Science Institute, University of Texas at Austin, PO Box 1267, Port Aransas, Texas, USA, 78373-1267.
BACTERIOPHAGE DECAY RATES AND PROCESSES RESPONSIBLE FOR VIRAL DECAY IN SEAWATER

We used three different marine bacteriophages to determine loss rates and loss processes for viruses in seawater. We examined decay of infectivity, as infection is responsible for most viral-mediated processes. In Texas coastal waters decay rates ranged from 0.011 to 0.025 h^{-1} and were similar among viruses. Viruses did not decay or decayed very slowly in 0.2-μm filtered, autoclaved or ultracentrifuged seawater. Decay rates were more rapid in 3-μm filtered water and there was no difference between 8-μm filtered and unfiltered seawater. Infectivity was very sensitive to sunlight. In full sunlight decay rates were 0.4-0.8 h^{-1}, but decreased 4 to 6 fold when UV was blocked. Exposure to 20% surface irradiance increased decay rates 4-8 fold. As well, high concentrations of microflagellate grazers increased viral decay rates approximately 10 fold.

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González, J. M. [1], C. Suttle [2], E. B. Sherr [1] and B. F. Sherr [1]. [1]College of Oceanography, OSU, Oceanogr. Admin. Bldg. #104, Corvallis, OR 97331-5503, USA. [2]Marine Science Institute, University of Texas at Austin, P.O. Box 1267, Port Aransas, TX 78373-1267, USA.

GRAZING ON VIRUSES BY MARINE PHAGOTROPHIC PROTISTS.

Grazing on viral-size particles by protists was studied using both 50 nm fluorescent microspheres and fluorescently labelled viruses (FLV). Ingestion and digestion of FLV was analyzed. Uptake of viral- and bacterial-size particles were compared. Phagotrophic flagellates and scuticociliates ingested both viral particles and bacteria. Natural populations of choanoflagellates showed high ingestion rates on viral-size particles. Clearance rates on bacteria by phagotrophic flagellates and scuticociliates were about 10-fold that on viruses. A significant relationship between the initial concentration and protist ingestion rates of viral-size particles was obtained. For viral and bacterial concentrations of $5 \times 10^7 \cdot ml^{-1}$ and $10^6 \cdot ml^{-1}$, respectively, we estimated viruses can represent roughly about 27% of the C, 34% of the N, and 87% of the P that phagotrophic nanoflagellates obtain from bacteria. The ecological implications of these findings are discussed.

Matthew T. Cottrell, Curtis A. Suttle, University of Texas at Austin, Marine Science Institute, Port Aransas, TX 78373

Wide-Spread Occurrance and Clonal Variation in Viruses Which Lyse the Marine Micro-Alga Micromonas Pusilla

Seven clonal isolates of viruses that cause lysis of the eukaryotic, naked, photosynthetic flagellate, Micromonas pusilla (Prasinophyceae) were isolated from the coastal waters of New York, Texas, California and British Columbia, as well as the oligotrophic waters of the central Gulf of Mexico. The viruses are large polyhedrons (ca. 115 nm dia.) that lack tails. Restriction fragment analysis demonstrated that each clone is genetically different from the other six clones. In contrast, only four different types of viruses could be recognized based on the molecular weights of the major proteins. We measured concentrations of these viruses in situ that ranged from not detectable to 4.6×10^6 infective units l^{-1}. Our results demonstrate that this genetically-diverse group of viruses are widespread in nature. These viruses may constrain the maximum density that M. pusilla reaches in nature. If viruses infecting other phytoplankton taxa are similarly widespread they are might contribute to the maintainance of diversity in phytoplankton communities.

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Viruses, Viral Dynamics and Phytoplankton Productivity.
C.A. SUTTLE. Marine Science Institute, The University
of Texas at Austin, P.O. Box 1267, Port Aransas TX 78373-1267.

Viruses in seawater infect a number of important groups of phytoplankton including diatoms, cyanobacteria and flagellates. The viruses we have examined are host-specific, geographically widespread, variable in titer and genetically diverse. A cyanophage which infects *Synechococcus* sp. did not infect 12 other strains of *Synechococcus* tested. Seven clones of a virus which infects the photosynthetic flagellate *Micromonas pusilla* were isolated from the Pacific, Atlantic and Gulf of Mexico; although the viruses were morphologically similar they were genetically distinct from each other, and based on major proteins included several phenotypes. These viruses ranged in titer from < 20 to 4.6×10^6 L^{-1}. Viruses decayed rapidly in seawater with infectivity decreasing *ca.* 50 % per day. Addition of concentrated material from the viral-size fraction (20-200 nm) to phytoplankton communities caused rapid and sizable decreases in photosynthesis and chlorophyll fluorescence. Autoradiography indicated that phytoplankton > 3 μm were most affected by the addition of this material. These data indicate that viruses potentially affect phytoplankton community composition and are a diverse and dynamic element of nutrient cycles in the sea.
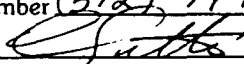
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S. thermophilus is a widely used industrial microbe in dairy fermentations. The development of gene transfer systems and construction of bifunctional shuttle vectors require detailed knowledge of

Chan, Amy M. and Curtis A. Suttle, Marine Science Institute, The University of Texas at Austin, P.O. Box 1267, Port Aransas, Texas, USA, 78373-1267

HOST SPECIFICITY, OCCURRENCE AND MORPHOLOGY OF CYANOPHAGES INFECTING MARINE SYNECHOCOCCUS

Nine different *Synechococcus*, including phycoerythrin- and phycocyanin-containing strains, were screened with 31 natural virus communities taken from 3 locations in south Texas coastal waters, at different times of the year. Cyanophages were generally present, but the frequency with which they were detected depended upon the strain of *Synechococcus* that was screened. Six cyanophages were cloned from natural virus communities and investigated in more detail. They varied in host-specificity. For example, one clone infected a single *Synechococcus* strain of 12 tested, whereas, another infected 4 of 9 strains tested. Photosynthesis was not affected until about 9 hours following infection and the virus burst cycle was complete about 19 h post infection. The viruses were morphologically similar to those infecting freshwater *Synechococcus*.

1

HIGH CONCENTRATIONS OF VIRUSES IN SEAWATER INFECTING MARINE SYNECHOCOCCUS

Curtis A. Suttle

and

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1   Cyanobacteria of the genus *Synechococcus* are among the most important primary

2   producers in oceanic environments, accounting for a substantial portion of the carbon

3   fixation in many regions of the sea (1, 2). We have collected data since 1989 on the

4   abundance and distribution of a number of viruses in seawater, and in every sample

5   examined, viruses (cyanophages) infecting *Synechococcus* spp. were present, and frequently

6   very abundant. In the coastal waters of Texas and along a transect in the western Gulf of

7   Mexico we measured cyanophage concentrations ranging from a few to $> 10^5 ml^{-1}$. The

8   abundance of cyanophages also fluctuated seasonally, with the lowest concentrations

9   associated with lower temperatures and salinities. Our results provide further evidence

10   that viruses are important mortality agents for *Synechococcus* in marine systems, and

11   consequently may have a significant effect on nutrient and energy cycling in the sea.

12

13   The existence of cyanophages in freshwater has been known for some time (3, 4).

14   Yet, despite the importance of cyanobacteria as major primary producers (1, 2) and

15   nitrogen fixers (5) in the sea, there have been few studies on cyanophages in marine

16   systems. Recent reports that a significant portion of *Synechococcus* cells in natural marine

17   planktonic communities are infected by viruses (6), and that viruses infecting

18   *Synechococcus* can be readily isolated from seawater (7) indicate that cyanophages are

19   potentially an important cause of cyanobacterial mortality in the oceans. In addition, the

20   observation of high concentrations of viruses in seawater (6, 8) has provided impetus for

21   understanding the role of viral-mediated processes in the cycling of nutrients and energy in

22   marine ecosystems.

23

24   In order to determine the distribution and abundance of cyanophages in the marine

25   environment we routinely assayed their concentration in the coastal waters of Texas and

26   along a transect in the western Gulf of Mexico. The approach was to estimate the number

27   of infectious units that cause lysis of representative strains of marine *Synechococcus*

28   (SYN48, SNC2, SNC1, 838BG and DC2). DC2, SYN48 and SNC2 were phycoerythrin-

29   containing red strains; whereas, SNC1 and 838BG were green strains, characterized by

30   having phycocyanin as their dominant photosynthetic pigment. Open ocean *Synechococcus*

2

1   communities arc almost entirely made up of cells containing phycoerythrin. DC2, SYN48

2   and 838BG were isolated from the North Atlantic, tropical Atlantic and southern Gulf of

3   Mexico, respectively, and were obtained from the Provasoli-Guillard Culture Collection at

4   Bigelow Laboratory in Maine, USA. We isolated SNC1 and SNC2 from Laguna Madre, a

5   hypersaline lagoon in southern Texas, separated from the Gulf of Mexico by a barrier

6   island. The concentration of cyanophages was titered in seawater collected from the pier

7   of the Marine Science Institute of The University of Texas at Austin, and along a cruise

8   track extending from the Institute to 80 km out into the lower productivity waters of the

9   Gulf of Mexico (< 0.1 μg chlorophyll a l^{-1}).

10

11   The concentration of cyanophages was determined by dispensing aliquots of

12 exponentially-growing cultures of *Synechococcus* into the wells of 96-well microtiter plates,

13 followed by subsamples from a dilution series of the natural virus community to be titered.

14 Each dilution was replicated 8 times and each assay was duplicated for a total of 16

15 replicates at each dilution. Controls received no virus addition. The most-probable

16 number (MPN) of infective viruses was estimated from the number of wells in which lysis

17 occurred over the 7-10 d following the addition. The concentration of infective units and

18 the error associated with these estimates were determined using a computer program

19 written in BASIC (9). We confirmed that viruses were responsible for cell lysis by using

20 transmission electron microscopy to examine representative wells of the microtiter plates

21 containing lysed cells; in every instance lysis was the result of viruses.

22

23   Along the Gulf of Mexico transect infective cyanophages were detected in every

24 sample, for all of the assayed hosts (Fig. 1). The concentration of viruses that infected each

25 host generally decreased with increasing distance offshore and below depths of 30 m.

26 Cyanophages that infected two of the red strains of *Synechococcus* (DC2 and SYN48) were

27 most abundant. They ranged in titer from about 10^2 ml^{-1} at 97 m and 83 km offshore, to

28 3.0×10^5 ml^{-1} at the surface, 11 km offshore. Viral abundance appeared to be related to

29 the distribution of *Synechococcus* cells with the highest concentrations of both in a region

30 of lower salinity near shore.

1

2   The concentration of cyanophages in seawater collected from the pier varied

3   seasonally and depended strongly on the host that was screened (Fig. 2). Nevertheless,

4   infective cyanophages were detected in every sample and occurred over the entire range of

5   salinities (18-37.5 ppt) and temperatures (12-30 $^{\circ}$C) that we assayed. Similarly to the

6   samples collected on the transect, in almost every instance the concentrations of viruses

7   infecting (DC2 and SYN48) were higher than for the other cyanobacteria, and ranged from

8   a few per ml to 1.9×10^5 ml^{-1}. Although the absolute abundance of viruses varied

9   depending on the host that was screened the relative change in concentration among the

10   cyanophages was similar. In every case the lowest titers occurred in the spring of 1990 and

11   1991, coincident with lower water temperatures and salinities.

12

13   The cyanophages infecting the phycoerythrin-containing oceanic *Synechococcus*

14   isolates (DC2 and SYN48) were always present in the highest concentrations, irrespective

15   of the origin of the virus community. In contrast, viruses infecting a phycoerythrin-

16   containing strain isolated inshore (SNC2) were about an order of magnitude less abundant,

17   and present in similar concentrations to one of the green strains (SNC1). Although our

18   data are limited, they suggest that oceanic strains may be more susceptible to viral infection

19   than coastal strains. This makes intuitive sense as *Synechococcus* cells tend to occur at

20   lower concentrations in oligotrophic offshore waters than in more productive coastal waters

21   of similar temperature (10 and Fig. 2). As rapid viral propagation requires high host

22   densities (11), a more dilute *Synechococcus* community in oligotrophic waters will result in

23   less efficient phage propagation and, therefore, a lower probability of encountering a viral

24   pathogen.

25

26   It is also significant that hosts from widely separated geographical regions were

27   susceptible to infection by viruses from the coastal and nearshore waters of Texas. We

28   have also found that water from the coast of New York contains viruses which infect hosts

29   isolated from Texas. Similar observations made with algal viruses (12) and bacteriophages

30   (13) have indicated that viruses infecting specific hosts can be widely occurring in the

4

1   marine environment; however, the highest concentrations of infective viruses observed in

2   these studies were several orders of magnitude less than reported here.

3

4   The viruses which infected marine *Synechococcus* spp. belonged to the same three

5   families that have been observed to infect freshwater cyanobacteria (Fig. 3) (14). The

6   Siphoviridae (formerly Styloviridae) have long noncontractile tails, the Podoviridae have

7   short tails and the Myoviridae have contractile tails . We have only cloned podoviruses and

8   styloviruses that infected green strains of *Synechococcus*, whereas, myoviruses were

9   observed to infect both red and green strains. One of the virus isolates belonging to the

10   Myoviridae (Fig. 3a) has several filaments originating from between the tail and capsid. As

11   such structures are extremely rare but have been observed in several freshwater

12   cyanophages (15), it suggests that marine and freshwater cyanophages may be of common

13   ancestry.

14

15   Our data demonstrate that viruses infecting *Synechococcus* spp. are widely occurring

16   and very abundant in the marine environment. Proctor and Fuhrman (6) have suggested,

17   based on observations of infected *Synechococcus* cells in the marine environment, that a

18   large portion of cyanobacterial mortality may result from viral infection. Our observations

19   support their conclusions and suggest that viral-induced mortality may be particularly

20   important in nearshore environments. Furthermore, viral lysis of *Synechococcus* cells

21   results in photosynthetically-fixed carbon being lost to the dissolved and colloidal organic

22   carbon pools,from which it can only re-enter the foodweb via heterotrophic processes. This

23   results in a much less efficient transfer of nutrients and energy to higher trophic levels. As

24   cyanobacteria are major primary producers (1, 2, 10) and nitrogen-fixers (5) in much of the

25   world's oceans, it is essential to elucidate the role of high concentrations of cyanophages on

26   nutrient cycling and trophic transfer in marine ecosystems.

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FIG. 1 Salinity (ppt), concentration of unicellular cyanobacteria (cells ml^{-1}), and abundance of cyanophages (viruses ml^{-1}) infecting 4 strains of *Synechococcus* along a transect in the western Gulf of Mexico. Virus abundances were determined directly in seawater samples collected using Niskin bottles suspended from a rosette. Assays for cyanophage concentration were done on board ship, and completed within an hour of sample collection. Salinity was determined from instruments on the rosette and hooked up to the ships flowing seawater system. Cyanobacteria were enumerated by epifluorescent microscopy using standard procedures (10).

FIG. 2 Temperature ($^{\circ}$C), salinity (ppt) and concentration of cyanophages infecting 5 strains of marine *Synechococcus* in coastal seawater collected from the pier of the Marine Science Institute. Virus abundances were determined in natural virus communities that had been concentrated from seawater using a 30,000 MW cutoff spiral wound ultrafiltration cartridge (Amicon) as previously described (7, 16). Comparisons of cyanophage abundances in seawater and in ultrafiltration retentate indicated that the recovery efficiency of the cyanophages varied between 13.3 and 60.4 %. As concentration efficiencies were not available for most dates, it was assumed that the viruses were recovered with 100 % efficiency for the purposes of calculations. Consequently, the cyanophage concentrations in water collected from the Institute pier are probably underestimated by 1.7 to 7.5 times.

FIG. 3 Electron micrographs of cyanophages infecting marine strains of *Synechococcus*: a) S-PWM4 (Myoviridae), b) S-BBP1 (Podoviridae), c) S-BBS1 (Siphoviridae). Scale bar represents 50 nm. Arrows indicate neck filaments, a relatively rare feature observed on some Myoviridae. Viral lysates were 0.2 μm filtered and concentrated by ultracentrifugation in a swinging-bucket rotor at 20 $^{\circ}$C and 146000 x *g* for 2.75 h. The pellets were resuspended in about 200 μl of artificial seawater. The viral

1   particles were transferred to 400-mesh, carbon-coated copper grids by floating the

2   grids on drops of the resuspended virus solutions for about 20 min. The grids were

3   stained by floating on a drop of 1% aqueous uranyl acetate for 20-30 s. The excess

4   stain was wicked off with filter paper and the grids were viewed at 80 kV using a

5   Philips EM301 transmission electron microscope.

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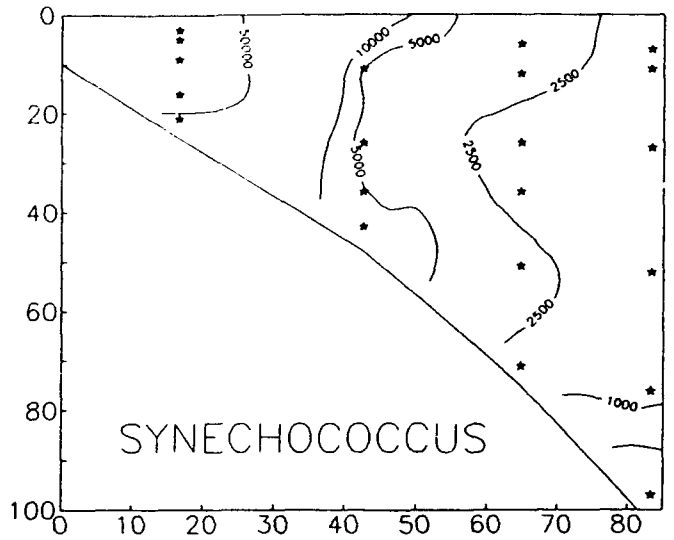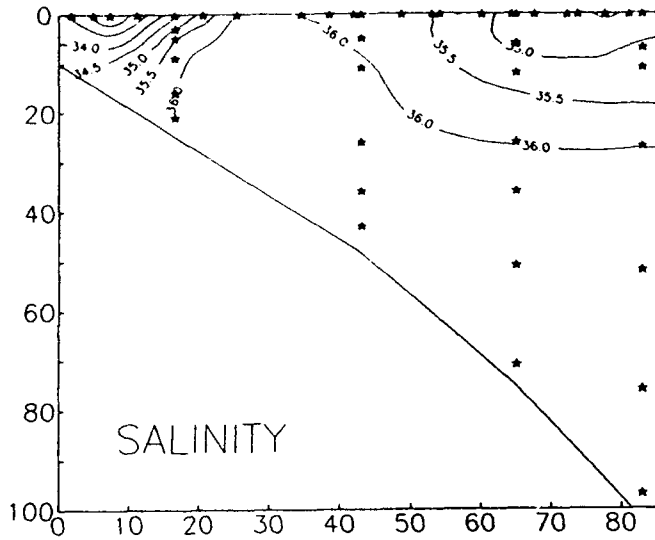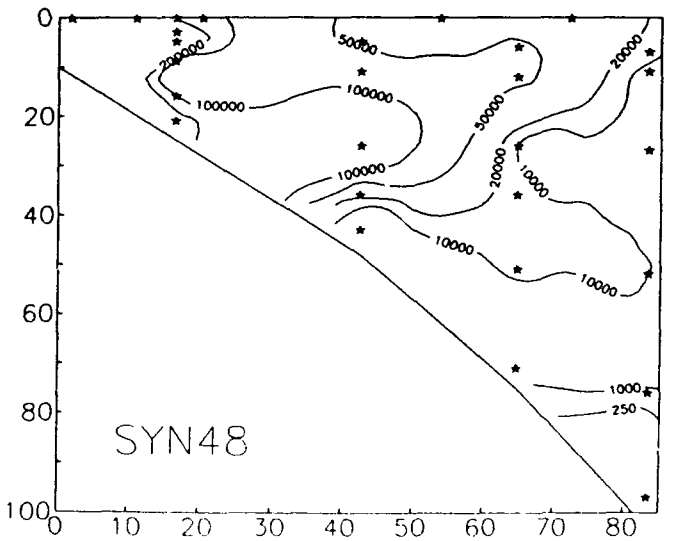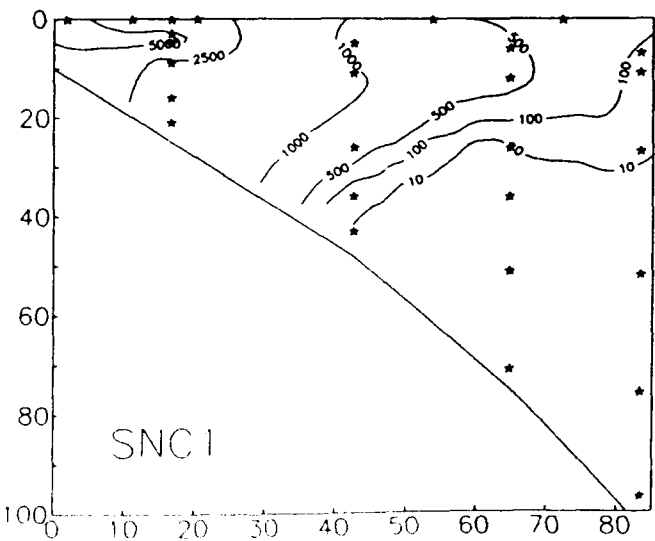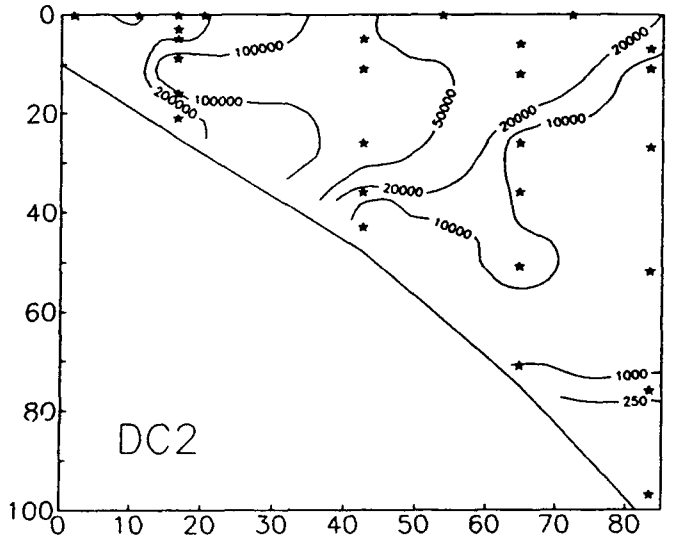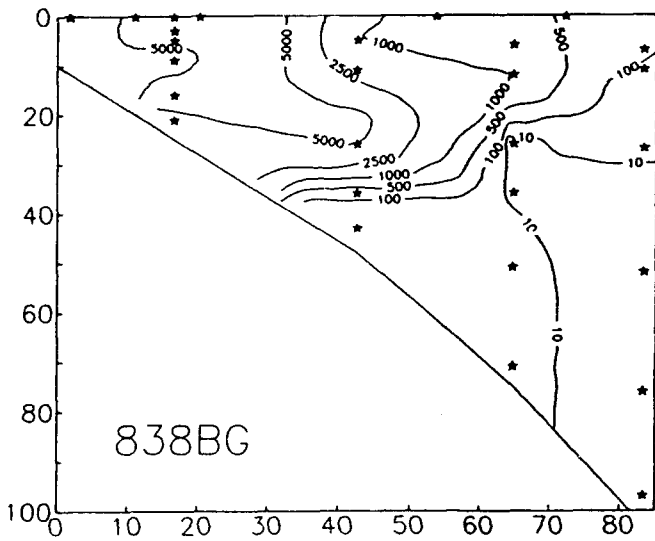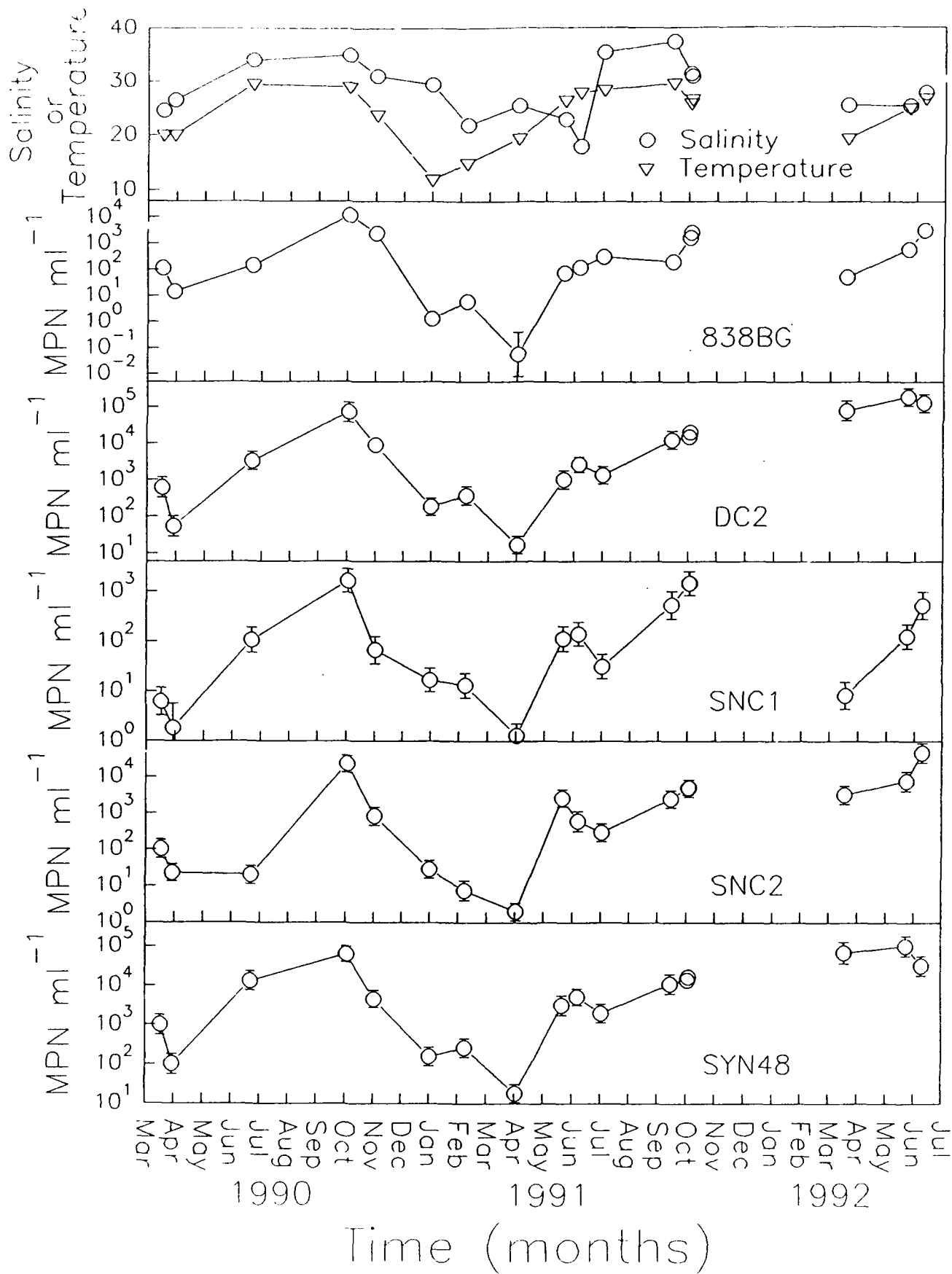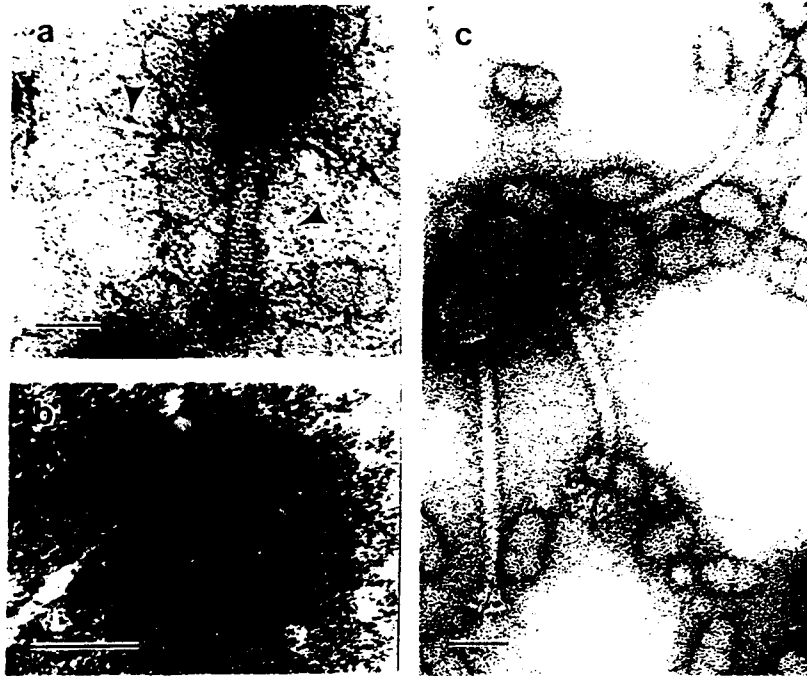
FIGURE 3   C A Suh6; A M Chan   * ORIGINAL FIGURE

1   **MARINE CYANOPHAGES INFECTING OCEANIC AND COASTAL STRAINS OF**

2   **SYNECHOCOCCUS: ABUNDANCE, MORPHOLOGY, CROSS-INFECTIVITY AND**

3   **GROWTH CHARACTERISTICS**

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9   Curtis A. Suttle

10

11   and

12

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1

2   ABSTRACT:

3

4   Eight different phycoerythrin- and phycocyanin-containing strains of *Synechococcus*

5   and one strain of *Anacystis* were screened against 29 natural virus communities taken from

6   3 locations in south Texas coastal waters, at different times of the year. In addition, one

7   sample was screened from Peconic Bay New York. Cyanophages were detected in all

8   samples, but the frequency with which they were detected and their abundance depended

9   upon the strain of *Synechococcus* that was screened. Viruses that infected red

10   *Synechococcus* strains were particularly common. In some instances concentrations

11   infecting a single *Synechococcus* strain were in excess of 10^5 ml^{-1}. The seven cyanophages

12   that were cloned belonged to the same three families of viruses that have been observed to

13   infect freshwater cyanobacteria, namely the Siphoviridae (formerly Styloviridae),

14   Myoviridae and Podoviridae. The cyanophage clones varied in host-specificity. For

15   example, one clone infected a single *Synechococcus* strain of 12 tested, whereas, another

16   infected 4 of 9 strains tested. Growth characteristics of one of the virus clones was

17   determined for a single host. Photosynthesis in *Synechococcus* was not affected until near

18   the onset of cell lysis and the virus burst cycle was complete about 17 h post infection. The

19   burst size was approximately 250 infective particles. The high abundance of cyanophages

20   in the natural environment provides further evidence that viruses are probably important

21   regulators of phytoplankton dynamics in marine systems.

22

INTRODUCTION

The existence of cyanophages in freshwater that infect both unicellular and filamentous strains of cyanobacteria has been recognized for some time (*e.g.* Safferman & Morris 1967, Stewart & Daft 1977, Martin & Benson 1988). Cyanophages have been implicated in the prevention of bloom formation and control of community structure in freshwater systems (Stewart & Daft 1977, Martin & Benson 1988). It is surprising, given the importance of cyanobacteria in marine systems as major primary producers (Li et al. 1983, Murphy & Haugen 1985) and nitrogen fixers (Carpenter & Romans 1991), that there has been little systematic effort to document the occurrence and importance of cyanophages in marine systems. It has only recently been reported that up to 1.5 % of marine *Synechococcus* spp. contain mature phage (Proctor and Fuhrman 1990) and that infective cyanophage can be readily isolated from seawater (Suttle et al. 1990). These observations suggest that viruses could be a significant cause of mortality for marine *Synechococcus*.

In order to determine the extent to which cyanophages exist in seawater we investigated the occurrence and abundance of viruses which cause lysis of oceanic and coastal strains of *Synechococcus* spp. and *Anacystis marina*. We also investigated cyanophages in terms of host-specificity, adsorption kinetics, time until bursting, burst size and changes in photosynthetic rates following infection, as these parameters are potentially important in dictating the impact of viruses on the cyanobacteria community. In addition, we characterized a number of virus clones by transmission electron microscopy (TEM) to ascertain if marine cyanophages are morphologically similar to their freshwater counterparts.

MATERIALS AND METHODS

2

3 **Cyanobacteria isolates.** Eight marine isolates of *Synechococcus* spp. and one of

4 *Anacystis marina* (UTEX2380) were screened against natural virus communities. The

5 cyanobacteria were isolated from the coastal waters of Texas (BBC1, BBC2, SNC1, SNC2)

6 or obtained from culture collections at Bigelow Laboratory (DC2, SYN48, 838BG) and

7 The University of Texas at Austin (UTEX 1634, UTEX 2380). In addition, 4 strains of

8 freshwater *Synechococcus* spp. (*S. leopoliensis*, UTEX 625, UTEX 2434; *S. cedorum*, UTEX

9 1191; *S. elongatus*, UTEX 563) and one strain of *Microcystis aeruginosa* (UTEX 2385 =

10 NRC-1) were used for host-range studies.

11 **Study sites.** The natural virus communities for the screening experiments were

12 concentrated from surface seawater samples collected from four locations in Texas coastal

13 waters and one station on the eastern end of Long Island, New York (Peconic Bay). In

14 addition, the abundance of cyanophages and cyanobacteria were estimated directly from

15 surface seawater samples taken at 7 stations along a seaward transect originating at 1 km

16 and ending 41 km offshore from the Marine Science Institute, on 8 April 1982. The

17 locations of the Marine Science Institute, Laguna Madre and Gulf of Mexico sampling

18 stations are reported elsewhere (Suttle *et al.* 1991). Laguna Madre is a hypersaline lagoon

19 which is separated from the Gulf of Mexico by a barrier island. The Gulf of Mexico station

20 was located in oligotrophic waters (< 0.1 μg chlorophyll a l^{-1}) approximately 70 km

21 offshore from Port Aransas, Texas. The concentration of cyanophages was determined in

22 17 seawater samples collected from the Marine Science Institute pier between March 1990

23 and June 1992. Depending on tide, rainfall, wind and season, the water off of the pier can

24 vary from low salinity (< 20 ppt) and estuarine (3.0-10.0 μg chlorophyll a l^{-1}) to high

25 salinity (37 ppt) and oligotrophic (0.1-1.0 μg chlorophyll a l^{-1}). The Brownsville Channel is

26 located 174 km south of Port Aransas and is a major pass between Lower Laguna Madre

27 and the Gulf of Mexico. The samples at this location were collected during a dinoflagellate

1   bloom. Peconic Bay is a small, vertically well mixed estuary with salinities varying between

2   about 23.5 and 29.5 ppt (Bruno *et al.* 1980).

3   **Isolation of virus communities and cyanophage distribution.** The 30 natural virus

4   communities that were screened for the presence of cyanophages were concentrated from

5   seawater using ultrafiltration as previously described in (Suttle *et al.* 1991). Briefly, 20 to

6   100 l of seawater were gently filtered through 142-mm-diameter glass-fiber (MFS GC50,

7   1.2 μm nominal pore-size) and 0.22 or 0.45 μm pore-size low-protein-binding Durapore

8   membrane filters (Millipore) to remove zooplankton, phytoplankton and most bacteria.

9   Subsequently, the viruses in the filtrate were concentrated up to 1100 times using a 30,000-

10   MW-cutoff spiral-wound ultrafiltration cartridge (Amicon S1Y30). Virus concentrates

11   were stored in the dark at 4° C until use.

12   Aliquots from the virus communities were added to exponentially-growing cultures

13   of the cyanobacteria. The cyanobacteria were grown under continuous irradiance (40-50

14   μmol quanta $m^2 s^{-1}$) at 25 ^{O}C in artificial seawater (ESAW, Harrison *et al.* 1980) and

15   13 x 100 or 25 x 150 mm borosilicate glass tubes with polypropylene screw caps. The

16   amount of concentrate added to each potential host was chosen to be equivalent to the

17   number of viruses that would be in 500 ml of seawater, assuming that the viruses were

18   concentrated with 100 % efficiency. The *in vivo* chlorophyll fluorescence of these cultures

19   was monitored daily for 7-10 d to look for signs of lysis; lysis was taken to be evidence of

20   the presence of a viral pathogen. In cultures which lysed, the presence of a viral pathogen

21   was confirmed by propagating 50 μl of 0.2-μm-filtered lysate to replicate tubes of

22   exponentially growing cultures. For tubes that did not lyse the presence or absence of a

23   pathogen was tested by adding 500 μl of unfiltered culture to replicate tubes of

24   exponentially growing cells. On occasion, when the initial viral titer was low, viral

25   pathogens were detected by this subsequent amplification step. For samples in which lytic

26   viruses were detected the concentration of cyanophages was estimated as outlined below.

27   **Estimation of cyanophage and cyanobacteria concentrations.** The concentrations of

1   cyanophages infecting particular hosts were estimated by adding aliquots of exponentially-

2   growing cyanobacteria into each well of a 96-well microtiter plate, followed by one of

3   several dilutions of the sample to be titered. A similar protocol has been used to estimate

4   cyanophage concentrations in waters off of the northeast coast of the United States

5   (Waterbury pers. comm.). The concentration of viruses was determined directly from

6   natural seawater samples (offshore transect) or in natural virus communities that had been

7   concentrated from seawater by ultrafiltration, as outlined above. Each dilution was

8   replicated 8 times and each assay was duplicated for a total of 16 replicates at each

9   dilution. Controls received no virus addition. The plates were incubated under continuous

10   irradiance (10-15 μmol quanta m^{-2} s^{-1}) at 25 $^{\circ}$C and the wells monitored daily for 7-10

11   days, for evidence of cell lysis. Wells that did not clear after 10 days were propagated into

12   fresh exponentially-growing cultures and monitored for another 7 days. Cultures which still

13   did not clear after propagation were scored as negative for the presence of lytic viruses.

14   The number of wells in which lysis occurred or did not occur was scored, and the

15   concentration of infective units and the error associated with these estimates were

16   determined using a computer program written in BASIC (Hurley and Roscoe 1983). We

17   confirmed that viruses were responsible for cell lysis by using TEM to examine

18   representative wells of the microtiter plates containing lysed cells; in every instance lysis

19   was the result of viruses. Estimates obtained for concentrated natural virus communities

20   were converted to ambient abundances assuming that the viruses were concentrated from

21   seawater with 100 % efficiency. Consequently, any loss of viruses due to the concentration

22   procedure would result in titer being underestimated. We have estimated the

23   concentration efficiency of cyanophages infecting several hosts by titering natural seawater

24   before and after concentration by ultrafiltration. Viruses infecting SYN48, SNC2, SNC1,

25   838BG and DC2 were concentrated with 38.2, 60.4, 40.2, 13.3 and 26.6 % efficiency,

26   respectively, from an offshore Gulf of Mexico sample. A comparison made using water

27   collected from the MSI pier indicated that viruses infecting SNC2 were concentrated with

1   58.6 % efficiency.

2   Epifluorescent microscopy was used to enumerate cyanobacteria in water samples

3   taken along the offshore transect (Waterbury *et al.* 1986). Seawater subsamples (10-25 ml)

4   were filtered onto 25 mm, 0.2 μm pore-size, black polycarbonate filters (Poretics), and the

5   number of yellow (red strains) and red (green strains) autofluorescent cyanobacteria

6   counted in a minimum of 20 microscope fields. Ideally, a minimum of 200 cells of each

7   type were counted, although red fluorescing cyanobacteria were frequently so scarce as to

8   make this impractical.

9   **Isolation and cloning of cyanophages.** Seven cyanophages were cloned from natural

10   virus communities. S-BBS1 was isolated by plaque purification using ESAW solidified with

11   1 % agar; the other viruses were cloned in liquid using a dilution assay (Cottrell and Suttle

12   1991). In this procedure a small aliquot of lysed culture was filtered through a 0.2-μm-

13   pore-size polycarbonate filter and transferred into an exponentially-growing culture of the

14   host. The pathogen was propagated in this manner several times to dilute out non-

15   replicating viruses. The virus was cloned by determining the most probable number of

16   infective units (Taylor 1962) and adding 0.2 infective units to twenty susceptible host

17   cultures. The cultures in which lysis occurred were assumed to have a single infective virus;

18   the probability that more than a single virus occurred in a given culture is 0.0176.

19   **Electron Microscopy.** Viral lysates were 0.2 μm filtered and concentrated by

20   ultracentrifugation in an AH-629 swinging-bucket rotor (Sorvall) at 20 $^\circ$C and 28,500 rpm

21   (146000 x g) for 2.75 h. The pellets were resuspended in about 200 μl of ESAW. Viruses

22   were transferred to 400mesh, carbon-coated copper grids by floating the grids on drops of

23   the resuspended virus solutions for about 20 min. The grids were stained by floating on a

24   drop of 1% aqueous uranyl acetate for 20-30 s. The excess stain was wicked off with filter

25   paper and the grids were viewed at 80 kV using either a Philips EM301 or a JOEL-100CX

26   transmission electron microscope.

27   **Host Range Studies.** Host specificity of S-BBP1 through S-PWP1 was tested in

7

1   liquid culture against 8 strains of marine *Synechococcus* spp. and one strain of *Anacystis*

2   *marina*. Cyanophages were screened for host specificity using culture lysate from a

3   susceptible cyanobacterial culture to which cloned cyanophage was added. The lysate was

4   0.2 μm-filtered and 50 μl was added to each of quadruplicate 5 ml exponentially-growing

5   cultures containing a potential host. Controls included cultures to which no virus was

6   added and cultures to which host culture filtrate was added. The effect of the added virus

7   on the potential host was determined by monitoring *in vivo* chlorophyll fluorescence.

8   Cyanobacteria cultures that had not lysed 4 days after reaching stationary phase were

9   considered to be unsuitable hosts for the virus. Lysates from strains that were sensitive to

10   the pathogen were 0.2-μm filtered and reinoculated into a new culture to ensure that the

11   effect could be propagated.

12   In addition to being tested against 9 strains of marine cyanobacteria, S-BBS1 was

13   tested against *M. aeruginosa* and four strains of freshwater *Synechococcus* spp. Most of

14   these host-range studies were done using triplicate plaque assays. Typically, an

15   exponentially-growing cyanobacteria culture (*ca.* 10^7 cells ml^{-1}) was concentrated about

16   100 fold by centrifugation (15 min, 10,000 x g, 18 $^{\circ}$C) and the cells resuspended in marine

17   (ESAW) or freshwater (Allen 1968) growth medium, or a combination of the two (1:1

18   ESAW:Allen's), as appropriate. A 500 μl aliquot of this cell suspension (*ca.* 5 x 10^8 cells)

19   was combined with 500 μl or less of a solution of the virus to be tested for host specificity,

20   and the viruses allowed to adsorb for 45 to 60 min. This mixture was added to 2.5 ml of

21   0.6 % molten (46 $^{\circ}$C) purified agar (Sigma) in growth medium, mixed quickly by vortexing

22   and poured directly onto a solidified bottom layer made with 1.0 % purified agar and

23   growth medium. After the top-agar hardened the plates were inverted and incubated at

24   25 $^{\circ}$C under continuous illumination at 36 μmol quanta m^2 s^{-1}. Plaques appeared in 3 - 5

25   d. As a control, S-BBS1 was also assayed against BBC1 grown on the 3 types of solid

26   media.

27   For potential hosts of S-BBS1 which did not grow well in top agar, screening was

1 done in liquid media. These included DC2, SYN48 and 838BG, which were tested in

2 ESAW, and *M. aeruginosa* which was tested in Allen's medium. As a control, S-BBS1 was

3 screened against BBC1 grown in both types of liquid media. Treatments were in triplicate.

4 The 0.2 μm-filtered (polycarbonate, Poretics) S-BBS1 was added to the exponentially

5 growing potential hosts at a virus to host ratio of about 0.01. About 3 to 5 days after the

6 cells reached stationary growth aliquots of the cultures were plaque assayed against BBC1

7 to test for amplification of S-BBS1 by the other hosts.

8   **Adsorption kinetics and growth cycle.** Standard protocols (Adams 1959) were used

9 to determine the adsorption kinetics for cyanophage S-BBS1 cultured on strain BBC1,

10 growing in ESAW. Approximately 9×10^6 cells ml^{-1} from an exponentially-growing culture

11 of BBC1 were combined with S-BBS1 at a virus to host ratio of 0.01. Following mixing

12 triplicate samples were immediately removed, diluted 10 fold with ice cold ESAW and

13 centrifuged for 5 min at $5200 \times g$ at 5 °C to remove cells with attached viruses. The

14 supernatant was promptly titered for unabsorbed viruses. Additional samples were assayed

15 for unabsorbed viruses at 15, 30, 45 and 60 min. The adsorption rate constant ($time^{-1}$) was

16 determined by multiplying the slope of a semi-log plot of the percent of free virus

17 remaining in solution versus time by 2.3. This was divided by the host concentration to

18 express the constant in the traditional units of ml min^{-1}.

19   Growth of the virus in the host cell was determined by one-step growth experiments

20 (Adams 1959, Van Etten *et al.* 1983). Triplicate cultures of BBC1 (*ca.* 2.3×10^7 cells ml^{-1})

21 were inoculated with S-BBS1 virus at a virus to host ratio of 0.02 and allowed to adsorb for

22 60 minutes at 25 °C. Each host-virus solution was centrifuged briefly. The pellet was

23 washed once with ESAW, resuspended, and diluted 400 x and 40,000 x in ESAW. At

24 predetermined intervals duplicate aliquots were withdrawn and titered by plaque assay as

25 described above, to determine the total number of infective centers. The infected cultures

26 were incubated at 25 °C at an irradiance of 36 μmol $m^{-2} s^{-1}$.

27   **CO_2 Fixation.** Cyanophage clone S-BBS1 was added to triplicate 60 ml

1   exponentially-growing cultures of *Synechococcus* strain BBC1 at a virus to host ratio of

2   about 1, and 10 μCi of ^{14}C-bicarbonate was added. The cultures were incubated under

3   fluorescent lights at an irradiance of 100 μmole m^{-1} s^{-2}. Rates of isotope incorporation

4   were compared to control cultures to which viruses were not added. At predetermined

5   times, 10 ml-samples were filtered onto 0.2-μm pore-size, cellulose-nitrate membranes,

6   acid fumed overnight and the radioactivity on the filters determined by liquid scintillation

7   counting. Standard errors of the ratio of uptake rates in the treatment and control cultures

8   were calculated as described in Yates (1981).

9

10   **RESULTS**

11   **Occurrence and concentration**

12   Viruses that infect *Synechococcus* spp. are widely occurring in the marine

13   environment. We detected lytic cyanophages in every virus community that we screened   Table 1

14   (Table 1). Cyanophages that caused lysis of the red strains (DC2, SYN48, and SNC2) and

15   some of the green strains (SNC1 and 838BG) were detected in almost all of the virus

16   communities; whereas, those that infected some of the green strains were much less

17   common. For example, lytic pathogens of UTEX 1634 and 2380 were not detected. As

18   well, < 10 % and < 30 % of the virus communities contained viruses which caused lysis of

19   BBC1 and BBC2, respectively.

20   The abundance of cyanophages was temporally variable, ranging from undetectable

21   to as high as 1.9 x 10^5 ml^{-1}, and strongly depended on the host that was screened (Table 2).

22   In almost every instance the viruses infecting the red strains DC2 and SYN48 were present   Table

23   in much higher concentrations than for the other cyanobacteria. The viruses occurred over   2

24   the entire range of salinities (18-70 ppt) and temperatures (12-30.4 $^{\circ}$C) that we screened,

25   although the lowest titers for samples taken from the Institute pier were recorded for the

26   spring of 1990 and 1991, coincident with lower water temperatures and salinities.

27   However, when the data for all sampling dates were combined cyanophage concentrations

1   were only weakly dependent on temperature (range in r^2, 0.53 to 0.70) (Fig. 1).

2   In nearshore stations along a transect in the western Gulf of Mexico, cyanophage

3   concentrations infecting DC2 and SYN48 were in excess of 10^4 ml^{-1} (Fig. 2). Moreover,

4   the distribution of red *Synechococcus* strains (yellow-fluorescing) appeared to be coupled to

5   the distribution of viruses infecting DC2 and SYN48. Cell and cyanophage concentrations

6   dropped by about half and 10-fold, respectively, as the ship moved between two visibly

7   different water types, indicated by a 5 ppt increase in salinity. Viruses infecting SNC1 and

8   SNC2 followed a similar pattern, although they were considerably less abundant. The

9   green strains (red fluorescing) were much less abundant and not as obviously tied to the

10   change in water type. As well, concentrations of viruses that infected 838BG (a green

11   strain originally isolated from the Gulf of Mexico) were similar on both sides of the salinity

12   change.

13

14   **Morphology of cyanophages**

15   The seven cyanophages that we cloned from marine waters all had tails and

16   icosahedral heads. The viruses fell into three distinct groups. S-BBS1 is about 290 nm in

17   overall length and has a long and flexible noncontractile tail (*ca.* 230 nm) (Fig. 3a), while

18   S-BBP1 and S-PWP1 are characterized by their very short tails (Fig. 3b and 3c). As well,

19   S-PWP1 appears to possess a contractile sheath. Both S-BBP1 and S-BBS1 have relatively

20   small capsids of similar diameter (*ca.* 50 nm); whereas, the capsid of S-PWP1 is somewhat

21   larger (*ca.* 65 nm). The third group (S-PWM1, S-PWM2, S-PWM3, S-PWM4) had

22   contractile tails and showed considerable structural variation (Fig. 3c-f). Of particular

23   interest is the obvious neck filaments found on S-PWM4.

24

25   **Host range**

26   As the detection of cyanophages in natural virus communities depended upon the

27   strain of *Synechococcus* spp. that was screened it indicated that all cyanophages could not

1   infect all strains of *Synechococcus*. As might be expected the two cyanobacterial strains for

2   which the highest titer of infectious viruses was routinely recorded (DC2 and SYN48) were

3   also the isolates which were infected by more than a single virus clone (Table 3). As well,

4   the viruses ranged in host specificity. S-PWM3 infected 4 and S-PWM4 infected 2 of the 9

5   cyanobacterial strains screened, while the five other virus clones only infected a single   Table 3

6   strain of cyanobacteria. It is interesting that S-PWM3 infected a green *Synechococcus*

7   strain, as well as, three of the red strains. No relationship was evident between host origin

8   and the viruses to which they were susceptible. For example, S-PWM3 infected coastal and

9   oceanic cyanobacterial strains. Similarly, the pigment composition of the host was not tied

10   to infection by a particular viral family, at least for the green strains, which were infected by

11   the three virus families. However, the four viruses that we cloned which infected the red

12   strains appeared to belong to the same virus family (Myoviridae); whereas, the

13   Syphoviridae and Podoviridae only infected green strains.

14   Given the morphological similarities among cyanophages isolated from marine and

15   fresh waters it seemed plausible that the marine isolates might also infect freshwater

16   cyanobacteria. As green strains of *Synechococcus* spp. are commonly observed in lakes,

17   and as Syphoviridae have been isolated which infect a number of freshwater *Synechococcus*

18   strains, we chose S-BBS1 for more detailed cross-infectivity experiments. S-BBS1 was not

19   able to infect any of four freshwater strains of *Synechococcus* spp. or one strain of

20   *Microcystis aeruginosa*. This was despite the fact that lytic viral pathogens had been

21   previously isolated to three of the four *Synechococcus* spp. and to the *M. aeruginosa* strain.

22

23   **Adsorption kinetics, photosynthetic rates and the viral growth cycle.**

24   The adsorption of S-BBS1 to BBC1 did not deviate substantially from a log-linear

25   relationship ($r^2 = 0.94$) for the 60 min over which the kinetics were determined (Fig. 4).   Fig 4

26   The average percentage of free viruses remaining at 15, 30, 45 and 60 min post-addition

27   were 46.9, 22.0, 18.5 and 11.3 %, respectively. The average adsorption rate constant over

1 the 60 min incubation was 0.035 min^{-1} (3.94×10^{-9} ml min^{-1}).

2   Data from the one-step growth experiment indicated that onset of cell lysis began

3 about 9 h following infection and was complete by 17 h post infection (Fig. 5).   Fig 5

4 Approximately 250 progeny viruses were produced per infected host cell when lysis

5 occurred.

6   Photosynthetic rates in infected cultures of BBC1 were similar to those in non-

7 infected controls until near the onset of cell lysis (Fig. 5). At 21 h after infection

8 photosynthetic rates in the infected cultures were < 4 % of those in the controls and by

9 26 h inorganic carbon uptake was not detectable in the cultures to which viruses were

10 added.

11

12

13 **DISCUSSION**

14 **Cyanophage abundance and distribution**

15   Clearly, cyanophages infecting *Synechococcus* spp. are abundant and widely

16 occurring in the marine environment. Cyanophages were abundant in natural virus

17 communities from a hypersaline lagoon (Laguna Madre), nearshore and neritic waters of

18 the Gulf of Mexico and in a sample from Peconic Bay, New York and frequently occurred

19 in titers in excess of 10^4 ml^{-1} for some hosts. Invariably, the virus communities included

20 cyanophages that infected red cyanobacteria strains, while viruses infecting some green

21 strains were less frequently detected and when present were less abundant (Tables 1 and

22 2).

23   Along the transect in the western Gulf of Mexico the highest viral titers were

24 associated with the greatest concentrations of yellow-fluorescing *Synechococcus* cells (red

25 strains) for 4 of the 5 hosts screened (Fig. 2), suggesting a coupling between cyanophage

26 and host abundance. As well, cyanophage concentrations occasionally reached > 10^5 ml^{-1}

27 in inshore samples (Table 2, Fig. 1). Although the titers were extremely variable and host

1   dependent, the lowest concentrations were generally associated with cooler water as

2   indicated by the weak correlations between cyanophage abundance and water temperature

3   (Fig. 1). Culture studies with freshwater phytoplankton communities have shown that the

4   contribution of cyanobacteria to total phytoplankton biovolume increases with increasing

5   water temperature (Tilman and Kiesling 1983). Consequently, *Synechococcus* abundances

6   were likely greater when water temperatures were warmer and the highest concentrations

7   of cyanophages were recorded.

8   The highest titers of cyanophages were obtained for viruses that infected red strains

9   of *Synechococcus*, which are the numerically dominant cyanobacteria in oceanic waters.

10   Furthermore, the cyanobacteria for which the highest titers of cyanophages were routinely

11   recorded (DC2 and SYN48) were open ocean isolates; DC2 originated from the North

12   Atlantic ($33^{\circ}45$'N, $67^{\circ}30$'W), while SYN48 was isolated from the tropical Atlantic

13   ($08^{\circ}44$'N, $50^{\circ}50$'W). Viruses infecting a red strain from Laguna Madre (SNC2) were also

14   widely occurring, but not as abundant as the oceanic strains. Although it is difficult to

15   extrapolate from such a limited data set, it suggests that oceanic strains of *Synechococcus*

16   are more sensitive to phage infection than coastal strains.

17   It is significant that viruses in seawater from a hypersaline lagoon, the coastal waters

18   of Texas and New York, and the Gulf of Mexico were able to infect hosts that were isolated

19   from the North and tropical Atlantic, as well as, Texas. Obviously, a single isolate of

20   *Synechococcus* can be infected by viruses that occur in widely separated regions. This was

21   also reported for viruses infecting the eukaryotic photosynthetic flagellate, *Micromonas*

22   *pusilla* (Cottrell and Suttle 1991).

23   Other recent studies have demonstrated high concentrations of cyanophages in

24   seawater. Waterbury and Valois (1992) reported cyanophages concentrations of 3.9×10^4

25   in Woods Hole Harbor and 2.1×10^3 in slope water, and Suttle and Chan (1992) recorded

26   abundances > 10^5 ml^{-1} in nearshore regions of the western Gulf of Mexico.

27

Cyanophage morphology

The cyanophages that we isolated belong to the same three families of viruses that have been observed to infect freshwater cyanobacteria, namely the Siphoviridae (formerly Styloviridae), Myoviridae and Podoviridae (see reviews by Padan & Shilo 1973, Stewart & Daft 1977, Safferman *et al.* 1983, Ackermann & DuBow 1987, Martin & Benson 1988). Safferman *et al.* (1983) have further classified the cyanophages into three genera. The cyanophages that we most frequently isolated were *Cyanomyovirus* spp. They showed considerable structural diversity suggesting that there may be several species of marine cyanophages within this genus. Neck filaments, that were observed on S-PWM4, have been reported for a number of viruses which infect freshwater filamentous cyanobacteria (*e.g.* Adolph & Haselkorn 1973, Padan & Shilo 1973), but not unicellular forms. As such structures are extremely rare (Ackermann and DuBow 1987), it suggests that these freshwater and marine cyanophages may share a common ancestry. The long and flexible tail of S-BBS1 (*ca.* 230 nm) is considerably longer than has been reported for other *Cyanostylovirus* spp. (*e.g.* Fox *et al.* 1976), but otherwise the virus appears similar to freshwater isolates. As well, the marine *Cyanopodovirus* spp. were morphologically similar to their freshwater counterparts (Safferman *et al.* 1983), although there is evidence that S-PWP1 may possess a contractile sheath.

Host range

In order to determine the host specificities of the marine cyanophages, we tested each of the viruses against eight strains of *Synechococcus* and one strain of *Anacystis* isolated from seawater. The isolates included coastal and oceanic strains, and strains with phycoerythrin or phycocyanin as their dominant pigment. With the exceptions of S-PWM3 and S-PWM4 the viruses infected only one of the nine cyanobacteria hosts screened (Table 3). The pattern was complicated by the fact that the host range of the phages overlapped. For example, S-PWM3 and S-PWM4 infected two oceanic red strains; however, S-PWM3

1   also infected a red and a green coastal isolate. Other studies have also revealed that host

2   range among cyanophages is complex. For example, cyanophage SM-1 (Safferman *et al.*

3   1969) infected two strains of chroococcoid cyanobacteria but not six other strains tested;

4   whereas, AS-1 (Safferman *et al.* 1972) infected three strains of eight tested. There was no

5   overlap in the host range of these cyanophages. Nonetheless, the relatively high host

6   specificity of the marine cyanophages suggests that the probability of a cyanophage

7   encountering a suitable host is small, thereby this may be a mechanism to explain the high

8   densities of cyanobacteria and cyanophages that are observed in seawater. As well, we

9   were unable to infect any of four freshwater strains of *Synechococcus* spp. or a strain of

10   *Microcystis aeruginosa* with S-BBS1, a stylovirus that infects a green *Synechococcus* strain

11   (BBC1) isolated from seawater. The results suggest that marine cyanophages may not

12   readily infect freshwater cyanobacteria.

13   The effect of viruses on *Synechococcus* in nature will be difficult to ascertain.

14   Although the number of cyanophages which infect a particular strain of *Synechococcus* can

15   be quantified, the variation within *Synechococcus* spp. in terms of phage resistance, and the

16   diversity in host range of cyanophages indicates that the results would only be applicable to

17   a subset of the *Synechococcus* community. Nonetheless, as rapid viral propagation occurs

18   at high host densities (Wiggins and Alexander 1985), and lysogeny of *Synechococcus* spp.

19   appears to be rare (Martin and Benson 1988), there must be strong selection for the

20   *Synechococcus* community to remain diverse in terms of viral resistance. Consequently,

21   infection may be an underlying mechanism maintaining high genetic diversity in

22   *Synechococcus* spp. (Wood & Townsend 1990).

23   Host range was not obviously tied to location from which the hosts and viruses were

24   isolated. Although the virus clones originated from the coastal waters of Texas they

25   infected strains of *Synechococcus* originating from the North and tropical Atlantic (FC2

26   and SYN48), Gulf of Mexico (838BG, data not shown), coastal waters of Texas (BBC1 and

27   BBC2) and Laguna Madre (SNC1 and SNC2). Most of the viruses were fairly host specific;

1   however, S-PWM3 was able to infect four strains of *Synechococcus*, including a red and a

2   green strain, indicating that cyanophage taxonomy was not closely coupled to the taxonomy

3   of the hosts. In contrast, viral taxonomy may influence the hosts which can be infected.

4   Viruses from all three families infected green strains of *Synechococcus*, but all of the virus

5   clones infecting the red strains belonged to the Myoviridae. As well, the stylovirus

6   (S-BBS1) and the podoviruses (S-BBP1 and S-PWP1) each infected a single *Synechococcus*

7   strain, suggesting that viruses from these families may be more host specific than those

8   from the Myoviridae.

9   **Virus-host interactions**

10   The adsorption rate constant that we measured for S-BBS1 adsorbing to

11   *Synechococcus* strain BBC1 (3.94×10^{-9} ml min^{-1}) was slow relative to those typically

12   observed for bacteriophages, but fell within the range reported for phages adsorbing to

13   freshwater unicellular cyanobacteria (Samimi and Drews 1978, Cseke & Farkas 1979, Amla

14   1981). These rate constants should be useful for determining if theoretical estimates of the

15   probability of virus and host encounter can be used to estimate actual rates of virus

16   adsorption to the surface of cyanophages. The large burst size that we observed (250) is

17   similar to that reported for a *Cyanostylovirus* sp. that infects freshwater unicellular

18   cyanobacteria (Martin *et al.* 1978), but is considerably greater than that reported for

19   *Cyanomyovirus* spp. (Safferman *et al.* 1972, Sherman & Connelly 1976).

20   The pattern of photosynthetic suppression was also similar to that observed when

21   freshwater *Synechococcus* strains are infected by viruses (Mackenzie & Haselkorn 1972).

22   Photosynthesis continues up until the point of cell lysis. This is in contrast to viruses which

23   infect filamentous freshwater cyanophages, where photosynthesis is suppressed shortly after

24   infection and the viral life cycle can continue to completion in the absence of light (Padan

25   *et al.* 1973).

26   **Implications**

27   This study in conjunction with others (Proctor & Fuhrman 1990, Suttle *et al.* 1990,

1   Waterbury and Valois 1992, Suttle and Chan 1992) demonstrates that infective

2   cyanophages are widespread and likely have considerable impact on natural marine

3   cyanobacteria communities. The high abundances of *Synechococcus* in the sea and the

4   apparently ubiquitous occurrence of viruses which infect them is somewhat enigmatic. It

5   suggests that most *Synechococcus* cells are entirely resistant to viral infection or that there

6   is great diversity within the genus in terms of resistance to infection. In either case the

7   implication is that viruses are a major factor structuring the marine cyanobacterial

8   community. Nonetheless, it will be difficult to ascertain the impact of viruses on the

9   population dynamics of *Synechococcus* because of the diversity of the cyanophages in terms

10   of host-range and of the cyanobacteria in terms of resistance.

11   *Synechococcus* is one of the most abundant photosynthetic cells in oligotrophic

12   oceanic environments where it can be responsible for 5-80 % of the total primary

13   productivity (*e.g.* Joint & Pomroy 1983, Li *et al.* 1983, Waterbury *et al.* 1986). If

14   cyanophages lyse a sizable portion of open ocean *Synechococcus* communities it implies

15   that a significant amount of the primary productivity will be lost as dissolved organic

16   matter, viral particles and cellular debris. Ultimately, bacterial processes will recover some

17   of these resources, but overall less nutrients and energy will be shunted from the primary

18   producers to consumers, resulting in an overall decrease in the efficiency of nutrient and

19   energy transfer to higher trophic levels.

20

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TABLE 1: Occurrence of cyanophages infecting *Synechococcus* spp.

Synechococcus Strain	Laguna Madre (8)	Marine Science Institute (15)	Station (number of samples) Gulf of Mexico (3)	Brownsville Channel (3)	Peconic Bay N.Y. (1)
DC2[1]	8	15	3	3	1
SYN48[1]	8	15	3	3	1
SNC2[1]	8	15	3	3	1
BBC1	2	1	0	0	0
BBC2	3	7	0	0	0
SNC1	8	15	3	3	1
838BG	5	15	3	3	1
UTEX1634	0	0	0	0	0
UTEX2380	0	0	0	0	0

[1] These are red strains.

Table 2: Mean concentrations and ranges (viruses ml^{-1}) of cyanophages which infect *Synechococcus* strains.

Location	n	*Synechococcus* Strain					
		838BG	DC2	SNC1	SNC2	SYN48	BBC2
Laguna Madre	8	61.5 (0.0-202)	20621.3 (0.6-15520)	125.3 (0.0-765)	571.5 (0.1-3170)	24167.0 (0.7-190080)	0.4 (0-2.9)
Brownsville	3	79.0 (50-135)	10930.0 (2670-4660)	65.0 (47-77)	911.7 (595-1110)	3018.3 (1435-4060)	0.0
MSI Pier	17	1409.3 (0.1-12140)	31640.4 (17-190080)	369.1 (0.0-1643)	5742.0 (2-46720)	20062.0 (19-64640)	n.d.
Gulf of Mexico	4	41.2 (0.8-107)	3259.5 (18-10740)	22.5 (0.4-77)	824.8 (9-1445)	1633.4 (26-4560)	0.0
Peconic Bay NY	1	0.0	2.3	0.2	1.9	2.4	n.d.

TABLE 3: Host-range of cyanophage clones.

Synechococcus Strain	Cyanophage Clone						
	S-BBS1	S-BBP1	S-PWM1	S-PWM2	S-PWM3	S-PWM4	S-PWP1
DC2[1]	.	.	+	+	+	+	.
SYN48[1]	.	.	.	.	+	+	.
SNC2[1]	.	.	.	.	+	.	.
BBC1	+	.	.	.	.	.	.
BBC2	.	+	.	.	.	.	.
SNC1	.	.	.	.	+	.	+
838BG	.	.	.	.	.	.	.
UTEX1634	.	.	.	.	.	.	.
UTEX2380	.	.	.	.	.	.	.

[1] These are red strains.

1   **FIGURE LEGENDS**

2

3   FIGURE 1: Relationship between temperature (^{O}C) and the concentration of

4   cyanophages in coastal seawater that infect strains of *Synechococcus*. The water was

5   collected from the MSI pier on 17 dates between 16 March 1990 and 9 June 1992.
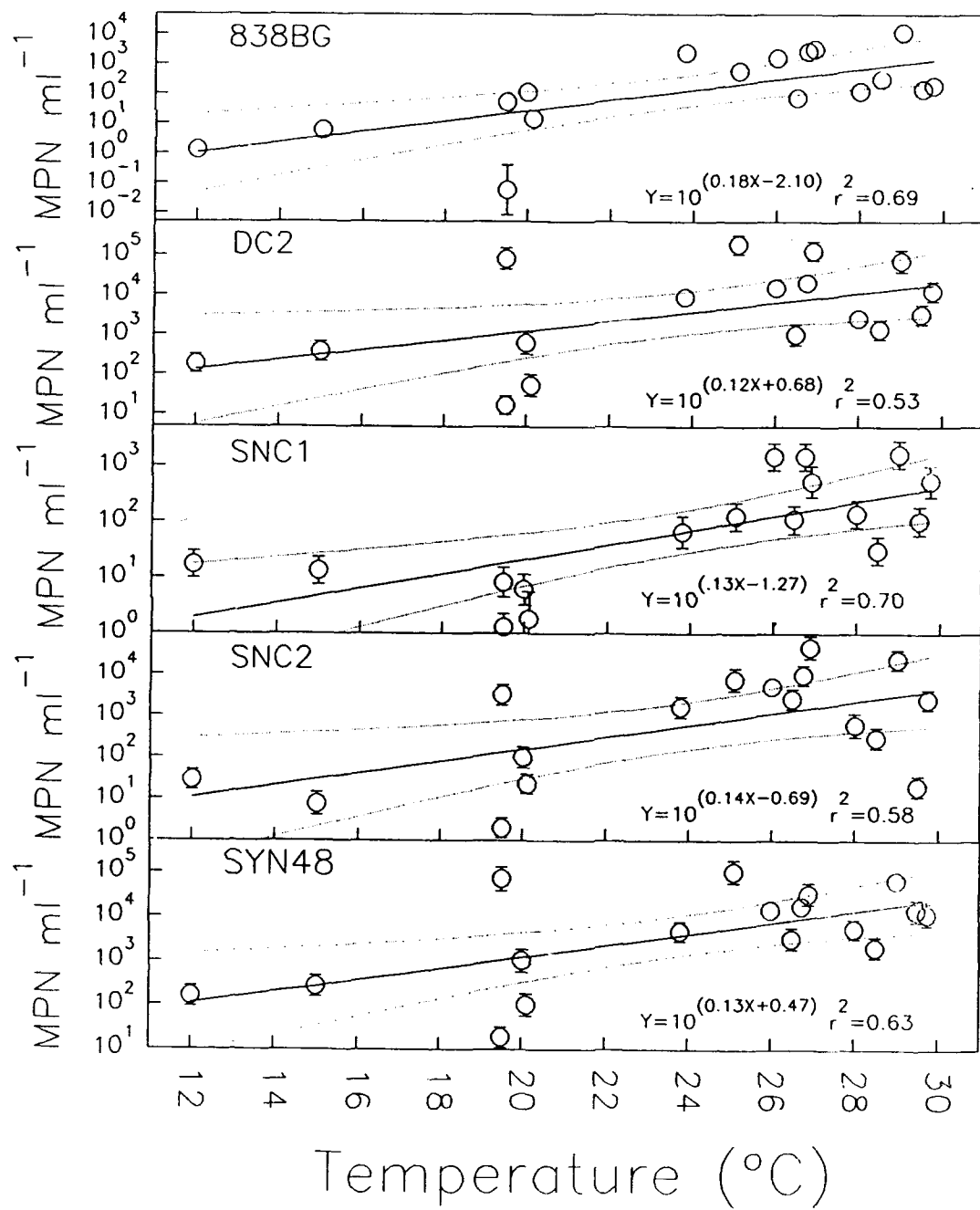
6

7   FIGURE 2: Salinity (ppt), temperature (^{O}C) and concentrations of cyanophages

8   (viruses ml^{-1}) infecting 5 strains of marine *Synechococcus* isolates along a transect in

9   the western Gulf of Mexico. The abundances of red (yellow fluorescing) and green

10   (red fluorescing) strains of unicellular cyanobacteria are also given.

11

12   FIGURE 3: Electron micrographs of cyanophages infecting marine strains of

13   *Synechococcus*. a) S-BBS1, Syphoviridae; b) S-BBP1, Podoviridae; c) S-PWP1,

14   Podoviridae; d) S-PWM1, Myoviridae; e) S-PWM2, Myoviridae; f) S-PWM3,

15   Myoviridae; g) S-PWM4, Myoviridae. Scale bar represents 50 nm. Arrows indicate

16   an apparent contracted sheath with exposed core in S-BBP1 (Fig. 3b) and neck

17   filaments in S-PWM4.

18

19   FIGURE 4:. Adsorption kinetics of cyanophage S-BBS1 on host *Synechococcus* strain

20   BBC1.

21

22   FIGURE 5: One step growth curve of S-BBS1 on *Synechococcus* host BBC1 and relative

23   photosynthetic rates (experimentals/controls) in cultures of *Synechococcus* (BBC1)

24   to which a cyanophage (S-BBS1) has been added.
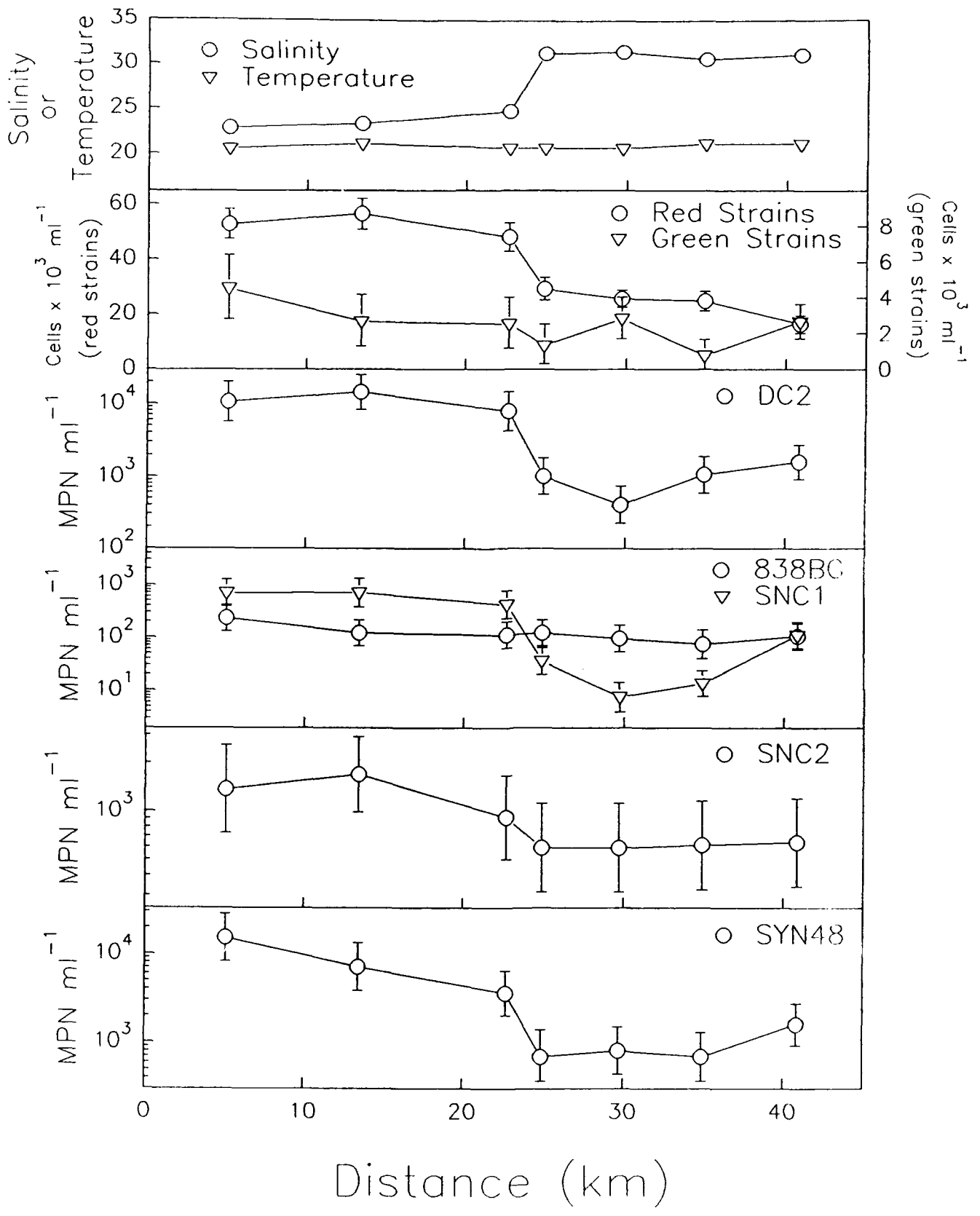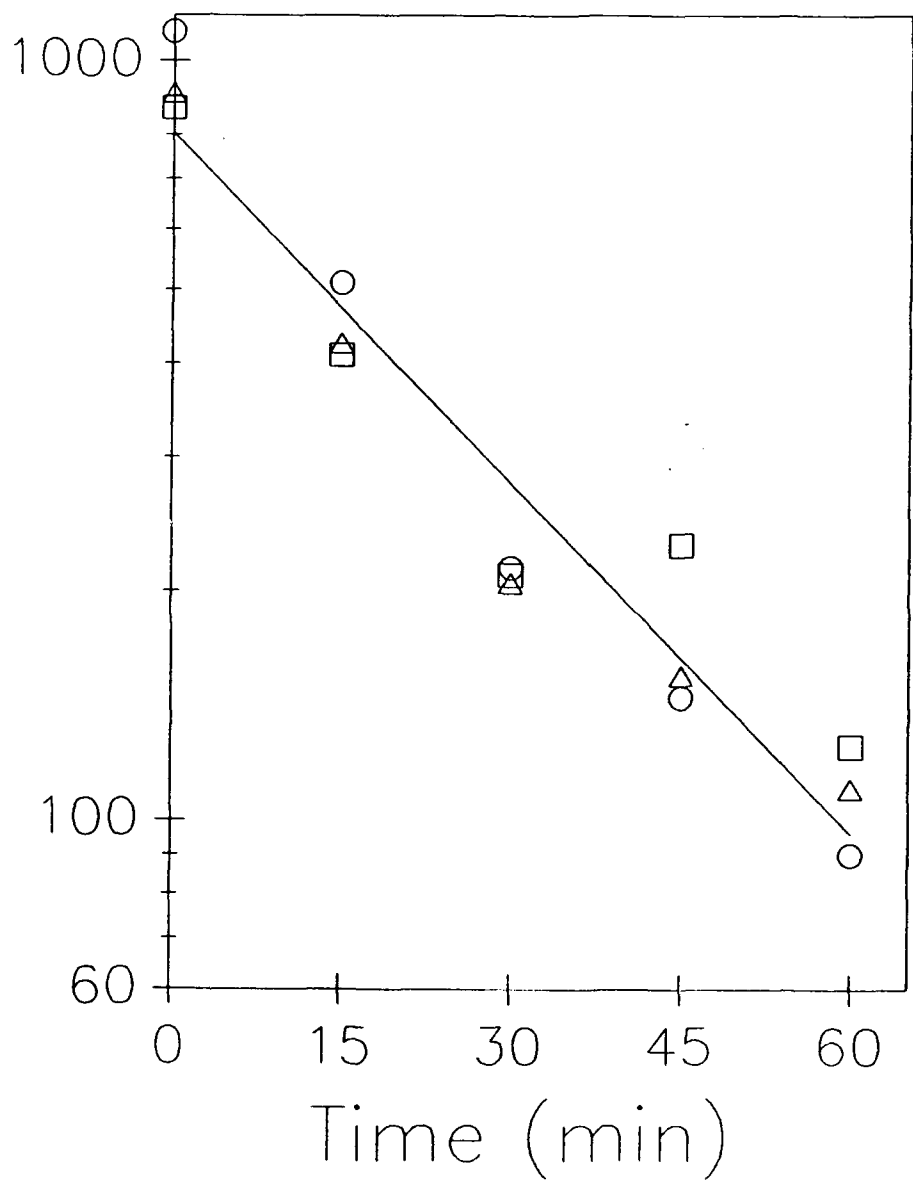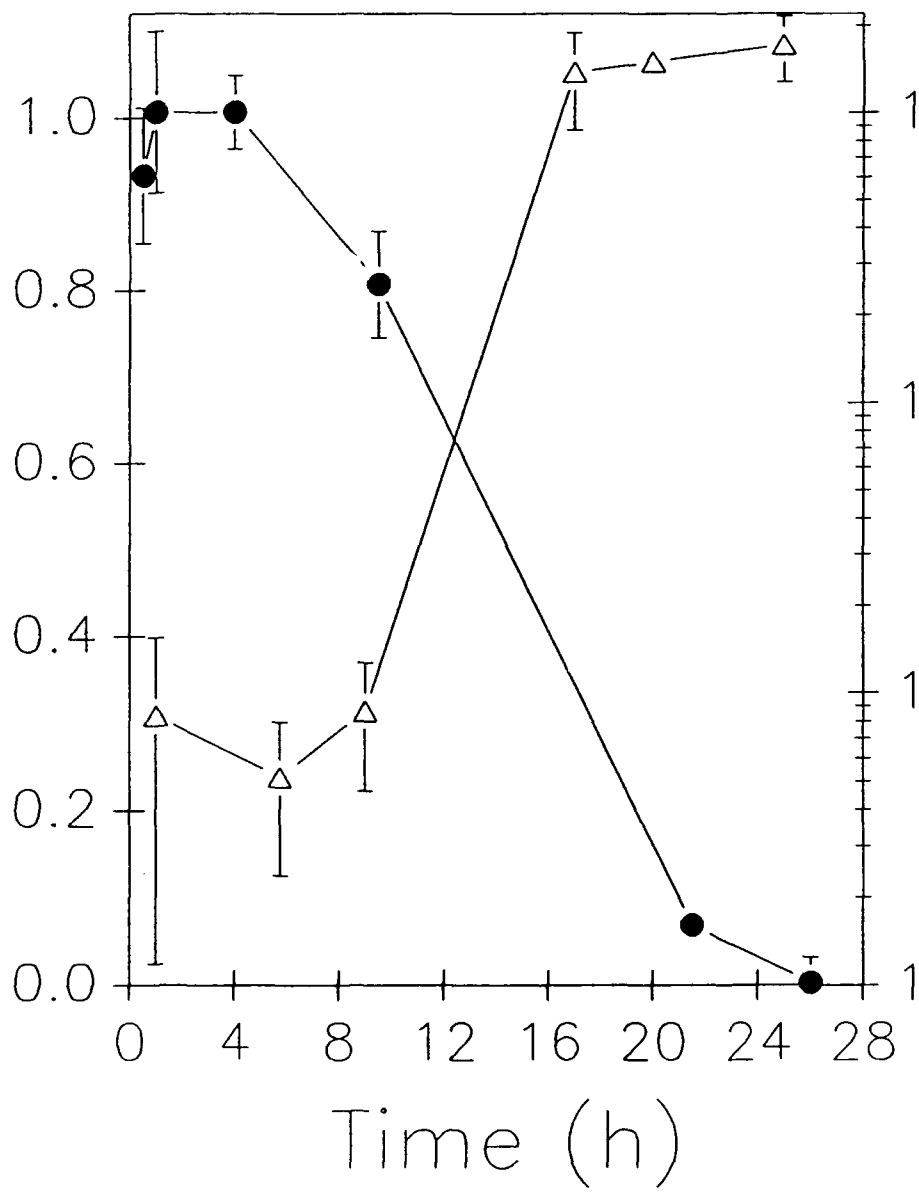
25

838BG

$Y=10^{(0.18X-2.10)}$ $r^2=0.69$

DC2

$Y=10^{(0.12X+0.68)}$ $r^2=0.53$

SNC1

$Y=10^{(.13X-1.27)}$ $r^2=0.70$

SNC2

$Y=10^{(0.14X-0.69)}$ $r^2=0.58$

SYN48

$Y=10^{(0.13X+0.47)}$ $r^2=0.63$

Temperature (°C)

MPN ml^{-1} MPN ml^{-1} MPN ml^{-1} MPN ml^{-1} MPN ml^{-1}

Fig 2