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

CURTIS A. SUTTLE,* AMY M. CHAN, AND MATTHEW T. COTTRELL

Marine Science Institute, The University of Texas at Austin, Port Aransas, Texas 78373-1267

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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* · liter^{-1}) to high salinity (37‰) and oligotrophic conditions (0.1 to 1.0 μg of chlorophyll *a* · 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

* Corresponding author.
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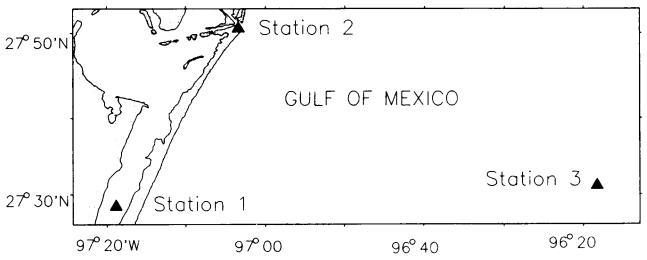

FIG. 1. The northwestern Gulf of Mexico, showing the locations of the sampling stations.

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

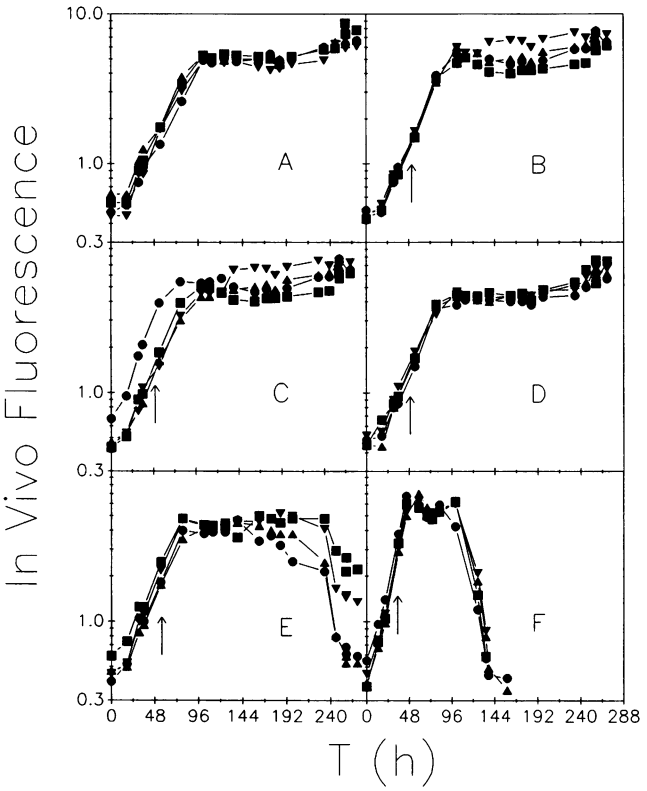

FIG. 2. An example showing how the fluorescence assay was used to screen natural virus communities for the presence of viral pathogens which infect the *Navicula* sp. Different symbols represent fluorescence measurements of quadruplicate cultures. The arrows indicate where viruses were added. (A) Control (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 responded 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.

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.

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	PVD	+	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.

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