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Discovery of a dsRNA virus infecting the marine photosynthetic protist *Micromonas pusilla*

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Abstract

We report the isolation of the first double-stranded (ds) RNA virus in the family Reoviridae that infects a protist (microalga *Micromonas pusilla*, Prasinophyceae). The dsRNA genome was composed of 11 segments ranging between 0.8 and 5.8 kb, with a total size of approximately 25.5 kb. The virus (MpRNAV-01B) could not be assigned to the genus level because host type, genome size, and number of segments smaller than 2 kb did not correspond to either of the two existing 11-segmented dsRNA genera *Rotavirus* and *Aquareovirus*. MpRNAV-01B has a particle size of 65–80 nm, a narrow host range, a latent period of 36 h, and contains five major proteins (120, 95, 67, 53, and 32 kDa). MpRNAV-01B was stable to freeze–thawing, resistant to chloroform, ether, nonionic detergents, chelating and reducing agents. The virus was inactivated at temperatures above 35 °C and by ionic detergent, ethanol, acetone, and acidic conditions (pH 2–5).

Keywords: dsRNA virus; 11-Segmented genome; *Micromonas pusilla*; Phytoplankton; Protist; Reoviridae

Introduction

It is well known that viruses are abundant in aquatic ecosystems (concentrations typically ranging from 10^5 to 10^8 ml^{-1}; Fuhrman, 1999; Proctor, 1997), and they are regarded as active and important members of the microbial food web (Fuhrman, 1999; Fuhrman and Suttle, 1993; Proctor and Fuhrman, 1991; Wilhelm and Suttle, 1999). Viruses have been found to affect population dynamics, horizontal gene transfer, as well as both energy and material fluxes (Chiura, 1997; Fuhrman, 1999; Gobler et al., 1997; Wilhelm and Suttle, 1999; Wommack and Colwell, 2000). In eukaryotic phytoplankton, an important group of organisms in natural aquatic communities, viruses can cause significant mortality (Bratbak et al., 1993; Brussaard et al., 1996; Suttle, 2000; Suttle and Chan, 1993; Suttle et al., 1990; Tarutani et al., 2000). Viruses or virus-like particles have been observed intracellularly in many different genera and classes of eukaryotic algae, varying from unicellular to multicellular, swimming to nonswimming, bloom-forming to non-bloom-forming, free-living to symbionts (Dodds, 1997; Garry et al., 1998; Proctor, 1997; Reisser, 1993; Sandaa et al., 2001; Van Etten et al., 1991).

About a dozen model systems of virus and photosynthetic host are presently in culture, including viruses infecting the chlorophyte *Micromonas pusilla* (Prasinophyceae). It was this phytoplankton species for which Mayer and Taylor (1979) provided the first evidence of infective algal viruses in seawater. *M. pusilla* is a naked, highly motile unicellular flagellate of very small size (1–3 μm in diameter). It is purely planktonic, widely distributed in both coastal and oceanic marine waters, and relatively abundant (Cottrell and Suttle, 1991; Sahlsten, 1998). The viruses so far isolated for *M. pusilla* are polyhedral, large in particle size (100–135 nm in diameter) and have DNA genomes (Cottrell and Suttle, 1991, 1995a; Mayer and Taylor, 1979; Sahlsten, 1998; Waters and Chan, 1982). In fact, almost all viral isolates infecting photosynthetic organisms have double-stranded (ds) DNA genomes, and belong to the virus family Phycodnaviridae (Pringle, 1999). Very recently, two instances of single-stranded RNA viruses infecting toxic bloom-forming phytoplankton (*Heterosigma akashiwo* and *Heterocapsa circularisquama*) have been described (Tai et al., 2003, and Tomaru, personal communication). Until now, there are no reports of double-stranded RNA viruses infect-

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ing marine photosynthetic organisms. In this study, we report the discovery and initial characterization of a dsRNA virus infecting the phytoplankter *M. pusilla*. The dsRNA virus has a genome consisting of 11 segments, similar to the Reoviridae. This is the first report of a 11-segmented dsRNA virus with a protist as host.

Results

Virus isolate

The newly isolated virus was found to co-occur in the original sample with a larger virus (particle size 100–140 nm) that was also able to infect *M. pusilla*. Only after passage through a 0.1-μm pore-size filter and end-point dilution we were able to remove the larger virus type. Ten virus clones were obtained from end-point dilution, all showing comparable behavior upon infection, as well as particle size and host specificity. The viruses infected none of the other algal species tested, including four species from the same class (Prasinophyceae, Table 1). Testing the viruses on five other *M. pusilla* strains also did not result in lysis, suggesting a high degree of strain specificity for the new virus isolates. The strain of *M. pusilla* on which the virus can be proliferated (LAC 38) originates from Norwegian coastal waters, whereas the other five strains were isolated from different locations in the English Channel, English coastal waters, or the Gulf of Main in the United States.

Table 1
List of phytoplankton used to screen for virus-induced lysis by MpRNAV-01B in the host range tests

Genus/species	Code[a]	Strain origin if not Scandinavian coastal waters	Lysis of strain
Prasinophyceae			
Micromonas pusilla	Mp LAC38[b]	Oslofjord, Norway	yes
Micromonas pusilla	Mp CCMP485[b]	Gulf of Maine, USA	no
Micromonas pusilla	Mp CCMP491[b]	English Channel	no
Micromonas pusilla	Mp CCMP1545[b]	English Channel	no
Micromonas pusilla	Mp UTEX991[c]	Plymouth 27, UK	no
Micromonas pusilla	Mp PLY27	English Channel	no
Pseudoscourfeldia marina	IFM		no
Pyramimonas cordata	IFM		no
Pyramimonas orientalis	IFM		no
Nephroselmis rotunda	IFM		no
Dinophyceae			
Katodinium rotundatum	IFM		no
Scripsiella tiochoidea	IFM		no
Prorocentrum minimum	IFM		no
Prymnesiophycea			
Phaeocystis globosa	IFM		no
Phaeocystis pouchetii	IFM		no
Chrysochromulina polylepis	IFM		no
Chrysochromulina ericinav	IFM		no
Emiliania huxleyi	IFM		no
Cyanophyceae			
Synechococcus sp.	WH 8103		no
Cryptophycea			
Hemiselmis sp.	IFM		no
Rhodomonas baltica	IFM		no
Pseudopedinella pyriformis	IFM		no

[a] LAC—Culture Collection of the Marine Center of Goteborg University; CCMP—Center of Culture of Marine Phytoplankton at Bigelow Laboratory for Ocean Sciences; UTEX—Culture Collection of Algae at the University of Texas at Austin; PLY—Plymouth Culture Collection; IFM—Culture Collection at the University of Bergen, Norway.
[b] Sahlsten (1998).
[c] Cottrell and Suttle (1991).

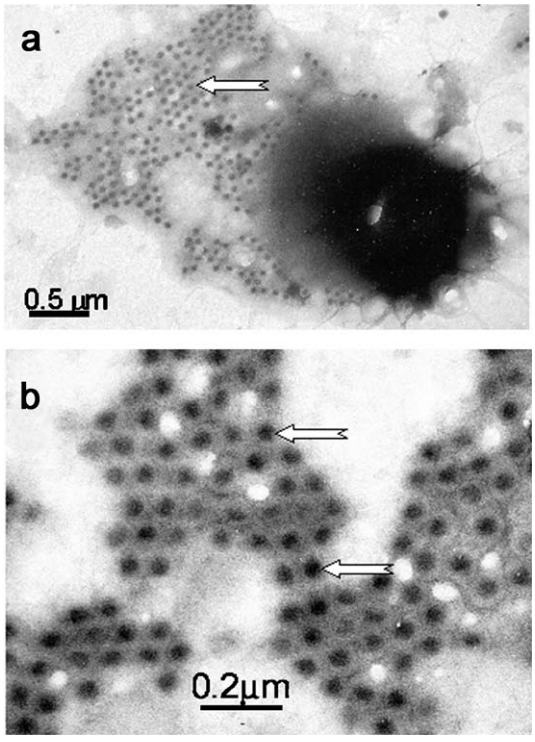

Fig. 1. Transmission electron micrographs of free viral particles of MpRNAV-01B infecting *M. pusilla*. The free viral particles in the culture lysate were pelleted by ultracentrifugation onto formvar grids and observed at ×25 000 magnification and an accelerating voltage of 60 kV. The arrows point at some of the released virus particles.

Transmission electron microscopy (TEM) preparations showed that the virus-like particles were nearly spherical, and approximately 65–80 nm in diameter (Figs. 1 and 2). We found only one type of particle, which did not appear to be enveloped. The viruses had a thick outer layer and a smaller electron-dense inner core (30–60 nm), and it seemed as if the capsid shell was composed of several layers (Fig. 2). The virus particles were in the cytoplasm of the algal host cells.

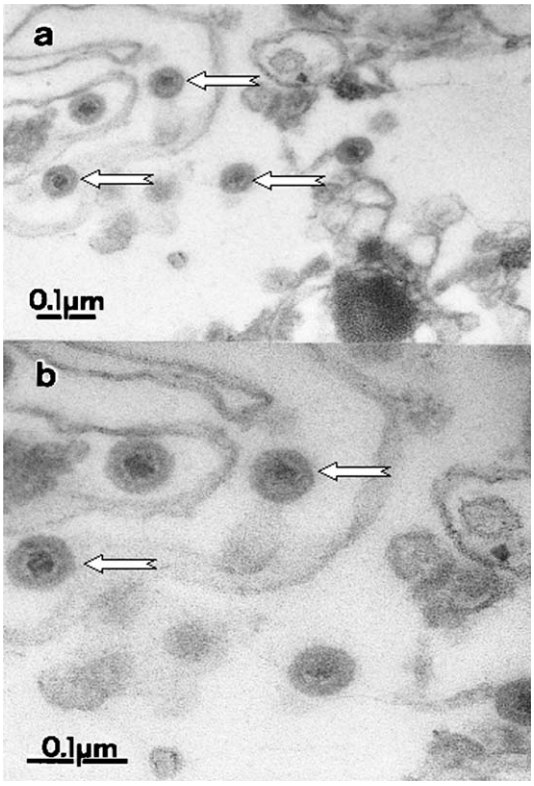

Fig. 2. Transmission electron microscope images of thin-sectioned *M. pusilla* cells infected with MpRNAV-01B. The arrows point at intracellular virus particles consisting of a thick outer layer and a smaller electron-dense inner core.

From the 10 viral clones originally isolated, only one was used for further study. It was designated MpRNAV01B: *M. pusilla* dsRNA virus, clone number 1, isolated in Bergen, Norway.

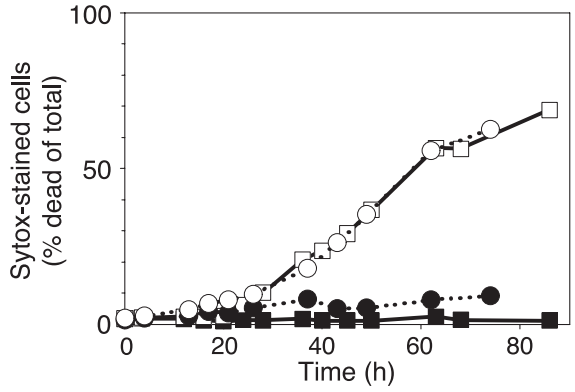

Fig. 4. The dynamics of the percentage of *M. pusilla* cells that stained fluorescent green with SYTOX Green. SYTOX Green stains cells with compromised cell membranes, defined as dead cells. Open symbols represent the infected cultures and the closed symbols the noninfected cultures. Squares stand for the undiluted experiment, and the circles for the diluted experiment (see legend Fig. 3).

Virus lytic cycle

To assure full lysis of the algal host in only one step, the lytic cycle of the virus was examined using a ratio virus to algal cell of 135 (Fig. 3). Determinations of the virus titer and the total virus and algal counts indicated that the ratio input virus to host cell should be >100 to ensure one-step viral growth curves (see Material and methods). To further support one-step experiments, parallel cultures were diluted 40-fold 4 h after infection. This way the collision rate between unadsorbed virus and algal host cells was reduced. Indeed, Fig. 3 shows evidence of one-step growth curves under both conditions (Burleson et al., 1992). The growth of *M. pusilla* was inhibited by the addition of virus within 24

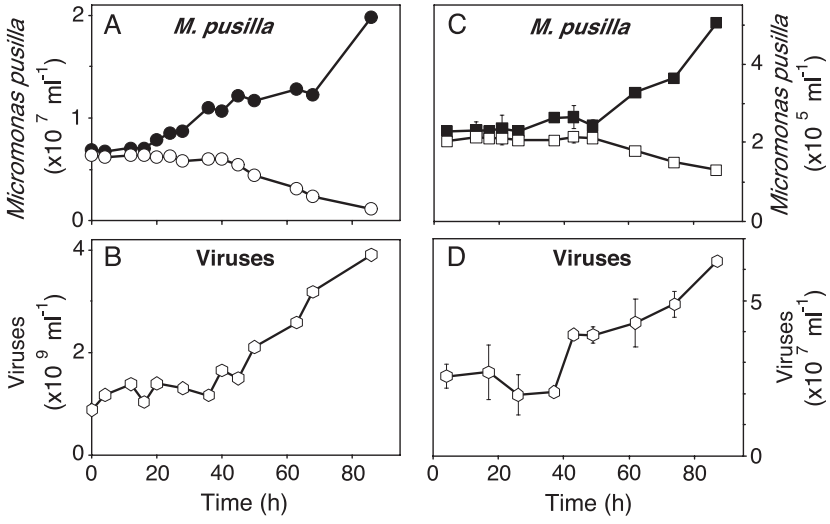

Fig. 3. Abundance of the algal host *M. pusilla* (A and C) and of free virus particles (B and D). Open symbols represent the viral infected cultures, and the closed symbols the noninfected control cultures. Exponentially growing cultures of *M. pusilla* were infected at a virus to host ratio of 135. In parallel, cultures were diluted 40-fold 4 h after adding viruses to promote one-step virus growth cycle by reducing the collision rate between unadsorbed viruses and algal host cells. Circles stand for the undiluted experiment (A and B), and squares for the diluted experiment (C and D, duplicate cultures). Error bars that are not visible are smaller than the symbol size.

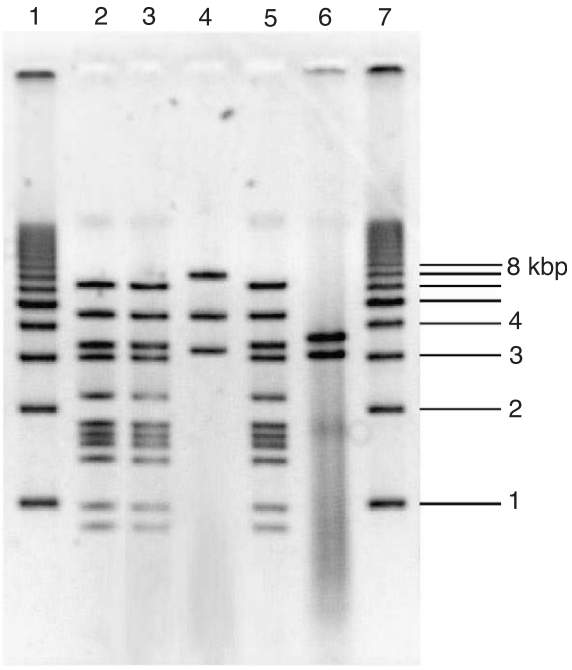

Fig. 5. Total nucleic acid patterns of MpRNAV-01B (lanes 2, 3, and 5). Molecular size standards: 1 kb dsDNA Molecular Ruler (lanes 1 and 7), dsRNA bacteriophage Phi-6 (lane 4; segments of 6374, 4074, 2948 bp), and dsRNA Infectious Bursal Disease Virus IBDV-V4 (lane 6; segments of 3260 and 2827 bp). Sizes of the 11 segments were estimated from several agarose gels and averaged against the two dsRNA markers: 800, 950, 1300, 1400, 1500, 1650, 2200, 2800, 3080, 4100, 5850 bp.

h (Fig. 3). A decline in algal cell numbers was observed 40 h after infection. The initial increase in free virus particles did not start till 36 h after infection. The increase in the percentage of algal cells with compromised cell membranes (fluorescently stained with the viability dye SYTOX Green, Fig. 4), by definition dead cells, occurred concomitantly with the increase of viruses released from the host cells. A steady increase in the percentage of dead cells was observed till the end of the experiment (>60% dead cells). The noninfected control cultures continued to grow over the entire course of the experiments and showed only very low percentages of dead cells. Based on the observed declines in algal cell abundance (assuming no algal growth) and the increases in virus particles from 40 h till the end of the experiment, the estimated burst size was 460–520 viruses per lysed algal cell. No significant differences in timing of lysis of the algal cells, nor latent period of the virus were found between the undiluted and the diluted cultures. The lytic cycle as well as the burst size were not affected by the light climate the alga was cultured at (continuous light or light–dark cycle of 14:10 h; data not shown).

Genome size and type

Electrophoretic separation of the nucleic acids purified from MpRNAV-01B revealed 11 distinct extra bands (Fig. 5). It is very unlikely, considering the stringent end-point dilution series we used to isolate virus clones, that this result was due to the presence of other viruses in our lysate. To rule this out, we checked nine virus clones, all showed identical banding pattern (data not shown). The segments could be divided into three size classes, with four large (>2.5 kb), five medium-sized (<2.5, >1 kb), and two small (<1 kb) segments. After correction of the fluorescence intensity of each segment for its size, the data suggested that each virion contained a single copy of the viral genome.

The segmented nature of the virus genome suggests that the genome type is RNA, which was confirmed by nuclease digestion tests (Fig. 6). When samples were digested with

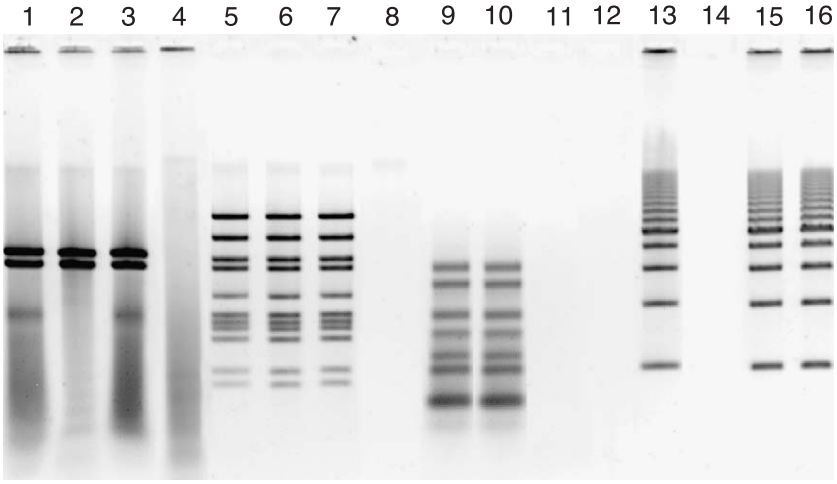

Fig. 6. Electrophoretograms of MpVRNAV-01B extracts after digestion with DNase, RNase under high ionic strength, and Rnase under low ionic strength. MpVRNAV-01B in lanes 5–8. The dsRNA Infectious Bursal Disease Virus IBDV-V4 (lanes 1–4), ssRNA Ladder (High Range Fermentas, lanes 9–12), and 1 kb dsDNA Molecular Ruler (lanes 13–16) were used as controls. The first lane of each series represents the nucleic acid extract pure (lanes 1, 5, 9, 13), the second lane contains the extract after digestion with DNase (lanes 2, 6, 10, 14), the third lane contains the extract after digestion with RNase under high ionic strength (lanes 3, 7, 11, 15), and the fourth lane contains the extract after digestion with RNase under low ionic strength (lanes 4, 8, 12, 16).

DNase or RNase (selectively digesting ssRNA) in high ionic strength (2× SSC) there was no effect on the bands, whereas dsDNA and ssRNA were digested by the respective enzyme treatments. Reducing the ionic strength of the RNase buffer (0.1× SSC) lowered the melting point, thus making the bands susceptible to treatment with RNase. This differential digestion indicated that the segments were dsRNA, which has never been reported for *M. pusilla*. The molecular weights corresponding to these bands ranged from 0.8 to 5.8 kb, determined by using dsRNA viruses of known size as molecular markers (Fig. 5). The total genome size of MpRNAV-01B was estimated to be about 25.5 kb.

Structural proteins

Two main bands were formed after isopycnic CsCl centrifugation, with densities of 1.36 and 1.37 g cm^{-3}. SDS-PAGE (Fig. 7) revealed that the number and size of the proteins was the same in both density fractions (not all bands may be visible in the gel shown), but the relative amount of proteins in the bands differed. In the heaviest fraction (1.37 g cm^{-3}), five major protein bands were detected of approximately 120, 95, 67, 53, and 32 kDa, and several minor and weak bands at about 210, 200, 150, 107, 88, 62, 60, 50, 40, 35, and 21 kDa. When normalizing the intensity of the major protein bands to the intensity of the 120 and 96 kDa bands in each lane, the amount of proteins in the 67-, 53-, and 32-kDa bands in the heaviest virus fraction (1.37 g cm^{-3}) were estimated to be, respectively, 4, 0.25, and 12 times the amount of protein in the corresponding bands in the lightest virus fraction (1.36 g cm^{-3}).

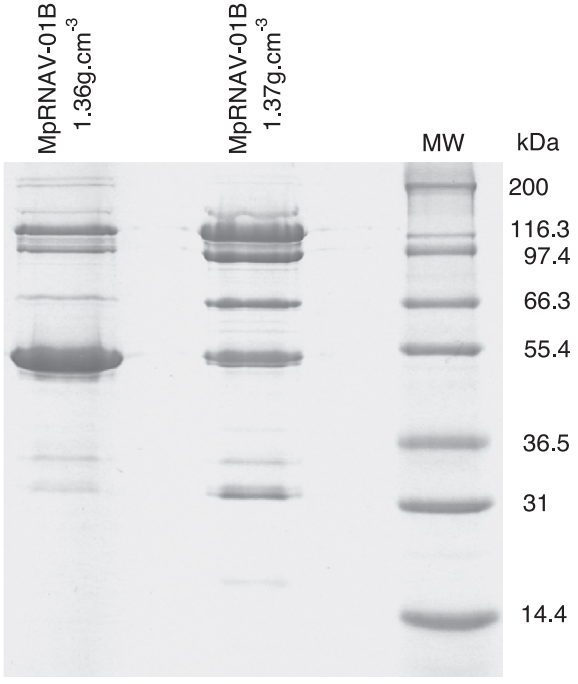

Fig. 7. SDS-PAGE of MpRNAV-01B proteins obtained from viral particles with density of 1.36 and 1.37 g cm^{-3} (determined by isopycnic cesium chloride centrifugation). Molecular weight marker (MW) is shown in the right lane (numbers are molecular size in kDa). The size of the five major proteins, 5, 107, 88, 62, 60, 50, 40, 35, and 21 kDa (not all bands may be visible in the gel shown).

Table 2
Sensitivity of MpRNAV-01B to a selection of treatments[a]

Treatment	Details[b]	Sensitivity[c]
Temperature (°C)	20	ns
	25	ns
	30	ns
	35	*
	40	***
	45–95	***
Freezing (°C)	−196	ns
	−80	ns
	−50	ns
	−20	ns
pH[d]	2	***
	2 readjusted	***
	5	***
	5 readjusted	***
	7	ns
	9	ns
	9 readjusted	ns
	10	ns
	10 readjusted	ns
Chloroform (%)	10 and 50	ns
Diethyl ether (%)	10 and 50	ns
Ethanol (%)	70	***
Acetone (%)	70	***
NP40 (%)	0.1–1	ns
Triton X-100 (%)	0.1–0.5	ns
	1	ps
Tween 80 (%)	1	ns
Sodium dodecyl sulfate (SDS) (%)	0.1–0.5	***
EDTA (mM)	0.1–100	ns
Dithiothreitol (DTT) (μM)	5–50	ns
L-cystine (μM)	5–50	ns

[a] Viral activity was assayed by a most probable numbers assay (MPN) using serial 10-fold dilutions (10–12) with five to eight replicates.
[b] Exposure temperature was set at 15 °C, except for the heating and freezing treatments.
[c] Sensitivity was classified as not sensitive (ns), 1–2 logs reduction in activity (*), and no activity remaining (***) when compared to the MPN value of untreated MpRNAV-01B (pH 8). Sensitivity was positively affected (ps) when infectivity was enhanced by at least 2 logs. The viral lysate was freshly made (15 °C) for each treatment. The deviance values were always smaller than the 1% significance level.
[d] Viral lysates were set to the specific pH and incubated for 1 h at 15 °C, after which MPN assays were performed for the adjusted lysate as well as for lysate for which the pH was readjusted to 8.

Sensitivity to physiochemical, chemical, and biochemical treatments

Heat inactivation of MpRNAV-01B was not evident at temperatures between 20 and 35 °C but heat treatment at temperatures between 40 and 95 °C for 10 min resulted in a complete inactivation of the virus (Table 2). Untreated lysates of this virus were still infective after storage for

more than a year in the dark at 4 °C. The virus was stable to freezing at −20, −50, −80, and −196 °C (liquid nitrogen) for 24 h (Table 2).

Transfer to darkness of the algal cultures to which viruses were added prevented viral replication (Table 2). Viral replication was restored when darkened cultures (up to 7 days) were set back into the light.

The virus was classified as sensitive to a treatment when a difference of at least 1 logarithm was observed in the activity of treated and untreated virus. In the case of MpRNAV-01B, loss of infection was detected following treatment with acidic pH (≤5), SDS, ethanol, and acetone (Table 2). No sensitivity was detected to treatment with chloroform or diethyl ether, Triton X-100, Tween 80 or NP40, EDTA, DTT or cystine (Table 2).

Discussion

Classification of virus isolate

So far, viruses that infect microalgae are typically large in particle size (>100 nm) as well as genome size (>300 kb), have a double-stranded DNA genome and belong to (or resemble) the virus family Phycodnaviridae (Bratbak et al., 1993; Cottrell and Suttle, 1991; Jacobsen et al., 1996; Mayer and Taylor, 1979; Nagasaki and Yamaguchi, 1997; Sandaa et al., 2001; Suttle and Chan, 1993; Van Etten et al., 1991). Only recently, two single-stranded RNA viruses infecting the harmful algal bloom species *H. akashiwo* and *H. circularisquama* have been isolated (Tai et al., 2003, and Tomaru, personal communication, respectively). To our knowledge, this is the first report of a photosynthetic protist being infected by a double-stranded RNA virus.

Viruses whose genomes contain a relatively high number of segments of dsRNA are classified into the family Reoviridae (Knipe and Howley, 2001). Reoviridae represent a group of viruses with 10–12 segmented linear dsRNA genomes, present in equimolar proportions, and with a total genome size between 19 and 32 kb (http://www.ncbi.nlm.nih.gov/ICTV/, and references therein). Each virion contains a single, full-length copy of the genome, and is not enveloped. There is only one type of particle. The nucleocapsids are 60–80 nm in diameter, consist of an inner core and several protein layers, and have a icosahedral symmetry, though they appear to be round. All these genomic and morphological characteristics agree with our findings for MpVRNAV-01B.

The MpRNA-01B virion contained five major structural proteins, ranging from 32 to 120 kDa and several additional minor polypeptides. The observations that MpRNAV-01B could be separated into subpopulations with different density (in cesium chloride) and that the protein composition in these populations were different resembles the rotaviruses (Estes, 1990). These viruses have an outer and an inner protein shell that may be absent in incompletely assembled particles or lost, giving the particles different density and protein composition. Purification of double-shelled rotavirus particles in gradients of cesium chloride has been reported to be difficult because of loss of the outer capsid (Estes, 1990). The MpRNAV system appears to be more complex however, as the lighter particles seem to have lost a significant portion of two main proteins (i.e. the 67 and 32 kDa) while the heavier particles seem to have lost a significant portion of one main protein (i.e. the 53 kDa). The alternative hypothesis that the lighter virus particles have lost two proteins and gained one, or that the heavier virus particles have lost one protein and gained two, is also an open possibility but the rationale for this is now elusive.

The number of segments is one of the factors that has been used to discriminate between the various genera of Reoviridae. So far, viruses with 11-segmented dsRNA genomes belong to the genus *Rotavirus* (Knipe and Howley, 2001) or the genus *Aquareovirus* (Winton et al., 1987). The rotaviruses have vertebrates (mammals and birds) as hosts, and are the major causes of severe dehydrating diarrhea in infants and young children worldwide (Glass et al., 1996). The hosts for aquareoviruses are aquatic vertebrates (fish) and invertebrates (shellfish). Other differences between the two groups relate to the wheel-like appearance of the virions (*Rotavirus* do and *Aquareovirus* do not have wheel-like virions), and the number of RNA species that are smaller than 2 kb (*Rotavirus* have five, while *Aquareovirus* have two). Typically, genomes of rotaviruses range between 16.5 and 21 kb; aquareoviruses have genomes of 22.5–23 kb. The newly isolated MpRNAV-01B is different from these two known genera of 11-segmented dsRNA viruses in the sense that its host is an eukaryotic microalga (protist), its genome size is somewhat larger (approximately 25.5 kb) and it has six RNA species smaller than 2 kb. Whether the presently newly isolated viral pathogen MpRNAV-01B represents a new genus of Reoviridae requires further research, for instance cross-hybridization studies.

It is likely that the photosynthetic protist *M. pusilla* is evolutionary older than the hosts of other 11-segmented reoviruses (Bhattacharya and Medlin, 1998; Cavalier-Smith, 2002; Doolittle, 1999). This makes the present virus–host model system very interesting and potentially important for phylogenetic analysis. Sequencing studies can be expected to reveal more insight in the origin of the genes of the 11-segmented Reoviridae.

The virus MpRNAV-01B was resistant to exposure to chloroform or ether, suggesting the lack of a lipid component in the structure of the virus. Rotaviruses and aquareoviruses are reported to be stable to treatment with chloroform and ether (Estes et al., 1979; Knipe and Howley, 2001; Rangel et al., 1999). MpRNAV-01B is resistant to exposure to nonionic detergents (infectivity even enhanced by Triton X-100 at 1% final concentration), but inactivated by ionic detergent SDS, as are the rotaviruses (Fields et al.,

1996). Also comparable to rotavirus, and the result of removal of the outer capsid (Fields et al., 1996), is the complete inactivation by ethanol. Acetone was found to have a comparable effect. The virus, furthermore, lost its infectivity after treatment at low pH (pH ≤ 5), but was resistant to reducing and chelating agents. Resistance to treatment with the calcium-chelating agent EDTA is contrary to the rotaviruses, loosing infectivity because of removal of the outer capsids by EDTA (Fields et al., 1996). MpRNAV-01B was readily inactivated at temperatures above 35 °C, and was stable to freeze–thawing. It is of ecological interest that growth of the algal host occurs within the temperature and pH ranges to which the virus was stable.

Ecological aspects

The host range test showed that MpRNAV-01B is highly species specific in its infectivity, as can be expected for viruses. The results also suggest stringent intraspecific host specificity, which seems to be a contradiction, given the cosmopolitan nature of this algal species (Cottrell and Suttle, 1991; Sahlsten, 1998). Strain specificity has been reported for the larger DNA viruses, belonging to the Phycodnaviridae, infecting *M. pusilla* (Cottrell and Suttle, 1991; 1995b; Mayer and Taylor, 1979; Sahlsten, 1998; Sandaa et al., 2001). The present study suggests that the observed strain specificity is related to the original geographical site of isolation of the algal host strains. This has important ecological implications because intraspecific host specificity may generate and maintain enhanced diversity in phytoplankton communities, as does interspecific host specificity. The earlier described DNA viruses infecting *M. pusilla* are geographically widespread and abundant (Cottrell and Suttle, 1991; Sahlsten, 1998). Whether this is also true for the presently reported dsRNA viruses remains to be seen.

The virus growth cycle was characterized by a latent period of 36 h, and a total lytic cycle of up to 80 h. This is longer than typically found for other viruses infecting phytoplankton species (between 7 and 19 h, Brussaard et al., 1999; Jacobsen et al., 1996; Nagasaki and Yamaguchi, 1997; Sandaa et al., 2001; Van Etten et al., 1991; Waters and Chan, 1982). Our data indicate that cell division does not occur after infection; this contrasts with the case for dsDNA virus infecting *M. pusilla* in which a gradual decline in rates of cell division and photosynthesis is found upon infection (Brussaard et al., 1999; Juneau et al., 2003; Waters and Chan, 1982). Darkness prevented lysis of the algal culture, indicating that viral replication requires active host photosynthesis. The release of virus was facilitated by drastic alterations in the plasma membrane permeability of the host cells as demonstrated by the strong increase in percentage of cells stained with the viability stain SYTOX Green. This dye only stains cells with compromised cell membranes, such as cells undergoing lysis.

The burst size was between 460 and 520 virus particles released per lysed algal cell, assuming no growth of the algal cells after the first signs of decline in algal cell number. Another way to estimate the burst size is by calculating the number of virus particles that a *M. pusilla* cell could contain (i.e. dividing volume of host cell by the volume of one virus particle). Assuming both algal and virus particles to be spheres, with the diameter of the virus particle to be 80 nm, the algal cell 1.5 μm in diameter, and 50% of the cell's volume typically occupied by viruses (based on TEM thin sectionings), we estimated a burst size of approximately 530 viruses per cell. This is in very good agreement with our experimental results. Compared to the DNA viruses infecting *M. pusilla*, the burst size is substantially larger (<100 viruses per cell, Brussaard et al., 1999; Waters and Chan, 1982). This can at least be partly explained by the larger particle size of the DNA viruses (100–140 nm, Cottrell and Suttle, 1991; Mayer and Taylor, 1979; Sahlsten, 1998).

The higher burst size of the dsRNA virus compared to dsDNA viruses infecting *M. pusilla* could theoretically explain the observed coexistence of MpRNAV-01B and a dsDNA virus with a latent period of only 12 h (unpublished results). However, the actual number of infectious MpRNAV-01B viruses released per *M. pusilla* cell is likely to be small due to the relatively low fraction of infectious viruses (approximately 1 out of 100). A high number of noninfectious particles can result from a high number of empty capsids as often found for rotaviruses and aquareoviruses (Van Regenmortel et al., 2000). An alternative explanation why MpRNAV-01B is not out-competed by the DNA virus might be that the adsorption rate of the dsRNA virus is higher than of the DNA virus and infection with other types of viruses is blocked by the dsRNA virus.

The fact that coexistence does occur and is stable over time suggests that dsRNA viruses might be of ecological importance for *M. pusilla* in the field. Moreover, the discovery of a dsRNA virus infecting a phytoplankter has important implications for our understanding of the role of viruses in the marine ecosystem, and their potential significance to mortality to marine eukaryotes. Clearly, investigators need to consider viruses other than Phycodnaviridae as pathogens of photosynthetic protists.

Material and methods

Algal cultures

The algal host *M. pusilla* (Butcher) Manton and Parke, was obtained from the Culture Collection of the Marine Research Center of Göteborg University (strain LAC 38). The algal culture was made axenic with a mixture of the antibiotics carbenicillin and cefotaxime (Sigma Chem. Co.,

Brussaard et al., 1999). The algal culture was checked for bacterial contamination using DAPI (adding 1:1 v/v of 0.2 μm pore size filtered 10 μg ml^{-1} stock solution, Sigma Chem. Co.) in combination with epifluorescence microscopy (Zeiss), and using transmission electron microscopy (Jeol 100 S, Bratbak and Heldal, 1993).

The algal cultures were grown in enriched artificial seawater (ESAW, Harrison et al., 1980) with the addition of Tris–HCl and Na_2SeO_3 according to Cottrell and Suttle (1991). The cultures were incubated at 15 °C under continuous white light from fluorescent tubes (50–100 μmol photons m^{-2} s^{-1}). The growth of the cultures was monitored via cell counting using flow cytometry (equipped with an air-cooled laser providing 15 mW at 488 nm and with the standard filter setup, Becton Dickinson FACSCalibur). Samples were diluted with 0.22-μm filtered culture medium, and the trigger was set on the red autofluorescence of chlorophyll (Brussaard et al., 1999).

For host specificity testing, unialgal cultures of representatives of different taxonomic groups (Table 1) were grown either in enriched ESAW or in enriched natural seawater medium f/2 (Guillard, 1975). Cultures were incubated at 8 or 15 °C under continuous light at 80 μmol photons m^{-2} s^{-1}.

Virus isolation

The sample the virus was isolated from was provided by Dr. E. Sahlsten, SMHI, Sweden, and originated from Norwegian coastal waters. Algal cultures were infected with 10% (v/v) of the original sample and incubated for 1 week, after which visual inspection for lysis followed (compared to noninfected control culture). Samples from the lysed cultures were filtered through 0.45 and 0.2 μm pore-size cellulose acetate filters (Schleicher and Schuell GmbH, Germany) and added to fresh exponentially growing algal cultures. Cultures showing lysis were repeatedly transferred to fresh algal cultures. The presence of viruses in the cultures that lysed was confirmed by transmission electron microscopy (TEM). To ensure that these viruses were not bacteriophages, fresh lysate was filtered through 0.1 μm pore-size filter and the filtrate was treated with antibiotics to eliminate any bacterial cells that might have passed through the filter. Continued presence of viruses was confirmed by TEM. Following this treatment, only one type of virus-like particle could be distinguished.

Viral clones were isolated by end-point dilution (12 tenfold dilution levels and up to 16 replicates) using exponentially growing culture of *M. pusilla* and fresh viral lysate (10% v/v). After 1 week incubation under conditions as described above, the most diluted lysed cultures were once more subjected to serial dilution. This procedure was repeated another three times but using 2-fold dilution steps (16 replicates for each dilution).

Host range

The host range for the virus was tested for 5 other *M. pusilla* strains and 16 different microalgal species (Table 1). A volume of 1 ml fresh lysate was added to each exponentially growing algal culture of 30–50 ml. Tests were performed in duplicate and during 14 days visually monitored comparing to noninfected control cultures of the different species. Non-lysed cultures were considered nonsusceptible for the lytic virus. Lysed cultures were inspected for virus proliferation using TEM (accelerating voltage 80 kV).

Electron microscopy

Samples for virus counting using transmission electron microscopy (TEM) were fixed with glutaraldehyde (25% EM grade, final concentration 2.5%) for several hours at 4 °C, after which the virus particles were harvested onto grids by cooled (8 °C) ultracentrifugation at 100 000 × *g* for 30 min (SW41 Beckman swing-out rotor). Grids were stained with uranyl acetate and analyzed on a Jeol 100 S TEM as described by (Bratbak and Heldal, 1993). For thin sectioning, samples were prefixed in 2.5% glutaraldehyde and concentrated by centrifugation (7000 × *g*, 30 min), resuspended and washed in 0.1 M cocodylate buffer (pH 7.3). After repeated centrifugation, the pellets were embedded in sodium alginate (1.5% w/v in cacodylate buffer) and gelled in 50 mM $CaCl_2$ (Tamponnet et al., 1988). Small pieces of alginate embedded material were postfixed in 2% osmium tetroxide in sodium cacodylate buffer for 1–2 h, dehydrated in a graded ethanol series until 96%. The material was embedded in LR White acrylic resin (Agar Scientific, Essex, UK) and sectioned with a diamond knife. The sections were post-stained with 2% uranyl acetate and lead citrate. Ultrathin sections were observed in an Jeol 100 S transmission electron microscope, operated at 60 kV accelerating voltage.

Virus growth cycle

Titer of the viruses was estimated before the virus growth experiments, using exponentially growing culture of *M. pusilla* and fresh viral lysate as a most probable number (MPN assay) with eight replicates and 10-fold dilutions (Cottrell and Suttle, 1991; Taylor, 1962). The cultures were incubated for 10 days during which the cultures were scored for lysis. The titer was determined with a BASIC program (Hurley and Roscoe, 1983). The deviance value was well below the 1% chi-squared significance level.

The virus growth cycle and the effect of viral infection on the algal host cells were studied during putative one-step growth curve experiments. The viral titer of infectious units at the start of the virus growth cycle experiments was 1.15×10^8 ml^{-1} (95% CI of 7.3×10^7 to 1.8×10^8

ml^{-1}). Estimates of the total count of viral particles (determined by TEM) for the same batch were 1×10^{10} ml^{-1}, thus 1 out of 100 viruses was infectious. To assure full lysis of the algal host in only one step, the multiplicity of infection (ratio of infectious viruses to algal host cells) should be at least 1 and consequently the ratio of input virus particles to algal host should be >100. Exponentially growing cultures (100 ml) of *M. pusilla* were infected with viruses at a ratio virus to host cell of approximately 135. In parallel, cultures were diluted 40-fold 4 h after adding viruses (virus to host ratio of 3). This way the collision rate between unadsorbed virus and algal host cell was reduced, further supporting one-step growth cycles. Noninfected algal cultures served as controls.

At regular time intervals (4–8 h) for the next 86 h, both the infected and the noninfected control cultures were sampled for algal and viral counts. Algal cells were counted using flow cytometry and viruses using TEM, as described above. Furthermore, a viability assay using SYTOX Green (Molecular Probes, Inc.) was performed on the algal cells according to Brussaard et al. (2001). The nucleic acid stain SYTOX Green easily penetrates cells with compromised plasma membranes but does not cross the membrane of live cells.

Genome type and size

Viruses were partially purified by removing cell debris and bacteria from fresh lysate using low-speed centrifugation in a swing-out centrifuge (Beckman J2-HS) at 7000 × *g* for 30 min at 4 °C. The supernatant was decanted and viral particles were subsequently concentrated by ultracentrifugation at 100 000 × *g* for 2 h at 8 °C (Beckman L8-M with SW28 rotor). The viral pellets were resuspended in 150 μl SM buffer [0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris–HCl, 0.005% (w/v) glycerin; Wommack et al., 1999]. Samples were either stored at −80 °C till use for RNA isolation.

For the isolation of viral nucleic acids, frozen virus concentrates were thawed in the presence of sodium dodecyl sulfate (1% final concentration) and then extracted with phenol/chloroform/isoamylalcohol (25:24:1), followed by treatment with chloroform. Nucleic acids were precipitated by adjusting the final aqueous phase to 0.3 M sodium chloride solution and addition of 3 volumes of ice-cold 95% ethanol, followed by incubation at and 20 °C overnight. After centrifugation (20 800 × *g* for 10 min at 4 °C), the pellet was washed with 70% of ethanol solution, dried and dissolved in water (PCR reagent, sterile and filtered, DNase and RNase nondetectable, Sigma-Aldrich Co).

Samples of virus extract were either loaded onto an agarose gel directly, or treated with various nucleases to identify the nature of the sample. These treatments were DNase RQ1 Rnase-Free DNase (Promega) and RNase ONE Ribonuclease (Promega) in 2× SSC and in 0.1× SSC medium (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). A recognized feature of dsRNA is its resistance to digestion by RNases under high salt conditions but not in lower ionic strength (Semancik et al., 1973). Electrophoretic fractionation of the samples was carried out in 0.7% (w/v) agarose gel (SeaKem LE agarose) in 1× TBE buffer at 75 V for 110 min. The gel was stained with ethidium bromide (1.3 μM final conc, Merck), destained for 20 min in Milli-Q (18.2 MΩ cm resistivity, Millipore Co.), and scanned with a BIO-RAD Fluor-S MultiImager. Size standards were a 1-kb Molecular Ruler (BIO-RAD EZ load, Z load), ssRNA Ladder (High Range, Fermentas), bacteriophage Phi-6, and Infectious Bursal Disease Virus IBDV-V4 (kindly provided by K. Wright and H. Boot, respectively; Boot et al., 1999; Mindich, 1988). As expected, the negative controls (extracts in nuclease buffer without the nucleases) gave identical banding patterns as the untreated extracts.

Proteins

Virus particles were harvested from lysate of 10 l *M. pusilla* culture by ultrafiltration (Vivaflow 200; 30 000 MWCO), after which the retentate was centrifugated at 4100 × *g* for 30 min at 4 °C to remove remaining bacteria and cell debris. The viruses in the supernatant were pelleted by centrifugation at 140 000 × *g* for 2 h at 8 °C (Kontron TFT55.38 rotor). The virus pellet was resuspended in 150 μl Tris (10 mM final concentration, pH 8). The resuspended pellets were pooled and stored at 4 °C till use (within 2 weeks). The viruses were purified by isopycnic centrifugation in CsCl with initial density 1.37 g cm^{-3}. The samples were centrifuged for 70 h at 150 000 × *g* and 7 °C in a Beckman SW41Ti rotor. The two main virus bands were removed with a sterile syringe, and the density was determined with a refractometer. The viruses were concentrated by centrifugation in a Sartorius Vivaspin concentrator (30 000 MWCO) and washed twice with PBS (pH 8) to remove the CsCl.

Purified virus particles were disintegrated by boiling for 5 min in SDS sample buffer and the proteins (3.5 μg protein per lane) were separated by SDS-PAGE (10% polyacrylamide gel) using a Mini Protean 3 Cell (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Protein molecular weight standards (Invitrogen, Mark 12, Carlsbad, CA, USA) were used for size calibration. The gel was fixed with 40% v/v ethanol and 10% v/v acetic acid and stained overnight with 0.1% Coomassie Blue, digitized and analyzed using a software program (Gel2K) designed by Svein Norland, Department of Microbiology, Bergen, Norway.

Stability against physiochemical treatments

Thermostability of the virus was tested at temperatures ranging from −196 (liquid nitrogen) to 95 °C. To test the

heat stability, viral lysate was dispensed in 1-ml aliquots into thin-walled glass tubes and placed in water baths maintained at 20–95 °C in steps of 5°. Tubes were incubated for 10 min followed by rapid chilling on ice. The degree of infectivity was determined using end-point dilution with five to eight replicates and 10–12 dilution levels (MPN assay mentioned above). Viral lysate at 15 °C was used as positive control, the temperature at which the host culture was kept.

The stability of the virus at low temperatures was tested by placing aliquots of lysate (0.5 ml) in cryovials at −20, −50 , −80, and −196 °C for 24 h. The usefulness of the cryoprotectants sucrose (10 and 20% w/v final concentration) and glycerol (10 and 20% v/v final concentration) was examined at −196 °C. Samples were thawed at 35 °C for a few minutes (<5 min) before immediate transfer to exponentially growing algal culture (10% v/v inoculate) as part of a MPN assay. As positive control, a nonfrozen sample was used. In additional, cryoprotectant glycerol or sucrose (both at final concentrations of 10% and 20%) was added to fresh virus lysate before deep freezing in liquid nitrogen.

The sensitivity of the virus to acidic or alkalic conditions was tested by adjusting the pH of 5-ml aliquots of viral lysate (pH 8) to pH 2, 5, 7, 9, and 10 by the addition of 0.1 N HCl or NaOH. The pH was not adjusted to pH levels greater than 10 because of flocculation. After 1 h incubation at 15 °C, the samples were inoculated to a fresh cultures of *M. pusilla* as part of a MPN assay. Furthermore, also samples that were readjusted to pH 8 by the addition of 0.1 N HCl or NaOH were analyzed. Medium aliquots that were treated in the same way as the samples were used as control.

To study the effect of darkness on the specific infectivity of the virus, 4-ml lysate aliquots were incubated in thin-walled glass tubes in the dark (rapped in Al foil). On days 1, 3, 5, and 7, tubes were unwrapped (new tubes each time) and measured immediately for loss in natural chlorophyll autofluorescence using a fluorometer (Turner Designs). Tubes infected with the virus that were kept in the light served as positive controls.

Sensitivity to chemical and biochemical agents

Organic solvents, detergent, reductants, and chelators were studied for their effect on the infectivity of the virus. Aliquots of 1 ml were used to test the sensitivity of the virus against treatment with organic solvents. For chloroform and diethyl ether, final concentrations of 10% and 50% (v/v) were used. Samples were vigorously shaken and incubated for 10 and 60 min at 15 °C. The chloroform was separated by centrifugation at 4100 × *g* for 5 min and the aqueous phase containing the viruses was recovered. The tubes were left overnight with the lids open at 4 °C to let any remaining chloroform evaporate. The diethyl ether was removed from the viral lysate by evaporation (overnight at 4 °C). The hydrophylic organic solvents ethanol and acetone were added to the cultures at a 70% v/v final concentration for 1 h at 15 °C. Acetone was evaporated overnight at 4 °C, whereas the ethanol-treated viruses were directly inoculated to algal cultures. The same specific procedures were completed for all solvents with equal volumes of medium and with nontreated viral lysates as controls. The virus samples were added to exponentially growing *M. pusilla* cells and 10-fold diluted (MPN assay) as described above.

Detergent sensitivity was examined using the neutral nonionic detergents Triton X-100, Nonidet P-40 (NP40), and Tween-80, as well as the anionic detergent sodium dodecyl sulfate (SDS) at final concentration (v/v) of 0.1%, 0.5%, and 1%. Virus aliquots (500 μl), containing the detergent of interest, were incubated for 1 h at 15 °C and subsequently inoculated to exponentially growing algal cultures for MPN assays. Similar treatments with medium served as controls.

To test whether the virus was sensitive to reducing agents, aliquots of viral lysate were treated with dithiothreitol (DTT, final concentration 5 μM–5 mM) and L-cystine (final concentration 5 μm–50 μM) for 1 h at 15 °C. These samples were used for MPN assays, using treated medium as controls.

Sensitivity to the chelating agent EDTA was tested for 0.1, 1, 10, and 100 mM final concentration in the same way as were the reducing agents.

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