

Seasonal abundance in Skagerrak-Kattegat coastal waters and host specificity of viruses infecting the marine photosynthetic flagellate *Micromonas pusilla*

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ABSTRACT: Seawater sampled in the Skagerrak and Kattegat coastal waters during the period October 1995 to September 1996 were screened for the occurrence of viruses lytic to marine microalgae. Viruses lytic to the photosynthetic marine picoflagellate *Micromonas pusilla* (Butcher) Manton & Parke (Prasinophyceae) were detected in all seawater samples screened. Evidence for viral lysis of any other of the 11 algal species tested was not obtained. Several viruses infecting different strains of *M. pusilla* were isolated. Ten isolated viruses which were tested for host specificity were found to be species specific to *M. pusilla* and even strain specific to 1–3 of the 6 strains of *M. pusilla* used in the experiment. In the Skagerrak and Kattegat the seasonal abundance of viruses infectious to a *M. pusilla* strain isolated from the Oslofjord, Norway, was at least 1 order of magnitude higher (average $2.5 \times 10^5 \text{ l}^{-1}$) than viruses infecting 2 *M. pusilla* strains isolated from Gulf of Maine, USA (average 2.2×10^4 and $4.6 \times 10^3 \text{ l}^{-1}$, respectively).

KEY WORDS: Virus · Microalga · Prasinophyceae · Strain specificity · Skagerrak · Kattegat

INTRODUCTION

Viral particles are found in high abundance in natural waters and they appear to be an active component of the marine ecosystem. Viruses infecting bacteria, bacteriophages, are the most thoroughly studied of the marine viruses (e.g. Børsheim 1993, Bratbak et al. 1994, Suttle 1994). The impact and abundance of viruses which infect marine microalgae were not recognised until recent years. Indications of virus infection in microalgae have been obtained with transmission electron microscopy (TEM) preparations of planktonic material collected from seawater (Proctor & Fuhrman 1991, Nagasaki et al. 1994). The electron micrographs showed heavily stained virus-like particles (VLPs) inside the cells of several groups of marine microalgae and bacteria. Algal viruses may be impor-

tant factors in algal communities, regulating nutrient cycling and influencing algal species succession in the pelagic system (e.g. Fuhrman & Suttle 1993). Experimental studies, increasing the number of viruses in seawater samples by adding natural viral concentrates in the size range 2 to 200 nm, have strengthened the conclusions that marine viruses may have an impact on the phytoplankton population and may inhibit their photosynthesis (Suttle et al. 1990, Suttle 1992).

Despite the fact that 2 species-specific viruses have been detected in samples containing the 2 bloom-forming haptophytes *Phaeocystis pouchetii* (Jacobsen et al. 1996) and *Emiliania huxleyi* (Bratbak et al. 1996) in Norwegian coastal waters, still not much is known about the impact of marine viruses on algal blooms.

Availability of experimental host-virus systems is the key to learning more about the ecological significance of algal viruses. Viruses have been found in algal cells from all major taxonomic classes but only a few have been brought into culture. Viruses infective to the pra-

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sinophyte *Micromonas pusilla* (Butcher) Manton & Parke (Mayer & Taylor 1979, Cottrell & Suttle 1991) (MpV) were some of the first marine viruses isolated, but still not much information has been collected on factors regulating the infection and replication process of the virus in the alga.

Species specificity of algal viruses may be a mechanism to maintain diversity in phytoplankton communities (Cottrell & Suttle 1991). The host range and specificity to different host strains may vary between different viral strains as has been found for *Synechococcus* phages (Suttle & Chan 1993, Waterbury & Valois 1993). Waterbury & Valois (1993) concluded that the acquisition and maintenance of resistance by *Synechococcus* spp. to their co-occurring phages permit these cyanobacteria to coexist stably with a diverse group of cyanophages that can be present at high titers in seawater.

The aim of the present study was to initiate investigations in Swedish coastal waters on the occurrence and dynamics of viruses that infect marine microalgae. The questions asked were: What species of microalgae are infected? To what degree are the viruses host-specific? Are there any seasonal dynamics? The aim was also to obtain viral isolates suitable for experimental studies in the laboratory on the host-virus system of a marine microalga and its specific lytic virus.

MATERIALS AND METHODS

Concentrating viruses from seawater. Large volumes (20 to 60 l) of surface seawater were collected from the Kattegat and Skagerrak North Atlantic coastal waters by 30 l Niskin bottles and kept dark at 4°C in polyethylene carboys. The water was pre-screened by pressure filtration through two 142 mm diameter filters (a GF/C glass fibre filter [Whatman] followed by a 0.45 µm pore size Durapore filter [Millipore]). The filtrate was concentrated approximately 100- to 300-fold by ultrafiltration with a 30 000 MW cut-off ultrafilter (Amicon Spiral Cartridge Model S1Y30) according to Suttle et al. (1991).

Algal cultures. Unialgal cultures of 12 marine microalgae, representing species common in the Kattegat-Skagerrak area and belonging to the taxonomic groups Prasinophyceae, Bacillariophyceae, Dinophyceae, Prymnesiophyceae, Cryptophyceae, and Chlorophyceae, were grown in artificial seawater (Harrison et al. 1980) enriched with f/2 nutrients (Guillard & Ryther 1962, Guillard 1975) in the laboratory using artificial light ('daylight fluorescent tubes', 80 µE s⁻¹ m⁻²) with a 12:12 h light:dark cycle. The growth medium for the 6 strains of the flagellate *Micromonas pusilla* (Prasinophyceae) was supplemented with 5 mM tris-HCl (pH =

7.7) and 10 nM Na₂SeO₃ according to Cottrell & Suttle (1991).

Screening tests for viral infection and lysis of algae.

The algae were grown in 10 ml polypropylene screw-capped glass tubes. The growth of the cultures was monitored by measuring the *in vivo* chlorophyll *a* fluorescence with a Turner Model 111 fluorometer equipped with a blue (Corning 560) primary filter and a red (Corning 2-64) secondary filter. The culture tubes fitted the cuvette holder of the fluorometer and measurements could thus be made directly without subsampling or removing the culture from the tubes. When the algal cultures reached exponential growth phase, an inoculate of 1 ml seawater concentrate was added to 5 parallel tubes, keeping 5 additional tubes as control. Virus infection indicated by lysis of the algae was seen as a drastic drop in fluorescence to near zero, while the fluorescence of the control cultures continued to stay high.

Abundance of infectious viruses in seawater. The number of infectious viruses in the seawater concentrates were tested on the 3 *Micromonas pusilla* strains CCMP490, CCMP494, and LAC38 (Table 1). An inoculum of 60 µl seawater concentrate was added to microtitre plates and mixed with 250 µl exponentially growing algal culture in 10-fold dilutions (Suttle 1993). The microtitre plates were inoculated in 10 parallel wells for each series of 10-fold dilutions up to 10⁴-fold dilution. The microtitre plates were incubated at 20°C under approximately 5 µE s⁻¹ m⁻² fluorescent light for about 4 wk and lysis was monitored every second day by measuring the *in vivo* chlorophyll *a* fluorescence (Cytofluor™ 2300 Fluorescent Measurement System, Millipore, excitation filter 460/40, emission filter 660/40). The number of infective particles, i.e. lytic viral particles, per ml was calculated by the MPN method using a BASIC program (Hurley &

Table 1. *Micromonas pusilla* strains used in the present study

<i>Micromonas pusilla</i> strain	Isolated from
CCMP ^a 490	Woods Hole, MA, USA (seawater tank debris)
CCMP485	Boothbay Hbr, Gulf of Maine, USA
CCMP494	Gulf of Maine, USA
CCMP1545	The English Channel
CCMP491	The English Channel (reclone of CCMP 1545)
LAC ^b 38	Oslofjord, Norway

^a*M. pusilla* strains named CCMP- were obtained from the Center for Culture of Marine Phytoplankton (CCMP) at Bigelow Laboratory for Oceanic Sciences
^bAlgal strain called LAC- was obtained from the Culture Collection of The Marine Research Center of Göteborg University

Roscoe 1983). An estimate of *in situ* abundance was made by assuming approximate 100-fold and 300-fold concentration factors from 20 and 60 l of seawater, respectively.

Virus isolation. Viruses were isolated from the Skagerrak and Kattegat by repeatedly transferring an inoculum from a lysed batch culture of *Micromonas pusilla* to an exponentially growing culture of the same strain at least 5 times. The resulting lysate was stored at 4°C and used as the stock virus sample. Observations by TEM of viruses collected on electron microscope grids and negatively stained (Bratbak et al. 1990) showed the virus particles (of the viral isolate MpVUF8-490) to be morphologically homogenous and hexagonally shaped. The nature of infection of 8 of the MpV isolates which were used in the present study was investigated, either by filtering through sterile 0.1 µm pore size membrane filter (Millex-VV, Millipore), or autoclaving (120°C, 20 min), before inoculating to *M. pusilla* host cultures in 10 replicate wells of microtitre plates and incubated as above. To examine bacterial growth inoculates of 100 µl were spread onto agar plates composed of algal medium enriched with organic substrates (per 1000 ml algal medium 1.0 g peptone, 0.5 g yeast extract, 0.5 g glucose, 0.5 g soluble starch, 0.01 g FeSO₄ · 7 H₂O, and 0.01 g Na₂HPO₄ were added) and 1.5% agar.

Host specificity. The host specificity for lytic infection was studied for 8 MpV isolates, originating from the Kattegat and Skagerrak. Species specificity was examined for the 12 microalgal species *Micromonas pusilla*, *Tetraselmis* sp., *Skeletonema costatum*, *Ditylum brightwellii*, *Thalassiosira weissflogii*, *Chaeto-*

ceros sp., *Phaeodactylum tricornutum*, *Prorocentrum minimum*, *Chrysochromulina polylepis*, *Prymnesium parvum*, *Rhodomonas* sp. and *Dunaliella tertiolecta*. Strain specificity was tested for 6 strains of *M. pusilla* (Table 1). In each experiment 50 µl of 0.2 µm filtered lysate from a MpV infected and lysed host culture of *M. pusilla* was inoculated to 300 µl algal culture in 10 parallel microwells, keeping 10 parallel controls. The microtitre plates were incubated and growth and lysis of the algal cells were monitored as above.

RESULTS

All of the seawater concentrates tested resulted in lysis of at least 1 strain of the prasinophycean flagellate *Micromonas pusilla*, but no other microalgal species were lysed.

Eight *Micromonas pusilla*-virus strains were isolated and found to be species specific to *M. pusilla* as no other algal species tested were visibly infected and lysed. In addition to the species specificity there was a pronounced strain specificity for lytic infection of 1 to 3 strains of the 6 *M. pusilla* strains tested (Table 2).

Seasonal dynamics in occurrence of MpV

The highest numbers of lytic agents against *Micromonas pusilla* were found during spring (Fig. 1a), although no significant seasonal dynamics could be drawn from the present data. In all samples examined there was a clear dominance of viruses infecting the *M. pusilla* strain LAC38, on average 2.5×10^5 viruses l⁻¹ (range 3.1×10^3 to 1.0×10^6 l⁻¹), with the highest abundance found in a surface water sample from the central Kattegat (station Fladen) in May 1996. Average abundance estimates of viruses infectious to *M. pusilla* strains CCMP494 and CCMP490 were respectively 2.2×10^4 l⁻¹ (range 8.2×10^1 to 5.6×10^4 l⁻¹) and 4.6×10^3 l⁻¹ (range 1.1×10^2 to 1.6×10^4 l⁻¹), i.e. 1 to 2 orders of magnitude less abundant than those infectious to strain LAC38.

MpV isolates

All of the 0.1 µm filtered lysates were infective and the respective *Micromonas pusilla* host strain cultures were generally lysed within a week after the inoculation. Bacterial growth on agar was not detected in any of the 0.1 µm filtrates. The results indicate that the infecting

Table 2. Strain specificity of *Micromonas pusilla* viral isolates from the Kattegat and Skagerrak, investigated with inoculates of viral isolates filtered through 0.2 µm filters. The first number in the name of the viral isolate refers to the seawater sample from which the virus was isolated and the last number refers to the *M. pusilla* host strain, in which the virus was first amplified. +: lysis, i.e. fluorescence decreased to less than 5% of control; (+): no clear lysis, i.e. fluorescence decreased to 5–50% of control; -: no lysis, i.e. no decrease/fluorescence higher than 50% of control

Viral isolate	<i>Micromonas pusilla</i> strain					
	LAC 38	CCMP 1545	CCMP 490	CCMP 485	CCMP 494	CCMP 491
MpVUF8-490	-	+	+	-	-	+
MpVUF8-1545	-	+	+	-	-	+
MpVUF9-38	+	-	-	+	-	-
MpVUF10-38	+	-	-	+	-	-
MpVUF11-38	+	(+)	-	+	-	-
MpVUF12-38	+	-	-	(+)	-	-
MpVUF15-490	-	+	+	(+)	-	+
MpVUF16-490	-	-	+	+	-	-

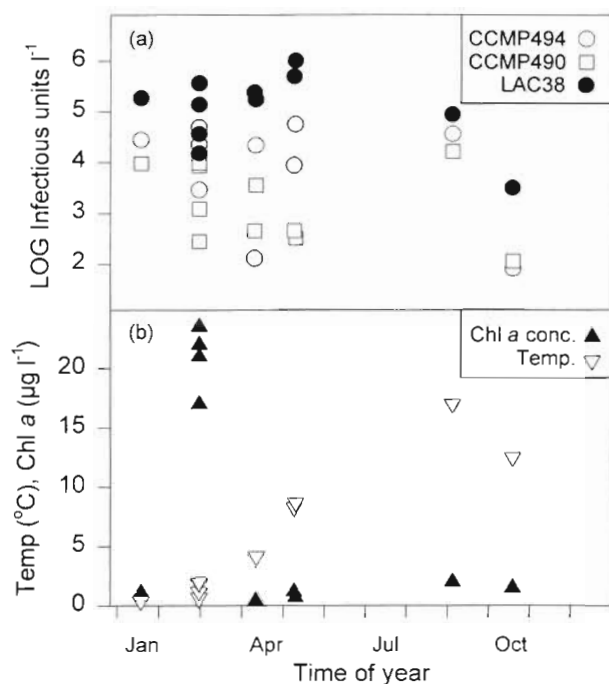


Fig. 1. Surface seawater samples from the Skagerrak and Kattegat. (a) Estimated number of infective lytic viruses, lysing the 3 different *Micromonas pusilla* strains CCMP494, CCMP490, and LAC38. (b) Ambient chlorophyll *a* concentration ($\mu\text{g l}^{-1}$) and temperature ($^{\circ}\text{C}$)

agents were not bacteria, but rather viruses small enough to pass the filter. The infectivity of the viruses was found to be destroyed by autoclaving as the autoclaved lysates did not lyse the algal cells. Preliminary studies of the negatively stained viruses (strain MpVUF8-490) in TEM showed homogenous hexagonal viruses of a size roughly estimated as 100 to 140 nm, which indicates that these viruses may be similar to other isolates of *Micromonas*-infecting viruses (Cottrell & Suttle 1991).

DISCUSSION

Viral nature of lytic agent

The filtration test of MpV isolates indicated that the lytic agents were viral particles. In the host-specificity test, the algal cultures were inoculated with viral lysates filtered through 0.2 μm membrane filters. It is known (e.g. Stockner et al. 1990) that 0.2 μm membrane filters contain some holes that are larger than the manufacturers' stated nominal pore size. This means that some bacteria may have passed through the filter and it cannot be excluded that the inoculate may have contained some bacteria in addition to the

viral particles. However, as all the original *Micromonas pusilla* host strains were lysed by 0.1 μm filtered inoculates, it is most likely that lysis of the algae in all of the experiments were due to viruses specific to *M. pusilla* (MpV).

Species and strain specificity of MpV

It is worth noting that the only alga which was clearly lysed by an inoculate of the seawater concentrates in this study was the picoflagellate *Micromonas pusilla*, and none of the other 11 algal species tested was clearly lysed. All the seawater samples which were screened for viruses were shown to contain agents lytic to *M. pusilla*. This sensitivity to virus infection found in *M. pusilla*, alternatively dominance of MpV among algal viruses, had already been noted by Mayer & Taylor (1979) when testing their isolated viral particles on 46 planktonic algal isolates representing 35 species from 5 classes, which resulted in *M. pusilla* being the only alga lysed. However, Suttle et al. (1990) were successful in isolating several other lytic pathogens of phytoplankton of diverse taxonomy. One possible reason for the success in finding viruses lytic to *M. pusilla* may be that this prasinophyte is ubiquitous in almost all marine waters with a very good adaptability to changing temperature and salinity (Thronsen 1976, Thronsen & Kristiansen 1991), and we have found it to be relatively easily kept in culture at the laboratory.

This is the first time that strain specificity has been reported in MpV. The data (Table 2) indicate some relationships between e.g. strains CCMP1545, CCMP490 and CCMP491 and between LAC38 and CCMP485, as several isolated viruses are lytic to the members in either of these 2 groups. The total agreement between CCMP1545 and CCMP491 strengthens the indications of strain specificity as these 2 cultures originate from the same strain, isolated in the English Channel. CCMP491 is a reclone made by C. Suttle 1992, from this strain. Although 5 of the 6 *Micromonas pusilla* strains used in these experiments (Table 1) were isolated far away from the Kattegat and Skagerrak, all of the strains were lysed by at least some of the viruses in our samples. At this moment we do not have any information about the genetic differences between the isolated viruses or between the algal strains used in the experiments. Some of the isolated viruses may be identical, e.g. the 2 viruses, MpVUF8-490 and MpVUF8-1545, which were shown to have the same strain specificity and isolated from the same surface seawater sample at station Fladen, Kattegat in October 1995 on the 2 *M. pusilla* strains CCMP490 and CCMP1545, respectively (Table 2). It is thus possible

that these could either be genetically identical or 2 separate viruses having the same host specificity. The specificity of viruses to different strains of *M. pusilla* has important ecological implications and is crucial when designing screening tests for viruses present in natural systems. The discrepancy in infectivity of viruses to the 3 tested strains of *M. pusilla* (Fig. 1a) indicated that a relatively local *M. pusilla* strain (LAC38 from the Oslofjord, Norway), was more suitable as a host to viruses collected in the Kattegat-Skagerrak area than algal strains isolated from the east coast of the Atlantic Ocean (CCMP494 and CCMP490 from coastal waters off Maine, USA). The abundance of MpV infective to *M. pusilla* strain LAC38 in spring 1996 in the Skagerrak and Kattegat (Fig. 1a) was in the same size range as the estimate of maximum abundance of *M. pusilla* algal cells (1×10^6 cells l^{-1}) at a Skagerrak coastal station in May 1993 (Karlson et al. 1996). Suttle & Chan (1994) even found the concentration of viruses to exceed that of their host cells, the cyanobacteria *Synechococcus*, by as much as 6 to 8 times. There may be an ecological parallel between MpV and cyanophages, as cyanophages also have variable host ranges and have been found in highest concentrations when the host abundance was greatest (Suttle & Chan 1994).

Unfortunately the abundance of *Micromonas pusilla* algal cells was not quantified in the present samples. Other studies in Skagerrak coastal waters (Kuylenstierna & Karlson 1994, Karlson et al. 1996), however, have shown *M. pusilla* to be most abundant in spring, i.e. at the same time as the highest numbers of infectious viruses (10^5 to 10^6 l^{-1}) was detected in this study (Fig. 1a, b). There was no correlation between total chlorophyll *a* concentration and abundance of lytic MpV (infectious units) which can be explained by the fact that the picoplankton *M. pusilla* only accounts for a smaller portion of the total phytoplankton biomass, and the high chlorophyll *a* concentrations in March (Fig. 1b) were due to a spring bloom dominated by diatoms.

Isolates of lytic algal viruses

The method used in this work for isolating algal viruses is only selective for any lytic virus which infects an alga that is kept in culture in the laboratory. This paper is focussed on lytic viruses, and any existence of latent infection has not been investigated. However, it cannot be excluded that the negative results in detecting lytic infection in the algal species other than *Micromonas pusilla* could have been because these algae may already have been latent infected by viruses, which may have made the algae immune to further

viral infection. Latent infection in microalgae has not yet been clearly proven, however.

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