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# Diversity in morphology, infectivity, molecular characteristics and induced host resistance between two viruses infecting *Micromonas pusilla*

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ABSTRACT: Two viruses infecting *Micromonas pusilla* (Prasinophyceae) were isolated from the Gulf of Naples (Mediterranean Sea). MpVN1 and MpVN2 had comparable sizes (100 to 130 nm) and a typical icosahedral shape. However, MpVN1 attached to the host with a tail of about 0.2 µm, which was absent in MpVN2. Infectivity varied between the 2 viruses tested on 11 *M. pusilla* isolates, with no relationships to the geographic provenance of the host strains, nor to their phylogenetic diversity. MpVN2 DNA was amplified using the AVS1/2 primers that are specific for the *pol* gene in the family *Phycodnaviridae*, whereas MpVN1 DNA was only amplified using specifically designed primers, which were found not to amplify MpVN2 DNA. AVS1/2-amplifiable viruses were present in ca. ⅓ of 33 infective *M. pusilla* virus samples collected over the spring of 1996, whereas the primers designed for MpVN1 DNA were effective only in 1 case. After the infection, host cultures recovered and became resistant to infection from the same virus, but MpVN1-resistant cultures were still susceptible to infection from MpVN2 and from natural virus populations. Immunity to viral infection was apparently not associated with chronic infection (pseudolysogeny) or with viral latency. It is proposed that acquired immunity could partially account for the intraspecific variability in susceptibility to viral infection and have important implications for the outcomes of host–virus interactions at sea.

KEY WORDS: Algal virus · *Micromonas pusilla* · Ultrastructure · Host range · Resistance



## INTRODUCTION

The number of marine phytoplankton species infected by viruses has been slowly but constantly growing since the first study of virus-like particles in a eukaryotic microalga (Pearson & Norris 1974). Over the last 10 yr, a dozen species have been added to the list, which initially comprised ca. 20 species (Van Etten et al. 1991, Zingone 1995), and now also includes a number of dinoflagellates (Tarutani et al. 2001, Onji et al. 2003, Tomaru et al. 2004a) and diatoms (Nagasaki et al. 2004a, 2005b, Bettarel et al. 2005). Ever since the discovery of viral infection in planktonic microalgae, studies have queried whether and to what extent these viruses are responsible for the demise of phytoplankton blooms in the sea (Brussaard 2004). Studies aimed at quantifying virus and host populations and viral infection rates in the natural environment in some cases have demonstrated that viruses can cause significant mortality in their host species populations (up to 88% infected cells in Nagasaki et al. 2004b) and eventually lead to the end of the bloom.

On the other hand, evidence has grown that host and viruses can coexist in the same environment. The existence of marine microalgae resistant to viral infection was first hypothesised for *Synechococcus*, to account for the contemporary presence and abundance of both host and phages in marine waters (Waterbury & Valois 1993). Subsequently, differences in sensitivity to viral infection were shown even within a single species, including the raphidophyte *Heterosigma akashiwo* (Nagasaki & Yamaguchi 1997, Lawrence et al. 2001),

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the dinoflagellate *Heterocapsa circularisquama* (Tarutani et al. 2001), the prasinophyte *Micromonas pusilla* (Sahlsten 1998, Zingone et al. 1999) and the diatom *Rhizosolenia setigera* (Nagasaki et al. 2004a). There is also growing evidence of remarkable diversity in viral particles infecting a given species, spanning from genotypic and phenotypic diversity within double strand (ds) DNA viruses (Cottrell & Suttle 1991, 1995b, Tomaru et al. 2004b) to single strand (ss) and dsRNA viruses (Tai et al. 2003, Brussaard et al. 2004, Tomaru et al. 2004a). The dynamics of distinct viral types interacting with hosts of different susceptibility may produce quite complex patterns in terms of host mortality and clonal composition during a bloom, as recently shown for *H. akashiwo* (Tomaru et al. 2004b).

*Micromonas pusilla* (Prasinophyceae) is an important and worldwide-distributed component of the picoeukaryotic phytoplankton (Throndsen & Zingone 1994, Not et al. 2004). An investigation conducted in the Gulf of Naples (GON, Mediterranean Sea) showed that temporal patterns of virus and host abundances were often unrelated, and that *M. pusilla* could, at times, survive despite relatively high virus concentrations (Zingone et al. 1999). In addition, the algicidal activity of natural virus populations varied among different host strains, in agreement with what has been reported for *M. pusilla* from other areas (Sahlsten 1998). To explore the nature and the extent of intraspecific diversity of viral infection, 2 viral strains were isolated and their morphology, molecular characteristics and host range were investigated using a number of different *M. pusilla* strains as hosts. This research also addressed the recovery and subsequent resistance that were generally observed after infection in host cultures, and included a molecular survey on *M. pusilla* viruses from natural samples.

Table 1. *Micromonas pusilla* strains used in this study. GON: Gulf of Naples

| Algal strain | Isolation date | Isolation place |
|---|---|---|
| Mp1 (CCMP1646) | 8 Apr 1993 | Stn MareChiara (MC), GON, Mediterranean Sea |
| Mp1res[a] | " | " |
| Mp2 | 24 Nov 1993 | Stn MC, GON, Mediterranean Sea |
| MpCO | 21 Nov 1996 | Stn Castel dell'Ovo, GON, Mediterranean Sea |
| MpPart | 8 Jan 1997 | Stn Partenope, GON, Mediterranean Sea |
| MpP7/1 (CCMP1723) | 15 May 1992 | Strait of Sicily, Mediterranean Sea |
| CCMP485 | 9 Sep 1989 | Boothbay Harbour, NW Atlantic Ocean |
| CCMP489 | 1 Nov 1988 | 28° 59′ N, 64° 22′ W, Sargasso Sea |
| MpNL | 1993 | The Netherlands, North Sea |
| MpUK (CCMP1545) | 1950 | English Channel, Plymouth, UK |
| Mp2/89 | Unknown | Unknown |
| MpOf | Unknown | Oslofjord, North Sea |

[a]Mp1 subculture resistant to MpVN1

## MATERIALS AND METHODS

**Host strains.** Eleven *Micromonas pusilla* strains were used as hosts in the infection experiments (Table 1). The Mediterranean strains were isolated by serial dilutions, and MpP7/1 and Mp1 were deposited at the Center for Culture of Marine Phytoplankton (CCMP) (Table 1). Strains from other areas were obtained from CCMP and from culture collections of various research institutions. The culture Mp1res was obtained by subculturing the strain Mp1 that had recovered after infection from MpVN1. Cultures were maintained at a salinity of 36 PSU in K-Si medium, at 18 to 22°C. Irradiance was 100 µmol quanta $m^{-2}$ $s^{-1}$ emitted from Osram cool white fluorescent tubes (Type L36W/20) in a 12:12 h light:dark cycle.

**Virus isolation and field studies.** Viral material analysed in this study was obtained during a series of extinction dilution experiments (Cottrell & Suttle 1991, 1995b) conducted in November 1993 to 1994 and in spring 1996 to 1998 in the GON. Detailed methods are provided in Zingone et al. (1999).

For MpVN1 isolation, a sample collected from GON surface waters in November 1994 was filtered on HT Tuffryn low-protein-binding polysulphonate membrane (0.22 µm pore-size) and incubated in exponentially growing Mp1 cultures in a 5-step series of 10-fold dilution steps, from 10 to $10^{-3}$ ml, at the same temperature and light conditions as indicated above. Cell lysis was recorded as a decline in fluorescence using a Turner fluorometer. The algal lysate was filtered on a 0.22 µm filter and then diluted in an 11-step series of 10-fold dilution steps. Aliquots (100 µl) of each dilution were added to 8 wells of a 96-well microtitre plate, each well containing 200 µl of exponentially growing Mp1 culture (Cottrell & Suttle 1995b). The microtitre plate was incubated as described for host strains and monitored for discolouration for 14 d. One of the wells in the most diluted series showing lysis, which in no case coincided with the last dilution step, was propagated in a tube containing Mp1 culture. After lysis, the content of the tube was filtered and re-incubated in a microtitre plate following the same procedure described above. The amplified virus sample obtained was filtered and stored at 4°C in the dark.

In March to April 1998, the resistant strain Mp1res was used along with Mp1 in extinction dilution experiments aimed at detecting viral strains with infective characteristics different from MpVN1. Seawater samples (0.22 µm filtered) were diluted with K medium in

a 5-step series of 10-fold dilution steps (from $10^{-1}$ to $10^{-5}$ ml), and 100 µl of each dilution was added to 2 replicates of 8 wells of a 96-well plate, each well containing 200 µl of exponentially growing Mp1 or Mp1res cultures as described in Zingone et al. (1999). Viral concentration estimates were obtained as most probable number from the number of discoloured cultures at the different dilution steps. A lysate obtained on 12 May 1998 was used to isolate MpVN2, which was made clonal by propagation on Mp1 cultures as described for MpVN1.

From 22 February to 2 July 1996, during weekly extinction dilution experiments with GON natural samples using Mp1 and P7/1 as hosts (Zingone et al. 1999), 1 lysate of the most diluted step was re-incubated with the corresponding host culture. The lysates were filtered and stored at 4°C in the dark. The 33 virus samples obtained were re-amplified several times through new infections of the corresponding host strains and then used in PCR experiments.

**Infection experiments.** In order to determine the intraspecific host specificity of MpVN1 and MpVN2, *Micromonas pusilla* strains were inoculated with the 2 viruses and monitored for lysis. Aliquots of 0.5 to 1 ml of viral stock were added to 13 ml of exponentially growing cultures of the host (in duplicate) at a ratio ranging from 1 to 10 viruses per cell. For each algal strain a third culture was used as control. *In vivo* autofluorescence was monitored daily to detect the lysis and the eventual recovery for up to 50 d. The experiment was repeated twice, and in some cases 3 times, for all strains. To detect differences in the length of the lytic cycle and in viral production, Mp1 was incubated with MpVN1 and MpVN2 at 2 different virus-to-host ratios (0.075 and 0.15) in duplicate. Cultures were monitored once a day for 5 d, and the total number of viruses was estimated through direct counts.

**Virus enumeration.** The abundance of *Micromonas pusilla* viruses used in infection experiments was calculated by direct epifluorescence microscopy counts. Viruses were fixed with 1% 0.02 µm filtered formalin and stained with DAPI or SYBR Green I. DAPI was added at a final concentration of 1 µg $ml^{-1}$ (Cottrell & Suttle 1995a). Samples were stored overnight at 4°C. A 5 µl drop of the stained sample was sandwiched between a glass slide and a 18 × 18 mm cover slip, and 300 to 900 particles were counted at a magnification of 1250×. Virus concentration was computed based on the number of counted fields, the area of the microscope field and the area of the cover slip. For SYBR Green I counts, samples were filtered on an Anodisc filter (Whatman; aluminium oxide, 0.02 µm pore size, 25 mm diameter) and stained with SYBR Green I for 15 min (Noble & Fuhrman 1998). All counts were performed within 30 min of the preparation of the slides.

**Electron microscopy.** For whole mount TEM preparations, the material was fixed with 1% glutaraldehyde or formaldehyde at room temperature for at least 1 h. A droplet was put on a Formvar-Carbon coated grid and allowed to dry. The grid was rinsed with distilled water and then stained with 0.5% uranyl acetate. To verify virus attachment to the host cells, exponentially growing cultures of Mp1, Mp1res and MpPart (ca. $1.2 \times 10^7$ cells $ml^{-1}$) were incubated with equal volume of MpVN1 stock ($2.3 \times 10^9$ virus $ml^{-1}$). The 3 cultures were fixed after 20 and 40 min from infection and prepared for whole mount as described above.

Ultrathin sections were prepared with host cultures infected with MpVN1 or MpVN2. To obtain information on different phases of infection in the same preparation, host cultures infected the day before were mixed with fresh uninfected culture material twice at ca. 2 h intervals and fixed in 0.5% glutaraldehyde 20 min after the last addition of fresh culture. The material was concentrated by centrifugation at 600 × *g* for 10 min (VEB MLW Zentrifugenbau Engelsdorf Model T52.1) at room temperature, post-fixed in 1% osmium tetroxide in seawater for 1 h, then dehydrated in ethanol series, transferred to propylene oxide and embedded in Epon. After polymerisation at 70°C for 24 to 35 h, the sections were cut with a Reichert Ultracut ultramicrotome. Sections were stained for 15 min with 1% uranyl acetate followed by 1 min with 0.3% lead citrate and rinsed with filter-deionised distilled water. Whole mounts and ultrathin sections were observed using a Philips EM 400 transmission electron microscope.

**UV light and Mitomycin-C treatment.** Exponentially growing cultures of the resistant strain Mp1res were exposed to 254 nm wavelength UV radiation (TUV G30 T8) for 30 s in a sterile Petri dish (5 ml $dish^{-1}$) or mixed with Mitomycin-C (Sigma Chemical) (Jiang & Paul 1996) at 3 final concentrations (0.5, 1 and 1.5 µg $ml^{-1}$) to induce viruses possibly present in a latent state. The treatments were repeated twice. Subsamples of the cultures were collected 4, 22 and 29 h after the treatments and checked for the presence of viruses with TEM and fluorescence microscopy after staining with DAPI and SYBR Green I. To detect infective particles, 1 ml of the treated cultures was filtered on the 0.22 µm pore size filter and incubated with Mp1 cultures in duplicate. Fluorescence was monitored daily.

**Molecular analyses.** For host DNA extraction, exponentially growing *Micromonas pusilla* cultures (1 l) were centrifuged for 20 min at 3010 × *g* (rotor GH-3.8A, Beckman Allegra 6-R centrifuge) at 4°C in 50 ml Falcon tubes. The pellet was resuspended in 750 µl of lysis mix (50 mM Tris-HCl pH 8; 100 mM EDTA; 100 mM NaCl; 1% SDS; 0.5 mg Proteinase K $ml^{-1}$) and incubated overnight at 55°C. Samples were mixed for

5 min on Thermomixer compact (Eppendorf) at 1400 rpm, with 300 µl of 5 M NaCl added, mixed as in the previous step, and centrifuged at 14 811 × *g* (rotor F45-24-11 Eppendorf 5417 centrifuge) for 10 min. Supernatant (ca. 750 µl) was precipitated with isopropanol, washed with 70% ethanol, and the dry pellet was resuspended in Tris-EDTA buffer. DNA extraction was performed with phenol:chloroform and ethanol precipitation (Maniatis et al. 1982), and the dry pellet was resuspended in water. For viral DNA extraction, lysates (50 ml) containing about $10^9$ viral particles $ml^{-1}$ were processed according to the lambda DNA Midi Kit (Qiagen) protocol. DNA (1 to 1.5 µg) was resuspended in sterile water.

Two sets of oligonucleotide primers were used for PCR. The primers AVS1 and AVS2, specific for the viral DNA polymerase gene (*pol*) in the family *Phycodnaviridae*, and the nested primer (POL), universal for B-family DNA *pol* genes, have been designed and widely used for PCR with isolated *Phycodnaviridae* viruses or natural virus communities (Chen & Suttle 1995). The primers AVS1/2 amplify a sequence of ca. 700 bp, while the nested primer POL gives a product of ca. 580 bp. Another set of oligonucleotide primers, the 1ET3 (5′-GAAATGTATGTAATATGGACTGAC-3′) and 1ET7 (5′-TAGTAATTCTAGTGATTGGTATGG-3′), was specifically designed for MpVN1 for a sequence of 150 bp within a fragment isolated through random subcloning of EcoRI-digested MpVN1 DNA.

For random subcloning, MpVN1 DNA was digested with EcoRI restriction enzyme (New England Biolabs, NEB). The entire EcoRI digestion was subcloned in pBS II KS+ (Stratagene). The vector was digested with EcoRI restriction enzyme (NEB) and dephosphorylated with calf intestinal phosphatase (Roche). The vector was purified according to the QIAquick gel extraction kit (Qiagen) protocol. Ligation reaction was performed with T4 DNA ligase (NEB). The plasmid DNA from selected clones was prepared according to the QIAprep spin miniprep kit (Qiagen) protocol. Cycle sequencing was performed with the CEQ dye terminator cycle sequencing start kit (Beckman) following the supplier's procedures. Capillary electrophoresis and sequence analysis were performed on the CEQ 2000 XL DNA analysis system (Beckman Coulter).

PCR was conducted with a minicycler (MJ Research) according to the following cycle parameters: denaturation at 98°C (5 min); 30 rounds of denaturation at 95°C (1 min), annealing at 48°C (1 min) and extension at 72°C (1 min); final extension at 72°C (5 min) in a 20 µl total volume PCR reaction mixture containing PCR assay buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each deoxyribonucleoside triphosphate, 1 pmol $µl^{-1}$ of each primer and 0.5 U of Taq DNA polymerase (Perkin Elmer). All reactions were tested several times under different stringency conditions. Control reactions were performed with only the forward or the reverse primer or without the template in order to eliminate possible PCR artefacts.

For Southern blotting, 5 µg of EcoRI-digested algal genomic DNA and 500 ng of viral DNA were electrophoresed on 1.5% agarose gel in 0.5× TBE buffer (Maniatis et al. 1982). Southern blotting was performed on Hybond-N+ membrane (Amersham Pharmacia Biotech) following the supplier's procedures. The hybridisation protocol was performed according to a modified Church/Gilbert procedure (Church & Gilbert 1984). DNA probes were prepared using 20 ng of gel-purified fragment DNA, radiolabelled according to the Random primed DNA labelling kit (Roche) protocol. Southern blotting was also performed on PCR products obtained with 1ET3/7 primers on resistant host strains.

## RESULTS

### Isolation

The viral strain MpVN1 infecting *Micromonas pusilla* strain Mp1 was obtained from GON surface waters on 7 November 1994. The abundances on that date were estimated as 3600 cells and 20 infective particles $l^{-1}$, i.e. close to the annual minima for both host and viruses (Zingone et al. 1999). The strain MpVN2 was isolated at the same site on 26 May 1998 using as host an Mp1 strain that had recovered from infection from MpVN1 and had become resistant to this latter virus (Mp1res, see below). The total host and virus abundance on that date was estimated as 253 cells and 3.4 infective particles $ml^{-1}$.

### Morphology

MpVN1 and MpVN2 show the hexagonal or pentagonal outlines typical for the icosahedral shape of most algal viruses (Figs. 1 to 4). Their size ranges from 100 to 130 nm, with no clear differences between the 2 viruses at the stage of free particles or virions within the infected cells. During the attachment phase, a tubular tail-like structure up to 77 nm long and 15 to 28 nm wide connects a vertex of MpVN1 to the host cell (Fig. 2A to E). The tail in some cases is much reduced (Fig. 2F). A thickened collar (up to 38 nm wide) is often evident at the boundary with the capsid (Fig. 2A,D,E). In MpVN2 (Fig. 3), a projection from the host cell membrane joins, at times, a vertex of the capsid (Fig. 3B,D), whereas in other cases the viral capsid adheres to the cell by a vertex apparently fusing with

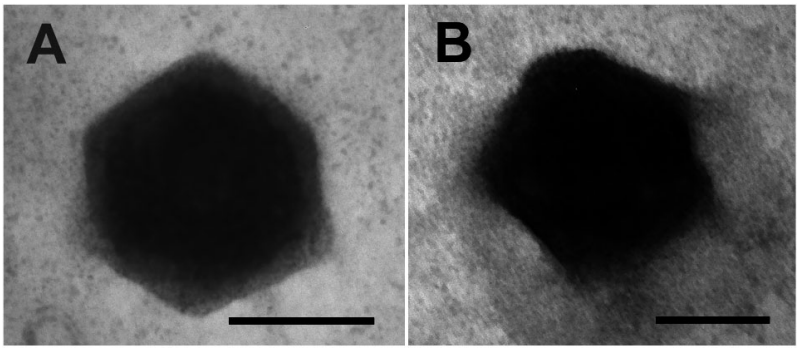


Fig. 1. *Micromonas pusilla* viruses. TEM micrographs, whole mounts. (A) MpVN1 and (B) MpVN2. Scale bars = 50 nm

the cell membrane (Fig. 3D,E). In both viral strains, additional thin fibres connect some vertexes of the capsid to the host cell membrane (Fig. 2E,G). In attached MpVN2 particles the nuclear material is often observed to be condensed and polarised in an elongated structure, with the longer axis perpendicular to the cell surface (Fig. 3A,D). Viruses may adhere to the host cell even in advanced phases of infection (Figs. 2B & 3A). Empty capsids are often observed attached to the cells (Figs. 2G & 3F), but no clear evidence of electron-dense material (DNA) passing through the tail was obtained. In a few cases an interruption of the membrane was seen in correspondence with the attachment point (Fig. 3C,D). The ultrastructural features of the infection were quite similar between the 2 viruses (Fig. 4). In the earliest stages of infection, the nuclear membrane maintains its integrity while the first signs of degeneration are observed in the cytoplasm, which appears less dense and filled with fibrillar material (Fig. 4A,B), or shows small (30 to 60 nm), circular or polygonal vesicles (Fig. 4A,C). First empty capsids are seen in the area between the chloroplast and the flagellar base (Fig. 4D,E). In more advanced stages of the infection, the nuclear membrane disappears and the whole cytoplasm is filled with empty capsids and mature virions (Fig. 4F,G). More than 40 complete and incomplete virions can be counted in a single cell section for both MpVN1 (Fig. 4F) and MpVN2 (Fig. 4G). The chloroplast does not seem damaged from the infection and maintains its integrity also in advanced stages of infection (Fig. 4H).

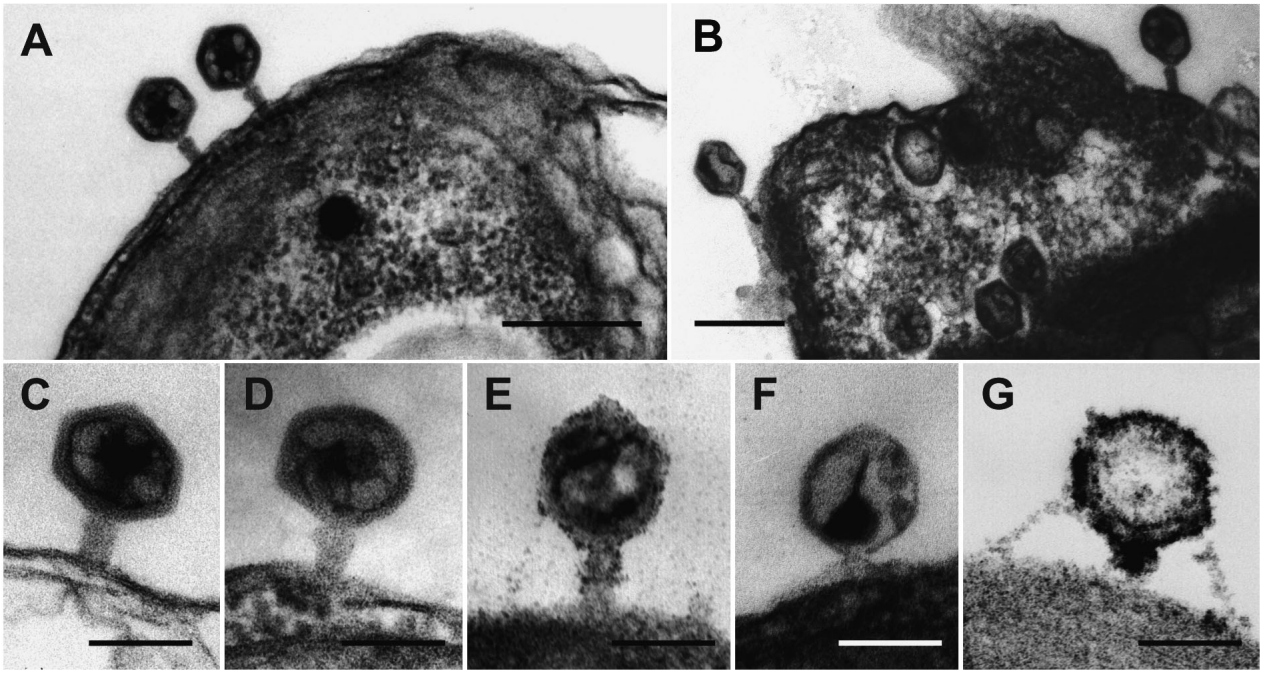


Fig. 2. *Micromonas pusilla* and MpVN1. TEM micrographs, thin sections of MpVN1 attached to the cell surface showing the tubular tail-like appendages in almost all cases. (A) Two viruses attached to the host cell membrane, with the thickened collar at the proximal end of the tail. Scale bar = 0.2 µm. (B) Two viruses attached to a cell with mature virions inside. Scale bar = 0.2 µm. (C) The nuclear material is condensed in the centre of the viral capsid. Scale bar = 0.1 µm. (D) A thickened collar is evident at the proximal end of the tail. Scale bar = 0.1 µm. (E) A thickened collar and thin fibres emerging from 2 vertexes of the viral capsid are visible. Scale bar = 0.1 µm. (F) The nuclear material is polarised near the capsid vertex joining the host cell. Scale bar = 0.1 µm. (G) Empty capsid with evident thin fibres. Scale bar = 0.1 µm

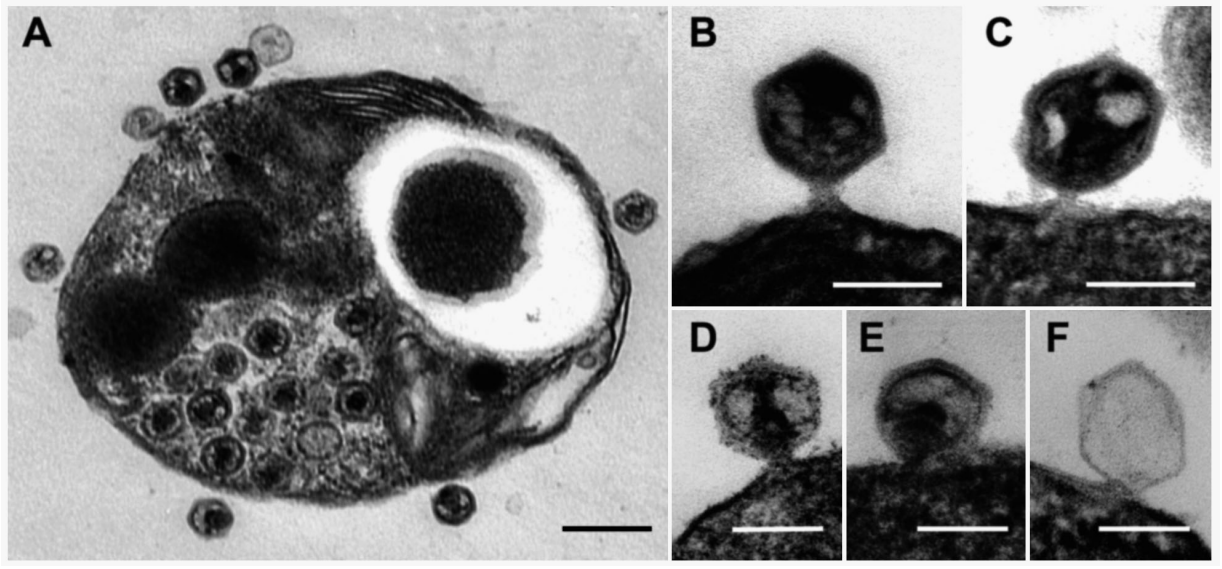


Fig. 3. *Micromonas pusilla* and MpVN2. TEM micrographs, thin sections. (A) Infected cell with several viruses attached to the surface. Scale bar = 0.2 µm. (B) The nuclear material is condensed in the centre of the viral capsid. Scale bar = 0.1 µm. (C) The cell membrane appears interrupted in correspondence with the virus attachment point. Scale bar = 0.1 µm. (D) The nuclear material is polarised in an elongated structure. Scale bar = 0.1 µm. (E) The nuclear material is polarised near the capsid vertex that joins the host cell. Scale bar = 0.1 µm. (F) Empty capsid. Scale bar = 0.1 µm

### Host range

The 2 viral strains examined had different infectivity characteristics towards the host strains tested (Table 2). Four strains, of which 2 were from the GON, 1 from the Sicily Channel and 1 from Plymouth, were susceptible to both viruses; 3 strains, of which 2 were from GON and 1 from the Oslo fjord, were sensitive to only 1 of the 2 viruses; and the other 4 were resistant to infection from both viruses. MpVN2 was capable of killing all the Mediterranean strains, while MpVN1 had a more restricted host spectrum. Both viruses were capable of infecting at least 1 strain from a distant geographic region. In the sensitive strain Mp1 incubated with MpVN1, viruses were attached to the host surface 20 min after the incubation, at times completely covering the cell (Fig. 5A), whereas hardly any particles were seen on cells of the resistant strain MpPart incubated with the same virus (Fig. 5B).

Table 2. *Micromonas pusilla*. Sensitivity of host strains to the lytic activity of MpVN1 and MpVN2. Lineages are from molecular analyses on concatenated markers (Šlapeta et al. 2006)

| Algal strain | MpVN1 | MpVN2 | Lineage |
|---|---|---|---|
| Mp1 (CCMP1646) | + | + | E |
| Mp1res | – | + | |
| Mp2 | – | + | |
| MpCO | + | + | |
| MpPart | – | + | |
| MpP7/1 (CCMP1723) | + | + | A |
| CCMP485 | – | – | |
| CCMP489 | – | – | A |
| MpNL | – | – | |
| MpUK (CCMP1545) | + | + | D |
| Mp2/89 | – | – | |
| MpOf | + | – | |

The time required to abate growth varied with both viral and algal strains (Fig. 6). With the same viral strain, and approximately at the same virus-to-host ratio, the infection process for some host strains was faster than for others. In all MpVN1-infected cultures except MpOf (Fig. 6E), fluorescence had decreased 1 d after the incubation of the virus, whereas the MpVN2 lysis rate was generally slower. In one case (Fig. 6G) fluorescence did not decrease until 3 to 4 wk after the incubation, the same host strain also showing delayed or null reactions in other experiments. The response of the host culture was generally consistent in repeated experiments, although less susceptible strains often showed variations in the time needed to abate fluorescence.

The incubation of Mp1 with MpVN1 and MpVN2 at 2 low virus-to-host ratios (0.075 and 0.15) confirmed that Mp1 cultures were abated quicker by MpVN1 than by MpVN2 (Fig. 7), with viral yields considerably higher in lysates from MpVN1 (Table 3).

### Recovery

All the strains were able to recover from viral infection from MpVN1 and MpVN2 (Fig. 6) between 7 and 45 d after the infection. Recovered strains often did not attain

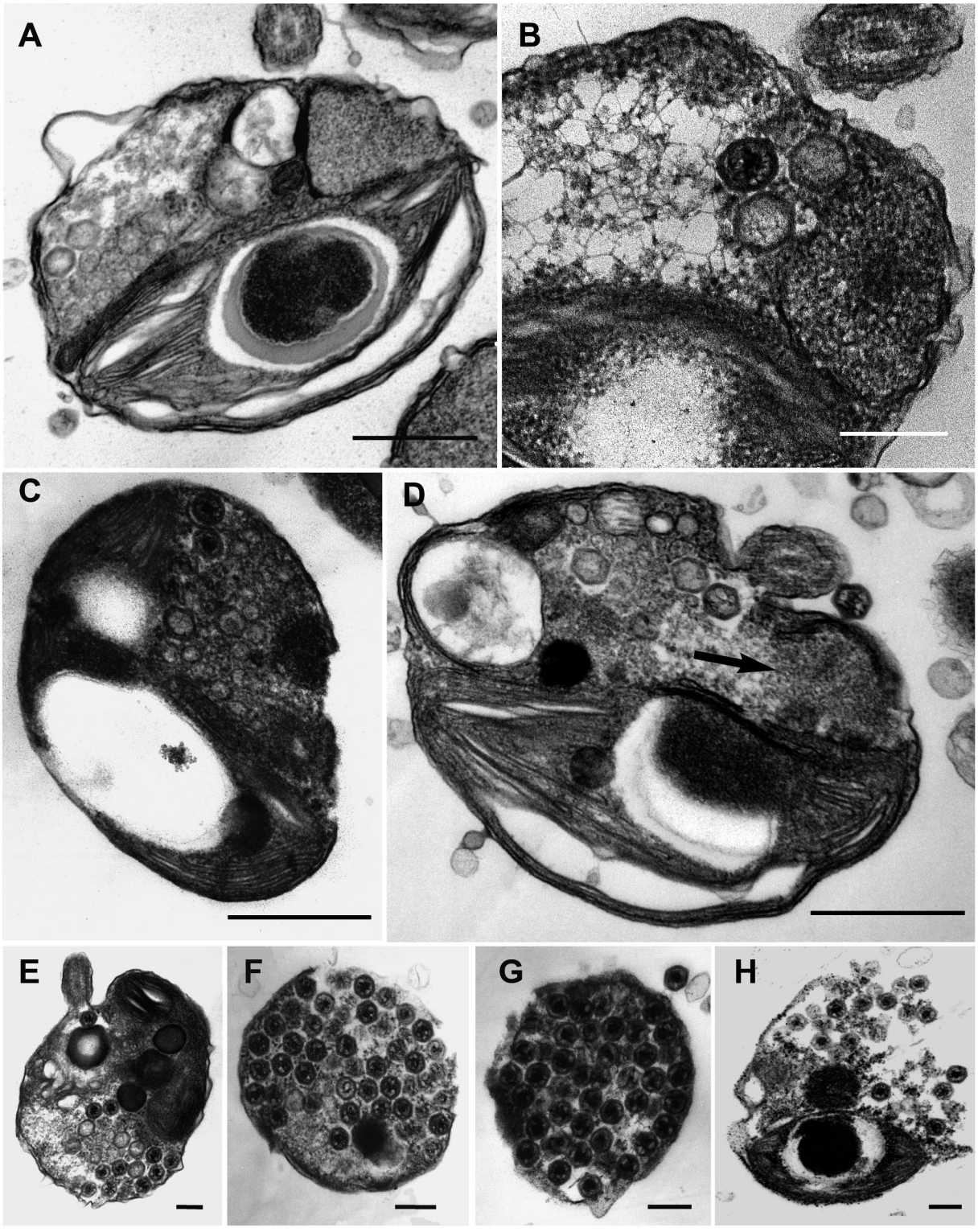


Fig. 4. *Micromonas pusilla.* TEM micrographs, ultrathin sections. Different stages of MpVN1 and MpVN2 infection. (A) MpVN1. Early effects of the infection. The cytoplasm is less dense, and a few vesicles and capsids are visible. Scale bar = 0.5 µm. (B) MpVN2. Detail of the cytoplasm filled with fibrillar material, some capsids and 1 mature virion. Scale bar = 0.2 µm. (C) MpVN1. The cytoplasm contains numerous small vesicles, some capsids and 2 mature virions. Scale bar = 0.5 µm. (D) MpVN1. Advanced stage of nuclear membrane degeneration (arrow). Scale bar = 0.5 µm. (E) MpVN1. Mature virions and empty capsids in the cytoplasm. Scale bar = 0.1 µm. (F) MpVN1. The cytoplasm is filled with virions. Scale bar = 0.1 µm. (G) MpVN2. The cytoplasm is filled with virions. Scale bar = 0.1 µm. (H) MpVN1. Lysis of the cell; the chloroplast maintains its integrity. Scale bar = 0.1 µm

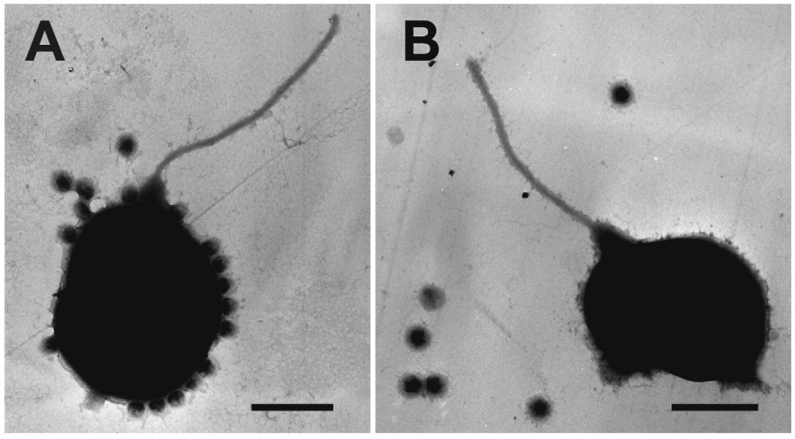


Fig. 5. *Micromonas pusilla* and viruses. TEM micrographs of whole mounts. (A) The susceptible strain Mp1 incubated with MpVN1. (B) The resistant strain MpPart incubated with MpVN1. Scale bars = 1 µm

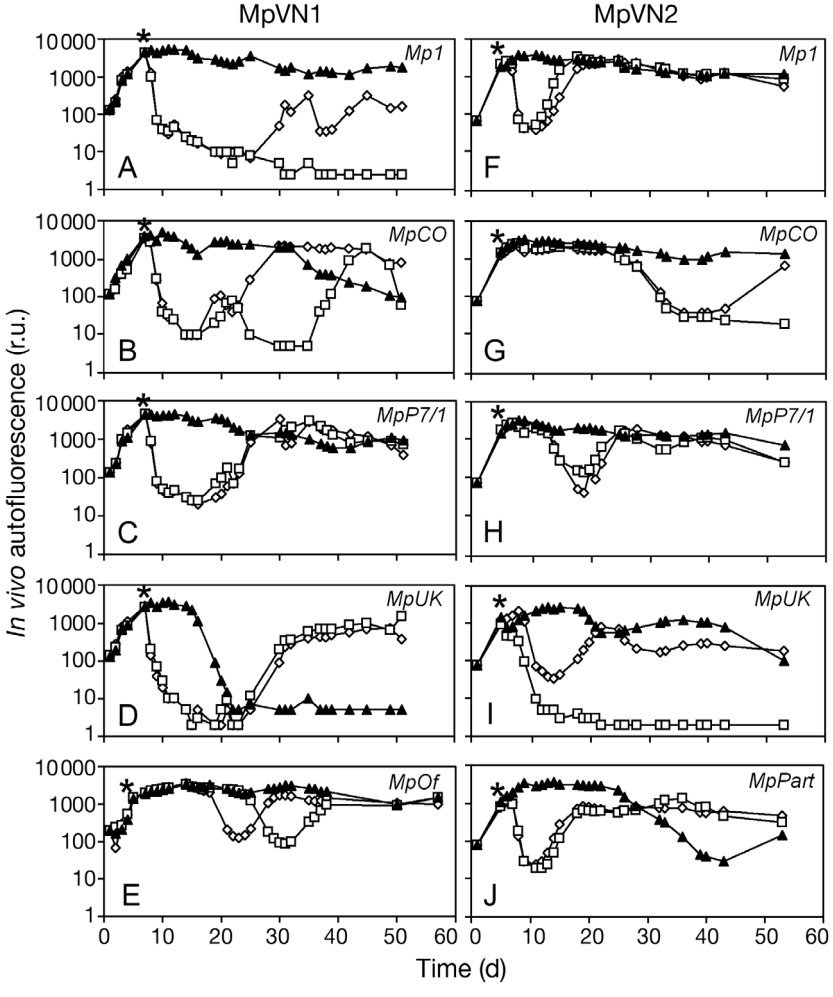


Fig. 6. *Micromonas pusilla*. Time course of *in vivo* autofluorescence (r.u.: relative units) of different strains incubated with (A–E) MpVN1 and (F–J) MpVN2. Cultures incubated with virus samples in duplicate are represented by open symbols; ▲: non-infected controls; ⋆: time of infection

the same fluorescence values as the uninfected control, while some cultures were not able to recover before the end of the experiment (Fig. 6G,I). Variations in recovery time and in the maximum recovery fluorescence occurred among different strains and, at times, also within the same strain. Occasionally, more than one instance of recovery followed by decline was observed within the same culture (Fig. 6B).

Attempts to cultivate recovered cultures were not always successful. An Mp1 culture recovered after MpVN1 infection, herein named Mp1res, was regularly transferred to fresh medium and used for further experiments. Like MpPart (Fig. 5B), this culture did not show viral particles attached to the cell membrane after 20 or 40 min from the addition of MpVN1. No infected cell was detected in ultrathin sections observed ca. 10 mo after the recovery, nor was the culture medium of Mp1res able to infect Mp1. After UV Mitomycin-C treatments of Mp1res, viruses were not observed in either light microscopy or electron microscopy, nor were they revealed by incubation with sensitive strains. Mp1res grew over 5 yr at almost the same rate, but attaining half the maximum fluorescence yield as Mp1.

### Re-infection experiments

Attempts to re-infect Mp1res cultures using MpVN1 always gave negative results. TEM observations after re-infection experiments showed free viral particle, rare viruses close to the cell surface and no infected cells. Mp1res was still resistant to MpVN1 3 yr after recovery, i.e. the last time it was tested.

Mp1res was susceptible to MpVN2, but was able to recover after infection. However, these re-recovered strains grew very slowly and it was not possible to test their presumable resistance to either MpVN1 or MpVN2. Incubation of an Mp1 culture recovered after infection from MpVN2 with MpVN1 also produced a decline in fluorescence values, which, however, was not clearly discernable from declines in the control

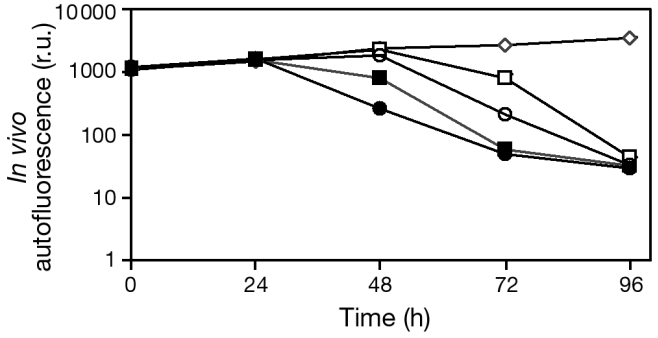


Fig. 7. *Micromonas pusilla*. Time course of *in vivo* autofluorescence (r.u.: relative units, average of 2 replicates) of Mp1 strain incubated at time 0 with MpVN1 (■: virus-to-host ratio 0.075; ●: virus-to-host ratio 0.15) and MpVN2 (□: virus-to-host ratio 0.075; ○: virus-to-host ratio 0.15). ◇: non-infected control

Table 3. Concentrations of MpVN1 and MpVN2 incubated with *Micromonas pusilla* strain Mp1 ($8 \times 10^6$ cells ml$^{-1}$) in the experiment shown in Fig. 7

| Viruses ml$^{-1}$ ($t$ = 0) | Virus-to-host ratio ($t$ = 0) | Viruses ml$^{-1}$ ($t$ = 96 h) |
|---|---|---|
| **MpVN1** | | |
| $6 \times 10^5$ | 0.075 | $5.9 \times 10^8 \pm 1.0 \times 10^8$ |
| $1.2 \times 10^6$ | 0.15 | $5.7 \times 10^8 \pm 1.6 \times 10^8$ |
| **MpVN2** | | |
| $6 \times 10^5$ | 0.075 | $9.9 \times 10^7 \pm 3.6 \times 10^7$ |
| $1.2 \times 10^6$ | 0.15 | $8.1 \times 10^7 \pm 4.3 \times 10^6$ |

tubes. Mp1res was susceptible to infection by natural virus samples from the GON, although it detected lower viral titres compared to Mp1 (Fig. 8).

### Molecular studies

Attempts to amplify the MpVN1 *pol* gene using the AVS1/2-POL primers gave negative results. Therefore, an MpVN1 DNA fragment obtained from the restriction analysis and subsequent subcloning was partially sequenced and the specific primers 1ET3/7 were designed to amplify a sequence of 150 bp. Upon isolation of MpVN2, the AVS1/2-POL primers were used in PCR amplifications of MpVN2 DNA, thus obtaining the 2 fragments of the *pol* gene of the expected length (Fig. 9A). In contrast, 1ET3/7 primers did not amplify MpVN2 DNA (Fig. 9B).

The possible latency of the virus in resistant strains was tested with PCR and Southern blot experiments aimed at detecting MpVN1 DNA integrated in the DNA of resistant host strains. Tests were conducted on both the host strains Mp1res and MpPart, a culture that was among those naturally

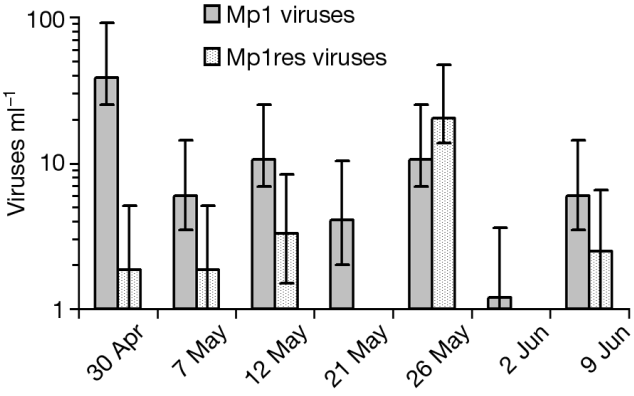


Fig. 8. Weekly variations of *Micromonas pusilla* viruses in the Gulf of Naples, spring 1998, estimated using Mp1 and Mp1res as host strains. Error bars represent ±SE

immune to MpVN1, using Mp1 as negative control. Neither amplifications with 1ET3/7 primers nor Southern blot experiments on resistant host DNA or on PCR products obtained with the above-mentioned amplification gave any positive results.

### Survey of viral strains in natural samples

PCR amplifications of the 33 samples of viral DNA collected in spring 1996 using the AVS1/2 primers produced a band of ca. 700 bp in 13 cases. In 6 cases a product of ca. 1600 bp was obtained instead (Fig. 10). In 2 cases both the 700 and the 1600 bp bands were seen in the same sample. Only in one case did the primers 1ET3/7 specific for MpVN1 give a product of 150 bp. In 12 cases neither primer pair was able to amplify the DNA of the viral sample.

## DISCUSSION

### Morphology

Free viral particles and intracellular virions of MpVN1 and MpVN2 are rather similar to each other and to other DNA Mp viruses (Mayer & Taylor 1979, Cottrell & Suttle 1991), but the tail shown by MpVN1 when attached to the host cell is a unique feature. Its origin is unclear, since it is neither evident in whole mounts nor in thin sections of free-living or intracellular viruses. Its role is unclear too, since genomic material passing through the tail has never been seen. MpVN1 viruses or empty capsids were also observed attached to the host through reduced projection from the cell, similar to what was observed for MpVN2. The tail could hence be used in the attachment and sub-

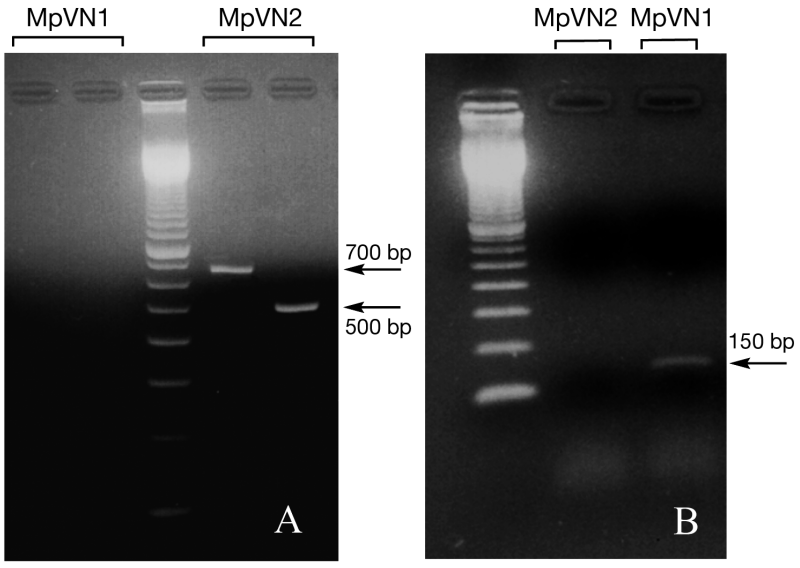


Fig. 9. Products from PCR performed on DNA from MpVN1 and MpVN2 viruses using: (A) AVS1/2/POL and (B) 1ET3/7 primer sets

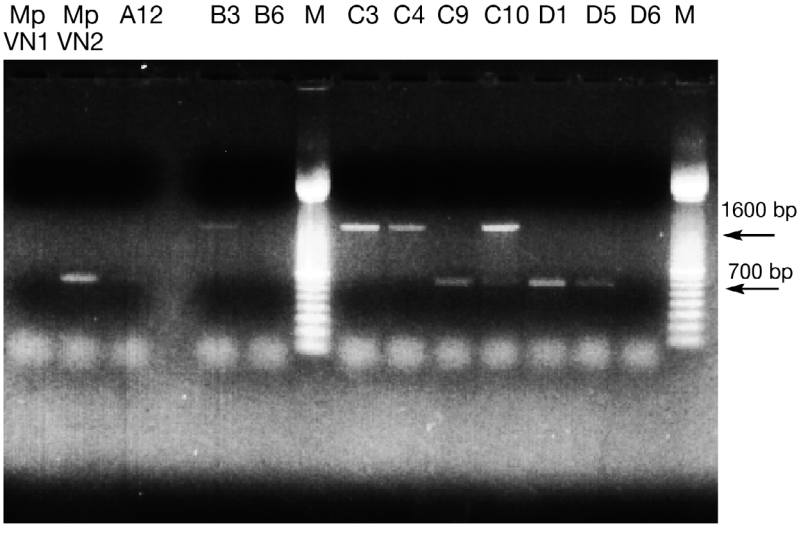


Fig. 10. PCR amplification with AVS1/2 primers on a subset of the host lysates obtained from infections with natural virus populations. A12 to D6: lysates from different sampling dates; M: molecular weight marker

sequently reabsorbed. A few cases of stalk- or tail-like structures at the attachment point have already been illustrated in the first description of viral infection in *Micromonas pusilla* (Mayer 1978), yet these were interpreted as projections from the host cell surface, being more similar to the structures observed in MpVN2.

A free viral particle with a tail, reported as the infective agent of the brown-tide pelagophyte *Aureococcus anophagefferens* (Milligan & Cosper 1994, Garry et al. 1998), was probably a phage, since there was no evidence of the tailed virus attached to that specific host. A tail in a eukaryotic algal virus is only documented for the freshwater chlorophyte *Chlorococcum minutum* (Gromov & Mamkaeva 1981), where it is invaginated into the capsid in free-living viruses and is projected outside from 1 vertex in the attachment to the host.

In the case of infection of *Chlorella* sp. by PBCV-1 (Meints et al. 1984), viral DNA is shown to enter the host cell through a hole in the cell wall, the capsid being left outside the cell. In *Micromonas pusilla*, the process is less clear due to the lack of a cell wall, yet interruptions in the cell membrane at the attachment point and empty capsids outside the cell suggest phage-like behaviour also in this case.

The thin, hair-like fibres emerging from the vertexes of the capsids in our viral strains are similar to those described in a *Chlorella* sp. virus, where they are interpreted as being responsible for the initial recognition and attachment of the virus to the cell and involved in orienting a specific virus vertex towards the host surface (Van Etten et al. 1991).

The next steps of the infection for both viruses investigated match the early ultrastructural description for viral infection in *Micromonas pusilla*, including the entirely cytoplasmatic morphogenesis of viral particles and the presence of membrane-bound vesicles embedded in the cytoplasm filled with large amounts of long, thin fibrils (Mayer 1978, Mayer & Taylor 1979). These phases are also similar to those illustrated for *Chlorella* sp. PBCV-1 (Meints et al. 1986) and *Aureococcus anophagefferens* viruses (Garry et al. 1998), although the virus-assembly centres that were described for the *Chlorella* sp. virus were not observed in our case. Interestingly, the chloroplast does not seem to be damaged directly by the infection and maintains its integrity even in advanced phases of infection, as also seen in *Chlorella* spp. viruses (Meints et al. 1981, 1986). This observation is in agreement with the apparent continuation of photosynthetic activity in infected *M. pusilla* until the end of the lytic cycle (Waters & Chan 1982). In contrast, the chloroplast of *A. anophagefferens* deteriorates at the latest stages of the infection, when the virioplasm expands to occupy much of the cell volume (Garry et al. 1998).

### Infectivity and strain specificity

The variations in the algicidal properties of the 2 viral strains examined against a set of potential hosts are the result of the interplay between viral infectivity and host susceptibility. The 2 viral strains clearly showed differences in the infection process, whereby MpVN1 took a lower time to abate host growth and produced a higher number of viruses. Further experiments are needed to establish whether these results were due to differences in infectivity, in the length of the latent phase, or in the burst size.

No relationship was found between susceptibility and geographic provenance of the potential host strains, nor with the season of collection of host and viruses, which would not support the hypothesis of co-evolutionary patterns to explain intraspecific variability in host susceptibility in this case. In contrast, previous studies on *Micromonas pusilla* (Sahlsten 1998, Brussaard et al. 2004) and *Heterosigma akashiwo* (Lawrence et al. 2001, Tai et al. 2003) reported susceptibility towards both DNA and RNA viruses only in host strains from the same location of the virus.

Intraspecific diversity in susceptibility to viral infection could be the reflection of cryptic diversity within the host species. In the case of *Micromonas pusilla*, a wide genetic diversity has been shown among strains collected at different places (Guillou et al. 2004, Šlapeta et al. 2006). Šlapeta et al. (2006) examined 4 strains used in the present study: the Mediterranean strains Mp1 (CCMP 1646) and Mp/P71 (CCMP 1723) were found to belong to 2 completely distinct lineages, whereas MpP7/1 showed a higher phylogenetic affinity with the Sargasso Sea strain CCMP489 (Table 2). The Plymouth strain CCMP1545 was found in a third distinct lineage. Interestingly, the genetically and geographically distant strains Mp1, MpP7/1 and CCMP1545 were all susceptible to MpVN1 and MpVN2, whereas the Sargasso Sea strain was not susceptible to either virus, despite its phylogenetic affinity to MpP7/1. Therefore, no match seems to exist between susceptibility to MpVN1 and MpVN2 and phylogenetic relatedness in *M. pusilla*.

In the present study, host strains demonstrated a yes-or-no response to the 2 viruses tested, whereby natural resistance is probably associated with the lack of specific receptors on the host cell membrane; however, there were also differences among the susceptible strains. For example, the response of the Oslo fjord strain to MpVN1 infection was rather slow, whereas MpCO was scarcely sensitive to infection from MpVN2, compared to the other susceptible host strains. The time required for the recovery process also varied among host strains. In addition, variations in the infection and recovery time were, at times, observed within the same host strain, which may reflect differences in physiological conditions of the host despite uniform growth conditions. Various environmental factors and the cell cycle stage may indeed influence the lytic process (reviewed in Brussaard 2004), although the underlying mechanisms for the variations observed are generally elusive.

### Recovery and resistance

Waters & Chan (1982) first reported incomplete lysis and subsequent growth of the infected cultures in *Micromonas pusilla*, with a complex pattern of resistant strains and viral mutants able to infect them. Resistant strains were interpreted as the result of a mutation/selection mechanism, which, however, would be extremely costly and would probably imply, like in bacteria, the loss or the impingement of some important physiological function (Lenski 1988). Several other microalgae have been reported to exhibit incomplete lysis and recovery from viral infection, among which are *Chlorella* sp. (Van Etten et al. 1991), *Emiliania huxleyi*, *Pyramimonas orientalis*, *Chrysochromulina ericina* and *Phaeocystis pouchetii* (Thyrhaug et al. 2003). In the case of *Chlorella* sp. the survival of infected cultures was attributed to a pseudolysogenic condition, whereby the virus is present in a carrier state, with a small fraction of the host population being infected continuously (Van Etten et al. 1991). The chronic infection would stimulate the production of specific or non-specific inhibitors that would protect the host cells from further infections by competing with viral particles for receptor sites (Thyrhaug et al. 2003). TEM observations of resistant cells with no viruses attached in *M. pusilla* would indicate that resistance depends on changed adhesion properties of viruses to the host membrane, in agreement with Waters & Chan (1982). However, differently from *P. pouchetii* (Thyrhaug et al. 2003), resistance in *M. pusilla* persisted for several years in the absence of viral production; had the protective agent been dependent on cell lysis, it would have been diluted with time. In addition, resistance induced by one viral strain did not protect from infection from another viral strain, which would suggest a highly specific protection mechanism. The induction of resistance in infected culture could derive from the virus entering a latent/lysogenic stage in the host cell, yet the negative results of tests aimed at detecting viral DNA or inducing the lytic cycle in resistant strains do not support this hypothesis. In *Chlorella* sp. viruses, DNA site-specific endonucleases and methyltransferases have been found, the biological function of which is unknown (reviewed in Van Etten 2003). Apparently, they are not

involved in protection from other viruses, yet their possible role in protection from the same virus deserves investigation.

## Molecular diversity

Despite a remarkable clonal variability (Cottrell & Suttle 1991, 1995b), *Micromonas pusilla* viruses known until recently belonged to a single, monophyletic virus family, the *Phycodnaviridae*. Within this family, the *pol* gene is generally amplified by the AVS sets of primers (Chen & Suttle 1995) and provides good phylogenetic resolution for the relationships among viruses infecting a single host and different hosts (Chen et al. 1996). Recently, the first dsRNA virus infecting a microalga has been isolated in *M. pusilla* (Brussaard et al. 2004), confirming a high phylogenetic diversity in algal viruses. The case of MpVN1 demonstrates that there is diversity also within DNA viruses infecting *M. pusilla*, some of which may not be amplified by AVS primers. This is also the case for viruses infecting *Chrysochromulina ericina* and *Pyramimonas orientalis* (Sandaa et al. 2001). Further molecular analyses are needed to ascertain whether these viruses still belong to the *Phycodnaviridae*, whereby the lack of amplification could be due to genetic differences in the primer region, like in the case of the *Heterosigma akashiwo* virus HaV (Nagasaki et al. 2005a).

Virus samples collected in spring 1996 presumably contained the most abundant viral strains isolated at each sampling date, since they were selected among the most diluted infective subsamples used in the extinction dilution experiments. These viruses had AVS-amplifiable *pol* (Chen & Suttle 1995) — producing fragments of expected length with the PCR technique — in only one-third of the cases, further indicating the need for complementary primers in the detection of algal viruses in the natural environment. As for the primers 1ET3/7, their effectiveness is uncertain, since the variability of the DNA fragment amplified is unknown.

## Concluding remarks

The remarkable diversity observed between 2 viral strains infecting *Micromonas pusilla* confirms results from other studies and further highlights the complexity of viral–algal interactions. One immediate implication of this diversity is the weakness of current methods used to detect specific viruses in the natural environment. The quantification of viral abundances based on extinction dilution experiments is clearly hampered by notable intraspecific variability in host susceptibility. In addition, the primers so far available for *M. pusilla* viruses are not effective against all DNA viruses, let alone RNA viruses. Finally, the wide variability in susceptibility to viral infection among host strains from the same area prevents the dynamics of the interactions between the 2 players in the game of viral infection from being traced, because their respective role as host and infective agent cannot be identified.

Another aspect of the complexity of virus–host relationships concerns the resistance that is regularly induced by viral infection. In the case of *Micromonas pusilla*, induced resistance appears to be a stable and inheritable feature of an algal strain. Relevant questions stemming from this observation are whether and to what extent this process also occurs in the natural environment. In the case where it does occur, the acquired resistance may play a role in shaping the host populations during a bloom and is possibly maintained in subsequent blooms, provided that it does not produce physiological impairments in the host. Acquired resistance could actually account for the widespread occurrence of non-sensitive strains within a species, i.e. acquired immunity and intraspecific variations in susceptibility could be 2 aspects of the same process. This hypothesis would also explain the increase of resistant strains that has been observed after the viral-induced termination of *Heterosigma akashiwo* blooms (Tarutani et al. 2000). It is clear that full elucidation of the molecular mechanisms underlying natural or acquired resistance is needed. In addition, the knowledge of possible side effects of induced resistance on the physiology of the host should be investigated in order to assess the actual influence of infection and recovery on the clonal composition and ecological performances of host populations. This information, coupled with more effective molecular tools to detect specific algal viruses, are mandatory prerequisites for quantifying and predicting the actual effect of viruses on algal dynamics in the natural environment.

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