

Response of marine viral populations to a nutrient induced phytoplankton bloom at different pCO₂ levels

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Abstract. During the PeECE III mesocosm experiment in 2005 we investigated how the viroplankton community responded to increased levels of nutrients (N and P) and CO₂. We applied a combination of flow cytometry, Pulsed Field Gel Electrophoresis and degenerate PCR primers to categorize and quantify individual viral populations, and to investigate their temporal dynamics. Species specific and degenerate primers enabled us to identify two specific large dsDNA viruses, EhV and CeV, infecting the haptophytes *Emiliania huxleyi* and *Crysochromulina ericina*, respectively. Some of the viral populations detected and enumerated by flow cytometry did not respond to altered CO₂-levels, but the abundance of EhV and an unidentified dsDNA virus decreased with increasing CO₂ levels. Our results thus indicate that CO₂ conditions, or the related change in pH, may affect the marine pelagic food web at the viral level. Our results also demonstrate that in order to unravel ecological problems as how CO₂ and nutrient levels affect the relationship between marine algal viruses and their hosts, we need to continue the effort to develop molecular markers used to identify both hosts and viruses.

1 Introduction

Increase in diffusion of CO₂ into the ocean has been proposed as a negative feedback mechanism, counteracting the observed, and future expected, increase in atmospheric CO₂ levels (McNeil et al., 2003). It is, however, uncertain to which extent such an increase will influence the marine microbial community, and how a possibly consequential change in the marine microbial composition will affect the oceans role as a sink for CO₂ in the future. Marine viruses may be

affected by altered CO₂ levels in the ocean, and may also influence their host organisms differently as the CO₂ concentrations changes. Viruses are known to be the most abundant biological entity on earth and although being considerably smaller than bacteria, their sheer number, ranging from 10⁵–10⁸ particles mL⁻¹ of seawater, makes them the second largest contributor to the global biomass (Suttle, 2005). They are thought to play an essential role in cycling of nutrients in the marine environment as well as in the population dynamics of phyto- and bacterioplankton (Fuhrman, 1999; Riemann and Middelboe 2002a; Fuhrman and Schwalbach, 2003; Brussaard, 2004; Suttle, 2005).

Polymerase Chain Reaction targeting conservative sequences of bacteriophages and more specifically cyanophages, have been used to study the diversity of these viral groups (Breitbart et al., 2004; Millard et al., 2004; Filèe, 2005; Sullivan, 2006). The impact that phages exercise on bacterioplankton composition and diversity have also been investigated both empirically (Hewson et al., 2003, 2006; Hewson and Fuhrman, 2006) and theoretically (Murray and Jackson, 1992; Thingstad and Lignell, 1997; Thingstad, 2000). There is increasing evidence that virus also influence the microbial community by acting as important agents of mortality for phytoplankton, causing release of nutrients bound in cellular biomass and relieving competitive pressure by dominant species (Larsen et al., 2001; Martinez-Martinez et al., 2006). The model describing the relationship between bacterial diversity and viruses, in which coexistence of competing bacterial species is maintained by viruses that “kill the winner” may thus also apply for algae and algal viruses (Thingstad, 2000). However, only a few complete algal virus genome sequences are available (Van Etten et al., 2002; Wilson et al., 2005) and the basis for designing general molecular probes that allow for detailed studies of native viral assemblages is limited. Many ecological studies of aquatic algal viruses hence rely on relatively coarse methods like flow cytometry (FCM)



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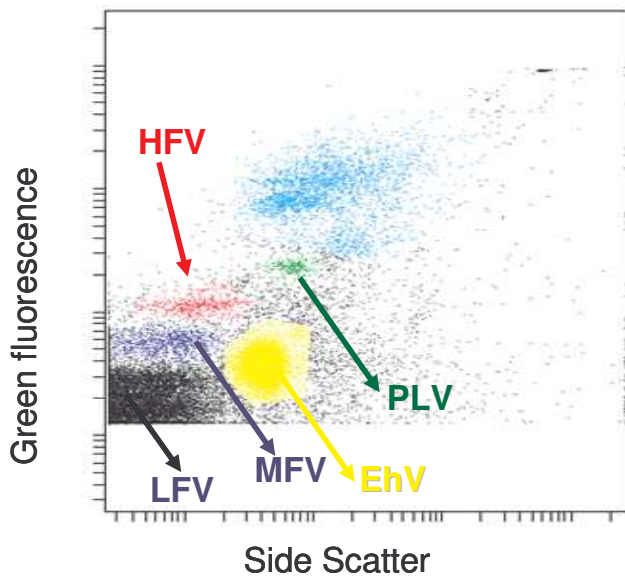


Fig. 1. Biparametric flow cytometric plots of viruses. Five different viral populations were discriminated on basis of side scatter signal (SSC) vs green fluorescence signal after staining with SYBR green I: Low Fluorescence Virus (LFV), Medium Fluorescence Virus (MFV), High Fluorescence Virus (HFV), *Emiliania huxleyi* virus (EhV) and Putative Large Virus (PLV). For further explanation see text. The current example is from a sample at day 15 from a 3×CO₂ mesocosm.

and pulsed field gel electrophoresis (PFGE) (Castberg et al., 2001; Larsen et al., 2001; Li and Dickie, 2001; Jacquet et al., 2002; Larsen et al., 2004; Baudoux et al., 2006; Martinez-Martinez et al., 2006; Sandaa and Larsen, 2006).

A notable exception to the problem of identifying specific virus populations and correlating them with host populations in natural ecosystems is *Emiliania huxleyi* and its associated virus EhV. One reason is that the full genome sequence is known for a EhV strain (Wilson et al., 2005), but maybe equally important, both host and virus gives distinct fluorescence and scatter signals in the flow cytometer allowing temporal dynamics to be followed in environmental samples (Castberg et al., 2001; Jacquet et al., 2002; Wilson et al., 2002; Martinez-Martinez et al., 2006). *Emiliania huxleyi* form dense blooms at temperate to sub-arctic latitudes and is in climate research considered to be a key phytoplankton species in marine carbon and calcite cycles. A mesocosm experiment, performed at the Marine Biological Field Station, University of Bergen, Norway, in May–June 2005, was therefore designed to investigate the effect of different pCO₂ pressures on blooms of *E. huxleyi* and co-occurring phytoplankton species. This experiment provided a unique opportunity to explore the effect of altered pCO₂ on EhV and *E. huxleyi* host-virus interactions. The effect on other phytoplankton viruses was assessed using flow cytometry and PFGE in combination with a new set of species specific and gen-

erate PCR primers allowing detection and identification of a more diverse group of large dsDNA viruses. The concurrent phytoplankton response in the same mesocosm experiment was studied by Egge et al. (2007) and Paulino et al. (2007).

2 Material and methods

2.1 Experimental setup

The mesocosm experiment consisted of nine seawater enclosures (2 m diameter and 9.5 m deep, volume 27 m³) mounted on floating frames along a raft located in a small bay in Raunefjorden (60°16' N, 5°13' E) outside Bergen, Norway. The seawater in the enclosures was manipulated to three different pCO₂ levels (1050 μatm (3×CO₂), 700 μatm (2×CO₂) and 350 μatm (1×CO₂)) with three replicate enclosures for each level. The mesocosms' headspace was kept at target CO₂ levels to simulate 1×, 2× and 3× atmospheric CO₂ levels. We collected samples from all nine mesocosms every day at 10 a.m. Subsamples for flow cytometric virus enumeration were obtained from all nine enclosures and from the adjacent seawater every second day for the first 6 days and then daily throughout the experiment. Samples for PFGE and PCR reactions were collected from one enclosure from each of the CO₂ levels: Enclosure 2 (3×CO₂), enclosure 5 (2×CO₂) and enclosure 8 (1×CO₂) on day 0, 6, 9, 11, 12, 15, 17, 19, 23, 25 and 27. For a full description of the experimental setup and sampling procedures, see Schulz et al. (2007).

2.2 Flow cytometry

Samples for enumeration of viruses were fixed with glutaraldehyde (final concentration 0.5%) for 30 min at 4°C, snap frozen in liquid nitrogen and stored at –70°C until further analysis. Thawed samples were diluted in 0.2 μm filtered 1×TE-buffer and stained with SYBR Green I (Molecular Probes Inc., Eugene, OR) for 10 min in the dark at 80°C. The samples were analysed by flow cytometer at event rates between 100–1000 viruses per second. The flow cytometer setup is described in Marie et al. (1999). The discrimination of virus populations was based on groups observed in scatter plots of side scatter (SSC) versus green DNA dye fluorescence signal (SYBR Green I, Fig. 1).

2.3 Pulsed Field Gel Electrophoresis

For PFGE 2 litres of sampled seawater was pre-filtered on 0.45 μm pore-size low-protein-binding Durapore membrane filters of 142 mm in diameter (Millipore, Billerica, MA, USA) in order to remove zooplankton, phytoplankton and some of the bacteria. The filtrate was then concentrated to ~45 mL using a QuixStand benchtop system with 100.000 pore size (NMWC) hollow fibre cartridges (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Five mL was stored at 4°C and used as template in PCR reactions

(see below), and 40 mL was pelleted by ultracentrifugation at 28 000 rpm for 1 h. Following ultracentrifugation, the supernatant was removed, and pellets resuspended overnight in 300 μ L SM-Buffer (0.1 M NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, 0.005% Glycerin) at 4°C. 200 μ L concentrate was used to make four PFGE plugs by adding 200 μ L of Insert agarose (FMC, ME, USA). Hence, each plug contained virus concentrate from 0.3 L of seawater. Lysis and washing of the plugs was performed as described by Larsen et al. (2001). The plugs were loaded onto a 1% Seakem GTG agarose gel (FMC, ME, USA), together with a ladder (λ size marker, Bio-Rad). Electrophoresis was done on a Bio-Rad DR-II electrophoresis unit, coupled to a Bio-Rad Chef DR-II drive and control module. Each gel was run in 1 \times TBE buffer (Tris-Buffered EDTA), at a total voltage of 6 V. Three different settings for switching time were used for separating DNA originating from small (SGV), medium (MGV) and large genome sized viruses (LGV). Pulses were set between 1 to 5 s for 20 h for separation of SGV: 0–150 kb, 8 to 30 s switch time for separation of MGV: 150–250 kb, and 20 to 40 s for 22 h for separation of LGV: 250–600 kb. Following electrophoresis the gels were stained for 30 min in a 10 000 dilution of SYBR green I in dH₂O, and visualised on a LAS-3000 imaging system (Fujifilm Life Science, Stamford, CT, USA).

2.4 PCR and sequencing

All bands occurring on the PFGE gels within each of the three size ranges (SGV, MGV, LGV) were pooled and excised from the gel. Specific single bands coinciding in size with previously isolated viruses were also cut out. All excised material was frozen at –20°C, and DNA was later extracted using GeneClean Turbo kit (BIO101) for extraction of large DNA fragments from agarose gel, following the manufacturer's instructions yielding approximately 10 ng/ μ L of DNA (total 30 μ L).

The presence of cyanophages was investigated using primers targeting the photosynthetic genes *psbD* on the DNA originating from the pooled PFGE bands (Clokic, 2006). The PCRs were carried out in a total volume of 50 μ L containing: sterile distilled water, PCR buffer (10 \times PCR buffer B, Promega, Madison, WI), dNTPs (each 200 nM), primers (each 0.5 μ M), 1.5 mM MgCl₂, 2.5 U Taq DNA polymerase (Promega) and template amplicon (1–2 ng). Amplification conditions were: 94°C for 5 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min.

DNA from the specific PFGE bands, which coincided in size with previously isolated viruses, were investigated using primers targeting the major capsid protein (MCP) in a standard PCR setup containing 30 cycles of denaturation at 94°C, annealing at 55°C and elongation at 72°C. The PCR primers targeting the *mcp* gene in EhV were designed from the genome sequence of EhV-86 (Wilson et al., 2005),

EhV163 (Allen et al., 2006) and EhV-99B1 (unpublished genome). They cover regions that are conserved in all three viral strains. Primers targeting CeV were designed from the *mcp* gene sequence of the CeV-01B strain. The gene region amplified using these primers appears to be conserved at the nucleotide level since we over a period of 7 years repeatedly have obtained sequences from concentrated seawater samples that are 100% identical to the *mcp* gene in CeV-01B. Primer sequences were 5'-TTT AAT TTC TCG GGC ATT GG-3' (forward) and 5'-GAG AAC GAG TAC GAG TAG ATG-3' (reverse) for "EhV size bands" and 5'-TGC CCT TCC TTT AAT TGC AC-3' (forward) and 5'-TAG AGT GAT GCC GCA ACA AG-3' (reverse) for "CeV-size bands".

Degenerate primers targeting the major capsid protein of Phycodnaviridae were used to amplify directly from the virus concentrate (see concentration procedure above). In order to design these primers *mcp* sequences were obtained from ongoing genome sequencing of the viruses PoV-01B, PpV-01, and CeV-01B and aligned together with currently available sequences from Phycodnaviridae in GenBank. Based on two conserved regions in the alignment a primer pair amplifying Chloroviruses, Prymesioviruses and Raphidoviruses, but not members of the Coccolitho- and Phaeovirus genera (EhV viruses, and FirrV-1 and EsV-1), was designed. The specificity of the primers was tested on viral concentrate from seawater samples, and on lysed algal cultures (own unpublished results). PCRs were performed with 2 μ L virus concentrate as template DNA in a total reaction volume of 20 μ L containing 10 μ L HotStar master mix (Quiagen, Germany), and 0.5 μ M of each of the primers 5'-GGY GGY CAR CGY ATT GA-3' (forward), and 5'-TGI ARY TGY TCR AYI AGG TA-3' (reverse). Amplification was done in a Bio-Rad I-Cycler programmed for an initial hotstart at 95°C for 15 min followed by a touchdown PCR containing 20 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s, and 35 additional cycles with annealing at 45°C. The run was terminated by 7 min incubation at 72°C. Amplicons were separated on a 2% agarose gel, and bands excised from the gel and sequenced according to standard procedures. Analysis of DNA sequences was carried out by alignment to the closest relative in the GenBank database using TBLASTX (Altschul et al., 1990). The obtained sequences have been submitted to GenBank with accession numbers as given in Table 1.

3 Results

3.1 Viral populations detected by flow cytometry

Using flow cytometry a total of five different viral populations were detected (Fig. 1). The flow cytometry signatures were compared with those from viruses we keep in pure cultures (own unpublished work) and with previously performed flow cytometry work (e.g. Larsen et al., 2001;

Table 1. Sequences obtained using degenerate primers targeting the Major Capsid Protein gene, and identified through TBLASTX search. Closest match are shown together with % identical residues. The sequences have been submitted to GenBank under the shown accession numbers.

Amplicon	Seq length AA	GenBank no.	Closest match in GenBank	Similarity
CeV/5/2005	172	EU006612	<i>C. ericina</i> CeV	100%
OTU/M05-01	115	EU086758	Chlorella virus NY2A (DQ491002.1)	50%
			Chlorella virus AR158 (DQ491003.1)	49%
OTU/M05-02	135	EU086759	<i>H. akashiwo</i> HaV-1 (AB198422.1)	49%
			Mimivirus (AY653733.1)	49%

Jacquet et al., 2002; Martinez-Martinez et al., 2006) but we did not include viral lysate in the samples as a reference. Three of the populations had similar SSC signals but were discriminated by differences in green fluorescence signal, and are in the following referred to as low fluorescence virus (LFV), medium fluorescence virus (MFV) and high fluorescence virus (HFV). One population exhibited SSC and green fluorescence intensities like those of previously detected EhV from similar samples (e.g. Jacquet et al., 2002; Martinez-Martinez et al., 2006) and of previously isolated EhV (Castberg et al., 2002). Moreover, a virus infectious to *E. huxleyi*, isolated from these samples displayed equal signals (own observations), and this population is thus denoted EhV. A fifth virus group, recognized by having substantially higher SSC and green fluorescence than any of the other four groups, is called PLV (putative large virus). Although viruses with genome sizes larger than the smallest known bacterial genomes have been isolated (Jacobsen et al., 1996; Sandaa et al., 2001; La Scola et al., 2003), these high fluorescence and SSC signals could be interpreted as a population of a single small bacteria species. We consider it more likely to represent a large virus, however, as the clearly defined size group is typical that of viral populations (own observations).

All the five virus groups reached concentrations considerably higher than what we observed in the fjord water (Fig. 2), and as such a response to increased primary and bacterial productivity caused by the nutrient addition was evident for all viral populations. The dynamics of LFV, EhV and HFV all followed a similar pattern with low initial concentrations followed by substantial increases from day 5–10, after which they remained at high concentrations throughout the experiment. LFV increased from $\sim 4 \times 10^7$ on day 6 to $\sim 1.4 \times 10^8$ virus mL⁻¹ at the end of the experiment (Fig. 2a), and EhV and HFV increased from background levels to ~ 1.1 – 1.4×10^7 viruses mL⁻¹ and 0.9 – 2.0×10^6 viruses mL⁻¹, respectively (Fig. 2b, c). MFV and PLV showed a different development (Fig. 2d, e) in that both were found at elevated concentrations compared to fjord water already from day 0 (MFV: $\sim 5.5 \times 10^6$ mL⁻¹, PLV: ~ 4 – 7.0×10^5 mL⁻¹). The concentration of MFV declined abruptly to $\sim 1.0 \times 10^6$ viruses mL⁻¹ on day 4–6 before increasing on day 12–18 to

$\sim 4.5 \times 10^6$ viruses mL⁻¹ and then remained at this concentration for the rest of the experiment. A small decline in PLV concentration was observed around day 2–4, it then increased from day 8 on, reaching a maximum concentration around 1 – 1.5×10^6 mL⁻¹ on day 16 before declining to initial levels at the end of the experiment.

The effect of the CO₂ manipulation was significant and consistent for HFV (Fig. 2c) with higher maximum concentrations in the $1 \times \text{CO}_2$ enclosures ($\sim 2.0 \times 10^6$ viruses mL⁻¹, day 15) than in the $2 \times \text{CO}_2$ and $3 \times \text{CO}_2$ ones ($\sim 1.5 \times 10^6$ mL⁻¹ (day 15) and 0.9×10^6 mL⁻¹ (day 18), respectively). A similar, although not significant CO₂ effect, was observed for EhV with a slightly higher abundance in the $1 \times \text{CO}_2$ enclosures than in the $2 \times \text{CO}_2$ and $3 \times \text{CO}_2$ enclosures (Fig. 2b). No clear effect of the CO₂ manipulation was seen for LFV, MFV and PLV (Fig. 2a, d, e).

3.2 Viral populations detected by Pulsed Field Gel Electrophoresis

A total of 21 viral populations, as defined by different genome sizes, could be identified on the PFGE gels (Fig. 3). Sixteen of the populations were common to all mesocosms (enclosure 2, enclosure 5, enclosure 8), while 5 (at ~ 26 , ~ 105 , ~ 110 , ~ 137 and ~ 440 kb) were observed in only one of them.

In total seven large genome sized viruses (LGV: >250 kb) were detected on the gels (Fig. 3). The largest genome size identified was ~ 510 kb, correlating with the size of a previously isolated virus (CeV-01B) infecting the prymnesiophyte *Chrysochromulina ericina* (Sandaa et al., 2001). This band was excised and PCR-amplified using allele specific primers, hereby verifying its origin as CeV. In the enclosure with the lowest CO₂ concentration (M2) CeV was present from the initiation of the experiment, and it was observed in all three mesocosms from day 10. A virus population with a genome size around 410 kb, thus similar in size to a previously isolated virus (EhV-99B1) infecting *Emiliania huxleyi* (Castberg et al., 2002), was present in all three mesocosms from day 7–8 to the end of the experiment. The origin of this band was identified as EhV by DNA extraction and allele specific PCR amplification using primers targeting the major

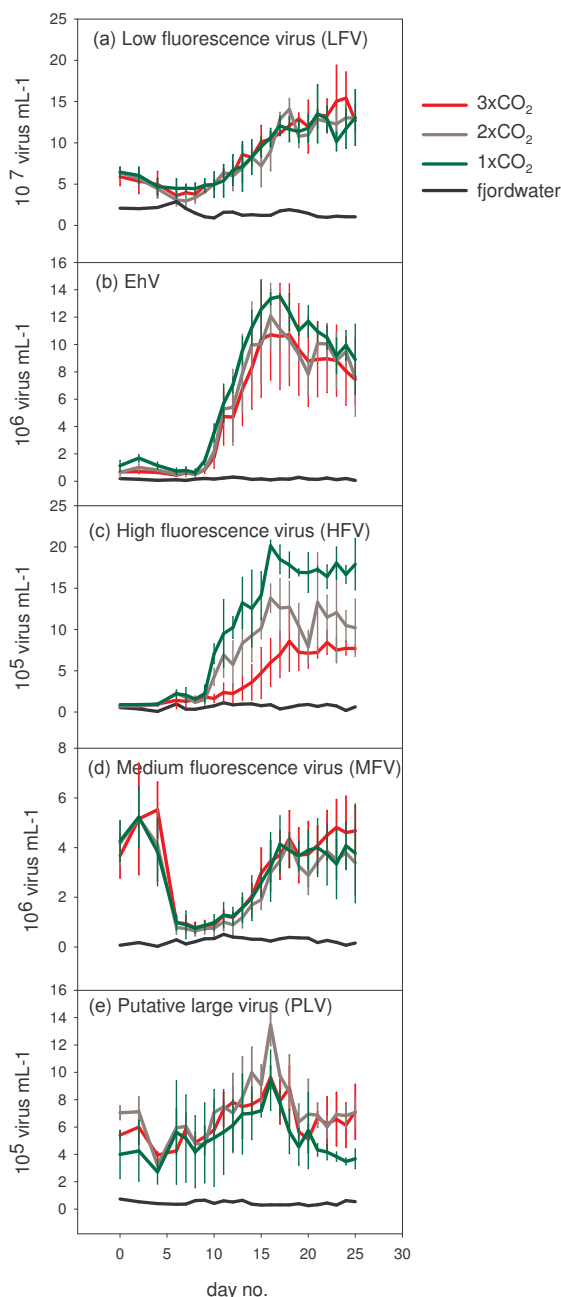


Fig. 2. Temporal distribution of viral populations quantified using flow cytometry. Figure (a) Low Fluorescence Viruses (LFV), (b) EhV (c) High Fluorescence Viruses (HFV), (d) Medium Fluorescence Viruses (MFV), and (e) Putative Large Virus (PLV). Each diagram shows a virus population at present (1×CO₂) and experimentally increased concentrations of atmospheric pCO₂ resembling projected levels in year 2100 (2×CO₂), and in 2150 (3×CO₂). Figures are average from three replicas, with error bars denoting ± standard deviation. Whenever standard deviations do not overlap the treatment may be considered to have a significant effect. The data are shown together with fjord water counts.

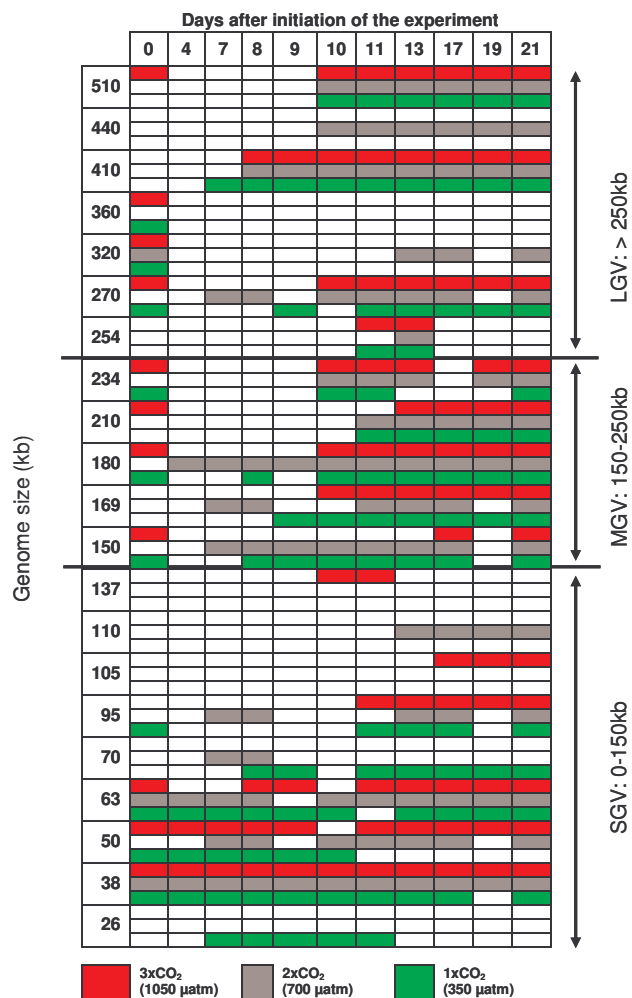


Fig. 3. Virus genomes present in the mesocosms during the course of the experiment, as investigated using Pulsed Field Gel Electrophoresis. The figure shows presence of virus bands at each of the days, for each of the three mesocosms tested (one mesocosm from each CO₂ concentration). Because of the limited sampling regime for these analyses the results are of a qualitative rather than a quantitative character.

capsid protein. A population of viruses with a genome size of ~270 kb was present in two of the mesocosms (1×CO₂ and 2×CO₂) at the initiation of the experiment. It disappeared but reappeared in all three mesocosms from day 7–10 (Fig. 3). A population of large dsDNA viruses with a genome size ~440 kb, only observed mesocosm 5 (2×CO₂) (Fig. 3), appeared at day 10 and was present throughout the experiment. We sporadically observed three other large virus genomes with sizes 254, 320 and 360 kb (Fig. 3).

A population maximum of five different viruses with medium sized genomes (MGV: 150–250 kb) was observed on the PFGE gels (Fig. 3). Their genome sizes were estimated to 150, 169, 180, 210, and 234 kb. Most of these viruses were present at the initiation of the experiment, but

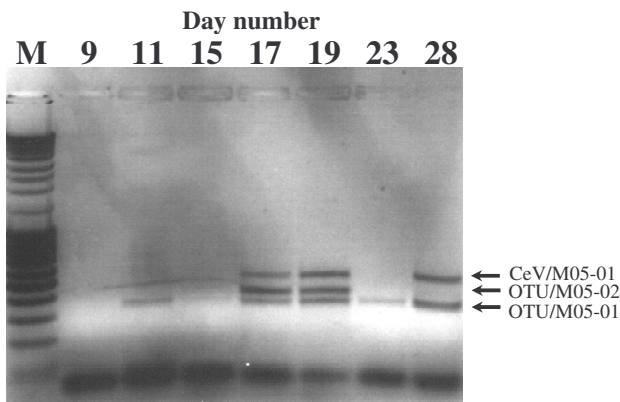


Fig. 4. Temporal diversity of viruses, investigated by amplification of a fragment of the viral major capsid protein gene sequence using degenerate PCR primers. PCR was performed with virus concentrate taken from a mesocosm subjected to a CO₂ concentration of 700 μ atm ($2\times$ CO₂). Sequencing of the amplicons identified the top band at 518 bp as being homologue to previously isolated CeV viruses, while the two smaller bands showed no homology to other viruses in the Genbank (TBLASTX). M – 100 bp ladder.

tended to disappear after the first few days before reoccurring again around day 7–10. PCRs using primers targeting the cyanophage *psbD* gene indicated the presence of cyanophages within this viral size group.

The most abundant group of viruses as determined by the intensity of the bands on the PFGE gel, was the group of small genome sized dsDNA viruses (SGV: 0–150 kb) (Fig. 3). Nine different genome sizes could be observed in this group during the course of the experiment, most being present from the start throughout the experiment (Fig. 3).

3.3 Viral identification by PCR using degenerate primers

The degenerate PCR primers targeting the phycodnaviridae *mcp* gene produced amplicons from the virus concentrate that were 347 bp, 407 bp, and 518 bp (Fig. 4). The amplicons were sequenced and recognized as MCP OTUs. The *mcp* primers target two of the eight conserved regions in the *mcp* gene. Outside these regions the *mcp* gene varies considerably with respect to sequence homology, inserts and deletions, and the size of the amplification products will thus vary. The 518 bp amplicon displayed a 100% homology to previously isolated CeV-01B (infecting *C. ericina*, Sandaa et al., 2001), and is therefore in the following called CeV/5/2005. Sequencing of the two other amplicons followed by a TBLASTX search, gave highest similarity score to members of the Chlorovirus genus for the product at 347 bp (Fig. 4, OTU/05-01) with highest similarity to the *Chlorella* viruses NY2A and AR158 (Table 1). The product at 407 bp (Fig. 4, OTU/05-02) showed highest similarity to the *Heterosigma akashiwo* infecting virus, HaV-1, and to the Mimivirus when comparing the aminoacid sequence of

the translated amplicon (Table 1). Each of the three amplicons was identified in the concentrate from all three pCO₂ setups.

4 Discussion

4.1 Identification of viral populations

Flow cytometry, PFGE and PCR were applied to monitor the viral community during the experiment. The great advantage of the flow cytometer is its ability to handle great quantities of viral samples with accuracy within reasonable time (Marie et al., 1999; Larsen et al., 2001; Li and Dickie, 2001; Jacquet et al., 2002; Larsen et al., 2004; Baudoux et al., 2006; Martinez-Martinez et al., 2006). It is therefore especially valuable when following the temporal development of various viral populations. The qualitative virus information is limited, however, and out of five different FCM-populations (LFV, MFV, HFV, EhV, PLV) only EhV, with its characteristic flow cytometry signature, could be identified (Castberg et al., 2001; Jacquet et al., 2002; Wilson et al., 2002; Martinez-Martinez et al., 2006). Some published works (Baudoux and Brussaard, 2005; Baudoux et al., 2006) and our own experience with cultivated viruses (unpublished) show that several large dsDNA algal virus have high green fluorescence signal when stained with SYBR Green I and give a signal comparable to HFV in the current investigation. On the basis of the ratio between bacteria and viruses of the LFV group (often around 1:10) it has previously been assumed that the majority of the LFV are bacteriophages (Marie et al., 1999; Wommack and Colwell, 2000; Castberg et al., 2001; Larsen et al., 2001; Zhong et al., 2002; Larsen et al., 2004). This assumption is reasonable also for the current investigation as heterotrophic bacteria were found in concentrations around 10% that of LFV and their dynamics were comparable (Paulino et al., 2007). FCM can in other words give us a coarse idea of the viral community composition, but differences in fluorescence signals between various viruses are often too subtle to allow their identification.

PFGE provides information of the genome size distribution, and to a certain extent concentration of specific populations within the viral community (Wommack et al., 1999; Steward et al., 2000; Larsen et al., 2001; Sandaa and Larsen, 2006). Genome size is an important character for establishing the identity of a virus and the PFGE data took us further in a qualitative description. Lately, this approach has been used in several studies to explore the dynamics in the communities of dsDNA viruses in the marine environment (Steward et al., 2000; Castberg et al., 2001; Larsen et al., 2001; Riemann and Middelboe, 2002b; Jiang et al., 2003; Ovreas et al., 2003; Larsen et al., 2004; Sandaa and Larsen, 2006). These studies have shown that the viral assemblage in the marine environment is distributed in a genome size range from approximately 20 to 560 kb, and that the most dominant

populations have genome sizes between 20 and 100 kb which is also the size range of most cultured marine bacteriophages with dsDNA genomes (Ackermann and DuBow, 1987; Jiang et al., 2003). Our PFGE results demonstrated that viral populations from 26 kb to 137 kb (SGV) were present in high concentrations during the entire experiment, with a dynamic similar to LFV. As such, it is plausible that the majority of SGV as well as LFV, consists mainly of bacteriophages.

Cultured cyanophages infecting *Synechococcus* are reported in the size range from 100–200 kb (Mann, 2003), and the presence of cyanophages in the MGV fraction (150–234 kb) was verified by amplifying excised gel fragments from this part of the PFGE gels using PCR primers targeting the cyanophage *psbD* gene (Clokic, 2006). Moreover, the intensity of the bands in this region on the PFGE gel showed a similar trend to that observed for *Synechococcus* spp. (Paulino et al., 2007), with a higher concentration compared to the fjord water at the initiation of the experiment, followed by a drop and reappearance of multiple bands around day 16. The dynamic of MFV (determined by FCM) followed the same trend, and it is therefore tempting to argue that both MGV and MFV contain cyanophages. It should be noted, however, that also a larger virus (270 kb) and other phytoplankton host groups (picoeukaryotes) displayed a similar dynamic (see below).

Most currently isolated algal viruses belonging to the family Phycodnaviridae have genomes >300 kb (Brussaard, 2004; Dunigan et al., 2006). Seven distinct populations within this size category (LGV) were revealed by the use of PFGE and four of these were genetically identified by allele specific or degenerate PCR primers. One was identified as EhV by combining expected genome size (\approx 410 kb) with allele specific amplification of DNA extracted from the bands observed on the PFGE gels. The dynamic observed by the intensities of bands confirm the dynamic of EhV with a peak somewhat delayed compared to that of the host (Paulino et al., 2007), which is also consistent with previous works showing increased viral concentrations after extensive mortality of *E. huxleyi* (Bratbak et al., 1993; Castberg et al., 2001; Larsen et al., 2001; Schroeder et al., 2003; Martinez-Martinez et al., 2006). LGV populations at \sim 510 kb and 440 kb were also revealed by PFGE. One of these was identified as CeV by allele specific PCR-amplification of the band at 510 kb. CeV appeared in high concentrations from day 15 coincidentally with crashes in nanoplankton populations, the phytoplankton group to which *C. ericina* belongs (Paulino et al., 2007). The presence of CeV in the mesocosms was further confirmed by an amplicon obtained and sequenced using degenerate primers, and the sequence showed 100% homology to a CeV isolated from the same area in 2001 (Sandaa et al., 2001). The 440 kb virus showed a similar temporal pattern to that of CeV. The exact identity of this band on the PFGE gel was not, however, tested with allele specific primers. The temporal development of both CeV and the 440 kb-virus is comparable to that of HFV as determined by

FCM and we suggest that large dsDNA viruses infecting algae constitute a major part of HFV. A fourth large dsDNA virus (270 kb) had a temporal appearance similar to that observed for MFV with high initial concentrations, a sharp decrease and a subsequent substantial increase. As such, it is possible that at least some of the viruses within MFV were the same as those giving rise to the 270 kb band on the pulsed field gel. The dynamic of the 270 kb-virus/MFV basically follows that of picoeukaryotes and to a certain extent *Synechococcus* (Paulino et al., 2007). As MFV concentrations were an order of magnitude (1/10) that of picoeukaryotes and *Synechococcus*, it is therefore tempting to suggest that the 270 kb-virus/MFV infected members of these two phytoplankton groups.

Genome sequencing of isolated viruses assigned to the Phycodnaviridae, have indicated high diversity within the family. Only fourteen core genes have been found to be shared in the currently sequenced genomes of Phycodnaviridae (Allen et al., 2006), and extensive differences in gene content exist even among members belonging to the same genus (Van Etten et al., 2002; Delaroque et al., 2003). Using degenerate primers targeting the *mcp* gene, we were capable of extracting DNA sequences from at least three different viruses. Interestingly, one of these was found to contain a 100% homologue DNA sequence to a previously isolated virus infecting *C. ericina*, isolated from field samples four years earlier (Sandaa et al., 2001). The occurrence of conserved sequences seemingly present over a period of years, suggest a high evolutionary pressure on this particular sequence or gene, beyond what could be expected from the generally short turnover time of viruses. Therefore, although the diversity of the gene content might be high within this family, the phylogenetic evolution might be considerably more constrained for the *mcp* gene in a genus of these viruses (Tidona et al., 1998). Besides the obtained product identical to CeV, two other putative Phycodnaviridae viruses were identified using degenerate PCR primers. One virus had a sequence closest in similarity to members of the Chloroviruses, whereas a second isolate showed strongest homology to the HaV-1 and Mimivirus (Table 1). The similarity to the Mimivirus is interesting in view of the fact that this virus previously have been assigned to a new family, the Mimiviridae, whose closest match was found in metagenome libraries from the Sargasso Sea (Ghedini and Claverie, 2005).

4.2 CO₂ effect on viral populations

The PFGE data revealed absence/presence of some viral populations as response to changed pCO₂ levels with one SGV and one LGV (110 kb and 440 kb) being observed exclusively at 3 \times CO₂, two SGVs (105 kb and 137 kb) solely at 1 \times CO₂, and two other SGVs (26 kb and 70 kb) at the 2x and 3 \times CO₂ only. The PFGE analysis were performed on material from only one mesocosm of each treatment (enclosure 2, enclosure 5, enclosure 8), and only a few of the populations

were identified and related to specific hosts. These results can therefore only indicate that CO₂ conditions in the ocean might affect viral production. The abundance of EhV and HFV, as revealed by flow cytometric analyses, decreased significantly with increasing pCO₂ while their hosts (*E. huxleyi* for EhV and nanoeukaryotes for HFV) showed the reverse response with a slight increase in abundance at higher CO₂ levels (Paulino et al., 2007). This negative effect on viral production is most clearly shown for *E. huxleyi*/EhV since specific concentrations of both host and virus are known. The EhV burst size can hence be calculated to 2890(+/-593), 2495 (+/-581) and 2032 (+/-339) virus per lysed cell for the 1×, 2× and 3×CO₂ treatments, respectively. The reduced viral concentration is thus not caused by lower host abundance but seems to be an effect of altered host-virus interaction or changes viral replication conditions. Moreover, the organisms in size group 5–10 μm (the size group to which *E. huxleyi* belongs) increased their primary production at high CO₂ levels (Egge et al., 2007) whereas viral production declined, supporting the idea that reduced viral concentration was caused by changes in host-virus interaction rather than being a secondary effect of reduced algal production.

If the coccoliths of *E. huxleyi* serve as a protection against viral attack (Young, 1994) it may be speculated that changes in the structure and calcite content of the shells may interfere with viral attachment and production. Increased pCO₂ has previously been demonstrated to cause undercalcification and malformation of coccoliths in *E. huxleyi* (Riebesell et al., 2000) and may suggest an increased rather than a decreased virus production as observed in this study. However, if virus attachment is related to the surface structure of the coccoliths we may also imagine a decreased virus production. *E. huxleyi*-blooms consist of genetically diverse *E. huxleyi* and EhV communities (Martínez-Martínez et al., 2007) and an alternative explanation to variable viral abundance could thus be that different genotypes, with varying ability to produce viruses, dominates at different CO₂ levels. The reduction in viral production with increasing CO₂ levels was more pronounced for HFV than for EhV. HFV is most likely a mixed group of several dsDNA viruses infecting different nanoeukaryotic algae, and since the nanoeukaryotes as a group showed a slight increase in abundance at higher CO₂ levels it is tempting to suggest that the reduced HFV production is due to an effect of CO₂ on the viruses or the host-virus interaction and not on the hosts or host abundance. We can however not exclude the possibility that the actual HFV hosts responded different from the nanoeukaryotes group as a whole and this limited resolution of community structure and host-virus relationships preclude further analysis of the effect of CO₂ level on HFV. It should also be noted that small differences in host abundances, not detectable by the methods used here, will be reinforced through the multiplication of viruses taking place in every host cell.

CO₂ manipulation did not have a discernible effect on LFV and MFV which consist of both bacterio- and

cyanophages. This result supports the observation that there was no difference in bacterial abundance or production (Allgaier et al., 2007). Our limited ability to resolve individual host and virus populations within the bacterioplankton, the LFV and the MFV, will presumably mask any effects of CO₂ that are not widespread and further analysis of the effects of CO₂ is hence also in this case precluded.

The observations discussed above support the idea that increased CO₂ levels may result in less effective phytoplankton viruses. However, increased CO₂ result in lower pH and the observations may thus also be interpreted as a pH effect. From the literature it is known that some viruses are unaffected by pH=3 or even less, whereas others are labile at pH<7 (Krueger and Fong, 1937; Weil et al., 1948; Jin et al., 2005). This difference in pH stability has in some cases been argued to represent adaption to a way of life (Rueckert, 1996). Weinbauer (2004) stated that pH can affect adsorption of phages in freshwater while marine phages are typically only affected by pH values deviating from that of seawater. Cyanophages in freshwater appears to have a broader tolerance for pH (5–11) than bacteriophages in general (Suttle, 2000). Brussaard et al. (2004) demonstrated loss of infection for MpRNAV-01B, a virus infecting the marine eukaryotic algae *Micromonas pusilla*, at pH≤5. We are not aware of additional studies that investigate the effect of pH on marine phytoplankton viruses and host-virus interactions. The pH in our mesocosms never dropped below 7.6 and the maximum difference in pH between the treatments was 0.47 pH units (7.64 in 3×CO₂ and 8.11 in 1×CO₂) (Bellerby et al., 2007). The pH in many of the above mentioned studies are hence out of range and of limited relevance to the present study, but it is nevertheless not inconceivable that phytoplankton host-virus interactions are affected by pH changes and that ocean acidification, at least when taken to an extreme, may affect the marine microbial food web at the viral level.

We have here demonstrated the possibility of identifying several dsDNA algal viral populations from environmental samples, and partially connect them to specific host populations. It is obvious, however, that there is a great need for a continued effort to develop molecular markers that can be used to identify both viruses and their host. It is also apparent that such identification will help us to a better understanding of the effect that environmental factor, like altered CO₂ and/or nutrient conditions, may have on the base of the pelagic food web.

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