The role of viruses in marine phytoplankton mortality
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Chapter 5

Phytoplankton losses in the North Sea during summer 2003

Anne-Claire Baudoux, Marcel J. W. Veldhuis, Anna A. M. Noordeloos, Govert van Noort, and Corina P. D. Brussaard

This study investigated microzooplankton grazing and virally mediated mortality of the eukaryotic (3 size classes) and prokaryotic (Synechococcus) picophytoplankton across the North Sea during summer 2003. Our results showed that the fate of picophytoplankton differed among algal groups and their area of occurrence. Highest viral lysis rates (0.16 – 0.23 d⁻¹) were recorded for specific picoeukaryotic groups in the coastal region and the station investigated at the DCM. The virally induced turnover rate of the host abundance was around 20% d⁻¹, which corresponded with the percentage dead cells observed for these respective groups (13 to 32%; using the live/dead dye SYTOX Green). Viral lysis was insignificant at the other stations, despite considerable percentage dead algal cells (up to 38%). Microzooplankton actively grazed upon picoeukaryotes (average per group 0.2 - 0.4 d⁻¹), independent of the region studied. Grazing on Synechococcus was restricted to the coastal waters (0.7 d⁻¹). Grazing by microzooplankton consumed on average 40 ± 27% of the carbon produced by picophytoplankton, constituting in general the main loss factor. Viral lysis released on average 8 ± 13% of the total picophytoplankton carbon production, and locally up to 32%.

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1. Introduction

Marine phytoplankton are responsible for up to half of the global primary production on Earth, and hence play a critical role in global carbon cycling (Geider et al. 2001). Traditionally, phytoplankton cells were treated as immortal unless they were preyed upon by zooplankton or lost by sedimentation through the water column. Over the past decades, our view of phytoplankton mortality has been transformed with the finding that algal cells can die by lysis. The reports of substantial algal cell lysis rates (Brussaard et al. 1995, 1996a, Agusti et al. 1998) and high fraction of dead phytoplankton cells (Veldhuis et al. 2001, Alonso-Laita & Agusti 2006) in marine environments emphasized the significance of cell death for phytoplankton mortality. Algal cell death by lysis can be caused by different mechanisms amongst which viral infection (Suttle et al. 1990, Brussaard 2004a) and environmental stresses, such as intense light (Berman-Frank et al. 2004), darkness (Berges & Falkowski 1998), or nutrient depletion (Brussaard et al. 1997, Berman-Frank et al. 2004).

The partitioning of phytoplankton mortality into sinking, grazing, and cell lysis is important because these loss factors affect the structure and the functioning of the pelagic microbial food web differently. Phytoplankton sedimentation results in the transfer particulate organic matter from the pelagic towards the benthic ecosystems (Smetack 1985), whilst the grazing will mostly channel phytoplankton biomass to the higher trophic levels in the pelagic food web. Through cell lysis, phytoplankton biomass is converted to dissolved organic matter that becomes available for bacteria, hence forcing the food web towards a more regenerative pathway (Wilhelm & Suttle 1999 and reference therein). Knowledge on the relative contribution of these loss factors for phytoplankton mortality is thus critical for an optimal understanding of the flow of energy and nutrient in marine environments.

Extensive field studies conducted in the North Sea indicated a pronounced temporal variation in the ecological relevance of these different loss factors (Riegman et al. 1993, Brussaard et al. 1995, 1996a, Kuipers & Witte 1999). During the eutrophic spring conditions in this temperate region, grazing by microzooplankton sets the limit of the small-sized phytoplankton whereas larger counterparts can form blooms while they escape grazing due to their size (Riegman et al. 1993, Brussaard et al. 1996a). Sedimentation was reported to be responsible for the termination of the diatom bloom in early spring, whereas algal cell lysis accounted for up to 75% of demise of the following Phaeocystis globosa bloom (Brussaard et al. 1995). Recent investigations indicated that viruses were the primary cause of cell lysis (Brussaard et al. 2005a, Baudoux et al. 2006).

The substantial nutrient consumption during these spring blooms forces the system towards a more oligotrophic status by summer. In summer, smaller-sized phytoplankton dominate the algal community (Kuipers & Witte 1999). These algal cells are not likely to sink considering their micrometer range size (Raven 1998), and microzooplankton is considered the major loss factor (Kuipers & Witte 1999). However, virtually no data exist on the significance of virally mediated mortality for phytoplankton
in the North Sea during summer (Brussaard et al. 1996b).

Our study compared the relative contribution of both viral lysis and microzooplankton grazing of the smaller-sized phytoplankton during oligotrophic summer conditions and explored the variability of these loss factors across the North Sea.

2. Material and methods

**Study area and sampling.** The data were obtained during the MOMAP-2 shipboard expedition in the North Sea from 8 to 20 July 2003, aboard the R.V. Pelagia (Fig. 1). During this cruise, 11 pelagic stations were sampled for physical and chemical parameters, microbial abundance, and phytoplankton viability (cell membrane integrity). Among these 11 stations, 5 main stations were also investigated for microzooplankton grazing and viral lysis. Samples were collected in 10 L NOEX bottles mounted on the Rosette sampler equipped with Seabird conductivity-temperature-depth (CTD). Typically, samples were collected at 6 different depths covering the upper 65 m of the water column (except for station 2 and 12, as max. depth was 45 m). The euphotic zone (1% light penetration) comprised the upper 23 to 45 m.

![Figure 1. Location of the sampling stations during the MOMAP-2 expedition conducted in the North Sea from 8 to 20 July 2003.](image)

**Physical and chemical parameters.** The salinity, temperature and light intensities were measured by the CTD Seabird mounted on the Rosette sampler equipped with a PAR detector. A PAR- detector was also mounted on deck to determine incident light. Nutrient samples (5 mL) were filtered through 0.2 µm pore-size polysulfone filters (Acrodisc, Gelman Sciences). Analyses were performed on board using a TrAAcs 800
autoanalyzer for dissolved orthophosphate, nitrate, nitrite, ammonium, and silicate as described in Baudoux et al. (2006). The limit of detection was 0.007 μM for phosphate, 0.15 μM for ammonium, 0.002 μM for nitrite, 0.03 μM for nitrate, and 0.02 μM for silicate.

**Phytoplankton community.** Chlorophyll a (Chl a) samples (typically 1.5 l) were filtered onto a GF/F filter (Whatman) and stored at -80°C until analysis. Chl a was extracted in 90% acetone and measured fluorimetrically.

Phytoplankton abundance from natural as well as experimental samples was enumerated directly after sampling using a Beckman Coulter XL-MCL flow cytometer. The instrument was equipped with a laser with an excitation wavelength of 488 nm (15 mW) and emission bands for the chlorophyll autofluorescence (> 630 nm) and phycoerythrin (PE 575 ± 20 nm). The discriminator for phytoplankton was the red chlorophyll autofluorescence. Flow rate (135 ± 7 μL min⁻¹) and machine drift were checked every day using calibrated beads (Flow-Check Fluorospheres, Beckman Coulter) as internal standard. Based on the pigment autofluorescence and forward scatter, we discriminated the prokaryotes *Synechococcus* spp. as well as 5 populations of eukaryotic cells. The division of the eukaryotes was based on their relative size as determined by flow cytometry using a series of fractionated samples according to Veldhuis & Kraay (2004). Using this procedure, the cyanobacterium *Synechococcus* had an equivalent spherical diameter (esd) of 1.1 μm. Three picoeukaryotic groups were discriminated with esd ranging between 1.1 and 1.5 μm (Pico I), 1.5 and 2.0 μm (Pico II), and 2.0 and 3.0 μm (Pico III). Furthermore, we defined 2 nanoeukaryotic groups, in lesser abundance, with esd ranging between 3.0 and 6.0 μm (Nano I), and 10 and 20 μm (Nano II). The determination of the cellular carbon content of each phytoplankton group was based on biovolume and assuming phytoplankton cells to be spherical. For *Synechococcus*, we used an averaged biovolume to carbon conversion factor of 260 fg C μm⁻³ derived *Synechococcus* WH8103 and WH8012 (average diameter 1.02 μm; Bertlisson et al. 2003). For the picoeukaryotes, we used a biovolume to carbon conversion factor of 239 fg μm⁻³, an average of the values obtained for Ostreococcus sp. CCE9901 (233 – 247 fg C μm⁻³; Worden et al. 2004) and *Micromonas pusilla* CCMP 489 (238 fg C μm⁻³; DuRand et al. 2002). A biovolume to conversion factor of 160 fg μm⁻³ was used for the nanoeukaryotes according to Verity et al. (1992). The resulting averaged cellular carbon content were 0.18 ± 0.004 pg cell⁻¹(mean ± SD) for *Synechococcus*, 0.22 ± 0.02 pg cell⁻¹ for Pico I, 0.52 ± 0.24 pg cell⁻¹ for the Pico II, 2.3 ± 1.1 pg cell⁻¹ for Pico III, and 8.0 ± 5.4 pg cell⁻¹ for Nano I and 321 ± 707 pg cell⁻¹ for Nano II.

The abundance of dead algal cells was determined using the nucleic acid-specific stain SYTOX Green (Molecular Probes, Invitrogen) to test cell membrane permeability (Brussaard et al. 2001). SYTOX Green can only penetrate and stain cells with a compromised plasma membrane (i.e., by definition dying cells). Briefly, samples were stained with SYTOX Green (final concentration 0.5 μM) for 15 min in the dark prior to flow cytometric analysis using a Beckman Coulter XL-MCL flow cytometer (see
above section for instrument specification). Within each phytoplankton subgroup, cells were separated in live and dead on the basis of their relative green fluorescence; dead cells had >5 times the green autofluorescence fluorescence of live cells. Only the numerically dominating picophytoplankton groups (Synechococcus, and Pico I, II, and III) allowed proper analysis (typically down to a depth of 45 m). For practical reasons, we restricted generally the analysis of live/dead algal cells to 1 station per region (region 1 was represented by station 10, region 2 by station 2, and region 3b by station 6). More stations (3, 4, 5, and 8) were, however, analyzed for region 3a in order to test the results consistency for different stations within one region.

**Virus community.** The abundance of putative algal viruses was determined on glutaraldehyde fixed samples (final concentration 0.5% glutaraldehyde, frozen in liquid nitrogen and stored at -80°C prior analysis) according to Brussaard (2004b), using a Beckton-Dickinson FACSCalibur flow cytometer equipped with a 15 mW 488 nm air-cooled argon-ion laser and a standard filter set up. The discriminator for virus enumeration was the green fluorescence. Thawed samples were 100-fold diluted in 0.2 µm filtered sterile TE-buffer (pH 8) and stained with 5 × 10^-5 of the commercial SYBR Green for 10 min at 80°C in darkness. Counts were corrected for the blank consisting of TE-buffer with sterile 0.2 µm filtered seawater in the correct dilution. Based on their relative green fluorescence and side scatter signature, we distinguished 4 virus subpopulations (V1, V2, V3, and V4, Fig. 2). Considering the viral FCM signatures of algal viruses brought in culture (Brussaard 2004b), the viral group V3 included to some extent putative algal viruses whereas group V4 consisted mainly of algal viruses.

![Figure 2. Cytogram of natural viral community. A maximum of four viral groups (V1, V2, V3, and V4) were discriminated based on the intensity of their green fluorescence after staining with the nucleic acid-specific dye SYBR Green I and the side scatter. V4 was assigned to putative algal viruses. V3 most likely also contains algal viruses. An internal standard (yellow green fluorescent 1 µm beads, Invitrogen – Molecular Probes) was added to the sample.](image)
Viral diversity was examined in the surface (5 m) for all stations and at the DCM of stations 2, 3, and 14 using pulsed field gel electrophoresis (PFGE) as described by Larsen et al. (2001). Summarizing, a concentrate of 2 liters water samples was plugged in molten InCert agarose (1.5 % (w/v), Cambrex Bioscience, Rockland, ME USA) and digested overnight at 30°C in a lysis buffer. Samples were loaded onto a 1% SeaKem GTG agarose gel (Cambrex Bioscience, Rockland, ME) and electrophored using a Bio-Rad DR-II CHEF Cell unit operating at 6 V cm⁻¹ at 14°C in 0.5× TBE tank buffer. Two pulse ramp settings were used for an optimal sizing of viral genomes (1) 1-6 s for 20 h to examine the smaller virus genomes ranging between 10 – 100 kb and (2) 8-30 s for 20 h to discriminate the larger virus genomes comprised 50 – 400 kb. After electrophoresis, gels were stained for 1 h with SYBR Green I (1×10⁻⁴ of commercial solution) and destained 10 min in MilliQ before a digital analysis for fluorescence using a FluorS imager (Bio-Rad Instruments). Viral genomes sizing was performed against a 5 kb lambda ladder or a lambda concatamers ladder (Bio-Rad, Richmond, CA). We determined the relative abundance of the different viral genome sizes using the Hyperladder VI (Gentaur) DNA standard with known size and amount of DNA. Large genome-sized dsDNA viruses are most likely algal viruses (Mann 2003, Brussaard 2004a); therefore viruses with genome sizes >100 kb were identified as putative algal viruses during this study. We realize that algal viruses may present genome size <100 kb, these smaller genome sized virus could not be included in the analysis because many of the virus with genome <100 kb viruses are phages infecting heterotrophic prokaryotes.

**Phytoplankton mortality assays.** Phytoplankton viral lysis and microzooplankton grazing rates were, for practical reasons, determined in surface (5 m) for one representative station per region only (region 1 was represented by station 10, region 2 by station 2, and region 3b by station 6, Table 1), except for region 3a for which 2 stations were investigated (stations 3 and 5) to allow intraregion-specific variation. Furthermore, only for station 3 an additional experiment could be performed at the deep chlorophyll a maximum depth (DCM, 37 m). Sample collection was performed by carefully siphoning from NOEX bottles into darkened carboys to prevent light stress when the sample was brought on deck. All material used for these experiments were acid cleaned (0.1N HCl), rinsed with MilliQ and finally with the same water as the sample. The experimental set up was performed in a controlled room at in situ temperature (10 - 19 °C) and in dimmed light.

We estimated viral lysis and microzooplankton grazing rates using an adapted dilution technique by Baudoux et al. (2006) which was based on the assay by Evans et al. (2003). The data analysis was restricted to the numerically dominating picophytoplankton groups (Synechococcus, Pico I, II, and III), as algal abundance needs to be high enough to allow detection after a 5-fold dilution (standard dilutions 20, 40, 70, and 100% of natural water). The natural seawater (<200 µm) was diluted with 0.2 µm pore-size filtered diluent to obtain the microzooplankton grazing rate (Landry & Hassett 1982). An additional dilution series with 30 kDa filtered natural sample was performed.
to provide the loss rate resulting from grazing and viral lysis. A detailed experimental design can be found in Baudoux et al. (2006). A 5 mL subsample was taken (T=0) from the soft polycarbonate incubation bottles (triplicate) upon filling, after which the bottles were closed without trapping air bubbles inside. All bottles were mounted on a slowly rotating (0.5 rpm) plankton wheel. Incubation temperature (10 – 19°C) and irradiance (3 – 100 µmol quanta m⁻² s⁻¹, light period of 14 h) were adjusted to the in situ conditions given by the CTD and PAR detectors. After 24 h incubation, another 5 mL subsample was taken to monitor phytoplankton growth using flow cytometry. The apparent growth rate (µₐₚₚ, d⁻¹) was calculated for each sample from the changes in abundance during the incubation. The regression coefficient of apparent growth rate vs. dilution factor for the 30 kDa series represents phytoplankton losses due to microzooplankton grazing and viral lysis (Mₐᵥ, d⁻¹), whereas the regression coefficient resulting from the 0.2 µm dilution series represents only the microzooplankton grazing rate (Mₐ, d⁻¹). Specific virally induced mortality rates (d⁻¹) were thus obtained from the difference between Mₐᵥ and Mₐ. Specific growth rates (µ, d⁻¹) were determined as the y-axis intercept value of the regression line obtained with the 30 kDa series. The significance (p) of each slope (Mₐ and Mₐᵥ) and the intercept (µ) were determined performing a t-test on the regression analysis. The significance (p) between the slopes of the regressions lines (i.e., significance of Mᵥ) was also estimated using a t-test.

### Table 1. Location and characteristics of the 5 main stations representative of the different water masses. Region 1 is represented by station 10; region 2 by station 2; region 3a by stations 3 and 5, and region 3b by station 6.

<table>
<thead>
<tr>
<th>Station</th>
<th>2</th>
<th>3</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>10</th>
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<td>55.83</td>
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<td>5</td>
<td>-0.02</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Temperature (°C)</td>
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<td>15.5</td>
<td>9.9</td>
<td>14.4</td>
<td>12.8</td>
<td>19.2</td>
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<tr>
<td>Salinity</td>
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<td>35.0</td>
<td>35.2</td>
<td>35.3</td>
<td>31.7</td>
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<tr>
<td>NO₃ (µM)</td>
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<td>0.06</td>
<td>0.35</td>
<td>0.24</td>
<td>2.33</td>
<td>0.06</td>
</tr>
<tr>
<td>NO₂ (µM)</td>
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<td>0.01</td>
<td>0.06</td>
<td>0.01</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>NH₄ (µM)</td>
<td>0.16</td>
<td>0.12</td>
<td>0.60</td>
<td>0.27</td>
<td>0.19</td>
<td>0.12</td>
</tr>
<tr>
<td>PO₄ (µM)</td>
<td>0.02</td>
<td>0.03</td>
<td>0.24</td>
<td>0.03</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>Si (µM)</td>
<td>0.04</td>
<td>0.81</td>
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<td>0.21</td>
<td>0.34</td>
<td>0.27</td>
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<tr>
<td>Chl a (µg L⁻¹)</td>
<td>0.51</td>
<td>0.25</td>
<td>0.83</td>
<td>0.28</td>
<td>1.77</td>
<td>0.68</td>
</tr>
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</table>
In parallel, the protistian grazing rates on cyanobacteria were also determined using the fluorescently labeled prey approach. Labeling of *Synechococcus* sp. S1 (~ 1 µm diameter, NIOZ culture collection) was performed prior to the cruise with 4,6-dichlorotriazin-2-yl aminofluorescein (DTAF, Sigma-Aldrich, St Louis, MO) according to Sherr and Sherr (1993). The fluorescently labeled cyanobacteria (FLC) were enumerated by epifluorescence microscopy (Zeiss, Axioplan 12500 X) and aliquots of known concentration were stored at -20°C until use. On board, FLC were diluted to <10% (v/v) of the natural cyanobacterial abundance by adding natural samples (triplicate, 1 l polycarbonate bottles). A 20 mL subsample (T=0) was taken upon filling and immediately fixed with 10% glutaraldehyde (1% final concentration). The fixed sample was filtered onto a 0.2 µm pore-size black membrane polycarbonate filter (Whatman) and stored at -20°C until analysis. Bottles were closed and incubated as described for the modified dilution assay. After 24 h incubation, another 20 mL subsample was taken and treated similarly for monitoring the abundance of FLC. The estimation of the grazing rates (d⁻¹) of *Synechococcus* was based on the decrease in abundance of FLC.

A carbon budget was determined combining the cellular carbon content estimates (see phytoplankton community section) and data of the dilution experiments. For each specific phytoplankton group, the carbon production (CP, in µg C L⁻¹ d⁻¹), losses due to grazing (G, µg C L⁻¹ d⁻¹) and, by adaptation, the losses due to viruses (V, in µg C L⁻¹ d⁻¹) were calculated using the formulas of Landry et al. (2000); CP = µ × Pm; G = Mg × Pm; V = Mv × Pm; and Pm = P₀ × [e(^µt) - Mv⁻^t + 1]/(µ - Mv⁻^t), where µ (in d⁻¹) is the dilution-based specific growth (y intercept of the 30 kDa regression); Mg and Mv are the dilution-based grazing and viral lysis rates (in d⁻¹), P₀ (in µg C l⁻¹) is the initial carbon biomass of picophytoplankton, Pm (in µg C l⁻¹) is the geometric mean carbon biomass of picophytoplankton during the incubation and t (in d) is the time of incubation.

### 3. Results

#### 3.1. Physical and chemical characterization of the studied area

Based on the T-S diagrams, 4 hydrographic regions were distinguished in the studied area (Fig. 3). Region 1 included the Norwegian coastal stations (9 and 10) and was characterized by large gradients in salinity (29 to 34.5) and temperature (6 to 19.5°C). Region 2 corresponded to the southeastern North Sea (stations 2, 12, and 14), characterized by intermediate salinity (34.2 to 34.5) and temperatures between 6 and 18°C. The central and northern North Sea waters (stations 3, 4, 5, 6, 8, and 13) defined a third region, characterized by the most saline waters (34.7 to 35.5). Within this latter region, a gradient of increasing salinity was observed in a northward direction. Based on
these criteria, we distinguished a region 3a including stations 3, 4, 5, 8, and 13 (salinity range within 34.7 - 35.2 and temperature within 7.1 and 16.6°C) and a region 3b represented by station 6 (salinity range within 35.0 - 35.4 and temperature within 9.4 and 12.7 °C). A thermal stratification was observed in regions 1, 2, and 3a but not in region 3b. Among these 3 stratified regions, only regions 2 and 3a presented a well defined surface mixed layer extending to about 15 m.

The surface (down to 20 m) concentrations of inorganic nutrients were <0.04 µM for PO₄, <0.35 µM for NO₃, <1 µM for Si and <0.33 µM for NH₄ in regions 1, 2, and 3a. In region 3b, the concentration of PO₄ and NO₃ in the surface waters (down to 15 m) were somewhat higher (0.13 and 2.3 µM, respectively), while Si and NH₄ levels were comparable to that of the other stations. Below these depths, all nutrient levels increased (max. 0.71 µM for PO₄, 9.9 µM for NO₃, and approx. 4.5 µM for Si and NH₄). The increase in PO₄ and NO₃ (0.20 and 0.49 µM, respectively) was, however, less pronounced in region 2.

### 3.2. Phytoplankton distribution

All stations presented a deep chlorophyll a maximum (DCM) of 0.8 - 2.1 µg Chl a L⁻¹, located between 15 and 40 m (Fig. 4). The total phytoplankton cell abundance exhibited a maximum typically located 5 to 10 m above the DCM (Fig. 4). The depth integrated phytoplankton cell abundance over the upper 45 - 65 m ranged from 0.7 × 10⁸ (region 2) to 3 × 10⁸ cells cm⁻² (regions 1 and 3a), of which 98 ± 2% were picophytoplankton cells, independent of the region. The phytoplankton community in the regions 1, 2, and 3a was numerically dominated by the cyanobacterium *Synechococcus,*
accounting for $82 \pm 8\%$ of the depth integrated phytoplankton abundance. The concurring picoeukaryotic groups comprised 4 to 7% and the nanoeukaryotes contributed for less than 2% of the depth integrated total abundance. The nutrient enriched region 3b presented a different phytoplankton community structure with a lower share of *Synechococcus* (23% of the depth integrated cell abundance). The Pico I, II, and III accounted for 21, 35, and 12% and the nanoeukaryotes comprised 10% of the total community.

![Depth profiles](image_url)

*Figure 4. Depth profiles of Chlorophyll a (Chl a, µg L$^{-1}$) and total picophytoplankton abundance (mL$^{-1}$) for the different geographical regions distinguished.*
The cell abundance of *Synechococcus* and Pico I distributed similarly for regions 1, 2, and 3a (Fig. 5). Both groups developed a subsurface peak (15 – 35 m) averaging $9 \pm 3 \times 10^4$ cells mL$^{-1}$ for *Synechococcus* and $10 \pm 5 \times 10^3$ mL$^{-1}$ for Pico I. By comparison, Pico II and III abundance peaked deeper (35 - 40 m, $2 \pm 1 \times 10^3$ mL$^{-1}$ for each group) in regions 2 and 3a. In region 1, the abundance of these latter groups was high in the surface ($10$ and $2.5 \times 10^3$ cells mL$^{-1}$ for Pico II and III, respectively) and decreased with depth. In region 3b, all picophytoplankton groups showed similar distributions with high abundance in the upper 15 m ($\sim 1 \times 10^4$ cells mL$^{-1}$ for *Synechococcus*, Pico I and II, and $3 \times 10^3$ cells mL$^{-1}$ for Pico III) and decreasing gradually with depth.

The distribution of % dead algal cells differed among the picophytoplankton groups and the area of occurrence (Fig 5). The % dead *Synechococcus*, Pico I, and Pico II increased (up to 75%) below 15 m in the coastal region 1. The % dead Pico III increased below 40 m to 60 to 80%, regardless of the region. Interestingly, we found a negative and significant relation between the fraction of dead *Synechococcus* and their cell abundance across the entire studied area ($r = -0.36$, $n = 40$, $p < 0.05$, Spearman rank order test).

In term of carbon biomass, the picophytoplankton groups comprised between 0.25 and 0.58 g C m$^{-2}$, which corresponded to $51 \pm 17$ % of depth integrated phytoplankton carbon biomass. Through the entire studied area, the carbon biomass of the picoeukaryotes equaled (54 ± 16%) that of the cyanobacterium *Synechococcus*.

### 3.3. Virus community

The virus community was discriminated into 4 groups based on their DNA green fluorescence and side scatter signature (Fig. 2). Total viral abundance was between $3$ and $7 \times 10^7$ mL$^{-1}$ in the top 15 - 30 m, below which it progressively declined (down to $1 – 3 \times 10^7$ mL$^{-1}$). The abundance of the putative algal virus group (V4) in the surface waters of region 3b was at least 4-fold higher ($8 \times 10^5$ mL$^{-1}$) than for the other regions (Fig. 6). The viral group V3, including some algal viruses, showed increased surface abundance at the northern stations of region 3a. Surface V3 abundance comprised up to 11% of the total virus abundance at station 5 while it was between 2 to 4% at the other stations.
Figure 5. Depth profiles of the abundance of the 4 picophytoplankton groups (solid circle) and their respective percentage of dead cells (% open circle) for each regions of North Sea. Dead cells were discriminated based on their membrane integrity using the nucleic acid stain SYTOX Green. Region 1 is represented by station 10; region 2 by station 2; region 3a by stations 3, 4, 5 and 8 (averaged in figure); and region 3b by station 6. Errors bars correspond to SD.
Figure 6. Depth profiles of viral group V4, assigned to putative algal viruses and group V3 most likely containing putative algal viruses (virus mL⁻¹) for the different geographical regions.
The virus diversity as determined by PFGE showed 5 to 20 distinct genome sizes per sample ranging from 30 to 280 kb (Fig. 7). All samples displayed viral genomes size ranging between 30 and 145 kb. Note that viral diversity in region 3b is likely underestimated as only 20% of the plugged viruses migrated (reason unknown). The viral community was dominated by small sized viral genomes (<100 kb) comprising on average 98 ± 1%. The largest viral genomes (105, 115, 145, 155, 165, and 180 kb) accounted for 0.4 to 5.5% of the viral community, with the greatest variability in richness and contribution found in region 3a. The viral diversity examined at the DCM (stations 2, 3, and 14) showed an increased richness and abundance of viral genomes ranging from 85 to 280 kb as compared to the surface sample.

**Figure 7.** Schematic outline of the relative abundance (indicated by the area of the dot) of viral genome sizes determined by PFGE fingerprinting. The PFGE was performed using two settings to optimally determine the small sized genomes (30 – 100 kb; basically bacteriophages) and the large sized genomes (50 - 400 kb, including the putative algal viruses).
3.4. Viral lysis and microzooplankton grazing

Viral lysis was substantial (0.16 - 0.23 d\(^{-1}\)) and significant (p < 0.05) in the surface the coastal Norwegian station 10 (region 1) and at the DCM of station 3, but only for the picoeukaryotic groups Pico I and Pico III (Fig. 8 and Table 2). In the surface waters of the offshore stations 2, 3, 5, and 6 (representatives of regions 2, 3a, and 3b), viral lysis could be detected but estimates were not statistically significant (p > 0.05).

Microzooplankton grazing rates ranged widely (from insignificant to 0.72 d\(^{-1}\)) among the different picophytoplankton groups and geographical area (Fig 8, Table 2). *Synechococcus* grazing was high at station 10 (0.72 d\(^{-1}\)), whilst it was considerably lower (0.05 ± 0.05 d\(^{-1}\)) at the offshore stations 2, 3, 5, and 6. This considerable variation in *Synechococcus* grazing rates was also reflected in the grazing rates obtained using the independent FLC assay. The two grazing methodologies showed a strong correlation (r = 0.98, n = 5, p < 0.05, Spearman rank order test). Conversely to *Synechococcus*, the picoeukaryotic groups were substantially grazed upon at all stations (average 0.13 ± 0.16 d\(^{-1}\) for Pico I, 0.18 ± 0.10 d\(^{-1}\) for Pico II, and 0.29± 0.16 d\(^{-1}\) for Pico III).

The algal growth rates in the surface waters varied considerably among the picophytoplankton groups (from 0.1 – 2.1 d\(^{-1}\), Table 2) without a clear geographical trend. The picocyanobacterium *Synechococcus* consistently presented the highest growth rates (0.81 d\(^{-1}\) to 2.1 d\(^{-1}\)) except for station 6 (region 3b, 0.17 d\(^{-1}\)) and station 3 at the DCM (-0.08 d\(^{-1}\)). The growth rates of the picoeukaryotes varied on average between 0.25 and 0.41 d\(^{-1}\) (0.25 ± 0.15 d\(^{-1}\), 0.34 ± 0.16 d\(^{-1}\), and 0.41± 0.20 d\(^{-1}\) for Pico I, II, and III, respectively).

3.5. Daily carbon production and losses

The total picophytoplankton carbon production (CP) varied greatly between stations (0.5 to 15 µg C L\(^{-1}\) d\(^{-1}\), Table 2) with the highest value found at the coastal station 10 (region 1, 15 µg C L\(^{-1}\) d\(^{-1}\)). Overall, the picoeukaryotic groups substantially contributed to the total picophytoplankton CP (on average 57 ± 35%).

The total picophytoplanktonic carbon losses due to viral lysis and microzooplankton grazing (0.3 to 8.4 µg C L\(^{-1}\) d\(^{-1}\)) balanced overall on average 49% of the picophytoplanktonic CP with considerable variation among picophytoplankton groups. Looking specifically at the picoeukaryotes, most of the CP was actually counterbalanced by microzooplankton grazing and viral lysis (on average per group 60 to 120%). For *Synechococcus* only 10% of the CP was lost. Analysis per station shows that only in the surface water of coastal station 10 (region 1) both viral lysis and microzooplankton grazing were responsible for the total carbon loss (contributing for 29 and 71%, respectively). For the other stations microzooplankton alone accounted for the total carbon loss in the surface waters (96 – 100%). At the DCM of station 3, however, virally mediated carbon loss (0.2 µg C L\(^{-1}\) d\(^{-1}\)) prevailed over grazing induced carbon loss (0.1 µg C L\(^{-1}\) d\(^{-1}\), comprising 32% of the picophytoplankton CP (0.5 µg C L\(^{-1}\) d\(^{-1}\)).
Figure 8. Plots of apparent growth rate vs. fraction of natural water in the parallel dilution experiment for the different picophytoplankton groups at the main stations. Region 1 is represented by station 10; region 2 by station 2; region 3a by stations 3 and 5, and region 3b by station 6. Parallel dilution experiments were performed in 30 kDa ultrafiltrate (no grazer, no virus) and 0.2 µm (no grazer) filtered seawater. Microzooplankton grazing rates correspond to the regression slope obtained with the 0.2 µm dilution series. Viral lysis rates correspond to the difference of regression coefficients of the 0.2 µm and 30 kDa series. For the readability of the figures, we averaged the triplicate apparent growth for each dilution level. This operation did not affect the estimated mortality rates. The error bars reflect the SD between measurements.
Table 2. Dilution-based specific growth ($\mu$, d$^{-1}$), microzooplankton grazing ($G$, d$^{-1}$), and viral lysis rates ($V$, d$^{-1}$) for the picophytoplankton groups and their abundance ($\times 10^3$ mL$^{-1}$) and fraction of dead cells (dead, %) at the onset of the experiment. Daily picophytoplanktonic carbon production (CP, $\mu$g C l$^{-1}$ d$^{-1}$) and the fraction of the CP consumed by microzooplankton (G : CP) and viruses (V : CP) was calculated for each experiment. Region 1 is represented by station 10; region 2 by station 2; region 3a by stations 3 and 5, and region 3b by station 6. Asterix correspond to significant values ($p \leq 0.05$).

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<th>Abundance</th>
<th>$\mu$</th>
<th>$G$</th>
<th>$V$</th>
<th>dead</th>
<th>CP</th>
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4. Discussion

4.1. Grazing and viral lysis

The phytoplankton community in the North Sea during summer distributed into 6 size-classes (1 to 20 µm), amongst which the picophytoplankton component (<3µm) dominated the community. The extent of the losses due to viral lysis and microzooplankton varied widely among the picophytoplankton groups and their geographical location. Microzooplankton were the main mortality agents (on average 0.19 ± 0.18 d⁻¹), but their impact differed among the picophytoplankton groups. *Synechococcus* was under the lowest grazing pressure (0.04 ± 0.05 d⁻¹) except at station 10 (0.72 d⁻¹). The strong correlation between the two independent grazing assays used during this study (dilution technique and FLC method; r = 0.98, n = 5, p < 0.05) implies that the low rates did not arise from experimental artefacts but were due to preferential grazing. An earlier study executed in the same site indicated that 1-2 µm sized phytoplankton were prone to lower grazing rates (0.07 d⁻¹) than the 2-3 µm size class (0.20 d⁻¹, Kuipers & Witte 1999). Another study conducted in oligotrophic surface waters has also consistently reported higher predation rates on picoeukaryotes (up to 3 fold) than *Synechococcus* (Worden et al. 2004). The differential grazing observed in offshore vs. coastal station could result from distinct predator communities at these stations that preferentially consumed certain picophytoplankton populations. For instance, *Synechococcus* was found to be selectively egested or digested by some heterotrophic nanoflagellates (Boenigk et al. 2001, Guillou et al. 2001) and ciliates (Christaki et al. 1999).

Significant viral lysis rates (up to 0.23 d⁻¹) were recorded along the Norwegian coast (region 1) for picoeukaryotic groups Pico I and Pico III, and at the DCM of the offshore station 3 for the picoeukaryote group Pico III (region 3). These results indicate that about 20% of the abundance of each group underwent viral lysis on a daily basis. Pigment analysis showed that prasinophytes distributed widely in the studied area, comprising 20 and 30% of the total phytoplankton Chl a as compared to 0.1 - 5% elsewhere (Brussaard C.P.D, unpubl. data). Interestingly, viruses infecting the prasinophyte *Micromonas pusilla* were isolated from all regions, but the highest titer (1 × 10³ mL⁻¹) was found in region 1 and 3a (Brussaard C.P.D., unpubl. data). Flow cytometry examination revealed a proliferation of viruses belonging to the group V3 in all lysates. These observations suggest that viruses may be responsible for the mortality in the *M. pusilla* population, which is consistent with an earlier mesocosm experiment carried out in Norwegian coastal waters during late spring (*M. pusilla* turnover rates of 9 - 25% d⁻¹; Evans et al. 2003). Also, viruses caused significant *M. pusilla* cell lysis along the coast of the oligotrophic Gulf of Mexico (turnover rates 2 - 10% d⁻¹; Cottrell & Suttle 1995).
The virally mediated turnover rates of about 20% of host abundance d\(^{-1}\) reported for the specific picoeukaryotic groups at stations 10 and 3 matched nicely with the fraction of dead algal cells in the respective groups (13% for the Pico I and 32% and 20% for Pico III at stations 10 and 3, respectively). Although this suggests that viral lysis may be the cause of cell membrane permeabilization of picoeukaryotes, the finding of relatively high % dead algal cells in combination with low viral lysis rates indicates that there is no general relationship between viral lysis and % dead cells per se. A buildup in standing stock of dead algal cells using the SYTOX Green live/dead assay cannot be ruled out but other factors causing cell lysis may also be involved (Franklin et al., 2006). Among these, our results indicated that water temperature might have altered *Synechococcus* viability as reported by Alonso-Laita & Agusti (2006). Increasing temperature significantly correlated to *Synechococcus* viability (r = 0.8, n = 6, p = 0.05, Spearman rank order test) and growth rate (r = 1, n = 6, p < 0.01, Spearman rank order test). The relatively low temperature at the DCM of station 3 (9.9°C) may have enhanced the % of dead *Synechococcus*, however the negative effect of light limitation on *Synechococcus* growth (Moore et al. 1995) and membrane integrity (Alonso-Laita & Agusti 2006) cannot be excluded.

Studies on viral lysis conducted in oligotrophic environments are, thus far, limited and mainly focused on *Synechococcus*. These studies consistently reported that *Synechococcus* experience relatively low viral lysis (Waterbury & Valois 1993, Suttle & Chan 1994, Garza & Suttle 1998). Our results support the relatively small impact of viruses on *Synechococcus* mortality in surface oligotrophic waters. In contrast to an earlier observation suggesting that dilution in virus-free water reduced the *Synechococcus* growth rates (Suttle 1996), we recorded similar growth rates for *Synechococcus* in both the 0.2 µm and the 30 kDa dilution series. These results, combined with a previous study carried out in the oligotrophic northeastern subtropical Atlantic Ocean (Baudoux et al. in press) show that the modified dilution method can be successfully used in oligotrophic environments.

The modified dilution method is, to date, the only method that provides viral lysis rates of phytoplankton mortality directly. It is, however, unclear whether this approach is sensitive enough to detect low viral lysis rates. In this study, viral lysis rates down to 0.01 - 0.06 d\(^{-1}\) were recorded but they were not statistically significant. The lowest significant (p < 0.05) virally induced mortality rate determined using this method is 0.1 d\(^{-1}\) (Evans et al. 2003, Baudoux et al. submitted, this study). Acknowledging this restriction, our results indicate differential viral control among picophytoplanktonic algal groups and their area of occurrence.

Different reasons can account for the lack of viral lysis even in presence of the relatively high standing stock of viral groups V4, assigned to putative algal viruses (8 × 10\(^{5}\) mL\(^{-1}\), station 6), and V3, likely containing algal virus (4 × 10\(^{6}\) mL\(^{-1}\), station 5). One reason for this observation could be that the viruses present in the sample were not specific to the co-occurring *Synechococcus* or picoeukaryote populations. The generally high genetic diversity of these hosts (Moon-van der Staay et al. 2001, Scanlan & West 2002), as well as the ability of *Synechococcus* to resist co-occurring viruses (Waterbury
& Valois 1993) could explain the absence of viral lysis. Another reason could be that the algal virus community included viruses infecting nanoeukaryotes rather than picoeukaryotes. Relatively high abundance of the prymnesiophytes *Chrysochromulina ericina* and *Emiliania huxleyi* have been reported in waters adjacent to station 6 (Riegman & Kraay 2001 and references therein). Both of these nanoeukaryotes can be subjected to viral lysis during summer blooms in the North Sea (Brussaard et al. 1996b). Interestingly, pigment analysis revealed that prymnesiophytes comprised most of the Chl *a* at station 6 (Brussaard C.P.D, unpubl. data). The FCM signature of the V4 viral group, furthermore, resembled that of *C. ericina* virus (Brussaard 2004b) or *E. huxleyi* virus (Jacquet et al. 2002). Finally, the absence of viral lysis could be caused by a reduced viral infectivity. In the surface layer of the ocean environmental factors such as high solar radiation, and particularly UV radiation (UVR), can severely alter the infectivity of algal viruses (Cottrell & Suttle 1995, Garza & Suttle 1998, Jacquet & Bratbak 2003). It is noteworthy that viral lysis was exclusively detected in light attenuated environments during the present study. The Norwegian coastal station had the shallowest euphotic zone (23 m, irradiance approximately half of that at other stations), whereas light intensity at the DCM at station 3 was very low (3 µmol quanta m⁻² s⁻¹). Viral lysis rates of picoeukaryotes at the DCM were not only enhanced as compared to the surface, but the richness and abundance of putative algal viruses (>100 kb) was also higher. Such shift in viral community structure was confirmed for the other stations where viral diversity was also studied at the DCM (stations 2 and 14). More detailed research is required to test whether differences in algal virus diversity between surface waters and the DCM are a more general feature. Also, it may be of ecological relevance to investigate whether picophytoplankton viral lysis at the DCM is generally higher as compared to the surface waters. Overall, our observations lead to speculate that the ambient light level may underlie differential impact of viruses on picophytoplankton mortality.

### 4.2. Implications for the carbon cycle

The distinction and quantification of the phytoplankton losses due to lysis and microzooplankton is essential for an optimal understanding of the carbon pathway in marine environments. Our results showed that viral lysis locally yielded substantial carbon release, amounting to 0.2 and 2.4 µg C L⁻¹ d⁻¹ at the DCM of station 3 and in the surface layer of station 10, respectively. The value obtained at the DCM compared well with an earlier study executed in the DCM waters of the subtropical northeastern Atlantic Ocean (0.1 – 0.3 µg C L⁻¹ d⁻¹, Baudoux et al. in press). In contrast, the amount of virally induced carbon release in the Norwegian coastal waters (2.4 µg C L⁻¹ d⁻¹) largely exceeds those reported in the surface coastal waters of the Gulf of Mexico (0.12 – 0.35 µg C L⁻¹ d⁻¹, Wilhelm & Suttle 1999 and references therein). These lower values may be caused by the limited number of potential host taken into account by these authors (only *M. pusilla* and *Synechococcus*); therefore the impact of algal viruses on carbon flow may be higher than previously assumed.
In addition to virally induced carbon release, the leakage of soluble compounds from the cells with compromised membrane may constitute another relevant source of dissolved organic carbon. However, the classification of cells as dead cannot be directly converted to cell lysis rates, as the elapsed time between the physiological death and the subsequent lysis is unknown. Therefore, the magnitude and quantification of this latter process needs further investigation.

The carbon losses generated by microzooplankton grazing were generally higher (range 0.1 – 6.0 µg C L⁻¹ d⁻¹) than those caused by viral lysis (range 0.2 - 2.4 µg C L⁻¹ d⁻¹), except at the DCM of station 3. The picoeukaryotes constituted the primary source of carbon for the microzooplankton, with on average 77% (range 55 - 140%) of the picoeukaryotic carbon production lost by microzooplankton grazing (as compared to 10% for Synechococcus). Our results substantiate earlier studies suggesting the potential of picoeukaryotes for the carbon transfer to the higher level of the pelagic marine food web (Worden et al. 2004). Overall, microzooplankton consumed 40 ± 27 % d⁻¹ of the total picophytoplankton carbon production (CP) which is slightly lower than the general estimate of CP consumption by microzooplankton of 59% d⁻¹ as reported for the temperate ecosystems (Calbet & Landry 2004). The analysis by Calbet & Landry (2004) includes, however, all seasons and different geographical study sites.

In summary, the present study shows an important spatial variability and algal group specificity of grazing and virally induced mortality rates. Such variability will affect the structure of the plankton community and the carbon cycling differently and should, therefore, be addressed in more detail in future analysis of phytoplankton mortality and carbon cycling.

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