Chapter 2

Virally induced mortality of *Phaeocystis globosa* during two spring blooms in temperate coastal waters

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This study reports virally mediated mortality rates of *Phaeocystis globosa* single cells in the southern North Sea during two consecutive spring blooms (2003 and 2004). An adapted dilution method was used to estimate simultaneously viral lysis and microzooplankton grazing. Parallel dilution experiments were performed with 30 kDa ultrafiltrate (virus and grazer-free diluent) and 0.2 µm filtered seawater (grazer-free, but virus-containing diluent). Specific viral lysis rates were calculated from the difference in *P. globosa* growth rates between the two dilutions series after 24 h incubation under natural conditions. The validity of this method was tested using a culture *P. globosa* infected with a known *P. globosa* virus (PgV). The field data show that virally induced mortality can be a substantial loss factor for *P. globosa* single cells (up to 0.35 d⁻¹), comparable to microzooplankton grazing (up to 0.4 d⁻¹). Viral lysis was the major cause of total *P. globosa* cell lysis. Assuming no loss due to sinking, viral lysis accounted for 5 to 66% of the total mortality of *P. globosa* single cells. Viral lysis and total putative PgV abundance increased concomitantly with *P. globosa* single cell abundance whilst the increase in infective PgV was delayed. This delay may be caused by the formation of transparent exopolymeric particles that are generated when *P. globosa* colonies disrupt and known to passively adsorb viruses. Viruses and microzooplankton were shown to be major controlling agents for *P. globosa* single cells, although their relative significance varied over the course of the bloom and between years.

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1. Introduction

With phytoplankton forming the basis of the pelagic marine food web, their dynamics critically influence the functioning of marine ecosystems. Traditionally, grazing and sinking are considered important source of phytoplankton mortality but over the last the decade, cell lysis has also been recognized as a significant loss factor for phytoplankton (Van Boekel et al. 1992, Brussaard et al. 1995, Agusti et al. 1998). Viral infection is a major cause of phytoplankton cell lysis, which affects population dynamics and diversity (for review see Brussaard 2004b). Successful infection depends on the encounter between the virus and the host, which is directly affected by their abundance. During algal bloom events, which are defined by high cell abundance, virally induced mortality has indeed been reported as a substantial loss factor (Bratbak et al. 1993, Brussaard et al. 1996b, Tomaru et al. 2004).

*Phaeocystis globosa* (Prymnesiophyte) is a world-wide distributed bloom-forming phytoplankter. This marine microphytoplankter is well-known for its complex polymorphic life cycle, including flagellated cells (5-7 µm in diameter) and colonies (up to 1-2 centimeters), which consist of colonial cells embedded in a polysaccharide (mucus) matrix. Typically in the southern North Sea, *P. globosa* develops high biomass spring blooms, which contribute for the bulk of local primary production (Lancelot & Billen 1984). These blooms also affect microbial food web dynamics and biogeochemical processes (Stefels & Van Boekel 1993, Brussaard et al. 1995, Brussaard et al. 1996a, Brussaard et al. 1999).

Viruses infecting *P. globosa* (PgVs) have been isolated and characterized (Brussaard et al. 2004, Baudoux & Brussaard 2005). A recent mesocosm experiment demonstrated that virally mediated mortality of *P. globosa* accounted for 30-100% of the total lysis of the *P. globosa* single cells. In contrast, cells embedded in a colonial matrix tend to escape viral infection (Brussaard et al. 2005a, Ruardij et al. 2005). To our knowledge, estimates of virally mediated mortality under natural *P. globosa* bloom conditions do not exist. The dynamics in viral abundance, virus to *P. globosa* ratio and total cell lysis rates of *P. globosa* during bloom events, however, suggest that viruses play an important ecological role (Brussaard et al. 2004, Brussaard et al. 2005a).

Direct methods for estimating viral lysis of phytoplankton are scarce, and to date, most of the viral lysis rates recorded in the literature rely on theoretical calculations or are based on indirect measurements (for review see Brussaard 2004b). Recently, an adaptation of the classical dilution approach (Landry & Hassett 1982) provided estimates of the viral lysis of the picophytoplankter *Micromonas pusilla* in mesocosms (Evans et al. 2003). The current study has applied this method to *P. globosa* under natural conditions. Our work aims to elucidate the relative significance of viral lysis as compared to microzooplankton grazing and total cell lysis of *P. globosa* single cells during two consecutive spring blooms.
2. Material and methods

**Study site and sampling.** Sampling of the coastal southern North Sea was performed twice a week between March (Day 60) and June (Day 180) in 2003 and 2004 from the jetty of the Royal Netherlands Institute for Sea Research (NIOZ). Because the jetty is located at the outer border of a major tidal inlet, samples were collected at incoming high tide. Samples containing freshwater run-off (salinity < 27 ‰) were not taken into account (1 out of 46 samples).

**Chemical parameters.** Nutrient samples (approx. 5 mL) were gently filtered through 0.2 µm pore-size polysulfone filters (Acrodisc, Gelman Sciences) and stored at -50°C (or 4°C for the reactive silicate) until analysis. Analyses were performed using a TrAAcs 800 autoanalyzer for dissolved orthophosphate (Murphy & Riley 1962), nitrogen (nitrate, nitrite and ammonium; Grasshoff 1983, Helder & De Vries 1979), and reactive silicate (Strickland & Parsons, 1968). The limit of detection was 0.03 µM for phosphate, 0.1 µM for ammonium, 0.01 µM for nitrite, 0.15 µM for nitrate, and 0.05 µM for silicate.

The concentration of transparent exopolymeric particles (TEP, in µg Equivalent Gum Xanthan L⁻¹) was measured according to Passow and Alldredge (1995). Replicate samples (30-75 mL) were filtered through 0.4 µm pore-size polycarbonate filters (Poretics). The particles retained on the filter were stained with 500 µL of a 0.02 % solution of Alcian blue prepared in 0.06 % acetic acid (pH 2.5). After staining (< 2 s), the filters were rinsed 3 times with MilliQ (Millipore) to remove excess dye. The filters were immediately transferred into 20 mL glass tubes and soaked for 3 h in a solution of 80 % H2SO4 with gentle agitation every 30 min. The samples were analyzed spectrophotometrically at 727 nm (U-3010 Hitachi).

**Microbial abundances.** Samples collected for phytoplankton pigments (150-700 mL) were filtered onto GF/F glassfiber filters (Whatman) and stored at −50°C. The extract from the filters was analyzed by high pressure liquid chromatography (HPLC) after extraction in 4 mL of 100 % methanol buffered with 0.5 mol L⁻¹ ammonium acetate and homogenized for 15 s. The relative abundance of the taxonomic group Prymnesiophyceae (specifically *Phaeocystis globosa* during our study) was determined using CHEMTAX (Mackey et al. 1996, Riegman & Kraay 2001)

*Phaeocystis globosa* single cells were enumerated in 50 µm-sieved and unfixed samples using a Beckman Coulter XL-MCL flow cytometer equipped with a 488 nm air-cooled laser. Special care was taken to avoid rupture of *P. globosa* colonies during sieving using a small volume of sample. Fixation of the sample resulted in the disintegration of the colonial matrix, therefore the total abundance of *P. globosa* cells (including both single and colonial cells) could be obtained from unfiltered samples that
were fixed to a 1 % final concentration with formaldehyde:hexamine solution (18 % v/v:10 % w/v). Fixation did not affect the P. globosa cell counts. These fixed samples were frozen in liquid nitrogen and stored at -80°C until flow cytometric analysis. P. globosa cells were discriminated on the basis of their natural red chlorophyll autofluorescence and forward scatter signal.

The abundance of virus-like-particles resembling P. globosa like viruses (PgV) was determined on glutaraldehyde fixed samples (final concentration 0.5 % glutaraldehyde, frozen in liquid nitrogen and stored at -80°C prior analysis) using a Beckton-Dickinson FACSCalibur flow cytometer, with a 15 mW 488 nm air-cooled argon-ion laser according to Brussaard (2004a). Thawed samples were diluted (dilution factor >25) in 0.2 μm filtered sterile TE-buffer (pH 8) and stained with the nucleic acid-specific dye SYBR Green I at a final concentration of 0.5×10^{-4} of the commercial stock (Molecular Probes, Eugene, OR). Putative PgV could be discriminated on the basis of the green fluorescence and side scatter signature (Fig. 1), which was identical to that of PgV isolates from the same geographical location and kept in culture at the Royal NIOZ (Brussaard et al. 2004).

![Figure 1. Flow cytometric signature of viruses infecting Phaeocystis globosa (PgV) from natural seawater. PgVs were detected based on their green fluorescence and side scatter upon staining with SYBR Green I.](image)

The abundance of infectious PgV was estimated using the end-point dilution approach (Most Probable Number, MPN, Suttle 1993). Natural seawater was filtered through an 1 μm polycarbonate filter (Poretics) and serial diluted (8 titers, 5 replicates) with exponentially growing P. globosa Pg-G (RUG culture collection, The Netherlands) and Pg-01MD06 (NIOZ culture collection). To screen for rare PgV in 2004, an additional natural sample was filtered through an 1 μm polycarbonate filter (Poretics),
concentrated approx. 40 times using a VivaFlow 200 ultrafiltration system (Vivascience), and added to a *P. globosa* host culture (20% v/v). Both *P. globosa* strains originated from the North Sea and were chosen for their different sensitivity to PgV, which was relatively broad for Pg-G and specific for Pg-01MD06 (Baudoux & Brussaard 2005). The algae were grown in a 1:1 mixture of f/2 medium (Guillard 1975) and enriched artificial seawater ESAW, (Harrison et al. 1980, Cottrell & Suttle 1991) completed with Tris-HCl and Na₂SeO₃ (Cottrell & Suttle 1991). The dilution series were incubated for 10 days at 15°C under a light:dark cycle of 16:8 h at 100 µmol photon m⁻² s⁻¹. Algal growth was monitored via in vivo chlorophyll fluorescence using a Turner Designs fluorometer and compared to noninfected controls. Those dilutions that showed signs of cell lysis were scored positive when PgV proliferation could be confirmed (using flow cytometry as described above). The positive scores were converted to abundance of infective PgV using a MPN assay computer program (Hurley & Roscoe 1983).

**Loss parameters of *P. globosa***. Total cell lysis rates of *P. globosa* (d⁻¹, unspecific cause) were estimated using the dissolved esterase activity (DEA) assay described in Brussaard et al. (1996a) and adapted by Riegman et al. (2002). Particulate esterase activity was obtained by subtracting the dissolved esterase activity (0.2 µm pore-size filtered) from the total esterase activity (unfiltered natural sample). The dissolved esterase activity was corrected for non-enzymatic hydrolysis of the substrate, as measured in natural sample filtered through 10 kDa (PES Vivaspin, Vivasciences), and for a decay of esterase activity in seawater using a half-life time of 48 h (Riegman et al. 2002). The *P. globosa*-specific particulate esterase activity was calculated by multiplying the total particulate esterase activity by the contribution of *P. globosa* to total chlorophyll based CHEMTAX pigment analysis described in the above section (Brussaard et al. 2004). Data points were occasionally omitted when an unrealistically high lysis rate was obtained from the ratio of low produced DEA to low *P. globosa* specific PEA. This may occur at the onset of the bloom when *P. globosa* biomass is still low.

Virally induced mortality of *P. globosa* single cells was estimated using the viral lysis dilution assay according to Evans et al. (2003). Parallel dilution series of natural seawater was performed with 0.2 µm filtered natural sample (Poretics, Millipore) to obtain microzooplankton grazing rate (Landry & Hassett 1982), and with 30 kDa filtered natural sample (polyether sulfone membrane, Pellicon filtration system, Millipore) to obtain grazing and viral lysis rates. Viral lysis rates were determined from the difference between the two dilutions series.

The viral lysis dilution assay has only been applied to *Micromonas pusilla* (Evans et al. 2003) therefore we checked the validity of this method for *P. globosa*. The test experiment was conducted using an exponentially growing *P. globosa* Pg-G (1×10⁵ cells mL⁻¹) in combination with the lytic virus PgV-07T (Brussaard et al. 2004) at a multiplicity of infection (MOI) of 10, as determined by MPN assay (described above). In order to simplify the interpretation of the test results no grazers were added, therefore
identical net growth rates were recorded for all dilutions with the 0.2 µm pore-size water, and thus the estimated grazing rate was not significantly different from zero (0.02 ± 0.07 d⁻¹, Fig. 2A). The dilution series with the <30 kDa diluent yielded a regression slope of 1.3 ± 0.07 d⁻¹, which corresponds to the viral lysis rate in this test since there was no grazing. Knowing that \textit{P. globosa} undergoes lysis 14-16 h after infection (Baudoux & Brussaard 2005), we conclude that the lysis rate obtained during the 24 h incubation originates from one lytic cycle. An independent one-step lytic growth cycle experiment using the same strain of \textit{P. globosa} and PgV validated the results of the dilution assay. The viral lysis rate (1.4 d⁻¹) calculated from this growth experiment were comparable to those obtained with the laboratory viral lysis dilution assay (1.3 d⁻¹). These tests demonstrated the utility and validity of viral lysis dilution assay, allowing this method to be applied in the field for \textit{P. globosa} (Fig. 2B).

\textbf{Figure 2.} Virally mediated mortality rates of \textit{P. globosa} for (A) a test experiment using the virus-host model system PgV-07T and Pg-G (no grazers were added) and (B) a typical field sample (Day 132 in 2003). Parallel dilution experiments were performed in 0.2 µm filtered seawater (grazer-free, but virus-containing diluent) and 30 kDa ultrafiltrate (virus and grazer-free diluent). The regression coefficient of apparent growth rate vs. dilution factors resulting from the 0.2 µm dilution series represents the microzooplankton grazing rate, and from the 30 kDa series represents microzooplankton grazing as well as viral lysis. Viral lysis rates (d⁻¹) were estimated from the difference in regression coefficient of the two set of dilutions. For the readability of the figures, we averaged the triplicate apparent growth for each dilution level. This operation does not affect the estimated mortality rates.
The experimental design of the dilution assay for field samples is described in Fig. 3. All materials (carboys, tubing, bottles) used for this assay were cleaned for 24 h with 0.1 N HCl, after which they were rinsed 3 times with MilliQ and once with the sample. To prevent losses of virus, grazers or disruption of *P. globosa* colonies, sieving and filtration were performed with special care by siphoning and avoiding air bubbling. Polycarbonate Poretics filters (47 mm, Millipore) were exclusively used and replaced frequently during the filtration to avoid loss of viral infectivity and abundance (Suttle et al. 1991). Samples were processed under dimmed light (to prevent light stress) and in situ temperature (4-18°C). The seawater used for the dilution (10 L) was collected and processed approx. 2 h before high tide in order to minimize the handling time of the natural water to be diluted. The 10 L sample was pretreated by reverse sieving through 200 and 50 µm mesh (20 cm diameter) to remove larger grazers and *P. globosa* colonies. Subsequently, the sample was filtered through 3 µm and 0.2 µm pore-size filters. A 5 L aliquot of the 0.2 µm filtrate was used for generating the 0.2 µm dilution series. The remaining 5 L were ultrafiltered through 30 kDa and used as diluent for the 30 kDa dilution series.

At high tide, 20 L of natural seawater was de novo collected. The salinity of the two batches of seawater used for the experiment was measured and found comparable in all cases (difference <0.5‰). The sample was sieved through 200 µm mesh by reverse sieving to remove mesozooplankton and immediately used to set up four levels of dilution (20, 40, 70 and 100 % sample) in 2 L polycarbonate bottles that already contained the 0.2 µm or the 30 kDa diluents. From the 2 L dilution bottle, 3 incubation bottles (250 mL polycarbonate bottles) were carefully filled by siphoning and 5 mL subsample were taken (T=0 h). These incubation bottles were refilled to the top with the original dilution waters (remaining from the 2 L bottles) in order to avoid any air bubbles being trapped inside upon closure. All bottles were incubated at near-surface depth (approx. 1-2 m) under natural light and temperature conditions in a basket in the NIOZ harbor (protected from wave-motion). Another sample of 5 mL was taken after a 24 h incubation period.

The set-up of the laboratory assay was similar to the field assay with the exception that a *P. globosa* culture free of virus and grazers was used. Viruses infecting *P. globosa* (0.2 µm filtered PgV-07T lysate, Poretics filters, Millipore) were added (MOI = 10) to the diluent directly after the 0.2 µm filtration step and to the *P. globosa* culture just prior to dilution. The *P. globosa* culture that had to be diluted was infected just before setting up the dilutions in the 2 L bottles. All bottles were incubated at the host culture’s growth conditions (15°C under light:dark cycle of 16:8 h at 100 µmol photons m⁻² s⁻¹).

For both field and laboratory assay, the single cells of *P. globosa* were enumerated directly upon sampling after gentle sieving through 50 µm mesh-size using a Beckman Coulter XL-MCL flow cytometer (three replicates of each sample). The apparent growth rate (µ in d⁻¹) of *P. globosa* single cells was calculated for each sample from the changes in abundance during the incubation according to the equation

\[ \mu = \ln N_{24} - \ln N_{0}, \]
where \( N_{t0} \) and \( N_{t24} \) are the abundance of \( P. \) globosa single cells at \( T=0 \) and \( T=24 \) respectively. A typical field example is presented Fig. 2B. The regression coefficient of apparent growth rate vs. dilution factors for the 0.2 µm dilution series represents the microzooplankton grazing rate (\( M_g \)), whereas the regression coefficient resulting from the 30 kDa series represents both microzooplankton grazing and viral lysis (\( M_{(g+v)} \)). Subsequently, mortality rate due to viral lysis (\( M_v \)) was calculated as \( M_v = M_{(g+v)} - M_g \). 

\( M_g \), \( M_{(g+v)} \) and their respective standard errors (\( SE_g \) and \( SE_{(g+v)} \)) were calculated using Sigma plot software. The standard error of \( M_v \) was calculated as the squared root of the sum of squared \( SE_g \) and \( SE_{(g+v)} \).

\[ \text{Figure 3. Experimental design of the viral lysis dilution assay (field assay). (1) Diluents were prepared approx. 2 h prior to (2) dilution with natural sample collected de novo at high tide. Samples were processed under dimmed light and at in situ temperature. Sample transfers were performed by siphoning or gentle pumping, avoiding damage to the organisms.} \]
3. Results

3.1. Chemical parameters

For the two consecutive years of study a comparable pattern in nutrient dynamics was recorded from Day 60 to 180 (Fig. 4). Nitrate concentration declined steadily from 60-80 µM at Day 60 to 0.3 µM at Day 107 in 2003 and 0.8 µM at Day 173 in 2004. For 2003, the nitrate concentration remained low until Day 119, after which it increased slightly again. In contrast, growth-limiting nitrate concentrations were not found during the sampling period in 2004. Inorganic phosphate concentrations declined sharply for both years, from 0.8-1 µM at Day 60 to around 0.4 µM during Days 90-95. The concentration of phosphate in 2003 did not increase until Day 112. In 2004 it increased quickly again (Day 99), but a second decline was detected from Days 117 to 125 (< 0.1 µM). Both years, ammonium concentrations ranged between 0.5 and 5.8 µM until Day 120, after which it increased substantially to maximum values of 9 and 16 µM in 2003 and 2004 respectively. Silicate concentrations decreased steadily from the beginning of the sampling period until Day 83 in 2003 and Day 92 in 2004, after which the concentration stayed low (0.4 to 6 µM) for both years.

![Figure 4. Concentrations of dissolved inorganic phosphate and nitrate during spring (A) 2003 and (B) 2004. Grey bars under the x-axis indicate the duration of the P. globosa bloom.](image-url)
The TEP concentration in 2004 (Fig. 5) increased steadily during the sampling period to a maximum of 1033 µg Equiv. GX L\(^{-1}\) on Day 128, after which it declined to a level comparable to the start of the sampling period (164 µg Equiv. GX L\(^{-1}\) on Day 160).

**Figure 5.** Concentration of TEP in µg Equivalent Gum Xanthan (GX) L\(^{-1}\) during the *P. globosa* spring bloom in 2004 as determined by the semi-quantitative method (Passow & Alldredge 1995). Grey bars under the x-axis indicate the duration of the *P. globosa* bloom in 2004.

### 3.2. *Phaeocystis globosa* bloom dynamics

The bloom of prymnesiophytes represented up to 70 % and up to 40 % of the total phytoplankton chlorophyll in 2003 and 2004 respectively (Figs. 6A and 6C). *P. globosa* was likely the dominant prymnesiophyte during the experimental period. The magnitude and the composition of the blooms differed between years. The bloom in 2003 occurred between Days 70 and 141, reached at the highest total cell abundance of \(7.6 \times 10^4\) cells mL\(^{-1}\), and was generally dominated by the colonial cell morph (Fig. 6B). Single cells dominated over cells embedded in a colonial matrix only during peak events (e.g. Day 102 and 119). In 2004, *P. globosa* bloom occurred between Days 92 and 159, reached only three fold lower cell abundance (\(2.1 \times 10^4\) cells mL\(^{-1}\)) as compared to 2003, and was dominated by the single cell morphotype (Fig. 6D).
Figure 6. Phaeocystis globosa bloom dynamics during spring 2003 and 2004. (A, C) Relative contribution of Prymnesiophyceae to the total phytoplankton community based on their pigment ratio to Chl a (Prymnesiophyceae are mostly, if not only, represented by P. globosa during the sampling periods. (B, D) Abundance of P. globosa single cells and total cells (single and colonial cells). Grey bars under the x-axis indicate the duration of the P. globosa bloom.
3.3. *Phaeocystis globosa* specific viruses

Despite the low abundance of *P. globosa* at the beginning of the sampling period, there was a substantial build-up of standing stock of putative PgVs both in 2003 and 2004 (Fig. 7). Putative PgV abundance increased concomitantly with the development of *P. globosa* biomass, largely corresponding to increased *P. globosa* single cells and/or reduced abundance of colonial *P. globosa* cells (Figs. 6B and 7A). PgV reached abundances > $4 \times 10^5$ mL$^{-1}$ for both years.

The numerical increase of the infective PgVs (MPN method using *P. globosa* strain Pg-G) was delayed compared to the total putative PgV abundance as detected by flow cytometry (Fig. 7). Although not visible in Fig. 7, infective PgVs were recorded in each sample tested using *P. globosa* strain Pg-G ($< 1.8 \times 10^7$ mL$^{-1}$ at Days 97, 105 and 113 in 2003; and $< 25$ mL$^{-1}$ from Days 60-120 in 2004). Maximum number of infective PgV was roughly comparable for 2003 and 2004 with $1.5-1.8 \times 10^4$ infective PgV mL$^{-1}$ at Days 133 and 145 respectively. For both years, the infective PgV accounted maximum for 5% of the total putative PgV population.

From a previous study, the *P. globosa* strain Pg-01MD06 was found to be specifically infected by only certain PgV isolates in culture (PgV Group II, Baudoux & Brussaard 2005). Therefore, this *P. globosa* strain was also assayed during 2004 to determine whether different PgVs coexisted in the field. Cell lysis of the host due to viral infection was recorded for Day 113 and 123 (Fig. 7B).

![Figure 7](image_url). Abundance of putative viruses infecting *P. globosa* (PgV) and infective PgV in (A) 2003 and (B) 2004. Total PgV (mL$^{-1}$) was obtained using flow cytometry, whereas the abundance of infective PgV (mL$^{-1}$) resulted from end-point dilution (MPN) using *Phaeocystis globosa* strain Pg-G (triangles) with a broad sensitivity to PgV and strain Pg-01MD06 with a narrow sensitivity to PgV (circles). Only positive scores of virally induced cell lysis of *P. globosa* are presented. Grey bars under the x-axis indicate the duration of the *P. globosa* bloom.
3.4. Viral lysis and grazing

The rates of viral lysis and grazing by microzooplankton of *P. globosa* single cells were estimated over the course of the blooms (Fig. 8). Virally mediated mortality rates in 2003 were low (0.01 to 0.03 d\(^{-1}\)) until the collapse of *P. globosa* single cells, when viral lysis was high (0.35 d\(^{-1}\) at Day 132, Fig. 8A). This enhanced viral lysis rate concurred with the highest concentrations of total putative PgV as well as infective PgVs (Figs. 7A and 8A). In contrast to viral lysis, microzooplankton grazing of *P. globosa* single cells was shown to be an important source of mortality during the entire bloom of *P. globosa* (Fig. 8A). During the course of the bloom, viral lysis accounted for 5 to 57% of the total mortality, assuming no losses of *P. globosa* single cells due to sinking.

Viral lysis rates were higher over the course of the bloom in 2004 (Fig. 8B, with values of 0.29, 0.19 and 0.24 d\(^{-1}\) at Days 123, 127 and 135 respectively) as compared to 2003 when viral lysis only increased at the bloom termination (0.35 d\(^{-1}\) at Day 132). These high *P. globosa*-specific viral lysis rates also coincided with increased abundances of total putative and infective PgV. Microzooplankton grazing in 2004 was another important loss factor for single cells of *P. globosa*, with rates ranging between 0.05 and 0.40 d\(^{-1}\). Viral lysis represented 44, 66 and 45% of total losses at Days 123, 127 and 135, respectively.

![Figure 8. Viral lysis and microzooplankton grazing rates of Phaeocystis globosa single cells during different stages of the P. globosa bloom in (A) 2003 and (B) 2004. Viral lysis (d\(^{-1}\)) is represented by the grey bars, and grazing (d\(^{-1}\)) by the white bars. Error bars correspond to standard error.](image-url)
The total *P. globosa* specific cell lysis varied over the course of the bloom for both years (Fig. 9). In 2003, total *P. globosa* cell lysis rates were $< 0.1 \text{ d}^{-1}$ until Day 121 and increased concomitantly with the collapse of the total *P. globosa* cell abundance ($0.2 \text{ d}^{-1}$ at Day 132). In 2004, total *P. globosa* cell lysis rates increased earlier (from Day 110). For both years total cell lysis rates reached maximum rates of about $0.2 \text{ d}^{-1}$.

**Figure 9.** Daily viral lysis and total cell lysis rates of *P. globosa* during the spring bloom in (A) 2003 and (B) 2004. Grey bars under the x-axis indicate the duration of the *P. globosa* bloom

### 4. Discussion

The present study revealed that virally mediated mortality, next to grazing, was a major source of loss for *P. globosa* cells during two consecutive spring blooms (in 2003 and 2004). Incidentally, viral lysis rates recorded during the blooms were higher than the microzooplankton grazing rates upon *P. globosa* cells. To our knowledge, this study is the first direct assessment of viral lysis rates in *P. globosa* under natural conditions. A recent study indicated that viruses can be significant mortality agents of *P. globosa* cells, but the experiment was conducted under controlled conditions in mesocosms and estimates were based on virus production and assumed burst size (Brussaard et al. 2005a).

The Landry and Hassett dilution method (Landry & Hassett 1982) was originally developed for the measurement of microzooplankton grazing and is now routinely applied in a broad range of aquatic environments (Landry & Calbet 2004). Recently, an extended version of the dilution method was successfully developed to
specifically estimate viral lysis of the picophytoplankter *Micromonas pusilla* (Evans et al. 2003). The suitability of the method was demonstrated for *Phaeocystis* using a cultured *P. globosa* host and virus model system. Thereby, this test experiment validated the two critical assumptions of the original dilution method; (1) that phytoplankton growth rate is independent of the dilution factor, and (2) losses are proportional to the dilution effect on the abundance of the predators (Landry & Hassett 1982, Landry et al. 1995). The obtained viral lysis rate (1.3 d⁻¹) using this assay was comparable from that of independent one-step lytic cycle experiment (1.4 d⁻¹). In order to estimate the number of *P. globosa* cells that underwent cell lysis during a 24 h period, the PgV produced in the non-diluted samples during the 24 h incubation were divided by a theoretical burst size ranging from 100-300 (Baudoux & Brussaard 2005). These values compared very well with those obtained by multiplying the cell abundance at the start of the incubation by the determined viral lysis rate, assuming no growth of the infected algal cells.

It is imperative to realize that this assay exclusively detects viral lysis of algal hosts that are newly infected within the 24 hours of incubation. This incubation period is essential to encompass the entire cellular diel cycle of phytoplankton that have synchronized cell cycles. The lytic nature of all known PgVs, as well as the 14 to 16 h required for *P. globosa* to undergo cell lysis (Baudoux & Brussaard, 2005) favor the detection of *P. globosa* viral lysis using this dilution approach. However, the time between successful viral infection of a *P. globosa* cell and its subsequent lysis is critical since late infection during the incubation period (later than 10 h) will no longer result in cell lysis within the duration of the incubation. The potential impact of viruses on the *P. globosa* population is, therefore, likely to be underestimated. The lysis of *P. globosa* cells happens 0-4 h after the first release of viral progeny of PgV (latent period of 10-16 h, Baudoux & Brussaard 2005). Thereby, a second round of infection by the newly produced viruses during the incubation period should not affect the viral lysis rates since the dilution method is governed by the enumeration of cells.

Viral lysis rates were compared with the *P. globosa* total cell lysis rates, which also includes lysis due to causes other than viral infection (e.g. environmental stress). This comparison reveals that virally induced mortality was the most important cause of lysis and thereby supports the findings of *P. globosa* studies conducted in mesocosms (Brussaard et al. 2005a). Differences between total cell lysis and viral lysis rates of *P. globosa* likely represent other forms of cell lysis, e.g. automortality of the colonial cells from nutrient depletion (Ruardij et al. 2005). Deviations due to the use of two different parameters to estimate viral lysis and total cell lysis can, of course, not be excluded. Viral lysis was determined from the difference in *P. globosa* cell abundance over 24 hours of incubation period using flow cytometry, whereas total cell lysis was estimated from the ratio of dissolved to particulate esterase activity. It may also be possible that the physiological status of cells influence the cellular esterase activity and thus the total lysis rates. Reduced percentages of dying cells were recorded for virally infected *P. pouchetii* cells in the early stationary growth phase upon staining with Calcein-AM, a fluorescent dye revealing intracellular esterase activity (Brussaard et al. 2001). We speculate that the lower total cell lysis rates than viral lysis rates at the end of the blooms might be influenced by such methodological variations.
The composition and magnitude of the *P. globosa* blooms differed for 2003 and 2004. The lower biomass of *P. globosa* colonies in 2003 and 2004 was likely due to the lower standing stock of inorganic phosphate in 2004 which was half the concentration of 2003. One possible reason could be that silicate became growth-limiting later in 2004 as compared to 2003 (Day 92 in 2004 as compared to Day 83 in 2003). Thus, dominance of diatoms was prolonged and subsequently the concentration of phosphate was reduced when *P. globosa* biomass finally developed (Jahnke 1989, Egge & Aksnes 1992). Another reason might be that the reduced mean water irradiance during the bloom period in 2004 (due to substantial cloud cover), limited colony formation (Peperzak 1993) and thus lowered *P. globosa* biomass.

Virally induced mortality of *P. globosa* cells as well as PgV abundance increased concomitantly with the development of the bloom, as can be expected since an increasing abundance of host enhances the rate of successful viral infection. Despite the higher *P. globosa* total cell abundance in 2003, the maximal abundance of PgV was comparable in both years. It is suggested that *P. globosa* cells that are embedded inside a colonial matrix are protected against viral infection (Brussaard et al. 2005a, Ruardij et al. 2005). Interestingly, our results show that the abundance of colonial cells was higher in 2003 than in 2004 but the abundance of single cells was comparable. This observation suggests that PgV was mainly produced by *P. globosa* single cells and thus corroborates the observations that colonies provide protection from viral infection. Hence, the morphotype composition of a *P. globosa* bloom is an important factor underlying the impact of viral infection for *P. globosa*.

When colonies disintegrate after experiencing nutrient depletion or light deprivation (Veldhuis et al. 1986, Peperzak 1993), cells are released in the surrounding waters and become readily infected (Brussaard et al. 2005a, Ruardij et al. 2005). In 2003, nitrate depletion (from Day 107 to 120) was most likely responsible for the major collapse in the abundance of colonial cells after Day 115, thus increasing the impact of viral lysis (0.35 d⁻¹ at the end of the bloom). Although the cause of colonial disintegration in 2004 is less obvious, the decline in colony abundance led to an increase in single cell abundance and enhanced viral lysis (0.29 d⁻¹ on Day 123).

The difference in colony abundance between the two years also likely influenced the dynamics of transparent exopolymeric particles (TEP), which are produced in high concentrations during colony disruption (Mari et al. 2005). TEP formation has recently been acknowledged as a major inhibitor for viral infection (Brussaard et al. 2005b, Ruardij et al. 2005). Viruses, like other microorganisms, tend to passively adsorb to TEP and therefore are not available to infect algal cells. In 2004, the period with the highest TEP concentration indeed coincided with a low abundance of infective PgV and reduced viral lysis rates. Interestingly, the disintegration of TEP concurred with an enhanced abundance of infective PgV. The concentrations of TEP recorded for 2004 were lower than those reported during a *P. globosa* bloom with higher colonial cell abundance (100 to 1000 µg equiv GX L⁻¹ as compared to 100 to 2000 µg GX L⁻¹; Mari et al. 2005). Thus, the higher abundance of colonies in 2003 induced, in all likelihood, a higher release of TEP and subsequently an enhanced impact of TEP on the
fraction PgV still infective, which in turn resulted in reduced viral lysis rates. Therefore it can be said that colonies play a controversial role acting as a potential viral lysis inhibitor with TEP production but also as an enhancing agent of virally induced mortality as they constitute a potential reservoir of single cells.

The present study, furthermore, indicates towards regulation of viral infection on a finer scale, as the results from the end-point dilution assay (MPN) suggest the co-existence of different PgVs. The PgV population infecting Pg-01MD06 appeared and disappeared within 2 weeks, implying that the PgV population in the field is diverse and dynamic. This has also been proposed by other authors for different algal viruses (Tarutani et al. 2000, Schroeder et al. 2003). The reason this PgV population is not maintained in the water column is not clear. Possible reasons may be the removal of the specific host from the water column or a loss of infectivity of this specific viral population, but this needs further investigation.

In summary, this study shows that viral lysis and grazing by microzooplankton are both major controlling agents for P. globosa single cells, although the relative significance of each of these factors can vary during the course of the bloom and between years. The application of the dilution method based on cell counts in combination with total algal cell lysis rates and abundance of infective algal viruses provides essential insight into the quantitative significance of viral lysis as compared to other loss factors. The present study also gives insight into on the ecological role of viral infection in relation to host population regulation, and some of the mechanisms controlling successful infection.

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**Literature cited**

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Suttle CA, Chan AM, Cottrel MT (1991) Use of ultrafiltration to isolate viruses from seawater which are pathogens of marine plankton. Appl Environ Microbiol 57:721-726


