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Both phosphorus and nitrogen limitation constrain viral proliferation in marine phytoplankton

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ABSTRACT

Through cell lysis, viruses shape phytoplankton community composition and stimulate biogeochemical cycling in the oceans. Earlier studies indicate that reduced phosphorus (P) availability can affect phytoplankton virus proliferation. The effects of nitrogen (N) availability are claimed to be weaker than for P, but this has not been thoroughly studied. Here we investigated how N-limiting growth conditions, resulting in altered algal elemental stoichiometry and physiology, affected virus proliferation in the phytoplankters *Micromonas pusilla* and *Phaeocystis globosa*. Algal cultures were adapted to balanced nutrient limited growth (N-, P- and NP-controlled) before infection with their respective viruses, i.e., MpV-08T and PgV-07T. The viral infection experiments were then performed in batch cultures to allow optimal one-step virus growth cycles. Compared to the nutrient replete cultures, infection of nutrient-controlled hosts resulted in elongated latent periods (time until first virus release) and reduced viral burst sizes (viruses lysed host cell\(^{-1}\)) for both MpV and PgV. For MpV, the viral burst size was reduced by 70%, independent of the type of nutrient. The burst size of PgV was most reduced under N-limitation, by as much as 92% (compared to 70% under P-limitation). Overall, our results demonstrate that algal virus production can be strongly impaired by N-limitation and that the effects are of a similar magnitude (or even larger) as for P. Our study indicates that viral control of natural phytoplankton populations might be strongly driven by both P- and N-availability.
INTRODUCTION

As the main primary producers in the marine environment, phytoplankton play a central role in biogeochemical cycling in the oceans. Overall phytoplankton production, community structure and food web dynamics are regulated by bottom-up (e.g. nutrients) and top-down (e.g. viral lysis) control (Dufour and Torréton 1996, Sterner 1989, Mojica et al. 2016). Both growth and cellular physiology (e.g. net carbon production, stoichiometry, etc.) are often limited by the availability of nitrogen (N) and of phosphorus (P) (Tilman et al. 1982, Moore et al. 2013). While P-limitation is prevalent in areas such as the Sargasso Sea and the eastern Mediterranean, N is regarded as the dominant limiting nutrient in large parts of the oligotrophic Pacific- and Atlantic Ocean. Furthermore, the actual limiting nutrient for phytoplankton in a certain area can depend on the season, and often the stoichiometry of both elements is such that phytoplankton may be limited by more than one nutrient, i.e., only the addition of both N and P would lead to increased production or biomass (Arrigo 2005, Moore et al. 2013). Viruses are important mortality agents for phytoplankton, and viral lysis drives phytoplankton community dynamics, succession and biogeochemical cycling (Wilhelm & Suttle 1999, Suttle 2007, Brussaard et al. 2008). Environmental factors such as nutrient availability have been found to influence algal host-virus interactions (Mojica & Brussaard 2014b). While several studies have shown that reduced P-availability of virally infected eukaryotic phytoplankton can result in elongated latent periods and reduced viral burst sizes (Bratbak et al. 1993, Clasen & Elser 2007, Maat et al. 2014), little is known about the influence of N-availability on algal virus proliferation. To date two studies of Emiliania huxleyi blooms under N-depletion showed either no effect (Bratbak et al. 1993) or merely a delaying effect on viral proliferation (Jacquet et al. 2002). However, as these results were obtained from mesocosm studies, nutrient conditions were difficult to control and other factors affecting algal and viral dynamics (e.g. viral decay) cannot be excluded. For a more
detailed understanding (i.e. a direct causal link) on the effects of N-availability on phytoplankton virus-host interactions, culture experiments are required. A laboratory study using *Phaeocystis pouchetti* showed that N-starvation of the host before infection resulted in reduced burst sizes as compared to nutrient replete cultures (Bratbak et al. 1998). Yet at the moment of infection, the cells were already in stationary phase for some days (in contrast, the nutrient replete cells were growing exponentially), which makes it hard to pin-point the exact causal factor of the reduced burst sizes. Jacquet et al. (2002) brought up the need for more work on this topic to clarify the effects of N-limitation on virus-phytoplankton interaction. Indeed, a better insight into which nutrients affect phytoplankton virus proliferation is pivotal because nutrient limited phytoplankton growth and cell physiology is ubiquitous in the marine environment (Moore et al. 2013) and viral lysis an important factor of phytoplankton mortality and major driver of marine biogeochemical cycling (Wilhelm & Suttle 1999, Baudoux et al. 2007, Mojica et al. 2016).

The aim of this study was to investigate how N-limited algal stoichiometry and physiology affect virus proliferation in the phytoplankton species *Micromonas pusilla* and *Phaeocystis globosa*. The effects of N were thereby compared to the effects of P and additionally mixed-nutrients (NP). Both algal species belong to genera with a global distribution (Schoemann et al. 2005, Slapeta et al. 2006). The picoeukaryotic photoautotroph *M. pusilla* is readily infected by viruses (Cottrell & Suttle 1995) and blooms of *P. globosa* have been shown to be controlled by viruses (Baudoux et al. 2006, Brussaard et al. 2005). The algal cultures were pre-grown under nutrient-controlled conditions, which synchronized the physiological state of the algal cells. As virus reproduction took place during the first half day post infection (p.i.), the nutrient status of the host cell at the moment of infection (i.e. preconditioning of nutrient limited growth) was expected to largely control virus growth characteristics (latent period and burst size). For clarity, the term (i) nutrient limitation is used...
as a general term that describes reduced growth or altered stoichiometry and physiology due to low nutrient availability (Moore et al. 2013), (ii) nutrient controlled growth refers to balanced algal growth rate which is dictated by the actual dilution rate (Quinlan 1986, MacIntyre & Cullen 2005), and (iii) nutrient starvation is the temporal unbalanced nutrient limited state of the cultures in batch mode (Parkhill et al. 2001), as encountered during the infection experiments (Maat et al. 2016).

MATERIALS AND METHODS

Culturing and experimental set-up

Axenic *M. pusilla* Mp-LAC38 (Culture collection Marine Research Center, Goteborg University) and *P. globosa* G(A) (Culture collection University of Groningen, The Netherlands) were cultured at 15°C under a 16:8h light:dark cycle with 100 μmol quanta m⁻² s⁻¹ irradiance during the light period (18W/965 OSRAM daylight spectrum fluorescent tubes; München, Germany). The medium used (Mix-TX) was a 1:1 mixture of modified f/2 medium (Guillard & Ryther 1962) and artificial seawater (ESAW; Harrison 1980), enriched with Tris-HCl and Na₂SeO₃ (Cottrell & Suttle 1991), and with Na₂-glycerophosphate omitted. Semi-continuous nutrient controlled culturing was chosen over full continuous (chemostat) cultures due to logistical considerations (for good comparisons we needed to handle 48 cultures simultaneously). Although different from chemostats, because the cultures are diluted once per day instead of continuously, this method yields comparable constant growth and physiology. With both types of culturing, the concentration of the limiting nutrient determines the algal abundances (biomass), while the medium dilution rate (supply of the limiting nutrient) determines the algal growth rate (µ) (Quinlan 1986, Nicklisch 1999). The concentrations of
NaNO₃ and Na₂HPO₄ in the Mix-TX medium (Table 1) were then chosen as such that the cell abundances of the nutrient controlled cultures were comparable. The maximum growth rates of the algal species were initially determined in replete (low abundance) batch cultures. In the nutrient limiting semi-continuous cultures, growth was then maximized under these specific conditions, i.e. maintained as close as possible to the $\mu_{\text{max}}$, i.e. highest possible without wash-out of cells. The maximum cell abundance that was reached in this manner was maintained (balanced growth) and steady state samples for nutrient concentrations and algal physiology were taken. In this way the nutrient limited cultivation allowed cells to grow at rates identical to chemostat culturing and potentially even near maximum growth rate ($\mu_{\text{max}}$, as under nutrient replete conditions; Quinlan 1986, Henry et al. 2008). Hence, on a daily basis the cells received a specific amount of limiting nutrient similar to the total cell quotas, i.e., just enough to maintain the set growth rate under the specific conditions. Although the nutrients in our semi-continuous cultures were taken up by the cells within the hour, the diel dynamics of cell growth and photosynthetic efficiency were similar to those under replete conditions and thus not affected by the discontinuous addition of medium (nutrients; Fig. S1). Growth of the semi-continuous cultures was either N-controlled, P-controlled or NP-controlled (i.e., daily addition of both nutrients to allow growth of the cultures). Nutrient replete cultures were used as control treatment, whereby the dilution rate was chosen to keep cell abundances at a fixed abundance (comparable to abundances reached in nutrient limited cultures), according to the turbidostat principle (MacIntyre & Cullen 2005). Cultures were considered to be at steady state (balanced growth) when growth rates and cell abundances prior to dilution maintained constant with time. Over the 4 weeks before the experiment, average (±s.d.) cell abundances were (1.2±0.06×10⁶, 1.4±0.07×10⁶, 1.2±0.05×10⁶ and 3.2±0.21×10⁵, 3.0±0.11×10⁵, 3.0±0.12×10⁵ for the N-, P- and NP-controlled cultures of *M. pusilla* and *P. globosa*, respectively). Steady state inorganic nutrient concentrations were additionally monitored to make sure that all of the daily added
nutrients were taken up by the cells. The average daily growth (and thus dilution) rates during steady state are depicted in Table 1. All treatments were performed in triplicate. The cultures were also sampled on a daily basis for flow cytometry (FCM) for the analysis of cell abundances, mean cellular forward scatter (FSC; indicator of cell size) and chlorophyll autofluorescence (RFL). Nutrient concentrations (sampled regularly during steady state and at the end of the viral infection experiments) were analyzed by colorimetry and the photosynthetic efficiency (Fv/Fm) was monitored by pulse amplitude modulated (PAM) fluorometry. Steady state cellular nutrient quotas of the nutrient controlled axenic phytoplankton cultures were calculated by dividing the decrease in the growth limiting nutrient in the culture over 24h (i.e. nutrients taken up; which for nutrient-controlled conditions equals the daily input as concentration after 24h was always below detection limit) by the increase in cell abundance over the same time period (Table 1; Veldhuis & Admiraal 1987). For the replete cultures the nutrient quotas were determined in the same way and under the same conditions. The nutrient limited state of the cells was demonstrated by alterations in cellular physiology, especially cellular nutrient quotas and Fv/Fm, both specific indicators of phytoplankton nutrient limitation (Beardall et al. 2001, Maat et al. 2014).

Two days before the experiment each culture was split into two cultures in order to accommodate the viral infection experiments (i.e. one control and one virally infected). As each treatment was cultured in triplicate, this yielded per species a total of 3 replicate non-infected controls and 3 replicate infected cultures per nutrient treatment. Viral infection experiments were started 3 h into the light period and performed in batch (dilution and hence supply of the limiting nutrient was stopped) in order to (i) optimize the conditions for successful one-step virus growth curve (from which the latent period and burst size are determined), and (ii) avoid virus proliferation simultaneous with the uptake of the added limiting resources, as this may influence virus growth characteristics. For the first 12 h Fv/Fm of the ‘batched’ non-infected
culture was identical to a nutrient controlled culture that still received the daily supply of limiting nutrient (Fig. S1) and only towards the end of the first day did Fv/Fm of the batch culture drop (by about 10%).

Cultures were inoculated with axenic viral lysate at a multiplicity of infection (MOI) of 10. Infectivity was determined by endpoint dilution of the algal virus lysates according to Suttle (1993) and the abundance of infectious viruses was found largely comparable to total virus count by FCM. Both axenic MpV-08T infecting *M. pusilla* LAC-38 (Martinez Martinez et al. 2015) and axenic PgV-07T infecting *P. globosa* G(A) (Baudoux & Brussaard 2015) are lytic dsDNA viruses and originate from the NIOZ culture collection. Viral lysates were obtained under the same conditions as the algal hosts, whereby the lysates for the N- and P-limited treatments were produced by at least three infection cycles on N-, P- or NP-controlled host. No detectable levels of limiting nutrient(s) were recorded in the lysates that were used for the viral infection experiments. The non-infected controls received sterilized 0.2 µm filtered (Sartorius A.G. cellulose acetate filters, Goettingen, Germany), aged natural seawater with N- and P-concentrations below the limit of detection. Sampling post infection (p.i.) took place every 3-6 h for algal and viral abundances and every 24 h for PAM fluorometry (the latter only for the non-infected cultures). Viral infectivity of the progeny viral lysates was determined at the end of the experiments using endpoint dilution assay (Suttle 1993) and compared to FCM total virus counts in order to obtain % infective. Inorganic nutrient concentrations were sampled at the start and end of the experiments. Algal and viral abundances were monitored during the infection experiment to determine the viral latent periods and burst sizes. Latent periods were determined as the time interval in which a clear increase in viral abundances was observed that continued during the following time-points. The burst sizes were determined by dividing the number of produced viruses by the maximum number of lysed host cells.
Both the algal cultures and viral lysates were regularly checked for axenity by epifluorescence microscopy (Porter & Feig 1980). In short, 1 ml samples were fixed with (0.1% final concentration) glutaraldehyde, stained with DAPI (4’,6-diamidino-2-phenylindole, dihydrochloride; Life Technologies Ltd. Paisley, UK) and filtered over a 0.2µm black polycarbonate filter (Whatman, Maidstone, UK). The cultures were axenic at all times.

**Analyses**

Flow cytometric determination of phytoplankton abundances was done on fresh samples according to Marie et al. (1999) using a BD Accuri™ C6 cytometer (BD Biosciences, San Jose, CA, USA) with the trigger on Chlorophyll $a$ red autofluorescence (RFL). The phytoplankton cells were distinguished in a scatter plot of RFL versus forward scatter (FSC). The mean cellular RFL and FSC signals were recorded as indicators for steady state cellular chlorophyll content and cell size (Shapiro 1988, DuRand et al. 2002).

Viruses were enumerated on fixed samples (according to Brussaard 2004) using a 488 nm argon laser-containing benchtop FacsCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with the trigger set on green fluorescence. In short, 1 ml samples were fixed with 25% gluteraldehyde (EM-grade, 0.5% final concentration; Sigma-Aldrich, St. Louis, MO, USA), incubated for 30 minutes at 4°C, flash frozen in liquid nitrogen and stored at -80°C. After thawing and prior to analysis, the samples were diluted 100-1000 fold in 0.2 µm filtered (FP 30/0,2 CA-S Whatman, Dasser, Germany) TE-buffer (pH=8), stained with SYBR Green I to a final concentration of $0.5 \times 10^{-4}$ of the commercial stock (Life Technologies Ltd., Paisley, UK) for 10 min at 80°C. Viruses were quantified on a scatter plot of green fluorescence versus side scatter (SSC). All flow cytometry data were analyzed using CYTOWIN 4.31 (Vaulot 1989).
Samples for Fv/Fm (2 ml) using PAM fluorometry (Water-PAM, Walz, Germany) were kept in the dark at in situ temperature for 15 min, after which the minimal (F0) and maximal (Fm) chlorophyll autofluorescence were measured. The variable fluorescence Fv was defined as Fm-F0 (see Maxwell & Johnson (2000)).

Nutrient samples (5ml) were filtered over 0.2µm Supor® membrane syringe filters (Pall Acrodisc®, Ann Arbor, MI, USA) into (Perkin Elmer, Shelton, USA) Pony vials™. Samples were stored at -20°C until analysis for NO₃⁻ and PO₄³⁻ on a TRAACS autoanalyzer 800+ (Bran+Luebbe, Norderstedt, Germany), according to Hansen and Koroleff (1999). The detection limits were 0.01 and 0.05 µM for PO₄³⁻ and NO₃⁻, respectively.

**Statistics**

Statistical analyses were carried out with either one-way ANOVAs or Kruskall Wallis tests (non-parametric) in the program Sigmaplot™ 12.0 (Systat software Inc, Chicago Il, USA). In the tables significant differences (p<0.05) between values are depicted by different letters (superscript), i.e. when two values have the same letter, they are not different.

**RESULTS**

**Steady state of preculturing phase**

For both species, the maximized steady state exponential growth rates under nutrient controlled semi-continuous culturing were not affected by the type of limitation (N-, P- or NP-control; Table 1). The nutrient controlled *M. pusilla* cultures were still able to reach near-maximum growth rate (µ_max) as under nutrient replete conditions, but for the nutrient controlled *P. globosa* cultures this was at best 73% of µ_max under replete conditions (Table 1). The cellular nutrient
quotas of the nutrient controlled cultures were strongly reduced as compared to the replete
treatment with excess nutrients (Table 1). For *M. pusilla* the N- and P-quotas under nutrient
controlled growth were both approximately 7-fold lower relative to nutrient replete. For *P. globosa* this was 11-fold for the N quota, but only 1.5-fold for the P quotas. The mean cellular
RFL in the N- and NP-controlled cultures was, in contrast to the P-controlled treatment,
significantly reduced for both phytoplankton species. Moreover, mean cellular FSC of *M. pusilla* was reduced under these conditions (Table 1). Finally, for both algal species the Fv/Fm
of the nutrient limited cultures at steady state was slightly, but significantly, reduced compared
to the replete cultures (Table 1).

**Viral infection experiments**

The viral infection experiments were carried out under batch conditions using the steady state
nutrient controlled and nutrient replete cultures. Growth of the non-infected nutrient replete
cultures did not change as a result of the batch mode, as can be expected under excess nutrient
conditions (Fig. 1&2). However, the algal growth rates of the nutrient limited non-infected *M. pusilla* and *P. globosa* cultures showed a respective 62±16 and 81±10 % decrease over the first
24 h p.i. No significant difference was found for the different types of limitations (N, P and NP;
one-way ANOVA, 0.438<p<0.653), demonstrating nutrient starvation upon the transition from
balanced nutrient controlled growth to batch conditions (Fig. 1&2). The Fv/Fm of the non-
infected nutrient limited *M. pusilla* cultures at 24 h p.i. showed a 33±0.02 % reduction in
comparison with steady state conditions, while this was a mere 12±0.04 % decrease for *P. globosa*. Time until full lysis of the infected *M. pusilla* cultures was equally fast for the three
nutrient treatments (48 h), but for infected *P. globosa* full lysis of the N- and NP-limited cultures
was reached approximately 24 h later than of the P-limited ones (48-72 h; Fig. 1 & 2).
The viral latent periods were not affected by the type of nutrient limitation, but overall both MpV and PgV latent periods were prolonged under nutrient limitation, i.e. 9-12 and 12-15h, respectively, compared to replete conditions (6-9 and 12-15h, respectively; Table 2, Fig. 3&4, Fig. S2). Also the rate of virus progeny increase (production) was lower under the nutrient limitations in both species (Fig. 3&4). For MpV this was 27% lower (for all nutrient limitations) than under nutrient replete conditions ($1.1 \times 10^6$ MpV ml$^{-1}$ h$^{-1}$), while for PgV this was 88% under N- and NP-limitation and 63% under P-limitation and (in comparison to $5.6 \times 10^6$ PgV ml$^{-1}$ h$^{-1}$ for nutrient replete). The higher total MpV yield in the N-limited cultures related to the slightly higher algal host abundance prior to lysis (Fig. 1&3). The MpV burst sizes under nutrient limiting conditions were reduced by 69$\pm$2 % as compared to nutrient replete conditions ($229$ viruses cell$^{-1}$), independent of the type of limitation (Table 2). For *P. globosa* this reduction was strongest under N- and NP-limitation ($>93\%$) and similar under P-limitation ($72\%$; compared to nutrient replete of $823$ viruses cell$^{-1}$; Table 1, Fig. 4). For both algal species, viral infectivity was not affected by the nutrient treatments.

**Discussion**

**Steady state of preculturing phase**

By using semi-continuous cultivation, the algal cultures were well-adapted to growth under nutrient limiting conditions. During steady state the cells showed clear nutrient limited physiology (Quinlan 1986, Nicklisch 1999; Moore et al 2013). This was most pronounced for the Fv/Fm and cellular nutrient quotas, both well-accepted indicators of phytoplankton nutrient limitation (Beardall et al. 2001). The reduction in Fv/Fm of nutrient limited phytoplankton is the result of a lowered photosynthetic rate, probably due to reduced concentrations of ATP and
reductants (Beardall et al. 2001). The stronger Fv/Fm reduction in N-controlled *P. globosa* (in comparison to the P-controlled cultures) may be attributed to a reduction of the photosystem II (PSII) reaction center protein D1 under N limitation (Geider et al. 1993). Furthermore, the reduction in cellular RFL under N-controlled growth (observed for both species) indicates chlorosis, a reduction in cellular chlorophyll *a* content. Chlorosis decreases the total demand of nutrients for photosynthesis and leads to reduced intracellular self-shading (package effect) with consequently a more efficient light harvesting per chlorophyll molecule (Berner et al. 1989).

The differences in N- and P-quota between nutrient replete and limited *M. pusilla* might be (partly) due to the accumulation, i.e. storage, of these elements under P-replete conditions. *M. pusilla* has been shown to have largely reduced concentrations chlorophyll *a* and phospholipids when grown under P-controlled growth (0.97 and 0.32μ*max*), compared to P-replete conditions (Maat et al. 2014, Maat et al. 2016). This demonstrates that the quota reductions are at least in part due to reductions of actual cellular components. Still, the reductions are within the ranges that are reported in the literature (Geider & LaRoche, 2002).

For *P. globosa*, the P-quota reduced to a stronger extent than the N-quota, suggesting that this nanophytoplankter copes better with N-limitation. This is supported by a study that showed a competitive advantage of *Phaeocystis* over other phytoplankton species under N-limiting conditions, while being outcompeted under P-limiting conditions (Riegman et al. 1992). NP-controlled growth led to decreased quota of both N and P in both species, suggesting that the cells were co-limited (Moore et al. 2013). It is, however, difficult at this stage to determine whether growth was ultimately inhibited by only one of the nutrients or whether both were involved (Arrigo 2005, Moore et al. 2013). Volume-specific nutrient quotas (based on a linear relation between cells size and FSC) showed a similar outcome (relative differences) as the quotas per cell (Table S1).
There were differences in the capability of the phytoplankton species to handle nutrient controlled growth, i.e. nutrient controlled *P. globosa* showed maximum growth rates that were reduced by approximately 30% in comparison to nutrient replete growth, whereas *M. pusilla* was able to reach near-maximum growth rates. Thus despite the poorer physiological state of the nutrient limited *M. pusilla* cells relative to the nutrient replete cells, they had just enough of the limiting nutrient to allow growth similar to the maximum growth rate observed under nutrient replete conditions. Similar results are reported for *M. pusilla* growing in P-limited chemostats, with net primary production, photophysiology and P:N:C stoichiometry strongly reduced (Maat et al. 2014). The ability of *M. pusilla* to still grow near $\mu_{\text{max}}$ implies that this species is better adapted to cope with low nutrient supply than *P. globosa*. This might be the result of its smaller cell size, i.e. 2 $\mu$m cell diameter as compared to 5 $\mu$m for *P. globosa*. Smaller-sized species are indeed thought to be better adapted to nutrient limitation, due to their higher surface to volume ratio, and smaller cell boundary layer (Raven 1998). The reduction in mean cellular FSC under N- and NP-controlled growth suggests that *M. pusilla* became even smaller in response to shortage of N. This could either be an adaptation to develop a more efficient nutrient uptake (more optimal surface to volume ratio), or a result of reduced intracellular components due to reduced total protein content (Geider et al. 1993).

**Virus infection experiments**

The one-step virus growth cycles of both MpV and PgV were strongly affected by N- and P-limitation, resulting in prolonged latent periods and strongly reduced burst sizes, independent of the type of nutrient. Similar effects on the viral latent periods and burst sizes were found in an independent pilot experiment 6 months in advance of this study (n=3; Table S2). The results are thus highly reproducible, showing that the observed effects are inherent to the tested species and conditions. The observed effects may, however, be underestimated as
theoretically the organic nutrients in the added viral lysate could have affected viral proliferation. Yet, considering the 10% (v/v) addition of viral lysate with 70% of the released cellular nutrients in the dissolved phase (Gobler et al. 1997) and an estimated 35% N and 70% P bioavailable (Lønborg & Álvarez-Salgado, 2012), the contribution to the total N and P in the cultures would have been a few percent at maximum.

Although the specific phytoplankton growth rates decreased when the medium supply was stopped, there were no significant differences for the type of nutrient limitation (N, P or NP). At the same time there were, however, nutrient type-specific differences in the speed and extent of PgV proliferation, i.e., slower host lysis, lower viral production and lower burst sizes for the N and NP treatment. This indicates that host growth rate as such was not the (sole) determinant for the outcome of infection and that the physiological history of the algal host (preculturing or preconditioning phase) also determines the effects on virus proliferation. Still, the species-specific relative reduction in growth rate under nutrient limitation (stronger for *P. globosa* than *M. pusilla*) seems to be reflected in the virus growth characteristics, i.e. stronger negative effects for *P. globosa*. The viral latent periods of both species were prolonged by all nutrient limitations (N, P or NP). This demonstrates that not only P (Bratbak et al. 1993, Wilson et al. 1996, Clasen & Elser 2007, Maat et al. 2014) but also N is needed for optimal virus proliferation. The observed elongations of the viral latent periods under P-limitation were also reported for MpV infecting *M. pusilla*, pre-grown in chemostats at 0.97μ_{max} (Maat et al. 2014).

Strikingly, our results show that N-limitation of the algal host can have similar (MpV) and even larger (PgV) adverse effects on virus production than P-limitation (i.e. rates of increase of extracellular progeny viruses and viral burst sizes). Even despite the finding that *P. globosa* was able to reduce its N-quotas to a stronger extent than its P-quotas (thus seems better able to cope with N limitation), viral progeny production and burst sizes were thus more strongly affected by N-limitation than P-limitation. Although the NP-controlled treatment resulted in
significantly higher nutrient quotas than the single-limitations of P or N for both species, the differences were very small, which might explain why no significant additional effect was found on viral proliferation of this treatment.

Previous studies on virus proliferation in nutrient depleted mesocosms suggest that a shortage of P is a more important inhibitor of viral replication in phytoplankton than N (Bratbak et al. 1993, Jaquet et al. 2002). The authors hypothesized that this is due to lower N:P ratios of viruses as compared to algal hosts. Here we show that not only P-, but also N-limitation negatively impacts viral proliferation. In the mesocosm experiments, *Emiliania huxleyi* and the virus EhV were the dominant host-virus system (Bratbak et al. 1993, Jaquet et al. 2002). It could be that the observed differences were due to specific responses of the different host-virus model systems. Alternatively, viral abundances could have been underestimated due to adsorption to aggregates (Brussaard et al. 2005, Mojica & Brussaard 2014), or the N-depletion under the semi-natural mesocosm conditions was not severe enough (i.e. nutrient availability or turnover were still too high to be truly limiting viral production in the infected host cells). Indeed, nitrate concentrations in the N-depleted mesocosms in the study of Bratbak et al. (1993) were not continuously low and showed regular spikes of nitrate up to 2 µM during the increase in EhV, which was likely enough to sustain host growth and EhV production.

Host characteristics are strongly affected by nutrient limitation, and viruses are strictly dependent on their host cells for the energy and elements needed for replication. Hence, the constraint on viral proliferation is likely the result of the physiological state of the host, i.e., decreased amount of enzymes and accessory molecules that are involved in viral replication. Nitrogen is a major component of the hosts’ enzymes that are necessary for processes in viral replication, such as transcription and translation of the viral genes and possibly photophosphorylation (Mackenzie & Haselkorn 1972, Baudoux & Brussaard 2008). As nutrient limitation has been shown to lower total protein content in phytoplankton cells, this might be
an even more important causal factor of nutrient limitation of viruses than the actual role of N as an element in the actual viral components. The same holds for P, which is an irreplaceable element in the energy metabolism of organisms and thus also in the above mentioned processes. Indeed, P-limitation has been shown to lead to reduced intracellular adenylate content (AMP, ADP and ATP) in phytoplankton (Theodorou et al. 1991). It has been suggested that some phytoplankton viruses recycle host nucleic acids, but it is not clear to what extent this would happen (Brown et al. 2007, Brown & Bidle 2014).

**Ecological implications**

Many questions are still unresolved about the precise role that phytoplankton viruses play in biogeochemical cycling in the oceans and how these processes are affected by the abiotic environment (Mojica and Brussaard 2014). Here we show that N-availability has the same potential as P-availability in its effects on phytoplankton growth and physiology, and consequently the ability of their lytic viruses to propagate (latent periods, burst sizes). Extrapolating this to natural ecosystems, the diminished virus production under N- (and/or P-) limitation will reduce the chance of new infections of phytoplankton (Levin & Lenski 1983, Murray & Jackson 1992, Bratbak et al. 1998, Mann 2003). The significance of N and P as regulatory factors seems high as these nutrients are often limiting marine phytoplankton production in many coastal and oceanic regions worldwide (Moore et al. 2013). Besides, nutrient limitation is expected to increase in space and time due to global climate change-induced warming of the surface oceans and subsequently strengthened vertical stratification (Sarmiento et al. 2004, Behrenfeld et al. 2006). Yet, to what extent nutrient limitation will control virus-host interactions under natural conditions will depend on several factors, such as the algal host species (possibly size class; this study), the nutrient supply rate during the
infection cycle and the type of limiting nutrient (this study). Further studies using different virus-host model systems are needed to elucidate host species-specificity and examine the consequences of virus replication under nutrient stress (N, P, but also micronutrients) on the flow of energy and matter.

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References


Table 1: Concentrations of inorganic nitrogen (N) and phosphorus (P) in the growth medium supplied to the axenic semi-continuous cultures of *Micromonas pusilla* and *Phaeocystis globosa* and the steady state exponential algal growth rates (µ), the cellular nutrient quota, forward scatter (FSC), Chl a autofluorescence by flow cytometry (RFL), and photosynthetic efficiency (Fv/Fm) under these growth conditions. All values are averages of triplicate cultures over two days (n=6). Significant differences (p<0.05) between the treatments (per species, per parameter) are depicted by different letters (a,b), i.e. numbers with the same letters are not statistically different. Note that these values also represent the state of the cells at the moment of infection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N (µM)</th>
<th>P (µM)</th>
<th>µ (d⁻¹)</th>
<th>µ (% of replete)</th>
<th>P-quota (fmol cell⁻¹)</th>
<th>N-quota (fmol cell⁻¹)</th>
<th>FSC (r.u.)</th>
<th>RFL (r.u.)</th>
<th>Fv/Fm (r.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. pusilla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-limitation</td>
<td>1</td>
<td>16</td>
<td>0.74 ±0.10 a</td>
<td>94</td>
<td>-</td>
<td>0.71 ±0.12 a</td>
<td>0.71 ±0.12 b</td>
<td>0.57 ±0.08 b</td>
<td>0.60 ±0.03 b</td>
</tr>
<tr>
<td>P-limitation</td>
<td>400</td>
<td>0.25</td>
<td>0.74 ±0.18 a</td>
<td>94</td>
<td>0.19 ±0.03 a</td>
<td>-</td>
<td>1.03 ±0.06 a</td>
<td>1.02 ±0.02 a</td>
<td>0.60 ±0.01 b</td>
</tr>
<tr>
<td>NP-limitation</td>
<td>1</td>
<td>0.25</td>
<td>0.76 ±0.14 a</td>
<td>96</td>
<td>0.22 ±0.03 a</td>
<td>0.88 ±0.09 b</td>
<td>0.76 ±0.05 b</td>
<td>0.62 ±0.05 b</td>
<td>0.60 ±0.03 b</td>
</tr>
<tr>
<td>Replete</td>
<td>400</td>
<td>16</td>
<td>0.79 ±0.19 a</td>
<td>100</td>
<td>1.23±0.38</td>
<td>5.29±0.71</td>
<td>1.00±0.10 a</td>
<td>1.00±0.02 a</td>
<td>0.64±0.01 a</td>
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<tr>
<td><strong>P. globosa</strong></td>
<td></td>
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<td></td>
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<tr>
<td>N-limitation</td>
<td>4</td>
<td>16</td>
<td>0.76 ±0.08 b</td>
<td>68</td>
<td>-</td>
<td>14.8±1.01 a</td>
<td>1.01±0.01 a</td>
<td>0.66±0.03 b</td>
<td>0.62±0.02 c</td>
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<tr>
<td>P-limitation</td>
<td>400</td>
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<td>73</td>
<td>3.62±0.17 a</td>
<td>-</td>
<td>1.09±0.02 b</td>
<td>0.93±0.05 a</td>
<td>0.65±0.02 b</td>
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<tr>
<td>NP-limitation</td>
<td>4</td>
<td>1</td>
<td>0.79 ±0.04 b</td>
<td>71</td>
<td>4.22±0.29 b</td>
<td>16.9±1.39</td>
<td>1.02±0.10 a</td>
<td>0.76±0.05 b</td>
<td>0.63±0.01 c</td>
</tr>
<tr>
<td>Replete</td>
<td>400</td>
<td>16</td>
<td>1.12 ±0.05 a</td>
<td>100</td>
<td>5.68±0.18</td>
<td>163.4±28.65</td>
<td>1.00±0.08 a</td>
<td>1.00±0.02 a</td>
<td>0.70±0.01 a</td>
</tr>
</tbody>
</table>
Table 2: The latent period (h), burst size (viruses produced per lysed host cell) and percentage reduction in burst size of MpV and PgV under N-, P- and NP-limitation and compared to nutrient replete treatment. Values are averages of triplicate cultures. Significant differences (p<0.05) between the treatments (per species) are depicted by different letters \(^{(a,b,c)}\), i.e. numbers with the same letters are not statistically different.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Latent period (h)</th>
<th>Burst size (viruses cell(^{-1}))</th>
<th>Burst size reduction relative to replete (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. pusilla</em></td>
<td>N-limitation</td>
<td>9-12</td>
<td>67 ±10(^{b})</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>P-limitation</td>
<td>9-12</td>
<td>69 ±4(^{b})</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>NP-limitation</td>
<td>9-12</td>
<td>77 ±4(^{b})</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Replete</td>
<td>6-9</td>
<td>229 ±2(^{a})</td>
<td></td>
</tr>
<tr>
<td><em>P. globosa</em></td>
<td>N-limitation</td>
<td>12-15</td>
<td>61 ±23(^{c})</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>P-limitation</td>
<td>12-15</td>
<td>230 ±62(^{b})</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>NP-limitation</td>
<td>12-15</td>
<td>49 ±19(^{c})</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Replete</td>
<td>9-12</td>
<td>823 ±46(^{a})</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: Abundances of non-infected (closed symbols) and infected (open symbols) *Micromonas pusilla* under nutrient limited (A) and replete (B) conditions.

Figure 2: Abundances of non-infected (closed symbols) and infected (open symbols) *Phaeocystis globosa* under nutrient limited (A) and replete (B) conditions.

Figure 3: Abundances of *Micromonas pusilla* virus (MpV) during the viral infection experiment under N-limited (closed triangles), P-limited (open circles), NP-limited (open triangles) and nutrient replete (closed circles) culture conditions.

Figure 4: Abundances of *Phaeocystis globosa* virus (PgV) during the viral infection experiment under N-limited (closed triangles), P-limited (open circles), NP-limited (open triangles) and nutrient replete (closed circles) culture conditions.
Figure 1

A

M. pusilla ($\times 10^6$ ml$^{-1}$)

Time post infection (h)

N-lim control
N-lim infected
P-lim control
P-lim infected
NP-lim control
NP-lim infected

B

M. pusilla ($\times 10^6$ ml$^{-1}$)

Time post infection (h)

Replete control
Replete infected
Figure 2

(A) Graph showing the Time post infection (h) vs. P. globosa (×10^6 ml⁻¹) with different conditions: N-lim control, N-lim infected, P-lim control, P-lim infected, NP-lim control, and NP-lim infected.

(B) Graph showing the Time post infection (h) vs. P. globosa (×10^6 ml⁻¹) with an upward trend over time.
Figure 3

The figure shows a graph plotting the change in MpV (in $\times 10^8$ ml$^{-1}$) over time post infection (in hours). The x-axis represents time post infection (h), ranging from 0 to 80 hours. The y-axis represents MpV (in $\times 10^8$ ml$^{-1}$), ranging from 0 to 2.0.

The graph includes data for various viruses, labeled as 'N-limited', 'P-limited', 'NP-limited', and 'replete'. Each virus type is represented by different symbols and error bars indicating variability.
Figure 4

[Graph showing the relationship between time post infection (h) and viruses (×10^8 ml^-1). The graph includes lines representing different conditions: N-limited, P-limited, NR-limited, and replete.]
Table S1: Volume specific cellular nutrient quotas of Phaeocystis globosa and Micromonas pusilla under N- limited, P-limited, NP-limited and nutrient replete conditions. Cell volume was calculated from the cell diameter, which was based on a linear relationship between phytoplankton cells size ($y$) and and mean cellular FSC ($x$) ($y=0.0075x+1.2373; r^2=0.9979$) of the three phytoplankton species P. globosa and M. pusilla and Heterosigma akashiwo.

<table>
<thead>
<tr>
<th></th>
<th>P. globosa</th>
<th>M. pusilla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P (fmol µm$^{-3}$)</td>
<td>N (fmol µm$^{-3}$)</td>
</tr>
<tr>
<td>N-limited</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>P-limited</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>NP-limited</td>
<td>0.05</td>
<td>1.85</td>
</tr>
<tr>
<td>replete</td>
<td>0.06</td>
<td>1.85</td>
</tr>
</tbody>
</table>

Table S2: Viral latent periods and burst sizes of the preceding pilot experiment (n=3). The experiment was carried out 6 months before the experiments in the presented study and according to the same methods. NP-limitation was not tested (n.d.; not determined)

<table>
<thead>
<tr>
<th></th>
<th>P. globosa</th>
<th>M. pusilla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Latent period (h)</td>
<td>Burst size (viruses cell$^{-1}$)</td>
</tr>
<tr>
<td>N-limited</td>
<td>12-16</td>
<td>72 ±6</td>
</tr>
<tr>
<td>P-limited</td>
<td>12-16</td>
<td>198 ±26</td>
</tr>
<tr>
<td>replete</td>
<td>8-12</td>
<td>801 ±112</td>
</tr>
</tbody>
</table>
Diel cycle of *M. pusilla* growth (A) and Fv/Fm (B) under P-controlled semi-continuous culturing. The diluted semi-continuous culture (grey circle) shows similar dynamics as the p-replete culture (black circle, indicating that discontinuous supply of nutrients does not affect balanced nutrient limited growth (i.e. no feast and famine, but rather continuous nutrient limited state). Phosphate added to the P-controlled (A; cross) culture at t0 are taken up (to below limit of detection) within 60 minutes after addition. Algal cells displayed synchronized cell division related to the dark period (shaded areas). Under batch condition (i.e. stopped supply of limiting nutrient to culture, inducing starvation) cell abundance stays constant over the 24h. Fv/Fm initially follows the diel pattern of the semi-continuous culture, but at the end of the 24h it declines due to P-starvation.
Figure S2

Viral abundances over time in the first day post infection to show the latent periods in more detail. The latent period was determined as the time interval with a clear increase in viral abundances, that continues throughout the following time-points. Error bars show standard deviations.