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Both phosphorus- and nitrogen limitation constrain viral proliferation in marine phytoplankton

Douwe S. Maat*, Corina P. D. Brussaard

NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Microbiology and Biogeochemistry, and Utrecht University, PO Box 59, 1790 AB Den Burg, Texel, The Netherlands

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 those 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, i.e. N-, P- and NP-controlled growth, before infection with their respective viruses MpV-08T and PgV-07T. The viral infection experiments were then performed in batch cultures to allow optimal 1-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 to or even larger than for P. Our study indicates that viral control of natural phytoplankton populations might be strongly driven by both P- and N-availability.

KEY WORDS: Phytoplankton · Algal virus · Nitrogen · Phosphorus · Nutrient limitation · *Phaeocystis globosa* · *Micromonas pusilla*

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 (Sterner 1989, Dufour & Torréton 1996, 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 (Dyhrman et al. 2007), N is regarded as the dominant limiting nutrient in large areas of the oligotrophic Pacific and Atlantic Oceans. 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 >1 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). Envi-

*Corresponding author: douwe.maat@nioz.nl

ronmental factors such as nutrient availability have been found to influence algal host–virus interactions (Mojica & Brussaard 2014). 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 2 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, such as viral decay, cannot be excluded. For a more detailed understanding of the effects of N-availability on phytoplankton virus–host interactions, culture experiments are required. A laboratory study using *Phaeocystis pouchetii* 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 are ubiquitous in the marine environment (Moore et al. 2013), and viral lysis is an important factor in phytoplankton mortality and a 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 (Brussaard et al. 2005, Baudoux et al. 2006). 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 was expected to largely control virus growth characteristics, i.e. the latent period and burst size. For clarity, (1) 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), (2) nutrient-controlled growth refers to a balanced algal growth rate which is dictated by the actual dilution rate (Quinlan 1986, MacIntyre & Cullen 2005) and (3) nutrient starvation is the temporally 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 *Micromonas pusilla* Mp-LAC38 (culture collection Marine Research Center, Goteborg University) and *Phaeocystis globosa* G(A) (culture collection University of Groningen, The Netherlands) were cultured at 15°C under a 16 h light:8 h dark cycle with 100 µmol quanta m^{-2} s^{-1} irradiance during the light period (18W/965 OSRAM daylight spectrum fluorescent tubes). The medium used (Mix-TX) was a 1:1 mixture of modified *f*/2 medium (Guillard & Ryther 1962) and artificial seawater (ESAW; Harrison et al. 1980), enriched with Tris-HCl and Na_2SeO_3 (Cottrell & Suttle 1991), and with Na_2-glycerophophate omitted. Semi-continuous nutrient-controlled culturing was chosen over full continuous (chemostat) cultures due to logistical considerations, as 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 or the supply of the limiting nutrient determines the algal growth rate (µ) (Quinlan 1986, Nicklisch 1999). The concentrations of $NaNO_3$ and Na_2HPO_4 in the Mix-TX medium (Table 1) were then chosen so that the cell abundances of the nutrient-controlled cultures were comparable. The maximum growth rates of the algal species were initially determined in replete batch cultures with low abundances. In the nutrient-limiting semi-continuous cultures, growth

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) by flow cytometry, chl *a* autofluorescence by flow cytometry (RFL) and photosynthetic efficiency (F_v/F_m) under these growth conditions. All values are averages (± standard error) of triplicate cultures over 2 d (n = 6). Significant differences ($p < 0.05$) between the treatments (per species, per parameter) are depicted by different superscripted letters, 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. r.u.: relative units

Treatment	N (μM)	P (μM)	μ (d^{-1})	μ (% of replete)	P-quota (fmol $cell^{-1}$)	N-quota (fmol $cell^{-1}$)	FSC (r.u.)	RFL (r.u.)	F_v/F_m (r.u.)
M. pusilla									
N-limitation	1	16	0.74 ± 0.04^a	94	–	0.71 ± 0.05^a	0.71 ± 0.05^b	0.57 ± 0.03^b	0.60 ± 0.01^b
P-limitation	400	0.25	0.74 ± 0.07^a	94	0.19 ± 0.01^a	–	1.03 ± 0.02^a	1.02 ± 0.01^a	0.60 ± 0.00^b
NP-limitation	1	0.25	0.76 ± 0.06^a	96	0.22 ± 0.01^a	0.88 ± 0.04^b	0.76 ± 0.02^b	0.62 ± 0.02^b	0.60 ± 0.01^b
Replete	400	16	0.79 ± 0.08^a	100	1.23 ± 0.16	5.29 ± 0.29	1.00 ± 0.04^a	1.00 ± 0.01^a	0.64 ± 0.00^a
P. globosa									
N-limitation	4	16	0.76 ± 0.03^b	68	–	14.8 ± 0.41^a	1.01 ± 0.00^a	0.66 ± 0.01^b	0.62 ± 0.01^c
P-limitation	400	1	0.82 ± 0.03^b	73	3.62 ± 0.07^a	–	1.09 ± 0.01^b	0.93 ± 0.02^a	0.65 ± 0.01^b
NP-limitation	4	1	0.79 ± 0.02^b	71	4.22 ± 0.12^b	16.9 ± 0.57	1.02 ± 0.04^a	0.76 ± 0.02^b	0.63 ± 0.00^c
Replete	400	16	1.12 ± 0.02^a	100	5.68 ± 0.07	163.4 ± 11.70	1.00 ± 0.03^a	1.00 ± 0.01^a	0.70 ± 0.00^a

was then maximized under these specific conditions (maintained as close as possible to the μ_{max}), i.e. highest possible without wash-out of cells. The maximum cell abundance that was reached in this manner was maintained, 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 the maximum growth rate (μ_{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 in the Supplement at www.int-res.com/articles/suppl/a077p087_supp.pdf). Growth of the semi-continuous cultures was either N-controlled, P-controlled, or NP-controlled, meaning that daily addition of, respectively, N, P, or N and P allowed growth of the cultures. Nutrient-replete cultures were used as control treatments, whereby the dilution rate was chosen to keep cell abundances at a fixed level which was comparable to the abundance reached in the nutrient-limited cultures and 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 remained constant with time. Over the 4 wk before the experiment, average (±SD) cell abundances were $1.2 \pm 0.06 \times 10^6$, $1.4 \pm 0.07 \times 10^6$, $1.2 \pm 0.05 \times 10^6$ and $3.2 \pm 0.21 \times 10^5$, $3.0 \pm 0.11 \times 10^5$, $3.0 \pm 0.12 \times 10^5$ 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 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 (F_v/F_m) 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 24 h by the increase in cell abundance over the same time period (our Table 1; Veldhuis & Admiraal 1987). For nutrient-controlled conditions, the nutrient uptake equaled the daily input, as concentration after 24 h was always below the detection limit. 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 F_v/F_m, 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 2 cultures, of which 1 was used as a non-infected control and the other was virally infected, in order to accommodate the viral infection experiments. 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, with dilution and hence supply of the limiting nutrient stopped, in order to (1) optimize the conditions for successful 1-step virus growth curve (from which the latent period and burst size are determined) and (2) avoid virus proliferation simultaneous with the uptake of the added limiting resources, as this may influence virus growth characteristics. For the first 12 h, F_v/F_m of the 'batched' non-infected culture was identical to a nutrient-controlled culture that still received the daily supply of limiting nutrients (Fig. S1 in the Supplement), and only towards the end of the first day did the F_v/F_m 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 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 2005) are lytic dsDNA viruses and originate from the Royal Netherlands Institute for Sea Research 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 3 infection cycles on N-, P-, or NP-controlled hosts. 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), aged natural seawater, with N- and P-concentrations below the limit of detection. Sampling post-infection 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. 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 intervals 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) and filtered over a 0.2 µm black polycarbonate filter (Whatman). 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) with the trigger on chlorophyll *a* red autofluorescence (RFL). The phytoplankton cells were distinguished in a scatter plot of RFL versus 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) 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), incubated for 30 min at 4°C, flash frozen in liquid nitrogen and stored at −80°C. After thawing and prior to analysis, the samples were diluted 100- to 1000-fold in 0.2 µm filtered (FP 30/0,2 CA-S Whatman) TE-buffer (pH = 8), stained with SYBR Green I to a final concentration of 0.5×10^{-4} of the commercial stock (Life Technologies Ltd) 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 F_v/F_m (2 ml) using PAM fluorometry (Water-PAM) were kept in the dark at *in situ* temperature for 15 min, after which the minimal (F_0) and maximal (F_m) chlorophyll autofluorescence were measured. The variable fluorescence F_v was defined as $F_m - F_0$ (see Maxwell & Johnson 2000).

Nutrient samples (5 ml) were filtered over 0.2 µm Supor® membrane syringe filters (Pall Acrodisc®) into (Perkin Elmer) Pony vials™. Samples were stored

at –20°C until analysis for NO_3^- and PO_4^{3-} on a TRAACS autoanalyzer 800+ (Bran+Luebbe), according to Hansen & Koroleff (1999). The detection limits were 0.01 and 0.05 µM for PO_4^{3-} and NO_3^-, respectively.

Statistics

Statistics were carried out with the program Sigmaplot™ 12.0 (Systat software, Chicago, IL). Either 1-way ANOVAs or non-parametric Kruskall-Wallis tests were used to test whether the nutrient treatments affected the steady state parameters or viral burst sizes. Pairwise multiple comparisons were then done by Holm-Šídák or Tukey tests.

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 *Micromonas pusilla* cultures were still able to reach a near-maximum growth rate (μ_{max}), comparable to nutrient-replete conditions, but for the nutrient-controlled *Phyeocystis 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 treatments. 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 F_v/F_m 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 (Figs. 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; 1-way ANOVA, $0.438 \leq p \leq 0.653$), demonstrating nutrient starvation upon the transition from balanced nutrient-controlled growth to batch conditions (Figs. 1 & 2). The F_v/F_m 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

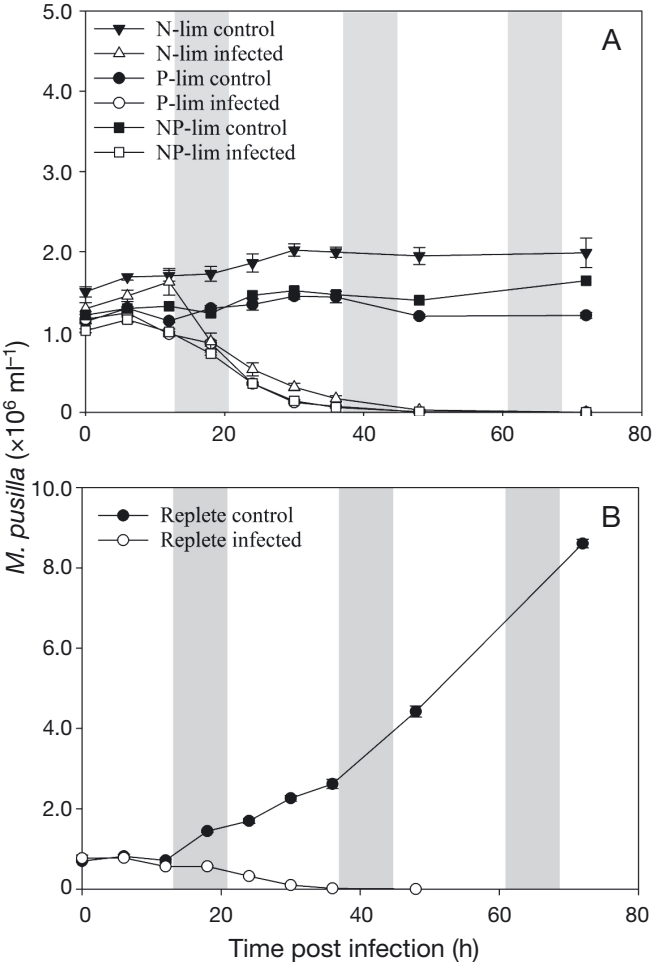

Fig. 1. Abundances of non-infected (control, closed symbols) and infected (open symbols) *Micromonas pusilla* under (A) nutrient-limited and (B) replete conditions. Shaded areas represent dark period (night). Error bars show standard errors

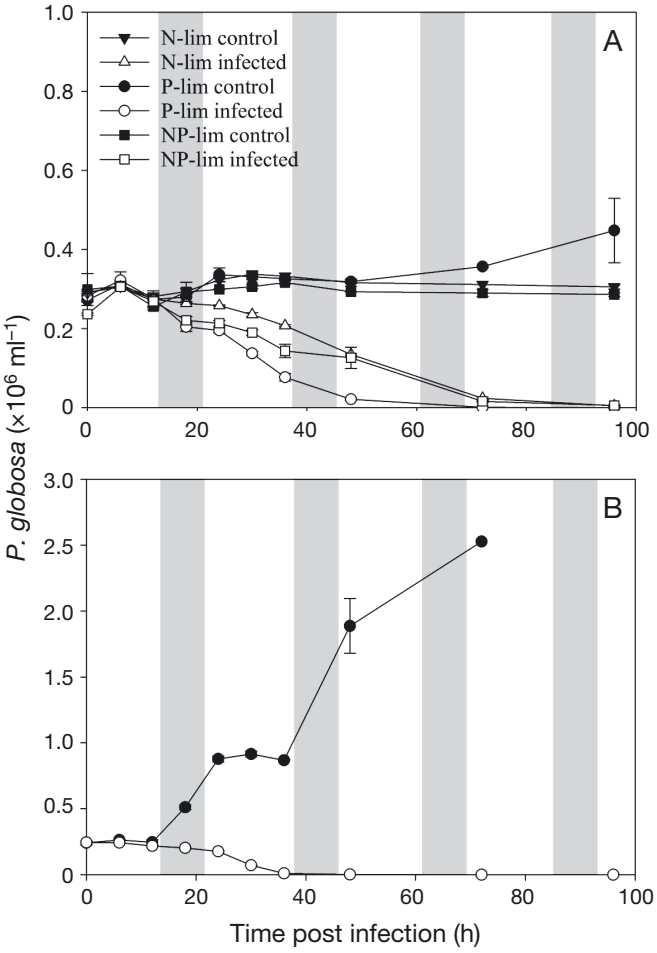

Fig. 2. Abundances of non-infected (control, closed symbols) and infected (open symbols) *Phaeocystis globosa* under (A) nutrient-limited and (B) replete conditions. Shaded areas represent dark period (night). Error bars show standard errors

the infected *M. pusilla* cultures was equally fast for the 3 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 that of the P-limited ones (48–72 h; Figs. 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 12–15 h, respectively, compared to under replete conditions, which were 6–9 and 12–15 h, respectively (Table 2, Figs. 3 & 4; Fig. S2 in the Supplement at www.int-res.com/articles/suppl/a077p087_supp.pdf). Also the rate of virus progeny increase (production) was lower under nutrient limitations in both species (Figs. 3 & 4). For MpV this was, for all nutrient limitations, 27% lower than under nutrient-replete conditions (11×10^6 MpV ml^{-1} h^{-1}), while, for PgV, this was 88% under N- and NP-limitation and 63% under P-limitation, in comparison to 5.6×10^6 PgV ml^{-1} h^{-1} for the nutrient-replete situation. The higher total MpV yield in the N-limited cultures related to the slightly higher algal host abundance prior to lysis (Figs. 1 & 3). The MpV burst sizes under nutrient-limiting conditions were reduced by 69 ± 2% as compared to under 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 conditions of 823 viruses cell^{-1}; Table 1, Fig. 4). For both algal species, viral infectivity was not affected by nutrient treatments.

Table 2. *Micromonas pusilla* and *Phaeocystis globosa*. 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 with comparison to the nutrient-replete treatment. Values are averages (± standard error) of triplicate cultures. Significant differences ($p < 0.05$) between the treatments (per species) are depicted by different superscripted letters, i.e. numbers with the same letters are not statistically different

Treatment	Latent period (h)	Burst size (viruses cell^{-1})	Burst size reduction relative to replete (%)
M. pusilla			
N-limitation	9–12	67 ± 6^b	71
P-limitation	9–12	69 ± 2^b	70
NP-limitation	9–12	77 ± 2^b	67
Replete	6–9	229 ± 1^a	
P. globosa			
N-limitation	12–15	61 ± 14^c	93
P-limitation	12–15	230 ± 36^b	72
NP-limitation	12–15	49 ± 11^c	94
Replete	9–12	823 ± 27^a	

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 F_v/F_m and cellular nutrient quotas, both well-accepted indicators of phytoplankton nutrient limitation (Beardall et al. 2001). The reduction in F_v/F_m of nutrient-limited phytoplankton is the result of a lowered photosynthetic

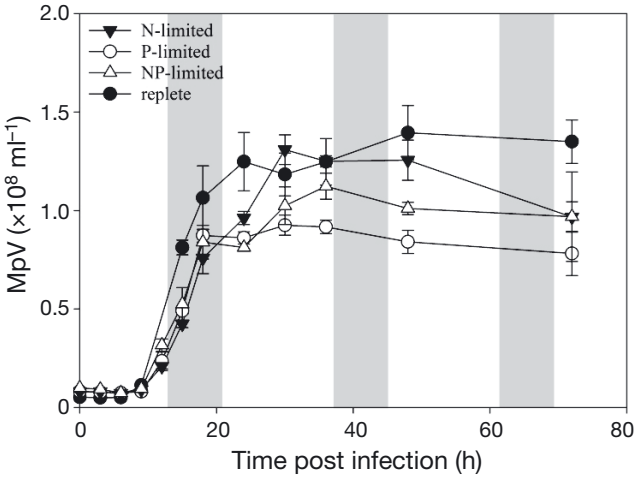

Fig. 3. Abundances of *Micromonas pusilla* virus (MpV) during the viral infection experiment under N-limited (▲), P-limited (○), NP-limited (△) and nutrient-replete (●) culture conditions. Shaded areas represent dark period (night). Error bars show standard errors

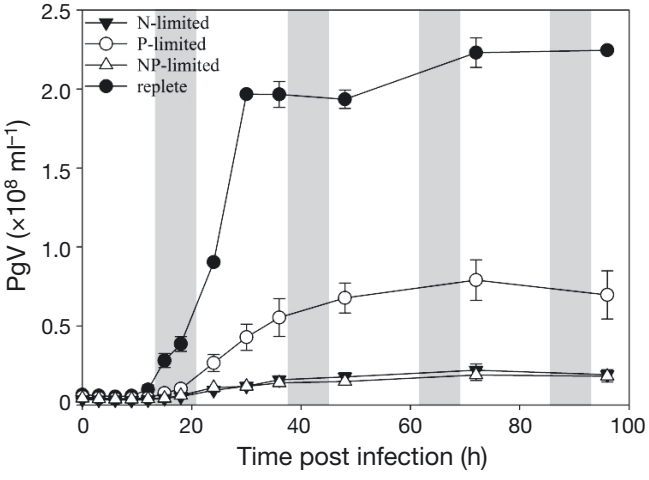

Fig. 4. Abundances of *Phaeocystis globosa* virus (PgV) during the viral infection experiment under N-limited (▲), P-limited (○), NP-limited (△) and nutrient-replete (●) culture conditions. Shaded areas represent dark period (night). Error bars show standard errors

rate, probably due to reduced concentrations of ATP and reductants (Beardall et al. 2001). The stronger F_v/F_m reduction in N-controlled *Phaeocystis globosa*, in comparison to 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, which was observed for both species, indicates chlorosis, a reduction in cellular chlorophyll *a* content. Chlorosis decreases the total demand upon nutrients for photosynthesis and leads to reduced intracellular self-shading, with more efficient light harvesting per chlorophyll molecule (Berner et al. 1989).

The differences in N- and P-quotas between nutrient-replete and nutrient-limited *Micromonas pusilla* might be partly or completely due to the accumulation, i.e. storage, of these elements under P-replete conditions. *M. pusilla* has been shown to have largely reduced concentrations of chlorophyll *a* and phospholipids when grown under P-controlled growth (0.97 and 0.32 μ_{max}) compared to P-replete conditions (Maat et al. 2014, 2016). This demonstrates that the quota reductions are at least in part due to the 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 N-quota reduced to a greater extent than the P-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 relationship between cell size and FSC, showed similar relative differences as the quotas per cell (Table S1 in the Supplement at www.int-res.com/articles/suppl/a077p087_supp.pdf).

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 have been 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 μ_{max} implies that this species is better adapted to cope with low nutrient supply than is *P. globosa*. This might be the result of its smaller cell size, i.e. 2 µm

cell diameter as compared to 5 µm 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, i.e. a higher 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 1-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 viral latent periods and burst sizes were found in an independent pilot experiment 6 mo in advance of this study (n = 3; Table S2 in the Supplement). 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 treatments. 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, or preconditioning phase, also determines the effects on virus proliferation. Still, the species-specific relative reduction in growth rate under nutrient limitation, which was stronger for *P. globosa* than for *M. pusilla*, seems to be reflected in virus growth characteristics, i.e. stronger negative effects for *P. globosa*. The viral latent periods of both species were prolonged by all nutrient limitations. 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*, pregrown in chemostats at 0.97 μ_{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 greater extent than its P-quotas and thus seems better able to cope with N-limitation, viral progeny production and burst sizes were thus more strongly affected by N-limitation than by 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 in 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, Jacquet 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, Jacquet 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 semi-natural mesocosm conditions was not severe enough, leaving nutrient availability or turnover 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 pro-

liferation is likely the result of the physiological state of the host, i.e. decreased amounts of enzymes and accessory molecules that are involved in viral replication. Nitrogen is a major component of the hosts' enzymes, which 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 oceans and how these processes are affected by the abiotic environment (Mojica & 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. 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 or size class (this study), the nutrient supply rate during the infection cycle and the type of limiting nutrient (this study). Further studies using different host–virus model systems are needed to elucidate host species specificity and examine the consequences of virus replication under macro- or micronutrient stress on the flow of energy and matter.

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