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1 **Research article (Aquatic Microbial Ecology)**

2

3 **Both phosphorus and nitrogen limitation constrain viral proliferation in marine**

4 **phytoplankton**

5

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14 Running title: Algal virus production inhibited by N-limitation

15

16 Key words: phytoplankton, algal virus, nitrogen, phosphorus, nutrient limitation, *Phaeocystis*

17 *globosa*, *Micromonas pusilla*

18

19

20 **ABSTRACT**

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

39

40

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

66 detailed understanding (i.e. a direct causal link) on the effects of N-availability on
67 phytoplankton virus-host interactions, culture experiments are required. A laboratory study
68 using *Phaeocystis pouchetii* showed that N-starvation of the host before infection resulted in
69 reduced burst sizes as compared to nutrient replete cultures (Bratbak et al. 1998). Yet at the
70 moment of infection, the cells were already in stationary phase for some days (in contrast, the
71 nutrient replete cells were growing exponentially), which makes it hard to pin-point the exact
72 causal factor of the reduced burst sizes. Jacquet et al. (2002) brought up the need for more work
73 on this topic to clarify the effects of N-limitation on virus-phytoplankton interaction. Indeed, a
74 better insight into which nutrients affect phytoplankton virus proliferation is pivotal because
75 nutrient limited phytoplankton growth and cell physiology is ubiquitous in the marine
76 environment (Moore et al. 2013) and viral lysis an important factor of phytoplankton mortality
77 and major driver of marine biogeochemical cycling (Wilhelm & Suttle 1999, Baudoux et al.
78 2007, Mojica et al. 2016).

79 The aim of this study was to investigate how N-limited algal stoichiometry and
80 physiology affect virus proliferation in the phytoplankton species *Micromonas pusilla* and
81 *Phaeocystis globosa*. The effects of N were thereby compared to the effects of P and
82 additionally mixed-nutrients (NP). Both algal species belong to genera with a global
83 distribution (Schoemann et al. 2005, Slapeta et al. 2006). The picoeukaryotic photoautotroph
84 *M. pusilla* is readily infected by viruses (Cottrell & Suttle 1995) and blooms of *P. globosa* have
85 been shown to be controlled by viruses (Baudoux et al. 2006, Brussaard et al. 2005). The algal
86 cultures were pre-grown under nutrient-controlled conditions, which synchronized the
87 physiological state of the algal cells. As virus reproduction took place during the first half day
88 post infection (p.i.), the nutrient status of the host cell at the moment of infection (i.e.
89 preconditioning of nutrient limited growth) was expected to largely control virus growth
90 characteristics (latent period and burst size). For clarity, the term (i) nutrient limitation is used

91 as a general term that describes reduced growth or altered stoichiometry and physiology due to
92 low nutrient availability (Moore et al. 2013), (ii) nutrient controlled growth refers to balanced
93 algal growth rate which is dictated by the actual dilution rate (Quinlan 1986, MacIntyre &
94 Cullen 2005), and (iii) nutrient starvation is the temporal unbalanced nutrient limited state of
95 the cultures in batch mode (Parkhill et al. 2001), as encountered during the infection
96 experiments (Maat et al. 2016).
97

98 MATERIALS AND METHODS

99

100 Culturing and experimental set-up

101

102 Axenic *M. pusilla* Mp-LAC38 (Culture collection Marine Research Center, Goteborg
103 University) and *P. globosa* G(A) (Culture collection University of Groningen, The Netherlands)
104 were cultured at 15˚C under a 16:8h light:dark cycle with 100 µmol quanta m^{-2} s^{-1} irradiance
105 during the light period (18W/965 OSRAM daylight spectrum fluorescent tubes; München,
106 Germany). The medium used (Mix-TX) was a 1:1 mixture of modified f/2 medium (Guillard &
107 Ryther 1962) and artificial seawater (ESAW; Harrison 1980), enriched with Tris-HCl and
108 Na_2SeO_3 (Cottrell & Suttle 1991), and with Na_2-glycerophophate omitted. Semi-continuous
109 nutrient controlled culturing was chosen over full continuous (chemostat) cultures due to
110 logistical considerations (for good comparisons we needed to handle 48 cultures
111 simultaneously). Although different from chemostats, because the cultures are diluted once per
112 day instead of continuously, this method yields comparable constant growth and physiology.
113 With both types of culturing, the concentration of the limiting nutrient determines the algal
114 abundances (biomass), while the medium dilution rate (supply of the limiting nutrient)
115 determines the algal growth rate (µ) (Quinlan 1986, Nicklisch 1999). The concentrations of

116 $NaNO_3$ and Na_2HPO_4 in the Mix-TX medium (Table 1) were then chosen as such that the cell
117 abundances of the nutrient controlled cultures were comparable. The maximum growth rates of
118 the algal species were initially determined in replete (low abundance) batch cultures. In the
119 nutrient limiting semi-continuous cultures, growth was then maximized under these specific
120 conditions, i.e. maintained as close as possible to the μ_{max}, i.e. highest possible without wash-
121 out of cells. The maximum cell abundance that was reached in this manner was maintained
122 (balanced growth) and steady state samples for nutrient concentrations and algal physiology
123 were taken. In this way the nutrient limited cultivation allowed cells to grow at rates identical
124 to chemostat culturing and potentially even near maximum growth rate ($\mu_{max;}$ as under nutrient
125 replete conditions; Quinlan 1986, Henry et al. 2008). Hence, on a daily basis the cells received
126 a specific amount of limiting nutrient similar to the total cell quotas, i.e., just enough to maintain
127 the set growth rate under the specific conditions. Although the nutrients in our semi-continuous
128 cultures were taken up by the cells within the hour, the diel dynamics of cell growth and
129 photosynthetic efficiency were similar to those under replete conditions and thus not affected
130 by the discontinuous addition of medium (nutrients; Fig. S1). Growth of the semi-continuous
131 cultures was either N-controlled, P-controlled or NP-controlled (i.e., daily addition of both
132 nutrients to allow growth of the cultures). Nutrient replete cultures were used as control
133 treatment, whereby the dilution rate was chosen to keep cell abundances at a fixed abundance
134 (comparable to abundances reached in nutrient limited cultures), according to the turbidostat
135 principle (MacIntyre & Cullen 2005). Cultures were considered to be at steady state (balanced
136 growth) when growth rates and cell abundances prior to dilution maintained constant with time.
137 Over the 4 weeks before the experiment, average (±s.d.) cell abundances were ($1.2\pm0.06\times10^6$,
138 $1.4\pm0.07\times10^6$, $1.2\pm0.05\times10^6$ and $3.2\pm0.21\times10^5$, $3.0\pm0.11\times10^5$, $3.0\pm0.12\times10^5$ for the N-, P- and
139 NP-controlled cultures of *M. pusilla* and *P. globosa*, respectively). Steady state inorganic
140 nutrient concentrations were additionally monitored to make sure that all of the daily added

141 nutrients were taken up by the cells. The average daily growth (and thus dilution) rates during
142 steady state are depicted in Table 1. All treatments were performed in triplicate. The cultures
143 were also sampled on a daily basis for flow cytometry (FCM) for the analysis of cell
144 abundances, mean cellular forward scatter (FSC; indicator of cell size) and chlorophyll
145 autofluorescence (RFL). Nutrient concentrations (sampled regularly during steady state and at
146 the end of the viral infection experiments) were analyzed by colorimetry and the photosynthetic
147 efficiency (Fv/Fm) was monitored by pulse amplitude modulated (PAM) fluorometry. Steady
148 state cellular nutrient quotas of the nutrient controlled axenic phytoplankton cultures were
149 calculated by dividing the decrease in the growth limiting nutrient in the culture over 24h (i.e.
150 nutrients taken up; which for nutrient-controlled conditions equals the daily input as
151 concentration after 24h was always below detection limit) by the increase in cell abundance
152 over the same time period (Table 1; Veldhuis & Admiraal 1987). For the replete cultures the
153 nutrient quotas were determined in the same way and under the same conditions. The nutrient
154 limited state of the cells was demonstrated by alterations in cellular physiology, especially
155 cellular nutrient quotas and Fv/Fm, both specific indicators of phytoplankton nutrient limitation
156 (Beardall et al. 2001, Maat et al. 2014).

157 Two days before the experiment each culture was split into two cultures in order to
158 accommodate the viral infection experiments (i.e. one control and one virally infected). As each
159 treatment was cultured in triplicate, this yielded per species a total of 3 replicate non-infected
160 controls and 3 replicate infected cultures per nutrient treatment. Viral infection experiments
161 were started 3 h into the light period and performed in batch (dilution and hence supply of the
162 limiting nutrient was stopped) in order to (i) optimize the conditions for successful one-step
163 virus growth curve (from which the latent period and burst size are determined), and (ii) avoid
164 virus proliferation simultaneous with the uptake of the added limiting resources, as this may
165 influence virus growth characteristics. For the first 12 h Fv/Fm of the 'batched' non-infected

166 culture was identical to a nutrient controlled culture that still received the daily supply of
167 limiting nutrient (Fig. S1) and only towards the end of the first day did Fv/Fm of the batch
168 culture drop (by about 10%).

169 Cultures were inoculated with axenic viral lysate at a multiplicity of infection (MOI) of
170 10. Infectivity was determined by endpoint dilution of the algal virus lysates according to Suttle
171 (1993) and the abundance of infectious viruses was found largely comparable to total virus
172 count by FCM. Both axenic MpV-08T infecting *M. pusilla* LAC-38 (Martinez Martinez et al.
173 2015) and axenic PgV-07T infecting *P. globosa* G(A) (Baudoux & Brussaard 2015) are lytic
174 dsDNA viruses and originate from the NIOZ culture collection. Viral lysates were obtained
175 under the same conditions as the algal hosts, whereby the lysates for the N- and P-limited
176 treatments were produced by at least three infection cycles on N-, P- or NP-controlled host. No
177 detectable levels of limiting nutrient(s) were recorded in the lysates that were used for the viral
178 infection experiments. The non-infected controls received sterilized 0.2 µm filtered (Sartorius
179 A.G. cellulose acetate filters, Goettingen, Germany), aged natural seawater with N- and P-
180 concentrations below the limit of detection. Sampling post infection (p.i.) took place every 3-6
181 h for algal and viral abundances and every 24 h for PAM fluorometry (the latter only for the
182 non-infected cultures). Viral infectivity of the progeny viral lysates was determined at the end
183 of the experiments using endpoint dilution assay (Suttle 1993) and compared to FCM total virus
184 counts in order to obtain % infective. Inorganic nutrient concentrations were sampled at the
185 start and end of the experiments. Algal and viral abundances were monitored during the
186 infection experiment to determine the viral latent periods and burst sizes. Latent periods were
187 determined as the time interval in which a clear increase in viral abundances was observed that
188 continued during the following time-points. The burst sizes were determined by dividing the
189 number of produced viruses by the maximum number of lysed host cells.

190 Both the algal cultures and viral lysates were regularly checked for axenity by
191 epifluorescence microscopy (Porter & Feig 1980). In short, 1 ml samples were fixed with (0.1%
192 final concentration) glutaraldehyde, stained with DAPI (4',6-diamidino-2-phenylindole,
193 dihydrochloride; Life Technologies Ltd. Paisley, UK) and filtered over a 0.2µm black
194 polycarbonate filter (Whatman, Maidstone, UK). The cultures were axenic at all times.
195
196 **Analyses**
197
198 Flow cytometric determination of phytoplankton abundances was done on fresh samples
199 according to Marie et al. (1999) using a BD Accuri^{tm} C6 cytometer (BD Biosciences, San Jose,
200 Ca, USA) with the trigger on Chlorophyll *a* red autofluorescence (RFL). The phytoplankton
201 cells were distinguished in a scatter plot of RFL versus forward scatter (FSC). The mean cellular
202 RFL and FSC signals were recorded as indicators for steady state cellular chlorophyll content
203 and cell size (Shapiro 1988, DuRand et al. 2002).
204 Viruses were enumerated on fixed samples (according to Brussaard 2004) using a 488
205 nm argon laser-containing benchtop FacsCalibur flow cytometer (BD Biosciences, San Jose,
206 Ca, USA) with the trigger set on green fluorescence. In short, 1 ml samples were fixed with
207 25% gluteraldehyde (EM-grade, 0.5% final concentration; Sigma-Aldrich, St. Louis, MO,
208 USA), incubated for 30 minutes at 4°C, flash frozen in liquid nitrogen and stored at -80°C.
209 After thawing and prior to analysis, the samples were diluted 100-1000 fold in 0.2 µm filtered
210 (FP 30/0,2 CA-S Whatman, Dasser, Germany) TE-buffer (pH=8), stained with SYBR Green I
211 to a final concentration of 0.5×10^{-4} of the commercial stock (Life Technologies Ltd., Paisley,
212 UK) for 10 min at 80°C. Viruses were quantified on a scatter plot of green fluorescence versus
213 side scatter (SSC). All flow cytometry data were analyzed using CYTOWIN 4.31 (Vaulot
214 1989).

215 Samples for Fv/Fm (2 ml) using PAM fluorometry (Water-PAM, Walz, Germany) were
216 kept in the dark at *in situ* temperature for 15 min, after which the minimal (F0) and maximal
217 (Fm) chlorophyll autofluorescence were measured. The variable fluorescence Fv was defined
218 as Fm-F0 (see Maxwell & Johnson (2000).

219 Nutrient samples (5ml) were filtered over 0.2µm Supor® membrane syringe filters (Pall
220 Acrodisc®, Ann Arbor, MI, USA) into (Perkin Elmer, Shelton, USA) Pony vials^{TM}. Samples
221 were stored at -20ºC until analysis for NO_3^- and PO_4^{3-} on a TRAACS autoanalyzer 800+
222 (Bran+Luebbe, Norderstedt, Germany), according to Hansen and Koroleff (1999). The
223 detection limits were 0.01 and 0.05 µM for PO_4^{3-} and NO_3^-, respectively.

224

225 **Statistics**

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

230

231 **RESULTS**

232

233 **Steady state of preculturing phase**

234

235 For both species, the maximized steady state exponential growth rates under nutrient controlled
236 semi-continuous culturing were not affected by the type of limitation (N-, P- or NP-control;
237 Table 1). The nutrient controlled *M. pusilla* cultures were still able to reach near-maximum
238 growth rate (μ_{max}) as under nutrient replete conditions, but for the nutrient controlled *P. globosa*
239 cultures this was at best 73% of μ_{max} under replete conditions (Table 1). The cellular nutrient

240 quotas of the nutrient controlled cultures were strongly reduced as compared to the replete
241 treatment with excess nutrients (Table 1). For *M. pusilla* the N- and P-quotas under nutrient
242 controlled growth were both approximately 7-fold lower relative to nutrient replete. For *P.*
243 *globosa* this was 11-fold for the N quota, but only 1.5-fold for the P quotas. The mean cellular
244 RFL in the N- and NP-controlled cultures was, in contrast to the P-controlled treatment,
245 significantly reduced for both phytoplankton species. Moreover, mean cellular FSC of *M.*
246 *pusilla* was reduced under these conditions (Table 1). Finally, for both algal species the Fv/Fm
247 of the nutrient limited cultures at steady state was slightly, but significantly, reduced compared
248 to the replete cultures (Table 1).
249

250 **Viral infection experiments**

251

252 The viral infection experiments were carried out under batch conditions using the steady state
253 nutrient controlled and nutrient replete cultures. Growth of the non-infected nutrient replete
254 cultures did not change as a result of the batch mode, as can be expected under excess nutrient
255 conditions (Fig. 1&2). However, the algal growth rates of the nutrient limited non-infected *M.*
256 *pusilla* and *P. globosa* cultures showed a respective 62±16 and 81±10 % decrease over the first
257 24 h p.i. No significant difference was found for the different types of limitations (N, P and NP;
258 one-way ANOVA, $0.438<p<0.653$), demonstrating nutrient starvation upon the transition from
259 balanced nutrient controlled growth to batch conditions (Fig. 1&2). The Fv/Fm of the non-
260 infected nutrient limited *M. pusilla* cultures at 24 h p.i. showed a 33±0.02 % reduction in
261 comparison with steady state conditions, while this was a mere 12±0.04 % decrease for *P.*
262 *globosa*. Time until full lysis of the infected *M. pusilla* cultures was equally fast for the three
263 nutrient treatments (48 h), but for infected *P. globosa* full lysis of the N- and NP-limited cultures
264 was reached approximately 24 h later than of the P-limited ones (48-72 h; Fig. 1 & 2).

265 The viral latent periods were not affected by the type of nutrient limitation, but overall
266 both MpV and PgV latent periods were prolonged under nutrient limitation, i.e. 9-12 and 12-
267 15h, respectively, compared to replete conditions (6-9 and 12-15h, respectively; Table 2, Fig.
268 3&4, Fig. S2). Also the rate of virus progeny increase (production) was lower under the nutrient
269 limitations in both species (Fig. 3&4). For MpV this was 27% lower (for all nutrient limitations)
270 than under nutrient replete conditions (11×10^6 MpV ml^{-1} h^{-1}), while for PgV this was 88% under
271 N- and NP-limitation and 63% under P-limitation and (in comparison to 5.6×10^6 PgV ml^{-1} h^{-1}
272 for nutrient replete). The higher total MpV yield in the N-limited cultures related to the slightly
273 higher algal host abundance prior to lysis (Fig. 1&3). The MpV burst sizes under nutrient
274 limiting conditions were reduced by 69±2 % as compared to nutrient replete conditions (229
275 viruses $cell^{-1}$), independent of the type of limitation (Table 2). For *P. globosa* this reduction
276 was strongest under N- and NP-limitation (>93%) and similar under P-limitation (72%;
277 compared to nutrient replete of 823 viruses $cell^{-1}$; Table 1, Fig. 4). For both algal species, viral
278 infectivity was not affected by the nutrient treatments.
279
280 **Discussion**
281
282 **Steady state of preculturing phase**
283
284 By using semi-continuous cultivation, the algal cultures were well-adapted to growth under
285 nutrient limiting conditions. During steady state the cells showed clear nutrient limited
286 physiology (Quinlan 1986, Nicklisch 1999; Moore et al 2013). This was most pronounced for
287 the Fv/Fm and cellular nutrient quotas, both well-accepted indicators of phytoplankton nutrient
288 limitation (Beardall et al. 2001). The reduction in Fv/Fm of nutrient limited phytoplankton is
289 the result of a lowered photosynthetic rate, probably due to reduced concentrations of ATP and

290 reductants (Beardall et al. 2001). The stronger Fv/Fm reduction in N-controlled *P. globosa* (in
291 comparison to the P-controlled cultures) may be attributed to a reduction of the photosystem II
292 (PSII) reaction center protein D1 under N limitation (Geider et al. 1993). Furthermore, the
293 reduction in cellular RFL under N-controlled growth (observed for both species) indicates
294 chlorosis, a reduction in cellular chlorophyll *a* content. Chlorosis decreases the total demand of
295 nutrients for photosynthesis and leads to reduced intracellular self-shading (package effect)
296 with consequently a more efficient light harvesting per chlorophyll molecule (Berner et al.
297 1989).

298 The differences in N- and P-quota between nutrient replete and limited *M. pusilla*
299 might be (partly) due to the accumulation, i.e. storage, of these elements under P-replete
300 conditions. *M. pusilla* has been shown to have largely reduced concentrations chlorophyll *a* and
301 phospholipids when grown under P-controlled growth (0.97 and 0.32μ_{max}), compared to P-
302 replete conditions (Maat et al. 2014, Maat et al. 2016). This demonstrates that the quota
303 reductions are at least in part due to reductions of actual cellular components. Still, the
304 reductions are within the ranges that are reported in the literature (Geider & LaRoche, 2002).
305 For *P. globosa*, the P-quota reduced to a stronger extent than the N-quota, suggesting that this
306 nanophytoplankter copes better with N-limitation. This is supported by a study that showed a
307 competitive advantage of *Phaeocystis* over other phytoplankton species under N-limiting
308 conditions, while being outcompeted under P-limiting conditions (Riegman et al. 1992). NP-
309 controlled growth led to decreased quota of both N and P in both species, suggesting that the
310 cells were co-limited (Moore et al. 2013). It is, however, difficult at this stage to determine
311 whether growth was ultimately inhibited by only one of the nutrients or whether both were
312 involved (Arrigo 2005, Moore et al. 2013). Volume-specific nutrient quotas (based on a linear
313 relation between cells size and FSC) showed a similar outcome (relative differences) as the
314 quotas per cell (Table S1).

315 There were differences in the capability of the phytoplankton species to handle nutrient
316 controlled growth, i.e. nutrient controlled *P. globosa* showed maximum growth rates that were
317 reduced by approximately 30% in comparison to nutrient replete growth, whereas *M. pusilla*
318 was able to reach near-maximum growth rates. Thus despite the poorer physiological state of
319 the nutrient limited *M. pusilla* cells relative to the nutrient replete cells, they had just enough of
320 the limiting nutrient to allow growth similar to the maximum growth rate observed under
321 nutrient replete conditions. Similar results are reported for *M. pusilla* growing in P-limited
322 chemostats, with net primary production, photophysiology and P:N:C stoichiometry strongly
323 reduced (Maat et al. 2014). The ability of *M. pusilla* to still grow near μ_{max} implies that this
324 species is better adapted to cope with low nutrient supply than *P. globosa*. This might be the
325 result of its smaller cell size, i.e. 2 µm cell diameter as compared to 5 µm for *P. globosa.*
326 Smaller-sized species are indeed thought to be better adapted to nutrient limitation, due to their
327 higher surface to volume ratio, and smaller cell boundary layer (Raven 1998). The reduction in
328 mean cellular FSC under N- and NP-controlled growth suggests that *M. pusilla* became even
329 smaller in response to shortage of N. This could either be an adaptation to develop a more
330 efficient nutrient uptake (more optimal surface to volume ratio), or a result of reduced
331 intracellular components due to reduced total protein content (Geider et al. 1993).
332

333 **Virus infection experiments**

334 The one-step virus growth cycles of both MpV and PgV were strongly affected by N-
335 and P-limitation, resulting in prolonged latent periods and strongly reduced burst sizes,
336 independent of the type of nutrient. Similar effects on the viral latent periods and burst sizes
337 were found in an independent pilot experiment 6 months in advance of this study (n=3; Table
338 S2). The results are thus highly reproducible, showing that the observed effects are inherent to
339 the tested species and conditions. The observed effects may, however, be underestimated as

340 theoretically the organic nutrients in the added viral lysate could have affected viral
341 proliferation. Yet, considering the 10% (v/v) addition of viral lysate with 70% of the released
342 cellular nutrients in the dissolved phase (Gobler et al. 1997) and an estimated 35% N and 70%
343 P bioavailable (Lønborg & Álvarez-Salgado, 2012), the contribution to the total N and P in the
344 cultures would have been a few percent at maximum.

345 Although the specific phytoplankton growth rates decreased when the medium supply
346 was stopped, there were no significant differences for the type of nutrient limitation (N, P or
347 NP). At the same time there were, however, nutrient type-specific differences in the speed and
348 extent of PgV proliferation, i.e., slower host lysis, lower viral production and lower burst sizes
349 for the N and NP treatment. This indicates that host growth rate as such was not the (sole)
350 determinant for the outcome of infection and that the physiological history of the algal host
351 (preculturing or preconditioning phase) also determines the effects on virus proliferation. Still,
352 the species-specific relative reduction in growth rate under nutrient limitation (stronger for *P.*
353 *globosa* than *M. pusilla*) seems to be reflected in the virus growth characteristics, i.e. stronger
354 negative effects for *P. globosa*. The viral latent periods of both species were prolonged by all
355 nutrient limitations (N, P or NP). This demonstrates that not only P (Bratbak et al. 1993, Wilson
356 et al. 1996, Clasen & Elser 2007, Maat et al. 2014) but also N is needed for optimal virus
357 proliferation. The observed elongations of the viral latent periods under P-limitation were also
358 reported for MpV infecting *M. pusilla*, pre-grown in chemostats at $0.97\mu_{max}$ (Maat et al. 2014).
359 Strikingly, our results show that N-limitation of the algal host can have similar (MpV) and even
360 larger (PgV) adverse effects on virus production than P-limitation (i.e. rates of increase of
361 extracellular progeny viruses and viral burst sizes). Even despite the finding that *P. globosa*
362 was able to reduce its N-quotas to a stronger extent than its P-quotas (thus seems better able to
363 cope with N limitation), viral progeny production and burst sizes were thus more strongly
364 affected by N-limitation than P-limitation. Although the NP-controlled treatment resulted in

365 significantly higher nutrient quotas than the single-limitations of P or N for both species, the
366 differences were very small, which might explain why no significant additional effect was
367 found on viral proliferation of this treatment.

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

382 Host characteristics are strongly affected by nutrient limitation, and viruses are strictly
383 dependent on their host cells for the energy and elements needed for replication. Hence, the
384 constraint on viral proliferation is likely the result of the physiological state of the host, i.e.,
385 decreased amount of enzymes and accessory molecules that are involved in viral replication.
386 Nitrogen is a major component of the hosts' enzymes that are necessary for processes in viral
387 replication, such as transcription and translation of the viral genes and possibly
388 photophosphorylation (Mackenzie & Haselkorn 1972, Baudoux & Brussaard 2008). As nutrient
389 limitation has been shown to lower total protein content in phytoplankton cells, this might be

390 an even more important causal factor of nutrient limitation of viruses than the actual role of N
391 as an element in the actual viral components. The same holds for P, which is an irreplaceable
392 element in the energy metabolism of organisms and thus also in the above mentioned processes.
393 Indeed, P-limitation has been shown to lead to reduced intracellular adenylate content (AMP,
394 ADP and ATP) in phytoplankton (Theodorou et al. 1991). It has been suggested that some
395 phytoplankton viruses recycle host nucleic acids, but it is not clear to what extent this would
396 happen (Brown et al. 2007, Brown & Bidle 2014).
397
398 **Ecological implications**
399
400 Many questions are still unresolved about the precise role that phytoplankton viruses
401 play in biogeochemical cycling in the oceans and how these processes are affected by the abiotic
402 environment (Mojica and Brussaard 2014). Here we show that N-availability has the same
403 potential as P-availability in its effects on phytoplankton growth and physiology, and
404 consequently the ability of their lytic viruses to propagate (latent periods, burst sizes).
405 Extrapolating this to natural ecosystems, the diminished virus production under N- (and/or P-)
406 limitation will reduce the chance of new infections of phytoplankton (Levin & Lenski 1983,
407 Murray & Jackson 1992, Bratbak et al. 1998, , Mann 2003). The significance of N and P as
408 regulatory factors seems high as these nutrients are often limiting marine phytoplankton
409 production in many coastal and oceanic regions worldwide (Moore et al. 2013). Besides,
410 nutrient limitation is expected to increase in space and time due to global climate change-
411 induced warming of the surface oceans and subsequently strengthened vertical stratification
412 (Sarmiento et al. 2004, Behrenfeld et al. 2006). Yet, to what extent nutrient limitation will
413 control virus-host interactions under natural conditions will depend on several factors, such as
414 the algal host species (possibly size class; this study), the nutrient supply rate during the

415 infection cycle and the type of limiting nutrient (this study). Further studies using different
416 virus-host model systems are needed to elucidate host species-specificity and examine the
417 consequences of virus replication under nutrient stress (N, P, but also micronutrients) on the
418 flow of energy and matter.
419

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426

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593

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.

	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.)	Fv/Fm (r.u.)
M. pusilla	N-limitation	1	16	0.74 ±0.10 ^{a}	94	-	0.71 ±0.12 ^{a}	0.71 ±0.12 ^{b}	0.57 ±0.08 ^{b}	0.60 ±0.03 ^{b}
	P-limitation	400	0.25	0.74 ±0.18 ^{a}	94	0.19 ±0.03 ^{a}	-	1.03 ±0.06 ^{a}	1.02 ±0.02 ^{a}	0.60 ±0.01 ^{b}
	NP-limitation	1	0.25	0.76 ±0.14 ^{a}	96	0.22 ±0.03 ^{a}	0.88 ±0.09 ^{b}	0.76 ±0.05 ^{b}	0.62 ±0.05 ^{b}	0.60 ±0.03 ^{b}
	Replete	400	16	0.79 ±0.19 ^{a}	100	*1.23±0.38*	*5.29±0.71*	1.00 ±0.10 ^{a}	1.00 ±0.02 ^{a}	0.64 ±0.01 ^{a}
P. globosa	N-limitation	4	16	0.76 ±0.08 ^{b}	68	-	*14.8 ±1.01* ^{a}	1.01 ±0.01 ^{a}	0.66 ±0.03 ^{b}	0.62 ±0.02 ^{c}
	P-limitation	400	1	0.82 ±0.07 ^{b}	73	*3.62±0.17* ^{a}	-	1.09 ±0.02 ^{b}	0.93 ±0.05 ^{a}	0.65 ±0.02 ^{b}
	NP-limitation	4	1	0.79 ±0.04 ^{b}	71	*4.22±0.29* ^{b}-	*16.9 ±1.39*	1.02 ±0.10 ^{a}	0.76 ±0.05 ^{b}	0.63 ±0.01 ^{c}
	Replete	400	16	1.12 ±0.05 ^{a}	100	*5.68±0.18*	*163.4±* $^{28.65}$	1.00 ±0.08 ^{a}	1.00 ±0.02 ^{a}	0.70 ±0.01 ^{a}

599 Table 2: The latent period (h), burst size (viruses produced per lysed host cell) and percentage
600 reduction in burst size of MpV and PgV under N-, P- and NP-limitation and compared to
601 nutrient replete treatment. Values are averages of triplicate cultures. Significant differences
602 ($p<0.05$) between the treatments (per species) are depicted by different letters ([a,b,c]), i.e. numbers
603 with the same letters are not statistically different.

604

605

606

Species	Treatment	Latent period (h)	Burst size (viruses $cell^{-1}$)	burst size reduction relative to replete (%)
M. pusilla	N-limitation	9-12	67 ±10 [b]	71
	P-limitation	9-12	69 ±4 [b]	70
	NP-limitation	9-12	77 ±4 [b]	67
	Replete	6-9	229 ±2 [a]	
P. globosa	N-limitation	12-15	61 ±23 [c]	93
	P-limitation	12-15	230 ±62 [b]	72
	NP-limitation	12-15	49 ±19 [c]	94
	Replete	9-12	823 ±46 [a]	

607

608

609 **Figure Legends**

610

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

613

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

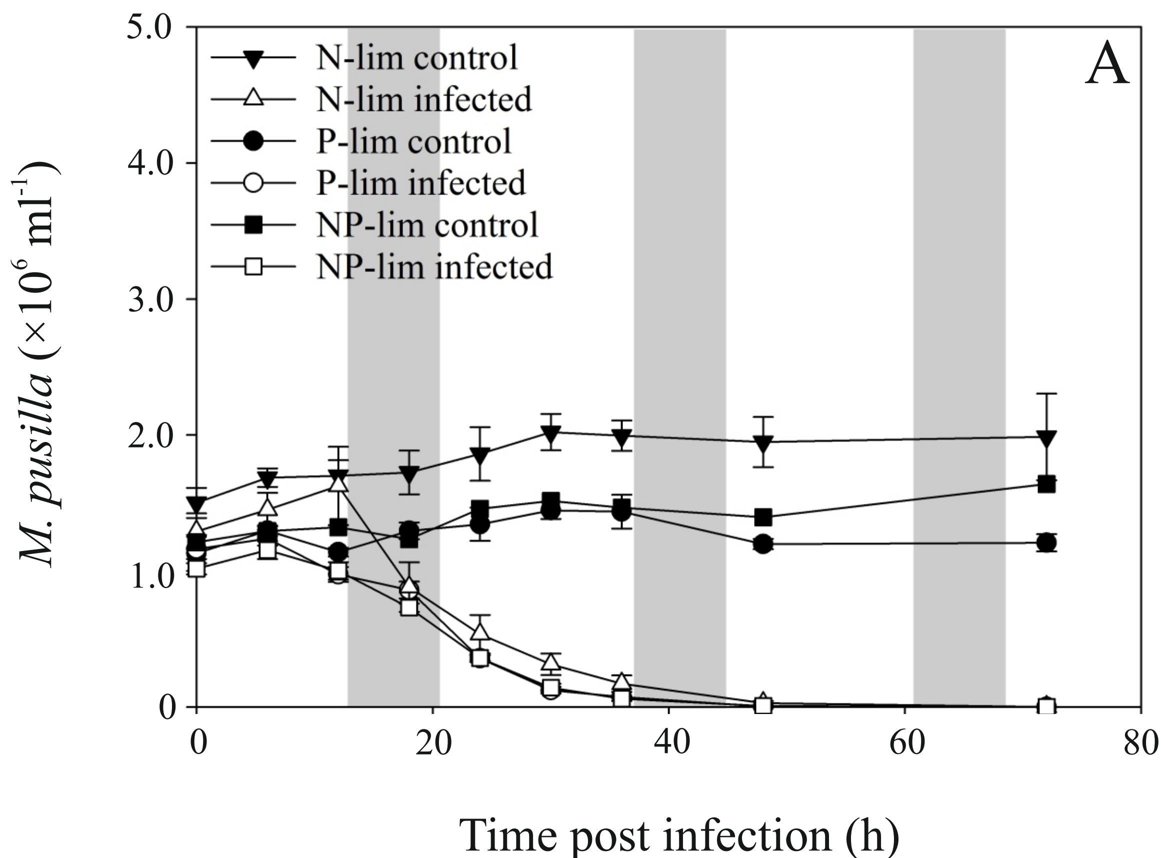
616

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

620

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

Figure 1



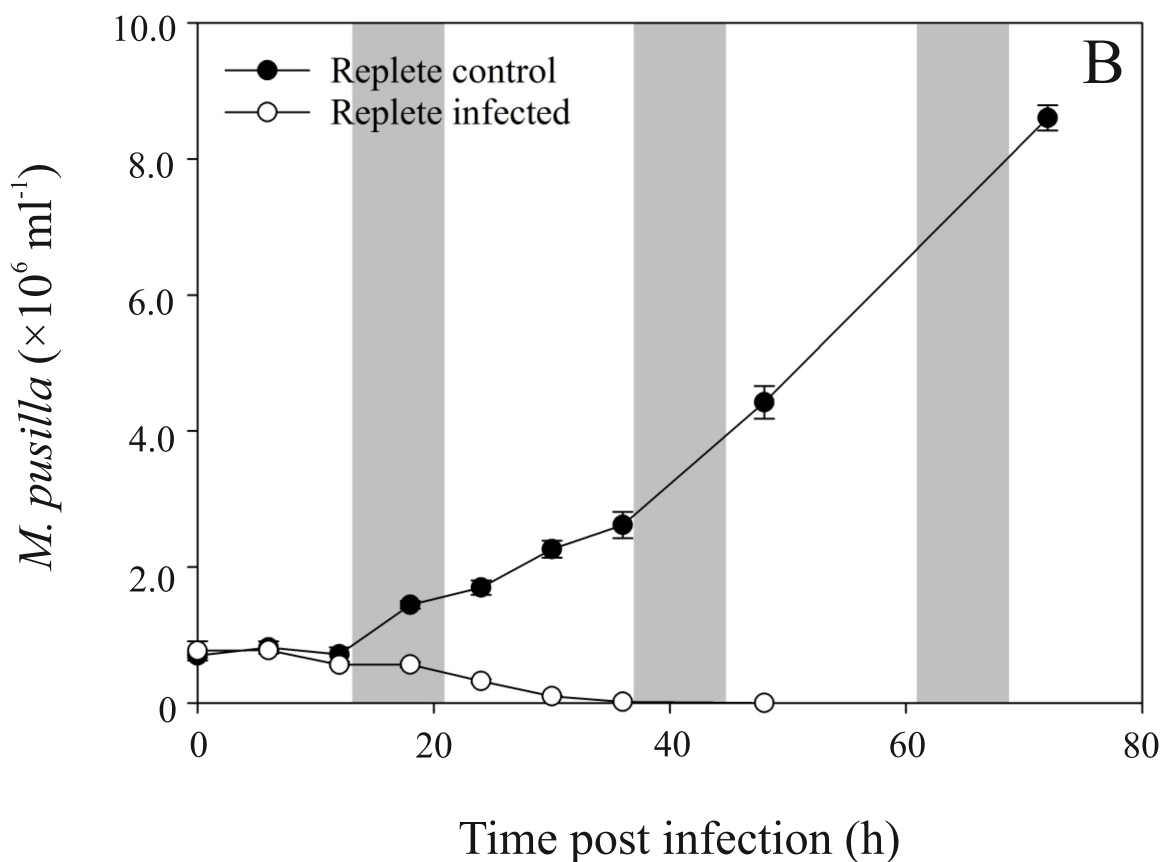
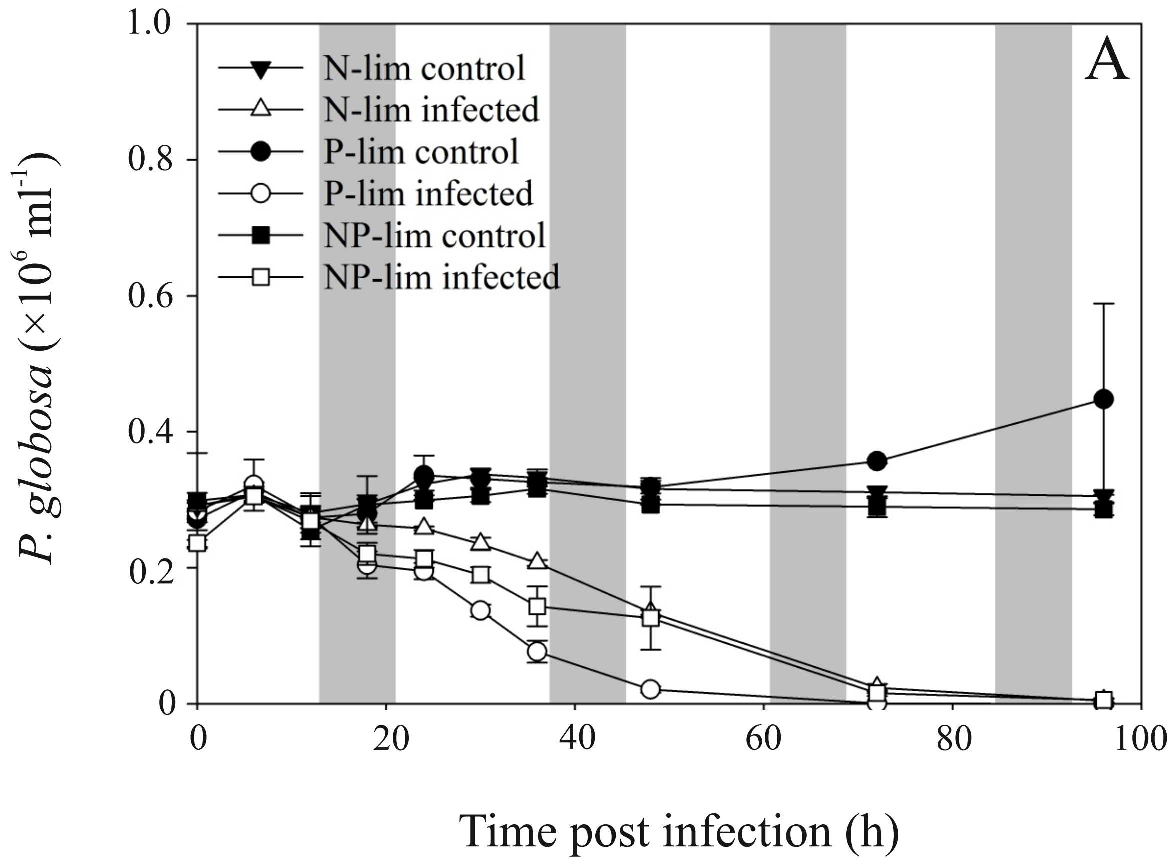


Figure 2



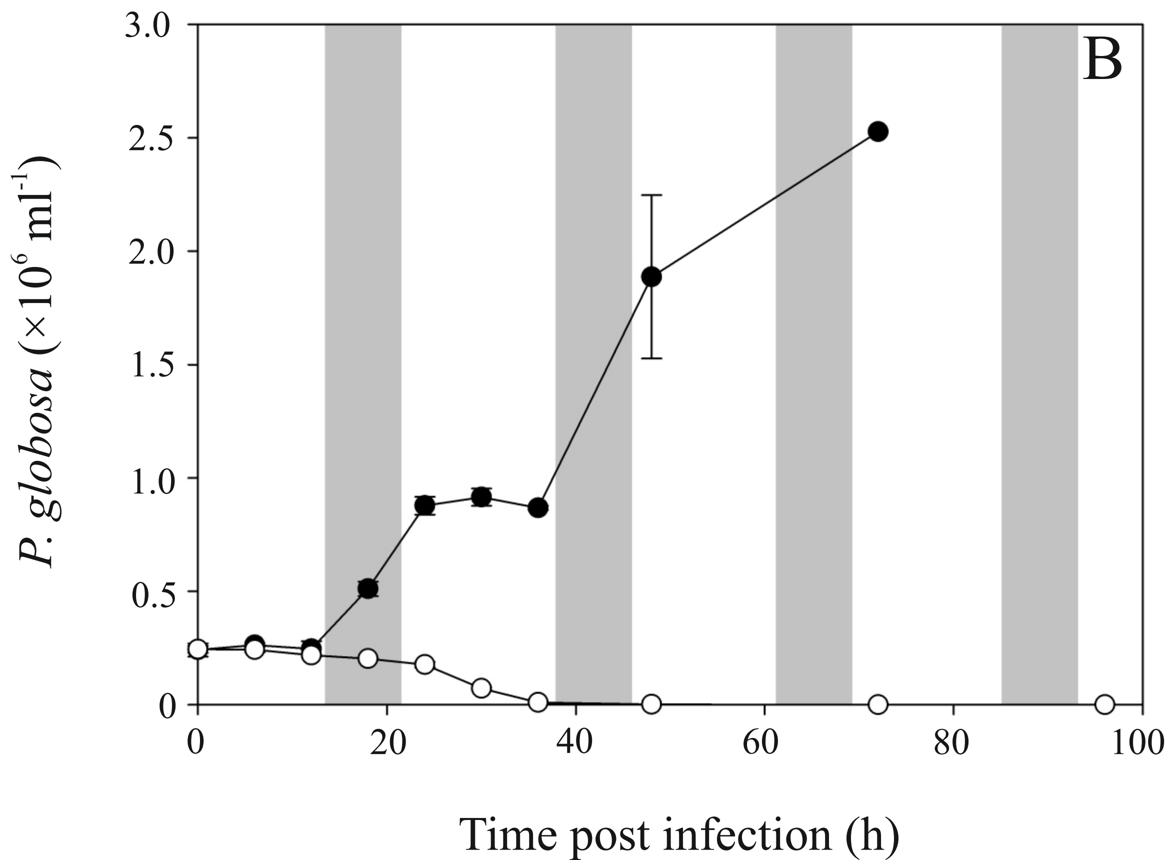


Figure 3

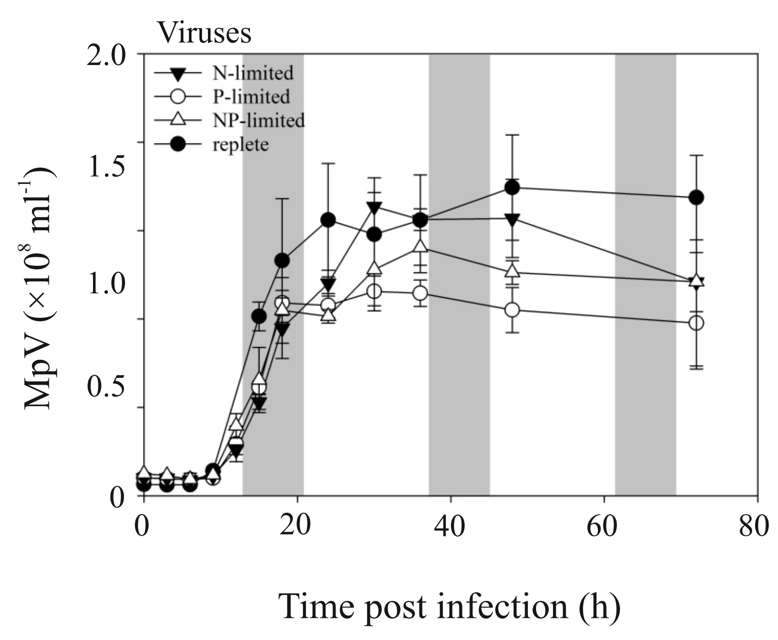


Figure 4

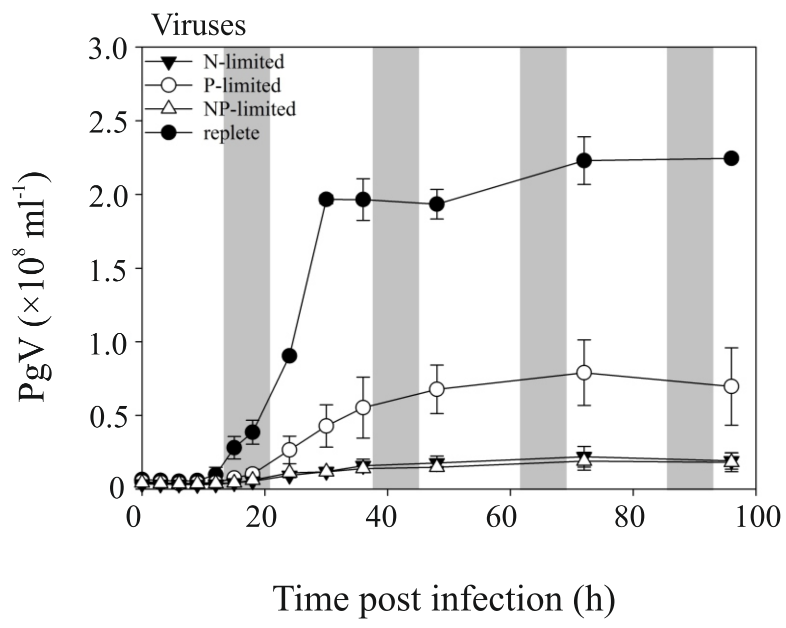


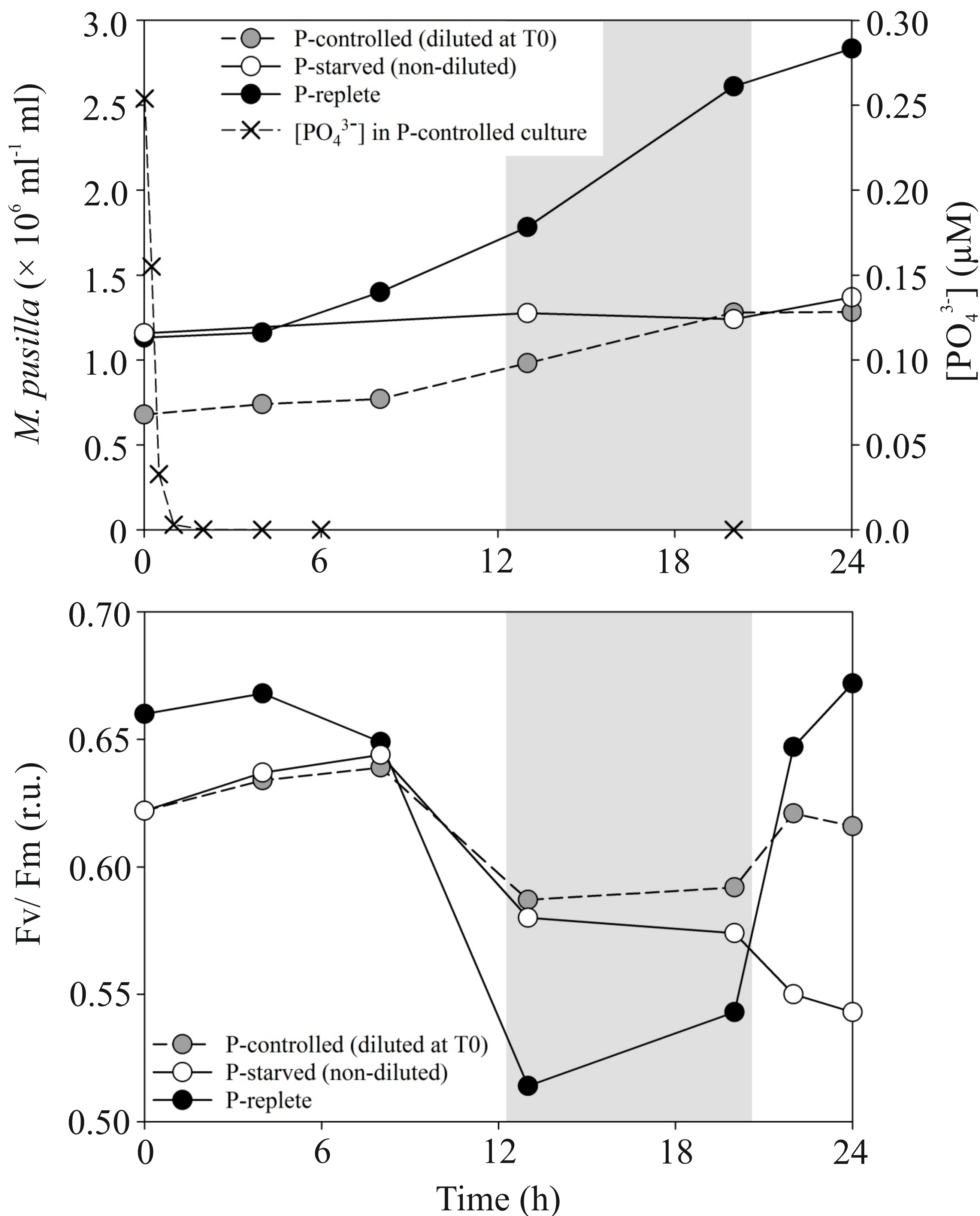
Table S1: Volume specific cellular nutrient quotas of Phaeocystis globosa and Micromonas pusilla under N- limited, P-limited, NP-limited and nutrient replete conditions. Cell volume was calculated from the cell diameter, which was based on a linear relationship between phytoplankton cells size (y) and 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.

	P. globosa		*M. pusilla*	
	P (fmol µm^{-3})	N (fmol µm^{-3})	P (fmol µm^{-3})	N (fmol µm^{-3})
N-limited		0.17		0.08
P-limited	0.04		0.02	
NP-limited	0.05	0.19	0.03	0.10
replete	0.06	1.85	0.12	0.52

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)

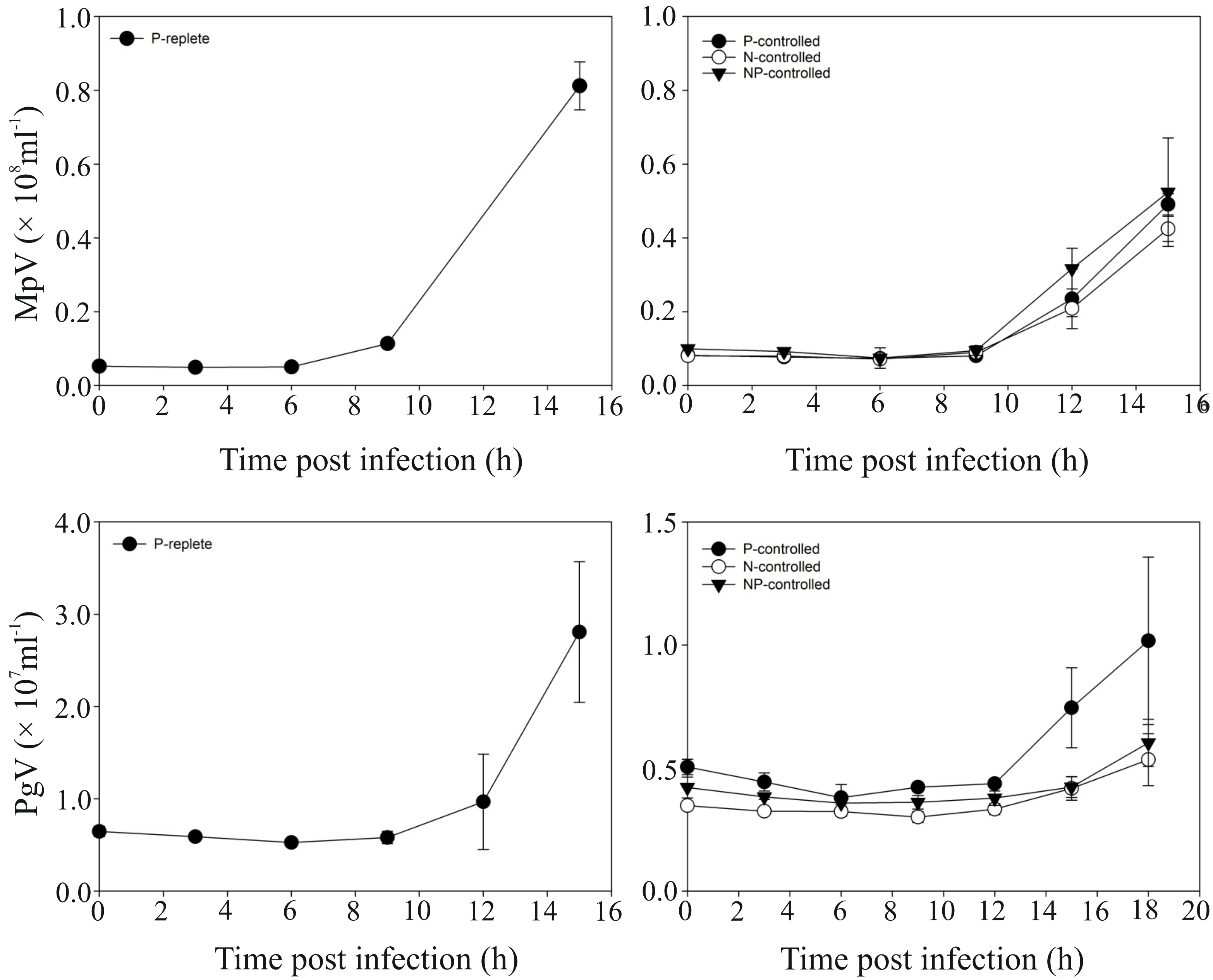
	P. globosa			*M. pusilla*		
	Latent period (h)	Burst size (viruses cell^{-1})	Burst size reduction (%)	Latent period (h)	Burst size (viruses cell^{-1})	Burst size reduction (%)
N-limited	12-16	72 ±6	91	8-12	62 ±3	72
P-limited	12-16	198 ±26	75	8-12	70 ±4	68
NP-limited	n.d.	n.d.		n.d.	n.d.	
replete	8-12	801 ±112		4-8	219 ±10	

Figure S1



Diel cycle of *M. pusilla* growth (A) and Fv/Fm (B) under P-controlled semi-continuous culturing.The diluted semi-continuous culture (grey cirlce) 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.