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RESEARCH ARTICLE

Virus production in phosphorus-limited *Micromonas pusilla* stimulated by a supply of naturally low concentrations of different phosphorus sources, far into the lytic cycle

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One sentence summary: Virus proliferation in phosphorus-limited phytoplankton is stimulated by supply of reactive and non-reactive phosphorus during infection, showing that remineralization and lysis of other cells maintain lysis in oligotrophic waters.

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ABSTRACT

Earlier studies show that the proliferation of phytoplankton viruses can be inhibited by depletion of soluble reactive phosphorus (SRP; orthophosphate). In natural marine waters, phytoplankton phosphorus (P) availability is, however, largely determined by the supply rate of SRP (e.g. through remineralization) and potentially by the source of P as well (i.e. the utilization of soluble non-reactive P; SNP). Here we show how a steady low supply of P (mimicking natural P recycling) to virally infected P-limited *Micromonas pusilla* stimulates virus proliferation. Independent of the degree of P limitation prior to infection (0.32 and 0.97$\mu_{\max}$ chemostat cultures), SRP supply resulted in 2-fold higher viral burst sizes (viruses lysed per host cell) as compared with no addition (P starvation). Delaying these spikes during the infection cycle showed that the added SRP was utilized for extra *M. pusilla* virus (MpV) production far into the lytic cycle (18 h post-infection). Moreover, P-limited *M. pusilla* utilized several SNP compounds with high efficiency and with the same extent of burst size stimulation as for SRP. Finally, addition of virus-free MpV lysate (representing a complex SNP mixture) to newly infected cells enhanced MpV production, implicating host-associated alkaline phosphatase activity, and highlighting its important role in oligotrophic environments.

Keywords: phytoplankton virus; phosphorus limitation; latent period; burst size; organic phosphorus; remineralization

INTRODUCTION

Phosphorus (P) is an important macronutrient for all organisms. As a component of lipids, sugars and nucleic acids it is involved in the structuring, metabolism and reproduction of cells. In many marine systems, relative shortage of this element is responsible for the limitation of phytoplankton productivity and biomass (Ruttenberg 2003; Dyhrman, Ammerman and Van Mooy 2007). The ecological importance of P limitation of phytoplankton is expected to increase as a consequence of enhanced vertical stratification of many oceanic regions due to global

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climate change (Karl *et al.* 1997; Sarmiento *et al.* 2004; Behrenfeld *et al.* 2006).

Reduced availability of soluble reactive phosphorus (SRP; orthophosphate) has been shown to lower the impact of viral infection on phytoplankton mortality (Bratbak, Egge and Heldal 1993; Wilson, Carr and Mann 1996; Bratbak *et al.* 1998; Clasen and Elser 2007; Maat *et al.* 2014). Thus far viral infection studies that tested the effects of nutrient availability have only focused on the effect of P depletion or P starvation. Although these conditions may represent the end of phytoplankton spring blooms (Ly, Philippart and Kromkamp 2014), they do not simulate the natural P-limiting growth conditions that can be found in oligotrophic waters. Phytoplankton P limitation is not solely the result of low P concentration, but is also defined by the supply rate of low concentrations of P (Harris 1986). In P-limited open ocean regions, phytoplankton growth completely depends on P recycling (Benitez-Nelson and Buesseler 1999; Benitez-Nelson 2000; Karl and Bjorkman 2002; Dyhrman, Ammerman and Van Mooy 2007). We hypothesize that a low supply of SRP, simulating P turnover as by remineralization, stimulates virus production under oligotrophic conditions. Under such conditions virally induced phytoplankton mortality rates in the natural environment might be less affected by P limitation than thus far conveyed (Bratbak, Egge and Heldal 1993; Wilson, Carr and Mann 1996; Bratbak *et al.* 1998; Clasen and Elser 2007; Maat *et al.* 2014).

The extent of P limitation depends on the capability of phytoplankton to utilize alternative P sources, such as short-chain polyphosphates (SCPs), phosphomonoesters (PMEs) and nucleotides (Cembella, Antia and Harrison 1984b; Casey *et al.* 2009), collectively called soluble non-reactive phosphorus (SNP; Benitez-Nelson 2000). The utilization of SNP requires special enzymes, such as extracellular alkaline phosphatases (APs; Cembella, Antia and Harrison 1984b, Dyhrman and Palenik 2003). These enzymes hydrolyze organic P compounds and release the orthophosphates, which can then be assimilated. In this way phytoplankton are able to utilize all sorts of molecules, ranging from nucleotides and phosphosugars to large compounds such as phospholipids and phytin (Cembella, Antia and Harrison 1984a). The concentration of SNP in the open ocean can be up to five times higher than the SRP fraction (Karl 2002), and thus a significant fraction of primary production is thought to be sustained by SNP (Benitez-Nelson and Buesseler 1999; Lomas *et al.* 2010). To date, there are no published data available on the possible utilization of SNP compounds by virally infected cells and the effects on virus proliferation. We hypothesize that SNP compounds can be utilized by the host cell during the viral infection cycle, thereby also enhancing viral production.

Here we examined three research questions: (i) does a steady supply of low concentrations of SRP to virally infected P-limited phytoplankton stimulate virus proliferation as compared with no addition, and to what extent is this dependent on the degree of P limitation? (ii) How far into the infection cycle can SRP supply be utilized for virus production? And (iii) is virus production positively affected by a low, but continuous, supply of SNP?

MATERIALS AND METHODS

Culturing

Experiments were performed with the picoeukaryotic photoautotroph *Micromonas pusilla*, pre-cultured axenically in P-limited chemostats at two different growth rates, representing different levels of P limitation. This ubiquitous phytoplankton species is found in coastal and oceanic regions worldwide (Not *et al.* 2004; Slapeta, Lopez-Garcia and Moreira 2006), copes well with low P availability (Maat *et al.* 2014), and is readily infected by lytic viruses (Mayer 1977; Cottrell and Suttle 1991; Baudoux and Brussaard 2008). Prior to culturing, all glassware and tubing was rinsed with 0.1 M HCl and ultrapure water before autoclaving. Axenic cultures of *M. pusilla* (MP Lac38; culture collection Marine Research Center, Goteborg University) were grown in 5 l P-limited chemostats at 15°C (using a Lauda Ecoline StarEdition RE104 water bath, and pumping water through water jackets of the borosilicate vessels). The cultures were gently stirred by a glass clapper above a magnetic stirrer, moving at 15 r.p.m. Irradiance at 100 μmol quanta m^{-2} s^{-1} was supplied by 18W/965 OSRAM daylight spectrum fluorescent tubes (QSL-2100; Biospherical Instruments Inc., San Diego, CA, USA) in a light–dark cycle of 16:8 h. Phosphate was the limiting nutrient (Na_2HPO_4, 0.25 μM final concentration) in filter-sterilized (0.1 μm Sartopore Midicap filter, Sartorius A.G., Germany) f/2 medium (Guillard and Ryther 1962), which was based on aged, nutrient-poor ocean water and modified to contain 40 μM $NaNO_3$ and 0.01 μM Na_2SeO_3 (Cottrell and Suttle 1991). The concentration of SRP in the aged sea water was below the detection limit of 0.02 μM. Final SRP concentrations of the medium were always verified using a colorimetric assay (see below). The chemostat dilution rate was adjusted to obtain two different growth rates, representing different degrees of P limitation. To allow comparison with P-replete growth conditions (exponential growth, $\mu_{max} = 0.72$ d^{-1}), separate dilution rates were set to the highest possible while avoiding washout (i.e. near-maximum growth rate of 0.97μ_{max}; MacIntyre and Cullen 2005) and to 0.32μ_{max} d^{-1} to allow testing of a stronger P limitation. After at least five volume changes, steady-state conditions (constant algal abundance) were maintained for another 3 months (demonstrating long term consistency). Algal alkaline phosphatase activity (APA) and photosynthetic capacity (variable/maximum chlorophyll fluorescence (F_v/F_m)) were stable over this period and SRP concentrations in the culture vessels were always below the detection limit (<0.02 μM).

Lysate of the lytic dsDNA virus *M. pusilla* virus (MpV)-08T (virus culture collection, Royal Netherlands Institute for Sea Research) was depleted in SRP by three recurring virus infection cycles of axenic algal host, cultured under P-starved culture conditions (batch cultures derived from the 0.97μ_{max} P-limited chemostats, deprived of SRP and thus P starved) prior to the experiments. Relatively high volumes of lysate of 10–15% v/v were used because the MpV stock under P-starved conditions (quantified by flow cytometry, see below) was relatively low and methods to concentrate the viruses (i.e. ultracentrifugation and tangential flow filtration) before addition led to decreased MpV infectivity (in a variable manner). The SRP concentration in the lysates used for the experiments was below the limit of detection (0.02 μM) and considered zero. Subsamples of glutaraldehyde-fixed (0.25% final concentration; Sigma-Aldrich, St Louis, MO, USA) algal cultures and viral lysates were regularly checked for axenity (containing no contaminating organisms) by epifluorescence microscopy (Axioplan, Oberkochen, Germany) using the DNA-stain DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Life Technologies Ltd, Paisley, UK) in combination with 0.2 μm pore size black polycarbonate filters (Whatman, Maidstone, UK) according to Porter and Feig (1980). The cultures and lysates were shown to be axenic at all times.

Experimental set-up

The viral infection experiments were performed with cultures that were collected from the P-limited steady-state chemostats.

However, dilution of the cultures strongly complicates the study of the one-step virus growth cycle as the virus–host contact rate will immediately change and non-growing infected host cells will be washed out. To allow for optimal infection (i.e. one-step infection allowing for the one-step virus growth cycle), the dilution of the cultures was stopped at the moment of infection and P was thereafter (during the infection cycle) supplied (spiked) by hand at fixed intervals and with similar overall rates as under chemostat culturing. This way we were able to keep the cells in a constant state of P limitation and thus maintain P-limited growth conditions for the non-infected cultures. Besides this P-spiked treatment, control cultures were taken from the same chemostats that (i) were deprived of SRP upon viral infection (P starved) and (ii) received directly upon infection 4 μM SRP (Na_2HPO_4) final concentration in order to overcome P limitation (P enriched). Additionally, P-replete control cultures (f/2 with 36 μM Na_2HPO_4, 882 μM $NaNO_3$) were grown at 1.0μ_{max} (0.72 d^{-1}) for 4 weeks in a semi-continuous fashion according to the turbidostat principle (regulated abundance of cells by daily dilution), facilitating non-limited cell physiology and constant exponential growth (MacIntyre and Cullen 2005). Under these optimal growth conditions, we expected the cultures to produce the maximum viral burst size (Maat *et al.* 2014).

For the viral infection experiments, duplicate subcultures were incubated in Erlenmeyer flasks under light and temperature conditions identical to the chemostats and inoculated with 0.2 μm filtered (polyethersulfone membrane filtration, Sartopore Midicap, Sartorius A.G., Goettingen, Germany) axenic MpV-08T lysate at a virus:host cell ratio of 10. Virus infectivity was close to 100%, as determined by comparing the most probable number of infective MpV (MPN endpoint dilution; Suttle 1993) with flow cytometry MpV total counts (Supplementary Fig. S1). The non-infected control cultures received an equal volume of 0.1 μm filtered (polyethersulfone membrane filtration, Sartopore Midicap) seawater with an SRP concentration below the detection limit. Algal and viral abundance samples were generally taken every 6 h or after longer time intervals later in the infection cycle. Algal samples were analyzed fresh and virus samples (1 ml) were fixed with glutaraldehyde-fixed (EM-grade, 0.5% final concentration; Sigma-Aldrich, St Louis, MO, USA) for 15–30 min, flash frozen in liquid nitrogen and stored at –80°C. Monitoring of the non-infected controls continued until the infected cultures were almost completely lysed. When lysis seemed to be delayed, sampling of the non-infected controls was also extended with at least another time point.

Addition of SRP to infected, P-limited *M. pusilla*

The cultures (derived from the respective chemostats) were spiked with SRP at concentrations that allowed similar growth as under 0.97 and 0.32μ_{max} chemostat culturing. The first 12 h post-infection (p.i.) spiking took place every hour (10.5 and 2.6 amol P $cell^{-1}$ h^{-1}, for the 0.97 and 0.32μ_{max} cultures, respectively), but thereafter (until full lysis of culture) the frequency was reduced to every 6 h as the viral latent periods (the time until first release of progeny viruses) were largely covered. The volume of added SRP stock ($[Na_2HPO_4] = 104.2$ μM) was adapted to the time period in between spiking, and at all times corrected for the change in cell abundance due to growth (non-infected controls) or cell lysis (infected cultures). This correction for cell abundance ensured that the non-infected controls and the infected cells obtained the same amount of SRP per algal cell. For example, the total concentration of spiked P to the 0.97μ_{max} roughly doubled per day for the non-infected controls, while it reduced for the lysing virally infected cultures according to the number of cells remaining. The replicate of the 0.32μ_{max} P-enriched treatment failed for technical reasons.

Finally, to study the possible effects of P limitation on viral genome production as a causal factor for potentially delayed and decreased viral production, the intracellular production of viral genomes in the 0.97μ_{max} cultures was monitored by quantifying MpV DNA polymerase gene copies (*DNApol*). The total MpV *DNApol* abundance per lysed host cell was calculated by dividing the increase in concentration of MpV *DNApol* copy number by the maximum decline in host cells over the entire one-step infection cycle (lysed host cells).

Delayed addition of SRP to infected, P-limited *M. pusilla*

To test how far into the virus growth cycle the supplied SRP can still be utilized by the host for virus production, the 0.97μ_{max} cultures received SRP additions at similar concentrations as for Experiment 1, but the administration was delayed for 1, 6, 12, 18, 24, 36 or 48 h p.i. To ensure that the potential effects on virus growth characteristics originated from the timing of spiking rather than the amount of P, the total amount of SRP at the first spiking event was increased with the period of delay and corrected for the remaining volume and *M. pusilla* abundance (thus equaling cellular P quota). Neither the infected nor the non-infected controls were negatively affected in their growth rate or F_v/F_m by this relatively large input of SRP (Supplementary Fig. S2).

Addition of SNP to infected, P-limited *M. pusilla*

To determine whether SNP availability affects viral proliferation in infected *M. pusilla* in a similar manner as SRP, the 0.97μ_{max} cultures were spiked with different SNP compounds at the same frequency and final P concentration as for Experiment 1. The compounds used were disodium glycerolphosphate hydrate (GP; Sigma-Aldrich, G6501), disodium adenosine 5′-monophosphate (AMP; Sigma-Aldrich, 01930) and pentasodium tripolyphosphate hexahydrate (PP; Sigma-Aldrich, T5633).

As viral lysate is an important source of SNP (Gobler *et al.* 1997; Haaber and Middelboe 2009), we tested whether the addition of the P-starved lysate that we added to the viral infection experiments was responsible for part of the virus production in our experiments. For this, 15% v/v virus-free MpV lysate (0.02 μm pore size Whatman Anodisc 25, UK) was added to P-limited 0.97μ_{max} cultures at T0. Note that all treatments received the same amount of lysate at the start of the experiment, which enables comparison across the different treatments.

Enumeration of algae, viruses and viral genomes

Algal abundances were analyzed on fresh samples by flow cytometry (Marie *et al.* 1999) using a BD Accuri^{TM} C6 cytometer (BD Biosciences, San Jose, CA, USA), triggered on chlorophyll *a* red autofluorescence. Viral abundance samples were thawed, diluted with TE buffer and stained with SYBRGreen I (final concentration of 0.5×10^{-4} of the commercial stock; Life Technologies Ltd, Paisley, UK) according to Brussaard (2004). Following a 10 min 80°C heat incubation, the samples were analyzed using a benchtop BD FacsCalibur (BD Bioscience) with the trigger set on green fluorescence. All flow cytometry data were analyzed using CYTOWIN 4.31 (Vaulot 1989). Viral burst size was determined by dividing the number of newly produced viruses (released by the host cells) by the maximum decline in host cells, i.e. number of lysed host cells.

Quantitative PCR was based on Brown, Campbell and Lawrence (2007), but included a heat treatment according to Short and Short (2008), new primers specific for strain MpV-08T, and the use of a calibration curve to allow absolute quantification of the number of *DNApol* copies μl^{-1}. Total MpV genome abundance, i.e. the sum of intra- and extracellular viral genomes in the sample, was determined on 1 ml samples that were stored at –20°C in 2 ml cryovials (Greiner Bio-One GmbH, Frickenhausen, Germany). One day before analysis the samples were thawed, diluted 1:5 in freshly prepared deionized water (18.2 MΩ), sonicated (MSE Soniprep 150, UK) for 3 × 10 s at amplitude 8, followed by a 5 min heat treatment at 80°C and storage at –80°C. This treatment was sufficient to break down all cells and viruses, as ascertained by flow cytometry (no cells or viruses could be detected any more by the protocols described above). The new forward (MpV08T_qF1: 5′-ATGGAAATATCGAAGGTATTA-3′) and reverse (MpV08T_qR1: 5′-ACCATATATCGAGTTCATTG-3′) primers targeted the viral DNA polymerase gene and produce a product of 220 bp. PCR reactions of 20 μl contained 1 μl template, 1u Picomax polymerase, 1× Picomax buffer (including 1.5 mM $MgCl_2$), 200 μM dNTPs, 0.2 μM of each primer, 1 mg ml^{-1} BSA and 0.2 × SYBR-green. After each run, a melting curve was constructed from a 5 s scan during stepwise increments of temperature from 72 to 95°C. All experimental samples were run along a calibration curve (constructed in duplicates from seven samples increasing from 10 copies to 10 million copies of the target) made from purified MpV product of newly designed strain-specific forward (MpV08T_Fa; 5′-AAGGGIGCITATTACACACC-3′) and reverse (MpV08T_Rt: 5′-GGCTTITTGAAIAGTGCACT-3′) primers, amplifying a fragment from 5 bases downstream of AVS1 to 46 bases upstream of AVS2 (AVS primers by Chen and Suttle 1995) producing a 589 bp product that included the target of the qPCR primers. The calibration curve was spiked with 10 million copies non-target SPUD-A DNA (Nolan *et al.* 2006; 5′-AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTGCACAAGCTATGGAACACCACGTAAGACATAAAACGGCCACATATGGTGCCATGTAAGGATGAATGT-3′). The efficiency of the qPCR reactions of experimental and calibration samples with primer pair MpV08T_qF1–MpV08T_qR1 was 96.1% ($R^2 = 0.994$).

Inorganic P concentrations, algal physiology and statistics

Concentrations of SRP were determined colorimetrically as described by Hansen and Koroleff (1999). Samples were filtered (0.2 μm, FP 30/0.2 CA-S Whatman, Dasser, Germany) into clean screw cap vials and stored at –20°C until analysis. The lower limit of detection was 0.02 μM. All chemostat cultures in steady state gave undetectable concentrations, demonstrating that all added P (in the medium) was directly taken up by the cells (this is standard practice for axenic phytoplankton cultures; e.g. Veldhuis and Admiraal 1987; Yao *et al.* 2011). The SRP concentrations were measured at the beginning and end of all infection experiments and were all shown to be below the limit of detection, with of course the exception for the P-replete and P-enriched treatments.

As an indicator of P limitation within the steady-state algal cultures (Healey and Hendzel 1979; Beardall, Young and Roberts 2001), alkaline phosphatase activity (APA) was determined fluorometrically according to Perry (1972). To a glass cuvette containing 2 ml of culture, 250 μl of 3-*O*-methylfluorescein phosphate (MFP; Sigma-Aldrich, M2629) was added to a final concentration of 595 μM. Emission at 510 nm was measured on a Hitachi F2500 fluorescence spectrophotometer (Hitachi Instruments, San Jose, CA, USA) for 60 s with an excitation wavelength of 430 nm. The rate of MFP conversion was determined from a standard curve of 3-*O*-methylfluorescein (Sigma-Aldrich, M7004). As expected, APA increased with the extent of P limitation, showing zero for the P-replete semi-continuous cultures and 9.5 ± 0.9 and 31 ± 0.1 amol P $cell^{-1}$ s^{-1} for the 0.97 and 0.32μ_{max} P-limited cultures, respectively.

Photosynthetic capacity F_v/F_m was determined by PAM fluorometry (Water-PAM, Walz, Germany). Samples (2 ml) were dark-adapted at culture temperature (15°C) for 15 min before analysis. All measurements were carried out with the same Water-PAM settings, whereby 0.2 μm filtered sea water was used as a blank. After determining the minimal (F_0) and maximum chlorophyll fluorescence (F_m), the variable fluorescence (F_v) was calculated as $F_m - F_0$ (see Maxwell and Johnson 2000).

Statistics were carried out using SigmaPlot^{TM} 12.0 (Systat Software Inc., San Jose, CA, USA). One-way ANOVA was used for testing the differences between the treatments, but when the assumptions were not met, non-parametric (Kruskall–Wallis or Mann–Whitney) tests were used. Values in tables and figures are the mean ± standard deviation (SD).

RESULTS

Addition of SRP to infected, P-limited *M. pusilla*

While growth in the P-starved cultures ceased, SRP addition allowed continued growth of the non-infected *M. pusilla* cultures (Fig. 1). The extent of P limitation determined the growth rates, i.e. the 0.32 and 0.97μ_{max} P-spiked cultures maintained growth at 0.30 ± 0.0 and 0.68 ± 0.0 d^{-1}, while this was 0.76 ± 0.0 and 0.53 ± 0.1 d^{-1} after receiving a surplus of 4 μM (SRP enriched). The level of limitation (0.97 and 0.32μ_{max}; starved, spiked or enriched) did not seem to affect the lysis dynamics of the infected cultures (Fig. 1b). All P-controlled cultures showed slightly slower lysis at the end of the first day post-infection (18–24 h) compared with the P-replete treatments (Fig. 1b).

The period to the release of the first progeny viruses (i.e. latent period of MpV) in the 0.97μ_{max} P-spiked algal host cultures was of similar length as in the P-enriched and P-replete cultures, i.e. 6–12 h (Fig. 1c). The MpV latent periods in all 0.32μ_{max} cultures were prolonged to 12–18 h. Independent of the effect on the latent period, the rates of increase in extracellular progeny viruses varied for the different treatments, i.e. rates reduced with increasing P stress (from 1.7 × 10^7 to 0.8–1.1 × 10^7 MpV ml^{-1} h^{-1} under replete and enriched conditions, and from 0.4–0.8 × 10^7 to 0.2 × 10^7 MpV ml^{-1} h^{-1} for the spiked compared with the P-starved treatment). MpV burst sizes of the P-starved 0.32μ_{max} cultures were not statistically different from the 0.97μ_{max} treatment (mean of 75 ± 1; Kruskall–Wallis ANOVA on ranks, $P = 0.667$, $n = 2$; Table 1). Independent (pilot) experiments with the same set-up and virus–host model systems (0.97 and 0.32μ_{max} SRP-starved cultures, $n = 15$ in total) gave similar results, i.e. average 74 ± 3 MpV per lysed host cell (data not shown). Likewise, the semi-continuously grown P-replete cultures showed good replication with an average MpV burst size of 310 ± 26 when including the results of other independent experiments ($n = 12$ over a period of 2 years). Furthermore, the 0.97μ_{max} P-starved treatment in all three experiments in the current study did not show significant differences in MpV latent period or burst size (mean of 75 ± 2; Kruskall–Wallis ANOVA on ranks, $P = 0.333$, total

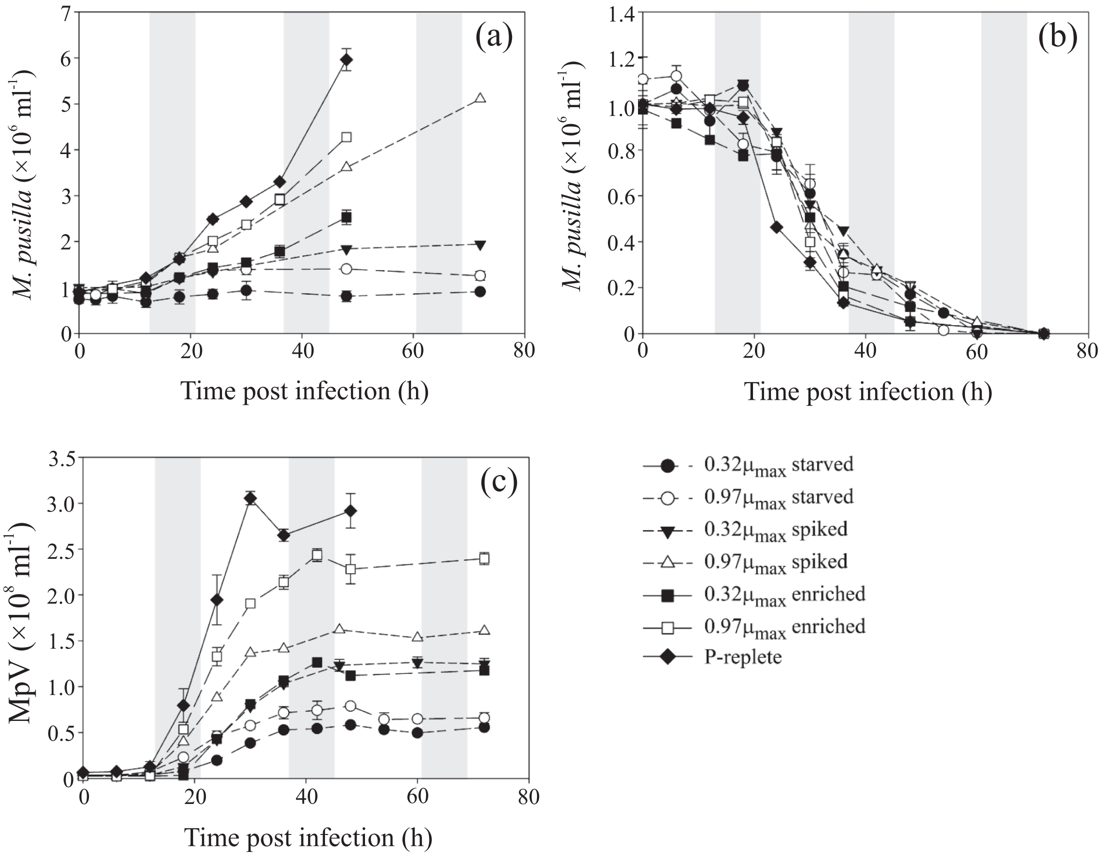

Figure 1. Temporal dynamics of *Micromonas pusilla* and viruses upon SRP addition to infected P-limited cells. Abundances of non-infected (**a**) and virally infected (**b**) *Micromonas pusilla* cultures, as well as the virus MpV (**c**) over time under different levels of P limitation. The viral infection experiments were carried out in Erlenmeyer flasks with cultures derived from P-limited chemostats at 0.97 and 0.32μ_{max}. Spiked cultures received SRP at an hourly rate until 12 h post-infection to maintain chemostat P supply (thereafter at a 6 h rate until all cells lysed). P-starved control cultures no longer received a P supply. Enriched cultures were spiked with 4 μM SRP to overcome the P limitation. P-replete cultures were taken along as positive control (non-limited cell physiology). Sampling times were 0, 3, 6, 12, 18, 24, 32, 36, 48 and 72 h post-infection). All treatments, except the 032μ_{max} enriched are means of duplicate cultures (± standard deviation).

$n = 6$; Table 1), illustrating that reproduction for our experiments was good. Independent of the degree of P limitation (0.97 *vs* 0.32μ_{max}), spiking of the infected cultures with SRP led to significantly increased MpV burst sizes compared with the P-starved cultures (on average a doubling; ANOVA, $P = 0.003$; Table 1). However, the burst sizes of the SRP-spiked cultures were still lower than for the P-enriched and P-replete treatments ANOVA, $P = 0.015$; Table 1). The enriched cultures still showed lower burst sizes than the replete treatment (i.e. 44 and 22% lower for the 0.32 and 0.97μ_{max} culture, respectively; Table 1).

Similarly to the MpV burst sizes in the 0.97μ_{max} spiked and starved treatments, the MpV *DNApol* copy number was also strongly affected by the strength of P limitation (Fig. 2), i.e. respectively 7- and 11-fold lower than P replete. Although the temporal dynamics of MpV *DNApol* copy number and viruses over the infection cycle were the same, the total number of viral *DNApol* per cell was 3-, 2- and 7-fold higher than the viral burst sizes of the 0.97μ_{max} P-starved, P-spiked and P-replete treatment, respectively (Table 1). Hence, under P-replete conditions the ratio of viral genomes to viruses was higher than under P-spiked and P-starved conditions.

Delayed addition of SRP to infected, P-limited *M. pusilla*

Delaying the addition (spiking) of SRP led to a lag in growth of the non-infected control cultures (i.e. 0.22 ± 0.0 d^{-1} until they were spiked, compared with 0.61 ± 0.1 d^{-1} for the continuously spiked culture), but growth quickly recovered upon spiking. The temporal dynamics of the infected cultures were not affected in comparison with the regularly spiked cultures (Fig. 3). Delayed SRP addition up to 18 h p.i. still gave comparable MpV production and burst sizes compared with continuous spiking from 0 h p.i. (Figs 1 and 3, Table 1). Starting the additions of SRP after 24 h p.i. gave similar results to the P-starved cultures (Mann–Whitney rank sum test, $P<0.001$, $n \geq 8$; Fig. 3c, Table 1). The 16:8 h light–dark synchronized growth of *M. pusilla* may theoretically have influenced the results of the delayed spiking experiment. However, at the moment of the 18 h p.i. SRP addition the cultures had already been in the dark for 5 h while still showing similar virus growth kinetics as the 0–12 h p.i. SRP supply treatments. Furthermore, the 24 h p.i. treatment started in the light and still showed lower MpV yield. The light–dark cycle did thus not affect the outcome.

Addition of SNP to infected, P-limited *M. pusilla*

Supplying the 0.97μ_{max} P-limited non-infected control cultures with the SNP compounds GP, AMP and PP resulted in maximum growth rates, similar to with SRP (0.72 ± 0.1 d^{-1}; average over 48 h experiment). Independent of SNP source, all infected algal cultures showed comparable lysis dynamics and MpV latent periods similar to SRP-spiked treatment (Fig. 4b and c, Table 1).

Table 1. Viral burst size, i.e. the number of newly produced *Micromonas pusilla* viruses (MpV) or viral genomes (*DNApol*) per lysed host cell under the different P treatments. Experiment 1 demonstrates the effect of spiking the infected cultures with soluble reactive phosphorus (SRP) at a rate similar to their chemostat growth rate (0.97 and $0.32\mu_{\max}$) or a surplus of P (enrichment). Experiment 2 demonstrates the influence of delayed SRP spiking (from 1 to 48 h post-infection) of the $0.97\mu_{\max}$ cultures. Experiment 3 demonstrates the effect of spiking with soluble non-phosphorus (SNP) compounds: adenosine monophosphate (AMP), glycerophosphate (GP) and polyphosphate (PP) and filtered (virus-free) lysate. Data are the mean ($n = 2$) ± standard deviation (SD).

Treatment	MpV (host cell^{-1})	*MpV-DNApol* (host cell^{-1})
Experiment 1 (SRP spiked)		
$1.0\mu_{\max}$ P replete	330 ± 16	2221 ± 174
$0.97\mu_{\max}$ P enriched	256 ± 4	
$0.32\mu_{\max}$ P enriched	186[a]	
$0.97\mu_{\max}$ P spiked	160 ± 8	341 ± 26
$0.32\mu_{\max}$ P spiked	141 ± 4	
$0.97\mu_{\max}$ P starved	74 ± 1	210 ± 34
$0.32\mu_{\max}$ P starved	76 ± 11	
Experiment 2 (delayed SRP spike)		
$0.97\mu_{\max}$ + 1 h	154 ± 17	
$0.97\mu_{\max}$ + 6 h	150 ± 18	
$0.97\mu_{\max}$ + 12 h	156 ± 8	
$0.97\mu_{\max}$ + 18 h	153 ± 6	
$0.97\mu_{\max}$ + 24 h	110 ± 32	
$0.97\mu_{\max}$ + 30 h	90 ± 6	
$0.97\mu_{\max}$ + 36 h	86 ± 1	
$0.97\mu_{\max}$ + 48 h	78 ± 2	
$0.97\mu_{\max}$ P starved	77 ± 1	
Experiment 3 (SNP spiked)		
$0.97\mu_{\max}$ + SRP	171 ± 3	
$0.97\mu_{\max}$ + AMP	165 ± 20	
$0.97\mu_{\max}$ + GP	162 ± 2	
$0.97\mu_{\max}$ + PP	177 ± 1	
$0.97\mu_{\max}$ + lysate	94 ± 3	
$0.97\mu_{\max}$ P starved	75 ± 2	

[a] Replicate failed for technical reasons.

All SNP compounds stimulated MpV production (as compared with SRP starved), PP even to the same degree as found for the SRP-spiked treatment (Fig. 4c). Although it took longer (about 24 h), the maximal yield of MpV under GP and AMP spiking was not significantly different from the PP and SRP treatments (Fig. 4c). Consequently, MpV burst sizes for the PP-, GP- and AMP-spiked treatments were not different from SRP spiked (Table 1).

The addition of virus-free lysate, to test whether lysate itself was a significant source of rapidly bioavailable SRP and subsequently MpV production in all infected cultures, led to a burst size slightly higher than the P-starved cultures (Fig. 4d, Table 1). This result was tested once more in a separate experiment ($n = 2$) with similar results. Overall, average burst size increase was 16 ± 5 MpV host cell^{-1} (t-test, $n = 4$, $P = 0.003$). This increase was clearly lower than the increase in MpV burst size obtained for the other SRP treatments, as the added total amount of P in the lysate was also lower than that for the other treatments.

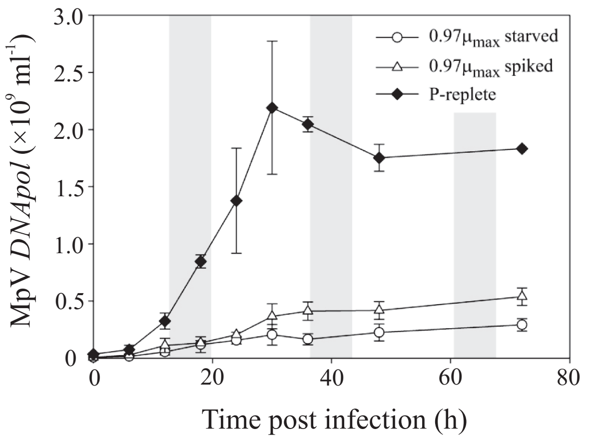

Figure 2. Viral genome production, represented by total MpV *DNApol* abundance, over time upon SRP addition to infected P-limited cells. Spiked cultures received SRP at a 1–6 hourly rate to maintain chemostat P supply. Gray bars represent dark (night) period. Sampling times were 0, 6, 12, 18, 24, 32, 36, 48 and 72 h post-infection). All treatments are means of duplicate cultures (± standard deviation).

DISCUSSION

In this study we tested the effects of low supply rates of SRP (simulating natural remineralization conditions) and SNP (simulating, for example, release of cell content by viral lysis) on the virus growth cycle of P-limited *M. pusilla*. Spiking with SRP during the infection cycle distinctly increased the viral burst sizes (with no effect on the latent period) around 2-fold as compared with the P-starved treatment. Since the algal host cells were still P limited, the burst sizes were not as high as those under P-replete or P-enrichment conditions (this study; Maat *et al.* 2014). Additionally, the strength of P limitation, obtained by adapting the algal host in P-limited chemostats to different growth rates prior to infection (i.e. 0.97 and low $0.32\mu_{\max}$), affected the burst size upon SRP spiking, with the strongest increase for the $0.97\mu_{\max}$ cultures.

Viruses are strictly dependent on their host for the substrate, enzymes and energy that are involved in virus replication, and hence the physiological state of the host can be expected to drive the outcome of infection. This was best illustrated by P enrichment (addition of 4 μM SRP to P-limited cultures), whereby SRP was no longer limiting *per se* and the difference in burst size between the 0.97 and $0.32\mu_{\max}$ pre-cultured *M. pusilla* was solely due to a constraint on the host's physiology prior to infection. Potential damage of photosynthetic machinery and/or additional components of the energy metabolism under P limitation could be such a constraint (Theodorou *et al.* 1991; Graziano, Roche and Geider 1996). Previous studies have shown the dependence of viral replication on photophosphorylation for several phytoplankton species (Padan, Ginzburg and Shilo 1970; Waters and Chan 1982; Vanetten *et al.* 1983; Juneau *et al.* 2003; Baudoux and Brussaard 2008). The strength of P limitation at steady state (prior to MpV infection) affected the F_v/F_m of *M. pusilla* negatively, i.e. 0.49 for $0.32\mu_{\max}$ cultures as compared with 0.61 for $0.97\mu_{\max}$ and 0.66 for P-replete cultures (Maat *et al.* 2014). Upon infection with MpV, SRP spiking, at least partly, counteracted the strong decline in F_v/F_m observed under P-starved conditions (Supplementary Fig. S3a). Spiking with P could thus stimulate host energy metabolism and hence viral proliferation. These data furthermore affirm the interplay of viral infection and environmental factors such as light and nutrient limitation on

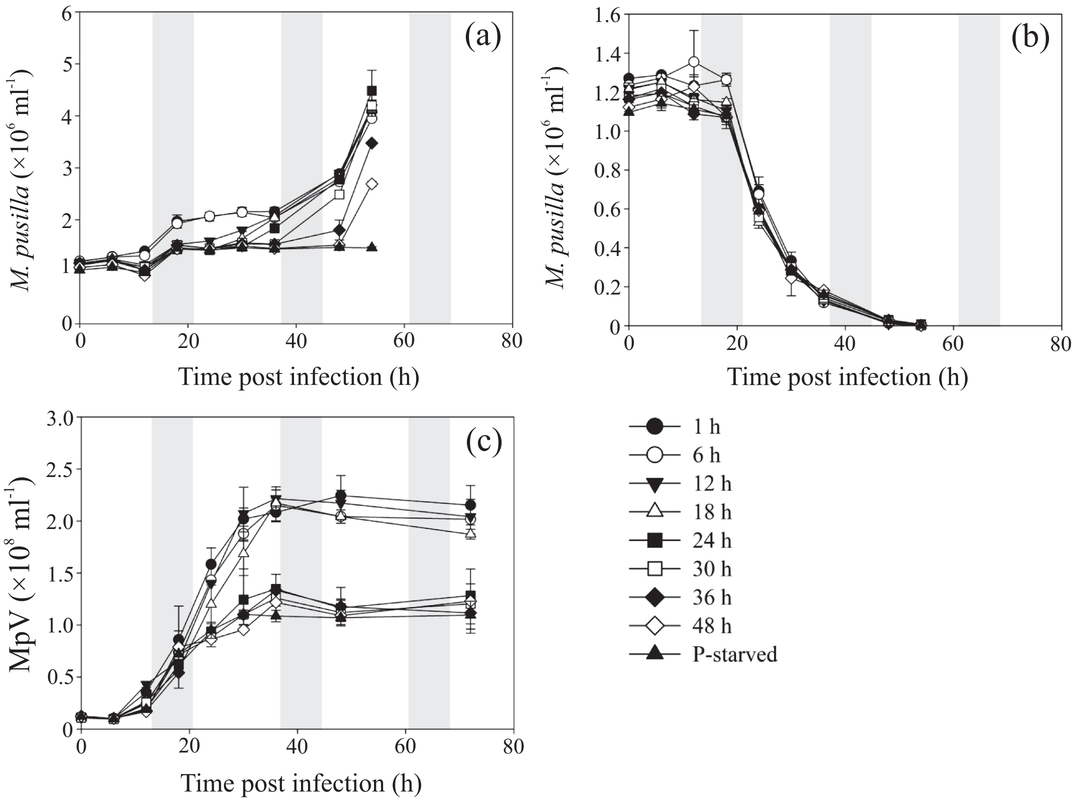

Figure 3. Temporal dynamics of *Micromonas pusilla* and viruses upon delayed SRP addition to infected P-limited cells. Abundances of non-infected (**a**) and virally infected (**b**) *Micromonas pusilla* cultures, as well as the virus MpV (**c**) under delayed SRP addition. The viral infection experiments were carried out in Erlenmeyer flasks with cultures derived from P-limited chemostats ($0.97\mu_{max}$). SRP was supplied similar to Experiment 1 but with a 6, 12, 18, 24, 30, 36 or 48 h delay (for proper comparison the concentration SRP added was corrected for the delay). As controls, P-limited cultures received either SRP addition like Experiment 1 (starting 1 h p.i.) or did not receive SRP addition (P starved). Sampling times were at 0, 6, 12, 18, 24, 32, 36, 48, 54 and 72 h post-infection). All treatments are means of duplicate cultures (± standard deviation).

the photosynthetic capacity of phytoplankton (Supplementary Fig. S3b; Kimmance *et al.* 2014).

However, the supplied P could also be (partly) allocated to the actual production of the viruses inside the host cell, e.g. synthesized into nucleic acids. Viruses have been found to recycle host nucleotides and it has been argued that the relative size of a phytoplankton genome is a good predictor of the viral burst size (Paul *et al.* 2002; Brown, Lawrence and Campbell 2006; Brown and Bidle 2014). Assuming non-limiting growth conditions of the host, a MpV genome of 208 kb (Martínez Martínez *et al.* 2015) and *M. pusilla* host genome of 22 Mbp (Worden *et al.* 2009), the predicted maximum viral burst size for our virus–host system would then be 106 MpV per lysed host cell, which is around 3-fold lower than experimentally recorded under P-spiked, -enriched and -replete conditions (this study; 141–330 MpV per lysed host cell). Thus at least part of the viral nucleotides has to have been the product of *de novo* synthesis.

Furthermore, there was a strong, 10-fold, overproduction of MpV *DNApol* per cell (as compared with MpV burst size) under P-replete conditions. Such overproduction of MpV genomes in comparison with released MpV progeny has been suggested to be a way to increase the speed of viral genome packaging in the dense host cytoplasm (Brown, Campbell and Lawrence 2007; Weidmann *et al.* 2011). Alternatively, Nissimov *et al.* (2016) argued that overproduction of genome copy numbers for *Emiliania huxleyi* viruses was due to a limitation in the availability of components of the protein capsid or lipid membrane. As the capsid of MpV-08T is also surrounded by a lipid membrane (Maat *et al.* 2016), either capsid proteins or lipids may potentially have been limiting MpV-08T production. Under P-limiting and P-starved conditions, both the viral burst sizes and the genome copy number per cell were lower than under P-replete conditions. It is likely that the availability of P affects the transcription of viral genomes directly, either because of its function in compounds that supply energy to the necessary enzymatic activity (Theodorou *et al.* 1991) or as element in viral nucleotides (besides potential recycling from the host; Wikner *et al.* 1993, Brown and Bidle 2014). Remarkably, the overproduction of MpV *DNApol* per cell compared with MpV burst size was only 2- and 3-fold under P-starved and P-limiting conditions, respectively. The packaging efficiencies (defined as the number of viruses divided by the number of virus genome copies) under P-starved and P-limiting conditions were thus higher than under P-replete conditions (47, 35 and 15%, respectively).

Delayed SRP spiking of the $0.97\mu_{max}$ cultures up to 18 h p.i. gave similar viral burst sizes to spiking from the start of infection. *Micromonas pusilla* was thus able to utilize the supplied SRP for additional MpV progeny far into the lytic cycle. Zheng and Chisholm (2012) showed that P addition to P-depleted and infected *Prochlorococcus* sp. led to a decrease in the transcription of a virally encoded gene that is likely involved in the uptake of P. Effects on the viral burst size were, however, not investigated in their study. Phytoplankton P uptake during the virus infection cycle has been the subject of discussion in several theoretical

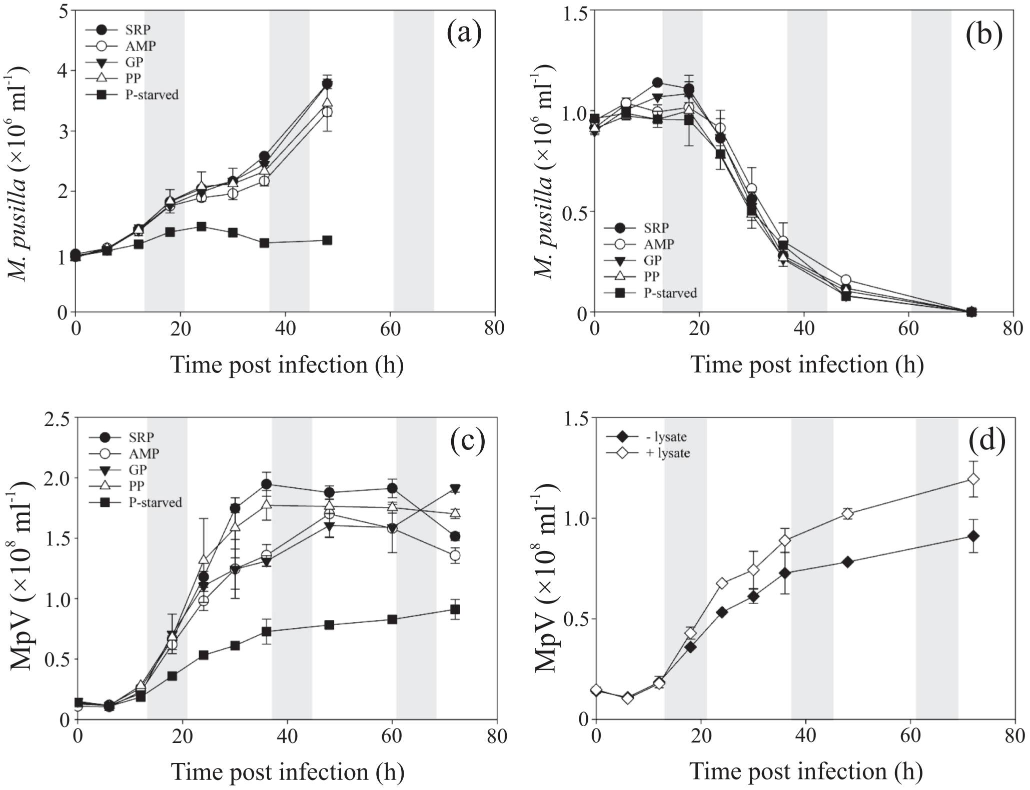

Figure 4. Temporal dynamics of *Micromonas pusilla* and viruses upon SNP addition to infected P-limited cells. Abundances of non-infected (**a**) and virally infected (**b**) *Micromonas pusilla* cultures, as well as the virus MpV (**c**) under SNP supply. The viral infection experiments were carried out in Erlenmeyer flasks with cultures derived from P-limited chemostats (0.97$\mu_{\max}$). The used SNP compounds glycerophosphate (GP; closed triangle), adenosine monophosphate (AMP; open circle) and polyphosphate (PP; open triangle) were compared with an SRP-spiked and -starved control (closed circle and square, respectively) whereby the supply rates were similar to those in Experiment 1. One-time supply (at T0) of 15% v/v virus-free and P-starved MpV lysate (**d**) was added as additional control treatment to test the potential stimulating effect on MpV production by SNP compounds in the lysate (note here the different scale of the Y-axis). Sampling times were 0, 6, 12, 18, 24, 32, 36, 48, 60 and 72 h post-infection). All treatments are means of duplicate cultures (± standard deviation).

studies, as it strongly affects the modelling results of the impact of viruses in natural nutrient-limited systems (Rhodes and Martin 2010; Jover *et al.* 2014). Overall, our results demonstrate that it is key for future research to consider SRP supply rate, i.e. P turnover rate under natural conditions, as a significant driver of virus proliferation and hence the way by which viruses structure host communities.

Viral lysis rates of different phytoplankton species (including *M. pusilla*) under natural oligotrophic conditions are found to be at least comparable to grazing rates (Cottrell and Suttle 1995; Baudoux *et al.* 2007; Mojica and Brussaard 2014). Besides, Lønborg, Middelboe and Brussaard (2013) showed that a high percentage of the dissolved cellular material released upon viral cell lysis of axenic *M. pusilla* is readily bioavailable. Such a steady supply of labile organic matter stimulates bacterial production and through remineralization SRP is constantly brought back into the system (Gobler *et al.* 1997; Brussaard *et al.* 2005; Haaber and Middelboe 2009). Our data illustrate that under P limitation a low supply of SRP during the infection cycle positively affects the production of MpV. Therefore the impact of viruses on phytoplankton mortality in oligotrophic ecosystems may be higher than previously anticipated (Wilson, Carr and Mann 1996; Bratbak *et al.* 1998; Clasen and Elser 2007; Maat *et al.* 2014).

Not only SRP, but also SNP compounds were found to be efficiently utilized by axenic *M. pusilla*. Growth and viral production dynamics of the non-infected and infected SNP-spiked cultures were similar to those under SRP supply. The ecological potential is substantial since SNP in the oligotrophic open ocean can be up to 5 times higher than SRP (Karl 2002). The more readily available SNP compounds such as PP and SCPs may accordingly be important stimulants not only of phytoplankton physiology and growth (Cembella, Antia and Harrison 1984a), but also of virus proliferation within infected algal host. The conversion of SNPs to SRP is likely to be catalyzed by APs and other enzymes (Chróst and Siuda 2002, Dyhrman and Palenik 2003). We show that *M. pusilla* APA in P-limited chemostats increases with decreasing growth rate. This increase continues when the chemostat pumps are stopped (P deprivation leading to starvation), and also in virally infected cultures (Maat *et al.* 2016; Supplementary Fig. S4). Additionally, by testing the effect of lysate as a source of SRP, we found that even relatively small amounts of lysate (of more complex composition) can directly stimulate growth of axenic non-infected *M. pusilla* as well as viral production in infected cultures. Literature suggests that around 38–49% of total P in the viral lysates is transferred back into the dissolved phase, whereby enzymatic activity from heterotrophic bacteria is suggested to be responsible for the conversion of total P to SRP (Gobler *et al.* 1997; Haaber and Middelboe 2009; Lønborg and Álvarez-Salgado 2012). We speculate that algal APs in our axenic algal cultures, potentially in combination with activity of other enzymes (e.g. nucleases), associated to lysed *M. pusilla* cells could have performed the same process (Chróst and Siuda 2002, Dyhrman and Palenik 2003). We were able to still detect APA in 1-week-old (axenic) P-starved cultures of *M. pusilla* (data not shown). This was probably due to APs bound to *M. pusilla* cell debris (lysed cells). Although some phytoplankton species excrete free APs to the environment (Chróst and Siuda 2002), we did not find APA in 0.2 μm filtered (lysed and non-lysed) *M. pusilla* cultures. Viral lysis-induced release of host cellular content seems an ecologically interesting source of bioavailable SNP that can be directly utilized and as such has the potential to stimulate MpV production of newly infected (but not yet lysed) neighboring cells. The reduction of P limitation induced by viral lysis and the subsequent stimulation of viral production implies a positive feedback process that maintains regeneration in P-limited oligotrophic ecosystems. We do however, realize that we used axenic phytoplankton cultures in our study, while in the natural environment heterotrophic bacteria may compete for the same sources of SNP (and even SRP that is made available by phytoplankton extracellular APA). Løvdal and coworkers (2008) showed that biomass-specific uptake of SRP and SNP was similar for phytoplankton and heterotrophic bacteria during an *Emiliania huxleyi* bloom in the coastal North Sea, which suggests that products of viral lysis are indeed available for phytoplankton and subsequently algal virus proliferation. In contrast, Hartmann *et al.* (2011) showed lower biomass specific uptake for small protists than for bacteria in the North Atlantic subtropical gyre, suggesting that the outcome of competition may depend on the (trophic) state of the marine system. However, the experimental incubations in their study were carried out in the dark, which could also have led to an underestimation of phytoplankton P uptake (Nalewajko and Lee 1983). We recommend forthcoming studies test the influence of potentially competing heterotrophic bacteria. Alternatively to taking up dissolved P, photosynthetic protists can display considerable bacterivory in the oligotrophic Atlantic Ocean (Hartmann *et al.* 2011, 2012). Also *M. pusilla* strains have been shown to be bacterivores, particularly under low P conditions (Gonzalez, Sherr and Sherr 1993; McKie-Krisberg and Sanders 2014) and as such potentially providing an additional source of P (next to SRP and SNP) that may be utilized to promote MpV production upon infection. Studies describing the effects of P availability on phytoplankton proliferation have thus far merely focused on the effects of SRP depletion (low ambient SRP concentrations at the moment of infection). We show here that besides low and relatively constant provision of P, also the source of P appears of ecological relevance for algal virus ecology. It will be interesting to test different algal species (including larger sized and theoretically more vulnerable to nutrient limitation) for their potential to utilize P (SRP and SNP sources) during viral infection.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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