Biogeosciences, 13, 1667–1676, 2016
www.biogeosciences.net/13/1667/2016/
doi:10.5194/bg-13-1667-2016

Biogeosciences Open Access EGU

Increasing P limitation and viral infection impact lipid remodeling of the picophytoplankter *Micromonas pusilla*

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Received: 3 September 2015 – Published in Biogeosciences Discuss.: 18 September 2015
Revised: 3 March 2016 – Accepted: 6 March 2016 – Published: 17 March 2016

Abstract. The intact polar lipid (IPL) composition of phytoplankton is plastic and dependent on environmental factors. Previous studies have shown that phytoplankton under low phosphorus (P) availability substitutes phosphatidylglycerols (PGs) with sulfoquinovosyldiacylglycerols (SQDGs) and digalactosyldiacylglycerols (DGDGs). However, these studies focused merely on P depletion, while phytoplankton in the natural environment often experience P limitation whereby the strength depends on the supply rate of the limiting nutrient. Here we report on the IPL composition of axenic cultures of the picophotoeukaryote *Micromonas pusilla* under different degrees of P limitation, i.e., P-controlled chemostats at 97 and 32 % of the maximum growth rate, and P starvation (obtained by stopping P supply to these chemostats). P-controlled cultures were also grown at elevated partial carbon dioxide pressure (pCO_2) to mimic a future scenario of strengthened vertical stratification in combination with ocean acidification. Additionally, we tested the influence of viral infection for this readily infected phytoplankton host species. Results show that both SQDG : PG and DGDG : PG ratios increased with enhanced P limitation. Lipid composition was, however, not affected by enhanced (750 vs. 370 µatm) pCO_2. In the P-starved virally infected cells the increase in SQDG : PG and DGDG : PG ratios was lower, whereby the extent depended on the growth rate of the host cultures before infection. The lipid membrane of the virus MpV-08T itself lacked some IPLs (e.g., monogalactosyldiacylglycerols; MGDGs) in comparison with its host. This study demonstrates that, besides P concentration, also the P supply rate, viral infection and even the history of the P supply rate can affect phytoplankton lipid composition (i.e., the non-phospholipid : phospholipid ratio), with possible consequences for the nutritional quality of phytoplankton.

1 Introduction

Intact polar lipids (IPLs) constitute an important component of phytoplankton cells, in which they function as a structural component (membranes), storage of carbon, and as signaling molecules (Volkman et al., 1998). IPLs comprise a large diversity in the structure of the polar head groups, the fatty acid (FA) tail lengths and number of FA double bonds, of which the actual cellular composition is found to be dependent not only on the phytoplankton species involved but also on growth-relevant environmental variables (Guschina and Harwood, 2009).

In the natural environment phytoplankton growth is subjected to a number of limitations (Harris, 1986). A widespread and important phytoplankton growth-regulating factor that limits growth in many marine systems is phosphorus (P) availability (Cloern, 1999; Dyhrman et al., 2007; Moore et al., 2013). Under low-P conditions, phytoplankton phospholipids (P lipids; i.e., lipids that contain P in their polar head groups) have been found to be substituted with non-P lipids (i.e., so-called lipid remodeling), thereby reducing the cellular P demand by 10–30 % (Sato et al., 2000; Van Mooy et al., 2009; Martin et al., 2011; Abida et al., 2015). In the heterogeneous marine environment the degree of P avail-

ability for phytoplankton is variable, and thus the trophic status of the system may affect the ratio of non-P lipids to P lipids. Data show that P lipid synthesis rates and P-lipid-to-non-P-lipid ratios are lower under low P availability (Van Mooy et al., 2009) and that an increasing fraction of P lipids is substituted by non-P lipids the longer the cells are deprived of P (Martin et al., 2011). The availability of P, and thus the degree of P stress to which a phytoplankton population is subjected, depend not only on P concentrations but also on the total P pool and P turnover or P supply rates in the water column (Harris, 1986). It would thus be interesting to clarify the relation between phytoplankton growth rates (as affected by P supply rates), physiological P limitation and IPL remodeling. As far as we know, P lipid remodeling in relation to phytoplankton growth rate and physiology has thus far not been described in the literature. Furthermore, the available studies on P lipid remodeling have focused merely on nano-sized phytoplankton (Sato et al., 2000; Van Mooy et al., 2009; Martin et al., 2011; Abida et al., 2015). As smaller phytoplankton species are generally considered to cope better with low nutrient availability (Raven, 1998) and make up a large proportion of open-oceanic phytoplankton communities (Marañón et al., 2001; Grob et al., 2007), it is appropriate to test the possible effects of P limitation on lipid remodeling for species from this size class of phytoplankton as well.

Investigations on the effects of P limitation on phytoplankton are timely as global warming is expected to lead to an expansion of the stratified areas of our world's oceans (Sarmiento et al., 2004). Besides the increased phytoplankton nutrient limitation due to the reduced mixing of nutrients to the surface of the water column, elevated partial CO_2 pressure (pCO_2) may affect phytoplankton growth, production and stoichiometry (Riebesell eand Tortell, 2011; Maat et al., 2014b). The effects of changing carbon dioxide (CO_2) conditions on the intact polar lipid composition of phytoplankton are still unknown.

Here we investigated the IPL composition of the picophytoplankton species *Micromonas pusilla* (Butcher; Manton and Parke, 1960; Prasinophyceae) under different degrees of P limitation (i.e., P-controlled chemostat growth at 97 and 32 % of the maximum growth rate and P starvation) and partial doubled pCO_2 as compared to present-day pCO_2 (370 µatm). *M. pusilla* is a globally distributed ubiquitous alga (Slapeta et al., 2006) that may represent significant fractions of phytoplankton communities (e.g., on average 22 % of total chlorophyll year-round in the English Channel; Not et al., 2004). The species has been shown to be readily infected by viruses (Cottrell and Suttle, 1991; Brussaard et al., 2004), whereby viral infection of P-limited *M. pusilla* prolongs the time of lysis of the algal host during which carbon fixation and P assimilation are still possible (Maat et al., 2014b). As viruses may influence phytoplankton lipid composition, the possible effects of viral infection on P-limitation-induced lipid remodeling in *M. pusilla* were studied as well.

2 Materials and methods

2.1 Culturing and treatments

The prasinophyte *Micromonas pusilla* Mp-LAC38 (culture collection Marine Research Center, University of Gothenburg; Sahlsten and Karlson, 1998), belonging to *Micromonas* clade A (Martínez Martínez et al., 2015), was pre-grown under P-replete and P-limited conditions as described in detail by Maat et al. (2014b). In short, axenic cultures were grown on a modified f/2 medium (Guillard and Ryther, 1962) with 0.01 µM Na_2SeO_3 (Cottrell and Suttle, 1991) in 5 L borosilicate culture vessels under 100 µmol quanta m^{-2} s^{-1} in a light : dark cycle of 16 : 8 h. The P-replete treatment (36 µM P) was obtained using semi-continuous cultures growing at maximum growth rate ($\mu_{max} = 0.72$ d^{-1}; Table 1) and diluted daily according to the turbidostat principle (MacIntyre and Cullen, 2005). Algal cells were counted by flow cytometry before and after dilution according to Marie et al. (1999). Balanced P-controlled (P = 0.25 µM) growth conditions were obtained by chemostats (i.e., continuous cultures under nutrient control), allowing cells to grow at a set dilution rate and hence at the same physiological state (MacIntyre and Cullen, 2005; Table 1). As chemostat dilution (growth) rates we chose nearly exponential growth (0.97 μ_{max}) and a more stringent level of P limitation at 0.32 μ_{max}. The strongest level of P limitation was then realized by stopping the P supply to the chemostat cultures, hence yielding P-starved (MacIntyre and Cullen, 2005) cultures with different growth history (i.e., 0.97 and 0.32 μ_{max}; Table 1). Increasing strength of P limitation was clearly visible in increased alkaline phosphate activity (APA) and reduced photosynthetic efficiency (F_v / F_m), both well-accepted indicators of phytoplankton nutrient limitation (Table 1; Beardall et al., 2001).

The P-replete and P-controlled cultures were maintained at present-day pCO_2 (370 µatm) as well as future pCO_2 (750 µatm, predicted for the year 2100; Meehl et al., 2007) by aerating the cultures with synthetic air (see for exact CO_2 conditions Maat et al., 2014b). The different culturing methods were solely used to create the different strengths of P limitation and CO_2 conditions. As all other culture conditions remained the same (e.g., type of vessels, aeration, irradiance, temperature), no additional (side) effects of the culturing method on *M. pusilla* growth, physiology or IPL composition are expected. During steady state of the P-replete and P-controlled cultures, 200 mL samples were taken for IPL analysis, GF/F-filtered, flash-frozen and stored at −80 °C. Samples for IPLs of the P-starved cultures were taken 30 h into batch state.

Viral infection experiments were carried out using the P-starved treatment (both 0.32 and 0.97 μ_{max}; present-day CO_2 concentration) and the double-stranded DNA virus MpV-08T (NIOZ culture collection, the Netherlands; Maat et al., 2014b). MpV lysate to infect the algal host cultures was de-

Table 1. P treatments used in the cultivation experiments of *M pusilla* and corresponding effects on growth rate (μ), doubling time (Td), alkaline phosphatase activity (APA) and photosynthetic efficiency (F_v / F_m). All experiments were carried out in the same vessels and under the same conditions (light, other nutrients, temperature, etc.). n/a is not applicable.

Culturing method	Treatment name	Effects on growth	μ (d^{-1})	Td (d)	APA (amol P $cell^{-1}$ h^{-1})	F_v / F_m (r.u.)
Semi-continuous turbidostat (daily dilution)	P-replete	Exponential growth at 1.0 μ_{max}	0.72	0.96	0.0	0.66
P-controlled chemostats	0.97 μ_{max} P-controlled	P-controlled growth at 0.97 μ_{max}	0.70	0.99	6.1	0.61
	0.32 μ_{max} P-controlled	P-controlled growth at 0.32 μ_{max}	0.23	3.01	24.3	0.50
Batch culturing once chemostat pump stopped; growth nears 0	0.97 μ_{max} P-starved	Starvation of the 0.97 μ_{max} P-controlled cultures	0.09	7.6	26.8	0.30
	0.32 μ_{max} P-starved	Starvation of the 0.32 μ_{max} P-controlled cultures	0.07	9.4	33.3	0.33
Batch culturing; chemostat pump stopped and infection with MpV-08T	0.97 μ_{max} P-starved, infected	Starvation of infected 0.97 μ_{max} P-controlled cultures	0.0	n/a	18.0	0.34
	0.32 μ_{max} P-starved, infected	Starvation of infected 0.32 μ_{max} P-controlled cultures	0.0	n/a	28.9	0.28

pleted in P by three cycles of lysis on a P-limited host. Infection took place at the start of the starvation using a virus : host ratio of 10 to obtain one-step infection cycles (82–100 % infectivity, as determined by most probable number end point dilution and flow cytometry; Suttle, 1993; Marie et al., 1999). Batch culturing avoided wash-out by dilution and consequently altered contact rates and inaccurate data analysis. Cell lysis of the P-replete cultures started after approximately 20 h, but took up to 36 h for the P-limited cultures (see Maat et al., 2014b; for more details). Therefore, samples for infected algal host IPL composition were taken 30 h post-infection (p.i.) to represent full P-starved conditions, while still being able to perform IPL analysis on intact algal cells.

Besides algal IPL analysis, we also examined the virus itself as it was shown to lose infectivity upon chloroform treatment, indicating that it possesses a lipid membrane (Martínez Martínez et al., 2014). The presence of a viral lipid membrane was confirmed by staining fresh MpV-08T with the lipophilic dye N-(3-triethylammoniumpropyl)-4-[4-(dibutylamino)styryl] pyridinium dibromide (FM 1-43) (Life technologies Ltd. Paisley, UK) in TE buffer (pH = 8) for 10 min at 4 °C and at a final concentration of 10 µM according to Mackinder et al. (2009). As a positive control, MpV-08T was stained with the nucleic acid stain SYBR Green I (Life Technologies Ltd., Paisley, UK) according to Brussaard (2004). FM1-43 and SYBR Green I stained viral particles were detected using a benchtop BD FACSCalibur equipped with a 488 nm argon laser (BD Biosciences, San Jose, USA; Marie et al., 1999; Brussaard, 2004).

For viral IPL extraction, the exponentially growing algal host was infected with MpV-08T (4 times 2.5 L), and upon full lysis of the host the virus was isolated according to Vardi et al. (2009). Approximately 10 L of lysate was pre-filtered on Whatmann GF/C filters (Maidstone, UK) in batches of 1 L and subsequently concentrated by 30 kD tangential flow filtration (Vivaflow 200, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The remaining volume was spun down into a 25 % OptiPrep™ (iodixanol; Axis-Shield, Dundee, 140 UK) solution on a Centrikon T-1080 (Kontron 141 Instruments ultracentrifuge, Watford, UK) in 12 mL ultraclear ultracentrifuge tubes (Beckman Coulter Inc., Brea, CA) in a swing-out rotor (SW41TI; Beckman Coulter, Palo Alto, USA) at approximately 100 000 × g for 2 h. The 25 % Optiprep™ layer with viruses was then transferred on top of prepared density gradient containing 30, 35, 40 and 45 % Optiprep™. After ultracentrifugation at 200 000 × g for 4 h, the white viral band was removed and gently filtered onto 0.02 µm Anodisc filters (25 mm diameter; Whatman, Maidstone, UK). Until analysis this sample was stored in 20 mL glass scintillation vials (Packard BioScience, Meriden, USA) at −80 °C.

2.2 Soluble reactive phosphorus (SRP) and indices of P limitation

Concentrations of SRP were determined by colorimetry according to Hansen and Koroleff (1999). SRP concentrations during chemostat culturing and the viral infection experiment were always at the detection limit (20 nM) and considered 0 µM, while in the P-replete cultures the concentrations of SRP never fell below 28 µM.

APA was determined according to Perry (1972). In 2 mL of culture the conversion rate of supplied (250 µL) 3-O-methylfluorescein phosphate (595 µM in 0.1 M Tris buffer with pH 8.5; Sigma-Aldrich, St. Louis, USA) was determined with an excitation and emission wavelength of 430 and 510 nm, respectively. After addition and gentle mixing, each sample was quickly and directly measured on a Hitachi F2500 fluorescence spectrophotometer (Tokyo, Japan) for 60 s by comparison of the values to a standard curve

of 3-O-methylfluorescein (Sigma-Aldrich, St. Louis, USA). Temperature was kept at 15 °C until the actual measurement, and all handling was carried out under dimmed light. Total APA was divided by the cell number to obtain APA in amol P cell^{-1} s^{-1}.

The photosynthetic efficiency (F_v / F_m) was determined by PAM fluorometry (Water-PAM, Walz, Germany). Samples were kept in the dark for 15 min at in situ temperature, after which the minimal (F_0) and maximal (F_m) chlorophyll autofluorescence were measured. The variable fluorescence F_v was calculated as Fm–F0 (Maxwell and Johnson, 2000).

2.3 Extraction and analysis of IPLs

The filters containing *M. pusilla* or MpV were freeze-dried, cut into small pieces and extracted with a modified Bligh and Dyer (BD) extraction as described by Pitcher et al. (2011). The single-phase solvent mixture of methanol (MeOH) : dichloromethane (DCM) : phosphate buffer (2 : 1 : 0.8, $v : v : v$) was added to the pieces of filter in a glass centrifuge tube. This mixture was then sonicated for 10 min, after which the extract and residue were separated by centrifuging at 1000 × g for 5 min. The solvent mixture was collected in a separate glass flask, and the whole process was repeated twice. The single-phase extract was supplemented with DCM and phosphate buffer to obtain a new ratio of MeOH : DCM : phosphate buffer (1 : 1 : 0.9, $v : v : v$) and to induce phase separation. After spinning down the extract at 1000 × g for 5 min, the DCM phase was collected in a round-bottom flask. The MeOH : phosphate buffer phase was then washed twice with DCM. The collected DCM phases were reduced under a stream of N_2. The same protocol was used for the Anodisc aluminum oxide filters containing MpV, although they could be ground directly in the BD solvent mixture in a glass tube using a spatula.

The extracts were dissolved in a hexane : isopropanol : water (72 : 27 : 1; $v/v/v$) injection solvent and filtered over a 0.45 µm regenerated cellulose filter (Grace, Deerfield, USA) just prior to analysis. Analysis was performed by high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS^n) using methods modified from Sturt et al. (2004). Separation was conducted on an Agilent 1200 series LC equipped with a thermostated autoinjector, coupled to a Thermo LTQ XL linear ion trap with Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, USA). Specifications on the gradient, column and ESI settings can be found in Sinninghe Damsté et al. (2011).

The IPLs were identified in positive ion mode (m/z 400–2000), whereby the four most abundant ions from each positive ion full scan were fragmented first to MS^2 (normalized collision energy (NCE 25, isolation width (IW) 5.0, activation Qz 0.175) and then to MS^3 (NCE 25, IW 5.0, Qz 0.175). The IPL structures were identified by comparison with fragmentation patterns of authentic standards as described in Brandsma et al. (2012). The absolute abundances of the different IPLs were not determined; instead IPL abundances were examined relatively to the total amount of IPLs, i.e., the IPL peak area divided by the total sum of peak areas. Ratios of IPL groups were calculated to detect relative changes and possible lipid substitutions between all pairs of IPLs. The IPL-bound fatty acids (IPL-FAs) were determined by the fragment ions and diagnostic neutral losses from the MS^2 spectra (Brügger et al., 1997; Brandsma et al., 2012). For the PGs, no FA combinations could be determined in this way, because there were no specific FA losses or FA fragments produced under positive ionization for this group. The combined chain lengths (x) and doubled bond equivalents (y) of the two FAs (acyl moieties) are depicted as ($C_{x:y}$).

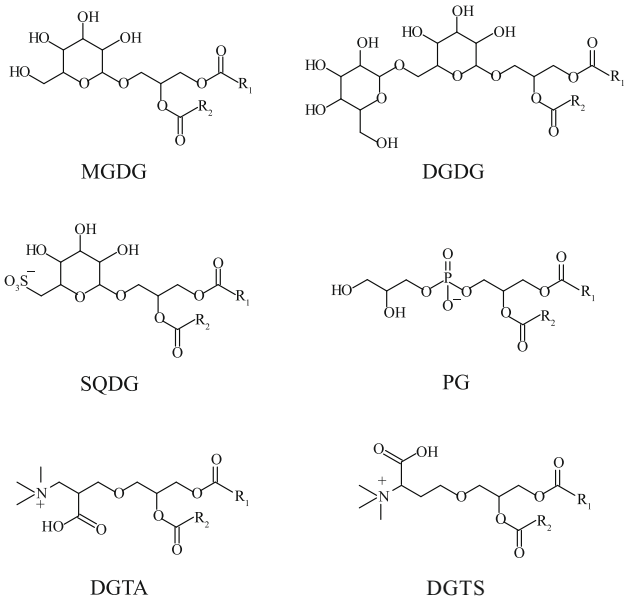

Figure 1. Structures of the detected IPL classes in *Micromonas pusilla*: the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG); the phospholipid phosphatidylglycerol (PG); and the betaine lipids diacylglyceryl-(N, N, N)-trimethylhomoserine (DGTS) and diacylglyceryl hydroxymethyltrimethyl-β-alanine (DGTA). R1 and R2 represent the acyl groups.

In all *M. pusilla* culture samples six different classes of IPLs were detected (Figs. 1 and 2): the three glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG); the phospholipid phosphatidylglycerol (PG); and the betaine lipids diacylglyceryl-(N, N, N)-trimethylhomoserine (DGTS) and diacylglyceryl hydroxymethyltrimethyl-β-alanine (DGTA). For comparison, the partial base peak chromatogram of the MpV-08T lipid extract is presented in Fig. 2b.

Table 2. Relative abundance of the IPL classes SQDGs, PGs, DGDGs, MGDGs, DGTSs and DGTAs in *Micromonas pusilla* LAC-38 as percentage of summed peak areas and per culturing treatment (horizontally sum 100 %). Treatments consisted of P-replete growth and four different strengths of P limitation, i.e., P-controlled exponential chemostat growth at 0.97 and 0.32 μ_{max} and 30 h P starvation of both P-controlled treatments. The P-starved cultures were additionally infected with the virus MpV-08T, whereby the cells were still intact after 30 h P starvation and infection. The P-replete and P-controlled cultures were subjected to 370 and 750 µatm CO_2.

Treatment	pCO_2 (µatm)	SQDG (%)	PG (%)	DGDG (%)	MGDG (%)	DGTS (%)	DGTAs (%)
1.0 μ_{max} P-replete	370	3.0	11	2.7	10	2.3	71
	750	2.9	10	2.8	8.9	3.1	72
0.97 μ_{max} P-controlled	370	8.4	7.3	7.2	5.2	5.5	66
	750	9.1	4.6	7.8	6.1	3.5	69
0.97 μ_{max} P-starved	370	6.2	1.3	4.8	4.1	1.6	82
0.97 μ_{max} P-starved, infected	370	4.5	1.1	3.0	3.7	0.8	87
0.32 μ_{max} P-controlled	370	9.0	4.5	9.6	7.0	2.1	68
	750	8.7	4.5	9.4	6.0	2.4	69
0.32 μ_{max} P-starved	370	4.9	0.9	3.6	4.2	1.1	85
0.32 μ_{max} P-starved, infected	370	4.6	2.4	4.1	3.9	2.1	83

2.4 Statistics

Statistics were carried out in SigmaPlot 13.0 (Systat Software Inc., Chicago, USA). Differences amongst the P treatments between the relative peak areas of the IPLs were tested by two-way ANOVAs ($n = 1$, significance level $p = 0.05$) and Holm–Šidák multiple comparisons. The relation of the SQDG : PG and DGDG : PG ratios with F_v / F_m were tested (separately for the 0.97 and 0.32 μ_{max} cultures) by linear regressions (significance level $p = 0.05$).

3 Results

The different P treatments could be discriminated based on *M. pusilla* growth rates, F_v / F_m and APA (Table 1), whereby APA showed increased rates and Fv / Fm decreased values with increased P limitation. In comparison to P-replete culturing conditions, P-controlled exponential growth at 0.97 and 0.32 μ_{max} (chemostats) and P starvation (stopping P supply to the chemostats) led to clear IPL remodeling (Table 2 and S1 in the Supplement). The SQDGs showed a relative increase (to the other IPLs) under all levels of P limitation as compared to P-replete conditions ($p < 0.03$), while for the DGDGs and the DGTAs this increase was only significant for the cultures that were P-starved for 30 h ($p < 0.044$). The total relative increase of the DGTAs in the P-starved cultures compared to the P-replete ones (1.2-fold) was, however, relatively small compared to those of the SQDGs and DGDGs (approximately 3-fold). In contrast to these IPLs, the relative abundance of the PGs decreased under all levels of P limitation (P-controlled growth and starvation; $p < 0.006$), while the MGDGs decreased only under P-controlled growth ($p = 0.029$), but not under P starvation. The change in MGDGs was smaller as compared to the change in PGs (1.4- vs. 6.8-fold). No significant trend was found for the relative abundance of the DGTSs under these conditions. Linear regressions showed increasing SQDG : PG or DGDG : PG ratios with decreasing photosynthetic efficiency (F_v / F_m; Fig. 3a and b; $p \leq 0.001$; $n = 6$; $r^2 = 0.96$; normality by Shapiro–Wilk test: $p = 0.781$ and 0.594 for the SQDG : PG and DGDG : PG ratio, respectively). Similar relations were found with APA as an indicator of the strength of P limitation, but here a clear distinction was found between the 0.97 and 0.32 μ_{max} (pre-grown) cultures (Fig. S1 in the Supplement). Moreover, the DGDG : MGDG ratio was 4-fold higher for the P-limited than for the P-replete cultures (Table 2). Elevated pCO_2 did not affect the relative abundance of the six IPL groups for any of the treatments (Table 3; $0.071 < p < 0.623$).

Similarly to the non-infected P-starved cultures, the PGs in the virally infected P-starved cultures decreased significantly compared to the P-controlled ones ($p = 0.007$). However, virally infected *M. pusilla* cells showed smaller increases in SQDG : PG and DGDG : PG ratios than the non-infected P-starved cells (Fig. 3a and b). The increase was however still larger for the 0.97 μ_{max} P-starved cultures than for the 0.32 μ_{max} P-starved cultures. The IPL composition of MpV showed some differences compared to *M. pusilla*; i.e., MGDGs could not be detected anymore, and the PGs were 6-fold lower relative to the DGTSs (Fig. 2b). These relative simplifications of MpV IPL composition as compared to its host were also reflected in a lower diversity of IPL-bound fatty acid groups (IPL-FAs) for all groups of MpV IPLs (IPL-FAs; Table 3). However, some FA combinations were only

Table 3. Fatty acid combinations (chain length : double bonds) for the SQDGs, DGDGs, MGDGs, DGTSs and DGTAs of *M. pusilla* and its virus MpV. The fatty acid combinations of the PGs could not be determined with the diagnostic tool. n.d. stands for non-detected.

	MGDGs	DGDGs	SQDGs	DGTSs	DGTAs
M. pusilla	16 : 0, 18 : 3	16 : 0, 18 : 3	14 : 0, 16 : 0	14 : 0, 18 : 4	14 : 3, 16 : 0
	16 : 0, 18 : 4	16 : 0, 18 : 5	14 : 0, 16 : 1	16 : 0, 18 : 1	14 : 0, 18 : 4
	16 : 0, 18 : 5	16 : 3, 18 : 3	16 : 0, 16 : 0	16 : 0, 18 : 2	16 : 0, 16 : 4
	16 : 2, 18 : 3	16 : 2, 18 : 4	16 : 0, 18 : 3	16 : 0, 18 : 3	16 : 0, 18 : 4
	16 : 3, 18 : 3	16 : 4, 18 : 3	16 : 0, 18 : 4	16 : 0, 18 : 4	16 : 1, 18 : 4
	16 : 4, 18 : 3	16 : 4, 18 : 5			16 : 4, 18 : 1
	16 : 4, 18 : 4				16 : 4, 18 : 4
	16 : 4, 18 : 5				14 : 0, 22 : 6
					16 : 4, 22 : 6
					18 : 4, 22 : 6
					22 : 6, 22 : 6
MpV	n.d.	16 : 0, 16 : 4	16 : 0, 16 : 0	14 : 0, 16 : 0	14 : 3, 16 : 0
			16 : 0, 16 : 1	14 : 0, 18 : 4	14 : 0, 18 : 4
			16 : 0, 18 : 3	16 : 0, 18 : 3	16 : 0, 16 : 4
			16 : 0, 18 : 4	16 : 0, 18 : 4	16 : 0, 18 : 4
				16 : 0, 22 : 6	16 : 1, 18 : 4
					16 : 4, 18 : 1
					16 : 4, 18 : 4
					14 : 0, 22 : 6
					16 : 4, 22 : 6
					18 : 4, 22 : 6
					22 : 6, 22 : 6

found in MpV and not in *M. pusilla*: DGDGs ($C_{32:4}$), SQDGs ($C_{32:1}$) and the DGTSs ($C_{30:0}$ and $C_{38:6}$).

4 Discussion

4.1 Lipid remodeling under P limitation and elevated pCO_2

The prasinophyte *M. pusilla* substituted P-containing IPLs with non P-containing IPLs under low P availability. Similar lipid remodeling has also been reported for some other phytoplankton groups (i.e., diatoms and cyanobacteria; Sato et al., 2000; Van Mooy et al., 2009; Martin et al., 2011; Abida et al., 2015). In addition to the replacement of PGs with SQDGs, changes were also observed for other IPLs, i.e., a large increase in DGDGs, a small increase in DGTAs and a small decrease in MGDGs. Increases in DGDGs and DGTAs with decreasing MGDGs were also observed in *Phaeodactylum tricornutum* under P depletion. However, for this diatom a larger (5-fold versus 1.2-fold in *M. pusilla*) increase of DGTAs was observed, presumably as a replacement for phosphocholines that are present in these cells (Abida et al., 2015). The decrease in *M. pusilla* MGDGs under P-controlled growth versus P repletion might be explained by their potential role as precursor for DGDGs (Heemskerk et al., 1988), which is in our study supported by the large increase in the DGDG : MGDG ratio from P-replete to P-limiting conditions.

The linear increase for SQDG : PG and DGDG : PG ratios with rising strength of P limitation (reflected in F_v / F_m and APA; Beardall et al., 2001) demonstrates that lipid remodeling was a continuous process, whereby SQDGs and DGDGs were increasingly substituting for the PGs. Furthermore, we show that the increase in the ratios of non-phospholipids to phospholipids under P starvation depended on the pre-growth condition; i.e., the SQDG : PG and DGDG : PG ratios of the $0.97\,\mu_{max}$ P-controlled cultures further increased 5- and 4-fold under P starvation, respectively, while this was 2.5- and 2-fold for the $0.32\,\mu_{max}$ P-controlled ones. Thus besides P concentrations, also the P supply rate (e.g., P remineralization rates in the natural environment) and even the history of the P supply rate may influence algal IPL composition. Whether this is a general feature requires further study. This is especially important in the light of climate-change-related processes; warming of the surface ocean will increase the degree of P limitation through strengthened vertical stratification (Sarmiento et al., 2004). Our study further showed that CO_2 enrichment of the surface ocean did not affect *M. pusilla* IPL composition.

Van Mooy et al. (2009) showed that the substitution of PGs by SQDGs was the only process of lipid remodeling under P starvation for five species of cyanobacteria. Yet the two eukaryotic diatom species (*Thalassiosira pseudonana*

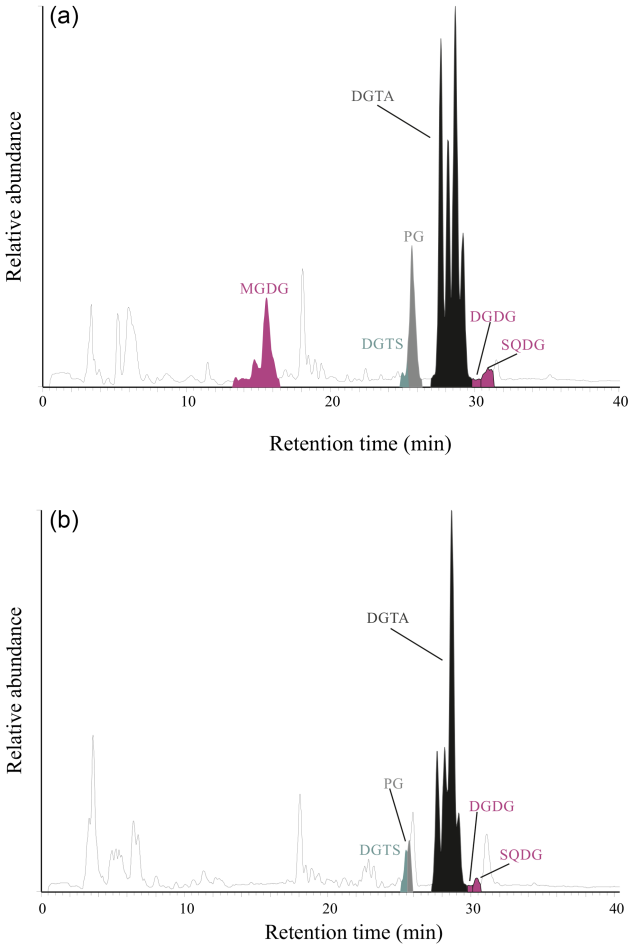

Figure 2. Partial base peak chromatogram (MS1, m/z 400–2000) of lipid extract of *M. pusilla* **(a)** and the virus MpV-08T **(b)** obtained by HPLC-MS analysis.

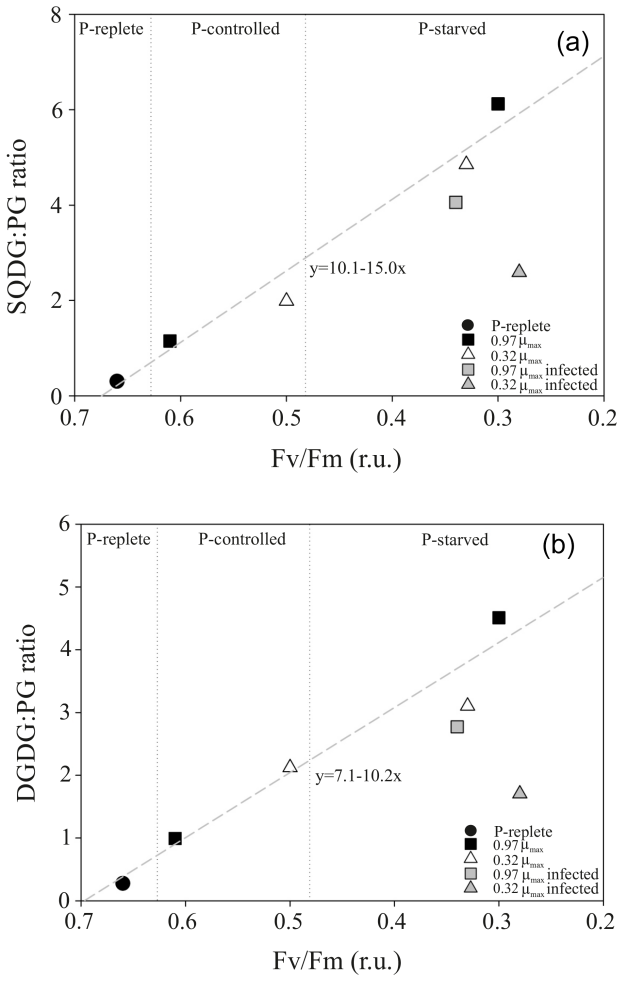

Figure 3. Relation of the SQDG : PG **(a)** and DGDG : PG **(b)** ratios with photosynthetic efficiency (F_v / F_m) of the P-replete (circles) and 0.97 (squares) and 0.32 μ_{max} (triangles) P-controlled cultures. The ratios of the virally infected cultures are depicted in grey. Dotted lines in the figures represent linear regressions for the SQDG : PG ($p < 0.001$, $r^2 = 0.98$) and DGDG : PG ratios ($p < 0.001$, $r^2 = 0.96$). The virally infected cultures were omitted from the regressions.

and *Chaetoceros affinis*) and the prymnesiophyte *Emiliania huxleyi* that were also studied showed additional substitution of the P lipids phosphatidylcholines with (N-containing) betaine lipids. The authors suggested that this makes these diatom species lesser competitors in the oligotrophic ocean than cyanobacteria. However, our results show only a minor increase in betaine lipids (i.e., DGTAs) for P-starved *M. pusilla*. Additionally, we did not detect the N-containing P lipids phosphatidylethanolamine or phosphatidylcholine (both thought to be common in eukaryotic phytoplankton; Van Mooy and Fredricks, 2010). In terms of lipid composition, the picoeukaryote *M. pusilla* seems thus less dependent on N than the other studied eukaryotic phytoplankters and, therefore, well adapted to cope with low nutrient availability under oligotrophic conditions. This might be a general characteristic of smaller photoeukaryotes (Raven, 1998).

4.2 Lipid remodeling under viral infection

Our findings demonstrate that viral infection lessened the substitution of phospholipids by non-phospholipids under P-starving conditions, particularly of the 0.32 μ_{max} P-starved *M. pusilla* cells (compared to the 0.97 μ_{max} P-starved cultures). This might be an active process, as phytoplankton viruses have been shown to alter or inhibit host metabolic pathways for the benefit of viral proliferation (Seaton et al., 1995; Rosenwasser et al., 2014). However, lipid remodeling still occurred and may have even contributed to viral replication under P-limited conditions by supplying P (derived from the replaced P lipids) to the infection process (as an elemental building block for viral particles or energy in the form of,

e.g., ATP). The exact mechanisms by which viruses interfere with IPL remodeling under P-limiting conditions require further study.

The membrane of MpV-08T was impoverished in IPL diversity compared to its host, as MGDGs were not detected anymore and the relative abundance of PGs was greatly reduced. Other glycolipids (DGDGs and SQDGs) seemed, however, unaffected. Maat et al. (2014a) showed a similar difference for viral IPLs of a virus (PgV-07T) that infects the prymnesiophyte *Phaeocystis globosa*, whereby the MGDGs were also greatly reduced in the virus compared to the host. In contrast to *M. pusilla*, however, the DGDGs were also partially lower and the SQDGs were not detected in PgV-07T. These IPLs are thought to be mainly associated with the chloroplast (Guschina and Harwood, 2009); because the chloroplast in infected *P. globosa* stays largely intact (see Maat et al., 2014a), these IPLs would then not be recruited by the virus. As *M. pusilla* is able to maintain primary production far into the infection cycle (Maat et al., 2014b), the chloroplast likely maintains its integrity as well. MpV might thus selectively recruit its lipids from other cellular compartments or produce them de novo. Several IPLs – such as certain SQDGs, DGDGs and DGTAs – were found in MpV but not in its host. Although at this point we cannot exclude the possibility that the host did contain these IPL-FAs in concentrations too low to detect, it is more likely that these compounds were produced by alteration of IPL-FAs during viral infection.

4.3 Ecological implications

Our results show substantial shifts in IPL composition of the picoeukaryote *M. pusilla* under P limitation, which seems different than for larger-sized eukaryotic phytoplankton (Sato et al., 2000; Van Mooy et al., 2009; Martin et al., 2011; Abida et al., 2015). At the same time, small-sized eukaryotic photoautotrophs such as *M. pusilla* have been shown to be favored under low-P conditions and under enhanced pCO_2 compared to larger phytoplankton size classes (Brussaard et al., 2013; Engel et al., 2008; Maat et al., 2014b). Phytoplankton-derived lipids are an important part of the nutrition of many aquatic organisms, including zooplankton grazers of phytoplankton, because of their auxotrophy for important lipid-associated compounds (e.g., PUFAs; Fraser et al., 1989; Breteler et al., 2005; Bell and Tocher, 2009). Hence, the intake by grazers of essential lipids depends on the available algal food source (abundance and species composition; Escribano and Pérez, 2010; Jones and Flynn, 2005) and indirectly on the environmental factors and viruses (Evans et al., 2009; Bale et al., 2015) to which the phytoplankton are subjected. Information on the IPL composition of these primary producers in relation to the trophic status of the environment and the level of viral control is therefore valuable to our understanding of organic carbon and energy transfer to higher trophic levels.

The Supplement related to this article is available online at doi:10.5194/bg-13-1667-2016-supplement.

Author contributions. Corina P. D. Brussaard and Douwe S. Maat designed the project. Douwe S. Maat performed culturing work and analyses, and Nicole J. Bale and Ellen C. Hopmans performed IPL analysis. Stefan Schouten, Jaap S. Sinninghe Damsté and Corina P. D. Brussaard provided expertise and supervised the project. Douwe S. Maat wrote the manuscript with contributions of all authors.

Acknowledgements. This project was funded through grants to Corina P. D. Brussaard by the Royal Netherlands Institute for Sea Research (NIOZ), an institute of the Netherlands Organisation for Scientific Research (NWO) and additionally by the Division for Earth and Life Sciences (ALW) as part of the NWO under contract no. 839.10.513. Nicole J. Bale was supported by NWO through grant 839.08.331 to Jaap S. Sinninghe Damsté as part of the National Ocean and Coastal Research Programme (ZKO). We thank the two anonymous referees for their helpful comments which improved the manuscript.

Edited by: G. Herndl

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