Biogeosciences Discuss., 12, 15583–15610, 2015
www.biogeosciences-discuss.net/12/15583/2015/
doi:10.5194/bgd-12-15583-2015

Biogeosciences Discussions
Open Access

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

D. S. Maat[1], N. J. Bale[2], E. C. Hopmans[2], J. S. Sinninghe Damsté[2], S. Schouten[2], and C. P. D. Brussaard[1,3]

[1]Department of Biological Oceanography, Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, the Netherlands
[2]Department of Marine Organic Biogeochemistry, Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, the Netherlands
[3]Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94248, 1090 GE Amsterdam, the Netherlands

Received: 3 September 2015 – Accepted: 3 September 2015 – Published: 18 September 2015

Correspondence to: D. S. Maat (douwe.maat@nioz.nl)

Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

The intact polar lipid (IPL) composition of phytoplankton is plastic and dependent on environmental factors. Previous studies have shown that phytoplankton under phosphorus (P)-stress substitute phosphatidylglycerols (PGs) with sulphoquinovosyldiacylglycerols (SQDGs) and digalactosyldiacylglycerols (DGDGs). However, these studies focused merely on P-depletion, while phytoplankton in the natural environment often experience P-limitation whereby the degree of limitation depends on the supply rate of the limiting nutrient. Here we demonstrate a linear increase in SQDG : PG and DGDG : PG ratios with increasing cellular P-stress in the picophotoeukaryote *Micromonas pusilla*, obtained by P-replete, P-limited (chemostat) and P-starved (no supply of P) culturing conditions. These ratios were not affected by the degree of the P-limiting conditions itself (i.e. 0.97 and 0.32 μ_{max} chemostats), suggesting there is a minimum requirement of PGs for the maintenance of cell growth. Viral infection reduced the increase in SQDG : PG and DGDG : PG ratios in P-starved cells, but the extent did depend on the growth rate of the cultures before infection. The membrane of *M. pusilla* virus MpV itself was lacking some IPLs compared to the host as, e.g. no monogalactosyldiacylglycerols could be detected. Growth of the phytoplankton cultures under enhanced CO_2 concentration did not affect the lipid remodeling results. The present study provides new insights into how the P-related trophic state of an ecosystem as well as viral infection can affect phytoplankton IPL composition, and therefore influence food web dynamics and biogeochemical cycling.

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

BGD
12, 15583–15610, 2015

double bonds, of which the actual cellular composition is found to be dependent on the phytoplankton species involved, but also on growth-relevant environmental variables (Guschina and Harwood, 2009).

An important factor affecting phytoplankton growth and physiology is phosphorus (P) availability (Moore et al., 2013). Under P-stress, phytoplankton phospholipids (P-lipids; lipids with the element P in the polar head group) have been found to be substituted with non P-lipids (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 heterogenic marine environment the degree of P-availability for phytoplankton is variable, hence 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, does not only depend on P-concentrations, but also on the total P-pool and P-turnover- or 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-stress and IPL remodeling. As far as we know, P-lipid remodeling in relation to phytoplankton growth rate and physiology is thus far not described in literature.

The intensity of P-stress that is experienced by a phytoplankton cell may depend on the species, as different species have different nutrient requirements (Hecky and Kilham, 1988). Also the effect of P-limitation on lipid composition has been shown to depend on the species, as two diatoms and a prymnesiophyte substituted P-lipids with nitrogen (N) containing betaine lipids, while smaller prokaryotic phytoplankton showed a substitution by sulfur containing lipids (Van Mooy et al., 2009). The authors suggested that prokaryotic phytoplankton are therefore more independent of nitrogen and have an advantage in oligotrophic areas. Eukaryotic phytoplankton can, on the other hand, be found in different size classes, whereby the smaller ones are thought to cope better

BGD
12, 15583–15610, 2015

with low resource conditions, due to their relatively large surface to volume ratio and smaller cell boundary layer (Raven, 1998). Smaller species would thus be able to cope better under P-limitation, which might be reflected in their lipid metabolism. It is so far unknown whether smaller eukaryotic phytoplankton cells show a similar independence to nitrogen as prokaryotic phytoplankton, in concordance with their assumed better adaptability to low resource environments.

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), consequently leading to increased phytoplankton nutrient limitation due to reduced mixing of nutrients to the surface of the water column. This is furthermore complicated by the rapidly increasing atmospheric CO_2 concentrations that elevate inorganic carbon concentrations in the ocean, possibly affecting phytoplankton growth, production and stoichiometry (Riebesell, 2011). To our knowledge, no information is published on the effects of ecologically relevant changing CO_2 conditions on the intact polar lipid composition of phytoplankton.

Many different phytoplankton species have been shown to be sensitive to viral infection (Brussaard, 2004a and references therein). Viruses are numerically abundant parasites that use their hosts' metabolism for progeny production, thereby changing host cellular composition before lysis, e.g. pigments, lipids and fatty acids (Evans et al., 2009; Bale et al., 2015; Llewellyn et al., 2007; Vardi et al., 2009). Viral infection of *Emiliania huxleyi* induced production of a glycosphingolipid that plays a role in the infection process (Vardi et al., 2009). However, studies on the possible effects of viral infection on the substitution of P-lipids for non P-lipids are thus far not reported.

Here we investigated the IPL composition of the picophytoplankton species *Micromonas pusilla* (Butcher; Manton and Parke, 1960; Prasinophyceae) under different degrees of P-stress (i.e. P-replete, increasing P-limitation at 0.97 and 0.32 μ_{max}, and P-starving) and elevated partial CO_2 pressure (pCO_2; 750 µatm) as compared to present-day pCO_2 (370 µatm). Cellular P-stress was thereby quantified as Alkaline Phosphatase Activity (APA), an indicator of phytoplankton P-limitation (Beardall et al.,

BGD
12, 15583–15610, 2015

2001). *M. pusilla* is globally distributed (Slapeta et al., 2006) and can represent significant fractions of phytoplankton chlorophyll *a* biomass (e.g. on average 22 % year round in the English Channel; Not et al., 2004). The species is also readily infected by viruses (Cottrell and Suttle 1991, Brussaard et al., 2004), whereby viral infection of P-limited *M. pusilla* prolongs the time to lysis of the algal host during which carbon fixation and P-assimilation is still possible (Maat et al., 2014b). The possible influence of viral infection on the substitution of P-lipids under P-limitation was investigated and also the IPL composition of the virus MpV-08T was compared to its host.

2 Materials and methods

2.1 Culturing and treatments

The prasinophyte *Micromonas pusilla* Mp-LAC38 (culture collection Marine Research Center, Göteborg University; Sahlsten and Karlson 1998) was pre-grown under several degrees of P-stress, as described in detail by Maat et al. (2014b). In short, duplicate axenic cultures were grown on 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-treatments comprised a P-replete growth condition (semi-continuous culturing; P = 36 µM; 1.0 μ_{max} = 0.72 d^{-1}), two P-limited growth conditions, i.e. chemostat culturing at 0.97 μ_{max} and 0.32 μ_{max} (P = 0.25 µM), and P-starvation for the P-limited cultures. Chemostats are continuous cultures, wherein cells maintain exponential growth, but are limited by a single nutrient. The concentration of that limiting nutrient (P in this study) in the growth medium determines total biomass, while the growth rate is directly driven by the dilution rate (MacIntyre and Cullen, 2005). The benefit of chemostats is that all algal cells have a similar and consistent physiological condition during steady state (this in contrast to batch cultures) that is determined by their growth rate. We chose near exponential growth (0.97 μ_{max}) and a more stringent level of P-limitation under slower growth (0.32 μ_{max}).

BGD
12, 15583–15610, 2015

P-starved conditions were obtained by stopping the dilution of the chemostats (batch culture), resulting in growth inhibition. The P-replete treatment (not limited, thus not in chemostats) was maintained semi-continuously, according to the turbidostat principle (daily dilution based upon abundances). The viral infection experiments were carried out under batch culture conditions (as for P-starved) to avoid wash-out by dilution and consequently altered contact rates and inaccurate data analysis. Batch cultures were obtained by halting medium supply whereby the algal cells went into P-starvation. Cultures were maintained at present day pCO_2 (370 µatm) as well as future pCO_2 (predicted for the year 2100; 750 µatm; Meehl et al., 2007) by bubbling the cultures according to Maat et al. (2014). During steady state of the P-replete and P-limited cultures (at abundances of 8×10^5 mL^{-1} for at least several weeks) 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. The moment the transfer of medium stopped, half of the P-starving cultures (at present day pCO_2) were infected with the *M. pusilla* virus MpV-08T (NIOZ culture collection, the Netherlands). P-limited MpV lysate was obtained by three cycles of lysis on a P-limited host. MpV was added at a virus : host ratio of 10 (100 % infectivity, as determined by most probable number endpoint dilution; Suttle 1993) to obtain one-step infection cycles. During the viral infection experiment, samples for algal host IPL-composition were taken 30 h post infection (p.i.) to represent full P-starved conditions while cell lysis was still minimal to allow IPL analysis on intact cells.

MpV-08T is a lytic double-stranded DNA virus shown to lose infectivity upon chloroform treatment, indicating that it possesses a lipid membrane (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

BGD
12, 15583–15610, 2015

SYBRGreen 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, 2004b).

For viral IPL extraction, 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 prefiltered 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 % OptiPrepTM (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 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 alkaline phosphatase activity (APA)

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.

The actual level of P-stress resulting from the different P-treatments, was determined by APA according to Perry (1972). In 2 mL of culture the conversion of supplied 3-O-methylfluorescein phosphate (595 µM; Sigma-Aldrich, St. Louis, USA) was determined with an excitation and emission wavelength of 430 and 510 nm, respectively. This was

BGD
12, 15583–15610, 2015

carried out on a Hitachi F2500 Fluorescence spectrophotometer (Tokyo, Japan) for 60s by comparison of the values to a standard curve of 3-O-methylfluorescein (Sigma-Aldrich, St. Louis, USA). Total APA was divided by the cell number to obtain APA cell^{-1}.

2.3 Extraction and analysis of IPLs

The filters containing either *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 ten minutes, 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.

Before analysis, 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). Analysis was done 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).

BGD
12, 15583–15610, 2015

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 relative abundances of the different IPLs (in respect to each other) could not be determined, due to the different ionization efficiencies. The changes in IPL abundances (per compound) amongst the different treatments are therefore shown as relative peak areas, i.e. the compound peak area divided by the total sum of peak areas.

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 fatty acid combinations could be determined in this way. The chain lengths (x) and doubled bonds (y) of the two FA moieties are depicted as ($C_{x:y}$).

2.4 Statistics

Statistics were carried out on Sigmaplot 13.0 (Systat software Inc., Chicago, USA). Differences amongst the P-treatments between the relative peak areas of the IPLs, were tested by 2-way ANOVAs ($n = 1$, significance level $p = 0.05$) and Holm-Sidak multiple comparisons. The relation of the SQDG : PG and DGDG : PG ratios with APA were tested (separately for the 0.97 and 0.32 μ_{max} cultures) by linear regressions (significance level $p = 0.05$).

3 Results

The APA results clearly demonstrate the increasing degree of P-stress of the different treatments, i.e. increasing from 0 amol cell^{-1} s^{-1} for the P-replete, to 6 for 0.97 μ_{max} and 24 for 0.32 μ_{max} P-limited, to 27–33 for the P-starved cultures.

BGD
12, 15583–15610, 2015

Lipid analysis of *M. pusilla* resulted in the detection of six different classes of IPLs (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).

The relative abundance of the six IPL groups was not affected by pCO_2 treatment (Table 1; $0.071 < p > 0.623$). However, P-limitation and starvation led to changes in the relative abundance of five of the six IPL compounds (Table 2). The SQDGs increased relatively to the other IPLs under P-limitation and starvation ($p < 0.03$) as compared to P-replete, while for the DGDGs and the DGTAs this increase was only significant for the starved cultures ($p < 0.044$). The total relative increase of the DGTAs from P-replete to P-starved (1.2-fold) was however relatively small compared to the SQDGs and DGDGs (approximately 3-fold). In contrast to these IPLs, the relative abundance of the PGs decreased under P-limitation and starvation ($p < 0.006$), and the MGDGs decreased only under P-limitation ($p = 0.029$). 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. Similarly to the non-infected P-starved cultures, the PGs in the virally infected P-starved cultures decreased significantly compared to the P-limited ones ($p = 0.007$).

We found positive linear correlations between APA and the SQDG : PG or DGDG : PG ratios for the 0.97 μ_{max} cultures (Fig. 3a; ($p \leq 0.005$). The 0.32 μ_{max} cultures showed a non-linear increase in these ratios with increasing APA (Fig. 3b). In the virally infected cultures, the SQDG : PG and DGDG : PG ratios also increased for the 0.97 μ_{max} pre-grown cultures (Fig. 3a) with increasing APA, and thus P stress, though in the 0.32 μ_{max} cultures the SQDG : PG and DGDG : PG ratios remained at the same level as for the 0.32 μ_{max} P-limited ones.

The IPL composition of MpV showed some differences compared to *M. pusilla*, i.e. MGDGs could not be detected anymore and the PGs showed a 6-fold decrease com-

BGD
12, 15583–15610, 2015

pared to the DGTSs (Fig. 4). These relative simplifications of MpV IPL composition as compared to its host were also reflected in a lower diversity of IPL-bound fatty acids (IPL-FAs) for all groups of MpV IPLs (IPL-FAs; Table 3). Several IPL-FAs belonging to the DGDGs ($C_{32:4}$), SQDGs ($C_{32:1}$) and the DGTSs ($C_{30:0}$ and $C_{38:6}$), were only found in MpV and not in *M. pusilla*.

4 Discussion

4.1 Lipid remodeling under P-limitation

As has been reported for some other phytoplankton species studied under P-stress (diatoms and cyanobacteria; Sato et al., 2000; Van Mooy et al., 2009; Martin et al., 2011; Abida et al., 2015), the prasinophyte *M. pusilla* also substituted P-lipids with non P-lipids under decreasing P-availability. In addition to the replacement of PGs by SQDGs, changes were observed for other lipids, 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 with a larger (5-fold) increase of DGTAs presumably as replacement for phosphocholines that are present in this diatom (Abida et al., 2015). The decrease in MGDGs might be explained by their potential role as precursor for DGDGs (Heemskerk et al., 1988).

Furthermore, using APA as quantitative indicator of P-stress (Beardall et al., 2001) we show for the 0.97 μ_{max} P-limited *M. pusilla* cells a positive relation between the SQDG : PG and DGDG : PG ratios with P-stress. This implies that lipid remodeling is a continuous process, whereby SQDGs and DGDGs are increasingly substituted for the PGs with increasing P-stress. The non-linear increase in these lipid ratios for the 0.32 μ_{max} cultures was most probably due to the already high APA under P-limiting conditions and lower maximum APA compared to the 0.97 μ_{max} cultures. Lowered cellular

BGD
12, 15583–15610, 2015

P-quota as specific adaptations to P-stress will result in a different relation between APA and the ratio of non-phospholipids to phospholipids (Maat et al., 2014b).

Although the degree of P-limitation (0.97 vs. 0.32 μ_{max} P-limited growth) affected APA as well as other physiological parameters such as photosynthetic efficiency and net primary production per cell (this study, Maat et al., 2014b), the level of PG substitution under 0.97 and 0.32 μ_{max} P-limited growth was found to be similar. In a natural system under P-limitation, the P-supply rate would define the gross growth rate and thus be of minor importance on the lipid composition. However, under conditions that lead to P-starvation, such as a reduced P-supply or competition for P with other organisms in the natural environment (Brown et al., 1981; Harris, 1986), the P-lipids would then show large decreases compared to the other IPLs. Yet, we show that the increase in ratios under P-starvation does depend on the pre-growth conditions. The SQDG : PG and DGDG : PG ratios of the 0.97 μ_{max} cultures further increased a respective 5 and 4-fold under P-starvation, while this was 2.5 and 2-fold for the 0.32 μ_{max} cultures. It can thus be anticipated that there is a minimum requirement of PGs in *M. pusilla* under P-stress as long as the cells maintain growth. When growth stops (P-starvation), the substitution of PGs by SQDGs and DGDGs continues, differing by the degree of P-limitation during pre-growth (0.97 or 0.32 μ_{max}). Likely this is mainly a survival strategy whereby P is channeled to basic cellular functions, when growth can no longer be maintained. SQDGs, DGDGs and PGs constitute the major part of thylakoid lipids (Guschina and Harwood, 2009). A replacement of the PGs by SQDGs under P-stress has been suggested to be necessary to maintain membrane charge balance under P-stress and the optimal functionality of these membranes is then likely affected by this substitution (Sato, 2004). P-lipid substitution might also take place in the photosynthetic membranes of *M. pusilla* as its photosynthetic efficiency is strongly reduced under P-starvation (Maat et al., 2014b).

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

BGD
12, 15583–15610, 2015

prymnesiophyte *Emiliania huxleyi* that were also studied, showed additional substitution of phosphatidylcholines with N-containing betaine lipids. The authors suggested that this makes cyanobacteria better competitors in the oligotrophic ocean than eukaryotic phytoplankton, as their lipid substitutions are independent of N. Yet, our results show only a minor increase in betaine lipids (DGTAs) for P-starved *M. pusilla*. Additionally, we did not detect the nitrogen containing P-lipids phosphatidylethanolamine or phosphatidylcholine, which are both common in eukaryotic phytoplankton (Van Mooy and Fredricks, 2010). In terms of lipid composition, the picoeukaryote *M. pusilla* is thus well adapted to cope with low nutrient-availability under oligotrophic conditions.

4.2 Lipid remodeling under viral infection

Viral infection interfered with the lipid remodeling in P-starving *M. pusilla*, whereby the replacement of phospholipids by glycolipids was lessened in the virally infected cultures, especially in the 0.32 μ_{max} ones. As viral replication requires energy and elemental building blocks from the host cell, there might be a competition for these resources between viral replication and lipid substitution. In the virally infected 0.97 μ_{max} cells, where intracellular resources are most likely more abundant than in the 0.32 μ_{max} pregrown cultures, lipids are substituted to a greater extent. Our findings demonstrate that P-limited, virally infected cells can still substitute phospholipids by non-phospholipids under P-starving conditions, a process that does not seem to be relevant for the production of viruses as such. This process might, however, facilitate a certain rate of photophosphorylation within the infected cells, providing energy for virus proliferation. Additionally, it may result in larger release of cellular carbon upon virally-induced cell lysis. As we show, this is dependent on growth conditions before infection (0.32 vs. 0.97 μ_{max}) and thus the trophic status of the marine ecosystem in the first place.

The membrane of MpV-08T was impoverished in IPLs as compared to its host, whereby the MGDGs were not detected anymore and PGs 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 prym-

BGD
12, 15583–15610, 2015

nesiophyte *Phaeocystis globosa*, whereby the MGDGs were also greatly reduced in the virus compared to the host. In contrast to in *M. pusilla*, however, the DGDGs also partially decreased and the SQDGs were not detected in PgV-07T. These IPLs are thought to be mainly associated with the chloroplast (Guschina and Harwood, 2009) and 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 IPL-FAs, belonging to the SQDGs, DGDGs and DGTAs, were found in MpV but not in its host. Although at this point we cannot exclude that the host did contain these IPL-FAs in too low concentrations to detect, it is possible that these compounds were produced by alteration of IPL-FAs during viral infection.

4.3 Ecological implications

Phytoplankton derived lipids are an important part of the nutrition of many aquatic organisms, including heterotrophic 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 food source (phytoplankton species present) and indirectly on the environmental factors to which the phytoplankton are subjected. Information on the IPL composition of these primary producers and how this is influenced by the environment (trophic status) is valuable to our understanding of organic carbon and energy transfer to higher trophic levels. This is especially important in the light of climate change related processes. For example, global warming will strengthen vertical stratification of the surface ocean (Sarmiento et al., 2004), which increases the degree of nutrient limitation and can consequently be expected to induce changes in phytoplankton lipid composition (present study). Even though we demonstrated that *M. pusilla* IPL composition was not affected by elevated pCO_2 conditions, other phytoplankton

BGD
12, 15583–15610, 2015

species or higher pCO_2 concentrations might show effects. Muradyan et al. (2004) showed an increase of the MGDG:DGDG ratio in *Dunaliella salina* under increased pCO_2. Although the CO_2 concentration that was used in their study (10 %), was approximately 250 times higher than the natural present day environment, it reveals that there are mechanisms that can induce these changes.

Our results indicate that picophytoplankton may show a different IPL composition and IPL related response to P-limitation as larger eukaryotic phytoplankton. At the same time, small-sized eukaryotic photoautotrophs such as *M. pusilla* have been shown to be favored under enhanced pCO_2 compared to larger phytoplankton size-classes (Brussaard et al., 2013) and under low P-conditions (Maat et al., 2014b). Hence, IPL dynamics might play a role in a better adaptability of picophytoplankton under such (future ocean) conditions. Knowledge of the role of IPLs in the response of phytoplankton to their environment and the interacting role of viruses can expand our understanding on food web dynamics and biogeochemical cycling.

Author contributions. C. P. D. Brussaard and D. S. Maat designed the project. DSM performed culturing work and analyses and N. J. Bale and E. C. Hopmans performed IPL analysis. S. Schouten, J. S. Sinninghe Damsté and C. P. D. Brussaard provided expertise and supervised the project. DSM wrote the manuscript with contributions of all authors.

Acknowledgements. This project was funded through grants to C. 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. N. J. Bale was supported by NWO through grant 839.08.331 to J. S. Sinninghe Damsté as part of The National Ocean and Coastal Research Programme (ZKO).

References

Abida, H., Dolch, L -J., Meï, C., Villanova, V., Conte, M., Block, M. A., Finazzi, G., Bastien, O., Tirichine, L., and Bowler, C.: Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in *Phaeodactylum tricornutum*, Plant Physiol., 167, 118–136, 2015.

BGD
12, 15583–15610, 2015

Bale, N. J., Maat, D. S., Hopmans, E. C., Mets, A., Sinninghe Damsté, J. S., Brussaard, C. P. D., and Schouten, S.: Fatty acid dynamics during viral infection of *Phaeocystis globosa*, Aquat. Microb. Ecol., 74, 85–94, 2015.

Beardall, J., Young, E., and Roberts, S.: Approaches for determining phytoplankton nutrient limitation, Aquat. Sci., 63, 44–69, 2001.

Bell, M. V. and Tocher, D. R.: Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions, in: Lipids in aquatic ecosystems, edited by: Arts, M. T., Kainz, M. J., Brett, M. T., Springer, Dordrecht, the Netherlands, 211–236, 2009.

Brandsma, J., Hopmans, E. C., Brussaard, C. P. D., Witte, H. J., Schouten, S., and Sinninghe Damsté, J. S.: Spatial distribution of intact polar lipids in North Sea surface waters: relationship with environmental conditions and microbial community composition, Limnol. Oceanogr., 57, 959–973, 2012.

Breteler, W., Schogt, N., and Rampen, S.: Effect of diatom nutrient limitation on copepod development: role of essential lipids, Mar. Ecol.-Prog. Ser., 291, 125–133, 2005.

Brown, E. J., Button, D. K., and Lang, D. S.: Competition between heterotrophic and autotrophic microplankton for dissolved nutrients, Microb. Ecol., 7, 199–206, 1981.

Brussaard, C., Noordeloos, A., Sandaa, R.-A., Heldal, M., and Bratbak, G.: Discovery of a dsRNA virus infecting the marine photosynthetic protist *Micromonas pusilla*, Virology, 319, 280–291, 2004.

Brussaard, C. P. D.: Viral Control of Phytoplankton Populations – a Review, J. Euk. Microbiol., 51, 125–138, 2004a.

Brussaard, C. P. D.: Optimization of procedures for counting viruses by flow cytometry, Appl. Environ. Microbiol., 70, 1506–1513, 2004b.

Brussaard, C. P. D., Noordeloos, A. A. M., Witte, H., Collenteur, M. C. J., Schulz, K., Ludwig, A., and Riebesell, U.: Arctic microbial community dynamics influenced by elevated CO_2 levels, Biogeosciences, 10, 719–731, doi:10.5194/bg-10-719-2013, 2013.

Cottrell, M. T. and Suttle, C. A.: Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter, *Micromonas-pusilla*, Mar. Ecol.-Prog. Ser., 78, 1–9, 1991.

Fraser, A. J., Sargent, J. R., Gamble, J. C., and Seaton, D. D.: Formation and transfer of fatty acids in an enclosed marine food chain comprising phytoplankton, zooplankton and herring (*Clupea harengus* L.) larvae, Mar. Chem., 27, 1–18, 1989.

BGD
12, 15583–15610, 2015

Graziano, L. M., Roche, J., and Geider, R. J.: Physiological responses to phosphorus limitation in batch and steady-state cultures of *Dunaliella tertiolecta* (chlorophyta): a unique stress protein as an indicator of phosphate deficiency, J. Phycol., 32, 825–838, 1996

Guillard, R. R. and Ryther, J. H.: Studies of marine planktonic diatoms. 1. *Cylotella nana hustedt*, and *Detonula convervacea* (cleve) *gran*, Can. J. Microbiol., 8, 229–239, 1962.

Guschina, I. A. and Harwood, J. L.: Algal lipids and effect of the environment on their biochemistry, in: Lipids in aquatic ecosystems, edited by: Arts, M. T., Kainz, M. J., and Brett, M. T., Springer, Dordrecht, the Netherlands, 1–24, 2009.

Hansen, H. P. and Koroleff, F.: Determination of nutrients, in: Methods of Seawater Analysis, 3rd Edn., edited by: Grasshoff, K., Kremling, K., and Erhardt, M., Wiley, Weinheim, Germany, 125–187, 1999.

Harris, G.: The concept of limiting nutrients, in: Phytoplankton ecology-structure, function and fluctuation, Chapman and Hall, London, United Kingdon, 137–164, 1986.

Hecky, R. and Kilham, P.: Nutrient limitation of phytoplankton in freshwater and marine environments: A review of recent evidence on the effects of enrichment, Limnol. Oceanogr., 33, 796–822, 1988.

Heemskerk, J. W., Bögemann, G., Helsper, J. P., and Wintermans, J. F.: Synthesis of mono- and digalactosyldiacylglycerol in isolated spinach chloroplasts, Plant. Physiol., 86, 971–977, 1988.

Llewellyn, C. A., Evans, C., Airs, R. L., Cook, I., Bale, N. J., and Wilson, W. H.: The response of carotenoids and chlorophylls during virus infection of*Emiliania huxleyi* (Prymnesiophyceae), J. Exp. Mar. Biol. Ecol., 344, 101–112, 2007.

Maat, D. S., Bale, N. J., Hopmans, E. C., Baudoux, A.-C., Sinninghe Damsté, J. S., Schouten, S., and Brussaard, C. P. D.: Acquisition of intact polar lipids from the prymnesiophyte Phaeocystis globosa by its lytic virus PgV-07T, Biogeosciences, 11, 185–194, doi:10.5194/bg-11-185-2014, 2014a.

Maat, D. S., Crawfurd, K. J., Timmermans, K. R., and Brussaard, C. P. D.: Elevated CO_2 and Phosphate Limitation Favor *Micromonas pusilla* through Stimulated Growth and Reduced Viral Impact, Appl. Environ. Microbiol., 80, 3119–3127, 2014b.

MacIntyre, H. L. and Cullen, J. J.: Using cultures to investigate the physiological ecology of microalgae, in: Algal culturing techniques, edited by: Anderson, R. A., Elsevier Academic press, Amsterdam, 287–327, 2005.

BGD
12, 15583–15610, 2015

Mackinder, L. C. M., Worthy, C. A., Biggi, G., Hall, M., Ryan, K. P., Varsani, A., Harper, G. M., Wilson, W. H., Brownlee, C., and Schroeder, D. C.: A unicellular algal virus, *Emiliania huxleyi* virus 86, exploits an animal-like infection strategy, J. Gen. Virol., 90, 2306–2316, 2009.

Marie, D., Brussaard, C. P. D., Thyrhaug, R., Bratbak, G., and Vaulot, D.: Enumeration of marine viruses in culture and natural samples by flow cytometry, Appl. Environ. Microbiol., 65, 45–52, 1999.

Martin, P., Van Mooy, B. A., Heithoff, A., and Dyhrman, S. T.: Phosphorus supply drives rapid turnover of membrane phospholipids in the diatom *Thalassiosira pseudonana*, The ISME J., 5, 1057–1060, 2011.

Meehl, G. A., Stocker, T. F., Collins, W. D., Friedlingstein, P., Gaye, A. T., Gregory, J. M., Kitoh, A., Knutti, R., Murphy, J. M., Noda, A., Raper, S. C. B., Watterson, I. G., Weaver, A. J., and Zhao, Z. C.: Global Climate Projections, in: Climate Change 2007: The Physical Science Basis, edted by: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., Tignor, M., and Miller, H. L.: Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge, United Kingdom and New York, USA, 749–844, 2007.

Moore, C., Mills, M., Arrigo, K., Berman-Frank, I., Bopp, L., Boyd, P., Galbraith, E., Geider, R., Guieu, C., and Jaccard, S.: Processes and patterns of oceanic nutrient limitation, Nature Geosci., 6, 701–710, 2013.

Muradyan, E., Klyachko-Gurvich, G., Tsoglin, L., Sergeyenko, T., and Pronina, N.: Changes in lipid metabolism during adaptation of the *Dunaliella salina* photosynthetic apparatus to high CO_2 concentration, Russ. J. P. Physiol., 51, 53–62, 2004.

Not, F., Latasa, M., Marie, D., Cariou, T., Vaulot, D., and Simon, N.: A single species, *Micromonas pusilla* (Prasinophyceae), dominates the eukaryotic picoplankton in the western English channel, Appl. Environ. Microbiol., 70, 4064–4072, 2004.

Perry, M. J.: Alkaline phosphatase activity in subtropical central north pacific waters using a sensitive fluorometric method, Mar. Biol., 15, 113–115, 1972.

Pitcher, A., Villanueva, L., Hopmans, E. C., Schouten, S., Reichart, G.-J., Sinninghe Damsté, J. S.: Niche segregation of ammonia-oxidizing archaea and anammox bacteria in the Arabian Sea oxygen minimum zone, ISME J., 5, 1896–1904, 2011.

Raven, J. A.: The twelfth Tansley Lecture, Small is beautiful: the picophytoplankton, Funct. Ecol., 12, 503–513, 1998.

BGD
12, 15583–15610, 2015

Rengefors, K., Pettersson, K., Blenckner, T., and Anderson, D. M.: Species-specific alkaline phosphatase activity in freshwater spring phytoplankton: application of a novel method, J. Plankton Res., 23, 435–443, 2001.

Riebesell, U. and Tortell, P. D.: Effects of Ocean Acidification on Pelagic Organisms and Ecosystems, in: Ocean Acidification, edited by: Gattuso, J. P. Hansson, L., Oxford University Press, Oxford, United Kingdom, 2011.

Riegman, R., Stolte, W., Noordeloos, A. A., and Slezak, D.: Nutrient uptake and alkaline phosphatase activity of*Emiliania huxleyi* (Prymnesiophyceae) during growth under N and P limitation in continuous cultures, J. Phycol., 36, 87–96, 2000.

Sahlsten, E. and Karlson, B.: Vertical distribution of virus-like particles (VLP) and viruses infecting *Micromonas pusilla* during late summer in the southeastern Skagerrak, North Atlantic, J. Plankton Res., 20, 2207–2212, 1998.

Sarmiento, J. L., Slater, R., Barber, R., Bopp, L., Doney, S. C., Hirst, A. C., Kleypas, J., Matear, R., Mikolajewicz, U., Monfray, P., Soldatov, V., Spall, S. A., and Stouffer, R.: Response of ocean ecosystems to climate warming, Global Biogeochem. Cy., 18, 1–23, 2004.

Sato, N.: Roles of the acidic lipids sulfoquinovosyl diacylglycerol and phosphatidylglycerol in photosynthesis: their specificity and evolution, J. Plant. Res., 117, 495–505, 2004.

Sato, N., Hagio, M., Wada, H., and Tsuzuki, M.: Environmental effects on acidic lipids of thylakoid membranes, Biochem. Soc. Trans., 28, 912–914, 2000.

Sinninghe Damsté, J. S., Rijpstra, W. I. C., Hopmans, E. C., Weijers, J. W. H., Foesel, B. U., Overmann, J., and Dedysh, S. N.: 13,16-Dimethyl octacosanedioic acid (iso-diabolic acid), a common membrane-spanning lipid of *Acidobacteria* subdivisions 1 and 3, Appl. Environ. Microb., 77, 4147–4154, 2011.

Slapeta, J., Lopez-Garcia, P., and Moreira, D.: Global dispersal and ancient cryptic species in the smallest marine eukaryotes, Mol. Biol. Evol., 23, 23–29, 2006.

Sturt, H. F., Summons, R. E., Smith, K., Elvert, M., and Hinrichs, K. U.: Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry – new biomarkers for biogeochemistry and microbial ecology, Rapid Commun. Mass Spectrom., 18, 617–628, 2004.

Suttle, C. A.: Enumeration and isolation of viruses, in: Current Methods in Aquatic Microbial Ecology, edited by: Kemp, P. F., Sherr, B. F., Sherr, E. F. and Cole, J. J., Lewis Publishers Boca Raton, Florida, 121–134, 1993.

BGD
12, 15583–15610, 2015

Van Mooy, B. A. and Fredricks, H. F.: Bacterial and eukaryotic intact polar lipids in the eastern subtropical South Pacific: water-column distribution, planktonic sources, and fatty acid composition, Geochim. Cosmochim. Acta., 74, 6499–6516, 2010.

Van Mooy, B. A. S., Fredricks, H. F., Pedler, B. E., Dyhrman, S. T., Karl, D. M., Koblizek, M., Lomas, M. W., Mincer, T. J., Moore, L. R., Moutin, T., Rappe, M. S., and Webb, E. A.: Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity, Nature, 458, 69–72, 2009.

Vardi, A., Van Mooy, B. A. S., Fredricks, H. F., Popendorf, K. J., Ossolinski, J. E., Haramaty, L., and Bidle, K. D.: Viral glycosphingolipids induce lytic infection and cell death in marine phytoplankton, Science, 326, 861–865, 2009.

Vincent, W. F.: Rapid physiological assays for nutrient demand by the plankton, II. Phosphorus, J. Plankton Res., 3, 699–710, 1981.

Volkman, J. K., Barrett, S. M., Blackburn, S. I., Mansour, M. P., Sikes, E. L., and Gelin, F.: Microalgal biomarkers: a review of recent research developments, Org. Geochem., 29, 1163–1179, 1998.

BGD
12, 15583–15610, 2015

Table 1. Effect of pCO_2 on the relative peak areas of the IPL classes SQDGs, PGs, DGDGs, MGDGs, DGTSs and DGTAs in *Micromonas pusilla* Mp-LAC38 as percentage of summed peak areas per P-treatment (horizontally sum 100 %).

	pCO_2 (µatm)	SQDGs (%)	PGs (%)	DGDGs (%)	MGDGs (%)	DGTSs (%)	DGTAs (%)
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} limited	370	8.4	7.3	7.2	5.2	5.5	66
	750	9.1	4.6	7.8	6.1	3.5	69
0.32 μ_{max} limited	370	9.0	4.5	9.6	7.0	2.1	68
	750	8.7	4.5	9.4	6.0	2.4	69

BGD
12, 15583–15610, 2015

Table 2. Relative abundance of the IPL classes SQDGs, PGs, DGDGs, MGDGs, DGTSs and DGTAs as percentage of summed peak areas per P-treatment (horizontally sum 100 %), i.e. P-replete 1.0 μ_{max}, P-limited 0.97 and 0.32 μ_{max}, and P-starving (pre-grown at 0.97 and 0.32 μ_{max}) *Micromonas pusilla* Mp-LAC38 cultures. The latter were also infected with MpV-08T, IPL samples taken 30 post infection (host cells still intact).

	SQDG (%)	PG (%)	DGDG (%)	MGDG (%)	DGTS (%)	DGTAs (%)
1.0 μ_{max} P-replete	3.0	11	2.7	10	2.3	71
0.97 μ_{max} limited	8.4	7.3	7.2	5.2	5.5	66
0.97 μ_{max} starved	6.2	1.3	4.8	4.1	1.6	82
0.97 μ_{max} starved infected	4.5	1.1	3.0	3.7	0.8	87
0.32 μ_{max} limited	9.0	4.5	9.6	7.0	2.1	68
0.32 μ_{max} starved	4.9	0.9	3.6	4.2	1.1	85
0.32 μ_{max} starved infected	4.6	2.4	4.1	3.9	2.1	83

BGD
12, 15583–15610, 2015

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. non-detected.

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

BGD
12, 15583–15610, 2015

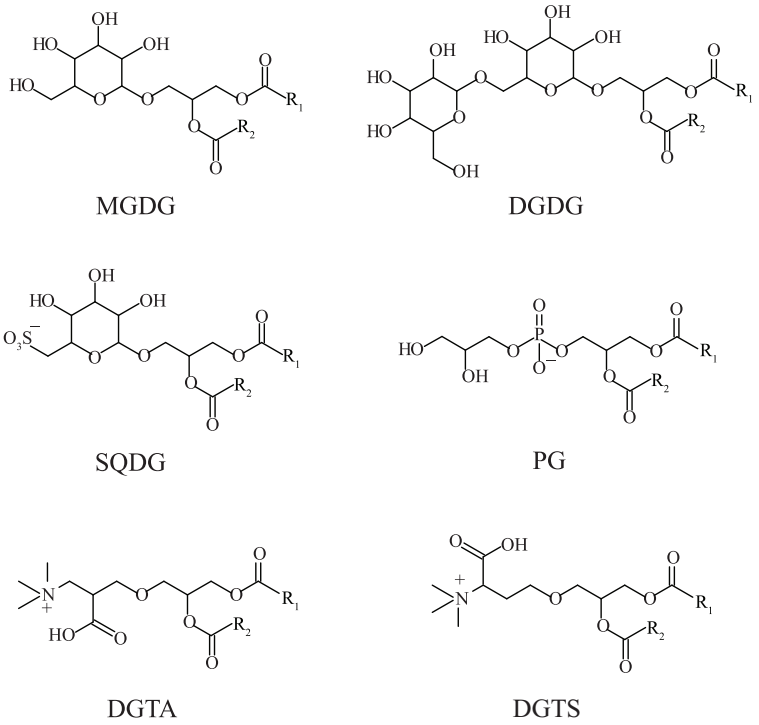


Figure 1. Structures of the detected IPL classes in *Micromonas pusilla*: the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG); the phospholipids phosphatidylglycerol (PG) and the betaine lipids diacylglyceryl-(N, N, N)-trimethylhomoserine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

BGD
12, 15583–15610, 2015

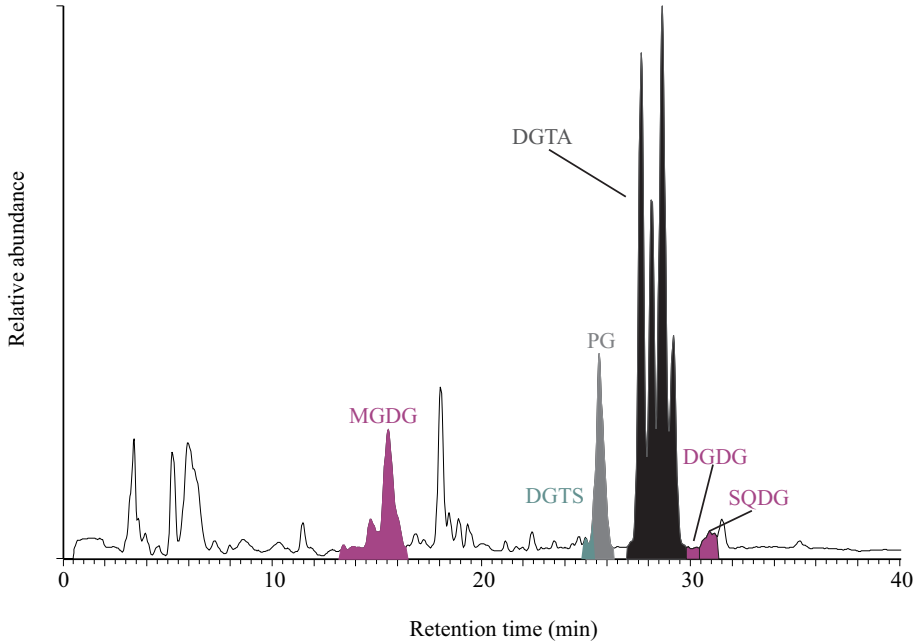
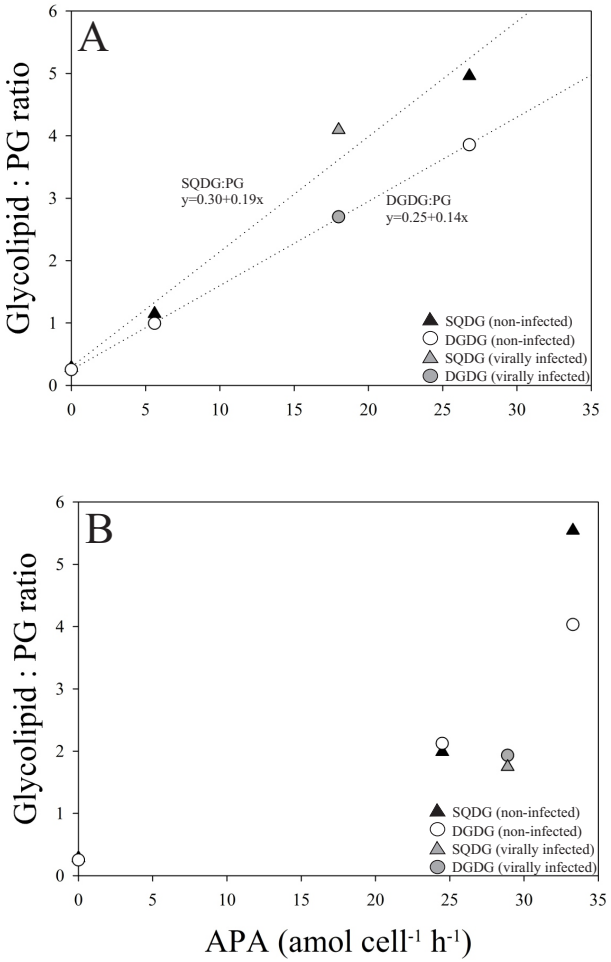


Figure 2. Partial base peak chromatogram (MS1, m/z 400–2000) of lipid extract of *M. pusilla* obtained by HPLC-MS analysis.

BGD
12, 15583–15610, 2015



BGD
12, 15583–15610, 2015

Lipids in P-stressed *Micromonas* and virus

D. S. Maat et al.

Figure 3. Relation of the SQDG : PG (black triangles) and DGDG : PG (white circles) ratios with alkaline phosphatase activity (APA) for the 0.97 **(a)** and 0.32 μ_{max} **(b)** cultures. The ratios of the virally infected cultures are depicted in grey. Lines in **(a)** represent linear regression for the SQDG : PG ($p = 0.011$, $r^2 = 0.98$) and DGDG : PG ratios ($p < 0.001$, $r^2 = 1.0$).

BGD
12, 15583–15610, 2015

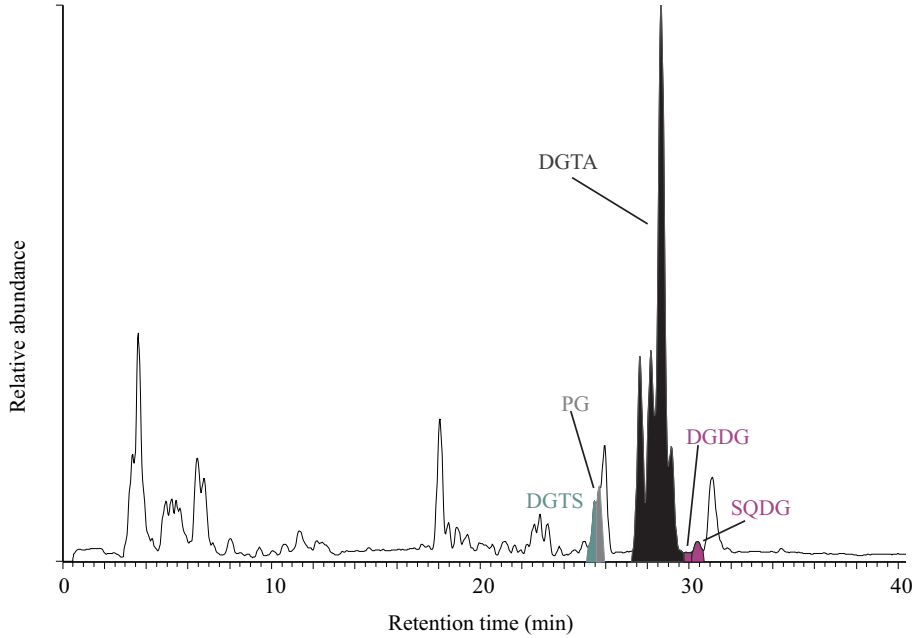


Figure 4. Partial base peak chromatogram (MS1, m/z400–2000) of lipid extract of virus MpV-08T obtained by HPLC-MS analysis.

BGD
12, 15583–15610, 2015