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### ► **To cite this version:**

D. S. Maat, N. J. Bale, E. C. Hopmans, Anne-Claire Baudoux, J. S. Sinninghe Damsté, et al.. Acquisition of intact polar lipids from the prymnesiophyte Phaeocystis globosa by its lytic virus PgV-07T . Biogeosciences, 2014, 11 (1), pp.185-194. <10.5194/bg-11-185-2014>. <hal-01253987>

**HAL Id: hal-01253987**

**<https://hal.science/hal-01253987v1>**

Submitted on 12 Jan 2016

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# Acquisition of intact polar lipids from the prymnesiophyte *Phaeocystis globosa* by its lytic virus PgV-07T

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Received: 26 June 2013 – Published in Biogeosciences Discuss.: 12 July 2013

Revised: 4 December 2013 – Accepted: 4 December 2013 – Published: 14 January 2014

**Abstract.** Recent studies showed changes in phytoplankton lipid composition during viral infection and have indicated roles for specific lipids in the mechanisms of algal virus–host interaction. To investigate the generality of these findings and obtain a better understanding of the allocation of specific lipids to viruses, we studied the intact polar lipid (IPL) composition of virally infected and non-infected cultures of the prymnesiophyte *Phaeocystis globosa* G(A) and its lytic virus PgV-07T. The *P. globosa* IPL composition was relatively stable over a diel cycle and not strongly affected by viral infection. Glycolipids, phospholipids and betaine lipids were present in both the host and virus, although specific groups such as the diacylglycerylhydroxymethyltrimethyl- $\beta$ -alanines and the sulfoquinovosyldiacylglycerols, were present in a lower proportion or were not detected in the virus. Viral glycosphingolipids (vGSLs), which have been shown to play a role in the infection strategy of the virus EhV-86, infecting the prymnesiophyte *Emiliania huxleyi* CCMP374, were not encountered. Our results show that the involvement of lipids in virus–algal host interactions can be very different amongst virus–algal host systems.

## 1 Introduction

Phytoplankton are the primary source of a large variety of organic compounds, including lipids. In the marine food web these lipids are not only an important nutritional source of carbon, but also contain substantial amounts of phosphorus and nitrogen. Several phytoplankton species belonging to the class Prymnesiophyceae are considered important players in organic carbon and sulfur cycling worldwide (Smith et al., 1991; Arrigo et al., 1999; Stefels and van Boekel, 1993; Moon-van der Staay et al., 2000). *Phaeocystis globosa* is an ecologically important species in temperate coastal regions (Schoemann et al., 2005), and can be a substantial source of intact polar lipids (IPLs) in particulate organic matter (Brandsma et al., 2012b). This species is found in a single-celled flagellate stage as well as in colonies, and can develop dense blooms (see review by Schoemann et al., 2005). Blooms of *P. globosa* have been reported to be terminated or even prevented by viral infection (Brussaard and Martínez Martínez, 2008). Phytoplankton viruses play a fundamental role in the marine food web. Through the lysis of their algal hosts, viruses release nutritional compounds from the cells into new pools of dissolved and particulate organic matter – a process called the viral shunt (Wilhelm and Suttle, 1999). Viral infection may also affect the cellular composition of the infected host. Evans et al. (2009) have shown

that viral infection of the prymnesiophyte *Emiliania huxleyi* CCMP1516 can affect the host fatty acid composition. Viruses can thus potentially alter the composition of lipids in phytoplankton.

Additionally, it has been shown that lipids can play a role in the interaction between viruses and their algal hosts as elegantly shown for the phytoplankton-virus model system *E. huxleyi* CCMP374 and its virus EhV-86 (Vardi et al., 2009). Viral infection induced the production of specific viral glycosphingolipids (vGSLs) in cells of *E. huxleyi* that seem to play a role in a programmed cell death mechanism of the host and the timing of cell lysis. These compounds are found in the virus itself as well. In a natural coccolithophore population, it was shown that these lipids may be used as biomarkers for viral infection of phytoplankton (Vardi et al., 2012). Members of the Group I *P. globosa* viruses (PgVs) belong to the nucleocytoplasmic large DNA viruses (NCLDVs) (Brussaard et al., 2004). Many NCLDVs are known to possess a lipid membrane (Van Etten et al., 2010), that have been found to span the inner side (Yan et al., 2005) or the outer side of the capsid (Mackinder et al., 2009). However, questions remain about the acquisition and composition of the lipids in these membranes.

In this study we investigated the composition of IPLs in the prymnesiophyte *P. globosa*, before and during viral infection with PgV-07T, the most intensively studied *P. globosa* virus strain to date (Baudoux and Brussaard, 2005, 2008; Sheik et al., 2013). Our aims were to detect possible changes of the host IPLs induced by the virus and, since many NCLDVs possess a membrane, clarify the IPL composition of the possible viral membrane as well. Additionally, we aimed to compare our findings to specific IPL dynamics in the phylogenetically related phytoplankton species *E. huxleyi*, in which vGSLs play an important role in the mechanisms of viral lysis.

## 2 Materials and methods

### 2.1 Culturing conditions

Axenic cultures of *Phaeocystis globosa* G(A) (culture collection of the University of Groningen, The Netherlands) were grown in batch cultures at 15 °C in a 1 : 1 mixture of *f/2* medium (Guillard, 1975) and modified artificial seawater (ESAW, Harrison, 1980), i.e. enriched with Tris-HCl and Na<sub>2</sub>SeO<sub>3</sub> (Cottrell and Suttle, 1991). Irradiance was supplied at 90 μmol quanta m<sup>-2</sup> s<sup>-1</sup> under a 16 h light: 8 h dark cycle. The lytic *P. globosa* virus, PgV-07T (Baudoux and Brussaard, 2005) was derived from the virus culture collection of the Royal Netherlands Institute for Sea Research. This double-stranded DNA (dsDNA) virus, belonging to PgV Group I with a genome size of 470 kbp and a diameter of approximately 150 nm (Baudoux and Brussaard, 2005), was cultured under the same conditions on exponentially growing

*P. globosa*. Both culture and lysate were regularly checked for axenicity by epifluorescence microscopy, using the nucleic acid stain 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Life technologies Ltd. Paisley, UK). The cultures were axenic at all times during the experiments.

### 2.2 Detection of viral membrane

The presence of a lipid membrane in PgV-07T was confirmed by staining fresh viruses 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) at a final concentration of 10 μM for 10 min. Viral particles were detected using a benchtop BD FACSCalibur equipped with a 488 nm argon laser (BD Biosciences, San Jose, USA), after setting the trigger on green fluorescence. Additionally, to test whether this lipid membrane was required for the infectivity of PgV, freshly produced viral lysate was treated with diethyl ether and chloroform (98 and 99 %, respectively; J. T. Baker). Both solvents have been shown to be effective indicators of lipids in viruses (Feldman and Wang, 1961), including those of viruses containing an inner membrane (Olsen et al., 1974; Bamford et al., 1995). Separate incubations with 10 or 50 % (*v/v* final concentration) of either of the two compounds were performed at room temperature for 60 min. After incubation, the solvent from the samples treated with diethyl ether was removed from the virus suspension just by evaporation (overnight at 4 °C). The chloroform treatments were centrifuged (Eppendorf 5810R, Hamburg, Germany) at 4100 × *g* for 5 min. The aqueous upper phase was recovered and remaining traces of chloroform were removed by evaporation (overnight at 4 °C). Positive controls, i.e. viruses not treated with solvent, underwent the same evaporation process. For all treatments, virus infectivity was tested by most probable number (MPN) endpoint dilution assay (Suttle, 1993).

### 2.3 Experimental procedures of host lipid dynamics

For the viral infection experiment, 2 L cultures of exponentially growing *P. globosa* (8.6 × 10<sup>4</sup> cells mL<sup>-1</sup>) were used. Duplicate cultures were inoculated with fresh 0.2 μm filtered (Minisart high flow Syringe Filter; Sartorius A.G., Göttingen, Germany) PgV lysate, obtaining a virus to host ratio of 55 : 1. The duplicate non-infected control cultures were inoculated with the same volume of autoclaved seawater. Samples for algal abundance (3.5 mL), PgV abundance (1 mL), and IPL composition (150 mL) were taken at regular intervals until the cultures were completely lysed. Algal samples were fixed with a 1 % final concentration of formaldehyde : hexamine solution (18 % *v/v* : 10 % *w/v*). Samples for virus enumeration were fixed with 25 % glutaraldehyde (EM grade; Sigma-Aldrich, St. Louis, USA) to a final concentration of 0.5 %, and incubated for 30 min at 4 °C, after

which the samples were flash-frozen in liquid nitrogen. IPL samples were filtered through 47 mm Whatman GF/F filters (nominal pore size 0.7  $\mu\text{m}$ ; Maidstone, UK), folded in aluminum foil and flash-frozen in liquid nitrogen. All samples were stored at  $-80^\circ\text{C}$  until analysis. A second experiment was conducted, with a similar set-up as described above, to allow isolation of PgV after complete host-cell lysis (48 h).

#### 2.4 Isolation of PgV for lipid extraction

Viruses were isolated and purified according to Vardi et al. (2009) with the following adjustments to the original protocol. The 10 L of lysate was gently filtered through Whatman GF/C filters (47 mm diameter; nominal pore size 1.0  $\mu\text{m}$ ; Maidstone, UK) in 10 separate batches of 1 L to prevent clogging. After concentration by 30 kDa tangential flow filtration (Vivaflow 200; Sartorius Stedim Biotech GmbH, Göttingen, Germany), the remaining 50 mL of concentrated lysate was spun down into a 25 % OptiPrep<sup>TM</sup> (iodixanol) (Axis-Shield, Dundee, UK) solution. This was carried out by ultracentrifugation (Centrikon T-1080; Kontron Instruments, 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  $100\,000 \times g$  for 2 h. Subsequently, the lower layer containing the 25 % OptiPrep<sup>TM</sup> and viruses was pipetted on top of prepared density gradients containing 30, 35, 40 and 45 % OptiPrep<sup>TM</sup>. After ultracentrifugation (as described above, but at  $200\,000 \times g$  for 4 h), the tubes were pierced with a sterile syringe needle to take out the clearly visible band containing PgV. The sample was gently filtered onto 0.02  $\mu\text{m}$  Anodisc filters (25 mm diameter; Whatman, Maidstone, UK), which were stored in 20 mL glass scintillation vials (Packard bioscience, Meriden, USA) at  $-80^\circ\text{C}$  until analysis. During each step viral abundance samples were taken to confirm the recovery of viruses during filtration and centrifugation.

#### 2.5 Algal and viral abundance

Samples to use for algal and viral enumeration were analysed using a benchtop BD FACSCalibur flow cytometer with a 488 nm argon laser (BD Biosciences, San Jose, USA). Algal abundances were determined without dilution by chlorophyll red autofluorescence and side scatter (Marie et al., 1999). Virus samples were, once thawed, diluted 100 to 10 000-fold in sterile TE buffer (pH = 8) according to Brussaard (2004). In short, PgVs were stained with the nucleic acid-specific dye SYBR Green I to a final concentration of  $0.5 \times 10^{-4}$  of the commercial stock (Life technologies Ltd. Paisley, UK) for 10 min at  $80^\circ\text{C}$  and discriminated by their green fluorescence and side scatter signal. All flow cytometry data were analysed using CYTOWIN 4.31 (Vaulot, 1989).

#### 2.6 Intact polar lipid analysis

After freeze-drying, the GF/F filters containing the infected *P. globosa* cells were cut into small pieces and extracted using a modified Bligh and Dyer (BD) extraction as described by Pitcher et al. (2011). All solvents used in the procedures described below were liquid chromatography/mass spectrometry (LC/MS) grade, with exception of the dichloromethane (DCM) which was high-performance liquid chromatography (HPLC) grade. A known volume of single-phase solvent mixture of methanol (MeOH):DCM:phosphate buffer (2:1:0.8,  $v:v:v$ ) was added to the sample in a glass centrifuge tube and placed in an ultrasonic bath for 10 min. The extract and residue were separated by centrifuging at  $1000 \times g$  for 5 min, and the solvent mixture collected in a separate glass flask; this was repeated three times. DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9,  $v:v:v$ ), and to induce phase separation. The extract was centrifuged at  $1000 \times g$  for 5 min. The DCM phase was collected in a round-bottom flask and the MeOH:phosphate buffer phase was washed two additional times with DCM. The combined DCM phases were reduced under a stream of  $\text{N}_2$ . The Anodisc filters containing the viral isolate were extracted in the same way, but were ground directly in the BD solvent mixture in a glass tube, using a spatula. The polypropylene ring surrounding the filter was removed after the first sonication step. A blank filter extraction showed no background IPL contaminants.

An aliquot of the BD extracts was dissolved in an injection solvent composed of hexane/isopropanol/water (72:27:1;  $v/v/v$ ) and filtered through a 0.45  $\mu\text{m}$  regenerated cellulose filter (4 mm diameter; Grace, Deerfield, USA) prior to analysis by HPLC electrospray ionization MS (HPLC-ESI-MS<sup>n</sup>) using methods modified from (Sturt et al., 2004). Briefly, HPLC 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, MA). For details of gradient, column and ESI setting see Sinninghe Damsté et al. (2011). The IPLs were identified using a positive ion mode ( $m/z$  400–2000). The four most abundant ions from each positive ion full scan were fragmented first to MS<sup>2</sup> (normalized collision energy (NCE) 25, isolation width (IW) 5.0, activation Qz 0.175) and then to MS<sup>3</sup> (NCE 25, IW 5.0, Qz 0.175). Structural identification of the IPLs was carried out by comparison with fragmentation patterns of authentic standards as described in Brandsma et al. (2012a). Due to the varying ionization efficiencies of the IPLs examined in this study we can not calculate the actual abundance of the different lipid groups. Instead, we compare the apparent abundance of the IPLs in the total ion chromatograms, which may not reflect the actual relative concentrations of the different IPLs in the cells. In addition to

**Table 1.** The relative distribution of the intact polar lipids (IPLs) in the control and infected *P. globosa* cultures at three time points and in the virus PgV-07T. Relative ionization of the compounds per treatment is depicted by plusses (+), or by “Tr.” (trace) when below 5 % of the total ionization. Minus (–) shows a complete absence. Due to their low abundance, detection of the PEs and DMPEs was not consistent; hence they were not included in the IPL sum.

		MGDGs	DGDGs	SQDGs	PC	PG	DGTA	DGCC	GSL
T0	Control	++	+	Tr.	+++	Tr.	+	++	Tr.
	Infected	++	+	Tr.	+++	Tr.	+	++	Tr.
T24	Control	+	+	Tr.	+++	+	+	++	Tr.
	Infected	+	+	Tr.	+++	+	+	++	Tr.
T48	Control	+	+	Tr.	+++	+	+	++	Tr.
	Infected	+	+	Tr.	+++	+	+	++	Tr.
	PgV	Tr.	Tr.	–	++++	+	+	++	Tr.

the characterization of routinely described glycolipids, phospholipids and betaine lipids, we surveyed extracts for the presence of viral glycosphingolipids (vGSL) using the mass spectral characteristics described by Vardi et al. (2009) and those of C<sub>18:2</sub>/C<sub>16:0</sub> glucosylceramide standard (Avanti Polar Lipids, Inc., Alabaster, USA). In order to ascertain that our instrument sensitivity was sufficient to detect these compounds, a positive control was carried out by analysis of an extract of *E. huxleyi* CCMP1516 (CCMP Culture collection, Bigelow) infected by EhV-86 (Wilson, personal communication, 2013). For this host–virus system we were able to detect the described viral glycosphingolipid in MS<sup>1</sup> as its protonated molecule ([M+H]<sup>+</sup>) at *m/z* 804, which gave rise to a diagnostic loss of 162 Da in MS<sup>2</sup> (data not shown), as in Vardi et al. (2009).

### 3 Results

#### 3.1 Algal growth and viral dynamics

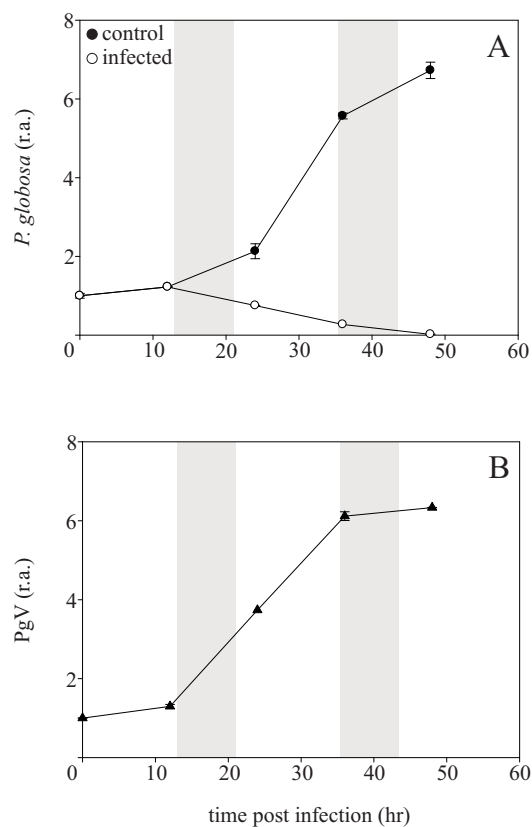
The non-infected *P. globosa* cultures grew at maximum growth rate (1.1 d<sup>−1</sup>) throughout the experiment, while viral infection led to a rapid decline of cell numbers and full lysis 48 h post-infection (pi; Fig. 1a). The one-step viral growth curve showed a release of newly produced viruses within 12 h pi (Fig. 1b) resulting in a burst size of 288 viruses cell<sup>−1</sup>. PgVs stained well with the lipophilic dye FM 1-43 (Fig. 2a). The abundance of the FM 1-43 stained PgVs was slightly lower (8 %) than the SYBR green I stained PgV abundance (Fig. 2b). The presence of a viral lipid membrane was, furthermore, confirmed by total loss of infectivity of PgV-07T upon treatment with 10 and 50 % (*v/v*) diethyl ether or chloroform.

#### 3.2 Intact polar lipids in *P. globosa* and PgV

Ten classes of IPLs (see Fig. 3 for structures) were detected in the cultures at the start of the experiment (Fig. 4a), i.e. the glycolipids monogalactosyldiacylglycerols (MGDGs), digalactosyldiacylglycerols (DGDGs), gly-

cosphingolipids (GSLs) and sulfoquinovosyldiacylglycerols (SQDGs); the phospholipids phosphatidylethanolamines (PEs), dimethylphosphatidylethanolamines (DMPEs), phosphatidylglycerols (PGs) and phosphatidylcholines (PCs) and the betaine lipids diacylglycerol hydroxymethyltrimethyl-β-alanines (DGTAs) and diacylglycerol carboxyhydroxymethylcholines (DGCCs). After 24 and 48 h the non-infected controls only showed minor changes in IPL composition, with a decrease of the relative contribution of the MGDGs and an increase of the PGs relative to the initial contributions (Table 1, Fig. 4b). Comparison between the control and virally infected cultures did not reveal any substantial differences (Fig. 4c, Table 1). The algal host DMPEs, which were detected in low levels in the control cultures at T48, were not detected in the infected cultures at T48, most likely due to the decrease in cellular material over the course of the viral infection. Glycosphingolipids with [M+H]<sup>+</sup> ions of *m/z* 780, 778, 766 and 764 were detected. These were all identified by the presence of a MS<sup>2</sup> product ion arising from a loss of 180 Da from the [M+H]<sup>+</sup> ion, and a less abundant product ion arising from loss of 162 Da. The MS<sup>3</sup> spectra of *m/z* 778 and 780 both contained product ions at *m/z* 257 and 275, the same as those ascribed to the C<sub>19:3</sub> alkyl chain of host-glycosphingolipids (hGSL) in *E. huxleyi* (Vardi et al., 2012). While the MS<sup>2</sup> and MS<sup>3</sup> spectra of both *m/z* 766 and 764 exhibited similar characteristics to those arising from the *m/z* 780 and 778 ions, full structural elucidation was not possible from ion-trap MS spectra. As all four glycosphingolipids were found equally present in both the infected and control cultures and were thus not affected by viral infection; further structural identification of the compounds fell outside of the scope of this study. Viral glycosphingolipids (vGSLs), with a dominant loss of 162 Da, as described by Vardi (2009), were not detected.

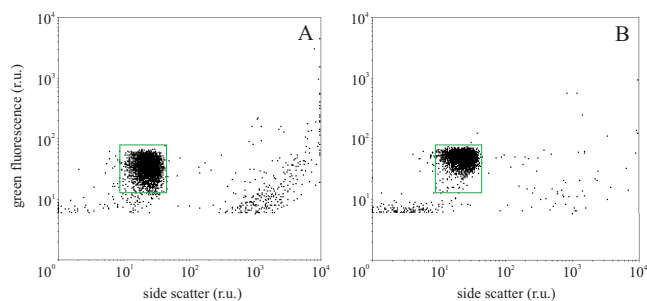
The fatty acid moieties of the IPLs (IPL-FAs), determined using the fragment patterns observed in the MS<sup>2</sup> spectra of the [M+H]<sup>+</sup> ions exhibited only minor changes in composition due to the viral infection (data not shown). For example, the MGDG-FA distribution did not change significantly in the control cultures over the 48 h experiment. However, in



**Fig. 1.** Abundances (normalized to T0) of algal host *Phaeocystis globosa* (A), and of the virus PgV-07T (B). Closed symbols represent the non-infected cultures, the open symbols the virally infected cultures. If standard deviation bar is not visible, it falls within the symbol. Shaded areas depict dark (night) period and r.a. stands for relative abundance.

the infected cultures there was a relative increase in  $C_{14:0, 18:1}$  and  $C_{16:0, 18:1}$  MGDGs, with a concomitant decrease or little change in the polyunsaturated MGDG-FAs and no change in the saturated MGDG-FAs (data not shown). Similarly, there were differences in the relative contribution of different PC-FAs (data not shown) between the infected and control cultures at T48. The infected cultures contained slightly more of the  $C_{32:4}$ ,  $C_{34:4}$ ,  $C_{34:5}$  and  $C_{40:11}$  PC-FAs, although somewhat less of the  $C_{40:10}$ ,  $C_{42:11}$  and  $C_{44:12}$  PC-FAs.

The IPL composition of PgV-07T was quite distinct from that of the host, with the PCs forming a much more substantial part of the IPLs than in the host. Of the other phospholipids (Fig. 4d, Table 1), PGs were also present, but PEs and DMPEs were not detected. Both betaine classes were detected in the viral isolate. The glycolipids were responsible for the largest difference in IPL composition between the virus and host. The contribution of the MGDGs and DGDGs to the sum of IPLs was reduced in the virus relative to the host and the SQDGs were not detected at all. As mentioned



**Fig. 2.** Flow cytometric signatures of PgV after staining with the green fluorescent lipid stain FM 1-43 (A) and the nucleic acid-specific dye SYBR Green I (B); r.u. stands for relative units.

above for the host, the vGSLs described by Vardi (2009), were also not detected in the virus.

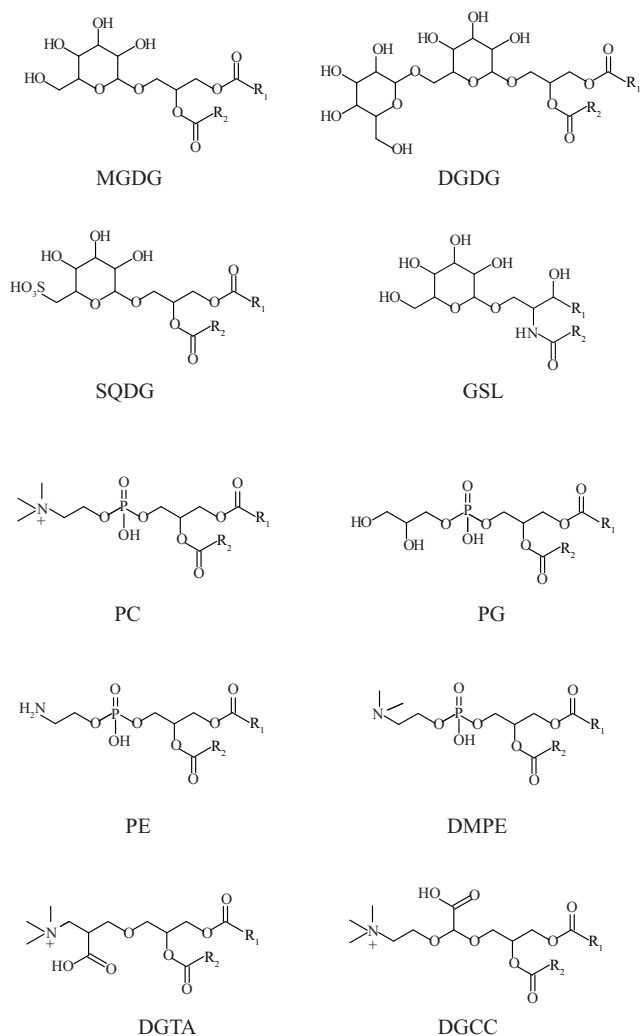
The IPL-FA composition of PgV-07T was for some IPL classes quite distinct from that of *P. globosa* (Tables S1 and 2). The MGDGs, DGDGs and DMPEs in the virus comprised a smaller range of fatty acids than those in the host while the PGs, DGCCs and DGTAs in the virus exhibited almost identical ranges of fatty acids as they had in the host. Only the virus PCs contained additional fatty acids that were not detected in the host:  $C_{34:7}$ ,  $C_{36:8-10}$ ,  $C_{38:4}$ ,  $C_{38:10}$ ,  $C_{42:12}$ ,  $C_{42:13}$  and  $C_{44:11}$  PC-FAs (Table S1 and 2).

## 4 Discussion

The present study showed that the IPL composition of the prymnesiophyte *P. globosa* G(A) was not substantially affected by viral infection, except for minor changes in the IPL-FA combinations. However, the IPL composition of the virus PgV-07T was different from the host's lipid composition, suggesting a selective acquisition of these compounds. We have not encountered any virus-specific lipids that were not detected in the non-infected host. This is in contrast to the finding of viral glycosphingolipids in another prymnesiophyte virus-host model system, i.e. EhV-86 and *Emiliania huxleyi* CCMP374. Phylogenetically related virus-phytoplankton systems may thus have very different lipid-related features during viral propagation.

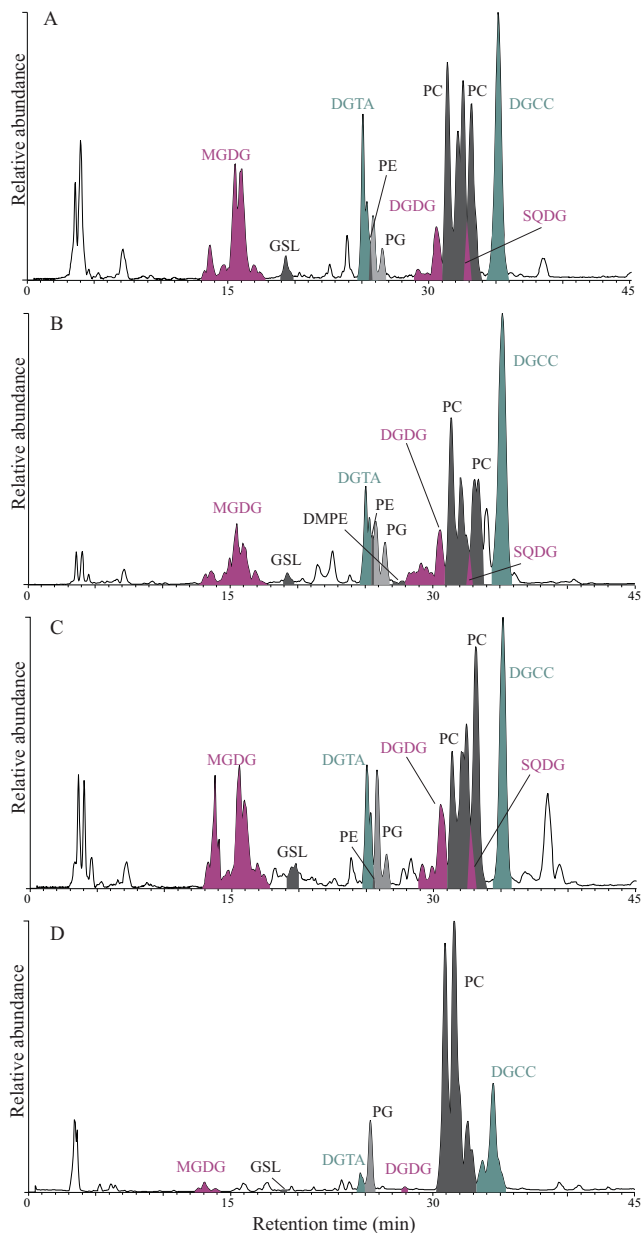
### 4.1 Impact of viral infection on host lipid composition

The IPL classes detected in *P. globosa* originate from all cellular components, including the cell membrane as well as intracellular membranes from the chloroplasts and other organelles. In previous studies, environmental factors such as irradiance (Guschina and Harwood, 2009), but also viral infection (Vardi et al., 2009) were shown to affect the composition of these host IPLs. For instance, it is known that viral infection typically induces a membranous rearrangement in the host to compartmentalize viral replication (den Boon et al., 2010). In our study we did not observe a strong impact



**Fig. 3.** Structural formulas of the main IPL classes belonging to the glycolipids monogalactosyldiacylglycerols (MGDGs), digalactosyldiacylglycerols (DGDGs), glycosphingolipids (GSLs) and sulfoquinovosyldiacylglycerols (SQDGs); the phospholipids phosphatidylethanolamines (PEs), dimethylphosphatidylethanolamines (DMPEs), phosphatidylglycerols (PGs) and phosphatidylcholines (PCs) and the betaine lipids diacylglycerol hydroxymethyltrimethyl- $\beta$ -alanines (DGTA) and diacylglycerol carboxyhydroxymethylcholines (DGCCs). The acyl groups are represented by R1 and R2.

by viral infection on the composition of IPL classes of *P. globosa*, nor did the diel cycle have an effect. Minor differences in the IPL-FA distribution of some of the IPLs associated with viral infection were observed. Evans et al. (2009) showed that viral infection of *E. huxleyi* CCMP1516 led to a relative decrease in polyunsaturated fatty acids. In our study, the changes in host IPL-FAs during infection were only minor, i.e. a decrease of certain FAs amongst the MGDGs, DGDGs and DMPEs.



**Fig. 4.** Partial base-peak chromatograms (MS1,  $m/z$  400–2000) of non-infected *P. globosa* at T0 (A) and T48 (B), virally infected *P. globosa* at T48 (C), and the virus PgV-07T (D).

#### 4.2 Acquisition of intact polar lipids from the host

By staining the viruses with a lipophilic dye, we have shown that PgV-07T possesses a lipid membrane. The complete loss of infectivity of PgV-07T after treatment with diethyl ether and chloroform indicates that this membrane plays a crucial role in the infection of *P. globosa*. Based on transmission electron microscopy (TEM) thin-section images, the Group I PgVs (i.e. 150 nm large NCLDV with a thin outer layer surrounding a layered inner core, to which PgV-07T

belongs), are assembled in the cytoplasm and leave the host after disruption of the cell membranes (Baudoux and Brussaard, 2005). Thus, in contrast with EhV-86 (Mackinder et al., 2009), PgV seems to obtain its lipid membrane in the host cytoplasm and not from the cell membrane. The lipids of PgV are then either allocated, newly synthesized or obtained from other membranes, such as the endoplasmic reticulum (E.R.) or the Golgi system (den Boon et al., 2010; Heaton and Randall, 2011). Meints et al. (1986) showed the appearance of membranous structures at the sites of virus assembly in *Chlorella* sp. This virus, called PBCV-1, belongs to the Phycodnaviridae and is thought to obtain these lipid membranes from the host endoplasmic reticulum (Wilson et al., 2009). The origin and patterns of PgV replication in the host cell, i.e. formation of virions in the cytoplasm and virus release in a single burst (Baudoux and Brussaard, 2005), show a strong resemblance to PBCV-1 (Meints et al., 1986). Using electron cryomicroscopy, Yan et al. (2000, 2005) confirmed that not only PBCV-1, but also the *Phaeocystis pouchetii* virus PpV01, contains a membrane covering the inside of the capsid. Regarding the similarity in patterns of infection between these viruses and PgV-07T, it is likely that PgV-07T also possesses an intracapsid membrane.

Of the viral IPL classes, the phospholipids appear to be acquired in higher proportions than the glyco- and betaine lipids. The PCs, considered to be the most dominant phospholipids in phytoplankton (Van Mooy et al., 2009) were well represented in *P. globosa* and, probably due to its ubiquity in all cellular membranes (Thompson, 1996), in the virus as well. Some viral PCs contained additional fatty acids that were not found in the host. While it is possible that these PCs were the products of de novo synthesis, it can also be that they were present in the host but were in too low a concentration to be detected. Indeed, in PgV they constitute only a minor fraction of the total amount of PCs (data not shown). The PgV extract contained a higher proportion of PCs than the host and may have also contained less sample matrix, factors which would make it easier to detect individual PCs that are present in low abundance.

The second most important group of phospholipids in *P. globosa*, the PGs, are known to be abundant in chloroplasts and other organelles, such as the endoplasmic reticulum (Schwertner and Biale, 1973). The source of the viral lipid membrane could be associated with such organelles. The PEs and DMPEs were not detected in the virus, but even in the infected cultures they were only present in trace amounts. These compounds have been shown to be precursors in the methylation pathway of PC in bacteria (Aktas and Narberhaus, 2009) and yeast (Kanipes and Henry, 1997), while this is the case for PEs in the phytoplankton species *Chlamydomonas reinhardtii* (Yang et al., 2004). PEs and DMPEs have furthermore been shown to be associated to small organelles such as mitochondria (Schwertner and Biale, 1973). For the infected lysing *P. globosa* cultures, such organelles

might have been lost by passing through the GF/F filter during sampling of the infected cultures.

The betaine lipids in the PgV membrane were relatively low in relation to their contribution to the total lipids in the algal host. Interestingly, DGTAs have been found in algal thylakoid membranes (Murata, 1998). TEM thin sections and epifluorescence microscopy of infected *P. globosa* cells (data not shown) illustrate that the integrity of the chloroplast of *P. globosa* is largely unaffected far into the viral growth cycle, leaving the chloroplast membranes intact. The DGTAs in *P. globosa* might also be mainly associated with the membranes of the unaffected chloroplast and therefore present in lower relative amounts in the membrane of PgV-07T.

The relative loss of glycolipids, including the apparently complete loss of SQDGs in the virus, is probably due to the same process. The four classes of glycolipids detected in *P. globosa* are generally associated with the chloroplast in algae and higher plants (Guschina and Harwood, 2009; Sato, 2009). With a roughly 50 % contribution, the MGDGs often comprise the majority of the chloroplast lipids, while the other half is composed of DGDGs, SQDGs and phospholipids. GSLs have not been found in chloroplasts, but their synthesis is directly related to chloroplast metabolism and vice versa; the disruption of sphingolipid biosynthesis was found to lead to the disruption of the chloroplast membranes in the vascular plant *Datura stramonium* (Abbas et al., 1992; Chen et al., 2009). Additionally, GSLs are also found in plant cell membranes, where they can account for up to 27 mol % of lipids (Chen et al., 2009). In this case both the association to the chloroplast and the cell membranes could account for the relative loss of GSLs in PgV-07T.

In summary, our results suggest that PgV-07T acquired its lipid membrane mainly in the host cytoplasm and that the chloroplast membranes as well as the cell membrane were not utilized. Since we did not find any group of IPLs in the infected cultures or the virus that were not also detected in the non-infected culture, we hypothesize that PgV-07T selectively recruits its lipids from existing membranes, such as the E.R. or the Golgi apparatus. Examination of the composition of these membranes could clarify this issue.

### 4.3 Glycosphingolipids as markers for viral infection

Although we detected a group of GSLs in both the non-infected and infected *P. globosa* cells, we did not observe the specific vGSLs that Vardi et al. (2009) described as being synthesized in infected *E. huxleyi* cells and being part of the viral membrane. Indeed, the four viral genes involved in the synthesis of this vGSL and transcribed during infection (Wilson et al., 2005) were found to be absent in the genome of PgV-016T (Santini et al., 2013). This virus belongs to the same group of PgVs (Group I), as PgV-07T as PgV-07T, as based on e.g. genome and particle size (Baudoux and Brussaard, 2005), but could still be different in other characteristics since it was found to belong to a different phylogenetic

cluster based on the DNA polymerase gene (Santini et al., 2013). The difference in infection mechanisms is especially interesting since *P. globosa* and *E. huxleyi* both belong to the same class of algae, namely the Prymnesiophyceae, and both PgV-07T and EhV-86 are NCLDV (Baudoux and Brussaard, 2005; Wilson et al., 2005). *E. huxleyi* has been extensively used as a model system to study specific features of algal virus-host interactions, such as caspase activity (Bidle et al., 2007), host entry, departure and viral envelope acquisition (Mackinder et al., 2009) and now the involvement of vGSLs (Vardi et al., 2009). Comparison of these and additional virological aspects with those of PgV-07T (Group I) and other known members of phytoplankton infecting NCLDVs, i.e. PgVs from Group II, PpV-01, PBCV-1, *Chrysochromulina ericina* virus (CeV-01B) and *Pyramimonas orientalis* virus (PoV-01B), suggests that the infection strategy of EhV-86 is rather uncommon amongst the known phytoplankton viruses (Yan et al., 2000; Sandaa et al., 2001; Baudoux and Brussaard, 2005; Mackinder et al., 2009; Van Etten et al., 2010). Thus, the use of lipid biomarkers for viral infection of phytoplankton (Vardi et al., 2012) might only be successful for specific virus-host systems such as *E. huxleyi* and EhV-86 and not be generally applicable. More data on lipids in other virus-phytoplankton systems must be acquired in order to address this issue.

**Supplementary material related to this article is available online at <http://www.biogeosciences.net/11/185/2014/bg-11-185-2014-supplement.pdf>.**

**Acknowledgements.** We thank two anonymous reviewers for their constructive comments which improved the manuscript. We dedicate this paper to Carlo Heip († 15 February 2013). D. S. Maat was supported by a grant to C. P. D. Brussaard of the Royal Netherlands Institute for Sea Research (NIOZ) for funding of this project. NIOZ is an institute of the Netherlands Organization for Scientific Research (NWO). N. J. Bale was supported by a grant (839.08.331) to J. S. Sinninghe Damsté of The National Ocean and Coastal Research Programme (ZKO) supported by NWO.

Edited by: G. Herndl

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