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Temporal variations in abundance and composition of intact polar lipids in North Sea coastal marine water

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Abstract

Temporal variations in the abundance and composition of intact polar lipids (IPLs) in North Sea coastal marine water were assessed over a one-year seasonal cycle, and compared with environmental parameters and the microbial community composition. Sulfoquinovosyldiacylglycerol (SQDG) was the most abundant IPL class, followed by phosphatidylcholine (PC), phosphatidylglycerol (PG) and diacylglyceryl-(*N,N,N*)-trimethylhomoserine (DGTS) in roughly equal concentrations, and smaller amounts of phosphatidylethanolamine (PE). Although the total concentrations of these IPL classes varied substantially throughout the year, the composition of the IPL pool remained remarkably constant. Statistical analysis yielded negative correlations between IPL concentrations and dissolved inorganic nutrient concentrations, but possible phosphorous limitation during the spring bloom did not result in changes in the overall planktonic IPL composition. Significant correlations between SQDG, PC, PG and DGTS concentrations and chlorophyll-*a* concentrations and algal abundances indicated that eukaryotic primary producers were the predominant source of IPLs at this site. However, whilst IPL concentrations in the water were closely tied to total algal abundances, the rapid succession of different algal groups blooming throughout the year did not result in major shifts in IPL composition. This shows that the most commonly occurring IPLs have limited chemotaxonomic potential, and highlights the need to use targeted assays of more specific biomarker IPLs.

1 Introduction

Intact polar lipids (IPLs) and their derived polar lipid fatty acids (PLFAs) are widely used in ecological and biogeochemical studies as biomarkers to determine the abundance and composition of extant microbial communities. These lipid molecules are mostly glycerol-based with a hydrophilic (polar) head group attached to the *sn*-3 position and a wide variety of fatty acid chains at the *sn*-1 and *sn*-2 positions (see Fahy et al., 2005,

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2009 for an overview and classification). As basic building blocks of cell membranes, lipids comprise 11–23 % of the organic carbon in marine plankton (Wakeham et al., 1997), and they often contain key elements such as nitrogen, phosphorous or sulphur. The characterization of the lipid content of marine microbes has shown that specific types of IPLs or PLFAs are synthesized predominately, or sometimes exclusively, by specific microbial groups. For example, the sulfur-bearing glycerolipid sulfoquinovosyldiacylglycerol (SQDG) is only found in thylakoid membranes of photosynthetic organisms (Benning, 1988; Frenzten, 2004), whilst long-chain polyunsaturated fatty acids (PUFAs) are typical for marine microalgae (Volkman et al., 1998; Guschina and Harwood, 2006). Although this primarily culture-based chemotaxonomic record is still far from comprehensive, specific IPLs or PLFAs may be used as biomarkers for the presence of their source organisms in different environments, with IPLs containing more structural information than their derived PLFAs (Shaw, 1974; Lechevalier and Lechevalier, 1989; Sturt et al., 2004). Moreover, IPLs are thought to be exclusively derived from living microbes, due to their comparatively rapid degradation upon cell death (White et al., 1979; Harvey et al., 1986), and IPL abundances are consequently used as a proxy for the extant microbial biomass in environmental samples (e.g., Petsch et al., 2001; Lipp et al., 2008; Zink et al., 2008). Finally, microbes have the ability to adjust the IPL composition of their membranes in response to changes in their environment, such as temperature or nutrient availability (e.g., Van Mooy et al., 2009), although such adaptations have mostly been studied in cultures maintained under controlled conditions (Minnikin et al., 1974; Benning et al., 1995; Pernet et al., 2003; Martin et al., 2010).

At present the number of studies into IPL dynamics in the marine water column is still limited. This is partly due to the comparatively recent development of suitable instrumentation for IPL analysis using multistage mass spectrometry coupled to high performance liquid chromatography by electrospray ionization interface (HPLC/ESI-MS^n; Brügger et al., 1997; Fang and Barcelona, 1998). Thus far, IPL compositions in marine waters have been determined in the Black Sea (Schubotz et al., 2009), the Sargasso Sea and Pacific Ocean (Van Mooy et al., 2006, 2009; Van Mooy and

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Fredricks, 2010), the western North Atlantic (Popendorf et al., 2011) and the North Sea (Brandsma et al., 2011a). At all of these sites the IPL composition is dominated by a relatively small number of IPL classes, which are the glycerolipids sulfoquinovosyldiacylglycerol (SQDG) and mono- and digalactosyldiacylglycerol (MGDG and DGDG), the glycerophospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), and the betaine lipids diacylglyceryl-(N,N,N)-trimethylhomoserine (DGTS), diacylglyceryl-hydroxymethyl-(N,N,N)-trimethylalanine (DGTA) and diacylglyceryl-carboxyhydroxymethylcholine (DGCC). Comparison with other parameters measured in the same waters yielded tentative relationships between the IPL composition and the microbial community composition (Van Mooy and Fredricks, 2010; Brandsma et al., 2011a; Popendorf et al., 2011), as well as nutrient availability (Van Mooy et al., 2006, 2009).

However, each of these studies presents a snapshot analysis, as all the data were collected within short amounts of time (several weeks at most), and thus the temporal variability of IPLs in marine waters has not yet been resolved in any detail. In this study we monitored the IPL abundance and composition of coastal North Sea surface waters during a one-year seasonal cycle. We compare this IPL time series with the microbial abundances, community composition and environmental conditions at the same site and time interval, in order to determine how these are reflected in the IPL composition and abundances.

2 Materials and methods

2.1 Study site and time series

From 1974 onwards, bucket water samples for environmental and microbial analyses have been collected from the NIOZ sampling jetty (53°00′06″ N 4°47′21″ E) at the entrance of the Marsdiep tidal inlet, which connects the North Sea and the westernmost basin of the Dutch Wadden Sea (Fig. 1). Sampling is performed at high tide, to assure

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that the water originates from the southeastern coastal North Sea (cf. Alderkamp et al., 2006), and includes measurements of salinity, water temperature and dissolved nutrients, as well as chlorophyll-*a* concentrations, phytoplankton and bacterial abundances, marine algal species composition and primary production. The sampling frequency is 40 to 60 times per year, varying from once or twice a month in winter up to twice a week during phytoplankton spring blooms (e.g., Cadée and Hegeman, 2002). The current study was synchronized with this long-term time series, and ran over a one-year time period from March 2007 to March 2008, comprising 28 sampling dates.

2.2 Microbial analyses

Chlorophyll-*a* concentrations were assessed from 0.5–1.0 l water samples (filtered over $MgCO_3$-coated filters, as per Cadée and Hegeman, 2002), and calculated from non-acidified values of chlorophyll-*a* according to Philippart et al. (2010). Primary production was measured in an incubator, kept at in situ temperature and constant light conditions, using the ^{14}C method of Cadée and Hegeman (1974) and including actual daily irradiation in the estimation model (Philippart et al., 2007). Phytoplankton samples were preserved with acid Lugol's iodine, and cells were counted with a Zeiss inverted microscope using 5 ml counting chambers. Most algae were identified to species level, but some were clustered into taxonomic and size groups (Philippart et al., 2000). Analysis of changes in the phytoplankton species composition covered the nine most numerous marine algal taxa, which together contributed more than 85 % to the total numbers of marine algae in the Marsdiep during the study period.

Samples for (cyano)bacterial abundances were preserved with formalin (final concentration 1.5 %) and snap-frozen in liquid nitrogen before storage at −80 °C. After thawing, the microbial community composition was analyzed with a bench-top flow cytometer (Beckman Coulter XL-MCL) with reduced sheath-flow to enhance the sensitivity of the instrument. Chlorophyll fluorescence (>630 nm) and phycoerythrin fluorescence (575 ± 20 nm) of the cyanobacteria were collected in separate photomultipliers (Veldhuis and Kraay, 2004) and used as the primary selection criteria for the presence

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of cyanobacterial cells. Total bacterial numbers were determined by flow cytometry after staining the cells with the green nuclear stain PicoGreen (MP, P-7581), according to Veldhuis et al. (1997). 10 µl of a working solution PicoGreen (100x diluted in TBS buffer) was added to 100 µl of sample and incubated for 15–30 min prior to analysis. Green fluorescence of the stained DNA (525 ± 20 nm) was as used as the primary selection criterion for the presence of bacterial cells.

2.3 Intact polar lipid analysis

Surface water samples for IPL analysis (~20 l) were taken with an acid-rinsed Nalgene bottle from a depth of less than 1 m. The water was filtered through precombusted 0.7 µm GF/F filters (142 mm diameter; Whatman, Clifton, NJ, USA), using a table-top filtration unit. All filters were then freeze-dried and extracted using a modified Bligh-Dyer procedure (Bligh and Dyer, 1959; Brandsma et al., 2011a), and IPL analysis of the extracts was performed by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC/ESI-MS^2) using chromatographic conditions as described by Jaeschke et al. (2009) and source and fragmentation parameters as described by Boumann et al. (2006) and Brandsma et al. (2011a). Initially, the extracts were analyzed in positive and negative ion mode (two separate runs) using a data dependent MS^2 routine in which a full scan (*m/z* 300–1000) was followed by fragmentation of the base peak of the resulting mass spectrum. Identification of the major IPL classes was based on diagnostic fragmentation patterns in the MS^2 mass spectra (Kato et al., 1996; Brügger et al., 1997; Keusgen et al., 1997; Fang and Barcelona, 1998). Subsequently, targeted mass spectrometric experiments were used to elucidate the structural diversity within each of the identified IPL classes, and for quantification of the IPL classes and their constituent species. IPLs with a phosphatidylcholine (PC) or diacylglyceryl-trimethylhomoserine (DGTS) head group were measured in positive ion mode by parent ion scanning (*m/z* 300–1000) of fragment ions diagnostic for their polar head groups (i.e., *m/z* 184 and *m/z* 236, respectively). IPLs with a phosphatidylglycerol (PG), phosphatidylethanolamine (PE) or sulfoquinovosyldiacylglycerol (SQDG) head group were measured by neutral loss scanning (m/z 300–1000) for losses of 189 Da, 141 Da and 261 Da, respectively. The carbon number and degree of unsaturation of the fatty acid moieties of the various IPLs were calculated using the m/z of the molecular species, and these are denoted as such below (i.e., $C_{32:2}$ PC refers to an IPL with a phosphatidylcholine head group and two fatty acids containing a total of 32 carbon atoms and two double bond equivalents; note that this does not include the glycerol moiety). Information on individual fatty acid compositions of the predominant IPL species were based on fragment ions or neutral losses diagnostic for fatty acids obtained in the data dependent MS^2 experiments (see Brügger et al., 1997).

For quantification of the PGs, PCs, PEs, SQDGs and DGTSs, the peak areas of each IPL class (total ion current) and their constituent IPL species (extracted ion chromatogram) were compared with the respective peak areas of known quantities of authentic standards. The standards used in this study were $C_{16:0}/C_{16:0}$ PC, $C_{16:0}/C_{16:0}$ PG and $C_{16:0}/C_{16:0}$ PE (all Avanti Polar Lipids, Alabaster, AL, USA), a mixture of SQDGs, which contained predominately $C_{16:1}/C_{18:2}$ SQDG (~60 %), but also small amounts of SQDGs with $C_{16:0-16:1}$, $C_{18:0-18:1}$ and $C_{20:5}$ fatty acid combinations (Lipid Products, Redhill, Surrey, UK), and a standard of $C_{14:0}/C_{18:1}$ DGTS, which was purified from IPL extracts of *Isochrysis galbana* (CCMP, 1323) as described by Brandsma et al. (2011a). Limits of detection were 50–100 pg on column for the glycerophospholipids, 100 pg on column for the DGTSs and 1 ng on column for the SQDGs. All IPL quantifications were reproducible within a 10 % error between duplicate runs, and the instrument response was monitored by repeated analysis of blanks and quantitative standards every 10 samples.

2.4 Statistical analyses

Relationships between the various datasets (IPL concentrations, environmental parameters, microbial abundances) were tested statistically in Systat 13 (Systat Software, San Jose, CA). The measures of association between different variables were determined by calculating their Spearman's rank correlation coefficients (ρ). This test was

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chosen as many of the variables showed a highly skewed distribution. Only variable dependencies having corrected probability values (p) of less than 0.05 were considered significant and are reported here. In addition, principal component analysis (PCA) was used to extract principal components that could explain the variance in the IPL dataset, as well as in datasets containing the IPL classes plus environmental parameters or microbial groups.

3 Results

3.1 Temporal variability of environmental parameters

During the time series the sea surface temperature in the Marsdiep varied from around 6 °C in winter to almost 19 °C in summer (Fig. 2a). Salinity was fairly stable at 26–31, although lower values (down to 23) were measured in December 2007 and in early spring. Levels of dissolved inorganic nutrients (P, N and Si) were highest at the end of winter, then decreased sharply at the onset of spring and remained low throughout most of the summer, before gradually increasing again through fall and winter (Fig. 2b). Dissolved inorganic phosphate (DIP) levels ranged from a maximum of 1.1 $\mu mol\,l^{-1}$ to <0.1 $\mu mol\,l^{-1}$, whilst silicate (DISi) levels ranged from 40–42 $\mu mol\,l^{-1}$ to less than 1 $\mu mol\,l^{-1}$, and nitrate concentrations ranged from 83 $\mu mol\,l^{-1}$ to 1 $\mu mol\,l^{-1}$. NO_3^- was the most abundant dissolved inorganic nitrogen (DIN) species in winter (>90 % of the DIN pool), but comprised only 30–50 % in spring and summer, concurrent with strong increases in NO_2^- (4–8 %) and NH_4^+ (30–65 %). The N:P ratio of dissolved inorganic nutrients was highest at the end of winter and in spring (generally around 80, but with brief maxima up to 722), and lowest in summer (generally around 30, but with a minimum of 13).

3.2 Microbial abundances and community composition

Primary production and chlorophyll-*a* concentrations varied strongly throughout the year in response to the environmental conditions (Fig. 2c). In winter the primary production was low at 3–6 $\mu g\,C\,l^{-1}\,h^{-1}$, but increased to 165 $\mu g\,C\,l^{-1}\,h^{-1}$ during the spring bloom. The same pattern was observed for the chlorophyll-*a* concentrations, which increased from 2 $\mu g\,l^{-1}$ to 55 $\mu g\,l^{-1}$. After the spring bloom the primary production and chlorophyll-*a* concentrations remained fairly high throughout the summer and fall, before decreasing to their low winter values.

Within the eukaryotic algae, a sequence of blooms was observed at various times in the year, with total cell numbers of the nine most numerous taxa reaching 1.0×10^5 cells ml^{-1} between mid-March and mid-May (Fig. 2d). The first and by far the most pronounced algal bloom occurred in spring and was formed by the Prymnesiophyte *Phaeocystis globosa*, with the colonial form predominating during the first part of the bloom and the solitary form during the second part (Fig. 3). Concurrently, blooms of the diatoms *Chaetoceros socialis*, *Skeletonema costatum* and *Pseudonitzschia delicatissima*, as well as other Prymnesiophytes and various unidentified flagellate algae were observed. The second and more moderate algal bloom occurred between mid-May and June and was formed by the diatoms *Thalassiosira* spp. and *Chaetoceros socialis*, together with the Cryptophyte *Plagioselmis* spp. and various unidentified flagellate algae (Fig. 3). Finally, the third and least pronounced algal bloom occurred during summer (July and October) and was again formed by the diatoms *Thalassiosira* spp. and *Chaetoceros socialis*, together with the Cryptophytes *Plagioselmis* spp. and *Hemiselmis* spp. (Fig. 3) and cyanobacteria (up to 3.2×10^5 cells ml^{-1}; Fig. 2d). Bacterial numbers were fairly constant throughout the year (3–5 $\times10^6$ cells ml^{-1}; Fig. 2d), but were lowest at during the algal spring bloom (1.5×10^6 cells ml^{-1}) and highest around its end (6.1×10^6 cells ml^{-1}).

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3.3 IPL composition and abundances

Five major IPL classes were detected in the surface waters of the Marsdiep (Fig. 4): SQDG, PC, PG, PE and DGTS. Although these classes comprised the greater part of the base peak chromatogram, the betaine lipids DGTA and DGCC, as well as trace amounts of the glycerolipids MGDG and DGDG, and a number of unidentified compounds were detected in some of the samples as well. Each of the identified IPL classes comprised a wide range of IPL species with differing fatty acid combinations (Table S1). The least variety was observed in the SQDGs and PGs (around 50 species each), followed by the PEs and DGTSs (around 90 species each), whilst the PCs were the most varied class (more than 120 species). Fatty acid chain lengths generally ranged from C_{12} to C_{22}, although C_{14} to C_{18} fatty acids predominated (Table S1). Whilst the majority of the fatty acids in each of the IPL classes had even chain lengths, some odd-carbon number fatty acids (C_{13} to C_{19}) were also detected, in particular in the PEs and PGs. Long-chain polyunsaturated fatty acids (PUFAs) were common in the PCs, but rare in the other glycerophospholipids and DGTSs, and not detected in the SQDGs. The average fatty acid chain length and degree of unsaturation within each of the IPL classes remained stable throughout the year. Average fatty acid chain lengths were highest in the PCs (34.8 ± 0.7 carbon atoms), followed by the PEs (33.3 ± 0.7), PGs (33.2 ± 0.2) and DGTSs (33.1 ± 0.7) and lowest in the SQDGs (31.0 ± 0.5). Similarly, the average degrees of unsaturation were highest in the PCs (2.5 ± 0.4 double bond equivalents), followed by the PGs, PEs and DGTSs (all 1.7 ± 0.2), and lowest in the SQDGs (0.8 ± 0.2).

Throughout the year the most abundant IPL class in coastal North Sea waters was SQDG, with concentrations ranging from $0.9\,\mu g\,l^{-1}$ in winter to almost $35\,\mu g\,l^{-1}$ at the peak of the spring bloom (Fig. 2e). The SQDGs were dominated by seven species ($C_{28:0}$, $C_{30:1}$, $C_{30:0}$, $C_{32:2}$, $C_{32:1}$, $C_{32:0}$ and $C_{34:1}$), which on average comprised $80 \pm 4\,\%$ of the total SQDG concentration throughout the year (Table S2). In winter, the most abundant SQDG species were $C_{32:1}$ SQDG ($21 \pm 3\,\%$) and $C_{28:0}$ SQDG ($15 \pm 4\,\%$),

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whilst during the spring bloom and in summer $C_{28:0}$ SQDG was the most abundant species (26 ± 4 %), followed by $C_{32:1}$ SQDG (16 ± 4 %), $C_{30:1}$ SQDG (14 ± 2 %) and $C_{30:0}$ SQDG (12 ± 3 %). In addition, $C_{32:3}$ and $C_{36:2}$ SQDG, which normally comprised <1 % of the total SQDGs, were present in elevated abundances during the spring bloom (each up to 10 %).

The glycerophospholipids (i.e., PC, PG and PE) detected in the coastal North Sea waters were always present in lower concentrations that the SQDGs. Summed glycerophospholipid concentrations ranged from 0.6 µg l^{-1} in winter to 9.6 µg l^{-1} at the peak of the spring bloom. PGs and PCs were present in more or less equal amounts, with concentrations ranging from 0.3 to 4.8 µg l^{-1} and 0.1 to 3.9 µg l^{-1}, respectively (Fig. 2f). The PEs were the least abundant of the quantified IPLs, with concentrations ranging from less than 10 ng l^{-1} to 1.0 µg l^{-1}.

The PGs were dominated by seven species ($C_{30:1}$, $C_{30:0}$, $C_{32:2}$, $C_{32:1}$, $C_{34:2}$, $C_{34:1}$ and $C_{36:2}$), which on average comprised 66 ± 5 % of the total PG concentration throughout the year (Table S2). $C_{32:1}$ PG was the most abundant species (17 ± 3 %), whilst the other species each comprised between 5 and 11 %. The PCs were the most diverse IPL class and did not contain any predominant species. The eleven on average most abundant PC species ($C_{28:0}$, $C_{30:1}$, $C_{30:0}$, $C_{32:2}$, $C_{32:1}$, $C_{34:2}$, $C_{34:1}$, $C_{36:6}$, $C_{36:5}$, $C_{36:2}$ and $C_{38:6}$) together comprised only 45 ± 4 % of the total PC concentration throughout the year (Table S2). The highest contribution measured was 8 ± 3 % ($C_{36:2}$ PC during the spring bloom), but generally each species constituted <5 % of the total PC. The PEs also contained a wide range of species, which was again reflected in the comparatively low average contribution of the five predominant species ($C_{30:1}$, $C_{32:2}$, $C_{32:1}$, $C_{34:2}$ and $C_{34:1}$) to the total PE concentration (49 ± 9 %; Table S2). The most abundant species were $C_{32:1}$ PE and $C_{34:2}$ PE (14 ± 4 % each), followed by $C_{32:2}$ PE (9 ± 4 %), and $C_{30:1}$ PG and $C_{34:1}$ PE (6 ± 3 % each). The relative abundances of the predominant glycerophospholipid species within their respective classes showed little temporal variation, with the same species predominating throughout the year. However, during the spring bloom and in summer a number of additional species were detected in elevated

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abundances. These included $C_{40:10}$ PC, $C_{42:11}$ PC, $C_{34:4}$ PG, $C_{35:0}$ PG, $C_{30:0}$ PE, $C_{38:6}$ PE and $C_{40:6}$ PE, which each temporarily constituted 5–11 % of the total concentration of their respective class, but typically <2 % during most of the year (Table S2).

DGTS was present in roughly equal amounts to the glycerophospholipids PC and PG, with concentrations ranging from 0.3 to 4.6 $\mu g\,l^{-1}$ (Fig. 2e). As with the PCs and PEs, the DGTSs contained a wide range of species, which was reflected in the comparatively low average contribution of the four predominant species ($C_{32:1}$, $C_{34:2}$, $C_{34:1}$ and $C_{36:2}$) to the total DGTS concentration throughout the year (only 36 ± 9 %; Table S2). Of these, $C_{34:1}$ DGTS was generally the most abundant species (11 ± 2 %). However, during the spring bloom the DGTS composition was more diverse, with ten predominant species ($C_{28:0}$, $C_{30:1}$, $C_{30:0}$, $C_{31:1}$, $C_{32:1}$, $C_{34:2}$, $C_{34:1}$, $C_{35:2}$, $C_{36:5}$ and $C_{36:2}$), of which only $C_{36:2}$ DGTS contributed more than 7 % to the total concentration.

In summary, the IPL pool in the surface waters of the Marsdiep contained a large number of IPL species (at least 400, but likely more), almost all of which could be detected throughout the year, but with only a limited number of species (less than 40) making up the largest part of the total IPL pool. These predominant species showed little temporal variation, constituting fairly constant fractions of their respective classes over time. However, during the spring bloom and in summer a number of IPL species were present in elevated abundances (typically around 5 %, rather than <1 %), and the IPL pool appeared to be somewhat less diverse than at other times.

4 Statistics relationships

The measures of dependence between each of the measured variables (Spearman's ρ, $n = 30$) are given in Table S3. Significant positive correlations were found between total SQDG, PC, PG, and DGTS concentrations, and chlorophyll-*a* concentrations ($\rho > 0.68$), primary production ($\rho > 0.68$) and algal abundances ($\rho > 0.53$), and the four classes were also strongly inter-correlated ($\rho > 0.75$). Scatter plots of the log-transformed data revealed the relationship between these IPL classes and the algal

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abundances to be linear, with R^2 values ranging from 0.45 for DGTS to 0.71 for SQDG ($n = 28$; Fig. 5). Furthermore, negative relationships were observed with the dissolved nutrient concentrations in the water ($\rho < -0.51$; Table S3). PE was the only IPL class that was not correlated with any environmental or microbial parameter measured here, or any other IPL class (Fig. 5; Table S3). The concentrations of the predominant individual IPL species were in general positively correlated with the total concentrations of their respective classes.

Principal component analysis (PCA) of the concentrations of the five IPL classes and microbial parameters yielded three principal components, explaining 86 % of the total variance (56 %, 17 % and 13 % for principal components 1, 2 and 3, respectively) in the dataset (Fig. 6 upper panel). PC, PG, SQDG and DGTS concentrations were positively loaded on the first axis, together with the algal abundances, chlorophyll-*a* concentrations and primary production, whilst PE was positively loaded on the second axis. The bacterial abundances were positively loaded on the third axis, whilst the cyanobacterial abundances were negatively loaded on the second axis, but positively on the third axis. PCA of the IPL classes and environmental parameters yielded three principal components, explaining 88 % of the variance (Fig. 6 lower panel). PC, PG, SQDG and DGTS concentrations were again positively loaded on the first axis (44 %), with salinity and temperature positively loaded on the second axis (32 %) and PE positively loaded on the third axis (12 %). The dissolved nutrient concentrations (DIP, DIN, DISi) were all negatively loaded on both the first and the second axis. Finally, a PCA of the most abundant IPL species in each class yielded only two principal components, which explained 66 % of the total variance (data not shown). Almost all IPL species were positively loaded on the first axis (55 %), whilst the PEs were the only class to load positively on the second axis (11 %).

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5 Discussion

The predominant IPL classes observed in the coastal North Sea waters were the glycerolipid SQDG, three glycerophospholipids (PG, PC and PE) and the betaine lipid DGTS. The same classes have so far been found to dominate the IPL composition in a range of marine waters, from the Pacific Ocean and Sargasso Sea (Van Mooy et al., 2006, 2009; Van Mooy and Fredricks, 2010) to the Black Sea (Schubotz et al., 2009), the western North Atlantic Ocean (Popendorf et al., 2011) and the North Sea and English Channel (Brandsma et al., 2011a). Whilst the glycerolipids MGDG and DGDG are often present in substantial quantities in some of these waters as well, they were only detected in trace amounts in the Marsdiep samples, similar to the observations made by Brandsma et al. (2011a) for the entire North Sea. In line with previous studies, the structural diversity in IPLs was large, comprising at least 400 different IPL species, but of these only a limited number made up the bulk of the total IPL pool.

Furthermore, despite the substantial changes in environmental conditions and microbial community composition (Figs. 2 and 3), the temporal variations in the IPL pool observed in the coastal North Sea waters were mostly quantitative and not qualitative. In other words, while the abundances of the IPL pool varied greatly throughout the year, its internal composition showed relatively little change, and was mostly limited to an increased contribution during the spring and summer blooms of several IPL species that were otherwise present in low concentrations. The principal component analyses and Spearman results both indicated a high degree of covariance between the SQDGs, PCs, PGs and DGTSs ($\rho > 0.77$), whilst the PEs were unrelated (Fig. 6 and Table S3). The cause for the different statistical behaviour of the PEs compared to the other IPL classes lies predominately in its behaviour during the spring bloom. Whilst IPLs in general increased in concentration from mid-March onward, PE concentrations remained at low values throughout this period (Fig. 2f). However, all IPLs reached maximum concentrations at the start of May, and PE concentrations behaved in much the same way as those of the other IPLs throughout the rest of the year. With this one significant exception, the general IPL composition in the coastal North Sea thus remained fairly stable throughout the year, unlike the variable environmental conditions and microbial community composition.

The IPL concentrations were statistically compared with the environmental data, in order to determine the influence of external parameters, such as temperature or nutrient concentrations. The results from the statistical tests all showed either a negative or no relationship between the IPL abundances and environmental parameters, with the exception of temperature (Fig. 6 lower panel and Table S3). Concentrations of SQDG, PG, PC and DGTS were all negatively correlated with the nutrient concentrations in the water, and positively with temperature. These are likely indirect relationships, with nutrients being incorporated into microbial biomass during the spring and summer blooms, which are triggered by rising temperatures and light availability. Culture and environmental studies have shown that marine phytoplankton can rapidly substitute glycerophospholipids with non-phosphorous IPLs (i.e., SQDG and betaine lipids) when phosphate is scarce (Benning et al., 1995; Van Mooy et al., 2009; Martin et al., 2010). However, in the coastal North Sea the ratios of SQDG to PG and DGTS to PC (as proposed by Van Mooy et al., 2009) remained fairly stable throughout the year (around 7.7 and 1.6, respectively), despite the strong decrease in DIP concentration during the spring bloom. Although there is a possibility that nitrogen rather than phosphorous was the most limiting nutrient during the spring bloom, due to the comparatively more rapid and complete recycling of the latter (e.g., Dodds, 2003), the high N:P ratios of dissolved inorganic nutrients measured in especially early April and late May (up to 722) indicate that phosphorus may have been a limiting factor for phytoplankton growth. If this was the case, then the nutrient scarcity did not lead to a rapid phytoplankton-wide adaptation of IPL compositions in this part of the North Sea.

The IPL concentrations were also statistically compared with chlorophyll-*a* concentrations, primary productivity and the microbial abundances and community composition. The significant correlations between SQDG, PG, PC and DGTS concentrations with the primary production rate, chlorophyll-*a* concentrations and algal abundances

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imply that the majority of the IPLs in the coastal North Sea were related to the biomass of the eukaryotic primary producers. This was also reflected in the PCA where these IPL classes grouped together with these parameters (Fig. 6 upper panel). Scatter plots of the log-transformed IPL and algal data showed that the relationship was linear and strongest for SQDG (Fig. 5), in agreement with its role as the main anionic IPL in thylakoid membranes of photosynthetic organisms (Benning, 1988; Janero and Barrnett, 1982; Frentzen, 2004). Like the total concentrations of their classes, the concentrations of the predominant SQDG, PC, PG and DGTS species were correlated with primary productivity, chlorophyll-*a* and algal abundances (Table S3). Indeed, studies of cultured *Thalassiosira* (Zhukova, 2004; Martin et al., 2010), *Chaetoceros* (Servel et al., 1993; Zhukova and Aizdaicher, 2001), *Skeletonema* (Berge et al., 1995), cryptophytes such as *Hemiselmis* (Chuecas and Riley, 1969) and prymnesiophytes such as *Phaeocystis* (Al-Hasan et al., 1990; Hamm and Rousseau, 2003), have shown that each of the different algal groups occurring in the coastal North Sea predominately synthesize PC, PG, SQDG and betaine lipids (Sato 1992; Dembitsky, 1996; Kato et al., 1996), containing combinations of $C_{14:0}$, $C_{16:4-16:0}$, $C_{18:5-18:0}$, $C_{20:5}$ and $C_{22:6}$ fatty acids. Exceptions were $C_{34:0}$ SQDG, $C_{34:4}$ PG and $C_{34:1}$ DGTS, which showed a better correlation with cyanobacterial abundances and may thus have been derived from that microbial group. However, cyanobacterial cell numbers did not correlate significantly with total concentrations of any of the IPL classes, and in the PCA results plotted on different axes than the IPLs (Fig. 6 and Table S3). Combined with the low abundances of MGDG and DGDG, which are common IPLs in cyanobacterial membranes (e.g., Murata and Nishida, 1987; Harwood and Jones, 1989), this suggests that cyanobacteria did not contribute substantially to the total IPL pool in the coastal North Sea. A likely reason for this is the small cell size of cyanobacteria compared to eukaryotes, which translates into a much lower total amount of IPLs per cell (see Veldhuis and Kraay, 2004 for a comparable argument on cell size and chlorophyll-*a* content).

The PE concentrations could not be related to any of the measured microbial abundances, despite the fact that PE is presumed to be the main glycerophospholipid in

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bacterial membranes (Shaw, 1974; Lechevalier and Lechevalier, 1989). The sharp increase in PE concentrations at the end of the spring bloom would point to a bacterial source, as maximum bacterial production rates in the Marsdiep are known to coincide with the collapse of the bloom (Van Boekel et al., 1992). However, bacterial abundances in the Marsdiep are strongly suppressed by heterotrophic nanoflagellate grazing (Brussaard et al., 1995), and it is therefore possible that this led to a mismatch between bacterial numbers and bacterially-produced IPLs (including PEs), or that the bacterial IPLs were rapidly transferred to higher trophic levels. Additionally, concentrations of two PE species containing the PUFA $C_{22:6}$ (i.e., $C_{38:6}$ PE and $C_{40:6}$ PE) were not related with the total PE concentrations, but rather with the concentrations of the other glycerophospholipids and DGTS. As those IPL classes and long-chain PUFAs are normally associated with eukaryotic algae (Gushina and Harwood, 2006), a non-bacterial origin for those two PE species is likely.

Despite the large number of IPL species quantified, general IPL analysis as performed in this study appears to lack the chemotaxonomic resolution to accurately differentiate within the microbial community, beyond the level of "marine algae", "phototrophs", or "(cyano)bacteria". The community composition analysis showed a rapid succession of algal species, with subsequent bloom periods throughout the year, which did not result in major changes in the IPL composition. However, the rapid fluctuations in IPL abundances were closely linked to changes in the total algal counts, showing that in this type of environment IPLs provide a good biomarker for living microbial biomass. The lack of large temporal variations in the IPL composition suggests that the IPL contents of the different algal groups occurring in the coastal North Sea must have been relatively similar. Indeed, studies of the main algal groups occurring in this region show that they all predominately synthesize PC, PG, SQDG and betaine lipids, containing combinations of $C_{14:0}$, $C_{16:4-16:0}$, $C_{18:5-18:0}$, $C_{20:5}$ and $C_{22:6}$ fatty acids. The prevalence of these IPLs across a wide range of algal groups and throughout the world's oceans (e.g., Schubotz et al., 2009; Van Mooy and Fredricks, 2010; Popendorf et al., 2011; Brandsma et al., 2011a) further suggests that general IPL screening of marine waters may yield little chemotaxonomic information, and in future studies it will therefore be necessary to target more specific biomarker IPLs, such as anammox bacterial ladderanes (Jaeschke et al., 2009; Brandsma et al., 2011b) or cyanobacterial glycerolipids (Bauersachs et al., 2009), in order to track the presence of specific microbial populations in the environment.

6 Conclusions

The coastal marine waters of the Marsdiep tidal inlet contain a wide range of IPLs, whose composition is comparable to that of the adjacent southern North Sea. Despite substantial variations in their abundances, the IPLs showed relatively little compositional changes over the year. Concentrations of SQDGs, PGs, PCs and DGTSs mostly co-varied, and their abundances were linked to the total algal biomass in the water. The origin of the PEs at this site remains unclear, although they may have been related to bacterial production at the end of the algal spring bloom. Intriguingly, the IPL species distribution through time did not reflect the succession of algal groups, implying that their IPL composition is generally similar. Finally, no direct influence of environmental conditions on the IPL composition was observed.

Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/8/8895/2011/ bgd-8-8895-2011-supplement.pdf.

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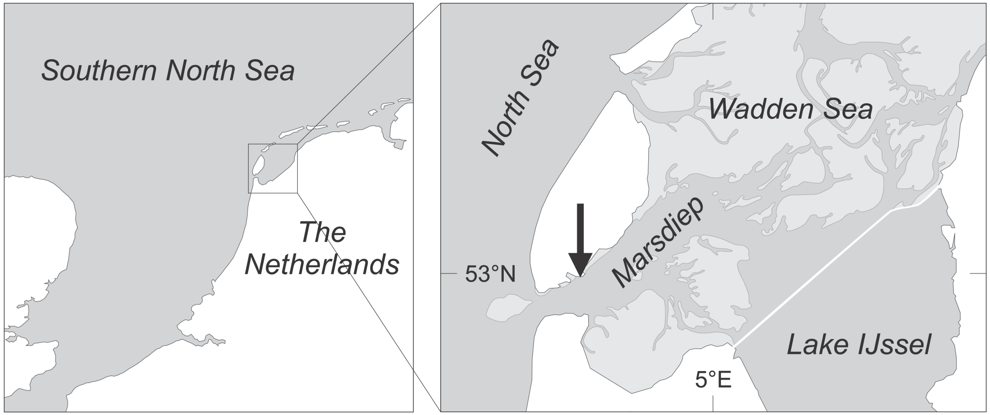

Fig. 1. Map of the southeastern North Sea and Wadden Sea; the arrow marks the sampling site at the entrance of the Marsdiep tidal inlet.

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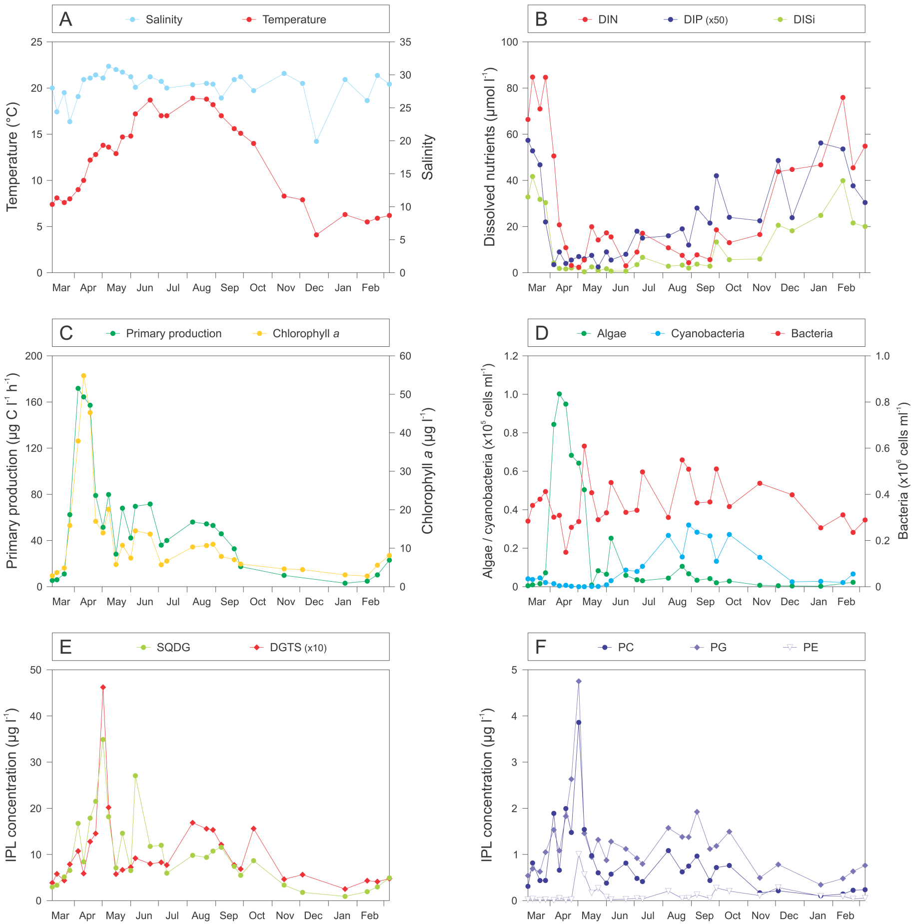

Fig. 2. Time series of: **(A)** salinity and temperature; **(B)** dissolved inorganic nutrient concentrations; **(C)** primary production and chlorophyll-*a* concentrations; **(D)** microbial abundances; **(E)** and **(F)** intact polar lipid concentrations (see Fig. 4 for acronyms).

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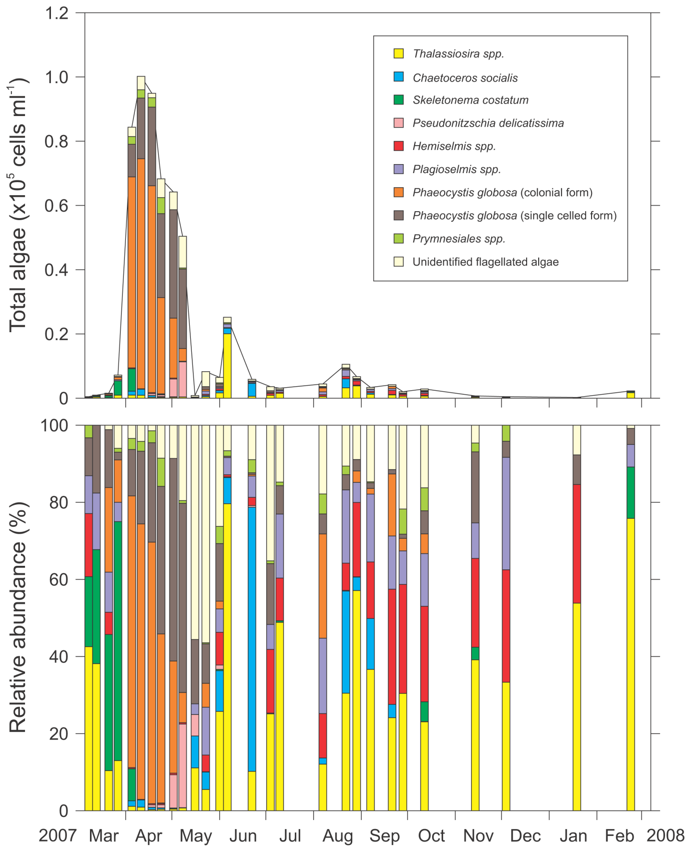

Fig. 3. Abundances of the different algal groups in the Marsdiep. The upper graph shows the absolute abundances, whilst the lower graph shows relative abundances (normalized to the total counts).

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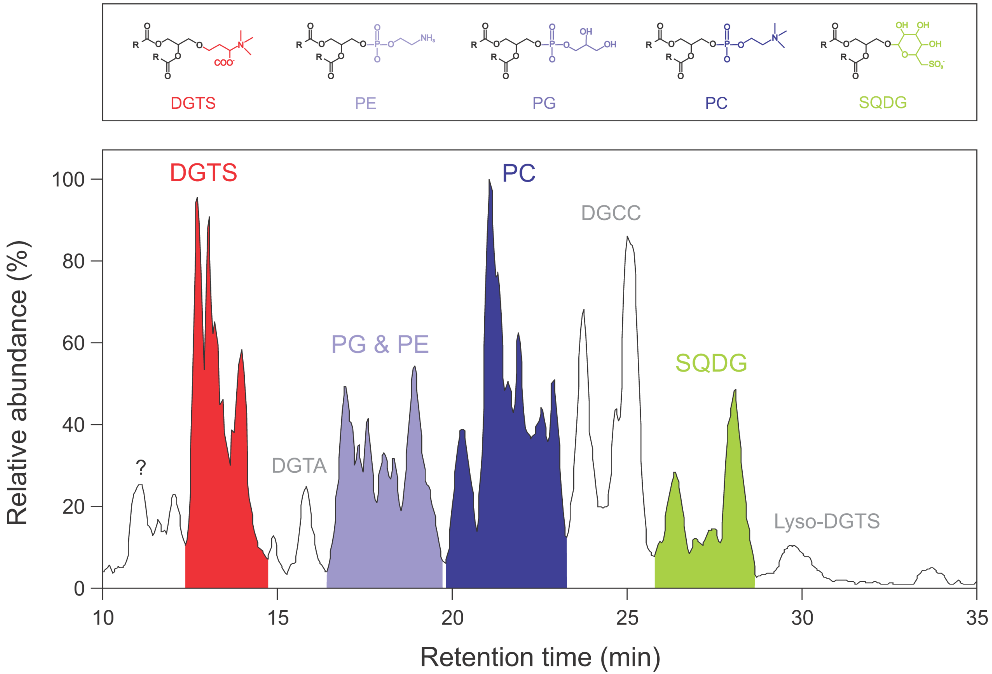

Fig. 4. Partial base peak chromatogram (positive ion – Gaussian smoothed) showing the IPL classes identified in Marsdiep water during the phytoplankton spring bloom in late April. Unidentified peaks are indicated with a question mark. Example structures are given for each of the quantified IPL classes: diacylglyceryl-trimethylhomoserine (DGTS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sulfoquinovosyldiacylglycerol (SQDG). Each peak comprises a wide range of IPLs with the same head group, but different fatty acids at the *sn*-1 and *sn*-2 positions (R′ and R″ in the example structures). Due to differences in response factors between the IPL classes, their relative abundances in the base peak chromatogram are not necessarily indicative of their respective absolute abundances.

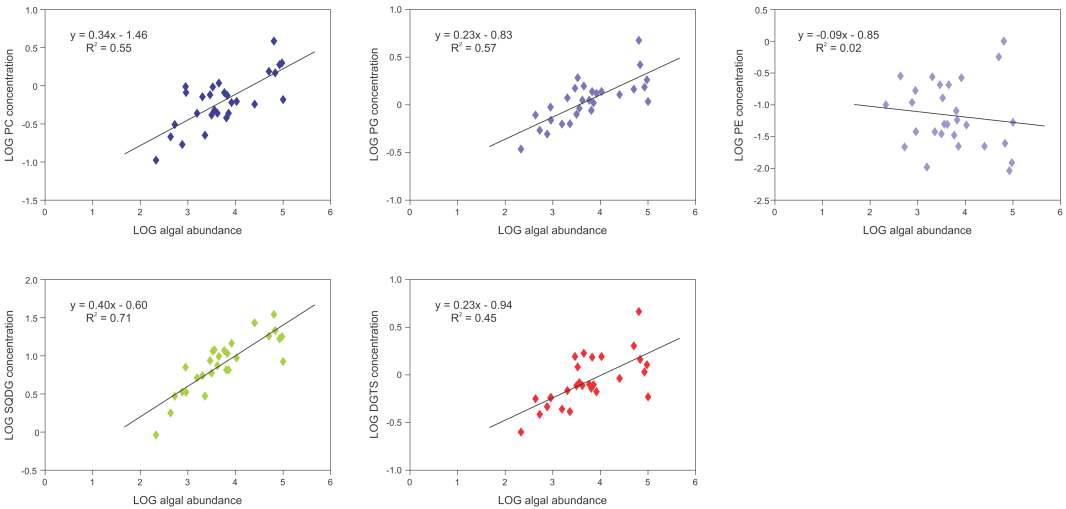

Fig. 5. Correlation plots between algal abundances and IPL concentrations in the Marsdiep.

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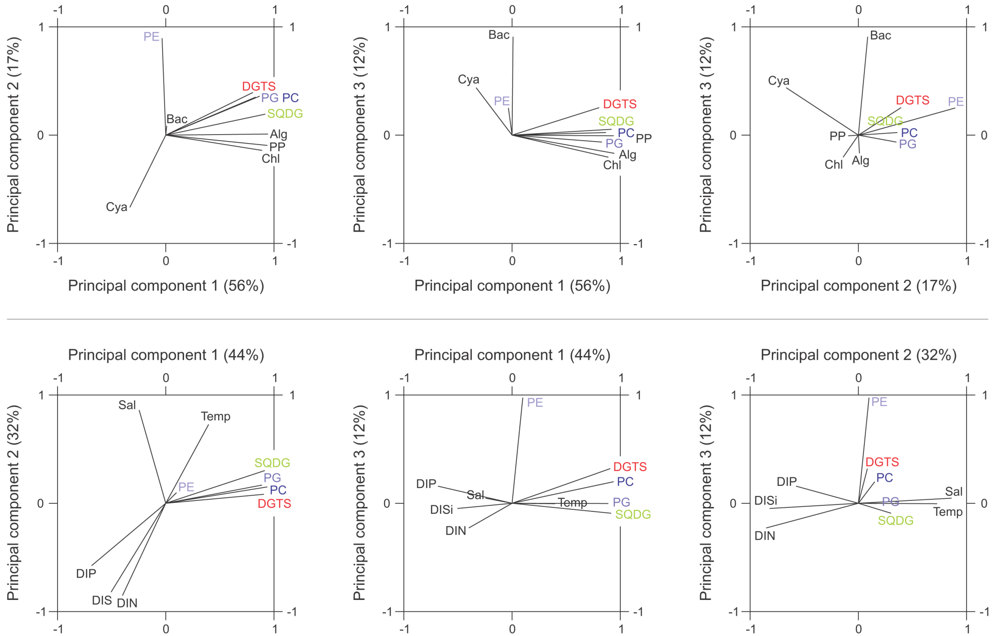

Fig. 6. Principal component analysis (PCA) plots for the total concentrations of the IPL classes with microbial abundances, chlorophyll-*a* concentrations and primary productivity (upper panel) and with environmental parameters (lower panel).

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