The origin and fate of intact polar lipids in the marine environment

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The origin and fate of intact polar lipids in the marine environment

De herkomst en het lot van intacte polaire lipiden in het mariene milieu

met een samenvatting in het Nederlands

Proefschrift

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For my family

Calm the muddy water:
it becomes clear

Lao Tzu
Tao Te Ching

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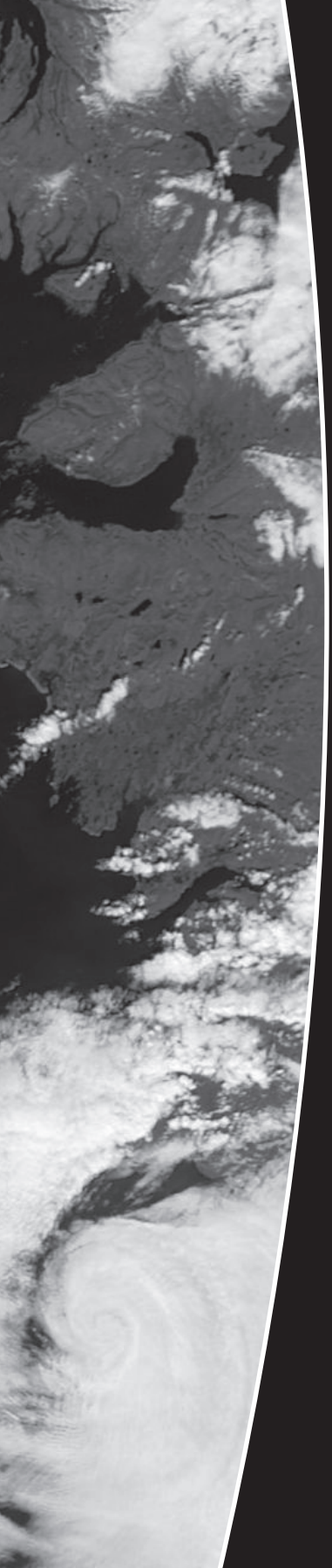


CHAPTER 1

INTRODUCTION

1.1 MICROBIAL DIVERSITY

The three domains of life, as defined by Woese *et al*. (1990), include the Bacteria and Archaea (the 'Prokaryota') and the Eukaryota. The vast majority of the prokaryotes and many eukaryotes are too small to see with the naked eye, and were only discovered after the invention of the microscope in 1675. Although the definition of these "micro-organisms" or "microbes" is not clearly defined, it generally indicates species that can exist as single cells while containing a replicable nucleic acid genome. This definition encompasses a wide range of small organisms, such as bacteria, fungi, protozoa and algae, but not viruses (see Rybicki, 1990). The size of microbes is normally in the µm range, but can be more than 1 mm in the case of the larger algae (and up to 1 m in the extreme case of *Caulerpa* macroalgae). Despite their generally small size, microbes are the most abundant organisms on Earth, and contain the bulk of the biosphere's organic carbon, nitrogen and phosphorous (Whitman *et al*., 1998). They are also the main drivers of the biogeochemical cycles. For example, in the nitrogen cycle atmospheric N_2 is fixed by a wide range of bacteria in the soil and oceans, but also by fungi and deep-sea archaea (Dekas *et al*., 2009), while it is released again by denitrifying bacteria (Cabello *et al*., 2004), as well as anaerobic ammonium oxidizing (anammox) bacteria (Jetten *et al*., 2009). Studying the composition and abundance of microbial communities is thus of fundamental importance for our understanding of Earth system functioning. Unfortunately, because microbes are relatively small and often lack obvious morphological characteristics (with the notable exception of algae), it is difficult to study them in their natural surroundings through direct observational analysis. A range of indirect methods is therefore employed to try to answer two basic questions in microbial ecology: who is there (diversity/taxonomy), and how many are there (abundance)?

Enrichment cultures can be used to determine the presence of a microbial species in the environment and most importantly, offer the possibility to study their physiology. However, enrichment cultures are severely limited, because only a small fraction of the microbial species present in the environment appears to be culturable (the so-called 'great plate count anomaly' of Staley and Konopka, 1985; see also Amann *et al*., 1995; Alain and Querellou, 2009; Vartoukian *et al*., 2010), despite the development of novel enrichment techniques (e.g., Zengler *et al*., 2002). The technique is also purely qualitative, as in principle only a single living cell is needed to generate a successful enrichment culture.

A widely-used approach to quantify microbial abundances is to count the cells directly in a small subsample and then extrapolate the results. To improve visibility of the cells, they are routinely stained with fluorescent dyes, such as DAPI (4',6-diamido-2-phenylindole) or acridine orange (*N,N,N',N'*-tetramethylacridine-3,6-diamine). Some of these dyes have the added advantage of being membrane-impermeable and thereby act as a viability assay (for example the nucleic acid probes ethidium bromide and

SYTOX Green). Alternatively, in a technique called fluorescence *in situ* hybridization (FISH), targeted fluorescent hybridization probes are used to bind to specific sequences of DNA on the chromosomes, after which the stained cells can be counted (DeLong *et al.*, 1989; Amann *et al.*, 2001; Wagner *et al.*, 2003; Amann and Fuchs, 2008). Counting of the cells can be done manually, using microscopy, or automated, using for example flow cytometry. The disadvantages of direct counting are that it is time-consuming, to some extent subjective, and can be difficult in some situations (for example when counting filamentous organisms, or when the sample contains an interfering matrix like sediment). Flow cytometry is rapid and relatively objective, but is restricted to counting cells in suspension solutions and is comparatively expensive.

The alternative to counting cells is to use a cellular component as a proxy for the cell itself. A well-known example is the analysis of pigments, such as carotenoids. Photosynthetic microbes are known to synthesize a wide range of pigments, and some of these are specific to certain microbial groups. These pigments can thus be used as 'biomarkers' for their source organism(s) and can be measured rapidly and with high sensitivity (e.g., Mackey *et al.*, 1996; Pinckney *et al.*, 2001), but they are restricted to photosynthetic microbes.

Proteomics has the potential to not only identify uncultured microbial groups based on their (projected) protein composition (e.g., Shevchenko *et al.*, 2001), but also to determine their metabolic capabilities and functioning in the environment (Phillips and Bogyo, 2005; Lacerda and Reardon, 2009). However, this technique is still under development and to date has seen only limited application in environmental microbiology (Valenzuela *et al.*, 2006; Maron *et al.*, 2007; Schweder *et al.*, 2008).

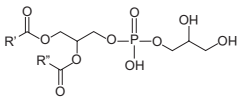
Most progress in characterizing microbial diversity has thus far been made by gene-based techniques (Eisen, 2007). As the blueprint for virtually all known organisms, except viruses, DNA provides the most specific proof that a microbe is present in an environmental sample. To date, most focus has been on the amplification of ribosomal RNA (rRNA) genes by polymerase chain reaction (PCR), which provides a method to phylogenetically place an organism on the 'Tree of Life' (phylotyping) (Olsen *et al.*, 1991; Pace, 1997; Cole *et al.*, 2009). Quantitative PCR can be used to determine the concentration of rDNA gene sequences present in a sample, and thereby estimate the number of microbial cells (e.g., Rehnstam *et al.*, 1993; Takai and Horikoshi, 2000) or even the level of gene expression by quantification of rRNA (e.g., Lam *et al.*, 2007; Frias-Lopez *et al.*, 2008). However, problems with selectivity of the PCR technique, variability in rDNA gene copy numbers between taxa, and the observation that other genes than rRNA can yield a better taxonomic resolution are leading to the development of more holistic approaches (Eisen, 2007). In particular shotgun metagenomics, in which random fragments of microbial DNA from an environmental sample are sequenced, yields massive and comprehensive datasets on the genetic diversity in the environment (e.g., Venter *et al.*, 2004; Sogin *et al.*, 2006).

1.2 INTACT POLAR LIPIDS AS TOOLS IN ENVIRONMENTAL MICROBIOLOGY

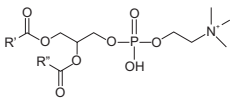
An alternative to DNA as information-carrying molecules for environmental microbiological studies are intact polar lipids (IPLs), and in particular glycerolipids. These polar molecules naturally form bilayers, making them the foundation for biological membranes, which provide a selectively permeable barrier between the cell and its environment. The classification and nomenclature for lipids has recently been standardized by Fahy *et al.* (2005, 2009), who identified eight lipid categories. Of these, the two main membrane-forming categories are the glycerolipids and the glycerophospholipids. Structurally the lipids in both categories are similar, and the glycerophospholipids are considered a subcategory of the glycerolipids (Fahy *et al.*, 2005). The central structural element in both lipid categories is a glycerol 'backbone', which can be acylated or alkylated at three positions. For example, the neutral diacyl- and triacylglycerides have fatty acyl chains attached to the *sn*-1, *sn*-2 and in the latter case also *sn*-3 positions. Naturally occurring IPLs have a polar 'headgroup' attached to the *sn*-3 position of the glycerol, which may contain any of a wide variety of structural elements, such as saccharides, carboxylic acid, sulfonic acid, trimethylamine, and many more. IPLs belonging to the glycerophospholipid subcategory have headgroups containing a phosphate moiety (see Fig. 1.1 for example structures of some common IPLs and their abbreviations). The remaining *sn*-positions of an IPL glycerol are normally occupied by one or two fatty acyl or alkyl 'tails'. These polar lipid fatty acids (PLFAs) vary widely in chain length and degree of unsaturation, and may contain structural elements like rings or side-chains. Within the prescribed structural framework of the glycero(phospho)lipids a large number of IPL configurations is possible and more than 10,000 have already been uploaded to the LIPID MAPS Structure Database (www.lipidmaps.org; 4 April 2011 update).

1.2.1 Chemotaxonomic value of IPLs

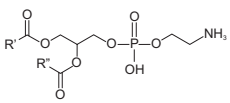
IPLs have several characteristics that make them attractive targets for environmental microbiological studies. The enormous variety in possible IPL structures makes it likely that many IPLs are synthesized predominately or uniquely by specific (groups of) organisms, and such biomarker IPLs can be used accordingly to indicate the presence of the source organism(s) in the environment. However, at present our knowledge of the IPL compositions of the multifarious microbial groups is still limited. To some extent this is due to the relatively recent development of adequate analytical techniques and instrumentation (see 1.3), but the main limiting factor is that we depend on enrichment cultures to determine in detail the IPL content of specific microorganisms. Nonetheless, there are a large number of studies available on the IPLs in bacteria, cyanobacteria, archaea, and especially algae.



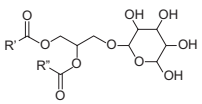
Phosphatidylglycerol (PG)



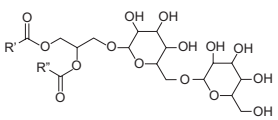
Phosphatidylcholine (PC)



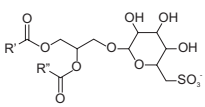
Phosphatidylethanolamine (PE)



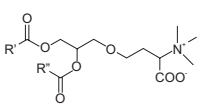
Monogalactosyldiacylglycerol (MGDG)



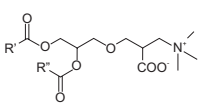
Digalactosyldiacylglycerol (DGDG)



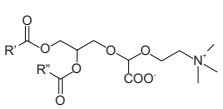
Sulfoquinovosyldiacylglycerol (SQDG)



Diacylglyceryl-trimethylhomoserine (DGTS)



Diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA)



Diacylglyceryl-carboxyhydroxymethylcholine (DGCC)

Figure 1.1 Glycerolipids and especially glycerophospholipids are the basic building blocks of biological membranes. Within the structural framework of a glycerol backbone, one or two fatty acyl or alkyl chains and a polar headgroup, a large number of configurations is possible. Example structures and abbreviations are given for the common microbial IPLs. Note that these are all diacyl-IPLs (fatty acid moieties attached to the glycerol are represented as R' and R"), whereas for example archaea synthesize dialkyl-IPLs.

Bacterial membranes predominately contain the glycerophospholipids PE and PG, but may also contain PC (Shaw, 1974; Sohlenkamp *et al.*, 2003 and references therein) (Table 1.1). Other common IPLs include glycosyldiradylglycerols, such as MGDG in gram-negative and DGDG in gram-positive bacteria (Kates, 1990). In addition, the betaine lipid DGTS has been found in a few photosynthetic genera (Klug and Benning, 2001; Elshahed *et al.*, 2007). Bacteria also commonly synthesize PLFAs with odd carbon numbers, particularly C_{15}, C_{17} and C_{19} fatty acids, and fatty acids containing side-chains, both of which are rare in the other microbial groups (Shaw, 1974; Kates, 1990; Řezanka and Sigler, 2009). Cyanobacteria differ from most other bacterial phyla in that they contain a large thylakoid (photosynthetic) membrane, and in many ways these prokaryotes resemble the larger eukaryotic algae. Cyanobacterial IPLs comprise large amounts of glycosyldiradylglycerols, including the thylakoid membrane-bound SQDG, but only one type of glycerophospholipid: PG (Quinn and Williams, 1983; Lechevalier and Lechevalier, 1989; Bauersachs *et al.*, 2009) (Table 1.1). They were recently shown to contain DGTS as well (Řezanka *et al.*, 2003). Cyanobacteria mostly synthesize shorter-chain, saturated or monounsaturated fatty acids, but some groups are characterized by their high C_{18} polyunsaturated fatty acid content (e.g., Kenyons, 1972).

Table 1.1 Occurrence of common IPL classes and fatty acids in cultured microbial groups.

Microbial group	IPL headgroups	Fatty acyl/alkyl chains
Bacteria	Mostly PE and PG, some PC MGDG, DGDG and other glycosyldiradylglycerols DGTS in some photosynthetic genera	On average shorter chain-lengths Odd carbon numbers (i.e., C_{15}, C_{17}, C_{19}) Chain methylation
Cyanobacteria	PG MGDG, DGDG and SQDG DGTS	Mostly shorter chain-lengths C_{18} PUFAs in some groups
Archaea	Wide range of glycerophospholipids, glycosyldiradylglycerols and mixed glycosylglycerophospholipids Sulfated headgroups	No fatty acyl chains Ether-bound C_{20} and C_{40} isoprenoid chains
Eukaryotic algae	Mostly PC and PG, but also other glycerophospholipids MGDG, DGDG and SQDG DGTS, DGTA and DGCC	On average longer chain-lengths C_{18}, C_{20} and C_{22} PUFAs

The archaea form a separate domain of life, and while they have prokaryotic cells, they are more closely related to the eukaryotes than the bacteria (Woese *et al.*, 1990). Although they were initially thought to thrive only in extreme environments, archaea are now recognized as an important microbial group throughout the marine environment (e.g., Wuchter *et al.*, 2006; Agogué *et al.*, 2008) and they may predominate in the deep biosphere (Lipp *et al.*, 2008). Archaeal membranes can be unusual in that some of them consist of a monolayer of isoprenoid tetraether lipids, rather than a bilayer of 'standard' polar lipids (Koga and Morii, 2005) (Table 1.1). Archaeal IPLs contain a wide variety of headgroups, including all of the common glycerophospholipids, glycosyldiradylglycerols and mixed glycosylglycerophospholipids, as well as sulfated forms of these (Koga and Morii, 2005; Biddle *et al.*, 2006; Schouten *et al.*, 2008; Chong, 2010).

The focus of research on eukaryotic microbes has almost entirely been on algae, and little is known about the IPL content of other eukaryotic groups, such as protozoa (Thompson and Nozawa, 1972). Eukaryotes have more complex cells than prokaryotes, and therefore potentially contain a larger diversity in IPLs. Thorough reviews on algal IPL compositions can be found in Volkman *et al.* (1998) and Guschina and Harwood (2006) (Table 1.1). As the algal chloroplast are thought to have originated from cyanobacteria through endosymbiosis (Mereschkowsky, 1905), part of their IPL content is similar to that prokaryotic group (Quinn and Williams, 1983). However, algal cells also contain a wide variety of glycerophospholipids, particularly PC (Dowhan, 1997), and glycosyldiradylglycerols (Wood, 1974; Lechevalier and Lechevalier, 1989; Guschina and Harwood, 2006). In addition, they contain all three betaine lipids DGTS, DGTA and DGCC (Sato, 1992; Dembitsky, 1996; Kato *et al.*, 1996). Another characteristic of eukaryotic algae is that they contain high amounts of long-chain polyunsaturated fatty acids (PUFAs), such as $C_{20:5}$ and $C_{22:6}$ (Shaw, 1974; Volkman *et al.*, 1998; Guschina and Harwood, 2006).

Within the wide variety of IPLs that have been identified, particular headgroups and fatty acyl or alkyl configurations thus appear to be specific to particular (groups of) organisms. These can in turn be used as biomarker IPLs to study their source organism(s) in the environment.

1.2.2 IPLs as a proxy for living microbial biomass

The second advantage of using IPLs in marine microbiological studies is their direct relationship with living cells. Unlike fatty acids, which can persist intact upon cell death and the disintegration of the cellular membranes and may be preserved on geological timescales (e.g., Eglinton *et al.*, 1991; Hedges and Prahl,1993), IPLs are thought to be relatively labile compounds. Experiments on the turnover of radioactive-labelled IPLs have shown that they loose their polar headgroup within a matter of hours or days (White *et al.*, 1979; Tollefson and McKercher, 1983; Harvey *et al.*, 1986; Petersen *et al.*, 1991; Moodley *et al.*, 2000). This means that IPLs are unlikely to exist outside living cells, and can thus be used as a proxy for living microbes. Based on these studies, IPL

quantities are used for estimates of living microbial biomass in a wide range of environments (e.g., Findlay *et al.*, 1989; Ringelberg *et al.*, 1997; Petsch *et al.*, 2001; Lipp *et al.*, 2008; Zink *et al.*, 2008).

However, this proxy depends critically upon the speed and completeness of the IPL degradation. In Figure 1.2 the degradation curve of ^{13}C-labelled PE measured by Harvey *et al.* (1986) is reproduced, which reveals that while the initial IPL degradation rates are very rapid, they slow down over time, and by the end of the experiment a significantly fraction of the original IPL pool remains intact. This prompts the question if IPL degradation is indeed always as rapid as is presumed. Intriguingly, it was also shown that factors such as oxygen concentration and organic matter content can substantially affect IPL (Harvey *et al.*, 1986), but these are seldom taken into account when IPLs are used as 'life markers'. While the success of its application provides circumstantial evidence that IPLs are indeed indicative of living cells, their exact degradation rate and the influencing factors need to be re-evaluated.

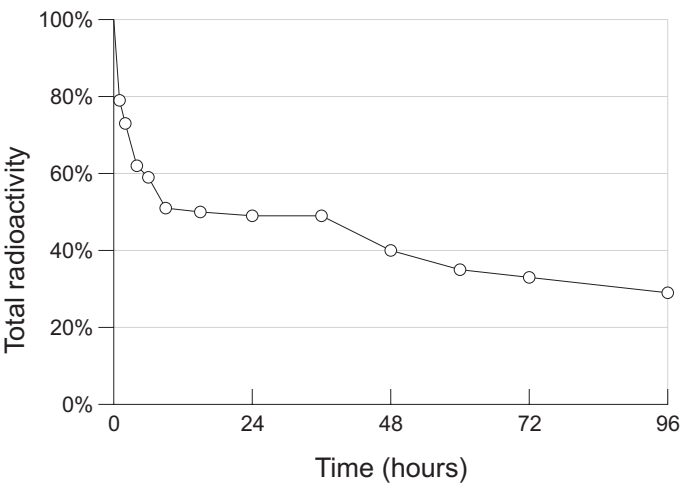


Figure 1.2 Aerobic degradation of ^{13}C-labelled phosphatidylethanolamine, incubated in beach sediments (reproduced from Harvey et al., 1986; their Fig. 3). While IPL degradation rates are initially very high, they slow down over time, and after several days a significant fraction of the original IPL pool remains intact. Similar results are found in all lab-based studies of IPL degradation (White et al., 1979; Tollefson and McKercher, 1983; Harvey et al., 1986; Petersen et al., 1991; Moodley et al., 2000).

1.2.3 Relationship between IPL composition and the environment

A final important observation is that microbes can adapt the IPL composition of their membranes to some extent, depending on their environmental conditions. The structural

configuration of a polar lipid molecule determines its behaviour within a membrane, which in turn influences the membrane's biophysical characteristics, such as its density, fluidity or charge. Microbes can actively optimize these properties by substituting certain IPLs in their membranes for others in a process called lipid remodelling. For example, both bacteria and eukaryotic algae can substitute glycerophospholipids with non-phosphorous glycosyldiradylglycerols or betaine lipids (Benning *et al.*, 1995; Martin *et al.*, 2010), but lipid remodelling can take place in the fatty acids as well. Ratios of IPLs thus potentially hold valuable information about the environmental conditions of their source organism(s), if this relationship can be quantified. However, lipid remodelling also means that the IPL composition of an organism is not constant, but will to some extent depend on external conditions or other possible factors, such as its growth stage. This can make the chemotaxonomic interpretation of IPL profiles in environmental samples more difficult when the relationships between the IPL composition of organisms present and their environmental conditions are unknown.

1.3 ANALYTICAL METHODS AND APPLICATION OF IPLS IN THE MARINE ENVIRONMENT

1.3.1 Methods for IPL analysis

IPL analysis involves a number of steps for which analytical procedures and instrumentation have been developed over the years. Until the late 1980s it was not possible to measure IPL molecules intact, and instead their constituent polar lipid fatty acids (PLFAs) were analyzed (e.g., White *et al.*, 1979). PLFA analysis involves extraction of the molecules from their associated matrix using organic solvents (normally following the methods of either Folch *et al.*, 1957 or Bligh and Dyer, 1959), hydrolyzing the IPLs to release the fatty acids and then measuring these by gas chromatography mass spectrometry (GC-MS). Although GC-MS analysis remains the method of choice for measuring fatty acids, it is not very suitable for measuring IPLs. This is because the IPL molecules are not sufficiently volatile, and therefore need to be broken down by hydrolysis into their constituent parts before GC analysis, whereby all potential chemotaxonomic information contained in the different combinations of apolar tails and headgroups is lost. In addition, this means that the measured PLFAs are derived from a mixed pool of polar lipids (e.g., Aries *et al.*, 2001). To omit this last problem the extract can be separated by solid-phase chromatography before hydrolysis, in order to remove all non-polar components (i.e., free fatty acids, neutral lipids, etc.). A well-known

separation technique is thin-layer chromatography (TLC) (Wagner *et al*., 1961; Touchstone, 1995), which has in recent year been replaced by various types of liquid chromatography (LC) techniques.

To address the problem of reduced specificity in determining microbial community structures and biomass deriving from the hydrolysis step in the PLFA method, analytical methods needed to be developed by which the IPL molecules could be measured directly (Haack *et al*., 1993; Fang and Barcelona, 1998). Fast atom bombardment mass spectrometry (FAB-MS) was the first method used to successfully measure intact glycerophospholipids (Jensen *et al*., 1986, 1987; Heller *et al*., 1987), but the chemical matrix background made mass spectral interpretation of especially glycerophospholipid mixtures difficult (Cole and Enke, 1994). This was overcome with the development of electrospray ionization (ESI) by Fenn *et al*. (1989), which made it possible to produce ions of IPL molecules that are not fragmented in the ionization source, without adding a matrix.

Coupled high performance liquid chromatography electrospray ionization (tandem) mass spectrometry (HPLC/ESI-MS^n) systems are now the most widely used analytical platform for IPL analysis of complex environmental samples (Kim *et al*., 1994; Fang and Barcelona, 1998; Rütters *et al*., 2002; Sturt *et al*., 2004). In this system IPL extracts are separated primarily according to headgroup polarity on the HPLC column. The molecular ions are then detected in the first stage of MS, after which they are fragmented and the fragment ions detected in the second stage of MS (Fig. 1.3). If required, targeted mass spectrometric experiments based on the known fragmentation patterns of the different IPL classes (e.g., Brügger *et al*., 1997) can be set up to elucidate the structural diversity within a class, such as the individual IPL species and their fatty acid contents.

1.3.2 IPLs in the marine environment

The development of new analytical techniques to identify IPLs in complex matrices allows for a much more rapid assessment of IPLs in the marine environment. However, as a result of this relatively recent development, the number of studies of IPLs in the marine environment is still limited. Thus far, the main focus of IPL studies in marine settings has been on subsurface sediments and in particular the deep biosphere. The observation that fatty acids are preserved in sediments (Eglinton *et al*., 1991; Hedges and Prahl,1993) led to the use of IPLs as more reliable 'life markers' for subsurface microbial communities (e.g., Rütters *et al*., 2002; Zink *et al*., 2003; Mangelsdorf *et al*., 2005). However, these biomarker IPL results are rarely compared with other methods for microbial detection, such as gene-based techniques, and generally do not take into account the potential differences in degradation rates of different IPLs.

Furthermore, in contrast to marine sediments there is a comparative lack of studies of IPLs in marine waters, and it has not yet been resolved which are the main IPLs in marine waters, and how well IPLs reflect the *in situ* microbial community composition and abundance. It is also unknown how IPL abundances in marine waters vary over

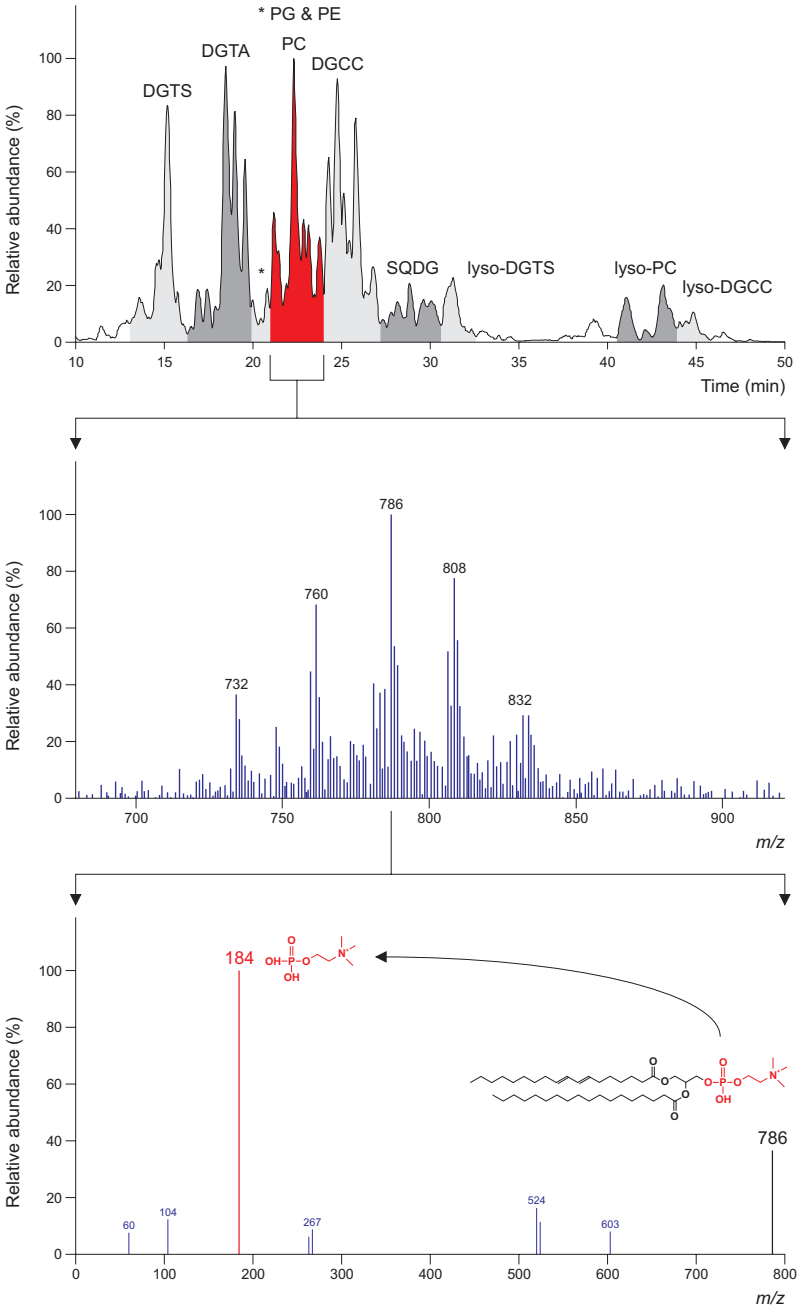


Figure 1.3 Example of IPL analysis by HPLC/ESI-MS^2 – IPL classes have different retention times on the HPLC column due to their different headgroups (base peak chromatogram in the upper panel). The first stage of MS measures all charged species eluting from the column (average mass spectrum in the middle panel), after which the most abundant species is fragmented and its fragment ions measured in the second stage of MS (mass spectrum in the lower panel). Note that with MS/MS no information is gained on the exact structure of the fatty acid moiety (i.e., the location of double bonds, side chains, rings, etc.).

time and if these changes correspond to changes in the abundances of different microbial groups, for example during a phytoplankton bloom and its subsequent crash.

Finally, it is known from enrichment cultures grown under variable conditions that microbes can adapt the IPL composition of their membranes in order to cope with changes in their environment (Rose, 1989; Benning *et al.*, 1995). Recent studies by Van Mooy *et al.* (2006, 2008, 2009) provide evidence that such lipid remodelling may occur on a phytoplankton-wide scale, and potentially has a large impact on biogeochemical cycles. It is therefore necessary to obtain more information the relationship between the IPL composition of the marine microbial communities and their environment.

1.4 FRAMEWORK OF THE THESIS

The development of analytical methods for direct IPL analysis, in particular HPLC/ESI-MS^n, now enables us to measure the IPL composition of environmental samples in great detail. The aim of this thesis is therefore to investigate the sources of IPLs in the marine environment, and especially the water column. Particular attention is given to the value of IPLs as biomarkers for living microbial cells, and how well the IPL composition reflects the *in situ* microbial community composition. In addition, the impact of environmental factors on the IPL composition of the microbial community is addressed.

In **Chapter 2** the use of IPLs as a proxy for living microbial cells is re-assessed, using decaying cultures of the marine diatom *Chaetoceros calcitrans* as a model system. Several experiments were performed in which the degradation of PC, PG and SQDG was monitored with and without the presence of bacteria. A similar decay in IPL concentrations was observed as in previous degradation experiments (e.g., White *et al.*, 1979; Harvey *et al.*, 1986). However, our results show that while IPL concentrations initially corresponded well with numbers of living cells, they overall correlated best with total cell numbers. A substantial amount of IPLs was retained in dead cells or cell fragments, implying that care should be taken in applying IPLs as a proxy for living microbial cells. The introduction of bacteria into the experiment led to an additional loss of diatom IPLs, but at the same time production of bacterial IPLs (in particular PG and PE). Finally, the ether-bound SQDG degraded at a lower rate than the glycerophospholipids PC and PG.

In **Chapter 3** IPL analysis is employed alongside a range of other techniques, including fatty acid analysis, activity measurements, FISH and quantitative PCR, to detect anaerobic ammonium oxidizing (anammox) bacteria in marine sediments of the Swedish Gullmar Fjord. The ladderane IPL profile in the sediment matched those of the activity measurements and the anammox bacterial cell abundances, as determined by FISH, while the profiles of the ladderane fatty acids did not. This provides empirical evidence for the use of specific biomarker IPLs as a proxy for living anammox bacterial cells.

In **Chapters 4 and 5** the spatial distribution of IPLs in the North Sea and several adjacent water masses is investigated and compared with the environmental conditions, microbial abundances and microbial community composition. Chapter 4 describes the summer distribution of microorganisms in the North Sea and its relationship to environmental factors, based on two research cruises in 2007 and 2008. This provides the environmental and microbial background data for Chapter 5, in which the IPL composition of North Sea surface waters is described in detail. The two datasets are then compared statistically in order to determine how spatial variations in microbial community composition and environmental parameters are reflected in the IPLs. Our results show that a suite of IPLs – namely SQDG, PC, PG, PE, DGTS, DGTA and DGCC – predominates in the North Sea, similar to what has been observed in other oceans. Spatial differences in the relative abundances of these IPLs did not correlate with any of the environmental parameters, but could tentatively be related to the abundances of the three major microbial groups: bacteria, cyanobacteria and eukaryotic algae. Furthermore, significant relationships were found between concentrations of SQGDs, PCs, PGs and DGTSs, and concentrations of chlorophyll *a*, implying that the majority of these IPLs were derived from photosynthetic primary producers. However, none of the relationships were particularly strong and unequivocal, leading to the conclusion that general IPL screening may be lacking in chemotaxonomic resolution.

In **Chapter 6** the temporal variability in the IPL composition at a coastal marine site (the Marsdiep in the southeastern North Sea) is examined over a one-year cycle. As in Chapter 5, variations in the IPLs are compared to the changes in environmental conditions and the microbial community. Our results show a strong seasonal variation in IPL abundances, with the highest concentrations measured during the spring bloom. No direct relationships between IPLs and environmental parameters were found, but concentrations of SQDGs, PCs, PGs and DGTSs could be related to algal abundances, chlorophyll *a* concentrations and primary productivity. However, the rapid succession of different algal groups that was observed was not reflected in the IPL composition, which remained remarkably similar throughout the year. This highlights the conclusion that care should be taken in inferring the microbial community composition in marine waters directly from the IPL composition.

In **Chapter 7** the outcomes of these studies are summarized and the use of IPLs as biomarkers in marine microbiology is discussed. While IPLs provide a better estimate of microbial abundances than fatty acids, their degradation rates may depend on environmental conditions, and under the right circumstances they could be preserved intact. This could lead to a biased view of microbial communities in for example anoxic sediments, and more work is therefore necessary to resolve the degradation of IPLs in detail. Specific IPLs were successfully used to trace anammox bacteria in marine sediments. However, the results from the spatial and temporal variability studies in the North Sea indicate a surprising lack of chemotaxonomic resolution in the IPLs. This indicates that care must be taken in directly inferring the microbial community composition from the IPL composition, and that specific biomarker IPLs should preferably be used in marine microbiological studies.

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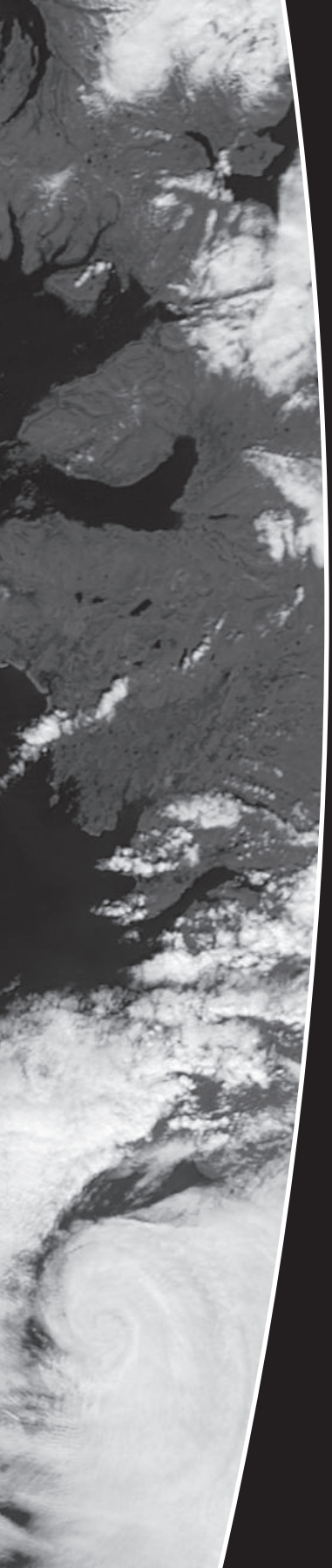

CHAPTER 2

A DEGRADATION STUDY OF INTACT POLAR LIPIDS OF A MARINE DIATOM *(CHAETOCEROS CALCITRANS)*

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ABSTRACT

Intact polar lipids (IPLs) and their derived polar lipid fatty acids (PLFAs) are widely used as a proxy for living microbial cells in environmental samples. We quantified the concentrations of four IPL classes – phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and sulfoquinovosyldiacylglycerol – in decaying cultures of the marine diatom *Chaetoceros calcitrans*, and compared these with the changes in cell numbers. IPL degradation rates were determined in three initially identical experimental setups: under axenic and non-axenic conditions, as well as in a mixed scenario where bacteria were introduced partway through the incubation. In the axenic experiments, the strong decrease in IPL concentrations over the first 16 days corresponded with a decrease in living cell numbers. However, after this point the number of living cells continued to decrease to zero, while the decrease in IPL concentrations became progressively less. At the end of the axenic experiment after 72 days, 25% of the initial phosphatidylcholine and phosphatidylglycerol concentrations, and 50% of the initial sulfoquinovosyldiacylglycerol concentration remained present. Overall, the IPL concentrations corresponded best with the total number of cells in the culture, instead of with the number of living cells. The introduction of bacteria into the experiments lead to additional losses of IPLs (20-50%), but nonetheless substantial IPL concentrations remained present. Within the IPLs, sulfoquinovosyldiacylglycerol appeared to be more stable than the glycerophospholipids. Our results imply that there may not be a direct relationship between IPL abundances and living microbial cells, in particular in settings where the (bacterial) breakdown of deceased cells is slow.

2.1 INTRODUCTION

Microbes are the drivers of many biogeochemical cycles and comprise one of the largest organic carbon pools on earth (Whitman *et al.*, 1998), making the quantification of living microbial biomass important for ecological and biogeochemical studies. Estimates of microbial biomass are typically obtained by determining the number of microbial cells in a sample and multiplying this by a certain conversion factor. Since direct counting of living cells is likely to underestimate the extant microbial community, especially in sediments (White, 1988; Amann *et al.*, 1995; Pearson, 2008), a range of more inclusive biochemical methods have been developed, each of which uses a different cellular compound as a proxy for living microbial cells (see White *et al.*, 1997 and references therein). One widely used biochemical approach targets the extractible intact polar lipids (IPLs), or their derived polar lipid fatty acids (PLFAs) (e.g., White *et al.*, 1979; Ringelberg *et al.*, 1997; Petsch *et al.*, 2001; Lipp *et al.*, 2008; Zink *et al.*, 2008). These basic constituents of cell membranes are mostly glycerol-based lipid molecules with a hydrophilic (polar) head group attached to the *sn*-3 position (Fahy *et al.*, 2005; 2009). When the amount of IPLs per cell is taken to be constant (see Balkwill *et al.*, 1988; Findlay *et al.*, 1989), the conversion from IPL quantities to microbial biomass is straightforward.

Another key assumption in the use of IPLs as indicators for living cells is that they degrade rapidly upon cell death and lysis. However, the evidence for this assumption is based on a relatively small number of incubation studies (White *et al.*, 1979; Tollefson and McKercher, 1983; Harvey *et al.*, 1986; Petersen *et al.*, 1991; Moodley *et al.*, 2000). In each of these, the observed IPL degradation rates were initially high, with 50% losses over the first hours to days. However, the degradation rates decreased progressively over time and as a consequence a significant fraction of the initial IPL pools (up to 50%) remained intact at the termination of the incubations (up to 80 days). Much lower degradation rates were also measured for IPLs with ether-bound alkyl moieties and a glycosidically bound head group (glycerolipids) than for glycerophospholipids (IGPs), which have ester-bound head groups (Harvey *et al.*, 1986). Furthermore, these studies were limited to bulk IPL degradation, as IPL quantities were inferred from enumerations of lipid-bound ^{32}P or ^{14}C, and were not measured directly. Degradation rates of IPLs with different molecular compositions (head groups and/or fatty acids) have thus not been studied in detail. However, the combination of high-performance liquid chromatography (HPLC) with electrospray ionization coupled to multistage mass spectrometry (ESI-MS^n) now enables the study of IPL compositions of complex extracts with high resolution and sensitivity (Brügger *et al.*, 1997; Fang and Barcelona, 1998; Rütters *et al.*, 2002; Sturt *et al.*, 2004).

In this study we determined the relationship between IPLs abundances and the presence of living microbial cells by quantifying various IPL classes in decaying cultures of the marine diatom *Chaetoceros calcitrans* under controlled conditions. Firstly, changes

in the IPL concentrations and viable cell numbers were enumerated in a culture incubated under axenic conditions. This provided the most straightforward, if least natural, comparison, as any IPL degradation had to be due to autolysis of the algal cells, without any bacterial breakdown or synthesis of IPLs taking place. Furthermore, the experiment was also performed under non-axenic conditions, allowing us to determine the influence of bacterial activity on the different IPL degradation rates, by comparing them with the purely autolytic rates. Several IPL classes were quantified simultaneously in the same culture by HPLC/ESI-MS^2, and thus potential differences in the degradation rates of these IPLs could be assessed. Together these measurements provide us with a better understanding of the degradation of different IPL types, both with and without bacterial activity, and their relationships with microbial cell numbers.

2.2 MATERIALS AND METHODS

2.2.1 Experimental setup and sampling

An axenic culture of the marine diatom *Chaetoceros calcitrans* (CCMP1315) was grown in artificial sea water supplemented with nutrients, trace elements and vitamins (ES:F/2 medium 1:1 v/v) to assure optimal growth conditions. When the culture reached its stationary growth phase it was subdivided into three separate cultures of 2 l each. One of these cultures was inoculated with a natural mixed bacterial community by adding 100 ml of sea water from the Marsdiep tidal inlet (surface water sample taken at high tide on 26 February 2007), which had been filtered through a 1.2 µm mesh GF/C filter (Whatman, Clifton, NJ, USA). The other two cultures were kept axenic, but were inoculated with 100 ml of artificial sea water (as above), in order to maintain comparable volumes. The culture bottles were then sealed in aluminium foil and incubated in the dark at 10°C for 72 days. The cultures were homogenized daily by gentle rotation of the bottles. When the amount of living cells in each culture fell below 1% (as assessed by flow cytometry; see below), a 10 ml aliquot was re-suspended in fresh medium and transferred to the original growth conditions. These re-growth experiments were used to determine if the culture had indeed reached full senescence, or if it could be revived. In addition, aliquots were checked by epifluorescence microscopy using a Zeiss Axioplan microscope.

At regular intervals (2-7 days) during the incubation period aliquots were taken for enumeration of diatom and bacterial cells, a viability assay of the diatoms, and IPL analysis. Diatom abundance samples (2 ml) were stored in the fridge at 10°C before analysis (typically within 30 min). Viability assay samples (0.5 ml) were stained with 5 µl of 50 µM SYTOX Green solution (MolecularProbes / Invitrogen, Eugene, OR, USA) and kept in the dark at room temperature for 10 min before analysis. Bacterial abundance samples (1 ml) were fixed with a final concentration of 0.5% glutaraldehyde (EM grade;

Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 30 min, before snap freezing in liquid nitrogen and storage at -80°C. Finally, IPL samples (50 ml) were filtered over 0.7 µm mesh GF/F filters (47 mm diameter, precombusted at 450°C for 12 h; Whatman, Clifton, NJ, USA), which were then freeze-dried and stored at -20°C.

All materials used in the incubation were sterilized before use, and all sampling was performed in a sterile environment (laminar air flow cabinet) to prevent contamination of the cultures. However, despite these precautions, one of the axenic cultures did become infected with bacteria partway through the incubation (see Results and Discussion).

2.2.2 Diatom cell counts and viability assay

The abundance of *C. calcitrans* was monitored in triplicate using a bench-top Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Brea, CA, USA), equipped with an air-cooled 15 mW 488 nm argon-ion laser (Veldhuis and Kraay, 2000). The discriminator for intact *C. calcitrans* cells was set at red chlorophyll autofluorescence >630 nm. In addition, a viability assay, based on the permeability of the cell membranes to the nucleic acid dye SYTOX Green (MolecularProbes/Invitrogen, Eugene, OR, USA) was used to determine the relative abundance of living and dead algal cells (Veldhuis *et al.*, 2001). In cells with a compromised outer membrane, SYTOX Green will bind with the nucleic acids and emit a strong green fluorescent signal at 515±20 nm. Consequently, in the *C. calcitrans* cultures only algal cells with high red autofluorescence and low green fluorescence signals were classified as 'living', while the remaining algal cells were classified as 'dead'. Absolute cell numbers, cell sizes, and relative green and red autofluorescence of the cells were analyzed with the software package FCS Express 3 (De Novo Software, Los Angeles, CA, USA).

2.2.3 Bacterial cell counts

Bacterial abundances in the cultures were determined following the method of Marie *et al.* (1997). Briefly, defrosted samples were diluted with autoclaved 0.2 ml filtered TE buffer (Tris:EDTA 10:1 v/v at pH 8.0) and stained with the nucleic acid dye SYBR Green I (MolecularProbes/Invitrogen, Eugene, OR, USA) at a final concentration of 1×10^{-4} of the commercial stock, in the dark and at room temperature for 15 min. Analysis was then performed on a bench-top FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), equipped with an air-cooled 15 mW 488 nm argon-ion laser. The trigger was set on the green fluorescence, and the readings were collected in logarithmic mode and analyzed with the freeware package CYTOWIN (http://www.sb-roscoff.fr/phyto/cyto.html).

2.2.4 Intact polar lipid analysis

Samples for IPL analysis were extracted using a modified Bligh-Dyer procedure (Bligh and Dyer, 1959; Vancanneyt *et al.*, 1996; Rütters *et al.*, 2002). Briefly, the filters were extracted ultrasonically three times for 10 min in a solvent mixture of methanol,

dichloromethane and phosphate buffer (2:1:0.8 v/v). After sonication, the supernatants were phase-separated by adding additional dichloromethane and buffer to a final solvent ratio of 1:1:0.9 (v/v). The organic phases containing the IPLs were then collected and the aqueous phases re-extracted three times with dichloromethane. Finally, the extracts were dried under a stream of nitrogen gas. Before analysis, the extracts were re-dissolved in a mixture of dichloromethane and methanol (9:1 v/v) at a concentration of 10 mg ml^{-1}, and aliquots were filtered through 0.45 µm True Regenerated Cellulose syringe filters (4 mm diameter; Grace Alltech, Deerfield, IL, USA).

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 conditions as described by Boumann *et al.* (2006). Initially the IPL extracts were analyzed using an MS routine where a positive ion scan (*m/z* 300-1000) was followed by a data dependent MS^2 experiment in which the base peak of the mass spectrum was fragmented (collision energy 30 V, collision gas 0.8 mTorr Ar). Identification of the major IPL classes was based on diagnostic fragmentation patterns in the MS^2 mass spectra (Bisseret *et al.*, 1985; Kato *et al.*, 1996; Brügger *et al.*, 1997; Keusgen *et al.*, 1997; Fang and Barcelona, 1998).

Four IPL classes were quantified using targeted mass spectrometric experiments. IPLs with a phosphatidylcholine (PC) head group were detected in positive ion mode by parent ion scanning (*m/z* 300-1000) of the polar head group moiety (*m/z* 184), while IPLs with a phosphatidylethanolamine (PE), phosphatidylglycerol (PG) or sulfoquinovosyldiacylglycerol (SQDG) head group were detected by neutral loss scanning (*m/z* 300-1000) for losses of 141, 189 and 261 Da, respectively. The MS^2 settings for detection of the intact glycerophospholipids (IGPs) were identical to those described above, but for detection of SQDG the collision energy for the neutral loss assay was set to 25 V. For quantification, the total ion current peak areas of each IPL were compared with those 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), and a mixture of SQDGs, which contained predominately $C_{16:1}/C_{18:2}$ SQDG (~60%), but also small amounts of SQDGs with $C_{16:0\text{-}16:1}$, $C_{18:0\text{-}18:1}$ and $C_{20:5}$ fatty acid combinations (Lipid Products, Redhill, Surrey, UK). Due to the limited amount of culture material, most samples could only be run as single analysis. Limits of detection were 50-100 pg on column for the IGPs and 1 ng on column for SQDG.

2.3 RESULTS AND DISCUSSION

A stock culture of axenically grown *Chaetoceros calcitrans* was split and allowed to autolyse for 72 days in three separate dark-incubations. In two of the experimental setups the cultures were incubated without additional treatment, whereas in the third experiment a natural mixed bacterial community was purposefully introduced by

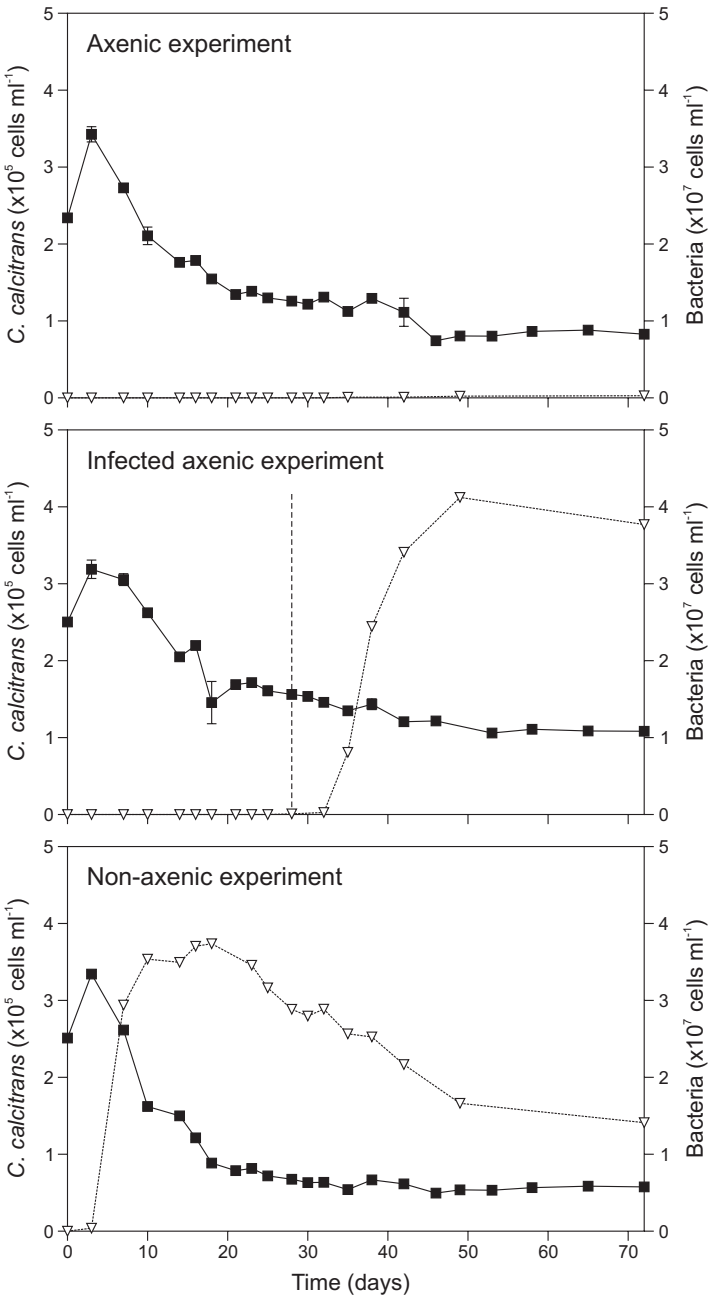

Figure 2.1 Total counts of *C. calcitrans* cells (filled squares) and bacterial cells (open triangles) in each of the experiments over time. The dashed line in the second plot indicates the moment of infection with bacteria.

adding (filtered) sea water to the culture. No bacteria were detected in either of the axenic experiments over the first 28 days. However, from this point onward bacteria were observed in one of the experiments, indicating that this culture had become bacterially infected, while the other culture remained axenic during the entire incubation period. In the non-axenic experiment bacterial abundances were high throughout almost the entire incubation period (Fig. 2.1). The experiments thus provided three different IPL degradation scenarios using essentially identical starting material: with and without the presence of bacteria, and a mixed scenario in which bacteria were only present in the second part of the incubation.

At the start of the incubations, the intact polar lipid composition of *C. calcitrans* comprised seven main IPL classes: the glycerophospholipids PC and PG, the glycerophosphosulpholipid phosphatidylsulfocholine (PSC), the sulphur-bearing glycerolipid SQDG, the glycerolipids mono- and digalactosyldiacylglycerol (MGDG and

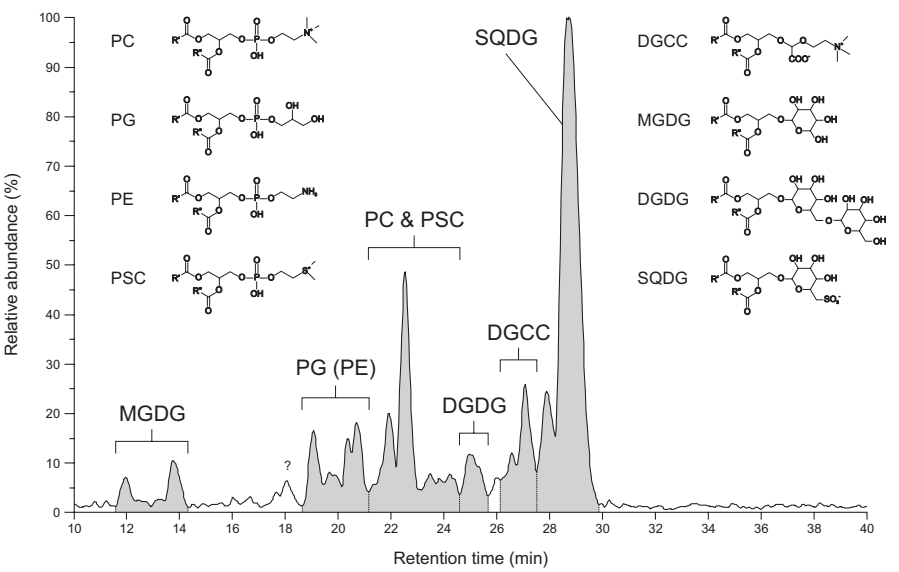

Figure 2.2 Partial base peak chromatogram (positive ion – Gaussian smoothed) showing the IPL composition of *Chaetoceros calcitrans* at the start of the incubation. Unidentified peaks are indicated with a question mark. Example structures are given for each of the detected IPL classes: phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE; see text), phosphatidylsulfocholine (PSC), diacylglyceryl-carboxyhydroxymethylcholine (DGCC), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) 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.

DGDG), and the betaine lipid diacylglyceryl-carboxyhydroxymethylcholine (DGCC) (Fig. 2.2). In addition, the non-axenic experiment contained the glycerophospholipid PE, which was not detected in the axenic experiments. Throughout the incubation period PC, PG and SQDG remained the predominant IPL classes in the cultures, while MGDG and DGDG also remained present. PSC and DGCC were only detected at the start of the incubations, but not anymore from day 7 onward, while PE remained present throughout the non-axenic experiment.

The changes in abundance of the IPL classes PC, PG, PE and SQDG were quantified using authentic standards, and compared with the number of living and dead *C. calcitrans* cells in the cultures. At the start of the incubation, each contained 27 ng ml^{-1} PC, 13 ng ml^{-1} PG, and 181 ng ml^{-1} SQDG, while the non-axenic experiment also contained 42 pg ml^{-1} PE. In order to facilitate comparison between the changes in each of the IPL classes, as well as between the three experiments, the IPL concentrations discussed below were normalized relative to these initial values.

2.3.1 Axenic degradation experiment

In the axenic degradation experiment the total diatom count continued to increase during the first few days of the dark incubation from 2.3 x 10^5 cells ml^{-1} to a maximum of 3.4 x 10^5 cells ml^{-1} (Fig. 2.1), indicating there was still a small amount of growth (less than one doubling) despite the absence of light. This short initial growth phase was followed by a rapid, but progressively slower, four-fold reduction in cell numbers to a final 8.3 x 10^4 cells ml^{-1} (day 72). Living cells made up approximately 99% of the total cell count at the start of the incubation, but decreased more or less linearly to less than 1% after 49 days (Fig. 2.3). Full senescence was confirmed by a re-growth experiment conducted at day 53, which failed to revive the culture when an aliquot was transferred to optimal growth conditions.

Relative to the start of the dark incubation the concentrations of PC and PG decreased by approximately 60% over the first 10 days (to 9 ng ml^{-1} and 5 ng ml^{-1}, respectively; Fig. 2.4). However, after day 10 the rate of decrease diminished substantially and at the end of the incubation approximately 25% of the initial PC and PG concentrations remained (6 ng ml^{-1} and 4 ng ml^{-1}, respectively). In contrast, the relative concentration of SQDG in this culture increased almost 3-fold during the first 7 days of the incubation (to 512 ng ml^{-1}), followed by a gradual decrease to approximately 140% of the initial concentration (250 ng ml^{-1}), or 50% of the maximum concentration at day 7.

Since no bacteria were present, any changes in the IPL concentrations in this experiment were the result of either *de novo* biosynthesis by living diatom cells (increase) or IPL degradation by autolysis (decrease). However, lipid remodelling (i.e., substitution of IPLs with one type head group for another type) in response to the stressing environmental conditions could also have resulted in the simultaneous decrease of one IPL class and increase of another class within the cells (Rose, 1989; Guschina and Harwood, 2009). Most of the IPL concentration changes took place during the first 16

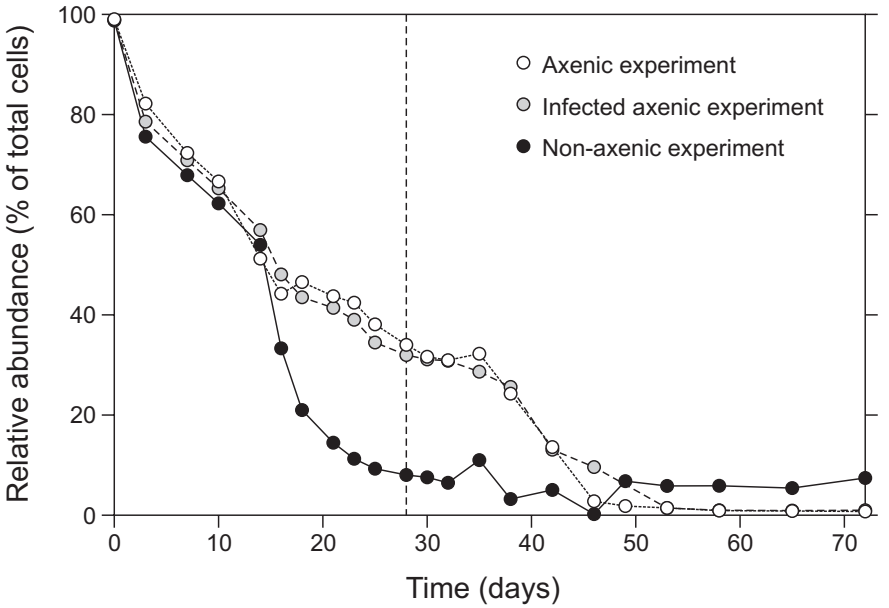

Figure 2.3 Relative abundance of living *C. calcitrans* cells in the three experiments over time. The dashed line indicates the moment of infection with bacteria of the second (initially) axenic experiment.

days of the incubation. The decrease in PG and PC during this time coincided with an increase in SQDG, which could suggest lipid remodelling. PG and SQDG are the predominant IPLs in thylakoid membranes, where they are known to substitute for each other (Janero and Barrnett, 1981; Frentzen, 2004; Martin *et al.*, 2010). PC on the other hand is common in the cellular membranes of most eukaryotes (Janero and Barrnett, 1981; Lechevalier and Lechevalier, 1989; Dowhan, 1997) and it can be substituted with the betaine lipid DGCC (Martin *et al.*, 2010), the glycerolipid digalactosyldiacylglycerol (DGDG; Tjellström *et al.*, 2008), or possibly its sulfonium analogue PSC (Anderson *et al.*, 1978; Bisseret *et al.*, 1985). However, the remodelling of IPGs into other IPLs in phytoplankton membranes appears to be predominately driven by phosphate limitation (Van Mooy *et al.*, 2006, 2009; Martin *et al.*, 2010), which is improbable in the nutrient-replete conditions of the current study. Although a shift from PG to SQDG due to light limitation cannot be excluded, such a remodelling of thylakoid IPLs has not been observed in previous light-limited incubations of algal cultures (i.e., a mixed community of *Navicula gelida* var. *antarctica*, *Fragilariopsis curta* and *Nitzschia mediocostricta*; Mock and Kroon, 2002; and *Tichocarpus crinitus*; Khotimchenko and Yakovleva, 2005), and is thus unlikely to have contributed substantially to the concentration changes in either PG or SQDG. In addition, DGCC and PSC were only observed in the

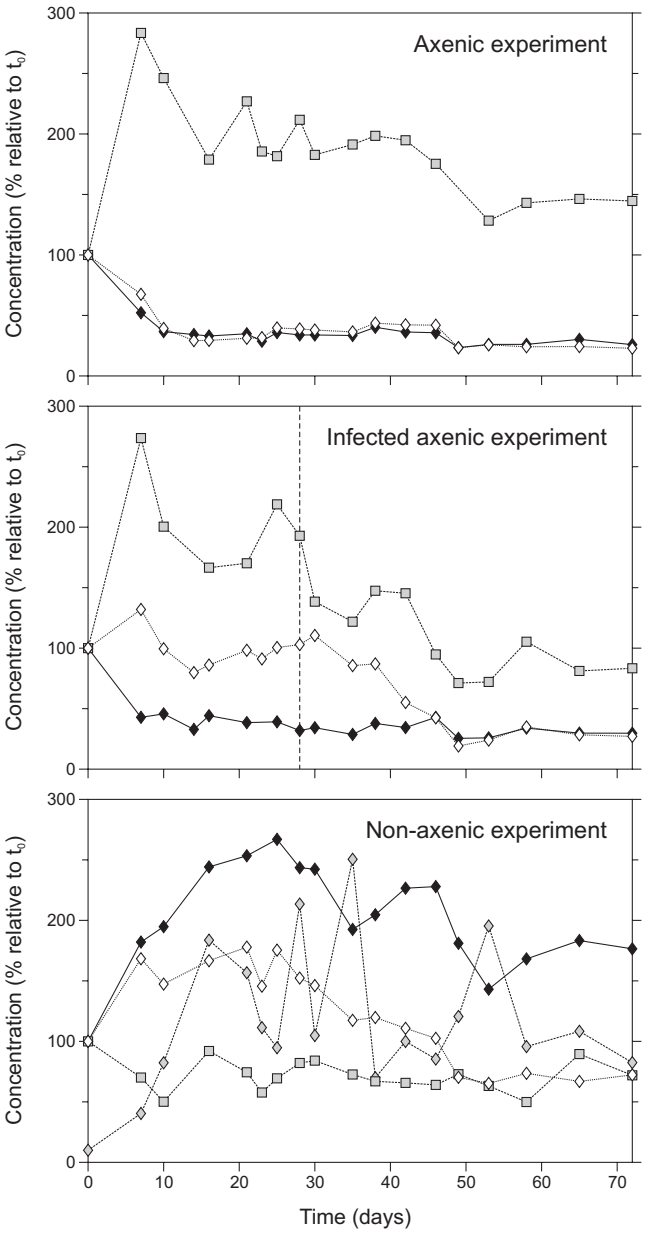

Figure 2.4 Concentration changes of total PG (black diamonds), total PE (shaded diamonds; values x 0.1), total PC (open diamonds) and total SQDG (shaded squares) in each of the experiments over time. The concentrations are normalized to the starting concentrations at day 0: 27 ng ml^{-1} PC, 13 ng ml^{-1} PG, 181 ng ml^{-1} SQDG and 42 pg ml^{-1} PE (only in the non-axenic experiment). The dashed line in the second plot indicates the moment of infection with bacteria.

culture at the start of the incubation, while no major changes in DGDG were observed. The lack of a significant increase in these substitute IPLs implies that lipid remodelling was not a major cause for the decrease in PC either.

If lipid remodelling is thus excluded, the most plausible reason for the decrease in the IGPs, as well as the decrease in SQDG from day 7 onward, is autolysis of algal cells. The initial increase in SQDG coincided with the final growth phase of the culture during the first few days of the experiment and could indicate enhanced biosynthesis of thylakoid membrane in response to darkness (Khotimchenko and Yakovleva, 2004, 2005). Over the first 16 days of the incubation the changes in IGPs correlated well with the changes in living cell numbers in the culture (linear correlation, $R^2 = 0.88$, n=5). However, from around day 16 onward the number of living cells continued to decrease, while levels of IGPs decreased much less and remained constant after day 46. This difference was most likely caused by the accumulation of dead cells in the culture, and as a consequence from day 25 onward the relative changes in the IGPs correlated well with the changes in total cell numbers (linear correlation, $R^2 = 0.90$, n=12). In a similar way, the relative changes in SQDG did not correspond with the changes in living cell numbers in the culture, but instead from day 7 onward correlated well with the changes in total cell numbers (linear correlation, $R^2 = 0.81$, n=18). Thus, it seems that, after the initial growth phase, a substantial amount of the IPLs measured was contained in dead cells or cell fragments (e.g., Harvey *et al*., 1986; Moriceau *et al*., 2009).

2.3.2 Infected axenic degradation experiment

The infected axenic degradation experiment represented a mixed model system in which part of the IPL degradation was initially induced by autolysis of algal cells, and part of the IPL degradation was subsequently mediated through bacteria. The bacteria were accidentally introduced partway into an originally axenic incubation experiment (day 28). Between days 32 and 49 the bacterial numbers increased rapidly to a maximum of 4.1×10^7 cells ml^{-1}, and they remained high until the end of the incubation (Fig. 2.1). The changes in total diatom counts over time were comparable to the axenic degradation experiment, with an increase from 2.5×10^5 cells ml^{-1} to a maximum of 3.2×10^5 cells ml^{-1} over the first few days, followed by a decrease from day 3 onward to a final 1.1×10^5 cells ml^{-1} (Fig. 2.1). No notable change in the rate of decrease of the total counts was observed after infection of the culture. Matching the observations of the axenic degradation experiment, the relative contribution of living cells decreased to less than 1% of the total cell count after 53 days (Fig. 2.3). A re-growth experiment initiated at this point failed to revive the culture, confirming full senescence.

In this experiment the relative concentration of PG showed a similar trend as in the axenic experiment, with approximately 27% of the initial concentration remaining at the end of the incubation (4 ng ml^{-1}; Fig. 2.4). There was no noticeable effect of the bacterial infection on the PG concentrations. The changes in SQDG concentration were comparable as well, although the overall decrease during the second half of the

incubation was larger than in the axenic experiment. Nonetheless, approximately 80% of the initial SQDG concentration remained at the end of the incubation (153 ng ml^{-1}), which amounts to 30% relative to the maximum at day 7. In contrast to the fully axenic experiment, PC showed an increase of 30% over the first 7 days of the incubation (to 36 ng ml^{-1}), before rapidly decreasing again by a similar amount. Around the time of the bacterial infection a second decrease in PC of approximately 70% occurred, and at the end of the incubation approximately 27% of the initial PC concentration remained (7 ng ml^{-1}). Relative to the maximum value at day 7, the total decrease amounted to 80%, of which approximately 50% took place after the bacterial infection.

These results show that the bacterial infection substantially enhanced IPL degradation, with PC and SQDG decreasing by an additional 50% and 20% compared to the axenic experiment. The smaller loss of SQDG compared to PC could indicate greater resistance of these glycosidically bound IPLs to bacterial action (see also Harvey *et al.*, 1986; Harvey and Macko, 1997). Surprisingly, the additional losses were not offset by bacterial synthesis of IPLs after day 32. In particular PE and PG are commonly found in bacterial membranes (Shaw, 1974; Lechevalier and Lechevalier, 1989; Dowhan, 1997), but no significant increases in these IGP concentrations were detected after bacterial infection. It is possible that these particular bacteria do not synthesize IGPs at all, or only in low quantities. However, it could also indicate that the total bacterial biomass in this experiment remained small compared that of the much larger diatoms.

As in the fully axenic degradation experiment, the relative changes in the IGPs correlated well with the changes in living cell numbers over the first 16 days (linear correlation, $R^2 = 0.88$, n=5), but rather with total cell numbers from day 21 onward (linear correlation, $R^2 = 0.83$, n=14). However, from approximately day 35 onward the levels of PC decreased at a higher rate than the number of total cells, concurrent with the bacterial breakdown of algal cells. The relative changes in SQDG behaved in a comparable way to the axenic degradation experiment until day 32. For the remainder of the experiment however, the decrease in SQDG was larger than that of the total cells, implying increased degradation of these IPLs due to bacterial activity. Nonetheless, the relative changes in SQDG again corresponded well with the overall changes in total cell numbers (linear correlation, $R^2 = 0.83$, n=18).

2.3.3 Non-axenic degradation experiment

In the non-axenic experiment, the culture was purposefully infected from the onset with a full suite of natural marine bacteria (but no grazers). Bacterial numbers in this culture increased exponentially over the first 10 days, and more gradually thereafter, to a maximum value of 3.7×10^7 cells ml^{-1} at day 18, before steadily declining again to 1.4×10^7 cells ml^{-1} (Fig. 2.1). The total diatom counts showed a more pronounced decrease than in the two previous experiments, from 2.5×10^5 cells ml^{-1} to 5.8×10^4 cells ml^{-1}, with 90% occurring in the first 21 days. In addition, the relative amount of living cells

decreased more rapidly as well, to 10% of the total cell count after 21 days. However, both the total cell count and the percentage of living cells in the culture stabilized after this point (Figs. 2.1 and 2.3). Intriguingly, the culture did not reach full senescence, and it was successfully revived by re-growth experiments initiated at days 53 and 72.

In this experiment the relative concentrations of the three IGPs (PC, PG and PE) increased over the first 25 days of the incubation (Fig. 2.4). PC concentrations almost doubled (to 50 ng ml^{-1}) before gradually decreasing again to 70% (20 ng ml^{-1}), while PG increased almost 3-fold (to 36 ng ml^{-1}) before decreasing to 170% (23 ng ml^{-1}), and PE increased 25-fold (to 1 ng ml^{-1}) before decreasing to around 10 times its initial concentration (400 pg ml^{-1}) in the final weeks of the incubation. The initial increases in these IGPs corresponded with the increase in bacteria in the culture, making them the likely source for these compounds. Although the peak bacterial numbers in this experiment were somewhat lower than in the infected axenic experiment, either their IGP content per cell, or their total biomass must have been much higher. In contrast to the (initially) axenic experiments, the relative concentrations of SQDG showed a 30% decrease over the first 7 days of the incubation (to 127 ng ml^{-1}), but remained more or less stable throughout the rest of the experiment. As the decrease in SQDG corresponded with the increase in bacteria, this implies microbial degradation of these IPLs. The relatively high proportion (70%) of SQDG escaping degradation again indicates that they are more resistant to bacterial action than the IGPs.

2.4 IMPLICATIONS

Our results agree with, and extend those of previous IPL degradation experiments (White *et al.*, 1979; Tollefson and McKercher, 1983; Petersen *et al.*, 1991; Harvey *et al.*, 1986; Moodley *et al.*, 2000). Although these were performed with purified IPL standards or different microbial cultures, we found a similar degradation pattern for the IPLs as in those studies, with a rapid decrease in concentration of up to 60% over the first two 10-16 days, followed by a much smaller decrease (<15%) over the remainder of the incubation time. This suggests that the observed IPL degradation patterns may be quite general and independent of the source organism.

While similar patterns were observed in both (initially) axenic experiments, the accidental introduction of bacteria substantially enhanced IPL degradation rates, with additional losses of both PC (50%) and SQDG (20%) occurring simultaneously with peak bacterial growth (see also Harvey and Macko, 1997). In the non-axenic experiment, the losses in algal IPLs were likely masked by the synthesis of bacterial IPLs, but an additional loss of SQDG (30%) was observed here as well. However, as in the previous studies the IPL concentrations in the *C. calcitrans* cultures did not drop below detection limit. Thus, a substantial fraction (20-30%) of the IPLs escaped even bacte-

rially-enhanced degradation, implying that IPLs can potentially be preserved on timescales of weeks to months, and perhaps even years (as extrapolated by Schouten *et al.*, 2010). In contrast to most of the previous studies our data also allow to infer degradation rates of IPL classes with different head groups under comparable circumstances. PC and PG behaved in a similar fashion, but SQDG degraded at an overall much slower rate, decreasing only half as much in concentration as the IGPs in the axenic experiment. Combined with the observations of Harvey *et al.* (1986), who found substantially slower degradation rates for glycosidic ether lipids than for ester-bound IGPs, this result indicate that glycolipids in general may be more stable and could persist longer in the environment than IGPs (see also Bauersachs *et al.*, 2010; Schouten *et al.*, 2010).

An important question that can be addressed with our experiments is whether IPL quantities accurately reflect living microbial cell numbers. Over the first 16 days of the incubations, the changes in IPL concentrations in the (initially) axenic cultures were indeed closely linked to the numbers of living cells. However, over time the IPL degradation rates decreased dramatically, and at the end of the incubations approximately 25% of the PC and PG, and approximately 50% of the SQDG remained intact, while the cultures had reached full senescence. In fact, from around day 21 onward the changes in IPL concentrations were correlated with total cell numbers, including dead cells and cell fragments, rather than with living cells. This implies that care has to be taken in interpreting IPLs, and especially glycolipids, to be exclusively derived from living cells, in particular in settings where the (bacterial) breakdown of deceased cells is slow, for example under low oxygen conditions (e.g., Harvey *et al.*, 1986, 1995; Boetius and Lochte, 2000), or where IPLs have the opportunity to bind to organic or inorganic substances (e.g., Harvey *et al.*, 1986; Moriceau *et al.*, 2009). Although it is unlikely that 'fossil' IPLs will constitute a significant amount of the total IPL pools in environments where large amounts of active living cells are present, such as the upper part of the water column or water-sediment interfaces, they could contribute substantially in environments where microbial communities are present in low abundance or have slow metabolisms, such as anoxic water columns or the deep biosphere.

2.5 CONCLUSIONS

Our IPL degradation experiments show that a substantial IPL pool persists in dead cells and cell fragments, and not exclusively in living cells. When bacteria are present, this residual IPL pool is greatly reduced, but not completely removed. Although IPL quantities appear to be a good proxy for living microbial cells in natural environments where the relative abundance of living cells is high, part of the IPL pool could be preserved over longer timescales (months to years) in some environments, in particular in settings with low microbial biomass or activity. In addition, glycosidically bound

glycerolipids appear to be more resistant to both autolytic and bacterially mediated degradation than glycerophospholipids, and are therefore less suitable as a proxy for living microbial cells.

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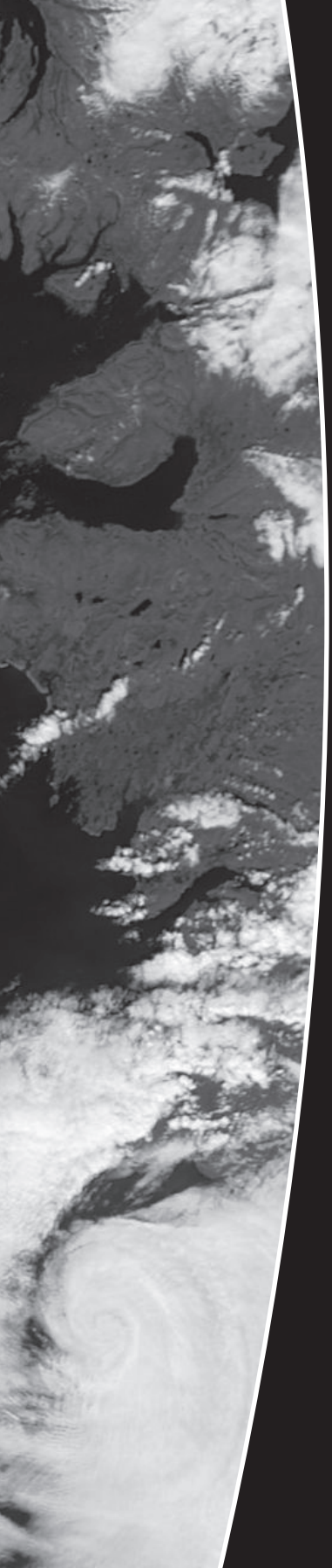

CHAPTER 3

A MULTI-PROXY STUDY OF ANAEROBIC AMMONIUM OXIDATION IN MARINE SEDIMENTS OF THE GULLMAR FJORD, SWEDEN

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is an important process for nitrogen removal in marine pelagic and benthic environments and represents a major sink in the global nitrogen cycle. We applied a suite of complementary methods for the detection and enumeration of anammox activity and anammox bacteria in marine sediments of the Gullmar Fjord, and compared the results obtained with each technique. ^{15}N-labeling experiments showed that nitrogen removal through N_2 production was essentially limited to the upper 2 cm of the sediment, where anammox contributed 23-47% of the total production. The presence of marine anammox bacteria belonging to the genus "*Candidatus* Scalindua" was shown by 16S rRNA gene sequence comparison. FISH counts of anammox bacteria correlated well with anammox activity, while quantitative PCR may have underestimated the number of anammox bacterial 16S rRNA gene copies at this site. Potential nitrogen conversion by anammox ranged from 0.6 to 4.8 fmol N cell^{-1} day^{-1}, in agreement with previous measurements in the marine environment and in bioreactors. Finally, intact ladderane glycerophospholipid concentrations better reflected anammox activity and abundance than ladderane core lipid concentrations, most likely because the core lipid fraction contained a substantial fossil component, especially deeper in the sediment.

3.1 INTRODUCTION

Anammox bacteria form a monophyletic group within the order of *Brocadiales*, which is affiliated to the *Planctomycetes* and currently includes five genera (Strous *et al.*, 1999a; Jetten *et al.*, 2009). This group specializes in the anaerobic oxidation of ammonium to dinitrogen gas, using nitrite as electron acceptor (Strous *et al.*, 1999a; Kartal *et al.*, 2007). The widespread occurrence of anammox bacteria in marine environments and the high contributions to N_2 production that have been measured in oxygen depleted systems, suggest that anammox represents a major sink in the global nitrogen cycle (Devol, 2003; Arrigo, 2005).

A variety of different techniques have been used to measure anammox activity and to quantify the abundance of anammox bacteria in natural environments (Schmid *et al.*, 2005; Rattray, 2008). Stable isotope labeling was first applied to confirm the biological origin of anammox (Van de Graaf *et al.*, 1995), and it remains the most frequently used method to quantify anammox activity. The method is based on the stoichiometry of the anammox reaction, which pairs one ammonium to one nitrite during N_2 formation. Consequently, the formation of $^{29}N_2$ ($^{15}N^{14}N$) gas following incubation with ^{15}N-labeled nitrite or nitrate is used to estimate the potential anammox activity (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al.*, 2004).

For the detection and enumeration of the responsible microorganisms, molecular techniques are employed that either enable the direct counting of anammox bacterial cells using fluorescence in situ hybridization (FISH), or the quantification of anammox bacterial DNA or RNA gene copies by quantitative polymerase chain reaction (q-PCR) (e.g., Schmid *et al.*, 2005; Hamersley *et al.*, 2007; Byrne *et al.*, 2009; Lam *et al.*, 2009). Using these techniques, "*Candidatus* Scalindua" is by far the most frequently observed anammox genus in the marine environment to date (e.g., Kuypers *et al.*, 2003; Penton *et al.*, 2006; Schmid *et al.*, 2007; but see Byrne *et al.*, 2009).

The presence of anammox bacteria can also be inferred from the detection and quantification of ladderane lipids. These unusual lipids contain a number of linearly concatenated cyclobutane rings and are specific biomarker compounds for anammox bacteria (Sinninghe Damsté *et al.*, 2002; Sinninghe Damsté *et al.*, 2005). Ladderane lipids are predominately enumerated as core lipid derivatives (Kuypers *et al.*, 2003, 2005; Hopmans *et al.*, 2006; Hamersley *et al.*, 2007; Jaeschke *et al.*, 2007; Byrne *et al.*, 2009), but are known to occur as intact ladderane glycerophospholipids (ladderane IGPs) within the cell (Boumann *et al.*, 2006). As abundances of ladderane IGPs, such as C_{20}-[3]-ladderane monoalkylether-phosphocholine, may more accurately reflect living biomass than ladderane core lipids, they could be more suitable biomarkers for viable anammox bacteria (e.g., Jaeschke *et al.*, 2009).

While the above techniques have mostly been applied individually or in pairs, to date no study has applied all five simultaneously at the same site. In this study ^{15}N-labeling,

FISH, q-PCR and ladderane core lipid and IGP analyses were combined for the first time to simultaneously determine the activity, phylogeny and distribution of anammox bacteria in marine sediments. The study site was the Gullmar Fjord on the Swedish Skagerrak coast, which has been extensively studied for marine nitrogen cycling, and where anammox was estimated to account for almost half of the total N_2 production (Engström *et al.*, 2005; Trimmer *et al.*, 2006). Furthermore, anammox bacteria belonging to the genus "*Candidatus* Scalindua" (Schmid *et al.*, 2007; Van de Vossenberg *et al.*, 2008), as well as ladderane core lipids (Hopmans *et al.*, 2006) have been detected in surface sediments from this site. Comparing the results obtained with each of the different techniques at the same site allows us to address some of their limitations and suitability for the study of anammox in marine sediments.

3.2 MATERIALS AND METHODS

3.2.1 Study site and sampling

All cores used for this study were obtained in April 2005 in the Gullmar Fjord on the Swedish Skagerrak coast. Samples were taken at the deepest part of the fjord (Alsbäck), at a water depth of 116 to 118 m. Sediment was collected with an Olausen box-corer, from which sediment cores (10 cm internal diameter) were sub-sampled. The cores were taken with Plexiglas tubes, which were driven into the sediment, sealed, removed from the box-corer and transported intact to a cold room (6ºC).

On land, one sediment core was used intact for oxygen microelectrode profiles, while the remaining cores were sectioned horizontally. One core was used for pore water inorganic nitrogen (NH_4^+, NO_3^- and NO_2^-), one core to extract DNA for 16S rRNA gene sequence comparison, and four cores for ladderane lipid analysis and extraction of DNA for q-PCR analysis. Finally, three cores were pooled per depth interval and used for activity measurements and FISH counting.

3.2.2 Pore water profiles

Sedimentary pore water concentrations of O_2 were measured in triplicate with the use of Clark-type microelectrodes (Revsbech, 1989). For concentration profiles of pore water NH_4^+, NO_3^- and NO_2^-, one sediment core was sectioned in 0.25 cm increments (0-2 cm depth) and 0.5 cm increments (2-4 cm depth). Each section was placed in a 60 ml centrifuge tube and centrifuged for 20 min at 11,000 G, after which the supernatant water was filtered through a 0.45 µm cellulose acetate filter. NH_4^+, NO_3^- and NO_2^- were analyzed with an automatic analyzer (TRAACs 800, Braun Luebbe, Norderstedt, Germany), following standard colorimetric procedures (Strickland and Parsons, 1972).

3.2.3 Activity measurements

Potential anammox and denitrification rates in the sediment were determined by ^{15}N-labeling experiments (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al.*, 2004). Per depth interval, a duplicate time series of 5 data points was set up. After sectioning, the sediment was homogenized and approximately 2 ml (exact weight was determined for each sample) was placed in a 6 ml Exetainer vial (Labco, High Wycombe, UK). The vials were sealed and the headspaces gently flushed with helium. The sediments were then pre-incubated in the dark at 6°C for 24 h to eliminate any oxygen or NO_x^-. Following pre-incubation, ^{15}N solutions were injected into the vials through a rubber septum, using a Hamilton syringe. The vials were handled under a stream of helium, in order to maintain strictly anoxic conditions during injection of the ^{15}N solutions and to minimize contamination with atmospheric N_2 (Risgaard-Petersen *et al.*, 2004). Three treatments were performed in which $^{15}NH_4^+$, $^{15}NH_4^+ + {}^{14}NO_3^-$, and $^{15}NO_3^-$ isotopes were added by introducing 0.1 ml of 1 mmol l^{-1} solutions (final concentrations of 50 nmol isotope (ml wet sediment)$^{-1}$). The reaction in each sample set was stopped by injecting 100 µl of 7 mol l^{-1} $ZnCl_2$, after 0, 1, 2, 3, and 4 h, respectively. ^{15}N-labeled N_2 gasses were then measured by gas chromatography mass spectrometry (GC-MS) on a RoboPrep-G+ in line with Tracermass (Europa Scientific, Crewe, UK), as described by Risgaard-Petersen and Rysgaard (1995).

Potential anammox and denitrification rates were calculated from $^{29}N_2$ and $^{30}N_2$ production rates in the $^{15}NO_3^-$ incubation (Thamdrup and Dalsgaard, 2002). The $^{15}NH_4^+$ treatment was used as a negative control to detect any oxidation of ammonium in the sediment without the addition of nitrate. Assuming all oxidants are removed during the pre-incubation, this treatment should not show any production of $^{29}N_2$ or $^{30}N_2$. In the parallel $^{15}NH_4^+ + {}^{14}NO_3^-$ treatment, $^{29}N_2$ production implied that the added nitrate was the oxidizing agent for ammonium. The combination of these two treatments thus establishes anammox activity in the samples.

3.2.4 16S rRNA gene sequence comparison

DNA was extracted from the sediment for anammox bacterial 16S rRNA gene sequence comparison. Sediment aliquots were suspended in 10 ml of DNA extraction buffer (100 mmol l^{-1} Tris/HCL (pH 8.0), 100 mmol l^{-1} sodium EDTA (pH 8.0), 100 mmol l^{-1} sodium phosphate (pH 8.0), 1.5 mol l^{-1} NaCl, 1% CTAB), and the total genomic DNA was extracted as described by Juretschko *et al.* (1998). Preferential PCR amplification of 16S rRNA genes of members of the *Planctomycetales* was performed with the forward primer Pla46F (*E. coli* positions 46-63; Schmid *et al.*, 2000), in combination with a universal reverse primer (*E. coli* positions 1529-1545; Juretschko *et al.*, 1998). PCR was performed with a Tgradient cycler (Biometra, Göttingen, Germany), as described by Schmid *et al.* (2000). Negative controls (no DNA added) and positive controls (DNA from a "*Candidatus* Brocadia anammoxidans" enrichment culture added) were

included in all sets of amplifications. The optimal annealing temperature for the primer set Pla46F/630R regarding yield and specificity was 60°C (Schmid *et al.*, 2003). The presence and size of amplification products were determined by agarose gel electrophoresis of 5 µl aliquots of the PCR products. The biofilm-derived 16S rRNA gene amplificates were cloned directly, using the TOPO TA Cloning kit (Invitrogen, Groningen, The Netherlands) according to the instructions of the manufacturer. Plasmid-DNA was isolated with the FlexiPrep kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Plasmids with an insert of the expected size were identified by agarose gel electrophoresis after EcoRI digestion (5 U, EcoRI buffer for 3 h at 37°C). Sequencing was done non-radioactively using the BigDye Terminator Cycle Sequencing v2.0 kit, and the reaction mixtures were analyzed with a 3700 DNA Analyzer (both Applied Biosystems, Foster City, CA, USA). The complete sequences of the 16S rRNA gene fragments were determined using M13 forward and reverse primers targeting vector sequences adjacent the multiple cloning site, as well as using the universal Eubacterial primers 609F (*E. coli* positions 785-806; 5'-TTA GAT ACC CC(A/G/T) GTA GT-3') and 699R (*E. coli* positions 1099-1114; 5'-AGG GTT GCG CTC GTT GC-3'), targeting the 16S rRNA gene.

The gene sequences retrieved in this study were added to the 16S rRNA gene sequence database of the ARB program package (Ludwig *et al.*, 2004). The sequences were then aligned automatically and corrected manually. Phylogenetic analysis of the 16S rRNA gene sequences was performed by applying neighbor-joining, parsimony and maximum likelihood analyses (fastDNAml; Maidak *et al.*, 1996) to different datasets.

3.2.5 Fluorescence In Situ Hybridization

Anammox bacterial cell numbers in the sediment were determined by FISH counting. Sediment aliquots were fixated according to Schmid *et al.* (2003; 2007), and in the last step of the fixation the samples were suspended in 1 ml of 50% ethanol in phosphate-buffered saline (pH 7.4). A mixture of several probes was used for hybridization: the anammox-specific probes S-G-Sca-1309-a-A-21 (Sca1309), S-*-Scabr1114-a-A-22 (Scabr1114), S-*-BS-820-a-A-22 (BS820) and S-*-AMX-0368-a-A-18 (Amx368) (Schmid *et al.*, 2003); the *Planctomycetes*-specific probe S-P-Planc-0046-a-A-18 (Pla46) (Neef *et al.*, 1998); and a mixture of the probes S-D-Bact-0338-a-A-18, S-D-Bact-0338-b-A-18 and S-D-Bact-0338-c-A-18 (Amann *et al.*, 1990; Daims *et al.*, 1999). Details about the probes used in this study can be found in Schmid *et al.* (2003) and on http://www.microbial-ecology.de/probase/ (Loy *et al.*, 2003). All probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) labeled derivatives (Interactiva, Ulm, Germany). The hybridization method and optimal conditions for hybridization probes were taken from Schmid *et al.* (2000; 2003). After hybridization and air-drying, the slides were embedded in Vectashield (Vector Laboratories, Burlingame, CA, USA) with 4',6-diamidino-2-phenylindole (DAPI) for total cell counts. For image acquisition a Zeiss axioplan 2 microscope (Zeiss, Jena, Germany) was used. Of each sample, ten photos were randomly selected and the counts were executed by at least two people.

3.2.6 Quantitative PCR analysis

DNA was extracted from the sediment for the quantification of anammox bacterial 16S rRNA gene copy numbers by quantitative PCR (q-PCR), according to Jaeschke *et al.* (2010). Total DNA was extracted from freeze-dried sediment aliquots (100 mg) using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Part of the total DNA extract was subjected to agarose gel electrophoresis to determine DNA quality and quantity. All extracts contained mainly higher molecular weight DNA (~20 kbp fragments) at a concentration of >10 ng μl^{-1}.

Undiluted, as well as 5 and 25 times diluted, DNA extracts were used for the q-PCR analysis. As minor inhibition was observed, only the data from the 5 times diluted series were used. Real-time PCR amplification was performed using the 16S rRNA gene specific primer set Brod541F (5'-GAG CAC GTA GGT GGG TTT GT-3') – Brod1260R (5'-GGA TTC GCT TCA CCT CTC GG-3') for detecting anammox bacteria in sediments (Penton *et al.*, 2006). Reaction mixtures (20 µl) contained 1 unit of Picomaxx high-fidelity DNA polymerase and 2 µl of 10x Picomaxx PCR buffer (both Stratagene, La Jolla, CA, USA), as well as 0.25 mmol each of dNTP, 8 µg of BSA, 0.25 µmol of each primer, 5 x 10^4 times diluted SYBR Green (Invitrogen / Molecular Probes, Carlsbad, CA, USA), a final concentration of 10 nmol fluorescein, 3 mmol $MgCl_2$, and ultrapure sterile water (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). All reactions involved initial denaturing (4 minutes at 95°C), followed by 35-40 cycles including denaturing (30 seconds at 94°C), primer annealing (40 seconds at 62°C), a photomoment (25 seconds at 80°C), and primer extension (60 seconds at 72°C). Real-time PCR was performed using an iCycler (Biorad, Hercules, CA, USA). Calibration of the samples was performed by using a dilution series (10^7 to 10^2 copies $reaction^{-1}$) of the 719 bp purified PCR product from an enrichment culture of "*Candidatus* Scalindua wagneri". Real-time PCR was followed by meltcurve analysis and gel electrophoresis to check for the presence of unspecific products and primer dimers, neither of which were detected. In addition, no signal was observed in the negative PCR control.

3.2.7 Ladderane lipid analysis

Concentrations of four ladderane core lipids (C_{18}-[5]-ladderane, C_{20}-[5]-ladderane and C_{20}-[3]-ladderane, as well as C_{20}-[3]-ladderane monoalkylether; Sinninghe Damsté *et al.*, 2005) and one intact ladderane glycerophospholipid (C_{20}-[3]-ladderane monoalkylether-phosphocholine; Boumann *et al.*, 2006) in the sediment were enumerated. Sediment samples were extracted for ladderane core lipid analysis, following the procedure of Hopmans *et al.* (2006). The freeze-dried and homogenized sediments were extracted five times ultrasonically in a solvent mixture of dichloromethane (DCM) and methanol (MeOH) (2:1 v/v). The bulk of the solvents was removed by rotary evaporation under vacuum, and the extracts were further dried over small Na_2SO_4 columns. An aliquot of the lipid extract was saponified with 1 N potassium hydroxide (KOH in MeOH 96%) at 100°C

for 1 h. Non-saponifiable lipids (neutral lipids) were extracted out of the basic solution (pH>13) three times with DCM. Fatty acids were obtained by acidifying the residue to pH 3, and subsequently extracting three times with DCM. The fatty acid fraction was then methylated by adding diazomethane (CH_2N_2), to convert the fatty acids into their corresponding fatty acid methyl esters (FAMEs). The excess diazomethane was removed by evaporation. Both the neutral and fatty acid fractions were dried over Na_2SO_4 columns. To remove polyunsaturated fatty acids from the fatty acid fractions, these were successively eluted with ethyl acetate over small columns filled with silica and $AgNO_3$ (5%) silica, yielding saturated fatty acids fractions. These fractions were finally re-dissolved in acetone and filtered through 0.45 µm PTFE filters (4 mm diameter) before analysis.

Ladderane core lipids were analyzed by high performance liquid chromatography, coupled to atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS^2), under conditions described by Hopmans *et al.* (2006). The setup consisted of an Agilent 1100 LC, containing an inline membrane degassing unit, thermostatted auto-injector and column compartment, coupled to a Quantum TSQ Ultra EM triple quadrupole mass spectrometer, equipped with an Ion max source with an APCI probe (Thermo, San Jose, CA, USA). Separation was achieved on two Zorbax Eclipse XDB-C8 columns (4.6 x 150 mm, 5 µm particles) (Agilent Technologies, Santa Clara, CA, USA), coupled in series and maintained at 30°C. Ladderane lipids were eluted with 0.4 ml min^{-1} MeOH, with a total runtime of 20 min. Detection was achieved by positive ion APCI and selective reaction monitoring (SRM) of four specific fragments for each measured ladderane lipid (Hopmans *et al.*, 2006). The source settings were: vaporizer temperature 475°C, discharge current 2.5 µA, sheath gas (N_2) pressure 50 (arbitrary units), auxiliary gas (N_2) pressure 5 (arbitrary units), capillary temperature 350°C, and source CID -10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of the ladderane lipids was done using external calibration curves of standards of isolated ladderane FAMEs and the C_{20}-[3]-ladderane monoalkylether (Sinninghe Damsté *et al.*, 2002; Hopmans *et al.*, 2006). A detection limit (defined by a signal-to-noise ratio of 3) of 30-35 pg injected on-column was achieved by this technique.

Sediment samples were also extracted for the analysis of the ladderane IGP C_{20}-[3]-ladderane monoalkylether-PC (Boumann *et al.*, 2006), using a modification of the method of Bligh and Dyer (1959). The freeze-dried and homogenized samples were extracted three times ultrasonically in a solvent mixture of methanol, dichloromethane and phosphate buffer (pH 7.4) (2:1:0.8 v/v/v). After extraction, further DCM and buffer were added to achieve phase separation (final ratio 1:1:0.9 v/v/v). The DCM phases were collected and the bulk of the solvents removed by rotary evaporation under vacuum. The extracts were further dried under a stream of N_2 gas. Aliquots were finally re-dissolved in DCM:MeOH (9:1 v/v) and filtered through 0.45 µm True Regenerated Cellulose filters (4 mm diameter; Grace Alltech, Deerfield, IL, USA) before analysis. The C_{20}-[3]-ladderane monoalkylether-PC was analyzed by high performance liquid chromatography, coupled to electrospray ionization tandem mass spectrometry (HPLC/ESI-MS^2), as described by Jaeschke *et al.*, 2009. The setup consisted of an Agilent 1100 LC

(see above) coupled to a Quantum TSQ Ultra EM triple quadrupole mass spectrometer, equipped with and Ion max source with an ESI probe (Thermo, San Jose, CA, USA). Separation was achieved on a LiChrospher diol column (2.1 x 250 mm, 5 µm particles) (Grace Alltech, Deerfield, IL, USA), maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 ml min^{-1}: 90% A + 10% B to 70% A + 30% B over 10 min, maintained for 20 min, then to 35% A + 65% B in 15 min, maintained for 15 min, then back to 100% A for 20 min to re-equilibrate the column. Solvent A consisted of a mixture of hexane, 2-propanol, formic acid and 14.8 M NH_3 (aq) in a ratio of 79:20:0.12:0.04 (v/v/v/v), while solvent B had the same constituents, but in a ratio of 88:10:0.12:0.04 (v/v/v/v). Detection of the C_{20}-[3]-ladderane monoalkylether-PC was achieved by positive ion ESI and SRM of the transition from *m/z* 530 $[M+H]^+$ to *m/z* 184 (corresponding to the PC head group), with 1.5 mTorr argon as collision gas and 20 V collision energy. Quantification of the ladderane IGP was done using an external calibration curve of an isolated C_{20}-[3]-ladderane monoalkylether-PC standard (for details see Jaeschke *et al.*, 2009). A detection limit of ~10 pg injected on-column was achieved with this technique.

3.3 RESULTS AND DISCUSSION

3.3.1 Pore water profiles and activity measurements

The Gullmar Fjord has a highly stratified water column, with stagnant bottom waters during most of the year and storm-induced winter mixing (Svansson, 1984). The sediments for this study were collected during spring from the deepest part of the fjord (Alsbäck) at a water depth of 116 to 118 m. The *in situ* temperature was 6°C, and the bottom water had an O_2 concentration of 210 µmol l^{-1} and a NO_3^- concentration of 15 µmol l^{-1}.

Free oxygen only penetrated the top of the sediment and was depleted (<0.02 µmol l^{-1}) below 0.7 cm depth (Fig. 3.1A). A strong NO_3^- concentration gradient was observed between the bottom water (15 µmol l^{-1}) and the surface sediment (5 µmol l^{-1}), and NO_3^- concentrations continued to decrease downcore to less than 0.5 µmol l^{-1} below 2.5 cm depth. Within the nitrate reduction zone (0-1.5 cm depth) a nitrite peak of 0.15 µmol l^{-1} was detected, below which NO_2^- concentrations gradually decreased with depth to 0.02 µmol l^{-1}. The NH_4^+ profile did not indicate a removal of ammonium by anammox, as has been observed in deep water sediments or oxygen minimum zones (Kuypers *et al.*, 2003, 2005; Engström *et al.*, 2009). Instead NH_4^+ concentrations increased linearly from 0.7 µmol l^{-1} at the surface to 15 µmol l^{-1} in the 2-2.5 cm depth interval, and continued to increase more gradually to 20 µmol l^{-1} at 4 cm depth.

^{15}N-labeled nitrate was used to estimate potential anammox and denitrification activity in the sediment (Fig. 3.1B). It should be noted that with this technique the potential activity is measured, while the actual *in situ* activity might be lower due to oxygen

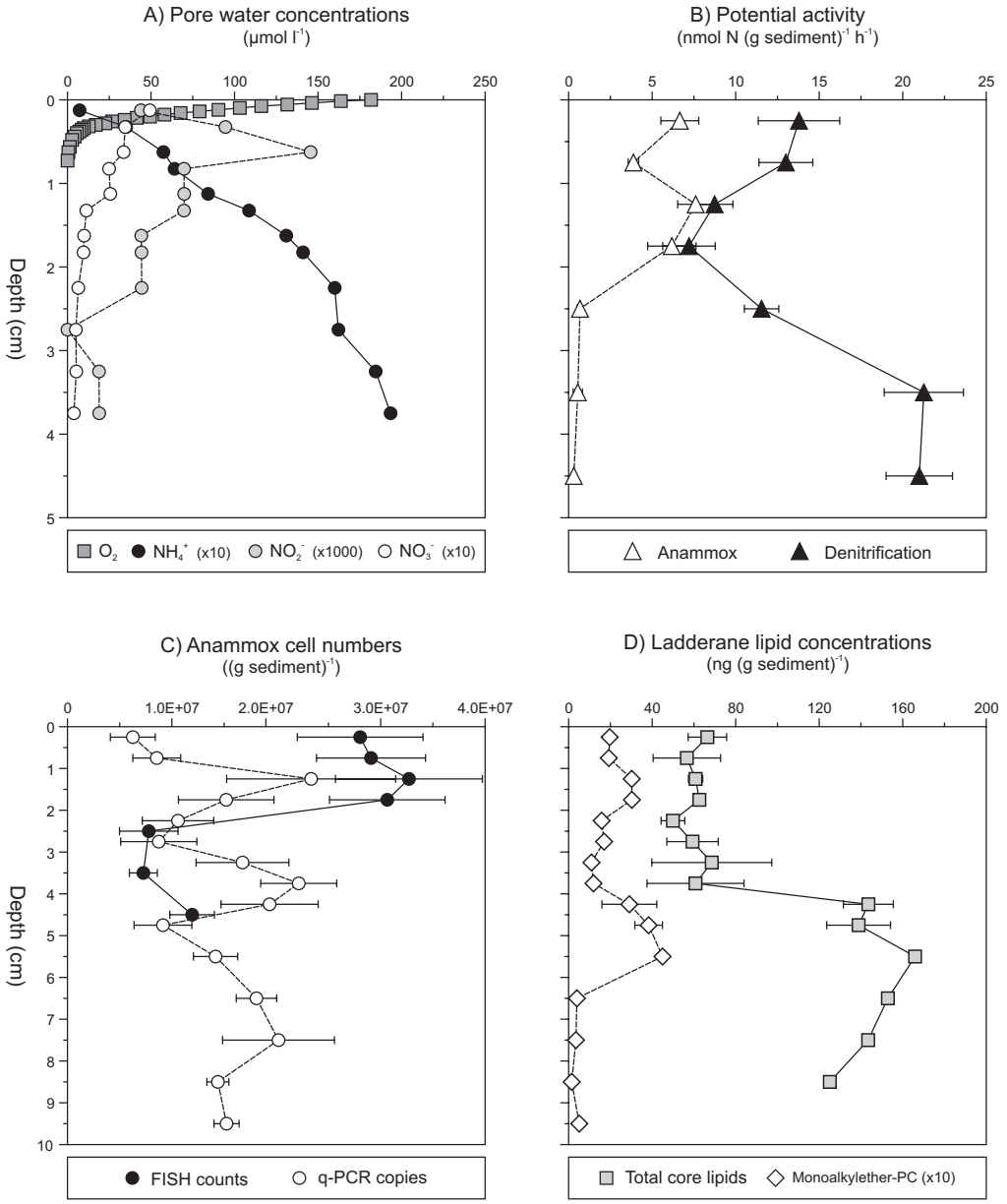

Figure 3.1 Depth profiles of (A) pore water concentrations of O_2, NH_4^+, NO_3^- and NO_2^-; (B) potential anammox and denitrification activity; (C) anammox cell numbers as determined by FISH and q-PCR, assuming one 16S rRNA copy per cell; (D) concentrations of summed ladderane core lipids (C_{18}-[5]-, C_{20}-[5]- and C_{20}-[3]-ladderane fatty acids, and C_{20}-[3]-ladderane monoalkylether) and ladderane IGP (C_{20}-[3]-ladderane monoalkylether-PC) in the Gullmar Fjord sediments. The error bars represent standard errors of the mean.

inhibition or substrate limitation (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al.*, 2004; Hamersley *et al.*, 2007). The potential anammox activity was highest in the upper 2 cm, with an average of 6.1±1.6 nmol N (g sediment)$^{-1}$ h^{-1}, and a maximum of 7.6 nmol N (g sediment)$^{-1}$ h^{-1}. Below 2 cm depth the anammox activity was less than 10% of the surface value, with an average of 0.5±0.2 nmol N (g sediment)$^{-1}$ h^{-1}. In several water column studies the highest potential anammox activities were found just below the oxic zone, coinciding with a distinct nitrite maximum (Kuypers *et al.*, 2003; Thamdrup *et al.*, 2006; Hamersley *et al.*, 2007). In the Gullmar Fjord sediments a similar pattern could be recognized, with the maximum potential anammox activity occurring in the 1-1.5 cm depth interval. The average activity value of 6.1 nmol N (g sediment)$^{-1}$ for the top of the sediment is comparable to previous measurements for the same depth interval at this site (3.5-6.0 nmol N (g sediment)$^{-1}$; Engström *et al.*, 2005; Schmid *et al.*, 2007). The pronounced anammox activity observed in the top 0.5 cm of the sediment may support the idea that anammox bacteria can thrive in (partly) oxygenated environments (Kuypers *et al.*, 2005; Thamdrup *et al.*, 2006; Jensen *et al.*, 2007; Jaeschke *et al.*, 2009), although it is likely that the *in situ* activity was lower than the measured potential activity.

The potential denitrification activity in the sediment generally ranged from 13.8 to 21.3 nmol N (g sediment)$^{-1}$ h^{-1}, with distinctly lower rates down to 7.2 nmol N (g sediment)$^{-1}$ h^{-1} in the 1-2 cm depth interval (Fig. 3.1B). The interval of decreased potential denitrification coincided with that of maximum potential anammox activity. Of the total N_2 production in the upper 2 cm of the Gullmar Fjord sediments, 23-47% was accounted for by anammox. This is in agreement with previous studies at this site, in which anammox was estimated to contribute 40-48% in the same depth interval (Engström *et al.*, 2005; Trimmer *et al.*, 2006). Deeper in the sediment denitrification was the dominant process, with anammox accounting for only 1-6% of the total sedimentary N_2 production. Since ammonium was present in high concentrations, a limitation in nitrite supply could have impeded anammox below 2 cm depth. Inhibition of anammox by hydrogen sulfide is less likely at this site, due to the high concentrations of metal (Fe, Mn) oxides in the sediment (Engström *et al.*, 2005).

3.3.2 Anammox phylogeny, cell abundance and distribution

16S rRNA gene sequences of the anammox bacteria in the Gullmar Fjord sediments formed two clusters, both of which fell within those containing the genus "*Candidatus* Scalindua". These sequences had high sequence identities with "*Candidatus* S. wagneri" (92.4%), "*Candidatus* S. sorokinii" (96.6%) and "*Candidatus* S. brodae" (96.7%) (Fig. 3.2). Our results thus support previous findings of anammox bacteria related to "*Candidatus* Scalindua" at this locality (Schmid *et al.*, 2007; Van de Vossenberg *et al.*, 2008).

Two molecular techniques, FISH counting and 16S rRNA gene quantification by q-PCR, were used to determine the anammox bacterial cell abundance and distribution in the sediment. FISH counts showed that the number of anammox bacteria was

highest in the upper 2 cm of the sediment, with an average of 3.0±0.2 x 10^7 cells (g wet sediment)$^{-1}$ (Fig. 3.1C). Below 2 cm depth the number of anammox bacteria was substantially lower, with an average of 9.0±2.6 x 10^6 cells (g wet sediment)$^{-1}$. Total bacterial counts (data not shown) averaged 3.3±0.4 x 10^8 (g wet sediment)$^{-1}$ in the upper 2 cm of the sediment, then dropped sharply to 1.6 x 10^8 (g wet sediment)$^{-1}$ in the 2-4 cm depth interval, and increased again to 4.7 x 10^8 (g wet sediment)$^{-1}$ below 4 cm depth. Of the total number of bacterial cells in the upper 2 cm of the sediment, anammox bacteria thus comprised up to 11%, which is 2-4 times higher than previously reported from Gullmar Fjord sediments (Schmid *et al.*, 2007).

The high number of anammox cells in the top layer corresponded with the high potential anammox activity in the upper part of the sediment, while in the underlying zone of low anammox activity the number of anammox cells also decreased sharply, to less than 30% of the surface value. However, this decrease was lower than the reduction in anammox activity, which could imply a downcore increase in the proportion of dormant cells.

In contrast, the q-PCR profile showed an increase in anammox bacterial 16S rRNA gene copy numbers from 6.3 x 10^6 copies (g sediment)$^{-1}$ at the surface to 2.3 x 10^7 copies (g sediment)$^{-1}$ in the 1-1.5 cm depth interval, before decreasing again downcore (Fig. 3.1C). A second maximum was observed in the 3-4.5 cm depth interval (1.9 x 10^7 copies (g sediment)$^{-1}$) and numbers were high in the deepest part of the sediment as well (up to 1.8 x 10^7 copies (g sediment)$^{-1}$). Assuming that anammox bacteria hold only one copy of the 16S rRNA gene per cell, the q-PCR-derived cell numbers thus differed substantially from the FISH counts, although both methods indicated that approximately 3 x 10^7 anammox cells were present in the most active part of the sediment.

There may be several explanations for the poor match between the two methods used to quantify anammox bacterial abundance. The q-PCR was performed on a different sediment core than the activity measurements and FISH counts, and variations in the spatial distribution of anammox bacteria and anammox activity between the cores cannot be excluded (e.g., Prosser, 2010). Secondly, different probes and primers are used in each quantification technique, which could result in different detection efficiencies for the various anammox species, and therefore make a direct comparison difficult (Schmid *et al.*, 2003; Penton *et al.*, 2006). Detection of cells by FISH can be challenging for cells with low activity, since these may have a lower number of ribosomes (Pavlekovic *et al.*, 2009). The cell counts in the zone of low anammox activity (below 2 cm depth; Fig. 3.1B) could therefore be an underestimate of the actual cell numbers. In q-PCR, the PCR reaction can be hampered by sedimentary organic matter in the sample (Lindberg *et al.*, 2007; Kallmeyer and Smith, 2009), which would lead to reduced amplification and an underestimate of the actual copy numbers by this technique. As the reactive organic matter content of the sediment decreases rapidly with depth (S. Hulth, unpublished data), this effect would be highest in the top few centimeters. Conversely, the presence of fossil DNA in the anoxic sediment cannot be excluded (e.g., Coolen and Overmann 1998; Coolen *et al.*, 2004), and could lead to an overestimate of the actual copy numbers by q-PCR.

One way to evaluate the two quantification methods is by combining the activity measurements with the estimated cell numbers. When combined with the FISH counts, the anammox rate was 4.8±1.4 fmol N $cell^{-1}$ day^{-1} in the upper 2 cm of the sediment, and rapidly decreased to a minimum of 0.6 fmol N $cell^{-1}$ day^{-1} in the 4-5 cm depth interval. This calculated maximum rate is similar to those found in several water column oxygen minimum zones (Kuypers *et al.*, 2003, 2005; Hamersley *et al.*, 2007; Rattray, 2008), although it is almost 5 times higher than anammox rates observed previously in the Gullmar Fjord surface sediments (Schmid *et al.*, 2007). When combined with the q-PCR-derived cell numbers, the anammox rate was at least 25 fmol N $cell^{-1}$ day^{-1} in the upper 0.5 cm of the sediment, and showed a downcore decrease to a minimum of 0.5 fmol N $cell^{-1}$ day^{-1}. This calculated maximum rate is several times higher than the maximum detected rate in the natural environment to date (6.7 fmol N $cell^{-1}$ day^{-1}; Rattray, 2008), and would be outside the range calculated for anammox cells growing more or less optimally in sequencing batch reactors (2-20 fmol N $cell^{-1}$ day^{-1}; Strous *et al.*, 1999b). However, it should be noted that all of those rates were calculated using FISH counts, and not q-PCR-derived cell numbers. Nonetheless, it appears that q-PCR may have underestimated the abundance of anammox bacterial 16S rRNA copy numbers in the active top layer of the Gullmar Fjord.

3.3.3 Ladderane lipids

Four ladderane core lipids, differing in chain length and number of cyclobutane moieties (i.e., the C_{18}-[5]-, C_{20}-[5]- and C_{20}-[3]-ladderane fatty acids, as well as the C_{20}-[3]-ladderane monoalkylether; Sinninghe Damsté *et al.*, 2005), were analyzed in the Gullmar Fjord sediments by HPLC/APCI-MS^2. The relative contribution of the three ladderane fatty acids to the total ladderane core lipid concentration decreased gradually with depth, from 70% to 30%, while the relative contribution of the C_{20}-[3]-ladderane monoalkylether increased accordingly. However, the relative abundances of the ladderane fatty acids were more or less constant over depth and dominated by the C_{18}-[5]-ladderane (78%), with smaller amounts of the C_{20}-[3]-ladderane (12%) and the C_{20}-[5]-ladderane (10%). Total ladderane core lipid concentrations increased with depth, from an average of 61±6 ng (g sediment)$^{-1}$ in the top 4 cm to an average of 145±14 ng (g sediment)$^{-1}$ below this depth (Fig. 3.1D). The values for the surface sediments are comparable to those reported previously from the same site (88 ng (g sediment)$^{-1}$; corrected from Hopmans *et al.*, 2006). Furthermore, they are within the range previously reported from surface sediments at shallow water depths or in near-coastal environments (up to 97 ng (g sediment)$^{-1}$), where conditions for anammox are most favorable (Jaeschke *et al.*, 2009; 2010).

However, the depth profile did not match those of the anammox cell numbers and potential anammox activity. Whereas these were high in the upper 2 cm of the sediment and low below that depth, ladderane core lipid concentrations remained more or less stable throughout the same depth interval. This difference may have been caused by

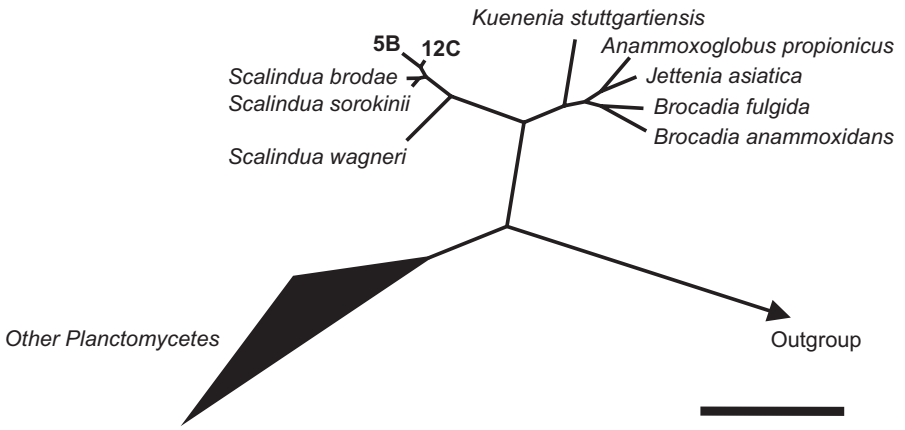

Figure 3.2 16S rRNA-based phylogenetic tree of anammox bacteria. The bacteria found in the Gullmar Fjord sediments (clones 5B and 12C, GenBank Accession Numbers EF602038 and EF602039, respectively) belong to the genus "*Candidatus* Scalindua", and are closely related to "*Candidatus* S. sorokinii" and "*Candidatus* S. brodae". The black bar indicates 10% sequence divergence.

preservation of ladderane core lipids in the anoxic sediments, leading to a fossilized component of the extractable lipid pool, as was noted previously in the Irish Sea (Jaeschke *et al.*, 2009).

To address this potential bias we quantified concentrations of the intact glycerophospholipid (IGP) C_{20}-[3]-ladderane monoalkylether-phosphocholine (PC) by HPLC/ESI-MS^2 at the same site. Anammox bacteria are known to synthesize a range of ladderane IGPs, consisting of a glycerol backbone with one or two ladderane fatty acids (the core lipid), and a phosphate-based headgroup (Boumann *et al.*, 2006; Rattray *et al.*, 2008). Upon cell death, IGPs are thought to be rapidly transformed into their core lipid derivatives due to the loss of their polar headgroup, and consequently they may represent a more accurate proxy for living microbial cells than the more stable core lipids (White *et al.*, 1979; Harvey *et al.*, 1986). The concentration profile of the C_{20}-[3]-ladderane monoalkylether-PC in the sediments of the Gullmar Fjord is shown in Figure 3.1D. In the upper 2 cm, the concentration varied between 2.0 and 3.0 ng (g sediment)$^{-1}$, with the highest values in the 1-2 cm depth interval. In the 2-4 cm depth interval the concentration decreased to 1.0 ng (g sediment)$^{-1}$, before increasing again to a second maximum of 4.5 ng (g sediment)$^{-1}$ at 6 cm depth. In the deepest part of the sediment, the concentration was very low at less than 0.5 ng (g sediment)$^{-1}$.

Since the C_{20}-[3]-ladderane monoalkylether-PC depth profile matches those of the anammox bacterial numbers and activity fairly well, it appears to be more suited as a biomarker for active anammox than ladderane core lipids. However, C_{20}-[3]-ladderane

monoalkylether-PC concentrations per cell did show a downcore increase from 0.08 fg cell^{-1} (0-2 cm depth) to 0.22 fg cell^{-1} (2-5 cm depth). As monoalkylether IGPs are potentially formed during cell lysis, by the loss of one fatty acyl or alkyl chain, increasing concentrations of this IGP with depth could indicate the presence of recently deceased cells. Furthermore, although they are known to degrade faster than core lipids, IGPs could potentially fossilize to some extent as well (e.g., Harvey *et al.*, 1986; Schouten *et al.*, 2010), and the presence of extracellular C_{20}-[3]-ladderane monoalkylether-PC in these sediments cannot be excluded. Finally, enrichment cultures of "*Candidatus* Scalindua" were recently shown to contain almost no C_{20}-[3]-ladderane monoalkylether-PC (Rattray *et al.*, 2008), while it was abundant in enrichment cultures of other anammox genera (Boumann *et al.*, 2006; Rattray *et al.*, 2008). Although its near absence in "*Candidatus* Scalindua" cultures may have depended on the applied cultivation conditions, the downcore increase in C_{20}-[3]-ladderane monoalkylether-PC could therefore also have been due to a predominance of different anammox genera deeper in the sediment.

3.4 CONCLUSIONS

A range of complementary methods was used to determine the activity, phylogeny and distribution of anammox bacteria in sediments from the Gullmar Fjord. Nitrogen removal through anammox could be detected in the upper 2 cm of the sediment, where anammox contributed 23-47% of the total N_2 production. Gene sequence comparison showed the presence of marine anammox bacteria belonging to the genus "*Candidatus* Scalindua", and high FISH counts in the top part of the sediment correlated well with potential anammox activity levels. Nitrogen conversion potentials for this site ranged from 0.6 to 4.8 fmol N cell^{-1} day^{-1}. The differences between the q-PCR results and other measurements imply that FISH counting may have been more accurate in the detection of anammox bacterial cells in the present setting. In addition, C_{20}-[3]-ladderane monoalkylether-PC concentrations were found to better reflect both anammox bacterial activity and abundance than ladderane core lipid concentrations, since the latter likely contained a substantial fossil component.

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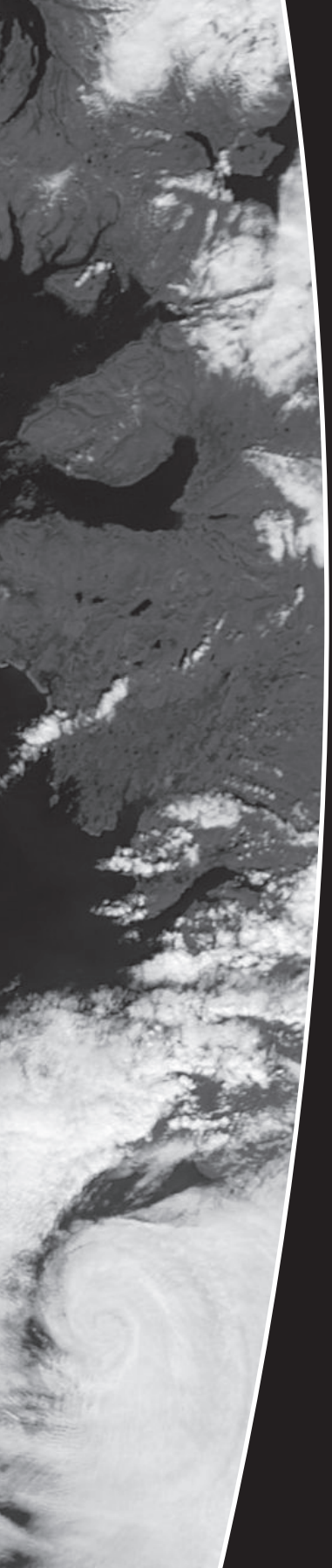

CHAPTER 4

SUMMER DISTRIBUTION OF MICROORGANISMS IN THE NORTH SEA

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ABSTRACT

Microbial organisms are vital for the functioning of all food webs and are the major drivers of the global biogeochemical cycles. However, not much is known about the distribution of the microbial community in the biologically highly active North Sea, a coastal sea on the northwestern European continental shelf. The physicochemical and microbial geographical distributions in the North Sea were studied during two summer cruises (2007 and 2008). Phytoplankton, bacterial and viral characteristics clustered largely (92% of total stations) into three main clusters, representing the main hydrographical regions. The largest microbial cluster, covering most of the central and western North Sea, was characterized by a thermally stratified water column and dominated by cyanobacteria and nanoeukaryotes. The second largest cluster, dominated by picoeukaryotes, was influenced by influxes of North Atlantic water via the English Channel and south of the Shetland Islands. The third cluster represented regions with riverine input, high nutrient concentrations and high heterotrophic bacterial and viral abundances. Our study shows that a small number of selective stations can be good representatives of the microbial biogeography of the North Sea. Microorganisms are key players in our study to understand and predict the Earth's response to global changes, and shelf seas like the North Sea are prone to many environmental and anthropogenic changes. The microbial community is highly active and typically has a high turnover. Therefore, we recommend monitoring the microbial community in the North Sea by sampling a reduced number of selective stations and study not only for standing stock but also for production and losses.

4.1 INTRODUCTION

Coastal and marginal seas are known for their biological richness and productivity, and contribute a disproportionately large fraction of the oceanic primary productivity (approximately 25%) in relation to their contribution to the total ocean surface area (<10%). Autotrophic processes play a key role in the global carbon cycle by linking the terrestrial, oceanic and atmospheric carbon reservoirs. Based on a study in the North Sea, globally extrapolated net uptake of carbon dioxide by coastal and marginal seas is 20% of the world's uptake of anthropogenic carbon dioxide (Thomas *et al.*, 2004).

The North Sea is a northwestern European temperate shelf sea bordered by densely populated, highly industrialized countries, which carry out intense commercial fishing (it has the longest known record of fisheries yield), oil and gas drilling, and sand and gravel extraction. In addition, the North Sea is one of the areas with the most ship traffic (it hosts two of the world's largest ports) and its coastal zone is used intensively for recreation.

Because of its biological, economic and recreational value the North Sea has been the subject of intense investigation. Many previous studies have documented sudden and substantial changes during the past few decades. Some of those changes were seen in relation to hydrographical and oceanographical parameters (Svendsen and Magnusson, 1992), biomass and diversity in phytoplankton and zooplankton communities (Southward *et al.*, 1995; Edwards *et al.*, 2002), fisheries (Ried *et al.*, 2001), bird communities (Swennen, 1991) and seal populations (Reijnders and Brasseur, 2003). In a study using existing long-term data from the North Sea, Weijerman *et al.* (2005) showed that significant regime shifts occurred in the marine ecosystem in 1979 and 1988. The most evident shifts were in the biological data series and it was concluded that they were triggered by a number of environmental factors. In particular, salinity and weather conditions were attributed as the main causes for the 1979 shift and temperature and weather conditions for the 1988 shift. Using data from the North Sea Continuous Plankton Recorder survey, Edwards *et al.* (2002) also concluded that the biological irregularities and large plankton community shifts recorded during those years were mostly due to changes in salinity and water temperature, caused by oceanic water incursions into the North Sea, rather than otherwise important trends in atmospheric oscillations and anthropogenic perturbations.

Since those changes were recorded, there has been increasing awareness of global climate change (e.g., ocean warming). A more recent study in the western English Channel over a 15 year period (1992-2007) showed significant long-term changes in algal composition, with decreased abundances of diatoms and *Phaeocystis* and increased abundances of coccolithophorids and dinoflagellates, which were related to the observed 0.5°C warming over the past 50 years (Widdicombe *et al.*, 2010). Changes in phytoplankton community composition and dominance may have profound ecological

consequences, as phytoplankton form the base of the marine food web.

Marine microorganisms (including phytoplankton) make up over 95% of the organic particulate carbon in the seas and are main drivers of the biogeochemical cycles. Microbes are potentially good indicators of changes (natural or anthropogenic) in their environment, mainly because of their short life cycles and the tight coupling between environmental change and population dynamics. Also, being free-floating organisms, they respond to changes in temperature and oceanic currents by rapidly increasing or reducing their distribution ranges (Hays *et al*., 2005). Microbial plankton communities can be utilized in impact assessment because their nonlinear amplified responses to environmental perturbations indicate change even better than environmental parameters themselves (Taylor *et al*., 2002). As the main drivers of biogeochemical cycles, changes in composition and distribution of microbial assemblages can significantly modify the ecology and biogeochemistry in the oceans.

Despite their potential importance, the response of planktonic microbes, other than phytoplankton, to changing conditions in the North Sea has barely been studied. Bacteria may be influenced directly by climate change due to shifts in parameters like temperature altering their metabolism and growth rates (Kritzberg *et al*., 2010) or changes to ocean currents altering their abundance and distribution (Evans *et al*., in press). They may also be influenced indirectly due to their coupling to primary production (Hoppe *et al*., 2008), with changes to phytoplankton communities influencing the extent and temporal availability of their substrates. Climate change is also likely to have implications to biological entities like viruses and fungi, which are infectious to other components of the plankton. Changes to the activity or abundance of hosts will have direct consequences for infection rates and could cause shifts in parasite life cycles, for example from lytic to lysogenic infection in viruses (Danovaro *et al*., 2010). Alternatively, factors such as ocean acidification could promote the decay of free viruses (Danovaro *et al*., 2010).

Without better knowledge of microbial abundances and distribution in the North Sea we cannot fully evaluate and monitor the impacts of gradual climate change or of potential anthropogenic environmental degradation or disaster. We investigated the summer distribution of microorganisms during two scientific cruises (MICROVIR and CarboOceans), covering on both occasions all of the representative areas within the North Sea. Compared to previous studies that focused on a discrete region within the North Sea or only one component of the microbial community, our study is more comprehensive as at each station phytoplankton, bacteria and virus communities were sampled alongside a number of physicochemical metadata parameters. The purposes of our study were: (1) to assess the distribution of the main components of the microbial community; (2) to determine the factors influencing the distribution and interactions of marine microbes in the North Sea; and (3) to provide microbial and environmental data against which future measurements can be compared.

4.2 MATERIALS AND METHODS

4.2.1 Study area and sampling

During the MICROVIR cruise (64PE217) onboard RV *Pelagia* in July 2007, a total of 21 stations were sampled (Fig. 4.1), covering representative areas of the North Sea, as well as the English Channel and North Atlantic Ocean (south and north of the Shetland Islands). During the CarboOceans cruise (64PE294) onboard RV *Pelagia* between 22nd August and 10th September 2008, a total of 92 stations were sampled (Fig. 4.1), providing a more intensive coverage of the North Sea waters.

Seawater samples were collected at a range of different depths (5-300 m) throughout the water column using 22 NOEX bottles (10 l each) mounted on a Rosette sampler equipped with Seabird conductivity-temperature-depth (CTD) sensors and a natural autofluorescence sensor.

Discrete samples for dissolved inorganic nutrients (5 ml) were gently filtered through 0.2-mm pore size polysulfone filters (Acrodisc, Gelman Sciences), after which samples for inorganic nitrogen and phosphorus were stored at -20°C and samples for reactive silicate (Si) were stored in the dark at 4°C until analysis. Phytoplankton pigment samples of typically 5 l were filtered onto a GF/F filter (Whatman) and stored at -80°C until analysis. Small samples (1-5 ml) were taken for phytoplankton, bacteria and virus counting using flow cytometry. Phytoplankton samples were either counted directly (MICROVIR cruise) or fixed to 1% final concentration with formaline:hexamine solution (18% v/v : 10% w/v), flash frozen in liquid nitrogen after 30 min fixation and stored at -80°C until analysis (CarboOceans cruise). Fixation and freezing did not affect the total phytoplankton counts. Samples for bacteria and viruses were fixed to a final concentration of 0.5% with glutaraldehyde (25% stock solution, EM-grade) for 30 min, after which they were flash frozen in liquid nitrogen and stored at -80°C until analysis in the home laboratory.

4.2.2 Analyses

Dissolved inorganic nutrient analyses were performed on board with a TrAAcs 800 autoanalyzer for dissolved orthophosphate (Murphy and Riley, 1962), inorganic nitrogen (nitrate + nitrite: NO_x) and ammonium (Helder and De Vries, 1979; Grasshoff, 1983), and reactive silicate (Strickland and Parsons, 1968).

For a restricted number of stations (all MICROVIR stations and stations CO04, 09, 12, 26, 37, 38, 46, 73, 74, 76 and 84 from the CarboOceans cruise), the extracts from the phytoplankton pigment filters were analyzed by high perfomance liquid chromatography (HPLC) after extraction in 4 ml of 100% methanol, buffered with 0.5 mol l^{-1} ammonium acetate and homogenization for 15 seconds. The relative abundances of different taxo-

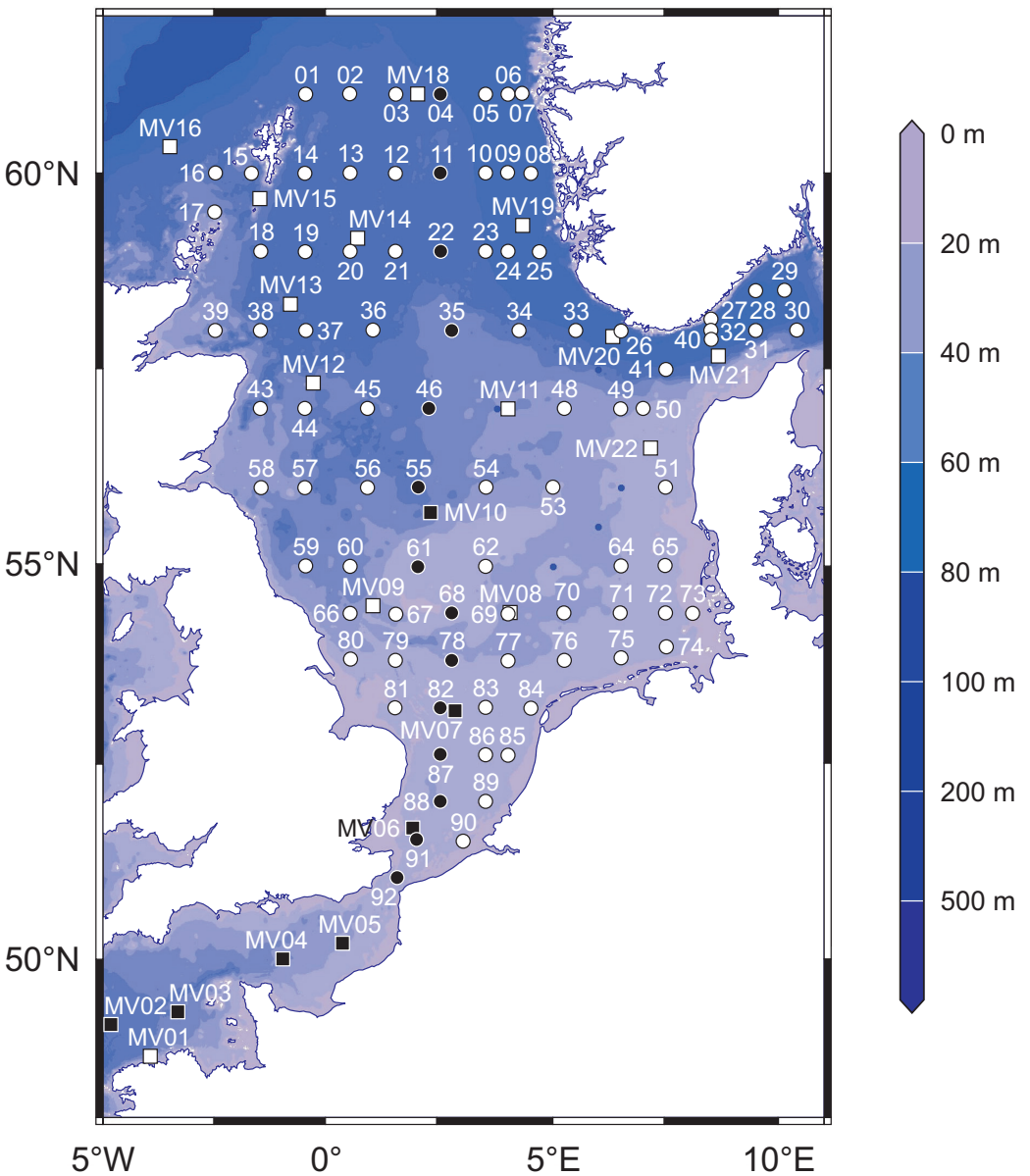

Figure 4.1 Bathymetry and the location and designation of sites sampled in the North Sea during the MICROVIR and the CarboOceans cruises. Stations sampled on the MICROVIR cruise are designated by the prefix MV and denoted by squares and stations sampled on the CarboOceans cruise are denoted by circles. Stations indicated by black symbols were selected to provide data for the south to north transect.

nomic groups were determined using CHEMTAX (Mackey *et al.*, 1996; Riegman and Kraay, 2001). Due to technical issues, we unfortunately lost the remaining CarboOceans HPLC samples.

Phytoplankton abundances (<20 µm) were enumerated using a Becton-Dickinson FACSCalibur flow cytometer (MICROVIR cruise) or a modified Beckman Coulter XL-MCL flow cytometer (CarboOceans cruise). Both benchtop flow cytometers were equipped with an air-cooled Argon laser with an excitation wavelength of 488 nm (15 mW). The discriminator for phytoplankton was set on the red chlorophyll autofluorescence. Approximately 1 ml of sample was counted in 10 min. Based on the red autofluorescence and scatter signal, we discriminated picoeukaryotic populations, as well as the nanoeukaryotes. The prokaryotic cyanobacteria were discriminated from the other phytoplankton on the basis of their orange autofluorescence, originating from the accessory pigment phycoerythrin. The division of the eukaryotes was based on their relative size obtained by gravity filtration of small volumes of sample (Veldhuis and Kraay, 2004).

Bacterial abundances were determined using the FACSCalibur flow cytometer, after the thawed samples were diluted in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stained with the nucleic acid-specific green fluorescent dye SYBR Green I (Molecular Probes Inc., Eugene, USA) to a final concentration of 1×10^{-4} of the commercial stock for 15 min in the dark at room temperature (Marie *et al.*, 1999). Viral abundances were determined with the FACSCalibur flow cytometer, as previously described by Brussaard (2004) on thawed samples. Briefly, thawed samples were diluted (enough to avoid coincidence) with Tris-EDTA buffer (pH 8.0) and stained with SYBR Green I at a final concentration of 0.5×10^{-4} of the commercial stock for 10 min in the dark at 80°C. Samples were cooled at room temperature for 5 min in the dark before they were run in the flow cytometer. As for bacteria, the different virus groups were discriminated on the basis of green fluorescence versus side scatter signal. Virus counts were corrected for a blank sample, consisting of Tris-EDTA buffer that was stained and analyzed in the same way as the viral samples. Listmode files were analyzed using CYTOWIN (Vaulot, 1989; freely available through: http://www.sb-roscoff.fr/Phyto/index.php?option=com_content&task=view&id=72&Itemid=123).

4.2.3 Statistical analysis

The geographical distributions of the microbial groups measured at 10 m depth were comparable to the averaged values for the total mixed layer (defined as the depth at which a temperature shift of more than 0.5°C occurs). Therefore only the 10 m depth measurements were used for statistical analysis. Furthermore, preliminary analysis (ANOSIM testing) of the data obtained during the two different cruises showed that the two datasets were not significantly different from each other (Global $R < 0.2$, $p = 0.079$), and could be taken together for further analysis.

Compositional similarities between the stations were determined by distance-based ordination, using the dedicated software package PRIMER 6 with the PERMANOVA+

add-on (both PRIMER-E Ltd, Lutton, UK). Similarity matrices of the stations were calculated based on their environmental composition or microbial abundances, and the results were plotted using multidimensional scaling (MDS) ordination. Cluster analysis was used to identify significantly different groups of stations with comparable environmental compositions (Global $R = 0.85$, $p < 0.001$) or microbial abundances (Global $R = 0.68$, $p < 0.001$). The measure of dependence between the two similarity matrices was determined by calculating their Spearman's rank correlation coefficients (RELATE test in PRIMER).

Finally, the measures of association (Spearman's rho; ρ) between each of the environmental and microbial parameters were determined using the software package Systat 13 (Systat Software, San Jose, CA).

4.3 RESULTS

4.3.1 Physicochemical characterization of study area

The physicochemical characteristics of the mixed surface waters (Fig. 4.2) and the water column over a transect from south to north (Fig. 4.3) show the complex hydrographic nature typical of the North Sea. The northwestern region of the North Sea has relatively cold (11.8-13.2°C), high saline (35.0-35.3) and inorganic nutrient-rich water (0.1-0.4 µM P, up to 5.4 µM NOx) originating from the northern North Atlantic Ocean. North Atlantic water also flows into the North Sea from the south, but this influx mixes directly with coastal waters in the English Channel. Along the shallow southeastern continental shelf of the North Sea the water is warmer (up to 18°C). Surface waters along the Norwegian, Danish and German coasts are also relatively warm (up to 17.2 °C), but less saline (down to 28.9). The high nutrient load in the German Bight is due to riverine input (up to 0.7 µM P, 2.8 µM N and 7.1 µM Si). The S-N transect (Fig. 4.3) clearly shows the transition from well-mixed waters in the south to the thermally stratified northern region of the North Sea. The thermocline is generally situated around 20-30 m depth, below which the inorganic nutrient concentrations increase strongly (maximum concentration 0.9 µM P, 14 µM N and 7 µM Si).

Cluster analysis of the physicochemical parameters of the well-mixed surface waters (10 m) resulted in three large clusters (together comprising 89 out of 108 stations), three minor clusters and an additional five clusters consisting of only 1-2 stations each (Fig. 4.4). The main clusters (#4-7; Fig. 4.4A) are primarily differentiated by salinity and temperature, and secondarily by inorganic nutrient concentrations. The remaining clusters (#1-3 and #8-11) are discriminated by their higher inorganic nutrient concentrations and secondarily by their higher temperature or lower salinity. Taking into account the depth profiles of the stations, the clusters largely correspond to the differ-

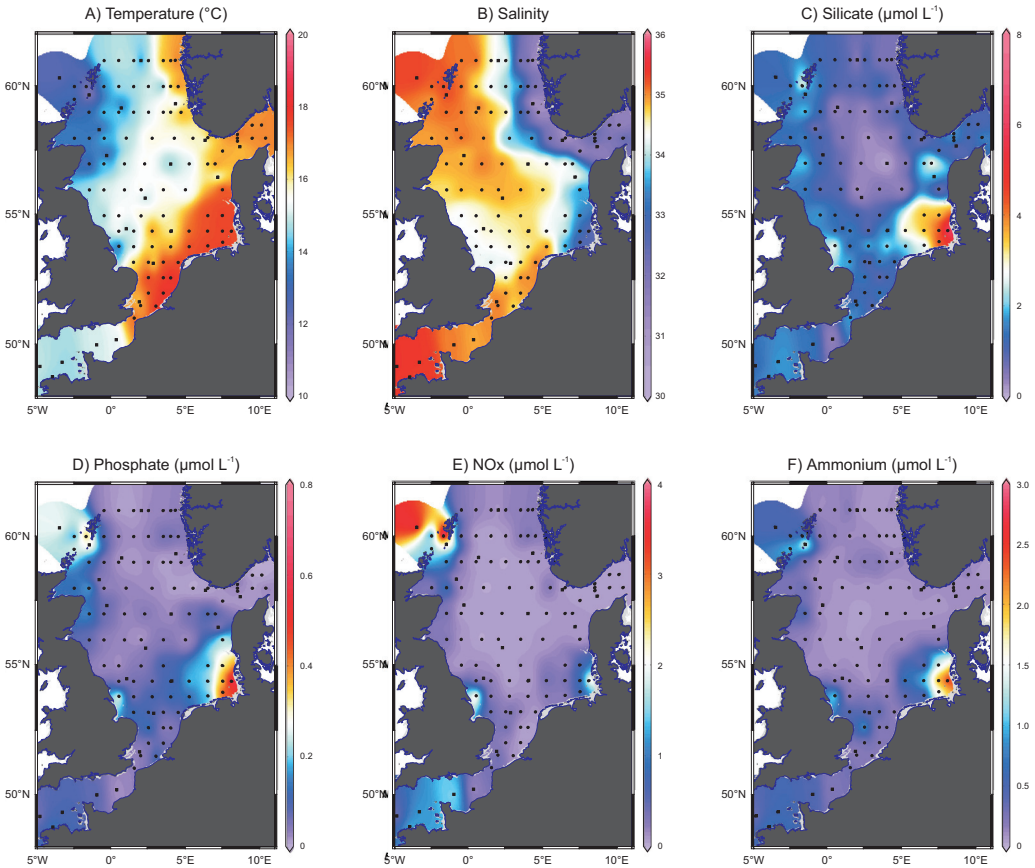

Figure 4.2 Composite plots of the physicochemical conditions in the upper mixed layer (depth 10 m) of the North Sea from both the MICROVIR 2007 and CarboOceans 2008 summer cruises. Black dots represent sampling sites and contour plots were created using the freeware package *Ocean Data View 4* (Schlitzer, 2010).

ent hydrographical regions as described by for example Otto *et al.* (1990) and Ducrotoy *et al.* (2000). The largest cluster (#7) represents the deeper northern and central North Sea, with T-S curves typical for thermally stratified waters (surface temperature 15-16°C decreasing to 7°C; and salinity increasing from 34 to 35.5). The second largest cluster (#6) shows a strongly bent T-S curve, representing thermally stratified Norwegian coastal waters (from around 17°C decreasing to 7°C) with less saline surface water (29-35). Cluster #5 shows the influence of English Channel water and Continental Coastal water, resulting in temperatures of 17-18°C and salinities of 34.9-35.2 in the south, and temperatures of 16-18°C and salinities of 33.2-34.8 towards the north. Cluster #4 shows

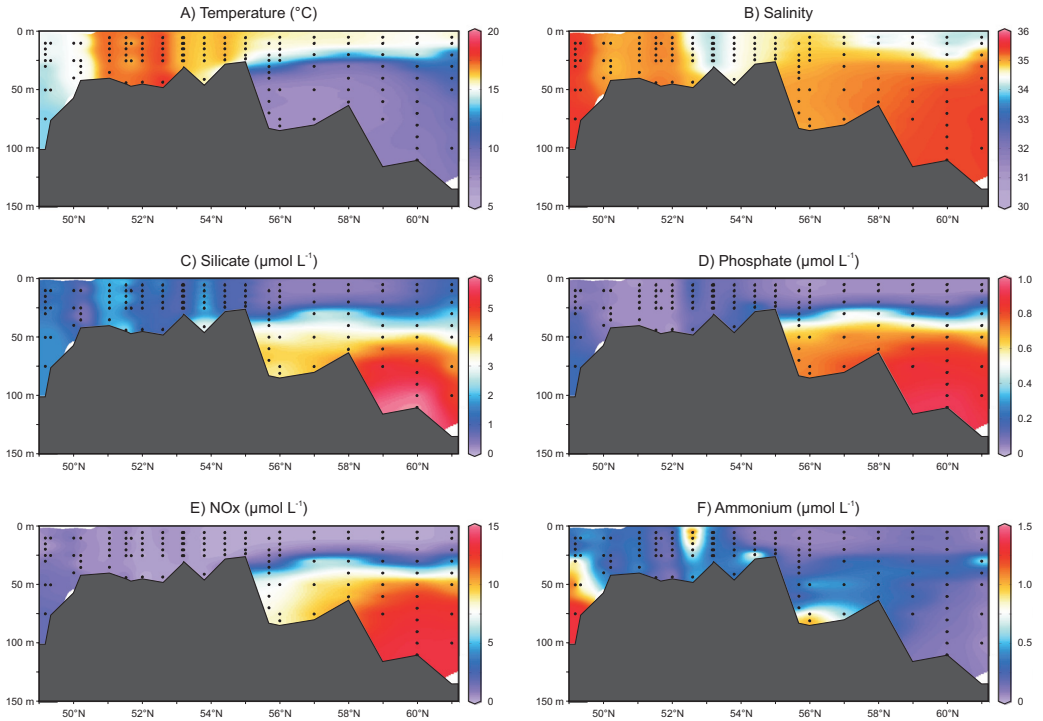

Figure 4.3 Composite plots of the physiochemical characteristics throughout the water column over a south to north transect of the North Sea from both the MICROVIR 2007 and CarboOceans 2008 summer cruises. Black dots represent sampling sites and contour plots were created using the freeware package *Ocean Data View 4* (Schlitzer, 2010).

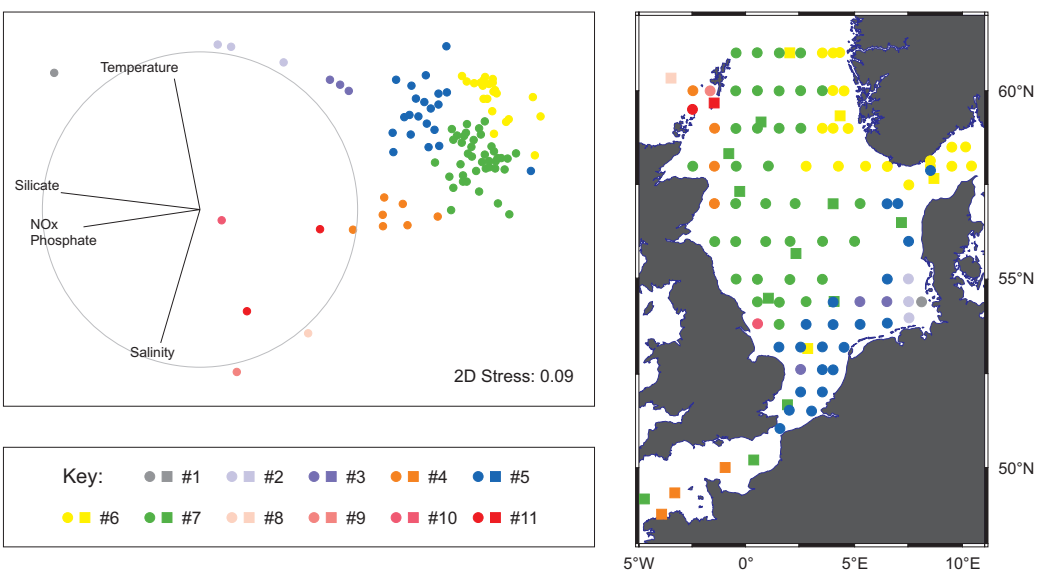

Figure 4.4 MDS plot of physicochemical data and map indicating the positions of stations located in each cluster.

the input of North Atlantic water via the Fair Isle current into the Scottish Coastal water (northwestern North Sea) and through the English Channel in the south. In these regions salinity varied between 34.8 and 35.4 and temperatures were relatively low (12-15 °C). The small clusters #2 and #3 (mainly German Bight) show the input of fresh river water. The other minor clusters appear to be intermediates of the major hydrographical regions.

4.3.2 Microbial distributions

The spatial distribution of the biological variables in the mixed surface waters is presented in Fig. 4.5 and their depth distribution in Fig. 4.6. The chlorophyll *a* autofluorescence (Chl *a*; Fig. 4.5A), an indicator of the total phytoplankton biomass, was found to be highest along the continental shelf, south of the Dogger Bank (55°N; 2°E) and in the northeast of the study area. The English Channel stations contained large chain-forming diatoms (>20 µm), as observed by light microscopy and indicated by HPLC pigment analysis, which likely explains the peak in fluorescence and the absence in <20 µm phytoplankton in this region (Fig. 4.5). Abundances of nanoeukaryotes (Fig. 4.5B; 2-20 µm diameter) were highest in the northwestern region of the study area (1.2 x 10^4 ml^{-1}), but were also high in the north (including a large portion of the Norwegian coast). The picoeukaryotes (Fig. 4.5C; <2 µm diameter) showed high abundances (up to 2.4 x 10^4 ml^{-1}) in the northwestern North Sea, the Continental Coastal waters, the Dogger Bank area, the English Channel and the Scottish Coastal waters. The central North Sea showed the lowest values for chlorophyll *a* autofluorescence and eukaryotic phytoplankton but the pico-prokaryotic cyanobacteria (*Synechococcus*) were prevalent there. Highest cyanobacterial abundances were observed in the mid-northern region (up to 9.6 x 10^4 ml^{-1}) and south of the Norwegian coast (up to 6.4 x 10^4 ml^{-1}).

Phytoplankton pigment analysis by HPLC showed that the Prymnesiophyceae dominated in the continental shelf area (55-72% of total Chl *a*) and were abundant along the Norwegian coast (17-35%). The Crysophyceae generally dominated in the northern half of the North Sea, with percentages of 80-86% in the northwest, 25-66% close to the Norwegian coast, 60% in the Skagerrak, and 50-60% in the eastern North Sea. The contribution of the Prasinophyceae ranged from a few percent to 14% along the continental shelf, 21% along the Scottish coast and 40% in the English Channel. The autotrophic Dinophyceae contributed about 10% in the English Channel and increased to 13-18% in the German Bight and towards southern Norway. As can be expected, the distribution of the Cyanophyceae matched the distribution of the cyanobacterial abundances obtained by flow cytometry. The maximum contribution of Cyanophyceae was between 15 to 22 % of total Chl *a*. Cryptophyceae were detected in the German Bight, the English Channel and the Scottish coast and made up 12-20% of the total. Chlorophyceae were only detected in significant numbers in the Skagerrak (10%), whereas the Bacillariophyceae were only detected in the English Channel (20%). Eustigma were present without a clear pattern and contributed typically between 0-10% of the total Chl *a* concentration.

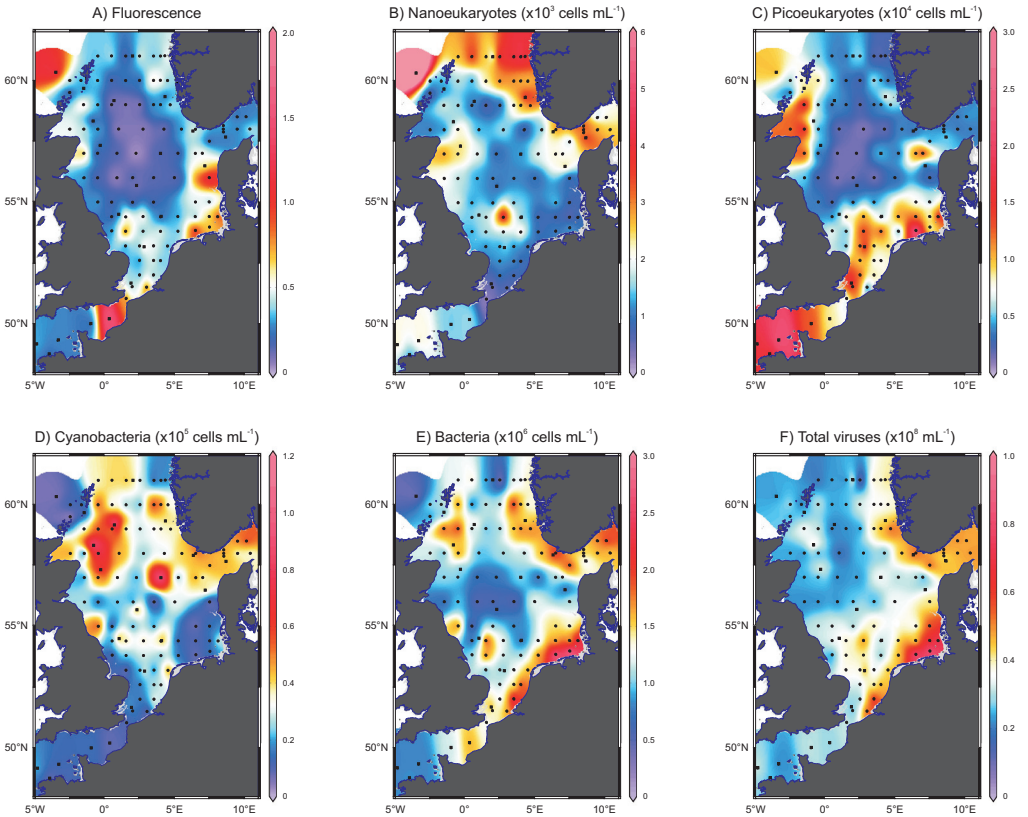

Figure 4.5 Composite plots of fluorescence and the distribution of nanoeukaryotic algae, picoeukaryotic algae, cyanobacteria, bacteria, and viruses in the upper mixed layer (depth 10 m) of the North Sea from both the MICROVIR 2007 and CarboOceans 2008 summer cruises. Black dots represent sampling sites and contour plots were created using the freeware package *Ocean Data View 4* (Schlitzer, 2010).

The heterotrophic bacteria (Fig. 4.5D) were most abundant along the continental shelf (particularly the Dutch and German coasts; up to 2.9 x 10^6 ml^{-1}), the Norwegian coast (2 x 10^6 ml^{-1}), south of the Shetland Islands, and around the Dogger Bank. Lowest bacterial abundances were detected in the north and upper northwest (1.5-4.0 x 10^5 ml^{-1}). The mixed surface water distribution of total virus abundances (Fig. 4.5E) largely matched that of the heterotrophic bacteria, their numerically dominant hosts. Highest viral abundances were observed in the German Bight (1.1 x 10^8 ml^{-1}), along the Norwegian coast (5.7 x 10^7 ml^{-1}) and around the Dogger Bank (5 x 10^7 ml^{-1}). Using flow cytometry, four populations of viruses could be detected based on their green fluorescence (after staining with a nucleic acid-specific green fluorescent dye) and side scatter. Populations V1 and V2 were the numerically dominant populations, making up 65±5% and 29±4% of the total, respectively. Population V3 (6±2%) showed its highest abundances along

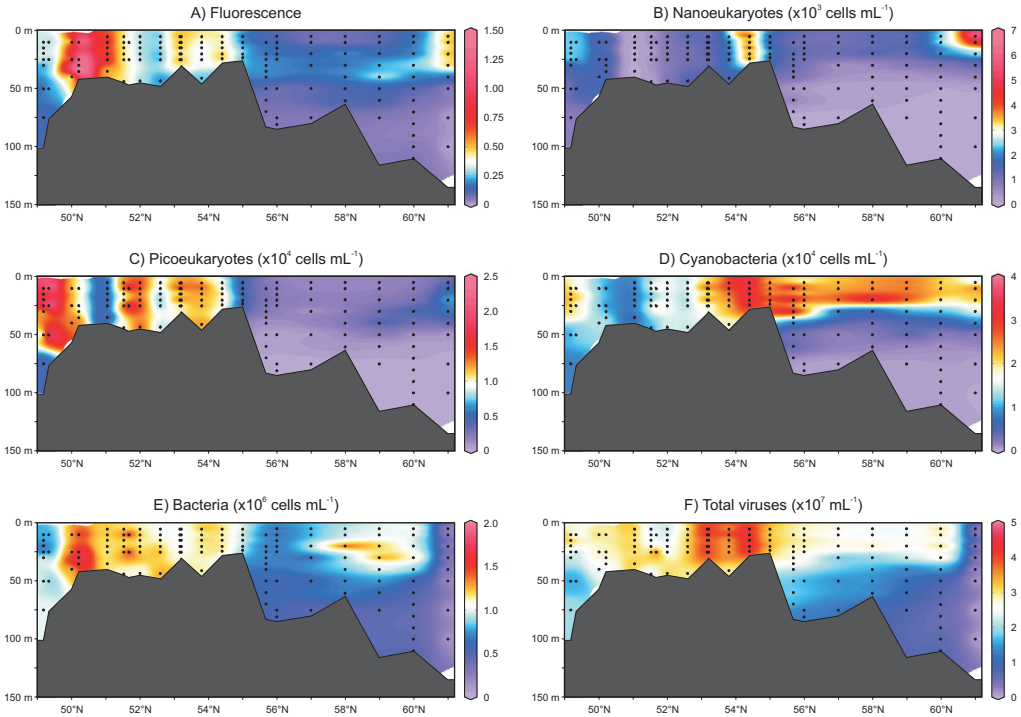

Figure 4.6 Composite plots of the fluorescence and microbial abundances throughout the water column over a south to north transect of the North Sea from both the MICROVIR 2007 and CarboOceans 2008 summer cruises. Black dots represent sampling sites and contour plots were created using the freeware package *Ocean Data View 4* (Schlitzer, 2010).

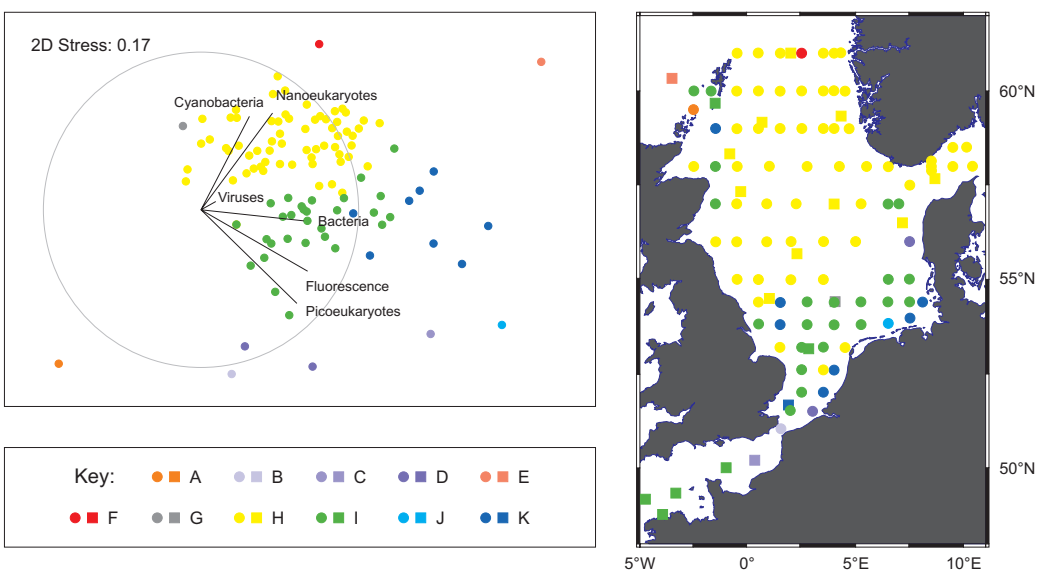

Figure 4.7 MDS plot of microbial data and map indicating the positions of stations located in each cluster.

the Dutch coast (2.0 x 10^7 ml^{-1}), whereas the highest abundance of population V4 (on average 1% of total) was detected in the German Bight (5.7 x 10^5 ml^{-1}). The total virus to bacterial host ratio (VBR) in the mixed surface waters ranged from 5 to 86, with an average of 30±12.

The S-N depth transect (Fig. 4.6) shows that in the stratified northern half of the North Sea the cyanobacteria were most abundant, with their maximum at the thermocline (20 m). The depth profile also shows that the North Sea is not uniformly populated with microbes. Instead, there is a large degree of geographical patchiness that can be recognized throughout the entire mixed layer. The strong peak in fluorescence in the English Channel (southern part of the transect) was mainly due to the presence of chain-forming diatom in that region (microscopic observation). The high number of picoeukaryotes in the south was not well reflected in the natural fluorescence signal, due to their very small cell sizes. In the southern North Sea the fluorescence signal was made up by nano- and picoeukaryotes, as well as cyanobacteria, with the nanoeukaryotes peaking on the shallow Dogger Bank. Further north in the stratified surface waters of the central North Sea cyanobacteria numerically dominated the phytoplankton community, while nanoeukaryotes showed increased abundances in the northern-most regions. It becomes clear from the depth profiles that the bacterial community distribution followed that of the phytoplankton community, with highest abundances in the southern part of the North Sea. Viral abundances followed the distribution of their numerically dominant hosts (bacteria and algae) and were highest south of the Dogger Bank. The VBR over depth for all stations was 27±14, in the same range as for the 10 m surface waters. No significant differences in VBRs were observed above and below the thermocline for the stations in the northern region of the North Sea. Interestingly, an approximately 1.5-fold increase in VBR was observed with depth for stations CO27-32, situated south of Norway in the Skagerrak. The depth distribution plots of the numerically dominant virus populations V1 and V2 were comparable to that of the total virus population. However, virus population V3 seemed to be highest at locations where picoeukaryote abundances were high and cyanobacteria were reduced, as compared to the adjacent areas. Finally, virus population V4 was clearly highest on the Dogger Bank area.

Cluster analysis of biological variables in the surface waters (10 m) resulted in two large clusters (comprising 91 out of 108 stations), an additional smaller cluster consisting of 8 stations, and eight clusters consisting of only 1-2 stations each (Fig. 4.7). The largest cluster (H) encompasses the largest part of the North Sea, as well as the Norwegian coastal waters (Fig. 4.7B). It is discriminated from the other clusters by its higher abundances of nanoeukaryotes and cyanobacteria. The second main cluster (I) contains stations located in the southern North Sea, the English Channel and waters around the Shetland Islands. The smaller cluster K consists mainly of stations along the German Bight and the southern Dutch and English coasts. The remaining minor clusters were found in the eastern English Channel, along the Danish coast, and in the north(west)ern North Sea (Fig. 4.7B).

4.4 DISCUSSION

The present study explored geographical summer distributions of important components of the microbial community in the North Sea. The distributions of phytoplankton, bacteria and viruses at 10 m resulted in the identification of eleven clusters, of which the main three were comprised 92% of the total number of stations sampled. These microbial clusters were clearly influenced by the different hydrographical regions (Otto *et al*., 1990; Ducrotoy *et al*., 2000). A rank correlation test of the spatial distributions of the microbial and environmental (temperature, salinity and inorganic nutrients) datasets indicated that there is a significant relationship between the two ($\rho = 0.32$, $p = 0.001$). However, the distribution of the largest microbial cluster (H) includes the distribution of the two largest physicochemical clusters (#6 and #7), covering the largest part of the North Sea. It includes the warmer and less saline Norwegian coastal waters, shown to have relatively high abundances of nanoeukaryotes. Algal pigment analysis showed that the nanoeukaryotes consisted mostly of Chrysophyceae and Prymnesiophyceae. Nevertheless, the nanoeukaryote abundance was not exclusively high in the Norwegian coastal waters. High numbers (with comparable predominance of Chrysophyceae and Prymnesiophyceae) were also found in the northern and northwestern North Sea. The prokaryotic cyanobacteria (*Synechococcus*) also showed high abundances along the southern part of the Norwegian coast and in open North Sea waters. These non-exclusive distributions contributed to the discrimination of only one microbial cluster versus two environmental clusters. Overall, cyanobacterial abundances correlated negatively with phosphate, nitrate and ammonia concentrations ($\rho = -0.5$, $p < 0.002$), indicating that they thrive in nutrient-poor waters such as the thermally stratified waters in the northern North Sea. *Synechococcus* can generally be found in a wide range of marine environments (Partensky *et al*., 1999), including seawater with somewhat lower salinity like the Norwegian coastal waters. Indeed, its highest abundances are typically found in stratified waters with a homogeneous distribution in the upper layer and a dramatic decrease in numbers below the thermocline.

The input of North Atlantic water via the English Channel and south of the Shetland Islands (Otto *et al*., 1990; Ducrotoy *et al*., 2000) resulted in a separate cluster (I), dominated by picoeukaryotes. Interestingly, no substantial numbers of picoeukaryotes were found in the deeper, thermally stratified and nutrient-poor central North Sea. Using probes targeting 18S rRNA, Masquelier *et al*. (in press) showed that during the MICROVIR cruise these picoeukaryotes were made up by Chlorophyta, and were dominated by Mamiellophyceae and particularly *Micromonas pusilla*. Picoeukaryotes, dominated by *M. pusilla*, have been shown to constitute up to 30% of the total chlorophyll biomass in the English Channel (Not *et al*., 2004). A study by Gescher *et al*. (2008) showed that around Helgoland (German Bight) the genus *Bathycoccus* was the major contributor to the prasinophyte composition. Our algal pigment data showed that within this cluster 10-40% (20% in northeastern section) of the total chlorophyll came from the Prasino-

phyceae, the class to which both *Micromonas* and *Bathycoccus* belong. Pigment analysis further implied that the major portion of the picoeukaryotes along the continental slope belonged to the Prymnesiophyceae. Recently, Prymnesiophyceae have been shown to be a relevant component of the picoeukaryote community (Lepère *et al.*, 2009; Kirkham *et al.*, 2011; Li Shi *et al.*, 2011).

The third largest microbial cluster (K) represents coastal waters influenced by riverine input (rivers Rhine, Ems and Thames), and showed the presence of Cryptophyceae (up to 19% of total chlorophyll) and a high bacterial and viral abundance. Cryptophytes have been documented to be an important and constant contributor to the phytoplankton community of the German Bight, especially in the picoplankton fraction (Metfies *et al.*, 2010).

The heterotrophic bacteria were particularly abundant along the continental slope and the southern Norwegian coast (Skagerrak), most likely because of enhanced dissolved organic matter load. The S-N transect clearly showed that the bacterial distribution coincided with the phytoplankton community. The high fluorescence in the English Channel, mainly made up by summer diatoms like *Guinardia* species (Weston *et al.*, 2008; Masquelier *et al.*, in press), resulted in significant concentrations of photosynthetic extracellular released (PER) organic compounds that can be consumed by the heterotrophic bacteria. To a lesser extent, due to their lower total biomass, pico- and nanoeukaryotes also produce PER that will be used by heterotrophic bacteria (Sintes *et al.*, 2010). Yet, organic matter can also be released due to sloppy feeding and particularly virally induced cell lysis, which results in relatively large quantities of readily available organic compounds (Brussaard *et al.*, 2005; Suttle, 2005). The virus population known to be almost exclusively made up of algal viruses (V4; Brussaard *et al.*, 2010) was most abundant along the northern Norwegian coast , where it was significantly correlated with the nanoeukaryotes in microbial cluster H ($\rho = 0.54$, $p < 0.001$) and north of The Netherlands (no significant correlation found). The virus population V3, known to contain bacteriophages (including cyanophages) and viruses infectious to picoeukaryotes (Brussaard *et al.*, 2010), showed highest abundances in Continental Coastal waters and was significantly correlated to the picoeukaryotes in microbial cluster I ($\rho = -0.45$, $p = 0.004$). The negative correlation represents strong viral control over the host community, resulting locally in the diminished presence of picoeukaryotes (see Fig. 4.5). Similar findings of V3 and V4 abundances were made for the water column over the S-N depth transect. Baudoux *et al.* (2006, 2007) reported substantial viral lysis of pico- and nanoeukaryotes (loss rates comparable to grazing). Viral lysis was responsible for 50-100% of the total cell losses of populations of picoeukaryotes in the open ocean, and up to 66% of the total mortality of single cells of the nanoeukaryote *Phaeocystis globosa* in the southern North Sea. During a summer study in the North Sea, Baudoux *et al.* (2008) also found that up to 38% of the picoeukaryotes in the surface waters were dead, although they could not always show that this was due to viral infection. Overall, these studies showed that the total carbon production by the pico- and nanophytoplankton community was balanced by combined losses due to viral

lysis and microzooplankton grazing. The typically high growth rates of the smaller-sized phytoplankton community were counterbalanced by the losses. Although these algae are small, they are generally abundant and can account for a significant share of the primary production (Riegman and Noordeloos, 1998). During the MICROVIR cruise primary production showed the highest rates at the stations influenced by North Atlantic waters, either via the English Channel to the Continental Coastal waters, or via the Shetland Islands. Primary production rates at these stations ranged from 1.1 to 2.0 g C m^{-2} d^{-1}, which is two to three-fold higher than found for the central and northwestern North Sea (0.5-0.9 g C m^{-2} d^{-1}; C. Brussaard, unpublished data). Generally, these high-productivity stations were numerically dominated by picoeukaryotes. However, east of the Shetland Islands nanoeukaryotes were also contributing to the enhanced primary production, whereas in the English Channel diatoms were also important.

Generally, the heterotrophic bacterial community undergoes high turnover by grazing and viral lysis as well (Winter *et al.*, 2004). Our data show that the geographical distribution of the viral community in the mixed surface layer was strongly correlated with the numerically most dominant host, i.e., bacteria ($\rho = 0.57$, $p < 0.001$). The S-N depth transect also displays a dynamic pattern of viral and bacterial abundances. The VBRs ranged between 5 and 86 in the mixed surface waters and between 1 and 90 over the entire water column, illustrating the dynamic relationship between virus and bacteria. The average VBR of 30±12 for surface waters is higher than reported for coastal environments in the review by Wommack and Colwell (2000), suggesting an active role for viruses in the North Sea. A locally enhanced VBR (e.g., Dogger Bank) would arise from enhanced virus production and hence more bacterioplankton lysis, whereas a reduced VBR (e.g., English Channel) indicates bacterial net growth or enhanced viral decay.

Coastal and marginal seas play a key role in the global carbon cycle by linking the terrestrial, oceanic and atmospheric carbon reservoirs. Microorganisms are vital to the functioning of all ecosystems and are key players in our study to understand and predict the Earth's response to global changes, either by anthropogenic or natural causes. As phrased elegantly by Fuhrman (2009), changes in the microbial structure in space and time are very informative, as they show what scales a particular sample represents. This is indeed crucial for extrapolating from individual samples to larger scales. It is clear that the microbial community is highly productive, and that therefore the standing stock does not represent the actual activity of the different microbial components. However, the microbial distribution and resultant microbial clusters presented in this study are based on the microbial standing stock, i.e., what is present in the water column at a specific depth. With this in mind, we recommend for future monitoring of the microbial community in changing coastal seas like the North Sea to reduce the number of stations studied, but to include production and loss measurements. Our study confirms that this suggestion is feasible, as the limited number of stations during the MICROVIR cruise were found to be representative of the different microbial clusters in the North Sea (as compared to the larger number of stations sampled during the CarboOceans cruise).

Microbial components of the plankton are particularly sensitive indicators of change (Taylor *et al.*, 2002), and their distribution (Hughes, 2000) and the timing of their proliferation (Hays *et al.*, 2005) will have significant ecological and biogeochemical influence. As these planktonic organisms constitute the base of the food web, changes in levels and timing of their productivity will have repercussions for the growth and reproduction of higher trophic levels. In turn, the functioning of the biological pump may be influenced, and thereby the capacity of seawater to absorb carbon dioxide from the atmosphere. Changes to the activity and abundance of bacteria and viruses will also have significant implications for factors, such as nutrient remineralization and element cycling (Wilhelm and Suttle, 1999; Danovaro *et al.*, 2010). The North Sea is prone to changes in various environmental parameters, and monitoring the microbial abundances and activity will help us to anticipate how microbial systems, and thus biogeochemical cycles, will shift in a changing world.

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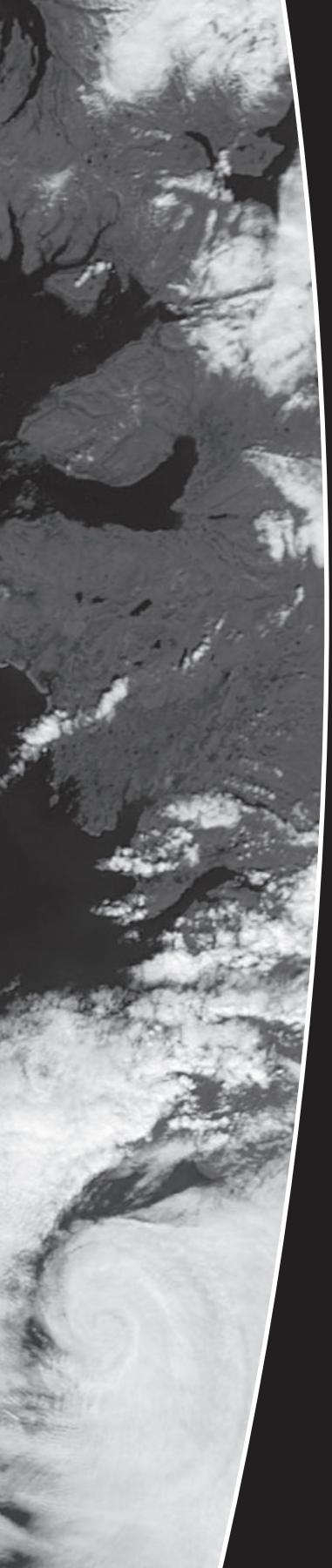

CHAPTER 5

SPATIAL DISTRIBUTION OF INTACT POLAR LIPIDS IN NORTH SEA SURFACE WATERS

relationship with environmental conditions and microbial community composition

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ABSTRACT

The intact polar lipid (IPL) composition of the surface waters of the North Sea was characterized and quantified, and potential relationships with environmental conditions, microbial abundances and community composition were investigated. The total IPL pool comprised at least 600 different IPL species in seven main classes: the glycerophospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), the sulfur-bearing glycerolipid sulfoquinovosyldiacylglycerol (SQDG) and the nitrogen-bearing betaine lipids diacylglyceryl-trimethylhomoserine (DGTS), diacylglyceryl-hydroxymethyltrimethylalanine (DGTA) and diacylglyceryl-carboxyhydroxymethylcholine (DGCC). Although no significant relationships were found between the IPL composition and environmental parameters, such as nutrient concentrations, distance-based ordination yielded distinct clusters of IPLs, which could in turn be tentatively related to the predominant microbial groups. SQDGs and PGs, as well as PC species containing saturated fatty acid moieties, were related to picoeukaryote abundances and PC species with polyunsaturated fatty acid (PUFA) moieties to nanoeukaryote abundances. The PEs were likely of mixed cyanobacterial-bacterial origin, while DGTAs and DGCCs were mainly associated with cyanobacteria. DGTSs were likely derived from either pico- or nanoeukaryotes, although the DGTS species with PUFAs also showed some relationship with cyanobacterial abundances. Concentrations of the algal-derived IPLs showed strong positive correlations with chlorophyll *a* concentrations, indicating they may be used as biomarkers for living photosynthetic microbes. However, direct relationships between the IPLs and microbial groups were relatively weak, implying that the predominant IPLs in marine surface waters are not derived from single microbial groups and that direct inferences of microbial community compositions from IPL compositions should be considered with care.

5.1 INTRODUCTION

Intact polar lipids (IPLs) are the basic building blocks of biological membranes and consequently occur ubiquitously in the natural environment. Their molecular structure typically encompasses a glycerol backbone with ester-linked fatty acids attached to the *sn*-1 and *sn*-2 positions (the core lipid), and a hydrophilic (polar) head group at the *sn*-3 position (Fahy *et al.*, 2005). Many of these head groups contain essential elements such as phosphorous, nitrogen or sulfur, and their biosynthesis may be related to nutrient availability to the organism (e.g., Minnikin *et al.*, 1974; Benning *et al.*, 1995; Martin *et al.*, 2010). Certain IPL classes or constituent fatty acids are synthesized predominately, or sometimes exclusively, by specific microbial groups and can thus be used as chemotaxonomic markers (Shaw, 1974; Lechevalier and Lechevalier, 1989; Sturt *et al.*, 2004). Furthermore, IPL molecules are thought to be degraded rapidly upon cell death and therefore predominately derived from living (microbial) cells (White *et al.*, 1979; Harvey *et al.*, 1986). Based on these two concepts, the distribution of IPLs in environmental samples should reflect the composition of the extant microbial community, and IPL analysis thus has the potential to provide valuable information complementary to that obtained by microbiological and molecular techniques.

The development of rapid and comprehensive methods for the direct analysis of IPLs (Brügger *et al.*, 1997; Fang and Barcelona, 1998; Rütters *et al.*, 2002a) has increased our knowledge of their sources and dynamics in the marine environment, in particular in subsurface sediments (e.g., Rütters *et al.*, 2002b; Zink *et al.*, 2003; Lipp and Hinrichs, 2009). However, the number of studies of IPL compositions of marine waters is still limited. Schubotz *et al.* (2009) showed the presence of various glycerophospholipids, betaine lipids and glycosyl-glycerolipids, as well as the sulfur-bearing glycerolipid sulfoquinovosyldiacylglycerol (SQDG), in surface waters of the Black Sea. The IPL assortment was inferred to represent a mixed community of eukaryotic algae, cyanobacteria and heterotrophic bacteria, but no direct comparison with the phytoplankton composition was made. Van Mooy and Fredricks (2010) analyzed IPLs in the euphotic zone of the eastern South Pacific. They observed a similar suite of IPLs as in the waters of the Black Sea, and tentatively identified broadly-defined phytoplankton groups as their likely sources. Popendorf *et al.* (2011) again observed similar IPLs in the western North Atlantic Ocean and used a combination of observational data, isotope tracing and culture incubations to link the production of IPLs to different planktonic groups. Finally, studies of IPL production under phosphate limitation in open ocean surface waters have shown that plankton can substitute glycerophospholipids with betaine lipids or SQDGs in settings where phosphate is scarce (Van Mooy *et al.*, 2006, 2009; Popendorf *et al.*, 2011), a mechanism that is well known from microbial cultures (Rose, 1989; Benning *et al.*, 1995; Martin *et al.*, 2010).

Although these studies have increased our knowledge of IPLs in marine waters, there is still a strong need for observational and comparative data to improve our

understanding of their distribution, sources and dynamics in the world's oceans and their chemotaxonomic potential. Here we present a detailed analysis of the IPL composition in the surface waters of the North Sea and distinct adjacent water masses in the northeast Atlantic Ocean, English Channel and Skagerrak. We statistically compare the IPL data with contemporaneous environmental and microbiological data (as described in Brandsma *et al.*, 2011) in order to determine if environmental conditions directly influence the IPL composition at this location, and to determine what relationships exist between the IPLs and the microbial community composition.

5.2 MATERIALS AND METHODS

5.2.1 Cruise and sampling

All samples for this study were taken during the RV *Pelagia* cruise MICROVIR (64PE217), which took place in July 2007 from Brest (France) to Texel (The Netherlands) and covered the entire North Sea, plus several adjacent water masses (Fig. 5.1). A total of 21 stations were sampled at a water depth of 10 m for temperature, salinity, dissolved inorganic nutrients and chlorophyll *a* (Table 5.1), as well as microbial abundances and phytoplankton composition (see Brandsma *et al.*, 2011 for details). In addition, particulate matter for IPL analysis was sampled by *in situ* filtration of the surface water over 0.7 µm mesh GF/F filters (292 mm diameter, precombusted at 450°C for 12 h; Whatman, Clifton, NJ, USA). All filters were stored frozen at -80°C for the duration of the cruise and until extraction in the lab.

5.2.2 Intact polar lipid analysis

The particulate matter filters were freeze-dried before the IPLs were extracted using a modified Bligh-Dyer procedure (Bligh and Dyer, 1959; Vancanneyt *et al.*, 1996; Rütters *et al.*, 2002a). Briefly, the filters were extracted ultrasonically three times for 10 min in a solvent mixture of methanol, dichloromethane and phosphate buffer (2:1:0.8 v/v). After sonication, the supernatants were phase-separated by adding additional dichloromethane and buffer to a final solvent ratio of 1:1:0.9 (v/v). The organic phases containing the IPLs were then collected and the aqueous phases re-extracted three times with dichloromethane. Finally, the extracts were dried under a stream of nitrogen gas. Before analysis, the extracts were re-dissolved in a mixture of dichloromethane and methanol (9:1 v/v) at a concentration of 10 mg ml^{-1}, and aliquots were filtered through 0.45 µm mesh True Regenerated Cellulose syringe filters (4 mm diameter; Grace Alltech, Deerfield, IL, USA).

IPL analysis of the extracts was performed by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC/ESI-MS^2), using

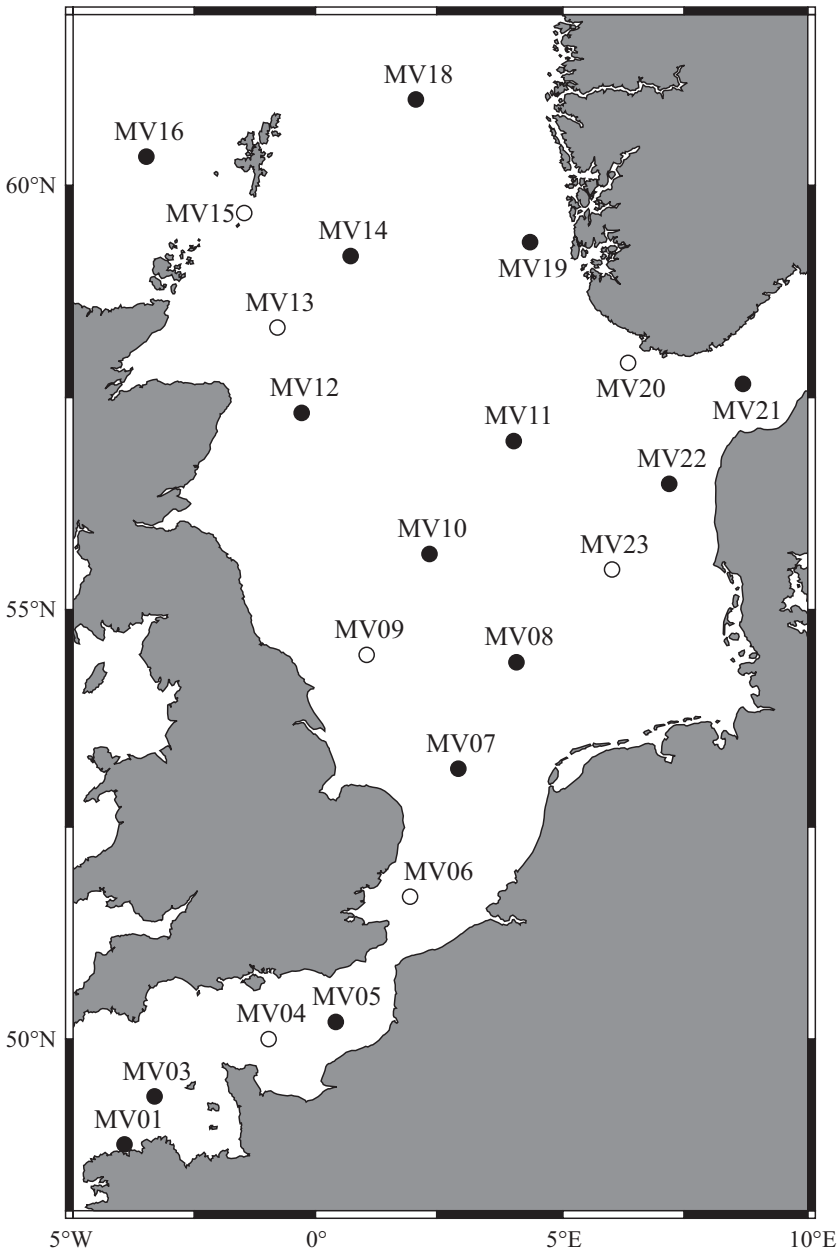

Figure 5.1 Station map of the RV Pelagia cruise MICROVIR (64PE217) in July 2007. Samples for environmental analyses, microbial abundances and IPL analysis were taken at all of the stations, with the exception of MV23 (no bacterial abundances). Chlorophyll *a* concentrations were only determined for stations with filled symbols. All sampling was performed at 10 m water depth.

Table 5.1 Location of sampling stations of the MICROVIR cruise in the North Sea, date of sampling, amount of water filtered for IPL analysis, chlorophyll *a* concentrations and summary of the environmental parameters (from Brandsma *et al.*, 2011).

Station	Latitude (°N)	Longitude (°W)	Date[a]	Sampling volume (l)	Temperature (°C)	Salinity	Chlorophyll *a* (µg L^{-1})[b]	Phosphate (µmol L^{-1})	Ammonium (µmol L^{-1})	NO_x (µmol L^{-1})	Silicate (µmol L^{-1})
MV01	48.7695	-3.9467	4 July	120	14.6	35.3	0.46	0.15	0.75	1.44	1.70
MV03	49.3299	-3.3299	5 July	60	14.7	35.4	0.71	0.09	0.56	1.19	1.72
MV04	50.0000	-1.0006	7 July	54	15.0	35.0	n.a.	0.08	0.47	1.47	1.43
MV05	50.2033	0.3305	8 July	196	15.1	34.9	9.24	0.02	0.21	0.16	0.30
MV06	51.6663	1.8834	9 July	60	15.7	35.0	n.a.	0.02	0.12	0.01	0.18
MV07	53.1698	2.8708	10 July	237	15.2	34.1	5.37	0.06	0.24	0.45	0.32
MV08	54.4127	4.0523	11 July	268	15.4	34.8	0.24	0.08	0.07	0.06	0.17
MV09	54.5003	0.9998	12 July	52	14.8	34.6	n.a.	0.03	0.09	0.03	1.00
MV10	55.6806	2.2796	13 July	237	14.8	34.9	0.22	0.05	0.09	0.04	0.29
MV11	57.0010	3.9995	14 July	231	14.7	34.5	0.44	0.02	0.08	0.03	0.08
MV12	57.3305	-0.3299	17 July	194	13.0	34.8	0.44	0.07	0.08	0.04	0.88
MV13	58.3298	-0.8295	17 July	66	13.0	35.0	n.a.	0.03	0.07	0.05	0.42
MV14	59.1698	0.6711	19 July	235	13.1	35.2	0.30	0.01	0.06	0.05	0.20
MV15	59.6700	-1.5011	19 July	52	11.1	35.3	n.a.	0.30	1.93	1.83	1.13
MV16	60.3302	-3.4993	20 July	166	12.3	35.3	0.54	0.27	0.49	3.77	1.23
MV18	61.0002	1.9989	22 July	91	13.5	34.0	0.34	0.02	0.07	0.04	0.09
MV19	59.3304	4.3302	24 July	106	14.6	30.4	0.52	0.01	0.07	0.05	0.03
MV20	57.9195	6.3292	25 July	47	14.9	32.2	n.a.	0.01	0.07	0.07	0.39
MV21	57.6699	8.6750	26 July	166	16.1	30.7	0.62	0.01	0.07	0.04	0.02
MV22	56.5007	7.1720	27 July	111	15.4	34.4	1.55	0.02	0.09	0.11	1.89
MV23	55.4999	5.9996	29 July	252	16.0	34.7	n.a.	0.01	0.06	0.04	<0.01

[a] all dates analyzed in 2007

[b] n.a. = not analyzed

chromatographic conditions as described by Jaeschke *et al.* (2009). 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. Source and fragmentation parameters for positive ion analysis were as described by Boumann *et al.* (2006). For analysis in negative ion mode the following source parameters were used: capillary temperature 250°C, sheath gas (N_2) pressure 49 (arbitrary units), auxiliary gas (N_2) pressure 21 (arbitrary units), spray voltage -4.2 kV, and source CID 7 V. The collision energy was set at -35 V with a collision gas (Ar) pressure of 0.8 mTorr for fragmentation

in negative ion mode. Identification of the major IPL classes was based on diagnostic fragmentation patterns in the MS^2 mass spectra (Vogel *et al.*, 1990; Benning *et al.*, 1995; 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), diacylglyceryl-(*N,N,N*)-trimethylhomoserine / diacylglyceryl-hydroxymethyl-(*N,N,N*)-trimethylalanine (DGTS / DGTA), or diacylglyceryl-carboxyhydroxymethylcholine (DGCC) 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, *m/z* 236 and *m/z* 178, respectively). DGTS and DGTA both produce a fragment ion at *m/z* 236, but were distinguished by their elution order on the HPLC, with the more polar DGTA eluting later (Dembitsky, 1996). IPLs with a phosphatidylglycerol (PG), phosphatidylethanolamine (PE) or sulfoquinovosyldiacylglycerol (SQDG) head group were measured in positive ion mode by neutral loss scanning (*m/z* 300-1000) for losses of 189 Da, 141 Da and 261 Da, respectively. The MS^2 settings for detection of these IPLs were identical to those described above, but for detection of SQDG the collision energy for the neutral loss assay was set to -25 V. 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., $C_{30:1}$ PG refers to an IPL with a phosphatidylglycerol head group and two fatty acids containing a total of 30 carbon atoms and one double bond equivalent; 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 (mass 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), and a mixture of SQDGs, which contained predominately $C_{16:1}/C_{18:2}$ SQDG, but also small amounts of SQDGs with $C_{16:0\text{-}16:1}$, $C_{18:0\text{-}18:1}$ and $C_{20:5}$ fatty acid combinations (Lipid Products, Redhill, Surrey, UK). In addition, a standard of $C_{14:0}/C_{18:1}$ DGTS was purified from IPL extracts of *Isochrysis galbana* (CCMP 1323) biomass using semi-preparative HPLC (Jaeschke *et al.*, 2009) and flow injection analysis – mass spectrometry (FIA-MS; Smittenberg *et al.*, 2002). Structural identification and purity of this standard (~70%) were confirmed by comparing its ^{1}H and ^{13}C NMR spectra with previously published spectra of DGTS (Evans *et al.*, 1982). As no standards were available for DGTA and DGCC, the concentrations of these IPL classes were estimated by comparing their peak areas (total ion current in the full scan data acquired as part of the positive ion data dependent MS^2 experiment) with those of DGTS, which has a similar molecular structure (Dembitsky, 1996; Kato *et al.*, 1996). Limits of detection were 50-100 pg on column for the glycerophospholipids, 100 pg

on column for DGTS and 1 ng on column for SQDG. 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.

5.2.3 Statistical analysis

Relationships between the IPL concentrations and environmental and microbial parameters were tested statistically in Systat 13 (Systat Software, San Jose, CA). The measure of association between each pair of variables was determined by calculating their Spearman's rank correlation coefficients (ρ). This test was chosen as many of the variables showed a highly skewed distribution. Only variable associations with corrected probability values (p) of less than 0.05 were considered significant and are reported here. In addition, the IPL compositional similarities between the stations were determined by distance-based ordination, using the dedicated software package PRIMER 6 with the PERMANOVA+ add-on (both PRIMER-E Ltd, Lutton, UK). Bray-Curtis similarity matrices of the stations were calculated based on their IPL composition (both for the total concentrations of the IPL classes and the concentrations of the IPL species), and the results were plotted using multidimensional scaling (MDS) ordination. Cluster analysis was used to group stations with comparable IPL compositions. The measures of dependence between the IPL similarity matrices and a similarity matrix based on the microbial community composition were determined by calculating their Spearman's rank correlation coefficients (RELATE test in PRIMER).

5.3 RESULTS

5.3.1 Structural diversity of IPLs

The IPL diversity in the surface waters of the North Sea and adjacent areas comprised seven main classes: the glycerophospholipids PC, PG and PE, the sulfur-bearing glycerolipid SQDG, and the nitrogen-bearing betaine lipids DGTS, DGTA and DGCC (Fig. 5.2). In addition, trace amounts of the glycerolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG) were detected as well. Each of the main IPL classes contained a large variety of IPL species with different fatty acid compositions. A considerable difference in the number of species was noted between PG and SQDG (around 40 each), and the other IPL classes (around 100 each). The fatty acid compositions of the predominant IPL species can be found in Table S5.1. Of the different classes, SQDG showed the least structural variation, containing mostly combinations of C_{14}, C_{16} and C_{18} saturated or mono-unsaturated fatty acids. For the other IPL classes, the variation in composition was larger, with fatty acid chain lengths generally ranging from C_{12} to C_{22}. Within the

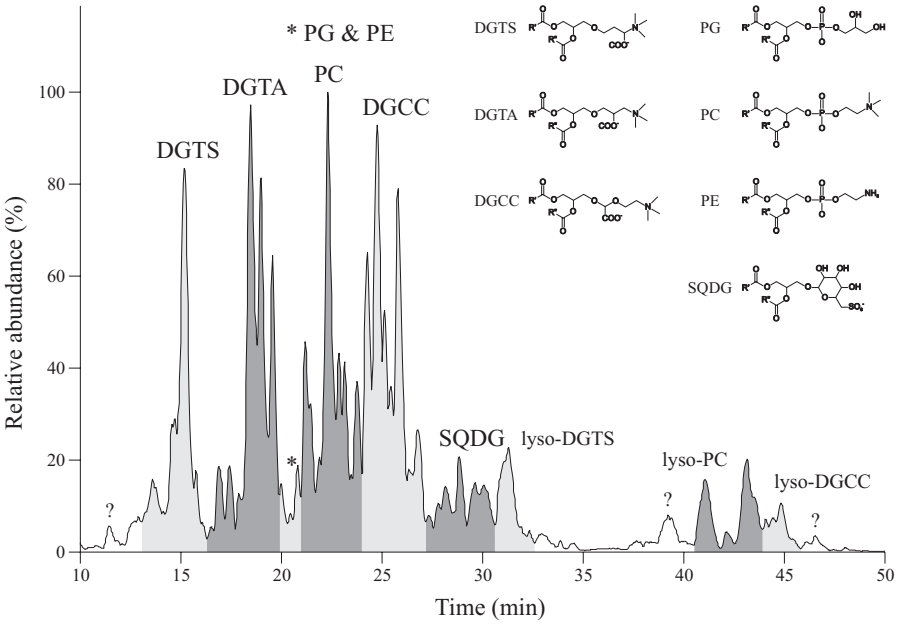

Figure 5.2 Partial base peak chromatogram (Gaussian smoothed) of the HPLC/ESI-MS^2 analysis of IPLs in the particulate matter sampled at station MV10, showing the IPL classes that predominate in marine surface waters throughout the North Sea. Unidentified peaks are indicated with a question mark. Example structures are given for each of the detected IPL classes: diacylglyceryl-trimethylhomoserine (DGTS), diacylglyceryl-hydroxymethyltrimethylalanine (DGTA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), diacylglyceryl-carboxyhydroxymethylcholine (DGCC) 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). Note that due to differences in mass spectral response factors between the IPL classes, their relative abundances in the base peak chromatogram are not necessarily indicative of their respective absolute abundances.

PGs and DGTSs the shorter chain lengths (C_{14} to C_{18}) predominated, while most of the DGCCs had longer-chain fatty acids (C_{18} to C_{22}). Although the majority of the fatty acids in each of the IPL classes had combinations of fatty acids with even chain lengths, some odd-carbon number fatty acids (C_{13} to C_{19}) were also detected. These were most common amongst the PEs and PCs, but absent in the DGCCs. Finally, long-chain C_{18} to C_{22} polyunsaturated fatty acids (PUFAs) were particularly predominant in the PCs, DGTAs and DGCCs, but absent in the SQDGs.

5.3.2 IPL concentrations and spatial distribution

Quantification of the IPLs at the different stations shows that overall SQDG was the most abundant IPL class (Fig. 5.3), with concentrations generally ranging from 0.5 to 5 µg l^{-1}, but up to 45 µg l^{-1} in the eastern English Channel (Fig 5.4A). $C_{28:0}$, $C_{30:1}$, $C_{30:0}$ and $C_{32:1}$ SQDG constituted the bulk of the total SQDG concentration throughout (70±5%; Table S5.2). At most stations the relative abundances of these four species were comparable, but elevated contributions of $C_{28:0}$ SQDG were measured around the Shetland Islands (up to 49% of the total SQDG) and in the southern North Sea (34%). In addition, elevated contributions of $C_{32:0}$ SQDG and $C_{34:1}$ SQDG were detected in the southeastern North Sea (11% and 6%, respectively), while in the eastern English Channel $C_{32:1}$ SQDG predominated (32%).

PC was generally the most abundant of the glycerophospholipids at all stations, followed by PG and PE (Fig. 5.3). PC concentrations generally ranged from 80 to 440 ng l^{-1}, but up to 1.2 µg l^{-1} in the eastern English Channel (Fig. 5.4B). There was no single predominant PC species, although ten species were on average more common (3-9% of

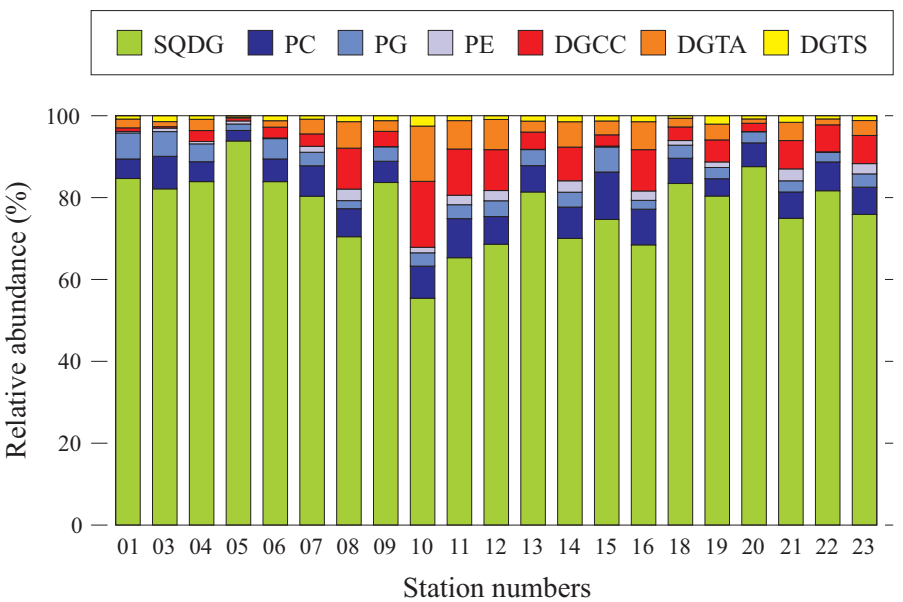

Figure 5.3 Relative abundances of the predominant IPL classes in the surface waters of the North Sea. The abundances are presented as a fraction of the total quantified IPL content at each station (i.e., within the mass window of *m/z* 300-1000 and excluding minor unidentified compounds, but including estimated concentrations of DGTA and DGCC).

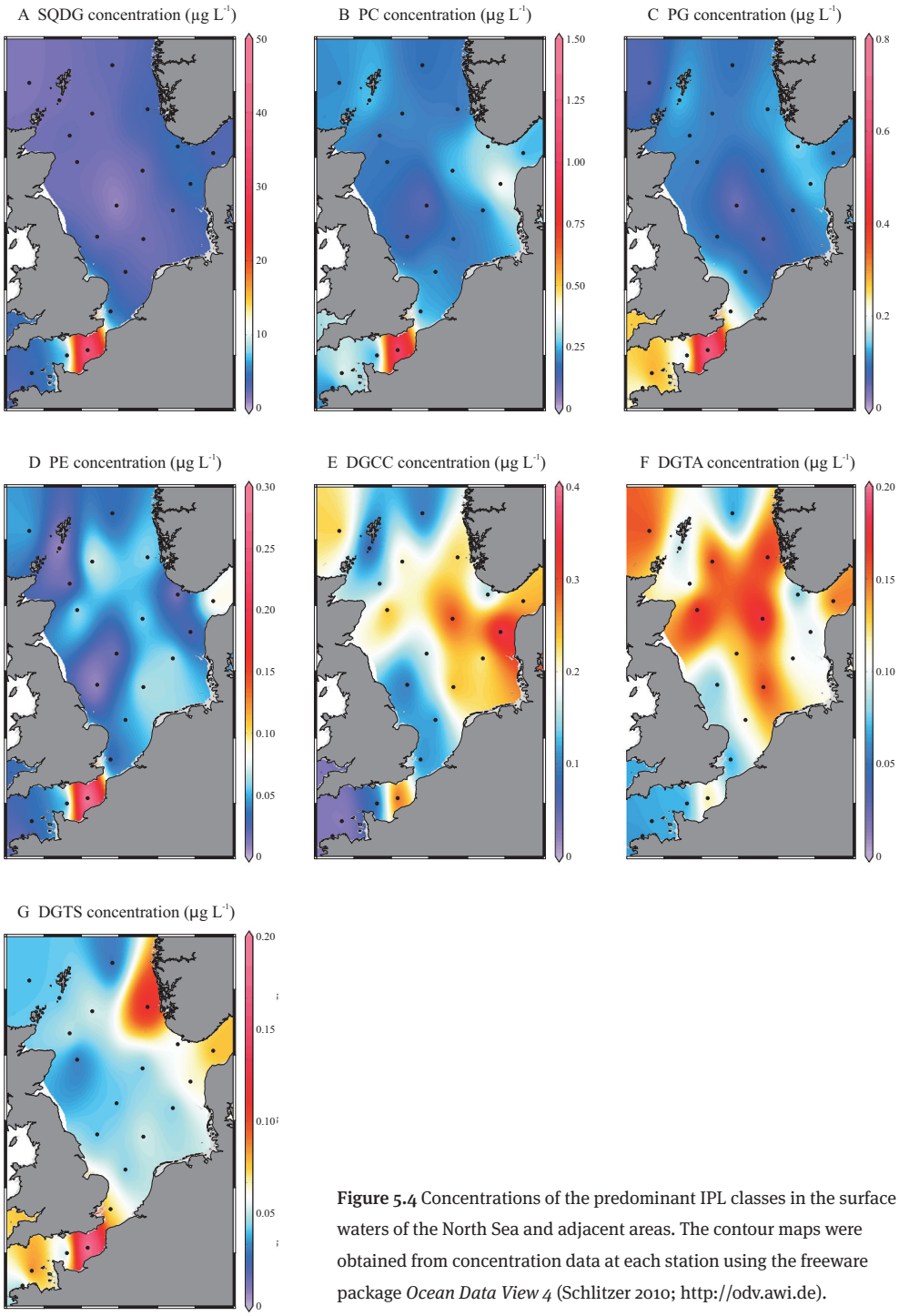

Figure 5.4 Concentrations of the predominant IPL classes in the surface waters of the North Sea and adjacent areas. The contour maps were obtained from concentration data at each station using the freeware package *Ocean Data View 4* (Schlitzer 2010; http://odv.awi.de).

the total PC; Table S5.2), with the PUFA-bearing $C_{38:6}$ PC having the highest abundance overall (7-22%). PG concentrations generally ranged from 30 to 320 ng l^{-1}, but up to 750 ng l^{-1} in the eastern English Channel (Fig. 5.4C). $C_{32:2}$, $C_{32:1}$, $C_{34:2}$ and $C_{36:2}$ PG constituted the bulk of the total PG concentration at the different stations (57±3%), with their relative abundances showing little variation (Table S5.2). In addition to these species, another six PGs comprising a wide range of $C_{12:0}$ to $C_{20:4}$ fatty acids were present in elevated amounts at some of the stations (up to 14%). Finally, PE concentrations generally ranged from 1 to 100 ng l^{-1}, with the stations in close proximity to the coast or in front of major river systems generally having the lowest values, and a maximum of 350 ng l^{-1} in the eastern English Channel (Fig. 5.4D). The species composition was more variable than in the PCs and PGs, with many species predominating at some of the stations, while having low abundances at the rest. Nonetheless, $C_{32:2}$, $C_{32:1}$, $C_{34:2}$, $C_{34:1}$ and $C_{38:6}$ PE were generally more common, constituting on average 47±2% of the total PE concentration throughout (Table S5.2).

Of the betaine lipids, DGTA and DGCC were more abundant than DGTS at most stations, although care should be taken as their concentrations could only be estimated due to the lack of authentic standards (see materials and methods). In general, DGCC was up to 2.5 times more abundant than DGTA (Fig. 5.3), with estimated concentrations ranging between 20 and 410 ng l^{-1} for DGCC, and between 60 and 190 ng l^{-1} for DGTA (Fig. 5.4E-F). $C_{34:5}$, $C_{36:6}$, $C_{36:5}$, $C_{38:6}$ and $C_{44:12}$ DGCC were the predominant DGCC species throughout, although $C_{44:12}$ DGCC was generally not detected in the English Channel. $C_{38:6}$ DGCC was the most abundant species in the central part of the North Sea, while elevated contributions of $C_{30:0}$ and $C_{32:0}$ DGCC were observed in the southeastern North Sea and the eastern English Channel. Within the DGTAs, the species containing short-chain saturated or mono-unsaturated fatty acids (particularly $C_{30:1}$ and $C_{33:1}$ DGTA) predominated in the English Channel and southern and western North Sea, while the species containing longer-chain PUFAs (particularly $C_{38:6}$, $C_{40:10}$, $C_{42:11}$ and $C_{44:12}$ DGTA) predominated in the rest of the North Sea, the Skagerrak and around the Shetland Islands. Finally, DGTS concentrations generally ranged from 15 to 90 ng l^{-1}, but up to 160 ng l^{-1} in the eastern English Channel (Fig. 5.4G). As with the PCs, no predominant species could be identified, although $C_{28:0}$, $C_{30:1}$, $C_{32:2}$, $C_{32:1}$ and $C_{34:2}$ DGTS were on average more common and comprised 33±2% of the total DGTS concentration at most stations (Table S5.2). While the relative abundances of these five species were mostly comparable throughout the study area, elevated contributions of $C_{30:1}$ DGTS were measured in the central North Sea (up to 29%). In addition, a further eleven DGTS species were present in moderate and highly variable amounts (up to 6%). In particular, elevated concentrations of $C_{34:1}$ DGTS were detected in the English Channel and southern North Sea (12%), while the PUFA-containing $C_{36:5}$ DGTS predominated around the Shetland Islands (24%).

5.4 DISCUSSION

5.4.1 Environmental and microbial biogeography

The environmental conditions and microbial biogeography of the North Sea during the MICROVIR have previously been discussed in Brandsma *et al.* (2011), but a summary of their results is given here and in Table 5.1. In general, the environmental parameters showed an east-west gradient from the 'Atlantic' stations (Shetland Islands and English Channel) to the stations in the North Sea. Temperature increased from 11°C to 18°C, while salinity decreased from 35.4 to 34.6 (Fig. 5.5A-B). The Skagerrak and Norwegian coastal area were further distinguished from the rest of the North Sea by their lower salinity (around 32). Concentrations of dissolved inorganic nitrogen, phosphorous and silicate were high at the Atlantic stations and near-coastal sites, but substantially lower over much of the North Sea (Fig. 5.5C-E). N:P ratios of dissolved inorganic nutrients ranged from around 16 at the Atlantic stations to less than 5 in the central North Sea, while high ratios (up to 25) were measured in the Skagerrak and the eastern English Channel (Fig. 5.5F).

Throughout most of the study area chlorophyll *a* concentrations were fairly low (0.2-0.5 µg l^{-1}), except in the eastern English Channel and southern North Sea, where concentrations reached 9.2 µg l^{-1} (Fig. 5.6A and Table 5.1). Within the microbial community, the distribution of the cyanobacteria was generally converse to that of the eukaryotes (Fig. 5.6B-D). Cyanobacteria were most abundant in the central North Sea (up to 9.6 x 10^4 cells ml^{-1}), while smaller picoeukaryotes were most abundant in the English Channel and southern and western North Sea (up to 2.4 x 10^4 cells ml^{-1}), and larger nanoeukaryotes were most abundant around the Shetland Islands and in the eastern North Sea and Skagerrak (up to 1.2 x 10^4 cells ml^{-1}). In addition, at station MV05 in the eastern English Channel a bloom of large diatoms (Bacillariophyceae) took place at the time of sampling. The bacterial distribution was generally unrelated to the distributions of the cyanobacteria and eukaryotes, although some of the highest abundances of heterotrophic bacteria (1.8 x 10^6 cells ml^{-1}) were found at the aforementioned diatom bloom (Fig. 5.6E).

5.4.2 Statistical relationships between IPLs and environmental and microbial parameters

To investigate the potential sources of IPLs and the effect of environmental conditions on the IPL composition, we statistically compared the IPL concentrations with the environmental and microbiological parameters measured in the same set of samples (Brandsma *et al.*, 2011). The measure of association (Spearman's correlation coefficient: ρ) between each pair of parameters is given in Table S5.3. None of the IPL abundances correlated with salinity, temperature or nutrient concentrations, but positive correlations were observed between the N:P ratio and total concentrations of SQDG, PG, PC

and DGTS (ρ > 0.54). Positive correlations were also observed between concentrations of chlorophyll *a* and SQDG, PG and PC (ρ > 0.69). Scatter plots revealed this relationship to be linear for the log-transformed data, with R^2 values of 0.45 for PG, 0.59 for PC and 0.61 for SQDG (n = 14). To some extent chlorophyll *a* concentrations correlated with DGTS as well (ρ = 0.48), but they did not with PE, DGTA or DGCC. Few significant correlations were found between microbial abundances and IPL concentrations. SQDG, PG and PC were weakly correlated with picoeukaryotic abundances (ρ > 0.45), while all IPL classes were weakly correlated with bacterial abundances (ρ > 0.40), except for DGTA, which was the only class to correlate with cyanobacterial abundances (ρ = 0.65).

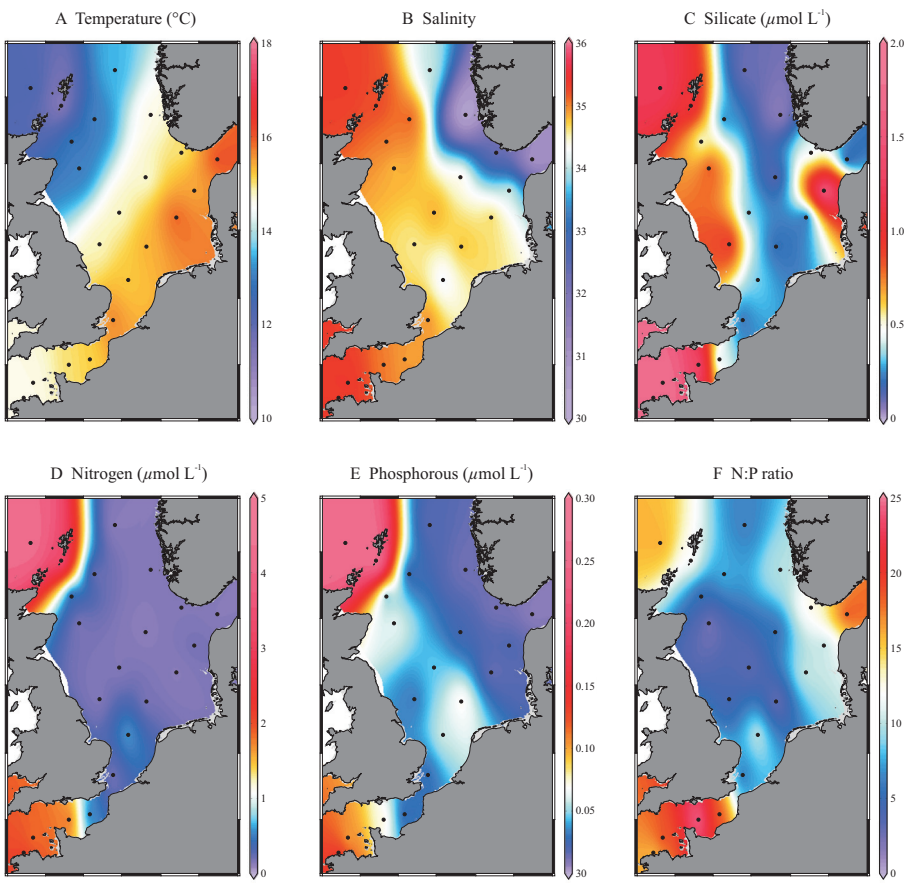

Figure 5.5 Temperature, salinity, concentrations of dissolved inorganic nutrients (Si, N and P) and N:P ratio in the surface waters of the North Sea (data obtained from Brandsma *et al.*, 2011). Contour maps were obtained using the freeware package *Ocean Data View 4* (Schlitzer 2010; http://odv.awi.de).

In general, the individual IPL species showed correlation patterns similar to their respective total IPL classes. The SQDGs and PGs, as well as most of the PCs and DGTSs, were correlated with chlorophyll *a* concentrations and bacterial and picoeukaryote abundances. The DGTSs showed the weakest correlations, and several DGTS species could not be related to any microbial group. Furthermore, the PC species containing long-chain PUFAs were correlated with nanoeukaryote, rather than with picoeukaryote abundances. The PEs were mostly correlated with bacterial abundances, but PE species containing either a $C_{20:5}$ or a $C_{22:6}$ fatty acid were not associated with any of the microbial groups.

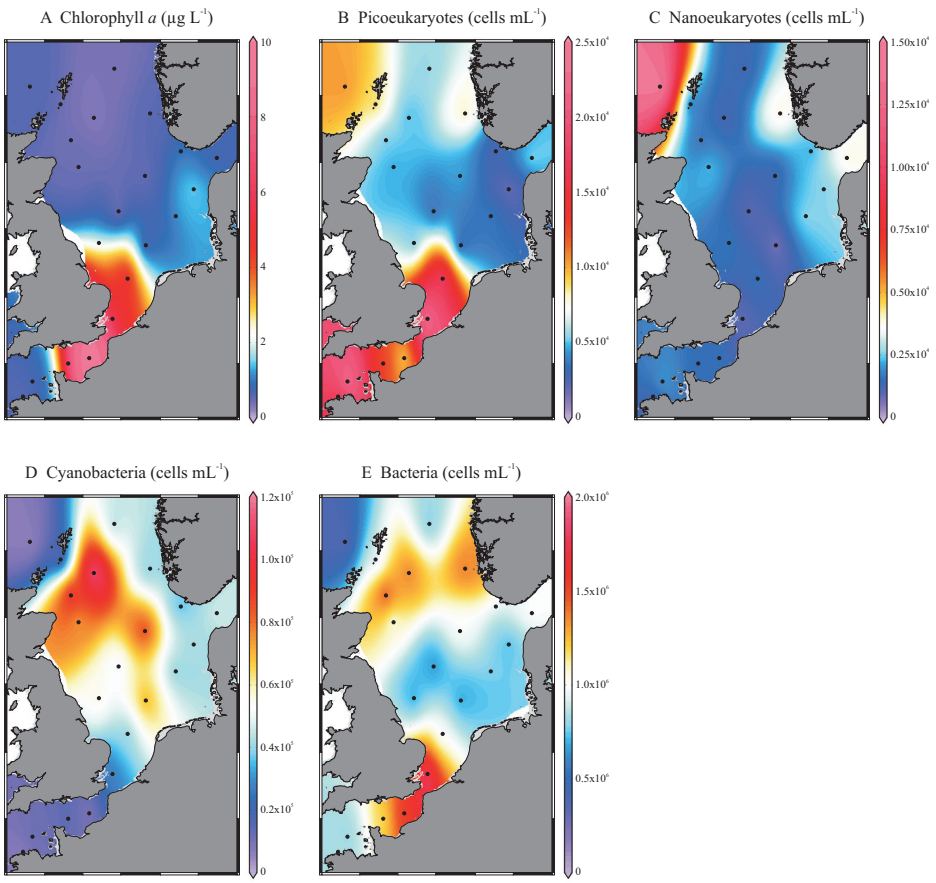

Figure 5.6 Chlorophyll *a* concentrations and abundances of the major microbial groups in the surface waters of the North Sea (data obtained from Brandsma *et al.*, 2011). Note that a bloom of large diatoms (Bacillariophyceae) was sampled in the eastern English Channel at station MV05. Contour maps were obtained using the freeware package *Ocean Data View 4* (Schlitzer 2010; http://odv.awi.de).

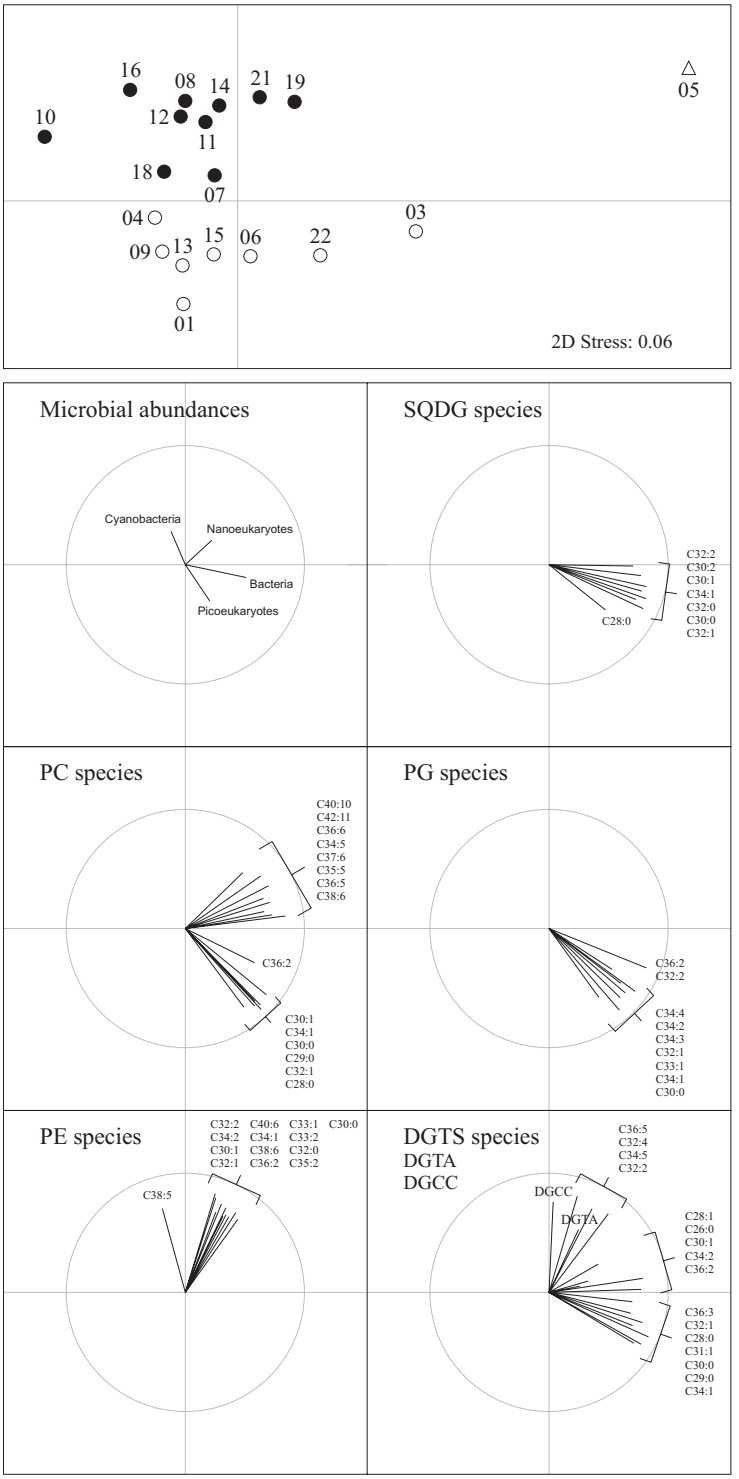

Figure 5.7 Distance-based ordination of the MICROVIR stations based on IPL species concentrations (Multidimensional scaling ordination of a Bray-Curtis similarity matrix). The distance between stations in the upper section of the figure is a measure of their compositional similarity, with most stations falling into two clusters (based on cluster analysis of the similarity matrix; threshold at >80% similarity), and one outlier station (MV05). The plots in the lower part of the figure show how the IPL concentrations correspond with the spatial distribution of the stations, and how they correlate with the microbial community composition.

An alternative statistical method to relate the IPL concentrations with the environmental and microbial data is to plot the stations according to their compositional similarities using distance-based ordination (in this case MDS ordination of Bray-Curtis similarity matrices) and cluster analysis (Fig. 5.7). Although the resulting plot appears to be skewed towards one particular station (MV05, where the diatom bloom produced very high IPL concentrations), analysis of the dataset while excluding this station resulted in comparable plots and identical clustering of the stations (data not shown). The distance-based ordination shows that the stations in the English Channel, western North Sea and off the Danish coast (cluster 1; open circles in Fig. 5.7) were characterized by high concentrations of SQDGs and PGs, while those in the central North Sea (cluster 2; filled circles in Fig. 5.7) were characterized by high concentrations of PEs, DGTAs and DGCCs. The PCs and DGTSs occupied an intermediate position between these two groupings, and were high in the eastern North Sea, the Skagerrak, and parts of the English Channel. These three IPL groupings roughly matched the clustering of the microbial community composition, with stations MV16 and MV22 being the most obvious exceptions, and a significant correlation was found between the two similarity matrices ($\rho = 0.40$, $p = 0.008$), However, no such correlation could be found between the IPL concentrations and the environmental data ($\rho = 0.17$, $p = 0.114$). Furthermore, the distributions of the PC and DGTS species were less uniform than those of the SQDGs, PGs and PEs, and depended on their fatty acid moieties (Fig. 5.7). High concentrations of PC species with short-chain saturated or monounsaturated fatty acids coincided with high concentrations of SQDGs and PGs, as well as high picoeukaryote abundances. In contrast, the PC species containing one or two long-chain PUFAs coincided with high nanoeukaryote abundances. The division in the DGTSs was less clear-cut with DGTS species occupying intermediate positions between the other IPL groupings. However, all DGTS species containing PUFAs coincided with high concentrations of PUFA PCs, PE, DGTA or DGCC, rather than with SQDG, PG or non-PUFA PCs.

5.4.3 Influence of environmental parameters on IPL distributions

Statistical analyses revealed no significant correlations between IPL concentrations and sea surface temperature or salinity, which indicates that these parameters are unlikely to affect IPL distributions directly (Table S5.3). Similarly, no correlations were found with the concentrations of dissolved inorganic nutrients (i.e., phosphate, ammonium, NO_x and silicate) in these waters. However, positive correlations were observed between concentrations of SQDG, PG, PC and DGTS and the N:P ratio. This ratio is generally seen as a measure for phosphate availability (Howarth, 1988), although it should be applied with care, as it does not take into account the more rapid and complete turnover of phosphorous compared to nitrogen, meaning that phosphate is not necessarily the limiting nutrient during phytoplankton blooms (e.g., Dodds, 2003). Martin *et al.*, (2010) recently showed that phytoplankton grown under phosphate limitation can rapidly

substitute the glycerophospholipids PG and PC in their cellular membranes with non-phosphorous SQDG and DGTS, and it was proposed that elevated ratios of SQDG:PG and DGTS:PC in marine waters reflect reduced phosphate availability to the phytoplankton (Van Mooy *et al.*, 2006, 2009). In our study area the N:P ratio ranged from less than 5 (potentially N-limited) to 25 (potentially P-limited), but despite this wide range of phosphate availability, no direct relationship of the N:P ratio with either the SQDG:PG ratio or the DGTS:PC ratio was observed ($R^2 < 0.03$, $n = 21$). Instead, concentrations of these four IPL generally co-varied, with especially SQDG and PGs, and PC and DGTS positively correlated with each other (Table S5.3). This co-variance contradicts the prediction that SQDG and DGTS should be found in elevated concentrations at stations with low N:P ratios, while PG and PC should be found in reduced concentrations (as per Van Mooy *et al.*, 2009). Together with the general lack of correlation between the IPL concentrations and ratios, and the nutrient concentrations and ratios, this implies that the nutrients were not a major controlling factor for the IPL diversity in the North Sea.

5.4.4 Potential sources of IPLs in the North Sea

Statistical analysis of the IPL concentrations with the microbial abundances yielded a number of significant relationships (Table S5.3 and Fig. 5.7), although the results were influenced to some degree by the comparatively high IPL concentrations at station MV05. The diatom bloom and associated heterotrophic bacteria at this site produced concentrations of SQDG, PG, PC, PE and DGTS that were on average 4-18 times higher than their concentrations over the rest of the North Sea (Fig. 5.4). Therefore, this site dominated the variability between the stations in the statistical analysis, despite pre-treatment of the data (log transformation). Nonetheless, the repeated analysis of the dataset showed that the results obtained from the distance-based ordination were comparable, whether station MV05 was included or not.

In the distance-based ordination plot three distinct sets of IPLs were distinguished and these could be tentatively related to the prevalent microbial groups (Fig. 5.7). The first set comprised SQDGs and PGs, as well as PCs containing saturated fatty acid moieties and several DGTSs, which were highest at stations in the English Channel, the southern and western North Sea and off the Danish coast, where picoeukaryote and bacterial abundances were highest (Figs. 5.6 and 5.7). The second set comprised the PUFA-PCs and PUFA-DGTSs, which were highest in the eastern North Sea, the Skagerrak and parts of the English Channel. These areas were characterized by high nanoeukaryote abundances, although surprisingly the station with the highest nanoeukaryote cell count (station MV16; Fig. 5.6C) did not show elevated IPL concentrations (Fig. 5.4). The third set of IPLs comprised the PEs, DGTAs and DGCCs, and possibly DGTSs containing PUFAs. These had the highest concentrations at stations in the central North Sea, which were characterized by high cyanobacterial and bacterial abundances (Figs. 5.6 and 5.7). The distance-based ordination analysis thus implicates the major microbial groups as predominant sources for discrete sets of IPLs.

The observed relationships can be substantiated by comparing them with IPL compositions of cultivated microbes. SQDG is restricted to photosynthetic organisms, where it forms the main anionic IPL in thylakoid membranes (e.g., Benning, 1988; Keusgen *et al.*, 1997; Frentzen, 2004). As SQDGs have to date not been found in heterotrophic bacteria, the correlations found with bacterial abundances are likely indirect, resulting from high numbers of heterotrophic bacteria occurring at sites of high photosynthetic phytoplankton biomass, in particular in the southern North Sea. Interestingly, SQDG concentrations were related to picoeukaryote, rather than nanoeukaryote abundances. Although this IPL probably occurs in both groups, it may be that the smaller picoeukaryotes contained comparatively more thylakoid membrane per cell, and thus contributed more to the total SQDG pool than the larger, but four times less abundant nanoeukaryotes.

In contrast to SQDG, the glycerophospholipids PC, PG and PE are common constituents of cellular membranes in most microorganisms, with PC mostly confined to eukaryotes and PE predominantly found in bacteria (e.g., Shaw, 1974; Lechevalier and Lechevalier, 1989; Sohlenkamp *et al.*, 2003 and references therein). PG is a more universal IPL class, as it is synthesized by both eukaryotes and prokaryotes, including cyanobacteria (e.g.,Wood, 1974; Lechevalier and Lechevalier, 1989; Dowhan, 1997), and is also found in small amounts in thylakoid membranes (Janero and Barrnett, 1981; Frentzen, 2004). Nonetheless, PG concentrations in the North Sea were mostly associated with picoeukaryote abundances, indicating that these were the predominant source of PGs. A similar relationship was found between picoeukaryotes and non-PUFA PCs, while the PCs containing PUFAs were rather associated with nanoeukaryote abundances. Although long-chain PUFAs are commonly found in both algal groups (e.g., Shaw, 1974; Volkman *et al.*, 1998; Guschina and Harwood, 2006), it thus appears that in the North Sea the larger nanoeukaryotes are the predominant source for PUFA PCs. The relationships found for the PEs were more ambiguous than for the other glycerophospholipids. The Spearman test yielded significant correlations between PE concentrations and bacterial abundances, which could for a large part be explained by the elevated presence of both at station MV05 (diatom bloom). On the other hand, the PE species containing PUFAs were not correlated with bacterial abundances, in line with the uncommon occurrence of such fatty acids in bacteria, while the distance-based ordination plots showed that overall the PEs were associated with stations with high cyanobacterial abundances (Fig. 5.7). Although PEs have thus far not been found in cultivated cyanobacteria (e.g., Lechevalier and Lechevalier, 1989; Van Mooy *et al.*, 2006), it is therefore possible that the PEs in the North Sea were partly derived from cyanobacteria, and partly from heterotrophic bacteria.

The betaine lipids DGTS, DGTA and DGCC are found in a wide range of eukaryotes (e.g., Sato, 1992; Dembitsky, 1996; Kato *et al.*, 1996), as well as in cyanobacteria (Řezanka *et al.*, 2003) and some photosynthetic bacteria (Klug and Benning, 2001; Elshahed *et al.*, 2007). Although betaine lipids are predominately found in cellular membranes, DGTS has also been found in minor amounts in thylakoid membranes (Janero and Barrnett, 1982; Mendiola-Morgenthaler *et al.*, 1985). In the North Sea, DGTA

and DGCC correlated with cyanobacterial abundances, implying that this microbial group was the predominant source. DGTS concentrations were not related to any single microbial group and in the distance-based ordination plots individual DGTS species occupied intermediate positions between the other IPL groupings, implying mixed sources for these IPLs. Most of the DGTS species appeared to be derived from either pico- or nanoeukaryotes, while some, including those containing PUFAs, were rather derived from either nanoeukaryotes or cyanobacteria.

5.4.5 IPL concentrations as a proxy for living biomass

Our statistical analysis shows that the IPL composition in the surface waters of the North Sea can be tentatively linked to the *in situ* microbial community composition. However, the overall measures of statistical dependence between the IPLs and microbial groups were relatively low, raising the question of well they represent the *in situ* microbial biomass. Abundances of IPLs or IPL-derived fatty acids are widely used as a proxy for microbial biomass in environmental and biogeochemical studies (e.g., Petsch *et al.*, 2001; Zink *et al.*, 2003; Lipp *et al.*, 2008), although the value of this proxy has recently been qualified to some extent (Schouten *et al.*, 2010). Statistical comparison of the IPL concentrations in the North Sea surface waters with the chlorophyll *a* concentration, a measure for photosynthetic phytoplankton biomass (Huot *et al.*, 2007), yielded good correlations for the eukaryotic IPL classes SQDG, PC, PG and possibly DGTS, but not for the (cyano)bacterial classes PE, DGTA and DGCC (Table S5.3). There thus appears to be a relationship between the amount of photosynthetic phytoplankton biomass and the concentrations of several IPL classes, which provides empirical evidence for the applicability of these compounds as a biomass proxy in marine waters. However, an exceptional situation was found at one of the stations (MV07), where chlorophyll *a* concentrations were high, but IPL concentrations were comparable to those of other stations. It is possible that this site represents the situation where a phytoplankton bloom has recently crashed, with the chlorophyll *a* molecules potentially having a longer residence time than the IPLs.

5.4.6 Comparison of the IPL composition of North Sea surface waters with other sites

Our study shows that there is a large diversity in intact polar lipids in the surface waters of the North Sea and adjacent areas. At least 600 different IPL species were recognized, and since each of these species may comprise multiple fatty acid compositions the total number of IPLs in a single surface water sample is likely to be much higher. Moreover, only the dominant IPLs in a mass window of 300-1000 Da were targeted here, and therefore the real structural diversity in IPLs could be even larger.

The IPL diversity within the current mass window was dominated by seven classes: SQDGs, glycerophospholipids (PC, PG and PE) and betaine lipids (DGCC, DGTA and

DGTS). Similar findings were made for the surface waters of the Black Sea (Schubotz *et al.*, 2009), the Sargasso Sea and the Pacific Ocean (Van Mooy *et al.*, 2006, 2009; Van Mooy and Fredricks, 2010), and the western North Atlantic Ocean (Popendorf *et al.*, 2011). However, mono- and digalactosyldiglycerides (MDGDs and DGDGs), which were abundant at these other sites, were only detected in minor amounts in the North Sea. The fatty acid compositions of the predominant IPL classes were generally comparable between the different locations. However, SQDGs and PGs containing C_{18} PUFAs were detected in the Black Sea (Schubotz *et al.*, 2009), but were rare (PGs) or not detected (SQDGs) in the North Sea and eastern South Pacific (Van Mooy and Fredricks, 2010). Furthermore, a comparison of the fatty acid chain lengths and degrees of unsaturation of the predominant IPL classes in the North Sea and eastern South Pacific showed that the IPLs in the North Sea had either similar or lower (PC) average fatty acid chain lengths, and a lower average degree of unsaturation (Table 5.2). These differences were most pronounced in the PCs, which in the North Sea contained comparatively more short-chain saturated or mono-unsaturated fatty acids than long-chain PUFAs. Although no quantitative data are available from the Black Sea, at all three sites SQDG, PG and DGTS had the shortest and most saturated fatty acid chains, while PC contained comparatively more long-chain PUFAs. DGTA and DGCC contained the longest and most unsaturated fatty acids in the North Sea and eastern South Pacific, but unfortunately no distinction was made between the three types of betaine lipid in the Black Sea (Schubotz *et al.*, 2009).

The general similarity between the IPL compositions observed in the North Sea and at other sites investigated to date suggests that the same suite of IPL classes, each with a broadly defined but distinct fatty acid profile, predominates in the surface waters of the world's oceans. The main difference between sites lies in the relative abundance of

Table 5.2 Average fatty acid chain lengths (number of carbon atoms) and degrees of unsaturation (double bond equivalents) of SQDG, PC, PG, PE and DGTS in surface waters of the North Sea (this study) and the eastern South Pacific (approximate values obtained from Van Mooy and Fredricks, 2010; their Figs. 8 and 9). Note that the values refer to the combined fatty acid moieties and do not include the glycerol moiety.

IPL class	North Sea		Eastern South Pacific	
	Average chain length	Average degree of unsaturation	Average chain length	Average degree of unsaturation
SQDG	30.3 ± 0.3	0.7 ± 0.1	30.3 ± 0.3	0.7 ± 0.1
PC	35.1 ± 1.0	3.2 ± 0.5	35.1 ± 1.0	3.2 ± 0.5
PG	33.2 ± 0.3	1.8 ± 0.1	33.2 ± 0.3	1.8 ± 0.1
PE	34.9 ± 0.9	2.7 ± 0.2	34.9 ± 0.9	2.7 ± 0.2
DGTS	32.5 ± 0.7	1.9 ± 0.5	32.5 ± 0.7	1.9 ± 0.5

these IPL classes and to some degree their fatty acid compositions. This is quite striking and somewhat surprising as the investigated sites comprise rather different environments (e.g., eutrophic vs. oligotrophic, tropical vs. temperate) and therefore presumably contain different microbial communities. Although there do appear to be some general relationships between the IPL composition in marine surface waters and the *in situ* microbial community composition, the overall low measures of statistical dependence, plus the fact that many of the IPLs do not appear to be specific for a single microbial group and predominate across a wide range of oceanographic settings, indicate that direct inferences of microbial community compositions from IPL compositions should be considered with care.

5.5 CONCLUSIONS

The intact polar lipid composition of the surface waters of the North Sea and adjacent areas showed many similarities with those detected at other sites, indicating that the same suite of IPL classes predominates in the surface waters of the world's oceans. The most abundant IPL species quantified in this study formed several statistically distinct groups, which could in turn be tentatively linked to the occurrence of the predominant microbial groups. Furthermore, concentrations of IPLs associated with eukaryotes correlated well with chlorophyll *a* concentrations, providing empirical support for the use of IPLs as a proxy for living microbial biomass. Finally, no evidence was found for direct influences of environmental parameters on the overall IPL composition in the North Sea surface waters. Our results show that while IPLs have the potential to be used as biomarkers for living microbial groups, they may be lacking in chemotaxonomic resolution and care should therefore be taken in inferring the microbial community composition in marine waters directly from the IPL composition.

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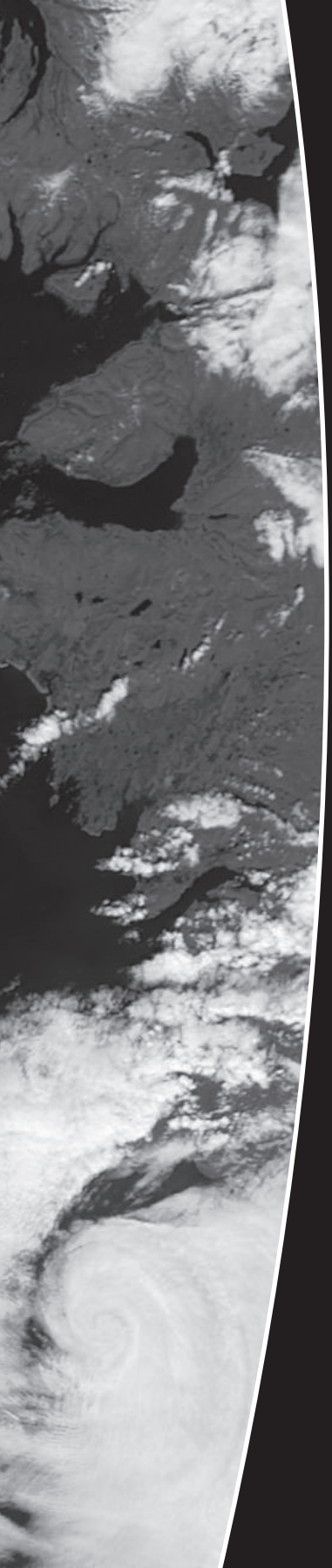

CHAPTER 6

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, while 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.

6.1 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, 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), while 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 Fredricks, 2010), the western North Atlantic (Popendorf *et al.*, 2011) and the North Sea (Brandsma *et al.*, 2011). 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 phosphati-

dylcholine (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-carboxyhydroxy-methylcholine (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.*, 2011; 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.

6.2 MATERIALS AND METHODS

6.2.1 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. 6.1). Sampling is performed at high tide, to assure 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.

6.2.2 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

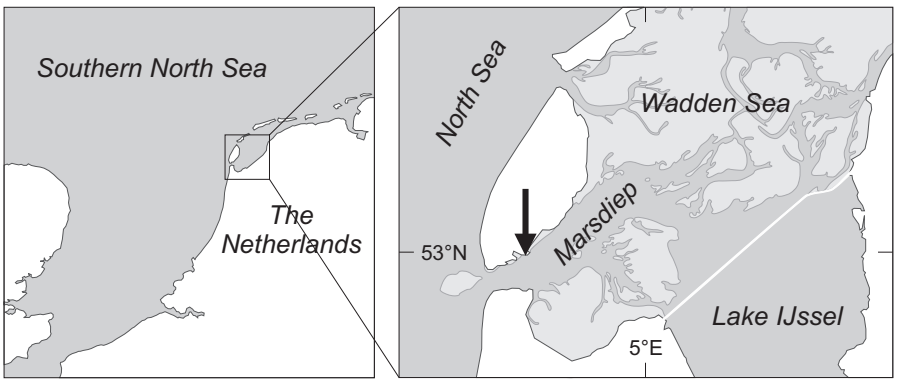

Figure 6.1 Map of the southeastern North Sea and Wadden Sea; the arrow marks the sampling site at the entrance of the Marsdiep tidal inlet.

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 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.

6.2.3 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 proce-

dure (Bligh and Dyer, 1959; Brandsma *et al*., 2011), 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*. (2011). 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 $C_{16:0\text{-}16:1}$, $C_{18:0\text{-}18:1}$ 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*. (2011). 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.

6.2.4 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 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.

6.3 RESULTS

6.3.1 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. 6.2A). 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. 6.2B). Dissolved inorganic phosphate (DIP) levels ranged from a maximum of 1.1 µmol l^{-1} to <0.1 µmol l^{-1}, while silicate (DISi) levels ranged from 40-42 µmol l^{-1} to less than 1 µmol l^{-1}, and nitrate concentrations ranged from 83 µmol l^{-1} to 1 µmol 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).

6.3.2 Microbial abundances and community composition

Primary production and chlorophyll *a* concentrations varied strongly throughout the year in response to the environmental conditions (Fig. 6.2C). In winter the primary production was low at 3-6 µg C l^{-1} h^{-1}, but increased to 165 µg C l^{-1} h^{-1} during the spring bloom. The same pattern was observed for the chlorophyll *a* concentrations, which increased from 2 µg l^{-1} to 55 µg 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 x 10^5 cells ml^{-1} between mid-March and mid-May (Fig. 6.2D). The first and by far the most pronounced algal bloom occurred in spring and was formed by the Prymnesiophyte

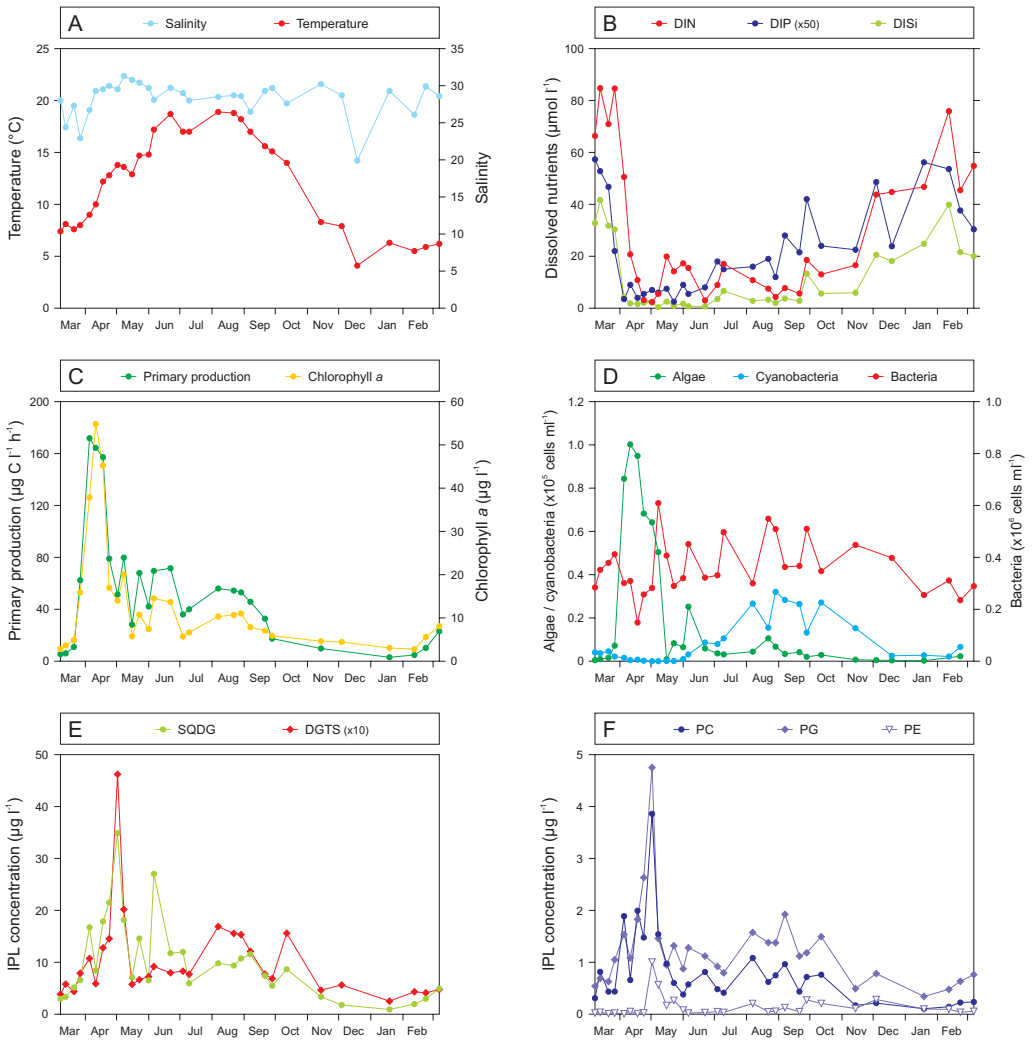

Figure 6.2 Time series of: A) salinity and temperature; B) dissolved inorganic nutrient concentrations; C) primary production and chlorophyll *a* concentrations; D) microbial abundances; E) and F) intact polar lipid concentrations (see main text for acronyms).

Phaeocystis globosa, with the colonial form predominating during the first part of the bloom and the solitary form during the second part (Fig. 6.3). 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*,

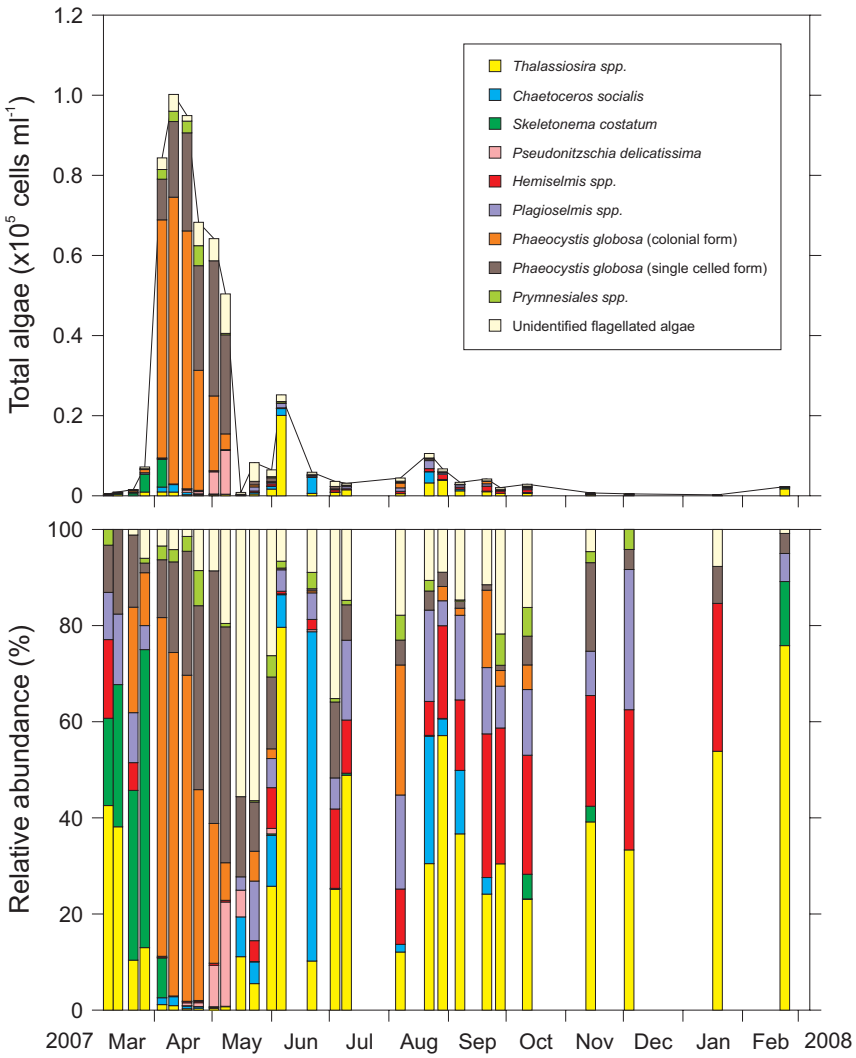

Figure 6.3 Abundances of the different algal groups in the Marsdiep. The upper graph shows the absolute abundances, while the lower graph shows relative abundances (normalized to the total counts).

together with the Cryptophyte *Plagioselmis spp.* and various unidentified flagellate algae (Fig. 6.3). 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. 6.3) and cyanobacteria (up to 3.2 x 10^5 cells ml^{-1}; Fig. 6.2D). Bacterial numbers were fairly constant throughout the year (3-5 x 10^6 cells ml^{-1}; Fig. 6.2D), but were lowest at during the algal spring bloom (1.5 x 10^6 cells ml^{-1}) and highest around its end (6.1 x 10^6 cells ml^{-1}).

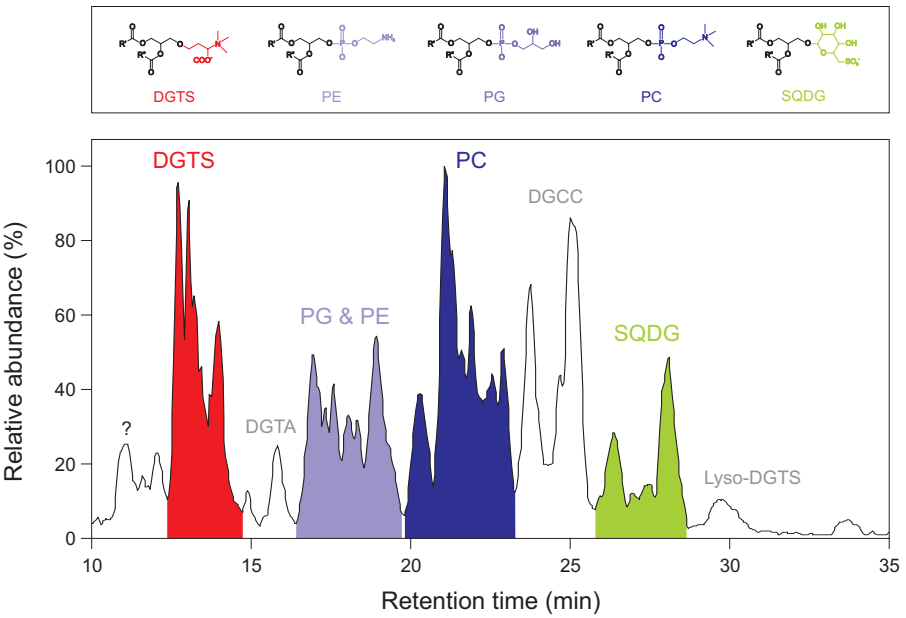

Figure 6.4 Partial base peak chromatogram (positive ion – Gaussian smoothed) showing the IPL classes identified in Marsdiep water during the phytoplankton spring bloom in late April. Unidentified peaks are indicated with a question mark. Example structures are given for each of the quantified IPL classes: 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.

6.3.2 IPL composition and abundances

Five major IPL classes were detected in the surface waters of the Marsdiep (Fig. 6.4): 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 S6.1). The least variety was observed in the SQDGs and PGs (around 50 species each), followed by the PEs and DGTSs (around 90 species each), while 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 S6.1). While 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 poly-unsaturated fatty acids (PUFAs) were common in the PCs, but rare in the other glycero-phospholipids 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 µg l^{-1} in winter to almost 35 µg l^{-1} at the peak of the spring bloom (Fig. 6.2E). 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±4% of the total SQDG concentration throughout the year (Table S6.2). In winter, the most abundant SQDG species were $C_{32:1}$ SQDG (21±3%) and $C_{28:0}$ SQDG (15±4%), while 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 glycero-phospholipid 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. 6.2F). 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 S6.2). $C_{32:1}$ PG was the most abundant species (17±3%), while 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 S6.2). 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 S6.2). 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 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 S6.2).

DGTS was present in roughly equal amounts to the glycerophospholipids PC and PG, with concentrations ranging from 0.3 to 4.6 µg l^{-1} (Fig. 6.2E). 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 S6.2). 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.

6.3.3 Statistics relationships

The measures of dependence between each of the measured variables (Spearman's ρ, n = 30) are given in Table S6.3. 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 abundances to be linear, with R^2 values ranging from 0.45 for DGTS to 0.71 for SQDG (n = 28; Fig. 6.5). Furthermore, negative relationships were observed with the dissolved nutrient concentrations in the water ($\rho < -0.51$; Table S6.3). PE was the only IPL class that was not correlated with any environmental or microbial parameter measured here, or any other IPL class (Fig. 6.5; Table S6.3). 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. 6.6 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, while PE was positively loaded on the second axis. The bacterial abundances were positively loaded on the third axis, while 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,

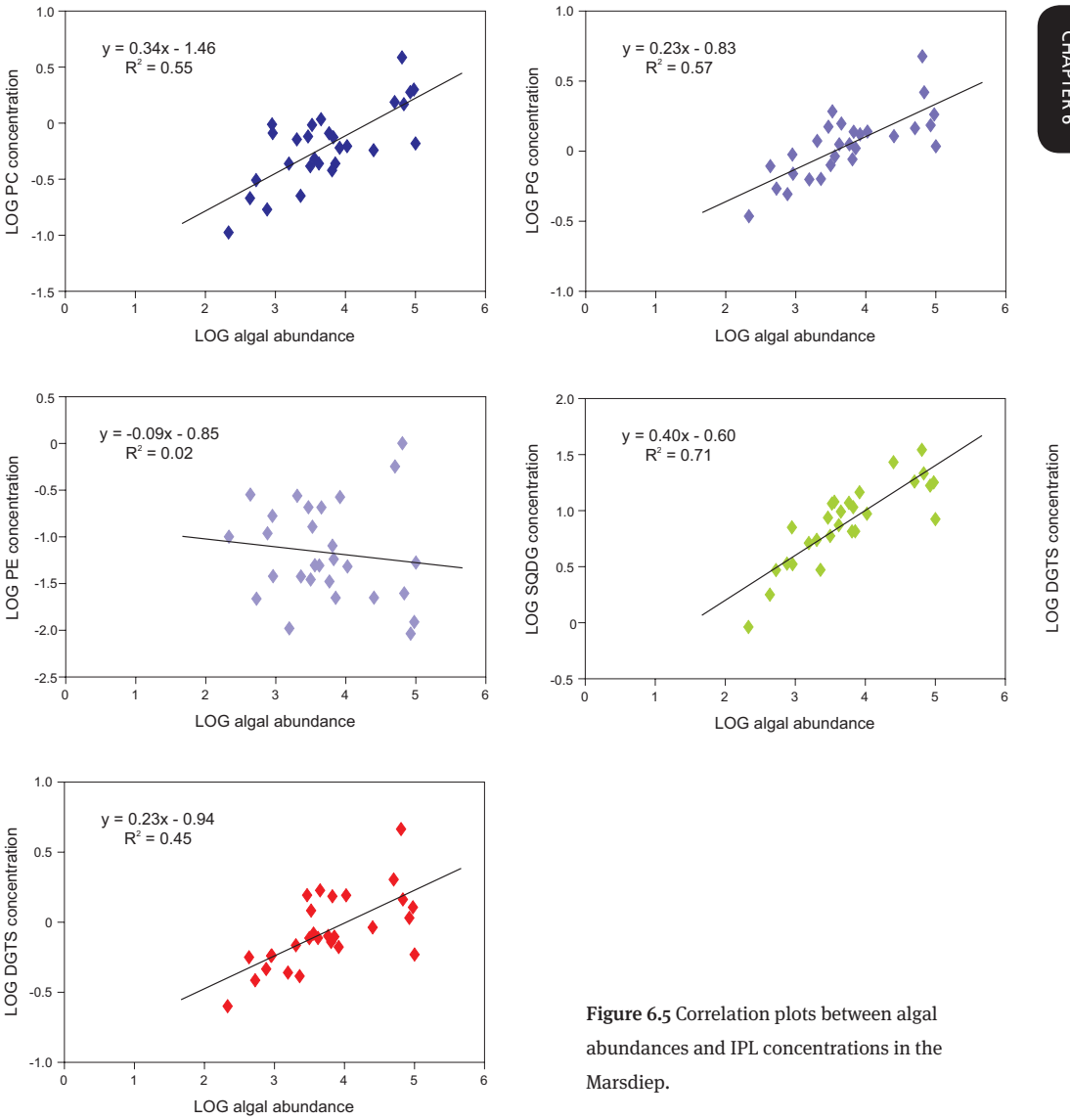

Figure 6.5 Correlation plots between algal abundances and IPL concentrations in the Marsdiep.

explaining 88% of the variance (Fig. 6.6 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%), while the PEs were the only class to load positively on the second axis (11%).

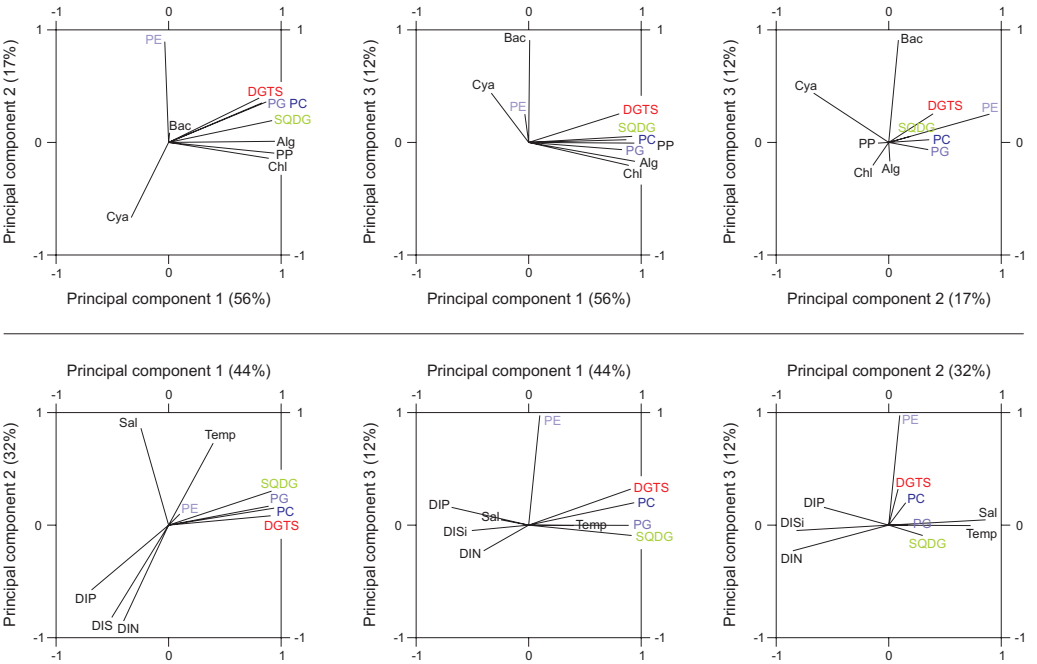

Figure 6.6 Principal component analysis (PCA) plots for the total concentrations of the IPL classes with microbial parameters (upper panel) and with environmental parameters (lower panel). Abbreviations: Alg = algal abundances, Cya = cyanobacterial abundances, Bac = bacterial abundances, Chl = chlorophyll *a* concentrations, PP = primary production, Temp = temperature, Sal = salinity, DIP/N/Si = dissolved inorganic phosphorous/nitrogen/silicate.

6.4 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.*, 2011). While 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.* (2011) 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. 6.2 and 6.3), 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 ($\rho > 0.77$), while the PEs were unrelated (Fig. 6.6 and Table S6.3). 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. While IPLs in general increased in concentration from mid-March onward, PE concentrations remained at low values throughout this period (Fig. 6.2F). 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. 6.6 lower panel and Table S6.3). 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 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. 6.6 upper panel). Scatter plots of the log-transformed IPL and algal data showed that the relationship was linear and strongest for SQDG (Fig. 6.5), 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 S6.3). 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 $C_{14:0}$, $C_{16:4\text{-}16:0}$, $C_{18:5\text{-}18:0}$, $C_{20:5}$ 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. 6.6 and Table S6.3). 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 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 $C_{14:0}$, $C_{16:4\text{-}16:0}$, $C_{18:5\text{-}18:0}$, $C_{20:5}$ 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.

6.5 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.

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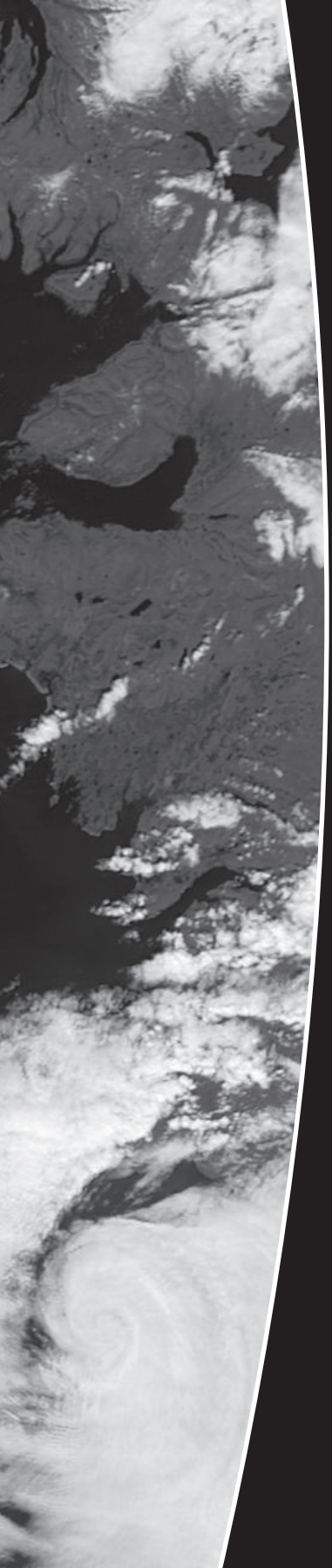

CHAPTER 7

SYNTHESIS AND OUTLOOK

7.1 INTRODUCTION

Ever since the discovery of microbes and the realization that they play an essential role in Earth system functioning, efforts have been made to identify, characterize and enumerate natural microbial communities. Initially, direct counting and enrichment cultures were used, and while these continue to yield important results, they are nowadays complemented by molecular methods, such as rDNA analysis, metagenomics and proteomics. The measurement of intact polar lipids (IPLs), the basic building blocks of biomembranes, also falls in the latter category. IPLs are complex molecules that are ubiquitous in the natural environment and have several characteristics that potentially allow them to be used as a proxy for living microbial cells and communities.

The aim of this thesis was to investigate the origins, dynamics and fate of IPLs in the marine environment and thereby test their use as a tool in marine environmental microbiology. Due to the relatively recent development of suitable instrumentation for direct IPL analysis, in particular HPLC/ESI-MS^n, most of our knowledge of polar lipids in the marine environment has been gained by studying polar lipid-derived fatty acids (PLFAs). However, the hydrolysis step necessary in analyzing PLFAs results in a considerable loss of structural information, in particular on the polar headgroups. By measuring IPLs directly, their structural diversity can now be examined in much greater detail, and as a result of this more detailed analysis the following questions are raised:

- How representative are IPL quantities of living microbial cells?
- To what extent can IPLs be used as chemotaxonomic markers for marine microbes?
- Are (changes in) environmental conditions reflected in microbial community-wide IPL compositions?

7.2 IPLS AS BIOMARKERS FOR LIVING MICROBIAL CELLS

The use of IPLs as biomarkers for living microbial cells depends critically upon the speed and completeness of their degradation upon cell death. However, the experimental evidence for this proxy is surprisingly scarce and may not be entirely accurate, as the IPLs were not measured directly. The first IPL degradation rates were determined by White *et al.* (1979), who observed rapid losses within the first few days of incubation. Similar results with half-times ranging from several hours to days were later obtained by a number of researchers (Tollefson and McKercher, 1983; Harvey *et al.*, 1986;

Petersen *et al.*, 1991; Moodley *et al.*, 2000), who all concluded that IPLs indeed degrade rapidly upon cell death, and can thus be used as a proxy for living microbial cells, and by extension microbial biomass (e.g., Balkwill *et al.*, 1988; Findlay *et al.*,

1989). However, upon examining their results it becomes clear that in each of the experiments IPL degradation rates slowed down progressively over time, and in none of the cases the IPL pool degraded completely.

In this thesis an attempt was made to provide both direct experimental evidence, as well as indirect evidence from the marine environment, for the use of IPLs as biomarkers for living microbial cells. The IPL degradation rates observed in decaying diatom cultures (*Chapter 2*) were comparable to those found in the previous experiments: initially high, but progressively slower over time. By using pure (axenic) cultures and enumerating the number of living and dead diatom cells in the incubations, it was shown that IPL concentrations correspond with the number of total cells, rather than living cells. The observation that IPLs are preserved in dead cells and cell fragments provides an explanation for the 'residual' IPL pool that is seen in all the previously reported degradation experiments. Although the introduction of bacteria into the system led to increased losses of IPLs, the residual pool was still substantial (~20%). Furthermore, IPLs with an ether-bound headgroup (i.e., SQDG) appear to degrade slower than the ester-bound glycerophospholipids (i.e., PC and PG) and are more resistant to bacterial degradation as well. This observation is in agreement with other degradation studies (Harvey *et al.*, 1986; Logemann *et al.*, 2011) and suggests that degradation rates are not uniform across the different types of IPLs.

The experimental evidence thus clearly suggests that, particularly compared to other biomolecular proxies like DNA and RNA (Dell'Anno and Corinaldesi, 2004), IPLs degrade neither as quickly, nor as completely as was previously assumed. This is surprising, as various studies have found good correlations between IPL (or rather PLFA) abundances and microbial cell numbers or biomass estimates obtained through other techniques (e.g., White *et* al., 1979; Ringelberg *et al.*, 1997). Indeed, our own results from the Gullmar Fjord (*Chapter 3*) indicate that ladderane IPLs can be used to track abundances of anammox bacterial cells in anoxic marine sediments, the environment with the highest IPL preservation potential (Harvey *et al.*, 1986; Logemann *et al.*, 2011). Furthermore, IPL concentrations in marine waters (*Chapters 5 and 6*) were correlated with phytoplankton cell counts and chlorophyll *a* concentrations, providing further evidence in favour of the proxy. It thus seems that under specific conditions IPLs could be preserved for prolonged periods of time, but that under most circumstances the majority of the IPL pool is degraded fairly quickly, and they can thus be assumed to correspond to living microbial cells. This is especially the case when there is a highly active microbial food web, such as in oxygen-rich environments where bacterial activity is high. Nonetheless IPL degradation requires further study, as the mechanisms that can potentially enhance or decrease degradation rates are not yet fully understood.

7.3 RELATIONSHIP BETWEEN IPLS AND MICROBIAL COMMUNITY COMPOSITION

The large structural diversity encountered in IPL molecules offers the potential for many biomarkers that are uniquely synthesized by particular organisms. Our current knowledge of biomarker IPLs and their fatty acid/alkyl constituents is based on analyses of enrichment cultures, and these are likely to provide more biomarkers in the future. An important aim of this thesis was to apply the available set of biomarker IPLs to the marine environment, and determine how well the IPL composition reflects the *in situ* microbial community composition. The ladderane IPLs measured in the Gullmar Fjord (*Chapter 3*) are an example of biomarker IPLs that are known to be highly specific to one particular group of bacteria (i.e., anammox bacteria; Sinninghe Damsté *et al*., 2002, 2005), and indeed their abundance in the sediment corresponded fairly well with that of anammox bacterial cell numbers and anammox activity.

In contrast, the lipidomics approach used in the surface waters of the North Sea (*Chapters 5 and 6*) provides more of a challenge for interpretation, as specificity is traded for comprehensiveness. This is illustrated by the large number of IPL species present in a single seawater sample (at least 600, not including variations in for example double bond positions), necessitating the use of statistical treatment to relate the occurrence of (sets of) IPLs to the occurrence of potential source organisms (see also Van Mooy and Fredricks, 2010). Concentrations of SQDGs, PGs, PCs and DGTSs were related to abundances of the photosynthetic primary producers, PEs to bacteria, and DGTAs and DGCCs possibly to cyanobacteria. The importance of cell size is illustrated by the much better correlation of the first set of IPLs with abundances of eukaryotic algae, but not cyanobacteria. While SQDG, PG, PC and DGTS are all known to be common in both microbial groups (Wood, 1974; Lechevalier and Lechevalier, 1989; Řezanka et al., 2003; Frentzen, 2004), it is likely that the larger eukaryotic cells contributed comparatively more to the total IPL pool, something that has also been observed for photopigments (Veldhuis and Kraay, 2004).

Surprisingly, despite the large number of IPLs analyzed, few unequivocal relationships were found with the microbial community composition and none of these relationships was particularly strong (*Chapter 5*). Furthermore, the rapid succession of algal groups blooming in the Marsdiep (*Chapter 6*) was not reflected in the species composition of the 'algal' IPLs, such as SQDG. Combined with the prevalence of these same IPLs at different marine sites (Schubotz *et al*., 2009; Van Mooy *et al*., 2009; Van Mooy and Fredricks, 2010; Popendorf *et al*., 2011), this suggests that they are the most widely synthesized, but also the least specific IPLs. General IPL screening may thus be comprehensive, but in comparison to other molecular methods like genomics or proteomics, it appears to lack the chemotaxonomic resolution to distinguish between microbial

groups in any detail, and care should be taken in inferring microbial community compositions directly from IPL compositions. However, with the current technology it is possible to look beyond these 'generic' IPLs and target more specific biomarkers, as is shown by the successful application of ladderane IPLs (*Chapter 3*).

7.4 RELATIONSHIP BETWEEN IPLS AND ENVIRONMENTAL FACTORS

Lipid remodelling in response to changing environmental conditions is a well-known process in microbial enrichment cultures, but its functioning and expression in the marine environment have not been verified in any detail. Van Mooy *et al*. (2006, 2008, 2009) were the first to suggest that lipid remodelling takes place on a plankton-wide scale in the oceans. They observed a shift away from phosphorous-containing glycerophospholipids in settings where phosphorous is scarce, resulting in elevated ratios of SQDG over PG and betaine lipids over PC. Such shifts have major implications, not only for the biogeochemical cycling of elements (such as P, N, S, etc), but also for example for the trophic value of primary producers for consumers in the marine food web.

One aim of this thesis was therefore to establish to what extent (changes in) environmental conditions are reflected in the IPL composition of the marine microbial community. This comparison was made on both a spatial (*Chapter 5*) and a temporal scale (*Chapter 6*) in the North Sea. The differences in environmental conditions throughout this region, such as the input of nutrient-rich waters from the North Atlantic Ocean, have a significant influence on the microbial distribution (*Chapter 4*). However, the environmental conditions do not appear to influence the overall IPL composition, beyond the fact that it changes the microbial community composition. This meant that almost no relationships were found between IPL concentrations and environmental factors. Furthermore, the IPL classes that were predicted by Van Mooy *et al*. (2006) to show contrasting behaviour under varying phosphate-availability (i.e., SQDG and betaine lipids increase, glycerophospholipids decrease), were rather found to co-vary (*Chapter 5*). In the Marsdiep time series the relative abundances of SQDGs, PGs, PCs and DGTSs remained fairly constant throughout the year (*Chapter 6*), while their concentrations were negatively related to concentrations of dissolved inorganic nutrients.

These results indicate that the abundances of the major IPL producing microbes are the driving force behind IPL abundances, and that environmental changes do not influence the IPL composition on a plankton-wide scale. It is possible that, although different microbes respond in different ways to environmental change, the fact that they seem to produce mostly 'generic' IPLs (see above) can buffer out the effects of lipid remodelling. These effects may however be observed more clearly in more specific biomarker IPLs.

7.5 OUTLOOK

The research presented in this thesis shows the power of modern analytical techniques, particularly HPLC/ESI-MS^n, to obtain both comprehensive and targeted IPL data from environmental samples. A large variety of IPLs is present in the marine environment, with thousands of different IPL species that could be used as potential biomarkers. Unfortunately it appears that many of the most abundant IPLs are non-specific. This makes it difficult to target specific microbial groups, or to determine the influence of the environment on their membrane composition by general IPL screening. The application of bioinformatics to lipid analysis is still in its infancy (Fahy *et al.*, 2007), but may soon allow for simultaneously comprehensive and in-depth analysis of IPLs in environmental samples ('meta-lipidomics'), as is already possible for genomics and proteomics data. Until then, it will probably be more effective to focus on IPLs that are known to be specific biomarkers for certain microbes or microbial groups, rather than trying to gain information from the overall picture. This does highlight the need for more of these specific biomarker IPLs, which will have to be obtained from microbial enrichment cultures (for example the IPLs measured in the newly isolated Thaumarchaeote "*Candidatus* Nitrosopumilus maritimus"; Schouten *et al.*, 2008; or glycosyldiradylglycerols in heterocyst cyanobacteria; Bauersachs *et al.*, 2009). Furthermore, most studies of IPLs in the marine environment to date have used MS^2 to acquire structural information down to the IPL species level. By using techniques such as MS^3 (e.g., Ekroos *et al.*, 2003), 2D-HPLC-MS (e.g., Guo and Lankmayr, 2010) or high-resolution / high mass-accuracy MS (VanLear and McLafferty, 1969; Schwudke *et al.*, 2011) additional structural information can be gained, which would further increase the resolution of IPL identification and increase the chances of finding specific biomarker IPLs.

In addition to the matter of chemotaxonomy, there are a number of issues that need to be addressed to better constrain the use of IPLs as 'life markers'. For example, anoxia and the presence of organic matter are two external factors that could slow down IPL degradation (Harvey *et al.*, 1986), while the presence of remineralizing bacteria seems to lead to additional losses (Harvey and Macko, 1987). The influence of factors like these should be quantified in incubation experiments and subsequently tested in the natural environment. One way to very specifically trace the fate of IPLs in such studies would be to combine biomarker IPL analysis with stable isotope labelling (e.g., Veuger *et* al., 2006; Popendorf *et* al., 2011). The second issue has to do with the IPLs themselves, namely the observation that different types of IPLs degrade at different rates (Harvey *et al.*, 1986; Schouten *et al.*, 2010; Logemann *et al.*, 2011). This difference seems to be headgroup-dependent, with the ether-bound glycosyldiradylglycerols degrading at a lower rate than the ester-bound glycerophospholipids, but this has not yet been resolved in any detail. Nonetheless, it is of particular importance for the study of IPLs in anoxic sediments (Biddle *et al.*, 2006; Lipp *et al.*, 2008; Pearson, 2008), the environ-

ment in which IPLs potentially have the best chance of being preserved for prolonged periods of time.

In conclusion, if the remaining issues with IPL degradation can be resolved, and the chemotaxonomic specificity can be increased, IPLs will provide a useful tool for detecting and potentially quantifying their source organism(s). With more representatives of microbial groups being brought into culture and the application of higher resolution mass spectrometric techniques, it is to be expected that new biomarker IPLs will be identified in the near future, while the development of meta-lipidomics should enable both in-depth and comprehensive analysis of the IPLs present in the marine environment.

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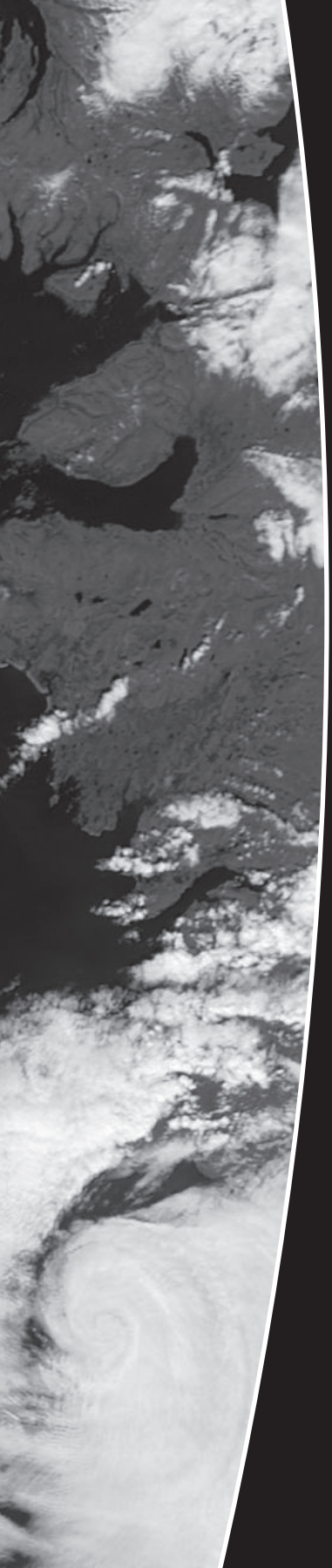


SUMMARY
SAMENVATTING

SUMMARY

Microorganisms, such as bacteria, archaea and algae, are the most abundant organisms on Earth and they contain the bulk of the biosphere's carbon, nitrogen and phosphor. They are also the main drivers of the biogeochemical cycles, and therefore the study of microbes in their environment (microbial ecology) is important for our understanding of Earth system functioning. Unfortunately, the very small size of microbes makes it difficult to study them *in situ*, and a range of methods is employed to determine their natural diversity, abundance and activity. One such approach is to measure molecular compounds present in a microbial cell. Rather than trying to obtain information about the organism through direct visual or morphological analysis, analysis of its pigments, proteins, DNA or lipids has been shown to contain a wealth of information about their source organism.

A promising method for the identification, characterization and enumeration of microbial communities in the natural environment is the measurement of intact polar lipids (IPLs), the basic building blocks of biomembranes. These complex molecules are ubiquitous in nature and have several characteristics that make them useful as proxies for living microbial cells. Within the large molecular diversity encountered in IPLs, certain types are uniquely synthesized by particular organisms, and these specific IPLs can consequently be used as biomarkers for those source organisms. Furthermore, IPLs are thought to degrade rapidly upon cell death, meaning they are indicative of living cells and can thus be used for estimates of viable microbial cell numbers or biomass. Finally, microbes can actively remodel the IPL composition of their biomembranes in adaptation to their environment, meaning that information about environmental conditions can potentially be obtained from IPL measurements.

However, the comparatively recent development of suitable analytical instrumentation for direct analysis of IPL molecules (i.e., the coupling of liquid chromatography to mass spectrometry through an electrospray ionization interface) means that only a limited number of studies have been done on IPLs in the marine realm, and in particular the water column. The aim of this thesis was therefore to investigate the origins, dynamics and fate of IPLs in the marine environment. First, the applicability of IPLs as a proxy for living microbial cells was re-assessed by determining IPL degradation rates in decaying diatom cultures, and in targeted study in which IPLs specific to anammox bacteria were measured in marine sediments in parallel with other proxies for anammox bacterial abundance. Subsequently, a comprehensive analysis of IPLs in surface waters of the North Sea was performed, both on a spatial and a temporal scale. These results were statistically compared with measurements of the environmental conditions and the microbial biogeography and their use as a tool in marine environmental microbiology was evaluated.

A laboratory study of IPL degradation in decaying diatom cultures showed that, while degradation rates were initially high, they slowed progressively over time. Furthermore, a substantial fraction of the total IPL pool remained intact for several weeks after the cultures had reached full senescence. Overall, IPL concentrations correlated with total cell counts (including both living and dead cells) rather than with living cell counts, implying that they do not exclusively reflect living cells. IPL degradation rates were enhanced when bacteria were introduced into the culture, but also in this scenario the IPL pool did not disappear completely. These results show that IPL degradation is not as uniformly rapid and complete as is currently assumed. While circumstantial evidence suggests that in most marine environments the majority of the IPL pool is degraded fairly rapidly, and most IPLs can be used as proxies for living microbial cells, care should be taking in settings where IPL degradation may be impeded. For example, in anoxic sediments with a high organic matter content the possibility exists that part of the IPL pool escapes degradation and becomes fossilized.

In marine sediments of the Gullmar Fjord in southwest Sweden, a specific biomarker IPL (C_{20}-[5]-ladderane monoalkylether phosphatidylcholine) was used to trace abundances of its source organisms: anaerobic ammonium oxidizing (anammox) bacteria. The good correlations found between ladderane IPL concentrations in the sediment and several other markers for ladderane bacterial abundances and activity show the applicability of this very specific biomarker. However, a discrepancy between concentrations of the ladderane IPL and ladderane fatty acids was noted, which increased progressively with sediment depth. This shows that these fatty acids are readily fossilized and IPLs provide a better indicator for living microbial cells.

To assess the value of IPLs as environmental and chemotaxonomic markers, a comprehensive analysis of the IPL composition, environmental conditions and microbial community composition of marine surface waters was performed in the North Sea. Strong environmental gradients between the North Sea and its adjacent water masses of the eastern North Atlantic Ocean and Skagerrak/Baltic Sea result in a number of distinct hydrogeographical regions. These in turn drive the spatial distribution of the diverse microbial community, resulting in biogeographic regions with distinct microbiological compositions during summer.

Comprehensive IPL analysis of the North Sea surface waters indicated the presence of a large structural variety, comprising thousands of different IPL species. The IPL pool was dominated by seven IPL classes, with the sulphur-bearing glycerolipid sulfoquinovosyl-diacylglycerol (SQDG) being the most abundant. The glycerophospholipids phosphatidylcholine (PC), -glycerol (PG) and -ethanolamine (PE), as well as the betaine lipids diacylglyceryl-trimethylhomoserine (DGTS), -trimethylalanine (DGTA) and -carboxyhydroxymethylcholine (DGCC) were present in smaller and roughly comparable amounts. The overall fatty acid compositions of the different IPL classes were quite distinct, with the SQDGs containing almost exclusively short-chain saturated fatty

acids, while the PCs, DGTAs and DGCCs contained many long-chain polyunsaturated fatty acids (PUFAs).

Statistical comparisons were made between the environmental and microbial parameters and the IPL compositions measured throughout the North Sea (spatial distribution) and measured in the Marsdiep tidal inlet over a one-year time series (temporal variation). Little evidence was found for a direct influence of environmental conditions on IPL composition, and it appears that in this area the microbial community composition is the dominant factor determining the IPL composition. Tentative microbial sources for the predominant IPL classes could be identified, but only with poor taxonomic resolution (e.g., small algae, cyanobacteria, diatoms), and none of the statistical relationships were particularly strong. This was particularly striking in the Marsdiep time series, where the IPL composition of the surface water remained relatively constant throughout the year, despite major shifts in the phytoplankton community composition. Combined with the observation in other studies that a comparable suite of IPLs predominates in marine waters at a variety of sites, it thus appears that the most abundant IPLs in the world's oceans are non-specific. This lack of chemotaxonomic resolution of the majority of the IPL pool makes it difficult to target specific microbial groups by general IPL screening.

To bring out the full potential of IPLs as biomarkers in (marine) environmental microbiology, it thus seems necessary to focus on specific biomarker IPLs, such as ladderanes for anammox bacteria. Unfortunately, general full scan IPL screening like that performed in the North Sea and at other marine sites, will detect the common, most abundant and least specific IPLs, which obscure the less abundant, but more specific IPLs. This deeper layer of information can be accessed by specifically targeting known biomarker IPLs, for example using selective reaction monitoring (SRM). Alternatively, it may be possible to detect these more relevant IPLs by applying techniques that yield more structural information, such as MS^3, 2D-HPLC-MS or high mass accuracy MS, particularly in combination with bioinformatics approaches to deal with the very large datasets (meta-lipidomics). Regardless of the techniques chosen, more specific biomarker IPLs will need to be obtained from microbial enrichment cultures. If the remaining issues with IPL degradation can be resolved, and their chemotaxonomic specificity can be increased, IPLs will be a useful tool for detecting and quantifying microorganisms in the marine environment.

SAMENVATTING

Microörganismen, zoals bacteriën, archaea of algen, zijn de meest voorkomende organismen op aarde, en zij bevatten de grootste hoeveelheid koolstof, stikstof en fosfor in de biosfeer. Ze zijn tevens een drijvende kracht in de biogeochemische kringlopen, en de studie van microörganismen in hun natuurlijke opgeving (microbiële ecologie) is daarom van groot belang voor ons begrip van het 'systeem aarde'. Helaas maakt de geringe grootte van microörganismen het moeilijk om ze direct *in situ* te bestuderen, en daarom zijn verschillende methodes ontwikkeld om hun natuurlijke diversiteit, aantallen en activiteit te bepalen. In bepaalde detectiemethoden worden moleculaire componenten, die van nature voorkomen in microbiële cellen, gemeten. In plaats van te proberen een microörganisme te bestuderen door middel van directe visuele of morfologische analyse, is aangetoond dat een grote hoeveelheid informatie verkregen kan worden door karakteristieke pigmenten, proteïnen, DNA of lipiden te analyseren.

Een veelbelovende methode voor het karakteriseren van microbiële levensgemeenschappen in het natuurlijk milieu is analyse van intacte polaire lipiden (IPLs), de essentiële bouwstenen van celmembranen. Deze complexe moleculen zijn wijdverspreid in de natuur en hebben een aantal kenmerken die ze bij uitstek geschikt maken om als proxies voor levende microbiële cellen gebruikt te worden. Binnen de enorme structurele diversiteit aan IPLs zijn er bepaalde lipiden die alleen geproduceerd worden door specifieke (micro)organismen. Dit soort specifieke IPLs kunnen dus als 'biomarker' gebruikt worden voor dat organisme. Verder wordt aangenomen dat IPLs zeer snel degraderen nadat een organisme sterft. Dit betekent dat IPLs alleen afkomstig kunnen zijn van levende cellen, en dat ze dus gebruikt kunnen worden voor schattingen van hoeveelheden levende microörganismen of microbiële biomassa. Daarnaast is bekend dat microörganismen actief de IPL samenstellingen van hun membranen kunnen aanpassen aan hun dynamische omgeving. IPLs kunnen dus ook waardevolle informatie bevatten over het milieu waarin een microörganisme leeft.

Omdat geschikte technologie voor de directe analyse van IPLs (de koppeling van vloeistofchromatografie aan massaspectrometrie door middel van een electrospray ionisatie interface) pas relatief recent ontwikkeld is, zijn er slechts enkele studies gedaan naar IPLs in het mariene milieu, en met name de waterkolom. Het doel van dit proefschrift was daarom om de herkomst, dynamiek en het lot van IPLs in het mariene milieu te onderzoeken. Ten eerste is de toepasbaarheid van IPLs als proxy voor levende microbiële cellen opnieuw bekeken, onder andere door degradatiesnelheden van IPLs in afstervende diatomeeënculturen te meten. Daarnaast zijn concentraties van specifieke IPLs, die alleen voorkomen in anammox bacteriën, gemeten in mariene sedimenten, en vergeleken met metingen van andere proxies voor anammox bacteriën. Vervolgens is de IPL samenstelling in het oppervlaktewater van de Noordzee in detail geanalyseerd, zowel met betrekking tot regionale verschillen als met betrekking tot

variaties in de tijd. Door de resultaten statistisch te vergelijken met metingen van de milieufactoren en microbiële biogeografie was het mogelijk de toepassing van IPLs voor de mariene microbiologie te toetsen.

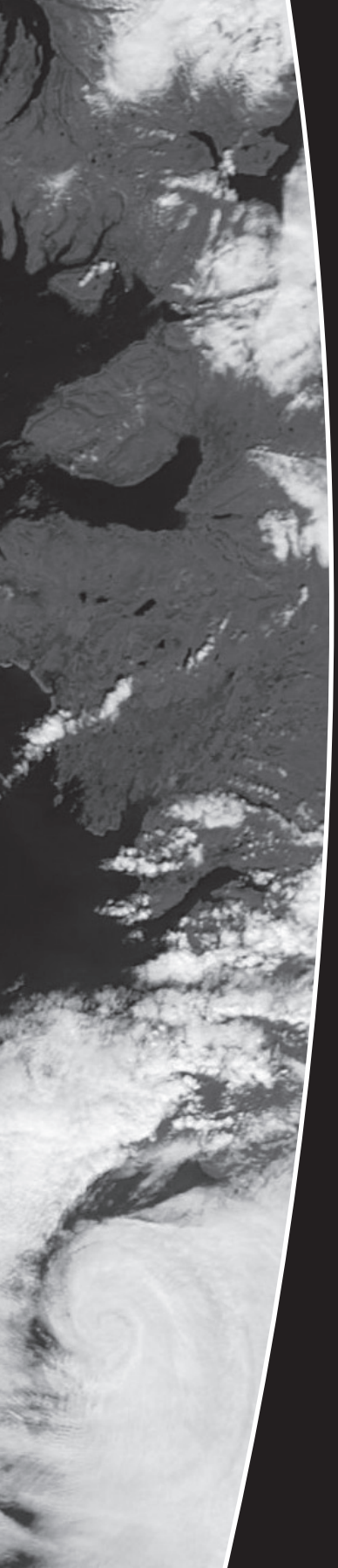
In een laboratoriumstudie van IPL degradatie in afstervende diatomeeëncultures werd aangetoond dat de degradatiesnelheden van IPLs in eerste instantie weliswaar hoog waren, maar dat ze, naarmate de tijd verstreek, steeds lager werden. Verder bleek dat enkele weken nadat de cultures volledig afgestorven waren een aanzienlijk deel van de totale hoeveelheid van de oorspronkelijk aanwezige IPLs nog steeds intact was. IPL concentraties kwamen het beste overeen met de totale hoeveelheden cellen (zowel dode als levende), hetgeen aangeeft dat ze dus niet exclusief voorkomen in *levende* cellen. De degradatiesnelheid van IPLs nam toe wanneer er bacteriën in de cultuur geïntroduceerd werden, maar ook in dit experiment verdwenen de IPLs niet volledig. Deze resultaten laten zien dat IPL degradatie niet zo uniform snel en volledig is als tot nog toe aangenomen werd. Hoewel indirect bewijs aangeeft dat in de meeste mariene milieus het grootste deel van de IPLs vrij snel degradeert, en de meeste IPLs dus inderdaad gebruikt kunnen worden als proxies voor levende microbiële cellen, is voorzichtigheid geboden in milieus waar IPL degradatie minder snel verloopt. In bijvoorbeeld zuurstofloze sedimenten met een hoog gehalte aan organisch materiaal bestaat de mogelijkheid dat een gedeelte van de IPLs niet gedegradeerd wordt maar fossiliseert.

In mariene sedimenten van het Gullmar Fjord in zuidwest Zweden is een specifieke IPL (C_{20}-[5]-ladderaan monoalkylether phosphatidylcholine) gebruikt om de aanwezigheid van anaërobe ammonium oxiderende (anammox) bacteriën te traceren. De goede correlaties die gevonden werden tussen concentraties van de ladderaan IPL in het sediment, en een aantal andere proxies voor de aanwezigheid en activiteit van anammox bacteriën, geven een goed voorbeeld van de toepasbaarheid van dit soort specifieke biomarkers. Interessant was daarnaast het verschil in de concentratieprofielen van de ladderaan IPL en de ladderaan vetzuren. De laatste namen steeds toe met sedimentdiepte, hetgeen aangeeft dat deze vetzuren relatief gemakkelijk fossiliseren en daardoor minder geschikt zijn als indicatoren voor levende microbiële cellen dan IPLs.

Om de waarde van IPLs als microbiëel ecologische indicatoren te testen, werd een gedetailleerde analyse uitgevoerd van de IPL samenstelling, milieufactoren en microbiële gemeenschap in het oppervlaktewater van de Noordzee. Door sterke verschillen in mileuomstandigheden tussen de Noordzee en de aangrenzende watermassa's van de noordelijke Atlantische Oceaan en de Oostzee ontstaan in dit gebied een aantal duidelijk verschillende hydrogeografische regio's. Deze bepalen op hun beurt de verspreiding van de microbiële gemeenschappen, hetgeen gedurende de zomermaanden een aantal verschillende biogeografische regio's oplevert. In een volledige analyse van de IPL samenstelling van het oppervlaktewater van de Noordzee werd de aanwezigheid van duizenden verschillende typen IPLs aangetoond. De IPLs werden gedomineerd

door zeven IPL klassen, waarvan de zwavelhoudende glycerolipide sulfoquinovosyl-diacylglycerol (SQDG) in de hoogste concentraties voorkwam. De glycerofosfolipiden phosphatidyl-choline (PC), -glycerol (PG) en -ethanolamine (PE), alsmede de betaine lipiden diacylgyceryl-trimethylhomoserine (DGTS), -trimethylalanine (DGTA) en -carboxyhydroxymethylcholine (DGCC) werden in lagere en min of meer vergelijkbare concentraties aangetroffen. De vetzuursamenstellingen van deze dominante IPL klassen waren over het algemeen verschillend. Daar waar de SQDGs bijna alleen maar korte verzadigde vetzuren bevatten, werden de PCs, DGTAs en DGCCs gekenmerkt door met name lange meervoudig onverzadigde vetzuurketens.
Statistische vergelijkingen tussen de milieu- en microbiële parameters en de IPL samenstelling in de Noordzee (ruimtelijke verdeling) en het Marsdiep (variatie in de tijd) leverden weinig bewijs op voor een directe invloed van de milieufactoren, en het lijkt erop dat in dit gebied de samenstelling van de microbiële gemeenschap bepalend is voor de IPL samenstelling. Hoewel het mogelijk was de belangrijkste bronnen voor de dominante IPL klassen te identificeren, kon dit slechts met een beperkte taxonomische resolutie (bijv. kleine algen, cyanobacteriën of diatomeeën), en de statistische verbanden waren over het algemeen zwak. Dit bleek bijzonder duidelijk in de Marsdiep tijdserie, waar de IPL samenstelling van het oppervlaktewater relatief constant bleef, ondanks sterke veranderingen in de samenstelling van de phytoplanktongemeenschap. Omdat hetzelfde assemblage IPLs dat wordt aangetroffen in de Noordzee ook domineert in de mariene waterkolom op andere locaties, lijkt het er op dat de meest voorkomende IPLs in de wereldzeeën weinig soort-specifiek zijn. Dit gebrek aan chemotaxonomische resolutie binnen een groot deel van het spectrum aan IPLs bemoeilijkt de studie van de microbiële samenstelling door middel van algemene IPL analyse.

Om het volledige potentieel van IPLs als biomarkers in (mariene) microbiële ecologie optimaal te benutten zal het dus nodig zijn om te focussen op specifieke biomarker IPLs, zoals bijvoorbeeld de ladderanen voor anammox bacteriën. Helaas worden in een algemene IPL analyse zoals hier toegepast voor de Noordzee met name de algemene, meest voorkomende en minst specifieke IPLs gemeten, terwijl de minder algemene, maar meer specifieke, IPLs verborgen blijven. Deze diepere laag met waardevolle informatie kan aangeboord worden met technieken als 'selective reaction monitoring' (SRM), waarbij alleen bekende zeer specifieke IPLs gemeten worden. Mogelijk kunnen de relevante IPLs ook gemeten worden met behulp van technieken die meer structurele informatie opleveren, zoals MS^3, 2D-HPLC-MS of hoge resolutie MS, in het bijzonder in combinatie met bioinformatica methoden voor de behandeling van de zeer grote datasets (meta-lipidomics). Onafhankelijk van de techniek die gekozen wordt, is het van belang dat meer specifieke biomarker IPLs gevonden worden in microbiële culturen. Aannemende dat de nog openstaande vragen over IPL degradatie beantwoord, en hun taxonomische nauwkeurigheid vergroot kunnen worden, biedt IPL analyse een veelbelovend middel voor de detectie en kwantificatie van microörganismen in het mariene milieu.



SUPPLEMENTARY TABLES

Table S5.1 Approximate fatty acid compositions of the predominant IPL species in the surface waters of the North Sea (number of carbon atoms and double bond equivalents). Note that the positions of the fatty acids on the glycerol backbone (*sn*-1 or s*n*-2) were not determined and that the carbon numbers of the IPL species do not include the glycerol moiety.

IPL species	Predominant fatty acids
SQDGs	
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0
C30:2	Unknown – probably C14:0/C16:2 or C14:1/C16:1
C30:1	C14:0/C16:1 and C14:1/C16:0
C30:0	C14:0/C16:0 plus small amounts of C12:0/C18:0 and C15:0/C15:0
C32:2	C16:1/C16:1 plus small amounts of C14:0/C18:2 and C16:2/C16:0
C32:1	C14:0/C18:1 and C16:1/C16:0
C32:0	C16:0/C16:0 and C14:0/C18:0 plus small amounts of C15:0/C17:0
C34:1	C16:0/C18:1
PCs	
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0 and C13:0/C15:0
C29:0	C14:0/C15:0 plus small amounts of C13:0/C16:0
C30:1	C14:0/C16:1 plus small amounts of C14:1/C16:0 and C15:1/C15:0
C30:0	C14:0/C16:0 and C15:0/C15:0 plus small amounts of C12:0/C18:0 and C13:0/C17:0
C32:1	C14:0/C18:1 and C16:1/C16:0 plus small amounts of C15:0/C17:1
C34:5	C14:0/C20:5, C16:1/C18:4 and C16:0/C18:5 plus small amounts of C14:1/C20:4
C34:1	C16:0/C18:1 plus small amounts of C17:1/C17:0
C35:5	C15:0/C20:5 plus small amounts of C13:0/C22:5, C15:1/C20:4 and C17:1/C18:4
C36:6	C14:0/C22:6, C16:1/C20:5 and combinations of C18:6-18:0
C36:5	C14:0/C22:5, C16:0/C20:5 and combinations of C18:5-18:0
C36:2	C18:1/C18:1 plus small amounts of C16:1/C20:1, C16:0/C20:2 and C18:2/C18:0
C37:6	C15:0/C22:6, C17:1/C20:5 and C18:0/C19:6 plus small amounts of C16:0/C21:6
C38:6	C16:0/C22:6, C18:1/C20:5 and C18:0/C20:6 plus small amounts of C19:6/C19:0
C40:10	C18:5/C22:5, C18:4/C22:6 and C20:5/C20:5
C42:11	Unknown – probably C20:5/C22:6
PGs	
C30:1	C14:0/C16:1 plus small amounts of C12:0/C18:1, C13:0/C17:1 and C15:1/C15:0
C30:0	C12:0/C18:0, C14:0/C16:0 and C15:0/C15:0
C32:2	C16:1/C16:1 plus small amounts of C14:0/C18:2, C15:1/C17:1 and C16:2/C16:0
C32:1	C14:0/C18:1 and C16:1/C16:0 plus small amounts of C15:0/C17:1

C33:1	Unknown – possibly C15:0/C18:1 or C16:0/C17:1
C34:4	C16:1/C18:3 and C16:0/C18:4 plus small amounts of C14:0/C20:4
C34:3	Unknown – probably C16:2/C 18:1 or C16:1/C 18:2
C34:2	C16:1/C18:1 and C16:0/C18:2 plus small amounts of C14:0/C 20:2 and C17:1/C 17:1
C34:1	C16:1/C18:0 and C16:0/C18:1 plus small amounts of C15:0/C 19:1 and C17:1/C 17:0
C36:2	C18:1/C18:1 plus small amounts of C14:0/C 22:2, C16:0/C 20:2 and C17:1/C 19:1
PEs	
C30:1	Unknown – possibly C14:0/C16:1 or C15:1/C15:0
C30:0	Unknown – possibly C14:0/C16:0 or C15:0/C15:0
C32:2	Unknown – possibly C14:0/C18:2 or C16:1/C16:1
C32:1	Unknown – possibly C14:0/C18:1 or C16:1/C16:0
C32:0	Unknown – possibly C14:0/C18:0 or C16:0/C16:0
C33:2	Unknown – possibly C15:1/C 18:1 or C16:1/C17:1
C33:1	Unknown – possibly C15:0/C 18:1 or C16:0/C 17:1
C34:2	C16:1/C 18:1 plus small amounts of C16:0/C 18:2 and C17:1/C 17:1
C34:1	Unknown – probably C16:0/C 18:1 or C17:1/C 17:0
C35:2	Unknown – possibly C16:1/C 19:1 or C17:1/C 18:1
C36:2	Unknown – possibly C16:0/C20:2 or C18:1/C18:1
C38:6	C16:0/C 22:6, C18:1/C 20:5 and C18:0/C 20:6
C38:5	C18:0/C 20:5
C40:6	C18:0/C 22:6 plus small amounts of C20:1/C 20:5
DGTSs	
C26:0	C12:0/C14:0
C28:1	C14:1/C14:0 plus small amounts of C12:0/C16:1
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0 and C13:0/C15:0
C29:0	C14:0/C15:0 plus small amounts of C13:0/C16:0
C30:1	C14:0/C16:1 plus small amounts of C12:0/C18:1, C14:1/C16:0 and C15:1/C15:0
C30:0	C14:0/C 16:0 and C15:0/C15:0
C31:1	Unknown – possibly C15:0/C16:1
C32:4	C14:0/C18:4
C32:2	C14:0/C18:2 and C16:1/C16:1 plus small amounts of C14:1/C18:1 and C16:2/C16:1
C32:1	C14:0/C18:1 and C16:1/C16:0 plus small amounts of C14:1/C18:0 and C15:0/C17:1
C34:5	Unknown – possibly C14:0/C20:5 or C16:0/C18:5
C34:2	C16:1/C18:1 and C16:0/C18:2 plus small amounts of C17:1/C17:1
C34:1	C16:0/C18:1 plus small amounts of C16:1/C18:0
C36:5	C16:0/C20:5 plus small amounts of C14:0/C22:5 and combinations of C18:5-18:0
C36:3	C18:2/C18:1 plus small amounts of C16:0/C20:3 and C18:3/C 18:0
C36:2	C18:1/C18:1 plus small amounts of C16:1/C20:1, C16:0/C20:2 and C18:2/C 18:0

DGTAs	
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0 and C13:0/C15:0
C30:1	C14:0/C16:1 plus small amounts of C12:0/C18:1, C14:1/C16:0 and C15:1/C15:0
C32:4	C14:0/C18:4 and C16:4/C16:0
C32:1	C14:0/C18:1 and C16:1/C16:0
C33:1	C15:0/C18:1 plus small amounts of C14:0/C19:1 and C16:0/C17:1
C34:4	C16:0/C18:4 plus small amounts of C14:0/C20:4 and C16:4/C18:0
C34:1	C16:0/C18:1 plus small amounts of C17:1/C17:0
C35:1	C17:0/C18:1 plus small amounts of C16:0/C19:1
C36:6	C14:0/C22:6 and C16:1/C20:5
C36:5	C16:0/C20:5 plus small amounts of C14:0/C22:5 and C18:5/C18:0
C36:2	C18:1/C18:1
C38:6	C16:0/C22:6 plus small amounts of C18:1/C20:5
C40:10	C18:4/C22:6 and C20:5/C20:5
C42:11	C20:5/C22:6
C44:10	C22:6/C22:6
DGCCs	
C30:0	C14:0/C16:0
C32:5	C14:0/C18:5
C32:4	C14:0/C18:4
C32:0	C16:0/C16:0
C34:5	C14:0/C20:5 and C16:0/C18:5
C36:6	C14:0/C22:6 plus small amounts of C16:0/C20:6, C18:5/C18:1 and C18:6/C18:0
C36:5	C14:0/C22:5 and C16:0/C20:5
C38:6	C16:0/C22:6 plus small amounts of C18:0/C20:6
C40:12	Unknown – possibly C18:6/C22:6
C40:10	Unknown – possibly C18:4/C22:6 or C20:5/C20:5
C40:6	C18:0/C22:6
C42:11	C20:5/C22:6
C44:12	C22:6/C22:6
C44:8	Unknown – possibly C22:6/C22:2

Table S5.2 Total concentrations of the IPL classes and relative abundances of the predominant IPL species of 1) SQDG, 2) PC, 3) PG, 4) PE, and 5) DGTS at the stations of the MICROVIR cruise. Note that the relative abundances of the IPL species are given as a percentage of the total concentration of their respective IPL class.

Station	Total SQDG (µg L-1)	Relative abundance SQDGs (%)							
		C28:0	C30:2	C30:1	C30:0	C32:2	C32:1	C32:0	C34:1
MV01	2.5	29	1	17	20	3	13	8	2
MV03	4.4	27	1	16	14	8	12	9	2
MV04	1.9	22	2	22	17	6	13	6	3
MV05	44.5	21	2	19	12	3	34	3	1
MV06	3.1	36	2	19	15	3	9	5	2
MV07	2.5	24	3	24	15	5	13	5	1
MV08	1.8	21	2	21	22	5	9	6	2
MV09	2.1	25	2	23	17	6	9	4	3
MV10	0.6	24	1	20	17	7	10	3	3
MV11	1.8	15	2	24	20	6	11	7	3
MV12	1.8	21	3	24	15	6	10	7	2
MV13	2.3	19	3	28	15	7	13	5	2
MV14	1.9	14	4	27	13	10	11	5	3
MV15	1.7	39	1	14	17	3	9	4	3
MV16	1.6	49	<1	9	17	2	7	2	3
MV18	2.3	28	2	20	18	5	7	4	2
MV19	3.5	19	2	20	20	6	10	6	2
MV20	4.7	23	2	22	19	5	10	7	3
MV21	2.6	17	2	24	18	6	10	7	3
MV22	5.1	14	1	14	22	3	10	11	6
MV23	2.1	20	3	22	23	4	8	7	2

Station	Total PC	Relative abundance PCs (%)							
	(ng L-1)	C28:0	C29:0	C30:1	C30:0	C32:1	C34:5	C34:1	C35:5
MV01	143	9	5	4	7	6	2	7	1
MV03	425	5	2	3	7	4	3	4	2
MV04	111	6	3	4	5	4	3	5	3
MV05	1216	2	1	2	3	2	3	3	7
MV06	206	4	2	4	4	5	4	4	3
MV07	233	4	1	2	4	3	3	2	4
MV08	178	2	1	2	2	2	4	1	5
MV09	131	9	3	4	8	6	3	4	1
MV10	78	4	1	2	3	2	5	1	3
MV11	264	1	1	2	2	2	5	1	4
MV12	178	2	1	2	2	2	6	1	2
MV13	183	7	3	5	5	4	2	3	4
MV14	207	3	2	3	2	2	4	1	4
MV15	268	5	3	3	5	4	3	3	3
MV16	206	2	1	1	1	1	5	2	3
MV18	167	3	2	2	2	2	4	2	5
MV19	181	3	2	2	3	2	4	2	2
MV20	313	4	2	3	5	4	3	3	4
MV21	227	3	1	2	3	2	3	2	4
MV22	437	2	2	1	5	1	4	2	3
MV23	185	2	2	1	1	2	4	1	4
	n.d. = not detected								

Station	Total PC	Relative abundance PCs (%)						
	(ng L-1)	C36:6	C36:5	C36:2	C37:6	C38:6	C40:10	C42:11
MV01	143	1	4	3	1	6	n.d.	<1
MV03	425	3	7	2	2	10	2	3
MV04	111	3	5	2	2	7	1	2
MV05	1216	4	7	2	3	9	3	4
MV06	206	3	4	3	3	7	1	1
MV07	233	4	5	2	5	8	3	2
MV08	178	4	5	1	5	9	2	3
MV09	131	3	4	2	3	12	1	2
MV10	78	5	5	1	5	11	3	4
MV11	264	5	4	1	5	12	2	3
MV12	178	5	4	2	4	10	4	5
MV13	183	2	5	2	4	8	1	2
MV14	207	4	4	1	5	9	2	3

MV15	268	3	5	3	3	9	1	2
MV16	206	4	5	3	3	7	3	3
MV18	167	3	4	3	4	7	2	2
MV19	181	5	4	2	3	9	3	5
MV20	313	4	5	2	5	13	1	2
MV21	227	4	6	2	5	11	2	3
MV22	437	6	5	1	4	22	1	2
MV23	185	5	5	1	4	10	3	4
	n.d. = not detected							

Station	**Total PG**	**Relative abundance PGs (%)**									
	(ng L-1)	C30:1	C30:0	C32:2	C32:1	C33:1	C34:4	C34:3	C34:2	C34:1	C36:2
MV01	190	7	5	9	19	3	4	3	13	10	9
MV03	322	6	3	11	16	3	6	4	13	9	10
MV04	100	6	4	12	16	3	5	3	12	10	10
MV05	749	6	14	7	24	1	2	1	8	4	7
MV06	183	13	5	10	16	3	5	3	10	7	9
MV07	104	8	8	13	15	1	8	4	11	4	7
MV08	51	6	3	20	16	1	2	2	15	5	13
MV09	89	7	4	7	19	2	1	3	13	9	14
MV10	32	5	2	16	21	1	2	3	13	5	12
MV11	94	12	n.d.	14	19	1	2	2	11	5	14
MV12	102	4	4	21	22	2	3	2	11	4	8
MV13	111	4	2	23	22	5	<1%	1	13	7	11
MV14	98	9	n.d.	20	16	3	2	2	12	5	13
MV15	141	10	7	10	16	2	3	4	13	7	9
MV16	51	3	4	8	13	1	2	2	17	8	16
MV18	86	10	5	14	19	1	2	1	15	7	14
MV19	118	4	2	17	21	2	1	1	13	5	16
MV20	146	6	5	13	17	2	1	2	15	8	15
MV21	96	6	3	15	17	2	1	2	16	6	16
MV22	147	5	4	10	13	2	2	10	14	8	13
MV23	90	9	6	20	23	1	1	1	10	5	10
	n.d. = not detected										

Station	Total PE	Relative abundance PEs (%)									
	(ng L-1)	C30:1	C30:0	C32:2	C32:1	C32:0	C33:2	C33:1	C34:2	C34:1	C35:2
MV01	11	5	4	n.d.	16	5	4	8	16	10	4
MV03	45	2	3	4	11	3	2	2	13	5	5
MV04	13	5	4	5	11	n.d.	5	6	14	7	2
MV05	351	6	2	7	10	2	2	4	12	3	3
MV06	7	6	4	6	8	3	2	5	14	9	3
MV07	46	3	2	4	7	1	1	2	10	8	2
MV08	73	2	1	7	10	1	2	2	14	5	1
MV09	1	n.d.	1	2	7	1	n.d.	2	19	7	2
MV10	13	2	1	7	10	2	1	2	16	4	2
MV11	64	5	2	5	7	1	2	2	9	4	1
MV12	66	2	1	10	11	2	3	4	16	5	3
MV13	2	2	2	3	16	3	1	5	11	8	5
MV14	75	3	1	8	9	1	3	4	13	5	4
MV15	5	1	1	6	7	1	4	4	15	5	2
MV16	54	2	1	5	8	3	1	1	12	6	1
MV18	30	5	3	7	12	3	1	2	17	7	1
MV19	58	2	1	8	10	2	3	4	18	6	4
MV20	3	1	4	3	5	5	n.d.	1	12	13	4
MV21	103	3	1	6	9	1	2	2	14	6	1
MV22	6	<1	1	8	11	2	1	1	6	5	3
MV23	70	3	2	6	8	2	1	3	12	4	1
	n.d. = not detected										

Station	Total PE	Relative abundance PEs (%)			
	(ng L-1)	C36:2	C38:6	C38:5	C40:6
MV01	11	3	4	1	<1
MV03	45	3	19	n.d.	7
MV04	13	3	8	2	3
MV05	351	2	11	n.d.	4
MV06	7	5	2	1	<1
MV07	46	1	19	3	4
MV08	73	2	11	3	5
MV09	1	3	14	4	2
MV10	13	2	15	3	5
MV11	64	2	11	3	4
MV12	66	2	10	2	3
MV13	2	6	12	2	2
MV14	75	2	7	2	3

MV15	5	5	14	7	4
MV16	54	1	13	3	3
MV18	30	4	9	1	2
MV19	58	3	7	1	2
MV20	3	2	8	6	1
MV21	103	4	11	2	4
MV22	6	2	18	2	1
MV23	70	3	9	3	1
	n.d. = not detected				

Station	**Total DGTS**	**Relative abundance DGTSs (%)**								
	(ng L-1)	C26:0	C28:1	C28:0	C29:0	C30:1	C30:0	C31:1	C32:4	C32:2
MV01	24	2	3	10	5	5	4	2	<1	1
MV03	77	2	3	8	3	5	3	2	<1	3
MV04	20	1	4	6	3	5	2	2	1	3
MV05	156	3	6	11	4	5	2	2	2	2
MV06	45	2	2	9	3	9	3	2	1	3
MV07	26	<1	1	8	4	4	4	2	1	3
MV08	38	1	3	6	1	13	2	1	6	5
MV09	31	2	3	6	1	29	4	4	1	5
MV10	25	2	4	5	1	24	3	2	3	7
MV11	33	1	2	7	2	16	3	2	3	5
MV12	24	2	3	5	1	9	1	1	5	4
MV13	38	2	4	10	4	24	4	6	1	3
MV14	40	1	6	6	2	13	2	2	5	6
MV15	30	5	6	13	6	9	3	2	<1	2
MV16	35	3	2	5	1	5	1	<1	2	8
MV18	16	1	1	16	4	15	4	2	2	5
MV19	88	9	3	4	1	9	2	1	4	4
MV20	42	3	2	10	3	9	3	2	2	5
MV21	56	4	2	7	2	10	2	2	3	5
MV22	48	1	2	6	2	11	3	2	2	4
MV23	33	4	3	9	2	7	2	1	3	3

Station	Total DGTS (ng L-1)	Relative abundance DGTSs (%)						
		C32:1	C34:5	C34:2	C34:1	C36:5	C36:3	C36:2
MV01	24	9	<1	3	12	<1	2	3
MV03	77	7	1	6	13	1	5	4
MV04	20	7	<1	7	12	1	5	6
MV05	156	4	4	4	4	2	2	2
MV06	45	7	1	6	11	<1	4	4
MV07	26	6	1	6	11	2	6	5
MV08	38	4	3	3	2	6	2	3
MV09	31	5	<1	8	3	1	<1	3
MV10	25	7	3	3	2	3	1	1
MV11	33	5	4	7	2	7	<1	3
MV12	24	6	3	6	4	9	2	2
MV13	38	7	<1	3	3	<1	<1	2
MV14	40	4	4	5	1	6	1	2
MV15	30	5	<1	4	6	2	2	3
MV16	35	6	2	4	3	4	1	2
MV18	16	6	2	4	2	4	1	3
MV19	88	4	3	4	3	4	2	3
MV20	42	6	2	6	4	4	1	3
MV21	56	4	3	5	2	5	1	3
MV22	48	7	2	5	7	3	4	5
MV23	33	4	4	5	6	4	4	3

Table S5.3 Spearman correlation coefficients (ρ) between IPL concentrations, microbial abundances and environmental parameters.

	Temperature	Salinity	Phosphate	Ammonium	NOx	Silicate	N:P ratio	Bacteria	Cyanobacteria
Salinity	-0,34								
Phosphate	-0,23	0,69							
Ammonium	0,01	0,60	0,75						
NOx	-0,11	0,59	0,64	0,67					
Silicate	-0,33	0,81	0,83	0,81	0,71				
N:P ratio	0,15	0,27	0,01	0,48	0,57	0,28			
Bacteria	0,13	-0,37	-0,68	-0,29	-0,24	-0,39	0,23		
Cyanobacteria	0,09	-0,32	-0,43	-0,72	-0,76	-0,56	-0,71	0,18	
Picoeukaryotes	-0,04	0,27	0,25	0,64	0,72	0,51	0,85	0,09	-0,81
Nanoeukaryotes	-0,23	-0,22	-0,11	0,11	0,14	0,02	0,51	0,21	-0,42
Chlorophyll *a*	0,22	0,00	0,04	0,51	0,49	0,27	0,83	0,38	-0,68
Total SQDG	0,32	-0,10	-0,29	0,11	0,32	0,01	0,69	0,58	-0,42
Total PG	0,04	0,13	-0,04	0,37	0,38	0,34	0,58	0,63	-0,42
Total PC	0,28	0,03	-0,16	0,17	0,15	0,01	0,58	0,51	-0,11
Total PE	0,25	-0,25	-0,46	-0,44	-0,22	-0,42	0,04	0,63	0,37
Total DGTS	0,33	0,01	-0,32	-0,04	0,22	-0,19	0,54	0,53	-0,11
Total DGTA	-0,14	-0,18	-0,10	-0,37	-0,41	-0,32	-0,55	0,22	0,65
Total DGCC	0,14	-0,28	-0,23	-0,26	-0,37	-0,39	-0,26	0,40	0,33
C28:0 SQDG	-0,05	0,26	0,29	0,52	0,70	0,42	0,76	0,06	-0,88
C30:2 SQDG	0,23	-0,32	-0,56	-0,25	0,02	-0,19	0,33	0,79	0,02
C30:1 SQDG	0,26	-0,31	-0,56	-0,12	0,03	-0,21	0,58	0,78	-0,17
C30:0 SQDG	0,32	-0,14	-0,14	0,18	0,33	-0,04	0,61	0,40	-0,54
C32:2 SQDG	0,18	-0,16	-0,48	-0,13	0,03	-0,12	0,49	0,79	-0,02
C32:1 SQDG	0,27	0,02	-0,17	0,29	0,35	0,14	0,65	0,69	-0,36
C32:0 SQDG	0,27	-0,05	-0,14	0,26	0,18	0,07	0,54	0,66	-0,31
C34:1 SQDG	0,19	0,09	-0,29	0,06	0,17	-0,14	0,70	0,60	-0,22
C30:1 PG	0,08	0,24	-0,10	0,22	0,18	0,19	0,52	0,51	-0,21
C30:0 PG	0,13	0,17	0,24	0,53	0,50	0,52	0,60	0,10	-0,73
C32:2 PG	-0,03	0,11	-0,22	0,10	0,10	0,20	0,38	0,74	-0,10
C32:1 PG	-0,06	0,15	-0,12	0,21	0,09	0,19	0,38	0,65	-0,20
C33:1 PG	-0,06	0,27	-0,09	0,25	0,32	0,31	0,58	0,64	-0,28
C34:4 PG	-0,11	0,36	0,26	0,46	0,40	0,60	0,36	0,33	-0,31
C34:3 PG	-0,01	0,34	0,21	0,55	0,42	0,54	0,53	0,48	-0,36
C34:2 PG	0,01	0,08	-0,23	0,16	0,27	0,14	0,68	0,59	-0,45
C34:1 PG	0,09	0,04	-0,25	0,19	0,21	0,04	0,70	0,59	-0,43
C36:2 PG	0,00	0,11	-0,24	0,18	0,21	0,02	0,71	0,62	-0,40
C28:0 PC	0,08	0,26	-0,04	0,36	0,54	0,37	0,80	0,46	-0,55
C29:0 PC	0,24	0,06	-0,18	0,25	0,40	0,15	0,69	0,59	-0,43
C30:1 PC	0,17	0,23	-0,16	0,20	0,27	0,21	0,61	0,59	-0,20

	Temperature	Salinity	Phosphate	Ammonium	NOx	Silicate	N:P ratio	Bacteria	Cyanobacteria
C30:0 PC	0,49	0,00	-0,05	0,37	0,37	0,13	0,66	0,48	-0,44
C32:1 PC	0,45	0,00	-0,06	0,37	0,31	0,13	0,67	0,47	-0,43
C34:5 PC	-0,15	0,21	0,06	0,16	0,07	0,20	0,23	0,39	-0,01
C34:1 PC	0,27	0,10	-0,03	0,48	0,39	0,21	0,84	0,44	-0,59
C35:5 PC	0,46	-0,24	-0,34	-0,15	-0,21	-0,30	0,33	0,35	0,09
C36:6 PC	0,09	0,08	-0,23	0,02	-0,09	-0,03	0,44	0,55	0,06
C36:5 PC	0,43	0,04	-0,04	0,21	0,22	0,06	0,60	0,34	-0,18
C36:2 PC	-0,09	0,21	0,08	0,38	0,49	0,28	0,84	0,14	-0,77
C37:6 PC	0,48	-0,27	-0,41	-0,19	-0,21	-0,30	0,23	0,56	0,25
C38:6 PC	0,34	-0,07	-0,26	0,05	-0,04	-0,06	0,46	0,63	0,06
C40:10 PC	-0,16	0,16	0,10	0,28	0,30	0,30	0,43	0,33	-0,32
C42:11 PC	0,01	-0,05	-0,26	0,00	-0,06	-0,02	0,38	0,63	-0,03
C30:1 PE	0,29	-0,38	-0,56	-0,53	-0,43	-0,55	-0,03	0,62	0,44
C30:0 PE	0,55	-0,14	-0,21	-0,03	0,02	-0,21	0,49	0,30	-0,07
C32:2 PE	0,06	-0,32	-0,47	-0,50	-0,31	-0,40	-0,08	0,62	0,35
C32:1 PE	0,15	-0,15	-0,34	-0,35	-0,10	-0,25	0,15	0,56	0,18
C32:0 PE	-0,17	0,08	-0,15	-0,01	0,16	0,03	0,56	0,36	-0,30
C33:2 PE	-0,04	-0,03	-0,35	-0,29	-0,04	-0,15	0,14	0,74	0,21
C33:1 PE	0,09	-0,16	-0,46	-0,31	-0,18	-0,25	0,10	0,85	0,32
C34:2 PE	0,13	-0,33	-0,47	-0,41	-0,18	-0,42	0,12	0,62	0,16
C34:1 PE	0,46	-0,41	-0,39	-0,27	0,06	-0,35	0,20	0,57	0,07
C35:2 PE	0,06	-0,02	-0,35	-0,18	0,10	-0,07	0,31	0,76	0,10
C36:2 PE	0,21	-0,25	-0,60	-0,46	-0,10	-0,42	0,36	0,68	0,10
C38:6 PE	0,57	-0,10	-0,09	0,11	0,13	-0,04	0,46	0,31	-0,09
C38:5 PE	0,26	-0,52	-0,15	-0,39	-0,32	-0,51	-0,32	-0,04	0,47
C40:6 PE	0,51	0,03	-0,16	-0,07	0,03	-0,17	0,34	0,34	0,14
C26:0 DGTS	0,17	0,08	-0,06	0,23	0,29	-0,03	0,54	0,27	-0,46
C28:1 DGTS	0,19	0,12	-0,36	-0,15	0,10	-0,18	0,34	0,51	0,04
C28:0 DGTS	0,32	-0,05	-0,40	-0,07	0,17	-0,19	0,68	0,49	-0,30
C29:0 DGTS	0,43	-0,03	-0,31	0,19	0,23	-0,01	0,71	0,65	-0,31
C30:1 DGTS	0,31	-0,31	-0,68	-0,42	-0,47	-0,63	0,00	0,50	0,31
C30:0 DGTS	0,56	-0,10	-0,32	0,19	0,23	-0,10	0,72	0,60	-0,31
C31:1 DGTS	0,50	-0,08	-0,51	0,01	-0,03	-0,22	0,57	0,69	-0,02
C32:4 DGTS	0,21	-0,41	-0,57	-0,55	-0,30	-0,59	-0,21	0,59	0,41
C32:2 DGTS	0,14	-0,08	-0,40	-0,18	0,09	-0,31	0,41	0,43	-0,08
C32:1 DGTS	0,17	0,29	-0,10	0,35	0,40	0,09	0,77	0,44	-0,46
C34:5 DGTS	0,25	-0,32	-0,67	-0,48	-0,38	-0,62	0,02	0,66	0,36
C34:2 DGTS	0,27	-0,09	-0,36	0,06	0,03	-0,13	0,54	0,71	-0,06
C34:1 DGTS	0,40	0,14	0,22	0,58	0,59	0,39	0,65	0,33	-0,56
C36:5 DGTS	0,01	-0,13	-0,24	-0,22	-0,02	-0,36	0,15	0,28	-0,01
C36:3 DGTS	0,44	0,04	0,11	0,39	0,54	0,27	0,54	0,25	-0,50
C36:2 DGTS	0,47	-0,14	-0,23	0,05	0,28	-0,17	0,58	0,47	-0,20

	Picoeukaryotes	Nanoeukaryotes	Chlorophyll *a*	Total SQDG	Total PG	Total PC	Total PE	Total DGTS	Total DGTA
Salinity									
Phosphate									
Ammonium									
NOx									
Silicate									
N:P ratio									
Bacteria									
Cyanobacteria									
Picoeukaryotes									
Nanoeukaryotes	0,47								
Chlorophyll *a*	0,82	0,65							
Total SQDG	0,62	0,26	0,69						
Total PG	0,66	0,24	0,69	0,84					
Total PC	0,45	0,38	0,72	0,48	0,50				
Total PE	-0,28	0,21	0,18	0,20	0,12	0,53			
Total DGTS	0,25	0,31	0,48	0,58	0,41	0,67	0,61		
Total DGTA	-0,64	0,05	-0,35	-0,47	-0,30	0,06	0,58	0,15	
Total DGCC	-0,49	0,12	0,03	-0,17	-0,12	0,31	0,79	0,30	0,72
C28:0 SQDG	0,79	0,44	0,75	0,67	0,60	0,36	-0,09	0,37	-0,55
C30:2 SQDG	0,29	0,25	0,50	0,75	0,69	0,57	0,51	0,62	-0,04
C30:1 SQDG	0,48	0,41	0,67	0,87	0,75	0,69	0,41	0,67	-0,21
C30:0 SQDG	0,55	0,24	0,63	0,90	0,69	0,36	0,09	0,51	-0,45
C32:2 SQDG	0,37	0,36	0,59	0,79	0,75	0,74	0,54	0,73	-0,01
C32:1 SQDG	0,64	0,21	0,72	0,91	0,95	0,61	0,23	0,61	-0,27
C32:0 SQDG	0,46	0,31	0,67	0,85	0,88	0,54	0,31	0,57	-0,11
C34:1 SQDG	0,41	0,31	0,56	0,72	0,59	0,69	0,46	0,81	-0,06
C30:1 PG	0,51	-0,09	0,47	0,67	0,75	0,58	0,09	0,26	-0,41
C30:0 PG	0,70	0,27	0,71	0,71	0,71	0,25	-0,17	0,06	-0,68
C32:2 PG	0,34	0,22	0,48	0,74	0,90	0,48	0,35	0,46	-0,04
C32:1 PG	0,37	0,08	0,43	0,72	0,89	0,39	0,14	0,36	-0,15
C33:1 PG	0,56	0,19	0,55	0,80	0,94	0,48	0,19	0,46	-0,22
C34:4 PG	0,58	0,00	0,52	0,53	0,82	0,50	-0,05	0,10	-0,31
C34:3 PG	0,65	0,18	0,67	0,62	0,91	0,60	0,09	0,28	-0,20
C34:2 PG	0,61	0,29	0,61	0,91	0,87	0,39	0,12	0,43	-0,47
C34:1 PG	0,62	0,24	0,62	0,89	0,83	0,48	0,09	0,45	-0,47
C36:2 PG	0,56	0,32	0,60	0,80	0,77	0,54	0,20	0,59	-0,28
C28:0 PC	0,81	0,23	0,74	0,84	0,85	0,53	0,07	0,39	-0,59
C29:0 PC	0,69	0,11	0,67	0,94	0,91	0,52	0,10	0,51	-0,49
C30:1 PC	0,58	-0,01	0,56	0,74	0,80	0,65	0,22	0,39	-0,38
C30:0 PC	0,65	0,09	0,73	0,88	0,82	0,59	0,12	0,46	-0,46
C32:1 PC	0,66	0,08	0,73	0,85	0,80	0,61	0,08	0,39	-0,50

	Picoeukaryotes	Nanoeukaryotes	Chlorophyll *a*	Total SQDG	Total PG	Total PC	Total PE	Total DGTS	Total DGTA
C34:5 PC	0,17	0,36	0,46	0,17	0,39	0,79	0,51	0,51	0,37
C34:1 PC	0,79	0,32	0,85	0,80	0,78	0,65	0,06	0,42	-0,51
C35:5 PC	0,12	0,16	0,46	0,34	0,17	0,79	0,53	0,38	-0,05
C36:6 PC	0,18	0,45	0,53	0,36	0,42	0,88	0,67	0,66	0,29
C36:5 PC	0,41	0,41	0,74	0,46	0,39	0,94	0,57	0,64	0,02
C36:2 PC	0,77	0,53	0,76	0,64	0,53	0,48	-0,01	0,34	-0,58
C37:6 PC	0,08	0,07	0,43	0,35	0,29	0,84	0,65	0,45	0,12
C38:6 PC	0,30	0,33	0,64	0,53	0,56	0,95	0,62	0,64	0,15
C40:10 PC	0,41	0,64	0,67	0,33	0,48	0,73	0,41	0,58	0,19
C42:11 PC	0,20	0,58	0,56	0,50	0,56	0,74	0,62	0,69	0,30
C30:1 PE	-0,28	0,01	0,13	0,21	0,09	0,55	0,83	0,35	0,36
C30:0 PE	0,24	0,23	0,56	0,49	0,25	0,81	0,56	0,57	-0,08
C32:2 PE	-0,35	0,29	0,12	0,17	0,12	0,37	0,95	0,45	0,59
C32:1 PE	-0,16	0,35	0,27	0,39	0,28	0,45	0,91	0,62	0,45
C32:0 PE	0,29	0,74	0,58	0,40	0,32	0,58	0,59	0,60	0,12
C33:2 PE	-0,09	0,25	0,23	0,41	0,47	0,47	0,85	0,66	0,51
C33:1 PE	-0,11	0,20	0,27	0,41	0,49	0,55	0,87	0,59	0,51
C34:2 PE	-0,19	0,45	0,27	0,34	0,20	0,42	0,90	0,67	0,51
C34:1 PE	0,01	0,30	0,45	0,42	0,26	0,54	0,85	0,64	0,32
C35:2 PE	0,15	0,30	0,41	0,60	0,63	0,63	0,73	0,78	0,31
C36:2 PE	0,04	0,32	0,33	0,66	0,40	0,53	0,75	0,78	0,14
C38:6 PE	0,25	0,36	0,66	0,41	0,29	0,84	0,65	0,61	0,10
C38:5 PE	-0,38	0,15	-0,15	-0,40	-0,52	0,05	0,40	-0,07	0,58
C40:6 PE	-0,01	0,17	0,39	0,30	0,14	0,77	0,79	0,73	0,30
C26:0 DGTS	0,27	0,41	0,43	0,52	0,37	0,22	0,26	0,71	-0,02
C28:1 DGTS	0,00	0,15	0,20	0,44	0,32	0,42	0,59	0,91	0,25
C28:0 DGTS	0,45	0,14	0,49	0,89	0,60	0,46	0,25	0,64	-0,46
C29:0 DGTS	0,59	0,14	0,67	0,93	0,83	0,59	0,23	0,58	-0,42
C30:1 DGTS	-0,36	-0,04	-0,10	0,21	0,01	0,18	0,45	0,62	0,26
C30:0 DGTS	0,58	0,17	0,70	0,89	0,73	0,67	0,25	0,70	-0,37
C31:1 DGTS	0,32	0,10	0,50	0,73	0,60	0,68	0,39	0,78	-0,15
C32:4 DGTS	-0,46	0,07	-0,07	0,12	0,01	0,20	0,80	0,61	0,63
C32:2 DGTS	0,06	0,46	0,32	0,34	0,14	0,45	0,62	0,90	0,29
C32:1 DGTS	0,52	0,31	0,58	0,65	0,58	0,51	0,25	0,81	-0,17
C34:5 DGTS	-0,36	0,13	0,03	0,20	0,05	0,36	0,79	0,72	0,52
C34:2 DGTS	0,33	0,46	0,64	0,60	0,60	0,85	0,59	0,86	0,19
C34:1 DGTS	0,69	0,28	0,80	0,77	0,81	0,54	0,12	0,53	-0,28
C36:5 DGTS	-0,18	0,42	0,18	0,00	-0,14	0,34	0,69	0,66	0,52
C36:3 DGTS	0,53	0,29	0,67	0,75	0,67	0,41	0,17	0,63	-0,24
C36:2 DGTS	0,44	0,32	0,62	0,75	0,55	0,72	0,42	0,87	-0,04

	Total DGCC	C28:0 SQDG	C30:2 SQDG	C30:1 SQDG	C30:0 SQDG	C32:2 SQDG	C32:1 SQDG	C32:0 SQDG	C34:1 SQDG
Salinity									
Phosphate									
Ammonium									
NOx									
Silicate									
N:P ratio									
Bacteria									
Cyanobacteria									
Picoeukaryotes									
Nanoeukaryotes									
Chlorophyll *a*									
Total SQDG									
Total PG									
Total PC									
Total PE									
Total DGTS									
Total DGTA									
Total DGCC									
C28:0 SQDG	-0,18								
C30:2 SQDG	0,11	0,26							
C30:1 SQDG	0,04	0,46	0,91						
C30:0 SQDG	-0,07	0,79	0,50	0,70					
C32:2 SQDG	0,14	0,35	0,93	0,95	0,57				
C32:1 SQDG	-0,07	0,58	0,74	0,84	0,78	0,81			
C32:0 SQDG	0,14	0,56	0,62	0,76	0,81	0,75	0,91		
C34:1 SQDG	0,20	0,53	0,49	0,72	0,71	0,72	0,74	0,76	
C30:1 PG	-0,13	0,45	0,46	0,62	0,57	0,60	0,75	0,64	0,65
C30:0 PG	-0,28	0,79	0,35	0,46	0,68	0,37	0,60	0,60	0,26
C32:2 PG	0,06	0,35	0,76	0,75	0,51	0,85	0,83	0,83	0,59
C32:1 PG	0,02	0,46	0,55	0,66	0,66	0,69	0,83	0,87	0,66
C33:1 PG	-0,14	0,50	0,65	0,71	0,60	0,77	0,89	0,83	0,68
C34:4 PG	-0,20	0,47	0,49	0,49	0,37	0,55	0,69	0,56	0,29
C34:3 PG	-0,09	0,50	0,50	0,56	0,46	0,63	0,83	0,75	0,49
C34:2 PG	-0,19	0,68	0,62	0,78	0,81	0,72	0,85	0,83	0,73
C34:1 PG	-0,14	0,67	0,55	0,78	0,85	0,69	0,86	0,84	0,81
C36:2 PG	-0,01	0,65	0,51	0,75	0,79	0,70	0,82	0,83	0,91
C28:0 PC	-0,32	0,68	0,61	0,71	0,64	0,66	0,83	0,65	0,59
C29:0 PC	-0,24	0,64	0,70	0,81	0,82	0,75	0,96	0,81	0,70
C30:1 PC	-0,16	0,42	0,62	0,71	0,52	0,72	0,82	0,65	0,65
C30:0 PC	-0,11	0,64	0,56	0,72	0,85	0,63	0,91	0,84	0,67
C32:1 PC	-0,12	0,62	0,52	0,71	0,81	0,61	0,87	0,81	0,66

	Total DGCC	C28:0 SQDG	C30:2 SQDG	C30:1 SQDG	C30:0 SQDG	C32:2 SQDG	C32:1 SQDG	C32:0 SQDG	C34:1 SQDG
C34:5 PC	0,50	0,27	0,40	0,43	0,12	0,59	0,36	0,41	0,48
C34:1 PC	-0,11	0,72	0,42	0,69	0,77	0,58	0,83	0,78	0,73
C35:5 PC	0,39	0,19	0,40	0,54	0,32	0,51	0,29	0,30	0,50
C36:6 PC	0,49	0,23	0,49	0,61	0,25	0,73	0,47	0,55	0,72
C36:5 PC	0,34	0,41	0,46	0,58	0,38	0,63	0,51	0,50	0,62
C36:2 PC	-0,15	0,91	0,25	0,53	0,70	0,41	0,51	0,50	0,62
C37:6 PC	0,43	0,02	0,58	0,61	0,21	0,64	0,41	0,35	0,46
C38:6 PC	0,38	0,24	0,65	0,74	0,37	0,82	0,65	0,65	0,69
C40:10 PC	0,35	0,51	0,49	0,52	0,28	0,63	0,44	0,47	0,46
C42:11 PC	0,46	0,32	0,65	0,71	0,39	0,82	0,57	0,69	0,66
C30:1 PE	0,69	-0,11	0,45	0,44	0,16	0,48	0,20	0,25	0,43
C30:0 PE	0,34	0,37	0,40	0,58	0,50	0,56	0,42	0,45	0,65
C32:2 PE	0,81	-0,08	0,50	0,38	0,08	0,50	0,15	0,29	0,34
C32:1 PE	0,65	0,15	0,54	0,48	0,30	0,62	0,33	0,49	0,53
C32:0 PE	0,40	0,53	0,39	0,51	0,36	0,57	0,32	0,44	0,63
C33:2 PE	0,58	0,10	0,64	0,54	0,26	0,71	0,49	0,56	0,59
C33:1 PE	0,62	-0,02	0,69	0,59	0,23	0,74	0,53	0,58	0,56
C34:2 PE	0,74	0,13	0,54	0,50	0,31	0,58	0,29	0,45	0,53
C34:1 PE	0,59	0,15	0,64	0,54	0,33	0,57	0,40	0,40	0,39
C35:2 PE	0,36	0,23	0,84	0,74	0,39	0,89	0,68	0,65	0,65
C36:2 PE	0,36	0,29	0,74	0,77	0,56	0,81	0,53	0,58	0,75
C38:6 PE	0,44	0,31	0,46	0,53	0,34	0,57	0,42	0,46	0,50
C38:5 PE	0,43	-0,47	-0,19	-0,23	-0,36	-0,22	-0,38	-0,31	-0,21
C40:6 PE	0,56	0,14	0,37	0,41	0,24	0,52	0,32	0,38	0,61
C26:0 DGTS	0,21	0,57	0,21	0,37	0,65	0,35	0,48	0,62	0,68
C28:1 DGTS	0,31	0,18	0,55	0,51	0,34	0,63	0,47	0,46	0,68
C28:0 DGTS	-0,12	0,60	0,58	0,78	0,86	0,69	0,73	0,70	0,84
C29:0 DGTS	-0,12	0,53	0,68	0,84	0,80	0,77	0,93	0,86	0,78
C30:1 DGTS	0,43	-0,15	0,35	0,38	0,23	0,39	0,18	0,26	0,48
C30:0 DGTS	-0,06	0,52	0,67	0,84	0,80	0,75	0,90	0,80	0,80
C31:1 DGTS	0,06	0,23	0,70	0,81	0,56	0,81	0,78	0,69	0,78
C32:4 DGTS	0,73	-0,19	0,49	0,32	0,10	0,41	0,14	0,21	0,30
C32:2 DGTS	0,41	0,25	0,45	0,48	0,31	0,53	0,31	0,33	0,64
C32:1 DGTS	0,05	0,59	0,36	0,56	0,64	0,55	0,71	0,69	0,87
C34:5 DGTS	0,70	-0,14	0,46	0,43	0,17	0,50	0,22	0,30	0,54
C34:2 DGTS	0,35	0,31	0,72	0,81	0,47	0,87	0,73	0,73	0,80
C34:1 DGTS	-0,08	0,68	0,51	0,59	0,72	0,58	0,85	0,82	0,53
C36:5 DGTS	0,73	0,16	0,14	0,17	0,13	0,21	-0,01	0,12	0,45
C36:3 DGTS	-0,07	0,63	0,58	0,59	0,70	0,59	0,73	0,72	0,45
C36:2 DGTS	0,10	0,51	0,65	0,77	0,73	0,76	0,75	0,71	0,79

	C30:1 PG	C30:0 PG	C32:2 PG	C32:1 PG	C33:1 PG	C34:4 PG	C34:3 PG	C34:2 PG	C34:1 PG
Salinity									
Phosphate									
Ammonium									
NOx									
Silicate									
N:P ratio									
Bacteria									
Cyanobacteria									
Picoeukaryotes									
Nanoeukaryotes									
Chlorophyll *a*									
Total SQDG									
Total PG									
Total PC									
Total PE									
Total DGTS									
Total DGTA									
Total DGCC									
C28:0 SQDG									
C30:2 SQDG									
C30:1 SQDG									
C30:0 SQDG									
C32:2 SQDG									
C32:1 SQDG									
C32:0 SQDG									
C34:1 SQDG									
C30:1 PG									
C30:0 PG	0,52								
C32:2 PG	0,66	0,52							
C32:1 PG	0,79	0,54	0,90						
C33:1 PG	0,78	0,57	0,93	0,91					
C34:4 PG	0,78	0,66	0,72	0,72	0,76				
C34:3 PG	0,78	0,64	0,81	0,80	0,87	0,92			
C34:2 PG	0,77	0,71	0,81	0,86	0,89	0,60	0,69		
C34:1 PG	0,84	0,64	0,72	0,86	0,83	0,57	0,68	0,96	
C36:2 PG	0,77	0,48	0,71	0,84	0,82	0,48	0,64	0,91	0,96
C28:0 PC	0,79	0,75	0,69	0,65	0,84	0,74	0,79	0,85	0,81
C29:0 PC	0,82	0,67	0,74	0,79	0,86	0,69	0,76	0,90	0,91
C30:1 PC	0,94	0,53	0,73	0,73	0,85	0,78	0,82	0,77	0,79
C30:0 PC	0,78	0,70	0,61	0,70	0,72	0,64	0,74	0,78	0,84

	C30:1 PG	C30:0 PG	C32:2 PG	C32:1 PG	C33:1 PG	C34:4 PG	C34:3 PG	C34:2 PG	C34:1 PG
C32:1 PC	0,84	0,71	0,59	0,70	0,71	0,66	0,75	0,78	0,85
C34:5 PC	0,38	0,12	0,50	0,42	0,39	0,51	0,54	0,22	0,25
C34:1 PC	0,80	0,71	0,56	0,67	0,71	0,61	0,75	0,80	0,88
C35:5 PC	0,50	0,20	0,19	0,17	0,14	0,24	0,24	0,23	0,35
C36:6 PC	0,47	0,14	0,58	0,47	0,49	0,36	0,51	0,39	0,44
C36:5 PC	0,44	0,34	0,37	0,25	0,36	0,38	0,50	0,32	0,38
C36:2 PC	0,56	0,72	0,34	0,44	0,49	0,42	0,46	0,71	0,74
C37:6 PC	0,52	0,09	0,34	0,24	0,26	0,35	0,37	0,21	0,30
C38:6 PC	0,60	0,26	0,63	0,51	0,56	0,52	0,64	0,45	0,51
C40:10 PC	0,23	0,33	0,51	0,37	0,42	0,47	0,53	0,32	0,30
C42:11 PC	0,35	0,26	0,74	0,59	0,58	0,39	0,53	0,51	0,48
C30:1 PE	0,36	-0,11	0,26	0,20	0,14	0,07	0,09	0,16	0,22
C30:0 PE	0,48	0,30	0,24	0,22	0,23	0,23	0,29	0,33	0,43
C32:2 PE	0,01	-0,10	0,40	0,19	0,18	-0,05	0,05	0,14	0,07
C32:1 PE	0,12	0,09	0,53	0,32	0,36	0,05	0,19	0,34	0,25
C32:0 PE	0,19	0,30	0,44	0,31	0,39	0,13	0,27	0,46	0,41
C33:2 PE	0,26	0,02	0,70	0,52	0,58	0,23	0,38	0,43	0,34
C33:1 PE	0,35	0,01	0,72	0,53	0,57	0,28	0,44	0,41	0,36
C34:2 PE	-0,01	-0,03	0,42	0,25	0,24	-0,12	0,05	0,27	0,21
C34:1 PE	0,05	0,10	0,31	0,08	0,19	0,03	0,15	0,18	0,14
C35:2 PE	0,39	0,17	0,80	0,58	0,70	0,42	0,53	0,54	0,46
C36:2 PE	0,35	0,15	0,59	0,45	0,50	0,09	0,19	0,58	0,54
C38:6 PE	0,28	0,30	0,30	0,14	0,23	0,25	0,37	0,21	0,25
C38:5 PE	-0,47	-0,54	-0,47	-0,58	-0,53	-0,53	-0,42	-0,57	-0,51
C40:6 PE	0,25	0,02	0,26	0,13	0,20	0,08	0,22	0,14	0,19
C26:0 DGTS	0,07	0,27	0,35	0,40	0,38	-0,09	0,17	0,50	0,50
C28:1 DGTS	0,14	-0,07	0,49	0,36	0,44	0,00	0,16	0,37	0,33
C28:0 DGTS	0,68	0,49	0,55	0,62	0,65	0,30	0,38	0,84	0,87
C29:0 DGTS	0,79	0,60	0,73	0,75	0,82	0,55	0,70	0,87	0,90
C30:1 DGTS	0,01	-0,31	0,21	0,22	0,07	-0,33	-0,21	0,15	0,21
C30:0 DGTS	0,69	0,49	0,59	0,62	0,68	0,44	0,60	0,74	0,81
C31:1 DGTS	0,60	0,23	0,63	0,58	0,64	0,33	0,49	0,62	0,68
C32:4 DGTS	-0,19	-0,36	0,23	0,08	0,03	-0,28	-0,18	-0,01	-0,04
C32:2 DGTS	-0,07	-0,13	0,26	0,12	0,21	-0,23	-0,03	0,24	0,23
C32:1 DGTS	0,46	0,30	0,51	0,56	0,64	0,22	0,47	0,66	0,70
C34:5 DGTS	0,00	-0,34	0,29	0,19	0,13	-0,27	-0,11	0,13	0,16
C34:2 DGTS	0,45	0,19	0,67	0,56	0,61	0,36	0,55	0,52	0,56
C34:1 DGTS	0,48	0,71	0,61	0,58	0,68	0,61	0,76	0,63	0,61
C36:5 DGTS	-0,24	-0,28	-0,02	-0,09	-0,11	-0,39	-0,22	-0,05	-0,02
C36:3 DGTS	0,24	0,64	0,55	0,44	0,54	0,41	0,52	0,54	0,47
C36:2 DGTS	0,42	0,28	0,47	0,44	0,51	0,28	0,42	0,53	0,59

	C36:2 PG	C28:0 PC	C29:0 PC	C30:1 PC	C30:0 PC	C32:1 PC	C34:5 PC	C34:1 PC	C35:5 PC
Salinity									
Phosphate									
Ammonium									
NOx									
Silicate									
N:P ratio									
Bacteria									
Cyanobacteria									
Picoeukaryotes									
Nanoeukaryotes									
Chlorophyll *a*									
Total SQDG									
Total PG									
Total PC									
Total PE									
Total DGTS									
Total DGTA									
Total DGCC									
C28:0 SQDG									
C30:2 SQDG									
C30:1 SQDG									
C30:0 SQDG									
C32:2 SQDG									
C32:1 SQDG									
C32:0 SQDG									
C34:1 SQDG									
C30:1 PG									
C30:0 PG									
C32:2 PG									
C32:1 PG									
C33:1 PG									
C34:4 PG									
C34:3 PG									
C34:2 PG									
C34:1 PG									
C36:2 PG									
C28:0 PC	0,72								
C29:0 PC	0,83	0,91							
C30:1 PC	0,72	0,89	0,87						
C30:0 PC	0,74	0,80	0,92	0,80					
C32:1 PC	0,74	0,81	0,91	0,83	0,99				

	C36:2 PG	C28:0 PC	C29:0 PC	C30:1 PC	C30:0 PC	C32:1 PC	C34:5 PC	C34:1 PC	C35:5 PC
C34:5 PC	0,39	0,24	0,22	0,38	0,23	0,25			
C34:1 PC	0,82	0,84	0,85	0,79	0,91	0,94	0,31		
C35:5 PC	0,32	0,30	0,31	0,48	0,46	0,52	0,53	0,49	
C36:6 PC	0,56	0,35	0,34	0,51	0,37	0,39	0,87	0,48	0,69
C36:5 PC	0,42	0,48	0,43	0,54	0,58	0,58	0,70	0,62	0,81
C36:2 PC	0,72	0,71	0,61	0,51	0,59	0,62	0,32	0,79	0,40
C37:6 PC	0,28	0,34	0,37	0,58	0,47	0,51	0,59	0,43	0,92
C38:6 PC	0,55	0,50	0,53	0,67	0,60	0,62	0,78	0,61	0,79
C40:10 PC	0,41	0,36	0,30	0,29	0,28	0,26	0,89	0,38	0,38
C42:11 PC	0,59	0,36	0,40	0,40	0,37	0,36	0,82	0,42	0,48
C30:1 PE	0,25	0,11	0,17	0,37	0,23	0,26	0,44	0,19	0,79
C30:0 PE	0,43	0,38	0,42	0,50	0,59	0,61	0,52	0,58	0,93
C32:2 PE	0,17	0,00	0,02	0,10	0,00	-0,03	0,49	-0,04	0,41
C32:1 PE	0,35	0,18	0,21	0,22	0,20	0,15	0,50	0,14	0,42
C32:0 PE	0,54	0,36	0,25	0,25	0,20	0,19	0,64	0,39	0,43
C33:2 PE	0,47	0,29	0,34	0,38	0,24	0,18	0,58	0,17	0,24
C33:1 PE	0,45	0,31	0,37	0,47	0,31	0,28	0,57	0,24	0,38
C34:2 PE	0,35	0,06	0,14	0,08	0,13	0,07	0,48	0,10	0,38
C34:1 PE	0,18	0,24	0,29	0,23	0,35	0,29	0,35	0,22	0,50
C35:2 PE	0,55	0,48	0,55	0,54	0,42	0,36	0,63	0,33	0,32
C36:2 PE	0,61	0,39	0,50	0,43	0,40	0,36	0,40	0,34	0,51
C38:6 PE	0,26	0,35	0,33	0,40	0,51	0,50	0,60	0,49	0,80
C38:5 PE	-0,47	-0,50	-0,48	-0,40	-0,29	-0,31	-0,08	-0,33	0,21
C40:6 PE	0,29	0,20	0,22	0,35	0,36	0,34	0,60	0,32	0,74
C26:0 DGTS	0,63	0,26	0,39	0,09	0,39	0,31	0,17	0,42	-0,02
C28:1 DGTS	0,50	0,25	0,36	0,27	0,24	0,16	0,40	0,18	0,14
C28:0 DGTS	0,84	0,70	0,82	0,68	0,76	0,75	0,14	0,72	0,46
C29:0 DGTS	0,84	0,84	0,95	0,86	0,93	0,92	0,21	0,88	0,43
C30:1 DGTS	0,34	-0,14	0,11	0,00	0,04	0,01	0,15	-0,01	0,20
C30:0 DGTS	0,78	0,74	0,89	0,76	0,91	0,89	0,25	0,85	0,50
C31:1 DGTS	0,70	0,58	0,73	0,70	0,69	0,67	0,35	0,64	0,49
C32:4 DGTS	0,10	-0,19	0,00	-0,09	-0,06	-0,14	0,28	-0,20	0,16
C32:2 DGTS	0,42	0,13	0,20	0,06	0,11	0,04	0,40	0,15	0,21
C32:1 DGTS	0,82	0,58	0,65	0,51	0,60	0,56	0,33	0,68	0,16
C34:5 DGTS	0,32	-0,08	0,09	0,06	0,04	-0,01	0,36	0,00	0,32
C34:2 DGTS	0,67	0,47	0,57	0,55	0,56	0,53	0,72	0,57	0,53
C34:1 DGTS	0,57	0,70	0,77	0,58	0,86	0,80	0,32	0,76	0,20
C36:5 DGTS	0,20	-0,18	-0,13	-0,21	-0,12	-0,17	0,44	-0,03	0,24
C36:3 DGTS	0,44	0,56	0,66	0,37	0,70	0,60	0,24	0,54	0,12
C36:2 DGTS	0,64	0,50	0,68	0,50	0,72	0,66	0,44	0,61	0,50

	C36:6 PC	C36:5 PC	C36:2 PC	C37:6 PC	C38:6 PC	C40:10 PC	C42:11 PC	C30:1 PE	C30:0 PE
C34:5 PC									
C34:1 PC									
C35:5 PC									
C36:6 PC									
C36:5 PC	0,82								
C36:2 PC	0,40	0,50							
C37:6 PC	0,72	0,80	0,19						
C38:6 PC	0,92	0,90	0,37	0,87					
C40:10 PC	0,77	0,69	0,50	0,41	0,68				
C42:11 PC	0,90	0,68	0,39	0,53	0,82	0,85			
C30:1 PE	0,60	0,54	0,07	0,83	0,64	0,21	0,45		
C30:0 PE	0,71	0,90	0,51	0,83	0,80	0,45	0,53	0,69	
C32:2 PE	0,59	0,41	-0,01	0,51	0,50	0,43	0,64	0,78	0,40
C32:1 PE	0,67	0,54	0,17	0,47	0,58	0,51	0,74	0,67	0,53
C32:0 PE	0,76	0,63	0,65	0,32	0,56	0,77	0,79	0,39	0,53
C33:2 PE	0,67	0,42	0,09	0,41	0,59	0,54	0,76	0,59	0,32
C33:1 PE	0,70	0,48	0,03	0,58	0,69	0,47	0,75	0,71	0,39
C34:2 PE	0,63	0,47	0,15	0,42	0,52	0,52	0,74	0,65	0,46
C34:1 PE	0,47	0,63	0,10	0,63	0,58	0,42	0,52	0,67	0,59
C35:2 PE	0,71	0,55	0,20	0,51	0,71	0,65	0,82	0,49	0,41
C36:2 PE	0,64	0,52	0,36	0,52	0,62	0,43	0,71	0,65	0,61
C38:6 PE	0,74	0,96	0,36	0,80	0,84	0,61	0,64	0,58	0,89
C38:5 PE	0,01	0,16	-0,43	0,25	0,06	-0,16	-0,11	0,38	0,19
C40:6 PE	0,78	0,87	0,22	0,75	0,78	0,50	0,62	0,67	0,85
C26:0 DGTS	0,34	0,28	0,47	-0,11	0,21	0,37	0,49	-0,05	0,24
C28:1 DGTS	0,55	0,37	0,14	0,25	0,45	0,45	0,62	0,26	0,31
C28:0 DGTS	0,40	0,44	0,67	0,37	0,48	0,20	0,42	0,33	0,60
C29:0 DGTS	0,46	0,53	0,56	0,48	0,64	0,25	0,47	0,29	0,54
C30:1 DGTS	0,35	0,08	-0,08	0,23	0,25	0,09	0,40	0,36	0,21
C30:0 DGTS	0,48	0,62	0,53	0,54	0,68	0,31	0,48	0,29	0,63
C31:1 DGTS	0,61	0,57	0,32	0,59	0,73	0,33	0,59	0,37	0,56
C32:4 DGTS	0,34	0,17	-0,25	0,32	0,29	0,26	0,46	0,56	0,19
C32:2 DGTS	0,55	0,44	0,26	0,24	0,41	0,53	0,62	0,28	0,37
C32:1 DGTS	0,51	0,46	0,58	0,15	0,46	0,43	0,53	0,04	0,37
C34:5 DGTS	0,54	0,30	-0,07	0,42	0,43	0,29	0,57	0,61	0,34
C34:2 DGTS	0,86	0,75	0,37	0,64	0,88	0,74	0,89	0,44	0,62
C34:1 DGTS	0,36	0,59	0,49	0,27	0,54	0,50	0,47	-0,03	0,42
C36:5 DGTS	0,49	0,37	0,17	0,20	0,28	0,49	0,50	0,42	0,35
C36:3 DGTS	0,28	0,50	0,40	0,17	0,42	0,50	0,49	-0,09	0,37
C36:2 DGTS	0,58	0,71	0,44	0,53	0,70	0,52	0,62	0,33	0,70

	C32:2 PE	C32:1 PE	C32:0 PE	C33:2 PE	C33:1 PE	C34:2 PE	C34:1 PE	C35:2 PE	C36:2 PE
C34:5 PC									
C34:1 PC									
C35:5 PC									
C36:6 PC									
C36:5 PC									
C36:2 PC									
C37:6 PC									
C38:6 PC									
C40:10 PC									
C42:11 PC									
C30:1 PE									
C30:0 PE									
C32:2 PE									
C32:1 PE	0,92								
C32:0 PE	0,63	0,74							
C33:2 PE	0,86	0,90	0,64						
C33:1 PE	0,86	0,85	0,53	0,96					
C34:2 PE	0,93	0,95	0,74	0,86	0,81				
C34:1 PE	0,78	0,79	0,49	0,68	0,71	0,80			
C35:2 PE	0,70	0,80	0,61	0,92	0,89	0,75	0,69		
C36:2 PE	0,73	0,85	0,70	0,80	0,75	0,84	0,69	0,82	
C38:6 PE	0,50	0,62	0,57	0,43	0,49	0,55	0,74	0,53	0,53
C38:5 PE	0,33	0,20	-0,05	0,03	0,12	0,25	0,42	-0,10	-0,01
C40:6 PE	0,61	0,73	0,58	0,59	0,60	0,65	0,71	0,60	0,65
C26:0 DGTS	0,24	0,46	0,57	0,43	0,29	0,54	0,31	0,42	0,52
C28:1 DGTS	0,50	0,64	0,53	0,73	0,62	0,68	0,50	0,78	0,75
C28:0 DGTS	0,17	0,39	0,44	0,37	0,34	0,36	0,32	0,50	0,75
C29:0 DGTS	0,12	0,32	0,30	0,39	0,46	0,25	0,37	0,56	0,58
C30:1 DGTS	0,42	0,40	0,23	0,43	0,42	0,56	0,29	0,40	0,58
C30:0 DGTS	0,09	0,29	0,29	0,33	0,40	0,28	0,44	0,54	0,58
C31:1 DGTS	0,23	0,36	0,31	0,46	0,53	0,37	0,40	0,64	0,65
C32:4 DGTS	0,80	0,71	0,34	0,74	0,71	0,84	0,72	0,64	0,66
C32:2 DGTS	0,54	0,64	0,69	0,63	0,52	0,76	0,58	0,65	0,73
C32:1 DGTS	0,12	0,35	0,53	0,45	0,37	0,38	0,26	0,53	0,53
C34:5 DGTS	0,75	0,69	0,46	0,72	0,71	0,82	0,61	0,63	0,73
C34:2 DGTS	0,48	0,60	0,63	0,68	0,70	0,63	0,57	0,82	0,71
C34:1 DGTS	0,02	0,28	0,29	0,31	0,30	0,20	0,42	0,50	0,30
C36:5 DGTS	0,66	0,64	0,68	0,57	0,46	0,80	0,58	0,43	0,55
C36:3 DGTS	0,10	0,37	0,34	0,35	0,28	0,33	0,50	0,56	0,43
C36:2 DGTS	0,26	0,49	0,46	0,48	0,46	0,50	0,60	0,68	0,70

	C38:6 PE	C38:5 PE	C40:6 PE	C26:0 DGTS	C28:1 DGTS	C28:0 DGTS	C29:0 DGTS	C30:1 DGTS	C30:0 DGTS
C34:5 PC									
C34:1 PC									
C35:5 PC									
C36:6 PC									
C36:5 PC									
C36:2 PC									
C37:6 PC									
C38:6 PC									
C40:10 PC									
C42:11 PC									
C30:1 PE									
C30:0 PE									
C32:2 PE									
C32:1 PE									
C32:0 PE									
C33:2 PE									
C33:1 PE									
C34:2 PE									
C34:1 PE									
C35:2 PE									
C36:2 PE									
C38:6 PE									
C38:5 PE	0,28								
C40:6 PE	0,90	0,29							
C26:0 DGTS	0,26	-0,25	0,35						
C28:1 DGTS	0,36	-0,22	0,59	0,70					
C28:0 DGTS	0,37	-0,38	0,40	0,57	0,53				
C29:0 DGTS	0,46	-0,35	0,39	0,46	0,44	0,86			
C30:1 DGTS	0,13	-0,11	0,36	0,52	0,75	0,41	0,25		
C30:0 DGTS	0,56	-0,25	0,48	0,51	0,51	0,84	0,96	0,36	
C31:1 DGTS	0,51	-0,27	0,55	0,47	0,70	0,75	0,85	0,62	0,90
C32:4 DGTS	0,29	0,27	0,48	0,38	0,70	0,16	0,07	0,73	0,15
C32:2 DGTS	0,44	0,03	0,59	0,74	0,89	0,44	0,29	0,70	0,41
C32:1 DGTS	0,34	-0,39	0,44	0,85	0,75	0,70	0,71	0,47	0,75
C34:5 DGTS	0,35	0,15	0,58	0,50	0,80	0,34	0,24	0,89	0,32
C34:2 DGTS	0,69	-0,08	0,69	0,54	0,74	0,56	0,67	0,52	0,74
C34:1 DGTS	0,56	-0,26	0,37	0,53	0,33	0,52	0,75	-0,07	0,76
C36:5 DGTS	0,40	0,28	0,58	0,60	0,64	0,15	-0,05	0,56	0,08
C36:3 DGTS	0,53	-0,27	0,38	0,62	0,50	0,53	0,64	0,12	0,69
C36:2 DGTS	0,68	0,00	0,66	0,59	0,64	0,73	0,73	0,37	0,84

	C31:1 DGTS	C32:4 DGTS	C32:2 DGTS	C32:1 DGTS	C34:5 DGTS	C34:2 DGTS	C34:1 DGTS	C36:5 DGTS	C36:3 DGTS
C34:5 PC									
C34:1 PC									
C35:5 PC									
C36:6 PC									
C36:5 PC									
C36:2 PC									
C37:6 PC									
C38:6 PC									
C40:10 PC									
C42:11 PC									
C30:1 PE									
C30:0 PE									
C32:2 PE									
C32:1 PE									
C32:0 PE									
C33:2 PE									
C33:1 PE									
C34:2 PE									
C34:1 PE									
C35:2 PE									
C36:2 PE									
C38:6 PE									
C38:5 PE									
C40:6 PE									
C26:0 DGTS									
C28:1 DGTS									
C28:0 DGTS									
C29:0 DGTS									
C30:1 DGTS									
C30:0 DGTS									
C31:1 DGTS									
C32:4 DGTS	0,34								
C32:2 DGTS	0,56	0,71							
C32:1 DGTS	0,74	0,23	0,70						
C34:5 DGTS	0,56	0,91	0,81	0,46					
C34:2 DGTS	0,84	0,48	0,71	0,71	0,64				
C34:1 DGTS	0,54	-0,01	0,24	0,62	-0,01	0,59			
C36:5 DGTS	0,18	0,74	0,85	0,44	0,78	0,48	0,00		
C36:3 DGTS	0,53	0,19	0,42	0,58	0,13	0,57	0,91	0,13	
C36:2 DGTS	0,76	0,37	0,63	0,69	0,44	0,81	0,72	0,38	0,75

Table S6.1 Approximate fatty acid compositions of the predominant IPL species in the surface water of the Marsdiep (number of carbon atoms and double bond equivalents). Note that the positions of the fatty acids on the glycerol backbone (*sn*-1 or *sn*-2) were not determined and that the carbon numbers of the IPL species do not include the glycerol moiety.

IPL species	Predominant fatty acids
SQDGs	
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0
C30:2	C14:0/C16:2 and C14:1/C16:1
C30:1	C14:0/C16:1 and C14:1/C16:0
C30:0	C14:0/C16:0 plus small amounts of C12:0/C18:0
C32:3	C16:2/C16:1 plus small amounts of C14:1/C18:2
C32:2	C16:1/C16:1 plus small amounts of C14:0/C18:2 and C16:2/C16:0
C32:1	C14:0/C18:1 and C16:1/C16:0
C32:0	C16:0/C16:0 and C14:0/C18:0
C34:2	C16:1/C18:1 plus small amounts of C16:0/C18:2
C34:1	C16:0/C18:1
C34:0	C16:0/C18:0
C36:2	C18:1/C18:1 plus small amounts of C18:2/C18:0
PCs	
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0 and C13:0/C15:0
C30:1	C14:0/C16:1 plus small amounts of C14:1/C16:0 and C15:1/C15:0
C30:0	C14:0/C16:0 and C15:0/C15:0 plus small amounts of C12:0/C18:0
C32:2	C14:0/C18:2 and C16:1/C16:1
C32:1	C14:0/C18:1 and C16:1/C16:0 plus small amounts of C15:0/C17:1
C34:2	C16:1/C18:1 and C16:0/C18:2 plus small amounts of C17:1/C17:1
C34:1	C16:0/C18:1 plus small amounts of C17:1/C17:0
C34:0	C16:0/C18:0 plus small amounts of C17:0/C17:0
C35:5	C15:0/C20:5 plus small amounts of C15:1/C20:4 and C17:1/C18:4
C36:6	C14:0/C22:6, C16:1/C20:5 and combinations of C18:6-18:0
C36:5	C14:0/C22:5, C16:0/C20:5 and combinations of C18:5-18:0
C36:2	C18:1/C18:1 plus small amounts of C16:1/C20:1, C16:0/C20:2 and C18:2/C18:0
C37:6	C15:0/C22:6, C17:1/C20:5 and C18:0/C19:6 plus small amounts of C16:0/C21:6
C38:6	C16:0/C22:6, C18:1/C20:5 and C18:0/C20:6 plus small amounts of C19:6/C19:0
C40:10	C18:4/C22:6 and C20:5/C20:5 plus small amounts of C18:5/C22:5
C42:11	C20:5/C22:6

PGs	
C30:1	C14:0/C16:1 plus small amounts of C12:0/C18:1, C13:0/C17:1 and C15:1/C15:0
C30:0	C12:0/C18:0, C14:0/C16:0 and C15:0/C15:0
C31:0	Unknown – probably C14:0/C17:0 or C15:0/C16:0
C32:2	C16:1/C16:1 plus small amounts of C14:0/C18:2, C15:1/C17:1 and C16:2/C16:0
C32:1	C14:0/C18:1 and C16:1/C16:0 plus small amounts of C15:0/C17:1
C34:4	C16:1/C18:3 and C16:0/C18:4 plus small amounts of C14:0/C20:4
C34:3	Unknown – probably C16:2/C 18:1 or C16:1/C 18:2
C34:2	C16:1/C18:1 and C16:0/C18:2 plus small amounts of C14:0/C 20:2 and C17:1/C 17:1
C34:1	C16:1/C18:0 and C16:0/C18:1 plus small amounts of C15:0/C 19:1 and C17:1/C 17:0
C36:2	C18:1/C18:1 plus small amounts of C17:1/C 19:1
PEs	
C30:1	Unknown – possibly C14:0/C16:1 or C15:1/C15:0
C30:0	Unknown – possibly C14:0/C16:0 or C15:0/C15:0
C31:1	Unknown – possibly C14:0/C17:1 or C15:0/C16:1
C32:2	Unknown – possibly C14:0/C18:2 or C16:1/C16:1
C32:1	Unknown – possibly C14:0/C18:1 or C16:1/C16:0
C32:0	Unknown – possibly C14:0/C18:0 or C16:0/C16:0
C33:2	Unknown – possibly C15:1/C 18:1 or C16:1/C17:1
C33:1	Unknown – possibly C15:0/C 18:1 or C16:0/C 17:1
C34:2	C16:1/C 18:1 plus small amounts of C16:0/C 18:2 and C17:1/C 17:1
C34:1	Unknown – probably C16:0/C 18:1 or C17:1/C 17:0
C36:2	Unknown – possibly C16:0/C20:2 or C18:1/C18:1
C38:6	C16:0/C 22:6, C18:1/C 20:5 and C18:0/C 20:6
C40:6	C18:0/C 22:6 plus small amounts of C20:1/C 20:5
DGTSs	
C28:0	C14:0/C14:0 plus small amounts of C12:0/C16:0 and C13:0/C15:0
C30:1	C14:0/C16:1 plus small amounts of C12:0/C18:1, C14:1/C16:0 and C15:1/C15:0
C30:0	C14:0/C 16:0 and C15:0/C15:0
C31:1	Unknown – possibly C15:0/C16:1
C32:2	C14:0/C18:2 and C16:1/C16:1 plus small amounts of C14:1/C18:1 and C16:2/C16:1
C32:1	C14:0/C18:1 and C16:1/C16:0 plus small amounts of C14:1/C18:0 and C15:0/C17:1
C34:3	C16:1/C18:2 plus small amounts of C16:2/C18:1
C34:2	C16:1/C18:1 and C16:0/C18:2 plus small amounts of C17:1/C17:1
C34:1	C16:0/C18:1 plus small amounts of C16:1/C18:0
C35:2	Unknown – possibly C17:1/C18:1
C36:5	C16:0/C20:5 plus small amounts of C14:0/C22:5 and combinations of C18:5-18:0
C36:3	C18:2/C18:1 plus small amounts of C16:0/C20:3 and C18:3/C 18:0
C36:2	C18:1/C18:1 plus small amounts of C16:1/C20:1, C16:0/C20:2 and C18:2/C 18:0

Table S6.2 Total concentrations of the IPL classes and relative abundances of the predominant IPL species of SQDG, PC, PG, PE and DGTS in the Marsdiep. Note that the relative abundances of the IPL species are given as a percentage of the total concentration of their respective IPL class.

Date	Relative abundance SQDGs (%)												
	C28:0	C30:2	C30:1	C30:0	C32:3	C32:2	C32:1	C32:0	C34:2	C34:1	C34:0	C36:2	Other
07-03-2007	12	1	8	9	1	11	26	6	2	12	1	0	11
12-03-2007	16	1	7	12	1	4	22	8	2	13	2	1	11
20-03-2007	15	2	9	10	1	9	17	6	3	15	2	1	11
26-03-2007	7	0	6	7	1	14	22	6	6	16	2	1	13
04-04-2007	30	2	13	9	6	5	16	5	1	4	0	0	9
10-04-2007	29	2	12	13	8	4	18	3	1	1	1	1	8
17-04-2007	32	1	10	11	10	3	15	4	1	2	0	2	10
23-04-2007	32	1	9	11	8	2	12	4	1	4	0	4	12
01-05-2007	25	1	11	8	4	3	17	3	2	2	0	9	14
07-05-2007	26	2	15	8	2	2	19	3	1	3	0	5	14
15-05-2007	25	5	17	13	1	3	11	3	1	4	0	1	15
22-05-2007	23	3	18	16	1	2	17	3	1	2	0	0	13
31-05-2007	27	2	11	17	1	3	17	5	1	2	1	1	12
05-06-2007	28	1	12	17	1	2	25	4	0	1	0	0	10
21-06-2007	32	2	11	22	0	2	11	7	0	2	2	0	9
03-07-2007	18	2	7	14	0	4	10	16	2	9	8	1	10
09-07-2007	18	2	9	16	1	2	12	15	1	6	7	0	11
06-08-2007	22	2	11	17	1	4	11	13	1	4	4	1	10
21-08-2007	25	2	11	17	1	3	16	9	1	2	1	0	12
28-08-2007	28	1	10	18	0	2	14	8	1	2	2	0	12
06-09-2007	25	2	13	12	1	4	15	8	1	3	2	1	13
20-09-2007	23	2	13	18	0	3	10	11	1	2	3	0	12
27-09-2007	16	1	14	16	1	5	21	6	1	3	1	1	15
11-10-2007	20	2	11	11	1	5	16	10	2	5	2	1	15
13-11-2007	17	2	13	10	0	4	22	10	1	5	1	1	13
03-12-2007	16	1	11	14	1	3	22	8	2	3	1	2	17
18-12-2007	6	1	9	7	1	8	28	17	3	9	2	1	10
18-01-2008	13	1	17	10	1	7	23	8	1	4	2	1	12
11-02-2008	18	1	12	10	1	5	23	10	1	5	0	0	15
22-02-2008	27	2	16	11	1	4	23	5	0	2	1	0	10
07-03-2008	29	2	15	8	1	3	20	5	1	2	0	0	13

Date	Relative abundance PCs (%)														
	C28:0	**C30:1**	**C30:0**	**C32:2**	**C32:1**	**C34:2**	**C34:1**	**C34:0**	**C36:6**	**C36:5**	**C36:2**	**C38:6**	**C40:10**	**C42:11**	**Other**
07-03-2007	2	4	3	3	6	6	5	2	2	2	9	2	1	1	53
12-03-2007	2	3	2	2	7	4	8	2	2	5	4	5	2	1	52
20-03-2007	3	3	3	3	6	7	5	1	2	3	8	2	2	1	52
26-03-2007	2	3	2	5	5	7	4	1	3	3	9	3	2	1	51
04-04-2007	3	5	5	2	10	3	5	1	2	4	4	3	1	1	51
10-04-2007	3	2	3	2	5	6	5	1	3	3	12	2	1	1	50
17-04-2007	2	3	4	2	7	4	5	1	4	6	6	6	1	1	48
23-04-2007	3	3	3	2	6	4	6	1	2	4	10	4	1	1	51
01-05-2007	4	3	3	2	5	4	4	1	3	4	6	4	1	1	55
07-05-2007	5	3	6	1	4	3	4	1	3	4	5	5	2	2	52
15-05-2007	5	3	3	1	4	4	4	6	2	3	5	4	1	1	52
22-05-2007	6	3	4	2	5	6	6	1	2	3	9	4	1	1	47
31-05-2007	4	3	5	2	5	7	7	1	1	2	9	3	1	1	48
05-06-2007	4	5	4	3	6	5	5	1	3	4	6	4	2	1	49
21-06-2007	4	3	8	2	6	3	8	2	2	3	3	4	1	1	49
03-07-2007	5	3	6	2	5	3	5	1	2	3	5	3	2	2	53
09-07-2007	3	3	5	2	4	3	5	1	3	5	3	5	3	3	51
06-08-2007	4	4	7	2	5	2	4	1	3	5	4	7	3	4	46
21-08-2007	2	3	3	3	4	2	3	1	5	4	3	6	3	3	54
28-08-2007	3	3	4	3	3	2	3	1	5	4	3	8	3	4	52
06-09-2007	3	3	3	2	4	2	3	1	5	5	3	9	3	4	49
20-09-2007	5	3	4	2	4	3	5	1	2	3	4	5	2	1	56
27-09-2007	2	2	4	2	3	2	3	1	5	6	2	8	5	6	48
11-10-2007	2	2	2	3	3	2	3	1	7	5	3	8	5	5	49
13-11-2007	3	3	3	3	5	5	6	2	2	2	6	2	2	1	54
03-12-2007	2	3	3	3	6	5	6	2	2	2	10	2	1	1	53
18-12-2007	1	3	2	3	6	8	6	1	3	4	8	3	3	1	49
18-01-2008	2	3	3	3	5	8	7	1	1	1	12	1	1	0	50
11-02-2008	3	4	3	4	7	9	6	1	2	1	9	1	1	0	49
22-02-2008	3	6	4	5	8	7	6	2	2	1	8	1	1	0	45
07-03-2008	4	6	5	5	7	7	6	2	1	2	8	1	1	0	46

Date	Relative abundance PGs (%)											
	C30:1	C30:0	C31:0	C32:2	C32:1	C34:4	C34:3	C34:2	C34:1	C35:0	C36:2	Other
07-03-2007	4	4	3	13	18	2	2	11	8	0	10	24
12-03-2007	5	4	3	9	18	3	3	10	10	0	10	26
20-03-2007	5	4	2	11	18	3	2	10	8	0	12	25
26-03-2007	3	3	2	17	16	4	6	9	7	0	9	25
04-04-2007	9	4	1	9	21	1	1	10	7	4	10	23
10-04-2007	5	2	1	9	15	1	1	12	7	5	16	27
17-04-2007	9	5	2	6	15	0	1	12	7	6	16	21
23-04-2007	8	6	1	6	16	0	1	13	7	4	17	22
01-05-2007	6	10	2	5	13	0	1	13	7	1	17	26
07-05-2007	6	7	2	7	14	1	1	13	6	1	17	26
15-05-2007	13	7	2	4	12	0	1	10	8	0	17	25
22-05-2007	22	9	2	4	12	0	1	8	6	0	12	22
31-05-2007	16	10	3	5	14	1	1	9	7	0	14	22
05-06-2007	9	7	2	5	20	2	1	8	8	0	9	29
21-06-2007	10	8	2	5	20	1	1	9	8	0	11	24
03-07-2007	6	5	3	8	18	1	2	8	10	0	11	27
09-07-2007	7	6	2	6	19	3	2	8	9	0	9	28
06-08-2007	8	7	2	5	18	3	2	9	8	0	10	28
21-08-2007	7	5	2	5	18	4	2	8	7	0	7	33
28-08-2007	9	6	2	6	14	5	2	8	8	1	8	32
06-09-2007	7	6	2	6	17	5	2	7	9	0	6	32
20-09-2007	7	6	2	7	16	5	2	8	9	0	7	30
27-09-2007	5	5	3	7	17	3	2	9	7	1	7	36
11-10-2007	7	4	2	6	15	6	3	8	7	1	7	34
13-11-2007	6	6	4	9	19	2	1	9	10	0	7	28
03-12-2007	5	7	5	7	18	0	0	10	11	0	9	28
18-12-2007	4	3	2	11	15	3	6	11	13	1	8	24
18-01-2008	5	4	3	11	18	1	1	13	9	0	10	27
11-02-2008	6	4	3	10	21	1	2	10	10	0	9	24
22-02-2008	7	4	2	9	23	1	1	9	8	0	9	26
07-03-2008	11	4	2	9	22	1	1	8	8	0	9	25

Date Relative abundance PEs (%)														
	C30:1	C30:0	C31:1	C32:2	C32:1	C32:0	C33:2	C33:1	C34:2	C34:1	C36:2	C38:6	C40:6	Other
07-03-2007	3	2	3	13	19	2	2	3	19	7	3	1	0	23
12-03-2007	4	1	2	16	17	2	1	7	16	2	4	1	0	28
20-03-2007	5	2	1	11	12	4	1	7	19	9	4	0	0	26
26-03-2007	6	2	3	13	13	1	5	3	19	3	6	0	1	26
04-04-2007	5	1	4	13	9	6	1	0	26	3	5	1	0	27
10-04-2007	7	3	5	8	19	1	2	6	11	11	5	1	0	20
17-04-2007	2	1	3	7	8	0	4	1	6	1	2	8	3	51
23-04-2007	3	9	3	5	13	0	3	1	9	9	4	0	0	41
01-05-2007	8	3	4	7	15	2	1	2	12	5	5	2	1	34
07-05-2007	9	2	6	6	11	2	1	3	9	5	3	3	1	38
15-05-2007	9	4	5	7	12	2	1	2	8	9	4	2	0	34
22-05-2007	17	6	4	6	11	2	2	4	12	5	3	1	0	28
31-05-2007	13	8	4	5	14	2	1	2	12	5	7	0	0	26
05-06-2007	6	6	2	6	18	3	3	4	16	8	1	1	0	24
21-06-2007	6	4	2	4	15	5	1	4	11	10	2	2	0	34
03-07-2007	6	5	4	6	14	4	1	4	12	13	6	2	1	22
09-07-2007	5	5	4	5	12	4	2	3	13	6	4	3	3	33
06-08-2007	6	3	2	4	11	3	1	3	11	5	3	7	11	31
21-08-2007	3	3	3	5	15	2	2	3	19	6	6	3	1	29
28-08-2007	4	5	3	5	11	4	1	3	12	6	5	4	4	33
06-09-2007	4	4	3	4	10	4	2	5	14	7	4	3	1	35
20-09-2007	3	6	3	5	13	6	2	5	12	9	4	2	0	30
27-09-2007	2	1	2	5	10	2	2	5	12	4	3	8	8	35
11-10-2007	3	2	2	7	12	2	2	4	16	4	3	5	4	33
13-11-2007	6	3	3	9	17	3	3	5	17	6	3	0	0	24
03-12-2007	5	3	4	10	18	3	2	5	17	5	3	1	0	23
18-12-2007	5	3	4	15	15	1	2	4	15	4	4	1	0	25
18-01-2008	6	3	3	19	14	4	2	2	18	5	3	1	0	20
11-02-2008	6	3	3	16	17	2	2	4	18	6	3	0	0	19
22-02-2008	6	3	5	13	12	2	2	3	18	5	6	0	0	23
07-03-2008	8	2	4	16	14	1	1	3	18	6	2	1	0	23

Date	Relative abundance DGTSs (%)													
	C28:0	C30:1	C30:0	C31:1	C32:2	C32:1	C34:3	C34:2	C34:1	C35:2	C36:5	C36:3	C36:2	Other
07-03-2007	1	3	2	3	4	11	1	8	11	2	1	5	14	35
12-03-2007	1	3	2	2	7	10	3	12	9	2	1	4	11	34
20-03-2007	1	5	2	2	4	11	3	10	11	2	1	5	11	32
26-03-2007	1	2	1	1	5	8	8	11	8	1	3	5	10	38
04-04-2007	4	4	2	2	4	8	2	9	10	1	5	4	9	37
10-04-2007	3	4	2	3	4	8	3	7	9	1	5	5	10	36
17-04-2007	6	3	2	2	2	6	4	5	7	1	6	3	9	43
23-04-2007	6	3	2	2	3	6	4	6	7	1	8	2	10	39
01-05-2007	2	3	2	2	3	6	1	18	6	9	3	3	14	30
07-05-2007	15	4	2	2	2	4	1	7	2	5	2	1	7	46
15-05-2007	4	15	8	14	5	7	1	3	2	1	0	1	4	35
22-05-2007	14	8	6	7	4	6	1	2	2	1	0	1	2	46
31-05-2007	5	6	7	9	4	8	2	5	5	1	1	3	7	37
05-06-2007	3	5	5	6	5	11	4	7	8	1	1	3	7	35
21-06-2007	4	5	6	5	2	8	2	5	11	1	1	3	7	40
03-07-2007	2	3	4	4	5	8	6	10	11	1	2	4	6	35
09-07-2007	3	3	4	4	3	7	6	8	11	1	2	3	7	39
06-08-2007	2	3	3	3	3	8	3	10	13	1	1	3	8	38
21-08-2007	4	3	4	4	3	7	2	6	11	1	1	3	8	43
28-08-2007	3	4	8	8	2	6	1	6	11	1	1	2	5	41
06-09-2007	2	3	4	4	2	6	3	8	14	1	2	3	6	41
20-09-2007	3	4	6	6	2	8	1	7	13	1	0	4	7	37
27-09-2007	2	2	3	3	3	10	2	8	13	1	1	4	13	35
11-10-2007	2	2	2	3	3	6	5	9	9	1	2	3	8	45
13-11-2007	2	3	3	3	2	9	1	12	13	2	0	2	10	38
03-12-2007	2	2	4	3	2	11	0	7	13	2	0	4	12	38
18-12-2007	0	1	1	2	6	8	2	23	12	2	1	2	10	30
18-01-2008	1	3	3	5	3	12	1	8	12	2	0	6	13	32
11-02-2008	1	2	2	4	6	9	2	14	11	2	0	4	13	30
22-02-2008	1	3	2	3	5	11	1	11	10	1	0	7	15	30
07-03-2008	2	3	3	4	4	10	1	11	11	1	0	5	13	31

Table S6.3 Spearman correlation coefficients (ρ) between IPL concentrations, microbial abundances and environmental parameters.

	Total SQDG	Total PC	Total PG	Total PE	Total DGTS	Temperature	Salinity	DIP	DISi	DIN
Total PC	0,87									
Total PG	0,77	0,83								
Total PE	0,02	0,20	0,07							
Total DGTS	0,84	0,91	0,75	0,22						
Temperature	0,50	0,47	0,41	0,04	0,55					
Salinity	0,16	0,04	0,10	0,26	-0,06	0,20				
DIP	-0,83	-0,63	-0,62	0,09	-0,57	-0,50	-0,43			
DISi	-0,76	-0,59	-0,54	-0,12	-0,56	-0,65	-0,58	0,87		
DIN	-0,68	-0,65	-0,51	-0,25	-0,68	-0,75	-0,48	0,58	0,77	
N:P ratio	-0,03	-0,18	-0,09	-0,30	-0,28	-0,45	-0,13	-0,22	0,06	0,63
Chlorophyll *a*	0,82	0,79	0,68	-0,17	0,73	0,40	0,18	-0,82	-0,73	-0,48
Primary production	0,84	0,78	0,68	-0,17	0,73	0,46	0,16	-0,85	-0,76	-0,49
Skeletonema costatum	0,03	-0,01	0,01	-0,50	0,06	-0,44	-0,47	0,09	0,42	0,41
Thalassiosira spp.	0,14	0,09	-0,08	-0,25	0,20	0,58	-0,12	-0,21	-0,27	-0,24
Chaetoceros socialis	0,61	0,49	0,45	-0,18	0,40	0,60	0,21	-0,71	-0,81	-0,54
Pseudonitzschia delicatissima	0,61	0,42	0,56	0,02	0,28	0,08	0,54	-0,80	-0,74	-0,36
Hemiselmis spp.	0,31	0,40	0,17	0,20	0,49	0,72	0,06	-0,21	-0,35	-0,64
Plagioselmis spp.	0,02	0,07	-0,19	-0,08	0,15	0,66	-0,22	0,02	-0,11	-0,24
Phaeocystis globosa (colony)	0,51	0,56	0,58	-0,07	0,42	0,10	0,12	-0,55	-0,45	-0,29
Phaeocystis globosa (single)	0,54	0,40	0,51	-0,10	0,42	0,16	0,23	-0,66	-0,47	-0,31
Prymnesiales	0,51	0,46	0,34	-0,20	0,43	0,25	0,09	-0,56	-0,51	-0,21
Other flagellates	0,83	0,65	0,60	0,08	0,60	0,49	0,41	-0,90	-0,86	-0,63
Total algae	0,73	0,59	0,55	-0,24	0,53	0,41	0,19	-0,81	-0,71	-0,48
Total cyanobactria	-0,20	-0,03	-0,14	-0,05	0,10	0,47	-0,29	0,38	0,19	-0,21
Total bacteria	0,05	0,02	0,01	0,16	0,23	0,38	-0,11	0,02	-0,06	-0,11
C28:0 SQDG	0,91	0,78	0,75	-0,06	0,70	0,60	0,33	-0,88	-0,86	-0,73
C30:2 SQDG	0,86	0,67	0,71	0,05	0,60	0,57	0,40	-0,86	-0,81	-0,66
C30:1 SQDG	0,94	0,85	0,77	0,11	0,74	0,47	0,27	-0,83	-0,79	-0,66
C30:0 SQDG	0,92	0,77	0,70	-0,07	0,74	0,71	0,25	-0,84	-0,84	-0,77
C32:3 SQDG	0,88	0,83	0,80	-0,02	0,71	0,34	0,14	-0,78	-0,65	-0,47
C32:2 SQDG	0,76	0,72	0,62	-0,16	0,67	0,15	-0,20	-0,49	-0,31	-0,24
C32:1 SQDG	0,93	0,85	0,70	0,03	0,78	0,34	0,05	-0,71	-0,66	-0,51
C32:0 SQDG	0,77	0,74	0,52	0,05	0,84	0,62	-0,24	-0,49	-0,43	-0,60
C34:2 SQDG	0,70	0,67	0,57	-0,02	0,74	0,22	-0,26	-0,41	-0,26	-0,32
C34:1 SQDG	0,60	0,49	0,54	-0,16	0,58	0,12	-0,33	-0,31	-0,07	-0,14
C34:0 SQDG	0,46	0,51	0,38	-0,03	0,65	0,64	-0,31	-0,19	-0,22	-0,50

C36:2 SQDG	0,75	0,73	0,81	0,15	0,70	0,24	0,07	-0,53	-0,46	-0,43
C28:0 PC	0,79	0,71	0,87	0,06	0,61	0,54	0,38	-0,81	-0,76	-0,62
C30:1 PC	0,81	0,81	0,94	0,03	0,71	0,40	0,13	-0,65	-0,59	-0,51
C30:0 PC	0,80	0,73	0,86	0,02	0,69	0,64	0,28	-0,75	-0,70	-0,67
C32:2 PC	0,74	0,86	0,86	-0,06	0,79	0,26	-0,17	-0,48	-0,34	-0,36
C32:1 PC	0,79	0,75	0,93	-0,05	0,66	0,30	0,08	-0,64	-0,52	-0,39
C34:2 PC	0,67	0,57	0,72	-0,13	0,44	-0,07	0,05	-0,60	-0,40	-0,09
C34:1 PC	0,78	0,72	0,91	-0,01	0,62	0,31	0,16	-0,70	-0,59	-0,41
C34:0 PC	0,63	0,65	0,90	0,03	0,52	0,29	0,16	-0,54	-0,43	-0,37
C36:6 PC	0,75	0,89	0,93	0,14	0,82	0,47	0,02	-0,55	-0,50	-0,55
C36:5 PC	0,76	0,87	0,97	0,08	0,81	0,46	0,02	-0,57	-0,50	-0,53
C36:2 PC	0,70	0,65	0,73	-0,05	0,52	0,03	0,06	-0,63	-0,47	-0,19
C38:6 PC	0,71	0,85	0,93	0,20	0,81	0,58	0,09	-0,55	-0,54	-0,63
C40:10 PC	0,67	0,83	0,87	0,21	0,81	0,54	-0,02	-0,43	-0,42	-0,55
C42:11 PC	0,63	0,80	0,83	0,22	0,80	0,66	0,05	-0,43	-0,46	-0,63
C30:1 PG	0,79	0,80	0,72	0,14	0,66	0,55	0,32	-0,79	-0,76	-0,67
C30:0 PG	0,83	0,86	0,72	0,23	0,79	0,63	0,29	-0,74	-0,77	-0,77
C31:0 PG	0,59	0,77	0,53	0,44	0,72	0,50	0,03	-0,32	-0,42	-0,61
C32:2 PG	0,62	0,77	0,64	-0,01	0,71	0,07	-0,28	-0,26	-0,15	-0,27
C32:1 PG	0,82	0,94	0,75	0,07	0,87	0,44	-0,08	-0,53	-0,48	-0,59
C34:4 PG	0,36	0,51	0,35	0,06	0,64	0,46	-0,46	0,01	-0,02	-0,32
C34:3 PG	0,47	0,66	0,53	0,04	0,74	0,33	-0,50	-0,08	0,00	-0,28
C34:2 PG	0,85	0,96	0,87	0,23	0,86	0,37	0,09	-0,65	-0,60	-0,56
C34:1 PG	0,83	0,95	0,78	0,18	0,91	0,50	-0,05	-0,54	-0,53	-0,68
C35:0 PG	0,73	0,84	0,73	0,05	0,77	0,26	0,02	-0,53	-0,47	-0,48
C36:2 PG	0,87	0,85	0,82	0,18	0,74	0,37	0,27	-0,80	-0,76	-0,57
C30:1 PE	0,01	0,12	0,01	0,96	0,13	0,03	0,35	-0,01	-0,18	-0,20
C30:0 PE	0,17	0,26	0,08	0,92	0,26	0,17	0,35	-0,12	-0,29	-0,37
C31:1 PE	0,00	0,17	0,04	0,98	0,17	-0,02	0,30	0,06	-0,10	-0,18
C32:1 PE	-0,20	-0,05	-0,12	0,90	-0,03	-0,30	0,12	0,30	0,18	0,10
C32:1 PE	-0,01	0,15	0,03	0,99	0,17	0,02	0,27	0,10	-0,10	-0,21
C32:0 PE	0,07	0,22	0,11	0,92	0,26	0,25	0,26	0,03	-0,18	-0,37
C33:2 PE	0,08	0,27	0,05	0,89	0,26	0,00	0,26	0,02	-0,14	-0,26
C33:1 PE	0,01	0,17	0,09	0,96	0,19	0,05	0,16	0,14	-0,07	-0,21
C34:2 PE	-0,07	0,15	-0,02	0,97	0,17	-0,04	0,16	0,20	0,03	-0,14
C34:1 PE	0,14	0,25	0,11	0,95	0,25	0,14	0,33	-0,04	-0,24	-0,36
C36:2 PE	0,01	0,20	0,08	0,96	0,22	0,04	0,27	0,07	-0,08	-0,21
C38:6 PE	0,26	0,47	0,42	0,78	0,50	0,41	0,18	-0,10	-0,29	-0,47
C40:6 PE	0,19	0,43	0,35	0,68	0,50	0,46	0,14	-0,07	-0,20	-0,47
C28:0 DGTS	0,81	0,80	0,73	0,17	0,76	0,65	0,41	-0,82	-0,84	-0,80
C30:1 DGTS	0,80	0,74	0,72	0,22	0,73	0,63	0,32	-0,79	-0,80	-0,69
C30:0 DGTS	0,66	0,66	0,54	0,37	0,73	0,80	0,29	-0,60	-0,76	-0,80
C31:1 DGTS	0,61	0,62	0,46	0,37	0,64	0,70	0,25	-0,59	-0,71	-0,70
C32:2 DGTS	0,71	0,71	0,67	0,14	0,81	0,28	-0,23	-0,46	-0,33	-0,30
C32:1 DGTS	0,71	0,84	0,66	0,11	0,92	0,48	-0,20	-0,38	-0,39	-0,55

C34:3 DGTS	0,69	0,73	0,61	-0,03	0,85	0,39	-0,33	-0,40	-0,29	-0,41
C34:2 DGTS	0,56	0,68	0,56	0,15	0,83	0,24	-0,38	-0,19	-0,10	-0,36
C34:1 DGTS	0,54	0,73	0,52	0,09	0,79	0,44	-0,32	-0,19	-0,19	-0,54
C35:2 DGTS	0,42	0,62	0,56	0,17	0,70	0,08	-0,26	-0,10	-0,03	-0,33
C36:5 DGTS	0,79	0,84	0,79	-0,05	0,84	0,37	-0,10	-0,58	-0,47	-0,48
C36:3 DGTS	0,53	0,75	0,57	0,07	0,80	0,29	-0,32	-0,19	-0,12	-0,35
C36:2 DGTS	0,52	0,77	0,62	0,19	0,78	0,14	-0,20	-0,20	-0,18	-0,34

	N:P ratio	**Chlorophyll *a***	**Primary production**	**Skeletonema costatum**	**Thalassiosira spp.**	**Chaetoceros socialis**	**Pseudonitzschia delicatissima**	**Hemiselmis spp.**	**Plagioselmis spp.**	**Phaeocystis globosa (colony)**
Total PC										
Total PG										
Total PE										
Total DGTS										
Temperature										
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*	0,17									
Primary production	0,19	0,96								
Skeletonema costatum	0,36	0,04	-0,06							
Thalassiosira spp.	-0,13	0,33	0,26	0,04						
Chaetoceros socialis	0,00	0,69	0,73	-0,26	0,45					
Pseudonitzschia delicatissima	0,28	0,69	0,65	-0,09	-0,11	0,57				
Hemiselmis spp.	-0,56	0,16	0,14	-0,25	0,46	0,33	-0,11			
Plagioselmis spp.	-0,22	-0,04	0,01	-0,13	0,59	0,22	-0,42	0,52		
Phaeocystis globosa (colony)	0,08	0,77	0,71	0,03	0,05	0,41	0,57	0,20	-0,20	
Phaeocystis globosa (single)	0,21	0,60	0,57	0,22	0,11	0,39	0,64	0,21	-0,18	0,63
Prymnesiales	0,29	0,69	0,77	0,00	0,20	0,53	0,50	0,13	0,02	0,44
Other flagellates	0,05	0,82	0,81	-0,18	0,28	0,74	0,78	0,37	-0,01	0,63
Total algae	0,13	0,96	0,93	0,05	0,44	0,74	0,68	0,27	0,04	0,75
Total cyanobactria	-0,60	-0,32	-0,23	-0,12	0,44	-0,11	-0,78	0,50	0,58	-0,34

Total bacteria	-0,17	-0,01	0,03	-0,14	0,30	-0,07	-0,28	0,20	0,33	-0,28
C28:0 SQDG	-0,06	0,83	0,84	-0,09	0,29	0,79	0,73	0,37	0,05	0,56
C30:2 SQDG	0,00	0,69	0,73	-0,18	0,17	0,70	0,69	0,33	0,03	0,45
C30:1 SQDG	0,00	0,78	0,78	-0,09	0,14	0,66	0,67	0,27	0,00	0,49
C30:0 SQDG	-0,14	0,74	0,77	-0,13	0,31	0,76	0,56	0,44	0,24	0,45
C32:3 SQDG	0,17	0,85	0,85	0,10	0,06	0,58	0,68	0,16	-0,12	0,66
C32:2 SQDG	0,16	0,69	0,67	0,38	-0,08	0,22	0,32	0,05	-0,09	0,55
C32:1 SQDG	0,09	0,87	0,84	0,14	0,13	0,56	0,57	0,20	-0,04	0,53
C32:0 SQDG	-0,29	0,52	0,55	0,08	0,32	0,33	0,01	0,56	0,37	0,17
C34:2 SQDG	0,01	0,53	0,54	0,39	-0,04	0,08	0,24	0,19	0,03	0,43
C34:1 SQDG	0,08	0,35	0,36	0,51	-0,19	-0,12	0,10	-0,03	-0,05	0,20
C34:0 SQDG	-0,47	0,33	0,33	-0,03	0,37	0,13	-0,30	0,51	0,52	0,12
C36:2 SQDG	-0,02	0,57	0,56	0,10	-0,21	0,26	0,52	0,12	-0,30	0,49
C28:0 PC	-0,04	0,70	0,73	-0,22	0,08	0,62	0,73	0,25	-0,12	0,63
C30:1 PC	-0,03	0,66	0,65	0,02	0,01	0,54	0,59	0,12	-0,15	0,48
C30:0 PC	-0,17	0,66	0,69	-0,18	0,15	0,58	0,60	0,40	-0,04	0,52
C32:2 PC	-0,03	0,74	0,68	0,31	0,06	0,33	0,35	0,14	-0,06	0,62
C32:1 PC	0,10	0,67	0,69	0,12	-0,14	0,47	0,64	-0,05	-0,24	0,52
C34:2 PC	0,46	0,66	0,63	0,32	-0,26	0,34	0,73	-0,30	-0,39	0,56
C34:1 PC	0,13	0,65	0,68	0,05	-0,15	0,48	0,72	-0,01	-0,26	0,53
C34:0 PC	0,00	0,48	0,49	-0,07	-0,27	0,32	0,55	-0,01	-0,30	0,44
C36:6 PC	-0,18	0,69	0,66	0,06	0,09	0,42	0,42	0,35	-0,05	0,54
C36:5 PC	-0,15	0,69	0,68	0,05	0,02	0,40	0,47	0,28	-0,11	0,57
C36:2 PC	0,38	0,70	0,70	0,23	-0,27	0,36	0,72	-0,21	-0,35	0,66
C38:6 PC	-0,27	0,61	0,59	-0,11	0,08	0,40	0,39	0,44	0,04	0,50
C40:10 PC	-0,30	0,56	0,54	0,00	0,11	0,28	0,24	0,43	0,06	0,41
C42:11 PC	-0,41	0,52	0,52	-0,16	0,17	0,32	0,18	0,56	0,14	0,40
C30:1 PG	-0,05	0,66	0,66	-0,14	0,18	0,69	0,61	0,47	0,17	0,50
C30:0 PG	-0,19	0,65	0,68	-0,21	0,12	0,60	0,54	0,48	0,25	0,46
C31:0 PG	-0,38	0,32	0,43	-0,19	-0,01	0,25	0,10	0,49	0,30	0,13
C32:2 PG	-0,12	0,67	0,59	0,31	0,01	0,14	0,18	0,12	-0,18	0,60
C32:1 PG	-0,23	0,76	0,72	0,07	0,20	0,47	0,31	0,37	0,14	0,47
C34:4 PG	-0,42	0,25	0,18	0,16	0,41	0,02	-0,41	0,44	0,51	0,05
C34:3 PG	-0,33	0,39	0,33	0,26	0,22	0,01	-0,24	0,40	0,27	0,31
C34:2 PG	-0,06	0,81	0,82	-0,05	-0,04	0,48	0,54	0,26	-0,13	0,64
C34:1 PG	-0,30	0,68	0,71	-0,01	0,08	0,45	0,27	0,45	0,12	0,42
C35:0 PG	-0,12	0,79	0,70	0,12	0,07	0,38	0,43	0,29	-0,23	0,65
C36:2 PG	0,09	0,81	0,83	-0,13	-0,08	0,60	0,78	0,12	-0,18	0,65
C30:1 PE	-0,16	-0,15	-0,14	-0,51	-0,24	-0,12	0,13	0,10	-0,10	-0,09
C30:0 PE	-0,26	-0,09	-0,05	-0,53	-0,23	-0,07	0,14	0,25	0,02	-0,03
C31:1 PE	-0,20	-0,16	-0,16	-0,46	-0,25	-0,20	0,05	0,13	-0,10	-0,07
C32:1 PE	-0,09	-0,34	-0,39	-0,29	-0,38	-0,40	-0,07	-0,11	-0,23	-0,20
C32:1 PE	-0,26	-0,20	-0,19	-0,50	-0,28	-0,16	0,02	0,17	-0,08	-0,11

C32:0 PE	-0,43	-0,18	-0,13	-0,57	-0,17	-0,13	-0,05	0,30	0,03	-0,10
C33:2 PE	-0,24	-0,03	-0,03	-0,38	-0,24	-0,22	0,06	0,13	-0,08	0,06
C33:1 PE	-0,32	-0,21	-0,19	-0,48	-0,26	-0,20	-0,03	0,14	-0,06	-0,08
C34:2 PE	-0,28	-0,26	-0,27	-0,35	-0,20	-0,26	-0,11	0,19	-0,02	-0,16
C34:1 PE	-0,33	-0,09	-0,04	-0,57	-0,30	-0,09	0,10	0,19	-0,09	-0,04
C36:2 PE	-0,26	-0,18	-0,15	-0,42	-0,26	-0,20	0,02	0,17	-0,11	-0,06
C38:6 PE	-0,42	0,07	0,09	-0,50	-0,13	-0,01	0,01	0,41	0,07	0,02
C40:6 PE	-0,47	0,04	0,08	-0,41	-0,03	-0,12	-0,13	0,49	0,18	0,03
C28:0 DGTS	-0,18	0,69	0,74	-0,22	0,21	0,69	0,62	0,57	0,15	0,54
C30:1 DGTS	-0,07	0,61	0,61	-0,19	0,18	0,66	0,59	0,41	0,16	0,36
C30:0 DGTS	-0,37	0,46	0,47	-0,35	0,36	0,60	0,32	0,62	0,38	0,15
C31:1 DGTS	-0,25	0,40	0,41	-0,33	0,32	0,59	0,31	0,56	0,37	0,08
C32:2 DGTS	0,08	0,55	0,55	0,31	0,12	0,27	0,31	0,18	0,08	0,29
C32:1 DGTS	-0,31	0,62	0,63	0,19	0,21	0,32	0,13	0,45	0,15	0,30
C34:3 DGTS	-0,12	0,63	0,62	0,32	0,17	0,16	0,14	0,28	0,18	0,38
C34:2 DGTS	-0,29	0,44	0,39	0,33	0,16	0,06	-0,02	0,34	0,05	0,29
C34:1 DGTS	-0,53	0,42	0,42	0,10	0,17	0,21	-0,13	0,51	0,20	0,24
C35:2 DGTS	-0,31	0,31	0,30	0,27	-0,16	-0,05	0,05	0,26	-0,12	0,35
C36:5 DGTS	-0,08	0,78	0,77	0,21	0,18	0,42	0,47	0,29	-0,11	0,64
C36:3 DGTS	-0,29	0,55	0,48	0,30	0,25	0,13	-0,05	0,39	0,09	0,41
C36:2 DGTS	-0,23	0,57	0,51	0,23	0,04	0,13	0,17	0,23	-0,08	0,42

	Phaeocystis globosa (single)	**Prymnesiales**	**Other flagellates**	**Total algae**	**Total cyanobacteria**	**Total bacteria**	**C28:0 SQDG**	**C30:2 SQDG**	**C30:1 SQDG**	**C30:0 SQDG**
Total PC										
Total PG										
Total PE										
Total DGTS										
Temperature										
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*										
Primary production										
Skeletonema costatum										
Thalassiosira spp.										

Chaetoceros socialis										
Pseudonitzschia delicatissima										
Hemiselmis spp.										
Plagioselmis spp.										
Phaeocystis globosa (colony)										
Phaeocystis globosa (single)										
Prymnesiales	0,42									
Other flagellates	0,75	0,59								
Total algae	0,74	0,64	0,87							
Total cyanobactria	-0,51	-0,25	-0,46	-0,37						
Total bacteria	-0,21	-0,03	-0,10	-0,13	0,38					
C28:0 SQDG	0,62	0,52	0,91	0,84	-0,22	-0,05				
C30:2 SQDG	0,61	0,37	0,87	0,69	-0,29	0,01	0,91			
C30:1 SQDG	0,48	0,42	0,82	0,68	-0,27	0,02	0,93	0,90		
C30:0 SQDG	0,51	0,42	0,83	0,76	-0,06	0,03	0,94	0,85	0,88	
C32:3 SQDG	0,69	0,56	0,81	0,78	-0,35	-0,02	0,84	0,81	0,85	0,75
C32:2 SQDG	0,44	0,42	0,47	0,51	-0,19	-0,01	0,56	0,53	0,64	0,58
C32:1 SQDG	0,49	0,53	0,74	0,71	-0,27	0,09	0,83	0,73	0,88	0,79
C32:0 SQDG	0,26	0,26	0,46	0,37	0,32	0,25	0,61	0,54	0,64	0,73
C34:2 SQDG	0,52	0,31	0,43	0,39	-0,11	0,07	0,47	0,46	0,52	0,52
C34:1 SQDG	0,38	0,15	0,21	0,19	-0,09	0,16	0,34	0,39	0,42	0,42
C34:0 SQDG	0,08	0,04	0,15	0,17	0,57	0,37	0,33	0,29	0,32	0,52
C36:2 SQDG	0,61	0,31	0,57	0,44	-0,23	0,12	0,65	0,64	0,68	0,60
C28:0 PC	0,62	0,34	0,82	0,69	-0,27	-0,08	0,87	0,89	0,84	0,81
C30:1 PC	0,48	0,33	0,64	0,58	-0,20	-0,07	0,82	0,75	0,86	0,75
C30:0 PC	0,58	0,38	0,76	0,66	-0,12	0,00	0,87	0,84	0,80	0,83
C32:2 PC	0,44	0,37	0,48	0,59	-0,09	-0,01	0,66	0,52	0,70	0,62
C32:1 PC	0,56	0,39	0,61	0,58	-0,30	-0,09	0,75	0,71	0,78	0,72
C34:2 PC	0,62	0,38	0,56	0,55	-0,66	-0,26	0,59	0,59	0,66	0,52
C34:1 PC	0,54	0,39	0,64	0,56	-0,35	-0,12	0,75	0,72	0,77	0,73
C34:0 PC	0,42	0,17	0,46	0,38	-0,26	-0,08	0,61	0,65	0,67	0,59
C36:6 PC	0,45	0,35	0,56	0,55	0,03	0,15	0,72	0,64	0,75	0,66
C36:5 PC	0,47	0,42	0,56	0,56	-0,04	0,09	0,73	0,64	0,75	0,68
C36:2 PC	0,67	0,43	0,62	0,60	-0,58	-0,22	0,62	0,60	0,67	0,57
C38:6 PC	0,42	0,29	0,54	0,49	0,08	0,16	0,71	0,65	0,72	0,67
C40:10 PC	0,36	0,30	0,44	0,41	0,19	0,25	0,63	0,56	0,66	0,60
C42:11 PC	0,34	0,29	0,46	0,39	0,29	0,29	0,61	0,57	0,61	0,61
C30:1 PG	0,51	0,34	0,78	0,62	-0,20	-0,15	0,87	0,81	0,85	0,82
C30:0 PG	0,42	0,38	0,74	0,59	-0,09	0,04	0,83	0,75	0,86	0,85
C31:0 PG	-0,01	0,17	0,31	0,15	0,20	0,16	0,48	0,38	0,56	0,58

C32:2 PG	0,34	0,36	0,35	0,47	-0,05	-0,03	0,44	0,33	0,51	0,42
C32:1 PG	0,28	0,47	0,56	0,57	0,03	0,00	0,73	0,59	0,79	0,74
C34:4 PG	-0,11	0,00	-0,01	0,06	0,62	0,57	0,20	0,12	0,29	0,30
C34:3 PG	0,13	0,10	0,12	0,22	0,38	0,28	0,26	0,19	0,33	0,35
C34:2 PG	0,50	0,51	0,70	0,63	-0,19	0,00	0,76	0,69	0,84	0,73
C34:1 PG	0,26	0,35	0,53	0,47	0,10	0,06	0,72	0,61	0,78	0,76
C35:0 PG	0,48	0,51	0,58	0,60	-0,09	0,07	0,67	0,54	0,69	0,57
C36:2 PG	0,61	0,53	0,83	0,68	-0,40	-0,10	0,82	0,79	0,86	0,79
C30:1 PE	-0,07	-0,18	0,15	-0,22	-0,17	0,08	-0,04	0,10	0,12	-0,05
C30:0 PE	-0,04	-0,14	0,23	-0,16	-0,09	0,16	0,08	0,20	0,24	0,10
C31:1 PE	-0,06	-0,19	0,10	-0,23	-0,14	0,10	-0,08	0,07	0,10	-0,11
C32:1 PE	-0,20	-0,31	-0,13	-0,40	-0,22	0,01	-0,31	-0,17	-0,12	-0,35
C32:1 PE	-0,12	-0,18	0,07	-0,26	-0,08	0,15	-0,09	0,05	0,08	-0,10
C32:0 PE	-0,21	-0,26	0,06	-0,26	0,17	0,24	0,01	0,14	0,16	0,04
C33:2 PE	-0,14	-0,10	0,09	-0,15	-0,04	0,18	-0,05	0,01	0,13	-0,05
C33:1 PE	-0,15	-0,25	0,01	-0,27	0,03	0,23	-0,08	0,04	0,09	-0,07
C34:2 PE	-0,18	-0,26	-0,04	-0,32	0,01	0,13	-0,16	-0,05	0,03	-0,18
C34:1 PE	-0,09	-0,14	0,18	-0,20	-0,06	0,18	0,05	0,21	0,22	0,06
C36:2 PE	-0,05	-0,18	0,09	-0,24	-0,05	0,18	-0,09	0,05	0,07	-0,08
C38:6 PE	-0,05	-0,10	0,15	-0,09	0,17	0,28	0,20	0,29	0,34	0,23
C40:6 PE	-0,04	-0,12	0,07	-0,08	0,32	0,34	0,12	0,18	0,20	0,18
C28:0 DGTS	0,64	0,47	0,85	0,71	-0,10	0,03	0,87	0,81	0,83	0,84
C30:1 DGTS	0,55	0,25	0,79	0,58	-0,17	0,21	0,83	0,83	0,86	0,83
C30:0 DGTS	0,30	0,15	0,62	0,40	0,15	0,32	0,70	0,68	0,70	0,76
C31:1 DGTS	0,21	0,06	0,57	0,33	0,07	0,21	0,65	0,65	0,68	0,71
C32:2 DGTS	0,49	0,40	0,53	0,46	-0,14	0,13	0,54	0,48	0,60	0,57
C32:1 DGTS	0,25	0,42	0,41	0,42	0,21	0,21	0,59	0,42	0,60	0,63
C34:3 DGTS	0,42	0,44	0,41	0,46	0,11	0,16	0,50	0,40	0,50	0,56
C34:2 DGTS	0,33	0,25	0,29	0,33	0,19	0,23	0,38	0,27	0,43	0,38
C34:1 DGTS	0,07	0,18	0,20	0,24	0,43	0,15	0,41	0,24	0,43	0,50
C35:2 DGTS	0,33	0,20	0,17	0,22	0,13	0,13	0,28	0,13	0,34	0,25
C36:5 DGTS	0,66	0,59	0,67	0,72	-0,11	0,07	0,70	0,59	0,67	0,65
C36:3 DGTS	0,29	0,27	0,29	0,39	0,23	0,08	0,40	0,26	0,42	0,41
C36:2 DGTS	0,25	0,40	0,30	0,38	0,02	0,03	0,41	0,21	0,47	0,37

	C32:3 SQDG	C32:2 SQDG	C32:1 SQDG	C32:0 SQDG	C34:2 SQDG	C34:1 SQDG	C34:0 SQDG	C36:2 SQDG	C28:0 PC	C30:1 PC
Total PC										
Total PG										
Total PE										
Total DGTS										

Temperature										
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*										
Primary production										
Skeletonema costatum										
Thalassiosira spp.										
Chaetoceros socialis										
Pseudonitzschia delicatissima										
Hemiselmis spp.										
Plagioselmis spp.										
Phaeocystis globosa (colony)										
Phaeocystis globosa (single)										
Prymnesiales										
Other flagellates										
Total algae										
Total cyanobactria										
Total bacteria										
C28:0 SQDG										
C30:2 SQDG										
C30:1 SQDG										
C30:0 SQDG										
C32:3 SQDG										
C32:2 SQDG	0,80									
C32:1 SQDG	0,89	0,81								
C32:0 SQDG	0,54	0,63	0,66							
C34:2 SQDG	0,69	0,86	0,69	0,70						
C34:1 SQDG	0,55	0,79	0,55	0,63	0,89					
C34:0 SQDG	0,30	0,44	0,36	0,82	0,58	0,55				
C36:2 SQDG	0,82	0,73	0,74	0,54	0,74	0,66	0,38			
C28:0 PC	0,79	0,51	0,65	0,47	0,44	0,37	0,30	0,71		
C30:1 PC	0,78	0,59	0,73	0,53	0,48	0,45	0,29	0,74	0,88	
C30:0 PC	0,72	0,51	0,65	0,60	0,49	0,43	0,43	0,70	0,94	0,86
C32:2 PC	0,79	0,77	0,79	0,58	0,68	0,60	0,43	0,72	0,64	0,83
C32:1 PC	0,80	0,68	0,73	0,49	0,59	0,59	0,30	0,81	0,84	0,94
C34:2 PC	0,78	0,72	0,71	0,25	0,61	0,59	0,04	0,71	0,66	0,73

C34:1 PC	0,77	0,60	0,70	0,47	0,56	0,55	0,29	0,77	0,87	0,89
C34:0 PC	0,66	0,52	0,54	0,37	0,46	0,53	0,29	0,69	0,83	0,85
C36:6 PC	0,81	0,62	0,75	0,59	0,59	0,50	0,45	0,78	0,75	0,86
C36:5 PC	0,78	0,62	0,73	0,60	0,60	0,55	0,46	0,78	0,80	0,90
C36:2 PC	0,84	0,78	0,74	0,31	0,70	0,60	0,12	0,77	0,68	0,71
C38:6 PC	0,73	0,52	0,65	0,60	0,54	0,45	0,49	0,73	0,79	0,84
C40:10 PC	0,70	0,56	0,65	0,65	0,56	0,50	0,57	0,72	0,67	0,78
C42:11 PC	0,64	0,48	0,56	0,65	0,51	0,43	0,63	0,69	0,69	0,73
C30:1 PG	0,74	0,43	0,70	0,54	0,45	0,25	0,28	0,57	0,82	0,75
C30:0 PG	0,70	0,50	0,74	0,66	0,54	0,35	0,39	0,58	0,76	0,75
C31:0 PG	0,43	0,38	0,55	0,64	0,46	0,30	0,44	0,49	0,41	0,53
C32:2 PG	0,68	0,82	0,71	0,56	0,71	0,60	0,49	0,66	0,41	0,58
C32:1 PG	0,72	0,69	0,80	0,75	0,61	0,47	0,54	0,60	0,61	0,77
C34:4 PG	0,24	0,42	0,38	0,69	0,48	0,49	0,79	0,27	0,12	0,29
C34:3 PG	0,44	0,65	0,49	0,71	0,68	0,63	0,78	0,47	0,26	0,42
C34:2 PG	0,89	0,73	0,85	0,62	0,67	0,50	0,41	0,81	0,76	0,83
C34:1 PG	0,73	0,66	0,76	0,80	0,64	0,49	0,59	0,71	0,64	0,77
C35:0 PG	0,80	0,73	0,77	0,57	0,58	0,43	0,39	0,76	0,60	0,68
C36:2 PG	0,90	0,68	0,83	0,53	0,63	0,45	0,31	0,81	0,84	0,80
C30:1 PE	-0,04	-0,19	0,00	-0,01	-0,05	-0,19	-0,11	0,09	0,09	0,00
C30:0 PE	0,08	-0,05	0,11	0,17	0,12	-0,06	0,04	0,20	0,18	0,06
C31:1 PE	0,01	-0,13	0,01	-0,01	0,00	-0,16	-0,09	0,13	0,06	0,01
C32:1 PE	-0,19	-0,24	-0,13	-0,20	-0,13	-0,18	-0,28	-0,05	-0,18	-0,14
C32:1 PE	-0,04	-0,17	0,01	0,01	-0,06	-0,18	-0,08	0,10	0,02	0,00
C32:0 PE	-0,05	-0,15	0,01	0,17	-0,02	-0,14	0,11	0,11	0,15	0,08
C33:2 PE	0,08	0,00	0,14	0,06	0,08	-0,11	-0,05	0,14	0,01	-0,02
C33:1 PE	-0,03	-0,14	0,02	0,07	-0,02	-0,10	0,04	0,16	0,06	0,05
C34:2 PE	-0,11	-0,19	-0,03	0,02	-0,05	-0,17	-0,07	0,02	-0,07	-0,04
C34:1 PE	0,08	-0,04	0,11	0,13	0,06	-0,08	0,05	0,24	0,17	0,08
C36:2 PE	0,02	-0,11	0,02	0,04	0,06	-0,10	-0,01	0,19	0,06	0,02
C38:6 PE	0,21	0,04	0,21	0,33	0,13	0,04	0,31	0,34	0,35	0,35
C40:6 PE	0,15	0,03	0,14	0,35	0,19	0,07	0,37	0,27	0,25	0,23
C28:0 DGTS	0,73	0,41	0,69	0,58	0,47	0,28	0,35	0,59	0,81	0,74
C30:1 DGTS	0,74	0,47	0,72	0,59	0,49	0,35	0,35	0,66	0,80	0,76
C30:0 DGTS	0,48	0,24	0,53	0,65	0,34	0,16	0,48	0,47	0,64	0,58
C31:1 DGTS	0,45	0,20	0,49	0,59	0,26	0,08	0,38	0,37	0,58	0,52
C32:2 DGTS	0,65	0,64	0,69	0,68	0,70	0,65	0,41	0,66	0,50	0,67
C32:1 DGTS	0,59	0,64	0,70	0,79	0,65	0,51	0,60	0,63	0,45	0,65
C34:3 DGTS	0,64	0,74	0,66	0,81	0,84	0,75	0,71	0,64	0,41	0,51
C34:2 DGTS	0,47	0,58	0,55	0,74	0,66	0,60	0,56	0,56	0,30	0,53
C34:1 DGTS	0,37	0,49	0,48	0,80	0,50	0,42	0,73	0,44	0,29	0,48
C35:2 DGTS	0,38	0,48	0,44	0,53	0,57	0,52	0,38	0,54	0,24	0,48
C36:5 DGTS	0,86	0,77	0,79	0,68	0,78	0,63	0,53	0,78	0,65	0,70

C36:3 DGTS	0,53	0,64	0,56	0,66	0,62	0,48	0,59	0,53	0,33	0,53
C36:2 DGTS	0,51	0,54	0,59	0,50	0,51	0,37	0,33	0,52	0,33	0,60

	C30:0 PC	C32:2 PC	C32:1 PC	C34:2 PC	C34:1 PC	C34:0 PC	C36:6 PC	C36:5 PC	C36:2 PC	C38:6 PC
Total PC										
Total PG										
Total PE										
Total DGTS										
Temperature										
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*										
Primary production										
Skeletonema costatum										
Thalassiosira spp.										
Chaetoceros socialis										
Pseudonitzschia delicatissima										
Hemiselmis spp.										
Plagioselmis spp.										
Phaeocystis globosa (colony)										
Phaeocystis globosa (single)										
Prymnesiales										
Other flagellates										
Total algae										
Total cyanobactria										
Total bacteria										
C28:0 SQDG										
C30:2 SQDG										
C30:1 SQDG										
C30:0 SQDG										
C32:3 SQDG										
C32:2 SQDG										
C32:1 SQDG										

C32:0 SQDG										
C34:2 SQDG										
C34:1 SQDG										
C34:0 SQDG										
C36:2 SQDG										
C28:0 PC										
C30:1 PC										
C30:0 PC										
C32:2 PC	0,67									
C32:1 PC	0,82	0,83								
C34:2 PC	0,56	0,69	0,85							
C34:1 PC	0,82	0,72	0,95	0,85						
C34:0 PC	0,80	0,67	0,88	0,74	0,90					
C36:6 PC	0,77	0,88	0,80	0,57	0,76	0,75				
C36:5 PC	0,84	0,89	0,87	0,63	0,84	0,83	0,96			
C36:2 PC	0,57	0,70	0,83	0,95	0,81	0,71	0,62	0,64		
C38:6 PC	0,83	0,81	0,77	0,48	0,75	0,78	0,97	0,96	0,53	
C40:10 PC	0,75	0,81	0,72	0,42	0,67	0,70	0,96	0,94	0,47	0,96
C42:11 PC	0,78	0,72	0,65	0,30	0,61	0,66	0,91	0,89	0,37	0,94
C30:1 PG	0,79	0,61	0,67	0,54	0,71	0,60	0,70	0,69	0,57	0,73
C30:0 PG	0,79	0,66	0,68	0,48	0,69	0,59	0,72	0,73	0,55	0,77
C31:0 PG	0,49	0,53	0,45	0,20	0,45	0,38	0,62	0,60	0,30	0,66
C32:2 PG	0,44	0,85	0,58	0,54	0,50	0,49	0,70	0,70	0,61	0,59
C32:1 PG	0,68	0,86	0,69	0,48	0,64	0,55	0,79	0,81	0,51	0,76
C34:4 PG	0,23	0,53	0,22	-0,03	0,14	0,16	0,52	0,47	0,02	0,49
C34:3 PG	0,36	0,71	0,41	0,24	0,33	0,38	0,65	0,62	0,30	0,60
C34:2 PG	0,74	0,85	0,82	0,69	0,79	0,72	0,89	0,88	0,77	0,84
C34:1 PG	0,69	0,79	0,69	0,45	0,67	0,58	0,83	0,81	0,53	0,81
C35:0 PG	0,60	0,79	0,63	0,52	0,58	0,51	0,81	0,78	0,60	0,72
C36:2 PG	0,77	0,71	0,84	0,79	0,86	0,74	0,77	0,78	0,85	0,74
C30:1 PE	0,02	-0,15	-0,06	-0,07	0,01	0,00	0,03	-0,01	-0,02	0,10
C30:0 PE	0,12	-0,09	-0,01	-0,05	0,08	0,07	0,12	0,07	0,05	0,21
C31:1 PE	-0,01	-0,09	-0,06	-0,07	-0,01	0,03	0,09	0,03	0,01	0,15
C32:1 PE	-0,25	-0,18	-0,19	-0,12	-0,16	-0,12	-0,08	-0,13	-0,10	-0,06
C32:1 PE	-0,03	-0,12	-0,07	-0,12	-0,03	0,00	0,08	0,03	-0,05	0,14
C32:0 PE	0,13	-0,06	-0,02	-0,22	0,01	0,07	0,17	0,12	-0,13	0,27
C33:2 PE	-0,06	0,02	-0,07	-0,09	-0,04	-0,05	0,18	0,09	0,02	0,20
C33:1 PE	0,00	-0,05	-0,01	-0,12	0,04	0,07	0,15	0,10	-0,05	0,21
C34:2 PE	-0,10	-0,07	-0,14	-0,19	-0,12	-0,07	0,08	0,01	-0,13	0,13
C34:1 PE	0,11	-0,07	0,01	-0,07	0,06	0,10	0,15	0,10	0,03	0,21
C36:2 PE	0,02	-0,05	-0,03	-0,08	0,01	0,05	0,15	0,08	0,01	0,20
C38:6 PE	0,38	0,23	0,23	-0,02	0,25	0,38	0,51	0,46	0,05	0,61
C40:6 PE	0,33	0,25	0,13	-0,15	0,12	0,23	0,49	0,42	-0,05	0,60

C28:0 DGTS	0,82	0,59	0,66	0,46	0,68	0,60	0,73	0,73	0,54	0,78
C30:1 DGTS	0,77	0,57	0,69	0,54	0,70	0,61	0,71	0,67	0,60	0,73
C30:0 DGTS	0,68	0,39	0,46	0,19	0,47	0,39	0,59	0,54	0,27	0,67
C31:1 DGTS	0,58	0,30	0,38	0,18	0,43	0,35	0,51	0,44	0,24	0,57
C32:2 DGTS	0,53	0,72	0,69	0,59	0,63	0,47	0,69	0,69	0,61	0,62
C32:1 DGTS	0,58	0,77	0,59	0,34	0,52	0,40	0,76	0,75	0,43	0,72
C34:3 DGTS	0,51	0,74	0,56	0,44	0,53	0,40	0,69	0,70	0,50	0,65
C34:2 DGTS	0,42	0,74	0,48	0,27	0,37	0,31	0,67	0,66	0,32	0,62
C34:1 DGTS	0,44	0,64	0,40	0,10	0,34	0,32	0,62	0,63	0,17	0,61
C35:2 DGTS	0,36	0,69	0,47	0,28	0,35	0,37	0,63	0,65	0,34	0,62
C36:5 DGTS	0,69	0,83	0,73	0,62	0,69	0,60	0,84	0,84	0,69	0,77
C36:3 DGTS	0,43	0,79	0,47	0,29	0,36	0,32	0,70	0,67	0,37	0,63
C36:2 DGTS	0,41	0,81	0,53	0,37	0,43	0,36	0,71	0,72	0,43	0,64

	C40:10 PC	**C42:11 PC**	**C30:1 PG**	**C30:0 PG**	**C31:0 PG**	**C32:2 PG**	**C32:1 PG**	**C34:4 PG**	**C34:3 PG**	**C34:2 PG**
Total PC										
Total PG										
Total PE										
Total DGTS										
Temperature										
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*										
Primary production										
Skeletonema costatum										
Thalassiosira spp.										
Chaetoceros socialis										
Pseudonitzschia delicatissima										
Hemiselmis spp.										
Plagioselmis spp.										
Phaeocystis globosa (colony)										
Phaeocystis globosa (single)										
Prymnesiales										
Other flagellates										
Total algae										

Total cyanobactria										
Total bacteria										
C28:0 SQDG										
C30:2 SQDG										
C30:1 SQDG										
C30:0 SQDG										
C32:3 SQDG										
C32:2 SQDG										
C32:1 SQDG										
C32:0 SQDG										
C34:2 SQDG										
C34:1 SQDG										
C34:0 SQDG										
C36:2 SQDG										
C28:0 PC										
C30:1 PC										
C30:0 PC										
C32:2 PC										
C32:1 PC										
C34:2 PC										
C34:1 PC										
C34:0 PC										
C36:6 PC										
C36:5 PC										
C36:2 PC										
C38:6 PC										
C40:10 PC										
C42:11 PC	0,97									
C30:1 PG	0,60	0,60								
C30:0 PG	0,66	0,66	0,91							
C31:0 PG	0,63	0,63	0,64	0,80						
C32:2 PG	0,66	0,59	0,34	0,41	0,43					
C32:1 PG	0,76	0,72	0,70	0,79	0,72	0,78				
C34:4 PG	0,63	0,61	0,15	0,32	0,45	0,54	0,57			
C34:3 PG	0,71	0,68	0,24	0,35	0,47	0,81	0,69	0,87		
C34:2 PG	0,81	0,77	0,75	0,79	0,68	0,78	0,86	0,38	0,59	
C34:1 PG	0,79	0,78	0,74	0,81	0,83	0,72	0,93	0,54	0,67	0,89
C35:0 PG	0,74	0,71	0,56	0,57	0,48	0,84	0,80	0,43	0,61	0,85
C36:2 PG	0,66	0,63	0,80	0,79	0,54	0,58	0,72	0,14	0,35	0,92
C30:1 PE	0,09	0,10	0,16	0,21	0,33	-0,15	-0,02	-0,09	-0,12	0,16
C30:0 PE	0,17	0,20	0,28	0,39	0,52	-0,06	0,10	0,02	-0,01	0,27
C31:1 PE	0,15	0,15	0,13	0,20	0,37	-0,02	0,03	-0,02	-0,01	0,22
C32:1 PE	-0,02	-0,08	-0,15	-0,07	0,17	-0,09	-0,15	-0,10	-0,09	0,00
C32:1 PE	0,16	0,16	0,08	0,18	0,38	-0,06	0,01	0,01	-0,02	0,19

C32:0 PE	0,27	0,31	0,16	0,30	0,50	-0,06	0,11	0,16	0,08	0,22
C33:2 PE	0,21	0,19	0,11	0,26	0,46	0,10	0,13	0,10	0,10	0,29
C33:1 PE	0,23	0,23	0,05	0,17	0,41	0,01	0,05	0,14	0,09	0,21
C34:2 PE	0,16	0,14	0,05	0,15	0,39	-0,02	0,04	0,11	0,06	0,14
C34:1 PE	0,21	0,25	0,18	0,28	0,46	0,02	0,11	0,05	0,03	0,29
C36:2 PE	0,22	0,23	0,12	0,20	0,41	0,01	0,03	0,05	0,08	0,24
C38:6 PE	0,61	0,63	0,34	0,44	0,58	0,19	0,35	0,33	0,34	0,47
C40:6 PE	0,60	0,65	0,28	0,42	0,59	0,17	0,31	0,38	0,39	0,39
C28:0 DGTS	0,67	0,69	0,88	0,91	0,61	0,35	0,67	0,23	0,29	0,78
C30:1 DGTS	0,64	0,64	0,86	0,86	0,59	0,29	0,59	0,28	0,29	0,73
C30:0 DGTS	0,59	0,65	0,75	0,81	0,67	0,17	0,56	0,40	0,32	0,58
C31:1 DGTS	0,49	0,54	0,77	0,76	0,65	0,09	0,51	0,30	0,24	0,54
C32:2 DGTS	0,66	0,58	0,50	0,56	0,52	0,59	0,67	0,48	0,63	0,71
C32:1 DGTS	0,76	0,74	0,53	0,67	0,75	0,73	0,87	0,66	0,73	0,77
C34:3 DGTS	0,71	0,67	0,42	0,52	0,50	0,74	0,73	0,64	0,81	0,68
C34:2 DGTS	0,71	0,65	0,30	0,44	0,49	0,74	0,72	0,71	0,81	0,62
C34:1 DGTS	0,67	0,68	0,40	0,52	0,69	0,69	0,80	0,71	0,78	0,62
C35:2 DGTS	0,67	0,60	0,25	0,44	0,50	0,67	0,61	0,54	0,65	0,59
C36:5 DGTS	0,80	0,75	0,57	0,60	0,44	0,82	0,78	0,46	0,70	0,86
C36:3 DGTS	0,71	0,68	0,36	0,43	0,52	0,86	0,79	0,69	0,87	0,69
C36:2 DGTS	0,70	0,63	0,39	0,52	0,61	0,80	0,82	0,54	0,68	0,74

	C34:1 PG	**C35:0 PG**	**C36:2 PG**	**C30:1 PE**	**C30:0 PE**	**C31:1 PE**	**C32:2 PE**	**C32:1 PE**	**C32:0 PE**	**C33:2 PE**
Total PC										
Total PG										
Total PE										
Total DGTS										
Temperature										
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*										
Primary production										
Skeletonema costatum										
Thalassiosira spp.										
Chaetoceros socialis										

Pseudonitzschia delicatissima										
Hemiselmis spp.										
Plagioselmis spp.										
Phaeocystis globosa (colony)										
Phaeocystis globosa (single)										
Prymnesiales										
Other flagellates										
Total algae										
Total cyanobactria										
Total bacteria										
C28:0 SQDG										
C30:2 SQDG										
C30:1 SQDG										
C30:0 SQDG										
C32:3 SQDG										
C32:2 SQDG										
C32:1 SQDG										
C32:0 SQDG										
C34:2 SQDG										
C34:1 SQDG										
C34:0 SQDG										
C36:2 SQDG										
C28:0 PC										
C30:1 PC										
C30:0 PC										
C32:2 PC										
C32:1 PC										
C34:2 PC										
C34:1 PC										
C34:0 PC										
C36:6 PC										
C36:5 PC										
C36:2 PC										
C38:6 PC										
C40:10 PC										
C42:11 PC										
C30:1 PG										
C30:0 PG										
C31:0 PG										
C32:2 PG										

C32:1 PG										
C34:4 PG										
C34:3 PG										
C34:2 PG										
C34:1 PG										
C35:0 PG	0,80									
C36:2 PG	0,76	0,72								
C30:1 PE	0,07	-0,06	0,20							
C30:0 PE	0,24	0,05	0,30	0,93						
C31:1 PE	0,13	0,03	0,20	0,97	0,92					
C32:1 PE	-0,09	-0,16	-0,04	0,89	0,76	0,91				
C32:1 PE	0,12	0,00	0,15	0,96	0,90	0,97	0,91			
C32:0 PE	0,24	0,00	0,16	0,89	0,89	0,89	0,77	0,91		
C33:2 PE	0,22	0,14	0,22	0,84	0,88	0,89	0,81	0,87	0,84	
C33:1 PE	0,17	0,02	0,15	0,89	0,86	0,92	0,87	0,96	0,92	0,84
C34:2 PE	0,11	-0,03	0,05	0,92	0,85	0,95	0,93	0,96	0,90	0,87
C34:1 PE	0,25	0,12	0,29	0,93	0,94	0,94	0,80	0,95	0,92	0,86
C36:2 PE	0,16	0,03	0,20	0,94	0,90	0,97	0,87	0,95	0,90	0,89
C38:6 PE	0,47	0,29	0,36	0,69	0,68	0,73	0,57	0,75	0,82	0,65
C40:6 PE	0,43	0,23	0,25	0,59	0,61	0,63	0,48	0,63	0,76	0,66
C28:0 DGTS	0,73	0,58	0,79	0,15	0,29	0,14	-0,15	0,13	0,23	0,16
C30:1 DGTS	0,68	0,54	0,80	0,25	0,37	0,21	-0,07	0,19	0,27	0,18
C30:0 DGTS	0,67	0,42	0,61	0,37	0,49	0,32	0,02	0,33	0,47	0,28
C31:1 DGTS	0,63	0,35	0,59	0,39	0,51	0,34	0,06	0,33	0,46	0,28
C32:2 DGTS	0,69	0,57	0,64	0,10	0,17	0,12	0,07	0,12	0,09	0,15
C32:1 DGTS	0,89	0,76	0,59	0,00	0,11	0,05	-0,10	0,07	0,17	0,14
C34:3 DGTS	0,73	0,67	0,59	-0,10	0,03	-0,05	-0,16	-0,07	-0,02	0,09
C34:2 DGTS	0,70	0,67	0,39	0,01	0,08	0,09	0,05	0,10	0,15	0,18
C34:1 DGTS	0,84	0,67	0,38	-0,07	0,06	-0,01	-0,14	0,03	0,17	0,10
C35:2 DGTS	0,62	0,61	0,35	0,00	0,08	0,09	0,07	0,13	0,15	0,20
C36:5 DGTS	0,76	0,85	0,78	-0,12	-0,01	-0,05	-0,21	-0,08	-0,06	0,06
C36:3 DGTS	0,76	0,77	0,45	-0,07	-0,01	0,04	-0,07	0,01	0,07	0,13
C36:2 DGTS	0,75	0,76	0,50	0,04	0,09	0,14	0,08	0,14	0,13	0,25

	C33:1 PE	**C34:2 PE**	**C34:1 PE**	**C36:2 PE**	**C38:6 PE**	**C40:6 PE**	**C28:0 DGTS**	**C30:1 DGTS**	**C30:0 DGTS**	**C31:1 DGTS**
Total PC										
Total PG										
Total PE										
Total DGTS										
Temperature										

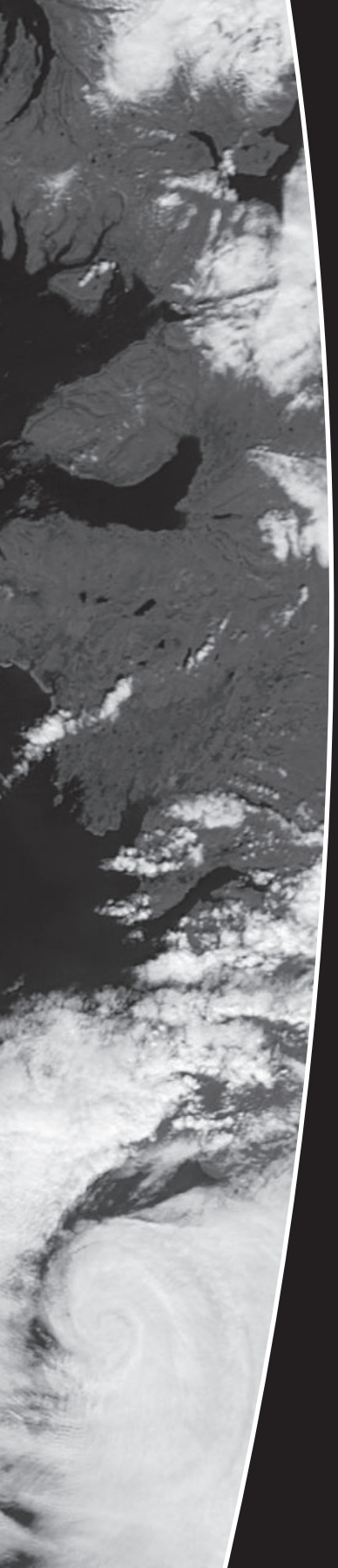
Salinity										
DIP										
DISi										
DIN										
N:P ratio										
Chlorophyll *a*										
Primary production										
Skeletonema costatum										
Thalassiosira spp.										
Chaetoceros socialis										
Pseudonitzschia delicatissima										
Hemiselmis spp.										
Plagioselmis spp.										
Phaeocystis globosa (colony)										
Phaeocystis globosa (single)										
Prymnesiales										
Other flagellates										
Total algae										
Total cyanobactria										
Total bacteria										
C28:0 SQDG										
C30:2 SQDG										
C30:1 SQDG										
C30:0 SQDG										
C32:3 SQDG										
C32:2 SQDG										
C32:1 SQDG										
C32:0 SQDG										
C34:2 SQDG										
C34:1 SQDG										
C34:0 SQDG										
C36:2 SQDG										
C28:0 PC										
C30:1 PC										
C30:0 PC										
C32:2 PC										
C32:1 PC										
C34:2 PC										
C34:1 PC										

C34:0 PC										
C36:6 PC										
C36:5 PC										
C36:2 PC										
C38:6 PC										
C40:10 PC										
C42:11 PC										
C30:1 PG										
C30:0 PG										
C31:0 PG										
C32:2 PG										
C32:1 PG										
C34:4 PG										
C34:3 PG										
C34:2 PG										
C34:1 PG										
C35:0 PG										
C36:2 PG										
C30:1 PE										
C30:0 PE										
C31:1 PE										
C32:1 PE										
C32:1 PE										
C32:0 PE										
C33:2 PE										
C33:1 PE										
C34:2 PE	0,92									
C34:1 PE	0,92	0,88								
C36:2 PE	0,91	0,94	0,92							
C38:6 PE	0,78	0,73	0,75	0,74						
C40:6 PE	0,65	0,66	0,63	0,70	0,90					
C28:0 DGTS	0,11	0,08	0,22	0,17	0,42	0,39				
C30:1 DGTS	0,17	0,13	0,30	0,23	0,44	0,36	0,88			
C30:0 DGTS	0,32	0,29	0,43	0,35	0,59	0,53	0,81	0,89		
C31:1 DGTS	0,29	0,30	0,42	0,35	0,57	0,47	0,74	0,86	0,95	
C32:2 DGTS	0,13	0,12	0,13	0,17	0,28	0,26	0,52	0,61	0,51	0,46
C32:1 DGTS	0,10	0,10	0,13	0,11	0,39	0,41	0,60	0,56	0,62	0,52
C34:3 DGTS	-0,01	-0,05	0,01	0,01	0,24	0,33	0,47	0,44	0,41	0,31
C34:2 DGTS	0,16	0,18	0,11	0,15	0,32	0,38	0,40	0,37	0,39	0,26
C34:1 DGTS	0,11	0,08	0,11	0,05	0,37	0,40	0,43	0,35	0,47	0,40
C35:2 DGTS	0,19	0,20	0,11	0,15	0,34	0,41	0,41	0,29	0,26	0,11
C36:5 DGTS	-0,04	-0,11	0,01	0,01	0,24	0,25	0,65	0,58	0,44	0,34
C36:3 DGTS	0,05	0,10	0,04	0,11	0,31	0,38	0,40	0,35	0,38	0,29
C36:2 DGTS	0,15	0,21	0,12	0,17	0,35	0,35	0,45	0,36	0,35	0,25

	C32:2 DGTS	C32:1 DGTS	C34:3 DGTS	C34:2 DGTS	C34:1 DGTS	C35:2 DGTS	C36:5 DGTS	C36:3 DGTS
Total PC								
Total PG								
Total PE								
Total DGTS								
Temperature								
Salinity								
DIP								
DISi								
DIN								
N:P ratio								
Chlorophyll *a*								
Primary production								
Skeletonema costatum								
Thalassiosira spp.								
Chaetoceros socialis								
Pseudonitzschia delicatissima								
Hemiselmis spp.								
Plagioselmis spp.								
Phaeocystis globosa (colony)								
Phaeocystis globosa (single)								
Prymnesiales								
Other flagellates								
Total algae								
Total cyanobactria								
Total bacteria								
C28:0 SQDG								
C30:2 SQDG								
C30:1 SQDG								
C30:0 SQDG								
C32:3 SQDG								
C32:2 SQDG								
C32:1 SQDG								
C32:0 SQDG								
C34:2 SQDG								
C34:1 SQDG								
C34:0 SQDG								
C36:2 SQDG								

C28:0 PC								
C30:1 PC								
C30:0 PC								
C32:2 PC								
C32:1 PC								
C34:2 PC								
C34:1 PC								
C34:0 PC								
C36:6 PC								
C36:5 PC								
C36:2 PC								
C38:6 PC								
C40:10 PC								
C42:11 PC								
C30:1 PG								
C30:0 PG								
C31:0 PG								
C32:2 PG								
C32:1 PG								
C34:4 PG								
C34:3 PG								
C34:2 PG								
C34:1 PG								
C35:0 PG								
C36:2 PG								
C30:1 PE								
C30:0 PE								
C31:1 PE								
C32:1 PE								
C32:1 PE								
C32:0 PE								
C33:2 PE								
C33:1 PE								
C34:2 PE								
C34:1 PE								
C36:2 PE								
C38:6 PE								
C40:6 PE								
C28:0 DGTS								
C30:1 DGTS								
C30:0 DGTS								
C31:1 DGTS								
C32:2 DGTS								

C32:1 DGTS	0,76							
C34:3 DGTS	0,81	0,79						
C34:2 DGTS	0,80	0,82	0,83					
C34:1 DGTS	0,52	0,86	0,71	0,79				
C35:2 DGTS	0,57	0,71	0,64	0,85	0,72			
C36:5 DGTS	0,74	0,76	0,86	0,74	0,63	0,63		
C36:3 DGTS	0,69	0,87	0,78	0,87	0,82	0,71	0,77	
C36:2 DGTS	0,68	0,86	0,66	0,82	0,74	0,81	0,70	0,87



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CURRICULUM VITAE

"A journey is best measured in friends, rather than miles"

This thesis has been a long time in the making, and over the years there have been a great many people who have, in some way or another, helped me finish it. I would like to take this opportunity to thank you all.

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CURRICULUM VITAE

Joost Brandsma was born on the 10th of September 1980 in Olst, the Netherlands. After finishing high school in 1998, he went on to study Earth Sciences at the Vrije Universiteit (Free University) in Amsterdam, specializing in Quaternary geology. For his thesis on the stratigraphic signature of prehistoric hurricanes he spent several months collecting and analyzing sediment cores in the coastal salt marshes of New England, under the guidance of the late Dr. Orson van der Plassche. In April 2004 he was awarded his Master of Science degree with distinction. As part of his undergraduate studies, Joost spent a year at the University of Iceland (Háskóli Íslands) between 2002 and 2003, studying Earth Sciences on a EU ERASMUS grant. Since then he has returned to Iceland almost yearly as a freelance tour leader, most recently to see the 2010 Eyjafjallajökull eruption.

From 2004 to 2010 Joost worked as a PhD student at the Royal Netherlands Institute for Sea Research (NIOZ) in the Department of Marine Organic Biogeochemistry, under the supervision of Prof.dr.ir. Jaap S. Sinninghe Damsté, Prof.dr.ir. Stefan Schouten and Dr.ir. Ellen C. Hopmans. His PhD research initially focused on the lipid geochemistry of benthic foraminifera and their hypothesized bacterial symbionts, but in 2006 this focus shifted to the origins and dynamics of intact polar lipids in the marine environment, in particular the North Sea. In September 2010 he joined a collaborative research project between the NIOZ Department of Biological Oceanography and the British Antarctic Survey (BAS). Under the supervision of Dr. Claire Evans he spent an austral summer at the BAS research station Rothera, helping to investigate the role of marine viruses in the microbial ecology of the Antarctic coastal zone.

Joost currently lives in England, where he works as a postdoctoral research fellow for the University of Southampton lipidomics research group.

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