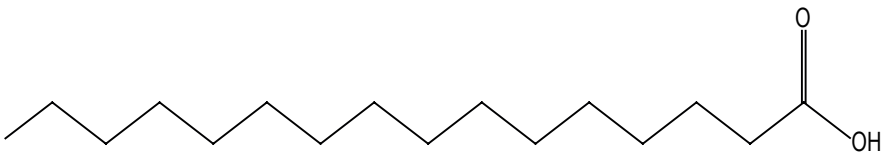
Metabolism of microbial communities in the environment

A compound-specific stable hydrogen isotope approach



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Metabolism of microbial communities in the environment:

A compound-specific stable hydrogen isotope approach

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Metabolism of microbial communities in the environment: A compound-specific stable hydrogen isotope approach

Component-specifieke stabiele waterstofisotopen als gereedschap voor de bepaling van het metabolisme van microbiële gemeenschappen in het milieu

(met een samenvatting in het Nederlands)

Metabolismus von mikrobiellen Gemeinschaften in der Umwelt: Ein komponentenspezifischer stabiler Wasserstoff Isotopen Ansatz

(mit einer Zusammenfassung in deutscher Sprache)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 21 december 2015 des middags te 4.15 uur

door

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„Keine Panik auf der Titanic“

Für meine Eltern

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Chapter 1

Introduction

1.1. Microbial metabolism and diversity

Microorganisms and their metabolic activity shape and affect the environment both on a global and local scale as they are key-players in all elemental cycles (Madigan et al., 2012). It has been estimated that microorganisms contribute up to 60 % to the biomass of the whole biosphere (Singh et al., 2009) with prokaryotic cells alone containing 350-550 Pg of cellular carbon, approximately 60-100% of the estimated total carbon in plants (Whitman et al., 1998). The marine water column and surface sediments contain a major part of all microorganisms (Whitman et al., 1998). One of the most important players in the carbon cycle are marine phytoplankton, contributing half the global primary production (Field et al., 1998) with one of the highest cellular production rates on earth (Whitman et al., 1998).

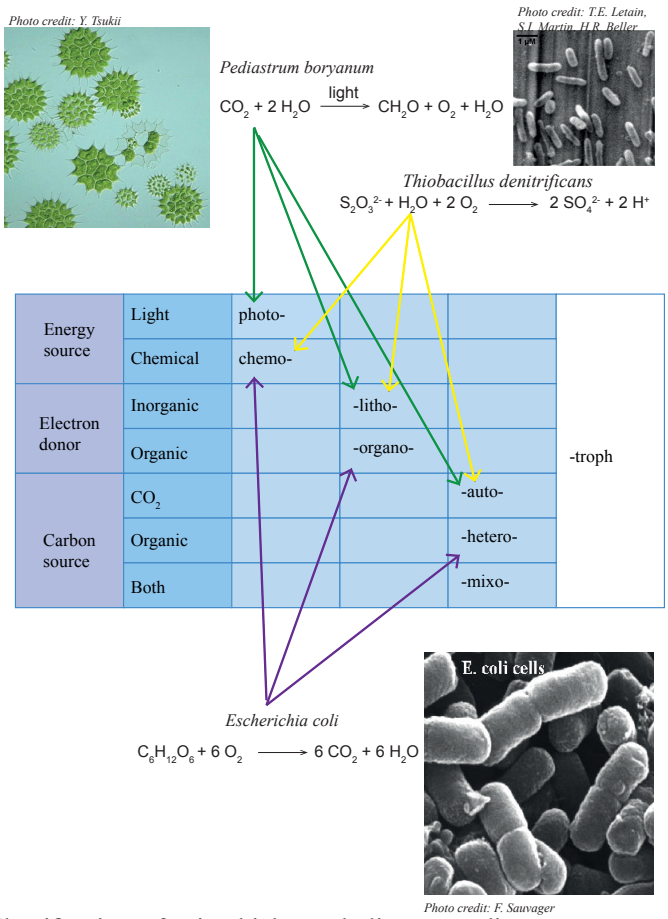

Figure 1: Classification of microbial metabolism regarding energy source, electron donor and carbon source.

Understanding the impact of microorganisms on their environment requires studying their metabolic capabilities and activities. The metabolism of microorganisms can be distinguished based on their energy source, electron donor and carbon source (Figure 1). Organisms can either be metabolically flexible and able to express different metabolic pathways depending on environmental conditions or be restricted to just one (Lengeler et al., 1999; Madigan et al., 2012). One of the first approaches to study microorganisms and their metabolism involved the isolation or enrichment of organisms from environmental samples using specific growth conditions, e.g. different energy and carbon sources and to study the physiological properties of the isolates in culture. While this has led to a large number of microorganisms available in pure culture (e.g. ~10600 species in the Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ] in 2015), it has nevertheless been estimated that only ~1% of all microorganisms in the environment can be cultivated with standard techniques (Amann et al., 1995). Additionally, those microorganisms that are brought into pure culture are not necessarily the most abundant microorganisms present in nature or do not play an important role in the environment (Overmann, 2006). Furthermore, microorganisms expressing novel metabolic pathways can be overlooked because of e.g. use of inappropriate selection conditions for cultivating efforts (Overmann, 2006; Madigan et al., 2012). Thus, studying activity, diversity and metabolism *in situ* is essential to be able to understand the complete picture of metabolic dynamics within microbial communities.

Over the past decades, a wide range of cultivation-independent approaches have been used to study and understand the metabolic activity of microbial communities in the natural environment. These techniques use a wide range of organic and inorganic molecules, including biomarker molecules like DNA, RNA and lipids. A selection of the most common techniques includes stable isotope probing (SIP), functional gene analysis, microelectrode profiling, incorporation of radiolabeled tracers into macromolecules (DNA or proteins), microautoradiography fluorescence in situ hybridization (MAR-FISH), and secondary ion mass spectrometry in situ hybridization (SIMSISH) (Table 1). However, several limitations for these methods have been observed including e.g. targeting only a small selection of microorganisms with specific metabolic capabilities, inducing artificial changes in microbial communities and cross labelling of secondary metabolites (e.g. Radajewski et al., 2000; Rastogi and Sani, 2011). In addition, methods targeting specific genes require knowledge of the DNA sequences for genes coding for enzymes involved in

Table 1: Selected examples for cultivation independent methods and their limitations to assess metabolic activity in microbial communities *in situ*.

Method	Target	Aim	Potential problems	Example literature
Stable isotope probing (SIP)	DNA, RNA, lipids	- identification of microorganism metabolising isotopic labelled substrate	- incubation time (incorporation in secondary metabolites, low signal) - concentration (artificial changes of microbial activity, low signal)	(Boschker et al., 1998; Radajewski et al., 2000; Radajewski et al., 2003; Dumont and Murrell, 2005)
Functional gene activity analysis	mRNA, 16S rRNA	- estimation of relative abundance of specific groups - estimation of metabolic activity	- pre-knowledge of gene sequences required	(Corredor et al., 2004; Henry et al., 2004; Jensen et al., 2008; Agrawal and Lal, 2009)
Microelectrode profiling	concentration respiration gases e.g. O_2, H_2S, H_2	- analysis of microbial processes like photosynthesis, respiration, sulfur reduction	- no identification of metabolic active organism possible - only on very small scale, does not assess the whole community	(Nielsen et al., 1990; Schramm et al., 1996; Pepper et al., 2014)
Incorporation of radiolabeled tracers into macromolecules	DNA, protein	- measuring metabolic activity of heterotrophic microorganisms by incorporation of radiolabeled thymidine or leucin	- limited to heterotrophic microorganisms - concentration, incubation time, nonspecific labelling of non-target molecules - not all targeted microorganisms take up exogenously supplied molecules	(Kirchman and Hoch, 1988; Tibbles and Harris, 1996; Pepper et al., 2014)
Microautoradiography fluorescence in situ hybridization (FISH- MAR)	DNA	- identification of active cells that metabolise the added radioactive substrate	- added substrate can be metabolised by non-target organisms - probes require pre-knowledge of gene sequence - natural radioactive isotopes with long half-life necessary	(Okabe et al., 2005; Overmann, 2006; Rogers et al., 2007; Rastogi and Sani, 2011)
Secondary ion mass spectrometry in situ hybridization (SIMSISH)	DNA	- direct observation and identification of active cells that metabolise added labelled substrate	- added substrate can be metabolised by non-target organisms - probes require pre-knowledge of gene sequence	(Behrens et al., 2008; Li et al., 2008; Rastogi and Sani, 2011)

different metabolic pathways, which makes it difficult to identify novel metabolic pathways. Furthermore, recent studies have shown that a higher transcriptional activity of a gene does not always correlate with a higher activity of the pathway in which the protein-coding gene is involved (Bowen et al., 2014). Therefore, it is desirable to develop additional techniques for studying microbial activity in the environment in order to complement existing ones.

1.2. Isotopic composition of microbial lipids

A possible alternative for DNA-based techniques is the use of the natural stable isotope abundance of different biomarker molecules, especially lipids. An advantage of lipids compared to DNA is their stability over geological timescales, making them ideal biomarkers also for paleostudies. Specific biomarker lipids have been used for taxonomic purposes, for example, alkenone lipids have been shown to be exclusively produced by some haptophyte algae (Volkman et al., 1998), while dinosterol is produced by approximately half of all dinoflagellate species (Mansour et al., 1999). Next to these specific biomarker lipids, fatty acid profiles of microorganisms grown in pure culture have been used to study microbial diversity (Shaw, 1974; Lechevalier, 1977; Bobbie and White, 1980). The advantage of fatty acids over specific biomarker lipids is that they are synthesized by all eukaryotes and bacteria and can thus be used to study the whole microbial community, apart from archaea, *in situ* (Shaw, 1974; Lechevalier, 1977; Bobbie and White, 1980). Furthermore, they occur as part of intact polar lipids (IPL) which are considered relatively labile, falling apart shortly after cell death (White et al., 1979; Harvey et al., 1986) , therefore, representing living cells. Intact phospholipids are of special interest since they are a major part of cell membranes. Therefore, phospholipid derived fatty acids (PLFA) should be derived from living and actively growing organisms, making them good target molecules for stable isotope research in relation to metabolism.

The natural abundance of ^{13}C in biomarker lipids and PLFA can provide information on carbon fixation or acquisition pathways. It has been used, for example to identify methanotrophs, which produce lipids that are strongly depleted in ^{13}C since their substrate, methane, is already depleted in ^{13}C (Summons et al., 1994). Cyanobacteria (Sakata et al., 1997) and other photoautrophs fixing CO_2 via RubisCO (Popp et al., 1998a; Popp et al., 1998b), produce lipids that are more depleted in ^{13}C compared to the inorganic carbon

source than organisms using the reversed tricarboxylic acid (TCA) cycle (van der Meer et al., 1998) or the 3-hydroxypropionate pathway (van der Meer et al., 2001). In sulphate reducing bacteria the $\delta^{13}C$ value of fatty acids allows for a discrimination between autotrophic, mixotrophic and heterotrophic growth (Londry et al., 2004).

1.3. Hydrogen isotopic composition of lipid biomarkers

In nature hydrogen occurs as two stable isotopes, protium or hydrogen (^{1}H or H) and deuterium (^{2}H or D), with a relative abundance of ~99.984 % and 0.016%, respectively (Coplen et al., 2002). However, small differences are observed and to express these differences the deuterium to hydrogen (D/H) ratio is applied using so-called δD values. δD values are expressed relative to Vienna standard mean ocean water (VSMOW):

$$\delta D_{sample} = \left(\frac{(D/H)_{sample} - (D/H)_{VSMOW}}{(D/H)_{VSMOW}} \right) * 1000 \qquad [1]$$

The δD of environmental water is controlled by both evaporation and precipitation (Craig and Gordon, 1965) and in marine environments also by the input from fresh water coming from rivers (Mook, 2001). The hydrogen isotopic composition of organic material reflects the hydrogen isotopic composition of the environmental water in which it was produced. Therefore, it provides information about environmental conditions such as evaporation, precipitation and salinity at the time of production. However, bulk organic material consists of a variety of organic molecules with a wide range of isotopic compositions. Their isotopic composition can depend on e.g. the biosynthetic pathway, the source organisms and the exchange of non-covalent bound hydrogen with the environment (Sessions et al., 1999; Hayes, 2001; Schimmelmann et al., 2006). Therefore, the development of environmental proxies based on stable hydrogen isotopes requires biomarker molecules containing non-exchangeable hydrogen atoms. In lipids the majority of the hydrogen atoms are covalently bound to carbon and therefore non-exchangeable. Consequently, the hydrogen isotopic composition in lipids can be preserved over a time scale of ~10^{6} y or more (Sessions et al., 2004). Lipids and their hydrogen isotopic composition can therefore be used as an indirect method for paleoenvironmental and paleohydrological reconstructions as so

called environmental proxies.

The majority of environmental proxies using lipid δD is based on photoautotrophic organisms (Sachse et al., 2012). In these organisms environmental water is the primary source for hydrogen during biosynthesis of organic matter and therefore the δD of lipids depends on the δD of the source water and the isotopic fractionation associated with biosynthetic reactions. This dependency between lipid and water δD allows for paleoenvironmental and paleohydrological reconstruction. For example, the δD of leaf-wax lipids, e.g. long-chain *n*-alkanes, of terrestrial plants correlates with the δD of meteoric water, indirectly providing information on evaporation and precipitation, and allowing to study climate variability both on recent and geological timescales (Sauer et al., 2001; Sachse et al., 2004; Schefuß et al., 2005). δD_{water} in aquatic environments has been reconstructed by analysing the hydrogen isotopic composition of algal lipids like sterols and short chain *n*-alkanes (Sauer et al., 2001; Sachse et al., 2004). Specific parameters, such as salinity and growth phase, have been shown to influence the hydrogen isotopic fractionation in aquatic organisms. The hydrogen isotopic composition of alkenones produced by haptophyte algae depends strongly on salinity (Schouten et al., 2006) and, therefore, allows for the reconstruction of past salinities in different marine environments (van der Meer et al., 2007; van der Meer et al., 2008). A similar relationship has been shown for cyanobacterial lipids (Sachse and Sachs, 2008) and dinosterol produced by dinoflagellates (Sachs and Schwab, 2011). Increasing salinity leads to a decreasing hydrogen isotope fractionation during lipid biosynthesis with δD values increasing by up to 3 ‰ per salinity unit (Schouten et al., 2006). Furthermore, changes in growth rate, which can be caused by changes in nutrient levels, also affect the δD of alkenones, leading to an increased fractionation with increasing growth rate during alkenone biosynthesis (Schouten et al., 2006). The hydrogen isotopic composition of alkenones extracted from cultures harvested in different growth phases can differ up to 30 ‰ in δD with an increase in fractionation at later growth phases (Wolhowe et al., 2009). The effect of growth rate and growth phase on the D/H ratio of lipids show that, in addition to environmental parameters, biological factors also influence the hydrogen isotopic fractionation. Therefore, it is likely that biological factors like metabolism and lipid biosynthetic pathways influence the D/H ratio of lipids.

1.4. D/H ratio as an indicator for microbial metabolism

The first indication that the δD of lipids could be used as an indicator for microbial metabolism was provided by the study of Zhang et al. (2009a). These authors showed in cultivation experiments that the D/H ratio of fatty acids produced by four different *Proteobacteria* (*Cupriavidus oxalaticus*, *Cupriavidus nectaor*, *Escherichia coli* and *Rhodopseudomonas palsustris*) depends on the metabolism expressed by those microorganisms during their growth (Figure 2). Furthermore, *C. oxalaticus* produced fatty acids significantly depleted in D when grown under chemoautotrophic conditions, while when grown as a heterotroph it produced fatty acids strongly enriched in D. When different microorganisms were grown under similar metabolic conditions, they produce fatty acids with similar D/H ratios. For example, photoautotrophically grown algae and bacteria, grown both under oxic or anoxic conditions, produce fatty acids which are similarly depleted in D relative to the growth medium (Figure 2) with the fractionation factor ε between lipid and water ($\varepsilon_{lipid/water}$):

$$\varepsilon = \left(\frac{1000+\delta D_{lipid}}{1000+\delta D_{water}} - 1\right) * 1000 \qquad [2]$$

ranging between -150 and -250 ‰ (Sessions et al., 1999; Chikaraishi et al., 2004; Zhang and Sachs, 2007; Zhang et al., 2009a; Zhang et al., 2009b). Chemoautotrophically grown microorganisms produce fatty acids, which were significantly depleted in D between -250 and -400 ‰ relative to the growth medium (Valentine et al., 2004; Campbell et al., 2009; Zhang et al., 2009a). In contrast, heterotrophic microorganisms grown on different organic substrates produce fatty acids which range between depleted and enriched in D relative to the growth medium with $\varepsilon_{lipid/water}$ ranging between -150 and +200 ‰ (Sessions et al., 2002; Zhang et al., 2009a) (Figure 2).

The differences in the D/H ratio of fatty acids of microorganisms with different core metabolisms are attributed to the hydrogen isotopic composition of nicotinamide adenine dinucleotide phosphate (NADP). NADP occurs in cells in two forms, a reduced form, NADPH, and an oxidized form, $NADP^+$, and serves in its reduced form as the source of hydrogen during biosynthesis of organic molecules including fatty acids (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003). Therefore, the hydrogen isotopic composition of the fatty acids will most likely reflect the hydrogen isotopic composition of the utilized NADPH. NADPH can, depending on the metabolism expressed

by the cell, be generated via different pathways (Figure 3), each associated with different hydrogen isotopic fractionation effect. In oxygenic photoautotrophic microorganisms the majority of the NADPH is produced in the light reaction during photosynthesis at the end of the electron transport chain by ferredoxin-$NADP^+$ reductase (Shin, 2005), while both anoxygenic photoautotrophs and chemoautotrophs form NADPH indirectly by generation of NADH by reversed electron transport from reduced quinones (Lengeler et al., 1999). In heterotrophs the main sources for NAPDH are the pentose phosphate (OPP) pathway, the tricaboxylic acid (TCA) cycle and the NADH-NADPH converting transhydrogenase reaction (Lengeler et al., 1999; White et al., 2007).

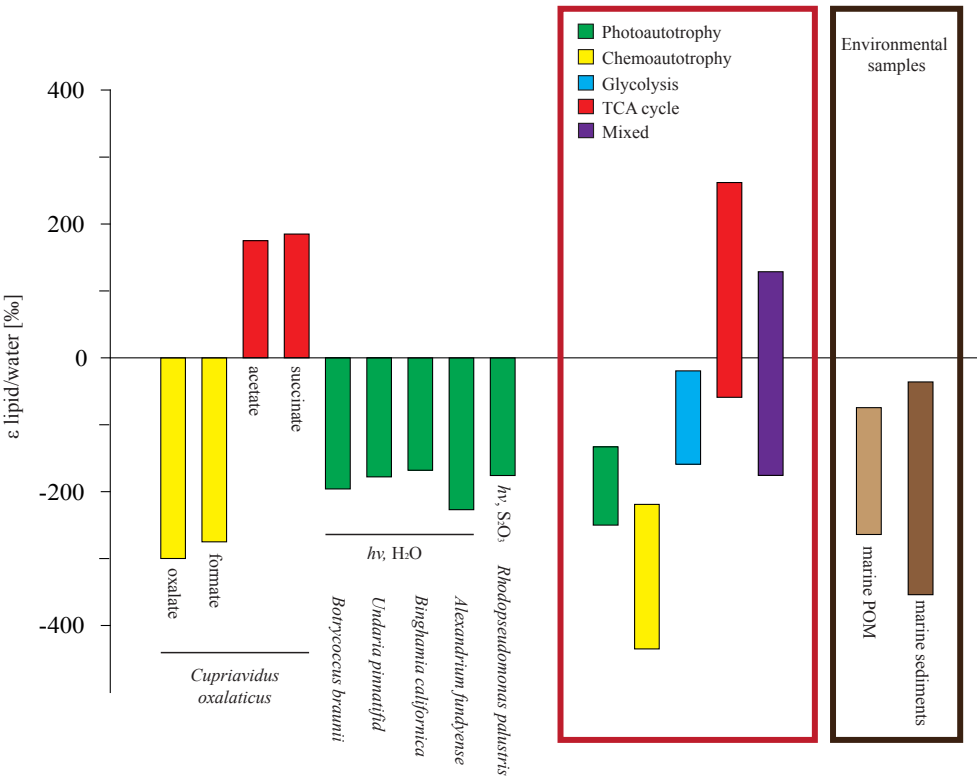

Figure 2: D/H ratio of fatty acids obtained from various cultures and environmental samples. Figure modified after a figure from A. Sessions presented at the International Meeting on Organic Geochemistry IMOG 2009 and based on the results of Zhang et al., 2009a.

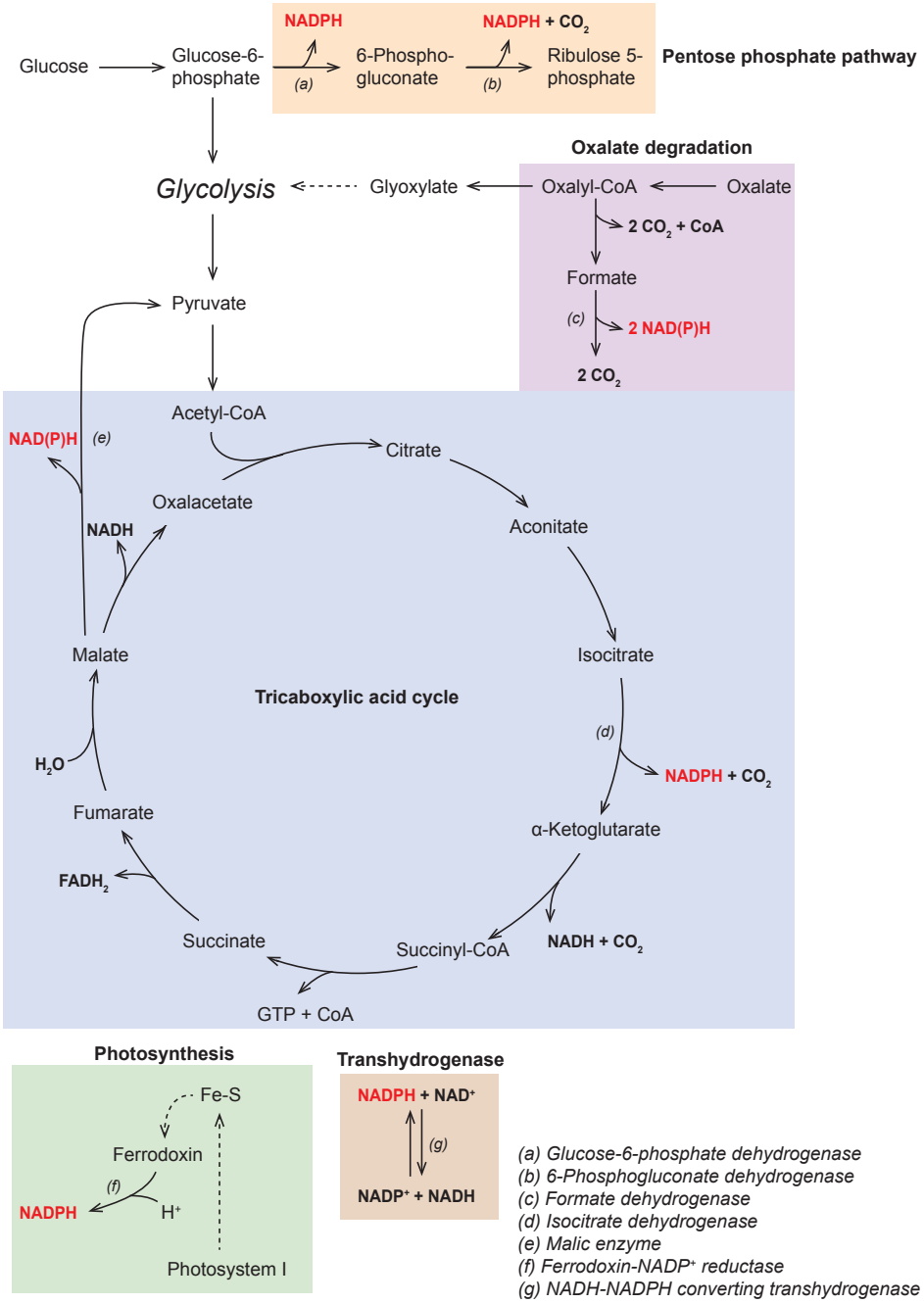

Figure 3: Scheme of the major biochemical pathways involved in the synthesis of NADPH. Figure modified after Zhang et al. (2009a).

Only few cultures have been studied up to now to show the significant influence of the general metabolism on the δD of fatty acids, which allows to distinct between chemoautotrophic, photoautotrophic and heterotrophic growth. In order to evaluate the applicability of the δD of fatty acids as an indicator for microbial metabolism, more culture studies are required. Additionally, the effect of other parameters than metabolism on the δD of fatty acids have to be studied in order to avoid any biases generated by factors other than metabolism. Finally, it is necessary to investigate the applicability of the hydrogen isotopic composition of fatty acids as a tool for studying core metabolism of microbial communities in environmental settings. While a wide range in hydrogen isotopic composition of fatty acids extracted from environmental samples has been observed (Figure 2) (Jones et al., 2008; Li et al., 2009), it has not been shown to what extent this is connected to the general metabolism of the microbial communities in those settings. A correlation between metabolism and δD of fatty acids derived from environmental microbial communities would allow for the applicability of this approach as a new cultivation independent method to assess *in situ* microbial metabolism in addition to already established methods, thereby reducing the potential biases of the different approaches when used individually. Finally, for geochemists this lipid based hydrogen isotope approach could be of interest as a paleo-proxy for studying metabolism of past microbial communities over geological timescale.

1.5. Scope of this thesis

The research presented in this thesis is focused on the influence of metabolism on the hydrogen isotopic composition of fatty acids produced by a range of bacterial and eukaryotic microorganisms in culture and the environment.

The work presented in this thesis is divided in two parts. The first part focuses on the dependency of the δD of fatty acids on the metabolism of microbes also in comparison with other biological and environmental factors like growth phase and salinity. The second part focuses on the application of the δD of fatty acids as a cultivation independent indicator for the general metabolism of microbial communities in two different marine settings. Additionally, this part contains the methodological framework for the environmental studies needed to assess only living and not dead or fossil microorganisms or biomass.

Part I: Testing the D/H ratio of fatty acids as a new method to assess microbial metabolism

Chapter 2 shows a comparison of the effect of growth phase relative to that of metabolism on the D/H ratio of fatty acids of five different microorganisms grown in pure culture. Three photoautotrophs, one chemoautotroph and one heterotroph were grown and harvested during exponential, stationary and death phase. While the different metabolism types lead to significant differences in hydrogen isotopic fractionation, the effect of growth phase is relatively small. This shows that growth phase is a minor factor compared to metabolism in determining the δD of fatty acids produced by microorganisms.

In **Chapter 3** the effect of salinity on the δD of the fatty acids produced by the photoautotrophic haptophyte algae *Isochrysis galbana* and the heterotrophic bacteria *Pseudomonas* strain LFY10 is studied. Salinity is known to affect the δD of alkenones produced by specific haptophytes, leading to a decrease in isotopic fractionation with increasing salinity. Fatty acids produced by *I. galbana* also show a decreased fractionation with increased salinity as observed previously for alkenones. The δD of fatty acids produced by *Pseudomonas* str. LFY10, on the other hand, does not seem to be affected by salinity. This suggests that salinity does not affect the hydrogen isotopic composition of fatty acids in all microorganisms in a similar fashion, but rather depends on the metabolism expressed. Additionally, the salinity effect is relatively small compared to the effect of metabolism.

Part II: Application of D/H ratio of fatty acids as an indicator of metabolism in natural settings

In **Chapter 4** the efficiency of a widely applied method for separating different intact polar lipid classes in order to distinguish between what used to be considered solely storage (glyco) and membrane (phospho) lipids from living biomass has been re-evaluated. The traditional method used dichloromethane (DCM), acetone and methanol (MeOH) to separate a Bligh-Dyer extract (BDE) on a silica column in a neutral, glyco- and phospholipid fraction, respectively. However, analysis by high performance liquid chromatography-mass spectrometry of these fractions showed an incomplete separation of glyco- and phospholipids between the acetone and MeOH fractions. Based on these results it is recommended to separate only the neutral lipids from the polar lipids by eluting with DCM and MeOH, respectively. Since the traditional third fraction (MeOH) contains not all and not only phospholipids but

also glycolipids it might be better to analyse the fatty acids derived from all IPLs avoiding live versus death and other possible biases.

In **Chapter 5** the hydrogen isotopic composition of fatty acids from pelagic microbial communities in the coastal North Sea was studied over the course of more than a year. The δD of fatty acids showed seasonal changes with an increased isotopic fractionation relative to North Sea water during the spring season. The changes in δD correlated with an increase in the chlorophyll *a* concentration, most probably reflecting increased photoautotrophy due to a phytoplankton bloom in spring. After the phytoplankton bloom, a decrease in isotopic fractionation correlates with an increased input of the heterotrophic bacterioplankton to the pelagic community. The results suggest that the δD of fatty acids can be used to study changes in the metabolism of the overall community in seasonally changing pelagic environments.

Chapter 6 shows the δD of fatty acids derived from sediments (0-8 cm) collected during two different seasons in the marine Lake Grevelingen (The Netherlands). The hydrogen isotopic compositions of the general fatty acids do not change significantly with depth which could potentially suggest only minor changes in the general metabolism of the sedimentary microbial communities, which are dominated by chemoautotrophic and heterotrophic microorganisms. The high relative abundance of the algal biomarker, *n*C20:5 polyunsaturated fatty acid, however, indicates that the δD of fatty acids in this case, might reflect an allochthonous contribution from the water column rather than the sedimentary microbial community. This shows that, when using the δD of fatty acids as an indicator for microbial metabolism, the origin of the fatty acids has to be considered especially when coming from a different environment (in this case the water column) dominated by microbes using a different metabolism.

In summary, the research presented in this thesis shows that metabolism is a major factor influencing the hydrogen isotopic composition of fatty acids, while other factors like salinity and growth phase only have a relatively minor influence. The environmental studies conducted here demonstrate that the hydrogen isotopic composition of fatty acids is a promising, cultivation independent, tool to study microbial metabolism *in situ*, but they also point out limitations of the method which should be a topic for future research.

Part I

Testing the D/H ratio of fatty acids as a new method to assess microbial metabolism

Chapter 2

Impact of metabolism and growth phase on the hydrogen isotopic composition of microbial fatty acids

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Abstract

Microorganisms are involved in all elemental cycles and therefore it is important to study their metabolism in the natural environment. A recent technique to investigate this is the hydrogen isotopic composition of microbial fatty acids, i.e. heterotrophic microorganisms produce fatty acids enriched in deuterium (D) while photoautotrophic and chemoautotrophic microorganisms produce fatty acids depleted in D compared to the water in the culture medium (growth water). However, the impact of factors other than metabolism have not been investigated. Here, we evaluate the impact of growth phase compared to metabolism on the hydrogen isotopic composition of fatty acids of different environmentally relevant microorganisms with heterotrophic, photoautotrophic and chemoautotrophic metabolisms. Fatty acids produced by heterotrophs are enriched in D compared to growth water with $\varepsilon_{lipid/water}$ between 82 ‰ and 359 ‰ when grown on glucose or acetate, respectively. Photoautotrophs ($\varepsilon_{lipid/water}$ between -149 ‰ and -264 ‰) and chemoautotrophs ($\varepsilon_{lipid/water}$ between -217 ‰ and -275 ‰) produce fatty acids depleted in D. Fatty acids become, in general, enriched by between 4 and 46 ‰ with growth phase which is minor compared to the influence of metabolisms. Therefore, the D/H ratio of fatty acids is a promising tool to investigate community metabolisms in nature.

2.1. Introduction

Microorganisms are key players in all elemental cycles and therefore have a huge impact on their immediate and the global environment (Conrad, 1996; Morel and Price, 2003; Arrigo, 2005; Falkowski and Godfrey, 2008; Muyzer and Stams, 2008; Hügler and Sievert, 2011; Orcutt et al., 2011). In order to comprehend their environmental impact, it is important to characterise and understand their metabolic activities. Several approaches help to understand microbial metabolisms present in different environments. One approach is the isolation or enrichment of microorganisms from a specific environment to test its growth on possible substrates and investigate its metabolic pathways. Unfortunately, the isolation of specific microorganisms can give a biased view of the composition of microbial communities as it has been estimated that only ~1% of all microorganisms can be enriched, isolated and cultivated by standard techniques (Amann et al., 1995). Often microorganisms with new metabolic capacities or that are present in the highest abundance have not been isolated (Overmann, 2006). Therefore, studying microbial activity *in situ* becomes necessary in order to understand metabolic dynamics within microbial communities.

For this purpose e.g. stable isotope probing (SIP) can be used to identify specific microorganisms which utilize particular substrates (Nold and Ward, 1996; Radajewski et al., 2000). The specific substrates have to be highly enriched in a stable isotope (e.g. D, ^{13}C, ^{15}N, ^{18}O) for the label to be incorporated by active microorganisms into biomarkers like DNA, RNA and lipids. The labelled biomarkers can be then purified and identified (Boschker et al., 1998; Manefield et al., 2002; Radajewski et al., 2003; Dumont and Murrell, 2005; van der Meer et al., 2005; Neufeld et al., 2007; van der Meer et al., 2007). The most common approach to characterise the metabolic activity of microbial communities is estimate activity rate measurements of a specific activity (Chapelle and Lovley, 1990; Phelps et al., 1994). An alternative to this is the characterisation of functional genes which are involved in different metabolic pathways using messenger RNA (mRNA) and 16S ribosomal RNA (Holmes et al., 2005). This approach allows not only for the identification of members of the community by gene sequence but also their relative abundance by determination of the copy number of that sequence and their metabolic activity by mRNA copy numbers (Corredor et al., 2004; Henry et al., 2004; Holmes et al., 2005; Sharma et al., 2007; Jensen et al., 2008; Agrawal and Lal, 2009; Blazejak and Schippers, 2011; Kong et al., 2012; Akerman et al., 2013).

However, all the approaches listed above have their limitations like isotopic cross-labelling, artificial change in both microbial diversity and activity as a result of experiment set-up of incubations, or requires pre-knowledge of gene sequences (Radajewski et al., 2000; Dumont and Murrell, 2005; van der Meer et al., 2005; Cebron et al., 2007; Bowen et al., 2014). An alternative is to use the natural isotopic composition of lipids. For example, carbon isotope discrimination ($\delta^{13}C$) can be used for identification of methanotrophs due to the fact that they produce lipids depleted in ^{13}C compared to other microorganisms (Summons et al., 1994).

Recently it has been shown that the ratio of deuterium to hydrogen (D/H or δD) of fatty acids reflects the central metabolism of microorganisms (Zhang et al., 2009a). Microbes grown under phototrophic conditions produce fatty acids depleted in D (ranging from -150 ‰ to -250 ‰) relative to the growth medium under both oxic and anoxic conditions (Sessions et al., 1999; Chikaraishi et al., 2004; Zhang and Sachs, 2007; Zhang et al., 2009a). Fatty acids of chemoautotrophs are even more depleted in D (ranging from -250 ‰ to -400 ‰) relative to the growth medium, independent of the electron donor (Valentine et al., 2004; Campbell et al., 2009; Zhang et al., 2009a). In contrast, organisms grown under heterotrophic conditions, e.g. grown with acetate or glucose as substrate, are relatively enriched in D and range from -150 ‰ to > +200 ‰ regardless of factors such as temperature (Sessions et al., 2002; Zhang et al., 2009a; Dirghangi and Pagani, 2013b; Fang et al., 2014). Zhang et al. (2009a) attributed these differences to the D/H ratio of nicotinamide adenine dinucleotide phosphate (NADPH), which is generated by a variety of different reactions in different metabolic pathways (each associated with different hydrogen isotopic fractionations) and subsequently used as the main H source in lipid biosynthesis (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003). The analysis of the D-composition of microbial fatty acids may thus yield insights into the metabolism of individual microbes or microbial communities. Furthermore, the persistence of lipids over geological time periods should allow for the study of microbial metabolisms in the past from sedimentary records. However, not many microbes have yet been analysed for the hydrogen isotopic composition of fatty acids. Furthermore, other factors than metabolism have been shown to influence the D/H ratio of lipids such as temperature (Zhang et al., 2009b; Dirghangi and Pagani, 2013b), lipid biosynthetic pathways (Fang et al., 2014), growth rate, growth phase and salinity (Schouten et al., 2006; Wolhowe et al., 2009; Chivall et al., 2014; M'Boule et al., 2014).

In order to improve the reliability of δD of fatty acids as an indicator for the metabolism of microorganisms we evaluated both the effect of metabolism (auto- vs heterotrophic) and of growth phase (exponential, stationary and death phase) on the δD values of fatty acids of different microorganisms which are mainly derived from aquatic environments with salinities ranging from almost freshwater to open marine. *Thiocapsa roseopersicina* and *Halochromatium glycolicum* are both anaerobic, phototrophic purple sulphur bacteria using hydrogen sulphide as electron donor and are found in microbial mats and saline lakes, respectively. *Isochrysis galbana* is an aerobic, phototrophic haptophyte algae using water as electron donor and common in coastal marine environments. *Thiobacillus denitrificans* is an anaerobic, chemolithoautotrophic β-*proteobacterium* using thiosulphate as electron donor and is common in aquatic environments from freshwater to marine. Finally, a recently isolated *Pseudomonas* str. LFY10 from Lake Fryxell, Dry Valleys, Antarctica, is investigated which is an aerobic, heterotrophic γ-*proteobacterium* using either glucose or acetate as carbon source.

2.2. Material and Methods

2.2.1. Cultures

The photoautotrophic purple sulphur bacteria *Thiocapsa roseopersicina* (DSM-217) and *Halochromatium glycolicum* (DSM-11080) were grown on a modified Pfenning's medium containing 0.34 g NH_4Cl, 0.34 g KH_2PO_4, 0.5 g $MgSO_4 \times 7\ H_2O$, 0.34 g KCl, 0.25 g $CaCl_2 \times 2\ H_2O$, 1.5 g $NaHCO_3$, 0.4 g $Na_2S \times 9\ H_2O$, 0.02 g vitamin B_{12}, and 1 mL trace element solution SL-12 (Pfennig, 1965) per liter of distilled water. The pH was adjusted with 1 M HCl to pH 7−7.5. The medium for *H. glycolicum* was additionally supplemented with 6% NaCl, 0.3% $MgCl_2 \times 6\ H_2O$ and 0.05% $Na_2S_2O_3$ (final concentration). The cultures were incubated in air tight bottles at 25 °C and a light intensity of ~ 1300 lux of a halogen lamp (16 h light, 8 h dark).

The chemolithoautotrophic sulphide oxidizer *Thiobacillus denitrificans* (DSM-12475) was grown on a medium containing 2 g KH_2PO_4, 2 g KNO_3, 1 g NH_4Cl, 0.8 g $MgSO_4 \times 7\ H_2O$, 2 mL trace element solution SL-4, 5 g $Na_2S_2O_3 \times 7\ H_2O$, 1 g $NaHCO_3$, 2 mg $FeSO_4 \times 7\ H_2O$, 1 mL 0.1 N H_2SO_4 per liter of distilled water (pH 7.0). The trace element solution SL-4 contained 0.5 g EDTA, 0.2 g $FeSO_4 \times 7\ H_2O$, 0.01 g $ZnSO_4 \times 7\ H_2O$, 3 mg $MnCl_2 \times 4$

H_2O, 0.03 g H_3BO_3, 0.02 g $CoCl_2 \times 6\ H_2O$, 1 mg $CuCl_2 \times 2\ H_2O$, 2 mg $NiCl_2 \times 6\ H_2O$, 3 mg $Na_2MoO_4 \times 2\ H_2O$ per liter of distilled water. *T. denitrificans* cultures were incubated at 25 °C.

The photoautotrophic eukaryote *Isochrysis galbana* (CCMP 1323) was grown at a salinity of 35.5 practical salinity units (psu) as previously described (M'Boule et al., 2014) in f/2 medium which contained 0.075 g of $NaNO_3$, 0.013 g of $Na_2HPO_4 \times 12\ H_2O$, 1 mL of a trace element solution and 1 mL of a vitamin solution per 1 L of sea water (Guillard, 1975). The trace element solution contained per liter of distilled water: 4.36 g EDTA, 3.15 g $FeCl_3 \times 6\ H_2O$, 0.01 g $CuSO_4 \times 5\ H_2O$, 0.02 g $ZnSO_4 \times 7\ H_2O$, 0.01 g $CoCl_2 \times 6\ H_2O$, 0.18 g $MnCl_2 \times 4\ H_2O$ and 4.8 mg $Na_2MoO_4 \times 2\ H_2O$. The vitamin solution contained per liter of distilled water: 0.5 mg biotin, 0.1 g vitamin B_1 and 0.5 mg vitamin B_{12}. The cultures were incubated at 15 °C and a light intensity of ~3000 lux of a cool white fluorescent light (16 h light, 8 h dark).

A recently isolated heterotrophic *Pseudomonas* str. LFY10 obtained from Prof. Matt Sattley (Indiana Wesleyan University, Marion, IN) was grown on an ammonium-glucose medium and an ammonium-acetate medium. The ammonium-glucose medium contained: 5 g glucose, 0.2 g $MgSO_4 \times 7\ H_2O$, 5 g NaCl, 1.3 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 2 mL trace element solution SL-4 per liter of distilled water (pH 7.1). The ammonium-acetate medium contained 5 g Na-acetate, 0.2 g $MgSO_4 \times 7\ H_2O$, 5 g NaCl, 1.3 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 2 mL trace element solution SL-4 per liter of distilled water. The pH was adjusted to 7.1. The cultures were incubated at 25 °C.

After inoculation cell densities, and thereby growth phase, were monitored regularly by flow cytometry (BD Accuri™ C6, San Jose USA) or by measuring the optical density (OD) at 600 nm with a spectrometer (Molecular Devices SpectraMax M2, Sunnyvale USA). Culture samples, including water samples for hydrogen isotope analysis, were taken during exponential, stationary and death phase. The water was stored with no headspace in 12 mL exertainers (Labco) in the dark at ~5 ºC until analysis. Biomass was collected by filtration over a 0.7 µm GF/F filter (Whatman, GE Healthcare Life Sciences, Little Chalfont, UK) or by centrifugation.

2.2.2. Lipid extraction

Bacterial biomass and filters were freeze dried and hydrolysed directly by base hydrolysis with 4 volumes of 1 N KOH in methanol (MeOH) solution

under reflux for 1 h at 190 °C. Afterwards the pH was adjusted to 4 with 2 N HCl/MeOH (1/1) and the liquid was transferred into a separatory funnel. The residues were further extracted once with MeOH/H_2O (1/1), twice with MeOH, and three times with dichloromethane (DCM) (2 volumes each). The extracts were combined and bidistilled H_2O (6 volumes) was added. The combined solutions were mixed and allowed to separate in a MeOH/H_2O and DCM phase, the DCM phase was removed and collected. The MeOH/H_2O layer was re-extracted twice with 3 mL DCM. The combined DCM layers were dried over a Na_2SO_4 column and the DCM was evaporated under a stream of nitrogen. The dried extracts were stored at 4 °C before further workup.

Fatty acids were methylated with a boron trifluoride-methanol solution (BF_3-MeOH) for 5 min at 60 °C. Then H_2O and DCM were added (1 mL each). The aqueous layer was washed three times with 1 mL DCM, and the combined DCM fractions were cleaned over a Na_2SO_4 column and dried under a stream of nitrogen. In order to obtain a fatty acid fraction, the methylated extract was separated over an aluminium oxide (AlOx) column, eluting the methylated fatty acids with DCM.

In order to identify the position of double bonds in unsaturated fatty acids, the methylated fatty acids were derivatized with dimethyldisulfide (DMDS) (Nichols et al., 1986). Hexane, DMDS and I_2/ether (60 mg/mL) were added to the fatty acids and incubated at 40 °C overnight. After adding hexane, the iodine was deactivated by addition of a 5% aqueous solution of $Na_2S_2O_3$. The aqueous phase was washed twice with hexane. The combined hexane layers were cleaned over a Na_2SO_4 column and dried under a stream of nitrogen. The dried extracts were stored at 4 °C before analysis.

2.2.3. Fatty acid analysis

The fatty acid fractions were analysed by gas chromatography (GC) using an Agilent 6890 gas chromatograph with a flame ionization detector (FID) using a fused silica capillary column (25 m x 320 µm) coated with CP Sil-5 (film thickness 0.12 µm) with helium as carrier gas. The temperature program was the following: initial temperature 70 °C, increase of temperature to 130 °C with 20 °C min^{-1}, and then to 320 °C with 4 °C min^{-1} for 10 min. Individual compounds were identified by GC/mass spectrometry (GC/MS) using a Agilent 7890A GC instrument and Agilent 5975C VL mass selective detector (MSD).

2.2.4. Hydrogen isotope analysis

Hydrogen isotope analysis of the fatty acids was performed by GC thermal conversion isotope ratio monitoring MS (GC/TC/irMS) using an Agilent 7890 GC connected via Thermo GC Isolink and Conflo IV interfaces to a Thermo Delta V MS according to Chivall et al. 2014. Samples were injected onto an Agilent CP-Sil 5 CB column (25 m × 0.32 mm ID; 0.4 µm film thickness; He carrier gas, 1.0 mL min^{-1}). The GC temperature program was 70 °C to 145 °C at 20 °C min^{-1}, then to 200 °C at 4 °C min^{-1} 320 °C for 15 min. Eluting compounds were converted to H_2 at 1420°C in a ceramic tube before introduction to the mass spectrometer. An internal standard, squalane (δD = -170 ‰), was co-injected with each fatty acid sample in order to monitor the precision of the measurements. The average δD of the internal standard was -167±3 ‰. The δD of the individual fatty acids was measured in duplicates and corrected for the added methyl group.

The hydrogen isotopic composition of fatty acids compared to water was expressed as $\varepsilon_{lipid/water}$ following:

$$\varepsilon = \left(\frac{1000+\delta D_{FA}}{1000+\delta D_{water}} - 1\right) * 1000$$

The δD of the water was determined by injecting at least 10 × 1 µL on an elemental analysis/thermal conversion/isotope ratio monitoring MS (EA/TC/irMS) using a Thermo Finnigan TC/EA interfaced via a Thermo Finnigan ConFlo III to a Thermo Finnigan Delta$^+$ XL mass spectrometer following the procedure described by M'Boule et al. (2014) with North Sea water (δD 5 ‰) and bidistilled water (δD -76 ‰) as standards.

The δD value of dry sodium acetate was also determined by EA/TC/irMS. NBS 22 mineral oil (δD -120.0 ‰) and polyethylene IAEA-CH-7 (δD -100.3 ‰) were used as standards. The δD value of acetate was measured in triplicate.

The hydrogen isotopic composition of the non-exchangeable hydrogen of the glucose substrate was determined by analysing the acetylated derivative of glucose. For this, glucose was acetylated using 0.5 mL acetic anhydride with a pre-determined δD value and 0.5 mL pyridine for 3 h at 75 ºC. Afterwards, 1 mL distilled H_2O was added and the water layer was washed three times with hexane. The hexane was evaporated and the acetylated glucose was dissolved in ethyl acetate and analysed by GC, GC-MS and GC/TC/irMS similar to the fatty acids. The δD value of the non-exchangeable hydrogen of

glucose was calculated by correcting for the added acetyl groups. The acetylated glucose was measured five times.

Statistical analysis was done via One Way ANOVA test with SigmaPlot Version 12.0 (Systat Software, Inc., San Jose, USA).

2.3. Results

2.3.1. *Thiocapsa roseopersicina*

Thiocapsa roseopersicina was grown photoautotrophically under anoxic conditions with CO_2 as sole carbon source and hydrogen sulphide as electron donor. Fatty acids that were present in all growth phases are *n*C16:1ω7, *n*C16:0 and *n*C18:1ω7. In addition, during the death phase traces of *n*C12:0, *n*C14:0 and *n*C17:1ω7 were also detected. The *n*C18:1 fatty acid (30−49 %) was the most abundant fatty acid in all growth phases followed by *n*C16:1 (21−32 %) and *n*C16:0 (~21%). Minor fatty acids were *n*C12:0, *n*C14:0 and *n*C17:1 fatty acids with abundances between 7 and 14 % (Table S1). All fatty acids were depleted in D relative to the growth medium (all δD values are summarized in Table S2) and the hydrogen isotopic fractionation expressed as $\varepsilon_{lipid/water}$ between the fatty acids and the growth water of the individual fatty acids ranged between -153 ‰ and -264 ‰ (Table 1). Fatty acids were most depleted during exponential growth and most enriched during the death phase (Figure 1A; Table 1).

2.3.2. *Halochromatium glycolicum*

Halochromatium glycolicum was grown photoautotrophically, under anoxic conditions with CO_2 as sole carbon source and hydrogen sulphide as electron donor. The fatty acids *n*C16:1ω7, *n*C16:0 and *n*C18:1ω7 were present in all growth phases with *n*C18:1 fatty acid being the most abundant (52−73 %) and *n*C16:1 the least abundant (7−9 %) (Table S1). In addition, a C19 fatty acid containing a cyclopropane moiety (C19:cyc) was only present in the death phase. All measured fatty acids were depleted in D relative to the growth medium (Table S2) and $\varepsilon_{lipid/water}$ for the individual fatty acids ranged between -159 ‰ and -230 ‰ (Table 1). The fatty acids were most depleted during exponential growth and most enriched during the death phase (Figure 1B; Table 1). The *n*C16:0 fatty acid was enriched in D by 40−50 ‰ compared to the other fatty acids in all growth phases.

Table 1: D/H fractionation between fatty acids and growth medium for fatty acids produced by different microorganisms under various metabolic conditions.

Organism	Substrate	$\delta D_{substrate}$ [‰]	δD_{water} [‰]	mean $\varepsilon_{lipid/water}$ [‰]								weight-ed. av. [‰]	GP
				C12:0	C14:0	C16:1*	C16:0	C17:cyc	C17:1$^{\varphi}$	C18:1$^{\phi}$	C19:cyc		
Thiocapsa roseopersicina	CO_2, light	-	-51±3			-264	-216			-259		-252	E
			-50±3			-260	-209			-254		-247	S
			-59±3	-204	-209	-221	-191		-153	-232		-210	D
Halochromatium glycolicum	CO_2, light	-	-50±2			-222	-187			-230		-221	E
			-51±2			-225	-175			-218		-210	S
			-61±2			-214	-159			-209	-187	-196	D
Isochrysis galbana	CO_2, light	-	4±2		-237		-233			-149		-225	E
			5±2		-215		-205			-179		-198	S
			9±1		-201		-200			-184		-192	D
Thiobacillus denitrificans	CO_2	-	-51±1			-262	-275	-228				-265	E
			-49±3			-250	-270	-229				-258	S
			-54±3			-252	-270	-217				-257	D
Pseudomonas str. LFY10	glucose	-8±11	-56±2			82	111			112		100	E
			-55±2			93	123			124		112	S
			-38±2			123	169	152		197	161	164	D
Pseudomonas str. LFY10.	acetate	-128±3	-57±2			265	278	359		309		289	E
			-57±3			249	261	328		290		270	S
			-44±2			247	294	307		304	323	300	D

*n*C16:1*: double bond at the ω7 position; *n*C17:1$^{\varphi}$: double bond at the ω7 position; *n*C18:1$^{\phi}$: double bond in all cultures except for *I. galbana* (ω9) at the ω7 position.; GP: growth phase; E= exponential, S= stationary, D= death

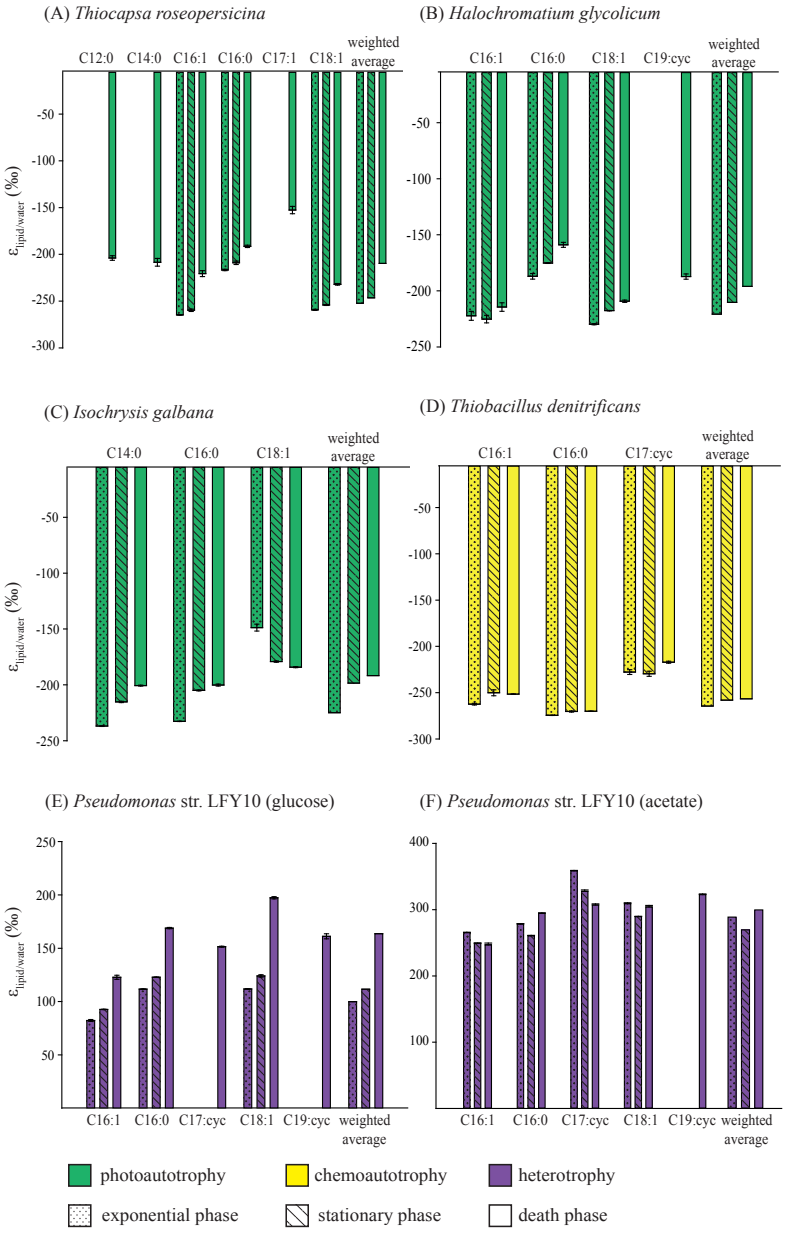

Figure 1: The D/H fractionation between fatty acids and culture medium observed in different growth phases during the culture experiments. Plotted are the mean ε values (lipid versus water). Error bars are the standard deviation of the duplicate measurements of the fatty acids. A. *Thiocapsa roseopersicina*, B. *Halochromatium glycolicum*, C. *Isochrysis galbana*, D. *Thiobacillus denitrificans* and *Pseudomonas* str. LFY10 grown on E. glucose and F. acetate. Also plotted is the weighted average $\varepsilon_{lipid/water}$ of the fatty acids.

2.3.3. *Isochrysis galbana*

Isochrysis galbana was grown photoautotrophically under oxic conditions with CO_2 as sole carbon source (M'Boule et al., 2014). In all growth phases, *n*C14:0, *n*C16:0 and *n*C18:1ω9 fatty acids were identified as well as various other unsaturated *n*C18:x and a polyunsaturated *n*C22 fatty acids. Either *n*C14:0 or *n*C18:1 fatty acids are in general the most abundant fatty acids (Table S1). All fatty acids were depleted in D compared to the growth medium (Table S2) and $\varepsilon_{lipid/water}$ values ranged from -149 ‰ to -237 ‰ (Table 1). The *n*C14:0 and *n*C16:0 fatty acids were most depleted in D during exponential growth and became enriched by up to 30 ‰ with increasing age of the culture. On the other hand *n*C18:1ω9 fatty acid was most enriched during exponential growth and became depleted in D with age of the culture by up to 35 ‰ (Figure 1C; Table 1). The D/H ratio of the other unsaturated *n*C18:x and a polyunsaturated *n*C22 fatty acids could not be measured with certainty due to either incomplete separation or low abundance.

2.3.4. *Thiobacillus denitrificans*

Thiobacillus denitrificans was grown chemoautotrophically with thiosulphate as electron donor. In all growth phases *n*C16:1ω7 and *n*C16:0 fatty acids were present in equal abundance. Minor amounts of C17:cyc were also detected (Table S1). All fatty acids were depleted in D relative to the growth medium (Table S2) with $\varepsilon_{lipid/water}$ of the individual fatty acids ranging between -217 ‰ and -275 ‰ (Table 1). In general, fatty acids were most depleted during exponential growth (Figure 1D). The *n*C16:0 fatty acid was depleted by 10−50 ‰ compared to the other fatty acids in all growth phases.

2.3.5. *Pseudomonas* str. LFY10

The *Pseudomonas* str. LFY10 strain was grown heterotrophically on either glucose or acetate as carbon source under oxic conditions. On both substrates, *Pseudomonas* str. LFY10 produced *n*C16:1ω7, *n*C16:0 and *n*C18:1ω7 fatty acids. The C17:cyc fatty acids was identified in all growth phases when grown on acetate but only during the death phase when grown on glucose. In the death phase, C19:cyc fatty acid was identified when grown on either substrate (Table S1). There were no differences in fatty acid distribution between exponential and stationary phase with *n*C16:1 and *n*C16:0 being the most abundant fatty acids when grown on glucose and *n*C16:0 being the most

abundant fatty acid when grown on acetate (Table S1). In the culture grown on glucose all fatty acids during death phase were enriched in D relative to the growth medium (Table S2) with $\varepsilon_{lipid/water}$ ranging between 82 ‰ and 197 ‰ (Table 1), as well as to the substrate, with $\varepsilon_{lipid/substrate}$ ranging between 30 ‰ and 161 ‰ (Table 2). All fatty acids became enriched with age of culture (Figure 1D). In the cultures grown on acetate, all fatty acids were significantly enriched compared to both the growth medium and the substrate. The $\varepsilon_{lipid/water}$ value of the individual fatty acids ranged between 247 to 359 ‰ (Table 1), while the $\varepsilon_{lipid/substrate}$ of the individual fatty acids was between 351 ‰ and 469 ‰ (Table 2). Fatty acids of *Pseudomonas* str. LFY10 grown on acetate did not show a general enrichment in D with progressing growth phase (Figure 1F).

Table 2: D/H fractionation between fatty acids and growth substrate for fatty acids produced by *Pseudomonas* str. LFY10

Substrate	δD_{water}	$\delta D_{substrate}$	$\varepsilon_{lipid/substrate}$ [‰]					GP
	[‰]	[‰]	C16:1*	C16:0	C17:cyc	C18:1ϕ	C19:cyc	
glucose	-56±2	-8±11	30	58		58		E
	-55±2		40	69		70		S
	-38±2		89	134	117	161	126	D
acetate	-57±2	-128±3	369	383	469	416		E
	-57±3		351	363	436	394		S
	-44±2		366	418	432	429	450	D

*n*C16:1*: double bond at the ω7 position; C18:1ϕ: double bond at the ω7 position. GP: growth phase; E= exponential, S= stationary, D= death

2.4. Discussion

2.4.1. Influence of metabolism on the δD of C16:0 fatty acid

The *n*C16:0 fatty acid is the most common and often most abundant fatty acid in bacterial and eukaryotic microorganisms (Gunstone et al., 2012). Indeed, all the cultures tested in this study synthesize at least the *n*C16:0 fatty acid, while other fatty acids were often either absent or present in lower amounts. Therefore, for comparing the hydrogen isotopic fractionation of the different microbes we focus on the *n*C16:0 fatty acid (Figure 2) and discuss the patterns in more detail below. Furthermore, the weighted average of all measured fatty acids for each culture showed the same trend as the *n*C16:0 fatty acid indicating that changes in the hydrogen isotopic composition of

individual fatty acids is not strongly affected by the relative abundance of the fatty acids (Table 1; Figure 1).

2.4.1.1 Photoautotrophs

The hydrogen isotopic fractionation for the *n*C16:0 fatty acid ($\varepsilon_{C16:0/water}$) of all photoautotrophs, *Thiocapsa roseopersicina*, *Halochromatium glycolicum* and *Isochrysis galbana*, is relatively similar and ranged from -187 to -233 ‰. The oxygenic photoautotroph *I. galbana* reduce $NADP^+$ to NADPH using H_2O as electron donor (Lengeler et al., 1999) and thus the sole source of hydrogen is water. The D/H ratio of fatty acids depends mainly on the fractionation associated with the splitting of water, the reduction of $NADP^+$ to NADPH, the transfer of H to the initial photosynthate and the transfer of H during fatty acid biosynthesis (Hayes, 2001). The anoxygenic photoautotrophs *Thiocapsa roseopersicina* and *Halochromatium glycolicum* both reduce $NADP^+$ via reverse electron transport (Imhoff, 2006). Depending on the pH, H_2S is soluble in water and forms S^{2-} and 2 H^+ with the latter being exchangeable with the protons of water. Since the amount of H_2S is relatively small compared to water (0.04%), the D/H ratio of water will not be substantially affected. The similar fractionation of anoxygenic and oxygenic photoautotrophs suggest that the steps leading to the production of NADPH under both conditions have similar fractionation factors. The same has been observed for various other algae and an anoxygenic, photoautotrophic bacterium (Zhang and Sachs, 2007; Zhang et al., 2009a).

2.4.1.2 Chemolithoautotrophs

The *n*C16:0 fatty acid of the chemolithoautotrophic *T. denitrificans* is more depleted in D compared to those of the photoautotrophs (Figure 2) even though water is also the most likely source for hydrogen here. *T. denitrificans* contains an electron transport chain to reduce NAD^+ to NADH by quinone-cytochrome b (Beller et al., 2006). The electron transport chain, the enzymes involved and the substrate reduced are different from photoautotrophic microorganisms, which could potentially explain the negative offset in fatty acid D/H ratios. Another possibility could be that *T. denitrificans* might, like *T. thioparus* to which it is physiologically similar (Kelly and Wood, 2000), rather use NADH than NADPH as hydrogen source during fatty acid biosynthesis. Matin and Rittenberg (1971) also suggested that in obligate chemolithoautotrophic *Thiobacilli* NADH, and not NADPH, is used for reducing power during biosynthesis. Additionally, at least some heterotrophs have been

shown to contain a NADH-NADPH converting transhydrogenase. In case of excess NADPH the enzyme leads to reduction of NAD^+ to NADH while oxidizing NADPH to $NADP^+$. This would leave the remaining NADPH pool enriched and the NADH strongly depleted in D (Zhang et al., 2009a). This could be an additional fractionation effect that potentially contributes to the relatively depleted fatty acids in chemoautotrophs which use NADH. However, the genome of *Thiobacillus denitrificans* does not contain a NADH-NADPH converting transhydrogenase (Beller et al., 2006), although we cannot exclude the possibility it contains enzymes with a similar function.

2.4.1.3. Heterotrophs

The heterotroph *Pseudomonas* str. LFY10 produces D-enriched fatty acids compared to D-depleted fatty acids in all autotrophs. Furthermore, similar to *Escherichia coli* (Zhang et al., 2009a), $\varepsilon_{lipid/water}$ of the individual fatty acids of *Pseudomonas* str. LFY10 is higher, when grown on acetate than on glucose. In heterotrophic organisms an important hydrogen source is the organic substrate used as carbon and energy source. All *Pseudomonas* species possess the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway (Moore et al., 2006) and possibly also a NADH-NADPH converting transhydrogenase (Louie and Kaplan, 1970; French et al., 1997). The observed enrichment in D is suggested to occur during $NADP^+$ reduction in the TCA cycle or due to conversion of excess NADPH to NADH via the NADH-NADPH converting transhydrogenase (Zhang et al., 2009a). Some of the supplied acetate might also be used as a direct building block during fatty acid biosynthesis and therefore some of the H of fatty acids would come directly from acetate. When *Pseudomonas* sp. is grown on glucose, $NADP^+$ will be reduced in the pentose phosphate pathway in addition to the TCA cycle. While $NADP^+$ reduction in the TCA cycle leads to NADPH enriched in D, the reduction in the pentose phosphate pathway might lead to more depleted NADPH. The mixed NADPH pool could thus explain why the fatty acids produced by *Pseudomonas* str. LFY10 are less enriched in D when grown on glucose compared to acetate.

2.4.2. Influence of growth phase on the δD of fatty acids

In addition to metabolism we investigated the effect of growth phase. Fatty acids produced by the different microorganisms are in general, but not exclusively, increasingly enriched in D with increasing age of the culture. From exponential to stationary phase, the $\varepsilon_{lipid/water}$ values of the *n*C16:0 fatty acids

of all but one culture increase by around 10 ‰. Only for *Pseudomonas* str. LFY10 grown on acetate a depletion of 15 ‰ was observed (Figure 2).

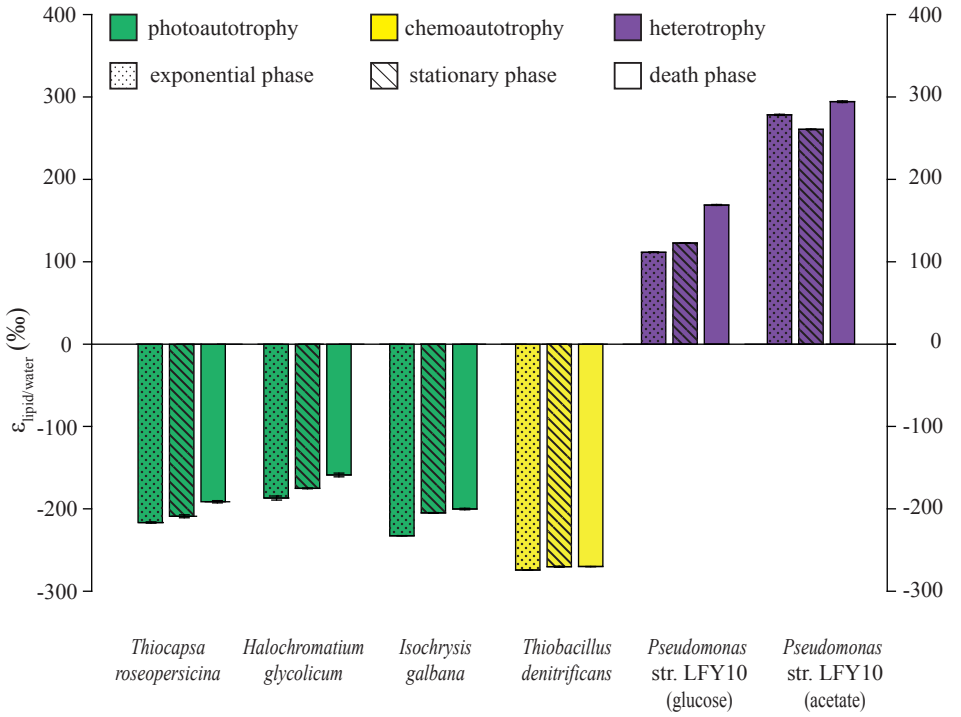

Figure 2: Impact of growth phase (exponential, stationary and death phase) on the hydrogen isotopic fractionation of the *n*C16:0 fatty acid of the different cultivated microbes. Plotted are the mean ε values of the duplicate measurements of the fatty acids and error bars are the standard deviation of the duplicate measurements of the fatty acids. Cultures are *Thiocapsa roseopersicina*, *Halochromatium glycolicum*, *Isochrysis galbana*, *Thiobacillus denitrificans* and *Pseudomonas* str. LFY10 grown on glucose and acetate.

Few studies have focused on the impact of growth phase on D/H ratios of lipids. Interestingly, Wolhowe et al. (2009) and Chivall et al. (2014) showed that alkenones produced by haptophyte algae are more depleted in D in stationary growth phase compared to exponential growth phase, which is in contrast to the enrichment observed here for the *n*C16:0 fatty acid as well as the weighted average of fatty acids in *I. galbana* (Figure 1C). This is interesting considering that the *n*C16:0 fatty acid is assumed to serve as a precursor for the synthesis of alkenones (Rontani et al., 2006; Wallace, 2012). A possible

explanation is that the *n*C16:0 is produced mainly in the chloroplast, while it is assumed that the alkenones are produced in the cytosol by chain elongation (Wallace, 2012). Therefore, two different NADPH pools could be used for the biosynthesis of alkenones and *n*C16:0 fatty acid, respectively. The observed isotopic difference with growth phase could be due to a decrease in structural lipid synthesis such as fatty acids since the algae are no longer growing and dividing as a result of nutrient limitation. At the same time NADPH is still produced during photosynthesis leading to a surplus of reducing power. This excess NADPH will then be used for the production of storage products, such as alkenones, which do not contain limiting elements like N or P (Wolhowe et al., 2009). Alternatively, when a smaller fraction of the relatively D depleted fatty acids are used for structural components such as membranes, they may be used for alkenone biosynthesis resulting in more D depleted alkenones with growth phase.

Zhang et al. (2009a) already reported that heterotrophic microorganisms like *Cupriavidus necator* and *C. oxalaticus* produce fatty acids during exponential growth that are more enriched in D compared to fatty acids produced during stationary phase ($\varepsilon_{lipid/water}$ 169 ‰ vs. 70 ‰ and 149 ‰ vs. 95 ‰, respectively) when grown on succinate. Like acetate, succinate is metabolized via the TCA cycle which may thus lead to production of NADPH enriched in D. Thus, for heterotrophs, growth on substrates that are directly involved in the TCA cycle apparently leads to a depletion in D of all fatty acids, including *n*C16:0, when shifting from exponential to stationary phase. In contrast, growth on substrates that are involved in the pentose phosphate pathway, like glucose, apparently leads to enrichment in D of fatty acids in stationary phase compared to exponential phase.

Although growth phase changes result in changes in D/H ratios of fatty acids these are relatively minor compared to the impact of metabolism on fatty acid D/H ratios. Therefore, changes in growth phase in microbial communities in the environment can be considered to have relatively minor impact on the overall isotopic signal of the fatty acid pool of the whole community. Additionally, lipid identity does not play a major role on the hydrogen isotopic composition of fatty acids compared to metabolism. The $\varepsilon_{lipid/water}$ values of the different fatty acids with the same initial biosynthetic pathways fall in a similar range and differences observed are due to the addition and/or removal of hydrogen atoms related with chain length and degree of unsaturation.

2.4.3. Application of δD of fatty acids as a population metabolism indicator

When we summarize all published (including this study) $\varepsilon_{C16:0/water}$ values of different microorganisms grown as either photoautotroph, chemoautotroph or heterotroph (Sessions et al., 1999; Sessions et al., 2002; Chikaraishi et al., 2004; Valentine et al., 2004; Zhang and Sachs, 2007; Campbell et al., 2009; Zhang et al., 2009a; Dirghangi and Pagani, 2013; Fang et al., 2014), the three metabolism types show distinct, but slightly overlapping ranges (Figure 3).

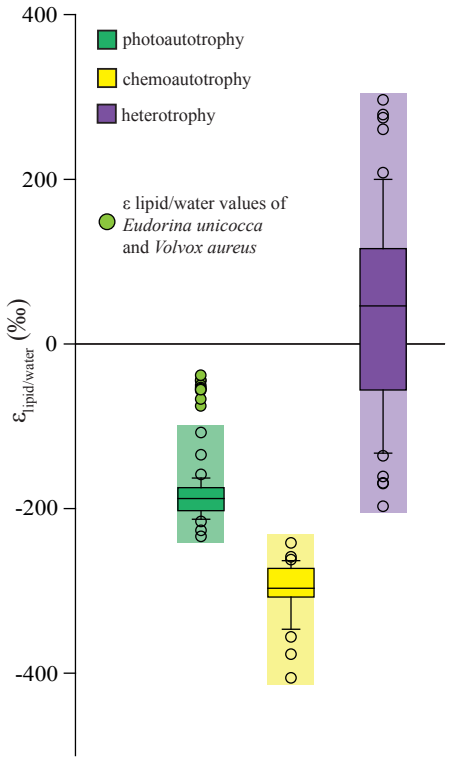

Figure 3: Box plots of D/H fractionations between the *n*C16:0 fatty acid and culture medium observed in different culture experiments. Cultures included from this study are *Thiocapsa roseopersicina*, *Halochromatium glycolicum*, *Isochrysis galbana*, *Thiobacillus denitrificans* and *Pseudomonas* str. LFY10. Additionally, published data for *Isochrysis galbana*, *Ascophyllum* sp., *Alexandrium fundyense*, *Methylococcus capsulatus*, *Saragassum filicinum*, *Undararia pinnatifida*, *Binghamia californica*, *Gelidium japonica*, *Sporomusa* sp., *Botrycoccus braunii*, *Eudorina unicocca*, *Volvox aureus*, *Desulfobacterium autotrophicum*, *Cupriavidus oxalaticus*, *Cupriavidus necator*, *Escherichia coli*, *Rhodopseudomonas palustris*, *Tetrahymena thermophile* and *Moritella japonica* DSK 1 have been included (Sessions et al., 1999; Sessions et al., 2002; Chikaraishi et al., 2004; Valentine et al., 2004; Zhang and Sachs, 2007; Campbell et al., 2009; Zhang et al., 2009a; Dirghangi and Pagani, 2013b; Fang et al., 2014).

Microorganisms grown as photoautotrophs produce fatty acids which are depleted in D relative to the growth medium with the majority of $\varepsilon_{C16:0/water}$ values ranging around -170 to -200 ‰. The only exceptions are the cultures of the freshwater algae *Eudorina unicocca* and *Volvox aureus* which produce D-enriched fatty acids compared to other photoautotrophs (Zhang and Sachs, 2007). A possible explanation could be that both *Eudorina unicocca* and *Volvox aureus* are colony forming algae which, unlike the colony forming algae *Botryococcus braunii* (Zhang and Sachs, 2007), consist of two different cell types, somatic cells and reproductive (gonidia) cells (Herron et al., 2009). Possible differences in the metabolism between these two different cell types could play a role in the relatively enrichment in D of the *n*C16:0 fatty acid compared to other photoautotrophs. Due to the fact that the $\varepsilon_{lipid/water}$ values of these two organisms do not seem to follow the pattern observed for the *n*C16:0 fatty acids produced by all photoautotrophs we have indicated them separately in our summary in Figure 3. Thus, C16:0 fatty acid of photoautotrophs have a mean $\varepsilon_{C16:0/water}$ value of -186 ‰ and range between -162 and -215 ‰ (95 % confidence interval, n=34). Chemoautotrophically grown microorganisms produce fatty acids with a mean $\varepsilon_{C16:0/water}$ value of -298 ‰ and ranging between -264 and -345 ‰ (95 % confidence interval, n=32) which is depleted by ca. 100 ‰ relative to fatty acids produced by photoautotrophs. Heterotrophically grown microorganisms typically show an enrichment in D of the lipids relative to the water (with a mean $\varepsilon_{C16:0/water}$ value of 39 ‰ and ranging between -133 and 199 ‰ (95 % confidence interval, n=53). The differences between the different metabolisms are significant ($P<0.001$) and should allow for the characterization of the dominant metabolism of microbial communities in the environment by analysing the isotopic difference between *n*C16:0 fatty acid and water. However, several issues should be kept in mind. Relatively few microorganisms have been analysed for their fatty acid hydrogen isotopic composition, and although most of them fit the general pattern it is possible that exceptions to this pattern arise once other microorganisms are analysed in the future. In the environment all microorganisms producing the *n*C16:0 fatty acid will contribute to the fatty acid pool in varying amounts depending on the amount produced in the cell. Therefore, the $\varepsilon_{C16:0/water}$ value in an environmental sample will not so much represent the average $\varepsilon_{lipid/water}$ value of the whole microbial community but will also be affected by the relative abundance of the *n*C16:0 fatty acid in the various contributing microbes. For instance, the pink streamer (PS) communities in Yellowstone National Park are dominated by chemoautotrophic *Aquificales* which mainly produce *n*C20:1, C21:cyc with *n*C18:0 and *n*C16:0 fatty acids occurring only in minor

amounts, while *n*C16:0 fatty acid is abundant in members of the co-occurring genus *Thermus* which are heterotrophic. Thus, the D/H ratio of the *n*C16:0 fatty acid reflects heterotrophy rather than chemoautotrophy in these PS communities despite the dominance of chemoautotrophs (Osburn et al., 2011).

Nevertheless, in order to obtain an idea of the dominating metabolism of a microbial community in the present, as well as in the past, the $\varepsilon_{C16:0/water}$ value is a promising approach, possibly combined with the $\varepsilon_{lipid/water}$ value of other fatty acids and potentially biomarker lipids with a more restricted origin.

2.5. Conclusion

The hydrogen isotopic composition of fatty acids produced by a range of different microorganisms depends on the general metabolism expressed during growth. Both photoautotrophs and chemoautotrophs produce fatty acids strongly depleted in D, while heterotrophs produce fatty acids enriched in D compared to the growth medium. Fatty acids produced during different growth phases become somewhat enriched in D with increasing age of the culture in most of the experiments described here. Thus, growth phase likely plays a minor role in controlling the D/H ratio of fatty acids relative to metabolism in the natural environment. Our results suggest that an overall characterization of community metabolism via the D/H ratio of fatty acids is potentially feasible.

Acknowledgment

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Chapter 3

Comparison of the effect of salinity on the D/H ratio of fatty acids of heterotrophic and photoautotrophic microorganisms

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Abstract

The core metabolism of microorganisms has a major influence on the hydrogen isotopic composition of their fatty acids. Heterotrophic microorganisms produce fatty acids with a deuterium to hydrogen (D/H) ratio either slightly depleted or enriched in D compared to the growth water, while photo- and chemoautotrophic microorganisms produce fatty acids which are heavily depleted in D. However, besides metabolism other biochemical and environmental factors (i.e. biosynthetic pathways, growth phase and temperature) have been shown to affect the D/H ratio of fatty acids and it is necessary to evaluate the magnitude of these effects compared to that of metabolism. Here we show that the effect of salinity on the D/H ratio of fatty acids depends on the core metabolism of the microorganism. While fatty acids of the photoautotroph *Isochrysis galbana* become more enriched in D with increasing salinity (enrichment of 30-40 ‰ over a range of 25 salinity units), no effect of salinity on the D/H ratio of fatty acids of the heterotrophic *Pseudomonas* str. LFY10 was observed ($\varepsilon_{lipid/water}$ of the *n*C16:0 fatty acid of ~ 120 ‰ over a range of 10 salinity units). This can likely be explained by the relative contributions of different H and NADPH sources during fatty acid biosynthesis.

3.1. Introduction

Over the last 15 years the hydrogen isotope composition (δD) of different biomarker lipids has increasingly been used to understand and reconstruct diverse environmental factors such as salinity and the precipitation/evaporation balance (e.g. Sauer et al., 2001; van der Meer et al., 2007; van der Meer et al., 2008; Berke et al., 2012; Garcin et al., 2012; Sachse et al., 2012; Coolen et al., 2013; van Soelen et al., 2013; Kasper et al., 2014). Several studies have also shown that the deuterium (D) to hydrogen ratio (D/H) of fatty acids reflects the core metabolism of microorganisms both in culture and in the environment (Zhang et al., 2009a; Osburn et al., 2011; Heinzelmann et al., 2015). Therefore, δD of lipids has attracted interest as a potential tool to study microbial activity *in situ* (Zhang et al., 2009a; Osburn et al., 2011). Microorganisms grown chemo- or photoautotrophically in culture produce fatty acids which are strongly depleted in D relative to the growth water, while heterotrophically grown organisms produce fatty acids enriched or only slightly depleted in D compared to the growth water. This also applies when the same microorganism is grown under different conditions expressing different core metabolisms (Zhang et al., 2009a), indicating that this effect is not species dependent but rather depends on metabolism.

The source of hydrogen is the most likely reason for this effect of metabolism on the hydrogen isotopic composition of fatty acids. The main source (~50 %) of hydrogen for fatty acid biosynthesis is nicotinamide adenine dinucleotide phosphate (NADPH) and thus the D/H ratio of fatty acids should reflect largely the D/H ratio of NADPH. Additional sources of hydrogen are the methyl group of acetyl-CoA (~25 %) and internal cell water (~25 %) (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003). Zhang et al. (2009a) suggested that the isotopic composition of hydrogen provided by NADPH depends on the pathway by which $NADP^+$ is reduced to NADPH. The main source of hydrogen for the reduction of $NADP^+$ in a heterotrophic microorganism is the organic substrate which gets oxidized via the oxidative pentose phosphate (OPP) pathway and/or the tricarboxylic acid (TCA) cycle, with both pathways fractionating against the heavier isotope. Additionally, the NADH-NADPH converting transhydrogenase, an important enzyme which regulates the NADPH-pool in case of excess NADPH by reducing of nicotinamide adenine dinucleotide (NAD^+) to NADH and oxidation of NADPH (Kim and Gadd, 2008), is associated with a strong fractionation effect and leaves the remaining NADPH-pool enriched in D. Oxygenic photoautotrophs

on the other hand, mainly reduce $NADP^+$ during photosynthesis using hydrogen from water via ferredoxin-$NADP^+$ oxidoreductase, and the resulting NADPH is approximately 600 ‰ depleted in D compared to water ($\alpha = 0.4$) (Schmidt et al., 2003; Zhang et al., 2009a).

Although compound-specific δD of fatty acids is a promising tool to characterize the metabolic activity of microbial communities in the environment, the possible impact of factors other than metabolism on the D/H ratio of fatty acids needs to be studied in more detail. Previous studies have shown that the fatty acid synthesis pathway influences the D/H ratio. For example, in the piezophilic, heterotrophic bacterium *Moritella japonica* DSK 1 long-chain polyunsaturated fatty acids, synthesized by the polykektide (PKS) pathway, were 120–210 ‰ more depleted in D than short-chain fatty acids, synthesized by the fatty acid synthase (FAS) pathway (Fang et al., 2014). Furthermore, temperature was also shown to influence the D/H ratio of some fatty acids leading either to depletion or enrichment in D by up to 30–40 ‰ and 20 ‰, respectively (Zhang et al., 2009b; Dirghangi and Pagani, 2013b).

One of the best studied environmental parameter affecting the hydrogen isotopic composition of aquatic microorganisms so far is salinity. It has been shown to affect the D/H ratio of long chain alkenones of haptophyte algae (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014) and of algal steroids such as dinosterol and brassicasterol (Sachse and Sachs, 2008; Sachs and Schwab, 2011; Nelson and Sachs, 2014). The D/H ratio of isoprenoid lipids derived from the heterotrophic euryarchaeote *Haloarcula marismortui* were also seemingly affected by salinity, although the salinity effect might have been indirect, with the original cause likely being growth rate rather than salinity itself (Dirghangi and Pagani, 2013a). Interestingly, the impact of salinity on the D/H ratio of fatty acids of microorganisms has not been studied so far and it is unknown how large this effect is compared to that imposed by metabolism. Therefore, the goal of this study is to evaluate the possible impact of salinity on the D/H ratio of fatty acids and compare it to the effect of metabolism. To this end, we studied the D/H ratios of fatty acids produced by a heterotrophic bacterium *Pseudomonas* str. LFY10 grown at three different salinities and compare them to changes in the D/H ratios of fatty acids produced by the photoautotrophic alga *Isochrysis galbana*, grown at six different salinities and harvested at three different growth phases (exponential, stationary and death phase). The salinity ranges from brackish to marine covering the range encountered in coastal and marine environments.

3.2. Material and Methods

3.2.1. Cultures

A freshwater *Pseudomonas* str. LFY10 was grown at 25 ºC on an ammonium-glucose medium at salinities of 6.4, 10.9 and 16.9 (Heinzelmann et al., 2015). The salinities were obtained by addition of different amounts of NaCl to the medium. Biomass samples and water samples for hydrogen analysis were collected during the stationary phase by filtration over a 0.7 µm GF/F filter. The photoautotrophic eukaryote *Isochrysis galbana* (strain CCMP 1323) was cultured previously at salinities of 10.3, 15.3, 20.2, 25.1, 30.2 and 35.5 in f/2 medium (Guillard, 1975) by M'Boule et al. (2014). The cultures were incubated at 15 °C and a light intensity of ~50 µE m^{-2} s^{-1} of a cool white fluorescent light (16 h light, 8 h dark). Biomass and water samples were collected during the exponential, stationary and death phase as previously described by Chivall et al. (2014) and Heinzelmann et al. (2015).

3.2.2. Fatty acid and hydrogen isotope analysis

Fatty acids were extracted by saponification of the freeze dried filters as described by Heinzelmann et al. (2015). Briefly, the filters were refluxed for 1 h in 1 N KOH in methanol (MeOH), after which the pH was adjusted to pH = 4 using 2 N HCl/MeOH (1/1, v/v). The solid phase was washed with MeOH/H_2O (1/1, v/v), MeOH and dichloromethane (DCM), the liquid phases were combined and washed with DCM. Fatty acids were derivatized by methylation with 13–15 % boron trifluoride in-methanol (BF_3-MeOH) after which a fraction containing the methylated fatty acids was obtained by eluting with DCM over an aluminium oxide column. In order to identify the position of double bonds in unsaturated fatty acids, an aliquot of the methylated fatty acids was derivatized with dimethyldisulfide (DMDS) (Nichols et al., 1986) and analysed by gas chromatography (GC) and mass spectrometry (MS).

The fatty acid fractions were analysed and identified by gas chromatography (GC) and GC/mass spectrometry (GC/MS) (as described by Heinzelmann et al. (2015). Hydrogen isotope analysis of the fatty acid fraction was performed by GC-thermal conversion-isotope ratio monitoring MS (GC/TC/irMS) using an Agilent 7890 GC connected via Thermo GC Isolink and Conflo IV interfaces to a Thermo Delta V MS according to Chivall et al. 2014. The H_3^+ correction factor was determined daily and was constant at 5.3±0.2. An

internal standard, squalane (δD = -170±4 ‰), was co-injected with each fatty acid sample in order to monitor the accuracy of the measurements. The mean measured δD of the internal standard was -165±3 ‰. The δD of the individual fatty acids was measured in duplicate and corrected for the addition of methyl hydrogen (δD_{ME} = -171±1 ‰; Heinzelmann et al. (2015).

The δD of culture medium and the non-exchangeable hydrogen of glucose, after derivatization with acetic anhydride, were determined by elemental analysis/TC/irMS (EA/TC/irMS) according to Chivall et al. (2014) and Heinzelmann et al. (2015), respectively.

3.3. Results

Pseudomonas str. LFY10 was grown heterotrophically with glucose as the substrate at three different salinities and was harvested during the stationary phase. It produced *n*C16:1ω7, *n*C16:0, *n*C18:1ω7 fatty acids and traces of C17:cyc and *n*C18:0 fatty acids at all three salinities. The hydrogen isotopic compositions of the most abundant fatty acids was determined and $\varepsilon_{lipid/water}$ and $\varepsilon_{lipid/glucose}$ values are summarised in Table 1, with ε expressing the hydrogen isotopic fractionation between lipid and water/substrate. All fatty acid did not show any difference in $\varepsilon_{lipid/water}$ with increasing salinity and no significant correlation was observed between $\varepsilon_{lipid/water}$ and salinity for the different fatty acids ($R^2 < 0.25$; p values > 0.7).

Table 1: ε values of fatty acids produced by *Pseudomonas* str. LFY10 at three different salinities.

Salinity	δD_{water} [‰]	$\delta D_{glucose}$ [‰]	$\varepsilon_{lipid/water}$ [‰]			$\varepsilon_{lipid/glucose}$ [‰]		
			C16:1*	C16:0	C18:1ϕ	C16:1*	C16:0	C18:1ϕ
6.4	-58±1	-8±11	96±5	122±2	121±1	35±5	60±2	59±1
10.9	-56±3	-8±11	92±1	116±2	116±3	34±1	57±2	56±3
16.9	-57±2	-8±11	97±2	118±4	118±2	38±2	58±4	57±2

*n*C16:1*: double bond is at the ω7 position; *n*C18:1ϕ: double bond is at the ω7 position.

Batch cultures of *Isochrysis galbana* were grown at six different salinities (M'Boule et al., 2014) and harvested at three different growth phases (Chivall et al., 2014). *I. galbana* produced *n*C14:0, *n*C16:0 and *n*C18:1ω9 fatty acids at all salinities and growth phases (Table 2). Low amounts of the *n*C18:0 and

other, unsaturated, fatty acids were also detected. Hydrogen isotope ratios were determined for the *n*C14:0, *n*C16:0 and *n*C18:1 fatty acids. The $\varepsilon_{lipid/water}$ values of fatty acids produced by *I. galbana* at the highest salinity (~35) has been reported previously (Heinzelmann et al., 2015). During all three growth phases $\varepsilon_{lipid/water}$ for individual fatty acids became less negative with increasing salinity (Table 2). The $\varepsilon_{lipid/water}$ values for all three fatty acids showed a strong correlation with salinity at every growth phase studied (Figure 1).

Table 2: ε values of fatty acids produced by *Isochrysis galbana* at different salinities and during different growth phases. δD_{water} and salinity values are the mean of measurements taken before inoculation and at the time of sampling (Chivall et al., 2014). $\varepsilon_{lipid/water}$ values of fatty acids produced at salinity ~35 were taken from Heinzelmann et al. 2015.

Growth phase	Salinity	δD_{water} [‰]	$\varepsilon_{lipid/water}$ [‰]		
			C14:0	C16:0	C18:1$^{\phi}$
exponential	10.3	-29±1	-269±0	-260±2	-192±1
	15.3	-22±1	-268±1	-254±1	-186±1
	20.3	-17±2	-260±1	-246±3	-170±2
	25.2	-9±2	-247±5	-234±6	-162±6
	30.3	-3±2	-239±0	-228±2	-161±1
	35.6	+4±2*	-237±1*	-233±0*	-149±4*
stationary	10.4	-27±1	-248±1	-242±2	-207±2
	15.3	-21±1	-251±1	-244±2	-203±0
	20.4	-15±1	-220±6	-225±1	-205±1
	25.3	-10±1	-229±2	-224±2	-196±2
	30.5	-2±1	-223±0	-216±1	-186±1
	35.8	+5±1*	-215±1*	-205±1*	-179±1*
death	10.7	-24±1	-242±1	-238±1	-195±1
	16	-19±2	-230±2	-233±1	-198±0
	21.1	-14±2	-228±0	-225±2	-196±1
	26.4	-5±2	-220±3	-217±2	-192±1
	31.8	+2±2	-216±2	-209±1	-182±3
	37.4	+9±1*	-201±1*	-200±1*	-184±1*

*n*C18:1$^{\phi}$: double bond is at the ω9 position. * data previously reported by Heinzelmann et al. 2015

3.4. Discussion

Fatty acids synthesized by the heterotrophically grown *Pseudomonas* str.

LFY10 are substantially enriched in D compared to the growth water, while fatty acids produced by *I. galbana* are depleted in D. This pattern is consistent with that previously observed for other heterotrophs and photoautotrophs (Sessions et al., 1999; Chikaraishi et al., 2004; Zhang and Sachs, 2007; Zhang et al., 2009a; Heinzelmann et al., 2015), which is related to their metabolism. In the following, we discuss the effect of salinity.

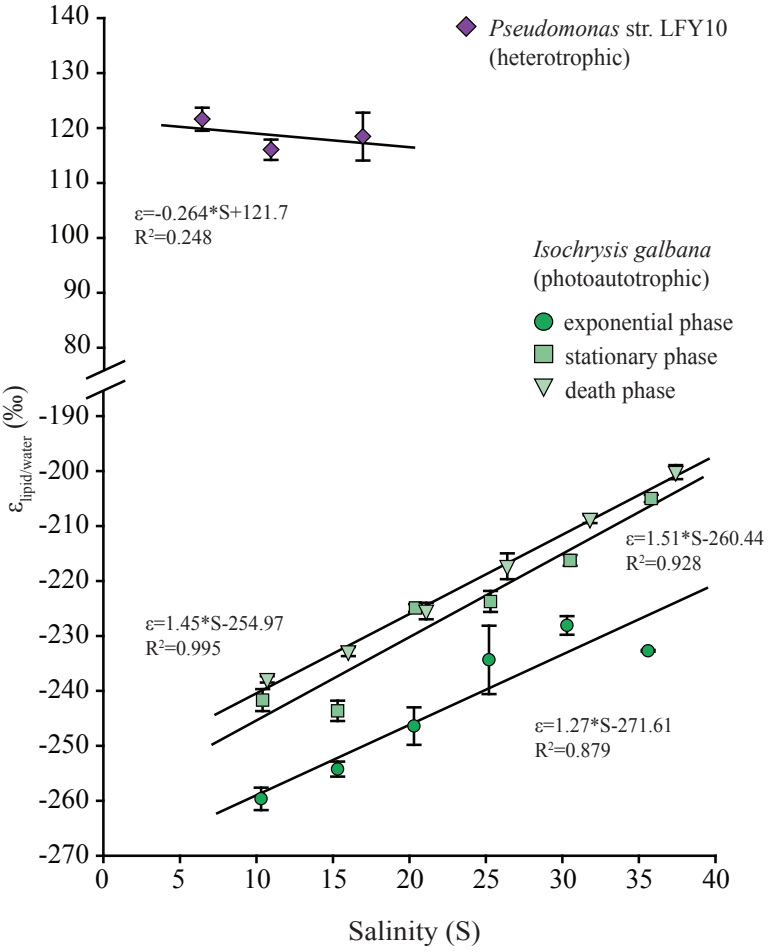

Figure 1: Hydrogen isotopic fractionation ($\varepsilon_{lipid/water}$ values) of the *n*C16:0 fatty acid produced by the heterotrophic *Pseudomonas* str. LFY10 and the photoautotrophic *Isochrysis galbana* against salinity.

Fatty acids of *I. galbana* are produced via the FAS pathway (Lengeler et al., 1999; Huerlimann and Heimann, 2013) and get enriched in D with

increasing salinity. There is a clear correlation between their $\varepsilon_{lipid/water}$ values and salinity with an increase of ~1.5 ‰ per salinity unit for all growth phases (Figure 1). A similar salinity effect has been previously observed for alkenones produced by various haptophyte algae (Schouten et al., 2006; Chivall et al., 2014; M'Boule et al., 2014). Furthermore, environmental data also seems to suggest that photoautotrophic organisms, including cyanobacteria, decrease hydrogen isotopic fractionation at increasing salinities (Sachse and Sachs, 2008), suggesting it may be a common phenomenon among photoautotrophs. The salinity effect was hypothesized to be caused by a reduction of water transport across the cell membrane with increasing salinity, which would lead to a D enrichment of the internal cell water relative to the medium and, therefore, a D enrichment in lipids (Sachse and Sachs, 2008; Sachs and Schwab, 2011). It has been estimated, that the main sources of hydrogen during biosynthesis of fatty acids in general are NADPH (~50 %), the methyl group of acetyl-CoA (~25 %) and internal cell water (~25 %) (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003). In photoautotrophic organisms both NADPH and acetyl-CoA ultimately gain their H from internal cell water during photosynthesis. Therefore, if a D enrichment of the internal cell water is indeed the cause of the D enrichment in alkenones with increasing salinity, then fatty acids should also be enriched in D with salinity, which we indeed observe. Our results, therefore, seem to confirm the hypothesis that the D enrichment of lipids with salinity in photoautotrophs is caused by a D enrichment of internal cell water. The fact that all the hydrogen in fatty acids derives ultimately from internal cell water, which gets enriched with increasing salinity, finally leads to enrichment in D of ~15 ‰ of fatty acids over a range of 10 salinity units in *I. galbana*.

None of the fatty acids produced by *Pseudomonas* str. LFY10 showed significant change of $\varepsilon_{lipid/water}$ over a range of 10 salinity units (Table 1). The lack of change agrees with the observations of Dirghangi and Pagani (2013a), who concluded that changes in the δD of archaeol in the heterotrophically grown euryarchaeote *H. marismortui* is mainly due to growth rate changes and not directly to changes in salinity. There are several possible explanations for this lack of change in $\varepsilon_{lipid/water}$ values with increasing salinity as discussed below.

Differences in hydrogen isotopic fractionation during biosynthesis leading to major differences in the $\varepsilon_{lipid/water}$ values of specific fatty acids have been attributed to different lipid biosynthetic pathways (Fang et al., 2014). However, similar to *I. galbana*, *Pseudomonas* str. LFY10 likely also expresses the FAS

pathway, since this pathway has been described to operate in other *Pseudomonas* species (Hoang and Schweizer, 1997). Therefore, differences in the salinity effect on the $\varepsilon_{lipid/water}$ values of fatty acids in both microorganisms are most likely not due to differences in the pathway of fatty acid biosynthesis.

Salt stress can lead to changes in enzyme activity in biochemical pathways. Danevčič and Stopar (2011) and Fu et al. (2014) observed that salt stress can lead to an up regulation of the glucose-6-phosphate dehydrogenase activity in the OPP pathway. This would lead to an increased contribution of NADPH produced in the OPP pathway, which is D-depleted with an $\varepsilon_{lipid/water}$ value of ~-250 ‰ compared to the general NADPH pool (Schmidt et al., 2003). Increased production of D-depleted NADPH by the OPP pathway at higher salinities could counter act the hypothetical increase in D of the internal cell water as a result of increasing salinity, potentially resulting in the absence of a salinity effect on hydrogen isotopic fractionation in fatty acids produced by *Pseudomonas* str. LFY10.

As previously mentioned, the $\varepsilon_{lipid/water}$ values of fatty acids from *I. galbana* increase by ~15 ‰ over a range of 10 salinity units with all hydrogen of the fatty acids originating from internal cell water. In *Pseudomonas* str. LFY10 on the other hand the majority of H originates from the substrate (~75 %), since NADPH (~50 %) gains its hydrogen from substrate and acetyl-CoA (~25 %), which is also derived from the substrate, and only ~25 % from internal cell water (Zhang et al., 2009a). The δD of glucose (-8±11 ‰) was the same at all salinities and therefore should not result in changes in the D/H ratio of fatty acids. Since approximately one quarter of the H should come from the internal cell water we would expect an D enrichment in fatty acids of one quarter of the magnitude observed in *I. galbana* assuming that both organisms modulate their internal cell water similarly. This should lead to an enrichment of ~3.8 ‰ in fatty acids over a range of 10 salinity units in *Pseudomonas* str. LFY10. Differences of ~3.8 ‰ are, however, close to the external precision of the measurement of 3 ‰ (as suggested by the precision in the measurement of the isotopic composition of our internal standard $\delta D_{Squalene}$ -165±3 ‰). Therefore, it is possible that there is an effect of salinity on the $\varepsilon_{lipid/water}$ values of fatty acids of *Pseudomonas* str. LFY10 but that it is too small to be detected. Alternatively, *Pseudomonas* str. LFY10 might not adapt its internal cell water as it is hypothesized for *I. galbana*.

Although the exact mechanism remains uncertain, we observe that the relationship between salinity and $\varepsilon_{lipid/water}$ values of fatty acids and the mag-

nitude of $\varepsilon_{lipid/water}$ may depend on the metabolism of the source organism. Importantly, it shows that salinity has a relatively small impact on hydrogen isotopic fractionation compared to metabolism. It will be necessary to extend this type of research to a wider range of microorganisms, including chemoautotrophic microorganisms and microorganisms which express a wide metabolic range. Additionally, it will be necessary to study how microorganisms adapt to changes in salinity i.e. changes in metabolic activity and water transport. This will increase our understanding of how environmental and biochemical parameters affect hydrogen isotopic fractionation in lipids and improve the application of the D/H ratio of fatty acids as an indicator for the general metabolism of microbial communities.

3.5. Conclusion

The D/H ratio of fatty acids produced by the heterotrophic microorganism *Pseudomonas* str. LFY10 showed no significant change in $\varepsilon_{lipid/water}$ values with increasing salinity, whereas fatty acids produced by the photoautotrophic algae *Isochrysis galbana* became increasingly enriched in D with increasing salinity. This suggests that the effect of salinity on the D/H ratio of fatty acids depends on the metabolism of the organism. This difference can likely be explained by the fact that hydrogen in fatty acids in heterotrophs are only for a small part derived from internal cell water whereas *I. galbana* gains all its hydrogen from internal cell water. Overall, our results shows that salinity has a relatively small to minor effect on hydrogen isotopic fractionation compared to metabolism.

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Part II

Application of D/H ratio of fatty acids as an indicator of metabolism in natural settings

Chapter 4

Critical assessment of glyco- and phospholipid separation using silica chromatography

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Abstract

Phospholipid derived fatty acids (PLFA) are commonly used to characterize microbial communities *in situ* and the phylogenetic position of newly isolated microorganisms. PLFAs are obtained through separation of phospholipids from glycolipids and neutral lipids using silica column chromatography. We evaluated the performance of this separation method for the first time using direct detection of intact polar lipids (IPL) with high performance liquid chromatography-mass spectrometry (HPLC-MS). We show that the phospholipid fraction does not, under standard or modified conditions, contain only phospholipids, but also other lipid classes such as glycolipids, betaine lipids and sulfoquinovosyldiacylglycerols. Thus, commonly reported PLFA compositions likely are not purely derived from phospholipids and perhaps may not be representative of fatty acids present in living microbes.

4.1. Introduction

Examining microbial communities *in situ* is one of the major challenges in microbial ecology. Traditionally, isolation and cultivation techniques were used, next to microscopic observations, to characterize microorganisms in environmental samples. This traditional approach has its limitations as it was estimated that, depending on the habitat, only about 0.001 to 1% of all microorganisms are cultivable by standard techniques (Amann et al., 1995). Over the last two decades, a number of cultivation-independent techniques for the examination of microbial community composition have been established, using in particular genomic techniques.

One of the earliest alternative approaches to study microbial communities independent of cultivation was the analysis of the composition and abundance of fatty acids in environmental samples and comparison to the fatty acid composition of pure cultures (Shaw, 1974; Lechevalier, 1977; Bobbie and White, 1980). Fatty acids do not occur as such in living biomass but as intact polar lipids (IPL) with the fatty acids esterified via either a glycerol or amide moiety to a polar head group. These fatty acids are released after cell death and can persist in natural environments. Therefore, fatty acids derived from living biomass have to be distinguished from those derived from dead and fossil biomass when analysing microbial communities *in situ*. One way to do this is to study phospholipid derived fatty acids (PLFA) as phospholipids are thought to be relatively labile and fall apart shortly after cell death (White et al., 1979; Harvey et al., 1986). Furthermore, they are the major lipids in cell membranes, and are rarely used as storage products. In contrast, glycolipids are generally classified as storage products (Kates, 1964), although it was recently shown, that they can also be major membrane lipid components in chloroplasts of plants, algae and in cyanobacteria (Hölzl and Dörmann, 2007). PLFAs are thus commonly used as indicators for living microbes. The interpretation of the PLFA patterns of environmental samples is done by comparison with the PLFA patterns of microorganisms grown in pure cultures (Rütters et al., 2002; Boschker, 2004; Dijkman et al., 2010). Furthermore, they are used to investigate metabolic activity *in situ* by stable isotope analysis in combination with labelling experiments (Boschker et al., 1998; Boschker and Middelburg, 2002). PLFA analysis is also often used as chemotaxonomic information to characterize the phylogenetic position of new microorganisms (Bodelier et al., 2009).

In order to obtain PLFAs rather than free fatty acids or fatty acids con-

tained in storage lipids, Vorbeck and Marinetti (1965) proposed a method to separate bacterial lipids in a 'neutral lipid', 'glycolipid' and a 'phospholipid' fraction by applying a silicic acid column and eluting the fractions with different mixtures of chloroform, acetone and methanol (MeOH). The obtained phospholipid fraction was subsequently hydrolysed to obtain phospholipid-derived fatty acids which could be analysed by gas chromatography. The efficacy of the silicic acid column separation was verified by measuring the phosphorus and the carbohydrate content in the different fractions using colorimetric methods (carbohydrates by the anthrone reaction after Radin et al. (Radin et al., 1955) and phosphorus content by the molybdenum blue method after Harris and Popat (Harris and Popat, 1954) and modified by Marinetti et al. (Marinetti et al., 1957). The separation method has subsequently over the years been modified by decreasing the volumes of eluents used, replacing chloroform with the less toxic dichloromethane (DCM) and the use of pure acetone and methanol, to obtain the neutral, glyco- and phospholipid fractions, respectively (King et al., 1977; Gehron and White, 1983; Guckert et al., 1985; Boschker, 2004). This modified separation method is now routinely used in environmental studies as well as for chemotaxonomy of microbes belonging to the bacteria and eukaryotes (White and Frerman, 1967; Rajendran et al., 1992; Zelles, 1999; Dijkman et al., 2010). However, after the initial studies, the performance of the separations has been rarely re-evaluated, particularly whether the PLFAs are truly derived from phospholipids only. Additionally, recent studies have shown, that both betaine lipids and sulfoquinovosyldiacylglycerols (SQDG) are abundant IPLs in the marine environment (Van Mooy et al., 2006; Van Mooy et al., 2009; Van Mooy and Fredricks, 2010; Brandsma et al., 2012a; Brandsma et al., 2012b). The fate of these IPLs after separation on a silica column is, to the best of our knowledge, unknown and thus it is unclear to what degree the fatty acids contained in these membrane lipids are accounted for in PLFA analysis.

In the last 15 years analytical techniques were developed that made it possible to directly analyse IPLs, including phospholipids, using high performance liquid chromatography mass spectrometry (HPLC-MS). Typically, IPLs are separated according to the polarity of the headgroup and identified by multistage mass spectrometry (Rütters et al., 2002; Zink et al., 2003; Sturt et al., 2004). However, this type of analysis does not allow detailed identification of the various fatty acids contained in the IPLs. For detailed identification, separation and hydrolysis of the lipid extract and subsequent GC(MS) analysis is still required. Additionally, for stable isotope probing, which requires

GC-amenable compounds, PLFAs are still needed. Therefore, PLFA analysis is still an important method within microbial ecology.

In this study we have re-assessed the composition of the different chromatographic fractions isolated by silica chromatography, using lipid extracts from different environmental samples. Through direct analysis of IPLs using HPLC-MS we accurately studied the fate of several environmentally important types of IPLs during one of the most used chromatographic fractionations and assessed whether PLFAs are truly representative of membrane lipids.

4.2. Material and Methods

4.2.1. Sampling

A marine sediment was collected in January 2012 during low tide in the Mokbaai on the Dutch Wadden Island Texel. A microbial mat sediment was collected in August 2010 on the Dutch Wadden Island Schiermonnikoog. Similar microbial mats from the same location were described by Bolhuis and Stal (Bolhuis and Stal, 2011). The microbial mat was sampled from the top of the sediment and immediately sealed. Sediment cores with a diameter of 7 cm were taken by hand, sliced on location and the first cm was collected. All samples were freeze dried, homogenized at stored at - 40 °C until extraction.

4.2.2. Extraction of intact polar lipids

The freeze dried samples were extracted using a modified Bligh-Dyer method (Bligh and Dyer, 1959; Rütters et al., 2002). In short, the samples were extracted with MeOH/DCM/Phosphate buffer 2/1/0.8 (v/v/v) ultrasonically three times for 10 min. The supernatants were collected to which DCM and phosphate-buffer were added to achieve a phase separation. The DCM fraction was transferred to a round bottom flask and the aqueous phase was washed three times with DCM. All DCM fractions were combined and dried using a rotary evaporator. The resulting Bligh Dyer Extract (BDE) was transferred into a vial with DCM/MeOH 9:1, further dried under a nitrogen flow and stored dry at -20°C.

4.2.3. Separation of different IPL-classes

The BDE was split into three equal, by volume, aliquots. All subsequent treatments were performed in triplicate. In order to obtain the so-called neutral, glyco- and phospholipid fractions the BDE was separated on a DCM pre-rinsed silica column (0.5 g; activated for 3 h at 150 °C) eluting with 7 mL DCM, 7 mL acetone and 15 mL MeOH, respectively (Boschker, 2004). The resulting fractions were dried under nitrogen and stored at -20 °C.

In order to test the effect of slightly different solvents mixtures the BDE was also separated on a pre-washed silica column (0.5 g) by eluting with 7 mL of DCM, 7 mL of acetone/MeOH (99/1) and 15 mL MeOH, respectively. These fractions were also dried under nitrogen and stored at -20 °C.

4.2.4. IPL analysis

For the IPL analysis, the original BDE and the different chromatographic fractions were dissolved and filtered in 250 µL of injection solvent (hexane/IPA/H_2O 718/271/10). IPLs were analysed directly afterwards on a high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC/ESI-MS^2) after Sturt et al. (Sturt et al., 2004) with some modifications. We used an Agilent 1200 series LC (Agilent, San Jose, CA), which was equipped with a thermostatted auto-injector and a column oven and coupled to a Thermo LTQ XL linear ion trap with an Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, MA). To each sample 5 µg/mL of 1-O-hexadecyl-2-acetoyl-sn-glycero-3-phosphocholine (C16 PAF) standard was added in order to monitor the performance of the machine. Separation was achieved on a Lichrosphere diol column (250 x 2.1 mm, 5 µm particles: Alltech Associates Inc., Deerfield, IL) maintained at 30 °C. The following elution program was used with a flow rate of 0.2 mL/min: 100% A for 1 min, followed by a linear gradient to 66% A: 34% B in 17 min, maintained for 12 min, followed by a linear gradient to 35% A: 65% B in 15 min, where A: hexane/2-propamol/formic acid/14.8 M NH_{3aq} (79/20/0.12/0.04 [v/v/v/v]) and B: 2-propanol/H_2O/formic acid/14.8 M NH_{3aq} (88/10/0.12/0.04 [v/v/v/v]). The total run time was 60 min with a re-equilibration period of 20 min in between runs. The settings for the ESI were: capillary temperature 275 °C, sheath gas (N2) pressure 25 arbitrary units (AU), auxiliary gas (N2) pressure 15 AU, sweep gas (N2) pressure 20 AU, spray voltage 4.5 kV. The lipid extract was analysed by positive-ion scanning (*m/z* 400 to 2000), which was

followed by a data-dependent MS^2 experiment where the four most abundant masses in the mass spectrum were fragmented (normalized collision energy 25; isolation width 5.0; activation Q 0.175).

For each IPL class, the individual IPL species were identified in the total BDE by their fragmentation pattern in MS^2 (Brügger et al., 1997). In order to evaluate the chromatographic behaviour of the different IPL classes during silica chromatography, the three to five most abundant IPL species within each IPL class were selected for quantification. The peak area of these IPL species were integrated in their MS^1 mass chromatograms. Since the same equivalent amount of extract was consistently injected, the peak areas of the total extract and the different fractions could be directly compared. To calculate the distribution of the IPL's over the fractions, the peak areas of the selected species within an IPL class in each fraction were summed and the percentage relative to the total amount in both the acetone and the methanol fraction was calculated. The final percentage is the average of the percentage in each of the triplicates. To determine the recovery, the peak areas of the selected species within an IPL class in each fraction was summed up and the percentage relative to the amount in the BDE could be calculated as identical injection volumes and concentrations were used for the fractions and BDE, respectively. The final value is an average of the triplicates. Due to different ionization efficiencies of different IPL classes, the different treatments for each IPL class were compared and not the amount of IPL classes with each other within a single fraction.

4.3. Results and Discussion

4.3.1. IPL composition

To evaluate the commonly used separation method in PLFA analysis, we subjected extracts from a microbial mat (Schiermonnikoog, the Netherlands) and a marine sediment (Mokbaai, the Netherlands) to the various silica chromatography protocols.

We first studied the IPL composition of the whole extract of both samples prior to chromatographic separation (Figure 1). This showed that the microbial mat sediment contained two types of glycolipids, monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) (see Figure 2 for structures). For both of these IPLs classes, the main sugar moiety has

been recognized as galactose which is why they are in general referred to as galacto-lipids (Van Mooy and Fredricks, 2010). Both of these glycolipids mainly contained C_{16} and C_{18} fatty acids with 0-3 double bounds in different combinations (Table 1).

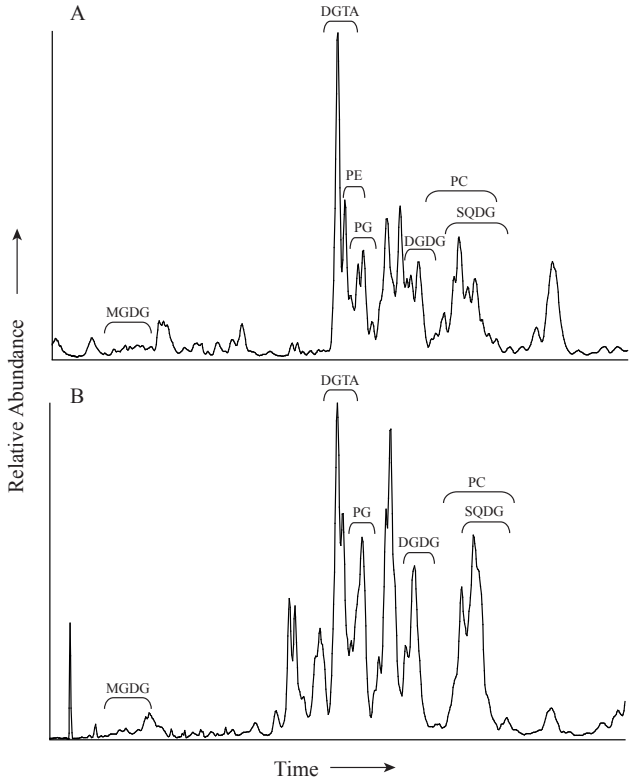

Figure 1: HPLC/ESI-MS base peak chromatogram of the Bligh Dyer extract of (A) the Schiermonnikoog microbial mat and (B) the Mokbaai sediment. monogalactosyldiacylglycerol (MGDG); digalactosyldiacylgylcerol (DGDG); phosphatidylglycerol (PG); phosphatidylcholine (PC); phosphatidylethanolamine (PE); sulfoquinovosyldiacylglycerol (SQDG); G. diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA). For structures of IPLs, see Figure 2.

In addition to glycolipids, the microbial mat sediment also contained three different phospholipid classes, phosphatidylglyceride (PG), phosphatidylcholine (PC) and phosphatidylethanolamines (PE) (Figures 1 and 2). The different phospholipid classes contained mainly two fatty acids with a combined total number of carbon atoms 30 to 40 and total amount of double bonds of 0-6 (Table 1). Due to the lack of specific fragments we were not able to assess the composition of individual fatty acids in these lipid classes. Sulfo-

quinovosyldiacylglycerols (SQDG) (Figure 2), which structurally resemble glycolipids but contain a sulphate group, were also found in the extract. The SQDGs contained mainly C_{16} and C_{18} fatty acids with 0-3 double bounds. Lipids with a diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA) head group (Figure 2), belonging to the class of betaine lipids, were also detected in the extract. The DGTAs contained mainly C_{16} and C_{18} fatty acids with 0-2 double bounds (Fig. 1 A).

Table 1: Fatty acid composition of different IPL classes in the BDE. For structures and acronyms of IPLs, see Figure 1 and 2.

	Fatty acid composition	
Lipid classes	Microbial mat	Marine sediment
MGDG	C14:0, C16:0-16:3, C18:0-18:4, C20:3-20:5	C14:0, C16:0-16:4, C17:0-17:1, C17:3, C18:1, C18:4, C20:4-20:5
DGDG	C14:0, C16:0-16:3, C18:3-18:4	C16:0-16:3, C20:4-20:5
PG*	C30:0-30:1, C31:1, C32:1-32:3, C33:1, C34:1-34:3, C36:2	C32:1-32:2, C33:1, C34:1-34:2, C36:2, C36:5-36:6
PC*	C30:0-30:1, C31:1, C32:0-32:4, C33:1, C34:1-34:2, C34:4-34:5, C35:1-35:2, C35:5, C36:2, C36:5-36:6, C37:2, C37:4-37:6, C38:2, C38:4-38:6, C39:5-39:6, C40:6	C31:8, C32:4, C33:8-33:10, C34:1, C34:4-34:5, C35:1, C35:4-35:5, C36:6-36:7, C37:5-37:6, C37:9, C37:12, C38:5-38:6, C38:9, C39:0, C39:2-39:3, C39:11, C40:5-40:6, C40:8, C41:0-41:4, C42:6
PE*	C32:1, C34:1-34:2	n.d.
SQDG	C14:0, C16:0-16:2, C18:0-18:3	C16:0-16:3, C20:4-20:5
DGTA	C14:0, C16:0-16:1, C18:0-18:2, C19:0-19:1, C20:5	C14:0-14:1, C16:0-16:1, C17:0, C18:1-18:2, C20:1

*combined total number of carbon atoms and total amount of double bound equivalents for both fatty acid moieties

The BDE extract of the Mokbaai sediment also contained MGDGs and DGDGs, both containing mainly C_{16} and C_{20} fatty acids with 0-5 double bounds (Table 1). Of the different phospholipid classes, both PGs and a large variety of different PCs could be identified, containing two fatty acids with a combined total carbon number of 31 to 42 and a total number of double bonds of 0-12. SQDGs were much less diverse in their fatty acid composition, containing mainly C_{16} fatty acids with 0-2 double bounds (Table 1). We also

found DGTAs in this sample, consisting mainly of C_{16} and C_{18} fatty acids with no or one double bound (Fig. 1 B).

Thus, both the microbial mat and the marine sediment contain a variety of IPLs including glycolipids (MGDG and DGDG) as well as phospholipids (PG, PC, and PE) making them suitable for evaluation of the commonly used silica separation method. In addition, they contained other IPLs such as SQDG and betaine lipids (DGTA) for which it is not known in which fraction they elute.

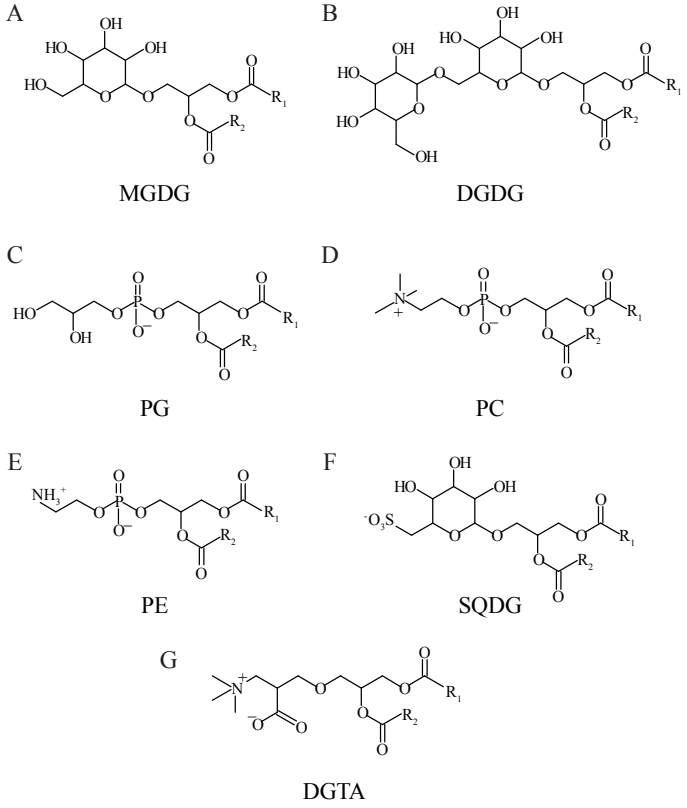

Figure 2: Overview of head groups of analyzed intact polar lipids (IPL). A. MGDG; B. DGDG; C. PG; D. PC; E. PE; F. SQDG; G. DGTA. R_1 and R_2 represent different fatty acid moieties. For acronyms of IPLs, see Figure 1.

4.3.2. Standard separation method

Analysis of the different chromatographic fractions obtained from the most

commonly used silica gel separation method showed for the microbial mat that MGDGs were distributed evenly between the acetone ('glycolipid') and the methanol ('phospholipid') fraction, 54±5% and 46±5%, respectively (Table 2). In contrast, all of the DGDGs were detected in the methanol fraction. Of the phospholipids only PCs eluted exclusively in the methanol fraction. Both PGs and PEs were also present in minor amounts in the acetone fraction, i.e. 9±1% and 11±1% in the acetone and 91±1% and 89±1% in the methanol fraction, respectively. Of the 'non-phospholipids', the majority (68±3) of the SQDGs eluted in the methanol fraction, although a relatively high percentage (32±1%) eluted in the acetone fraction. DGTAs were exclusively found in the methanol fraction (Table 2). We also assessed the overall recovery of the different IPLs by normalizing on the original amounts in the extract. Of the DGDGs, SQDGs, DGTAs, and PCs 75-98% could be recovered in the different fractions. All PGs could be recovered in the different fractions. Of all studied IPL classes the recovery of the PEs is the lowest, with 30% not recovered from the column (Figure 3 A). The recovery of the MGDGs is higher than 100%, which at first instance may be surprising. However, this could be due to ion suppression, i.e. the ionization of the early eluting MGDGs might be supressed due to matrix effects in the complex total BDE extract. This ion suppression may be much less in the cleaner chromatographic fractions leading to enhanced ionization and thus an increase in peak areas.

The distributions of the IPL classes over the different chromatographic fractions for the Mokbaai sediment extract were similar to those observed for the microbial mat (Table 2). The majority (61±5%) of the MGDGs eluted in the acetone fraction versus 39±5% in the methanol fraction. The DGDGs eluted nearly completely (96±5%) in the methanol fraction, with the remainder (4±5%) eluting in the acetone fraction. The PC and PGs eluted in the methanol fraction, with a small percentage of the PGs (9±2%) eluting in the acetone fraction. For the SQDGs, 67±3% eluted in the methanol and 33±3% in the acetone fraction. All DGTAs were found in the methanol fraction (Table 2). For recovery, similar results were found as for the microbial mat sediment. For example, 90-230% of the SQDGs, MGDGs and the DGTAs were recovered after separation, while about 20% of the PGs and PCs could not be recovered. The lowest recovery was for the DGDGs with 55% (Figure 3 B).

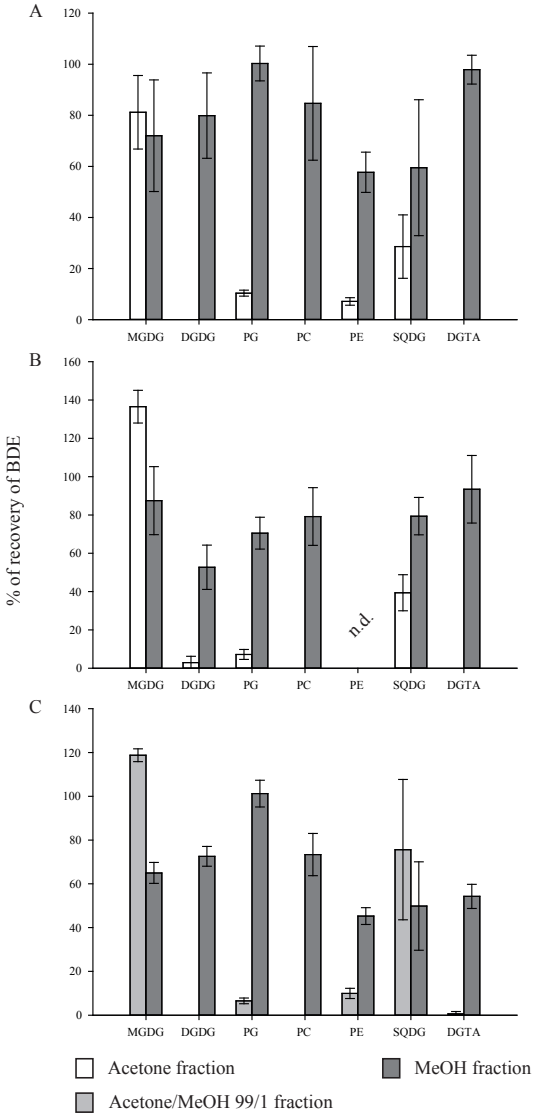

Figure 3: Recovery of different IPL classes after separation on a silica column of (A) + (C) the Schiermonnikoog microbial mat and (B) of the Mokbaai sediment normalized to the abundance in the original extract. For structures and acronyms of IPLs, see Figure 1 and 2.

Our results show that, independent of sample type, a large part of the phospholipids elute in the 'phospholipid' fraction as expected. However, crucially a major part of the glycolipids also elute in this fraction and not, as anticipated, in the glycolipid fraction. Furthermore, 'non' phospholipids like SQDGs and

DGTA also elute in the 'phospholipid' fraction while SQDGs are also found in the 'glycolipid' fraction. Both lipid classes contribute to the membrane lipid pool, especially under phosphate limitation (Van Mooy et al., 2006; Van Mooy et al., 2009) and therefore have to be considered when looking at membrane lipids as biomarkers for living biomass. Neither the 'glyco'- nor the phospholipid' fraction thus consist of glyco- or phospholipids only.

These results indicate that using this common separation method, a complete separation between glycolipid and phospholipid cannot be obtained and that the 'PLFA' reported are not exclusively derived from phospholipids but also from glycolipids, betaine lipids and SQDGs. Therefore, we experimented with a slightly modified elution scheme in an attempt to optimize separation.

4.3.3. Adaptation of separation method

To assess the possibility of obtaining better separation between glyco- and phospholipids we used a mixture of acetone and methanol (99/1,v/v) as second eluent. This slightly more polar solvent should result in a slightly different separation, with possibly more of the glycolipids, especially MGDGs, ending up in the second fraction. This modified elution scheme was tested on the microbial mat extract. Indeed, slight differences in the separation of the different lipid classes were observed. For example, the major part of the MGDGs now ended up in the acetone/methanol fraction and 35±1% in the methanol fraction (Table 2). The separation of DGDGs is the same compared to that of the standard method with all lipids eluting in the methanol fraction. As in the standard method all PCs eluted in the methanol fraction, with slightly less PGs (6±1% compared to 9±1%), but slightly more PEs in the acetone/methanol fraction (18±4% compared to 11±1%). The largest difference was observed for the SQDGs, with twice as much being found in the acetone/methanol fraction compared to the original acetone fraction, 60±8% vs. 32±1%. Concerning the DGTAs, 99±2% were still found in the methanol fraction (Table 2). Compared to the standard separation, recoveries for DGDGs, PCs, PEs and PGs were similar, but for MGDGs and SQDGs the recovery was increased from 153% to 184% respond from 75% to 120%, respectively. The apparently high recovery of SQDG, could again be due to ion suppression and therefore a suboptimal ionization of the SQDGs in the complex total BDE, similar to what is observed for the MGDGs. In contrast, the recovery of the DGTAs had decreased by 40% (Figure 3 C).

These results show that an increase in polarity of the second eluent chang-

es the elution pattern of some of the IPL classes, but does not result in an improved separation of glyco- and phospholipids. Further adaptations of the solvent mixture ratios are unlikely to succeed in providing a better separation as either more of the glycolipids will end up in the phospholipid fraction or phospholipids will elute in the glycolipid fraction. This is due to the fact, that the separation on a silica column is based on the polarity of the head groups. Therefore, MGDGs will always elute together with PCs and SQDGs.

Table 2: Elution of different IPL classes after separation of BDE on a silica column. For structures and acronyms of IPLs, see Figure 1 and 2.

Lipid classes	Microbial mat standard separation		Marine sediment standard separation		Microbial mat modified separation	
	Acetone fraction [%]	MeOH fraction [%]	Acetone fraction [%]	MeOH fraction [%]	Acetone fraction [%]	MeOH fraction [%]
MGDG	54±5	46±5	61±5	39±5	65±1	35±1
DGDG	0±0	100±0	4±5	96±5	0±0	100±0
PG	9±1	91±1	9±2	91±2	6±1	94±1
PC	0±0	100±0	0±0	100±0	0±0	100±0
PE	11±1	89±1	n.d.	n.d.	18±4	82±4
SQDG	32±1	68±1	33±3	67±3	60±8	40±8
DGTA	0±0	100±0	0±0	100±0	1±2	99±2

4.3.4. Implications

Our results show that it is not possible to obtain a complete separation between phospho- and glycolipids using the commonly used silica acid chromatography separation method. Furthermore, the recovery varies between different IPL classes, with some of the phospholipids (e.g. PE) not fully recovered in the 'phospholipid' fraction. Thus, previous studies examining PLFAs, might have analysed fractions that also contain fatty acids derived from glycolipids, betaine lipids and to some extent SQDGs, while missing out a certain amount of phospholipids and SQDGs. In the marine environment, different IPLs can contain different fatty acids (Schubotz et al., 2009; Van Mooy and Fredricks, 2010; Brandsma et al., 2012a; Brandsma et al., 2012b), and specific fatty acids do not necessarily derive always from the same lipid class. This will give a biased view of fatty acids present in microbial commu-

nities *in situ*. Furthermore, PLFA analysis of microbial isolates will also give an incomplete picture of the full diversity of fatty acids derived from IPLs. Therefore, microbial fingerprints basing on PLFA fractions are unlikely to reflect the true fatty acid pattern coming from phospholipids only. Future studies using PLFA fingerprints of environmental samples and microbial biomass have to be at least aware of this less than perfect separation and, preferably, combine this with direct analysis of IPLs. Alternatively, one can just separate a neutral and a polar fraction (using DCM and methanol) with latter containing the full suite of intact polar lipids.

4.4. Conclusion

Silica column chromatography results in incomplete separation of glyco- and phospholipids leading to the presence of 'non' phospholipids in the PLFA fraction. Therefore, studies of microbial activity using stable isotope analysis of PLFAs may be biased by fatty acids derived from glycolipids, SQDGs and betaine lipids. Because of the imperfect separation and the fact that other IPL classes such as SQDGs and betaines (and even MGDGs and DGDGs) are also part of the membrane lipids of living and active microorganisms a separation in only two fractions, a neutral fraction contain free fatty acids of dead biomass, and a polar fraction, containing all intact polar lipids, is preferable. The 'polar lipid' derived fatty acids can then be used to study microbial communities and their activity.

Acknowledgment

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Chapter 5

Seasonal changes in the D/H ratio of fatty acids of pelagic microorganisms in the coastal North Sea

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Abstract

Culture studies of microorganisms have shown that the hydrogen isotopic composition of fatty acids depends on their metabolism, but there are only few environmental studies available to confirm this observation. Here we studied the seasonal variability of the deuterium/hydrogen (D/H) ratio of fatty acids in the coastal Dutch North Sea and compared this with the diversity of the phyto- and bacterioplankton. Over the year, the stable hydrogen isotopic fractionation factor ε between fatty acids and water ranged between -172 ‰ and -237 ‰, the algal-derived polyunsaturated fatty acid *n*C20:5 being the most D-depleted and *n*C18:0 the least D-depleted fatty acid. The D-depleted *n*C20:5 is in agreement with culture studies, which indicate that photoautotrophic microorganisms produce fatty acids which are significantly depleted in D relative to water. The $\varepsilon_{lipid/water}$ of all fatty acids showed a transient shift towards increased fractionation during the spring phytoplankton bloom, as indicated by increasing chlorophyll *a* concentrations and relative abundance of the *n*C20:5 PUFA, suggesting increased contributions of photoautotrophy. Time periods with decreased fractionation (less negative $\varepsilon_{lipid/water}$ values) can be explained by an increased contribution by heterotrophy to the fatty acid pool. Our results show that the D is a useful tool to assess the community metabolism of coastal plankton.

5.1. Introduction

The hydrogen isotopic composition of fatty acids of microorganisms has been shown to depend on different factors like metabolism, salinity, biosynthetic pathways, growth phase and temperature (Zhang et al., 2009a; Zhang et al., 2009b; Dirghangi and Pagani, 2013b; Fang et al., 2014; Heinzelmann et al., 2015a; Heinzelmann et al., 2015b). While most of these factors lead to relatively small variations in the deuterium to hydrogen (D/H) ratio of fatty acids (10-20 ‰), differences in the central metabolism of microorganisms have a much more pronounced effect. Both photo- and chemoautotrophs produce fatty acids depleted in D compared to growth water with the stable hydrogen isotopic fractionation factor ε between fatty acids and water ($\varepsilon_{lipid/water}$) ranging between -150 ‰ to -250 ‰ and -250 ‰ and -400 ‰, respectively (Sessions et al., 2002; Chikaraishi et al., 2004; Valentine et al., 2004; Zhang and Sachs, 2007; Campbell et al., 2009; Zhang et al., 2009a; Heinzelmann et al., 2015a; Heinzelmann et al., 2015b). In contrast, heterotrophs produce fatty acids with either a relatively minor depletion or an enrichment in D compared to the growth water with $\varepsilon_{lipid/water}$ values ranging between -150 ‰ and +200 ‰ (Sessions et al., 2002; Zhang et al., 2009a; Dirghangi and Pagani, 2013b; Fang et al., 2014; Heinzelmann et al., 2015a; Heinzelmann et al., 2015b). The differences in hydrogen isotopic composition of fatty acids produced by organisms expressing different core metabolisms have been attributed to the D/H ratio of nicotinamide adenine dinucleotide phosphate (NADPH) (Zhang et al., 2009a). NADPH can be generated by a variety of different reactions in different metabolic pathways (each associated with different hydrogen isotopic fractionations) and is subsequently used as the main source of hydrogen in lipid biosynthesis (Saito et al., 1980; Robins et al., 2003; Schmidt et al., 2003).

Although the metabolism of a microorganism in pure culture is reflected by the D/H ratio of its fatty acids, it is not clear if the D/H ratio of fatty acids from environmental microbial communities can be used to assess the 'integrated' core metabolisms in nature. Culture conditions rarely represent environmental conditions since cultures are typically axenic and use a single substrate, they do not take into account microbial interactions, and they test a limited number of potential substrates, energy sources and core metabolisms. While previous studies have observed a wide range in the D/H ratio of lipids derived both from the water column and sediments (Jones et al., 2008; Li et al., 2009), only one study has been performed that links the D/H ratio of fatty acids from naturally occurring microbial communities to metabolisms possibly expressed by the members of those communities (Osburn et al., 2011). This study showed that different microbial communities from various

hot springs in Yellowstone National Park produce fatty acids with hydrogen isotopic compositions in line with the metabolism expressed by the source organism. The D/H ratio of specific fatty acids, which could be attributed to microorganisms expressing a specific core metabolism, was within the range expected for that metabolism. On the other hand, the D/H ratio of common or general fatty acids (e.g. *n*C16:0) allowed for assessing the metabolism of the main contributors of these more general fatty acid, but not necessarily the metabolism of the dominant community members (Osburn et al., 2011). These results show the applicability of this new method, but the ecosystem in which it was tested (hot spring microbial communities) is considered to be of relatively low diversity. Therefore, this method needs to be applied and evaluated in more complex and diverse microbial communities.

Here, we studied the seasonal variability of the hydrogen isotopic composition of fatty acids from coastal North Sea water sampled from the jetty at the Royal Netherlands Institute for Sea Research (NIOZ) in order to examine the relationship between hydrogen isotope fractionation in fatty acids and the general metabolism of the community. Time series studies have been previously performed at the NIOZ jetty to determine phytoplankton and prokaryotic abundances and composition (Brussaard et al., 1996; Philippart et al., 2000; Alderkamp et al., 2006; Philippart et al., 2010; Pitcher et al., 2011; Brandsma et al., 2012b; Sintes et al., 2013), lipid composition (Pitcher et al., 2011; Brandsma et al., 2012b), and chlorophyll *a* concentration (Philippart et al., 2010). Typically, the spring bloom in the coastal North Sea is predominantly formed by *Phaeocystis globosa*, followed directly by a bloom of various diatom species, a second moderate diatom bloom of *Thalassiosira spp.* and *Chaetoceros socialis* that occurs in early summer and an autumn bloom is formed by *Thalassiosira spp.*, *C. socialis*, cryptophytes and cyanobacteria (Cadée and Hegeman, 2002; Brandsma et al., 2012b), although the latter bloom seems to have weakened over the last years (Philippart et al., 2010). The abundance of bacteria co-varies with algal blooms and the bacteria are dominated by heterotrophs, e.g. bacteria belonging to the *Bacteroidetes* (Alderkamp et al., 2006), using released organic matter from declining phytoplankton blooms as carbon, nitrogen and phosphate sources. The intact polar lipid (IPL) composition of the microbial community was shown to be composed mainly of phospholipids, sulfoquinovosyldiacylglycerol and betaine lipids with a limited taxonomic potential (Brandsma et al., 2012b). The main source of those lipids was assumed to be the eukaryotic plankton.

This well studied site should allow us to trace the shift from an environment dominated by photoautotrophs during major phytoplankton blooms, towards

an environment with a higher abundance of heterotrophic bacteria following the end of the bloom. These shifts in the community structure should be reflected in the D/H ratio of fatty acids. We, therefore, analysed the D/H ratio of polar lipid derived fatty acids (PLFA) over a seasonal cycle and compared this with phytoplankton composition data and abundance and information on the bacterial diversity obtained by 16S rRNA gene amplicon sequencing.

5.2. Material and Methods

5.2.1. Study site and sampling

Surface water samples were collected from September 2010 until December 2011 from the NIOZ sampling jetty in the Marsdiep at the western entrance of the North Sea into the Wadden Sea at the island of Texel (53°00'06" N 4°47'21" E). Samples were taken during high tide to ensure that the water sampled was North Sea water.

For lipid analysis measured volumes of water (ca. 9-11 L) were filtered consecutively through pre-ashed 3 and 0.7 µm pore size glass fibre filters (GF/F, Pall; 142 mm diameter) and stored at -20 ºC until lipid extraction. For DNA analysis approximately 1 L seawater was filtered through a polycarbonate filter (0.2 µm pore size; 142 mm diameter; Millipore filters) and stored at -80 ºC until extraction.

Salinity measurements were done during the time of sampling with either an Aanderaa Conductivity/Temperature sensor 3211 connected to an Aanderaa datalogger DL3634 (Aanderaa Data Instruments AS, Norway) or a Refractometer/Salinometer Endeco type 102 handheld (Endeco, USA).

For chlorophyll *a* measurements 500 mL sea water was filtered through a 47 mm GF/F filter (0.7 µm pore size, Whatman, GE Healthcare Life Sciences, Little Chalfont, UK) and immediately frozen in liquid nitrogen. Samples were thawed and homogenised with glass beads and extracted with methanol. Chlorophyll *a* concentration was measured with a Dionex high-performance liquid chromatography (HPLC).

Water samples for salinity versus δD_{water} calibration (see below) were sampled weekly between March and September 2013 at high tide. Salinity was determined using a conductivity meter (VWR EC300) calibrated to IAPSO

standard seawater of salinities 10, 30, 35 and 37.

5.2.2. Polar lipid derived fatty acids

Filters were extracted for IPLs and eventually fatty acid analysis. The 0.7 µm filters did not yield enough total lipid extract for analysis. Therefore, only fatty acids obtained from the 3 µm filters were analysed. Due to fast clogging of the filters and a corresponding decrease of the pore size (Sørensen et al., 2013), the 3 µm filters will most likely contain most of the microorganisms present in North Sea water. Freeze dried filters were extracted via a modified Bligh-Dyer method (Bligh and Dyer, 1959; Rütters et al., 2002) with methanol (MeOH)/dichloromethane (DCM)/phosphate buffer (2:1:0.8, vol/vol/vol) using ultrasonication (Heinzelmann et al., 2014). Approximately 0.5 - 1 mg of the Bligh-Dyer extract (BDE) was separated into a neutral and polar lipid fraction using silica column chromatography (Heinzelmann et al., 2014). The BDE was added onto a DCM pre-rinsed silica column (0.5 g; activated for 3 h at 150°C) and eluted with 7 mL of DCM and 15 mL of MeOH. The resulting fractions were dried under nitrogen and stored at -20 °C. PLFAs were obtained via saponification of the MeOH fraction with 1 N KOH in MeOH (96%). The samples were refluxed at 140 °C for 1 h. Afterwards the pH was adjusted to 5 with 2 N HCl/MeOH (1/1), bidistilled H_2O and DCM were added. The MeOH/H_2O layer was washed twice with DCM, the DCM layers were combined and dried over Na_2SO_4. The sample was dried under nitrogen and stored in the fridge. The PLFAs were methylated with boron trifluoride-methanol (BF_3-MeOH) for 5 min at 60 °C. Afterwards H_2O and DCM were added. The H_2O/MeOH layer was washed three times with DCM, and potential traces of water were removed over a small Na_2SO_4 column after which the DCM was evaporated under a stream of nitrogen. In order to obtain a clean PLFA fraction for isotope analysis, the methylated extract was separated over an aluminium oxide (Al_2O_3) column, eluting the methylated PLFAs with three column volumes of DCM. For identification of the position of double bonds in unsaturated fatty acids, the methylated PLFAs were derivatised with dimethyldisulfide (DMDS) (Nichols et al., 1986). Hexane, DMDS and I_2/ether (60 mg/mL) were added to the fatty acids and incubated at 40 °C overnight. After adding hexane, the iodine was deactivated by addition of a 5% aqueous solution of $Na_2S_2O_3$. The aqueous phase was washed twice with hexane. The combined hexane layers were cleaned over Na_2SO_4 and dried under a stream of nitrogen. The dried extracts were stored at 4 °C.

5.2.3. Fatty acid and hydrogen isotope analysis

The fatty acid fractions were analysed by gas chromatography (GC) using an Agilent 6890 gas chromatograph with a flame ionization detector (FID) using a fused silica capillary column (25 m x 320 µm) coated with CP Sil-5 (film thickness 0.12 µm) with helium as carrier gas. The temperature program was as follows: initial temperature 70 °C, increase of temperature to 130 °C with 20 °C min^{-1}, and then to 320 °C with 4 °C min^{-1} which was kept for 10 min. Individual compounds were identified using GC/mass spectrometry (GC/MS) and the position of the double bonds in unsaturated fatty acids was determined after derivatisation with dimethyldisulfide (Heinzelmann et al., 2015b).

Hydrogen isotope analysis of the fatty acid fraction was performed by GC thermal conversion isotope ratio monitoring MS (GC/TC/irMS) using an Agilent 7890 GC connected via Thermo GC Isolink and Conflo IV interfaces to a Thermo Delta V MS according to Chivall et al. (2014). Samples were injected onto an Agilent CP-Sil 5 CB column (25 m × 0.32 mm ID; 0.4 µm film thickness; He carrier gas, 1.0 mL min^{-1}). The GC temperature program was 70 °C to 145 °C at 20 °C min^{-1}, then to 320 °C at 4 °C min^{-1} where it was kept for 15 min. Eluting compounds were converted to H_2 at 1420°C in an Al_2O_3 tube before introduction into the mass spectrometer. The H^{3+} correction factor was determined daily and was constant at 5.3±0.2. A set of standard *n*-alkanes with known isotopic composition (Mixture B prepared by Arndt Schimmelmann, University of Indiana) was analyzed daily prior to analyzing samples in order to monitor the system performance. Samples were only analyzed when the n-alkanes in Mix B had an average deviation from their off-line determined value of <5 ‰. An internal standard, squalane (δD = -170 ‰) was co-injected with each fatty acid sample fraction in order to monitor the precision of the measurements over time with δD = -164±4 ‰. The δD of the individual fatty acids was measured in duplicates and corrected for the added methyl group (Heinzelmann et al., 2015b).

δD of water samples was determined by elemental analysis/TC/irMS (EA/TC/irMS) according to Chivall et al. 2014.

5.2.4. Phytoplankton abundance and diversity

Phytoplankton samples were preserved with acid Lugol's iodine, and cells were counted with a Zeiss inverted microscope using 3 mL counting cham-

bers. Most algae were identified to species level, but some were clustered into taxonomic and size groups (Philippart et al., 2000). For each sampling date in the period from September 2010 to December 2011, the densities of the most abundant phytoplankton species or species' groups were calculated. The three most dominant algal species (or groups) together comprised, on average, more than 60% of the total numbers of marine algae in the Marsdiep during this study period.

5.2.5. DNA extraction

The 0.2 µm polycarbonate filters were defrosted and cut into small pieces with sterile scissors and then transferred into a 50 mL falcon tube. Filter pieces were lysed by bead-beating with ~1 g of sterile 0.1 mm zirconium beads (Biospec, Bartlesville, OK) in 10 mL RLT buffer (Qiagen) and 100 µL β-mercaptoethanol for 10 min. 1/60 volume RNase A (5 µg/µL) was added to the lysate, incubated for 30 min at 37 ºC and afterwards cooled down for 5 min on ice. The lysate was purified with the DNeasy Blood and Tissue kit (Qiagen, Hilden). DNA was eluted with 3x 100 µL AE buffer, the eluates pooled and reconcentrated. DNA quality and concentration was estimated by Nanodrop (Thermo Scientific, Waltham, MA) quantification.

5.2.6. 16S rRNA gene amplicon sequencing and analysis

The general bacterial diversity was assessed by 16S rRNA gene amplicon pyrotag sequencing. The extracted DNA was quantified fluorometrically with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, The Netherlands).

PCR reactions were performed with the universal (Bacteria and Archaea) primers S-D-Arch 0519-a-S-15 (5'-CAG CMG CCG CGG TAA-3') and S-D-Bact-785-a-A-21 (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth et al., 2012) adapted for pyrosequencing by the addition of sequencing adapters and multiplex identifier (MID) sequences. To minimize bias three independent PCR reactions were performed containing: 16.3 µL H_2O, 6 µL HF Phusion buffer, 2.4 µL dNTP (25 mM), 1.5 µL forward and reverse primer (10 µM; each containing an unique MID tail), 0.5 µL Phusion Taq and 2 µL DNA (6 ng/µL). The PCR conditions were following: 98 ºC, 30 s; 25× [98 ºC, 10 s; 53 ºC, 20 s; 72 ºC, 30 s]; 72 ºC, 7 min and 4 ºC, 5 min.

The PCR products were loaded on a 1% agarose gel and stained with SYBR® Safe (Life Technologies, The Netherlands). Bands were excised with a sterile scalpel and purified with Qiaquick Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. PCR purified products were quantified with Quant-iT^{TM} PicoGreen® dsDNA Assay Kit (Life Technologies, The Netherlands). Equimolar concentrations of the barcoded PCR products were pooled and sequenced on GS FLX Titanium platform (454 Life Sciences) by Macrogen Inc. Korea.

Samples were analyzed using the QIIME pipeline (Caporaso et al., 2010). Raw sequences were demultiplexed and then quality-filtered with a minimum quality score of 25, length between 250−350 bp, and allowing maximum two errors in the barcode sequence. Sequences were then clustered into operational taxonomic units (OTUs, 97% similarity) with UCLUST (Edgar, 2010). Reads were aligned to the Greengenes Core reference alignment (DeSantis et al., 2006) using the PyNAST_algorithm (Caporaso et al., 2010). Taxonomy was assigned based on the Greengenes taxonomy and a Greengenes reference database (version 12_10) (McDonald et al., 2012; Werner et al., 2012). Representative OTU sequences assigned to the specific taxonomic groups were extracted through classify.seqs and get.lineage in Mothur (Schloss et al., 2009) by using the Greengenes reference and taxonomy files. Sequence reads were submitted to SRA under the accession number XXXX.

5.2.7. Phylogenetic analyses

The phylogenetic affiliation of the 16S rRNA gene sequences was compared to release 119 of the Silva NR SSU Ref database (http://www. arb-silva. de/; Quast (2012)) using the ARB software package (Ludwig et al., 2004). Sequences were added to the reference tree supplied by the Silva database using the ARB Parsimony tool.

5.3. Results

Suspended particulate matter (SPM) of North Sea coastal water was obtained during a period from August 2010 - December 2011, covering a complete annual cycle, in approximately biweekly resolution.

5.3.1. Chlorophyll *a* concentration and phytoplankton abundance and diversity

Chlorophyll *a* concentrations ranged between 0.4 and 22.2 µg/L (Figure 1; Table S1). During late autumn, winter and early spring concentrations were low at ~4 µg/L. A peak in the chlorophyll *a* concentration occurred in the beginning of April and values stayed relatively high during this month, indicative of the spring bloom. Subsequently, the chlorophyll *a* concentration decreased again, reaching pre-bloom levels and stayed relatively constant.

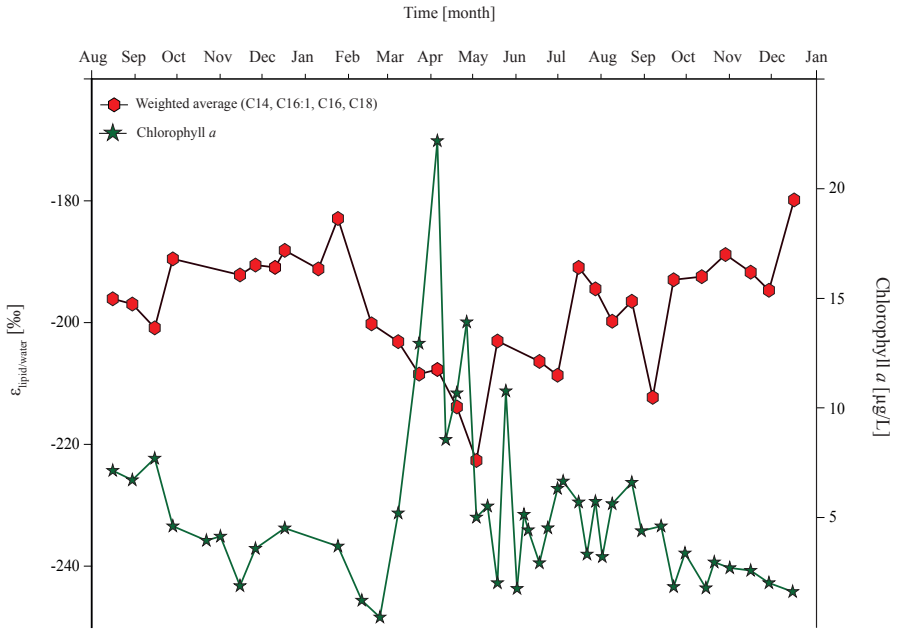

Figure 1: $\varepsilon_{average}$ values compared to chlorophyll *a* concentrations. $\varepsilon_{fattyacids}$ is the weighted average of *n*C14:0, *n*C16:1, *n*C16:0, *n*C18:0 fatty acids and the *n*C20:5 PUFA.

Phytoplankton diversity and abundance was determined using light microscopy and the two to three most abundant phytoplankton species were identified and counted (Table S2). The majority of the phytoplankton was composed of *Phaeocystis globosa*, diatoms and cyanobacteria (Figure 2), with the spring bloom primarily being made up of *P. globosa*. The highest abundance of diatoms was also during spring, while the cyanobacteria reached the highest abundance in the beginning of the sampling period from autumn until late winter and again during summer.

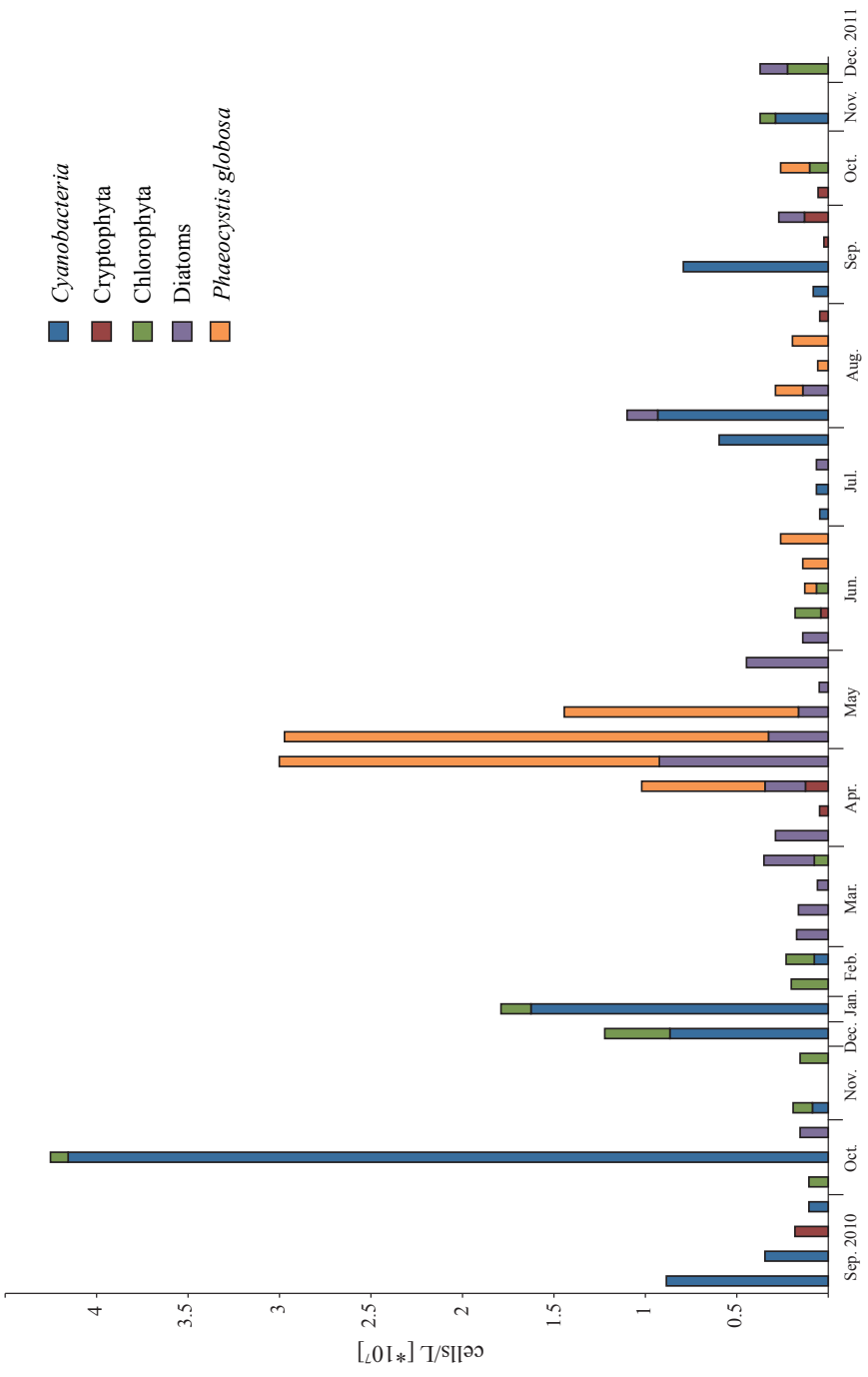

Figure 2: Phytoplankton diversity and abundance (measured in cells/L) observed in the coastal North Sea.

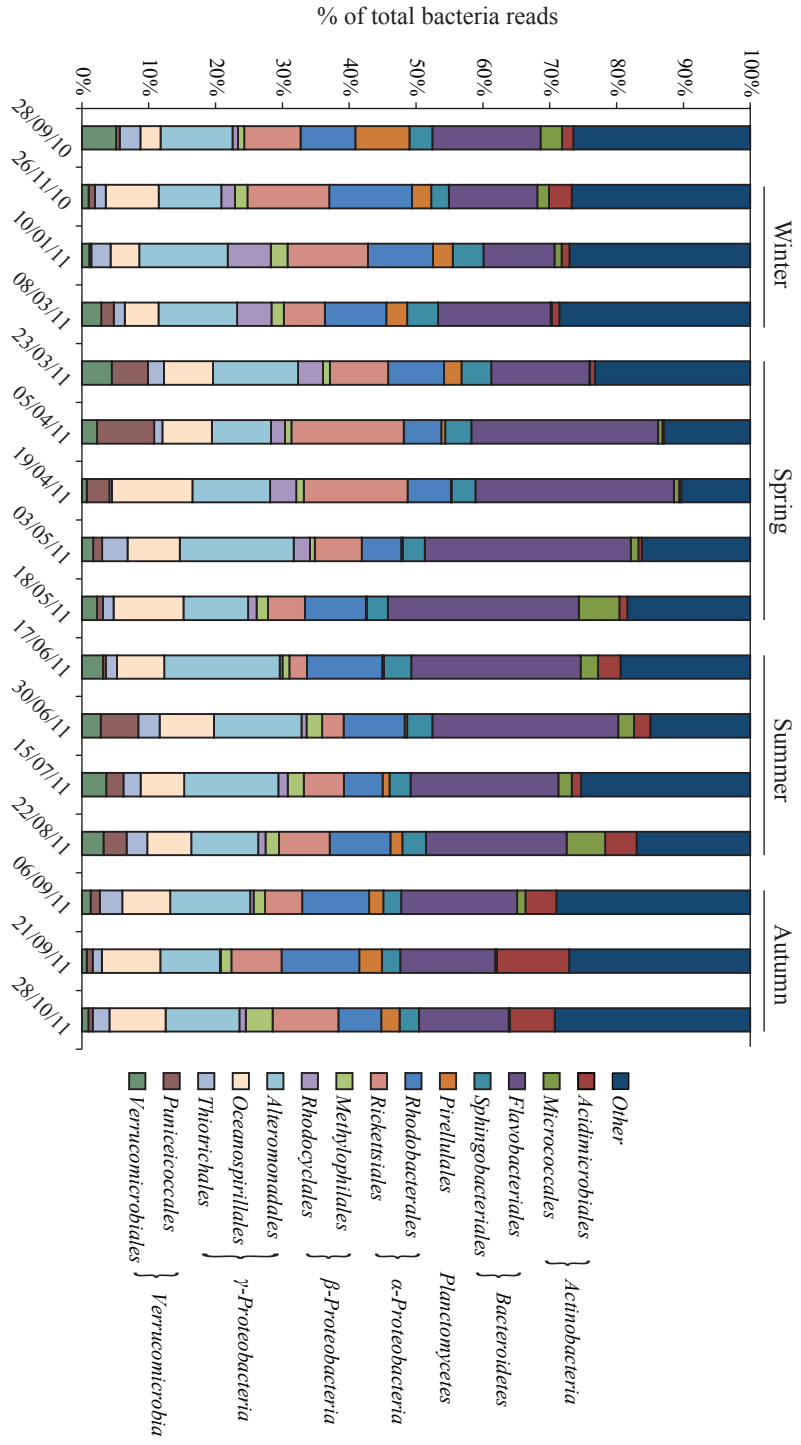

Figure 3: Order-level bacterial diversity and abundance in North Sea water based on the 16S rRNA gene sequence.

5.3.2. Microbial diversity

To assess bacterial diversity, 16S rRNA gene amplicon sequencing was performed on approximately half of the SPM samples (Table S3).

The bacteria detected consisted mainly of members of *Actinobacteria*, *Bacteriodetes*, *Planctomycetes*, α-*Proteobacteria*, β-*Proteobacteria*, γ-*Proteobacteria* and *Verrucomicrobia* (Figure 3; Table S3). The majority of the reads belonged to the orders of the *Flavobacteriales*, *Rhodobacteriales*, *Rickettsiales*, *Alteromonadales* and *Oceanospirillales*. The *Flavobacteriales* contributed between 12 to 32 % to the total bacterial reads with a relatively constant percentage of ~ 15 % during autumn and winter. The percentage of reads increased during early spring with the highest values from beginning of April until the end of May. The percentage of reads attributed to the *Flavobacteriales* decreased during summer and early autumn. Sequence reads affiliated to the *Rhodobacteriales* (6 to 12 %) and *Rickettsiales* (3 to 17 %) were the most represented within the α-*Proteobacteria.* The percentage of *Rhodobacteriales* reads was fairly constant with no obvious seasonal pattern. In contrast, the percentage of *Rickettsiales* reads followed a distinct seasonal pattern with a maximum in April (up to 17 %) and a minimum in June (3 %). *Alteromonadales* reads made up between 9 and 17 % of all bacteria reads and were fairly constant over the season. The percentage of *Oceanospirillales* reads were between 3 and 12 % of the total bacteria reads and show a clear maximum during mid-April (Figure 3; Table S3).

For a more accurate taxonomic classification of the bacterial groups, sequence reads of the *Bacteriodetes*, α-*Proteobacteria* and γ-*Proteobacteria* were extracted from the dataset and a phylogenetic tree was constructed (Figures S1-S3). Within the *Flavobacteriales* (*Bacteroidetes*) the majority of the reads fell either within the *Cryomorphaceae* or the *Flavobacteriaceae* with sequences clustering within *Fluviicola* and *Crocinitomix*, *Flavobacterium* and *Tenacibaculum*, respectively. Within the *Rhodobacterales* (α-*Proteobacteria*) most of the reads belonged to *Rhodobacteraceae* and sequences within this family were closely related to the genus *Octadecabacter*. Within the *Rickettsiales* most of the reads were affiliated to the *Pelagibacteraceae* (SAR11 cluster). The majority of the γ-*Proteobacteria* reads were classified within the *Alteromonadales* and *Oceanospirillales*. The *Alteromonadales* reads and sequences fell within the uncultured *HTCC2188*-isolate and *OM60*-clade and various members of the *Alteromonadaceae*-family. The *Halomonadaceae* family comprised most of the *Oceanospirillales* reads and additionally se-

quences clustered with various members of the *Oceanospirillaceae*.

5.3.3. Fatty acid distribution in North Sea SPM

Polar lipid derived fatty acids were comprised of *n*C14:0, *n*C16:1ω7, *n*C16:0, *n*C18:0, the polyunsaturated fatty acid (PUFA) *n*C20:5, and various unsaturated *n*C18 fatty acids (Figure 4; Table S4). The *n*C14:0 fatty acid followed a seasonal cycle with the lowest relative abundance during winter, and the highest from June to August (Figure 4a). The *n*C16:0 fatty acid was the dominant fatty acid (21-38 %) with no clear seasonal pattern (Figure 4c). The *n*C16:1 fatty acid was the next most abundant fatty acid (13–35 %) with a maximum from March to April (Figure 4b). Various unsaturated *n*C18:x fatty acids were observed throughout the season. Due to low abundance of the individual fatty acids and co-elutions the double bond positions could not be determined. These unsaturated fatty acids made up 9–30 % of all fatty acids (Figure 4d). The *n*C18:0 fatty acid had relative abundances varying between 2–18 % with the highest relative abundance during autumn months (10–18 %) and the lowest during spring, 2–6 % (Figure 4e). A *n*C20:5 PUFA (Figure 4f) was observed in most samples with the highest relative abundance during March and April (11–14 %) and early August (18 %). Trace amounts of *n*C15:0, *i*C15:0 and *ai*C15:0 fatty acids were also detected.

5.3.4. Hydrogen isotopic composition of fatty acids

δD values of *n*C14:0, *n*C16:1ω7, *n*C16:0, *n*C18:0 fatty acids and *n*C20:5 were obtained for most of the samples (Table S5). The D/H ratio of the other fatty acids could not be determined with high accuracy due to either incomplete separation or low abundance.

In general, *n*C14:0 and *n*C20:5 were the most depleted fatty acids with δD values ranging between -198 to -241 ‰ and -180 to -241 ‰, respectively. The *n*C18:0 was typically the fatty acid with the highest δD values ranging between -175 to -212 ‰ (Table S5).

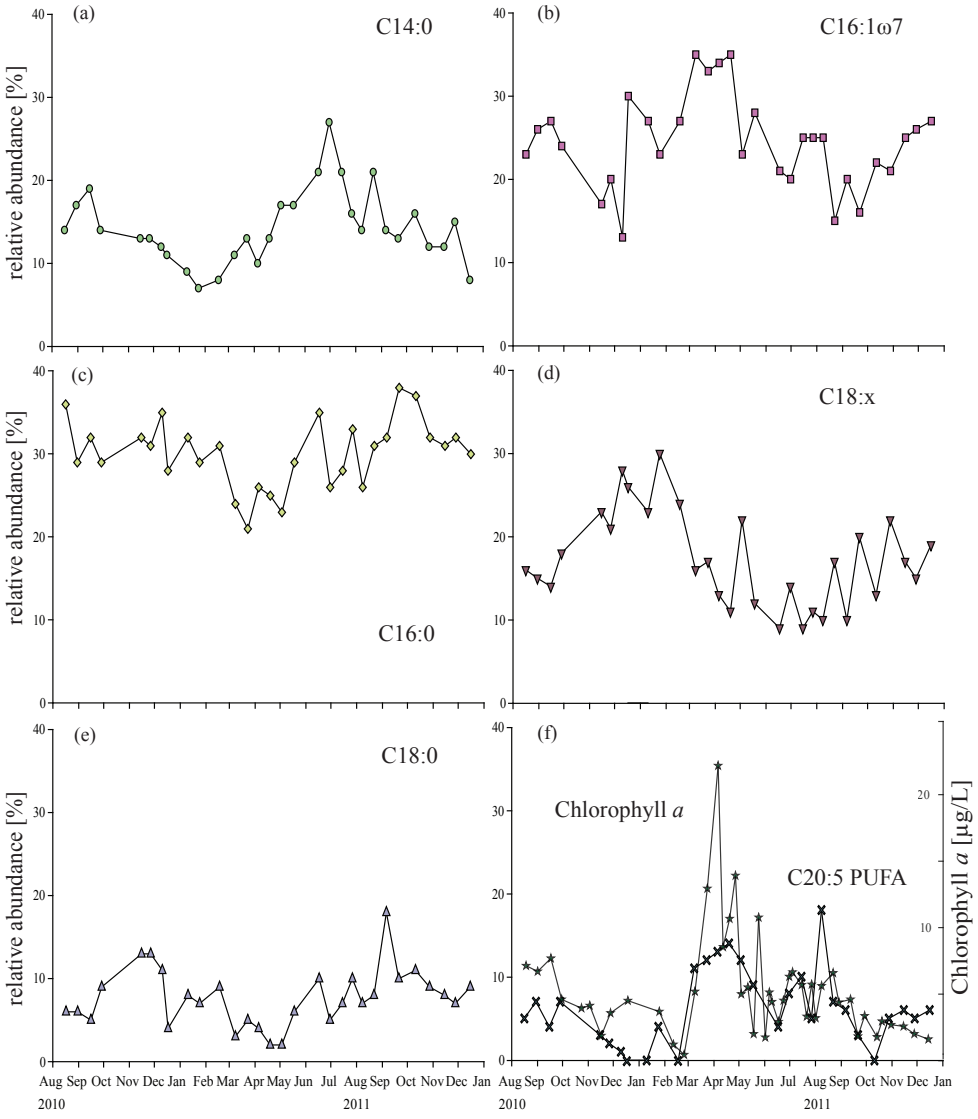

Figure 4: Relative abundance of fatty acids and chlorophyll *a* concentration in North Sea SPM. (a) *n*C14:0, (b) *n*C16:1, (c) *n*C16:0, (d) *n*C18:x, (e) *n*C18:0, (f) *n*C20:5 PUFA and chlorophyll *a*.

5.4. Discussion

5.4.1. Hydrogen isotopic fractionation expressed in fatty acids

For the proper assessment of the impact of metabolism on the hydrogen isotopic composition of fatty acids the hydrogen isotopic fractionation of the fatty acids versus water is required ($\varepsilon_{lipid/water}$). For this, the δD of the water (δD_{water}) at the time of sampling is needed. However, at the time of sampling of the SPM unfortunately no water samples were taken and preserved for δD analysis. Therefore, we used an alternative approach to estimate δD_{water} using the salinity of the water measured at the time of sampling. A strong correlation between salinity and δD_{water} is generally observed in marine environments since both parameters depend on evaporation, precipitation and freshwater influx (Craig and Gordon, 1965; Mook, 2001). To establish a local salinity - δD_{water} correlation, water samples were collected weekly during high tide (March to September 2013) and salinity and δD_{water} were measured. Indeed, a strong correlation between salinity and δD_{water} is observed (R^2=0.68; Figure S4). Using this correlation and the salinities measured, we reconstructed δD_{water} values at the time of sampling of the biomass (Table 1). The error in the estimate of $\varepsilon_{lipid/water}$ resulting from this approach is approximately 1.5 ‰, which is less than the error in the determination of δD of the fatty acids (1-12 ‰).

All fatty acids were depleted in D compared to water with the fractionation factor $\varepsilon_{lipid/water}$ ranging from -173 to -237 ‰, all following a similar seasonal trend with the highest degree of fractionation during spring to early summer, and early autumn (Figure 5; Table 1). The lowest degree of fractionation (most negative $\varepsilon_{lipid/water}$ values) was in general during late autumn and the winter months.

5.4.2. Source affects the hydrogen isotopic composition of individual fatty acids

The *n*C20:5 PUFA is the most specific fatty acid detected in North Sea SPM and is exclusively produced by algae (Carrie et al., 1998). The *n*C20:5 PUFA is one of the most D-depleted fatty acids (Figure 5), which is in agreement with culture studies that show that photoautotrophic microorganisms produce fatty acids that are depleted in D with $\varepsilon_{lipid/water}$ values between -162 and -215 ‰, while heterotrophic microorganisms on the other hand produce

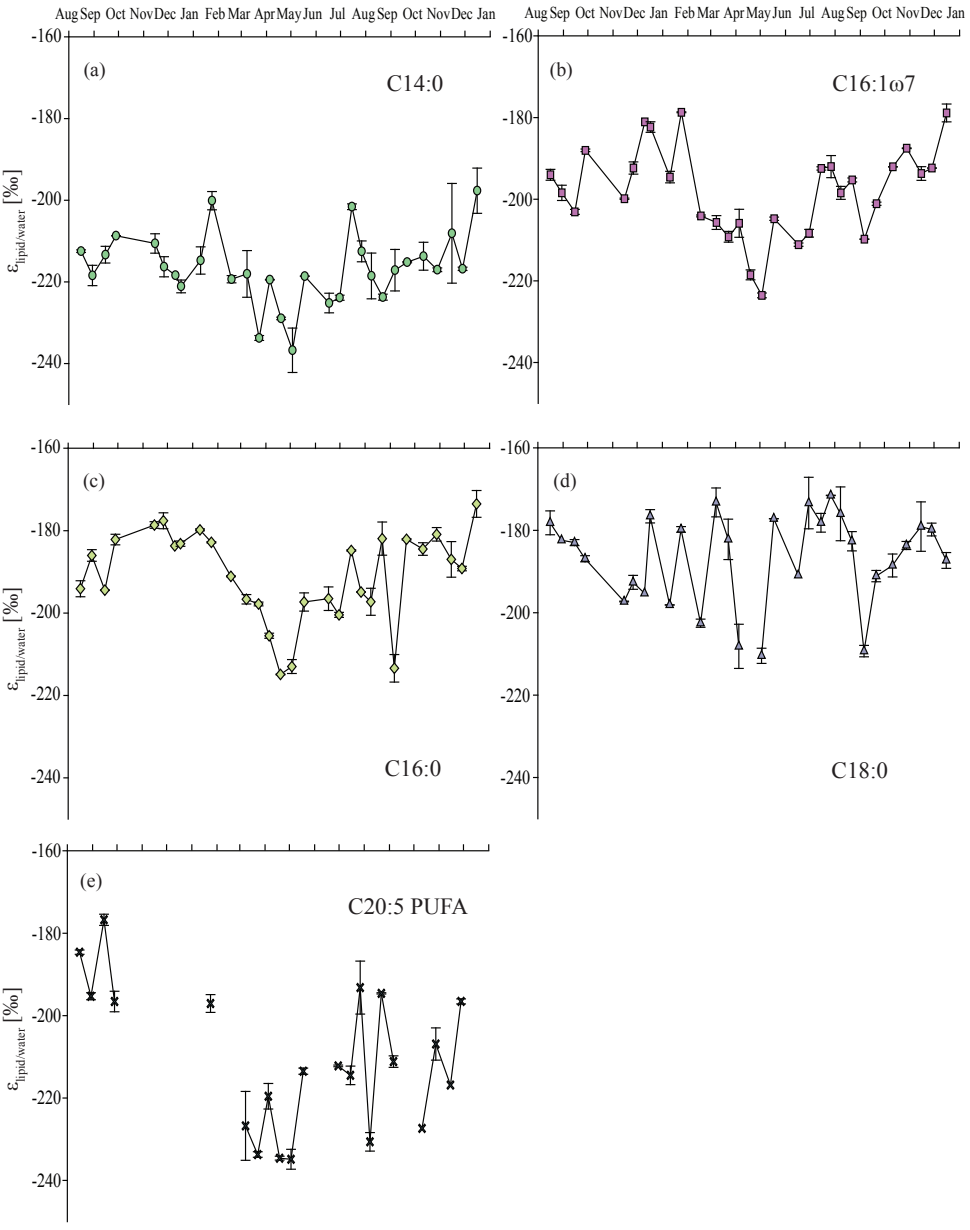

Figure 5: The D/H fractionation between fatty acids and North Sea water for fatty acids derived from suspended particulate matter in North Sea water samples. Plotted are the the $\varepsilon_{lipid/water}$ values of *n*C14:0, *n*C16:1, *n*C16:0, *n*C18:0 fatty acids and *n*C20:5 PUFA. Error bars are the standard deviation of the duplicate measurements of the fatty acids.

Table 1: D/H fractionation between fatty acids and North Sea water for fatty acids derived from suspended particulate matter in North Sea water samples.

Date	Salinity	δD_{water} [‰] (estimated)	$\varepsilon_{lipid/water}$ [‰]					$\varepsilon_{fattyacids}$ [‰]
			C14:0	C16:1	C16:0	C18:0	C20:5 PUFA	weighted average (C14, C16:1, C16, C18)
16/08/10	27.3	-8.2	-212±0	-194±1	-194±2	-178±3	-185±1	-196
30/08/10	29.7	-4.1	-218±2	-198±2	-186±1	-182±0	-196±1	-197
15/09/10	30	-3.6	-213±2	-203±1	-194±0	-183±1	-177±1	-201
28/09/10	24.7	-12.6	-209±0	-188±0	-182±1	-187±1	-197±2	-190
15/11/10	30	-3.6	-211±2	-200±0	-179±1	-197±0	N.D.	-192
26/11/10	24.8	-12.4	-216±2	-192±1	-178±2	-193±2	N.D.	-191
10/12/10	27.1	-8.5	-218±0	-181±0	-184±0	-195±0	N.D.	-191
17/12/10	24.1	-13.6	-221±2	-182±1	-183±1	-177±2	N.D.	-188
10/01/11	27.8	-7.3	-215±3	-195±1	-180±0	-198±0	N.D.	-191
24/01/11	23.0	-15.5	-200±2	-179±0	-183±0	-180±1	-197±2	-183
17/02/11	29.3	-4.8	-219±1	-204±0	-191±0	-203±1	N.D.	-200
08/03/11	25.8	-10.7	-218±6	-206±2	-197±1	-173±4	-227±8	-203
23/03/11	26.8	-9.0	-234±1	-209±1	-198±0	-182±5	-234±1	-208
05/04/11	29.2	-4.9	-219±0	-206±3	-205±1	-208±5	-220±3	-208
19/04/11	27.7	-7.5	-229±0	-219±1	-215±0	N.D.	-235	-214
03/05/11	31.1	-1.7	-237±5	-224±1	-213±2	-210±2	-235±2	-223
18/05/11	31.8	-0.5	-219±0	-205±0	-197±2	-177±0	-214±1	-203
17/06/11	32.0	0.7	-225±2	-211±0	-196±3	-191±0	N.D.	-206
30/06/11	31.2	-1.6	-224±1	-208±1	-200±1	-173±6	-213	-209
15/07/11	30.0	-3.6	-202±1	-192±0	-185±0	-178±2	-215±2	-191
27/07/11	26.3	-9.9	-213±3	-192±3	-195±0	-172±0	-194±6	-194
08/08/11	29.4	-4.6	-219±6	-198±2	-197±3	-176±7	-231±2	-200
22/08/11	26.9	-8.9	-224±1	-195±0	-182±4	-183±2	-195	-196
06/09/11	26.8	-9.0	-217±5	-210±0	-213±3	-209±1	-212±1	-212
21/09/11	30.1	-3.4	-215±0	-201±0	-182±0	-191±1	N.D.	-193
11/10/11	32.8	1.2	-214±3	-192±0	-184±2	-189±3	-227	-192
28/10/11	32.2	0.1	-217±0	-188±0	-181±2	-184±1	-207±4	-189
15/11/11	28.9	-5.5	-208±12	-194±2	-187±4	-179±6	-217	-192
28/11/11	31.7	-0.7	-217±0	-192±0	-189±1	-180±2	-197±1	-195
16/12/11	31.7	-0.7	-198±6	-179±2	-173±3	-187±2	N.D.	-180

*n*C16:1*: double bond at the ω7 position

fatty acids with $\varepsilon_{lipid/water}$ values ranging between -150 to +200 ‰. Furthermore, its concentration increased at the time of the phytoplankton bloom (Figure 4). Interestingly, after the phytoplankton bloom, when its abundance decreased (Figure 4), it became enriched in D (Figure 5). This enrichment might be due to changes in the relative contribution of source organisms. In diatoms *n*C20:5 PUFA can be one of the most abundant fatty acids, while *Phaeocystis* produces it in minor amounts only (Table S6). During the spring bloom both organisms will contribute to the fatty acid pool, while afterwards it will mainly derive from diatoms (Figure 2; Table S2). Another possible reason would be that after the bloom and due to nutrient limitation, phytoplankton uses storage products leading to an increased production of NADPH via other pathways than photosystem I. The NADPH produced by photoautotrophs via photosystem I is depleted in D (Zhang et al., 2009a), while NADPH produced via the pentose phosphate (OPP) pathway and the tricarboxylic acid (TCA) cycle is relatively enriched in D (Zhang et al., 2009a; Heinzelmann et al., 2015b). The utilization of storage products would lead to an increased production of NADPH via both the OPP pathway and the TCA cycle leading to more positive $\varepsilon_{lipid/water}$ values of the *n*C20:5 PUFA after the bloom.

Of all other fatty acids *n*C14:0 was generally the most D-depleted fatty acid, possibly suggesting a higher contribution of photoautotrophic organisms to this fatty acid. The quite similar $\varepsilon_{lipid/water}$ values of *n*C16:1 (-179 to -224 ‰) and *n*C16:0 (-178 to -215 ‰) relatively to each other suggest similar sources for the two fatty acids. The least negative $\varepsilon_{lipid/water}$ values for *n*C18:0 suggest that the sources of this fatty acid might differ from the other fatty acids i.e. with a higher contribution of heterotrophs compared to the other fatty acids.

Fatty acids profiles of representatives of most members of the phytoplankton and bacterial community observed at our site have been previously reported (Table S6) and can be used to assess the main sources of the different fatty acid pools. The main bacterial contributors to the *n*C16:0 and *n*C16:1ω7 fatty acids are most likely members of the *Alteromonadales* and the *Halomonadaceae*, while the majority of bacterial contributors to the *n*C14:0 and *n*C18:0 fatty acid are derived from the *Puniceicoccales* (Table S6). Both the *Flavobacteriales* and the *Rhodobacteriaceae,* which make up a large part of the total bacteria reads, will hardly contribute to the measured isotopic signal as they have been reported to produce only traces of *n*C14:0, *n*C16:0, *n*C16:1ω7 or *n*C18:0 fatty acids (Table S6). The observed phytoplankton species are main contributors to the *n*C14:0, *n*C16:0 and *n*C16:1ω7 fatty acids pools, but

contribute relatively little to the *n*C18:0 fatty acid pools. *Phaeocystis* produces mainly the *n*C14:0 and *n*C16:0 fatty acids (Nichols et al., 1991; Hamm and Rousseau, 2003).

Overall, the majority of the *n*C14:0 fatty acid pool will likely be predominately derived from photoautotrophs (Table S6), which potentially explains why the *n*C14:0 is almost always the most depleted fatty acid. The *n*C18:0 fatty acid on the other hand, will be mainly derived from heterotrophic bacteria (Table S6) resulting in more D enriched signal compared to that of the *n*C14:0 fatty acid.

Culture studies have shown that chemoautotrophs produce fatty acids, which are even more depleted in D compared to photoautotrophs. However, none of the fatty acids measured in the North Sea SPM have $\varepsilon_{lipid/water}$ values which fall in the range of those predicted for chemoautotrophs (-264 to -345 ‰; Heinzelmann et al. 2015b). This fits with the observation that sequence reads of chemoautotrophic bacteria accounted for < 3 % of the total bacterial reads (Figure 3; Table S3), and thus it is unlikely that this metabolism plays an important role in this environment.

5.4.3. Linking seasonal changes of hydrogen isotope fractionation to changes in community metabolism

All the fatty acids showed a similar seasonal trend with the most negative ε values in spring and the most positive ε values in the winter (Figure 5). In order to assess the dominant metabolism of the whole microbial community we calculated a weighted average ε of all measured fatty acids apart from the specific *n*C20:5 PUFA. The weighted average $\varepsilon_{lipid/water}$ ($\varepsilon_{\Sigma FA}$) followed the same seasonal trend as the $\varepsilon_{lipid/water}$ values of the individual fatty acids (Figure 1+5), and ranged between -180 and -225 ‰ with an average of -199 ‰.

Compared to the chlorophyll *a* concentration, the $\varepsilon_{\Sigma FA}$ followed an opposite seasonal trend i.e. when the chlorophyll *a* concentration increased in early April, $\varepsilon_{\Sigma FA}$ decreased (Figure 5). The chlorophyll *a* maximum in April-May indicates a spring bloom (Figure 2), which is known to occur annually in North Sea coastal waters (Philippart et al., 2010; Brandsma et al., 2012b) and corresponds with a shift towards more negative values for $\varepsilon_{\Sigma FA}$, as well as a high abundance of the algal-derived *n*C20:5 PUFA (Figure 4). It is likely that at least during the spring bloom the majority of the fatty acids are derived from the dominant algae, i.e. *Phaeocystis* and diatoms, which make up the

majority of the bloom, leading to a D depleted signal. Thus, the observation that the value of $\varepsilon_{\Sigma FA}$ was more negative during the spring bloom when the environment is dominated by photoautotrophic microorganisms (Figure 3) fits with an increased contribution by photoautotrophs relative to heterotrophic microorganisms to the fatty acid pool. At the end of the bloom more positive $\varepsilon_{\Sigma FA}$ values were observed, which is in agreement with an increased abundance of heterotrophic bacterioplankton in previous studies (Sintes et al., 2013), living on released organic material (Alderkamp et al., 2006).

Thus, $\varepsilon_{\Sigma FA}$ values reflect a mixed signal derived from mainly photoautotrophic and, to a lesser extent, heterotrophic microorganisms. Nevertheless, $\varepsilon_{lipid/water}$ values for all fatty acids remain in the range of photoautotrophic metabolism (Heinzelmann et al., 2015b), indicating that, overall, the fatty acids in this coastal seawater are mostly derived from phototrophic organisms. This is in accordance with the assumption that IPLs (containing fatty acids) in coastal North Sea waters over the annual cycle were predominantly derived from phytoplankton (Brandsma et al., 2012b). Our results show that it is possible to study whole community core metabolism in a natural environment by determining the weighted average D/H ratio of all fatty acids.

5.5. Conclusion

A seasonal study of fatty acids derived from the coastal Dutch North Sea shows that all fatty acids are depleted in D with δD ranging between -174 and -241 ‰. The most negative values were observed during the spring bloom, when the biomass is dominated by photoautotrophic microorganisms. The subsequent higher relative contribution of heterotrophs to the general fatty acid pools leads to shift in $\varepsilon_{lipid/water}$ towards more positive values by up to 20 ‰. This shift towards more positive values is in agreement with observations from culture studies where heterotrophic organisms fractionate much less or even opposite to photoautotrophic organisms. This study confirms that hydrogen isotopic fractionation as observed in general fatty acids can be used to study the core metabolism of complex environments and to track seasonal changes therein.

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Chapter 6

Assessing the metabolism of sedimentary microbial communities using the hydrogen isotopic composition of fatty acids

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Abstract

The hydrogen isotopic composition of fatty acids has previously been shown to reflect the general metabolism of microorganisms in pure culture, but has rarely been tested in the environment. Here, we report the abundance and hydrogen isotopic composition of fatty acids extracted from surface sediments in the saline Lake Grevelingen (The Netherlands) sampled at two different stations and during two seasons. These data are compared with the bacterial diversity revealed by 16S rRNA gene amplicon sequencing. All measured fatty acids were depleted in deuterium relative to the bottom water by 103 to 267 ‰. Fatty acids associated with heterotrophic bacteria (*i*C15:0 and *ai*C15:0) showed the smallest fractionation (-103 to -185 ‰) while those derived from phytoplankton (*n*C20:5) living in the surface water of the lake showed the largest fractionation (-230 to -267 ‰). The overall relatively large hydrogen isotope fractionation reflected in the majority of the more commonly occurring fatty acids (*n*C14:0, *n*C16:0, *n*C16:1ω7) is relatively large, -172 to -217 ‰, and relatively constant with depth. Together with the high relative abundance of the *n*C20:5 this suggests a substantial contribution from dead pelagic biomass settling from the water column to the sedimentary polar lipid derived fatty acid pool and not from the *in situ* microbial communities. Therefore, the majority of the isotope signal in the fatty acids from surface sediments might not represent the general metabolism of the active sedimentary communities. This suggests that the input of pelagic biomass into sedimentary environments may bias the information contained in the hydrogen isotopic composition of fatty acids. In such settings it could therefore be more useful to target group or species-specific fatty acids or other lipid biomarkers for isotopic analysis.

6.1. Introduction

In the past decades, different approaches have been applied to assess the metabolism of environmental microbial communities. Two of the most common approaches are stable isotope probing (SIP) (Boschker et al., 1998; Radajewski et al., 2003), and measurements of specific gene activity (Chapelle and Lovley, 1990; Phelps et al., 1994). The SIP method assesses the microbial metabolism by addition of isotopically labelled substrate to an environmental sample and determines the subsequent label incorporation into cellular biomarkers like DNA, RNA and lipids. The identification of the labelled biomarkers allows the coupling between metabolism and microbial identity, specifically when label is incorporated into lipids with taxonomic value (Boschker et al., 1998; Manefield et al., 2002; Radajewski et al., 2003; Wuchter et al., 2003; Dumont and Murrell, 2005; van der Meer et al., 2005; Neufeld et al., 2007; van der Meer et al., 2007). While this approach allows for a cultivation-independent identification of metabolically active microorganisms in the environment, it also introduces certain possible biases that have to be taken into account. Both incubation time and concentration of the labelled substrate have to be carefully considered in order to avoid cross-labelling by secondary metabolites, insufficient incorporation of the label into the targeted biomarker molecules, and artificial changes of both microbial diversity and activity (Radajewski et al., 2000; Dumont and Murrell, 2005; van der Meer et al., 2005; Cebron et al., 2007). Microbial activity can potentially also be assessed by looking at the gene expression of certain key genes involved in metabolic pathways (Moran et al., 2013). Additionally, quantifying the abundance of the 16S rRNA gene also enables an assessment of both microbial identity and abundance (Blazewicz et al., 2013). However, in order to draw conclusions about both the diversity and metabolic activity using functional gene analysis, both a database of sequences of the targeted gene, as well as knowledge of the involvement of the gene-coding enzyme in the metabolic function are required. This is especially a disadvantage when assessing novel or less well studied metabolic pathways or when the gene sequences are too diverse to allow for the development of a genetic-based screening method (Rastogi and Sani, 2011). Moreover, a higher transcriptional activity of a gene has been shown to not necessarily correlate with a higher activity of the pathway in which the protein-coding gene is involved (Bowen et al., 2014).

Recently, a new method using the natural hydrogen isotopic composition, i.e. the deuterium to hydrogen (D/H) ratio, of fatty acids has been shown to

reveal the core metabolism of microorganisms in pure culture and to distinguish between heterotrophic, chemoautotrophic and photoautotrophic growth (Sessions et al., 2002; Chikaraishi et al., 2004; Valentine et al., 2004; Zhang and Sachs, 2007; Campbell et al., 2009; Zhang et al., 2009a; Dirghangi and Pagani, 2013b; Fang et al., 2014; Heinzelmann et al., 2015a; Heinzelmann et al., 2015b). A similar effect of the core metabolism on the hydrogen isotopic composition of fatty acids has been observed in the natural environment, i.e. hot spring microbial communities (Osburn et al., 2011) and the pelagic microbial community in a coastal marine site (Heinzelmann et al., 2015c). However, the number of environmental applications is limited and it is, therefore, necessary to study a diverse range of environments in order to better constrain the limitations and benefits of this approach.

Here we tested this new approach on sedimentary microbial communities. Sedimentary bacteria play an important role in all elemental cycles i.e. carbon, oxygen, sulfur and nitrogen cycle (Alongi, 1994; Boetius and Lochte, 1997; Arnosti et al., 1998; Muyzer and Stams, 2008; Middelburg and Levin, 2009; Orcutt et al., 2011). The diversity of sedimentary bacterial communities is generally higher than that of pelagic communities (Lozupone and Knight, 2007), and depends on amongst others oxygen concentration of the overlying water (Orcutt et al., 2011). They are found to express a wide range of different metabolisms, including e.g. aerobic heterotrophy, chemoautotrophy, fermentation and sulphate reduction (Nealson, 1997). This strongly depends on the availability of oxygen, e.g. hypoxic/anoxic bottom waters lead to changes in the overall metabolic activity of microorganisms in the underlying sediment (Bartoli et al., 2009; Reese et al., 2012) and to an increased activity of anaerobic pathways compared to aerobic pathways (Middelburg and Levin, 2009).

In order to test the application of the D/H ratio of fatty acids as a tool to study microbial metabolism, we studied the D/H ratio of fatty acids of the microbial communities in sediment cores obtained from Lake Grevelingen, a marine lake. Previously, a wide range in the D/H of lipids extracted from Santa Barbara basin sediments has been observed (Li et al., 2009).We studied cores from two different stations, a shallow station with oxic bottom water in spring (March) and hypoxic bottom water in summer (August) and a deep station with oxic bottom water in spring and anoxic bottom water in summer. This should allow us to study spatial and seasonal differences in microbial metabolism due to changing oxygen concentrations and compare this to changes in the bacterial diversity as obtained by 16S rRNA gene amplicon sequencing.

6.2. Material and Methods

6.2.1. Study site and sampling

Lake Grevelingen is a former Rhine-Meuse estuary located in the south of the Netherlands between the provinces of Zeeland and Zuid-Holland. The lake was formed after the Rhine-Meuse estuary was closed by two dams in 1964 and 1970. In order to avoid permanent stratification and anoxic conditions in the water column, a connection to the North Sea was re-established in 1978. This connection to the North Sea is opened during winter and this has led to a rise in the salinity, which since then varies between 29-32. The lake has a mean water depth of 5.3 m with the deepest point being 48 m deep (Bannink et al., 1984; Kamermans et al., 1999). The main basin of Lake Grevelingen (Den Osse Basin) is up to 34 m deep and is prone to hypoxia/anoxia during summer due to stratification, which leaves the bottom water and sediment at the deepest point completely anoxic. Lake Grevelingen has been studied previously regarding both macro flora (Kamermans et al., 1999) and phytoplankton population (Bakker and De Vries, 1984) after reconnection with the North Sea. The phytoplankton is dominated by diatoms and some flagellates (Bakker and De Vries, 1984) and the main phytoplankton bloom occurs in July, while a minor bloom occurs already in early spring (March). The decaying summer bloom is thought to contribute to the hypoxia/anoxia in the water column at that time (Hagens et al., 2015). The microbial community of the sulfidic sediment has been the topic of only a limited amount of studies. *Desulfobulbaceae* filaments capable of electrogenic sulphide oxidation have been shown to be present in the Den Osse Basin (Malkin et al., 2014; Vasquez-Cardenas et al., 2015) and heterotrophic denitrifiers and anammox bacteria play a role in the nitrogen cycle in the sediments (Lipsewers et al., 2015).

Sediment cores were taken on board of the R/V Luctor in March and August 2012 at two different stations. Station 1 was located at the deepest point of the lake with a water depth of 34 m (51.747 ºN, 3.890 ºE) and station 3 had a water depth of 17 m (51.747 ºN, 3.898 ºE) (Hagens et al., 2015). Sediment cores were taken with an Uwitec corer (Uwitec, Austria) (length 60 cm; diameter 60 mm). The overlying water was removed and the core was sliced with 1 cm resolution. Samples were immediately stored on dry ice and later at -80 ºC in the lab until further extraction. Water overlying the sediment was sampled for δD_{water} measurements and stored air tight, without headspace, in

glass tubes at 4 ºC until measurement.

6.2.2. Polar lipid-derived fatty acids

The first 8 cm of the sediment cores sampled at both stations 1 and 3 were extracted for intact polar lipids (IPL). The freeze dried sediment (0.4–2.7 g) were extracted via a modified Bligh-Dyer method (Bligh and Dyer, 1959; Rütters et al., 2002) with methanol (MeOH)/dichloromethane (DCM)/phosphate buffer (2:1:0.8, vol/vol/vol) using ultrasonication as previously described by Heinzelmann et al. (2014). Subsequently, approximately 0.5–1 mg of the Bligh-Dyer extract (BDE) was separated into a neutral and a polar lipid fraction using silica column chromatography, eluting the polar lipids with MeOH according to Heinzelmann et al. (2014). Polar lipid-derived fatty acids (PLFA) were generated, methylated and cleaned up as previously described in Heinzelmann (2015c) by saponification with 1 N KOH in MeOH (96%), methylation with a boron trifluoride-methanol solution (BF_3-MeOH) and clean up over an aluminium oxide (AlOx) column, eluting the methylated PLFAs with DCM. The position of the double bonds in unsaturated fatty acids was determined via the derivatization with dimethyldisulfide (DMDS) (Nichols et al., 1986). The PLFA extracts were stored at 4 °C. PLFA are indicated here as Cx:yωz, where x designates the total number of carbons, v the number of double bonds and z the position of the double bond relatively to the aliphatic end (ω) of the molecule. The prefixes *n*, *i* and *ai* refer to a normal alkyl chain and *iso* and *anteiso* methyl branching of the alkyl chain, respectively.

6.2.3. Fatty acid and hydrogen isotope analysis

The PLFA fractions were analysed by gas chromatography (GC) using an Agilent 6890 gas chromatograph with a flame ionization detector (FID) using a fused silica capillary column (25 m × 320 µm) coated with CP Sil-5 (film thickness 0.12 µm) with helium as carrier gas. The temperature program was previously described in Heinzelmann et al. (2015b). Individual compounds were identified using GC/mass spectrometry (GC/MS) using a Agilent 7890A GC instrument and Agilent 5975C VL mass selective detector (MSD).

Hydrogen isotope analysis of the fatty acid fraction was performed by GC thermal conversion isotope ratio monitoring MS (GC/TC/irMS) using an Agilent 7890 GC connected via Thermo GC Isolink and Conflo IV interfaces to a Thermo Delta V MS according to Chivall (2014), with the temperature pro-

gram as previously described in Heinzelmann et al. (2015b). The H_3^+ correction factor was determined daily and was relatively constant at 5.3±0.2. A set of standard n-alkanes with known isotopic composition (Mixture B prepared by Arndt Schimmelmann, University of Indiana) was analysed daily prior to analysing samples in order to monitor the systems performance and samples were only analysed when the n-alkanes in Mix B had an average deviation from their off-line determined value of <5‰. An internal standard containing squalane (δD = -170 ‰) was co-injected with each fatty acid sample in order to monitor the precision (average δD -162±2 ‰) and the δD of the individual fatty acids was measured in duplicates and corrected for the added methyl group (Heinzelmann et al., 2015b).

The hydrogen isotopic composition of fatty acids compared to water was expressed as $\varepsilon_{lipid/water}$ following:

$$\varepsilon_{lipid/water} = \left(\frac{1000+\delta D_{FA}}{1000+\delta D_{water}} - 1\right) * 1000$$

The δD of water samples was determined by TC/elemental analysis/irMS (TC/EA/irMS) according to Chivall (2014).

6.2.4. DNA extraction

Sediments for DNA extraction were defrosted and centrifuged (3,000 *g*, 10 min) to remove excess water and then extracted (~0.2 g) with the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, USA) following the manufacturer's instructions. DNA quality and concentration were estimated by Nanodrop (Thermo Scientific, Waltham, MA) quantification.

6.2.5. 16S rRNA gene amplicon sequencing and analysis

The general bacterial diversity was accessed by 16S rRNA amplicon pyrotag sequencing. The extracted DNA was quantified fluorometrically with Quant-iTTM PicoGreen® dsDNA Assay Kit (Life Technologies, Netherlands). PCR reactions were performed with the universal (Bacteria and Archaea) primers S-D-Arch 0519-a-S-15 (5'-CAG CMG CCG CGG TAA-3') and S-D-Bact-785-a-A-21 (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth et al., 2012) adapted for pyrosequencing by the addition of sequencing adapters and multiplex identifier (MID) sequences. PCR reactions, conditions and workup were as previously described by Heinzelmann et al. (2015c). Equimolar concentrations of the barcoded PCR products were pooled and sequenced on GS FLX Titanium platform (454 Life Sciences) by

Macrogen Inc. Korea.

Sequencing reads were analysed as described in Heinzelmann et al. (2015c) using the QIIME pipeline (Caporaso et al., 2010) and taxonomy was assigned based on the Greengenes taxonomy and a Greengenes reference database (version 12_10) (McDonald et al., 2012; Werner et al., 2012). Representative OTU sequences assigned to the specific taxonomic groups were extracted through classify.seqs and get.lineage in Mothur (Schloss et al., 2009) by using the greengenes reference and taxonomy files. Data was submitted to SRA with the following accession numbers XXXX.

6.2.6. Phylogenetic analyses

The phylogenetic affiliation of the 16S rRNA gene sequences was compared to release 119 of the Silva NR SSU Ref database (http://www. arb-silva. de/; Quast (2012)) using the ARB software package (Ludwig et al., 2004). Sequences were added to the reference tree supplied by the Silva database using the ARB Parsimony tool.

6.3. Results

Sediment cores were taken at two stations: Station 1 (34 m water depth, deep station) and Station 3 (17 m water depth, shallow station). Water column oxygen concentration was measured by CTD and was between 299, close to the sediment, and 353 µM at the water surface at Station 1 in March and between 0 (i.e. not detected) at the bottom and 306 µM at the water surface in August (Figure S1) (Lipsewers et al., 2015). At Station 3 water column oxygen concentrations were not determined, but similar concentrations and distributions are expected to exist. The oxygen penetration depth at Station 1 was 1.5 mm in March and 0 mm (i.e. completely anoxic sediment) in August and 1.5-2.2 mm at Station 3 in March and 1.0 mm in August (Lipsewers et al., 2015). The sulphide concentration increased with sediment depth from not detected to 818 µmol/L at Station 1 in March and from 725 to 2893 µmol/L in August, and at Station 3 in March from 0 to 2 µmol/L, but decreased with sediment depth from 224 to 2 µmol/L in August (Table S1) (Lipsewers et al., 2015).

6.3.1. Fatty acid abundance and composition

A variety of fatty acids were observed in the sediments analysed, including *n*C14:0, *i*C15:0, *ai*C15:0, *n*C15:0, *n*C16:1ω7, *n*C16:0, *n*C18:0 fatty acids, a

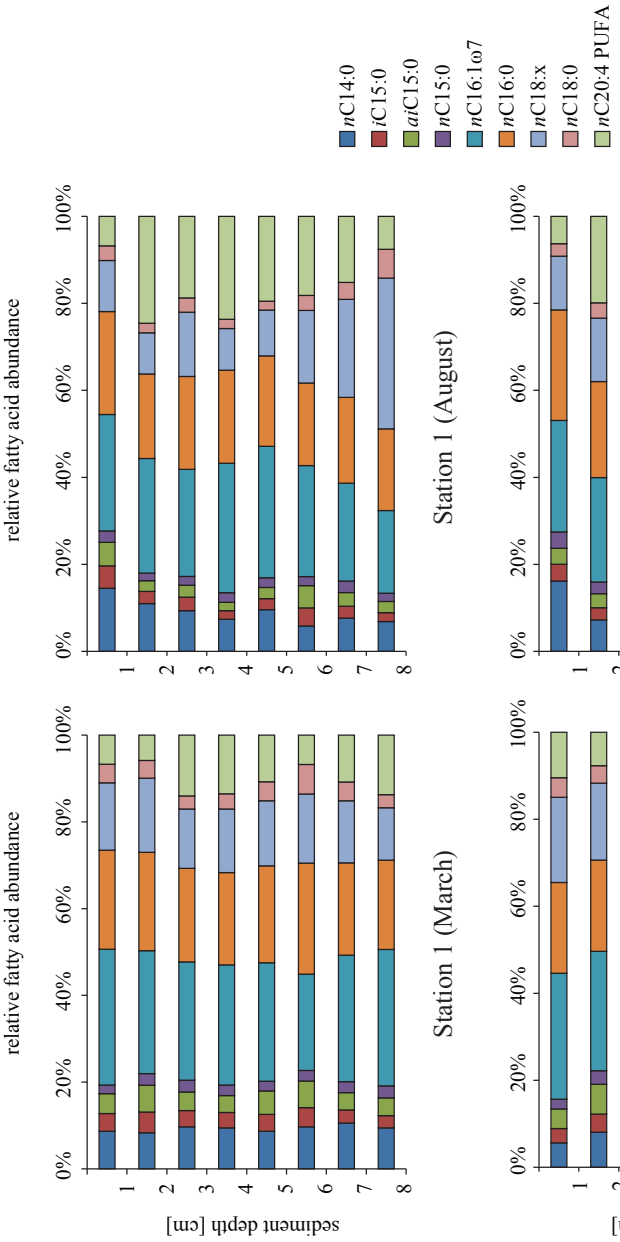

Figure 1: Relative abundance of fatty acids at Station 1 and Station 3 in March and August.

*n*C20:5 polyunsaturated fatty acid (PUFA) and various unsaturated *n*C18:x fatty acids (Figure 1; Table S2). Due to incomplete separation, the *n*C18:x fatty acids had to be integrated as one peak. The fatty acids distributions were similar in both stations and the relative abundance of most fatty acids stayed constant with depth (Figure 1; Table S2).

6.3.2. D/H ratio of fatty acids

The δD values of *n*C14:0, *i*C15:0, *ai*C15:0, *n*C16:1ω7, *n*C16:0, *n*C18:0 fatty acids and *n*C20:5 PUFA were obtained for most sediment layers (Table S3). The D/H ratio of the cluster of *n*C18:x fatty acids could not be measured with certainty due to either incomplete separation or low abundance.

All fatty acids were substantially depleted in D compared to the bottom water overlying the sediment (δD_{water} -1.8±3.3 to 0.1±2.8 ‰) with fractionation factor $\varepsilon_{lipid/water}$ values ranging between -103 and -267 ‰ (Table 1; Figure 2). The *n*C20:5 PUFA, was the most depleted fatty acid followed by the *n*C14:0, while the *i*C15:0 was usually the most D-enriched fatty acid (Table S3). The two different bacterial fatty acids *i*C15:0 and *ai*C15:0 differ by up to 70 ‰, with the *ai*C15:0 having similar $\varepsilon_{lipid/water}$ values as the *n*C16:0 fatty acid. The non-specific fatty acid *n*C18:0 generally also shows a smaller degree of fractionation compared to the other non-specific fatty acids and the *ai*C15:0, varying between -140 to -200 ‰.

Substantial differences in $\varepsilon_{lipid/water}$ values were observed between different depth intervals and cores for some of the fatty acids. At Station 1 in March, the $\varepsilon_{lipid/water}$ values for the *n*C16:1 fatty acid are variable with an overall trend from relatively small $\varepsilon_{lipid/water}$ values of ~-190 from 0 to 2 cm depth to ~-215 between 6 and 8 cm depth, while for August no major depth trend is observed. The $\varepsilon_{lipid/water}$ values for *n*C14:0 at Station 1 in August tend towards smaller fractionation (~20 ‰), with increasing depth, while no major trend with depth was observed in March. The $\varepsilon_{lipid/water}$ value for the *n*C16:0 fatty acid, varies from -170 to -196 ‰ with no particular trend with depth in either month. In March at Station 1 the $\varepsilon_{lipid/water}$ value for the *n*C18:0 fatty acid is ~ -190 ‰ at 0 to 2 cm depth, becomes significantly more positive (~ -150 ‰) at the 2 to 3 cm depth interval after which it slowly decreases again to ~-180 ‰ at 5-6 cm depth and increases further down to ~-160 ‰. In August, the $\varepsilon_{lipid/water}$ for the *n*C18:0 fatty acid is relatively stable at ~-140 to -148 ‰ from 0 to 6 cm depth, although some layers did not contain enough *n*C18:0 for a reliable measurement, and decreases to ~-186 ‰ at 7-8 cm depth (Table 1; Figure 2). At Station 3 in March no trend with depth was observed for *n*C16:1 and *n*C16:0 fatty acids. However, the $\varepsilon_{lipid/water}$ value of *n*C14:0, although vari-

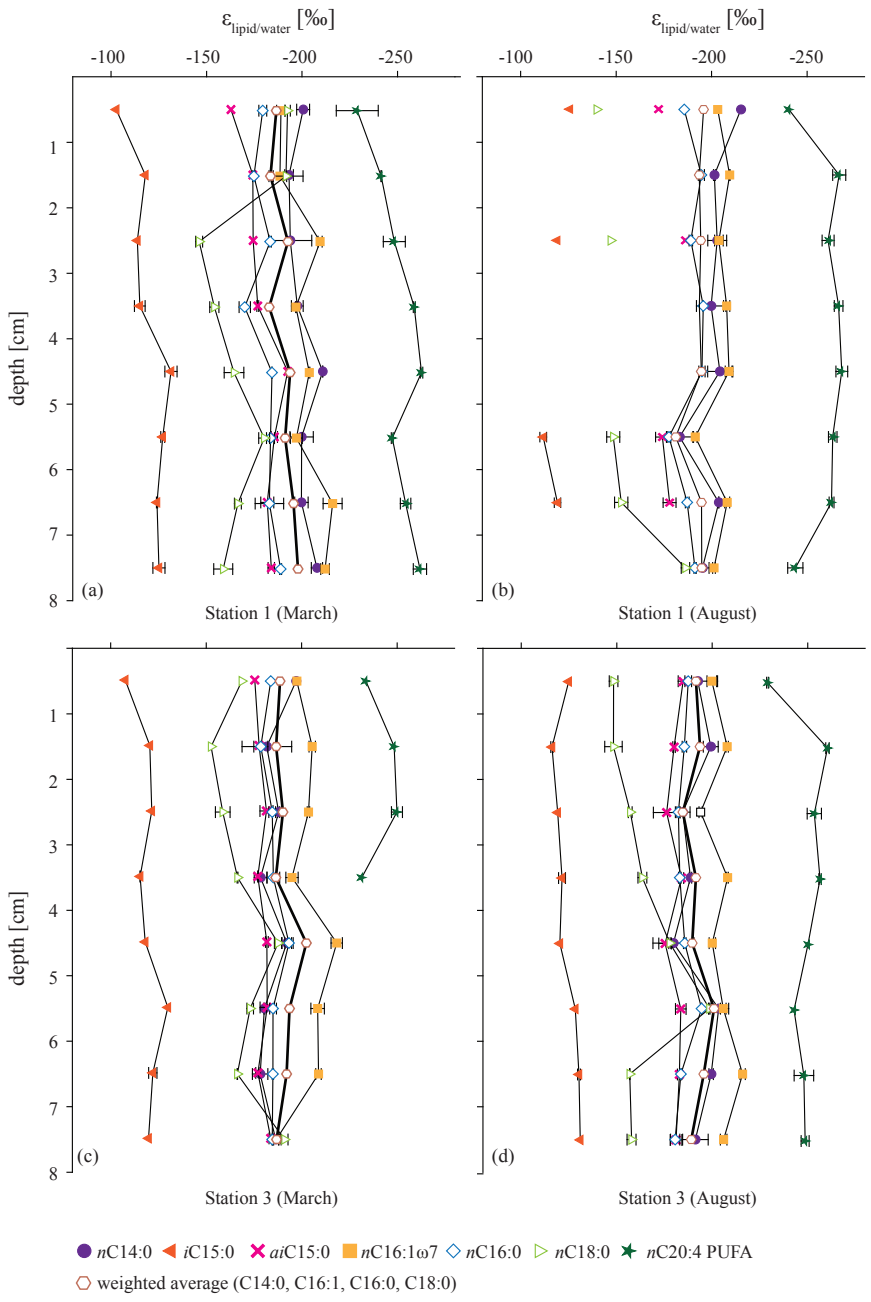

Figure 2: The D/H fractionation between fatty acids and overlying water for fatty acids derived from sediments. (a) Station 1 in March, (b) Station 1 in August. Plotted are the mean ε values (lipid versus water) and weighted average of the $\varepsilon_{lipid/water}$ values of *n*C14:0, *n*C16:1, *n*C16:0 and *n*C18:0 fatty acids. Error bars are the standard deviation of the duplicate measurements of the fatty acids.

Table 1: D/H fractionation between fatty acids and overlying water

Depth [cm]	δD_{water} [‰]	$\varepsilon_{lipid/water}$ [‰]							
		C14:0	*i*C15:0	*ai*C15:0	C16:1*	C16:0	C18:0	C20:5 PUFA	weighted av. (C14, C16:1, C16, C18)
Station 1 (March)									
0–1		-200±3	-103±0	-162±0	-188±2	-180±2	-191±1	-229±12	-186
1–2		-193±2	-118ϕ	-174ϕ	-188±3	-175±1	-190±9	-243±0	-183
2–3		-193±11	-114ϕ	-174ϕ	-209±1	-184±1	-145±2	-248±6	-192
3–4	-1.8±3.3	-197±3	-115±3	-176±0	-196±0	-170±3	-153±2	-258±1	-182
4–5		-210±0	-131±3	-192±0	-203±0	-185±0	-163±5	-262±1	-193
5–6		-199±6	-127±1	-185±2	-197±3	-184±2	-179±3	-247±0	-191
6–7		-199±3	-124±0	-181±3	-216±5	-183±7	-165±2	-254±3	-195
7–8		-207±3	-125±3	-183±2	-212±2	-189±1	-158±5	-261±3	-197
Station 1 (August)									
0–1		-215ϕ	-126ϕ	-171	-203ϕ	-186ϕ	-140ϕ	-240ϕ	-196
1–2		-201±0	N.D.	N.D	-209±0	-195±2	N.D.	-267±3	-194
2–3		-203±5	-119ϕ	-185	-204±2	-189±1	-147ϕ	-261±3	-194
3–4	0.1±2.8	-200±8	N.D	N.D	-208±2	-196±0	N.D.	-266±2	-194
4–5		-204±6	N.D	N.D	-209±2	-195±2	N.D.	-268±3	-195
5–6		-183±7	-112±2	-173±4	-191±1	-178±1	-148±3	-263±2	-181
6–7		-204±0	-120±2	-177±3	-208±1	-187±1	-153±3	-263±1	-195
7–8		-195±3	N.D	N.D	-201±1	-191±1	-186±2	-244±4	-195
Station 3 (March)									
0–1		-197ϕ	-108ϕ	-176ϕ	-197ϕ	-184ϕ	-169ϕ	-234ϕ	-189
1–2		-182±13	-120ϕ	-178±0	-206±2	-179±4	-153ϕ	-248ϕ	-187
2–3		-188±1	-121ϕ	-182±3	-204±1	-185±2	-159±4	-250±3	-190
3–4	-1.6±2.6	-179±3	-115±0	-177±0	-195±3	-185±3	-166±0	-231ϕ	-186
4–5		-192±3	-118ϕ	-182±1	-218±3	-193±2	-188±2	N.D.	-202
5–6		-181±0	-130ϕ	-182±4	-208±4	-185±2	-173±2	N.D.	-194
6–7		-178±4	-122±2	-177±1	-209±1	-185±0	-166±2	N.D.	-192
7–8		-186±1	-120ϕ	-184±1	-187±1	-185±0	-191±2	N.D.	-187
Station 3 (August)									
0–1		-192±10	-125ϕ	-185ϕ	-200±3	-187±2	-148±2	-229±0	-191
1–2		-199±4	-116±1	-180±1	-208±1	-185±1	-148±5	-260±1	-194
2–3		-185±4	-119ϕ	-176±7	-194±1	-182±1	-157±1	-253±4	-185
3–4	-1.7±4.1	-189±1	-121±2	-184±1	-208±0	-183±1	-163±2	-256±1	-191
4–5		-180±11	-120ϕ	-175±3	-200±2	-186±1	-178±1	-250±0	-190
5–6		-203±5	-128ϕ	-184±3	-206±1	-194±0	-199±0	-243±0	-201
6–7		-199±1	-130±1	-183±1	-216±1	-184±1	-157±0	-253±2	-196
7–8		-191±7	-131ϕ	-181±3	-206±2	-181±2	-158±2	-249±2	-189

* double bond at ω7 position; ϕ only one ε value existing

able, seems to show an overall trend towards more positive values by up to 10 ‰ from the surface to 8 cm depth, while the $\varepsilon_{nC18:0/water}$ shows an overall trend towards more negative values by up to 22 ‰. In August, no visible trend with depth could be observed in the $\varepsilon_{lipid/water}$ values for *n*C14:0, *n*C16:1 and *n*C16:0 fatty acids, all of which had similar $\varepsilon_{lipid/water}$ values as in March. The *n*C18:0 fatty acid on the other hand got more depleted compared to water with increasing depth by up to 50 ‰ from the surface layer to the 5-6 cm interval after which $\varepsilon_{lipid/water}$ decreased again by ~ 40 ‰ at 6-7 and 7-8 cm depth and is in general more enriched in D in August than in March (Table 1; Figure 2).

6.3.3. Bacterial diversity

The isotopic fractionation of the fatty acids shows a larger difference between the two different stations than between the different seasons, with the largest difference between the two stations in August. Therefore, the bacterial diversity of sediment cores taken in August was studied using 16S rRNA gene amplicon sequencing. The phylogenetic diversity at Station 1 (Figure 3; Table S4a) consisted of diverse members of the *Bacteroidetes*, *Planctomycetes* and *Proteobacteria* phyla. The main contributors to the total bacterial reads belonged to the order of the *Bacteroidales*, *Desulfobacterales*, *Alteromonadales* and *Thiotrichales*. The percentage of total bacteria reads attributed to the *Bacteroidales* varied from 5.7 to 11.9 % and tended to increase with depth, while of those of the *Desulfobacterales* remained fairly constant at ~13 %. The same was true for reads assigned to the *Alteromonadales*, which remained relative constant at ~6 %. On the other hand, the percentage of the *Thiotrichales* reads peaked at 11.7 % between 4 to 6 cm depth. In addition, the percentage of reads of various other orders decreased to nearly zero with increasing depth while others increased to up to 4.5 % (Figure 3; Table S4a).

Most of the orders observed in Station 1 were also present at Station 3 (Figure 3; Table S4b). The *Desulfobacterales* were the main contributor to the bacteria reads (up to 23 %) and the percentage of reads decreased with depth (down to 15.5 %). Additionally, both the *Bacteroidales* and the *Thiotrichales* contributed to more than 5 % of the total bacteria reads, with the percentage of reads of the *Bacteroidales* decreased to 3.5 % with depth, while the *Thiotrichales* remained fairly constant at ~10 % with depth (Figure 3; Table S4b).

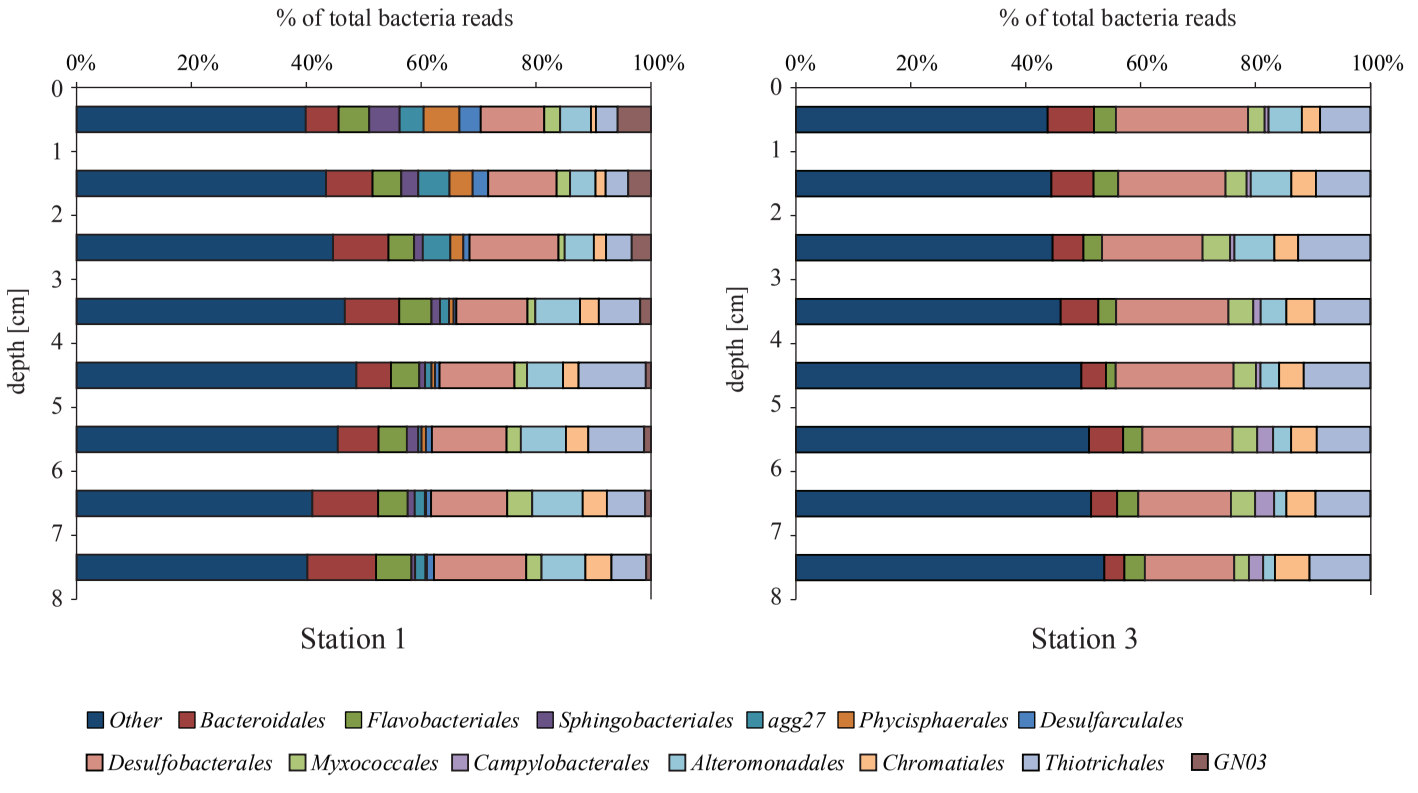

Figure 3: Bar plot of the bacterial diversity in August obtained via 16S rRNA gene amplicon sequencing.

6.4. Discussion

6.4.1.Hydrogen isotopic composition of source-specific fatty acids

Most of the fatty acids commonly occur in bacteria and eukaryotes (e.g. *n*C14:0, *n*C16:1ω7, *n*C16:0 and *n*C18:0), but some are more specific. Both the *i*C15:0 and *ai*C15:0 fatty acids are known to derive from bacterial sources (Gunstone et al., 2012), while the *n*C20:5 PUFA is mainly produced by algae and only in trace amounts by some bacteria (Volkman et al., 1989; Carrie et al., 1998; Iizuka et al., 2003) and therefore considered an algal biomarker. Of all fatty acids, the *n*C20:5 PUFA showed the highest degree of hydrogen isotope fractionation ($\varepsilon_{lipid/water}$ between -230 and -268 ‰) and the *i*C15:0 showed the smallest degree of fractionation ($\varepsilon_{lipid/water}$ between -103 and -131 ‰) (Figure 2). The $\varepsilon_{lipid/water}$ values obtained for the *n*C20:5 PUFA fall within the range previously associated with photoautotrophic growth (Heinzelmann et al., 2015b), in agreement with its algal source.

The $\varepsilon_{lipid/water}$ values of the *i*C15:0 fatty acid fall well within the range of those produced by heterotrophic microorganism which in general produce fatty acids that range between depleted (-133 ‰) up to heavily enriched (+200 ‰) in D compared to the growth medium (e.g. Zhang et al., 2009a; Heinzelmann et al., 2015b). Indeed, the majority of the sequences obtained by 16S rRNA gene amplicon sequencing belonged to heterotrophic bacteria involved in the degradation of high molecular weight biomass coming from the water column (*Bacteroidetes*) and in the sulfur cycle (*Desulfobacterales*). The heterotrophic *Bacteroidetes* are most likely the dominant source of the *i*C15:0 fatty acid (Table S5). Additionally to the *i*C15:0, *ai*C15:0 is also a known bacterial biomarker. Interestingly, this fatty acid was more depleted in D compared to *i*C15:0 by up to 70 ‰. This could possibly be explained by a difference in source organism for these fatty acids. While both *i*C15:0 and *ai*C15:0 fatty acids are produced by the *Bacteroidetes*, *ai*C15:0 is more dominant in species of the *Desulfobacterales* (Table S5). A recent study by Dawson et al. (2015) showed that the sulphate reducing bacterium *Desulfococcus multivorans* (belonging to the *Desulfobacterales*) produces, when grown as a heterotroph both in pure culture and in co-culture with a methanogen, fatty acids which are relatively depleted in D with $\varepsilon_{lipid/water}$ values between -102 and -188 ‰ depending on the substrate (Dawson et al., 2015). These values are more negative than those associated with heterotrophic growth in general, and are closer to what is associated with (photo)autotrophic growth. A contribution of the *Desulfobacterales* to the *ai*C15:0 fatty acid pool could thus explain the higher degree of hydrogen isotopic fractionation observed for this fatty acid compared to *i*C15:0. Additionally, the percentage of total bacterial

reads of the *Desulfobacterales* was higher in Station 3, suggesting a higher contribution to the fatty acid pool and possibly explaining the higher degree of fractionation of the *i*C15:0 fatty acid compared to Station 1.

6.4.2. Hydrogen isotopic composition of common fatty acids

While the hydrogen isotopic composition of group-specific fatty acids provides a clear idea of the metabolism expressed by the source microorganisms, it does not necessarily represent the whole microbial community. In order to study the whole microbial community, we calculated a weighted average ε ($\varepsilon_{\Sigma FA}$) of the non-specific fatty acids (*n*C14:0, *n*C16:1ω7, *n*C16:0 and *n*C18:0) (Table 1, Figure 2). At Station 1, $\varepsilon_{\Sigma FA}$ values were between -182 and -197 ‰ in March and between -181 and -195 ‰ in August. At Station 3, $\varepsilon_{\Sigma FA}$ values were between -186 and -202 ‰ in March and between -185 and -201 ‰ in August. The overall stable $\varepsilon_{\Sigma FA}$ values suggest only minor changes in the general metabolism of the sedimentary microbial communities assuming that the majority of the fatty acids derive from *in situ* production. This agrees with the 16S rRNA gene based diversity analysis of our study that shows relatively minor changes in the overall bacterial community with depth and no apparent depth trend of the distribution of the fatty acids.

Interestingly, however, the $\varepsilon_{\Sigma FA}$ values fall, in both stations, within the range associated with photoautotrophic growth (Zhang et al., 2009a; Heinzelmann et al., 2015a and references therein) although the $\varepsilon_{\Sigma FA}$ values were in general relatively positive compared to previous studies of photoautotrophic microorganisms (Osburn et al., 2011; Heinzelmann et al. 2015c). This is unexpected as photoautotrophs are not expected to be an active part of these sedimentary communities, but rather contribute to the biomass in the water column. It is possible that the hydrogen isotopic composition of the fatty acids are reflecting an averaging between the relatively D depleted signal from chemoautotrophy and the relatively D enriched signal from heterotrophy. While fatty acids produced by heterotrophs in general show only a small degree of fractionation, chemoautotrophs produce fatty acids which are significantly enriched with observed $\varepsilon_{lipid/water}$ values up to -404 ‰ (Valentine et al., 2004). Depending on the relative contribution of heterotrophs versus chemoautotrophs, this could lead to a fatty acid pool significantly more depleted in D than expected for predominantly heterotrophic sourced fatty acids and might explain the observed $\varepsilon_{lipid/water}$ values observed in the Grevelingen sediment samples. Indeed, besides sequences of heterotrophic bacteria belonging to *Bacteroidetes* and *Desulfobacterales*, sequences of chemoautotrophic members of the sulfur cycle (*Chromatiales*/*Thiotrichales*) were observed, which suggests that the sedimentary microbial communities consists of a mixture

of heterotrophic and chemoautotrophic microorganisms. However, it should be noted that not all of the *Chromatiales* are chemoautotrophic, some of the reads belong to the photoautotrophic purple sulfur bacteria. It is unlikely that they grew photoautotrophically in the sediment but rather inhabit the water column in periods of anoxia and reduced sulfur compounds presence in the photic zone of the water column. Besides, only few reads associated with photoautotrophic bacteria were observed, suggesting that *in situ* bacterial photoautotrophy plays a relatively small role in these sediments. Thus, the observed depleted $\varepsilon_{\Sigma FA}$ values of the unspecific, but abundant, fatty acids relative to those of the *i*C15:0 could be due to an admixture of chemoautotrophic bacteria. Alternatively, an important part of the sedimentary population consisted of the *Desulfobacterales*, belonging to the sulphate reducing bacteria, representatives of which seem to produce relatively depleted fatty acids even when growing heterotrophically (Dawson et al., 2015). This could potentially contribute to the general depletion in D of the fatty acids relative to the range typically associated by heterotrophic growth.

6.4.3. Pelagic contributions to the sedimentary fatty acid pool

Although the sedimentary microbial community is dominated by heterotrophic and chemoautotrophic microorganisms, potentially explaining the relatively low δD values of the most common sedimentary fatty acids, there is no real change in $\varepsilon_{\Sigma FA}$ values with depth which corresponds with changes in microbial diversity and specific redox zones. Interestingly, the relatively high abundance of the *n*C20:5 PUFA (i.e. up to 25 %; Figure 1) indicates a major input of algal biomass derived from the water column to the sedimentary fatty acid pool. In the sediments of both stations the relative abundance of *n*C20:5 PUFA increased in August compared to March (Figure 1) which might due to the phytoplankton blooms during spring and summer (Hagens et al., 2015). The high sedimentation rate at the site of >2 cm/y (Malkin et al., 2014), and the fact that anoxic conditions lead to reduced degradation rate of organic matter (Middelburg and Levin, 2009) would explain the high relative abundance of this fatty acid also in deeper layers of the sediment, i.e. preservation of algal-derived fatty acids during sediment burial.

The high relative abundance of *n*C20:5 PUFA suggest it is likely that also a major part of the more ubiquitous *n*C14:0, *n*C16:0 and *n*C16:1 fatty acids might originate from photoautotrophic organisms living in the water column and are thus not derived from the sedimentary microbial community. In fact, diatoms have been shown to dominate the phytoplankton in Lake Grevelingen (Bakker and De Vries, 1984) and are known to mainly produce *n*C14:0, *n*C16:0, *n*C16:1ω7 fatty acids and *n*C20:5 PUFA and only traces of *n*C18:0

(Table S5). It would thus be expected that these fatty acids in the Grevelingen sediments would also contain a significant contribution from pelagic phytoplanktonic biomass. Further support for this hypothesis comes from the $\varepsilon_{lipid/water}$ values for the *n*C18:0 fatty acid, which is only produced in traces by diatoms (Table S5). The generally more positive *n*C18:0 $\varepsilon_{lipid/water}$ values could indicate a relatively high *in situ* contribution from heterotrophic bacteria. Furthermore, the $\varepsilon_{nC18/water}$ value is much more variable in depth and between stations and seasons than $\varepsilon_{lipid/water}$ values for most other fatty acids suggesting a much higher contribution of microorganisms with other metabolisms, e.g. heterotrophy and chemoautotrophy.

Our results thus suggest that a large part of the more general or non-specific fatty acids, especially the *n*C14:0, *n*C16:0 and *n*C16:1ω7 fatty acids, are derived from algae living in the water column and only a minor fraction comes from *in situ* production of the sedimentary microbial population. This predominantly photoautotrophic origin of the non-specific fatty acids is the most likely explanation for the relatively low and stable $\varepsilon_{lipid/water}$ values for these fatty acids.

6.5. Conclusion

The sedimentary microbial community of the Lake Grevelingen consisted of heterotrophic and chemoautotrophic microorganisms. However, the hydrogen isotopic composition of the most abundant fatty acids seem to mainly reflect photoautotrophy, suggesting that these fatty acids are mainly derived from the phytoplankton present in the water column and deposited after cell death on the sediment surface. The effect of the deposition and slow diagenesis of organic matter coming from different aquatic and sedimentary microbial communities under anoxic conditions could lead to a bias in the hydrogen isotopic composition of fatty acids as a tool to study the metabolism of microbial communities *in situ*. It would be therefore beneficial in settings with a high contribution of allochthonous material, relative to *in situ* sedimentary production, to study the hydrogen isotopic composition of group or species specific fatty acids and potentially other lipid classes.

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Summary

Samenvatting

Zusammenfassung

Summary

Microorganisms have been shown to be the most abundant organisms on Earth and are crucial in all elemental cycles. Assessing their diversity, metabolic potential and activity is necessary in order to understand their role in the environment and ecosystem functioning as a whole. Isolation and cultivation of microorganisms allow to better understand the metabolic potential and activity of individual microorganisms *in vitro*. Unfortunately, it has been shown that the majority of environmental microorganisms cannot be isolated with standard cultivation methods. Therefore, it is necessary to develop cultivation-independent methods to bridge the gap between activity in culture and nature and to account for organism that cannot be isolated and cultured. Cultivation-independent methods (e.g. stable isotope probing, functional gene activity analysis, fluorescence in situ hybridization) allow to study specific metabolic activities *in situ*. However, these approaches have their limitations and usually do not allow to study the general metabolism of a microbial community as a whole. The hydrogen isotopic composition (δD value) of fatty acids reflect the metabolism expressed by the source organism and allows to differentiate between photoautotrophy, chemoautotrophy and heterotrophy. While metabolism seems to be the main factor influencing the δD value of fatty acids, the impact of other parameters is not yet completely known. Additionally, the results so far are mainly based on culture studies and it has to be shown if this approach is also applicable to different environmental settings allowing to study the general metabolism of microbial communities. In this thesis additional biological and physical parameters, besides metabolism, and their effect on the hydrogen isotopic composition of fatty acids were tested using culture studies. Furthermore, the D/H ratio of fatty acids derived from marine waters and sediments were analysed in order to study the general metabolism of aquatic microbial communities.

Several different microorganisms were grown under different metabolic conditions and sampled during different growth phases. The different fatty acids were either enriched or depleted in D in accordance with their metabolism (i.e. photoautotrophic, chemoautotrophic and heterotrophic) expressed by the microorganism. In comparison to that, fatty acids from cultures harvested in different growth phases are in general enriched in D when harvested at later growth phases by 4 to 46 ‰. The only exception was a heterotroph grown on a substrate directly involved in the tricarboxylic acid cycle. Nevertheless, this enrichment related to growth phase is relatively minor compared to the effect of metabolism on the δD value of the fatty acids. The reason for this growth phase dependent enrichment is yet unclear and will require further investigation. Salinity also has a significantly smaller effect on the hydrogen isotopic composition of fatty acids (0 to 40 ‰) than metabolism. While fatty acids of photoautotrophs get enriched in deuterium with increasing salinity (up to 40

‰), the hydrogen isotopic composition of fatty acids derived from heterotrophic microorganism does not seem to change with salinity. The mechanism behind the effect of salinity on the hydrogen isotopic composition of lipids is unknown up to date. It is possible that heterotrophic and photoautotrophic microorganisms deal with increasing salinity differently, leading to different changes in the hydrogen isotopic composition of fatty acids. Another possibility is that the difference in the hydrogen source between photoautotrophic and heterotrophic microorganisms, i.e. internal cell water and organic substrate, respectively, leads to the observed different response to salinity.

Culture studies suggest that the D/H ratio of fatty acids could be a suitable approach to study the general metabolism of microbial communities in the environment. However, the extractable lipid pool in environmental settings consists both of fossil lipids set free after cell death and those of living biomass. In the past phospholipid derived fatty acids were used to study living microbial communities as it was assumed that they degrade quickly after cell death. The most common used method to separate intact phospholipids from other lipid classes using silica chromatography was revaluated. This showed that the separation of an intact polar lipid extract on a silica column with dichloromethane, acetone and methanol results in an incomplete separation of phospho- and glycolipids. This leads to potential biases when characterizing microbial communities and their metabolism if only the traditional "phospholipid" fraction is analysed as it does not contain all fatty acids derived from the different membrane lipids. Therefore, it is recommended to separate intact polar lipid extract only into neutral and polar lipids and to analyse the fatty acids from all polar lipids.

In order to verify the applicability of the δD fatty acids to study the general metabolism of microbial communities, two different marine settings were investigated, the coastal North Sea and a saline lake in the south of the Netherlands. The pelagic microbial community of the coastal North Sea is characterised by a major phytoplankton bloom in spring. Analysis of the δD of the fatty acids derived from suspended particulate matter showed that the most negative fractionation, $\varepsilon_{\text{fatty acid/water}}$ values, were observed during spring coinciding with a peak in chlorophyll a concentration, a high abundance of an algal biomarker, the polyunsaturated fatty acid (PUFA) *n*C20:5, and high abundance of *Phaeocystis* all indicating the spring bloom. Following the spring bloom the fatty acids got more enriched in D. This fits with an expected increase of heterotrophic microorganisms following the demise of the phytoplankton bloom. The hydrogen isotopic composition of fatty acids clearly reflect the increase in photoautotrophy during the spring phytoplankton bloom in the coastal North Sea, exactly as expected.

Lake Grevelingen is a saline lake located in the south of the Netherlands. The former estuary suffers from anoxic bottom water in summer due to a reduced in- and outlet of water. Changes in the oxygen concentrations should therefore lead to changes in the redox horizons in the sediment. All measured sedimentary fatty acids were depleted in D relative to the bottom water by 103 to 267 ‰. While the bacterial biomarker fatty acids (*ai*C15:0 and *i*C15:0) derived likely from the sedimentary microbial community, the high relative abundance of the algal biomarker *n*C20:5 PUFA indicate a significant contribution of dead algal biomass coming from the water column to the sedimentary pool of the more commonly occurring fatty acids (*n*C14:0, *n*C16:0, *n*C16:1ω7). Therefore, the stable $\varepsilon_{fatty\ acid/water}$ of the individual fatty acids observed with depth in the sediments might be explained by A) a mixture of D depleted fatty acids from chemoautotrophic and D enriched fatty acids from heterotrophic microbes in the sediment which keeps the average values relatively constant or B) a dilution of the sedimentary signal by fatty acids from photoautotrophic microbes in the water column which obscures the signal from sedimentary microbes.

The results described in this thesis show a dominant effect of metabolism compared to other parameters on the D/H ratio of microbial fatty acids. Furthermore, the hydrogen isotopic composition of fatty acids can be used in the marine environment to study the general metabolism of entire microbial communities. However, the input of allochtonous fatty acids from dead biomass has to be considered as a potential complicating factor.

Micro-organismen zijn de meest voorkomende organismen op aarde, zowel in aantal als totale biomassa en leveren een cruciale bijdrage aan alle elementcycli. Om te begrijpen wat hun rol is in het milieu en in het functioneren van ecosystemen moeten de diversiteit, metabolische capaciteiten en activiteit van deze organismen in kaart gebracht worden. Het isoleren en kweken van micro-organismen maakt dat we de metabolische capaciteiten en de activiteit van individuele micro-organismen beter kunnen bestuderen en begrijpen. Helaas is het ook duidelijk dat het overgrote deel van micro-organismen in de natuur niet geïsoleerd kan worden met de bestaande kweekmethoden. Daarom is het essentieel om cultivatie-onafhankelijke methoden te ontwikkelen die de activiteit van micro-organismen in de natuur die niet geïsoleerd en gecultiveerd kunnen worden te bepalen. Met behulp van cultivatie-onafhankelijke methoden (zoals labelen met stabiele isotopen, de analyse van functionele genen en in situ hybridisatie met fluorescerende labels) kan specifieke metabolische activiteit *in situ* bestudeerd worden. Deze benaderingen hebben, echter, ook allemaal hun beperkingen en kunnen in het algemeen niet de activiteit van de gehele microbiële gemeenschap bepalen. De waterstofisotopenverhouding (δD waarde) van vetzuren is gerelateerd aan het metabolisme van de organismen die deze vetzuren maken en dit maakt het mogelijk om fotoautotrofie, chemoautotrofie en heterotrofie te onderscheiden. Hoewel metabolisme de belangrijkste factor lijkt te zijn die de δD waarde van vetzuren bepaald, zijn de mogelijke effecten van andere factoren nog onvoldoende onderzocht. Daarnaast zijn bijna alle resultaten tot nu toe gebaseerd op cultuurstudies en moet nog aangetoond worden in hoeverre deze methode gebruikt kan worden om het algemene metabolisme van microbiële gemeenschappen te bepalen in verschillende milieus. In dit proefschrift zijn naast metabolisme ook de effecten van een aantal andere biologische en fysische factoren op de waterstofisotopenverhouding van vetzuren onderzocht.

Een aantal verschillende micro-organismen zijn gekweekt onder verschillende metabolische condities en bemonsterd in verschillende groeifases. De verschillende vetzuren waren of wel relatief verrijkt dan wel verarmd in deuterium (D) in overeenstemming met het metabolisme (i.e. fotoautotroof, chemoautotroof en heterotroof) van de gekweekte micro-organismen. Vetzuren van microben bemonsterd in verschillende groeifases zijn daarentegen over het algemeen 4 tot 46 ‰ verrijkt in D wanneer zij bemonsterd zijn in latere groeifases. De enige uitzondering was een organisme dat heterotroof gekweekt was op een substraat dat rechtstreeks betrokken is in de citroenzuurcyclus. De verrijking in D in latere groeifases is relatief klein ten opzichte van het effect van metabolisme op de δD waarde van vetzuren. Het achterliggende mechanisme voor deze groeifase-afhankelijke verrijking is nog onbekend en vergt nader onderzoek. Het zoutgehalte of de saliniteit van water heeft ook een sig-

nificant maar kleiner effect op de waterstofisotopenverhouding van vetzuren (0 tot 40 ‰) dan metabolisme. Daarnaast worden de vetzuren van fotoautotrofe organismen in toenemende mate verrijkt in deuterium als de saliniteit toeneemt (tot 40 ‰) terwijl de vetzuren van heterotrofe organismen niet lijken te veranderen met veranderend zoutgehalte. Het precieze mechanisme achter dit effect van saliniteit op de waterstofisotopenverhouding is tot op heden niet bekend. Mogelijk gaan heterotrofe en fotoautotrofe micro-organismen anders om met toenemende saliniteit hetgeen resulteert in verschillende saliniteitseffecten op de waterstofisotopenverhouding van de vetzuren. Een alternatieve verklaring is dat de verschillende waterstofbronnen voor fotoautotrofe en heterotrofe micro-organismen, respectievelijk intracellulair water en het organische substraat, de verschillende saliniteitseffecten kan verklaren.

Cultuurstudies suggereren dus dat de D/H ratio van vetzuren een goede benadering voor het bestuderen van het algemene metabolisme van microbiële gemeenschappen in het milieu zou kunnen zijn. Een probleem is echter dat extraheerbare lipiden in het milieu bestaan uit zowel lipiden afkomstig van levende organismen als van fossiele lipiden die vrijkomen na de dood van cellen. In het verleden werden vetzuren gebonden in fosfolipiden gebruikt om levende microbiële gemeenschappen te bestuderen met het idee dat fosfolipiden snel na de dood uit elkaar vallen. De meest gebruikte methode om intacte fosfolipiden te scheiden van andere lipideklassen is silica chromatografie. Nader onderzoek van deze methode liet echter zien dat de scheiding van intacte polaire lipiden op een silicakolom met dichloormethaan, aceton en methanol als loopmiddelen resulteert in een onvolledige scheiding van fosfo- en glycolipiden. Dit zou potentieel tot systematische fouten kunnen leiden wanneer microbiële gemeenschappen en hun metabolisme onderzocht zouden worden door alleen de traditionele "fosfolipiden" fractie te analyseren omdat die niet de vetzuren van alle verschillende membraanlipiden bevat. Daarom is het aan te raden om intacte polaire lipiden extracten te scheiden in alleen neutrale en polaire lipiden en vervolgens de vetzuren gebonden in alle polaire lipiden te analyseren.

Om te onderzoeken of de δD van vetzuren gebruikt kan worden om het metabolisme van microbiële gemeenschappen te bestuderen zijn twee verschillende mariene systemen onderzocht, de Noordzee dicht bij de kust van Texel en een zoutwatermeer in het zuiden van Nederland. De pelagische microbiële gemeenschap van de Noordzee wordt gekarakteriseerd door een grote fytoplanktonbloei in het voorjaar. Analyse van de δD van de vetzuren afkomstig van gesuspendeerd particulair materiaal uit in de waterkolom laat de meest negatieve fractionering, $\varepsilon_{vetzuur/water}$ waardes, zien in het voorjaar tezamen met een piek in chlorofyl *a* concentraties, een grote hoeveelheid *Phaeocys-*

tis cellen en een grote hoeveelheid van het meervoudig onverzadigd vetzuur *n*C20:5, een karakteristiek vetzuur afkomstig van specifieke algen, allemaal indicaties voor de voorjaarsbloei. Na de voorjaarsbloei raakten de vetzuren meer verrijkt in D. Dit is in overeenstemming met de verwachte toename in heterotrofe micro-organismen na het afsterven van de fytoplanktonbloei. De δD waarden van de vetzuren gedurende de jaarcyclus laten dus duidelijk de toename in fotoautotrofie gedurende de voorjaarsfytoplanktonbloei in de Noordzee dicht bij de kust zien, precies zoals verwacht.

Het Grevelingenmeer is een zoutwatermeer in het zuiden van Nederland. Het voormalige estuarium wordt vandaag de dag gekarakteriseerd door de aanwezigheid van zuurstofloos bodemwater gedurende de zomer als gevolg van een gereduceerde in- en uitstroom van water. Veranderingen in de zuurstofconcentraties zouden daarom moeten leiden tot veranderingen in redox zones in het sediment en dus tot een successie van microbiële gemeenschappen met wellicht verschillende "overall" metabolismen. De vetzuren in het sediment waren verarmd in D ten opzichten van het bodemwater met 103 tot 267 ‰. Terwijl de specifieke bacteriële vetzuren (*ai*C15:0 en *i*C15:0) waarschijnlijk afkomstig zijn van de sedimentaire microbiële gemeenschap, geeft de grote hoeveelheid van het specifieke algen vetzuur *n*C20:5 aan dat er een substantiële bijdrage van meer algemene vetzuren (*n*C14:0, *n*C16:0, *n*C16:1ω7) is van dood algen materiaal afkomstig van de waterkolom aan de sedimentaire vetzuren. De constante $\varepsilon_{\text{vetzuur/water}}$ waarden van de individuele vetzuren met toenemende diepte in het sediment kan mogelijk verklaard worden door A) een mix van D-verarmde vetzuren afkomstig van chemoautotrofe en D-verrijkte vetzuren van heterotrofe microben in het sediment waardoor de gemiddelde waarde relatief constant blijft of B) een verdunning van het sedimentaire signaal door vetzuren afkomstig van fotoautotrofe microben in de waterkolom waardoor het signaal van sedimentaire microben niet zichtbaar is.

De resultaten beschreven in dit proefschrift laten een overheersend effect van metabolisme zien op de D/H ratio van microbiële vetzuren in vergelijking tot andere factoren. Daarnaast is aangetoond dat de δD van vetzuren gebruikt worden om het "overall" metabolisme van microbiële gemeenschappen in het marine milieu te bestuderen. De mogelijke inbreng van allochtone vetzuren uit dode biomassa moet dan wel meegenomen worden als een potentieel complicerende factor.

Mikroorganismen sind die am meisten verbreitesten Lebensformen auf der Erde und kritischer Bestandteil in allen Elementarkreisen. Um ihre Rolle in der Umwelt zu verstehen ist es notwendig, ihre Diversität, ihr Stoffwechselpotential und ihre Stoffwechselaktivität zu untersuchen. Die Isolierung und die Kultivierung von Mikroorganismen helfen dabei, das Stoffwechselpotential und die Stoffwechselaktivität einzelnener Mikroorganismen besser *in vitro* zu verstehen. Bedauerlicherweise kann der Größtteil der Mikroorganismen in der Umwelt nicht mittels Standardkultivierungsmethoden isoliert werden. Daher ist es notwendig, kultivierungsunabhängige Methoden zu entwickeln die es erlauben, diese Lücke zwischen Aktivität in Kultur und in der Natur zu überbrücken und Mikroorganismen zu berücksichtigen, die weder isoliert noch kultiviert werden können. Mittels kultivierungsunabhängiger Methoden (z.B. stabile Isotopen Sondierung, funktionelle Genaktivitäts Analyse, Fluoreszenz *in situ* Hybridisierung) ist es möglich, spezifische Stoffwechselaktivitäten *in situ* zu studieren. Allerdings, diese Anwendungen haben Limitierungen und erlauben es meistens nicht, den generellen Stoffwechsel einer mikrobiellen Gemeinschaft als ein Ganzes zu studieren. Die Wasserstoffisotopenkomposition (δD) von Fettsäuren reflektiert den Stoffwechsel des Ursprungsorganismus und erlaubt es, zwischen Photoautotrophie, Chemoautotrophie und Heterotrophie zu unterscheiden. Während der Stoffwechsel vermutlich der Hauptfaktor ist, der die δD Werte von Fettsäuren bestimmt, ist der Einfluss von anderen Parameteren bisher kaum bekannt. Außerdem, sämtliche Ergebnisse basieren bisher auf Kulturstudien und es muss sich zeigen, in wie weit dieser Ansatz auch in unterschiedlichen Umweltstandorten anwendbar ist um den generellen Stoffwechsel von mikrobiellen Gemeinschaften zu studieren. In dieser Arbeit werden, neben Stoffwechsel, weitere biologische und physikalische Parameter und ihr Effekt auf die Wasserstoffisotopenkomposition von Fettsäuren in Kulturvierungsexperimenten untersucht. Desweiteren wurde das D/H Verhältnis in Fettsäuren, extrahiert aus Meerwasser und Sedimenten, analysiert um den generellen Stoffwechsel von aquatischen mikrobiellen Gemeinschaften zu studieren.

Mehrere unterschiedliche Mikroorganismen wurden unter unterschiedlichen metabolischen Bedingungen kultiviert und Proben wurden während verschiedener Wachstumsphasen genommen. Die verschiedenen Fettsäuren waren, endsprechend des Stoffwechsels des Ursprungsmikroorganismus (z.B. Photoautotrophie, Chemoautotrophie und Heterotrophie), entweder in Deuterium an- oder abgereichert. Im Vergleich dazu sind Fettsäuren aus Kulturen die in unterschiedlichen Wachstumsphasen gewonnen wurden, im Generellen um 4 bis 46 ‰ in D angereichert wenn die Proben in späteren Wachstumsphasen genommen wurden. Die einzige Ausnahme war ein Heterotropher, der auf einem Substrat angezogen wurde, welches direkt im Zitronensäurezyklus

involviert ist. Nichtsdestotrotz, diese Anreicherung bezüglich der Wachstumsphase ist relativ gering im Vergleich zum Effekt, den der Stoffwechsel auf den δD von Fettsäuren hat. Der Grund für diese Wachstumsphasenabhängige Anreicherung ist noch unklar und verlangt weitere Nachforschungen. Im Vergleich zum Stoffwechsel hat Salinität einen signifikant kleineren Effekt auf die Wasserstoffisotopenkomposition von Fettsäuren (0 bis 40 ‰). Während Fettsäuren, die von Photoautotrophen produziert wurden, mit steigender Salinität an Deuterium angereichert wurden (bis zu 40 ‰), scheint sich die Wasserstoffisotopenkomposition von Fettsäuren, welche von heterotrophen Mikroorganismen produziert wurden, nicht von änderder Salinität beeinflusst zu sein. Der Mechanismus der Wirkung der Salinität auf die Wasserstoffisotopenkomposition von Lipiden ist zu diesem Zeitpunkt unbekannt. Es ist möglich, das heterotrophe und photoautotrophe Mikroorganismen unterschiedlich auf steigende Salinität reagieren was zu unterschiedlichen Änderungen in der Wasserstoffisotopenkomposition. Eine weitere Möglichkeit ist, dass die unterschiedlichen Wasserstoffquellen zwischen photoautotrophischen und heterotrophischen Mikroorganismen, z.B. internes Zellwasser bzw. organisches Substrat, zu den beobachteten Unterschieden in der Reaktion auf Salinität führen.

Kultivierungsstudien legen nahe, dass das D/H Verhältnis von Fettsäuren ein geeigneter Ansatz ist, um den generellen Stoffwechsel von mikrobiellen Gemeinschaften in der Umwelt zu studieren. Allerdings, der extrahierbare Lipidpool in der Umwelt besteht sowohl aus fossilen Lipiden, welche nach dem Zelltod freigesetzt wurden, und aus Lipiden, welche von lebender Biomasse stammen. In der Vergangenheit wurden Fettsäuren, die von Phospholipiden stammen genutzt um lebende mikrobielle Gemeinschaften zu studieren, da angenommen wurde, dass die Phospolipide schnell nach dem Zelltod degradieren. Die gebräuchlichste Methode um intakte Phospholipide von anderen Lipidklassen zu separieren ist die Silikatchromatographie und wurde hier reevaluiert. Es zeigte sich, dass die Trennung von einem Extrakt von intakten polaren Lipiden auf einer Silikatsäule mit DCM, Aceton und Methanol zu einer unvollständigen Trennung von Phospho- und Glykolipiden führt. Dies führt zu möglichen Abweichungen beim Charakterisieren von mikrobiellen Gemeinschaften und ihren Stoffwechseln wenn man nur die traditionelle „Phospholipid“-Fraktion analysier,t da diese nicht sämtliche Fettsäuren beinhaltet die von den verschiedenen Membranlipiden stammen. Membranlipide gelten anders als Vorratslipide als Indikator für lebende Biomasse. Daher ist es empfehlenswert, den Extrakt aus intakten polaren Lipiden nur in eine neutrale (z.B. fossile) und eine polare Lipidfraktion aufzuspalten.

Um die Anwendbarkeit des δD von Fettsäuren für das Studium des generel-

len Stoffwechsels von mikrobiellen Gemeinschaften zu verifizieren, wurden zwei unterschiedliche marine Standorte untersucht, die küstennahe Nordsee und ein saliner See im Süden der Niederlande. Die pelagiale mikrobielle Gemeinschaft in der küstennahen Nordsee wird von einer großen Phytoplanktonblüte im Frühling bestimmt. Die Analyse des δD von Fettsäuren, welche von im Wasser suspendierter partikulärer Materie stammen, zeigte, dass die negativsten $\varepsilon_{\text{Fettsäure/Wasser}}$ Werte im Frühling auftraten. Diese korrelierten mit einem Peak in der Chlorophyll *a* Konzentration, einer hohen Abundanz des Algenbiomarkers der mehrfachungesätigen Fettsäure (PUFA) *n*C20:5 und einer hohen Abundanz von *Phaeocystis* welche alle auf die Frühlingsblüte hinweisen. Der Frühlingsblüte folgend kommt es zu einer Anreichung der Fettsäuren in D. Dies stimmt mit dem zu erwartenen Anstieg von heterophen Mikroorganismen nach dem Ende der Phytoplanktonblüte überein. Die Wasserstoffisotopenkomposition von Fettsäuren reflektiert eindeutig den Anstieg von Photoautotrophie währen der Frühlingsphytoplanktonblüte in der küstennahen Nordsee.

Der saline See Grevelingen liegt im Süden der Niederlanden. Das ehemalige Mündungsgebiet leidet an anoxischem Bodenwasser im Sommer aufgrund eines reduzierten Zu- und Abflusses von Wasser. Änderungen in der Sauerstoffkonzentration sollten daher zu Änderungen im Redoxhorizont im Sediment führen. Alle gemessenden sedimentären Fettsäuren sind abgereichert an D relativ zum Bodenwasser (zwischen 103 und 267 ‰). Während die bakteriellen Biomarkerfettsäuren (*ai*C15:0 and *i*C15:0) höchstwahrscheinlich von den sedimentären mikrobiellen Gemeinschaften stammen, weist die relative hohe Abudanz des Algenbiomarkers *n*C20:5 PUFA auf einen signifikanten Beitrag von toter Algenbiomasse, welche aus der Wassersäule stammt, zu dem sedimentären Fettsäurepool der allgemeiner vorkommenen Fettsäuren (*n*C14:0, *n*C16:0, *n*C16:1ω7) hin. Daher wäre die unverändert stabilen $\varepsilon_{\text{Fettsäure/Wasser}}$ Werte der einzelnen Fettsäuren, welcher im Sediment beobachtet wurde, erklärbar durch entweder A) eine Mischung D abgereicherten Fettsäuren (Chemoautotrophie) und in D angereicherten Fettsäuren (Heterotrophie) im Sediment oder B) einer Abschwächung des sedimentären Signals durch Fettsäuren (Photoautotrophie) aus der Wassersäule, was das Isotopensignal der sedimentären Mikrooganismen verschleiert.

Die Ergebnisse, die in dieser Arbeit dargestellt sind, zeigen einen dominaten Einfluss des Stoffwechsels, im Verleich zu anderen Parametern, auf das D/H Verhältnis in mikrobiellen Fettsäuren. Desweiteren kann die Wasserstoffisotopenkomposition von Fettsäuren dazu genutzt werden, um in der marinen Umwelt den generellen Stoffwechsel von ganzen mikrobiellen Gemeinschaften zu studieren. Wie auch immer, der Beitrag von allochtonen

Fettsäuren aus toter Biomasse muss als potentialer verkomplizierender Faktor berücksichtigwerden.

Supplementary Data

Table S1: Fatty acid abundance in different microorganisms grown under various metabolic conditions.

Organism	Fatty acid abundance [%]								GP
	C12:0	C14:0	C16:1*	C16:0	C17:cyc	C17:1$^{\varphi}$	C18:1$^{\phi}$	C19:cyc	
Thiocapsa roseopersicina	-	-	32.4	20.0	-	-	47.7	-	E
	-	-	30.5	20.7	-	-	48.8	-	S
	14.3	7.3	20.9	20.5	-	7.4	29.6	-	D
Halochromatium glycolicum	-	-	6.9	19.8	-	-	73.3	-	E
	-	-	8.4	18.9	-	-	72.7	-	S
	-	-	9.3	18.4	-	-	52.4	19.9	D
Isochrysis galbana	-	55.5	-	32.5	-	-	12.0	-	E
	-	36.6	-	23.4	-	-	39.9	-	S
	-	23.6	-	23.0	-	-	53.4	-	D
Thiobacillus denitrificans	-	-	47.2	43.6	9.2	-	-	-	E
	-	-	47.3	46.2	6.6	-	-	-	S
	-	-	46.6	44.3	9.1	-	-	-	D
Pseudomonas str. LFY10	-	-	39.8	35.0	-	-	25.2	-	E
(glucose)	-	-	38.3	35.9	-	-	25.8	-	S
	-	-	6.9	47.8	25.1	-	10.6	9.6	D
Pseudomonas str. LFY10	-	-	29.2	44.4	12.3	-	14.1	-	E
(acetate)	-	-	29.1	44.6	11.9	-	14.3	-	S
	-	-	4.7	40.4	39.9	-	9.2	5.7	D

*n*C16:1*: double bond at the ω7 position; *n*C17:1$^{\varphi}$: double bond at the ω7 position; *n*C18:1$^{\phi}$: double bond in all cultures except for *I. galbana* (ω9) at the ω7 position.; GP: growth phase; E= exponential, S= stationary, D= death

Table S2: Mean δD values of fatty acids produced by different microorganisms under various metabolic conditions.

Organism	growth rate	δD_{water} [‰]	mean δD [‰]								GP
			C12:0	C14:0	C16:1*	C16:0	C17:cyc	C17:1$^{\varphi}$	C18:1$^{\phi}$	C19:cyc	
Thiocapsa roseopersicina	0.11	-51±3			-302	-256			-297		E
	n.d.	-50±3			-297	-249			-291		S
	n.d.	-59±3	-251	-255	-267	-239		-203	-277		D
Halochromatium glycolicum	0.12	-50±2			-261	-228			-268		E
	n.d.	-51±2			-264	-217			-257		S
	n.d.	-61±2			-262	-210			-257	-237	D
Isochrysis galbana	0.62$^{\varphi}$	4±2		-234		-229			-145		E
	n.d.	5±2		-211		-201			-175		S
	n.d.	9±1		-193		-193			-177		D
Thiobacillus denitrificans	0.93	-51±1			-300	-312	-267				E
	n.d.	-49±3			-287	-306	-267				S
	n.d.	-54±3			-292	-309	-259				D
Pseudomonas str. LFY10	n.d.	-56±2			22	50			50		E
(glucose)	n.d.	-55±2			32	61			62		S
	n.d.	-38±2			81	125	108		152	118	D
Pseudomonas str. LFY10	n.d.	-57±2			194	206	282		235		E
(acetate)	n.d.	-57±3			179	189	252		216		S
	n.d.	-44±2			192	237	250		247	265	D

*n*C16:1*: double bond at the ω7 position; *n*C17:1$^{\varphi}$: double bond at the ω7 position; *n*C18:1$^{\Phi}$: double bond in all cultures except for *I. galbana* (ω9) at the ω7 position.; GP: growth phase; E= exponential, S= stationary, D= death; $^{\varphi}$ value previously reported by Chivall et al. 2014

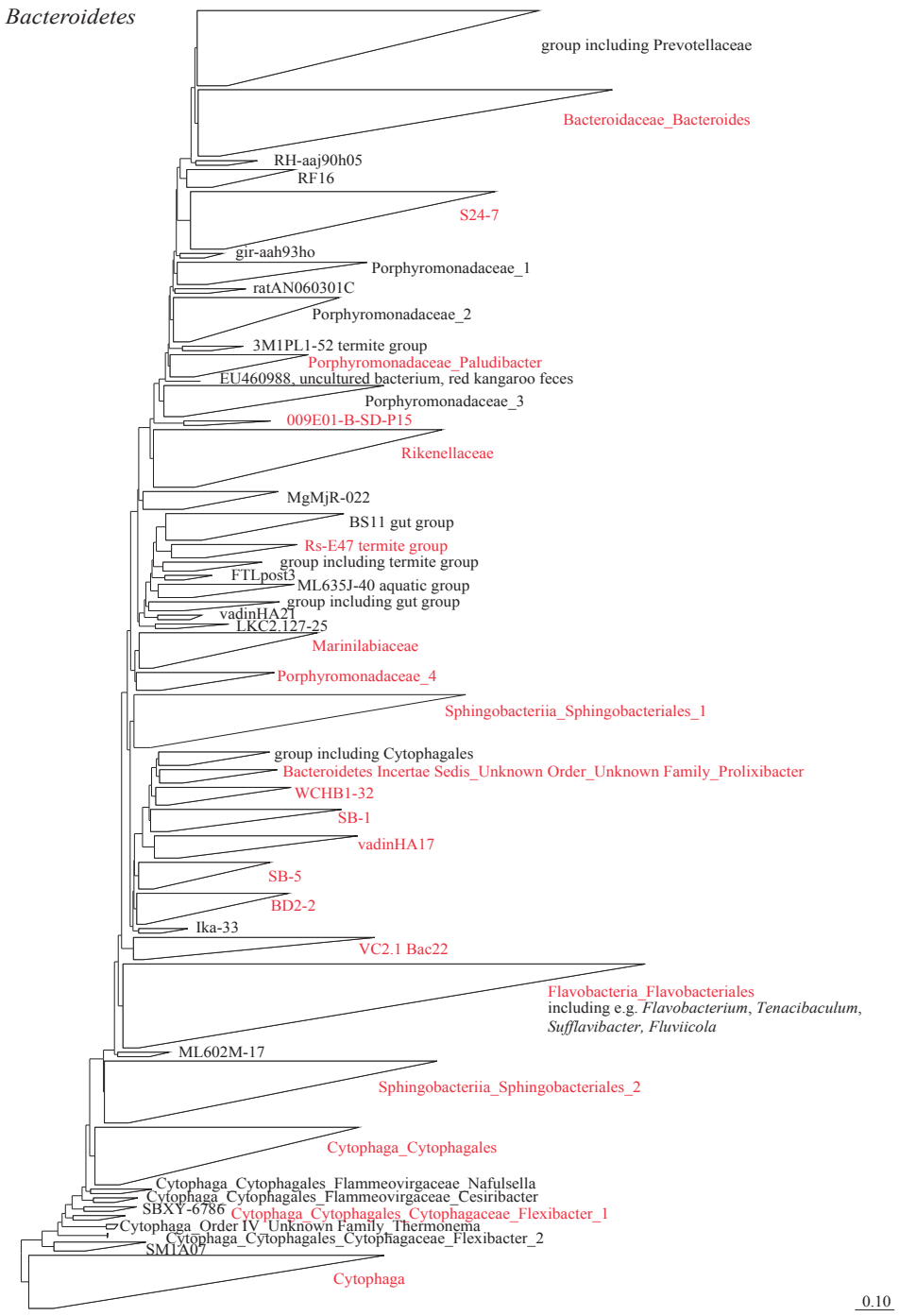


Figure S1: Phylogenetic tree of 16S rRNA gene sequence reads assigned to Bacteroidetes. Scale bar indicates 0.10 % estimated sequence divergence. Groups containing sequences are highlighted.

Alphaproteobacteria

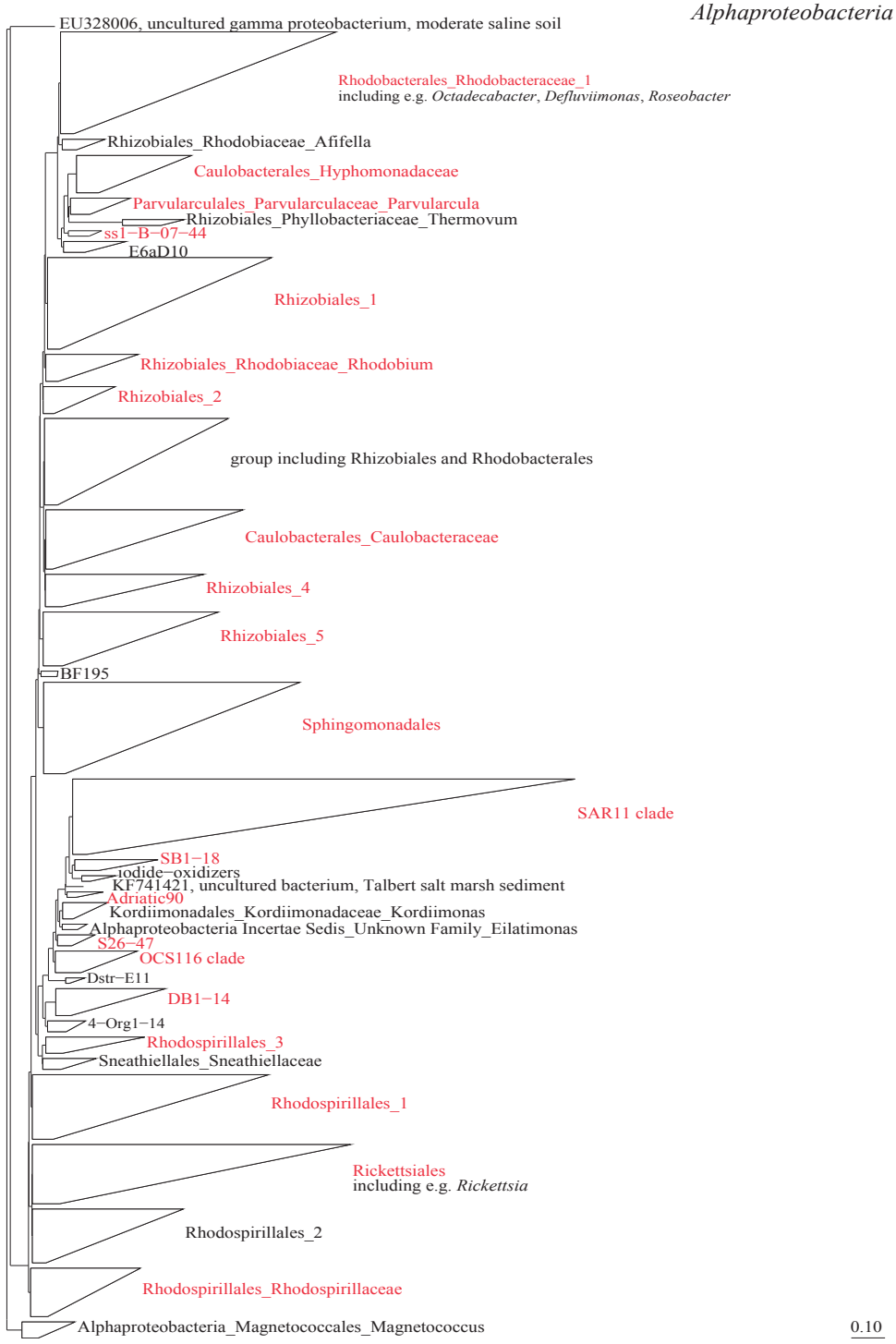
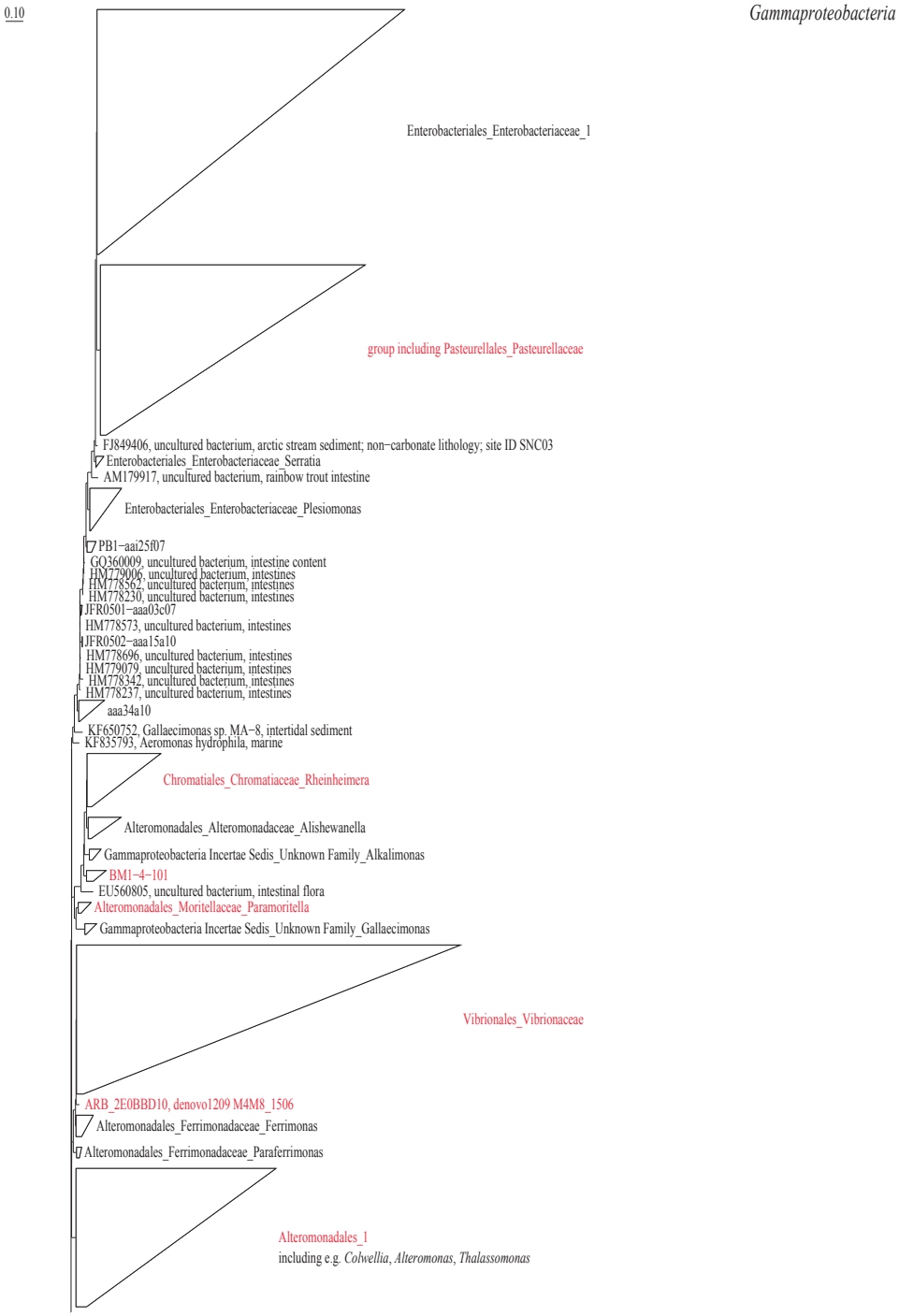
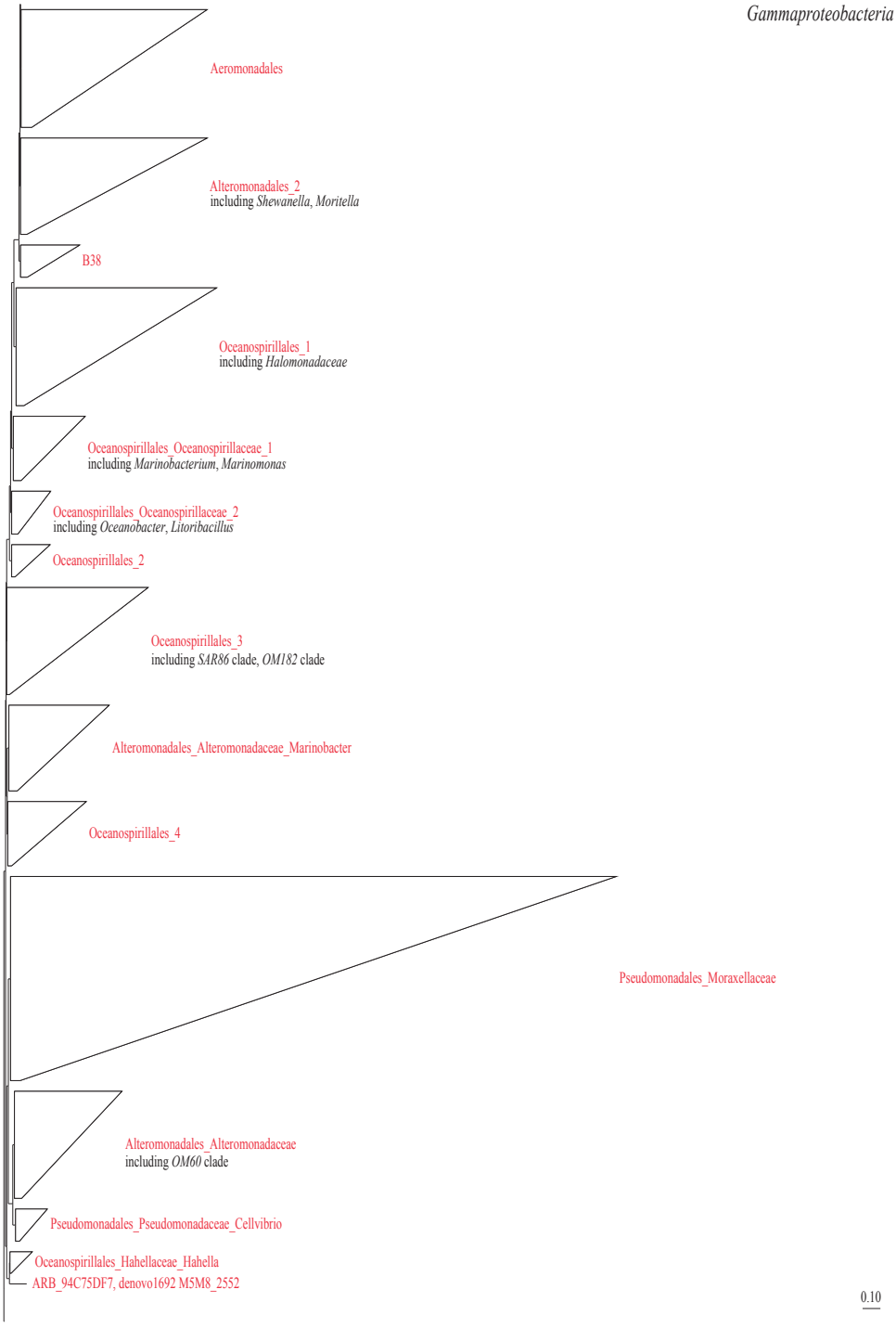


Figure S2: Phylogenetic tree of 16S rRNA gene sequence reads assigned to Alphaproteobacteria. Scale bar indicates 0.10 % estimated sequence divergence. Groups containing sequences are highlighted.


0.10
Gammaproteobacteria
Enterobacteriales_Enterobacteriaceae_1
group including Pasteurellales_Pasteurellaceae
FJ849406, uncultured bacterium, arctic stream sediment; non−carbonate lithology; site ID SNC03
Enterobacteriales_Enterobacteriaceae_Serratia
AM179917, uncultured bacterium, rainbow trout intestine
Enterobacteriales_Enterobacteriaceae_Plesiomonas
PB1−aai25f07
GQ360009, uncultured bacterium, intestine content
HM779006, uncultured bacterium, intestines
HM778562, uncultured bacterium, intestines
HM778230, uncultured bacterium, intestines
JFR0501−aaa03c07
HM778573, uncultured bacterium, intestines
JFR0502−aaa15a10
HM778696, uncultured bacterium, intestines
HM779079, uncultured bacterium, intestines
HM778342, uncultured bacterium, intestines
HM778237, uncultured bacterium, intestines
aaa34a10
KF650752, Gallaecimonas sp. MA−8, intertidal sediment
KF835793, Aeromonas hydrophila, marine
Chromatiales_Chromatiaceae_Rheinheimera
Alteromonadales_Alteromonadaceae_Alishewanella
Gammaproteobacteria Incertae Sedis_Unknown Family_Alkalimonas
BM1−4−101
EU560805, uncultured bacterium, intestinal flora
Alteromonadales_Moritellaceae_Paramoritella
Gammaproteobacteria Incertae Sedis_Unknown Family_Gallaecimonas
Vibrionales_Vibrionaceae
ARB_2E0BBD10, denovo1209 M4M8_1506
Alteromonadales_Ferrimonadaceae_Ferrimonas
Alteromonadales_Ferrimonadaceae_Paraferrimonas
Alteromonadales_1
including e.g. Colwellia, Alteromonas, Thalassomonas

Gammaproteobacteria




ARB_94C75DF7, denovo1692 M5M8_2552
Gammaproteobacteria
Oceanospirillales_Oceanospirillaceae_Pseudospirillum
Oceanospirillales_Alcanivoracaceae_Alcanivora
Pseudomonadales_Pseudomonadaceae
including Pseudomonas
BD3−1
Gammaproteobacteria_Cardiobacteriales_Cardiobacteriaceae
Gammaproteobacteria_Xanthomonadales_Xanthomonadaceae
Gammaproteobacteria_Chromatiales_Halothiobacillaceae
MACA−EFT26
Proteobacteria Incertae Sedis_Unknown Order_Unkown Family_Candidatus Allobeggiatoa
Xanthomonadales
Gammaproteobacteria Incertae Sedis_Unknown Family_Thiohalorhabdus
Chromatiales_Chromatiaceae_Nitrosococcus
Chromatiales_Ectothiorhodospiraceae_1
Chromatiales_Ectothiorhodospiraceae_Acidiferrobacter
Acidithiobacillales
Thiotrichales_Thiotrichaceae
1013−28−CG33
0.10

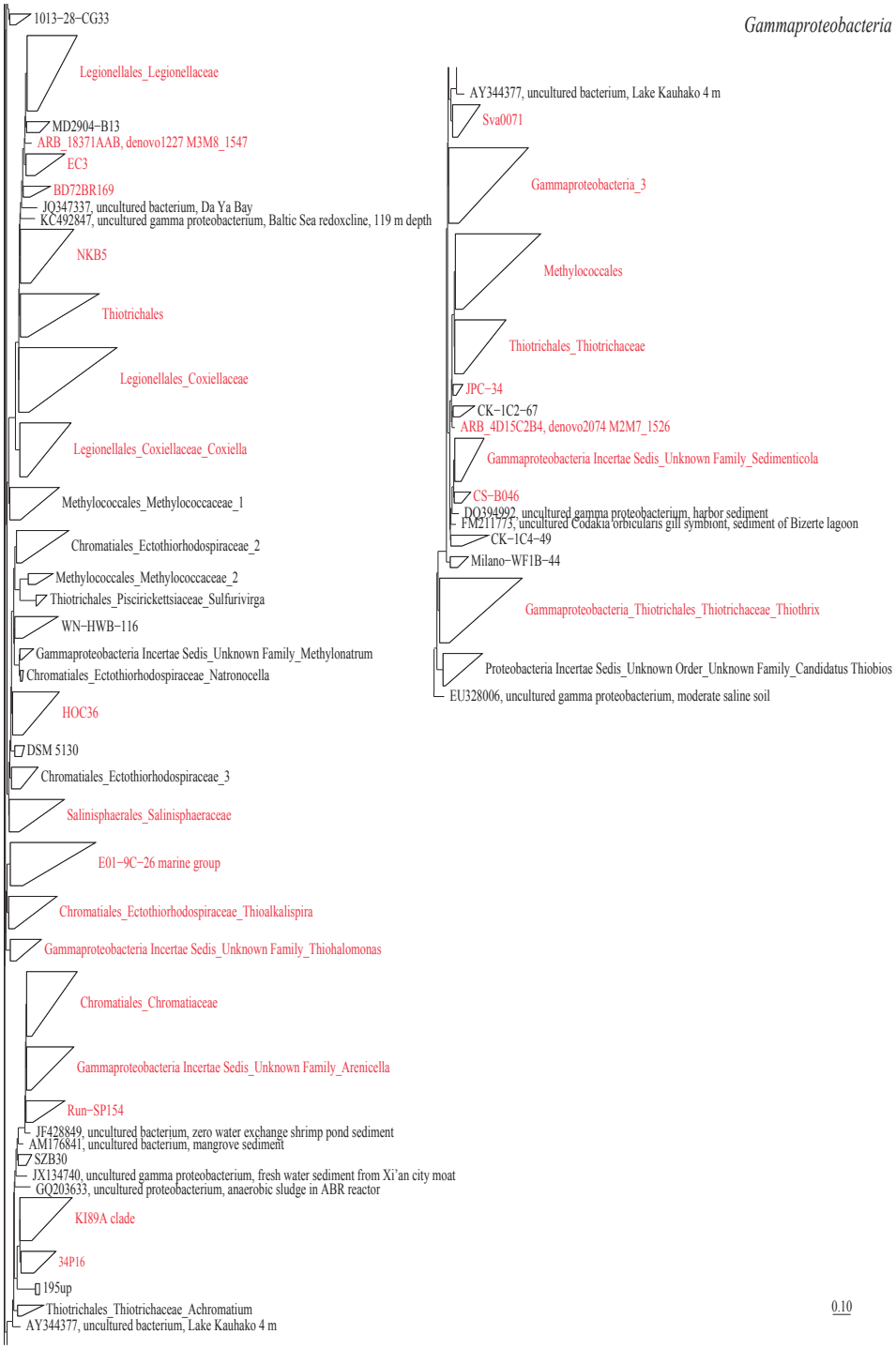


Figure S3: Phylogenetic tree of 16S rRNA gene sequence reads assigned to *Gammaproteobacteria*. Scale bar indicates 0.10 % estimated sequence divergence. Groups containing sequences are highlighted.

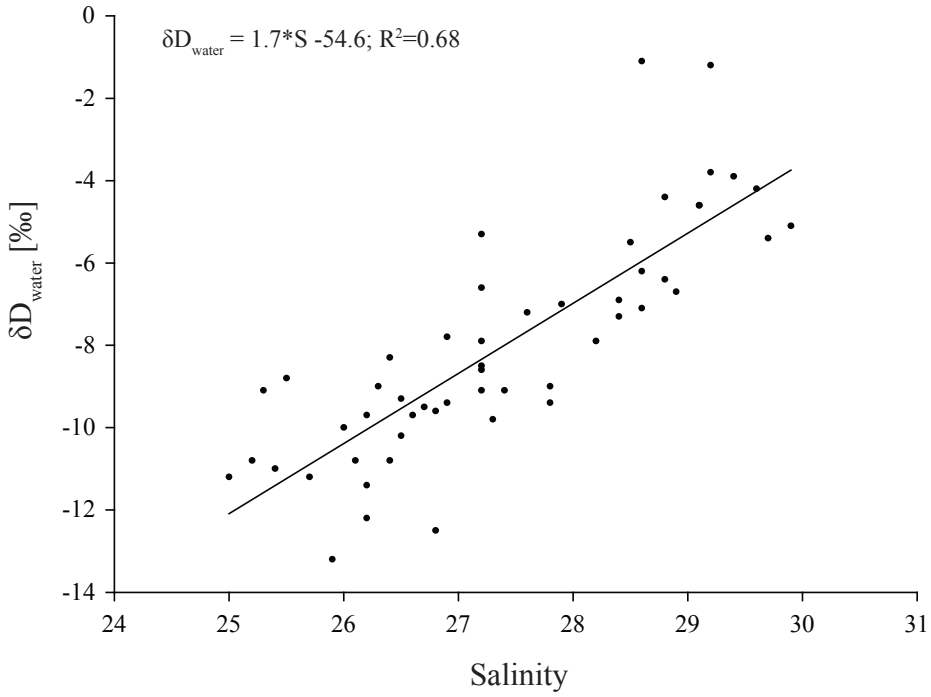


Figure S4: δD_{water} versus salinity of North Sea SPM sampled in 2013.

Table S1: Chlorophyll *a* concentration measured between the 16/08/10 and the 15/12/11 in µg/L.

Date	c Chlorophyll *a* [µg/L]	Date	c Chlorophyll *a* [µg/L]
16/08/10	7.1	06/06/11	5.1
30/08/10	6.7	09/06/11	4.4
15/09/10	7.7	17/06/11	2.9
28/09/10	4.6	23/06/11	4.5
22/10/10	4.0	30/06/11	6.3
01/11/10	4.1	04/07/11	6.7
15/11/10	1.9	15/07/11	5.7
26/11/10	3.6	21/07/11	3.3
17/12/10	4.5	27/07/11	5.7
24/01/11	3.7	01/08/11	3.2
10/02/11	1.2	08/08/11	5.6
23/02/11	0.5	22/08/11	6.6
08/03/11	5.2	29/08/11	4.4
23/03/11	12.9	12/09/11	4.6
05/04/11	22.2	21/09/11	1.8
11/04/11	8.6	29/09/11	3.4
19/04/11	10.7	14/10/11	1.8
26/04/11	14.0	20/10/11	3.0
03/05/11	5.0	31/10/11	2.7
11/05/11	5.5	15/11/11	2.6
18/05/11	2.0	28/11/11	2.0
24/05/11	10.8	15/12/11	1.6
01/06/11	1.8		

Table S2: Phytoplankton diversity and abundance. Abundance measured in cells/L.

Dates	cells/L [x10^6]															
	Cyanobacteria					Crypto-phyta		Chlo-rophyta		Diatoms					Hapto-phyta	Total
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
08/09/10	6	3														9
15/09/10			3													3
23/09/10						2										2
28/09/10		1						1								2
08/10/10								1								1
14/10/10			42													42
22/10/10								1		2						3
01/11/10		1														1
15/11/10								2								2
26/11/10								2	2							4
17/12/10			9						2							11
24/01/11			3	13					2							18
10/02/11								2								2
23/02/11			1													1
08/03/11										2						2
15/03/11										2						2
23/03/11								1			1					2
28/03/11										2	1					3
05/04/11											3					3
11/04/11						0.5										0.5
19/04/11						1				2					7	10
26/04/11												9			21	30
03/05/11												3			26	29
11/05/11													2		13	15
18/05/11														0.5		0.5
24/05/11										2	3					5
06/06/11								1		1						2
09/06/11							0.5	1								1.5
17/06/11															1	1
23/06/11															1	1
30/06/11															3	3
04/07/11		0.5														0.5
15/07/11		1														1
21/07/11											1					1

27/07/11	6															6
01/08/11	9									2						11
08/08/11										1					1	2
15/08/11															1	1
22/08/11															2	2
29/08/11						0.5										0.5
12/09/11					1											1
15/09/11	8															8.2
21/09/11							0.2									2
29/09/11						1				1						2
14/10/11						1		1								2
20/10/11															2	2
31/10/11								1								1
15/11/11		3														3
28/11/11								2								2
15/12/11								2		1						3

Cyanobacteria: A *Chroococcaceae*, B *Chlorococcales*, C *Cyanophyta*, D *Snowella*, E *Microcystis*; Cryptohyta: F *Hemiselmis*, G *Plagioselmis*; Chlorophyta: H *Prasinophyceae*, I Chlorophyta; Diatoms: J *Bacillariales*, K *Thalassiosiraceae*, L *Chaetoceros*, M *Pseudo-nitzschia delicatissima*, N *Cylindrotheca closterium*; Haptophyta: O *Phaeocystis globosa*

Table S3: Order-level bacterial diversity and abundance in North Sea SPM based on the 16S rRNA gene amplicon sequencing. Percentage based on total bacteria reads excluding Chloroplast reads.

bacterial groups	% of total bacteria reads															
	28/09/2010	26/11	10/01/2011	08/03	23/03	05/04	19/04	03/05	18/05	17/06	30/06	15/07	22/08	06/09	21/09	28/10/2011
Acidimicrobiales	1.7	3.4	1.1	1.1	0.8	0.2	0.3	0.5	1.2	3.3	2.4	1.4	4.7	4.6	10.8	6.7
Micrococcales	3.2	1.7	1.1	0.2	0.1	0.6	0.8	1.1	6.1	2.6	2.4	2.0	5.8	1.2	0.3	0.2
Flavobacteriales	16.2	13.2	10.6	16.8	14.7	27.9	29.7	30.8	28.6	25.3	27.8	22.1	21.0	17.3	14.2	13.4
Sphingobacteriales	3.4	2.7	4.6	4.6	4.5	3.9	3.6	3.3	3.2	4.1	3.8	3.1	3.5	2.7	2.7	2.9
Pirellulales	8.1	2.9	2.9	3.1	2.6	0.6	0.1	0.3	0.1	0.3	0.4	1.0	1.8	2.1	3.4	2.7
Rhodobacterales	8.2	12.4	9.7	9.2	8.4	5.6	6.5	5.9	9.1	11.2	9.1	5.8	9.1	10.0	11.6	6.4
Rickettsiales	8.4	12.2	12.0	6.1	8.7	16.8	15.5	7.0	5.5	2.6	3.2	6.0	7.6	5.6	7.5	9.9
Methylophiales	0.9	1.8	2.5	1.9	1.0	1.0	1.2	0.7	1.7	1.0	2.3	2.4	2.0	1.7	1.6	4.0
Rhodocyclales	0.9	2.1	6.5	5.1	3.7	2.1	3.9	2.5	1.3	0.4	0.7	1.4	1.1	0.5	0.1	0.9
Alteromonadales	10.7	9.3	13.2	11.8	12.7	8.8	11.6	17.0	9.7	17.3	13.1	14.1	10.0	12.0	8.9	11.1
Oceanospirillales	3.0	7.9	4.3	5.1	7.3	7.4	12.0	7.8	10.5	7.1	8.1	6.5	6.6	7.2	8.7	8.4
Thiotrichales	3.1	1.6	2.9	1.6	2.4	1.2	0.4	3.8	1.6	1.7	3.2	2.6	3.1	3.4	1.4	2.5
Puniceicoccales	0.6	1.0	0.3	1.9	5.4	8.5	3.4	1.3	0.9	0.4	5.6	2.6	3.5	1.4	0.9	0.7
Verrucomicrobiales	5.1	1.0	1.1	2.9	4.5	2.3	0.7	1.7	2.3	3.2	2.8	3.7	3.3	1.3	0.8	1.0

Actinobacteria: *Acidimicrobiales*, *Micrococcales*; *Bacteroidetes*: *Flavobacteriales*, *Sphingobacteriales*; *Planctomycetes*: *Pirellulales*; α-*Proteobacteria*: *Rhodobacterales*, *Rickettsiales*; β-*Proteobacteria*: *Methylophiales*, *Rhodocyclales*; γ-*Proteobacteria*: *Alteromonadales*, *Oceanospirillales*, *Thiotrichales*; *Verrucomicrobia*: *Puniceicoccales*, *Verrucomicrobiales*

Table S4: Fatty acid abundance in North Sea water samples.

Date	Fatty acid abundance [%]					
	C14:0	C16:1*	C16:0	C18:x	C18:0	C20:5 PUFA
16/08/10	14	23	36	16	6	5
30/08/10	17	26	29	15	6	7
15/09/10	19	27	32	14	5	4
28/09/10	14	24	29	18	9	7
15/11/10	13	17	32	23	13	3
26/11/10	13	20	31	21	13	2
10/12/10	12	13	35	28	11	1
17/12/10	11	30	28	26	4	0
10/01/11	9	27	32	23	8	0
24/01/11	7	23	29	30	7	4
17/02/11	8	27	31	24	9	0
08/03/11	11	35	24	16	3	11
23/03/11	13	33	21	17	5	12
05/04/11	10	34	26	13	4	13
19/04/11	13	35	25	11	2	14
03/05/11	17	23	23	22	2	12
18/05/11	17	28	29	12	6	9
17/06/11	21	21	35	9	10	4
30/06/11	27	20	26	14	5	8
15/07/11	21	25	28	9	7	10
27/07/11	16	25	33	11	10	5
08/08/11	14	25	26	10	7	18
22/08/11	21	15	31	17	8	7
06/09/11	14	20	32	10	18	6
21/09/11	13	16	38	20	10	3
11/10/11	16	22	37	13	11	0
28/10/11	12	21	32	22	9	5
15/11/11	12	25	31	17	8	6
28/11/11	15	26	32	15	7	5
16/12/11	8	27	30	19	9	6

*n*C16:1*: double bond at the ω7 position

Table S5: δD values of fatty acids.

Date	$\delta D_{fatty\ acid}$ [‰]				
	C14:0	C16:1*	C16:0	C18:0	C20:5 PUFA
16/08/10	-219	-201	-201	-185	-191
30/08/10	-222	-202	-189	-186	-199
15/09/10	-216	-206	-197	-186	-180
28/09/10	-219	-198	-192	-197	-207
15/11/10	-213	-203	-182	-200	N.D.
26/11/10	-226	-202	-188	-203	N.D.
10/12/10	-225	-188	-191	-202	N.D.
17/12/10	-232	-193	-194	-188	N.D.
10/01/11	-221	-200	-186	-204	N.D.
24/01/11	-212	-191	-195	-192	-209
17/02/11	-223	-208	-195	-206	N.D.
08/03/11	-226	-214	-205	-182	-235
23/03/11	-241	-216	-205	-190	-241
05/04/11	-223	-210	-209	-212	-223
19/04/11	-235	-224	-221	N.D.	-240
03/05/11	-238	-225	-214	-212	-236
18/05/11	-219	-205	-198	-178	-214
17/06/11	-225	-211	-196	-190	N.D.
30/06/11	-225	-210	-202	-175	-213
15/07/11	-204	-195	-188	-181	-217
27/07/11	-220	-200	-203	-180	-201
08/08/11	-222	-202	-201	-180	-234
22/08/11	-231	-202	-189	-190	-202
06/09/11	-224	-217	-221	-216	-218
21/09/11	-218	-204	-185	-194	N.D.
11/10/11	-213	-191	-183	-188	-226
28/10/11	-217	-187	-181	-184	-207
15/11/11	-212	-198	-191	-184	-221
28/11/11	-217	-193	-190	-180	-197
16/12/11	-198	-179	-174	-188	N.D.

*n*C16:1*: double bond at the ω7 position

Table S6: Fatty acid profiles of the bacterio- and phytoplankton observed via 16S rRNA gene amplicon sequencing and microscopy.

Organism	Fatty acid profile	Literature
Acidimicrobiales	OCS155: uncultured	
	Microthrixaceae: fatty acid profile not determined	
Micrococcales	*Microbacteriaceae*: mainly *ai*C15:0, *i*C16:0, *a*C17:0	(Evtushenko and Takeuchi, 2006)
Flavobacteriales	*Flavobacteriaceae*: mainly *i*C15:0, *ai*C15:0, C15:0; only traces of C16:0, C18:0, C14:0	(Van Trappen et al., 2004b; Khan et al., 2006; Heindl et al., 2008)
	Cryomorphaceae: mainly *i*C15:0, *i*C15:1ω10, C15:0; only traces of C16:0, C18:0, C14:0	(Bowman et al., 2003; O'Sullivan et al., 2005)
Sphingobacteriales	*Sphingobacteriaceae*: mainly *i*C15:0, iC17:0 3-OH, *i*C17:1ω9 and potentially C16:1ω7; minor amounts of C16:0 and C14:0	(Gallego et al., 2006)
	NS11-12: uncultured	
Pirellulales	*Pirellulaceae*: mainly C16:0, C18:1ω9; minor amounts of C14:0, C16:1ω7 and C18:0	(Kerger et al., 1988; Schlesner et al., 2004)
Rhodobacterales	*Rhodobacteraceae*: mainly C18:1ω7, C16:0, C16:1ω7, C18:0	(Yoon et al., 2007a; Venkata Ramana et al., 2009; Jung et al., 2010; Park and Yoon, 2014)
Rickettsiales	SAR11 cluster: fatty acid profile not determined	
Methylophiales	*Methylophilaceae*: mainly C16:0 and C16:1ω7; traces of C18:0	(Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012)
Rhodocyclales	*Rhodocyclaceae*: mainly C16:0, C16:1*cis*-9, C18:1; only traces of C14:0 and C18:0	(Reinhold-Hurek et al., 1993; Anders et al., 1995)

Alteromonadales	*Alteromonadaceae*: mainly C18:1ω7, C16:0, C16:1ω7; only traces of C14:0 and C18:0	(Bowman et al., 1998; Van Trappen et al., 2004a; Tang et al., 2008; Lee et al., 2012; Teramoto and Nishijima, 2014)
	SAR92 clade: fatty acid profile not determined	
	OM60 clade: mainly C16:0, C16:1ω7, C16:1ω6, C18:1ω7; only traces of C14:0 and C18:0	(Spring et al., 2009; Spring et al., 2013)
Oceanospirillales	*Halomonadaceae*: mainly C16:0, C18:1ω7; minor amounts of C14:0, C16:1ω7; traces of C18:0	(Sánchez-Porro et al., 2009; Long et al., 2013)
Thiotrichales	*Piscirickettsiaceae*: mainly C16:0, C16:1ω7; minor amounts of C14:0; traces of C18:0	(Doronina et al., 2003; Kim et al., 2007; Antony et al., 2012)
	Thiotrichaceae: mainly C18:1ω7, C16:1ω7, C16:0; minor amounts of C14:0, C18:0	(Aruga et al., 2002)
Puniceicoccales	*Coraliomargarita*: mainly C14:0, C18:1ω9, C18:0	(Yoon et al., 2007b)
Verrucomicrobiales	*Verrucomicrobiaceae*: mainly C16:0, some also C14:0 and C16:1ω7	(Yoon et al., 2008)
Chlorophyta	*Mamiellales*: mainly C16:0, C16:1ω7, C18:1ω7, C18:1ω9; only traces of C14:0 and C18:0	(Martínez-Fernández et al., 2006; Vaezi et al., 2013)
Stramenopiles (diatoms)	*Thalassiosira*: mainly C14:0, C16:0, C16:1ω7; only minor amounts of C18:0	(Viso and Marty, 1993)
	Chaetoceros: mainly C14:0, C16:0, C16:1ω7; only minor amounts of C18:0	(Viso and Marty, 1993; Zhukova and Aizdaicher, 1995)
Haptophyta	*Phaeocystis*: mainly C14:0, C16:0, C18:1ω9; minor amounts of C16:1ω7, C18:0 and C20:5 PUFA	(Al-Hasan et al., 1990; Nichols et al., 1991; Hamm and Rousseau, 2003)
Cryptophyta	*Chroomonas*: mainly C16:0, C18:1ω9 C20:5 PUFA; only minor amount of C14:0, C16:1ω7 and C18:0	(Viso and Marty, 1993; Zhukova and Aizdaicher, 1995)

Chapter 6

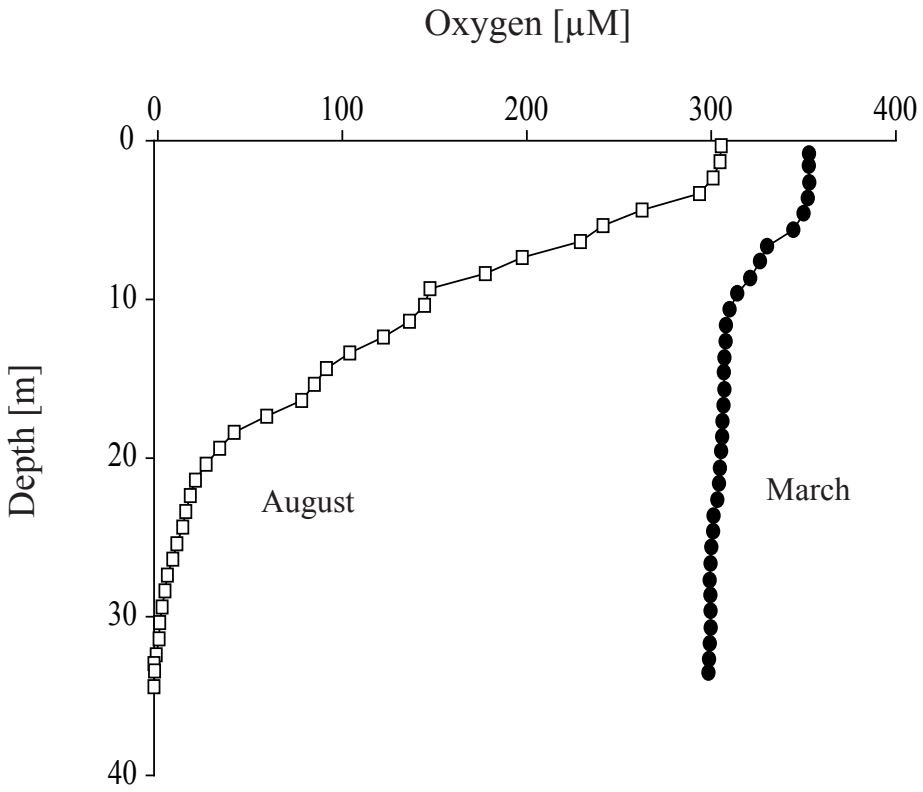


Figure S1: Oxygen concentration of water column at Station 1 in March and August in µM.

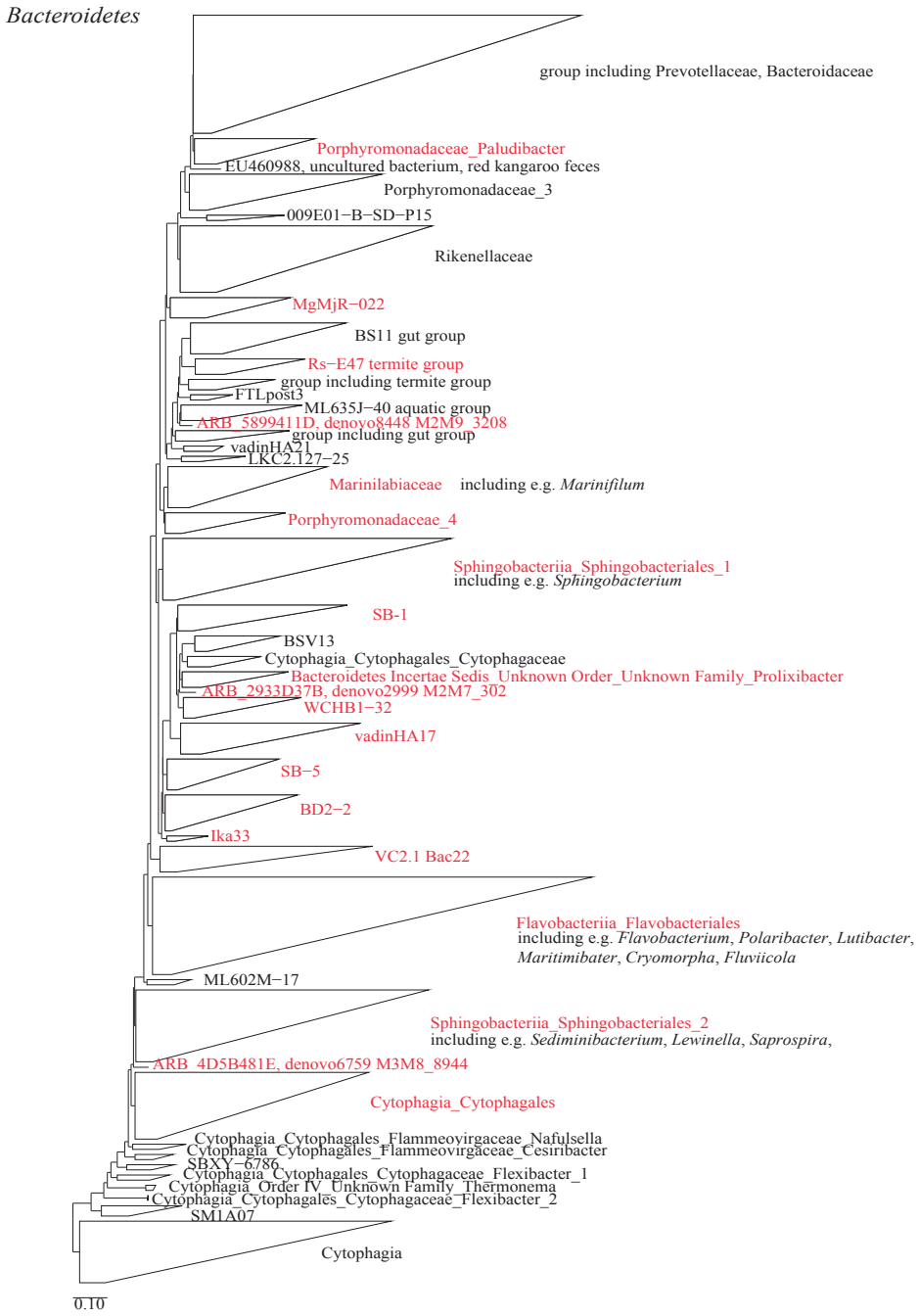


Figure S2: Phylogenetic tree of 16S rRNA gene sequence reads assigned to *Bacteroidetes.* Scale bar indicates 0.10 % estimated sequence divergence. Groups containing sequences are highlighted.

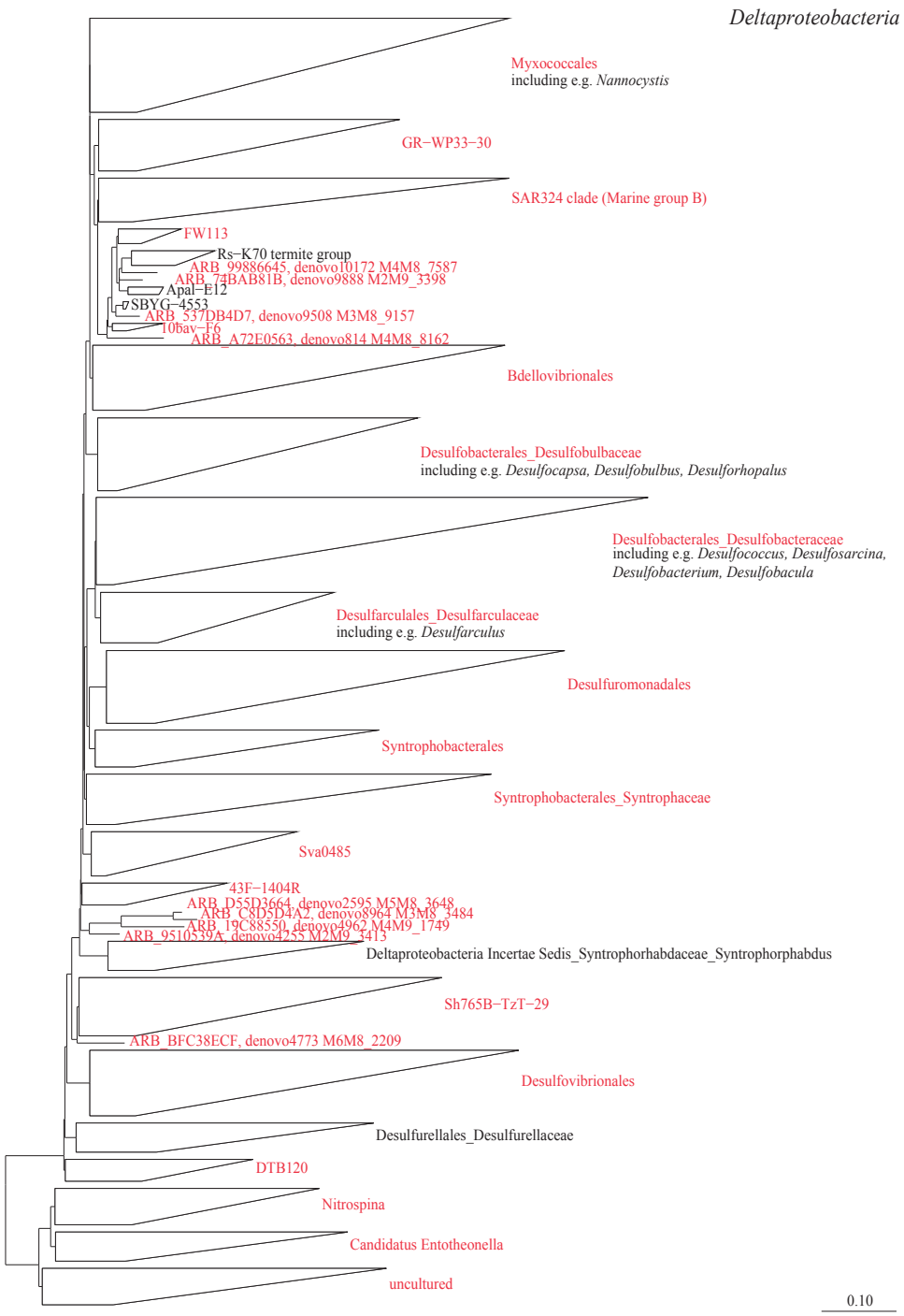
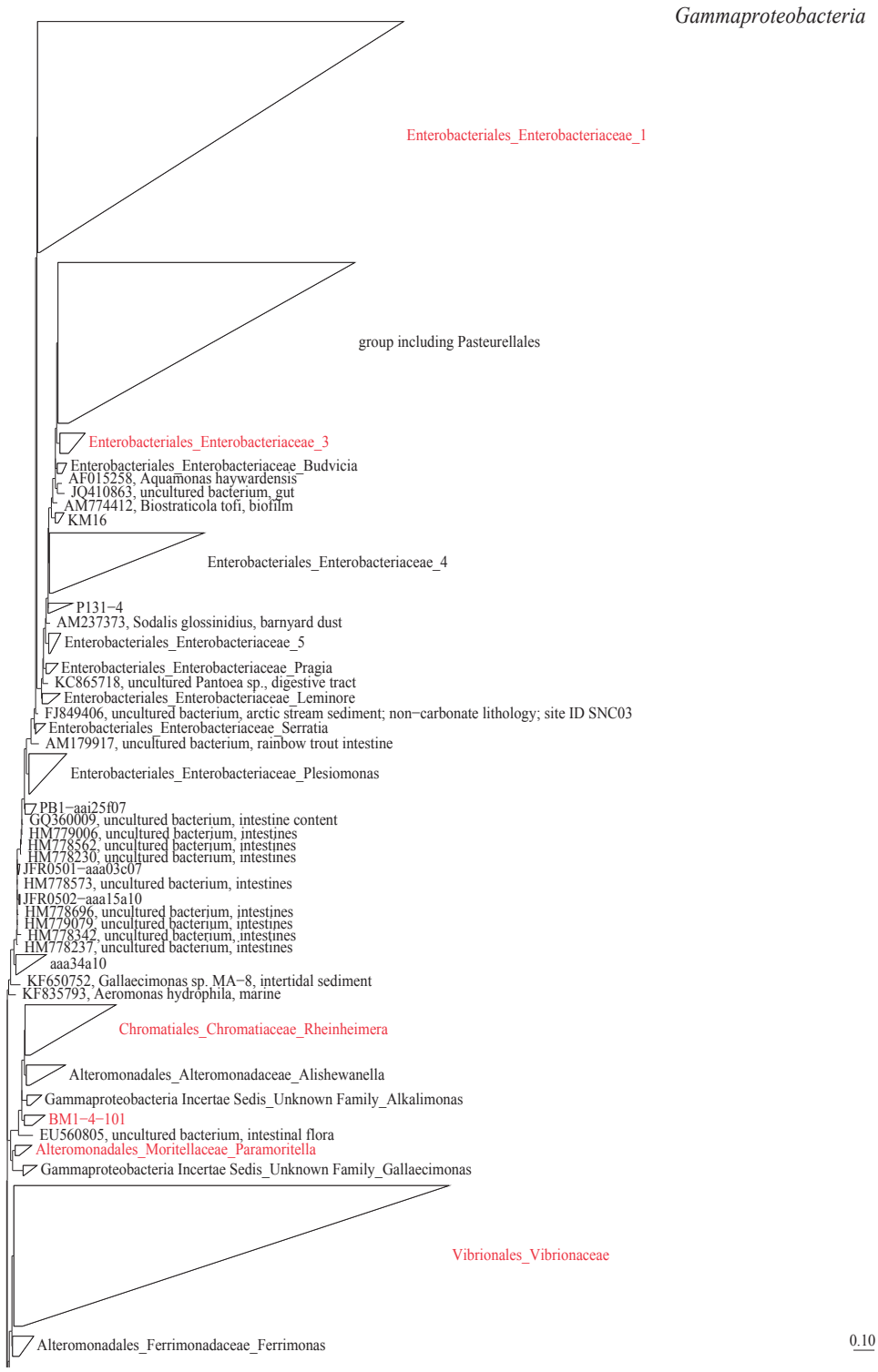
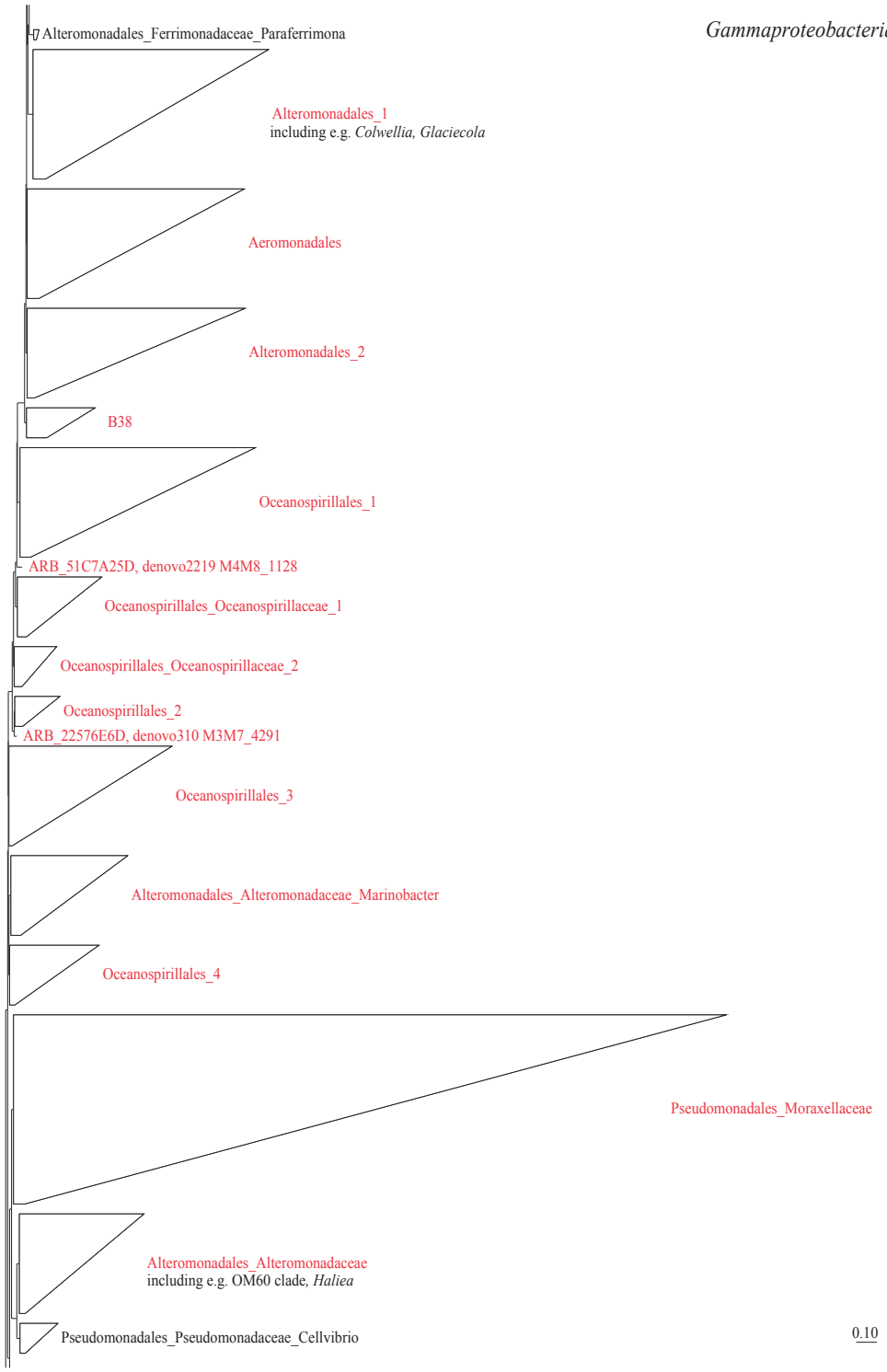
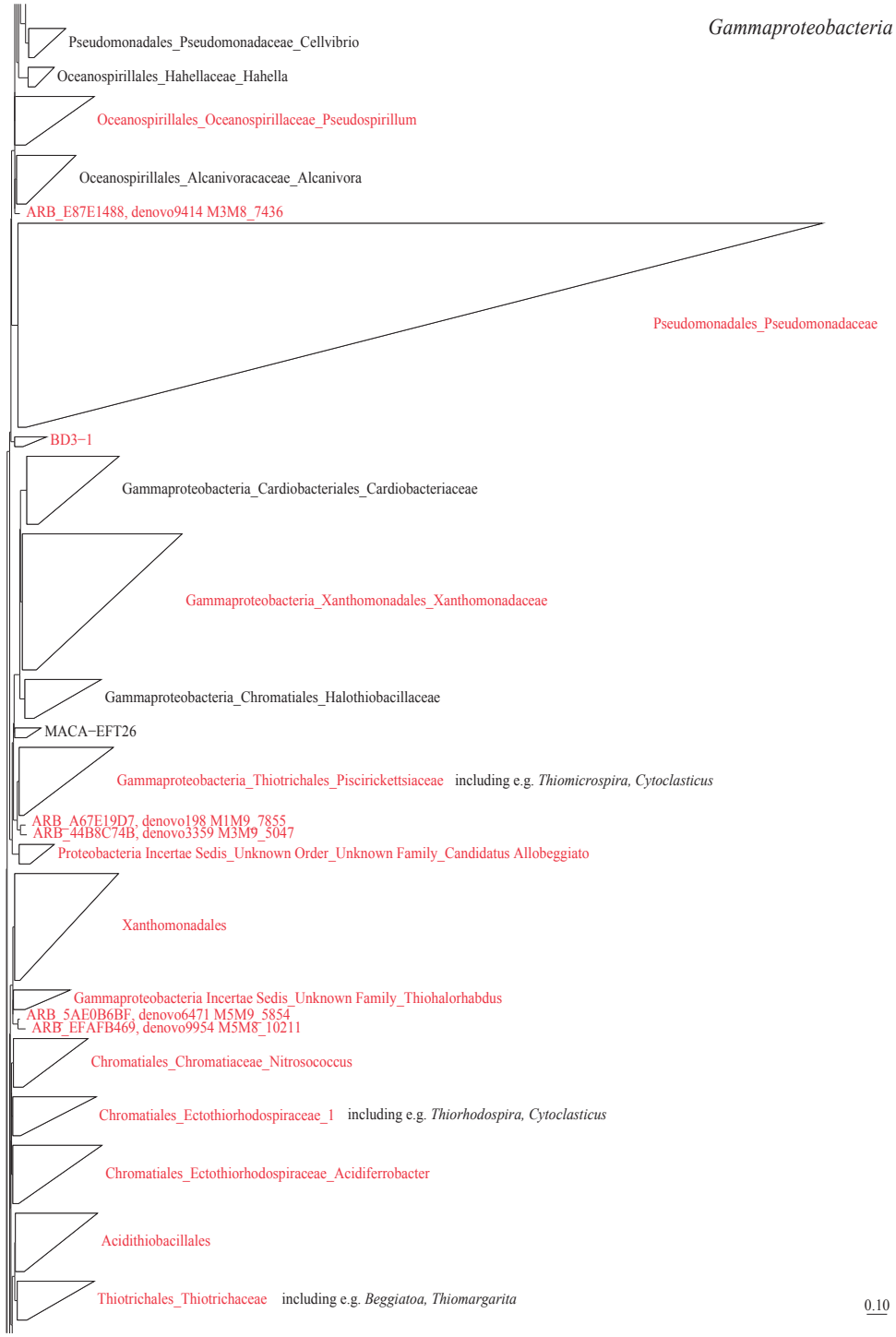


Figure S3: Phylogenetic tree of 16S rRNA gene sequence reads assigned to *Deltaproteobacteria.* Scale bar indicates 0.10 % estimated sequence divergence. Groups containing sequences are highlighted.


Gammaproteobacteria
Enterobacteriales_Enterobacteriaceae_1
group including Pasteurellales
Enterobacteriales_Enterobacteriaceae_3
Enterobacteriales_Enterobacteriaceae_Budvicia
AF015258, Aquamonas haywardensis
JQ410863, uncultured bacterium, gut
AM774412, Biostraticola tofi, biofilm
KM16
Enterobacteriales_Enterobacteriaceae_4
P131−4
AM237373, Sodalis glossinidius, barnyard dust
Enterobacteriales_Enterobacteriaceae_5
Enterobacteriales_Enterobacteriaceae_Pragia
KC865718, uncultured Pantoea sp., digestive tract
Enterobacteriales_Enterobacteriaceae_Leminore
FJ849406, uncultured bacterium, arctic stream sediment; non−carbonate lithology; site ID SNC03
Enterobacteriales_Enterobacteriaceae_Serratia
AM179917, uncultured bacterium, rainbow trout intestine
Enterobacteriales_Enterobacteriaceae_Plesiomonas
PB1−aai25f07
GQ360009, uncultured bacterium, intestine content
HM779006, uncultured bacterium, intestines
HM778562, uncultured bacterium, intestines
HM778230, uncultured bacterium, intestines
JFR0501−aaa03c07
HM778573, uncultured bacterium, intestines
JFR0502−aaa15a10
HM778696, uncultured bacterium, intestines
HM779079, uncultured bacterium, intestines
HM778342, uncultured bacterium, intestines
HM778237, uncultured bacterium, intestines
aaa34a10
KF650752, Gallaecimonas sp. MA−8, intertidal sediment
KF835793, Aeromonas hydrophila, marine
Chromatiales_Chromatiaceae_Rheinheimera
Alteromonadales_Alteromonadaceae_Alishewanella
Gammaproteobacteria Incertae Sedis_Unknown Family_Alkalimonas
BM1−4−101
EU560805, uncultured bacterium, intestinal flora
Alteromonadales_Moritellaceae_Paramoritella
Gammaproteobacteria Incertae Sedis_Unknown Family_Gallaecimonas
Vibrionales_Vibrionaceae
Alteromonadales_Ferrimonadaceae_Ferrimonas
0.10

Gammaproteobacteria




Gammaproteobacteria
Pseudomonadales_Pseudomonadaceae_Cellvibrio
Oceanospirillales_Hahellaceae_Hahella
Oceanospirillales_Oceanospirillaceae_Pseudospirillum
Oceanospirillales_Alcanivoracaceae_Alcanivora
ARB_E87E1488, denovo9414 M3M8_7436
Pseudomonadales_Pseudomonadaceae
BD3−1
Gammaproteobacteria_Cardiobacteriales_Cardiobacteriaceae
Gammaproteobacteria_Xanthomonadales_Xanthomonadaceae
Gammaproteobacteria_Chromatiales_Halothiobacillaceae
MACA−EFT26
Gammaproteobacteria_Thiotrichales_Piscirickettsiaceae including e.g. *Thiomicrospira, Cytoclasticus*
ARB_A67E19D7, denovo198 M1M9_7855
ARB_44B8C74B, denovo3359 M3M9_5047
Proteobacteria Incertae Sedis_Unknown Order_Unknown Family_Candidatus Allobeggiato
Xanthomonadales
Gammaproteobacteria Incertae Sedis_Unknown Family_Thiohalorhabdus
ARB_5AE0B6BF, denovo6471 M5M9_5854
ARB_EFAFB469, denovo9954 M5M8_10211
Chromatiales_Chromatiaceae_Nitrosococcus
Chromatiales_Ectothiorhodospiraceae_1 including e.g. *Thiorhodospira, Cytoclasticus*
Chromatiales_Ectothiorhodospiraceae_Acidiferrobacter
Acidithiobacillales
Thiotrichales_Thiotrichaceae including e.g. *Beggiatoa, Thiomargarita*
0.10

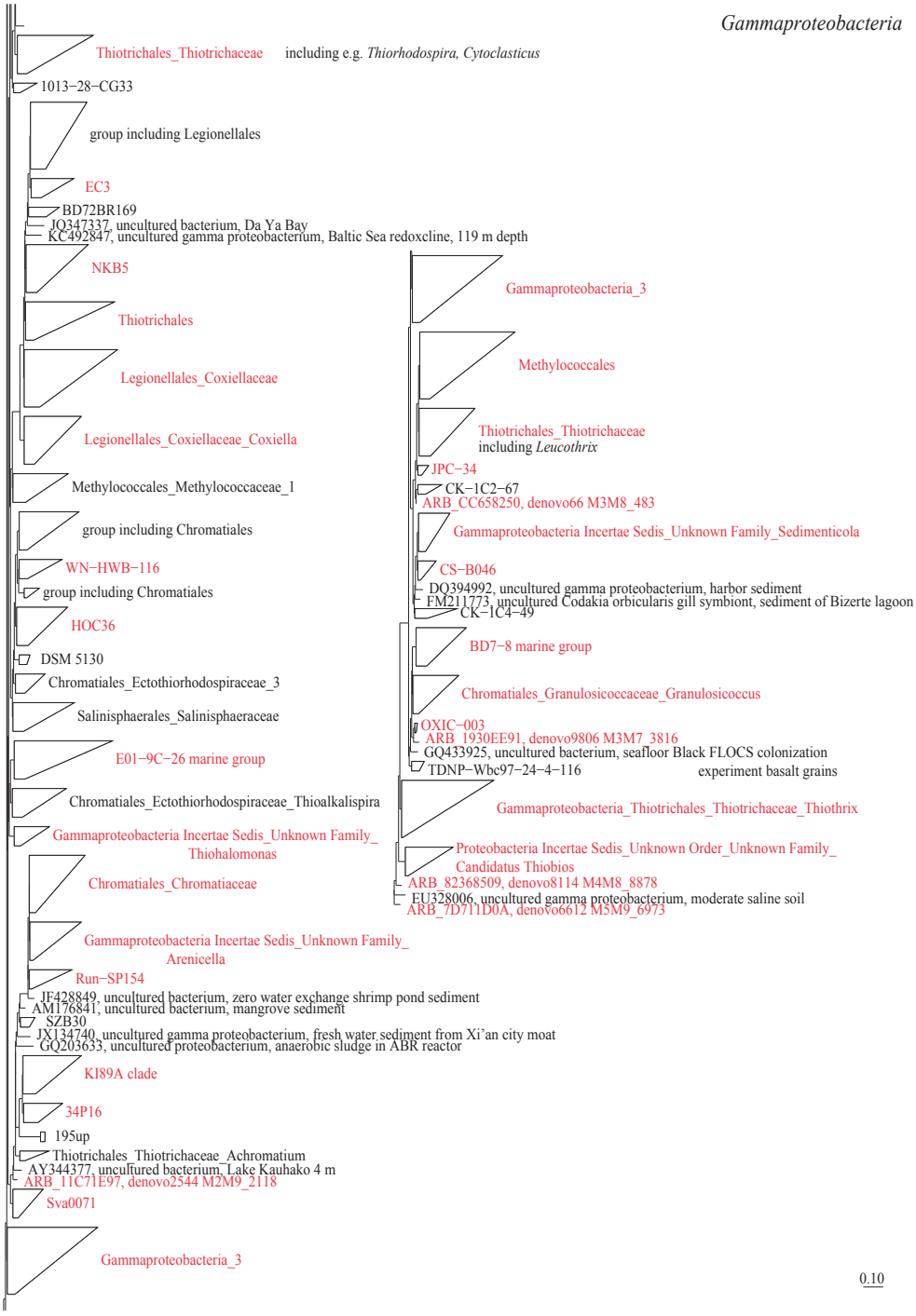


Figure S4: Phylogenetic tree of 16S rRNA gene sequence reads assigned to *Gammaproteobacteria.* Scale bar indicates 0.10 % estimated sequence divergence. Groups containing sequences are highlighted.

Table S1: Sulphide concentration in the sediment at Station 1 and 3 (Lipsewers et al., submitted)

Depth [cm]	Station 1		Station 3	
	Sulphide [µmol/L]		Sulphide [µmol/L]	
	March	August	March	August
0.25	n.d.	725	n.d.	224
0.75	n.d.	896	n.d.	199
1.25	n.d.	1374	n.d.	154
1.75	n.d.	1631	n.d.	199
2.25	n.d.	1382	n.d.	80
2.75	n.d.	1896	n.d.	211
3.25	n.d.	1855	n.d.	112
3.75	n.d.	2068	n.d.	105
4.25	1	2178	n.d.	170
4.75	65	1948	n.d.	15
5.25	78	2293	1	4
5.75	262	3014	2	46
6.25	182	2228	1	20
6.75	380	2893	1	14
7.25	398	2809	1	2
7.75	818	2807	1	15

Table S2: Fatty acid abundance in Lake Grevelingen sediment samples.

Depth [cm]	Fatty acid relative abundance [%]								
	C14:0	*i*C15:0	*ai*C15:0	C15:0	C16:1*	C16:0	C18:x.	C18:0	C20:5 PUFA
Station 1 (March)									
0–1	9	4	5	2	31	23	16	4	7
1–2	8	5	6	3	28	23	17	4	6
2-3	10	4	4	3	27	22	14	3	14
3–4	9	4	4	2	28	21	15	3	14
4–5	9	4	5	2	27	22	15	4	11
5–6	10	4	6	2	22	26	16	7	7
6–7	11	3	4	3	29	21	14	4	11
7–8	9	3	4	3	31	21	12	3	14
Station 1 (August)									
0–1	15	5	5	3	27	24	12	3	7
1–2	11	3	2	2	26	19	9	2	25
2–3	9	3	3	2	25	21	15	3	19
3–4	7	2	2	2	30	21	10	2	24
4–5	10	3	3	2	30	21	11	2	19
5–6	6	4	5	2	26	19	17	3	18
6–7	8	3	3	3	23	20	23	4	15
7–8	7	2	3	2	19	19	35	7	8
Station 3 (March)									
0–1	6	3	5	2	29	21	20	4	10
1–2	8	4	7	3	27	21	18	4	8
2–3	9	4	7	3	25	21	17	4	9
3–4	8	4	9	3	25	24	18	6	3
4–5	6	3	6	2	17	22	30	9	4
5–6	8	5	11	0	19	29	21	8	0
6–7	9	5	10	3	17	25	21	7	5
7–8	9	4	10	3	13	29	20	9	2
Station 3 (August)									
0–1	16	4	4	4	26	25	12	3	6
1–2	7	3	3	3	24	22	15	4	20
2–3	6	3	4	2	19	18	32	4	12
3–4	7	3	6	3	20	20	26	5	11
4–5	8	4	7	2	18	22	25	8	7
5–6	7	3	6	2	16	22	31	9	5
6–7	8	4	7	3	25	23	18	5	8
7–8	7	3	6	2	23	21	27	5	5

* double bond at ω7 position

Supplementary Data

Table S3: δD values of fatty acids obtained from sediments at Station 1 and 3 in March and August.

Depth [cm]	δD_{water} [‰]	$\delta D_{fatty\ acid}$ [‰]						
		C14:0	*i*C15:0	*ai*C15:0	C16:1*	C16:0	C18:0	C20:5 PUFA
Station 1 (March)								
0–1	-1.8±3.3	-201±3	-104±0	-164±0	-190±2	-181±2	-193±1	-231±12
1–2		-194±2	-120ϕ	-175ϕ	-189±3	-177±1	-192±9	-244±0
2–3		-195±11	-115ϕ	-175ϕ	-210±1	-185±1	-146±2	-249±6
3–4		-198±3	-117±3	-178±0	-198±0	-172±3	-154±2	-260±1
4–5		-212±0	-133±3	-193±0	-205±0	-186±0	-165±5	-263±1
5–6		-201±6	-129±1	-187±2	-198±3	-185±2	-180±3	-248±0
6–7		-201±3	-126±0	-183±3	-217±5	-185±7	-167±2	-255±3
7–8		-209±3	-127±3	-185±2	-213±2	-191±1	-159±5	-263±3
Station 1 (August)								
0–1	0.1±2.8	-215ϕ	-126ϕ	-171	-203ϕ	-186ϕ	-140ϕ	-240ϕ
1–2		-201±0	N.D.	N.D	-209±0	-195±2	N.D.	-267±3
2–3		-203±5	-119ϕ	-185	-204±2	-189±1	-147ϕ	-261±3
3–4		-200±8	N.D	N.D	-208±2	-196±0	N.D.	-266±2
4–5		-204±6	N.D	N.D	-209±2	-195±2	N.D.	-268±3
5–6		-183±7	-112±2	-173±4	-191±1	-178±1	-148±3	-263±2
6–7		-204±0	-120±2	-177±3	-208±1	-187±1	-153±3	-263±1
7–8		-195±3	N.D	N.D	-201±1	-191±1	-186±2	-244±4
Station 3 (March)								
0–1	-1.6±2.6	-198ϕ	-109ϕ	-177ϕ	-199ϕ	-185ϕ	-170ϕ	-235ϕ
1–2		-183±13	-122ϕ	-179±0	-207±2	-180±4	-154ϕ	-250ϕ
2–3		-189±1	-123ϕ	-183±3	-205±1	-186±2	-160±4	-251±3
3–4		-180±3	-117±0	-178±0	-196±3	-187±3	-168±0	-233ϕ
4–5		-194±3	-119ϕ	-183±1	-220±3	-195±2	-189±2	N.D.
5–6		-182±0	-131ϕ	-183±4	-210±4	-186±2	-174±2	N.D.
6–7		-180±4	-123±2	-178±1	-210±1	-186±0	-168±2	N.D.
7–8		-188±1	-121ϕ	-185±1	-188±1	-186±0	-192±2	N.D.
Station 3 (August)								
0–1	-1.7±4.1	-194±10	-126ϕ	-186ϕ	-201±3	-189±2	-150±2	-230±0
1–2		-201±4	-118±1	-181±1	-209±1	-187±1	-150±5	-262±1
2–3		-186±4	-121ϕ	-178±7	-195±1	-184±1	-158±1	-255±4
3–4		-190±1	-123±2	-185±1	-209±0	-184±1	-165±2	-258±1
4–5		-181±11	-122ϕ	-177±3	-201±2	-187±1	-179±1	-251±0
5–6		-205±5	-130ϕ	-185±3	-207±1	-196±0	-200±0	-244±0
6–7		-201±1	-132±1	-184±1	-217±1	-185±1	-158±0	-254±2
7–8		-193±7	-132ϕ	-182±3	-207±2	-182±2	-159±2	-250±2

* double bond at ω7 position; ϕ only one ε value existing

Table S4: Order-level bacterial diversity and percentage of total bacteria reads obtained in August at (a) Station 1 and (b) Station 3.

(a)

Organism	% of total bacteria reads							
	0-1 cm	1-2 cm	2-3 cm	3-4 cm	4-5 cm	5-6 cm	6-7 cm	7-8 cm
Bacteroidales	5.7	8.1	9.6	9.5	6.0	7.1	11.5	11.9
Flavobacteriales	5.3	5.0	4.5	5.6	5.0	4.9	5.2	6.2
Sphingobacteriales	5.9	3.1	1.7	1.5	1.2	2.0	1.3	0.7
agg27	4.2	5.5	4.8	1.6	1.1	0.6	1.8	1.8
Phycisphaerales	6.2	4.0	2.3	0.8	0.6	0.8	0.2	0.3
Desulfarculales	3.7	2.7	1.1	0.5	0.8	1.0	0.9	1.2
Desulfobacterales	11.1	12.0	15.5	12.4	13.1	13.0	13.3	16.1
Myxococcales	2.8	2.3	1.1	1.4	2.2	2.5	4.4	2.7
Alteromonadales	5.4	4.4	5.1	7.8	6.3	7.8	8.8	7.6
Chromatiales	0.9	1.7	2.1	3.3	2.7	3.9	4.2	4.5
Thiotrichales	3.8	4.0	4.5	7.2	11.7	9.7	6.7	6.0
GN03	5.8	3.9	3.3	1.9	0.9	1.2	1.0	0.8

(b)

Organism	% of total bacteria reads							
	0-1 cm	1-2 cm	2-3 cm	3-4 cm	4-5 cm	5-6 cm	6-7 cm	7-8 cm
Bacteroidales	8.1	7.4	5.4	6.6	4.3	5.9	4.6	3.5
Flavobacteriales	3.8	4.3	3.2	3.1	1.7	3.4	3.7	3.6
Desulfobacterales	23.0	18.7	17.5	19.6	20.5	15.7	16.1	15.5
Myxococcales	2.9	3.7	4.8	4.3	3.9	4.3	4.2	2.6
Campylobacterales	0.7	0.7	0.8	1.3	0.8	2.8	3.3	2.5
Alteromonadales	5.8	7.1	6.9	4.4	3.2	3.1	2.1	2.1
Chromatiales	3.2	4.3	4.2	4.9	4.3	4.5	5.1	6.0
Thiotrichales	8.7	9.5	12.5	9.7	11.6	9.3	9.5	10.6

Bacteroidetes: *Bacteroidales*, *Flavobacteriales*, *Sphingobacteriales*; *Planctomycetes*: *agg27*, *Phycisphaerales*; δ-*Proteobacteria*: *Desulfarculales*, *Desulfobacterales*, *Myxococcales*; ε-*Proteobacteria: Campylobacterales*; γ-*Proteobacteria*: *Alteromonadales*, *Chromatiales*, *Thiotrichales*; WS3: GN03

The sequence reads belonging to the *Bacteroidetes*, *δ-Proteobacteria* and *γ-Proteobacteria* were extracted from the dataset and added to a phylogenetic tree (Figures S2-S4). *Bacteroidetes* sequences clustered mainly within the *Marinilabiaceae* (*Bacteroidales*), the *Flavobacteriaceae* (*Flavobacteriales*) and the *Saprospiraceae* (*Sphingobacteriales*). Within the *δ-Proteobacteria* sequences belonging to the *Myxococcales* clustered mainly with uncultured representatives of the order. The majority of the *Desulfobacterales* reads fell within the *Desulfobacteraceae* and *Desulfobulbaceae* and sequences clustered within i.e. *Desulfococcus* and *Desulfobulbus*, respectively. *Desulfarculales* reads belonged entirely to the *Desulfarculaceae* and clustered mainly with uncultured representatives. The majority of the *Alteromonadales* reads and sequences fell within the OM60-clade. *Thiotrichales* reads belonging to the *Piscirickettsiaceae* clustered with i.e. *Cycloclasticus* and *Thiomicrospira*. Sequences belonging to the *Chromatiales* clustered with members of the *Chromatiaceae*, *Ectothiorhodospiraceae* and *Granulosicoccaceae*.

Table S5: Fatty acid profiles of the sedimentary bacterial community detected by 16S rRNA gene amplicon sequencing and the phytoplankton present in Lake Grevelingen according to Bakker and De Vries (1984)

Organism	Fatty acid profile	Literature
Bacteroidales	*Porphyromonadaceae*: mainly *ai*C15:0, C15:0, *ai*C17:0; only minor amounts of C14:0, C16:0 and C18:0	(Ueki et al., 2006)
	Marinilabiaceae: mainly *i*C15:0, *ai*C15:0	(Shalley et al., 2013)
Flavobacteriales	*Flavobacteriaceae*: mainly *i*C15:0, *ai*C15:0, C15:0; only traces of C16:0, C18:0, C14:0	(Van Trappen et al., 2004b; Khan et al., 2006; Heindl et al., 2008)
	Cryomorphaceae: mainly *i*C15:0, *i*C15:1ω10, C15:0; only traces of C16:0, C18:0, C14:0	(Bowman et al., 2003; O'Sullivan et al., 2005)
Sphingobacteriales	*Sphingobacteriaceae*: mainly *i*C15:0, *i*C17:0 3-OH, *i*C17:1ω9 and potentially C16:1ω7; minor amounts of C16:0 and C14:0	(Gallego et al., 2006)
	NS11-12: uncultured	
agg27	uncultured	
Phycisphaerales	*Phycisphaeraceae*: mainly C16:0 and C14:0; minor amounts of C18:0	(Yoon et al., 2014)
Desulfarculales	*Desulfarculaceae*: mainly *ai*C15:0, C14:0, C18:1ω7; minor amounts of C16:0 and C18:0	(Sun et al., 2010; An and Picardal, 2014)
Desulfobacterales	*Desulfobacteraceae*: varying amounts of C14:0, C16:0, C16ω7, C15:0 and *ai*C15:0; traces of C18:0	(Taylor and Parkes, 1983; Knoblauch et al., 1999; Tarpgaard et al., 2006; Duldhardt et al., 2010)
Myxococcales	*Nannocystaceae*: varying amounts of C14:0, *i*C15:0, *i*C16:0, C16:0, C16:1 and C20:4	(Iizuka et al., 2003; Shimkets et al., 2006)
	Haliangiaceae: mainly C16:0; minor amounts of C14:0, C16:1 and C18:0	
Campylobacterales	*Heliobacteraceae*: mainly C16:0, C16:1; minor amounts of C14:0	(Inagaki et al., 2003)
	Campylobacteraceae: mainly C16:0, C16:1 and C18:0; minor amounts of C14:0	(Finster et al., 1997)

Alteromonadales	*Alteromonadaceae*: mainly C18:1ω7, C16:0, C16:1ω7; only traces of C14:0 and C18:0	(Bowman et al., 1998; Van Trappen et al., 2004a; Tang et al., 2008; Lee et al., 2012; Teramoto and Nishijima, 2014)
	SAR92 clade: fatty acid profile not determined	
	OM60 clade: mainly C16:0, C16:1ω7, C16:1ω6, C18:1ω7; only traces of C14:0 and C18:0	(Spring et al., 2009; Spring et al., 2013)
*Chromatiale*s	*Chromatiaceae*: mainly C16:1ω7, C16:0, C18:1ω7; minor amounts of C12:0	(Shivali et al., 2011; Divyasree et al., 2014; Heinzelmann et al., 2015b)
	Ectothiorhodospiraceae: mainly C16:0, C16:1ω7; minor amounts of C18:0; traces of C14:0	(Thiemann and Imhoff, 1996; Park et al., 2011)
Thiotrichales	*Piscirickettsiaceae*: mainly C16:0, C16:1ω7; minor amounts of C14:0; traces of C18:0	(Doronina et al., 2003; Kim et al., 2007; Antony et al., 2012)
	Thiotrichaceae: mainly C18:1ω7, C16:1ω7, C16:0; minor amounts of C14:0, C18:0	(Aruga et al., 2002)
GN3	uncultured	
Stramenopiles	*Thalassiosira*: mainly C14:0, C16:0, C16:1ω7; only minor amounts of C18:0	(Viso and Marty, 1993)
	Chaetoceros: mainly C14:0, C16:0, C16:1ω7; only minor amounts of C18:0	(Viso and Marty, 1993; Zhukova and Aizdaicher, 1995)

I came to Texel in the beginning of 2011 and now more than four years later it is time to move on. But before I do, there is one thing left to finish this thesis of mine (or my freaky little bastard as I called it in the final weeks), and that would be the acknowledgement. While doing all the research contained in this thesis required lots of dedication and long hours both in the lab and the office, writing a decent acknowledgment seems even harder. Preferably it would be something like 'Thank you and I'm out of here', but that might be a tiny bit too short.

So let's start with the obvious. One key to a successful PhD is good supervision, and that is the one thing I cannot complain about for I had four supervisors. Marcel, I still have no idea why you offered me the position, but thank you for doing so. I learnt a lot from you, starting from lipid work and hydrogen isotopes, to how to stay sane while writing. It was great working with you on a daily basis and you are a pretty good supervisor. Next there would be Laura who shared my love for microorganisms. Without you and your large knowledge in molecular biology and microbial cultivation it would have been a lot harder. Thank you for adopting me as your PhD student during lab work in the molecular lab, conferences and paper writing. Stefan, you have the unbelievable talent to see sense in any kind of data I showed you. This thesis would have been impossible without you and your immense knowledge. Finally, Jaap, your input to all manuscripts was invaluable, thank you for it.

The great thing about BGC are the people who are more than willing to share their expertise. Nicole and Ellen, thank you for teaching me everything I still know or might have already forgotten about intact polar lipids, how to extract and analyse them. Jort, Monique, Anchelique, Marianne and Irene your expertise with the machines and data analysis made life so much easier.

I haven't spent all my time at the NIOZ just in the lipid lab, and sometimes it felt like I was in more than one place at once. Anna Noordeloos and Cees van Sloten were of great help while working at the Accurri. Judith van Bleijswijk and Harry Witte I want to thank for hosting me in the Molecular Lab.

A lot of the samples I worked with during my PhD were obtained with help of a lot of people. Daniela and Dave allowed me to use their precious *Isochrysis* filters. Sampling at the Jetty would have been impossible without the help of Nicole, Yvonne, Elisabeth, and Kasia (that one stormy night in March). Also, I would like to acknowledge all participants of the Grevelingen field campaign, but especially Dorina Seitaj, Diana Vasquez-Cardenas, Filip Meysman and Eric Boschker from Yerseke.

Down in the dungeon, there is the home of the δD group of Marcel. I might have been somewhat of a black sheep in this group by not working on the

salinity proxy and actually sitting upstairs, but nevertheless it were great four years with you guys. Danielle, I lost count on just how many water samples you measured for me, but be certain I am grateful for every single one of them. Dave, thanks for always having time for δD related question and I'm truly sorry to inform you that, while I might drink it on occasions, I still don't really like Gin and Tonic. Sebastian, eine Pizza mit wenig Käse und Cocktails wären mir dann doch lieber.

Finally, the BGC department. There were a lot of people during my time here and great thanks goes to all of them. But the ones I want to mention especially are the ones of my PhD generation. Together we went through four years of ups, downs and literature discussions and I wouldn't trade it for the world. Thank you Cindy, Sebastian, Yvonne, Elisabeth, Lisa and Martina.

Then there is of course the thing that is quite special about the NIOZ, the fantastic office situation. Dorien, Petra, Marijke, Yvonne and Cecile thanks for putting up with my weird perception of temperature in the office.

In order to stay sane on Texel it is essential to find great friends, it made everything so much more enjoyable. I had the luck to make new friends both at the NIOZ (John, Sebastian, Laura K., Kasia, Els, Kevin, Daniela and Loes) as also outside of it in Den Helder (Mariette, Bas, Teo and Bart). Thanks for the great time. While making new friends was great, having some of the old ones tagging along was even better. Thanks for all the visits (I know, for some reason Texel is pretty much at the end of the world), phone calls and free sofas, floors and other sleeping arrangements (especially Tanja, Sahra, Iraia, Netti, Astrid, Aina, and I-An).

Zum guten Ende, meine Familie. Mama und Papa, das ich bin wer und wo ich bin, habe ich euch zu verdanken. Ohne eure Unterstützung und euer Vertrauen in mich wäre es unmöglich gewesen. Danke für absolut alles, ihr seid die Besten. Tanja, mein Schwesterherz, ich kann mir keine bessere große Schwester auf der Welt vorstellen.

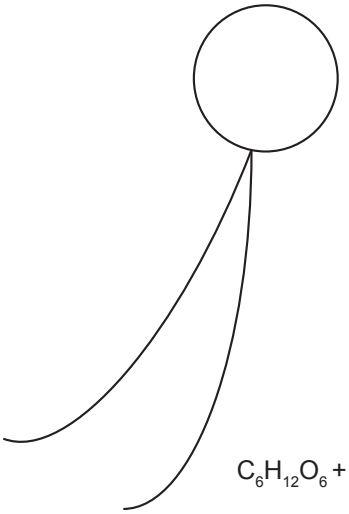
That's it.

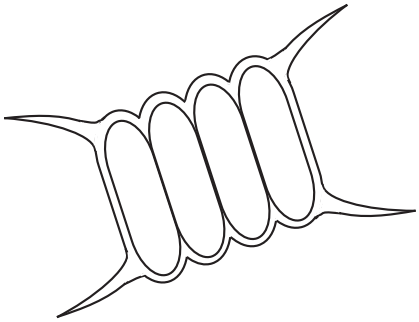
Thank you…..and I'm out of here!

About the Author

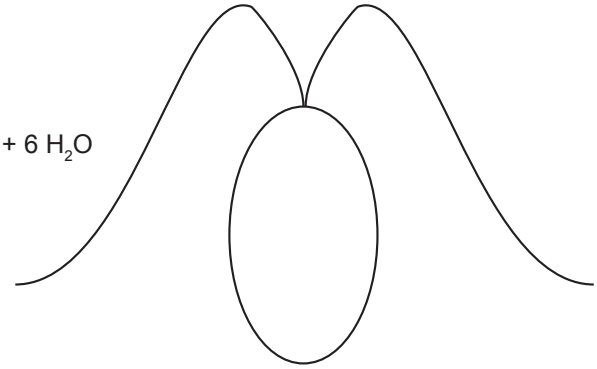
Sandra Heinzelmann was born on the 18th of May 1985 in Mainz, Germany. After finishing secondary school, she studied one semester Physics (thanks to the improvable educational system in Germany) before finally studying Biology at the Georg-August University of Göttingen from 2004 until 2010. During this time her focus shifted towards microbial ecology and molecular biology. A year studying abroad at the University of Helsinki sparked her interest in pursuing a PhD degree outside of Germany. In February 2011 she started as a PhD student at the Department for Marine Organic Biogeochemistry at the Royal Netherlands Institute for Sea Research under the supervision of Dr. Marcel van der Meer.

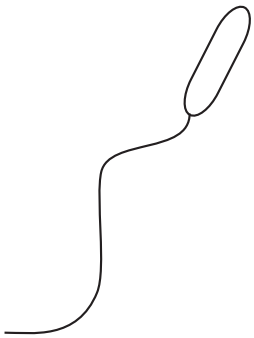
$$CO_2 + 2\,H_2O \xrightarrow{\text{light}} CH_2O + O_2 + H_2O$$



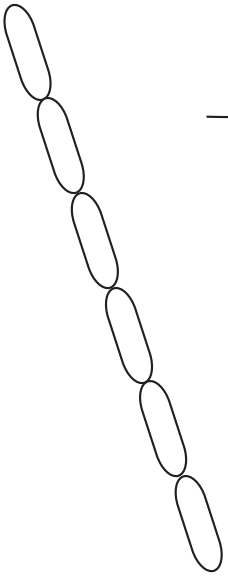


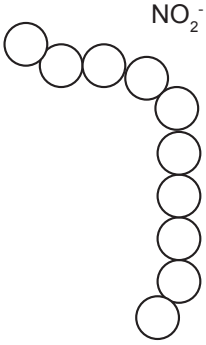
$$C_6H_{12}O_6 + 6\,O_2 \longrightarrow 6\,CO_2 + 6\,H_2O$$

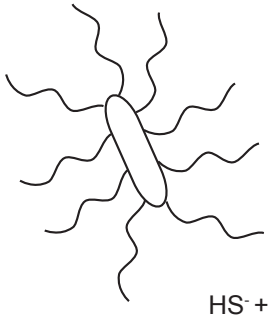




$$NO_2^- + \tfrac{1}{2}\,O_2 \longrightarrow NO_3^-$$

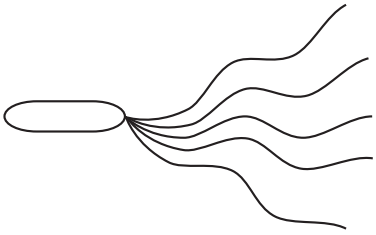


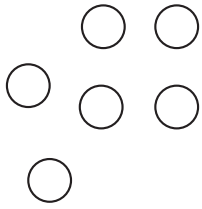




$$HS^- + H^+ + \tfrac{1}{2}\,O_2 \longrightarrow S^0 + H_2O$$

$$S_2O_3^{2-} + H_2O + 2\,O_2 \longrightarrow 2\,SO_4^{2-} + 2\,H^+$$





$$CH_3COO^- + SO_4^{2-} + 3\,H^+ \longrightarrow 2\,CO_2 + H_2S + 2\,H_2O$$