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 (FAs) has previously been shown to reflect the metabolism of microorganisms in pure culture, but has rarely been tested in the environment. Here, we report the abundance and hydrogen isotopic composition of polar lipid derived FAs extracted from surface sediments of the saline Lake Grevelingen (The Netherlands), at two different stations and during two seasons with oxic bottom water conditions during spring and hypoxic to anoxic conditions during late summer. These data are compared with the bacterial diversity revealed by 16S rRNA gene amplicon sequencing. All measured FAs were depleted in deuterium relative to the bottom water by 103 to 267‰. FAs associated with heterotrophic bacteria (i-15:0 and ai-15:0) showed the smallest fractionation (–103 to –185‰) while those derived from pelagic photoautotrophic phytoplankton (20:5) showed the largest fractionation (–230 to –267‰). Overall, the hydrogen isotope fractionation reflected in the majority of the more commonly occurring FAs (14:0, 16:0, 16:1ω7) is relatively large (–172 to –217‰). Together with the high relative abundance of the 20:5 FA, this suggests a substantial contribution from dead pelagic biomass settling from the water column to the sedimentary polar lipid derived FA 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. Therefore, the input of pelagic biomass into sedimentary environments may bias the information contained in the hydrogen isotopic composition of FA.

Key words

hydrogen isotopes, fatty acids, metabolism, chemoautotrophy, heterotrophy, photoautotrophy, sediment, saline lake, 16S rRNA gene sequencing, microorganisms

1. Introduction

In the past decades, several approaches have been developed 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). SIP assesses the microbial metabolism by addition of isotopically labelled substrate to an environmental sample and subsequent determination of label incorporation into cellular biomarkers like DNA, RNA, proteins and lipids. The identification of the labelled biomarkers allows the coupling between metabolism and microbial identity, specifically when label is incorporated into molecules with taxonomic value (Boschker et al., 1998; Manefield et al., 2002; Wuchter et al., 2003; Radajewski et al., 2003; Dumont and Murrell, 2005; van der Meer et al., 2005; 2007; Neufeld 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., 2006). Additionally, targeting the 16S rRNA and functional genes 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 two requirements have to be met (1) a database of sequences of the targeted gene and (2) knowledge of the involvement of the gene-coding enzyme in the metabolic function. 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). Additionally, targeted gene studies will not provide information on novel metabolic pathways as they only target known genes.

Recently, a new method using the natural hydrogen isotopic composition, i.e. the deuterium to hydrogen (D/H) ratio, of fatty acids (FAs) has been shown to reveal the general 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., 2009; Dirghangi and Pagani, 2013; Fang et al., 2014; Heinzelmann et al., 2015a; 2015b). The observed difference in the D/H ratio in FAs of microbes with different metabolism has been attributed to differences in the D/H ratio of nicotinamide adenine dinucleotide phosphate (NADPH; Zhang et al., 2009). These differences are caused by the different metabolic pathways (e.g. oxidative pentose phosphate pathway vs. light reactions of photosynthesis) used to generate it. A similar effect of metabolism on the hydrogen isotopic composition of FAs 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., 2016). 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 FA D/H ratio approach on sedimentary microbial communities. Sedimentary bacteria play an important role in all elemental cycles, i.e. those of carbon, oxygen, sulphur and nitrogen (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, among many other factors, on oxygen

concentration of the overlying water (Orcutt et al., 2011). Sedimentary bacteria express a wide range of different metabolisms, including aerobic heterotrophy, chemoautotrophy, fermentation and sulfate reduction (Nealson, 1997). It strongly depends on the availability of oxygen for example hypoxic/anoxic bottom waters lead to changes in the overall metabolic activity and diversity 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). Li et al. (2009) examined the hydrogen isotopic values of lipids as a tool for studying the metabolism of sedimentary microbial communities. They observed a wide range in the D/H of lipids extracted from Santa Barbara basin sediments with bacterial FAs being more enriched in D compared to those derived from algae. Jones et al. (2008) did not observe any significant differences in the D/H ratio of lipids between different sampling locations or at the same location with sediment depth. However, they did not compare their hydrogen isotope results with community composition and metabolic potential in the analyzed sediments. It has also been shown that FAs specific to sulfate reducing bacteria in sediments from methane seep settings have similar hydrogen isotopic values as those obtained from pure cultures (Dawson et al., 2015), suggesting that their metabolism expressed in vitro is reflected in their lipid isotope signatures in environmental settings.

In order to further test the application of the D/H ratio of FAs as a tool to study microbial metabolism, we studied the D/H ratio of FAs of the microbial communities in surface sediments obtained from Lake Grevelingen, a marine influenced lake. We studied 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 (Lipsewers et al., 2016; 2017). This allows us to study spatial and seasonal differences in microbial metabolism due to changing oxygen concentrations. Changes in the

D/H ratio of FAs will be compared to changes in the bacterial diversity as obtained by 16S rRNA gene amplicon sequencing.

2. Materials and Methods

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. The connection between Lake Grevelingen and the North Sea is re-established during winter, which has led to a rise in the salinity, now varying between 29 to 32 PSU. 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 for both macro-flora (Kamermans et al., 1999) and phytoplankton population (Bakker and De Vries, 1984) following its reconnection to the North Sea. The phytoplankton community is dominated by diatoms and some flagellates (Bakker and De Vries, 1984), the major phytoplankton bloom occurs in July, while a minor bloom occurs in early spring (March). The decaying biomass of the summer bloom is thought to contribute to the hypoxia/anoxia in the water column during late summer (Hagens et al., 2015). Recently, the microbial community of the sulfidic sediment has been the topic of several studies (Malkin et al., 2014; Seitaj et al., 2015; Vasquez-Cardenas et al., 2015; Rao et al., 2016; Sulu-Gambari et al., 2016a; 2016b; Lipsewers et al., 2016; 2017). Desulfobulbaceae filaments capable of electrogenic sulfide oxidation (Malkin et al., 2014; Vasquez-Cardenas et al., 2015) and nitrate-

123 accumulating Beggiatoaceae (Seitaj et al., 2015) have been shown to be present in the Den Osse
124 Basin. Furthermore, heterotrophic denitrifiers and anammox bacteria play a role in the nitrogen
125 cycle in the sediments (Lipsewers et al., 2016). The seasonal shift from oxic to hypoxic/anoxic
126 conditions leads to a community shift from chemoautotrophic Gamma- and
127 Epsilonproteobacteria to sulfate reducing Deltaproteobacteria and a decrease in
128 chemoautotrophic inorganic carbon fixation rates (Lipsewers et al. 2017)

129 Sediment cores were taken on board of the R/V Luctor in March and August 2012 at two
130 different stations. Station 1 had a water depth of 34 m (51.747 ºN, 3.890 ºE) and station 3 had
131 a water depth of 17 m (51.747 ºN, 3.898 ºE) (Hagens et al., 2015). Sediment cores were taken
132 with an Uwitec corer (Uwitec, Austria) (length 60 cm; diameter 60 mm). The overlying water
133 was removed and the core was sliced with 1 cm resolution. Samples were immediately stored
134 on dry ice and later at –80 ºC in the lab until further extraction. Water directly overlying the
135 sediment was sampled for δD_{water} measurements and stored air tight, without headspace, in glass
136 tubes at 4 ºC until measurement.

137 Sediment oxygen and sulfide depth profiles were measured using commercial
138 microelectrodes (Unisense A.S., Denmark) and a motorized micromanipulator. The procedure
139 is described in detail by Malkin et al. (2014). Sulfide concentrations of the pore water were
140 measured according to Sulu-Gambari et al. (2016). Water column oxygen concentration was
141 measured by CTD at Station 1 (Hagens et al., 2015).

142 *2.2. Polar lipid-derived FAs*

143 The first 8 cm of the sediment cores sampled at both stations 1 and 3 were extracted for
144 intact polar lipids (IPL). The freeze dried sediments (0.4–2.7 g) were extracted via a modified
145 Bligh-Dyer method (Bligh and Dyer, 1959; Rütters et al., 2002) with methanol
146 (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 gel column chromatography, eluting the polar lipids with MeOH according to Heinzelmann et al. (2014). Polar lipid-derived fatty acids (PLFA) were generated, methylated and separated into a FA fraction as previously described in Heinzelmann et al. (2016). The BDE was saponified with 1 N KOH in MeOH (96%), methylated with a boron trifluoride-methanol solution (BF_3–MeOH) and separated over an aluminium oxide (AlOx) column. The methylated PLFAs were eluted with DCM. The position of the double bonds in unsaturated FAs 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 x:yωz, where x designates the total number of carbons, y the number of double bonds and z the position of the double bond relatively to the aliphatic end (ω) of the molecule. The prefixes i and ai refer to *iso* and *anteiso* methyl branching of the alkyl chain, respectively.

2.3. FA 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 FA 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), with the temperature program 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 in order to monitor the performance of the GC-TC-irMS. 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 FA sample in order to monitor the precision (average δD – 162 ± 2‰) and the δD of the individual FAs was measured in duplicates and corrected for the added methyl group (Heinzelmann et al., 2015b). The isotopic value of the methyl group was determined via the derivatization of phathalic acid with a known isotopic composition.

The hydrogen isotopic composition of FAs 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 et al. (2014).

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.

2.5. 16S rRNA gene amplicon sequencing and analysis

The general bacterial diversity was assessed 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. (2016). 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. (2016) 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. The 16S rRNA gene amplicon reads (raw data) for Station 1 and 3 2–8 cm have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject no. PRJNA404017, while data for the first cm of Stations 1 and 3 have been published previously in Lipsewers et al. (2017).

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.

3. Results

Water column oxygen concentration at Station1 ranged between 299 and 353 µM in March and between 0 (i.e. not detected) and 306 µM in August (Fig. S1) (Hagens 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 (Seitaj et al., 2015; Lipsewers et al., 2016). The sulfide concentration increased with sediment depth up to 818 µmol/L at Station 1 in March and ranged from 725 to 2893 µmol/L in August. At Station 3 in March it ranged from 0 to 2 µmol/L, but decreased with sediment depth from 224 to 2 µmol/L in August (Supplementary Table 1) (Seitaj et al., 2015; Lipsewers et al., 2016). The sedimentation rate at the site is > 2 cm/y (Malkin et al., 2014) suggesting that the first eight cm of the sediment cores represent ca. 4 yr of deposition.

3.1. FA abundance and composition

A variety of polar lipid-derived FAs were observed in the sediments analysed, including 14:0, i-15:0, ai-15:0, 15:0, 16:1ω7, 16:0, 18:0 FAs, a 20:5 polyunsaturated FA (PUFA) and various unsaturated 18:x FAs (Fig. 1; Supplementary Table 2). Due to incomplete separation, the 18:x FAs had to be quantified as their sum. The FA distributions were similar for both stations and did not change substantially with season or sediment depth (Fig. 1; Supplementary Table 2).

3.2. D/H ratio of FAs

The δD values of 14:0, i-15:0, ai-15:0, 16:1ω7, 16:0, 18:0 FAs and 20:5 PUFA were obtained for most sediment layers (Supplementary Table 3). The D/H ratio of the cluster of 18:x FAs could not be measured with certainty due to either incomplete separation or low abundance.

All FAs were substantially depleted in D compared to the bottom water overlying the sediment, which had relatively constant δD values (δD_{water} –1.8 ± 3.3 to 0.1 ± 2.8‰) (Supplementary Table 3) with values for the fractionation factor $\varepsilon_{lipid/water}$ ranging between –103 and –267‰ (Table 1; Fig. 2). The 20:5 PUFA was the most depleted FA followed by the 14:0, while the i-15:0 was usually the most D-enriched FA (Supplementary Table 3). The two

different bacterial FAs i-15:0 and ai-15:0 differ by up to 70‰, with the ai-15:0 having similar $\varepsilon_{lipid/water}$ values as the 16:0 FA. The non-specific FA 18:0 generally also shows a smaller degree of fractionation compared to the other non-specific FAs and the ai-15:0, varying between –140 to –200‰.

Substantial differences in $\varepsilon_{lipid/water}$ values were observed between different depth intervals and cores for some of the FAs. At Station 1 in March, the $\varepsilon_{lipid/water}$ values for the 16:1 FA 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 trend with depth is observed. The $\varepsilon_{lipid/water}$ values for 14:0 at Station 1 in August tend towards smaller fractionation (by ca. 20‰ ranging from –191 to –215 ‰), with increasing depth, while no major trend with depth was observed in March. The $\varepsilon_{lipid/water}$ value for the 16:0 FA, 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 18:0 FA 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 18:0 FA is relatively stable at ~ –140 to –148 ‰ from 0 to 6 cm depth, although some layers did not contain enough 18:0 for a reliable measurement, and subsequently decreases to ~ –186 ‰ at 7–8 cm depth (Table 1; Fig. 2). The 20:5 PUFA got more depleted with depth in March (from –229 ‰ to –261 ‰). In comparison to March, it was more depleted in August and the $\varepsilon_{lipid/water}$ showed little variability between 1 and 7 cm depth (from –261 ‰ to –268 ‰).

At Station 3 in March no trend with depth was observed for 16:1 and 16:0 FAs. However, the $\varepsilon_{lipid/water}$ value of 14:0, although variable, seems to show an overall trend towards more positive values by up to 10‰ from the surface to 8 cm depth, while the $\varepsilon_{18:0/water}$ 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 14:0, 16:1 and 16:0 FAs, all of which had

similar $\varepsilon_{lipid/water}$ values as in March. The 18:0 FA on the other hand became 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, by 10–20 ‰, in August than in March (Table 1; Fig. 2). In March $\varepsilon_{lipid/water}$ values of the 20:5 PUFA could only be measured in the first four cm and the values were comparable to Station 1 (–231 to –250 ‰). In August the 20:5 PUFA showed a slightly larger degree of fractionation (–229 to –260 ‰). Similar to Station 1, $\varepsilon_{lipid/water}$ values were higher in the first and last cm with $\varepsilon_{lipid/water}$ of –229 ‰ and –249 ‰, respectively.

3.3. Bacterial diversity

The isotopic fractionation of the FAs shows a larger difference between the two different stations than between the different seasons, with the largest difference between the two stations in August (when considering individual FAs like 14:0). Therefore, the bacterial diversity of sediment cores taken in August was studied using 16S rRNA gene amplicon sequencing. The phylogenetic data for the first cm in both Stations 1 and 3 has been previously reported by Lipsewers et al. (2017) and here we report data for depth interval of the first 8 cm. In order to assess only the bacterial reads, chloroplast reads were removed. The phylogenetic diversity at Station 1 (Table 2a; Fig. S2) 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 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% (Table 2a; Fig. S2).

293 Most of the orders observed in Station 1 were also present at Station 3 (Table 2b; Fig S2).
294 The Desulfobacterales were the main contributor to the bacterial 16S rRNA gene reads (up to
295 23%) and the percentage of reads decreased with depth (down to 15.5%). The Bacteroidales
296 and the Thiotrichales contributed to more than 5% of the total bacterial 16S rRNA gene reads.
297 The contribution of the Bacteroidales decreased to 3.5% with depth, while the Thiotrichales
298 remained fairly constant at ~ 10% with depth (Table 2b; Fig. S2).

299 **4. Discussion**

300 *4.1. Hydrogen isotopic composition of source-specific FAs*

301 Most of the FAs detected in the sediment cores of Lake Grevelingen commonly occur in
302 bacteria and eukaryotes (e.g. 14:0, 16:1ω7, 16:0 and 18:0), but some are more specific. Both
303 the i-15:0 and ai-15:0 FAs are known to derive from bacterial sources (Gunstone et al., 2012),
304 while the 20:5 PUFA is mainly produced by algae and only in trace amounts by some bacteria
305 (Volkman et al., 1989; Carrie et al., 1998; Iizuka et al., 2003) and is, therefore, considered an
306 algal biomarker. Of all the FAs, the 20:5 PUFA showed the highest degree of hydrogen isotope
307 fractionation ($\varepsilon_{lipid/water}$ between –230 and –268‰) and the i-15:0 showed the lowest degree of
308 fractionation ($\varepsilon_{lipid/water}$ between –103 and –131‰) (Fig. 2). The $\varepsilon_{lipid/water}$ values obtained for the
309 20:5 PUFA fall within the range previously associated with photoautotrophic growth
310 (Heinzelmann et al., 2015b), in agreement with its algal source.

311 The $\varepsilon_{lipid/water}$ values of the i-15:0 FA fall well within the range of those produced by
312 heterotrophic microorganisms. Heterotrophic microorganisms in general produce FAs that
313 range between depleted (–133‰) up to heavily enriched (+200‰) in D compared to the growth
314 medium (e.g. Heinzelmann et al., 2015b; Zhang et al., 2009). Indeed, the majority of the
315 sequences obtained by 16S rRNA gene amplicon sequencing belonged to heterotrophic bacteria
316 involved in the degradation of high molecular weight biomass coming from the water column

(*Bacteroidetes*) and in the sulphur cycle (Desulfobacterales). The heterotrophic *Bacteroidetes* are most likely the dominant source of the i-15:0 FA (Supplementary Table 4). In addition to the i-15:0, ai-15:0 is also a known bacterial biomarker. Interestingly, this FA was more depleted in D compared to i-15:0 by up to 70‰. This could possibly be explained by a difference in source organism for these FAs. While both i-15:0 and ai-15:0 FAs are produced by the *Bacteroidetes*, ai-15:0 is more dominant in species of the Desulfobacterales (Supplementary Table 4). Recent studies by Dawson et al. (2015) and Osburn et al. (2016) showed that different sulfate reducing bacteria produce, when grown as heterotrophs, FAs depleted in D. For example, *Desulfococcus multivorans* (belonging to the Desulfobacterales) produce, when grown as a heterotroph both in pure culture and in co-culture with a methanogen, FAs 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 (Zhang et al. 2009; Heinzelmann et al. 2015b), and are closer to what is associated with (photo)autotrophic growth. Osburn et al. (2016) cultured sulfate reducers under heterotrophic and autotrophic conditions. They did not report significant differences in isotopic fractionation between autotrophic and heterotrophic growth as FAs were consistently depleted in D by up to –352‰. A contribution of the Desulfobacterales, growing either heterotrophically or autotrophically, to the ai-15:0 FA pool could thus explain the higher degree of hydrogen isotopic fractionation observed for this FA compared to i-15:0 Additionally, the percentage of total bacterial reads of the Desulfobacterales was higher in Station 3, suggesting a higher Desulfobacterales contribution to the FA pool and possibly explaining the higher degree of fractionation reflected in the ai-15:0 FA compared to Station 1.

4.2. Hydrogen isotopic composition of non-specific FAs

While the hydrogen isotopic composition of (group-) specific FAs clearly indicates the metabolism expressed by the source microorganisms, it does not necessarily represent the whole

342 microbial community. In order to study the whole microbial community, we calculated a
343 weighted average ε ($\varepsilon_{\Sigma FA}$) of the non-specific FAs (14:0, 16:1ω7, 16:0 and 18:0) (Table 1, Fig.
344 2). At Station 1, $\varepsilon_{\Sigma FA}$ values were between –182 and –197‰ in March and between –181 and –
345 195‰ in August. At Station 3, $\varepsilon_{\Sigma FA}$ values were between –186 and –202‰ in March and
346 between –185 and –201‰ in August. The overall stable $\varepsilon_{\Sigma FA}$ values suggest only minor changes
347 in the general metabolism of the sedimentary microbial communities assuming that the majority
348 of the FAs derive from *in situ* production. This agrees with the 16S rRNA gene based diversity
349 analysis of our study that shows relatively minor changes in the overall bacterial community
350 with depth and no apparent depth trend of the distribution of the FAs.

351 Using ^{13}C stable isotope probing of phospholipid derived FAs, Lipsewers et al. (2017)
352 report a shift from chemoautotrophic metabolism in March to heterotrophic metabolism in
353 August. This shift was inferred from a variation in the relative abundance of individual FAs,
354 i.e. an increase of iso, anteiso and branched FAs in the surface sediment in August, between the
355 different seasons and a change in the incorporation of labelled bicarbonate in phospholipid
356 derived FAs. However, we did not observe these changes in relative abundance of individual
357 FAs in our core. One possible explanation for the differences between this study and Lipsewers
358 et al. (2017) is that the latter isolated phospholipid derived FAs following the method of Guckert
359 et al. (1985). It has been shown this procedure results in a FA fraction which might not contain
360 all phospholipids (i.e. loss of ~10 % of phosphatidylglycerides (PG) and
361 phosphatidylethanolamines (PE)) and does contain other polar lipid classes like glycolipids (e.g.
362 50% of monogalactosyldiacylglycerols (MGDG), 70% of sulfoquinovosyldiacylglycerols
363 (SQDG) and nearly 100% of digalactosyldiacylglycerols (DGDG)) and betaine lipids (up to
364 100% of diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA)) (Heinzelmann et al., 2014).
365 In contrast, our FAs are derived from the full range of polar lipids, such as phospholipids,
366 glycolipids, betaine lipids and sulfoquinovosyldiacylglycerol lipids. Indeed, the reported FA

composition of Lipsewers et al. (2017) is different from ours, in that the first cm in their sediment cores was characterised by relatively high concentrations of 16:1 and 18:1, and the ai-15:0 FA increased in abundance in deeper layers of the sediment. In our study, however, the 16:1 FA concentration stays relatively constant throughout the whole core and a higher abundance of ai-15:0 FA in deeper layers was only observed at Station 3 in March. Additionally, Lipsewers et al. (2017) observed an increase of iso, anteiso and branched FAs from March to August which is not visible in the results shown here. Furthermore, we did not observe higher concentrations of 16:0 and 14:0 FAs in deeper layers in August compared to March.

Interestingly, the $\varepsilon_{\Sigma FA}$ values for both stations fall within the range associated with photoautotrophic growth (Zhang et al., 2009a; Heinzelmann et al., 2015a and references therein) although slightly more positive compared to previous studies of photoautotrophic microorganisms (Osburn et al., 2011; Heinzelmann et al., 2016). 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 FAs are reflecting an average of the relatively D depleted signal from chemoautotrophy, a process demonstrated to occur in these sediments (Lipsewers et al., 2017), and the relatively D-enriched signal from heterotrophy. While FAs produced by heterotrophs in general show only a small degree of fractionation, chemoautotrophs produce FAs which are significantly depleted in D with observed $\varepsilon_{lipid/water}$ values up to –404‰ (Valentine et al., 2004; Zhang et al. 2009). Depending on the relative contribution of heterotrophs versus chemoautotrophs, this could lead to a FA pool significantly more depleted in D than expected for FAs predominantly derived from heterotrophs and might explain the $\varepsilon_{lipid/water}$ values observed in the Grevelingen sediment. 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 sulphur bacteria. As photoautotrophic growth in the sediment is unlikely, it can be assumed that they might inhabit the water column during periods of anoxia and reduced sulfur compounds in the photic zone of the water column. In addition, only few reads associated with photoautotrophic bacteria were observed, suggesting that *in situ* bacterial photoautotrophy has a relatively small effect on the lipids in these sediments. Furthermore, PLFA-SIP experiments showed substantial uptake rates of dissolved inorganic carbon in the dark into i-15:0, ai-15:0 and 14:0 FAs suggesting a predominance of chemolithoautotrophy in the spring (Lipsewers et al., 2017). Thus, the observed depleted $\varepsilon_{\Sigma FA}$ values of the unspecific, but abundant, FAs relative to those of the i-15:0 FA could be due to a mixed contribution of chemoautotrophic and heterotrophic bacteria to the FA pool. Alternatively, an important part of the sedimentary population consisted of the Desulfobacterales, sulfate reducing bacteria, representatives of which seem to produce relatively depleted FAs even when growing heterotrophically (Dawson et al., 2015; Osburn et al., 2016). This could also contribute to the general depletion in D of the FAs relative to the range typically associated by heterotrophic growth.

A shift from a FA pool containing FAs derived from a mixture of chemoautotrophic and heterotrophic bacteria in March towards one dominated by FAs produced by heterotrophic sulfate reducing bacteria in August could potentially explain the absence of a shift in the hydrogen isotopic signal despite that Lipsewers et al. (2017) observed a reduction in chemoautotrophic activity. This reduction in chemoautotrophic activity was inferred from a reduced ^{13}C incorporation into FAs and a decreased abundance of genes involved in carbon fixation pathways.

4.3. Pelagic contributions to the sedimentary FA pool

Although the sedimentary microbial community is dominated by heterotrophic and chemoautotrophic microorganisms, 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 20:5 PUFA (i.e. up to 25%; Fig. 1), a FA characteristic of photoautotrophic algae indicates a major input of algal biomass derived from the water column to the sedimentary FA pool. This observation is supported by the presence of substantial amounts of chloroplast reads throughout the sediment core. In the sediments of both stations the relative abundance of 20:5 PUFA increased in August compared to March (Fig. 1) which might be due to the phytoplankton blooms during spring and summer (Hagens et al., 2015). The high relative abundance of 20:5 PUFA could be explained by the high sedimentation rate at the site of > 2 cm/y (Malkin et al., 2014), and the fact that anoxic conditions lead to a reduced degradation rate of organic matter (Middelburg and Levin, 2009), including that of intact polar lipids. IPLs are in general considered to represent living biomass as they degrade shortly after cell death. However, their lifespan in anoxic sedimentary environments is not known and therefore could be longer than expected. It has been shown that degradation of phospholipids decreases by 40% when comparing anoxic with oxic sediments, with 70% of the intact phosholipids degrading within 96 h (Harvey et al., 1986). This could therefore lead to a preservation of algal-derived FAs during sediment burial which can affect the hydrogen isotopic ratio of the PLFAs studied in this environmental setting. However, the degradation rate in the studied sediments is not known.

The high relative abundance of 20:5 PUFA also suggests that part of the more ubiquitous 14:0, 16:0 and 16:1 FAs may also originate from oxygenic 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 community in Lake Grevelingen

(Bakker and De Vries, 1984) and are known to mainly produce 14:0, 16:0, 16:1ω7 FAs along with 20:5 PUFA and only traces of 18:0 (Supplementary Table 4). It would thus be expected that these FAs 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 18:0 FA, which is only produced in trace amounts by diatoms (Supplementary Table 4). The generally more positive 18:0 $\varepsilon_{lipid/water}$ values could indicate a relatively high *in situ* contribution from heterotrophic bacteria. Furthermore, the $\varepsilon_{18/water}$ value shows considerably more variability with depth and between stations and seasons than $\varepsilon_{lipid/water}$ values for most other FAs. This suggests a higher contribution of microorganisms with other metabolisms, e.g. heterotrophy and chemoautotrophy.

Our results thus suggest that part of the more general or non-specific FAs, especially the 14:0, 16:0 and 16:1ω7 FAs, are derived from algae living in the water column and a smaller fraction comes from *in situ* production by the sedimentary microbial population. This predominantly photoautotrophic origin of the non-specific FAs is a likely explanation for the relatively low and stable $\varepsilon_{lipid/water}$ values for these FAs. This has previously also been suggested by Li et al. (2009) who observed that bacterial FAs in Santa Barbara Basin sediments were more enriched in D than even numbered FAs which most likely derived from phytoplankton biomass precipitating from the water column. Our findings do not necessarily contradict the results of Lipsewers et al. (2017), who found label incorporation in FAs in the same sediments. Because of the sensitivity of the SIP, small amounts of incorporation can be easily detected despite a large background of potentially fossil PLFA and only a limited number of FAs showed substantial incorporation of ^{13}C. As SIP is not impacted by a fossil biomass contribution, unlike the D/H ratio of the sedimentary FA pool, this technique can give a better idea on the microbial activity in the sediment. The D/H ratio of the sedimentary FA pool on the other hand can give a general idea of the community metabolism. This will include autotrophy and heterotrophy,

and potentially, depending on the setting, the impact of allochthonous organic material. Therefore, while the SIP experiment focused on chemoautotrophic microorganisms our study also indicated contributions of heterotrophic and photoautotrophic microorganisms.

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 FAs seem to mainly reflect photoautotrophy, suggesting that these FAs 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, especially IPLs, coming from different aquatic and sedimentary microbial communities under anoxic conditions could lead to a bias in the hydrogen isotopic composition of FAs as a tool to study the metabolism of microbial communities *in situ*. It would, therefore, be 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 FAs and potentially other lipid classes.

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Table 1: D/H fractionation between FAs and overlying water

Depth [cm]	δD_{water} [‰]	$\varepsilon_{lipid/water}$ [‰]							$\varepsilon_{\Sigma FA}$
		14:0	iso-15:0	ai-15:0	16:1*	16:0	18:0	20:5 PUFA	weighted av. 14:0, 16:1, 16:0, 18:0
Station 1 (March)									
0–1	–1.8±3.3	–200±6	–103±4	–162±4	–188±5	–180±5	–191±4	–229±13	–186
1–2		–193±5	–118±4	–174±4	–188±6	–175±5	–190±10	–243±5	–183
2–3		–193±12	–114±4	–174±4	–209±4	–184±4	–145±4	–248±7	–192
3–4		–197±5	–115±5	–176±4	–196±4	–170±5	–153±4	–258±4	–182
4–5		–210±4	–131±5	–192±4	–203±4	–185±4	–163±6	–262±4	–193
5–6		–199±8	–127±4	–185±4	–197±6	–184±5	–179±5	–247±5	–191
6–7		–199±5	–124±4	–181±5	–216±6	–183±8	–165±4	–254±5	–195
7–8		–207±5	–125±5	–183±4	–212±4	–189±4	–158±6	–261±5	–197
Station 1 (August)									
0–1	0.1±2.8	–215±3	–126±3	–171±3	–203±3	–186±3	–140±3	–240±3	–196
1–2		–201±3	N.D.	N.D	–209±3	–195±4	N.D.	–267±5	–194
2–3		–203±6	–119±3	–185±3	–204±4	–189±3	–147±3	–261±4	–194
3–4		–200±8	N.D	N.D	–208±4	–196±3	N.D.	–266±4	–194
4–5		–204±7	N.D	N.D	–209±4	–195±4	N.D.	–268±4	–195
5–6		–183±8	–112±4	–173±5	–191±3	–178±3	–148±5	–263±4	–181
6–7		–204±3	–120±4	–177±5	–208±3	–187±3	–153±5	–263±3	–195
7–8		–195±5	N.D	N.D	–201±3	–191±3	–186±4	–244±5	–195
Station 3 (March)									
0–1	–1.6±2.6	–197±3	–108±3	–176±3	–197±3	–184±3	–169±3	–234±3	–189
1–2		–182±13	–120±3	–178±3	–206±3	–179±5	–153±3	–248±3	–187
2–3		–188±3	–121±3	–182±5	–204±3	–185±3	–159±5	–250±4	–190
3–4		–179±5	–115±3	–177±3	–195±5	–185±5	–166±4	–231±3	–186
4–5		–192±4	–118±3	–182±3	–218±4	–193±4	–188±4	N.D.	–202
5–6		–181±3	–130±3	–182±5	–208±5	–185±3	–173±3	N.D.	–194
6–7		–178±5	–122±4	–177±3	–209±3	–185±3	–166±3	N.D.	–192
7–8		–186±3	–120±3	–184±3	–187±3	–185±3	–191±3	N.D.	–187
Station 3 (August)									
0–1	–1.7±4.1	–192±11	–125±4	–185±4	–200±5	–187±5	–148±5	–229±4	–191
1–2		–199±6	–116±4	–180±4	–208±4	–185±4	–148±6	–260±4	–194
2–3		–185±6	–119±4	–176±8	–194±4	–182±4	–157±4	–253±6	–185
3–4		–189±4	–121±5	–184±4	–208±4	–183±4	–163±5	–256±4	–191
4–5		–180±12	–120±4	–175±5	–200±5	–186±4	–178±4	–250±4	–190
5–6		–203±7	–128±4	–184±5	–206±5	–194±4	–199±4	–243±4	–201
6–7		–199±4	–130±4	–183±4	–216±5	–184±4	–157±4	–253±5	–196
7–8		–191±8	–131±4	–181±5	–206±5	–181±5	–158±5	–249±5	–189

* double bond at ω7 position

Table 2: 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

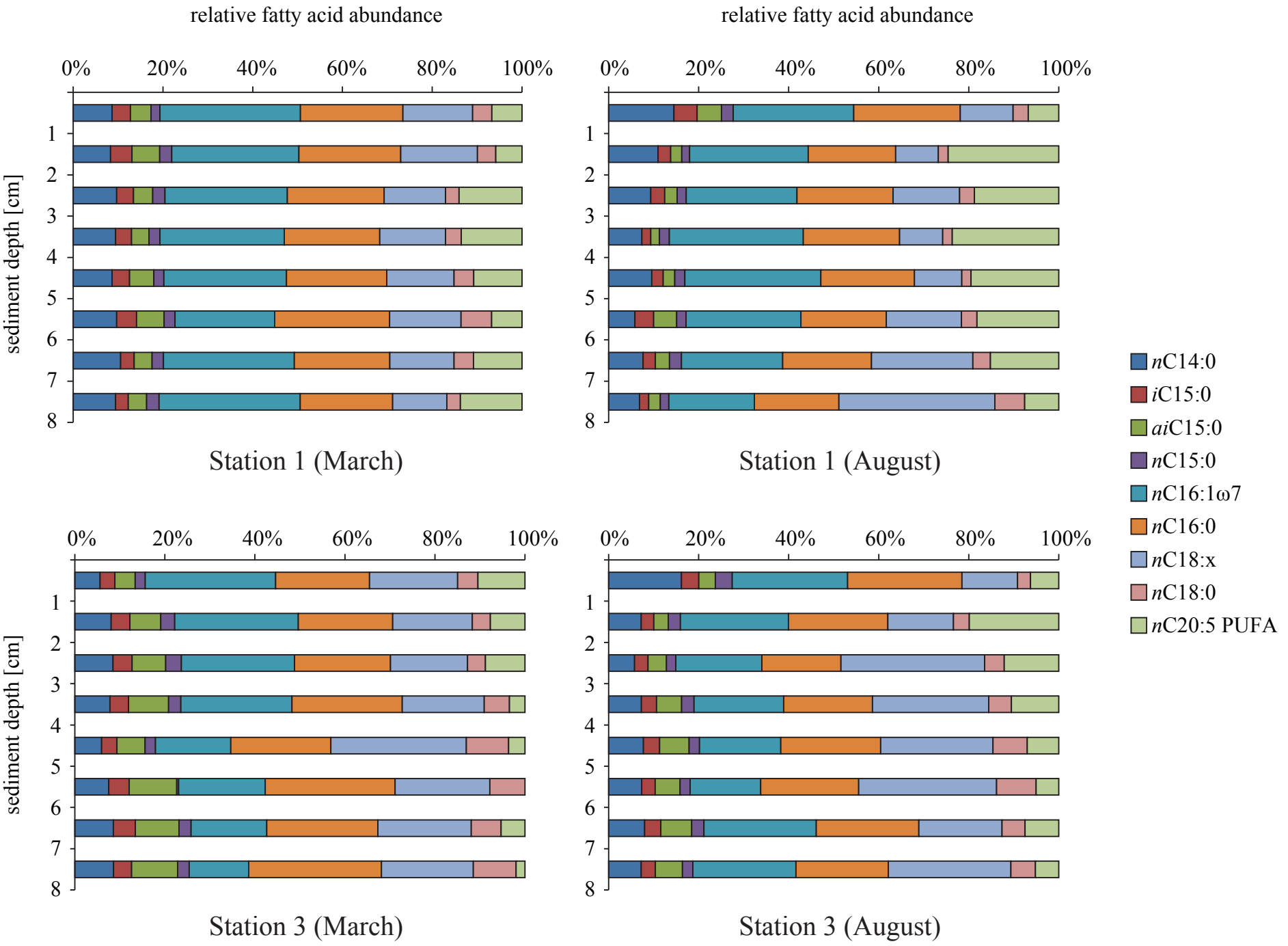
The sequence reads belonging to the Bacteroidetes, δ-Proteobacteria and γ-Proteobacteria were extracted from the dataset and added to a phylogenetic tree (Figures S2-S4). Bacteroidetes sequences clustered mainly within the Marinilabiaceae (Bacteroidales), the Flavobacteriaceae (Flavobacteriales) and 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.

Figure Legends

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

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.


relative fatty acid abundance
0%
20%
40%
60%
80%
100%
sediment depth [cm]
1
2
3
4
5
6
7
8
Station 1 (March)
relative fatty acid abundance
Station 1 (August)
Station 3 (March)
Station 3 (August)
nC14:0
iC15:0
aiC15:0
nC15:0
nC16:1ω7
nC16:0
nC18:x
nC18:0
nC20:5 PUFA