



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-to-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 ($\varepsilon_{\text{lipid/water}}$) ranged between -172 and -237‰ , the algal-derived polyunsaturated fatty acid $n\text{C}20:5$ generally being the most D-depleted (-177 to -235‰) and $n\text{C}18:0$ the least D-depleted fatty acid (-172 to -210‰). The in general highly D-depleted $n\text{C}20:5$ is in agreement with culture studies, which indicates that photoautotrophic microorganisms produce fatty acids which are significantly depleted in D relative to water. The $\varepsilon_{\text{lipid/water}}$ of all fatty acids showed a transient shift towards increased fractionation during the spring phytoplankton bloom, indicated by increasing chlorophyll *a* concentrations and relative abundance of the $n\text{C}20:5$ polyunsaturated fatty acids, suggesting increased contributions of photoautotrophy. Time periods with decreased fractionation (less negative $\varepsilon_{\text{lipid/water}}$ values) can potentially be explained by an increased contribution of heterotrophy to the fatty acid pool. Our results show that the hydrogen isotopic composi-

tion of fatty acids is a promising tool to assess the community metabolism of coastal plankton potentially in combination with the isotopic analysis of more specific biomarker lipids.

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 (Dirghangi and Pagani, 2013; Fang et al., 2014; Heinzelmann et al., 2015a, b; X. N. Zhang et al., 2009; Z. Zhang et al., 2009). While most of these factors lead to relatively small variations in the deuterium-to-hydrogen (D / H) ratio of fatty acids ($10\text{--}20\text{‰}$), differences in the central metabolism of microorganisms have a much more pronounced effect (X. N. Zhang et al., 2009). Both photo- and chemoautotrophs produce fatty acids depleted in D compared to growth water with the stable hydrogen isotopic fractionation factor ε between the $n\text{C}16:0$ fatty acid, the most commonly occurring fatty acid in microorganisms, and water ($\varepsilon_{\text{lipid/water}}$) ranging between -160 to -220‰ and -250 and -400‰ , respectively (Campbell et al., 2009; Chikaraishi et al., 2004; Heinzelmann et al., 2015a, b; Sessions et al., 2002; Valentine et al., 2004; X. N. Zhang et al.,

2009; Zhang and Sachs, 2007). In contrast, heterotrophs produce *n*C16:0 fatty acid with either a relatively minor depletion or an enrichment in D compared to the growth water with $\varepsilon_{\text{lipid/water}}$ values ranging between -150 and $+200\text{‰}$ (Dirghangi and Pagani, 2013; Fang et al., 2014; Heinzelmann et al., 2015a, b; Sessions et al., 2002; X. N. Zhang et al., 2009). It has been speculated that the differences in hydrogen isotopic composition of fatty acids produced by organisms expressing different core metabolisms (i.e. heterotrophy and photo- and chemoautotrophy) are mainly due to the D / H ratio of the H added to nicotinamide adenine dinucleotide phosphate (NADP^+) and the hydrogen isotope fractionation associated with the reduction of NADP^+ to NADPH , which is used during fatty acid biosynthesis (X. N. Zhang et al., 2009). NADP^+ is reduced to NADPH during a number of different reactions in multiple different metabolic pathways (each associated with different hydrogen isotopic fractionations) and is subsequently used as a major source of hydrogen in lipid biosynthesis (Robins et al., 2003; Saito et al., 1980; 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 whether the D / H ratio of fatty acids from environmental microbial communities can be used to assess the “community-integrated” core metabolisms and changes therein 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, which have been shown to affect the hydrogen isotopic composition of lipids (Dawson et al., 2015) and they test a limited number of potential substrates, energy sources and core metabolisms. Previous studies of environmental samples observed a wide range in the D / H ratio of lipids in both the marine water column and sediment (Jones et al., 2008; Li et al., 2009), suggesting inputs of organisms with a variety of metabolisms. Osburn et al. (2011) 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 first environmental results indicate the applicability of this new method, which clearly indicates the limitation of looking only at general occurring fatty acids.

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 microbial community. Time series studies have been previously performed at the NIOZ jetty to determine phytoplankton and prokaryotic abundances and composition (Alderkamp et al., 2006; Brandsma et al., 2012; Brussaard et al., 1996; Philippart et al., 2000, 2010; Pitcher et al., 2011; Sintes et al., 2013), lipid composition (Brandsma et al., 2012; Pitcher et al., 2011), and chlorophyll *a* concentration (Philippart et al., 2010). Typically, the spring bloom in the coastal North Sea is predominantly comprised of *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. An autumn bloom is formed by *Thalassiosira* spp., *C. socialis*, cryptophytes and cyanobacteria (Brandsma et al., 2012; Cadée and Hegeman, 2002). However the autumn bloom seems to have weakened over the last years (Philippart et al., 2010). The abundance of bacteria increases following the algal blooms and the bacteria are dominated by heterotrophs, e.g. bacteria belonging to *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., 2012). 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 (Brandsma et al., 2012). 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 (PLFAs) 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.

2 Material and methods

2.1 Study site and sampling

From September 2010 until December 2011 water samples were taken from the NIOZ sampling jetty in the Marsdiep at the western entrance of the North Sea into the Wadden Sea near the island of Texel ($53^{\circ}00'06''\text{N}$, $4^{\circ}47'21''\text{E}$). Surface water samples (depth $\max \pm 50$ cm below surface) were collected for suspended particulate matter (SPM) biweekly during high tide to ensure that water sampled was from the North Sea.

For lipid analysis measured volumes of water (ca. 9–11 L) were filtered consecutively, without pre-filtration, through pre-ashed 3 and $0.7\text{ }\mu\text{m}$ pore size glass fibre filters (GF/F,

Whatman; 142 mm diameter) and stored at -20°C until lipid extraction. For DNA analysis approximately 1 L seawater was filtered through a polycarbonate filter ($0.2\text{ }\mu\text{m}$ pore size; 142 mm diameter; Millipore filters) and stored at -80°C until extraction.

Salinity measurements were done during the time of sampling with either an Aanderaa conductivity/temperature sensor 3211 connected to an Aanderaa data logger 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 ($0.7\text{ }\mu\text{m}$ pore size, Whatman, GE Healthcare Life Sciences, Little Chalfont, UK) and immediately frozen in liquid nitrogen. Samples were thawed and homogenized with glass beads and extracted with methanol. Chlorophyll *a* concentration was measured with a Dionex high-performance liquid chromatograph (HPLC) (Philippart et al., 2010).

Water samples for salinity versus $\delta\text{D}_{\text{water}}$ 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.

2.2 Polar-lipid-derived fatty acids

Filters were extracted for IPLs and eventually fatty acid analysis. The $0.7\text{ }\mu\text{m}$ filters did not yield enough total lipid extract for analysis. Therefore, only fatty acids obtained from the $3\text{ }\mu\text{m}$ filters were analysed. Due to fast clogging of the filters and a corresponding decrease of the pore size (Sørensen et al., 2013), the $3\text{ }\mu\text{m}$ filters will most likely contain most of the microorganisms present in North Sea water, although it cannot be excluded that the microorganisms retained on the filter are biased towards a larger cell size. 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, *v/v/v*) 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 combined and water removed using Na_2SO_4 . The sample was dried under nitrogen and stored in the fridge. The PLFAs were methylated with boron trifluoride-methanol ($\text{BF}_3\text{-MeOH}$) 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 derivatized with dimethyldisulfide (DMDS) (Nichols et al., 1986). Hexane, DMDS and I_2 / ether (60 mg mL^{-1}) 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 $\text{Na}_2\text{S}_2\text{O}_3$. 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 .

2.3 Fatty acid and hydrogen isotope analysis

The fatty acid fractions were analysed by gas chromatography (GC) using an Agilent 6890 GC with a flame ionization detector (FID) using a fused silica capillary column ($25\text{ m} \times 320\text{ }\mu\text{m}$) coated with CP Sil-5 (film thickness $0.12\text{ }\mu\text{m}$) with helium as carrier gas. The temperature programme was as follows: initial temperature 70°C , increase of temperature to 130°C with $20^{\circ}\text{C min}^{-1}$, and then to 320°C with $4^{\circ}\text{C min}^{-1}$, which was kept for 10 min. Individual compounds and double bond positions (see above) were identified using GC–mass spectrometry (MS) (Schouten et al., 1998).

Hydrogen isotope analysis of the fatty acid fraction was performed by GC thermal conversion (TC) isotope ratio monitoring (ir) MS 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 ($25\text{ m} \times 0.32\text{ mm ID}$; $0.4\text{ }\mu\text{m}$ film thickness; He carrier gas, 1.0 mL min^{-1}). The GC temperature programme was 70 to 145°C at $20^{\circ}\text{C min}^{-1}$, then to 320°C at $4^{\circ}\text{C min}^{-1}$, 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 analysed daily prior to analysing samples in order to monitor the system performance. Samples were only analysed when the *n*-alkanes in Mix B had an average deviation from their offline determined value of $<5\text{ ‰}$. An internal standard, squalane ($\delta\text{D} = -170\text{ ‰}$), was co-injected with each fatty acid sample fraction in order to monitor the accuracy of the measurements over time with $\delta\text{D} = -164 \pm 4\text{ ‰}$. 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 (EA)/TC/irMS according to Chivall et al. (2014).

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 chambers. Most photoautotrophic microorganisms 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 phytoplankton species (or groups) together comprised, on average, more than 60 % of the total numbers of marine phytoplankton in the Marsdiep during this study period.

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 ($5 \mu g \mu L^{-1}$) was added to the lysate, incubated for 30 min at 37 °C 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 $3 \times 100 \mu L$ AE buffer, the eluates pooled and reconcentrated. DNA quality and concentration was estimated by NanoDrop (Thermo Scientific, Waltham, MA) quantification.

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-iTTM PicoGreen[®] dsDNA Assay Kit (Life Technologies, the Netherlands).

PCRs were performed with the universal (Bacteria and Archaea) primers S-D-Arch 0519-a-S-15 (5'-CAGCMGCCGCGGTAA-3') and S-D-Bact-785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2012) adapted for pyrosequencing by the addition of sequencing adapters and multiplex identifier (MID) sequences. To minimize bias three independent PCRs were performed containing the following: 16.3 μL H₂O, 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 ($6 ng \mu L^{-1}$). The PCR conditions were the following: 98 °C, 30 s; 25 \times [98 °C, 10 s; 53 °C, 20 s; 72 °C, 30 s]; 72 °C, 7 min and 4 °C, 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 puri-

fied with Qiaquick Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. PCR purified products were quantified with Quant-iTTM 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., South Korea.

Samples were analysed 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 and 350 bp, and allowing a maximum of 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. The 16S rRNA gene amplicon reads (raw data) were deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA293285.

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.

3 Results

3.1 Chlorophyll *a* concentration and phytoplankton abundance and diversity

Chlorophyll *a* concentrations ranged between 0.4 and 22.2 $\mu g L^{-1}$ (Fig. 1; Table S1 in the Supplement). During late autumn, winter and early spring concentrations were low at $\sim 4 \mu g L^{-1}$. 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 thereafter.

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 (Fig. 2), with the spring bloom primarily being made up of *P. globosa*.

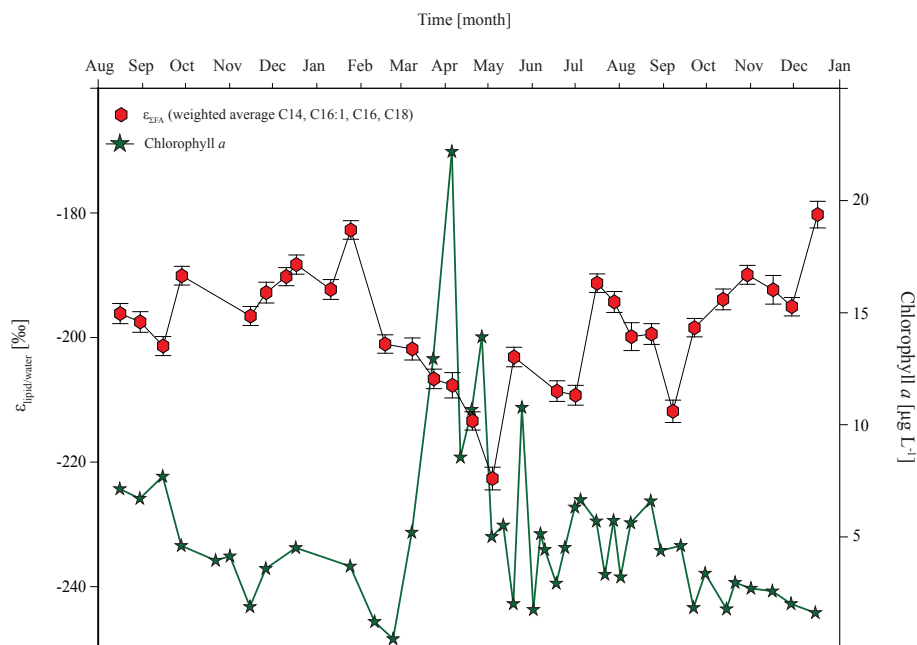


Figure 1. Values of ε_{FA} compared to chlorophyll *a* concentrations. ε_{FA} is the weighted average of *n*C14:0, *n*C16:1, *n*C16:0, *n*C18:0 fatty acids and the *n*C20:5 PUFA from jetty samples taken from August 2010 to December 2011.

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.

3.2 Bacterial diversity

To assess bacterial diversity, 16S rRNA gene amplicon sequencing was performed on approximately half of the SPM samples (Table S3a, b).

The bacteria detected consisted mainly of members of *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, α -*Proteobacteria*, β -*Proteobacteria*, γ -*Proteobacteria* and *Verrucomicrobia* (Fig. 3; Table S3a). The majority of the reads belonged to the orders of *Flavobacteriales*, *Rhodobacterales*, *Rickettsiales*, *Alteromonadales* and *Oceanospirillales*. The *Flavobacteriales* clade were one of the main contributors (12 to 32 %) to the total bacteria reads with a maximum from beginning of April until the end of May. The percentage of reads attributed to *Flavobacteriales* decreased during summer and early autumn. Sequence reads affiliated to *Rhodobacterales* (6 to 12 %) and *Rickettsiales* (3 to 17 %) were the most represented within the α -*Proteobacteria*. *Alteromonadales* reads made up between 9 and 17 % of all bacteria reads, and the percentage of *Oceanospirillales* reads was between 3 and 12 % of the total bacteria reads (Fig. 3; Table S3b).

For a more accurate taxonomic classification of the bacterial groups, sequence reads of *Bacteroidetes*, α -*Proteobacteria* and γ -*Proteobacteria* were extracted from

the dataset and a phylogenetic tree was constructed (Figs. S1–S3a, b). Within *Flavobacteriales* (*Bacteroidetes*) the majority of the reads fell either within *Cryomorphaceae* or *Flavobacteriaceae* with sequences clustering within *Fluviicola* and *Crocinitomix*, *Flavobacterium* and *Tenacibaculum*, respectively. Within *Rhodobacterales* (α -*Proteobacteria*) most of the reads belonged to *Rhodobacteraceae* and sequences within this family were closely related to the genus *Octadecabacter*. Within *Rickettsiales* most of the reads were affiliated to *Pelagibacteraceae* (SAR11 cluster). The majority of the γ -*Proteobacteria* reads were classified within *Alteromonadales* and *Oceanospirillales*. The *Alteromonadales* reads and sequences fell within the lineage of 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 sequences clustered with various members of *Oceanospirillaceae*.

3.3 Fatty acid distribution in North Sea SPM

Polar-lipid-derived fatty acids were comprised of *n*C14:0, *n*C16:1 ω 7, *n*C16:0, *n*C18:0, the polyunsaturated fatty acid (PUFA) *n*C20:5, and various unsaturated *n*C18 fatty acids (Fig. 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 (Fig. 4a). The *n*C16:0 fatty acid was the dominant fatty acid (21–38 %) with no clear seasonal pattern (Fig. 4c). The *n*C16:1 fatty acid was the next most abundant fatty acid (13–35 %) with a maximum from

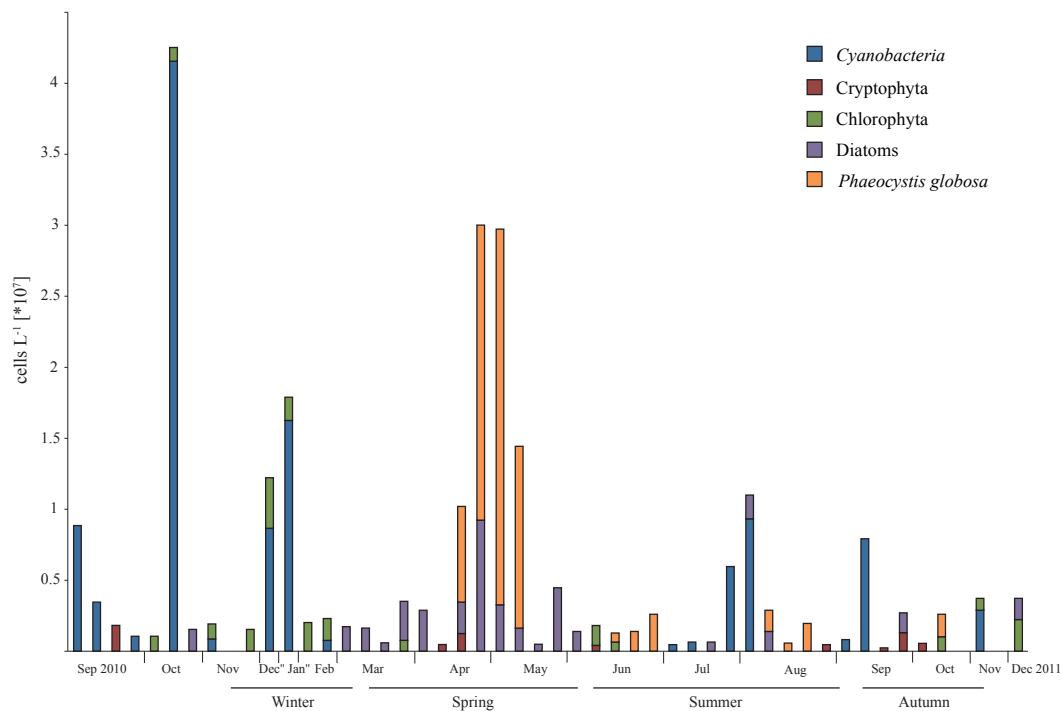


Figure 2. Phytoplankton diversity and abundance (measured in cells L⁻¹) observed in the coastal North Sea between August 2010 and December 2011.

March to April (Fig. 4b). Various unsaturated *n*C18: × 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 (Fig. 4d). The *n*C18:0 fatty acid had relative abundances varying between 2 and 18 % with the highest relative abundance during autumn months (10–18 %) and the lowest during spring, 2–6 % (Fig. 4e). A *n*C20:5 PUFA (Fig. 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.

3.4 Hydrogen isotopic composition of fatty acids

δD values of *n*C14:0, *n*C16:1 ω 7, *n*C16:0, *n*C18:0 and *n*C20:5 fatty acids were obtained for most of the samples (Table S5). The D / H ratio of the other fatty acids could not be determined with sufficient 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 and -212 ‰ (Table S5).

4 Discussion

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 ($\epsilon_{\text{lipid/water}}$). 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 ($R^2 = 0.68$; Fig. 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 δD_{water} values 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 ‰) and

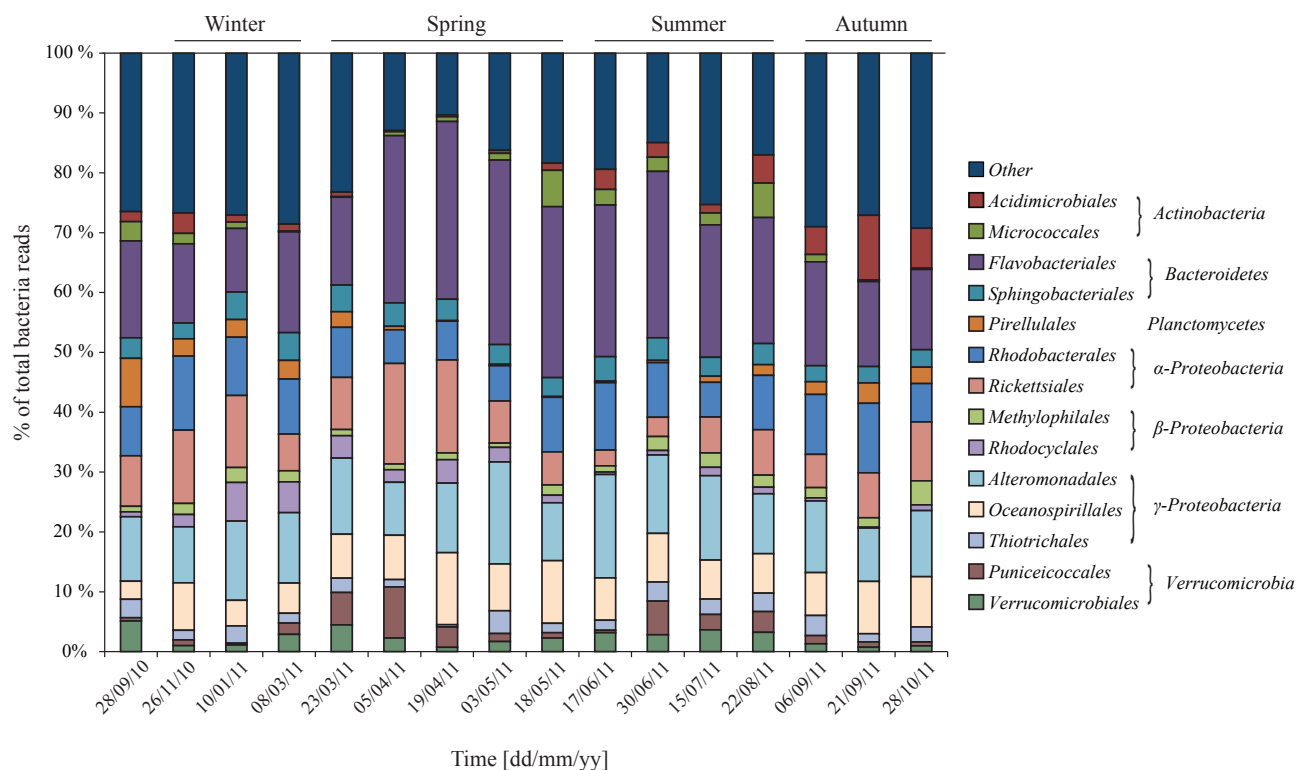


Figure 3. Order-level bacterial diversity and abundance in North Sea water based on the 16S rRNA gene sequence.

minor compared to the entire range in fatty acid δD (-174 to -241 ‰) and $\varepsilon_{\text{lipid/water}}$ (-173 to -237 ‰).

All fatty acids were depleted in D compared to water with the fractionation factor $\varepsilon_{\text{lipid/water}}$ 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 (Fig. 5; Table 1). The lowest degree of fractionation (most positive $\varepsilon_{\text{lipid/water}}$ values) was in general during late autumn and the winter months.

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). Here the *n*C20:5 PUFA is generally one of the most D-depleted fatty acids (Fig. 5), which is in agreement with culture studies that show that photoautotrophic microorganisms produce fatty acids that are depleted in D with $\varepsilon_{\text{lipid/water}}$ values between -160 and -220 ‰ (Heinzelmann et al., 2015b, and references therein), while heterotrophic microorganisms on the other hand produce fatty acids with $\varepsilon_{\text{lipid/water}}$ values ranging between -150 and $+200$ ‰ (Heinzelmann et al., 2015b, and references therein). Furthermore, its concentration increased at the time of the phytoplankton bloom (Fig. 4). Interestingly, after the phytoplankton bloom, when the abundance of pelagic algae

had decreased (Fig. 4), it became more enriched in D (Fig. 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 diatoms are the main source (Fig. 2; Table S2). A changing contribution from different species could potentially affect the hydrogen isotopic composition of a fatty acid even if the source organisms are all photoautotrophic phytoplankton. For instance, colony-forming algae such as *Eudorina unicocca* and *Volvox aureus* have been shown to fractionate much less against D than other algae (Zhang et al., 2007; Heinzelmann et al., 2015b). Indeed, *Phaeocystis*, although belonging to a different phylum, is also a colony-forming algae. However, if this were the reason for the changing D content of the *n*C20:5 PUFA following the spring bloom, it would then be expected to become more D-depleted with a reduced contribution from *Phaeocystis*, not D-enriched. A potential reason for the relatively D-enriched *Phaeocystis* lipids could be excretion of large amounts of D-depleted organic matter, leading directly or indirectly, through the isotopic composition of cell water, to D-enriched lipids (Sachs et al., 2016), as has been observed for colony-forming algae. Increased organic matter excretion by phytoplankton at the end of the bloom could therefore be another mechanism explaining the D enrichment of the *n*C20:5

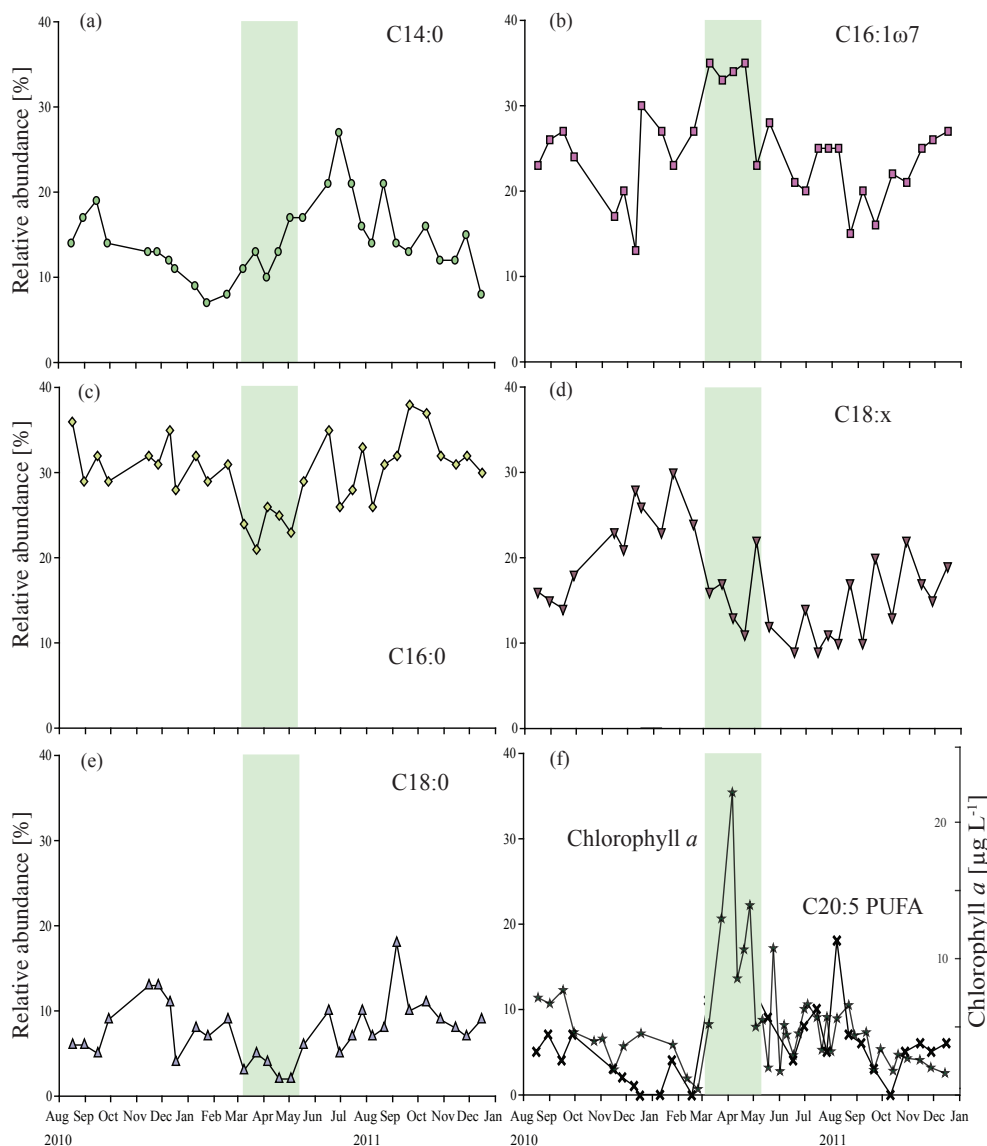


Figure 4. Relative abundance of fatty acids and chlorophyll *a* concentration in North Sea SPM. (a) *n*C14:0, (b) *n*C16:1, (c) *n*C16:0, (d) *n*C18:x, (e) *n*C18:0, (f) *n*C20:5 PUFA and chlorophyll *a*.

PUFA. Another possible reason could be that after the bloom and due to nutrient limitation, phytoplankton hypothetically might use more storage products potentially leading to an increased production of NADPH via other pathways than photosynthesis. The NADPH produced by photoautotrophs via photosystem *I* is depleted in D (X. N. Zhang et al., 2009), while NADPH produced via the pentose phosphate (OPP) pathway and the tricarboxylic acid (TCA) cycle is relatively enriched in D (Heinzelmann et al., 2015b; X. N. Zhang et al., 2009). The utilization of storage products would lead to an increased reduction of NADP^+ to NADPH via both the OPP pathway and the TCA cycle leading to more positive $\varepsilon_{\text{lipid/water}}$ values of the *n*C20:5 PUFA after the bloom. In batch culture with increasing nutrient limitation fatty acids

of algae became enriched in D with increasing age of the culture (Heinzelmann et al., 2015b) potentially due to a shift in the origin of NADPH or the excretion of organic matter or a combination of multiple factors.

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. However, it has been reported that fatty acids in general seem to become more enriched in D with chain length both in cultures and in environmental samples, which might play a minor role here as well (Jones et al., 2008; Campbell et al., 2009; Osburn et al., 2011). The quite similar $\varepsilon_{\text{lipid/water}}$ values of *n*C16:0 (−179 to −224‰) and *n*C16:1 (−173 to −215‰) suggest similar sources for the two fatty acids. The least negative

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. N.D. indicates not determined. Date format is DD/MM/YY

Date	Salinity	δD_{water} (‰) (estimated)	$\varepsilon_{\text{lipid/water}}$ (‰)					$\varepsilon_{\Sigma \text{FA}}$ (‰)
			C14:0	C16:0	C16:1*	C18:0	C20:5 PUFA	weighted average C14, C16:1, C16, C18
16/08/10	27.3	−8.2	−212 ± 2	−194 ± 3	−194 ± 3	−178 ± 4	−185 ± 2	−196 ± 2
30/08/10	29.7	−4.1	−218 ± 3	−198 ± 3	−186 ± 3	−182 ± 2	−195 ± 2	−197 ± 2
15/09/10	30	−3.6	−213 ± 3	−203 ± 2	−194 ± 2	−183 ± 2	−177 ± 3	−201 ± 2
28/09/10	24.7	−12.6	−209 ± 2	−188 ± 2	−182 ± 2	−187 ± 2	−197 ± 3	−190 ± 2
15/11/10	30	−3.6	−211 ± 3	−200 ± 2	−179 ± 2	−197 ± 2	N.D.	−196 ± 2
26/11/10	24.8	−12.4	−216 ± 3	−192 ± 3	−178 ± 3	−193 ± 3	N.D.	−193 ± 2
10/12/10	27.1	−8.5	−218 ± 2	−181 ± 2	−184 ± 2	−195 ± 2	N.D.	−190 ± 1
17/12/10	24.1	−13.6	−221 ± 3	−182 ± 2	−183 ± 2	−177 ± 3	N.D.	−188 ± 2
10/01/11	27.8	−7.3	−215 ± 4	−195 ± 3	−180 ± 2	−198 ± 2	N.D.	−192 ± 2
24/01/11	23.0	−15.5	−200 ± 3	−179 ± 2	−183 ± 2	−180 ± 2	−197 ± 3	−183 ± 2
17/02/11	29.3	−4.8	−219 ± 2	−204 ± 2	−191 ± 2	−203 ± 2	N.D.	−201 ± 1
08/03/11	25.8	−10.7	−218 ± 6	−206 ± 3	−197 ± 2	−173 ± 4	−227 ± 9	−202 ± 2
23/03/11	26.8	−9.0	−234 ± 2	−209 ± 3	−198 ± 2	−182 ± 5	−234 ± 2	−207 ± 2
05/04/11	29.2	−4.9	−219 ± 4	−206 ± 5	−205 ± 4	−208 ± 6	−220 ± 5	−208 ± 2
19/04/11	27.7	−7.5	−229 ± 2	−219 ± 2	−215 ± 2	N.D.	−235 ± 2	−213 ± 1
03/05/11	31.1	−1.7	−237 ± 6	−224 ± 2	−213 ± 3	−210 ± 3	−235 ± 3	−223 ± 2
18/05/11	31.8	−0.5	−219 ± 2	−205 ± 2	−197 ± 3	−177 ± 2	−213 ± 2	−203 ± 2
17/06/11	32.0	0.7	−225 ± 3	−211 ± 2	−196 ± 4	−191 ± 2	N.D.	−209 ± 2
30/06/11	31.2	−1.6	−224 ± 2	−208 ± 2	−200 ± 2	−173 ± 7	−212 ± 2	−209 ± 2
15/07/11	30.0	−3.6	−202 ± 2	−192 ± 2	−185 ± 2	−178 ± 3	−215 ± 3	−191 ± 2
27/07/11	26.3	−9.9	−213 ± 3	−192 ± 3	−195 ± 2	−172 ± 2	−193 ± 7	−194 ± 2
08/08/11	29.4	−4.6	−219 ± 7	−198 ± 4	−197 ± 5	−176 ± 7	−231 ± 4	−200 ± 2
22/08/11	26.9	−8.9	−224 ± 2	−195 ± 2	−182 ± 5	−183 ± 3	−195 ± 2	−199 ± 2
06/09/11	26.8	−9.0	−217 ± 6	−210 ± 2	−213 ± 4	−209 ± 3	−211 ± 3	−212 ± 2
21/09/11	30.1	−3.4	−215 ± 2	−201 ± 2	−182 ± 2	−191 ± 3	N.D.	−198 ± 1
11/10/11	32.8	1.2	−214 ± 4	−192 ± 2	−184 ± 3	−189 ± 4	−227 ± 2	−194 ± 2
28/10/11	32.2	0.1	−217 ± 2	−188 ± 2	−181 ± 3	−184 ± 2	−207 ± 4	−190 ± 2
15/11/11	28.9	−5.5	−208 ± 12	−194 ± 3	−187 ± 5	−179 ± 6	−217 ± 2	−192 ± 2
28/11/11	31.7	−0.7	−217 ± 2	−192 ± 2	−189 ± 2	−180 ± 3	−197 ± 2	−195 ± 1
16/12/11	31.7	−0.7	−198 ± 7	−179 ± 4	−173 ± 5	−187 ± 4	N.D.	−180 ± 2

*n*C16:1*: double bond at the ω 7 position.

$\varepsilon_{\text{lipid/water}}$ 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. Alternatively, as discussed above fatty acids become more enriched in D with increasing chain length both in cultures and environmental samples (Jones et al., 2008; Campbell et al., 2009; Osburn et al., 2011), which could be part of the reason why the *n*C18:0 is relatively enriched in D.

Fatty acid 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*C16:1 ω 7 fatty acids are most likely members of *Alteromonadales* and *Halomonadaceae*, while the majority of bacterial contributors to the *n*C14:0 and *n*C18:0 fatty acid are derived from *Puniceococcales* (Table S6). Both *Flavobacteriales* and

Rhodobacteraceae, 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*C16:1 ω 7 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*C16:1 ω 7 fatty acid 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 (Hamm and Rousseau, 2003; Nichols et al., 1991).

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 largely derived from heterotrophic bacteria (Table S6) resulting in more D-enriched signal compared to that of the *n*C14:0 fatty acid. However, the hydrogen isotopic composition of the *n*C18:0 fatty acid still falls within

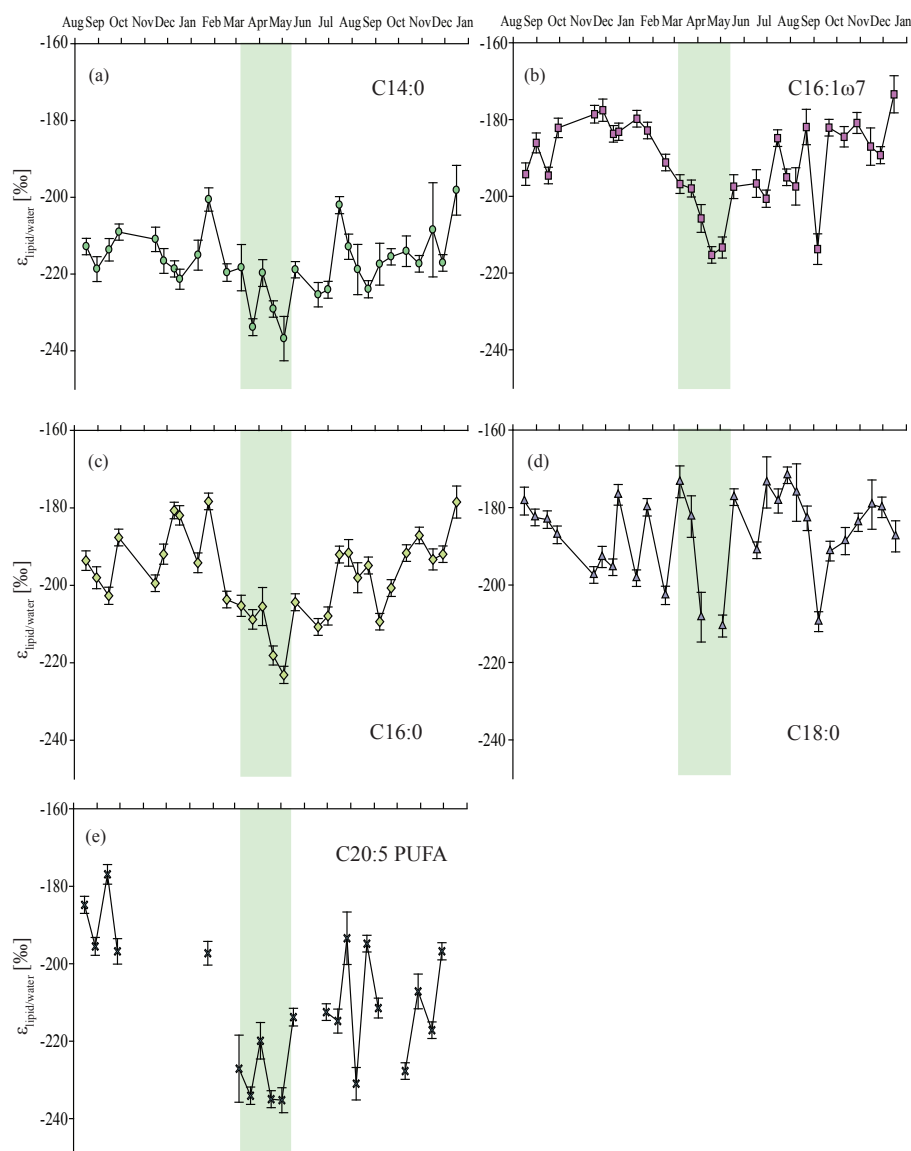


Figure 5. The D / H fractionation between fatty acids and North Sea water for fatty acids derived from suspended particulate matter in North Sea water samples. Plotted are the $\varepsilon_{\text{lipid/water}}$ values of *n*C14:0, *n*C16:1, *n*C16:0, *n*C18:0 fatty acids and *n*C20:5 PUFA.

the range for photoautotrophic organisms, albeit at the higher end, suggesting that although it is only produced in minor amounts by phytoplankton, a relatively high abundance of phytoplankton could still determine its isotopic composition.

None of the fatty acids measured in the North Sea SPM have $\varepsilon_{\text{lipid/water}}$ values which fall in the range of those predicted for chemoautotrophs (−264 to −345 ‰; Heinzelmann et al., 2015b, and references therein). This fits with the observation that sequence reads of chemoautotrophic bacteria accounted for < 3 % of the total bacterial reads (Fig. 3; Table S3a, b), and thus it is unlikely that this metabolism plays an important role in this environment.

4.3 Linking seasonal changes of hydrogen isotope fractionation to changes in community metabolism

In general most fatty acids showed a similar seasonal trend with the most negative ε values in spring and the most positive ε values in the winter (Fig. 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 $\varepsilon_{\text{lipid/water}}$ ($\varepsilon_{\Sigma\text{FA}}$) followed the same seasonal trend as the $\varepsilon_{\text{lipid/water}}$ values of the individual fatty acids (Figs. 1, 5) and ranged between −180 and −225 ‰ with an average of −199 ‰.

Compared to the chlorophyll *a* concentration, the $\varepsilon_{\Sigma\text{FA}}$ followed an opposite seasonal trend; that is, when the chlorophyll *a* concentration increased in early April, $\varepsilon_{\Sigma\text{FA}}$ decreased (Fig. 5). The chlorophyll *a* maximum in April–May indicates a spring bloom (Fig. 1), which is known to occur annually in North Sea coastal waters (Brandsma et al., 2012; Philippart et al., 2010) and corresponds with a shift towards more negative values for $\varepsilon_{\Sigma\text{FA}}$, as well as a high abundance of the algal-derived *n*C20:5 PUFA (Fig. 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 $\varepsilon_{\Sigma\text{FA}}$ was more negative during the spring bloom when the environment is dominated by photoautotrophic microorganisms (Fig. 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 $\varepsilon_{\Sigma\text{FA}}$ 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). This fits well with previous observations by Brandsma et al. (2012), who studied both the phytoplankton and bacterial communities during a period of 12 months. Cell counts contained in their study showed that the bacterial cell counts were lowest during the spring bloom, increased right after and stayed more or less stable throughout the rest of the sampling period. Phytoplankton cell counts on the other hand were highest during the bloom and dropped extremely afterwards with only the cyanobacteria cell counts increasing slightly in late summer/early autumn. At the same time they showed that in general the bacteria cell count was 10 times higher than the phytoplankton cell count. This suggests that the whole environment after the bloom might be dominated by heterotrophic bacteria. The relative stability of the system throughout the last decades (Philippart et al., 2000) suggests that the situation might have been similar during the sampling period described here.

Thus, $\varepsilon_{\Sigma\text{FA}}$ values reflect a mixed signal derived from mainly photoautotrophic and, to a lesser extent, heterotrophic microorganisms. Nevertheless, $\varepsilon_{\text{lipid/water}}$ values for all fatty acids remain in the range of photoautotrophic metabolism (Heinzelmann et al., 2015b, and references therein), 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., 2012). 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 Conclusions

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 subsequently higher relative contribution of heterotrophs to the general fatty acid pools leads to shift in $\varepsilon_{\text{lipid/water}}$ 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, although we cannot exclude the possibility of nutrient limitation affecting phytoplankton growth, leading to a shift in NADPH origin or excretion of organic matter, for instance, which could also affect the hydrogen isotope signal. 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. The combination of hydrogen isotope analysis of general fatty acids with those of more specific biomarker lipids might help further improve the assessment of core metabolisms in present and past environments.

6 Data availability

Data are available on Pangaea under doi:10.1594/PANGAEA.859031.

The Supplement related to this article is available online at doi:10.5194/bg-13-5527-2016-supplement.

Author contributions. N. J. Bale helped by providing samples and helped with sampling; L. Villanueva helped with carrying out sequencing experiments and analysis of subsequent data; D. Sinke-Schoen helped with measuring the hydrogen isotopic composition of North Sea water samples; C. J. M. Philippart provided chlorophyll *a* and phytoplankton data; J. S. Sinninghe Damsté, S. Schouten and M. T. J. van der Meer helped design experiments and contributed to the manuscript as supervisors of S. M. Heinzelmann; S. M. Heinzelmann prepared the manuscript with contributions of all co-authors.

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