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

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Abstract

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

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; Heinzelmann et al., 2015b; Zhang et al., 2009a; Zhang et al., 2009b). While most of these factors lead to relatively small variations in the deuterium to hydrogen (D/H) ratio of fatty acids (10-20 ‰), differences in the central metabolism of microorganisms have a much more pronounced effect. Both photo- and chemoautotrophs produce fatty acids depleted in D compared to growth

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47 water with the stable hydrogen isotopic fractionation factor ε between fatty acids and water
48 ($\varepsilon_{lipid/water}$) ranging between -150 ‰ to -250 ‰ and -250 ‰ and -400 ‰, respectively (Campbell
49 et al., 2009; Chikaraishi et al., 2004; Heinzelmann et al., 2015a; Heinzelmann et al., 2015b;
50 Sessions et al., 2002; Valentine et al., 2004; Zhang et al., 2009a; Zhang and Sachs, 2007). In
51 contrast, heterotrophs produce fatty acids with either a relatively minor depletion or an
52 enrichment in D compared to the growth water with $\varepsilon_{lipid/water}$ values ranging between -150 ‰
53 and +200 ‰ (Dirghangi and Pagani, 2013; Fang et al., 2014; Heinzelmann et al., 2015a;
54 Heinzelmann et al., 2015b; Sessions et al., 2002; Zhang et al., 2009a). The differences in
55 hydrogen isotopic composition of fatty acids produced by organisms expressing different core
56 metabolisms have mainly been attributed to the D/H ratio of nicotinamide adenine dinucleotide
57 phosphate (NADPH) (Zhang et al., 2009a). NADPH can be generated by a variety of different
58 reactions in different metabolic pathways (each associated with different hydrogen isotopic
59 fractionations) and is subsequently used as the main source of hydrogen in lipid biosynthesis
60 (Robins et al., 2003; Saito et al., 1980; Schmidt et al., 2003).

61 Although the metabolism of a microorganism in pure culture is reflected by the D/H ratio of its
62 fatty acids, it is not clear if the D/H ratio of fatty acids from environmental microbial
63 communities can be used to assess the 'integrated' core metabolisms in nature. Culture
64 conditions rarely represent environmental conditions since cultures are typically axenic and use
65 a single substrate, they do not take into account microbial interactions, and they test a limited
66 number of potential substrates, energy sources and core metabolisms. Previous studies observed
67 a wide range in the D/H ratio of lipids in both water column and sediment (Jones et al., 2008;
68 Li et al., 2009), suggesting inputs of organisms with a variety of metabolisms. So far, one
69 environmental study has been performed that links the D/H ratio of fatty acids from naturally
70 occurring microbial communities to metabolisms possibly expressed by the members of those
71 communities (Osburn et al., 2011). This study showed that different microbial communities

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

82 Here, we studied the seasonal variability of the hydrogen isotopic composition of fatty acids
83 from coastal North Sea water sampled from the jetty at the Royal Netherlands Institute for Sea
84 Research (NIOZ) in order to examine the relationship between hydrogen isotope fractionation
85 in fatty acids and the general metabolism of the community. Time series studies have been
86 previously performed at the NIOZ jetty to determine phytoplankton and prokaryotic abundances
87 and composition (Alderkamp et al., 2006; Brandsma et al., 2012; Brussaard et al., 1996;
88 Philippart et al., 2010; Philippart et al., 2000; Pitcher et al., 2011; Sintes et al., 2013), lipid
89 composition (Brandsma et al., 2012; Pitcher et al., 2011), and chlorophyll *a* concentration
90 (Philippart et al., 2010). Typically, the spring bloom in the coastal North Sea is predominantly
91 formed by *Phaeocystis globosa*, followed directly by a bloom of various diatom species, a
92 second moderate diatom bloom of *Thalassiosira spp.* and *Chaetoceros socialis* that occurs in
93 early summer and an autumn bloom is formed by *Thalassiosira spp.*, *C. socialis*, cryptophytes
94 and cyanobacteria (Brandsma et al., 2012; Cadée and Hegeman, 2002), although the autumn
95 bloom seems to have weakened over the last years (Philippart et al., 2010). The abundance of
96 bacteria co-varies with algal blooms and the bacteria are dominated by heterotrophs, e.g.

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97 bacteria belonging to the *Bacteroidetes* (Alderkamp et al., 2006), using released organic matter
98 from declining phytoplankton blooms as carbon, nitrogen and phosphate sources. The intact
99 polar lipid (IPL) composition of the microbial community was shown to be composed mainly
100 of phospholipids, sulfoquinovosyldiacylglycerol and betaine lipids with a limited taxonomic
101 potential (Brandsma et al., 2012). The main source of those lipids was assumed to be the
102 eukaryotic plankton.

103 This well studied site should allow us to trace the shift from an environment dominated by
104 photoautotrophs during major phytoplankton blooms, towards an environment with a higher
105 abundance of heterotrophic bacteria following the end of the bloom. These shifts in the
106 community structure should be reflected in the D/H ratio of fatty acids. We, therefore, analysed
107 the D/H ratio of polar lipid derived fatty acids (PLFA) over a seasonal cycle and compared this
108 with phytoplankton composition data and abundance and information on the bacterial diversity
109 obtained by 16S rRNA gene amplicon sequencing.

110 **2. Material and Methods**

111 **2.1. Study site and sampling**

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

116 For lipid analysis measured volumes of water (ca. 9-11 L) were filtered consecutively, without
117 pre filtration, through pre-ashed 3 and 0.7 µm pore size glass fibre filters (GF/F, Whatman; 142
118 mm diameter) and stored at -20 ºC until lipid extraction. For DNA analysis approximately 1 L

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seawater was filtered through a polycarbonate filter (0.2 µm pore size; 142 mm diameter; Millipore filters) and stored at -80 ºC until extraction.

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

For chlorophyll *a* measurements 500 mL sea water was filtered through a 47 mm GF/F filter (0.7 µm pore size, Whatman, GE Healthcare Life Sciences, Little Chalfont, UK) and immediately frozen in liquid nitrogen. Samples were thawed and homogenised with glass beads and extracted with methanol. Chlorophyll *a* concentration was measured with a Dionex high-performance liquid chromatography (HPLC) (Philippart et al., 2010).

Water samples for salinity versus δD_{water} calibration (see below) were sampled weekly between March and September 2013 at high tide. Salinity was determined using a conductivity meter (VWR EC300) calibrated to IAPSO standard seawater of salinities 10, 30, 35 and 37.

2.2. Polar lipid derived fatty acids

Filters were extracted for IPLs and eventually fatty acid analysis. The 0.7 µm filters did not yield enough total lipid extract for analysis. Therefore, only fatty acids obtained from the 3 µm filters were analysed. Due to fast clogging of the filters and a corresponding decrease of the pore size (Sørensen et al., 2013), the 3 µm filters will most likely contain most of the microorganisms present in North Sea water. Freeze dried filters were extracted via a modified Bligh-Dyer method (Bligh and Dyer, 1959; Rütters et al., 2002) with methanol (MeOH)/dichloromethane (DCM)/phosphate buffer (2:1:0.8, vol/vol/vol) using ultrasonication (Heinzelmann et al., 2014). Approximately 0.5 - 1 mg of the Bligh-Dyer extract (BDE) was separated into a neutral and polar lipid fraction using silica column chromatography

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(Heinzelmann et al., 2014). The BDE was added onto a DCM pre-rinsed silica column (0.5 g; activated for 3 h at 150°C) and eluted with 7 mL of DCM and 15 mL of MeOH. The resulting fractions were dried under nitrogen and stored at -20 °C. PLFAs were obtained via saponification of the MeOH fraction with 1 N KOH in MeOH (96%). The samples were refluxed at 140 °C for 1 h. Afterwards the pH was adjusted to 5 with 2 N HCl/MeOH (1/1), bidistilled H_2O and DCM were added. The MeOH/H_2O layer was washed twice with DCM, the DCM layers were combined and dried over Na_2SO_4. The sample was dried under nitrogen and stored in the fridge. The PLFAs were methylated with boron trifluoride-methanol (BF_3-MeOH) for 5 min at 60 °C. Afterwards H_2O and DCM were added. The H_2O/MeOH layer was washed three times with DCM, and potential traces of water were removed over a small Na_2SO_4 column after which the DCM was evaporated under a stream of nitrogen. In order to obtain a clean PLFA fraction for isotope analysis, the methylated extract was separated over an aluminium oxide (Al_2O_3) column, eluting the methylated PLFAs with three column volumes of DCM. For identification of the position of double bonds in unsaturated fatty acids, the methylated PLFAs were derivatised with dimethyldisulfide (DMDS) (Nichols et al., 1986). Hexane, DMDS and I_2/ether (60 mg/mL) were added to the fatty acids and incubated at 40 °C overnight. After adding hexane, the iodine was deactivated by addition of a 5% aqueous solution of $Na_2S_2O_3$. The aqueous phase was washed twice with hexane. The combined hexane layers were cleaned over Na_2SO_4 and dried under a stream of nitrogen. The dried extracts were stored at 4 °C.

2.3. Fatty acid and hydrogen isotope analysis

The fatty acid fractions were analysed by gas chromatography (GC) using an Agilent 6890 gas chromatograph with a flame ionization detector (FID) using a fused silica capillary column (25 m x 320 µm) coated with CP Sil-5 (film thickness 0.12 µm) with helium as carrier gas. The temperature program was as follows: initial temperature 70 °C, increase of temperature to 130 °C with 20 °C min^{-1}, and then to 320 °C with 4 °C min^{-1} which was kept for 10 min. Individual

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compounds were identified using GC/mass spectrometry (GC/MS) and the position of the double bonds in unsaturated fatty acids was determined after derivatisation with dimethyldisulfide (Heinzelmann et al., 2015b).

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

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

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 algae were identified to species level, but some were clustered into taxonomic and size groups (Philippart et al., 2000). For each

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192 sampling date in the period from September 2010 to December 2011, the densities of the most
193 abundant phytoplankton species or species' groups were calculated. The three most dominant
194 algal species (or groups) together comprised, on average, more than 60% of the total numbers
195 of marine algae in the Marsdiep during this study period.

196 **2.5. DNA extraction**

197 The 0.2 µm polycarbonate filters were defrosted and cut into small pieces with sterile scissors
198 and then transferred into a 50 mL falcon tube. Filter pieces were lysed by bead-beating with ~1
199 g of sterile 0.1 mm zirconium beads (Biospec, Bartlesville, OK) in 10 mL RLT buffer (Qiagen)
200 and 100 µL β-mercaptoethanol for 10 min. 1/60 volume RNase A (5 µg/µL) was added to the
201 lysate, incubated for 30 min at 37 ºC and afterwards cooled down for 5 min on ice. The lysate
202 was purified with the DNeasy Blood and Tissue kit (Qiagen, Hilden). DNA was eluted with 3x
203 100 µL AE buffer, the eluates pooled and reconcentrated. DNA quality and concentration was
204 estimated by Nanodrop (Thermo Scientific, Waltham, MA) quantification.

205 **2.6. 16S rRNA gene amplicon sequencing and analysis**

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

209 PCR reactions were performed with the universal (Bacteria and Archaea) primers S-D-Arch
210 0519-a-S-15 (5'-CAG CMG CCG CGG TAA-3') and S-D-Bact-785-a-A-21 (5'-GAC TAC
211 HVG GGT ATC TAA TCC-3') (Klindworth et al., 2012) adapted for pyrosequencing by the
212 addition of sequencing adapters and multiplex identifier (MID) sequences. To minimize bias
213 three independent PCR reactions were performed containing: 16.3 µL H_2O, 6 µL HF Phusion
214 buffer, 2.4 µL dNTP (25 mM), 1.5 µL forward and reverse primer (10 µM; each containing an

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unique MID tail), 0.5 µL Phusion Taq and 2 µL DNA (6 ng/µL). The PCR conditions were following: 98 ºC, 30 s; 25× [98 ºC, 10 s; 53 ºC, 20 s; 72 ºC, 30 s]; 72 ºC, 7 min and 4 ºC, 5 min.

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

Samples were analyzed using the QIIME pipeline (Caporaso et al., 2010). Raw sequences were demultiplexed and then quality-filtered with a minimum quality score of 25, length between 250−350 bp, and allowing maximum two errors in the barcode sequence. Sequences were then clustered into operational taxonomic units (OTUs, 97% similarity) with UCLUST (Edgar, 2010). Reads were aligned to the Greengenes Core reference alignment (DeSantis et al., 2006) using the PyNAST_algorithm (Caporaso et al., 2010). Taxonomy was assigned based on the Greengenes taxonomy and a Greengenes reference database (version 12_10) (McDonald et al., 2012; Werner et al., 2012). Representative OTU sequences assigned to the specific taxonomic groups were extracted through classify.seqs and get.lineage in Mothur (Schloss et al., 2009) by using the Greengenes reference and taxonomy files. The 16S rRNA gene amplicon reads (raw data) have been 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

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239 software package (Ludwig et al., 2004). Sequences were added to the reference tree supplied
240 by the Silva database using the ARB Parsimony tool.

241 **3. Results**

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

245 **3.1. Chlorophyll *a* concentration and phytoplankton abundance and diversity**

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

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

258 **3.2. Microbial diversity**

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

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261 The bacteria detected consisted mainly of members of *Actinobacteria*, *Bacteriodetes*,
262 *Planctomycetes*, *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria* and *Verrucomicrobia*
263 (Fig. 3; Table S3). The majority of the reads belonged to the orders of the *Flavobacteriales*,
264 *Rhodobacteriales*, *Rickettsiales*, *Alteromonadales* and *Oceanospirillales*. The *Flavobacteriales*
265 contributed between 12 to 32 % to the total bacterial reads with a relatively constant percentage
266 of ~ 15 % during autumn and winter. The percentage of reads increased during early spring
267 with the highest values from beginning of April until the end of May. The percentage of reads
268 attributed to the *Flavobacteriales* decreased during summer and early autumn. Sequence reads
269 affiliated to the *Rhodobacteriales* (6 to 12 %) and *Rickettsiales* (3 to 17 %) were the most
270 represented within the *α-Proteobacteria*. The percentage of *Rhodobacteriales* reads was fairly
271 constant with no obvious seasonal pattern. In contrast, the percentage of *Rickettsiales* reads
272 followed a distinct seasonal pattern with a maximum in April (up to 17 %) and a minimum in
273 June (3 %). *Alteromonadales* reads made up between 9 and 17 % of all bacteria reads and were
274 fairly constant over the season. The percentage of *Oceanospirillales* reads were between 3 and
275 12 % of the total bacteria reads and show a clear maximum during mid-April (Fig. 3; Table S3).

276 For a more accurate taxonomic classification of the bacterial groups, sequence reads of the
277 *Bacteriodetes*, *α-Proteobacteria* and *γ-Proteobacteria* were extracted from the dataset and a
278 phylogenetic tree was constructed (Fig. S1-S3). Within the *Flavobacteriales* (*Bacteroidetes*)
279 the majority of the reads fell either within the *Cryomorphaceae* or the *Flavobacteriaceae* with
280 sequences clustering within *Fluviicola* and *Crocinitomix*, *Flavobacterium* and *Tenacibaculum*,
281 respectively. Within the *Rhodobacterales* (*α-Proteobacteria*) most of the reads belonged to
282 *Rhodobacteraceae* and sequences within this family were closely related to the genus
283 *Octadecabacter*. Within the *Rickettsiales* most of the reads were affiliated to the
284 *Pelagibacteraceae* (SAR11 cluster). The majority of the *γ-Proteobacteria* reads were classified
285 within the *Alteromonadales* and *Oceanospirillales*. The *Alteromonadales* reads and sequences

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fell within the uncultured *HTCC2188*-isolate and *OM60*-clade and various members of the *Alteromonadaceae*-family. The *Halomonadaceae* family comprised most of the *Oceanospirillales* reads and additionally sequences clustered with various members of the *Oceanospirillaceae*.

3.3. Fatty acid distribution in North Sea SPM

Polar lipid derived fatty acids were comprised of *n*C14:0, *n*C16:1ω7, *n*C16:0, *n*C18:0, the polyunsaturated fatty acid (PUFA) *n*C20:5, and various unsaturated *n*C18 fatty acids (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 March to April (Fig. 4b). Various unsaturated *n*C18:x fatty acids were observed throughout the season. Due to low abundance of the individual fatty acids and co-elutions the double bond positions could not be determined. These unsaturated fatty acids made up 9–30 % of all fatty acids (Fig. 4d). The *n*C18:0 fatty acid had relative abundances varying between 2–18 % with the highest relative abundance during autumn months (10–18 %) and the lowest during spring, 2–6 % (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*C16:1ω7, *n*C16:0, *n*C18:0 fatty acids and *n*C20:5 were obtained for most of the samples (Table S5). The D/H ratio of the other fatty acids could not be determined with sufficient accuracy due to either incomplete separation or low abundance.

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

312 **4. Discussion**

313 **4.1. Hydrogen isotopic fractionation expressed in fatty acids**

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

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

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degree of fractionation (most positive $\varepsilon_{lipid/water}$ 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). The *n*C20:5 PUFA is 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 $\varepsilon_{lipid/water}$ values between -162 and -215 ‰, while heterotrophic microorganisms on the other hand produce fatty acids with $\varepsilon_{lipid/water}$ values ranging between -150 to +200 ‰. 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). Another possible reason could be that after the bloom and due to nutrient limitation, phytoplankton might use more storage products leading to an increased production of NADPH via other pathways than photosynthesis. The NADPH produced by photoautotrophs via photosystem I is depleted in D (Zhang et al., 2009a), while NADPH produced via the pentose phosphate (OPP) pathway and the tricarboxylic acid (TCA) cycle is relatively enriched in D (Heinzelmann et al., 2015b; Zhang et al., 2009a). The utilization of storage products would lead to an increased production of NADPH via both the OPP pathway and the TCA cycle leading to more positive $\varepsilon_{lipid/water}$ values of the *n*C20:5 PUFA after the bloom.

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

360 Fatty acids profiles of representatives of most members of the phytoplankton and bacterial
361 community observed at our site have been previously reported (Table S6) and can be used to
362 assess the main sources of the different fatty acid pools. The main bacterial contributors to the
363 *n*C16:0 and *n*C16:1ω7 fatty acids are most likely members of the *Alteromonadales* and the
364 *Halomonadaceae*, while the majority of bacterial contributors to the *n*C14:0 and *n*C18:0 fatty
365 acid are derived from the *Puniceicoccales* (Table S6). Both the *Flavobacteriales* and the
366 *Rhodobacteriaceae*, which make up a large part of the total bacteria reads, will hardly contribute
367 to the measured isotopic signal as they have been reported to produce only traces of *n*C14:0,
368 *n*C16:0, *n*C16:1ω7 or *n*C18:0 fatty acids (Table S6). The observed phytoplankton species are
369 main contributors to the *n*C14:0, *n*C16:0 and *n*C16:1ω7 fatty acid pools, but contribute
370 relatively little to the *n*C18:0 fatty acid pools. *Phaeocystis* produces mainly the *n*C14:0 and
371 *n*C16:0 fatty acids (Hamm and Rousseau, 2003; Nichols et al., 1991).

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

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

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

Compared to the chlorophyll *a* concentration, the $\varepsilon_{\Sigma FA}$ followed an opposite seasonal trend i.e. when the chlorophyll *a* concentration increased in early April, $\varepsilon_{\Sigma FA}$ decreased (Fig. 5). The chlorophyll *a* maximum in April-May indicates a spring bloom (Fig. 2), 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 $\varepsilon_{\Sigma FA}$, 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 $\varepsilon_{\Sigma FA}$ was more negative during the spring bloom when the environment is

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401 dominated by photoautotrophic microorganisms (Fig. 3) fits with an increased contribution by
402 photoautotrophs relative to heterotrophic microorganisms to the fatty acid pool. At the end of
403 the bloom more positive $\varepsilon_{\Sigma FA}$ values were observed, which is in agreement with an increased
404 abundance of heterotrophic bacterioplankton in previous studies (Sintes et al., 2013), living on
405 released organic material (Alderkamp et al., 2006). Interestingly, analysis of suspended
406 particulate matter from the California borderland basins also showed that typical bacterial fatty
407 acids were generally enriched in D while algal fatty acids were more depleted in D (Jones et al.,
408 2008), similar to what we observed here.

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

417 **5. Conclusion**

418 A seasonal study of fatty acids derived from the coastal Dutch North Sea shows that all fatty
419 acids are depleted in D with δD ranging between -174 and -241 ‰. The most negative values
420 were observed during the spring bloom, when the biomass is dominated by photoautotrophic
421 microorganisms. The subsequent higher relative contribution of heterotrophs to the general fatty
422 acid pools leads to shift in $\varepsilon_{lipid/water}$ towards more positive values by up to 20 ‰. This shift
423 towards more positive values is in agreement with observations from culture studies where
424 heterotrophic organisms fractionate much less or even opposite to photoautotrophic organisms.

425 This study confirms that hydrogen isotopic fractionation as observed in general fatty acids can
426 be used to study the core metabolism of complex environments and to track seasonal changes
427 therein.

428 **Data availability**

429 Data is available on Pangea under doi:10.1594/PANGAEA.859031

430 **Author Contribution**

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

438

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597

598 **Figure and tables legends**

599 **Figure 1**

600 $\varepsilon_{average}$ values compared to chlorophyll *a* concentrations. $\varepsilon_{fattyacids}$ is the weighted average of
601 *n*C14:0, *n*C16:1, *n*C16:0, *n*C18:0 fatty acids and the *n*C20:5 PUFA from Jetty samples taken
602 from August 2010 – December 2011.

603 **Figure 2**

604 Phytoplankton diversity and abundance (measured in cells L^{-1}) observed in the coastal North
605 Sea between August 2010 – December 2011.

606 **Figure 3**

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

609 **Figure 4**

610 Relative abundance of fatty acids and chlorophyll *a* concentration in North Sea SPM. (a)
611 *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*.

612 **Figure 5**

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

617 **Table 1**

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

620

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Table 1

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

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

Biogeosciences Discuss., doi:10.5194/bg-2016-125, 2016
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Published: 20 April 2016

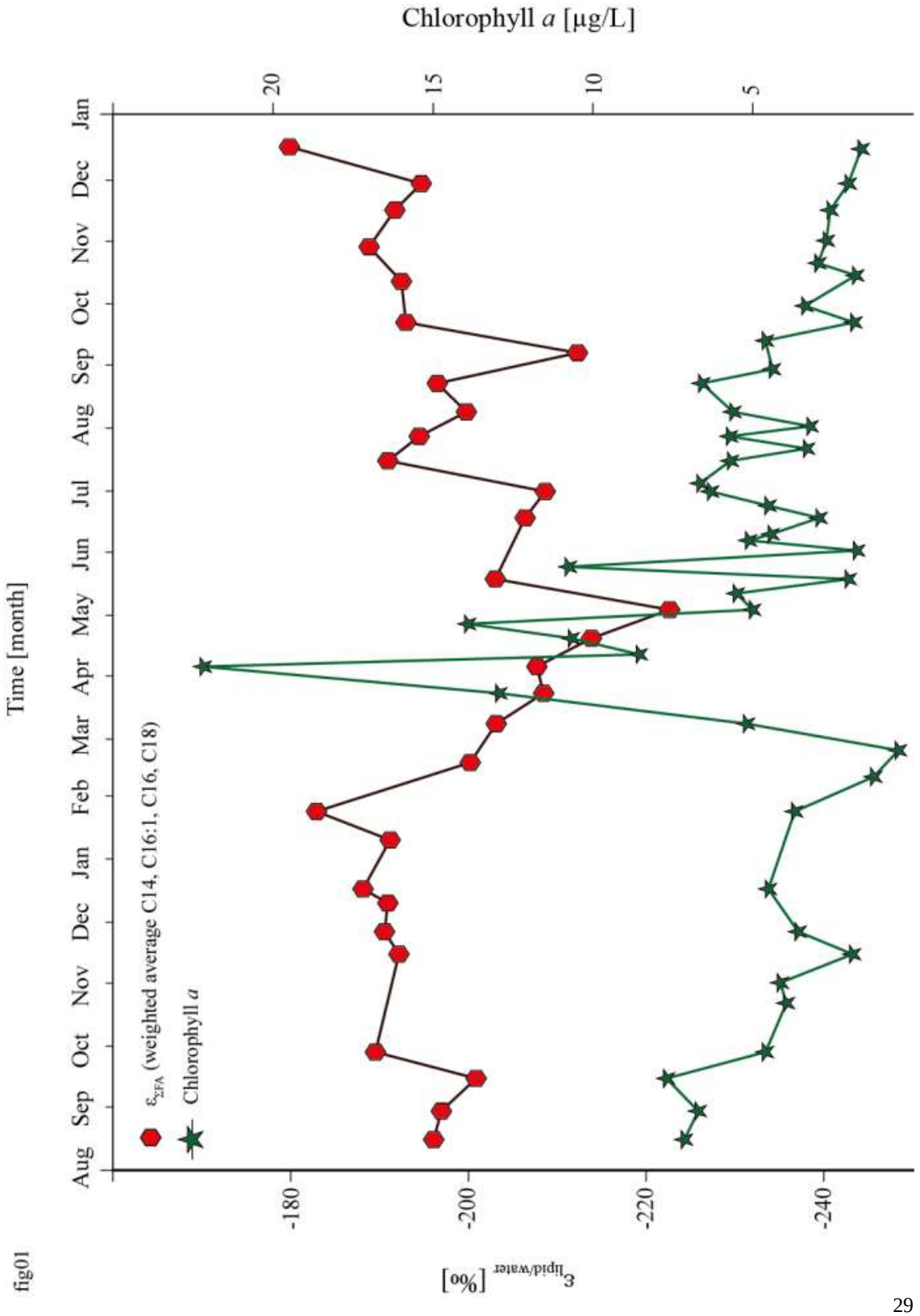

fig01

Biogeosciences Discuss., doi:10.5194/bg-2016-125, 2016
Manuscript under review for journal Biogeosciences
Published: 20 April 2016

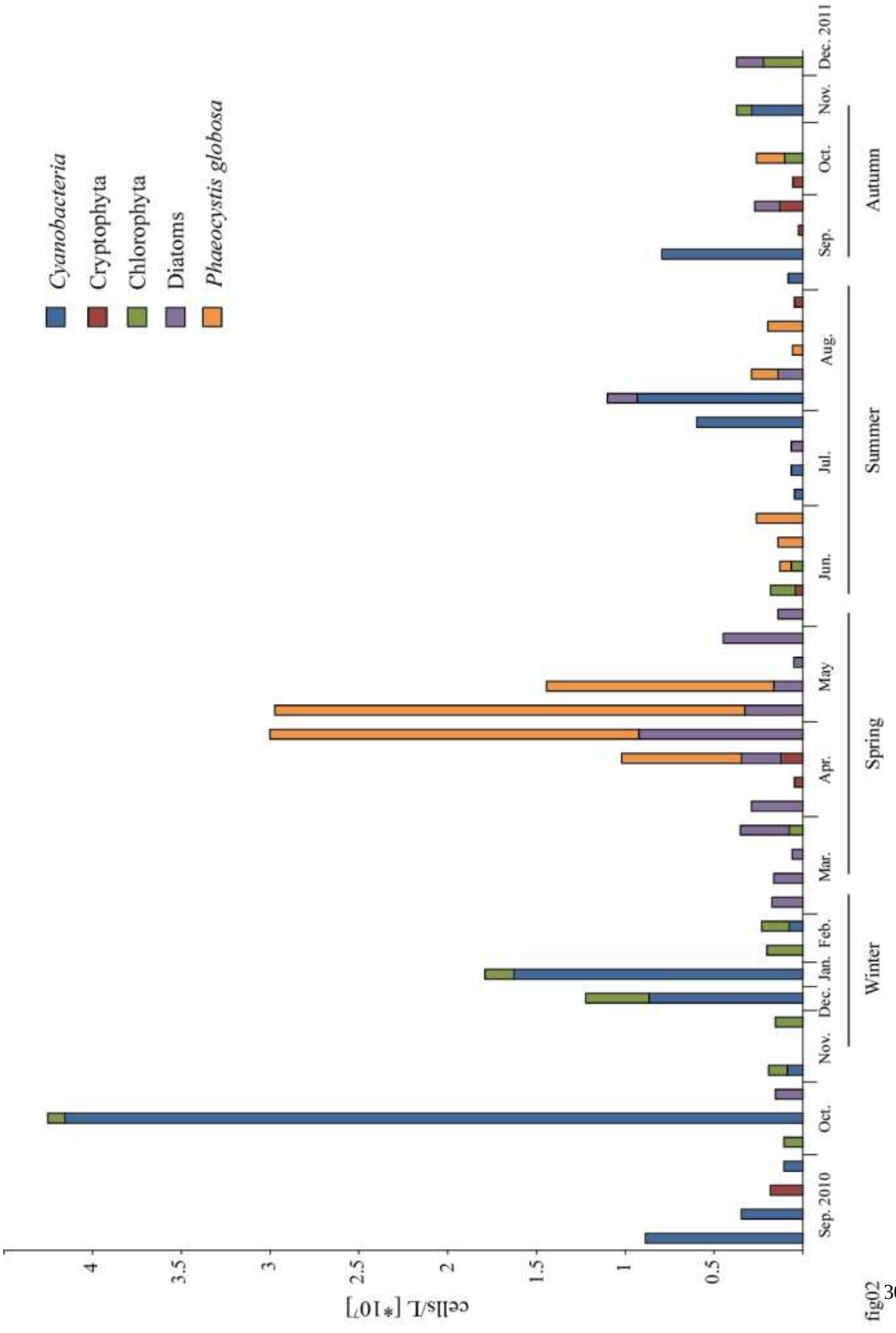

fig02

Biogeosciences Discuss., doi:10.5194/bg-2016-125, 2016
Manuscript under review for journal Biogeosciences
Published: 20 April 2016

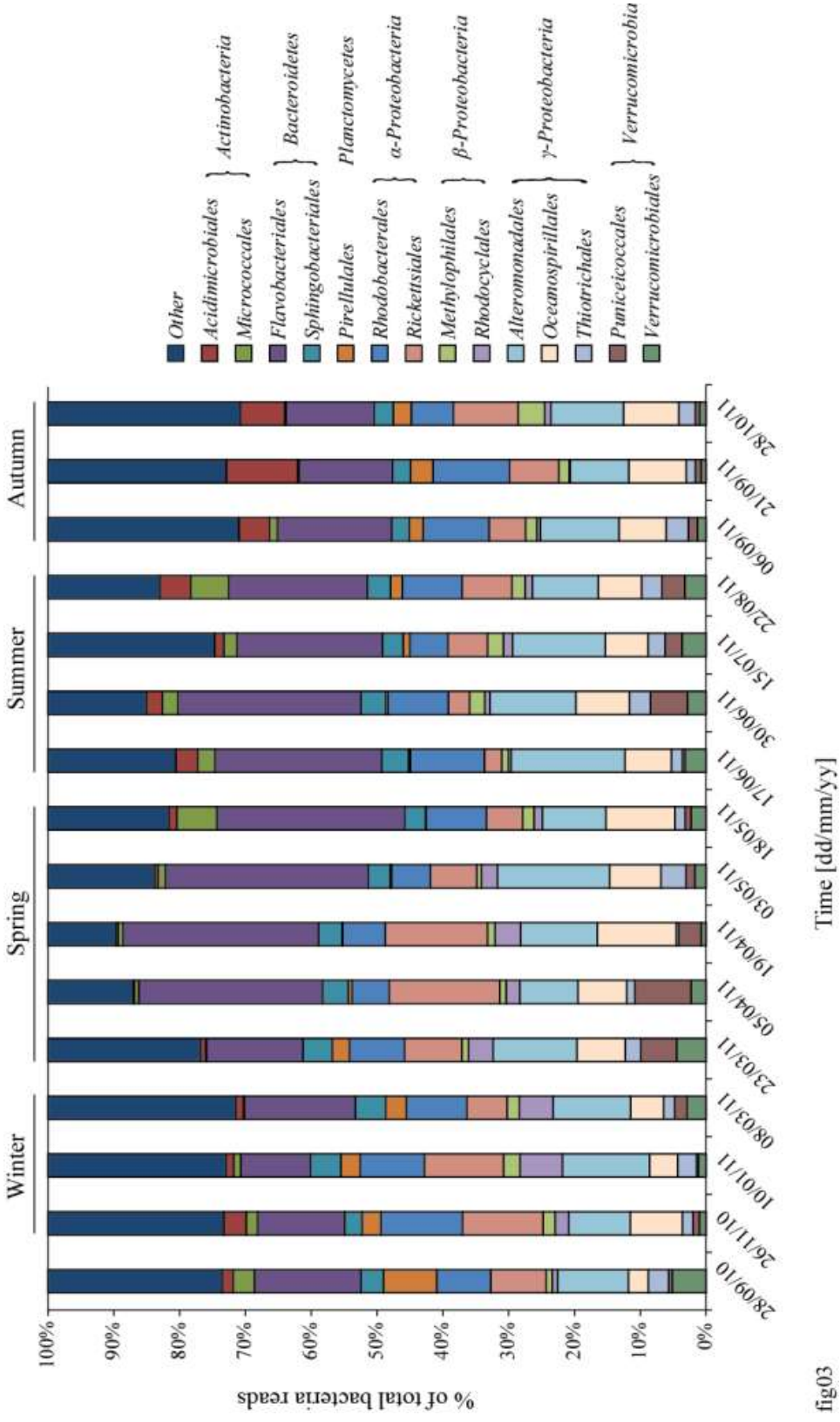

fig03

Biogeosciences Discuss., doi:10.5194/bg-2016-125, 2016
Manuscript under review for journal Biogeosciences
Published: 20 April 2016

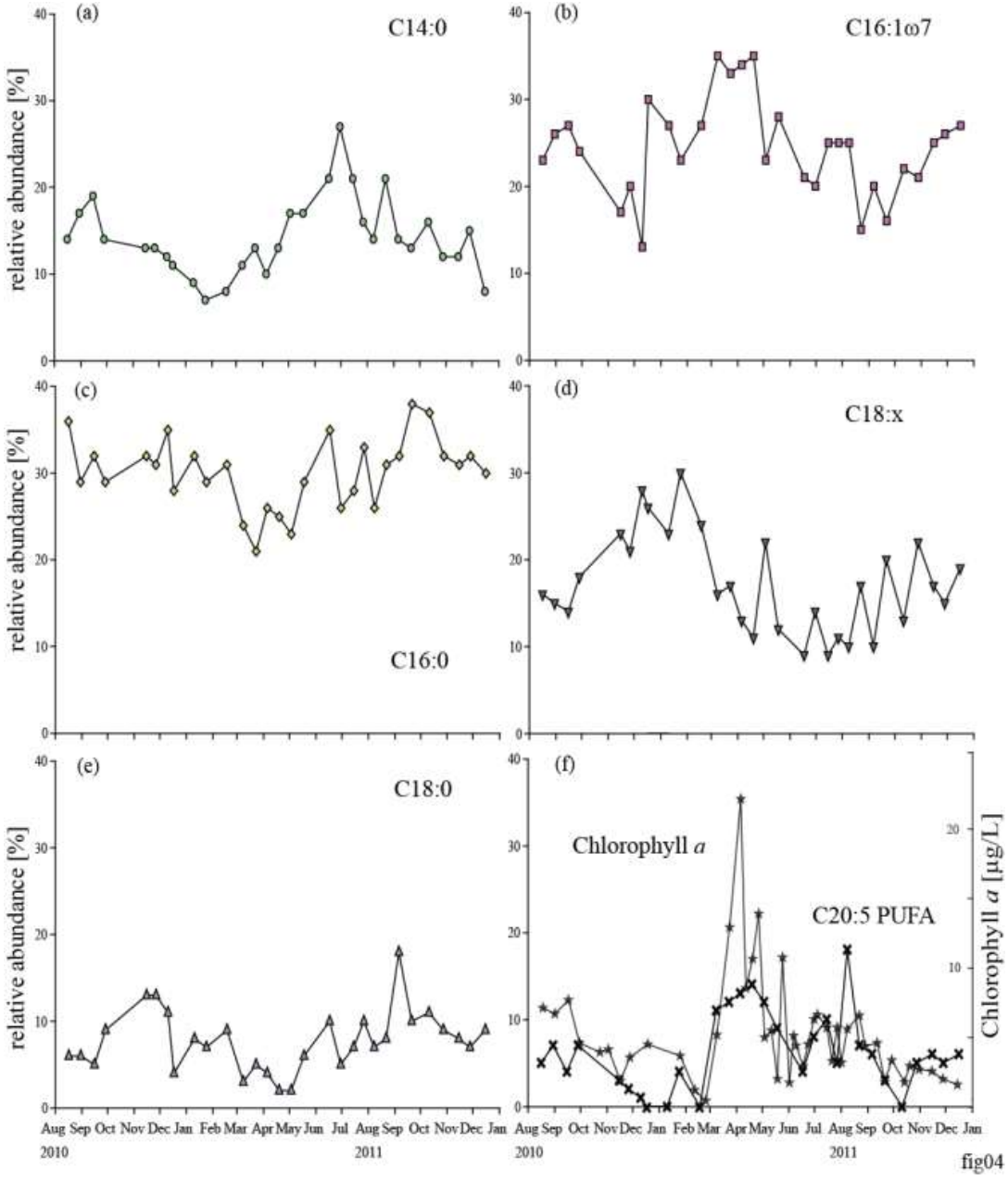

fig04

Biogeosciences Discuss., doi:10.5194/bg-2016-125, 2016
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