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# *Interactive comment on* "Seasonal changes in the D/H ratio of fatty acids of pelagic microorganisms in the coastal North Sea" *by* Sandra Mariam Heinzelmann et al.

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Reviewer 1 This manuscript provides an interesting environmental test to the hypothesis that changes in the net metabolism of microbial ecosystems are reflected in the hydrogen isotope composition of constituent lipid biomarkers. The authors collected a time series of lipid, DNA, and cell count samples from the North Sea over the course of an annual cycle. The authors do observe changes in both average and compound-specific fractionation and attribute those changes to varying abundances of heterotrophic vs. autotrophic microorganisms. While the dominant photoautotrophs are quantified, no data is presented on the abundance of heterotrophic bacteria. The sequencing data presented does suggest that heterotrophs are present and their rel-



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ative abundance varies, but the reader is left to speculate on the true balance of autotrophy and heterotrophy in the system through time. As this is the proposed driver of isotopic variability, this is a problematic omission. In addition, there are substantial problems with the quantification and presentation of errors which appear to significantly underestimate the final uncertainty on isotope values. I suggest revision prior to publication of this manuscript.

We want to thank the reviewer for her/his constructive comments. We are aware that unfortunately we do not have data about the actual abundance of heterotrophic bacteria in our time series. While we can say something about the total abundance of photoautotrophs and the relative contribution of different (heterotrophic) bacteria species to the bacterial fatty acid pool, the actual contribution of the bacteria to the total fatty acid pool is left to speculation. However, the NIOZ Jetty system has been subjugated to intensive sampling and study over the past decades and its microbial population dynamics at the jetty is therefore well known. Furthermore, it has been shown that the system is quite seasonally stable throughout the whole time it has been studied. Brandsma et al. 2012 for example studied both the phytoplankton and bacterial community from 2007 to 2008. Their study included cell counts for algae, cyanobacteria and bacteria. They showed that the cell number of algae increased during spring forming the known spring bloom, the cell number of cyanobacteria was in general low except for late summer/early autumn. This fits with our observation. At the same time they showed that the total cell number of the bacteria was ten times as high as for the diverse phytoplankton. Additionally, they showed that bacterial cell numbers were lowest during the spring bloom and increased right after. During the rest of their sampling period this cell number stayed more or less constant. This suggested that the microbial community was dominated by the heterotrophic bacterioplankton following the spring bloom. While especially the autumn bloom weakened over the last years the general shift in the microbial community most likely stayed the same. Due to the stability of this systems throughout the last decades we expect similar dynamics during our sampling period and are therefore confident that the shift between photoautotrophy and heterotrophy is





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the driver behind changes in the hydrogen isotopic composition of fatty acids sampled throughout our sampling period. We will include this background information in the manuscript to make things more clear. Concerning the quantification and presentation of errors, we will revise this in the manuscript.

Substantial comments: 85: What does 'general' metabolism mean? Perhaps 'Integrated' or 'Net' this is a problem throughout the manuscript. Please define exactly what you mean, then stick to a single term.

With general or core metabolism we mean photoautotrophic, chemoautotrophic or heterotrophic metabolism. We will clarify this in the manuscript.

300: Is this relative to total fatty acid? I would much rather see these written per liter of sample. With the way it is presented it is impossible to tell if 18:0 went down because 20:5 went up, or because it actually went down. You could show both in a split figure if necessary.

The relative abundance of the fatty acids is indeed relative to the total fatty acid that are mentioned in the manuscript. Since the hydrogen isotopic composition of the fatty acids also reflects the relative contributions from different metabolisms absolute amounts or fatty acid concentrations are not relevant for the main focus of this manuscript.

324-327: This is not that strong a correlation and I am suspect you are underestimating the error associated with your water isotope estimates. Just looking that the plot a salinity around 27 could yield dDwater of anywhere from -5 to -10. Please justify the 1.5 permil number and revise as necessary. Also there are no errors shown on the water isotope measurements themselves, but all of the errors need to be propagated to the water isotope estimates. What do the errors on dD of fatty acid measurements mean? Does it include error on internal and external standards? 1 permil would be an exceptionally low error for compound specific isotope measurements: : : in fact lower than those for individual compounds in the Arndt B standard mix. Both water isotope errors and dD lipid errors need to be propagated into your epsilon values.





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We agree with the reviewer that the propagated error including the salinity water isotope error on the $\varepsilon$ values need to be calculated and will be revised in the manuscript, however the observed trend in $\varepsilon$ versus time is already present in the hydrogen isotopic composition of the fatty acids, a value that is not affected by the uncertainty in the salinity vs. water $\delta$D value. Therefore we think that our discussion is correct and our conclusions can be drawn even though there is scatter in the salinity $\delta$D water relationship.

346-353: This is an interesting suggestion, but needs better support. What data exists on the use of storage products among algae? How long does and individual alga live (and thus is a seasonal physiological pattern reasonable)? I have heard of dark reactions becoming a dominant metabolic process on the diurnal, but not seasonal, timescales. What is the resonance time of a 20:5 in a diatom? Its not clear from your reference list which Zhang 2009 is 2009a, but Xinning Zhang's paper is not a good reference here as it does not include new data for phototrophs, metabolic switching, or concurrent metabolisms.

The utilization of storage products by algae during nutrient limitation is a possible explanation for changes in the hydrogen isotopic composition of the algae biomarker fatty acid C20:5 PUFA. We will make clear in the manuscript that this is a hypothesis. It is also possible that the situation with the spring bloom behaves comparable to that of a batch culture in the sense that with increasing nutrient limitation more and more cells will be in "stationary" growth phase and later even in the declining or death phase. It has been previously shown that the fatty acids of algae became enriched in D with increasing age of the culture. This factor might also play a role in the fact that fatty acids derived from the same organisms become enriched following the phytoplankton bloom.

365-368: What do they make instead? Table 6 says that Rhodobacteria make mainly 18:1, 16:0, 16:1, and 18:0, so this statement seems to be both factually and logically inconsistent. If Flavobacteria dominate reads and make all 15:0 and branched 15, why





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don't you see these lipids? Either the lipid quantification or attribution must be wrong.

We apologize for the mistake in Table S6, Rhodobacteria mainly produce C18:1 and only, in comparison, minor amounts of C16:0 and C18:0. We will correct this error in the revised manuscript.

388-391: I don't agree with excluding 20:5 from the average. Algae are also contributing to the other pools. Also I am not sure that I agree with the average following the same trends as compound specific measurements. Perhaps plotting the average behind the C-S data would strengthen this statement. We excluded the C20:5 PUFA from the average since we wanted to present an average of the general fatty acids without a specific source in order to have a more unbiased look at the whole community. However we also calculated the average including the C20:5 PUFA and the values only differ between 1 to 4 ‰ from the values excluding the C20:5 PUFA. Therefore, we don't think that it is necessary to include the C20:5 PUFA in the average. We agree with the reviewer that potentially in the future the approach might be to look at general fatty acids and specific biomarkers for different metabolisms to account for contributions from different organisms, investigating what works and what doesn't is exactly the purpose of this study.

408: This discussion needs to be more quantitative as you have most of the necessary data. The biggest hang up is a lack of data on the true bacterial abundance. I am not sure that you can really speak to the relative importance of autotrophy vs. heterotrophy at a given moment without at least an estimate. The presence of an algal bloom is not enough as we know that heterotrophic populations increase in response.

As we mentioned above, Brandsma et al. 2012 showed changes in the total number of bacteria and phytoplankton cells with time, with the bacterial abundance being lowest during the spring bloom. We are therefore confident that during our sampling period similar changes happened.

You do have estimates of relative microbial abundances and estimates of which fatty





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acids they makeâËŸAËĞ TIf you estimate the expected FA abundances based on these data, do they match the observed FA pattern? If not does that provide insight into the abundance of heterotrophs? Then since you know the metabolism of each organism (broadly) and therefor have an expectation of fractionation, can you predict what the fractionation should be based on community and evaluate match to the measurements?

This would be an interesting exercise. Unfortunately, absolute amounts of fatty acids produced by the various bacteria are also subject to growth conditions and potentially species, strain or eco type specific. The strains and species analysed grown in pure culture and under optimal conditions might not reflect what is going on in nature. Therefore it won't be possible to make any precise calculations.

Minor comments: 17-18: The running title is just as long as the original title.

We will shorten the running title as requested.

46: references are needed after 'effect'

We will add a reference.

47-53: Be clear through this discussion if you are talking about all FAs, just C16, or an average fractionation.

Thank you for the remark. We will make it clear in the manuscript in order to avoid any misunderstandings.

57-60: This is a little misleading. 'Main' implies the majority whereas NAPDH donates 50% of H to fatty acid . H2O is the ultimate source of H to autotrophs.

We will rephrase it in the revised manuscript.

63: I am not sure what 'core' metabolism is in the context.

As mentioned above we mean with general or core metabolism photoautotrophic,





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chemoautotrophic or heterotrophic metabolism. We will clarify this in the manuscript.

65: Dawson et al. (2015) recently tested the effect of microbial interaction on D/H.

We appreciate the comment from the review and apologize for not mentioning Dawson et al.. It will be changed in the manuscript.

66-68: Specify that these were targeting marine samples

We will specify this in the manuscript.

71-81: The discussion of Osburn et al. 2011 could be more concise. Also, the bigger issue related to the extrapolation of these results more broadly is not that it was a low diversity environment, but rather that is one full of highly unusual (hyper)thermophilic organisms. The Aquificales are not exactly cosmopolitan microbes.

We thank the reviewer for the remark and we will shorten this part of the introduction and point out that the diversity of this study site is rather unusual.

95: Your data do not show an autumn bloom at all.

As mentioned in the introduction Philippart et al. 2010 showed that the autumn bloom weakened over the last years. Thus it is not surprising that the autumn bloom might not be so obvious in our data.

95-96: Is there data on bacterial abundance from this site? This is necessary complementary information to your phytoplankton counts.

We understand that this might be one of the major limitations of our data set, but unfortunately there is no data on bacterial abundance. Nevertheless, we think that our interpretation of the data correct especially taking all the previous work at this site into account, but we will point out other possibilities as requested from the reviewers.

112: What was the sampling depth? Was it consistent between samples? Is turbulence of the water a concern? Some of the changes in bacterial abundance that you note

could be influenced by incorporation of sediment into the sample.

The samples were surface samples (depth max 50 cm below surface) and always taken during high tide. Therefore, the water is considered to be North Sea water. While we cannot rule out that turbulences might have led to an input of the sediment to the samples, the sample site is next to an artificial dyke which is stabilized by basalt and concrete and therefore the amount of sediment at the surface is likely limited. This is also evident by the clear seasonality in our signals which would be lost if we had a dominant sediment input. We will provide a more detailed description in the revised text.

117: 0.7 is not very small compared to many marine heterotrophic bacteria. The clogging noted in 135-138 is also troubling. Was any microscopy performed to confirm what was actually analyzed? How can you be sure the bacterial population was captured?

We are aware that there are bacteria which will be smaller than the 0.7 $\mu$m and will therefore not be captured by the filters we used. Unfortunately, for lipid analysis the filters used have to be plastic free and organic solvent resistant and at the time of sampling there were no suitable filters available with a smaller pore size. The clogging of the 3 $\mu$m and the rather low fatty acid yield of the 0.7 $\mu$m filters lead to the assumption that we captured the majority of the biomass in the water samples. Pitcher et al. 2011 (Limnology and Oceanography) also showed in their work a good signal for the small Thaumarchaeota in 3 $\mu$m filters. Of course we cannot rule out that we still have a discrimination towards bigger cells and that a portion of the smaller cells might not have been captured and therefore analysed. Unfortunately, at the moment there is no methodological possibility to change those biases.

186: Strange formatting on your instrumentation abbreviation protocol throughout 2.3. 'elemental analysis/TC/irMS (EA/TC/irMS)' is rather repetitive

We will change that in the manuscript.

189-195: Cyanobacteria are not usually considered algae. I find it strange that you counted the cyanobacteria, but did not do a total cell count.

We will change algae to photoautotrophic microorganisms in the manuscript.

242-244: This belongs in the methods.

We will move this sentence to the method section.

247: 4 is not particularly low given the range you present. What is the error on these measurements? The graph seems to show a trend during this time period rather than a constant low.

Unfortunately the exact error for these measurements is not known, but the reliability of the HPLC approach has been previously described in Philippart et al. 2010.

258: Microbial Diversity would include that of the Archaea. You discuss only bacterial diversity.

We will change the header in the manuscript.

289: The 'Other' group makes up a large percentage of your samples, You should add a section documenting any taxa that demonstrate seasonable variability.

Our figure shows the bacterial diversity on an order level. These orders contributed to at least 3% of the reads with at least one point during sampling. The other group consists of all the other bacterial orders which at no point in sampling contribute to at least 3% of the total bacteria reads. There was only small seasonal variability seen in these orders. Nevertheless, we will add a table to the supplementary with the relative contribution to the reads on a phyla level because that would also include orders which contribute less than 3% to the total bacterial reads.

335: Misleading. At times this is among the most D-enriched compound.

In general, the C20:5 is the most depleted fatty acid. We will clarify this in the text.

337-339: It becomes unclear at times if you are discussing your own data or that from the literature. I believe this is the literature and therefor requires references. Try to clarify this throughout the discussion.

We thank the reviewer for pointing out this and will add references when necessary throughout the discussion.

342-344: But why would Phaeocystis produced enriched lipids? Any why does it matter of 20:5 is a trace or major constituent? To my knowledge there is no published information relating the relative abundance of a lipid to its relative D-enrichment or D-depletion.

The same fatty acid will have a different isotopic value depending on the source organism, even when both express the same metabolism. We do not know the actual value for the C20:5 PUFA produced either by diatoms or Phaeocystis, however changes in the abundance of either species will have an effect on the isotopic value of the fatty acid in the samples. Therefore, it is possible that changes in the isotopic value are due to changes in the abundance in the different source organisms. Additionally, Phaeocystis is a colony forming algae which potentially affects their metabolism, the type of lipids they produce, the recycling of organic matter, the production of osmolytes, exchange of water over the cell membrane and potentially different water pools, other than just North Sea water, being used during fatty acid and NADPH synthesis (the colonies form a “ball” around an internal water pool). All these factors could potentially affect the hydrogen isotopic composition of an individual lipid, on top of the integrated metabolism signal. At lower concentrations of a specific fatty acid the contribution from a single source will be more easily picked up than at higher concentrations.

354: I think on average it is the most D-depleted

Indeed, the C14:0 fatty acid seems to be one of the most depleted fatty acid.

372-376: The table needs to be reformatted in order to make these more general

conclusions.

It is unclear to us what the reviewer exactly means.

386: This is not evident for 18:0

We will change that in the manuscript to most fatty acids.

392-394: Can the correlation of trends be shown statistically?

As mentioned in the manuscript, figure 1 shows that the chlorophyll a concentration and the $\varepsilon$ value s of the weighted averages follow opposing trends. Additionally, the chlorophyll a measurements have not always been at the same time points as the isotopic measurements. Therefore, a statistical correlation would require an extrapolation for the chlorophyll a data, which would be rather imprecise. So, it is impossible to add a statistical significance.

418-419: This could be said for fatty acids from almost all environments.

This is indeed true, however in this study we only look at our sample set and don't necessarily compare it with other studies.

422: Except that 20:5 also becomes very enriched and is derived from autotrophs.

As discussed above the PUFA has multiple sources that might changes during the course of a bloom as well as potential "growth phase" changes of the organisms present during the course of a bloom also resulting in changes in hydrogen isotope fractionation. Additionally, photoautotrophic microorganisms have been shown to produce fatty acids with $\varepsilon$ values ranging between -150 and -250 ‰Ṫhe isotope values of the C20:5 PUFA clearly fall within this range.

Figure 1: Error bars on epsilon measurements (propagated as discussed above). If you come up with a measure of heterotrophic abundance, I would like to see it plotted here as well.

We will add the error bars to the graph.

Figure 3: The 'Other' category is way to big. The usual standard is to lump phyla that comprise <1% of the total. This could be pushed to maybe 3 or 5% here since the trace constituents probably are not important. I would be particularly interested in any phyla with transient high abundances.

The Other category contains all microorganisms on an order level which contribute less than 3 % to the total bacteria reads. We will add an additional table for the contribution on a phylum level.

Figure 4 and 5: It would be helpful to have some shaded bars delineating seasons to guide the eye between these subplots.

We will add this to the graphs.

Table S4: Please report concentrations instead of relative abundance. Table S6: This would be far more useful reformatted in either tabular or heatmap form. It should be possible to scan down a column for a single FA and identify those that make it and those that don't. For the abundances that you discuss in the text, the original reference should be cited rather than the table itself.

Since we did not use an internal standard we cannot calculate the actual concentration of the fatty acids.

Figures S1-S3: I am not sure these trees are necessary.

The trees show to which bacterial genera our sequences are related. This allows us to see what the most likely fatty acid compositions of the bacteria in our samples are and is we can associate the isotopic signal of the individual fatty acids to the specific metabolic potential of these genera. The figures are supplied as supplemental information and do not form the core of the manuscript, only those readers who are interested in this type of information can look at it. Therefore, we prefer to keep these supplemental figures.





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Figure S4: Error bars on each input measurement. As noted above you need to include more information on the error associate with the extrapolated water isotope values. Perhaps confidence intervals on this figure would be useful in that context. It would be also useful to color code these points by date to illustrate seasonal trends in salinity and water isotopes that might be underlying the organic isotope trends.

As mentioned above we agree with the reviewer that the propagated error including the salinity water isotope error on the $\varepsilon$ values need to be calculated and will be revised in the manuscript, however the observed trend in $\varepsilon$ versus time is already present in the hydrogen isotopic composition of the fatty acids, a value that is not affected by the uncertainty in the salinity vs. water $\delta$D value. Therefore we think that our discussion is correct and our conclusions can be drawn even though there is some scatter in the salinity $\delta$D water relationship. Additionally, the samples were taken during a rather short timescale and it is unlikely that seasonal influences would lead to changes in the salinity/$\delta$D relationship of the water.

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