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# *Interactive comment on* "Benthic Archaea as potential sources of tetraether membrane lipids in sediments across an oxygen minimum zone" *by* Marc A. Besseling et al.

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We thank the reviewer for the constructive comments. We are pleased that that he/she liked the sequencing part of our study. The reviewer raises several concerns regarding our IPL analysis to which we would like to respond below.

1. I urge the authors to re-analyze their data using more appropriate reversed phase HPLC-MS methods. Previous studies have shown that diol column HPLC-MS and other normal phase methods lead to severe underestimation of the abundances of glycosidic GDGTs (Wörmer et al., 2013; Zhu et al., 2013). This would explain the unusually high abundances of HPH-GDGT reported in the present study and the lack



of other ubiquitous compounds such as hydroxylated and unsaturated GDGT (Liu et al., 2012; Zhu et al., 2014b) and BDGT/PDGT (Becker et al., 2016; Meador et al., 2015; Zhu et al., 2014a). Alternatively, re-analysis of the samples along with authentic standards would help to correct for different ionization efficiencies based on headgroup types. If the authors did perform such a correction (and if they did not do this) they need to acknowledge this in the methods and discussion sections

We are familiar with the reversed phase HPLC-MS method published by Wörmer et al., (2013). This is certainly an elegant addition to the lipidomics toolbox, however, we do not agree with the complete dismissal of the LC-MS method originally introduced by Sturt et al. (2014) using a diol column. We have introduced several improvements on the original method (see response to comment below for details), which have considerably improved the performance of the method. The reviewer hints at a systematic bias in the used diol column HPLC-MS method that would lead to an extreme overrepresentation of HPH-GDGTs vs. glycosidic GDGTs. Indeed it is well known that identical core lipids with different head groups can have very different response factors. Additional drivers of response factors are structural features such as length of the core lipid, degree of unsaturation, number of rings or functionalities like hydroxylations. Van Mooy and Fredricks, (2010) already published an estimation of these response differences using the diol column-MS method, based on diacylglycerols with various head groups. Interestingly, they report very similar response factors for diacyls with either a phosphoglycerol or a hexose headgroup, while a diacyl with a dihexose head group has a response factor of one third of the PG and half of the monohexose. While we acknowledge that HPH-GDGTs are likely to have a higher response factor than MH-GDGTs or DH-GDGTs, these differences are unlikely to explain the 40 fold difference in apparent relative abundance we observed in e.g. the surface sample at 3000 m depth. The other IPLs mentioned, i.e. unsaturated GDGT and BDGT/PDGT core lipids with varying head groups, have been reported to be absent in several settings (Becker et al., 2016; Zhu et al., 2014b) or were linked to archaeal producers not detected in our reported archaeal community, and it is, therefore, not a given fact that we should

detect them in the Arabian Sea sediments analysed in our study. Unsaturated GDGT IPLs are not commonly detected; Zhu et al. (2014) reported that unsaturated GDGT IPLs were not detected in sediments in the upwelling region of NW Africa and only in trace amounts in the eastern Mediterranean Sea. The absence of unsaturated GDGT IPLs in our samples is therefore not unsuspected and certainly does not prove that our analysis method is inappropriate as stated by the referee. We screened our extracts for a variety of IPL BDGTs and PDGTs with an extensive list of possible polar head group combinations as indicated in our supplementary Table S1. However none of these IPL compounds were detected. Becker et al., (2016) reported that BDGTs and PDGTs were only detected in Methanomassiliicoccales and not in the other studied archaeal lipidomes. Meador et al., (2015) detected MH-GDGTs in an estuarine sediment sample and linked this to a high relative abundance of MCG. However the notable 13C depletion of the BDGTs reported by Meador et al., (2015) links this compound to relatives of members of Methanomassiliicoccales, members of Methanomassiliicoccales were not detected in our sequencing data. Becker et al., (2015) did not detect BDGTs in samples with relative low concentrations of total organic carbon (TOC), our samples have relative low TOC which can explain the absence of BDGTs. Regarding hydroxylated GDGT-IPLs, we routinely detect these in biomass as well as in environmental samples (data not yet published). We are therefore confident that, would they have been present, we would have detected them. The reviewer suggests to reanalyse the samples against standards to aid in quantification. We have purposefully refrained from quantifying the detected GDGT-IPLs because authentic quantitative standards truly representative of the GDGT-IPLS are not available and quantifying these compounds based on standards that have very different core lipids and much less complex head groups, in our opinion, does not lead to a valid quantification. We do use standards to continuously monitor the performance of the analytical system. We will add text to the materials and methods section, clarifying that the reported abundances are based on peak responses that are not corrected for differences in response factors between various IPL-types.



Finally, re-analysis of the samples at this time would probably lead to biased results due to the storage time of 2-3 years. In our hands, samples that are re-analysed after such a lengthy storage period do show very different ratios for several IPL-GDGTs.

2. Judging from the supplementary spreadsheet, the authors may have considered a wider range of archaeal lipids, not exclusively traditional intact polar tetraethers, than reported in the manuscript. If so, where are these data? However, it is questionable if this comprehensive analysis is possible with the author's choice of chromatography. A more comprehensive analysis of the archaeal lipidome (e.g., by analyzing intact polar archaeols including core lipid structural modifications) in these samples would enhance source assignments and comparison to previous studies (Meador et al., 2015; Yoshinaga et al., 2015), and provide new biomarkers or source assignment for groups such as MCG and Woesearchaeota. This analysis would then satisfy the author's conclusion that the known diversity of archaeal IPLs was significantly expanded.

For our analysis, we now make use of high resolution MS with a mass range of 375-2000 m/z. Our analytical routine involves a data dependent analysis of the 10 most abundant masses in each MS1 spectrum with a dynamic exclusion window of 6 sec (we will add this information to the method section), maximizing our capacity to identify lipids. In addition, our method works with an inclusion list, where we specify masses of compounds that, if detected, will be targeted for a product spectrum. This inclusion list contains the exact mass of most GDGT and archaeol-IPLs known at the time of analysis of this sample set. We have used all this available data to search for the compounds in the inclusion list and for additional unknown IPLs with a GDGT or archaeol core. We have successfully detected additional GDGTs as well as archaeol-based IPLs in other environmental sample sets and archaeal biomass (data not yet published). We are of the opinion that there is also sometimes information in the absence of a certain set of compounds and therefore we reported our inclusion list to indicate the breadth of our search. In contrast to what the referee suggests, we do not conclude that we are expanding the known diversity of archaeal IPLs. We wrote that we expanded the

screening for IPLs in our lab based on the use of UHPLC chromatography and high resolution mass spectrometry (quadrupole-orbitrap hybrid). We will adjust the manuscript to make this point more clear.

3. Further, because the authors used a column different from the one described in their method reference, the suitability of the chromatography for IPL analysis cannot be assessed. The authors should provide annotated TIC or EIC traces for representative samples in supplementary figures. Importantly, the type of chromatography used here does not provide chromatographic separation of IPLs by core lipid type (e.g., GDGT-0, GDGT-1), but the way the authors present their data implies the contrary. If the different core lipid types of each IPL co-eluted in their analyses, the relative abundances reported would be questionable. At the very least the authors would need to do an isotope peak correction and then report in detail how this was done (correction factors for each IPL-core lipid combination). It would be much better to use a method than can actually chromatographically separate IPL-GDGTs by core lipid structure (Zhu et al., 2013). Additionally, the method used for this study likely cannot distinguish between the core lipid crenarchaeol and it's regioisomer. However, crenarchaeol more abundant than the regioisomer in MH GDGT and HPH GDGT but lower than its regioisomer in DH GDGT in thaumarchaeal cultures (Elling et al., 2017). This limitation needs to be addressed either by using a different chromatographic method or at least by discussing this issue in the manuscript

Indeed, the column used for the work described here is a different column from the original method. It is a UHPLC silica based diol column used with the same solvent system as originally described by Sturt et al., (2014) and the overall chromatographic patterns have thus not changed but the resolution of the chromatography has significantly improved. We will add information on the improved chromatography to the supplemental information. Due to the improved separation power of the column, we can separate GDGT-IPLs with different cores but the same head group. The suggested Isotopic corrections are therefore not necessary. The reviewer is correct that we cannot



separate crenarchaeol from crenarchaeol regio-isomer with identical head groups. We are aware that Zhu et al. (2013) reported a separation between IPL crenarchaeol and crenarchaeol regio-isomer with the same head group. With our method this is not feasible, we will add to the material and method section that IPL crenarchaeol is the sum of crenarchaeol and crenarchaeol regio-isomer. Lengger et al., (2012) reported core lipid content in these samples and did not find dominance of crenarchaeol regio-isomer over crenarchaeol. We therefore do not think that the culture study the reviewer refers to is relevant. We will add TIC and EIC traces to the supplementary data to clarify our peak separation and identification.

4. Given these fundamental limitations, major parts of the methods, results, and discussion sections need to be revised. However, HPLC-MS (re-)analysis with a different method could be achieved within a day or two, given that the current study only includes 8 samples. I hope that the authors will agree that this approach has the potential to greatly expand the significance of their work with only moderate additional effort.

As discussed above, we do not agree with the dismissal of the diol column based HPLC-MS method. As explained above, we feel that reanalysis of the samples at this time with the reversed phase method would not lead to valid results due to the age of the extracts. We respectfully disagree with the reviewer that our results, and therefore our interpretation and discussion, are invalid.

5. Other comments: Line 27: DeLong et al. report only on Antarctic samples. You may want to choose a reference that discusses a more diverse set of environments (e.g. DeLong and Pace, 2013; Schleper et al., 2005) or a collection of references e.g., (DeLong, 1992; Fuhrman et al., 1992; Teske and Sørensen, 2008).

The addition of literature would benefit the introduction, we will modify this.

6. Line 31: Lloyd et al. only provide data on two archaeal groups. You may rather cite a paper that actually discusses archaeal diversity, such as Teske and Sørensen (2008) or Teske (2013) some of the more recent literature, e.g., Hug et al. (2016) or Spang et

al. (2017).

We will modify the manuscript and add some of the proposed literature references. However, we feel that it is also important to cite original references where pioneering efforts have been described.

7. Line 40-42: What are the metabolisms of the other archaeal groups?

We mention the metabolism of detected groups in our discussion, however for most of the detected groups metabolism is unknown as neither cultures nor metagenomes are available to predict their metabolic capabilities. For the rest i.e. MGD-B and E, potential metabolism is derived from (meta)genome information as shown already in lines 314-315.

8. Line 59-61: What about the Lincoln et al. (Lincoln et al., 2014) paper?

Lincoln et al. (2014) indeed reported the marine group II as potential producers of crenarchaeol. However there is still an ongoing discussion on this hypothesis (Lincoln et al., 2014b; Schouten et al., 2014). We will add this literature and discussion into our introduction (lines 59-61).

9. Line 86: Why was this standard added? Was it used for any correction?

This standard (PAF; 1-O-hexadecyl-2-acetoyl-sn-glycero-3-phosphocholine) was added to continuously monitor the performance of the MS response and to correct for matrix effects. We also corrected peak areas accordingly. We will clarify this in the materials and methods section.

10. Line 90: Specify the modifications.

The modifications are already described in detail in the text after the referral to the original Sturt et al. (2004) method, lines 90-110. We will add more details as mentioned above.

11. Line 159-161: Two DH isomers were also reported earlier by Elling et al. (2014;



2017).

Indeed, Elling et al. (2014 reported an “early eluting “ DH-GDGT and Elling et al. (2017) reports early eluting isomers of DH- and DH-OH-GDGTs. We will change our text to acknowledge these reports. However, although Elling et al. (2017) speculates on the nature of the isomerisation, no spectral details are given. We show, for the first time, spectral data on these isomers and based on these spectra, interpret the nature of the isomerization. We therefore feel it is worthwhile to include this information in the supplementary information.

12. Line 166-171: Did you detect crenarchaeol regioisomer? If not, why not? Co-elution with crenarchaeol? This should be pointed out here or elsewhere.

As stated above, the crenarchaeol regio-isomer IPLs co-elude with crenarchaeol with the same head group. We will clarify this in the manuscript.

13. Line 172-173: The way the % values are used in this section is very confusing (. . .37.6% of 36.6%) etc. It could help to provide a further table in addition to Table 2 that shows the samples arranged by depth or headgroup type instead of sorting by core GDGT type.

We will clarify this in the manuscript and will consider how to alter our tables to make our data more clear.

14. Line 196: What does “p=1.00” represent?

The correlation between our IPL derived CL-GDGTs and the ones reported by Lengger et al., (2012). We will clarify this in the manuscript and note this differently.

15. Line 254-260: How were the transcripts analyzed? I did not find this information in the methods section. How long were the samples stored before analysis?

The transcripts were analysed as described in Pitcher et al., (2011). We refer to this in our material and method section. As mentioned, the sediments were sliced on board

and directly stored at -80C. They were kept at -80C until lipid and DNA/RNA extractions. After the extractions the DNA/RNA and the cDNA were stored at -80C until analyses.

16. Line 270-274: This sentence is a bit long and complex. Revise?

The referee is correct, we will modify this sentence in our manuscript.

17. Line 281: “Differences”?

The referee is correct, it should be differences instead of difference.

18. Line 297: Rather “overlying”?

The referee is correct, it should be overlying.

19. Line 319-321: Could you point out whether there is any evidence for the occurrence of Woesearchaeota in the water column and whether you would expect this group and the other archaeal groups to be present in oxic vs. anoxic environments (or both).

There is evidence for Woesearchaeota in marine pelagic oxygenated environments, most recently by (Liu et al., 2017). The Arabian Sea contains an oxic environment and an oxygen minimum zone, so far it is uncertain if the Woesearchaeota could reside in the anoxic pelagic zone.

20. Line 353-357: I disagree with the statement that the diversity of detected (detectable) IPLs was greatly enhanced by this study. There are two IPL types described in addition to those reported by (Lengger et al., 2012). However, the diversity of IPL-GDGTs reported by previous studies is much higher, e.g. Yoshinaga et al. (2015), with respect to both the headgroup types as well as the structural modifications in the core lipid such as hydroxylation, unsaturation, methylation, monoalkylation, trialkylation, or substitution of glycerol with butanetriol or pentanetriol. Many of these would likely be detectable in the presented samples using different methods.

In contrast to what the referee suggests, we do not conclude that we are expanding the known diversity of archaeal IPLs (see remark on point 2). However we can make this



more clear in the manuscript. We will rewrite the sentences.

21. Line 359-360: GDGT-0 and crenarchaeol are also dominant core lipids of MH-GDGT in Thaumarchaeota (Elling et al., 2014; Elling et al., 2015; Elling et al., 2017; Schouten et al., 2008). MH-GDGT also appears to be the dominant membrane lipid in many Thaumarchaeota. Also, how does your interpretation fit to the results of Lincoln et al. (2014) who suggested production of crenarchaeol by other archaeal groups?

We also see a high relative abundance of IPL GDGT-0 and IPL crenarchaeol in the surface sediment samples coinciding with a high relative abundance of thaumarchaeota. Lincoln et al. (2014) suggested that marine group II (MGII) may be significant contributors of crenarchaeol in oceanic surface waters based on the combination of core lipid (CL)-GDGT and metagenomic analyses. However, the lack of culture representatives of this group and the difference in the resilience times between CL-GDGTs (attributed to dead biomass) and DNA weakens the arguments of MGII as important GDGTs producers (Lincoln et al., 2014b; Schouten et al., 2014). Members of the MGII are, so far, only found in the pelagic and not in the benthic environment therefore we did not include this in our manuscript. The archaeal groups that we detected are, currently, lacking cultured representatives and therefore their membrane lipid composition is still uncertain.

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