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Interactive comment on "Optimizing sample pretreatment for compound-specific stable carbon isotopic analysis of amino sugars in marine sediment" *by* R. Zhu et al.

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General Comments

"The manuscript contains many ideas how to optimize and validate the various steps (hydrolysis, purification and derivatization) and each of them was tested carefully. Thus, the methodological tests look targeted and carefully performed and I'm glad about such a systematic method evaluation, which would be needed much more frequently in biogeochemistry. Due to this, in my view, this is a substantial contribution to scientific progress in the field of biogeoscience: working with well-developed and evaluated methods is the fundament of scientific work in that field and I strongly support

the scientific relevance of method publications."

We agree with the reviewer who stresses the importance of methodological studies for biogeochemistry.

"... However, finally it remains the editor's decision, whether to consider method comparison and evaluation studies for publication in biogeoscience."

We trust that the issue of general suitability has been considered during editorial screening of our manuscript.

"Method evaluations are precisely described and well performed and I suggested only minor comments, how this could be additionally improved for further method studies. Some aspects have to be mentioned more clearly and I wrote a respective comment wherever I realized a gap in information (see specific comments). Especially, in the discussion, it has to set a bit more focus on the comparison of HPLC versus GC based method for isotope determination: HPLC has the clear advantage of a higher isotopic precision and accuracy (see Bodé et al and Dippold et al.). However, GC-method have a much lower detection limit and thus are the only option to measure amino sugars in trace amounts – as they occur in sediments. This has to be clearly stated, because if not it's not really clear, why you go back from new-developed LC methods towards the "old" GC-C-IRMS methods, for your amino sugar d13C determination."

We have added the reason for using a GC-based method: "Compared with LC-IRMS, the GC-based method is less sensitive to adverse effects of the sample matrix on detection (McCullagh, 2010; Morrison et al., 2010; Rinne et al., 2012) and requires smaller amounts of amino sugars. The accuracy and precision of the GC-based method, though compromised by the introduction of carbon during derivatization (Glaser and Gross, 2005), has been shown to sufficiently resolve molecular isotopic differences caused by diverse biogeochemical processes in marine sediments (cf. Lin et al., 2010). Therefore, we consider GC-IRMS more appropriate than LC-IRMS for the isotopic analysis of amino sugars in deep marine sediments." in the introduction P595

L29. In the discussion, we briefly compared the precision of the GC- and LC-based methods and stated once more why we optimized our method based on GC-IRMS starting from P606 L16: “The standard errors of repeated injections for GC-IRMS were less than 1‰ but the total errors were up to 1.4‰ i.e. about 1‰ greater than those reported for the HPLC-based method developed for soils (Bodé et al., 2009). The total errors, which are derived from addition of C atoms and fractionation during derivatization, impose constraints on the isotopic resolving power of our method and should be taken into account during data interpretation. Despite this disadvantage, the much lower requirement of carbon (see below) renders the GC-based method an attractive avenue for the analysis of amino sugars in trace amounts.”

“In addition, the opportunities which arise from the possibility to measure amino sugar d13C in sediment samples is not really worked out – without a final conclusion in this direction, the manuscript is missing it’s highlight: the reader is not only interested in the fact, that he can now measure d13C of amino sugars in sediments, but also, which new perspectives arise from that possibility. I really enjoyed having a brief look into first results with a brief interpretation (and this is in my view fully sufficient for a method publication) but I missed a bit the perspective of your method (some ideas see in specific comments) – and this information will strongly improve the scientific relevance of your study.”

We have emphasized the perspective of our method starting from P609 L2: “. . . these observations of putative indigenous bacterial signals in the form of MurA make this compound a valuable biomarker target for the isotopic analysis of microbial biomass in the deep marine biosphere, which so far has been based on lipids and intact cells and biased towards detection of signals of the Archaea (cf. Biddle et al., 2006). The combined analysis of hydrolysable sugar-derived biomarkers with lipid biomarkers, with each derived from different biosynthetic pathways and representing different preservation mechanisms, holds great potential to provide a more balanced view for the study microbial life in subseafloor sediment.” We also modified the abstract to highlight the

new perspective offered by our method.

Specific Comments:

p. 595 l. 26: The comparison of GC versus LC methods here is very subjective: Surely, most LC-methods suffer from matrix effects and have to be adapted separately, to new matrix types. However, also GC-methods suffer from the “matrix” which frequently disturbs the dervatization step – and the reproducibility of this step is essential for d13C analysis in the range of natural abundance. In addition, an important disadvantage of GC-C-IRMS methods is not mentioned here: the introduction of derivatization C – which clearly restricts precision and accuracy of GC-C-IRMS methods for d13C determination. In addition, there are new instrument couplings like IC-O-IRMS (recently published in RCM), which overcome many of the classical problems of LC-O-IRMS (single run measurement, less matrix problems,. . .). However, I fully agree that LC-O-IRMS has further problems, e.g. the higher concentrations needed, which are even worse to be extracted from marine sediments compared to soils,... To summarize: the introduction section has to be more detailed at this point: the sentence: “GC-based method is advantageous. . .” is not true – here a more detailed introduction into that topic is needed.

A series of purification steps including desalting with ENVI-CarbTM Plus SPE cartridges can minimize interference of sedimentary matrix to the derivatization of amino sugars. Although the Dippold et al. (2014) method overcame some LC-based disadvantages in soil samples, marine sedimentary matrix has not been tested using this method yet. In the revised manuscript, we deleted “(GC-based method is) advantageous . . .” and changed our text to “Compared with LC-IRMS, the GC-based method is less sensitive to adverse effects of the sample matrix on detection (McCullagh, 2010; Morrison et al., 2010; Rinne et al., 2012) and requires smaller amounts of amino sugars.” Additionally, we added the reason for using the GC-based method in P596 L2: “The accuracy and precision of the GC-based method, though compromised by the introduction of carbon during derivatization (Glaser and Gross, 2005), has been shown

to sufficiently resolve molecular isotopic differences caused by diverse biogeochemical processes in marine sediments (cf. Lin et al., 2010). Therefore, we consider GC-IRMS more appropriate than LC-IRMS for the isotopic analysis of amino sugars in deep marine sediments."

p. 596 l. 19: Evaluation is not only needed with regard to recovery, but also regarding the following aspects: - amount of introduced derivatization C - reproducibility of the derivatization (matrix dependency) - fractionation caused by the derivatization

Isotopic fractionation caused by the derivatization has been systematically assessed in previous studies (cf. Lin et al., 2010) and is therefore not the focus of the present work. We have made it clear in the revised manuscript by slightly changing the text in P603 L4-6: "3-O-methyl-D-glucopyranose (3-O-Me-Glc) was applied as an internal standard to correct the isotopic fractionation that is known to occur during acetylation of the hydroxyl groups (Lin et al., 2010)." Moreover, text on P596 L19, has been changed to "... a systematic evaluation of these various methods with regard to the product recovery and pretreatment reproducibility is necessary."

p. 598 l.17: I strongly approve the use of an internal standard at this step: however, I don't think, myo-inositol is the best internal standard for amino sugars, as it contains no amino group and may behave strongly different to amino sugars in some of the purification steps. I don't know your samples, but in none of the samples I ever measured, methyl-Glucamine was present. This substance is an internal standard structurally and chemically more similar to amino sugars and thus might be the better choice for future samples.

Thank you for your suggestion. Our method was optimized on the basis of previously published methods. Therefore, we selected myo-inositol as an internal standard according to some earlier studies, such as Zhang and Amelung (1996), Glaser and Gross (2005). We appreciate the advice that methyl-Glucamine is an attractive alternative due to its structural and chemical similarities to amino sugars and will monitor whether this

compound is absent in marine sediment samples in future samples to further consider its use.

p. 600 l. 9: Are the abbreviations already introduced? Nevertheless, I suggest to write here once the full name of the derivatives, because it's likely, they are no longer in the mind of the reader.

The abbreviations of AA and ANA here have been introduced in the introduction (P 596 L16). Nevertheless, we added the abbreviations again in P600 L9 in the revised manuscript.

p. 601 l. 9 I'm not sure, this approach is helpful: although, there are no visible peaks in the sample, it is difficult to define a sample "MurA"-free – it is just under the detection limit. However, even small contributions to a mixture, if deviating strongly from the ïA ÌÍd'13C of the spiked standard substance, can significantly contribute to the peak's ïA ÌÍd'13C-value. Therefore, a standard-addition line, where by a linear regression the "true ïA ÌÍd'13C-value of unspiked soil" and the ïA ÌÍd'13C-value of pure standard can be fitted is more reliable.

We did not describe clearly enough how we produced the "MurA-free" sedimentary matrix here. Our "MurA-free" sedimentary matrix was produced by preparative HPLC and monitored by GC/MS on SIM mode, which has a detection limit of less than 0.05 ng of MurA on column. If there would be residual MurA in the samples, the concentrations must be below the detection limit (< 0.05 ng), which should be at least 400 to 2800-fold lower than the spiked MurA. Such low fractional contribution, even if it were from isotopically "exotic" residual MurA, will hardly have an effect on the isotopic composition of the spiked MurA. Indeed the δ13C values of pure MurA at respective concentration generally matched the spiked δ13C-MurA values, which suggest insignificant contributions of residual MurA. In order to avoid misunderstandings, we have made clarification under Section 2.6: "... pure ManN or MurA was spiked to the ManN-free or MurA-free sedimentary matrix to validate the δ13C values for realistic conditions. These sedi-

mentary matrices were obtained from the preparative HPLC and monitored by GC/MS on SIM mode, with concentration of the corresponding compound below the detection limit (< 0.05 ng on column)."

p. 603 l. 20 This is not true: the reason for low monosaccharide recoveries with HCl is a dehydration reaction: HCl is water-attracting and thus causes a dehydration of the monosaccharides. This reaction doesn't occur with amino sugars – the NH2-group cannot be split of by this reaction.

Thank you for your constructive comment. We have revised the manuscript as follows: "We observed that the HCl method yielded lower recoveries of neutral sugars compared with the other two protocols (data not shown), a result in agreement with the previous finding of Amelung et al. (1996) and suggesting the occurrence of a dehydration reaction between HCl and the monosaccharides."

p. 604 l.17 There's a newly published SPE column comparison paper from Indorf et. al out, where an SPE purification column is tested: you should briefly mention there result here and compare their SPE column with your resins.

Thank you for pointing out this new paper. We agree that it is relevant and mentioned this reference in the revised manuscript (P604 L17): ". . ., further evaluation should be performed in future work using a recently optimized cation exchange-based procedure (Indorf et al., 2013)."

p. 604 l.25 This is one of the most important results: long-term stability is one of the most crucial requirements for an isotope determination in the natural abundance range (an aspect, that many people using e.g. silylation do not consider). You should emphasize this result also in conclusion and/or abstract!

The long-term stability of the derivatives has been previously demonstrated by Guerrant and Moss (1984), which is not owing to the improvement of our pretreatment procedure. Although it is crucial for isotope analysis, long-term stability is not our original

finding. Nevertheless, given its importance, we added a sentence "Such long-term stability of derivatives is essential for isotope determination in the natural abundance range." in Section 3.3. We also addressed the importance of long-term stability in Conclusions (P609 L18): "The conversion of amino sugars to ANA derivatives offers GC-amenable analytes that are stable over a long-term period and suitable for isotopic analysis."

p. 605 l. 9 Add here one sentence like: However, it has to be tested for new samples with deviating matrix peaks, which of the columns will give the best performance / peak separation / . . .

We have inserted "However, it is better to be tested for new samples with deviating matrix peaks, which column will generate the best peak separation." in the revised manuscript.

p. 606 l. 17 Add here the reason: the clear disadvantage, of GC-C-IRMS methods, is a higher total error which is mainly a result of the introduced derivatization C (and presumably matrix-dependent fractionation processes during derivatization). Although, you optimize a GC-IRMS method here, you clearly have to discuss, that this is the obvious disadvantage of GC-C-IRMS compared to LC-O-IRMS.

We added the suggested text in a slightly different way in the revised manuscript (P606 L19): "The total errors, which are derived from addition of C atoms and fractionation during derivatization, impose constraints on the isotopic resolving power of our method and should be taken into account during data interpretation. Despite this disadvantage, the much lower requirement of carbon (see below) renders the GC-based method an attractive avenue for the analysis of amino sugars in trace amounts."

p. 607 l. 1-11 It's not clear what the "ng" refer to: if it is ng in the final sample before injection or the amount that is injected by one injection (i.e. underlying the peak)?

The "ng" refers to the amount of injected amino sugars on column (see Fig. 4). We

have clarified it to “20 to 140 ng per injection” in the revised manuscript.

p. 609 l. 8 I fully agree – and this is also an highly important aspect of your manuscript which you should strongly highlight: it’s not only the fact, that you are focusing on other members of the microbial community with amino sugars compared to lipid biomarkers, but in addition: 1) you have biomarkers which are preserved by a completely different mechanism (polymerization versus hydorphobicity) – and this may be a great advantage for specific research questions 2) you have biomarkers, which are derived from a completely different biosynthetic pathway (gluconeogenesis versus lipid synthesis) and consequently other metabolic precursors – consequently a big potential lies in the combined use of “hydrolysable sugar-derived biomarkers” with “lipid biomarker” → and this study is the prework for such approaches in sediment samples → this should clearly be highlighted here! Mention these aspects either here or in the conclusion section!

We have highlighted in the abstract (P594 L17): “The δ13C analysis of amino sugars provide a valuable addition to the biomarker-based characterization of microbial metabolism in the deep marine biosphere, which so far has been lipid-oriented and biased towards the detection of archaeal signals.” as well as in the conclusion (P609 L25): “. . . provides a valuable addition to the lipid-based characterization of microbial metabolism in the deep marine biosphere.”

Fig. 1 Figure Caption is misleading: I expected the ANA-derivative of muramic acid as structure V – but it was pure muramic acid: I suggest: first show the four structures of underivatized amino sugars – then show the structure of one derivatized hexosamine and then add the structure of the ANA-derivative of muramic acid.

Combined with comments from the other two reviewers, we finally deleted this figure in the revised version.

Interactive comment on Biogeosciences Discuss., 11, 593, 2014.