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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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"... A quite high importance is given to MurA as "purely bacterial" marker, though according to Parsons et al. 1981, MurN should not be used as a marker for bacterial residues in sediments and estuarine soils as cell walls of blue-green algae contain muramic acid in concentrations up to 50% of the dry weight (Sharon, 1965; Drews, 1973). This, information might be obsolete, but should be discussed in the MS."

Blue-green algae or cyanobacteria also belong to Bacteria, and therefore the assignment of MurA as a bacterial marker remains valid. The recent work of Benner and Kaiser (2003) showed that muramic acid is found in an assemblage of marine bacteria

but not in marine phototrophic algae. Their work has been cited in the Introduction (P595, L9). Moreover, we mentioned in the revised manuscript (P595 L11): "Planktonic bacterial sources may contribute to the sedimentary MurA pool, but they may complicate the use of MurA as biomarker for sedimentary bacteria."

"There are a couple of methodological tests In this MS the authors compare different hydrolisation, purification, derivatisation procedures described in literature, though in multiple cases the author adapted the original procedure in a way that, I expect, is likely to lesser the efficiency of the original procedure, the reason of the deviation should be discussed: In the hydrolisation tests, I do not understand why the H2SO4 samples where evaporated to dryness (P598, L16) (this drying step is commonly used to remove the volatile acids, HCl and TFA, but will only concentrate the H2SO4) before being redissolved in small amount of water to be neutralized with Ba(OH)2. This is not according to the referred method (Cowie and Hedges 1987). Dying the hydrolate have 2 potential problems 1) the increased H2SO4 might alter the liberated sugars and 2) the precipitation of BaSO4 in this small volume probably increases the amount of co-precipitated (amino)sugars."

We did not evaporate H2SO4-treated hydrolysates and have made it clear in the revised manuscript: "... the hydrolysates obtained from treatments (b) and (c) were evaporated to dryness with a rotary evaporator".

"In the Neutralization and desalting part, in the description of method according to Zhang and Amelung (1996) the samples are brought to pH (6.6-6.8) by adding this was mainly intended to remove the Fe, Mg by precipitation, centrifugation. Though here the precipitate did not appear to be separated by centrifugation?"

We also centrifuged the samples after neutralization, which has been made clear in the revised manuscript (P599 L3): "The acidic solution was adjusted to pH 6.5-7.0 with 1 M potassium hydroxide (KOH) and centrifuged to remove the precipitates. The supernatant was evaporated to dryness under N2, the condensates were re-dissolved

in 2 ml methanol (MeOH), and amino sugars were collected in the supernatant after centrifugation."

"Starting on page 599 L13 the authors describe a purification using a cation exchange resin. It is a bit strange to refer to Amelung, 1996. In that paper Amelung et al. used the cation exchange column re move cationic impurities, which are retained on the resin while there analytes NEUTRAL AND ACIDIC sugars are not retained. Amino sugars are retained on the resin together with the salts. And will be eluted together with the salts when eluted with 2M NH4+. So this procedure is definitely not a desalting procedure. It is a clean-up procedure though as it will remove neutral and anionic (acidic) contaminants. It would make much more sense to refer to Indorf et al., 2013 (also a method paper on purification of AS extract) or Bodé, 2013 who also used the same resin to purify AS in soil extracts for 13C determination. The author should also mention that the cation resin was in "H+" now the reader has to get the pre-conditioning in Amelung, 1996 to know this."

Our test using cation exchange resin was performed according to the procedure describe by Amelung et al. (1996), and that is why we referred to this reference. Although Indorf et al. (2013) showed promising results after cleanup with the cation exchange resin, it is now difficult for us to make a direct comparison given different elution procedures we performed. We have 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)."

"The definition of the lower limit for isotopic measurement seems rather odd to me P607 L 7 "< 20 ng indistinct peaks precluded proper evaluation of the isotopic composition" from my experience much more than "distinctive peaks" are needed to have an reliable isotopic measurement (usually peak hight of 50 or 100 mV are used as limit of isotopic determination, though it is better t to determine it experimentally looking at the deviation of isotopic measurement and increase of sdev). It would also be interesting to have a limit of isotopic determination for sediment samples (expressed as mass per mass DW)

with and without the prep HPLC purification method. It would be good to have this data in a table (e.g. table 4)."

A peak height of 50-100 mV is usually used to determine the minimum concentration required for isotopic analysis. Moreover, the accuracy and precision of the analysis were assessed by the mean and standard deviations of triplicate measurements (Fig. 4). We have restricted our statement for the limit of isotopic determination in the revised manuscript (starting from P607 L7): "At injected quantities below 20 ng, the peak height of respective compound was usually below 100 mV, which precluded proper determination of the isotopic composition." The limit of isotopic determination for sediment samples was shown in Fig. 4 (see standard in sediment).

Minor remarks:

"I am quite surprised not to find references to Amelung 2001 ("Methods using amino sugars as markers for microbial residues in soil", in "Assessment Methods for Soil Carbon, Advanced Soil Science" (an extensive review of methods for amino sugars ion soils."

We have added this reference in the revised manuscript (P595 L6).

"All the uncertainties on measurements are given as standard errors. For a method evaluation I expect to see standard deviation, as here we are not interested in the values of the measurement, but on the precision of the method (except for the results of the of the selected marine samples. . ...), leaving the choice of the number of replicates to the user of the method."

For evaluation of the pretreatment procedure, we indeed presented average values $\pm$ standard deviations of the parallel triplicate experiments, which we have modified and clarified both in the text (P603 L2, P606 L16) and table captions (Tables 1, 2, 3, 4).

"The term "Hexosamine" is used to indicate GlcN, GalN and ManN, and make the differentiation with MurA. Indeed these three are hexosamines while MurA not strictly

speaking, though it is also derived from a hexose. Using this term to make the discrimination give the expectation that if the other AS (not hexosamine) are derived from a sugar with another C nr. While the difference is that there is a acidic group attached for MurA. I would recoment to talk about basic AS and MurA."

We agree that MurA is not strictly defined. In some earlier papers, the authors even used amino sugars (including GlcN, GalN and ManN) and MurA to distinguish them. However, your suggestion is more appropriate. We have changed "hexosamine" to "basic amino sugars" in the revised version.

"Section 2.5 to 2.7 should be restructured, it is very difficult to follow, and titles of the sections are very confusing. The author should first describe the "Purification by prep HPLC", stating that this is only used in cases where the concentration was to low or matrix effect to strong, this part should also include the description of the instrumentation used for this. Next the "Quantification and compound specific stable isotope analysis of amino sugars" with description of the GC-MS and GC-IRMS method and including the part about determining the range."

We have changed the title of Sections 2.5 to "Preparation of amino sugar-enriched fractions by preparative HPLC". On the basis of your comment, we have reconstructed these sections, particularly for 2.7, in the revised manuscript. At the beginning of 2.5, we inserted one more sentence: "For samples containing low concentrations of amino sugars and/or elevated sedimentary matrix, amino sugar extracts could be separated and enriched via preparative HPLC." However, we prefer to describe all the instrumentation including preparative HPLC, GC/MS and GC-IRMS in one section. Therefore, the paragraph for description of preparative HPLC instrument has been moved to P602 L20 in the revised manuscript.

Specific comments:

P594 L14: Not clear, rewrite in the sort of; "Compound specific 13C analysis of amino sugars obtained from extractions of selected marine sediment samples indicated that.

. .. . .

We have revised it as follows: "Compound specific stable carbon isotopic analysis of amino sugars obtained from marine sediment extracts indicated that. . ."

P595 L 1: The sentence that starts with "As amino sugars..." is not clear, is not because they are present as biopolymers that they are used as mic contribution to organic matter. It is because they are present as biopolymer that they are preserved in soil, and are used as indicators of contribution of microbial residues to OM. The reason that they are used as microbial proxys is that the contribution of meso/macro organisms is considered to be very small see Simpson et al., 2004.

We have split the sentence into two in the revised manuscript: "Amino sugars are preserved in the form of biopolymers such as peptidoglycan, chitin, and lipopolysaccharides. Because of the minor contribution of amino sugars from meso- and macroorganisms (Simpson et al., 2004), the amount of amino sugars has been frequently used as a proxy for microbial contributions to soil organic matter, . . ."

P596 L 1: The comparison between LC-IRMS and GC-IRMS for AS is rather limited. It would be nice to expand this a bit. It should also be stated that the need of derivatisation increases the uncertainty on the isotopic value due to the need of correcting for the added C atoms and fractionation during derivatisation. . ...

We have revised here as well as in the discussion. P596 L2: "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)." 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."

P600 L 25, It is strange to have the NH4OH expressed as a volume ? (should be in masses or mole)

NH4OH is often expressed in masses or mole in solution. However, many references introduced this component as a constituent of LC eluents using volumetric ratios.

P600 L 25: Totally not clear what is meant with the sentence which starts with " NH4OH and formic acid were included. . .. . ."

In our case NH4OH and formic acid are not only served as buffer solution for online monitoring of amino sugars by MS, but also used to impede partially retention of amino sugars on the stationary phase of the column. In order to avoid misunderstandings, we deleted the sentence you refer to.

P601 L17: what is meant with "online detection" here?

After separating by an Econosphere NH2 column, the majority of the sample was collected in the fraction collector for further treatment, only a very small amount of the sample was directly transported to the MS system to monitor if amino sugars were well separated or not. However, the latter process is only performed during the method development. We therefore delected "online" in the revised manuscript to avoid misunderstandings.

P602 L27: equation, description and R of reff are wrong it is: δ13C =(Rsample/Rstandard-1)x1000‰ with Rsample and Rstandard being the ratio's 13C/12C for sample and reference standard respectively. The reference standard was the international reference Vienna Pee Dee belemnite (RVPDB = 0.011180$\pm$0.000028).

Thank you very much for pointing out our mistakes. We have corrected it in the revised version.

P603 L6: Equation for correction should be given, "F is a compound-specific correction factor for fractionation due to the derivatisation, and was determined experimentally using ANA derivatives of seven. . .. . .

The F factor and the related equation have been introduced in great detail by Glaser and Gross (2005). To maintain the brevity of the text, we did not provide the equation but cited the paper (Glaser and Gross, 2005) from which the F factor was proposed and used. We rewrote the text as: "F, a compound-specific correction factor for fractionation due to the derivatization, has been introduced by Glaser and Gross (2005). We determined the F factor experimentally using ANA derivatives of seven ..."

P603 L20 should be referred to Amelung et al. 1996 for the loss of neutral sugars with harsh HCl conditions.

Indeed, Amelung et al. (1996) did not mention the reason for loss of neutral sugars. Nevertheless, we have revised here: "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."

P604 L17: When it is evaporated it is NH3 not NH4OH. . ..

NH3 is easy to evaporate. By contrast, NH4OH hydrate can be formed during evaporation, which is relatively difficult to evaporate completely. Therefore, the residue, if present, should be in the form of NH4OH hydrate.

P605 L 20: Do the author mean "irreversible adsorption" ?

Preferential adsorption is one possibility we considered for the loss of amino sugars. We are not sure if the loss is "irreversible" or not, because we did not perform additional experiments to prove that.

P605 L21: I do not think that "particularly sensitive" is right here, "hampered by" is probably what is meant here? Though this was not observed in the test with the Dowex

50x resin wher much higher ammonia concentration were used ? So I do not belive this is a valid hypothesis.

Yes, “hampered by” is more appropriate here and was used in the revised version. In response to the latter comment: For example, the recovery of ManN after Dowex 50wx8 resin is ∼71.6% (Table 2), which is quite similar to the recovery of ManN here. Therefore, we infer that presence of NH4OH or NH4COOH could be one possibility for the loss of amino sugars.

P606 L23: Do the HPLC prep separation of really help when GalN is high ? Looking at Fig 2,b it appear that MurA is very well separated from GalN, ManN is not well separate so here it might help but the Prep separation do not separate these two compounds. . .. . .

As we stated, ManN unfortunately cannot be well separated by the preparative HPLC using our current procedure. However, the major advantage of the preparative HPLC procedure is MurA can be well isolated from the basic amino sugars. Therefore, we could theoretically concentrate the sample to a desired level for isotopic analysis without worrying about overloading of GalN, which is usually one to two orders of magnitude more concentrated than MurA in environmental samples.

P608 L25 add a reff about low 13C in mehtanotrophs

We have cited Hinrichs et al. (1999) in P608 L28, about 13C-depleted microbial biomass in marine sediment containing methanotrophs.

Table 2: The DOWEX 50WX8 H2O procedure is not described in the text, I assume it is elution with water? If the resin is in H form I would really not expect any AS to elute, so I wonder why this was taken a one of the possible procedures?

Dowex 50wx8 H2O is obtained by elution with MilliQ water, which has been described in P599 L16. The resin we used is H+ form. The usage of the resin could remove neutral and acidic contaminants, therefore we considered it for evaluation.

Table 3: should also give the LoD for isotopic composition

The LoD for isotopic composition has been briefly mentioned in the text (P607 L7) as well as shown in Fig. 4 (20 ng).

Fig 1: Not really needed

We have removed it in the revised manuscript.

Fig 2 Why Is the GC-Method not stopped after 1000 min?

1000 seconds were sufficient for the separation of amino sugars on GC system. However, there are some unknown compounds with different polarities which could be eluted later at higher temperature. Therefore, we extended the program for 10 min to make sure that those compounds were eluted from the column.

Fig 5: The way the AS concentration are presented, require the use of color, I do not believe this really needed here as it could easily be presented in another way.

Yes, it is not the only way to present the AS concentration data. However, the way we present is convenient for direct comparison of the samples.

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