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Biogeosciences Discussions

Interactive comment on "Stable carbon isotope discrimination and microbiology of methane formation in tropical anoxic lake sediments" *by* R. Conrad et al.

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We are grateful for the constructive and detailed expert comments of referee #1, which will help to improve our manuscript. In the following we will consecutively address each point raised and, if appropriate, will make suggestions how to change the ms.

The referee criticizes that we made several assumptions early in the manuscript that the discussion on the isotopic composition of acetate needs to be reorganized and substantiated, that the mcrA gene community composition needs to be presented in more detail, and that the 16S rRNA gene data would not be necessary. We will first address these points. As general reply, we would like to point out that the objective of our

study was the comparison of 16 different tropical lake sediments with respect to many different characteristics involved in carbon isotope discrimination during methane production. We had only a one-time access and limited amount of sediment samples and therefore, applied routine and standard procedures for measurement and evaluation. This required making compromises with respect to in-depth investigation of microbial communities and C-flow pathways.

[1] We made two assumptions early in the manuscript to simplify evaluation of the isotopic data. The first was assuming zero isotope fractionation between methyl-acetate and methane in acetoclastic methanogenesis, which was based on the observation that acetate concentrations were very low and that acetate accumulated only if acetoclastic methanogenesis was inhibited by methyl fluoride. The low acetate concentrations let us assume that the acetate-methyl was not or only little fractionated during the conversion to CH4. Explicitly, in an open system in which a substrate (input) is converted to a product, we have the following relationship (Fry 2006):

δ13Cproduct = δ13Cinput + ε (1-f)

with input = acetate-methyl, product = CH4 formed from acetate-methyl, ε = fractionation factor for acetate-methyl converted to CH4, and f = fraction of the acetate-methyl reacted. If most of the acetate-methyl produced by fermentation is reacted to CH4, the value of f will be close to unity and δ13Cproduct = δ13Cinput. Unfortunately, we do not know the value of f, and thus have to make an assumption. Because of the low acetate concentrations, it is unlikely that Methanosarcina species play a substantial role, as they require more than a hundred micromolar acetate concentration for growth and activity (Jetten et al. 1992). Hence, it must be Methanosaeta converting acetate-methyl to CH4. Methanosaeta has a relatively small fractionation factor, i.e., ε $\approx$ -7- -10‰ (Valentine et al. 2004; Penning et al. 2006). Hence, δ13CH4-ma may be smaller than δ13Cac-methyl , but by not more than 10‰ likely less. If we thus add 10 to nominator and denominator of equation (4), the fCO2,CH4 values in Fig. 6 would be lower (maximum 10%) than indicated (as mentioned on p. 8633, line 26-28). This will

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not change the interpretation of the data. We suggest presenting such data explicitly in a figure (Fig. 1) similar to original Fig. 6C and address the sensitivity of values of fCO2,CH4 to εac,CH4 in the Discussion (compare reply [12]).

[2] The second assumption was that all methane is produced via CO2 reduction and acetoclastic methanogenesis only. Since we detected in the sediments archaea ressembling Methanosarcinaceae, a family which does not only contain acetoclastic but also methylotrophic methangens, it can a priori not be ruled out that CH4 is also produced from C1-compounds, methanol in particular. We agree with the referee that this is a serious point. Unfortunately, methylotrophic methanogenesis is not easily quantified by experimentation or by using δ13C values. In fact, CH4 production from methanol can reliably be quantified only by measuring the turnover of methanol using isotopic labeling, or by measuring the accumulation of methanol when methylotrophic methanogens and acetogens are inhibited (e.g. using chloroform). We did not do such measurements in the present study as they were not feasible under the logistic circumstances. Therefore, we cannot explicitly determine the extent of methylotrophic methanogenesis. We can only discuss this point in a revision of the manuscript. Such discussion would address the following facets using literature data. (1) Whenever methylotrophic methanogenesis was quantified in freshwater environments by tracer experiments, it was found to contribute less than 5% and thus was considered negligible (e.g., Conrad & Claus 2005; Lovley & Klug 1983). This is different in marine sediments, where methanogenesis is largely replaced by sulfate reduction except for utilization of methylated compounds, so-called non-competitive methanogenic substrates (Oremland & Polcin 1982). (2) Anaerobic degradation of polysaccharides and other plant residues results in the production of stoichiometric amounts (equation 7 in our manuscript) of acetate, CO2 and H2. Production of methanol is only likely from the methyl groups of pectin, which are less than stoichiometric to the number of galacturonic acid monomers. Thus the ratio of acetate to CH3OH produced from pectin is expected to be larger than two, and only 3/4 CH4 are produced from one CH3OH. Therefore, it is reasonable to assume that methanol does not play a quantitatively im-

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portant role in CH4 production. (3) Only a limited number of methanogenic species are able to utilize methanol. These species need to be present in the sediment. In our study we cannot rule out that such methanogens were present. Recently, methanol-utilizing methanogens were found in rather large number in wetlands of the Tibet plateau, and the sediments exhibited a remarkable potential producing CH4 from methanol (Zhang et al. 2008). This sediment would be the first example where CH4 production would be dominated by methanol metabolism. However, the authors used enrichment conditions for their experiments, and appropriate tracer studies to prove the occurrence of substantial methylotrophic methanogenesis in undiluted sediment are still missing.

[3] Referee #1 recommends presenting actual phylogenetic information for the mcrA gene community composition of the different lakes. This is not possible, since we made no phylogenetic evaluation of the composition of the community, but only an analysis of the diversity. For this we used T-RFLP as fingerprint analysis technique. This technique gives an electropherogram in which each peak represents a gene fragment of a particular length and its relative abundance. It does not contain any phylogenetic information, which can only be obtained by cloning, sequencing and tree construction, which we did not perform for the lake sediments studied. Also the relative abundance of the peaks is biased in case of the mcrA gene, since the primary PCR reaction is using degenerated primers. Therefore, the relative abundance of the individual T-RF peaks of the mcrA gene is not exact (Lueders & Friedrich 2003). Although we cannot derive phylogenetic information or a community composition from the T-RFLP fingerprints of the mcrA genes, we can give the diversity indices for the different mcrA gene fragments detected (i.e., the most common ones) as shown in Table 4 of our manuscript. In addition, explorative multivariate statistics such as canonical correspondence analyses (CCA) were applied for our T-RFLP datasets to discover the community composition patterns among the different lake sediments, as shown in Fig. 8 of our manuscript. Since it was our objective to compare the patterns of microbial communities among the different lakes (without knowing anything about their phylogenetic affiliation), we deemed it desirable using more than one gene. Shifts in such explorative multivariate

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statistics based on more than one gene underline the environmental effect on the microbial community composition as recently shown in soil type vs. methane emission from rice roots (Conrad et al. 2008). Therefore, we also assayed the bacterial and archaeal 16S rRNA genes in addition to the mcrA genes. We also would like to point out that CH4 production is the result of a microbial community which consists not only of methanogenic archaea but also of fermenting bacteria. Therefore, analysis of bacterial genes is warranted. The 16S rRNA gene is the one universal to all life and therefore, the plausible target gene. Therefore, we think that 16S rRNA gene data are useful information and do not agree with the referee on this point.

Although we do not dare concluding phylogenetic data or even community composition from the T-RFLP data of the mcrA genes, we are confident enough doing so for the archaeal 16S rRNA genes. We have many years of experience using this gene for analyzing archaeal communities in various methanogenic environments. We always obtained only a limited number of different T-RF peaks having the same T-RF length. These T-RFs were very reproducible irrespectively of the environment tested. They also were repeatedly assigned to phylogenetic groups using cloning/sequencing of archaeal 16S rRNA genes from various methanogenic environments. In contrast to the mcrA gene, T-RFLP patterns of the 16S rRNA genes allow calculating the relative abundance of the individual restricted gene fragments, as the PCR reaction is using non-degenerated primers (Lueders & Friedrich 2003). Therefore, we can reasonably well calculate the relative composition of the archaeal community and putatively assign each T-RF peak to a phylogenetic group. It is these data to which we referred in our manuscript. Although archaea do not equate with methanogens, it is common experience that the archaeal community in anoxic methanogenic environments is dominated (>50%) by methanogens (e.g., see Conrad et al. 2010). In a revised manuscript, we would present a table in which the relative abundance of the different archaeal T-RF peaks is listed together with their putative phylogenetic affiliation.

Reply to the general comments: (It seems that the page numbers used by the referee

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were sometimes confused and actually were one page higher)

[4] Page 8627, line 23-25, Page 8630, line 23-26: We agree that the assumption of zero fractionation between acetate-methyl and CH4 can be questioned as any other assumption. See reply [1] above.

The referee points out that the data of Table 3, which show differences of δ13C values for acetate-methyl in the presence and absence of methyl fluoride, are not consistent with zero fractionation during acetoclastic methanogenesis. We are grateful for this comment. Indeed, the observation of δ13C being larger in the absence than the presence of methyl fluoride indicates that the lighter carbon was preferably consumed during acetoclastic methanogenesis thus leaving behind the heavier isotope. This was indeed observed in several of the lake sediments (those with positive differences in Fig. 5). Therefore, we propose discussing this point in a revised manuscript. However, we also observed the opposite, i.e., that δ13C of acetate was lower in the absence than in the presence of methyl fluoride (see reply [7] below).

We cannot exclude the operation of syntrophic acetate oxidation and have mentioned this point on page 8634, line 14-18. Sulfate concentrations were very low, except in lakes #8,9,10, where sulfate reduction probably operated at the beginning of the sediment incubation. Later on, however, and in the other sediments, sulfate reduction is unlikely, as the incubation conditions did not allow regeneration of sulfate from reduced sulfur. Acetate consumption by biomass synthesis can also only be minor. In fact, we made in the manuscript (page 8641, line 2-20) a rough calculation of the amount of microbial biomass that can be maintained by the energy generated and found that it was larger than expected suggesting the most of the microbes are dormant or dead.

[5] We indeed claimed having detected archaeal 16S rRNA T-RFs putatively affiliated to Methanosaeta and Methanosarcina. This should not be confused with mcrA genes of these phylogentic groups, which we did not claim having detected. Nevertheless, we agree to discuss possible effects of isotopic fractionation by acetoclastic methanogen-

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esis (see our reply [1] above).

[6] We completely agree with the referee that Methanosaeta species are the more likely acetate consumers than Methanosarcina species because of the lower threshold of the former ones (see our reply [1] above). Methanosarcina species, and other members of the family Methanosarcinaceae, can use other substrates than acetate, not only methylated compounds, but in particular H2/CO2. Therefore, the presence of Methanosarcinaceae is no indication for the importance of methylotrophic methanogenesis. Nevertheless, we agree that the possibility of methylotrophic methanogenesis should be discussed (see our reply [2] above).

[7] Data in Table 3 show that δ13C of acetate sometimes increased in the methyl fluoride treatments (compare reply [4] above). This observation cannot be explained by fractionation during acetoclastic methanogenesis. We had observed this phenomenon before on a German lake sediment (Conrad et al. 2009) and explained it in our manuscript by a side effect of methyl fluoride on acetate-production during fermentation (page 8636, line 9 to page 8637, line 14). We are grateful to referee #1 pointing out that acetate could have been utilized by other processes, such as syntrophic acetate oxidation, cryptic sulfate reduction or biosynthesis. We dismiss sulfate reduction and biosynthesis as important processes (see reply [4] above), but syntrophic acetate oxidation is indeed a possibility, provided it is not inhibited by methyl fluoride (which needs to be tested by future work). This is unclear, since acetate always accumulated in its presence, meaning that syntrophic acetate oxidation, if active at all, had a much lower capacity for acetate consumption than the inhibited acetoclastic methanogenesis. If we nevertheless assume that syntrophic acetate oxidation was active to at least some extent in the presence of methyl fluoride, it would presumably prefer the light carbon and leave relatively heavy residual acetate behind. Hence, it might result in the higher δ13C of acetate and acetate-methyl in the presence than in the absence of methyl fluoride, such as observed in some of the lake sediments (see Fig. 5 of our manuscript). We suggest changing the discussion accordingly in a revised manuscript.

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The principle trend was the same for acetate (Fig. 5a) as well as for acetate-methyl (Fig. 5b), albeit it was more obvious for the former. This is mentioned in the manuscript (page 8636, line 10-12). However, we have at the moment no conclusive explanation why the phenomenon was more obvious for total acetate than for acetate-methyl. We think it would be premature starting with speculations.

[8] The comment that data in Table 3 (also Table 2 and 4) are probably not normally distributed is valid. Therefore, we will change the standard errors shown at the bottom of the tables for each column to standard deviations (basically by multiplication with four, i.e., square root of n=16) in a revised manuscript.

[9] Page 8629, line 18, Fig. 4a: The referee correctly points out that the δ13C of acetate in the controls and the methyl fluoride treatments had on the average a 1:1 relationship. However, this is only true when calculating a regression over all the lake sediments tested. The individual lake sediments, on the other hand, show sometimes a marked deviation from the regression line (see Fig. 4a). Therefore, we should not use these data for claiming that fractionation during acetoclastic methanogenesis was zero for each individual lake sediment. In a revised manuscript, we will discuss this point in more detail (see our reply [1] above).

[10] Referee #1 addresses our observation that the fraction of hydrogenotrophic methanogenesis (fCO2,CH4) calculated from isotope data was not equal (or at least proportional) to the residual CH4 production after methyl fluoride inhibition (Fig. 6c) and suggests that this may be because we assumed zero-fractionation for acetoclastic methanogenesis. If we calculate fCO2,CH4 assuming a -10‰ fractionation instead of zero-fractionation, the data points in Fig. 6C would all shift to a 10% lower value on the y-axis (we suggested presenting such a figure (Fig. 1) in our reply [1]). A change in our assumption would therefore still result in non-proportionality between the two data sets.

As alternative option, referee #1 suggests considering methylotrophic methanogene-

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sis and the abundance of the potentially methylotrophic Methanosarcinaceae in the different lake sediments. More methylotrophic methanogens may be indicative for a larger deviation between fCO2,CH4 and the residual CH4 production in the presence of CH3F. This suggestion is indeed very interesting. We tested it by plotting the difference between fCO2,CH4 and the residual CH4 production rates against the relative abundance of the 186-bp T-RF of the archaeal 16S rRNA genes. This T-RF is characteristic for mostly Methanosarcinaceae (to a minor extent also for Crenarchaeota). However, the plot showed no correlation (R2 = 0.02). This result indicates that either the methylotrophic methanogens were not correctly represented by the relative abundance of the chosen T-RF, or there is no such correlation. We suggest mentioning this briefly in the discussion of a revised manuscript.

[11] Page 8633, line 15-21: We agree in principle with the statement of the referee that the difference between the δ13C of acetate in the controls and the methyl fluoride treatments should be smallest in those lake sediments where acetate concentrations were below the detection limit. However, this was not the case as seen from Table 3. Note that our statement in the manuscript concerned not individual lake sediments but the correlation analysis concerning all lake sediments together. Even if the correlations were significant (low P value) and explained a large percentage (r2-value) of the observed variance, there was obviously quite some variance as shown by the deviation of individual lake sediments from the regression.

[12] Page 8633, line 26-28: Here we mentioned in our manuscript that values of fCO2,CH4 would range between 40 and 80% if a fractionation factor of -10‰ for acetoclastic methanogenesis would be applied. We propose extending this discussion as suggested by the referee and in our reply [1].

[13] Page 8635, line 1-8: Thank you for the comment. In fact, we do not know whether methyl fluoride inhibits methylotrophic methanogenesis as well as acetoclastic methanogenesis or as little as hydrogenotrophic methanogenesis. This should be tested in future. As suggested above (reply [10]), we will extend the discussion includ-

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ing the consideration of methylotrophic methanogenesis in a revised manuscript.

[14] Page 8636, line 1-8: Yes, referee #1 correctly points out that the more negative values of δ13C of acetate-methyl may be caused by chemolithotrophic acetogenesis. This will be added to the discussion of a revised manuscript. Thank you for the reference of Lever et al. (2010).

[15] Page 8636, line 9-17: We have already addressed this comment (see our reply [4] and [7] above). The discussion will be changed accordingly in a revised manuscript.

[16] Page 8635, line 10-25, p. 8636, line 9-21: Revision of the Discussion will try to eliminate repetitive parts. The hint to methyl fluoride-insensitive syntrophic acetate oxidation is appreciated.

[17] Page 8636, line 1-15: We will try combining the discussion in a revised manuscript. However, note that the entire paragraph is on acetate only.

[18] Page 8636, line 25 to page 8637, line 14: This discussion refers to the intramolecular difference of acetate. We appreciate the comments of the referee and the reference to Lever et al (2010) (the paper we did not yet know). We will sharpen the discussion with respect to the different types of acetogenesis. Concerning organic acetogenesis, we had only one lake sediment (#15, Paca), in which total acetate and acetate-carboxyl was more 13C-depleted than acetate-methyl.

[19] Page 8642, line 6-27: As stated in our reply [3], we would add a table in which the archaeal community composition with respect to the T-RFs of 16S rRNA are shown. Of course, we could add a table showing the T-RFs of the mcrA gene also, but these would be just fragment length without any assignment to phylogeny.

The technical corrections will all be adopted in a revised manuscript.

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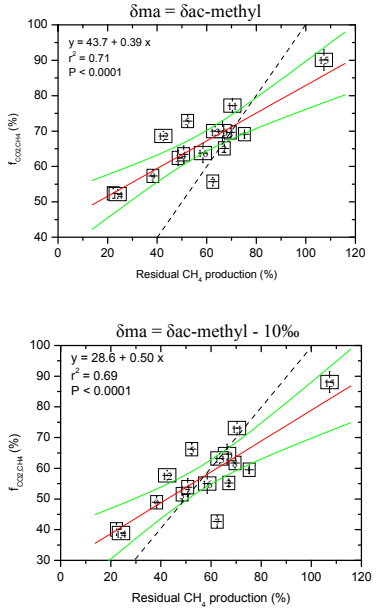

Fig. 1. Linear regression of the percent fraction (fCO2,CH4) of hydrogenotrophic methanogenesis against the residual rate (percent of the uninhibited rate) of CH4 production measured in the presence of CH3F

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