

## ***Interactive comment on “Anaerobic oxidation of methane in grassland soils used for cattle husbandry” by A. Bannert et al.***

**A. Bannert et al.**

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My main concern relates to the chemotaxonomic interpretation of PLFA, particularly of 16:1!7. 16:1!7 (syn. 16:1\_9) is in fact a ubiquitous FA in microbial systems and can not serve as biomarker strictu sensu. Anyway, despite the fact that some of the conclusions made by Raghoebarsing et al. (2006) were wrong (meaning the co-existence between archaea and bacteria), the result, which they gained from their  $^{13}\text{CH}_4$  labelling experiment, can be used also for the interpretation of the data presented here. Although 16:1!7 is very common, it was also observed as most enriched FA after  $^{13}\text{CH}_4$  addition by Raghoebarsing et al (similarities hold also for  $n_{14}:0$ , 18:1!7 etc.). This can be used as a support for nitrate/nitrite based AOM and the very weak line of evidence favoring growth of aerobic MO should be omitted (as other marker PLFA of type I and type II

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methanotrophs – e.g. 16:1!8 and 18:1!8 - are obviously lacking).

The reviewer is completely right and we omitted the weak hypothesis of a context between aerobic and anaerobic methane oxidation based on our PLFA analyses. Although we want to state, that biochemical relation between these two processes was shown for 'Candidatus Methylomirabilis oxyfera' which oxidizes methane via the classical aerobic methane oxidation pathway under anaerobic conditions by metabolizing nitrite into oxygen and dinitrogen gas. However to confirm the presence of this group in our samples there is a need for in depth molecular analysis.

The following paragraphs should be modified according to my concern. 4921, line 14 ff.: to draw a relationship between aerobic and anaerobic methanotrophs from the  $^{13}\text{C}$ -incorporation into the unspecific 16:1!7 is not correct. An uptake into an unspecific PLFA, which, however, in concert with the decrease in nitrate and the publication of Raghoebarsing et al. 2006 can be read as indication for the anaerobic oxidation of methane by relatives of candidatus Methylomirabilis oxyfera.

We rewrote the paragraph of the Abstract according to the reviewer's suggestions:

" $^{13}\text{C}$ -PLFA analyses clearly showed the utilization of  $\text{CH}_4$  as nutrient source mainly by organisms harbouring 16:1 $\omega$ 7 PLFAs. These lipids were also found as most  $^{13}\text{C}$  enriched fatty acids by Raghoebarsing et al. after addition of  $^{13}\text{CH}_4$  to an enrichment culture coupling denitrification of nitrate to anaerobic oxidation of methane. This might be an indication for anaerobic oxidation of methane by relatives of 'Candidatus Methylomirabilis oxyfera' in the investigated grassland soil under the conditions of the incubation experiment."

Page 4932, line 15 ff. This paragraph must be rewritten according to the general remark mentioned above. As described, particularly the 16:1!7 is ubiquitous and has, strictly spoken, no biomarker value. I would here rather use the absence of significant uptakes into 16:1!8 or 18:1!8 (e.g. Bowman et al., 1991) as indication that common aerobic methanotrophs of the type I and II cluster are unlikely to explain the observed uptake

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pattern. In the end the data of Raghoebarsing et al. 2006 can be carefully used that they are mostly in line with the high uptakes observed here.

We included the reviewer's suggestions concerning the missing biomarkers for aerobic methane oxidation and excluded the link between 16:1 $\omega$ 7 and aerobic methanotrophs, while making the link to the study of Raghoebarsing et al. (2006).

4934, line 4 ff: this paragraph should be rewritten according to the comment above.

The paragraph was rewritten.

Table 2: I know that comparable lists are commonly used in respective publications, but think they are not helpful and often, here as well, biased. There are indeed several PLFA with biomarker value, but others lack a biosignature function. For instance, the list for 16:1 $\omega$ 7 sources includes aerobes, anaerobes, type I methanotrophs, *Methylococcus* (which is in fact a type I methanotroph), etc. This is not helpful information and the authors should seriously take into account omitting this biased selection and the Table at all.

The table was omitted as suggested by the reviewer. It is right that the table is not absolutely necessary for understanding the results of PLFA analysis. The most important parts of the table are given in the Results and the Discussion part.

My second point is more a comment, perhaps to be considered in future experiments. Denitrification rates were obviously similar independent of the addition of methane. In fact, this does not exclude that nitrate-based AOM was occurring, but it calls for a careful interpretation in terms of other explanations and/or modifications of the experimental protocol. One option would be to also analyse nitrate (and nitrite) concentrations obtained during the experiments. The other would be to integrate genetic work or stable isotope probing using RNA-SIP to find other indications for nitrate-dependent AOM in soils.

The reviewer is completely right, that we cannot distinguish between denitrification and

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AOM by measuring nitrate consumption in our incubation system. RNA-SIP could be used with labeled carbon. But as nitrate is only electron acceptor, a labeling with  $^{15}\text{N}$  makes no sense, because it would not be incorporated. Analyzing nitrate/nitrite during the experiment is technical not easy, because we would risk getting oxygen in our anaerobic system by taking samples and the amount of soil in each bottle is too low for frequent nitrate/nitrite measurements.

Specific comments To complete the still short list of publications dealing with the anaerobic oxidation of methane, the authors should also add Rasigraf et al. (2012) and include the main result in the introduction.

Rasigraf and colleagues investigated isotope fractionation factors for carbon and hydrogen during methane oxidation by an enrichment culture of 'Candidatus *M. oxyfera*'. Values found were similar to those found for aerobic and other anaerobic methanotrophs. The results show that biological methane oxidation has a narrow range of fractionation factors for carbon and hydrogen irrespective of the underlying biochemical mechanism. We included the results of Rasigraf et al. in the introduction part. However, as we worked with highly enriched  $\text{CH}_4$  (20.2%  $^{13}\text{C}$ -enriched) fractionation plays only a minor role for the results of our experiment. If  $^{12}\text{CH}_4$  was really consumed faster, this would only have led to a small delay in time, as the added methane was almost completely consumed (91.3%).

A recent and non-cited publication deals with changes in microbial compositions as response of cattle husbandry (Elhottova et al. 2012; Applied Soil Ecology). Are these data related to the study here? If yes, it should be discussed in the light of the findings of the current study.

This study was carried out on the same sampling site, but did not focus on methane oxidation. However, interestingly it could be shown that the microbial community in the SI soil is different from that in the NI soil, which is called control plot (CO) in our publication. In addition, the work of Elhottová et al. confirms the several-fold increase of the

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microbial biomass in SI. These two points were added to our publication in the Discussion part and the paper of Elhottová et al. was cited as suggested by the reviewer.

One recommendation for future experiments is to also calculate uptake rates (as concentrations were also analysed). If just taking  $\delta^{13}\text{C}$ -values, changes in the microbial community composition resulting in changing PLFA abundances are not sufficiently expressed. In other words, a compound with low concentration, where the source organism is triggered by the substrate addition will show a high change in  $\delta^{13}\text{C}$ -values, while real uptakes can be in fact low. On the other hand, a compound with high abundances and low turnover time, will show only slight changes in  $\delta^{13}\text{C}$  although 'real' uptakes can be in fact high (expressed as compound specific e.g.  $(^{13}\text{C})$  uptake in  $\text{ng g}^{-1}$  soil (dw)).

The reviewer is right, although we think that high  $^{13}\text{C}$  integration in a low abundant fatty acid resulting in a low absolute uptake in comparison to the total amount of  $^{13}\text{CH}_4$  added does not necessarily mean that the organism harboring this PLFA does not play a role in anaerobic methane oxidation. As our question is mainly a qualitative one: which organisms might play a role in AOM in the grassland soil, we kept the relative  $^{13}\text{C}$  integration values. However, in addition we calculated now the compound specific absolute uptake of carbon out of methane in  $\text{nmol g}^{-1}$  soil dry weight. This pronounces even more the important role of organisms harboring 16:1 $\omega$ 7 PLFAs in the anaerobic oxidation of the total methane added. The unsaponifiable 16:0 PLFAs, which are at position two and three in the relative ranking do not appear here in the absolute calculation as they are low abundant. Important is also to consider, that not all of the oxidized carbon is incorporated into biomass. A big part is converted to  $\text{CO}_2$ . We think both views are valid and improve our understanding on the transformation of the methane. Consequently we included both types of calculation in the revised version. However, we want to state, that the used approach in this experiment does not allow differentiating if the metabolized methane is used by the corresponding microbes for an increase in biomass or changed ecophysiological properties (and consequently

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changed PLFA profiles). Furthermore, it is unclear how much of the applied methane is transformed into CO<sub>2</sub> or other metabolites which are not integrated into the cell derived carbon. Therefore, an absolute quantitative assessment of the process (calculating uptake rates) is not possible based on our data. Only molecular methods measuring label incorporation into the DNA and investigation of the ratio of label incorporation into DNA and lipids might allow differentiating between biomass formation and a change in ecophysiology.

4922, line 5: change “methanogenic” to “methanotrophic”

Was changed according to the reviewer’s comment.

4926, line 23: I tried to understand the definition for “unsaponifiable non-ester linked fatty acids”, but it remained unclear to me. Is it a plasmalogen or a free fatty acid? Please clarify if possible

These are non-ester linked unsubstituted fatty acids. We changed this in the revised version. Here is an explanation of Zelles (1997): "The NEL-PLFAs are components of sphingolipids, ornithine lipids, plasmalogens and other aminolipids. Sphingolipids have been found in the Bacteroides/Flavobacterium branch (Shah 1992), while plasmalogens are mainly present in Clostridia (Tunlid and White 1992). Anaerobic bacteria contain relatively large amounts of plasmalogens which have both ester and ether linkages. Only very few aerobic and facultative anaerobic bacteria contain plasmalogens (Harwood and Russel 1984). It was recently found that a number of Gram-negative polychlorophenol-degrading bacteria, described as a new species of the genus Sphingomonas, contained sphingolipids (Nohynek et al. 1996).

Zelles, L.: Phospholipid fatty acid profiles in selected members of soil microbial communities, *Chemosphere*, 35, 275-294, 1997.

4927, line 4 ff: Please clarify. Double bond positions can not be identified by EI mass spectra (if DMDS- or other derivatives were not used; see Buser et al. 1983). Did the

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authors perform co-elution experiments using commercially available standards (e.g. Supelco FAME 37, or BAME)?

We agree to the reviewer's comment, that the description in the method section might be misleading. The lipid fraction of monounsaturated fatty acid was measured underivatized to obtain a correct isotopic signal. After the first measurement, the sample was subjected to a DMDS-derivatisation to identify the position of the double bond. We made this clearer in the revised version of the manuscript.

4930, line 11ff. How can methanogenesis and methanotrophy be separated? With the data shown, it allows to characterise methanotrophy in the soil.

Methanotrophy could be detected by measuring isotope values of CO<sub>2</sub> and the methane consumption. An indicator of methanogenesis is the isotope value of CH<sub>4</sub>, which should decline when a lot of CO<sub>2</sub> is consumed. This was only the case at the end of the experiment and also only for some of the samples. But the reviewer is right that methanogenesis can no longer be separated when produced <sup>13</sup>CO<sub>2</sub> (by methanotrophy) is consumed during the experiment by methanogenesis. However, measuring methane production was not the focus of this work.

Line 4931, line 2: The detection limit for sulphate is different here to that given in the result section. Please correct where necessary.

The reviewer is right. This was a mix up with the detection limit for nitrate and we corrected it in the revised version.

4932, line 13: The authors should remain at discussing PLFA, which also demonstrated <sup>13</sup>C-enrichments and not only increases (the latter is not easy to be explained if only methane addition is the difference between both approaches). This relates for instance to the 22 and 24 FAs, which were not enriched (Fig. 4) and there is therefore no need to discuss them. Even if the source was autotrophic and use the CO<sub>2</sub> they should be enriched through cross-feeding (from <sup>13</sup>C-enriched CO<sub>2</sub> from methanotrophy). Can

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heterogeneities really excluded (also to explain the concentration increase in 18:1 $\omega$ 9)? Is biomass increase in that amount feasible (considering the often low doubling times of anaerob methanotrophs; e.g. for NC10 Ettwig et al. 2010 described it with 1-2 weeks)?

The reviewer is completely right, that  $^{13}\text{C}$ -enriched PLFAs are a much more reliable hint than just an increase in the amount of a certain PLFA after  $\text{CH}_4$  addition. Therefore, we concentrated on this in the revised version. Heterotroph organisms cannot be excluded and it might be an explanation for the higher abundance of 18:1 $\omega$ 9 without  $^{13}\text{C}$  enrichment in SI $\text{CH}_4$ . The amount of PLFA 16:1 $\omega$ 7, for example, increased around 4-fold between SI and SI $\text{CH}_4$ . This is possible as the cells might use the carbon to synthesize new lipids incorporating the label without the formation of new biomass due to a change in ecophysiology.

4932, line 16-18: What is the difference between "type I and II methanotrophs" and "methanotrophic bacteria"? Both terms are used as synonyms with different specificity!? Anyway, the authors should modify this paragraph according to my comment above.

This paragraph was anyhow rewritten according to the reviewer's comments above.

4932, line 23: I can't find information about the length of hydroxy-FA in archaea (in Gattering et al., 2002) and considering the lack of  $^{13}\text{C}$ -uptake the statement should be deleted here. To test for archaeal growth the authors should consider changing their protocol, which also allows for analysing GC-amenable archaeal lipids (e.g. archaeol).

According to the estimation that AOM in soil might be driven by bacteria, we concentrated with our PLFA analysis on bacteria and did not investigate archaea. The discussion of  $\alpha$ 23:0,  $\alpha$  $\beta$ 24:0 was deleted according to the reviewer's comment.

4933, paragraph starting at line 19 better should be omitted, as this is very speculative. Or the authors should add a reference demonstrating metabolism without substrate uptake (or that of the resulting  $^{13}\text{C}$ -enriched  $\text{CO}_2$ ).

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The paragraph was omitted in the revised version as we focused now on  $^{13}\text{C}$ -enriched PLFAs.

4934, line 1 and 3: exchange “nutrient” with “carbon”

Was exchanged according to the reviewer’s comment.

Figure 4: Just a comment. The high  $\delta^{13}\text{C}$ -values for NEL-ubr16:0 are (perhaps) an example for a situation I have described above. Concentrations appear to be low (Fig. 3) and thus a push in the growth of an originally minor abundant source organism would have shifted the  $\delta^{13}\text{C}$  considerably, although the uptake in  $^{13}\text{C}$ -carbon  $\text{ng g}^{-1}$  soil (dw) could have been low!?

The reviewer is completely right with his/her assumption: the total amount of NEL-ubr16:0 is low (0.13 respectively 0.11  $\text{nmol g}^{-1}$  dw in  $\text{SiCH}_4$ ; in comparison for 16:1 $\omega$ 7 the total amount is 19.2  $\text{nmol}$ ). But the  $^{13}\text{C}$  uptake in comparison to the total amount of a certain PLFA remains high for NEL ubr16:0. Thus, organisms harboring NEL ubr16:0 might not play the key role in AOM rates in the investigated incubation experiment, but might also be able to oxidize methane under anaerobic conditions.

Figure 3: If possible, data points should be slightly shifted from the fatty acid names (they appear to overlap). Some of the names look also odd.

The Figure was anyhow omitted.

Figure 4: Please avoid showing two decimal places for data (one is sufficient), which were obtained with an accuracy not higher than 0.2 (which is the case for  $\delta^{13}\text{C}$  analyses).

The Figure was changed according to the reviewer’s comment.

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