Reply by van Grinsven et al. to anonymous Referee #1

Referee comment on "Methane oxidation in the waters of a humics-rich boreal lake stimulated by photosynthesis, nitrite, Fe(III) and humics" by Sigrid van Grinsven et al., Biogeosciences Discuss., https://doi.org/10.5194/bg-2021-3-RC1, 2021

This interesting study investigates the biogeochemical methane cycle in a relatively shallow, eutrophic boreal lake using a wide range of chemical, microbiological and molecular techniques. The authors show that the lake Lovojärvi has a very active methane cycle mostly driven by upwards diffusing methane from the sediment (produced by methanogenesis) but also provide evidence for an additional source of methane within the water column near the Chla maxium. Methane seems to be efficiently consumed by the microbial community, in particular at the oxic-anoxic interface as well as in the anoxic hypolimnion. Using 13C-methane incubations, the authors show that methane oxidation in the anoxic hypolimnion seems to be coupled to in situ production of oxygen at shallower depths, while some of the tested electron acceptors appear to stimulate methane oxidation in the dark anoxic hypolimnion. Furthermore, FISH and 16S rRNA amplicon analyses indicate that alpha- and gammaproteobacterial methanotrophs appear to be the dominant methanotrophs.

Overall I find this study very interesting. Considering that boreal lakes are quite poorly characterized in respect to methane cycling, I believe that this study is a valuable addition to the current literature. The manuscript nicely highlights that light-driven methane oxidation as well as AOM coupled to other electron acceptors can be important processes in the anoxic hypolimnion of shallow boreal lakes. The evidence for photosynthesis-fueled methanotrophy appears robust und the authors do a good job discussing some of the observed anomalies (e.g. +O2 vs. light, 3 m vs. 4 m). However, I'm more skeptical about proposed stimulation of MOR by some of the amended substrates, in particular AQDS, and I feel that the authors should be more careful not to overstate the results of their incubation experiments (see point #1). Other than that, I have only minor suggestions. The manuscript is generally well written and understandable, and the Methods are rather brief but for the most part adequately described. The introduction could be more focused on methane cycling in boreal lakes in general (see point #2) and it would be helpful if the authors could provide some context around why Lovojärvi was studied (see point #3).

Re: Thank you for your kind words! We have added replies below to the separate comments.

Specific points:

#1 Stimulation of MOR in incubations

For some incubations, there is a clear increase in MOR (e.g. light) and the data looks robust to me. However, for other incubations the stimulation is much less pronounced (e.g. Fe, humic acid) or even so small that the difference is in my opinion within the margin of error (for AQDS). Without independent biological replicate incubations, which I don't think the authors did (please correct me if I'm wrong), I am not entirely convinced that the presented data for AQDS (and possibly Fe and humic acid) conclusively show a stimulation of MOR. As it is an interesting and important conclusion, I encourage the authors to provide some additional data (e.g. statistical tests) to support their claims.

Re: To provide the reader with data that are easier to compare, also statistically, we created a barplot including error bars (Fig. 4). We agree that in the first version of the manuscript, it was difficult to determine, whether the differences between the treatments were significant. We believe this issue is solved by the addition of the new figure. We have removed the statement that AQDS stimulated methane oxidation at 9 m, because the differences between the treatments are indeed small and the error relatively large. We also checked the mentioning of a stimulating effect of AQDS on MOR in the text, to make sure the conclusions on this part were not too strongly phrased. We however believe that it is mentioned in a correct way, without speculations on the importance of this electron acceptor, and without overemphasizing, or overly stating, the effect of AQDS on methane oxidation.

#2 Introduction could be more focused on boreal lakes

In its current form, the introduction is very general. While I agree that boreal lakes are not excessively studied, I believe that more can be said in the introduction than that "studies [...] are relatively scant". I encourage the authors to expand their introduction with more information about biological methane oxidation in boreal lakes (e.g. what is known about humic substances and why are they important, availability of other TEA, are they often Fe- and Mn-rich?).

Re: We agree that the introduction itself was relatively scant. We have now improved it strongly: we took out part of the general description of methane oxidation, especially the parts that were not relevant to the paper. We also added more information of other publications on boreal/northern lakes, including recent papers. We believe the introduction is now more targeted towards the use of different TEAs by methanotrophs on the one hand, and boreal lakes on the other hand.

#3 Boreal lakes and Lovojärvi

Lovojärvi strikes me as a quite unique lake (presence of halocline, extreme CH4 concentration above the sediment, meromixis). Is this a typical boreal lake with typical physico-chemical features? Since the authors use their findings to make general conclusions regarding the biological methane filter and the emission potential in boreal lakes (e.g. lines 14-18), it would be important to include some discussion/description on how representative Lovojärvi is for boreal lakes in general.

Re: We have searched through literature to compare these characteristics to other boreal lakes. The number of boreal lakes investigated is relatively low, and to what we found, they differ regarding quite a large number of different characteristics: stratification, Fe-content, water color etc. It is therefore difficult to set the characteristics of a 'typical boreal lake' and also to assess, whether specific characteristics have a stronger effect on the methane oxidation process than others. We, however, consider none of the 'special' characteristics of Lovojärvi (halocline, high [CH4] in lowest layer, meromixic) to be of major or special interest for the observed methane oxidation results, also because the incubation experiments of this study were performed with water from more 'average' water layers. We therefore consider the effect of the bottom layer relatively small. Another recent study on this lake (10.1093/femsec/fiaa252) has also not classified Lovojärvi as an outlier between other (boreal) lake systems.

Minor points:

Line 64: "aerobic MOB" sounds counterintuitive in this context. Please rephrase.

Re: We have rephrased the sentence

Line 73: There is definitely more literature available on methane oxidation in boreal lakes (e.g. Rissanen et al. 2017, https://doi.org/10.1093/femsec/fix078)

Re: We have now added more references and also discussed those in more detail.

Line 164: How much water was typically filtered? Re: 15 mL, this is added to the text now.

Line 174: How many cells were counted? Re: Added to the text now: *(260 - 550 cells counted per sample, distributed over 20 randomly chosen fields of view).*

Line 191: Include some information regarding sequencing depth (either here or as a table)

Re: Below a table with the sequencing depth. This table could be added to the manuscript as supplemental table, but we don't consider this directly necessary.

Depth	Reads
2m	26810
2.5m	29076
3m	30087
3.5m	33399
4m	32198
5m	27523
6m	30009
7m	32379
11m	123313
13m	35670
17m	23820

Line 233: The NOx profiles are quite stunning. I assume that the nitrate and nitrite peak close to the base of the oxycline are due to microbial ammonia oxidation. But what could be the source of nitrite in the bottom water?

Re: We have not included information about the N-profiles in the manuscript because it is not the scope of the paper. The nitrate profile is indeed consistent with nitrate regeneration through ammonium oxidation (producing the peak), as well as upward diffusion into the photic zone and downward diffusion into the denitrification zone, where it is being consumed in both locations. Nitrite is expected to occur as a reaction intermediate. We have, however, not determined which NOx-related processes occur in the water column as this was not within the scope of our research.

lines 292: The meaning of 'other Methylococcaceae' is unclear to me. Please specify.

Re: We have now clarified this in the revised text: "*At 3.5, 13 and 17 m, respectively 0.3, 0.1 and 0.3 % of 'other Methylococcaceae', specified as 16S rRNA sequence assigned to the family Methylococcaceae but not to the above-mentioned genera, were found.*"

lines 292-296: I'm confused. Methylocystaceae abundance seems low but this sentence suggests to me that they might be high since you detected unknown Rhizobiales bacteria? Please clarify.

Re: We have rephrased here to clarify: "*30 – 35 % of the Alphaproteobacterial reads at 2 – 3 m depth were, however, assigned to unknown bacteria of the Rhizobiales order, the order to which the alpha-MOB belong (Fig. S4). Possibly, part of these unknown Rhizobiales-assigned sequences belongs to methane oxidizing bacteria.*"

lines 283-301: It's not clear to me according to what logic the abundances of different methanotrophs, methylotrophs and some seemingly random taxa (Acidoferax, Planctomycetaceae, Rhizobiales) are listed one after the other. Please restructure. Also, some of these groups are never discussed and it's not clear why they are specifically mentioned here.

Re: We have listed the abundances of gamma-MOB and alpha-MOB, one after an other here, following the same order as in the previous paragraph on the FISH-results. Furthermore, we have added the relative abundance of other groups that we considered relevant for the reader (i.e.methylotrophs). We originally also included the other highly abundant microbial groups to provide a more complete picture of the microbial community in general, but we agree that it may be better to focus on the taxa that are most relevant in the context of this methane paper, which we do now.

Line 290: Were you able to observe any filamentous gamma-MOB using FISH?

Re: We did not find any evidence of filamentous gamma-MOB in the samples analyzed with the specified CARD-FISH probes. Both hybridized gamma- and alpha MOB were circular in shape with an approximate average cell size of 2 µm and 1 µm, respectively.

line 311: "natural conditions" suggests that different light intensities were used for incubations from 3m and 7, please clarify.

Re: We agree this was unclear, and we have now adapted it to "control", which is the same naming as used in the graphs. "*Methane oxidation under "control" conditions (dark, starting concentration ~50 µM CH_4 after $^{13}CH_4$ addition) peaked at the oxycline (3 m) and at 7 m depth (1.0 and 0.9 □M d^{-1}, respectively; Fig. 1B)."*

Line 319: Given the uncertainties in Table S3, AQDS 5m MOR increase does not look significant.

Re: We have now added Fig. 4, which allows for an easier comparison between the different treatments compared to the previous Fig. 1. Now, it is quite obvious that at 5 and 7 m, the uncertainties are relatively small. At 4 m and 9 m, there is indeed not enough certainty to make a robust statement on the effect of AQDS, so we have removed it from the text. We have also taken out 'in the hypolimnion', as 5 and 7 m do not cover the whole hypolimnion. *"Additions of AQDS, humic substances, and Fe(III) increased the methane oxidation rate at 5 and 7 m depth (Fig. 1)."*

line 348: What is meant by a concentration of +/- 0.5 uM ?

Re: We used this +/- because the O2 concentration gradient is very steep around 3m depth. But we recognize it is unclear and as the concentration is 0.5 uM at 3m, we have now removed the +/-.

line 392-401: It would be interesting if the authors could slightly expand on methanogenesis by phototrophs by including some brief speculation what cyanobacterial groups could be responsible for this (using the amplicon data).

Re: We are not aware of any studies that show that certain groups of cyanobacteria can produce methane, and others cannot. We therefore refrain from adding more speculation than needed to the manuscript.

Lines 486-488: The contribution of methanotrophs is indeed important, however, I suppose the halocline also plays an important role?

Re: We have adapted the text to include this: "*, it is likely not a major source of methane to the atmosphere due to effective methane consumption in the water column, combined with limited gas diffusion from the deep water layers.*"

Fig 1: This is quite a busy figure that could use some improvement. I suggest that change the scale of the x-axis for oxygen to highlight the O2 dynamics the lower concentration range (as shown in Fig. S1). In panel A, it looks as if oxygen concentration increases slightly in the hypolimnion, please comment (also in Fig. 2). In panel C, value for MOR – NO2 at 7 m is clearly <1.5 while table S3 shows a value of 1.54. Please explain error bars in legend.

Overall I suggest that the authors revise it to improve clarity. For example: i) not all x- axis same length (panesl C and D) or ii) error bars sometimes not visible.

Re: We agree that this figure was not clear enough and the information was hard to interpret. We have therefore taken the MOR-data out of this figure, and now show them in a separate barplot (Fig. 4). We have decided to leave the full oxygen profile in Fig. 1, but we have moved the zoomed-in plot of Fig. S1 to the main text. It is now combined within Fig. 2.

We have also looked into the O2 concentration data in more detail. We used two different oxygen sensors (described in the method section), and in the original figure the data from the higher-level sensor was used, also for the hypolimnion values. This sensor is, however, not suitable for the low concentrations below the oxycline. We have now adapted the figures in the manuscript to show the correct datasets, and have also clarified this in the figure's caption.

Fig 2: In my opinion, the y-axis could be limited to 10 m in order to focus on the upper water column.

RE: We agree and have adapted Fig. 2. Now, the details of the upper water column are better visible. Thank you for mentioning.

Fig 3. Only cosmetic, but there is an offset between lines and symbols.

Re: Adapted