Reply of van Grinsven et al. to Anonymous reviewer 2

The manuscript by van Grinsven et al "Methane oxidation in the waters of a humics-rich boreal lake stimulated by photosynthesis, nitrite, Fe (III) and humics" presents a detailed study on the magnitude and control of methane (CH4) oxidation in a small humic lake in Southern Finland. This study revisits some of the previously shown mechanisms regulating CH4 oxidation in lakes –light, oxygen, nitrite, Fe (III), and humic substance. Although all these factors are known to stimulate CH4 oxidation in freshwater lakes, assessing their roles and extent in a single lake water column is rarely attempted. Moreover, authors combined multiple tools – physicochemical profiling, stable carbon isotopic (δ13C-CH4) signature, near-ambient incubations with and without the addition of the above stimulants of CH4 oxidation, and molecular assessment on the dynamics of CH4 oxidizing bacteria (MOB). This article is well-written, and methods are adequately described. However, I feel that Introduction needs little more clarity. I suggest to re-write the introduction in such a way that objectives of the study are stated clearly and contextualized better. Moreover, I am missing a thorough discussion on the effect various factors on CH4 oxidation; for example, light is stimulating CH4 oxidation in this brown water lake – which is opposite to our general understanding that CH4 oxidation is inhibited by light (Murase and Sugimoto, 2005, Dumestre et al 1999, Shelly et al 2017). I would suggest authors to build the discussion based on these previous studies. Probably, light play differently across depths within a lake and across the lakes based on the extent of "browning" (increase of DOC in aquatic systems) and if so, what could be the effect of ongoing "browning" on future CH4 oxidation?

Re: We have revised the introduction. 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 aimed towards the use of different TEAs by methanotrophs on the one hand, and boreal lakes on the other hand.

Although there are indeed papers showing light inhibition of methanotrophy, there is a larger number of papers by now that show light-stimulated methane oxidation, via the pathway of coupled photosynthesis-aerobic MO (first discovered by Milucka et al. 2015, but also shown by Oswald et al.2015, Kallistova et al. 2019, Savvichev et al. 2019). The finding of light-stimulated MO in this lake therefore fits well within the expectation based on previous publications, and is strongly supported by the observed peak in chlorophyll. The light intensity in the lake at the methane oxidation peak (3 m), is also already strongly decreased compared to the surface (Fig. 2, also stated in the text: *". A strong peak in chlorophyll a concentration was observed at 3 – 4 m depth, where the light intensity was 0.3 – 1.14 µE m^{-2} s^{-1} "*), which may also prevent light inhibition.

To properly address the topic of browning, additional incubations would need to be performed. With the incubation experiment data in hand (only one light intensity), we have no information on the effect of diminishing light. We also don't have specific information on the oxygen demand of methane oxidation, so we can't perform a proper calculation to assess how much photosynthesis/O2 production would be required to still sustain methane oxidation, and at what rate. We therefore consider it too speculative to discuss the effect of ongoing browning on CH4 oxidation in the manuscript.

Specific comments L7:
I do not see any discernible CH4 oxidation "hotspot" in the anoxic water column based on the 13C-CH4 profile, although evident at ~3.0 m (Figure 2B).

Re: The 13C-CH4 profile shows, besides the distinct peak at 3 m, two smaller peaks at 7-8 m and 11-12 m. However, the reviewer is right in that the word 'hotspot' is perhaps too strong. We have therefore adapted the sentence: *"$\delta^{13}C$-CH_4 profiling of the water column revealed a methane-oxidation hotspot just below the oxycline and zones of methane oxidation within the anoxic water column."*

L53: …and classical MO – methane oxidation (MO)?
Re: adjusted, it now reads *"The hybrid metabolism of Methylomirabilis oxyfera combines partial denitrification (NO_2^- to NO) and classical aerobic methane oxidation, fueled by internal O_2 generation"*

L69-70: Please rephrase
Re: adapted, to: "*While oxygen supplied by episodic down-welling of cold O_2-laden water (Blees et al., 2014), or low-light photosynthesis (Milucka et al., 2015; Oswald et al., 2015) may explain this phenomenon to some degree, methane oxidation may also be coupled to the reduction of other electron acceptors than O_2, such as NO_X (Deutzmann et al., 2014; Graf et al., 2018; Oswald et al., 2016b), Fe(III) (Norði et al., 2013; Sivan et al., 2011), Mn(IV) (Crowe et al., 2011; Oswald et al., 2016a) and humic substances (Valenzuela et al., 2019) ."*

L71-73: I feel that the problem is not very well-defined.
Re: This part of the introduction has been completely changed in the revised version of the manuscript.

L79-83: I would suggest rephrasing of this entire sentence to bring clarity.
Re: This part of the introduction has completely changed in the revised version of the manuscript.

L82: TEAa
RE: adapted

L127: Please give little more details on the headspace equilibration; for example, how did you transfer the headspace gas into vials (?). Did you add water into the bottle to replace and take out the 20 ml headspace? Please mention how exactly you did it.

Re: We have expanded the text to include more details on the procedure. "*A headspace was created by exchanging 20 mL lake water with 20 mL N_2 gas. The bottles were then left for at least 24 hours to equilibrate the gas content between the gas and water phase. Afterwards, headspace gas samples were used to measure the CH_4 concentration by gas chromatography (GC; Agilent 6890N, Agilent Technologies) using a Carboxen 1010 column (30 m x 0.53 mm, Supelco), a flame ionization detector and an auto-sampler (Valco Instruments Co. Inc.). Resulting headspace concentrations were converted to dissolved water-phase CH_4 by applying calculated Bunsen solubility coefficients (Wiesenburg and Guinasso, 1979)*"

L137-142: I do not see any results on the fraction oxidized (f) or fractionation factor (α). Either you may provide the results or remove it from the Methods.
Re: Thank you for noticing. We have removed it from the Methods.

L206: Please provide the reasons for selecting these depths – or why you did not consider surface layers for oxidation measurements?
Re: We have added the following sentence to the text, to clarify our choice for these depths: *"These depths were selected based on their expected relevance for the methane cycle: previous research has*

repeatedly shown the highest methane oxidation rates to occur around the oxycline (Blees et al., 2014; Mayr et al., 2020; Milucka et al., 2015; Oswald et al., 2015; Panganiban et al., 1979; Sundh et al., 2005)."

L230: Please provide the detection limit of the LI-CORE in the methods – I doubt how much we can rely on 0.01µE m-2 s -1 .

Re: We have now adapted this statement to: "Light diminished between 5 and 6.6 m (0.05 – 0.01 µE m^{-2} s^{-1}; Fig. 2)."

L262: How do you define epilimnion – if it is the well-mixed surface zone, it surprising to see such a large variability in δ13C-CH4 (-50‰ to -35‰) within the epilimnion. Or do you think the first value (1m?) is erroneous? Please check.

Re: We define the epilimnion to 3 meters, which is the depth where oxygen becomes depleted. The measured δ13C-CH4 value of -50 is close to the atmospheric value of -47. Methane may also have been transported from the littoral zone, or come from below. We therefore don't think that the values are erroneous or problematic.

L311: "Potential Methane Oxidation Rates" instead of "Methane oxidation rates incubations"?. If you are providing mass-balance based estimation of oxidation (based on δ 13C-CH4), it makes sense to have "Methane oxidation rates – incubations" followed by "Methane oxidation rates – isotopic mass balance"
Re: Paragraph title adjusted

L333: Sentence reads strange to me – "….down to a depth of 3.1 m (oxycline) in the surface.."
Re: adjusted: *"Oxygen was detected down to a depth of 3.1 m (oxycline) within Lake Lovojärvi (Fig. 1A and XX2)"*

L341: "control methane oxidation rates were…" rephrase the sentence.
RE: adapted

L345: "…shallow stratified lakes,…" Please be careful with the cited references – Lake studied by Blees et al 2014 (Lake Lugano) is 288 m deep, not a shallow lake. Similarly, check other references too.
Re: Thank you for noticing. We have now removed the word "shallow", because it is not important whether the lakes are shallow; the preceding text is about the oxic-anoxic transition zone, which occurs both in deep and shallow lakes.

L357: "pending light availability"? Re: adapted

L363: 0.99 ± 0.06 – space, similarly L365. Re: adapted

L364-365: It seems to me that O2 is consumed within a short period of time, far before the termination of incubation around 48 hrs since the initial O2 concentration is only 15µM (Table S2), while light incubations continue to provide O2 through photosynthesis throughout the incubation. Please look into the time course of 13C-DIC and see whether the pattern is linear or not. Please consider this aspect.

Re: The methane oxidation rate is determined based on the linear increase in 13C-DIC, as is also described in the material and methods section ("Methane oxidation rates were estimated by linear

regression of the change of ^{13}C-DIC over the experimental interval,"). In none of the incubations with O2 did we observe a non-linear trend in 13C-DIC production over the 48 hour incubation period. This suggests that oxygen-limitation did not occur and that substrate oxygen was not used up over the course of the experiment. The data of 13C-DIC over time is currently not shown in the manuscript, but could be included as a supplementary figure, at the discretion of the AE. We, however, think it is not needed.

L371: I am not convinced – Table S2 suggest CH4 concentration is 15 µM, which seems to be the "optimal concentration" for highest methanotrophic activity (see, Thottathil et al 2019), not at a level to induce O2 inhibition.
Re: In this paragraph, we present several possible options for the observed difference in MOR between the oxic and light incubation. We do not state that inhibition is *the* explanation. But we think that it is worth mentioning, among others, the possibility of O2 inhibition, as for several methanotrophs, their ideal oxygen conditions are not known yet.

L377-378: "..perhaps attributable to the smaller methanotrophic community" – this is in contrast to L484-485 where you states that "water column methane oxidation rates therefore seems not necessarily coupled to methanotroph cell number, but rather to cell activity rates instead"
Re: We have removed the first sentence from the discussion.

L388-389: The local peak of CH4 cannot be attributed to aerobic CH4 production – for example, see Donis et al 2018 which showed that such metalimnetic peaks can only be a "physically driven accumulation".
Re: There is a lot of ongoing debate on this topic, and both papers on physically driven accumulation and cyanobacterial production are being published ((https://doi.org/10.1002/lno.11557, https://www.nature.com/articles/s41467-021-21215-2, https://www.nature.com/articles/s41467-021-21216-1, https://www.nature.com/articles/s41598-018-36530-w), including a paper by the same author D. Donis, stating "internal methane production in well-oxygenated surface water is an important source for surface-water methane" https://www.nature.com/articles/s41467-019-13320-0. Because of the still ongoing debate, we decided to display both possibilities, of in situ production and of lateral transport (*"Another reasonable explanation for the observed methane peak could be lateral transport of methane produced in sediments in the littoral zone")* to allow the reader to also judge on this matter, as we cannot be certain, which process is responsible for the methane profile that we observe.

L399-401: You are suggesting two contradictory (and much debated) processes to explain the same phenomena of sub-surface CH4 peaks. Based on the data from L. Lovojärvi and other similar systems, what is the most probable explanation?
Re: Based on the data in hand from Lovojärvi, we cannot define which process is leading to the observed CH4 accumulation (or whether both mechanisms are at work simultaneously). We therefore discuss both possibilities in the text. Overall, the co-occurrence between the phytoplankton peak and the methane maximum is striking, but it there not sufficient conclusive evidence to suggest in the manuscript that cyanobacterial methane production is indeed occurring and is the sole mechanism explaining the CH4 conentration peak.

L432: "It may be reasonably to assume…" requires rephrasing? Re: adjusted

L454-459: I am not quite understanding why do pulses of CH4 (that too bubble fluxes!) require to support alpha-MOB in the surface layers. In fact, recent studies have shown that AlphaMOB are known to be regulated by oxygen concentration, particularly Alpha MOB shows high abundance at high O2 concentration of ~200µM (see Reis et al 2019). Please look into those possibilities. Also, I

doubt whether ebullition occurs at depth of 17.5 m (sampling location) to support the hypothesized bubble pulses. If bubbles are rising from 17.5 m depth, why does bubble dissolution in the water column support only the MOB at the surface layers?.
Re: The reason why we specifically mention ebullition in relationship with the alpha-MOB in the surface layers, is that little to no methane seems (based on the analysis of concentration profiles) to reach the surface water layer via diffusion. At the depths where gamma-MOB were observed, diffusion seemed to be the main methane-delivering process. We do not mean to imply that bubble dissolution only occurs in the surface; if it happens, it happens everywhere, yet only in the surface waters, it is may be only way of methane delivery to the MOB. We have now adjusted the text to make this clearer: *"Possibly, these methanotrophs are supported by methane that reaches the upper water column via ebullition, in contrast to the continuous methane supply by diffusion to MOB in the lower water layers."* We have also added a sentence that includes the option that methane is delived to the alpha MOB laterally, from the littoral zone: "*Another possibility is the influx of methane from the littoral zone, via lateral transport.*" The relationship between O2 and alpha-MOB was already mentioned in the text ("*Alpha-MOB are known to predominantly occur at higher O_2 levels, whereas gamma-MOB tend to prefer high CH_4 levels (Amaral and Knowles, 1995; Crevecoeur et al., 2017). This zonation is visible in the Lake Lovojärvi water column, with alpha-MOB abundance peaking at 2 m ($6.8 \cdot 10^4$ cells mL^{-1}, Fig. 1D).")*

References

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