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Discussions

Interactive comment on "Lake mixing regime selects methane-oxidation kinetics of the methanotroph assemblage" *by* Magdalena J. Mayr et al.

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Reply to Reviewer 2:

Mayr, Zimmermann and colleagues studied the methane oxidation kinetics in the epiand hypolimnion of a eutrophic lake during autumn/winter lake overturn and report changing methane uptake kinetics. Likewise, changes in pmoC, A and B gene expression profiles were observed, indicating adjustments in the active methanotrophic community in dependence on methane availability. I see value in the presented work, but also limitations and open questions that would have to be clarified.

Answer: Thank you for your valuable comments. In the following we try to answer and clarify all open questions and limitations raised.

First of all, I request the authors to point out that they measured apparent methane oxidation kinetics. This should be clearly indicated throughout the manuscript.

Answer: We agree. In a revised manuscript we would change to "apparent methane oxidation kinetics" throughout the text.

In this study, all conclusions are derived from 1 - 2 l of water per sample, taken at C1 four different time points of the epilimnion and hypolimnion, respectively. However, no replicate samples were taken per layer. I find this hardly acceptable. How representative are the findings of 1 l water for the whole stratification layer of a lake? Can the authors be sure that the differences they see are indeed related to the respective water bodies? Already the molecular analysis of a second filter, which is a methodological replicate that was included for one sample, shows some differences (Fig. 3). Thus, I find it largely impossible to relate differences in the active methanotrophic community to stratification, especially in December and January and especially for pmoB and C (Fig. 3; statements l. 266-267), without knowing anything about the biological variation within a layer. The repeated measurements over time provide some evidence, but do not solve this issue when it comes to minor differences between specific samples.

Answer: Horizontal mixing in lakes is strong (Lerman and Chou, 1995, page 86), especially in stratified lakes and horizontal variation within a lake (excluding near-shore water or parts with limited water exchange) is expected to be small (Yannarell and Triplett 2004) in comparison with the vertical variation during stratification and temporal variation of the mixed layer. Taking one profile, usually close to the deepest point is therefore common practice for studies in smaller lakes or lake surveys (Oswald et al. 2017). Sample volume per depth is then typically decided by the requirements of the analytical methods rather than from concerns about the volumes representativeness.

In our earlier study on Rotsee we investigated the general population structure around

the oxycline and later the community composition change over time during lake overturn in considerable detail. We found that especially the mixed surface layer is a very homogeneous environment considering both environmental conditions and the methanotroph assemblage (Mayr et al. 2020a). Therefore, we think that the epilimnion measurements are indeed representative for the mixed layer (epilimnion in this study) and only very small variabilities would be expected from multiple measurements. The focus of this study was not on variability within the mixed layer or within hypolimnion, but rather to demonstrate that differences in kinetic parameters occur at all, in contrasting microbial communities within one lake which has not been demonstrated before. Therefore, although we did not study the variation within a layer in this study, we had considerable previous knowledge on the same lake from the years before and designed the study on this basis.

The hypolimnion during stratification by definition shows very different conditions to the epilimnion, but gradients of methane and other parameters can be observed within the hypolimnion. Therefore, whereas the hypolimnion is less homogeneous than the epilimnion, the difference to the epilimnion is clear based on both, the environmental conditions (orders of magnitude higher methane concentrations) and a different methanotrophic assemblage (this study and Mayr et al. 2020a, 2020b).

Regarding the request for (biological) replication of the kinetic measurements, we have to note that, although measuring different depths and or replicates from the layers would be favourable, we had to adjust the amount of incubations to our handling limit of incubations. We decided to improve the replication for the respective depths to have enough data points to determine the affinity of the respective depth (see our answer on outlier elimination to the other reviewer). Thus, in order to have enough different methane concentrations and technical replication we could only analyse 2 depths. This decision was also based on the results obtained in our previous study of the lake overturn period as discussed above (Mayr et al. 2020a, 2020b). Therefore, we could not have analysed more replicates or depths on one date due to handling

limitation of number of incubations, but we believe that the multiple time points provide confidence in our measurement. The reviewer may note that the existing studies on apparent MO kinetics in lakes were indeed done on lower numbers of samples.

We agree that the difference between epi- and hypolimnion become smaller in December and January, which is expected as the epilimnion is increasing in size and stratification of the lake weakens. In January the hypolimnion is almost gone and only small differences remain between epi- and hypolimnion, both in terms of methane concentration and methanotroph composition. We agree that the characteristics of the epi- and hypolimnion change over time (which is expected and was part of our research question) and use the terms epi- and hypolimnion more to clarify where the sample was taken, rather than to imply homogeneity within the category. Because there is a temporal variation, we also do not give average values for the epi- or hypolimnion. We agree that the term hypolimnion for the bottom sample in January might be misleading, because the lake has almost completely mixed, and the strong difference seen between epi- and hypolimnion before are not there anymore.

We will make the different characteristics of epi- and especially hypolimnion clearer in a revised manuscript. We will also tone down and clarify in methods and/or discussion section that the hypolimnion before lake overturn has some internal gradients. Further, in a revised manuscript we will better link and explain our study design to our previous work, in which we did a comprehensive analysis of methanotrophs during lake overturn with multiple measurements of the same layer.

Besides, the reason for including one experimental replicate (January, hypolimnion) or the conclusions derived from this sample are not mentioned anywhere.

Answer: We included this replication in order to increase confidence in our measurements. It is not easy to obtain enough RNA for metatranscriptomics from our lake samples, which we tried to optimize as much as possible considering the trade-off between 1) filtration time and 2) obtaining enough sample for RNA extraction and subsequent

sequencing. Because both effort and costs are high, we included only one replicate, although we agree more would be favourable. Typically, RNA based measurements show a somewhat larger variability than DNA based sequencing results, therefore we think that our replicate shows that the results are very reproducible for a metatranscriptomics sample, but that small differences between samples should not be overemphasized. That the three January samples are basically indistinguishable based on metatranscriptomics is not surprising as the mixing process is almost complete in January and the epi- and hypolimnion depths are similar at this date (see answer above). We will add a short explanation to a revised manuscript and add conclusions derived from this sample to a revised manuscript.

How do the authors know that they had a representative sample from the hypolimnion at the last sampling date? There is no change in temperature evident and the decline in oxygen concentrations does not reach oxygen concentrations as low as at the earlier time points. Likewise, methane concentration in this sample is not as high as in the other samples from the hypolimnion. Thus, it appears that the sample was not taken at appropriate depth to be comparable with the others.

Answer: During lake overturn the mixed layer (corresponding to epilimnion in our study) increases over time due to gradual cooling and the resulting density change. By January this mixing process is almost complete and the conditions in the remaining "hypolimnion" differ from those earlier in the season. The water at the lake bottom however still shows a small differences of e.g. methane and oxygen concentration to the water above, but the temperature gradient is gone (Figure 1d, Supplementary Fig. 1d). The lake has a max. depth of 16 m and our sample was taken from 15 m, the maximum depth that can be sampled with the niskin bottle without disturbing the sediment. Therefore, if the sample in January is s truly a "hypolimnion" sample is indeed somewhat debatable but refers to the bottom location of the sample as for the other sampling dates. As mentioned above we will better explain the term "hypolimnion sample" in a revised manuscript and describe the caveats that applies to the bottom sample in Jan-

uary.

The conclusion about the specific enrichment of well-adapted methanotrophs with particular methane oxidation kinetics (l. 23) is conceivable, but should be drawn more carefully, because it remains unclear whether the observed kinetics are indeed adaptations of particular competitive methanotrophs under oligotrophic conditions, especially with regard to affinity. As only apparent parameters could be estimated, it remains unclear whether the methane monooxygenase of the respective organisms has indeed a higher affinity (lower Km) and is thus more competitive. It should be kept in mind in this context that a low apparent Km is not necessarily a specific adaptation to low methane concentrations, but can be the result of starvation (see Dunfield and Conrad 2000, AEM).

Answer: It is of course true that we only measured the (apparent) kinetic properties of the whole MOB assemblage. Our conclusion is based on the observation that changes in the apparent kinetic properties of the assemblage are accompanied by changes in the community composition and changes in the expression level of pmoABC variants of the assemblage. In this respect, we draw conclusions on an average trend on the community level but we are well aware that there might be additional mechanisms, e.g. on individual species level as well. In a revised manuscript we will formulate the discussion in a more differentiated manner and discuss additional explanations. We will include in the discussion section that changes in Km can be a result of starvation. We also note that Dunfield and Conrad 2000 observed a constant specific affinity (a0), which is in contrast to our results. In our case both Km and a0 change with depth and with time, which may suggest a different mechanism than observed in Dunfield and Conrad (2000). Whether the kinetic differences, assuming they have a genetic basis, have an influence on the competitiveness of a species is indeed a very interesting question. We are currently preparing a manuscript that discusses a modelling approach in which we investigate the interplay of MOB populations consisting of "species" with varying Km and Vmax and their competitiveness in the Rotsee setting in detail.

To determine methane uptake kinetics (Fig. 2), the samples were apparently incubated at the temperatures measured in the epilimnion. However, samples from the hypolimnion encounter much lower temperatures in autumn. How does that affect comparability of the obtained results and conclusions about in situ conditions? This should be taken into account.

Answer: We incubated at an intermediate temperature between epi- and hypolimnion. Our approach was not designed to obtain the most realistic in-situ rates, but to get a valid comparison of the apparent kinetics in the different communities of the sampled water layers. To be able to compare the measured kinetics between epi- and hypolimnion at each given date, we measured them at the same temperature, since temperature influences the solubility of gases (methane). We tried to treat them as similarly as possible. A multi-temperature approach would have been prohibitive for the same logistical reasons discussed in the context of replication above. We propose to make the rationale for our approach more clear in the revision and to mention the caveats that arise from this choice.

Related to this point: Considering that altered temperature and oxygen conditions were used to characterize the methane uptake kinetics in vitro, to what extent can the findings be translated to in situ conditions, considering that these factors can affect the measured Km and Vmax (see the study of Thottathil et al 2019, who report that increasing oxygen concentrations in lake water can reduce maximum methane oxidation rates; doi.org/10.1007/s10533-019-00552-x). Is it conceivable that Vmax in the hypolimnion is underestimated when determining oxidation rates at higher oxygen concentrations in vitro?

Answer: Altered temperature and oxygen may influence methane uptake kinetics, but to our knowledge not much is known about these influences. Influence on methane oxidation rates does not necessarily also mean an influence on apparent methane affinity. Further, a previous study did not find that oxygen has an inhibiting effect in culture studies (Ren et al. 1997) on the other hand some species seem to be microaerophilic

(Rahalkar et al. 2007). In an attempt to measure methane affinities under as standardized conditions as possible we measured the methane oxidation kinetics under similar conditions to the best of our knowledge.

There were additional, methodological reasons to bubble the samples with air prior to incubations. One reason was to remove any background methane. The other reason was that 3H-CH4 is susceptible to some exchange of H with H2S, and purging with air also removes the H2S from the samples. Background exchange with H2S can be also be accounted for by killed controls, but this approach lowers the sensitivity of the method and high sensitivity is very important when measuring methane affinity. One could think about other methods like incubations with 13C-CH4 or 14C-CH4 but these methods likewise do not allow the very high sensitivity which is needed to measure methane oxidation rates at low methane oxidation activities, which is why we chose 3H-CH4 the most sensitive tracer (Bussmann et al. 2015). Another advantage of 3H-CH4 is that due to the high activity of this tracer only small amounts have to be added, which is also very important, because to measure methane affinity the methane oxidation rates have to be measured accurately also at very low methane concentrations.

In a revised manuscript we will add this rationale behind the methane affinity experiments to the methods section. Further, we will add that altered temperature and oxygen concentration as compared to the in-situ conditions may influence the result as a limitation of our study to the discussion section.

I find it very unfortunate that the identification of methanotrophs stops at the level “type Ia, type Ib, type II”. The sequence information should provide more detailed information about the identity of the methanotrophs. At least for pmoA comprehensive datasets are available covering besides cultivated strains diverse groups of uncultivated taxa, so that more information could have been extracted here to identify conspicuous taxa.

Answer: In a revised manuscript we will provide an improved classification of pmoA based on available databases (Wen et al. 2016). We note that the pmoABC sequences

are available as fasta files via our data repository.

Specific comments: l. 19 and 291: According to the data in table S1, the difference in Km is 20-fold, not 2 orders of magnitude

Answer: That's correct, we will change this in a revised manuscript.

l. 25: Where in the presented work is it shown or discussed that 90% of the methane are removed? It appears that this is not a conclusion that is derived from the presented work.

Answer: This is a result from our work on the overturn of the same lake one year prior to the present study (Zimmermann et al. 2019), and is based on using mass balance analysis and modelling. We will provide a clear reference for the statement in question.

l. 65: Metagenomic data were used as a basis for the metatranscriptomic data analysis, but are not presented independently; thus, I would not emphasize the metagenomics approach here for the analysis of MOB assemblages.

Answer: In a revised manuscript we will deemphasize the metagenomics part.

l. 73: Five campaigns in autumn 2017 does not appear correct (three samplings in 2017 and one in 2018 according to the presented results)

Answer: Yes, we will change this accordingly in a revised manuscript.

l. 74-75: More measured parameters are given here than presented; harmonize.

Answer: We will harmonize the methods part accordingly in a revised manuscript.

l. 78: I do not find any helpful information about the radio isotope tracer technique in Steinle et al 2015. While the cited references enabled me to understand how methane oxidation rates were determined, they do not allow me to evaluate whether/how this procedure can be used to survey methane oxidation kinetics.

Answer: The radio tracer technique is discussed in detail in Bussmann et al. (2015)

and in the supplements to Steinle et al. (2015). Methane oxidation kinetics are derived from rate measurements and we describe this in detail on line 86 – 108. To our knowledge there is no reference available where methane oxidation kinetics were determined using tritiated methane. In a revised manuscript we will add a reference to Lofton et al. (2014), who derived methane oxidation kinetics from methane oxidation rates that were measured using 14C-CH4. As described in Bussmann et al. 2015, the 3H-CH4 tracer technique is more sensitive than the 14C-CH4 technique and therefore allows shorter incubation times and rate determination at low CH4 concentrations.

l. 80: How much methane was in this mixture?

Answer: The specific activity of 3H-CH4 is 0.74 TBq mmol-1. The 200 μL of gaseous 3H-CH4/N2 mixture contained 108 pmol 3H-CH4 which corresponds to an activity of 80 kBq. In comparison, the 500 μL gas bubble with the lowest concentration of unlabelled methane, contained 17 nmol CH4.

l. 100-106: The authors describe different criteria that were used to identify and eliminate outliers here. Point four states that data points were removed in case less then two replicates remained. According to l. 93, duplicates were prepared. Does that mean that data for a specific methane concentration were lost each time one of the two replicates was identified as outlier? In this context, it is also unclear what Fig. 1 e-h shows. Do the presented data points represent individual measurements or are these mean values of the two replicates? Sometimes, I see two data points at a specific concentration, but sometimes I see only one point. Please clarify.

Answer: In a revised manuscript we will improve clarity and the level of detail of the affected methods sections. We prepared incubations in triplicates except for the first sampling campaign where we only prepared duplicates. Because we only had incubation duplicates for the first sampling date, it was not possible to detect outliers based on 2σ. For the incubation duplicates and incubation triplicates we measured each replicate twice and averaged the measurement duplicates. The data points in Fig. 1

e-h represent these averaged values for each individual incubation replicate. In those cases where it seems that there is only one point, the two or three datapoints are so close that they overlap. We will provide additional detail in a revised manuscript to clarify these points. Please also refer to our detailed reply to the other reviewer in this discussion.

It would be valuable to know how many high-quality reads the authors generated per sample in the metagenomic and metatranscriptomic analysis, respectively.

Answer: For metagenomics we generated approx. 31-37 million reads and for metatranscriptomics 26.6 – 34 million reads. In a revised manuscript we will provide this information in the methods section.

l. 157: Why three samples in October; to my understanding there should be one from the epilimnion and one from the hypolimnion per point of time.

Answer: We measured one additional depth in October in between epi- and hypolimnion, which is included in the data repository but is currently not discussed in the manuscript. We did not pursue the intermediate sample in later campaigns since we concluded that focusing our effort on the Epilimnion and Hypolimnion (e.g. continuing with measurement triplicates) would be better. We did however use the metagenomics data from this sample for the assembly. We propose to stick to the two-depth presentation for the main manuscript and figures as it keeps the story focused. However, if it is deemed desirable, we could add information on this additional sample in the supplementary material. We will also provide information on this sample in the methods section.

l. 163: Can a few words be added to describe this custom database? How was it set up? What type of data does it include?

Answer: We prepared three custom databases, one for each gene: pmoA-like, pmoB-like, pmoC-like. We extracted pxmABC, pmoCAB2, pmoCAB from both alpha- and

gammproteobacterial genomes manually. Note that pmoCAB2 is only known from alphaproteobacterial methanotrophs as yet. We used amino acid sequences with diamond blastp to find the genes in our metagenome and transcriptome. The custom database was especially important for pmoC which was not included in the available prokka databases. A short description will be provided in the manuscript upon revision, and the databases will be made publicly available. See Fig. 1.

l. 202-205, l. 295 and perhaps elsewhere: wording: do the authors refer to Km or a0 here when talking about affinity?

Answer: We refer to Km when talking about “affinity”. We refer to a0 when we talk about “specific affinity”. We will make sure that these terms are clearly defined on first use, and we think putting the kinetic parameter terms in italics throughout might further help to distinguish them in the text.

l. 204-207: I cannot follow argumentation here. And how do the authors explain that the organisms with the higher Vmax and lower Km disappear in January (Fig. 1h), although they should have a competitive advantage?

Answer: The fact that certain types disappear when they should have a competitive advantage with respect to the methane oxidation kinetics (at least insofar as we can conclude on the traits of individual species from the measurements performed on consortia) indeed leads us to the conclusion that one would need to consider additional traits to explain the observed abundance pattern and its dynamics. As stated on line 205 we believe that there have to be additional important differences in other traits (i.e. temperature optimum or specific adaptations to low or high oxygen concentrations). We already have strong indications from our previous work that these factors are indeed important (Mayr et al. 2020a, 2020b) Nonetheless, we do observe significant differences and changes in the methane oxidation kinetics which are related to the differences and changes in the environment. We propose to improve this section for a revised version by incorporating the information above.

l. 241: A range of 1 – 40 is a bit outdated. Atmospheric methane oxidizers in soil are meanwhile known to have a0s values with up to 195 x 10-12 L/cell*h (Tveit et al) and in upland soils, estimates are ranging up to 800 x 10-12 L/cell*h (Kolb et al 2005; doi:10.1111/j.1462-2920.2005.00791.x)

Answer: Thank you, we will update this in a revised version of the manuscript.

l. 248-250: I find the 25% and 93% values critical here, because huge differences are observed at the individual time points. Especially the 93% value appears to be strongly affected by the huge difference observed in December.

Answer: Yes, we will give more differentiated information upon revision of the manuscript. Individual percentages can be calculated from the individual values for the maximum methane oxidation rate as well as the in-situ methane oxidation rate in Supplementary Table 1. In the epilimnion there might indeed be a trend over time, however all values are below 50%. In the hypolimnion we observe less variation and all values are above 67%. Therefore, we do believe that it is a valid conclusion that MOB in the epilimnion were generally more limited than MOB in the hypolimnion.

l. 254: What do the authors mean with aggregate properties here? What aggregates do they refer to?

Answer: “Thus, the reported kinetics reflect aggregate properties of the respective assemblage.” Aggregate was used in the sense of "formed or calculated by the combination of several separate elements”, in this case of all the species and individuals in the community, i.e. we meant to express that the reported kinetics reflect the (apparent) properties of the MOB assemblage which is present at the depth and date of sampling. In a revised manuscript we will try to find a less ambiguous term.

l. 256 – 258: It would be very valuable if the described findings could be seen in Figure 3.

Answer: In a revised manuscript we will try to improve this and highlight type Ia Ib and

II in Fig. 3.

l. 292-293: The transcription of genes does not relate to enzyme affinity or apparent Km values; thus, I cannot follow argumentation here.

Answer: The association is only correlational so far, this is true. In a revised manuscript we will tone these statements down or clearly state them as hypotheses.

l. 301: I do not necessarily agree to the term “entirely” in the context with “kinetic traits”; other environmental conditions may have affected the kinetic parameters. Please keep in mind that you can only measure apparent parameters, not enzyme kinetics.

Answer: We will remove the “entirely” there. Yes, it is true that other parameters likely influence kinetic traits. We keep in mind that we do not measure enzyme kinetics but apparent kinetics. Nevertheless, our metatranscriptomics data clearly suggest that pMMO was by far the most expressed methane oxidizing enzyme. Therefore, according to our data most of the methane which is oxidized is oxidized by pMMO and not other enzymes.

l. 303-304: Please note that Methylocapsa gorgona does not possess a second pmoA gene for “high-affinity oxidation” despite being able to live on very low methane concentrations (Tveit et al).

Answer: Yes, we are aware of this interesting study and cite it elsewhere in the manuscript. Methylocapsa gorgona can live and grow on very low methane concentrations, but it does not have a very low apparent Km (4.9 μM), but it has a very high specific affinity. But many methanotrophs possess sMMO and pMMO, and sMMO has a lower affinity than pMMO. To our knowledge Methylocapsa gorgona does not have sMMO.

References: The reference list does not allow to differentiate publications (e.g. Mayr et al 2019a, b, c). The reference list lacks information about the year the work has been published and the indices a,b,c.

Answer: Here we followed the author guidelines of the Journal and added the year + a,b,c at the end of the reference.

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Mayr, M. J., Zimmermann, M., Dey, J., Brand, A. and Bürgmann, H.: Growth and rapid succession of methanotrophs 431 effectively limit methane release during lake overturn, bioRxiv, doi:https://doi.org/10.1101/707836, 2019b.

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Figure 1: The axis showing oxygen concentrations should have a more increments.

Answer: We will provide an axis with more increments in a revised manuscript.

Figure 2: explain error bars

Answer: As described in the figure caption we plot 95% confidence intervals as light green and light orange vertical bars. Average values are plotted as dark green and dark orange lines. In a revised manuscript we will improve clarity of the figure caption.

Figure 3: The distinction by color is difficult in plots a1-c1; why not choosing more distinct colors / a broader range of colors per plot? This is of particular importance, as the relative abundances cannot be taken from Table S2 without additional calculations. It is currently impossible to identify type Ib or type II methanotrophs based on the color code and without further invest. However, as pointed out above, it would be even more valuable if more taxonomic information could be provided.

Answer: We have a hard time coming up with a better and still colour-blind proof palette. We decided that taking a clear brightness-based palette with additional colour

information would be best. If the reviewer or editor have concrete suggestions for better colour palettes, we would greatly appreciate it. We will definitely highlight type Ia, Ib and II in the figure in a revised manuscript. We will provide the supplementary table with the underlying data as comma separated file instead of pdf to make it more accessible. The fasta files of all variants are available in the provided in a data repositors (“Data availability”). In a revised manuscript we can provide a taxonomic classification of pmoA based on available databases.

Table S3: Provide reference for Knief et al 2015.

Answer: We will provide the reference in a revised manuscript.

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pmoA-like
>WP_085769855.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylocystis bryophila][pxmA]
>AMK75760.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylomonas denitrificans][pxmA]
>AMK77276.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylomonas denitrificans][pmoA]
>WP_085772041.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylocystis bryophila][pmoA2]
>WP_085772257.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylocystis bryophila][pmoA1]
>WP_085772257.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylocystis bryophila][pmoA1b]
>WP_033157542.1 MULTISPECIES: ammonia monooxygenase [Methylomonas][pxmA]
>WP_033159227.1 methane monooxygenase/ammonia monooxygenase subunit A [Methylomonas sp. LW13][pmoA]
>OOO02385.1 Particulate methane monooxygenase beta subunit [Chromatiales bacterium USCg_Taylor][pmoA_USCg]
>CCJ08278.1 Particulate methane monooxygenase subunit A [Methylocystis sp. SC2]
>CCJ08985.1 Particulate methane monooxygenase subunit A [Methylocystis sp. SC2]
pmoB-like
>ACE95891.2 particulate methane monooxygenase subunit B-like protein [Methylomonas sp. LW13] [pxmB]
>ACE95892.2 particulate methane monooxygenase subunit B-like protein [Methylomonas methanica] [pxmB]
>WP_085769854.1 methane monooxygenase/ammonia monooxygenase subunit B [Methylocystis bryophila][pxmB]
>AMK75759.1 methane monooxygenase/ammonia monooxygenase subunit B [Methylomonas denitrificans][pxmB]
>AMK77277.1 methane monooxygenase/ammonia monooxygenase subunit B [Methylomonas denitrificans][pmoB]
>WP_085772042.1 methane monooxygenase/ammonia monooxygenase subunit B [Methylocystis bryophila][pmoB2]
>WP_085772258.1 methane monooxygenase/ammonia monooxygenase subunit B [Methylocystis bryophila][pmoB1]
>WP_085772258.1 methane monooxygenase/ammonia monooxygenase subunit B [Methylocystis bryophila][pmoB1b]
>WP_033157541.1 MULTISPECIES: methane monooxygenase/ammonia monooxygenase subunit B [Methylomonas][pxmB]
>WP_033159226.1 MULTISPECIES: methane monooxygenase/ammonia monooxygenase subunit B [Methylomonas][pmoB]
>OOO02386.1 Particulate methane monooxygenase alpha subunit [Chromatiales bacterium USCg_Taylor][pmoB_g]
pmoC-like
>WP_085769853.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylocystis bryophila] [pxmC]
>AMK75758.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylomonas denitrificans] [pxmC]
>AMK77275.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylomonas denitrificans] [pmoC]
>WP_085772039.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylocystis bryophila][pmoC2]
>WP_085771887.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylocystis bryophila][pmoC1]
>WP_085771887.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylocystis bryophila][pmoC1b]
>WP_033157540.1 MULTISPECIES: methane monooxygenase/ammonia monooxygenase subunit C [Methylomonas][pxmC]
>WP_033159228.1 MULTISPECIES: methane monooxygenase/ammonia monooxygenase subunit C [Methylomonas][pmoC]
>OOO02384.1 hypothetical protein USCGTAYLOR_01391 [Chromatiales bacterium USCg_Taylor][pmoC_g]
>CCJ06178.1 Methane monooxygenase subunit C [Methylocystis sp. SC2] [pmoC_cis]
>CCJ06303.1 Particulate Methane Monooxigenase subunit C [Methylocystis sp. SC2] [pmoC_cis]
>CCJ08277.1 Particulate methane monooxigenase subunit C [Methylocystis sp. SC2][pmoC]
>CCJ08984.1 Particulate Methane Monooxigenase subunit C [Methylocystis sp. SC2][pmoC]
>CCJ05653.1 Particulate methane monooxigenase subunit C, PmoC2 protein [Methylocystis sp. SC2] [pmoC2]
>AMK77275.1 methane monooxygenase/ammonia monooxygenase subunit C [Methylomonas denitrificans]

Fig. 1.

Reply to Reviewer 2:

Mayr, Zimmermann and colleagues studied the methane oxidation kinetics in the epiand hypolimnion of a eutrophic lake during autumn/winter lake overturn and report changing methane uptake kinetics. Likewise, changes in pmoC, A and B gene expression profiles were observed, indicating adjustments in the active methanotrophic community in dependence on methane availability. I see value in the presented work, but also limitations and open questions that would have to be clarified.

Answer: Thank you for your valuable comments. In the following we try to answer and clarify all open questions and limitations raised.

First of all, I request the authors to point out that they measured apparent methane oxidation kinetics. This should be clearly indicated throughout the manuscript.

Answer: We agree. In a revised manuscript we would change to "apparent methane oxidation kinetics" throughout the text.

In this study, all conclusions are derived from 1 - 2 l of water per sample, taken at C1 four different time points of the epilimnion and hypolimnion, respectively. However, no replicate samples were taken per layer. I find this hardly acceptable. How representative are the findings of 1 l water for the whole stratification layer of a lake? Can the authors be sure that the differences they see are indeed related to the respective water bodies? Already the molecular analysis of a second filter, which is a methodological replicate that was included for one sample, shows some differences (Fig. 3). Thus, I find it largely impossible to relate differences in the active methanotrophic community to stratification, especially in December and January and especially for pmoB and C (Fig. 3; statements l. 266-267), without knowing anything about the biological variation within a layer. The repeated measurements over time provide some evidence, but do not solve this issue when it comes to minor differences between specific samples.

Answer: Horizontal mixing in lakes is strong (Lerman and Chou, 1995, page 86), especially in stratified lakes and horizontal variation within a lake (excluding near-shore water or parts with limited water exchange) is expected to be small (Yannarell and Triplett 2004) in comparison with the vertical variation during stratification and temporal variation of the mixed layer. Taking one profile, usually close to the deepest point is therefore common practice for studies in smaller lakes or lake surveys (Oswald et al. 2017). Sample volume per depth is then typically decided by the requirements of the analytical methods rather than from concerns about the volumes representativeness.

In our earlier study on Rotsee we investigated the general population structure around the oxycline and later the community composition change over time during lake overturn in considerable detail. We found that especially the mixed surface layer is a very homogeneous environment considering both environmental conditions and the methanotroph assemblage (Mayr et al. 2020a). Therefore, we think that the epilimnion measurements are indeed representative for the mixed layer (epilimnion in this study) and only very small variabilities would be expected from multiple measurements. The focus of this study was not on variability within the mixed layer or within hypolimnion, but rather to demonstrate that differences in kinetic parameters occur at all, in contrasting microbial communities within one lake which has not been demonstrated before. Therefore, although we did not study the variation within a layer in this study, we had considerable previous knowledge on the same lake from the years before and designed the study on this basis.

The hypolimnion during stratification by definition shows very different conditions to the epilimnion, but gradients of methane and other parameters can be observed within the hypolimnion. Therefore, whereas the hypolimnion is less homogeneous than the epilimnion, the difference to the epilimnion is clear based on both, the environmental conditions (orders of magnitude higher methane concentrations) and a different methanotrophic assemblage (this study and Mayr et al. 2020a, 2020b).

Regarding the request for (biological) replication of the kinetic measurements, we have to note that, although measuring different depths and or replicates from the layers would be favourable, we had to adjust the amount of incubations to our handling limit of incubations. We decided to improve the replication for the respective depths to have enough data points to determine the affinity of the respective depth (see our answer on outlier elimination

Fig. 2.