Response to referee #4 (our answers in blue):

In this study, the role of AOM in Lake Kinneret sediment incubations was explored. Several incubations tested which terminal electron acceptors accounted for AOM activity. The main findings were that:

- pre-incubation with methane for 3 months significantly increased AOM
- hematite seemed the most likely iron mineral used as terminal electron acceptor (TEA) for AOM although it did not stimulate AOM and other iron minerals could have inhibited AOM
- natural humic acids and black coffee could be TEA for AOM
- sulfate-AOM was determined neglectable
- BES inhibition indicated that archaea mediated AOM, which was supported by metagenomic and 13C-lipid analyses

Major comments

It will improve the study to have its goals clarified in the introduction by L84-95. What were the specific research questions and knowledge gaps addressed here? What is this study addressing that was not known from your previous studies? This was still not clear to me after reading the entire manuscript. I think this is reflected in the title: “Modification of methane oxidation pathways during long-term incubations of methanic lake sediments” - I could not understand which modification occurred (bacterial methanotrophs did not thrive? TEA changed?). Think about a more specific title that summarizes the main key message of the study.

We thank the reviewer for the thorough and constructive review. As mentioned above, we realize that the paper was not clear enough. In the revised version, we clarified in the introduction the gap of knowledge, the aims and the hypothesis. We also edited the paper throughout. We also accept the comment about the title and changed it to: “Long-term incubations provide insights into the mechanism of AOM in methanogenic Lake Kinneret sediments.” This title summarizes the changes from oxygen and iron-oxides being the electron acceptors for methane oxidation, used by bacterial methanotrophs and methanogens, to potentially iron oxides and humic substances used by methanogens.

The materials and methods section needs major improvements for experiment reproducibility - adding amounts, concentrations, units, calculations etc. Sequencing data must be made available and an accession number must be provided. Data that has been already published and is here reproduced must be made clear.

In the revised version, we supply more details on the methods, so every treatment could be reproducible and clear. We accepted all comments below and edited the methods section completely. We moved Table S3 containing the details of the experiments to the main text, and a protocol for each experiment was added to the supplementary material.

All these incubations were done but no methane oxidation rates are provided in the manuscript, so calculating them and presenting them would add a lot of value.

We accept and appreciate this comment. We have added a new table with the different rates of AOM in all the experiments where methane concentrations were measured.

Metagenomics results were barely used (same goes for lipid data). Consider doing metabolic reconstruction of the MAGs recovered here or use this data for another study that explores metabolic potential and mechanisms of potential taxa responsible for Fe-AOM.

We now present in the main text the microbial data and discuss it more thoroughly. We added a principal component analysis biplot to the main text to discuss the difference between the three types of experiments and indicate the dominant taxa. Based on comments of another referee we decided not to explore the metabolic potential based on MAGs here but will do that in a separate publication.

Detailed comments

Review grammar of the manuscript. The manuscript has been edited by a native English speaker collaborator.

L42 - ANME between parentheses. Parentheses were added.

Intro: add background on the black coffee experiment - what was the hypothesis and the literature background? The black coffee was coffee grinds that were added as an organic source, however this treatment was removed from the manuscript, as suggested by the other reviewers.

2.1 add geographical coordinates of sampling site Coordinates were added.

2.2 indicate that concentrations of substrates in pre-incubated sediment experiments are provided in table S1 but bring this table to main text given that it is vital for the manuscript and experimental reproducibility. Also, add to this table similar details about the other two types of experiments (semi-bioreactor and incubation with recently collected material) which are so far missing from the methods section. Indicate if substrates were bought or synthesized (especially for minerals) with manufacturers / synthesis protocols. The methods section was re-organized as was similarly suggested by the other referees; the detailed table (Table S2) was moved to the main text. Details about the freshly collected sediments experiment and the semi-bioreactor were added to that table. The substrate data was added.

2.3 Name it “Porewater and gas analyses”? The name was changed altogether to “analytical methods”.

L202 can you provide methane detection limit in total amount (µmol) instead of concentration (µM)? Also, add the volume of gas injected into the GC? The detection limit was provided in total amount and the volume injected to the GC was added.

Eq 1 and 2: provide units for each term, label eq (1) in L205 and (2) in L206; invert eq (1) so it will be ð• • ¹ð• • ·ð• • ¼13ð□□¶ð□“ = ð□‘¥ × ð• • ¹13ð• • ¶ð• • »4 + (1 − ð• ‘ ¥) × ð• • ¹ð• • ·ð• • ¼13ð• • ¶ð• ‘ – Units were added. The equations labels were added. There seems to be a problem with the referee’s text, we could not read the suggested equation.

Also, can you add what was the final time used to calculate rates? Were rates derived from the slope or from the difference between T0 and T-final? Yes, all details appear now in the text and the protocols.

L161, section 2.2.2 - add bioreactor volume and manufacturer information? The bioreactor’s volume was 0.5 L and the bioreactor’s manufacturer is LENZ, Weinheim, Germany with custom-made lids. This detail was added to the text in the methods section of the semi-bioreactor section.

2.4 at L215 needs more details for experimental reproducibility: what was the sample exactly

(sediment? how many g?), concentrations of added compounds and steps - protocol format given that a modification of Sturt et al., 2004 was used. Suggestion: release the step-by-step protocol as supplemental material or zenodo link with doi number. Deposit sequencing data and add a data availability statement. Thanks for pointing this out. We added information on the amount and type of sample chosen for lipid extraction as well as the number of internal standards used. The text now reads: “A total lipid extract (TLE) was obtained from 0.4 to 1.6 g of the freeze-dried sediment or incubated sediment slurry using a modified Bligh and Dyer protocol (Sturt et al., 2004). Before extraction, 1 µg of 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine and 2-methyloctadecanoic acid were added as internal standards.” The extraction protocol in Sturt et al. is a modification of the original Bligh and Dyer method (1959), which itself is a modification of the preceding Folch et al. method (1957).

2.5 How were counts per million reads calculated? Add formula to methods here. Also, can you briefly list all tools that produced data part of this manuscript and are part of the SqueezeMeta pipeline? For instance, what did you use for MAG taxonomic classification? And for genome annotation / gene search? We now use coverage values instead of TPM, as suggested by the reviewer and used GTDB to classify MAGs. This is now clarified in the text as follows: “GTDB-Tk was used to classify the metagenome-assembled genomes (MAGs), based on Genome Taxonomy Database release 95 (Parks et al. 2021)”. Following suggestions elsewhere, we now refrain from any analyses involving annotations/gene searches and will discuss them in a separate publication.

I could not fully understand if and which results presented in this manuscript are already published (i.e. L115-117, L249-253). Can you please clarify this? Also, given that a number of different incubations were performed, I suggest numbering them consistently in text, tables and figures to facilitate tracking. The results that were published are the batch incubation experiment with the freshly collected sediments (the batch long term and the fresh bioreactor are new). This was clarified in the text. The different incubation experiments have been numbered.

L265-273 & Figure 2 = the most useful to me would be a plot of methane oxidation rates as a figure and, in the text, something like this: “treatment X or addition of X increased methane oxidation rates (in nmol/dry g sed/day to allow comparisons with other studies/settings) by X% relative to controls”. Also, in Fig 2, what is the difference between blue, red and yellow? Add this information in the legend. We added this information to the legend. Following the comment we also added a table where the rates from each experiments are presented.

Fig 2, 3 and 4 = Is it possible to improve the quality? Also, it would be great to have methane oxidation rates in the text or as a figure - from all these different incubations, the only number provided is “3-8 % of the 13C-methane” in L454, which should be presented as a rate - this information I would find most valuable from this study and would allow comparisons with data from other environments, which could be added to the discussion. The quality of the figures is low because they are embedded in the word file. The quality of the original figures is much better, and we will upload them as figure files. The data “3-8% of methanogenesis” is provided to show that even though the net process is methanogenesis, there is still substantial methane oxidation in the slurries. We wanted to compare to other studies that showed methane oxidation in a net methanogenesis environment. However, we agree with the referee, and we added a table with the rates of methane oxidation.

3.2 I suggest showing metagenomic results in the main manuscript. My suggestion is to make a heat map with MAG coverage normalized by metagenome size (instead of RPKM values) and add to this figure the info of Table S3. Also, instead of binscore, use MAG completeness and contamination (in %). Would also be good to know how many MAGs were reconstructed and which ones represent candidate iron reducers - FeGenie could be useful for that: https://doi.org/10.3389/fmicb.2020.00037. We now show a principal component

analysis biplot that shows changes in beta diversity and indicates the dominant taxa (Figure 5 in the main text), and describe it in much more details. Based on comments elsewhere, we decided not to include the metabolic potential based on MAGs, and we discuss it in a separate publication.

Table S4 I was surprised that mcrA and pmoA are not in this table! I think including these and iron reduction and extracellular electron transfer genes would be better use of your metagenomic datasets, which could be extensively better explored in this study. The updated table includes taxonomy only of much more MAGs (~195). As mentioned previously, we will explore the metabolism in a separate manuscript.

L328-331 The numbers here do not match Table 1, which shows more data than discussed here. Maybe this table is not so important and could go to supplemental materials? The lipid biomarker data are important for the study. To clarify the misunderstanding of the reviewer between relative ^{13}C-enrichment and absolute δ^{13}C values given in Table 1, we rewrote the text that now combines the two lines of information.

Table 1 = Can you clarify what exactly each incubation is and what are killed controls potentially present here? We clarified which incubations are presented in Table 1. Killed controls were tested for lipids in our previous study (Bar-Or et al., 2017) and showed indeed no enrichment. They are not presented in this table.

MAG coverages indicate Bathyarchaeota could be mediating Fe-AOM or play an indirect important role given that they are more abundant than ANME-1 - here the metabolic reconstruction of these MAGs would be fundamental! No mcrA was found in Bathyarchaeota - did you use an HMM that could find divergent sequences? what about other genes in reverse methanogenesis? what is Bathyarchaeota's metabolic potential in your incubations? We agree! As proper analyses of MAGs are expected to inflate this paper drastically, we are keeping this discussion for the follow-up study. We indeed intend to use HMM profiles.

From table S1 I assume hematite is the dominant iron mineral in lake sediments, is it? Then I find curious that this most promising terminal electron acceptor did not stimulate Fe-AOM while other iron minerals could have even inhibited AOM. Can these results alone be taken as evidence for Fe-AOM? I find them insufficient. More discussion is needed to hypothesize about what is happening and how to improve experimental conditions. The Fe-AOM was suggested in our previous studies with fresh methanogenic sediments of Lake Kinneret (Sivan et al., 2011; Bar-Or et al., 2017). In our current study, we examine if and how the methane oxidation changed in the two-stage incubated sediments (meaning that by the time the experiments were set up, the sediments are no longer fresh). In the two-stage incubation, we do not see a difference in the $\delta^{13}C_{DIC}$ between treatments with or without hematite, suggesting that either there is enough hematite to sustain the Fe-AOM, or that it is not the electron acceptor used for the AOM in these slurries. We agree that these results are insufficient to say that this is Fe-AOM, however, we cannot rule this option out. We elaborated the discussion regarding what is happening and added to the text ways to improve the experimental conditions.

In the semi-bioreactor experiment, why was little methane provided (when the methane headspace was replaced by anoxic liquid)? For how long were these semi-bioreactors operated? ~600 days? Also, any particular reason for calling them "semi" and not simply "bioreactors"? Finally, know that from our experience shaking biomass/sediments disrupts

AOM activity (related to L166-7). So, shaken and with little methane, I am not surprised to see in Fig 2 that there was no AOM detected in the bioreactor. In this manuscript, there is no discussion of bioreactor results, so I suggest to add something. We called it “semi” because it represents “semi-continuous flow” as porewater was exchanged weekly to biweekly during sampling. The initial dissolved methane concentration was established by temporarily creating a headspace of $^{13}CH_4$. After 24h equilibration time the headspace was replaced by anoxic porewater. This was the only way to add labeled methane to the reactor. The reactors operated for 677 days. We shook the system at the beginning when the methane head space was created, and after that only before sampling to make sure that the concentrations of the different constituents are homogenous. We did the same before sampling the bottles of our batch experiments and we never experienced any problem. We added more discussion regarding the bioreactor results.

L423 To enrich the discussion on 13C assimilation into lipid, I suggest addressing your results in the context of these findings and potentially more:

Wegener G, Niemann H, Elvert M et al. . Assimilation of methane and inorganic carbon by microbial communities mediating the anaerobic oxidation of methane. Environ Microbiol. 2008;10:2287–98.

Kellermann MY, Wegener G, Elvert M et al. . Autotrophy as a predominant mode of carbon fixation in anaerobic methane-oxidizing microbial communities. Proc Natl Acad Sci. 2012;109:19321–6.

Julia M Kurth, Nadine T Smit, Stefanie Berger, Stefan Schouten, Mike S M Jetten, Cornelia U Welte, Anaerobic methanotrophic archaea of the ANME-2d clade feature lipid composition that differs from other ANME archaea, FEMS Microbiology Ecology, Volume 95, Issue 7, July 2019, fiz082.

We thank the reviewer for these suggestions to support the fact of DIC assimilation by ANMEs/methanogens in our case. We have added these to the manuscript.

L426 move to results It was moved.

L426 Just because ANME are not very abundant it does not mean they are not (very) active. Here abundance is expressed as “< 1.5 %” - specify what this number refers to (relative abundance? how was this calculated? add to methods). We now report the abundance as coverage (~1 for ANME in all the metagenomic libraries). Their abundance is indeed low, but substantial (in top 27 of 195 MAGs, now presented in the Results). We certainly consider them as performing the AOM, and we suggest it in the text. For example, in the discussion we state that “ANME-1 are the likely mediators of AOM in these sediments, although some methanogens may be capable of oxidizing methane too through reverse methanogenesis (Elul et al. 2021).”

L443 I think it’s appropriate to tune this down: “we hypothesize Methanothrix could be involved in Fe-AOM”. High potential when ANME-1 is present and other archaea are more abundant is a bit stretching; but it would be nice to see some actual physiological evidence for the involvement of Methanothrix in Fe-AOM in the future. Here your back flux inferences also support ANME-1’s role being much larger than Methanothrix. We removed the statement regarding Methanothrix, and as mentioned above, emphasize the potential

involvement of ANME-1.

L469 Table S6 is for the first time mentioned here in the discussion. It presents qPCR results that have not been mentioned in the methods, so these must be added and the mention must be moved to results. Methanogenesis rates are expressed in µM/day, which I found cryptic and does not allow comparisons to other studies - please convert to n or µmol/dry g sed/day. The qPCR results of the mcrA gene were taken from the Bar-Or et al., 2017 study in order to provide a general order of magnitude estimation. The rates were calculated in this study. This was clarified in the text. The rates of methanogenesis were converted as suggested.

L470 I am missing and thus suggest adding a sentence hypothesizing about the key microorganisms (ANME-1) accounting for 3-8 % of 13C-methane oxidation to CO2 in these incubations. Also, what is this number referring to? Hematite-AOM? Humic acid-AOM? I would love to see rate comparisons between those! The sentence was added to the text as suggested. The number is referring to the slurries without any electron acceptor addition. We wanted to calculate how much of the produced methane is being oxidized in the basic environment of the slurries. However, as the referee suggested, we added the rates of oxidation in other treatments as well.

L481-8 I find this insufficient to explain why putative bacterial methanotrophs disappeared in long-term incubations if oxygen could be generated via methanobactins. However, this must be stated at hypothesis level, we don't know if iron reduction and methane oxidation were coupled via methanobactin-produced oxygen. I think it's better to offer other explanations or simply say it's unclear why bacterial methanotrophs disappeared. We do not know if O_2 is in fact produced in the natural sediments via methanobactins or another method, however, the fact that we do not see enrichment in their biomass in our two-stage experiments suggests that aerobic methanotrophy is no longer occurring in those slurries. The metagenomic analysis shows that their copy numbers are very low and do not increase with time. This implies that they are not active. Indeed, we do not know if the reason for that is lack of O_2, even though that is the most reasonable answer. This was clarified in the text.