Dear anonymous referee #‎‏4‏

We appreciate the time and effort that you dedicated to providing feedback on our manuscript and are ‎grateful for the insightful comments and suggestions. We hereby present point-by-point answers to the ‎issues raised (after each comment you will find a response paragraph).‎‏ ‏

We hope that the manuscript will now be suitable for publication in Biogeosciences‏.‏

Sincerely yours‏,‏

Michal Elul, on behalf of all co-authors‏ ‏

Rev#4‎

The manuscript by Elul et al reports the results of 16s amplicon and shotgun metagenomic analysis of a ‎narrow sediment horizon from Lake Kinneret. These DNA analyses were conducted on freshly sampled ‎sediment and sediment that had undergone the incubations characterized in detail in Bar-Or et al 2017. The ‎authors focus their attention on enzyme systems that may be associated with iron or methane cycling. The ‎authors provide information on the phylogenetic composition of the microbial community in general, as ‎well as assign phylogenetic composition to specific enzymes by BLASTing the metagenome reads against ‎the RefSeq database.‎

Response: We thank the reviewer for this thorough review. ‎

Major concerns: ‎

‎1) Insufficient information is given about the incubations which is needed to fully evaluate the likelihood of ‎the conclusions presented in the current work (most crucially, these incubations are methanogenic).

‎

Response: As requested by all the referees, we added section 3.1, named “Geochemical ‎evidence for iron coupled AOM in Lake Kinneret iron-rich methanic sediments”. In this ‎section, we describe the change in ferrous iron, δ13CDIC and methane concentrations with ‎time in the incubations. This section also includes a description of the concentrations of ‎relevant elements (methane, dissolved iron, manganese, nitrate, and sulfate) in this ‎investigated sedimentary methanogenic zone. We added also that these incubations are ‎indeed methanogenic (see more below). ‎

‎2) The suggestion that Methanothrix may carry out a methane oxidizing metabolism breaks with everything ‎that is known about this group, and the claim is not supported by any experimental data. This suggestion ‎should be removed.‎

Concerns 1&2: This manuscript is framed as a study that will draw significant insight from incubations. ‎Incubations with specific substrates or inhibitors can be very powerful tools in environmental ‎microbiology, particularly when the microbial community responds to the incubation conditions, and when ‎care is taken to clearly describe the bulk geochemical processes that have occurred in the incubations. ‎Unfortunately, this is not the case in this study, while I understand that the bulk of the description of the ‎incubations was previously published, a few key pieces of information have been left out of the current ‎manuscript. It would likely appear to a reader that these are incubations in which methane oxidation is the ‎dominant process since so much emphasis is put on AOM as compared to methanogenesis. AOM is the ‎most discussed metabolism in the abstract, and a major conclusion is the surprising attribution of AOM ‎metabolism to Methanothrix. However, these incubations are NOT carrying out the net oxidation of ‎methane, they are net methanogenic (see Figure 2b of Bar-Or 2017 “Positive methane concentrations ‎reflect net methanogenesis during iron-coupled AOM.”). To put the results more plainly: sequencing of ‎methanogenic incubations reveals a dominant archaeon that is a well-known methanogen. When stated in ‎this way, I cannot support the publication of such a speculative assignment of AOM activity to ‎Methanothrix. The simplest explanation is that the dominant methanogen is growing via the dominant ‎methane cycling process, i.e. methanogenesis. The justification for any discussion of AOM relies heavily on ‎the previous publication that found 13C methane was converted into 13C CO2, and this activity was ‎inhibited by BES. Methanogens carry out backflux of isotopic label from methane to CO2, and the authors ‎have cited the classic paper that shows this (Zehnder and Brock, 1979). Methanothrix could indeed be ‎responsible for the conversion of 13C methane into 13C CO2, but this observation does not constitute ‎evidence that they carry out net AOM in the environment or in these incubations. It is crucially important ‎for metabolisms that are so close to equilibrium for the authors to be very clear about whether they are ‎suggesting an organisms is making energy for growth by carrying out AOM, or whether the organism may ‎simply be responsible for the equilibration of isotope labels in the opposite direction of the process they ‎are using for energy generation. Another line of evidence for AOM is reaction-diffusion modeling that was ‎carried out on Lake Kinneret sediments (Adler et al 2011), which concluded that there was peak ‎methanogenesis 5-12cm below the sediment surface, and there was deeper AOM region under that. But ‎microbial 16s profiling carried out in Bar-Or et al 2015, did not show a significant change of methanothrix ‎‎(there referred to as methanosaeta) between the methanogenic and the methane oxidation zones. This is ‎a big claim the authors are trying to make, and it would require some sort of direct evidence like: 1) if there ‎was an incubation where AOM was the dominant processes and the authors were able to show that ‎methanothrix was the only organism present with the seven step methanogenesis pathway; 2) or better ‎yet that Methanothrix was enriched under these conditions vs. conditions without methane/Fe addition; 3) ‎or, upon the addition of methane (and Fe?) there was a positive reaction of methanothrix based on ‎metatranscriptome analyses, 4) or, at the very least that in nature there was a correlation between ‎methanothrix abundance and the horizons where methane oxidation is occurring. Unfortunately, the ‎community did not significantly change under any incubation condition (line 45), and there is no correlation ‎presented from the natural distribution of species, so there is no valid justification for assigning a novel ‎role to an organism that could just be making methane. Unless stronger evidence exists, all claims like the ‎one in line 375: "Our data hints that Methanothrix, which has not been considered to be involved in Fe-‎AOM previously, has the potential to be involved in methane oxidation, as presented in figure 5" should be ‎removed. ‎

Response

We thank the reviewer for this through discussion and fully agree and aware that in cases of ‎incubations with net methanogenesis a plausible explanation for the involvement of ‎methanogens (not the bacteria of course) can be through a back flux of the methanogenesis ‎process. Part of the work of our lab these days in several sets of incubations from different ‎settings is to try to separate between active (“true”) AOM and back flux of methanogenesis, ‎but it is beyond the scope of this biological study. This point regarding the methanogens was ‎probably not clear and discussed enough in the original manuscript, and is clarified and ‎discussed now in the revised version. Considering this, the methanogens that are involved in ‎the methane oxidation, in case it is back flux, can be indeed the main players in the system ‎which increased with depths or incubation time. We agree that due to the limited sample ‎size, statistical analyses of Bar-Or et al. 2015 results are impossible, but this study still ‎shows a trend, suggesting an increase in the read abundance of Methanothrix with depth and ‎time. However, we agree that we need to be much more careful at this stage, and in the ‎revised text, we use very cautious language when considering the involvement of ‎methanogens in this process (we write now methanogenic archaea in general). ‎

‎3) The authors do not carry out any calculations to support their claim that traditional ANME are not ‎abundant enough to carry out the trace AOM they claim to observe, and no effort is made to engage with ‎the thermodynamic feasibility of the processes they are proposing, which is fairly straightforward and ‎should be done.‎

Concern 3: If the authors reject the isotope backflux idea (there is not a clear quantitative argument ‎against this, even in Bar-Or et al 2017), and insist that there must be an organism subsisting on AOM in ‎their incubations, then it is unclear why the minor, traditional ANME organisms will not suffice. In the ‎abstract the authors write (lines 23-24) that “bonafide [sic] anaerobic oxidizers of methane (ANME) and ‎denitrifying methanotrophs Methylomirabilia (NC10) were scarce”, discounting their role in AOM in these ‎sediments. But then they highlight on line 25-26 “We show that putative aerobes, such as methane-‎oxidizing bacteria Methylomonas and their methylotrophic syntrophs methylotenera. . . can be involved in ‎the oxidation of methane. . .”. It is not at all clear why the authors feel that ANME should be discounted ‎while aerobic methanotrophs should be accepted as being responsible for methane oxidation. On line 176 ‎the authors say that 0.3-0.8% of their reads map to ANME-1. And the very next paragraph the authors ‎discuss the type I methanotrophs which are found to be 0.4-1.8% of the community. There is no ‎meaningful difference between 0.3-0.8% and 0.4- 1.8% in terms of abundance, so why do they feel ‎comfortable highlighting the possible role of aerobic methanotrophs at this abundance and not the ‎anaerobic ones? Why have the aerobic methane oxidizers made it into Fig 5 but the bona fide ANME have ‎not? AOM is not the dominant process, so its seems reasonable that if there is a small methane oxidizing ‎community that it could be carried out by normal methane oxidizers that are in low abundance. The only ‎way to rule this out is to determine the rate of AOM, try to estimate what 0.3-0.8% read mapping may ‎correspond to in terms of cell numbers, and then calculate a cell specific rate and show that this rate ‎seems far too high when compared to values present in the literature for ANME rates. None of this work is ‎done. When discussing possible metabolisms and their putative relative importance, it is very helpful to ‎discuss the thermodynamic feasibility of these reactions. But in the summary line 380-381 the authors ‎write “. . .whether this process [methanothrix AOM] is justified from the thermodynamic and kinetic ‎perspectives, remains to be elucidated.”. Doing the thermodynamic analysis should be a bare minimum ‎requirement when suggesting a remarkable new metabolism for an organism. What are the relative free ‎energies associated with acetoclastic methanogenesis and then Fe-AOM vs. acetate oxidizing iron ‎reduction? For a study that is essentially just a single metagenomic analysis (since there is no noteworthy ‎difference between any of the samples), the authors should at least attempt to supplement their discussion ‎with thermodynamic discussions. ‎

Response: We accept the comment, and based on our low AOM rates (~10-14 mol/cm3sec), ‎ANME-1 may be indeed involved in the AOM process despite its low numbers, and we now ‎state it in the abstract and all along. Please note that the involvement of Methanothrix in ‎AOM has been also previously suggested, ‎‎(https://www.sciencedirect.com/science/article/pii/S0048969720352062, ‎https://aem.asm.org/content/aem/83/11/e00645-17.full.pdf). ‎

Regarding the thermodynamics, active Fe-AOM is a possible competitive process in this zone ‎based on calculations that were done already in our previous studies. In short, it can be ‎shown that acetoclastic methanogenesis + Fe-AOM compared to acetoclastic iron reduction, ‎or hydrogenotrophic methanogenesis (more dominant at this depth, (Adler, 2016)) + Fe-AOM ‎compared to hydrogenotrophic iron reduction result in more or less the same negative Gibbs ‎energy of around -200 kJ/mol (see the excel calculation in the attached file). We ‎added the thermodynamic considerations to the revised version.‎

To summarize our response to the major comments, we are not rejecting the role of the back ‎flux. On the contrary, in our current lab work, we investigate it. Thus, we thank the reviewer ‎for the strong suggestion and encouragement to discuss it also in this paper and to be more ‎careful regarding the type of methanogens involved in methane oxidation. We, therefore, ‎write “methanogenic archaea” instead of “Methanothrix” when discussing AOM.‎

Minor comments: ‎

‎“Consortium” should not be used interchangeably with “community” especially in the context of AOM ‎research where “consortium” is very commonly used to refers to a physical, presumably syntrophic ‎association between two microorganisms. Since no evidence is provided about actually association ‎between any organisms described in this study “consortium” should be replaced throughout with ‎‎“community”. ‎

Response: We replaced “consortium” with “community” as suggested.‎

Line 361: “Our results show that in general, the phylogenetic diversity is a good predictor of the functional ‎diversity in these samples”. This is too broad of a statement for a paper that has a fairly narrow focus on ‎iron and methane cycling. ‎

Response: Although we highlight methane and iron cycling, we explored a wide array of ‎functions (section “General metabolic potential”, Fig. 2, Supplementary database 3). We, ‎however, agree that this statement is not necessary and remove it. ‎

Line 20: not clear what “intrinsic” means in this context. Are any organisms in this sample not intrinsic? ‎

Response: We removed “intrinsic” as suggested.‎

Line 63: Assigning Thermodesulfovibrio to a carbon oxidizing, iron reducing metabolism is wildly ‎speculative and should be removed unless more work is done to support the claim. The authors cite Spring ‎et al 1993 (indirectly, by way of BarOr et al 2015) for this claim. Spring et al does not make this claim, they ‎suggest as a throw-away hypothetical in the discussion section that it could be possible that ‎Magnetobacterium could gain energy from sulfide oxidation coupled to iron reduction. They had no ‎evidence for that claim, just suggested it was possible because Magnetobacterium has magnetosomes and ‎lives in sulfidic environments. If the authors want to follow up this speculation with analysis, then they ‎could look for the magnetosome genes in their metagenomes and see if they are phylogenetically aligned ‎with Magnetobacterium (see Lin et al 2014 for the genes in magnetobacterium, ‎https://www.nature.com/articles/ismej201494). If these thermodesulfovibrio have magnetosomes then ‎maybe its worth mentioning this, but even then, it is probably worth noting that there is no actual evidence ‎that these organisms can grow in this way. ‎

Response: The ability of some Themodesulfovibiro to grow using iron as electron acceptor ‎has been shown experimentally – for example, Frank et al. 2016 indicate that: “Besides ‎sulfate, strain N1 could also use sulfite, thiosulfate and Fe(III) as electron acceptors. However, ‎growth with Fe(III) as electron acceptor was slow.” ‎‎(https://www.frontiersin.org/articles/10.3389/fmicb.2016.02000/full). T. yellowstonii was ‎also considered previously as a potential iron reducer ‎‎(https://onlinelibrary.wiley.com/doi/abs/10.1111/gbi.12173). We added these citations to ‎the manuscript. ‎

Line 143-145: Here the use of “limiting nutrient” is confusing. This term often refers to something that is a ‎growth requirement because it is needed for the production of biomolecules or cofactors, P, N, Fe, etc. ‎This is a different concept than iron being used for the purpose of an electron acceptor, which seems to be ‎the focus of this study. Clarification is needed.‎

Response: Thank you for pointing this out. To avoid this issue, we changed “nutrient” to ‎‎“electron acceptor”.‎

Line 151: three groups are listed and then “3-6% read abundance, respectively”. Incorrect usage of ‎respectively, not clear what each groups abundance is. ‎

Response: We removed “respectively” from this sentence.‎

Line 158: class-level phylogenetic information should not be taken as evidence for the pH optimal for a ‎group (the authors actually cite a paper that describes how a different species of thermodesulfovibrio is ‎alkaliphilic as compared to other species in that genera). This is definitely is not evidence for acidic/basic ‎microenvironments.

‎

Response: ‎ Please note that we suggest that Thermodesulfovibrio are either neutrophilic or alkaliphilic. ‎We now add an additional citation to Sekiguchi et al. 2008 ‎‎(https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.2008/000893‎‎-0#tab2), which shows pH optima between 6.5 and 7.5 for various Thermodesulfovibrio ‎lineages. Candidatus Acidulodesulfobacterales, is often associated with pH <3 ‎‎(https://www.nature.com/articles/s41396-019-0415-y). In this sentence, we used careful ‎language (“hints”), as we agree that our findings don’t provide direct evidence for the ‎presence of microenvironments.‎

Line 378: “positive correlation between Methanosarcinales abundance and concentrations of reduced iron ‎in the deep sediment sections (Bar-Or et al 2017)”. This is a very strange claim and I cannot find any ‎significant data that supports it. Bar-Or 2017 does not include pore water profiles or depth profiles of ‎Methanosarcinales, so maybe this reference is supposed to be Bar-Or et al 2015? Even so, the data ‎presented in Bar-Or et al 2015 Figure 4 is single replicate from three depth points. It looks like the ‎difference between 6-9cm and 29-32cm for methanosarcinales is 50% -> 55% at most? With this level of ‎replication this is not a significant correlation that should be taken as evidence supporting ‎methanosarcinales being responsible for iron reduction.‎

Response: ‎Thank you for pointing out the mistake in the reference, this indeed refers to Bar-Or et al., ‎‎2015. As stated above, the number of samples in this study is indeed limited, yet a vertical ‎gradient in the abundance of Methanothrix was observed. In general, this paragraph uses a ‎now very careful language, as mentioned above. ‎

Figure 4: something is wrong with the description, or the data presented. For OmcS LK-2017 the number ‎next to the bar is 4, which the caption says corresponds to the number of total reads mapped to a gene. ‎That bar shows very fine delineations, “Deltaproteobacteria” is maybe 1/20th of the total area of the bar? ‎How can you get 1/20th with only 4 reads mapped? This comment applies to other bars in the OmcS figure. ‎Maybe worth revisiting how these were calculated? ‎

Response: These numbers are normalized per million reads, we adjusted the legend accordingly. ‎

Line 389: “Another possible explanation for the methylated compound leakage is the reversibility of the ‎enzymes involved in AOM, in particular methyl-CoM reductase”. Mcr does not may methylated compounds ‎like the ones the authors are referring to in the forward or reverse direction, so the reversibility of this ‎enzyme has nothing to do with this discussion.

‎

Response: As suggested, we removed “in particular methyl-CoM reductase” from the sentence.‎

Figure 5. The schematic in the top left shows iron reduction (Fe(III) -> Fe(II)) producing electrons‎

Thank you for pointing this out, we adjusted Figure 5 so the electron is either transferred to ‎Fe (III) or methanogens for methanogenesis. ‎