Biogeosciences Discuss.,
https://doi.org/10.5194/bg-2020-329-AC5, 2020
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Biogeosciences Discussions
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Interactive comment on "Metagenomic insights into the metabolism of microbial communities that mediate iron and methane cycling in Lake Kinneret sediments" *by* Michal Elul et al.

Michal Elul et al.

elul@post.bgu.ac.il

Received and published: 26 November 2020

Dear anonymous referee #âĂŐâĂŔ4âĂŔ We appreciate the time and effort that you dedicated to providing feedback on our manuscript and are âĂŐgrateful for the insightful comments and suggestions. We hereby present point-by-point answers to the âĂŐissues raised (after each comment you will find a response paragraph).âĂŐâĂŔ âĂŔ We hope that the manuscript will now be suitable for publication in BiogeosciencesâĂŔ.âĂŔ Sincerely yoursâĂŔ,âĂŔ Michal Elul, on behalf of all co-authorsâĂŔ âĂŔ

Rev#4âĂŐ The manuscript by Elul et al reports the results of 16s amplicon and shot-

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gun metagenomic analysis of a âĂŐnarrow sediment horizon from Lake Kinneret. These DNA analyses were conducted on freshly sampled âĂŐsediment and sediment that had undergone the incubations characterized in detail in Bar-Or et al 2017. The âĂŐauthors focus their attention on enzyme systems that may be associated with iron or methane cycling. The âĂŐauthors provide information on the phylogenetic composition of the microbial community in general, as âĂŐwell as assign phylogenetic composition to specific enzymes by BLASTing the metagenome reads against âĂŐthe RefSeq database.âĂŐ

Response: We thank the reviewer for this thorough review. âĂŐ

Major concerns: âĂŐ âĂŐ1) Insufficient information is given about the incubations which is needed to fully evaluate the likelihood of âĂŐthe conclusions presented in the current work (most crucially, these incubations are methanogenic). âĂŐ Response: As requested by all the referees, we added section 3.1, named “Geochemical âĂŐevidence for iron coupled AOM in Lake Kinneret iron-rich methanic sediments”. In this âĂŐsection, we describe the change in ferrous iron, δ13CDIC and methane concentrations with âĂŐtime in the incubations. This section also includes a description of the concentrations of âĂŐrelevant elements (methane, dissolved iron, manganese, nitrate, and sulfate) in this âĂŐinvestigated sedimentary methanogenic zone. We added also that these incubations are âĂŐindeed methanogenic (see more below). âĂŐ

âĂŐ2) The suggestion that Methanothrix may carry out a methane oxidizing metabolism breaks with everything âĂŐthat is known about this group, and the claim is not supported by any experimental data. This suggestion âĂŐshould be removed.âĂŐ Concerns 1&2: This manuscript is framed as a study that will draw significant insight from incubations. âĂŐIncubations with specific substrates or inhibitors can be very powerful tools in environmental âĂŐmicrobiology, particularly when the microbial community responds to the incubation conditions, and when âĂŐcare is taken to clearly describe the bulk geochemical processes that have occurred in the incubations. âĂŐUnfortunately, this is not the case in this study, while I understand that the bulk

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of the description of the âĂŐincubations was previously published, a few key pieces of information have been left out of the current âĂŐmanuscript. It would likely appear to a reader that these are incubations in which methane oxidation is the âĂŐdominant process since so much emphasis is put on AOM as compared to methanogenesis. AOM is the âĂŐmost discussed metabolism in the abstract, and a major conclusion is the surprising attribution of AOM âĂŐmetabolism to Methanothrix. However, these incubations are NOT carrying out the net oxidation of âĂŐmethane, they are net methanogenic (see Figure 2b of Bar-Or 2017 “Positive methane concentrations âĂŐreflect net methanogenesis during iron-coupled AOM.”). To put the results more plainly: sequencing of âĂŐmethanogenic incubations reveals a dominant archaeon that is a well-known methanogen. When stated in âĂŐthis way, I cannot support the publication of such a speculative assignment of AOM activity to âĂŐMethanothrix. The simplest explanation is that the dominant methanogen is growing via the dominant âĂŐmethane cycling process, i.e. methanogenesis. The justification for any discussion of AOM relies heavily on âĂŐthe previous publication that found 13C methane was converted into 13C CO2, and this activity was âĂŐinhibited by BES. Methanogens carry out backflux of isotopic label from methane to CO2, and the authors âĂŐhave cited the classic paper that shows this (Zehnder and Brock, 1979). Methanothrix could indeed be âĂŐresponsible for the conversion of 13C methane into 13C CO2, but this observation does not constitute âĂŐevidence that they carry out net AOM in the environment or in these incubations. It is crucially important âĂŐfor metabolisms that are so close to equilibrium for the authors to be very clear about whether they are âĂŐsuggesting an organisms is making energy for growth by carrying out AOM, or whether the organism may âĂŐsimply be responsible for the equilibration of isotope labels in the opposite direction of the process they âĂŐare using for energy generation. Another line of evidence for AOM is reaction-diffusion modeling that was âĂŐcarried out on Lake Kinneret sediments (Adler et al 2011), which concluded that there was peak âĂŐmethanogenesis 5-12cm below the sediment surface, and there was deeper AOM region under that. But âĂŐmicrobial 16s profiling carried out in Bar-Or et al 2015, did not show a sig-

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nificant change of methanothrix âĂŐâĂŐ(there referred to as methanosaeta) between the methanogenic and the methane oxidation zones. This is âĂŐa big claim the authors are trying to make, and it would require some sort of direct evidence like: 1) if there âĂŐwas an incubation where AOM was the dominant processes and the authors were able to show that âĂŐmethanothrix was the only organism present with the seven step methanogenesis pathway; 2) or better âĂŐyet that Methanothrix was enriched under these conditions vs. conditions without methane/Fe addition; 3) âĂŐor, upon the addition of methane (and Fe?) there was a positive reaction of methanothrix based on âĂŐmetatranscriptome analyses, 4) or, at the very least that in nature there was a correlation between âĂŐmethanothrix abundance and the horizons where methane oxidation is occurring. Unfortunately, the âĂŐcommunity did not significantly change under any incubation condition (line 45), and there is no correlation âĂŐpresented from the natural distribution of species, so there is no valid justification for assigning a novel âĂŐrole to an organism that could just be making methane. Unless stronger evidence exists, all claims like the âĂŐone in line 375: "Our data hints that Methanothrix, which has not been considered to be involved in Fe-âĂŐAOM previously, has the potential to be involved in methane oxidation, as presented in figure 5" should be âĂŐremoved. âĂŐ

Response We thank the reviewer for this through discussion and fully agree and aware that in cases of âĂŐincubations with net methanogenesis a plausible explanation for the involvement of âĂŐmethanogens (not the bacteria of course) can be through a back flux of the methanogenesis âĂŐprocess. Part of the work of our lab these days in several sets of incubations from different âĂŐsettings is to try to separate between active (“true”) AOM and back flux of methanogenesis, âĂŐbut it is beyond the scope of this biological study. This point regarding the methanogens was âĂŐprobably not clear and discussed enough in the original manuscript, and is clarified and âĂŐdiscussed now in the revised version. Considering this, the methanogens that are involved in âĂŐthe methane oxidation, in case it is back flux, can be indeed the main players in the system âĂŐwhich increased with depths or incubation time. We agree that due

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to the limited sample âĂŐsize, statistical analyses of Bar-Or et al. 2015 results are impossible, but this study still âĂŐshows a trend, suggesting an increase in the read abundance of Methanothrix with depth and âĂŐtime. However, we agree that we need to be much more careful at this stage, and in the âĂŐrevised text, we use very cautious language when considering the involvement of âĂŐmethanogens in this process (we write now methanogenic archaea in general). âĂŐ

âĂŐ3) The authors do not carry out any calculations to support their claim that traditional ANME are not âĂŐabundant enough to carry out the trace AOM they claim to observe, and no effort is made to engage with âĂŐthe thermodynamic feasibility of the processes they are proposing, which is fairly straightforward and âĂŐshould be done.âĂŐ Concern 3: If the authors reject the isotope backflux idea (there is not a clear quantitative argument âĂŐagainst this, even in Bar-Or et al 2017), and insist that there must be an organism subsisting on AOM in âĂŐtheir incubations, then it is unclear why the minor, traditional ANME organisms will not suffice. In the âĂŐabstract the authors write (lines 23-24) that “bonafide [sic] anaerobic oxidizers of methane (ANME) and âĂŐdenitrifying methanotrophs Methylomirabilia (NC10) were scarce”, discounting their role in AOM in these âĂŐsediments. But then they highlight on line 25-26 “We show that putative aerobes, such as methane-âĂŐoxidizing bacteria Methylomonas and their methylotrophic syntrophs methylotenera. . . can be involved in âĂŐthe oxidation of methane. . .”. It is not at all clear why the authors feel that ANME should be discounted âĂŐwhile aerobic methanotrophs should be accepted as being responsible for methane oxidation. On line 176 âĂŐthe authors say that 0.3-0.8% of their reads map to ANME-1. And the very next paragraph the authors âĂŐdiscuss the type I methanotrophs which are found to be 0.4-1.8% of the community. There is no âĂŐmeaningful difference between 0.3-0.8% and 0.4- 1.8% in terms of abundance, so why do they feel âĂŐcomfortable highlighting the possible role of aerobic methanotrophs at this abundance and not the âĂŐanaerobic ones? Why have the aerobic methane oxidizers made it into Fig 5 but the bona fide ANME have âĂŐnot? AOM is not the dominant process, so its seems reasonable that if there is a small methane oxidizing âĂŐcommu-

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nity that it could be carried out by normal methane oxidizers that are in low abundance. The only âĂŐway to rule this out is to determine the rate of AOM, try to estimate what 0.3-0.8% read mapping may âĂŐcorrespond to in terms of cell numbers, and then calculate a cell specific rate and show that this rate âĂŐseems far too high when compared to values present in the literature for ANME rates. None of this work is âĂŐdone. When discussing possible metabolisms and their putative relative importance, it is very helpful to âĂŐdiscuss the thermodynamic feasibility of these reactions. But in the summary line 380-381 the authors âĂŐwrite ". . .whether this process [methanothrix AOM] is justified from the thermodynamic and kinetic âĂŐperspectives, remains to be elucidated.". Doing the thermodynamic analysis should be a bare minimum âĂŐrequirement when suggesting a remarkable new metabolism for an organism. What are the relative free âĂŐenergies associated with acetoclastic methanogenesis and then Fe-AOM vs. acetate oxidizing iron âĂŐreduction? For a study that is essentially just a single metagenomic analysis (since there is no noteworthy âĂŐdifference between any of the samples), the authors should at least attempt to supplement their discussion âĂŐwith thermodynamic discussions. âĂŐ

Response: We accept the comment, and based on our low AOM rates (∼10-14 mol/cm3sec), âĂŐANME-1 may be indeed involved in the AOM process despite its low numbers, and we now âĂŐstate it in the abstract and all along. Please note that the involvement of Methanothrix in âĂŐAOM has been also previously suggested, âĂŐâĂŐ(https://www.sciencedirect.com/science/article/pii/S0048969720352062, âĂŐhttps://aem.asm.org/content/aem/83/11/e00645-17.full.pdf). âĂŐ Regarding the thermodynamics, active Fe-AOM is a possible competitive process in this zone âĂŐbased on calculations that were done already in our previous studies. In short, it can be âĂŐshown that acetoclastic methanogenesis + Fe-AOM compared to acetoclastic iron reduction, âĂŐor hydrogenotrophic methanogenesis (more dominant at this depth, (Adler, 2016)) + Fe-AOM âĂŐcompared to hydrogenotrophic iron reduction result in more or less the same negative Gibbs âĂŐenergy of around -200 kJ/mol (see the excel calculation in the attached file). We âĂŐadded the thermodynamic

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considerations to the revised version.âĂŐ

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

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

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

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

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

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

Response: We removed “intrinsic” as suggested.âĂŐ

Line 63: Assigning Thermodesulfovibrio to a carbon oxidizing, iron reducing metabolism is wildly âĂŐspeculative and should be removed unless more work is done to support the claim. The authors cite Spring âĂŐet al 1993 (indirectly, by way of BarOr

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et al 2015) for this claim. Spring et al does not make this claim, they âĂŐsuggest as a throw-away hypothetical in the discussion section that it could be possible that âĂŐMagnetobacterium could gain energy from sulfide oxidation coupled to iron reduction. They had no âĂŐevidence for that claim, just suggested it was possible because Magnetobacterium has magnetosomes and âĂŐlives in sulfidic environments. If the authors want to follow up this speculation with analysis, then they âĂŐcould look for the magnetosome genes in their metagenomes and see if they are phylogenetically aligned âĂŐwith Magnetobacterium (see Lin et al 2014 for the genes in magnetobacterium, âĂŐhttps://www.nature.com/articles/ismej201494). If these thermodesulfovibrio have magnetosomes then âĂŐmaybe its worth mentioning this, but even then, it is probably worth noting that there is no actual evidence âĂŐthat these organisms can grow in this way. âĂŐ

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

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

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

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

Response: We removed “respectively” from this sentence.âĂŐ

Line 158: class-level phylogenetic information should not be taken as evidence for the pH optimal for a âĂŐgroup (the authors actually cite a paper that describes how a different species of thermodesulfovibrio is âĂŐalkaliphilic as compared to other species in that genera). This is definitely is not evidence for acidic/basic âĂŐmicroenvironments. âĂŐ Response: âĂŐ Please note that we suggest that Thermodesulfovibrio are either neutrophilic or alkaliphilic. âĂŐWe now add an additional citation to Sekiguchi et al. 2008 âĂŐâĂŐ(https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.2008/000893âĂŐâĂŐ-0#tab2), which shows pH optima between 6.5 and 7.5 for various Thermodesulfovibrio âĂŐlineages. Candidatus Acidulodesulfobacterales, is often associated with pH <3 âĂŐâĂŐ(https://www.nature.com/articles/s41396-019-0415-y). In this sentence, we used careful âĂŐlanguage (“hints”), as we agree that our findings don’t provide direct evidence for the âĂŐpresence of microenvironments.âĂŐ

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

Response: âĂŐThank you for pointing out the mistake in the reference, this indeed

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refers to Bar-Or et al., âĂŐâĂŐ2015. As stated above, the number of samples in this study is indeed limited, yet a vertical âĂŐgradient in the abundance of Methanothrix was observed. In general, this paragraph uses a âĂŐnow very careful language, as mentioned above. âĂŐ

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

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

Line 389: “Another possible explanation for the methylated compound leakage is the reversibility of the âĂŐenzymes involved in AOM, in particular methyl-CoM reductase”. Mcr does not may methylated compounds âĂŐlike the ones the authors are referring to in the forward or reverse direction, so the reversibility of this âĂŐenzyme has nothing to do with this discussion. âĂŐ Response: As suggested, we removed “in particular methyl-CoM reductase” from the sentence.âĂŐ Figure 5. The schematic in the top left shows iron reduction (Fe(III) -> Fe(II)) producing electronsâĂŐ Thank you for pointing this out, we adjusted Figure 5 so the electron is either transferred to âĂŐFe (III) or methanogens for methanogenesis. âĂŐ

Please also note the supplement to this comment:
https://bg.copernicus.org/preprints/bg-2020-329/bg-2020-329-AC5-supplement.zip

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Interactive comment on Biogeosciences Discuss., https://doi.org/10.5194/bg-2020-329, 2020.

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