

## ***Interactive comment on “Intersecting Methane Production and Oxidation Zones in Freshwater Sediments” by Xueping Chen et al.***

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Reviewer 1 General comments: The paper of Chen et al investigated the pathways of methane production and oxidation in sediment of a freshwater reservoir. The authors collected several datasets on biogeochemical parameters, incubations and molecular inventory. The data are potentially interesting, but the analysis and interpretation are poor. The authors did not consider including in the analysis several important microorganism groups (e.g. ANME), nitrate concentrations were not measured; the molecular analysis was in general relatively superficial. The discussion section contained only few references and did not put the results into perspective with already available literature. The figures were of poor quality and partly showed false claims (Fig4). The authors should consider asking a native speaker to correct the language of the manuscript. I

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suggest re-writing the manuscript which should include an in-depth analysis of molecular data, stoichiometric calculations (see comment below) and careful integration of all data. The authors should also make themselves familiar with recent literature available of AOM.

Thanks for the comments. We would reanalysis the functional groups based on the Illumina sequences. In the discussion section, we would focus on the complex microbial community involved in methane cycle and the controlling parameters as another reviewer suggested. We would ask a native speaker to polish the English. We have explained in detail below. Again, we would re-analysis the data to integrate of all data, and rewrite the manuscript.

Specific comments: Line 76: the authors should cite Haroon et al 2013 for nitrate dependent AOM. Thanks. We would supplement the reference of “Haroon M F, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, Yuan Z and Tyson G W 2013 Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage, Nature 500 567–70.”

Line 131: a subsample of what? It would be “a subsample of the sediment”

Line 137: At an in-situ pH of 8, formic acid/ acetic acid/ propionic acid would be present as formate/ acetate/ propionate. Thanks for the comments. We confirmed that it should be formate, acetate and propionate.

Please correct this Lines 152-157: It is unclear to me how and in what form acetate was added to the sediment. Was the sediment slurried with liquid in order to achieve homogenous distribution? Sorry for the vague statement. The water content of the sediment ranged from 56.5%-84.5%, which look like slurry. The acetate (30 mmol L<sup>-1</sup>) was added as solution (0.5 mL each bottle) as final concentration of 3.0 mmol kg<sup>-1</sup> dry sediment. After the acetate added, the bottles were vortex softly. We would describe the process in detail in the text.

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Line 155: "thick butyl rubber stoppers": what kind? Please indicate the manufacturer and type. It is known from previous studies that black rubber stoppers inhibit anaerobic methane oxidation. Since the authors used same stoppers for AOM experiments, the obtained results might be false. Thanks for reminding, and we would use alternative stopper for future study. In this study, we indeed used the black rubber stoppers for incubation, which are used for repeated sampling the headspace by syringe inject during the whole incubation period. We have set control of sterilized sediment for the AOM activity, and subsequently the AOM was inhibit. Could you please kindly tell us the literature on the inhibit effect of the black rubber stoppers, we didn't find it regretfully.

Lines 159-160: Instead of indicating what volume was added, indicate the final headspace concentration, headspace volume and headspace pressure. I assume non-labelled methane was added? Thanks. We would supplement the final headspace concentration of methane, which was non-labelled.

Lines 184-185: How were the data normalized? How many sequences per sample were used for final analysis? The data obtained by MiSeq sequencing is paired-end sequence data. The data were first optimize for effective data statistics, and then remove the sequence to screen out unmatched sequences using FLASH and Trimmomatic softwares. Furthermore, according to different similarity levels, all sequences are divided into OTUs, and bioinformatics statistical analysis is usually performed on OTUs at 97% similarity level by Uparse Software platform (vsesion 7.1 <http://drive5.com/uparse/>). Specific steps are as follows, 1) Extract non-repetitive sequences from optimized sequences to reduce the amount of redundant calculations in the middle of analysis (<http://drive5.com/usearch/manual/dereplication.html>); 2) Remove single sequences that are not repeated (<http://drive5.com/usearch/manual/singletons.html>); 3) Perform OTU clustering on non-repetitive sequences (excluding single sequences) according to 97% similarity, remove chimeras in the clustering process, and obtain representative sequences of OTU; Finally, we obtained 561 817 (2155 OTU) and 2 067 765 (1885

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OTU) unique sequences for bacteria and archaea, respectively. Since the number of sequences in each sample are different, we flattened all samples for further analysis, and the number of the sequences used in analysis were set as 19373 and 19693 per sample for bacteria and archaea, respectively. We would describe in detail in the text

Line 187: Please indicate the settings used in the pipeline. In order to obtain the species classification corresponding to each OTU, the RDP classifier is Each OTU representative sequence used to perform taxonomic analysis by alignment database Silva (Release132 <http://www.arb-silva.de>) at the 97% similar level based on Bayes algorithm . The software and algorithm are QIIME platform ([http://qiime.org/scripts/assign\\_taxonomy.html](http://qiime.org/scripts/assign_taxonomy.html)), RDP Classifier (version 2.2 <http://sourceforge.net/projects/rdp-classifier/>), and the default confidence threshold is 0.7.

Line 192: Silva108 is an old release dating back to 2011. Why was such an old version used? Sorry for the misleading information. It should be Silva132.

Lines 193-195: It is not clear what was done. All steps and settings should be specified. The obtained data should be reproducible by other researchers. Sanger is an online biometric analysis website developed by Shanghai Meiji Biotechnology Co., Ltd. It contains various R language packages for biometric analysis, and users can perform various operations through simple operations. It includes biometric analysis including dilution curve, diversity index analysis, statistical analysis of community structure at each taxonomic level, and community composition, multi-sample species distribution, principal component analysis (PCA), and redundancy I analysis (RDA)... We would specified the analysis used in this study.

Lines 201-203: What are these primers? No reference, no information of specificity, no information on binding sites Line 222/Fig1: What is the oxygen diffusion depth in this sediment core? Why was nitrite measured but not nitrate? What form of iron was measured, I suppose Fe(II)? The primers were coding the functional genes (mcrA) for

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methanogens, we would supplement the reference for the primers. It is a pity we didn't measure the oxygen diffusion, however, the penetration depths of O<sub>2</sub> were less than 4 mm, with the mean value of 1.3 mm in summer and 3.2 mm in winter according to the work of Wang et al., (2016). We have verified that the nitrite should be instead of nitrate with the analytical center. Dissolved iron was determined by inductively coupled plasma mass-spectrometry, which were not distinct the valence of iron, however, we totally agree with you that is supposed to be ferrous under the weakly alkaline environment. Jingfu, Wang, Jingan, et al. Effects of seasonal hypoxia on the release of phosphorus from sediments in deep-water ecosystem: A case study in Hongfeng Reservoir, Southwest China.[J]. Environmental Pollution, 2016.

Lines 272-274: What is 100 The relative abundance of methanogens were calculated based on the ratio of reads numbers of methanogens to all the archaea reads numbers. We would explain in the figure legend.

Lines 291-292: I suppose NC10 includes all sequences assigned to this phylum? I hope the authors are aware that so far only some members from clade A were shown to perform AOM. Other members of this phylum were never shown to perform AOM and are likely to have different metabolisms. Thanks for your comments. We discussed with other researchers and we would improve the precision of the figures by constructing a local library of functional groups, however, it would need more time to construct the library.

Line 293: Nitrate reductase? The method section does not describe any analysis of nitrate reductases. The authors refer to Figure 4D where the relative abundance of NC10 sequences is shown. What does this mean? Figure4: What do percentages refer to, total bacteria? Why are some groups analyzed down to genus level and others not? NC10 comprises the whole phylum, what is the relative abundance of Methyloirabilis sequences? The percentages refer to total bacteria. As mentioned above, we would revised the figure 4 by reanalysis the data.

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Line 310: Can this claim be backed up by molecular data on known aerobic methanotrophs? I missed that data in the results section Yes, we agree with you. However, we have tried to quantify the functional gene of aerobic methanotrophs (e.g. pmoA gene) and which were poorly amplified. We would verify the abundance of aerobic methanotrophs.

Line 315: Figure 1 does not contain data on nitrate concentrations. I also miss nitrate data for proper interpretation of AOM driven by N oxides Sorry for misleading information. We have verified that the nitrite should be instead of nitrate with the analytical center.

Line 316: Authors claim that nitrite-dependent AOM took place in deeper layers. Where does the nitrite come from if oxygen only penetrates within the upper cm of the sediment profile? Sorry for misleading information. We have verified that the nitrite should be instead of nitrate with the analytical center. It is known from literatures that both nitrification and denitrification (Kuypers M M M , Marchant H K , Kartal B. The microbial nitrogen-cycling network [J]. Nature Reviews Microbiology, 2018, 16(5).) are active in the sediment. The Hongfeng reservoir in this study is eutrophic, and we have found quite high amount of functional bacterial involved in nitrification and denitrification in the surface sediment by quantity PCR, and thus we suppose that there would be nitrate and nitrite in the sediment. However, the oxygen only penetrates in the upper mm of the sediment profile (Wang et al., 2016), the amount of nitrate and the intermediate products of nitrite are quite low along the sediment profile

Lines 314-316: This is very vague Sorry for the vague statement. As mentioned above, we didn't detected the aerobic methanotrophs. We would delete the sentence.

Lines 325-328: The authors discuss very high AOM activities based on bottle incubations. The authors did not add any extra electron acceptors into those bottles which means that methane oxidation is sustained by in-situ electron acceptors. It is in my view an essential step to calculate the stoichiometries: how many electron

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acceptors were present in respective samples and how do concentrations correlate to methane consumption activities? Thanks for your comments. The potential electron acceptors include sulfate, nitrate and metals.  $\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O}$   
 $3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O}$   $5\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 5\text{CO}_2 + 4\text{N}_2 + 14\text{H}_2\text{O}$   
 $\text{CH}_4 + 4\text{MnO}_2 + 7\text{H}^+ \rightarrow \text{HCO}_3^- + 4\text{Mn}^{2+} + 5\text{H}_2\text{O}$   $\text{CH}_4 + 8\text{Fe}(\text{OH})_3 + 15\text{H}^+ \rightarrow \text{HCO}_3^- + 8\text{Fe}^{2+} + 21\text{H}_2\text{O}$  The sulfate concentration of sulfate, iron and nitrate was ranged in 0.1-0.6 mM, 10-44  $\mu\text{M}$ , and 5.9-24.8  $\mu\text{M}$ , respectively, and the methane oxidation rate was ranged in 1.37- 6.47  $\mu\text{g CH}_4 \text{ g}^{-1} \text{ sediment d}^{-1}$  (0.085-0.40  $\mu\text{M g}^{-1} \text{ sediment d}^{-1}$ ), which could be sustained by in-situ electron acceptors. We would supplement the stoichiometries analysis in the text.

Lines 340-342: The authors should be very careful with such statements. Activity does not directly correspond with the number of genes. Thanks, we would revise the sentence.

Line 348: I don't understand the sentence. Please rewrite Thanks, we would revise the sentence.

Lines 351-354: This is very vage. Stating the metabolism type based on 16S characterization at order level is farfetched. I expect characterization at genus level. Also, there are no references! Thanks, we would supplement the methanogens at genus level and references.

Line 363: Methanosarcina uses acetate, not acetic acid. This is basic physiology. Thanks, we would revise the sentence.

Lines 378-379: I did not see evidence for this claim from the presented data. Fig6: The figure contains several false claims. Not all NC10 perform AOM but so far only one genus, Methyloirabilis; Methyloirabilis does not use nitrate; how are metal oxides supposed to catalyze sulfate reduction to sulfide? The ANME abbreviation stands for anaerobic methane oxidizing archaea which were not discussed in this study at all. Why are NOB mentioned here? Sorry for the misleading of figure 6. As mentioned

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above, we would reanalysis the functional groups

Line 406: "undetected iron minerals". Were any analyzes performed? We performed X-ray diffraction (XRD) to determine the mineral composition of the sediments and only signal of carbonate were presented, without obvious signal of iron minerals.

Line 415: "high abundance of nitrate reductase". What does this supposed to mean? We have analysis the amount of nitrate reductase, and which supposed to be connected with nitrate reduction. However, we did not present the data in the manuscript. We would revise the sentence.

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Interactive comment on Biogeosciences Discuss., <https://doi.org/10.5194/bg-2020-330>, 2020.

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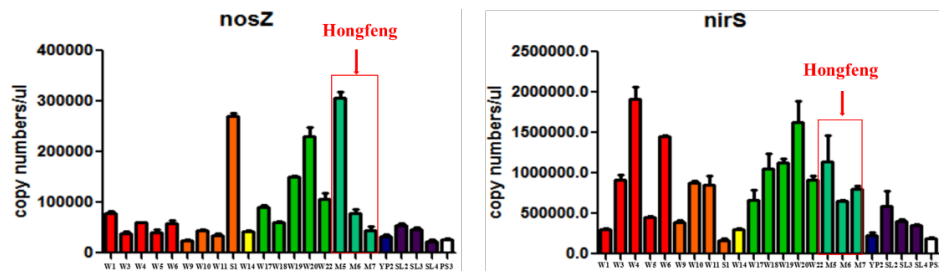


Fig. 1.