



EGUsphere, author comment AC3
<https://doi.org/10.5194/egusphere-2022-65-AC3>, 2022
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Reply on RC2

Cordula Nina Gutekunst et al.

Author comment on "Effects of brackish water inflow on methane-cycling microbial communities in a freshwater rewetted coastal fen" by Cordula Nina Gutekunst et al., EGU sphere, <https://doi.org/10.5194/egusphere-2022-65-AC3>, 2022

Dear reviewer2,

Many thanks for your feedback, which we find constructive and very valuable. Especially, we thank you for spotting that the methods used to compare microbial community abundance and composition with earlier studies were not well described. First of all, we want to assure you that the methods in the different studies are comparable and in fact we put a lot of emphasis on comparability of the approaches being aware of potential flaws associated with DNA extraction methods, PCR protocols, sequence data processing etc. All microbial analyses were done in the same lab, using the same DNA and RNA extraction kits and the same primer combinations for the qPCR. The PCR used to amplify and tag the individual samples for sequencing was also done in the same way as the previous sister study. Only the specific bacterial primer used earlier (S-D-Bact-0341-b-S-17/S-DBact-0785-a-A-21) was replaced by a universal primer targeting both bacteria and archaea with, however, the same resolution for bacteria. In all studies, archaea were amplified separately to get an in-depth analysis of archaeal community composition. When re-editing the manuscript, we will try to make the comparability of the three studies clearer by including the following statements into the manuscript:

l. 144: "The microbial analysis was conducted in the same laboratory and strictly followed the same protocols regarding DNA and RNA extraction and the usage of the primer combinations during sequencing and qPCR. Minor adaptations due to improved technologies are marked accordingly in the relevant subchapter of the method section."

l. 273: "Please note that Wen et al. (2018) and Unger et al. (2021) used a specific bacterial primer combination (S-D-Bact-0341-b-S-17/S-DBact-0785-a-A-21) instead of the universal primer we used here. We decided for the universal primer, because it has equal resolution for bacteria, but covers both, bacteria and archaea providing some back-up of the sequencing and qPCR data."

l. 307: "All sequencing reads, including those from Wen et al. (2018) and Unger et al. (2021) were merged into a common ASV file which provided the basis for all following analyses."

We would also like to draw your attention to existing lines in the manuscript, where we describe the normalization process of all data used (Wisconsin double standardization) to create the bubble plots (Fig. 4) in l. 328 and to create the NMDS ordination (Fig. 6) in l.

333.

Regarding your question concerning the data depository, please see our reply to reviewer1's second major comment:

'Yes, you are totally correct, the accession numbers will be included into the text. The reason for the incomplete statement in the manuscript under "Data availability" is that the manuscript submission was done before the uploading of the data. We are planning to include the following paragraph into the manuscript in l. 680 and replace the sentence, which is currently there:

"The data for all 97 post-inflow samples have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB52161 (with sample accession numbers ERS11559347-ERS11559443). Baseline2014 data can be found at EBI under the BioProject PRJNA356778 (accession numbers are SRR5118134 - SRR5118155 and SRR5119428 - SRR5119449) and Drought2018 data were deposited at ENA under BioProject accession number PRJEB38162 (sample accession number ERS4542720-ERS4542735, ERS4542752-ERS4542767, ERS4542784- ERS4542800 and ERS4542822- ERS4542837). Depth profile data can be provided by the corresponding authors upon request and will be uploaded to the Pangaea database in the near future." '

Line comments:

Line 33: I find this sentence a bit confusing to read. Perhaps remove "also"?

Reply: Thank you for your comment. The "also" was there to emphasize the fact that the drought lead to the results we see IN ADDITION to the brackish water inflow. We would like to suggest the following change: "We found that both, the inflow effect of brackish water and the preceding drought increased the sulfate availability in the surface and pore water."

Line 46: do the authors intend "loose" instead of "lose"?

Reply: Yes, thank you very much for the careful read. We meant "loose" and will change the text accordingly.

Line 112-114: sentence a bit confusing to read, do the authors intend "Thus" instead of "This"? In particular the " and, therefore can explain" is causing some befuddlement.

Reply: Thank you very much for the hint. We meant "this", because it links to the previous sentence and refers to the increase of SRB at the expense of methanogens. This increase together with an increase of ANMEs should lead to a decrease of methane production. We will try to improve the sentence and suggest the following: "The increase of SRB in conjunction with an anticipated increasing abundance of sulfate-dependent anaerobic methanotrophic archaea (ANMEs) should decrease methane production and, therefore can explain the reported decrease in methane emissions."

Line 198: Can you please clarify how samples were kept cold and if they were kept

anoxic? I would be concerned that collecting the soils, cooling them on ice (in a cooler?), then later placing the samples in a -80 freezer would not preserve the RNA as it was in the field, especially as there may have been an influence of O in the previously anoxic depths as the cores were stored in falcon tubes. Please indicate the length of time between sampling and freezing, if greater than a few hours, then the RNA data may be more reflective of the storage conditions and not the in-situ conditions.

Reply: This, indeed, a well justified concern. Samples for RNA extraction (only one site, HC2, out of four) of this study were stored in a cooler box first and frozen within three hours after sampling. We agree that we cannot exclude some level of RNA degradation during this period unlike in the previous study by Unger et al. (2021), where samples were stored in a dry shipper immediately after sampling. Nevertheless, RNA extraction and cDNA synthesis were timely and successful. Given that samples were stored at very low temperatures as soil samples, i.e. in their original matrix, a shift in community composition also of the active fraction in such a short time is unlikely. This corresponds with our results which show a large level of similarities between the two cDNA datasets. However, taking your concern into account, we decided to delete parts of the manuscript stressing taxa that were less represented in our study since in fact such a decrease may have resulted from decay of RNA rather than from real changes in the active community. We will therefore delete the sentences in the following lines:

l. 462: "Methylospirales were not detected in the cDNA-based extractions".

l. 465-466: "However, classes such as Syntrophobacteria and Desulfobulbia showed higher cDNA-based abundances only after the drought in Post-inflow Sping2019 in the surface peat layers."

l. 468-470: "These findings can however not be confirmed with the data on cDNA-based abundances, suggesting no active role of *Candidatus* Methanoperedens except during Drought2018 in the deepest peat layer at HC2."

l. 472-473: "According to the cDNA analysis, active ANME-3 were little abundant in the surface peat layers during the Drought2018 (black, Fig. 4c)."

Since there is very little data on the active communities in rewetted fens and since the cDNA data do not form the core of our study but rather serve as additional information, we would like to leave the cDNA in the manuscript. In order to avoid overinterpretation of the data, we decided to make the reader more aware of the different preservation procedures, though. Therefore, we will state the following in the caption of Fig. 4 in l. 1210: "Please also note that preservation methods differed slightly between the studies."

Line 274: which PCR buffer? What was the final reaction volume? Was the same amount of DNA added to each reaction?

Reply: Many thanks for these considerate additions. We used 10x Pol Buffer C by OptiQ DNA Polymerase (Roboklon). 50 µl was the final volume and 5 µl were used from each sample. Also considering comment no. 3 by the first reviewer, we suggest to change the sentence to: "For the PCR (Thermal Cycler, T100, Biorad, Feldkirchen, Germany) we added 10x Pol PCR-Buffer C (OptiQ DNA Polymerase, Roboklon, Berlin, Germany), 1.25 U OptiQ DNA Polymerase, 0.2 mM dNTP, 0.5 mM MgCl₂ and 0.5 µM of each primer to 5 µl of the purified sample. Using sterile water, we filled the mixture to a final volume of 50 µl." We also apologize for the error (final volume of 25 µl) made in the reply to the first reviewer. We checked again and found that 50 µl was the correct final volume.

In case your comment addressed the comparability of the amount of DNA between the three studies, we want to emphasize that the patterns between copies per g soil or copies per ng DNA (which we calculated additionally) are consistent and show the same trends, so that the unit (copies/ g soil) represents the amount of DNA contained.

Line 292: Was the same amount of DNA added to each reaction? How was this normalized? What is the final reaction volume? What was the primer concentration?

Reply: Thank you for pointing this out. The same volume of purified DNA (4 μ l) was used for each reaction. Since absolute copy numbers per gram soil were calculated, it was not necessary to use the same weight of DNA as starting material. Especially, as shown above, the normalizations against gram of soil and ng of DNA reveal the same trends. In reaction to your comment about the final reaction volume, we would modify the sentence further in addition to the changes suggested by reviewer1 (primer concentrations): "According to the in-house protocol, we used 10 μ l of SYBR Green, 0.08 μ l of each primer (with a concentration of 100 μ M each), 4 μ l template per reaction and 5.84 μ l sterile water, resulting in a total final volume of 20 μ l."

Line 296: standard curve was based on a series of dilutions of what? Please indicate brief methods, even though detailed in the sister studies.

Reply: Yes, we will give some brief details on the dilution method and suggest the following detail addition: "The standard curve was typically based on a series of dilutions of known numbers of concentrations in the range of 10^3 – 10^8 copies as specified in Winkel et al. (2018), with starting concentrations being 2.5×10^8 for 16S rRNA, 2.9×10^7 for *mcrA*, 3.2×10^7 for *pmoA* and 6.69×10^7 for *dsrB*."

Line 296: How were gene copy numbers normalized to the amount of soil used for DNA extractions? Based on the x-axes in Figure 5 this appears to be the case, but please clarify in the text.

Reply: Thank you very much for this important feedback. All absolute gene copy numbers (copies/ μ l) were multiplied by the final DNA extraction elution volume (50, 60 or 100 μ l), the dilution factor (mostly 10 or 100, sometimes 1) and divided by the initial fresh weight of the individual soil sample. In order to normalize the different soil water content values, a dry weight factor was determined (wet weight/dry weight) and multiplied with the gene copy numbers to get the gene copies number per g dry soil. We intend to add the following after l. 299: "All absolute gene copy numbers are given per gram dry soil and were calculated by normalizing them over their initial fresh weight taking into account a dry weight factor, the elution volume and the dilution factor. For better visualization, we log₁₀ transformed the data."

Line 330: how were ordination vectors constructed?

Reply: Do you mean, the environmental variables? We used the function *envfit* from *vegan* package. Thanks to reviewer1, we intend to add the following sentence between the sentence in l. 333 and l. 334: "We used the function *envfit()* of package *vegan* (Oksanen et al., 2020) in order to add environmental variables on the NMDS ordination

configurations.”

Line 386 “Drought”

Reply: Thank you very much! We will correct the tipping error.

Line 371 (and elsewhere): please indicate if these are average +/- standard error, perhaps in the methods? Or indicate the first time mentioned.

Reply: Yes, good point. We suggest to modify the sentence in l. 341 as follows: “To display average values for different subgroups (usually mean values with standard error if not indicated otherwise), we used the psych package (Revelle, 2020).”

Line 425 (editorial comment, can be ignored): to my eye, the colours appear more yellow than orange in the online version.

Reply: Thanks for the comment. Colours may appear different to every individual reader. Since we assume that the chosen colour palette will make sure that colour can be differentiated from each other (even for people with colour vision deficiencies) and it is clear, which colour is meant with “orange” we would like to keep the colour description as it is.

Line 475: 16S rRNA gene

Reply: Ok, we will change this accordingly.

Line 475: as mentioned above, please provide details on how these comparisons were made (i.e. were all of the same methods followed? Comparison of reactions efficiencies, same extraction kits, normalization to gene copy number to g of soil, etc.)

Reply: Thanks for the comment. Please find a detailed reply on the method comparison topic below your major comment above.

Line 492: were the ordinations made only on the DNA data? Were ordinations of cDNA data similar? Perhaps these could be presented in the supplemental data if they add to the story?

Reply: Thank you for this idea. Yes, the ordinations were only done for the DNA data, because they are available from all locations at all points in time. We believe that ordinations for the cDNA data would provide no additional insights into time-dependent community changes since cDNA was only obtained from location HC2 in the drought and post-inflow year, not for the baseline conditions and not for any of the other sampling sites. This means, that these ordinations would not enable us to show the effect of the brackish water inflow and the drought, because they cannot be compared to the previous

conditions. In addition, there are only 23 data points of cDNA, which we consider to be too little for a meaningful ordination.

Figure 1: I did not find a reference to this figure in the manuscript? It's an excellent figure and should be included.

Reply: Yes, you are right, we should definitely find a place for references. We suggested to include it at the following lines:

l. 33: "We found that both, the inflow effect of brackish water and in parts also the preceding drought increased the sulfate availability in the surface and pore water (see Fig.1)."

l. 557: "Therefore, the drought cannot be the only source for the observed increase in pore water ion concentrations and hence, we can assume that both, brackish water inflow and not only the legacy effect of the drought in 2018 changed sulfate concentrations in the surface and pore water and was critical for the methane dynamics and the microbial community composition (Fig. 1)."

l. 607: "If anaerobic CO₂ production had been a result of methane oxidation, it had to happen in an area outside the scope of our analysis, namely the water column or the fresh litter layer above the peat soil (Fig. 1)."

l. 653: "As discussed earlier, though, methane oxidation most likely occurred in the standing water above the peat (Fig. 1) given the substantial drop in methane emissions despite the fact that methanogenesis seemingly occurred besides alternative anaerobic pathways of carbon respiration, mostly sulfate reduction."

l. 669: "It remains unresolved, however, why methane emissions decreased to a new minimum since rewetting more than a decade ago, while methanogenic absolute abundances and methane concentrations overall did not change or even decreased (Fig. 1)."