

The authors have done a lot of excellent work both responding to reviewers comments that will assist readers to understand their work. It is nicely rounded and very well placed into context.

I have a few points for consideration on the manuscript.

1. The authors have an incorrect reference for the Bacterial reverse primer. Please see the following quotes from the Klindworth paper cited for the reverse primer: “Per sample, two separate PCR reactions were performed in order to test two bacterial primer pairs for 16S rDNA amplification. Primer pairs were: (i): S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3' (32), and S-D-Bact-0785-a-A-21, 5'-GACTACHVGGGTATCTAATCC-3 (32);”

“32

Herlemann D.P.R., Labrenz M., Juergens K., Bertilsson S., Waniek J.J., Andersson A.F.. Transition in bacterial communities along the 2000 km salinity gradient of the Baltic Sea, *ISME J*, 2011, vol. 5 (pg. 1571-1579)“

2. In previous work by the authors in which these primers were tested they used a temperature gradient PCR to select 60° C. It is not stated what the authors observed at 60°C that helped them choose this temperature. Could the authors please include this information in the methods as it is relevant to the interpretation of the results and might help others in their lab work.

3. Generally an annealing temperature of +/- 5° from the mean Tm of the primer pair, and as low as possible to achieve amplification is chosen. Typically this particular primer pair is run with annealing temperatures between 48°C and 56°C. The use of a higher annealing temperature could increase amplification bias reducing the phylogenetic range of template DNA amplified. Please carefully phrase any statements about the absence of detection of any clade as the absence could, more than usual, be due to amplification bias.

-<https://academic.oup.com/femsec/article/60/2/341/584515>

-<https://academic.oup.com/nar/article/41/1/e1/1164457#119410076>

-[http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.short?](http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.short?casa_token=h2nXrvzrHDIAAAAA:4IzeOXuwzgz48-voKEUGXXMXq7jPIHPUUAkmR8WSgcMNN3B79NXvMPf_nkNgxkTC7SbgD5TJQ5I)

[casa_token=h2nXrvzrHDIAAAAA:4IzeOXuwzgz48-](http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.short?casa_token=h2nXrvzrHDIAAAAA:4IzeOXuwzgz48-voKEUGXXMXq7jPIHPUUAkmR8WSgcMNN3B79NXvMPf_nkNgxkTC7SbgD5TJQ5I)

[voKEUGXXMXq7jPIHPUUAkmR8WSgcMNN3B79NXvMPf_nkNgxkTC7SbgD5TJQ5I](http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.short?casa_token=h2nXrvzrHDIAAAAA:4IzeOXuwzgz48-voKEUGXXMXq7jPIHPUUAkmR8WSgcMNN3B79NXvMPf_nkNgxkTC7SbgD5TJQ5I)

4. Please provide standard details of the PCR e.g. volumes, concentrations etc of reactants and template, or provide a reference where these details are given.

5. I have trouble reconciling the NMDSs in fig S7 which show a random placement of OTUs and figure 6 which shows that there is not just a difference in archaea and bacterial assemblages between sites but that differences can be seen at family and higher taxonomic levels and so should be very obvious at OTU level. Please provide details of how the NMDS were plotted as this difference seems strange. The

statement that no difference in structure was observed according to the NMDSS while an accurate description of what is plotted (though because sites are not plotted on the NMDSS the statement is only partially supported), does not fit with the other analyses which show clear differences at sites in communities and different depths.

6. Multiple contradictions were found in the following lines. Could the authors please fix these statements regarding the presence/absence of methanotrophs and methanogens so they are consistent.

Line 497-8 "Molecular analysis showed that both methanogens and methanotrophs were present at all four assessed sites Fig 6"

Line 666 "No aerobic or anaerobic methanotrophic prokaryotes were found in these peat deposits"

Line 610-611 "We conclude that in the observed absence of methanogenic and methanotrophic microbial populations, the in situ CH₄ observed in this study are trapped pockets of millennia old CH₄."

Line 672-4 "The absence of methanotroph activity is congruent with their absence in the results of 16S rRNA gene amplicon sequencing and confirms that methanotrophic species are most likely not present or active in this environment."

Line 780-782 "Whilst the source of CH₄ remains unconfirmed, we conclude that in the observed absence of methanogenic and methanotrophic microbial populations, the in situ CH₄ observed in this study are trapped pockets of millennia old CH₄."

Other contradictions:

Line 600-601 "Further, microbial analyses show that neither aerobic or anaerobic methanotrophic prokaryotes were activated by oxic or anoxic incubations. Therefore, we did not observe processes where biogenic CH₄ may have been produced in the present day."

7. Methanosarcinales are mentioned in the discussion but not in the results.

BTW: There are several recent studies showing the release of methane by bacteria and others.

-<https://www.nature.com/articles/ngeo2837>

-<https://www.nature.com/articles/s41564-017-0091-5>

-[https://advances.sciencemag.org/content/6/3/eaax5343?](https://advances.sciencemag.org/content/6/3/eaax5343?utm_source=TrendMD&utm_medium=cpc&utm_campaign=TrendMD_1)

[utm_source=TrendMD&utm_medium=cpc&utm_campaign=TrendMD_1](https://advances.sciencemag.org/content/6/3/eaax5343?utm_source=TrendMD&utm_medium=cpc&utm_campaign=TrendMD_1)