Dear Editor,

We would like to thank anonymous reviewers for useful comments and constructive suggestions.

Please accept a resubmission of our revised manuscript entitled "Metagenomic analyses of the late Pleistocene permafrost – additional tools for reconstruction of environmental conditions". We took in to account all suggestions and comments provided by reviewers, and we feel that manuscript was significantly improved upon the revision. We reviewed additional studies, added table with physicochemical measurements, gave needed references, and extended discussion as reviewers suggested. Point by point response to reviewers is below.

Point by point responses to reviewers

REVIEWER #1

Q1. The main finding of the study is that the microbial composition in the two samples are different. Furthermore, the sample with high methane content had a higher abundance of genes involved in methane production. The authors present in the introduction some background information, previous findings and the rationale of their work. However, they mainly consider their own work and miss previous work from other groups.

> **A1. We acknowledged studies on the similar subject from other groups (see L 49-53 and L 64)**

Q2. The absence of methane and methanogenic Archaea in late Pleistocene Ice Complex is not a general feature since studies of different groups in the Lena River Delta and the Kolyma lowlands have shown the opposite. Therefore, the basic question of the presented work should be revised.

> **A2. Studies from other groups showing the low concentration of methane in late Pleistocene Ice Complex (L 49-53), and isolation of methanogenic Archaea previously from permafrost-affected soils (L 64) were reviewed.**

Q3. Furthermore, the advantage of a metagenomic analysis in the context of the work should be explained more clearly. E.g., if the focus of the work is on the reason for a lack of methane and methanogenic activity in one of the samples (L56ff), is it really necessary to analyze the whole metagenome or is it sufficient to look for functional genes of methanogens and methane oxidizers?

> **A3. The advantage of a metagenomic analysis was explained as suggested. The paper by Fierer et al 2012, which concludes a possibility of using metagenomic approaches for prediction of variations in microbial diversity and functions across terrestrial biomes was reviewed (L 419-422).**

Q4. The Material and Methods parts lacks a detailed description of the settings where the samples were collected. If the data of the metagenomic analysis should be used for environmental reconstructions, all available information on the environmental

conditions at the sampling sites during the deposition of the organic matter should be given.

A4. We added Table with physicochemical measurements for studied samples (now it is Table 1)

Q5. The central part of the manuscript, the section Results and Discussion, mainly presents the relative abundance of different taxonomical units and functional genes among the millions of sequences retrieved from the samples. This section is very descriptive and there is almost no discussion on the reasons for the differences between the samples. E.g. the striking difference in the relative abundance of Proteobacteria and Actinobacteria, the most abundant groups of bacteria in the two samples, is ignored. If the analysis shall be used for pale-environmental reconstruction, the reader expects a discussion on the potential relation between obtained sequences and environmental conditions. Currently this section is more an inventory of isolated sequences. Only in the following conclusions the data are discussed on the background of previous work.

A5. We added discussion as suggested.

Q6. Some of the central conclusions are not backed by the data. E.g. the conclusion on metagenomic analysis as central key for paleo-reconstructions should be revised.

A6. See our answer above (A5).

Q7. Indeed the study has shown, that the abundance of genes related to methane production is higher in methane bearing sediments. No attempt was made to correlate the metagenomic data with the paleo-environment since no data on environmental conditions during deposition of the sampled sediment are presented. Was the climate 30,000 years ago at the sampling sites colder and dryer or similar as today? Furthermore, the description of the formation of the Ice Complex sediments is an oversimplification(see specific comments). The authors suggest, that the single sample from the Ice Complex they studied is representative for the whole Ice Complex sediments. This obviously cannot be the case.

A7. The correlation between metagenome data and phisycochemical measurements were done as suggested.

Q8. Many abbreviations are not explained in the text Specific comments: line 31: should read "of the sampled late Pleistocene..."

A8. Changed

Q9. l44ff: Please explain epigenetic and syncryogenic, since not all readers will be familiar with these two terms.

A9. Explained as suggested (see lines 44-48)

Q10. l43: Not only the findings of the authors should be considered, but also work from other groups (see general comments)

A10. See response on the comments A1 and A2.

Q11. l57: This general research question is not supported by recent literature. Sediments of the late Pleistocene Ice Complex may contain methane and methanogenic activity (after thaw).

A11. We reviewed studies from other researchers.

Q12. l58: It is unclear why metagenomic analysis should answer the question raised. Metagenomic analysis may be used to describe the microbial community present in the samples (as done in this study). If there is no methanogenic activity you would expect a low abundance or absence of methanogens (as shown in the current analysis). But the analysis will not tell you why this is the case.

A12. Metagenome analysis would show pathways present in the analyzed community. We provided the Table with physicochemical data, to show that differences in the community depend on environmental characteristics.

Q13. l 67:Please quote the respective study.

A13. Done

Q14. l75f: Please explain in more detail, why the presented descriptive results will help evaluating the community response to permafrost thaw and global warming.

A14. Changed wording

Q14a. l91: Could you give the concentration in the pore water? 1.2 mmol kg-1 seems above saturation concentrations of CH4 in water (depending on the amount of water in 1 kg).

A14a. Gas from soil was sampled using the modification of the technique of degasation through the phase equilibration (McAuliffe, 1971). Gas samples were collected in the field by degassing 50 g sub-samples of frozen cores in a 150-ml syringe under a 99,999% nitrogen atmosphere. The gas mixture was then transferred through a needle to the rubber septa sealed vials filled with saturated NaCl solution. At that point, brine was substituted by gas with the excess water flowing out through a second needle. Gas samples were kept in the vials until analyzed in the laboratory. CH_4 concentrations were measured using a KhPM-4 gas chromatograph (Chromatek, Russia) equipped with a flame ionization detector. Hydrogen was used as the carrier gas. The sensitivity limit of the methane detection was 10 ppm and the results were reproducible within 15%. The concentration then was recalculated on the weight of the degassed permafrost sample. This method has been repeatedly described in the cited papers.

Q15. l95ff: Please describe how CH4 was measured and give the detection limit of CH4.

A15. It was done above. The detection limit of gas chromatography was 10 ppm.

Q16. l145: It is surprising to me that the diversity of Eukaryotes is higher than of Bacteria. Has this been found before? How is the situation in active layer soils? And what couldbe the reason for high Eukaryotic diversity in anoxic soils, that should be almost free of Eukaryotes? Please discuss this finding. How can you be sure to detect all Bacterial species if you have only sequences with an average length of 150 base pairs?

A16. We revised text as suggested

Q17. l155: What means "significantly dominate"?

A17. Removed word "significantly"

Q18. l156ff: ditto

A18. See response above

Q19. l158:Please do not use a statement as header

A19. Changed as suggested

Q20. l240: Please explain SEED

A20. Done

Q21. l248: Please explain KEEG

A21. Done

Q22. l254: How did you test significant differences

A22. We used p-value in STAMP

Q23. l264: What means features in this context? Do you mean genes or sequences?

A23. Corrected

Q24. l351: This is an oversimplification. The authors of the cited study consider the Ice Complex development to MIS2 (last glacial maximum) and MIS3 (interstadial). Yedoma sediments from MIS3 are characterized by higher TOC and less decomposed organic matter than MIS2 deposits indicating anaerobic conditions during deposition.

A24. Our view of the amount of carbon in Yedoma is not entirely in accord with the view of the reviewer. We did not discuss differences in carbon concentration found in mentioned by reviewer sediments, because this is not a subject of the submitting manuscript.

Q25. l364ff: This is not a conclusions from the data presented but well established knowledgethat might be presented in the introduction with the respective citations.

A25. The conclusion was revised

Q26. l366 ff: The last sentence is unclear. Why will a method, which only describes the status quo help to understand how a community will respond to climate change?

A26. Revised

REVIEWER #2

The authors mentioned that they deposited the sequencing data to the MG-RAST, but authors did not provide MG-RAST ID number. These numbers should be provided.

A27. MG-RAST ID indicated see L132

P12097, L4-7 What data sets were used to get the input into the STAMP analyses? Please clarify.

A28. Indicated L145

Authors used both styles: P12099, L19 – □-Proteobacteria and L13 Alphaproteobacteria and L19 Gammaproteobacteria – the first style is preferable, please correct throughout the text.

A29. Corrected L207 and L213

P.12101, L5. In the text, Geobacter genus is classified as sulfate-reducing bacteria, whereas they are metal-reducing bacteria, and they are not considered to reduce sulfate.

A30. Corrected L256

P. 12101, L. 14. It is not obvious to include the Desulfitobacterium genus to sulfate - reducing bacteria, since they are able reduce only sulfite and thiosulfate.

A31. Corrected L265-267

P12102, L6 – Additionally to suggestions of the first reviewer to spell out SEED and KEGG, please explain what does the EC number mean?

A32. Spelled out L280, L294

P. 12103, L. 5-6 Alkaliphilus metalliredigens is not valid bacterial name and should be written as 'Alkaliphilus metalliredigens'

A 33. Corrected L317-318

References in the text must be listed in chronological order.

A34. Corrected

Please check the List of References – not all references are correctly abbreviated in accordance with the ISI Journal Title Abbreviations Index.

A35. Corrected

P.12111, L. 7 desulfitobacterium should be written as Desulfitobacterium

A36. Corrected: L267

REVIEWER #3

However, whether those genes are ultimately expressed is an entirely different matter. Much more information would be needed before a predictive capability can be achieved with respect to microbial community function after permafrost thaw.

A37. We added text L288-293, and L443-445

It appears the eight subsamples from each core were extracted and combined into a single run on the Illumina flow cell for IC4 or IC8. Figure 1 indicates these pseudoreplicates were taken at roughly the same depth. Is that correct or were samples collected over a broader range, say up to 0.5 m?

A38. We added clarification: L115 and L118

Unfortunately, most metagenome studies lack replication. How reproducible are the extractions and the Illumina sequencing of DNA in the authors' hands? What is the variability between samples? Can the authors indicate their DNA yield for all of the 8 pseudo-replicates? Were there differences in yield between the IC4 and IC8 samples after the Zymo Concentrator kit that can explain the observed changes in gene abundance?

A39. We referred our previous paper where we discussed performance of PowerSoil kit and compared community structure obtained from triplicate isolations. L155-158; and L164-167.

Most of the microbial community differences are reported to 2 or 3 significant figures (such as page 12099 line 27 and 12100 line 7). Without replicates and an understanding of the variability within the analysis for these particular samples, it could be that all observed differences between IC4 and IC8 are artifactual. Until the authors can prove the ability to discern these small differences in samples, reporting to 3 significant figures is unwarranted.

A40. Percentages expressed to 3 significant figures were removed as suggested, L244-245; L251.

I suspect that not all ORFs or 16S rRNA genes within the Illumina dataset were identified with a strong degree of confidence. What proportion of the ORFS that were detected are annotated as hypothetical proteins or very loosely affiliated with known genes? That data seems to have been eliminated from the manuscript and could represent a significant fraction of the reads.

A41. We added requested information L290-292.