Estimation of microbial metabolism and co-occurrence patterns in fracture groundwaters of deep crystalline bedrock at Olkiluoto, Finland

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Abstract

The microbial diversity in oligotrophic isolated crystalline Fennoscandian Shield bedrock fracture groundwaters is great but the core community has not been identified. Here we characterized the bacterial and archaeal communities in 12 water conductive fractures situated at depths between 296 m and 798 m by high throughput amplicon sequencing using the Illumina HiSeq platform. A sequencing depth of up to 1.2×10^6 reads per sample revealed that up to 95% and 99% of the bacterial and archaeal sequences obtained, respectively, belonged to only a few common species, i.e. the core microbiome. However, the remaining rare microbiome contained over 3 and 6 fold more bacterial and archaeal taxa. The metabolic properties of the microbial communities were predicted using PICRUSt. The approximate estimation showed that the metabolic pathways included commonly fermentation, fatty acid oxidation, glycolysis/gluconeogenesis, oxidative phosphorylation and methanogenesis/anaerobic methane oxidation, but carbon fixation through the Calvin cycle, reductive TCA cycle and the Wood-Ljungdahl pathway was also predicted. The rare microbiome is an unlimited source of genomic functionality in all ecosystems. It may consist of remnants of microbial communities prevailing in earlier environmental conditions, but could also be induced again if changes in their living conditions occur.

1 Introduction

Identifying and understanding the core microbiome of any given environments is of crucial importance for predicting and assessing environmental change both locally and globally (Shade and Handelsman, 2012). In a previous study (Bomberg et al., 2015) we showed by 454 amplicon sequencing that the active microbial communities in Olkiluoto deep subsurface were strictly stratified according to aquifer water type. Nevertheless, more rigorous sequencing efforts and more samplings have shown that an archaeal core community consisting of the DeepSea Hydrothermal Vent Euryarchaeotal Group 6 (DHVEG-6), ANME-2D and Terrestrial Miscellaneous Group (TMEG) archaea may exsists in the anaerobic deep groundwater of Olkiluoto (Miettinen et al., 2015). The bacterial core groups in Olkiluoto deep groundwater include at least members of the Pseudomonadaceae, Commamonadaceae and Sphingomonadaceae (Bomberg et al., 2014; 2015; Miettinen et al., 2015). The relative abundance of these main groups varies at different depths from close to the detection limit to over 90% of the bacterial or archaeal community (Bomberg et al., 2015; Miettinen et al., 2015). However, both the archaeal and the bacterial communities contain a wide variety of smaller bacterial and archaeal groups, which are distributed unevenly in the different water conductive fractures.

The rare biosphere is a concept describing the hidden biodiversity of an environment (Sogin et al., 2006). The rare biosphere consists of microbial groups that are ubiquitously distributed in nature but often present at low relative abundance and may thus stay below the limit of detection. Due to modern high throughput sequencing techniques, however, the hidden diversity of rare microbiota has been revealed. These microorganisms are the basis for unlimited microbial functions in the environment and upon environmental change specific groups can readily activate and become abundant. Access to otherwise inaccessible nutrients activate specific subpopulations in the bacterial communities within hours of exposure (Rajala et al., 2015) and enrich distinct microbial taxa at the expense of the original microbial community in the groundwater (Kutvonen, 2015). Mixing of different groundwater layers due to e.g. breakage of aquifer boundaries and new connection of separated aquifers may cause the microbial community to change and activate otherwise dormant processes. This has previously been shown by Pedersen et al. (2013), who indicated increased sulphate reduction activity when sulphate-rich and methane-rich groundwater mixed. The stability of deep subsurface microbial communities in isolated deep subsurface groundwater fractures are

assumed to be stable. However, there are indications that they may change over the span of several years as slow flow along fractures is possible (Miettinen et al., 2015; Sohlberg et al., 2015).

The microbial taxa present in an environment interact with both biotic and abiotic factors. Co-occurrence network analyses and metabolic predictions may help to understand these interactions. Barberan et al. (2012) visualised the co-occurrence networks of microbial taxa in soils and showed novel patterns connecting generalist and specialist species as well as associations between microbial taxa. They showed that specialist and generalist microbial taxa formed distinct and separate correlation networks, which also reflected the environmental settings. Metagenome predicting tools allows us to estimate microbial metabolic functions based on NGS microbiome data. Using the PICRUSt tool (Langille *et al.,* 2013) Tsitko et al. (2014) showed that oxidative phosphorylation was the most important energy producing metabolic pathway throughout the 7 m depth profile of an *Acidobacteria*-dominated nutrient poor boreal bog. Cleary et al. (2015) showed that tropical mussel-associated bacterial communities could be important sources of bioactive compounds for biotechnology. This approach is nevertheless hampered by the fact that only little is so far known about uncultured environmental microorganisms and their functions and the PICRUSt approach is best applied for human microbiome for which it was initially developed (Langille *et al.,* 2013). However, metagenomic estimations may give important indications of novel metabolic possibilities even in environmental microbiome studies.

Using extensive high throughput amplicon sequencing in this study we aimed to identify the core microbiome in the deep crystalline bedrock fractures of Olkiluoto Island and also to identify the rare microbiome. We aimed to show the interactions between the taxa of the rare biosphere and the surrounding environmental parameters in order to validate the factors that determine the distribution of the rare taxa. Finally, we aimed to estimate the prevailing metabolic activities that may occur in the deep crystalline bedrock environment of Olkiloto, Finland.

2 Materials and methods

2.1 Background

The Olkiluoto site has previously been extensively described (Posiva, 2013) and is only briefly described here. The Island of Olkiluoto situating on the west coast of Finland has

approximately 60 drillholes drilled for research and monitoring purposes. Studies on the chemistry and microbiology of the groundwater have been on-going since the 1980s. The groundwater is stratified with a salinity gradient extending from fresh to brackish water to a depth of 30 m and the highest salinity concentration of 125 g L^{-1} total dissolved solids (TDS) at 1000 m depth (Posiva, 2013). The most abundant salinity causing cations are Na^{2+} and Ca^{2+} and anions Cl^{-}. Between 100 and 300 m depths, the groundwater originates from ancient (pre-Baltic) seawater and has high concentrations of SO_4^{2-}. Below 300 m the concentration of methane in the groundwater increases and SO_4^{2-} is almost absent. A sulphate-methane transition zone (SMTZ), where sulphate-rich fluid replaces methane-rich fluid, is located at 250 − 350 m depth. Temperature rises linearly with depth, from ca. 5 – 6 °C at 50 m to ca. 20 °C at 1,000 m depth (Ahokas et al., 2008). The pH of the groundwater is slightly alkaline throughout the depth profile. Multiple drillholes intersect several groundwater-filled bedrock fractures, including larger hydrogeological zones such as HZ20 or HZ21 (Table 1). The bedrock of Olkiluoto consists mainly of micagneiss and pegmatitic granite type rocks (Kärki & Paulamäki, 2006).

This study focused on 12 groundwater samples from water conductive fractures situated at between 296 m and 798 m below sea level bsl and originating from 11 different drillholes in Olkiluoto (Figure 1). The samples represented brackish sulphate waters and saline waters (as classified in Posiva, 2013). The samples were collected between December 2009 and January 2013 (Table 1). The physicochemical parameters of the groundwater samples have been reported by reported by Miettinen et al. (2015), but have for clarity been collected here (Table 1).

2.2 Sample collection

The collection of samples occurred between December 2009 and January 2013 (Table 1) as described previously (Bomberg et al., 2015; Miettinen et al., 2015; Sohlberg et al., 2015). The samples were obtained from 11 different permanently packered or open drillholes equipped with removable inflatable packers. The position and direction of the drillholes are indicated in Figure 1. Shortly, in order to obtain indigenous fracture fluids, the packer-isolated fracture zones were purged by removing stagnant drillhole water by pumping for a minimum of four weeks before the sample water was collected. The water samples were collected directly from the drillhole into an anaerobic glove box (MBRAUN, Germany) via a sterile, gas-tight poly acetate tube (8 mm outer diameter). Microbial biomass DNA extraction was concentrated

from 1000 mL samples by filtration on cellulose acetate filters (0.2 µm pore size, Corning) by vacuum suction inside the glove box. The filters were immediately extracted from the filtration funnels and frozen on dry ice in sterile 50 ml cone tubes (Corning). The frozen samples were transported on dry ice to the laboratory where they were stored at -80°C until use.

2.3 Nucleic acid isolation

Community DNA was isolated directly from the frozen cellulose-acetate filters with the PowerSoil DNA extraction kit (MoBio Laboratories, Inc., Solana Beach, CA), as previously described (Bomberg et al., 2015). Negative DNA isolation controls were included in the isolation protocol. The DNA concentration of each sample was determined using the NanoDrop 1000 spectrophotometer.

2.4 Estimation of microbial community size

The size of the microbial community was determined by epifluorescence microscopy of 4',6 diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, MO, USA) stained cells as described in Purkamo et al. (2013). The size of the bacterial population was determined by 16S rRNA gene targeted quantitative PCR (qPCR) as described by Tsitko et al. (2014) using universal bacterial 16S rRNA gene-targeting primers fD1 (Weisburg et al., 1991) and P2 (Muyzer et al., 1993), which specifically target the V1- V3 region of the bacterial 16S rDNA gene. The size of the archaeal population in the groundwater was determined by using primers ARC344f (Bano *et al*., 2004) and Ar744r (reverse compliment from Barns *et al*., 1994) flanking the V4-V6 region of the archaeal 16S rRNA gene.

The qPCR reactions were performed in 10µL reaction volumes using the KAPA 2 × Syrb® FAST qPCR-kit on a LightCycler480 qPCR machine (Roche Applied Science, Germany) on white 96-well plates (Roche Applied Science, Germany) sealed with transparent adhesive seals (4titude, UK). Each reaction contained 2.5 µM of relevant forward and reverse primer and 1 µL DNA extract. Each reaction was run in triplicate and no-template control reactions were used to determine background fluorescence in the reactions.

The qPCR conditions consisted of an initial denaturation at 95 °C for 10 minutes followed by 45 amplification cycles of 15 seconds at 95 °C, 30 seconds at 55 °C and 30 seconds at 72 °C with a quantification measurement at the end of each elongation. A final extension step of

three minutes at 72 °C was performed prior to a melting curve analysis. This consisted of a denaturation step for 10 seconds at 95 °C followed by an annealing step at 65 °C for one minute prior to a gradual temperature rise to 95 °C at a rate of 0.11 °C s^{-1} during which the fluorescence was continuously measured. The number of bacterial 16S rRNA genes was determined by comparing the amplification result (Cp) to that of a ten-fold dilution series (10^1-10^7 copies μL^{-1}) of *Escherichia coli* (ATCC 31608) 16S rRNA genes in plasmid for bacteria and a dilution series of genomic DNA of *Halobacterium salinarum* (DSM 3754) for archaea. The lowest detectable standard concentration for the qPCRs was 10^2 gene copies/reaction. Inhibition of the qPCR by template tested by adding 2.17×10^4 plasmid copies containing fragment of the morphine-specific Fab gene from *Mus musculus* gene to reactions containing template DNA as described in Nyyssönen *et al*. (2012). Inhibition of the qPCR assay by the template DNA was found to be low. The average Crossing point (Cp) value for the standard sample (2.17×10^4 copies) was 28.7 (± 0.4 sd), while for the DNA samples Cp was 28.65 - 28.91 (± 0.03-0.28 sd). Nucleic acid extraction and reagent controls were run in all qPCRs in parallel with the samples. Amplification in these controls was never higher than the background obtained from the no template controls.

2.5 Amplicon library preparation

This study is part of the Census of Deep Life initiative, which strives to obtain a census of the microbial diversity in deep subsurface environment by collecting samples around the world and sequencing the 16S rRNA gene pools of both archaea and bacteria. The extracted DNA samples were sent to the Marine Biological Laboratory in Woods Hole, MA, USA, for preparation for HiSeq sequencing using the Illumina technology. The protocol for amplicon library preparation for both archaeal and bacterial 16S amplicon libaries can be found at http://vamps.mbl.edu/resources/faq.php. Shortly, amplicon libraries for completely overlapping paired-end sequencing of the V6 region of both the archaeal and bacterial 16S rRNA genes were produced as previously described (Eren et al., 2013). For the archaea, primers A958F and A1048R containing Truseq adapter sequences at their 5' end were used, and for the bacteria primers B967F and B1064R for obtaining 100 nt long paired end reads (https://vamps.mbl.edu/resources/primers.php). The sequencing was performed using a HiSeq 1000 system (Illumina).

2.6 Sequence processing and analysis

Contigs of the paired end fastq files were first assembled with mothur v 1.32.1 (Schloss et al., 2009). Analyzes were subsequently continued using QIIME v. 1.8. (Caporaso et al., 2010). Only sequences with a minimum length of 50 bp were included in the analyses. The bacterial and archaeal 16S rRNA sequences were grouped into OTUs (97% sequence similarity) using both the open reference and closed reference OTU picking strategy and classified using the GreenGenes 13_8 16S reference database (DeSantis et al., 2006). The sequencing coverage was evaluated by rarefaction analysis and the estimated species richness and diversity indices were calculated. For comparable α- and β-diversity analyses the data sets were normalized by random subsampling of 17,000 sequences/sample for archaea and 140,000 sequences/sample for bacteria. Microbial metabolic pathways were estimated based on the 16S rRNA gene data from the closed OTU picking method using the PICRUSt software (Langille *et al.,* 2013) on the web based Galaxy application (Goecks et al., 2010; Blankenberg et al., 2010; Giardine et al., 2005). The predicted KO numbers were plotted on KEGG pathway maps (http://www.genome.jp/kegg/) separately for the bacterial and archaeal predicted metagenomes, with a threshold of a minimum of 100 genes in total estimated from all samples. The sequence data has been submitted to the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) under study SRP053854, Bioproject PRJNA275225.

2.7 Statistical analyses

Non-metric multi-dimensional scaling plots using Chord's similarity index were calculated separately for the archaeal and bacterial communities using PAST3 (Hammer and Harper, 2001). The samples were also hierarchically clustered based on community similarity using the UPGMA clustering with Bray-Curtis similarity index and 100 bootstrap repeats with PAST3. A co-occurrence network was calculated using the Gephi software (Bastian et al., 2009) using the Fruchtermann-Feingold layout with the betweenness centrality algorithm for identifying microbial taxa with numerous connections and the Louvain method (Blondel et al., 2008) for identifying closely associated groups of microbes. The calculations were based on Spearman's rank correlation calculations obtained by the out.association command in mothur and only pairs with $r > 0.6$ and $p < 0.01$ were included in the network analysis and nodes with a degree range of less than 10 were excluded from the graph. The p values were not corrected and the Spearman's rank correlations were only used to form pairs between taxa for the network visualization.

3 Results

3.1 Microbial community size

The total number of microbial cells detected by epifluorescence microscopy of DAPI stained cells was between 2.3×10^4 and 4.2×10^5 cells mL^{-1} groundwater (Figure 2, Table 1). The concentration of bacterial 16S rRNA gene copies mL^{-1} varied between 9.5×10^3 and 7.0×10^5 and that of the archaea 2.6×10^1 and 6.3×10^4 (Figure 2, Table 1).

3.2 Sequence statistics, diversity estimates and sequencing coverage

The number of bacterial v6 sequence reads from the 12 samples varied between $1.4 - 7.8 \times 10^5$ reads, with a mean sequencing depth of 2.9×10^5 ($\pm$ 1.8×10^5 standard deviation) reads/sample (Table 2). The archaeal v6 sequence reads ranged from $0.17 - 12.1 \times 10^5$ reads with a mean sequencing depth of 4.1×10^5 ($\pm$ 3.5×10^5 standard deviation) reads/sample. The numbers of observed operational taxonomic units (OTUs) represented on average 82.6% (± 12.5%) of the Chao1- and 78.1 % (± 13.4%) of the ACE-estimated numbers of bacterial OTUs (Table 2ab). The archaeal communities were slightly better covered, with on average 88.5% (± 11.5%) of the Chao1 and 84.8% (± 12.6%) of the ACE estimated number of OTUs detected. Shannon diversity index H', calculated from 140,000 and 17,000 random sequence reads per sample for the bacteria and archaea, respectively, was high for both bacterial and archaeal communities. High H' values and climbing rarefaction curves (Figure S1) indicated high diversity in the microbial communities in the different deep groundwater fracture zones of Olkiluoto. The bacterial H' was on average 13 (±0.74), ranging from 11 to 14 between the different samples. The archaeal H' was on average 11 (±1.2) ranging from 9 to 12 between the samples.

3.3 Microbial communities

From the bacterial v6 sequences 49 different bacterial Phyla were detected (Appendix 1). These phyla included 165 bacterial classes, 230 orders, 391 families and 651 genera. The greatest number of sequences, between 21.83% and 47.94% per sample, clustered into an undetermined bacterial group (Bacteria, Other), which may be due the fact that sequences of poorer quality may be difficult to classify, especially as the sequences are short.

Only 31 of the identified genera represented at least 1% of the bacterial sequence reads in any sample (Figure 3a).

The archaea were represented by two identified phyla, the Euryarchaeota and the Crenarchaeota (Appendix 2). These included 21 classes, 38 orders, 61 families and 81 genera. Between 4.7% and 35.0% of the archaeal sequences of each sample were classified to unassigned Archaea, with a general increase in unassigned archaeal sequences with increasing depth. 15 archaeal genera were present at a minimum of 1% relative abundance in any of the samples (Figure 3b).

The bacterial core community, i.e. the taxa detected in all the tested samples, constituted of 95 out of 651 identified bacterial genera (Appendix 3). These genera accounted for 80.78 – 95.81% of all the bacterial sequence reads in the samples. The archaeal core community consisted of 25 of the 81 identified genera and accounted for 95.05 – 99.75% of the total number of sequence reads in each sample (Appendix 4).

3.4 Environmental parameters driving the microbial communities

The microbial community profiles of the different samples were clustered in a UPGMA tree (Figure 4). The samples were loosely clustered according to depth with the deeper samples generally more associated with each other and the samples from shallower depths associating with each other. A similar trend was seen in the NMDS plots (Figures 6a and b), although the bacterial communities clustered the samples more tightly into three groups compared to the archaeal communities. No clear environmental factor was identified to drive the communities. However, the deepest bacterial communities were affected by the increasing salinity and the communities from the shallower depths were affected by the concentration of sulphides and the alkalinity, according to the NMDS plots. The archaeal communities, on the other hand, were affected by the concentration of sulphate, sulphur, sulphide, iron, bicarbonate and magnesium at 415 m and 510 m depth.

3.5 Co-occurrence network

The co-occurrence network (Figure 6, Figure S2) indicated specific bacterial taxa are central to the whole microbial community. In the network the size of the nodes indicates centrality (small node = low centrality, big node = high centrality) and the colour indicates the degree of connections (colour scale blue-green-yellow-red indicates increasing degree). Numerous

microbial groups with specific functions, such as sulphate and sulphur reduction (e.g. *Desulfomonile*, *Desulfobacteraceae*, *Desulfovibrionales*, *Desulfurispora*, *Planctomycetes*), oxidation of reduced sulphur compounds (e.g. *Sulfuricurvum*, *Sulfurimonas*, *Thiohalorhabdales*, *Thiobacterales*, *Sulfobacillaceae*), methylotrophy (*Methylophaga*. *Methylosinus*, *Methylococcales*, *Methyloversatilis*), nitrogen cycling (e.g. *Nitrospira*, *Rhizobiaceae*), syntrophic bacteria (e.g. *Syntrophaceae*, *Syntrophobacteraceae*) showed relatively high degrees of centrality and number of connections in the network. However, the majority of the taxa with the highest degree of centrality were heterotrophic bacteria capable of fermentation, such as the Elusimicrobia, Exiguobacterium, Gordonia, Planctomycetes, and taxa capable of degradation of recalcitrant organic molecules, such as Kordiimonadales.

3.6 Predicted metabolic functions of the deep subsurface microbial communities

The putative metabolic functions of the microbial communities at different depth was predicted using the PICRUSt software, which compares the identified 16S rRNA gene sequences to those of known genome sequenced species thereby estimating the possible gene contents of the uncultured microbial communities. The analysis is only an approximation, but may give an idea of the possible metabolic activities in the deep biosphere. In order to evaluate the soundness of the analysis a nearest sequenced taxon index (NSTI) for each of the bacterial and archaeal communities was calculated by PICRUSt. An NSTI value of 0 indicates high similarity to the closest sequenced taxon while NSTI=1 indicates no similarity. The NSTI of the bacterial communities at different depths varied between 0.045 in sample OL-KR44 and 0.168 in sample OL-KR13 (Figure 7). The NSTI for archaea were much higher ranging from 0.141 in sample OL-KR9 at depth of 432 m and 0.288 in OL-KR44. This indicates that the metagenomic estimates are only indicative. The estimated microbial metabolism did not differ noticeably between the different depths (Figure 8a and b). The most important predicted metabolic pathways included membrane transport in both bacterial and archaeal communities. The most common pathways for carbohydrate metabolism were the butanoate, propionate, glycolysis/gluconeogenesis and pyruvate metabolism pathways for the bacteria and glycolysis/gluconeogenesis and pyruvate metabolism pathways for the archaea (Figure 9). Glucose is converted into pyruvate and further to Acetyl-CoA by both bacteria and archaea. The bacterial community may produce and utilize acetate. Both the bacterial and archaeal communities fix carbon via the Wood-Ljungdal (WL) reverse Citric acid cycle (rTCA) and Calvin pathways. Methane is produced from methylamines, CO_2 and methanol by

the methanogenic archaea. Based on the predicted metagenomes the bacterial community is not able to oxidize methane or hydrolyze methanol, but the methylotrophs present may use formic acid and trimethylamines.

The most abundant energy metabolic pathway in the bacterial communities was the oxidative phosphorylation (Figure S3) while for the archaea the methane metabolism was the most important (Figure9). Utilization of propanoate and butanoate (Figure 9) by the bacterial communities as well as well covered fatty acid biosynthesis and degradation pathways indicate that the bacterial community is capable of fermentation (Figure S4a and b). Nitrate is reduced both through dissimilatory nitrate reduction to ammonia and through denitrification to nitrous oxide by the bacteria (Figure S5). In addition, nitrogen is fixed to ammonia by both archaea and bacteria. The ammonia is then used as raw material for L-glutamate synthesis (Figure S5). Sulfur metabolism was not a major pathway in either the bacterial or the archaeal communities according to the predicted number of genes. However, assimilatory sulphate reduction was indicated in both the bacterial and archaeal communities, while dissimilatory sulphate reduction and sulphur oxidation was indicated only in the bacterial communities (Figure S6).

Several amino acid synthesis pathways were predicted (Figure 8), of which the most prominent were the alanine, aspartate and glutamate synthesis, arginine and proline synthesis, cysteine and methionine synthesis, glycine, serine and threonine synthesis, phenylalanine, tyrosine and tryptophan synthesis and the valine, leucine and isoleucine synthesis pathways.

Different types of membrane transport (ABC transporters) was identified where sulphate and iron (III) were taken up by the bacteria and tungstate, molybdate, proline, zink, cobalt and nickel was taken up by both archaea and bacteria (Figure S7). The estimated number of genes for both the purine and pyrimidine metabolism was more than two times higher in the archaeal community than in the bacterial community (Figure 8a and b).

4 Discussion

The phenotypic characteristics of the Fennoscandian Shield deep subsurface microbial communities are still largely unknown although specific reactions to introduced environmental stimulants have been shown (e.g. Pedersen et al., 2013; 2014; Rajala et al., 2015; Kutvonen 2015). Nevertheless, the connection of these microbial responses to specific

microbial groups is still only in an early phase. Metagenomic and gene specific analyses of deep subsurface microbial communities have revealed prominent metabolic potential of the microbial communities, which appear to be associated with the prevailing lithology and physicochemical parameters (Nyyssönen et al., 2014; Purkamo et al., 2015). It has also been shown with fingerprinting methods with ever increasing efficiency that the bacterial and archaeal communities are highly diverse in the saline anaerobic Fennoscandian deep fracture zone groundwater (Bomberg et al., 2014; 2015; Nyyssönen et al., 2012; 2014; Pedersen et al., 2014; Miettinen et al, 2015; Sohlberg et al., 2015). Nevertheless, the concentration of microbial cells in the groundwater is quite low (Figure 2, Table 1). Most of the microbial communities at different depth in Olkiluoto bedrock fractures consist of bacteria. However, at specific depths (328 m, 423 m) the archaea may contribute with over 50% of the estimated 16S rRNA gene pool (Table 1). The major archaeal group present at these depths were the ANME-2D archaea indicating that nitrate-mediated anaerobic oxidation of methane may be especially common (Haroon et al., 2013). The high abundance of archaea in Olkiluoto is special for this environment. Archaea have also been quantified from the Outokumpu deep scientific borehole (Purkamo et al., 2016), but unlike the situation in Olkiluoto the archaeal community was less than 1% of the total community at best.

Previously, using 454 amplicon sequencing, we have observed OTU numbers of approximately 800 OTUs per sample covering approximately 550 bacterial genera (or equivalent groups) and approximately 350 archaeal OTUs including approximately 80 different genera (or equivalent groups) (Miettinen et al., 2015). Miettinen et al. (2015) defined the OTUs 97% sequence homology and the number of sequence reads per sample was at most in the range of 10^4. In contrast, our sequence read numbers were 10- to 100-fold higher and the number of OTUs per sample in general 100-fold higher. This indicates that a greater sequencing depth increases the number of taxa detected from the subsurface environment and allows us a novel view of the so far hidden rare biosphere. Nevertheless, in comparison to the high number of OTUs detected the number of identified genera, 651 and 81 bacterial and archaeal genera, respectively, seems low. On the other hand, this indicates that the sequencing depth has been sufficient to detect most of the prokaryotic groups present. Nevertheless, the obtained numbers of OTUs per sample in this study were huge (Table 2). This may reflect the high level of variability in the short sequence reads of the v6 region used in this study. As discussed by Huse et al. (2008), short sequence reads very often match several different full-length 16S rRNA reads. As shown in our study taxonomic assignments, such as

'Proteobacteria_other' were common and may be due to multiple matches for the individual sequence reads obtained in the identification step of the analysis.

In general, the microbial communities at different depth grouped loosely into clusters according to the groundwater chemistry (Figure 5). Salinity diverged the bacterial communities of the two deepest samples (OL-KR44 and OL-KR29) from the rest of the samples and sulphate, sulphur and sulphide moved the more shallow samples from depths between 296 m and 347 m to the right of the NMDS plot. Sulphate reducers were not among the most common bacterial taxa in these samples (Figure 3), but several sulphur and sulfide oxidizing taxa were detected, such as the *Sulfuricurvum* and members of the *Thiobacterales*. The archaeal communities were evenly distributed throughout the NMDS plot. The archaeal communities did not change dramatically with depth and Euryarchaeota_Other, ANME-2D and Thermoplasma_E2 groups dominated throughout the depth profile. Previuos studies on the Finnish deep biosphere has shown that the microbial communities at different sites vary strongly from each other. Purkamo et al. (2015) investigated the bacterial and archaeal communities of different fracture zones of the Outokumpu deep scientific borehole and found that the majority of the bacterial populations at depths between 180 m and 500 m depth consist of Betaproteobacteria belonging to the *Commamonadaceae* and the archaeal communities consist of *Methanobacteriaceae* and *Methanoregula*.

The core communities, defined as taxa present in all the studied samples, accounted for between 80 – 97% and 95 − > 99% of the bacterial and archaeal communities, respectively. This is a considerable frequency of common microbial taxa. Nevertheless, the number of rare taxa detected from the sample set was 3.3 to 6.8 fold higher than the number of core taxa on genus level. Our results agree with Sogin et al. (2006) and Magnabosco et al. (2014), who showed that a relatively small number of taxa dominate deep-sea water and deep groundwater habitats, respectively, but a rare microbiome consisting of thousands of taxonomically distinct microbial groups are detected at low abundances. What this means for the functioning of the deep subsurface is that the microbial communities have the capacity to respond and change due to changes in environmental conditions. For example, Pedersen et al. (2014) showed that by adding sulphate to the sulphate-poor but methane-rich groundwater in Olkiluoto the bacterial population changed over the span of 103 days from a non-SRB community to a community dominated by SRB. In addition, a change in the geochemical environment induced by H_2 and methane impacted the size, composition and functions of the microbial

community and ultimately led to acetate formation (Pedersen et al., 2012; Pedersen, 2013; Pedersen et al., 2014). This is also in accordance to the network analysis (Figure 6), which indicated a great diversity in the metabolic functions of the most central microbial taxa detected in this environment.

The metabolic pathways predicted by PICRUSt are far from certain when uncultured and unculturable deep subsurface microbial communities are concerned. The NSTI values for both the bacterial and well as the archaeal communities were high indicating that closely related species to those found in our deep groundwater have yet to be sequenced. This is in accordance with Langille et al. (2013), who showed that environments containing a high degree of unexplored microbiota also tend to have high NSTI values. Staley et al. (2014) also showed in a comparison between PICRUSt and shot gun metagenomic sequencing of riverine microbial communities that PICRUSt may not be able to correctly assess rare biosphere functions. Nevertheless, Langille et al. (2013) showed that PICRUSt may predict the metagenomic content of a microbial community more reliably than shallow metagenomic sequencing. Thus, on higher taxonomical level common traits for specific groups of microorganisms may be revealed.

Energy metabolism. Deep subsurface environments are often declared energy deprived environments dominated by autotrophic microorganisms (Hoehler and Jorgensen, 2013). However, recent reports indicate that heterotrophic microorganisms play a greater role than the autotrophic microorganisms in Fennoscandian deep crystalline subsurface environments (Purkamo et al., 2015). Heterotrophic communities with rich fatty acid assimilation strategies have been reported to fix carbon dioxide on the side of e.g. fermenting activities in order to replenish the intracellular carbon pool, which otherwise would be depleted. Wu et al. (2015) also found by metagenomic analyses that fermentation was a major metabolic activity in the microbial community of Swedish deep groundwater. Our results agree with Purkamo et al. (2015) that a greater proportion of the microbial community is involved in carbohydrate and fatty and organic acid oxidation than in fixation of inorganic carbon. Nevertheless, autotrophic carbon fixation pathways were predicted in the analysis with PICRUSt, indicating that both the archaeal and bacterial communities include autotrophic members, although these microorganisms might not be obligate autotrophs. It is also likely that heterotrophic and chemilitotrophic microorganisms coexist in the Olkiluoto deep fracture zones forming networks as shown in Figure 6 for the benefit of the whole microbial community. Such

cooccurrences have been suggested by e.g. Osburn et al. (2014). It was also noted that even though evidence for methane oxidation could not be inferred from the PICRUSt predictions (no *pmo*A genes), the bacterial community may oxidize formate, which is in agreement with the findings reported by Wu et al. (2015).

Several carbon fixation pathways were predicted in the metagenomes, the Calvin cycle, reductive TCA (rTCA) cycle and Wood-Ljungdahl (WL) pathway. The WL-pathway is considered the most ancient autotrophic carbon fixation pathway in bacteria and archaea (Fuchs 1989, Martin et al. 2008, Berg et al. 2010; Hügler and Sievert, 2011) and was found in both the bacterial and the archaeal communities. In the archaeal community the Calvin cycle and the rTCA were especially pronounced in the samples from 296 m, 405 – 423 m and somewhat lower at 510 – 527 m depth. The bacterial communities are predicted to fix CO_2 at almost all depths with the exception of 405 m and 559 m depth. Nevertheless, our results agree with Nyyssönen et al. (2014), who showed my metagenomic analysis that the microbial communities at different depth of the Outokumpu scientific deep drill hole may fix carbon in several ways, of which the rTCA, the WL pathway and the Calvin cycle were identified. Magnabosco et al. (2016) showed that the WL pathway was the dominating form of carbon fixation in metagenomes of 3 km deep Precambrian crust biospheres in South Africa. Dong et al. (2014) also suggested that microorganisms in low-energy deep subsurface environment may have several strategies for e.g. carbon fixation, as shown in the *Halomonas sulfidaeris*, in order to access as many resources as possible. The predicted methane metabolism (methane and methyl compound consumption) and oxidative phosphorylation were equally strong in the bacterial community. Sulphur metabolism was not a common pathway for energy in either the archaeal or the bacterial communities, but bacteria with either assimilative or dissimilative sulphate reduction were present. Sulphur oxidation through the *sox* system was in general not predicted, but the *sox*D gene was predicted and oxidation of thiosulphate to sulphate may be possible (Figure S6). Nitrate is reduced both through dissimilatory nitrate reduction to ammonia and through denitrification to nitrous oxide by the bacteria. In addition, nitrogen is fixed to ammonia by both archaea and bacteria. The ammonia is then used as raw material for L-glutamate synthesis.

Oxidative phosphorylation was one of the most prominent energy generating metabolic pathways in the bacterial community. This indicates that ATP is generated by electron transfer to a terminal electron acceptor, such as oxygen, nitrate or sulphate. In the archaeal

community the oxidative phosphorylation was not as strongly indicated, but this may be due to missing data on archaeal metabolism in the KEGG database.

The main energy metabolism of the archaeal communities appeared to be the methanogenesis, especially at 296 m and 405 m. Methanogenesis was common also at all other depths except 330 m – 347m, 415 m and 693 m – 798 m. Methane is produced from CO_2-H_2 and methanol, and from acetate, although evidence for the acetate kinase enzyme was lacking. Methanogenesis from methylamines may also be possible, especially at 296 m and 405 m. Methane oxidation using methane monoxygenases and methanol dehydrogenases does not occur in either bacterial or archaeal communities.

Carbohydrate metabolism. Glycolysis/gluconeogenesis is one of the most common carbohydrate-metabolizing pathways predicted for both the archaeal and bacterial communities (Figure 9). Pyruvate from glycolysis is oxidized to acetyl-CoA by both archaea and bacteria and used in the TCA cycle. The TCA cycle provides for example raw material for many amino acids, such as lysine and glutamate. The butanoate and propanoate metabolisms were also common in the bacterial communities, indicating fermentative metabolism and capability of fatty acid oxidation.

Amino acid metabolism. Non-essential amino acids, such as alanine, aspartate and glutamate are produced from ammonia and pyruvate or oxaloacetate especially in the archaeal populations. In the archaeal population proline appears to be produced from glutamate. Despite the low use of sulphate as energy source in the microbial communities sulphate and other sulphur compounds are taken up for the production of the amino acids cysteine and methionine by both the archaeal and the bacterial communities. A higher predicted relative abundance of genes involved in aromatic amino acid synthesis (phenylalanine, tyrosine, tryptophane) was seen in the archaeal than in the bacterial communities. Both the archaeal and the bacterial communities synthesise branched chained amino acids (isoleucine, leucine and valine), but only the bacteria degrade them. Especially proteobacteria have been shown to be able to use the branched chained amino acids (isoleucine, leuscine and valine) and short chained fatty acids (acetate, butyrate, propionate) as sole energy and carbon source (Kazakov et al., 2009). The branched chained amino acids function as raw material in the biosynthesis of branched chained fatty acids, which regulate the membrane fluidity of the bacterial cell. In salt stress conditions, the proportion of branch-chained fatty acids in the membranes decreases.

Membrane transport. According to the predicted metagenomes, the microbial cells transport sulphate into the cell, but do not take up nitrate. Nitrogen is taken up as glutamate but not as urea. Iron is taken up by an Fe(III) transport system and an iron complex transport system in the bacterial communities, but generally only by the iron complex transport system in archaea. However, Fe(III) transport system may also exist in the archaeal communities at 405 m to 423 m depth, where also some manganese/iron transport systems could be found. Molybdate and phosphate is transported into the cell by molybdate and phosphate ATPases, respectively. Nickel is taken up mainly by a nickel/peptide transport system but also to some extent by a cobalt/nickel transport system. Zink is taken up to some extent by a zink transport system, but transport systems for manganese, manganese/iron, manganese/zink/iron, or iron/zink/copper are negligent. Ammonia is taken up by an Amt transport system.

5 Conclusions

The wide diversity of microbial groups in the deep Fennoscandian groundwater at the Olkiluoto site revealed that the majority of the microbial community present belong to only a few microbial taxa while the greatest part of the microbial diversity is represented by low abundance and rare microbiome taxa. The core community was present in all tested samples from different depths, but the relative abundance of the different taxa varied in the different samples. Specific rare microbial groups formed tight co-occurrence clusters that corresponded to different environmental conditions and these may become more abundant if the environmental conditions change. Fermentation or oxidation of fatty acids was a common carbon cycling and energy harvesting metabolic pathways in the bacterial communities whereas the archaea may either produce or consume methane. Glycolysis/gluconeogenesis was predicted to be common in both the archaeal and bacterial communities. In addition both the bacterial and archaeal communities were estimated to contain several different common carbon fixation pathways, such as the Calvin cycle and the reductive TCA and the Wood-Ljungdahl pathway.

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Table 1. Geochemical and microbiological measurements from 12 different water conductive fractures in the bedrock of Olkiluoto, Finland. The different drillholes are presented at the top of the table. The data is compiled from Posiva (2013) and Miettinen et al. (2015)

Drillhole	OL-KR13	OL-KR6	OL-KR3	OL-KR23	OL-KR5	OL-KR49	OL-KR9	OL-KR9	OL-KR2	OL-KR1	OL-KR44	OL-KR29
Sampling date	3/11/2010	18/5/2010	29/8/2011	15/12/2009	16/10/2012	14/12/2009	31/10/2011	29/8/2011	27/1/2010	26/1/2010	15/1/2013	18/5/2010
Depth (m)	296	328	340	347	405	415	423	510	559	572	693	798
Alkalinity												
mEq/L	2.19	0.37	0.47	0.05	0.27	0.16	0.18	0.13	0.29	0.23	0.49	0.13
Ec mS/m	897	1832	1047	2190	2240	2670	2300	2960	4110	3770	6690	7820
pH	7.9	7.9	7.9	7.5	7.9	8.1	7.7	8.1	8.6	7.8	7.5	7.3
NPOC mg L^{-1}	10	0	12	5.1	19	3	5.1	6.6	11	5	110	10
DIC mg L^{-1}	27	4.1	4.1	3.9	0	3	3	0	3.75	3.75	6.5	81
HCO_3 mg L^{-1}	134	22.6	25	17.1	16	9.8	11.6	7.3	17.7	14	30	424
N_{tot} mg L^{-1}	0.71	0	1.1	0.42	1.2	0.16	0.38	0.66	1.1	0.41	10	3.1
NH_4^+ mg L^{-1}	0.07	0.03	0.03	0	0	0	0.05	0	0.02	0.04	0.08	0.08
S_{tot} mg L^{-1}	31	130	12	1.7	1.7	0	4.8	0	0	0	4	0
SO_4^{2-} mg L^{-1}	79.5	379	32	2.9	3	1.4	13.7	0.9	0.5	0.5	9.6	2
S_2^- mg L^{-1}	5.1		0.38	0.62	2	0.02	0.36	0	0.02	0.13	0.02	0.02
Fe_{tot} mg L^{-1}	0.0042	0.0037	0.022	0.062	0.2	0.71	0.036	0.02	0	0.49	1.2	560
Fe(II) mg L^{-1}	0	0	0.02	0.08	0.21	0.53	0.06	0.02	0.02	0.04	1.2	0.46
TDS mg L^{-1}	4994	10670	5656	12710	12880	15900	13430	18580	25500	23260	37410	53210
K mg L^{-1}	8.2	9.3	7.6	8.3	18	27	12	17	19	20	24	27
Mg mg L^{-1}	35	77	17	55	68	19	32	41	18	52	33	136
Ca mg L^{-1}	460	1100	290	2100	1750	2700	2260	2930	4600	3700	7680	10000
Cl mg L^{-1}	2920	6230	3400	7930	7950	9940	8220	11500	15700	14600	22800	33500
Na mg L^{-1}	1320	2800	1850	2530	2990	3110	2790	3970	4980	4720	6570	9150
TNC ml^{-1}	4.2×10^5	1.0×10^5	2.4×10^5	2.5×10^5	2.1×10^5	1.5×10^4	na	2.9×10^4	5.9×10^4	8.7×10^4	5.5×10^4	2.3×10^4
16S qPCR ml^{-1}												
bacteria	7.0×10^5	9.5×10^3	2.0×10^4	3.6×10^5	4.9×10^4	1.3×10^4	7.2×10^4	1.5×10^5	1.4×10^5	1.9×10^4	3.2×10^4	1.5×10^4
archaea	5.8×10^3	2.0×10^4	9.9×10^3	6.3×10^4	6.2×10^3	1.5×10^2	4.4×10^4	5.2×10^2	7.5×10^2	3.0×10^3	2.6×10^1	2.8×10^2

Table 2a. The total number of sequence reads, observed and estimated (Chao1, ACE) number of OTUs, number of singleton and doubleton OTUs, and Shannon diversity index per sample of the bacterial 16S rRNA gene data set. The analysis results are presented for both the total number of sequence reads per sample as well as for data normalized according tot he sample with the lowest number of sequence reads, i.e. 140,000 random sequences per sample.

Bacteria		All sequences						Normalized to 140,000 sequences					
Sample	Number of sequence reads	Observed OTUs	Chao1	ACE	Singles	Doubles	Shannon	Observed species	Chao1	ACE	Singles	Doubles	Shannon
OLKR13/296m	786,346	79,527	87,188	91,360	18,025	21,203	13	37,045	74,288	84,530	22,445	6,762	13
OLKR3/318m	345,433	52,381	53,238	54,961	5,789	19,557	14	39,309	57,793	64,021	19,287	10,061	13
OLKR6/328m	188,812	29,411	35,018	37,269	9,209	7,561	13	26,442	34,964	37,626	10,420	6,369	13
OLKR23/347m	485,154	33,257	37,175	38,895	8,000	8,166	11	20,494	34,268	37,305	10,641	4,109	11
OLKR49/415m	184,052	38,275	49,758	53,525	14,799	9,535	13	34,117	48,804	52,938	15,372	8,043	13
OLKR9/423m	175,295	36,412	44,452	47,571	12,357	9,494	14	33,596	44,496	48,161	13,489	8,345	14
OLKR5/435m	141,886	40,445	70,520	78,340	22,166	8,167	14	40,145	70,288	78,232	22,086	8,090	14
OLKR9/510m	241,312	41,545	51,348	54,535	14,251	10,357	13	33,208	49,115	53,631	15,592	7,640	13
OLKR2/559m	257,789	45,456	72,269	78,325	22,550	9,481	13	32,600	62,318	69,573	19,071	6,118	12
OLKR1/572m	210,659	29,804	35,362	37,491	9,197	7,607	12	25,703	34,934	37,682	10,650	6,142	12
OLKR44/750m	303,058	31,410	31,589	32,188	2,005	11,200	12	25,937	33,448	36,295	10,346	7,124	12
OLKR29/798m	221,524	37,989	45,126	48,042	11,991	10,071	13	31,911	44,957	48,533	14,078	7,594	13

Table 2b. The total number of sequence reads, observed and estimated (Chao1, ACE) number of OTUs, number of singleton and doubleton OTUs, and Shannon diversity index per sample of the archaeal 16S rRNA gene data set. The analysis results are presented for both the total number of sequence reads per sample as well as for data normalized according tot he sample with the lowest number of sequence reads, i.e. 17,000 random sequences per sample.

Archaea		All sequences						Normalized to 17,000 sequences					
Sample	Number of sequence reads	Observed OTUs	Chao1	ACE	Singles	Doubles	Shannon	Observed OTUs	Chao1	ACE	Singles	Doubles	Shannon
OLKR13/296m	507,373	27,111	29,516	30,699	5,835	7,076	10	3,957	13,380	15,062	2,867	435	10
OLKR3/318m	271,699	25,491	32,299	34,231	9,205	6,221	11	4,955	15,044	17,238	3,546	622	10
OLKR6/328m	446,380	21,597	22,930	23,781	3,861	5,588	10	3,776	11,705	14,020	2,748	475	9
OLKR23/347m	395,339	20,800	22,403	23,214	4,083	5,199	10	3,919	11,855	13,323	2,755	477	9
OLKR49/415m	210,545	22,600	23,372	24,004	2,975	5,733	12	7,023	17,088	19,874	4,738	1,114	12
OLKR9/423m	697,360	22,014	22,527	23,082	2,381	5,520	9	3,180	9,617	10,586	2,224	383	9
OLKR5/435m	769,026	21,127	22,235	23,078	3,515	5,574	9	2,596	10,114	10,078	1,852	227	9
OLKR9/510m	169,142	12,709	12,782	12,960	713	3,488	11	4,879	11,205	13,215	3,148	782	11
OLKR2/559m	100,101	15,359	24,950	27,026	7,840	3,203	11	5,119	14,497	16,488	3,548	670	11
OLKR1/572m	1,213,360	28,884	33,207	34,832	7,846	7,118	9	2,273	9,233	9,923	1,631	190	9
OLKR44/750m	17,716	6,436	8,748	9,750	2,890	1,805	12	6,325	8,743	9,804	2,921	1,763	12
OLKR29/798m	98,770	15,641	16,720	17,483	3,158	4,617	12	6,951	14,655	17,184	4,483	1,303	12

Table 2b. The total number of sequence reads, observed and estimated (Chao1, ACE) number of OTUs, number of singleton and doubleton OTUs, and Shannon diversity index per sample of the archaeal 16S rRNA gene data set. The analysis results are presented for both the total number of sequence reads per sample as well as for data normalized according tot he sample with the lowest number of sequence reads, i.e. 17,000 random sequences per sample.

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OLKR23/347m	395,339	20,800	22,403	23,214	4,083	5,199	10	3,919	11,855	13,323	2,755	477	9
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OLKR5/435m	769,026	21,127	22,235	23,078	3,515	5,574	9	2,596	10,114	10,078	1,852	227	9
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OLKR44/750m	17,716	6,436	8,748	9,750	2,890	1,805	12	6,325	8,743	9,804	2,921	1,763	12
OLKR29/798m	98,770	15,641	16,720	17,483	3,158	4,617	12	6,951	14,655	17,184	4,483	1,303	12

1

1

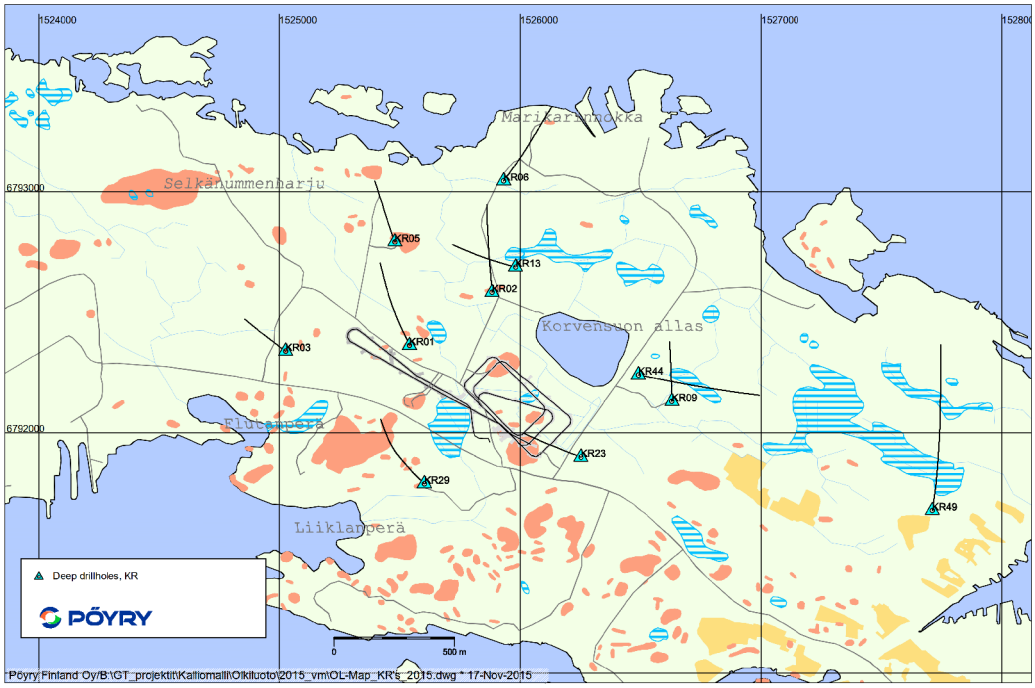


Figure 1. Map of Olkiluoto. The boreholes used in this study are marked with a turquoise triangle and the attached black line depicts the direction of the borehole. (with courtesy of Pöyry Oy, Nov 17th, 2015 by Eemeli Hurmerinta)

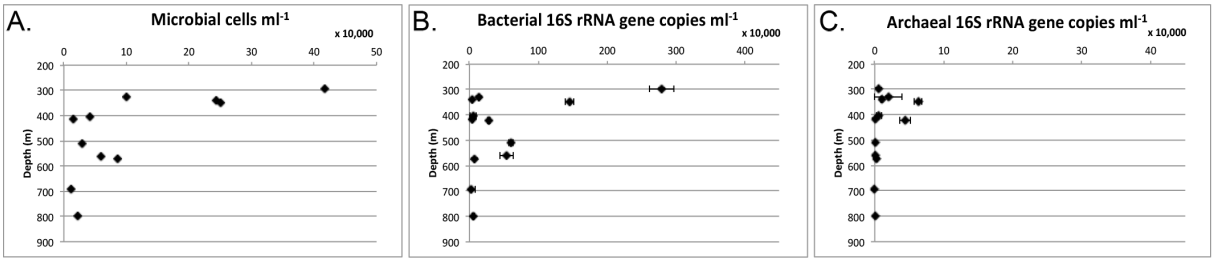


Figure 2. The concentration of A) microbial cells mL^{-1} determined by epifluorescence microscopy and the estimated concentration of B) bacterial and C) archaeal 16S rRNA gene copies mL^{-1} groundwater determined by qPCR in water conductive fractures situated at different depths in the Olkiluoto bedrock.

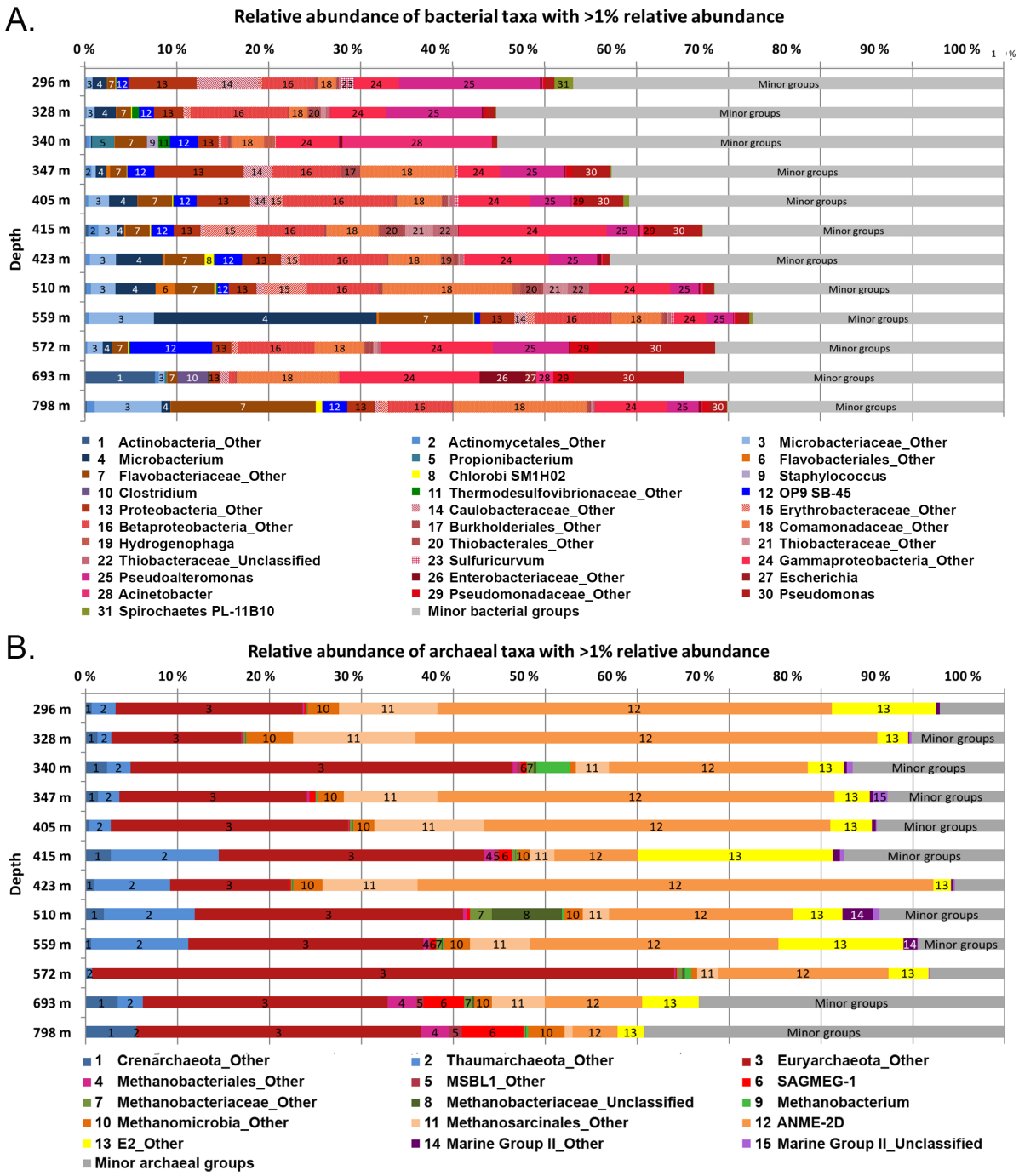


Figure 3. The most abundant A) bacterial and B) archaeal taxa representing at least 1% of the sequence reads in any of the samples. The number in each series indicate the taxon number in the list below the figures.

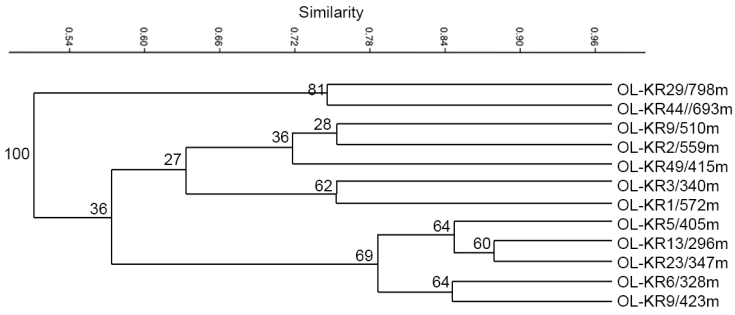


Figure 4. A Bray-Curtis UPGMA cladogram clustering the studied samples according to the detected taxonomy of the microbial communities. The bacterial and archaeal community profiles were combined. The branch support values were calculated from 100 bootstrap repeats.

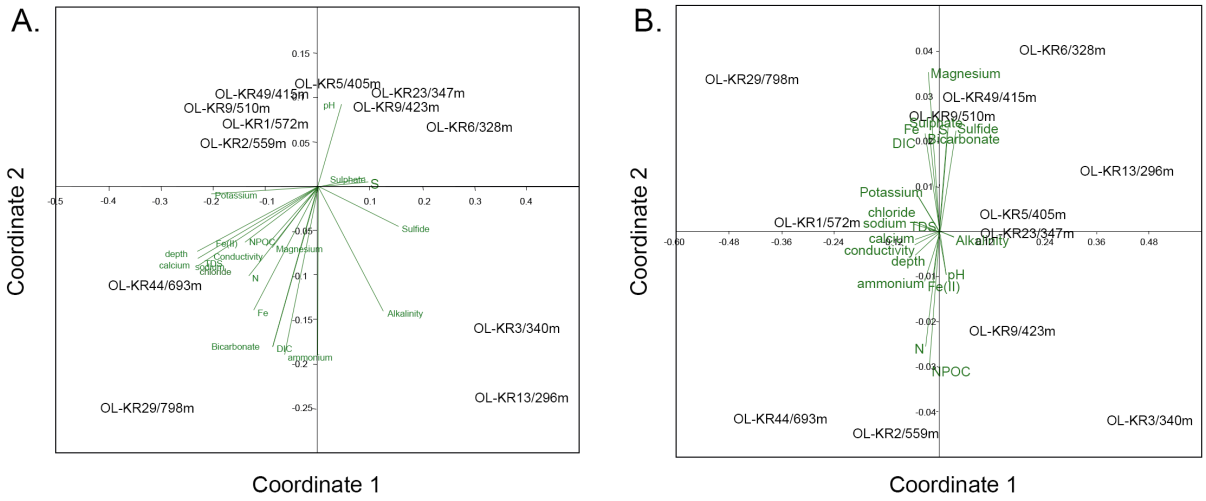


Figure 5. Non-metric multidimensional scaling analysis based on the A) bacterial and B) archaeal communities detected in the samples. The triplot (green) indicates directionality of the environmental variables.

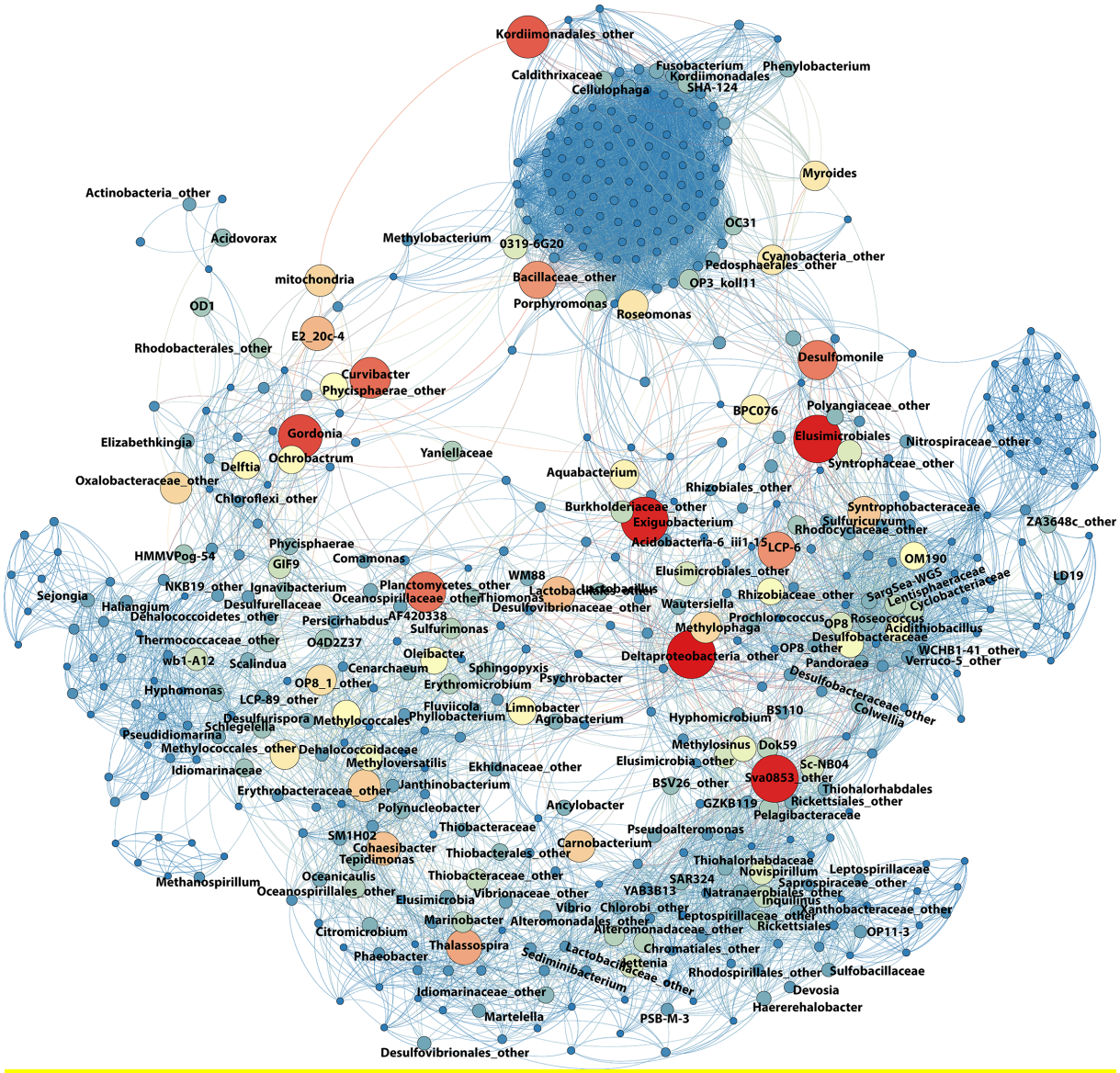


Figure 6. Network of co-occurring microbial taxa based on Spearman's rank correlation values between pairs of taxa. correlation ($R>0.7$, $p<0.01$) between different taxa. Each circle (node) represents a taxon and the size of the node is proportional to the number of connections (Spearman correlation value) of the node. The colour of the nodes indicates degree of centrality of the taxon, with low centrality shown as blue, increasing centrality by green to yellow to orange and highest centrality as red. Taxa with less than 10% centrality range (<2 connections) were filtered out. The most prominent nodes are indicated by taxon names. In Figure S2 the names for all nodes are shown.

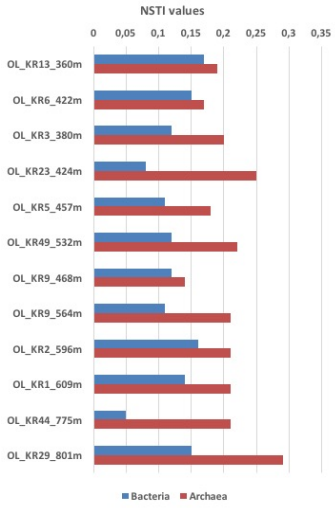
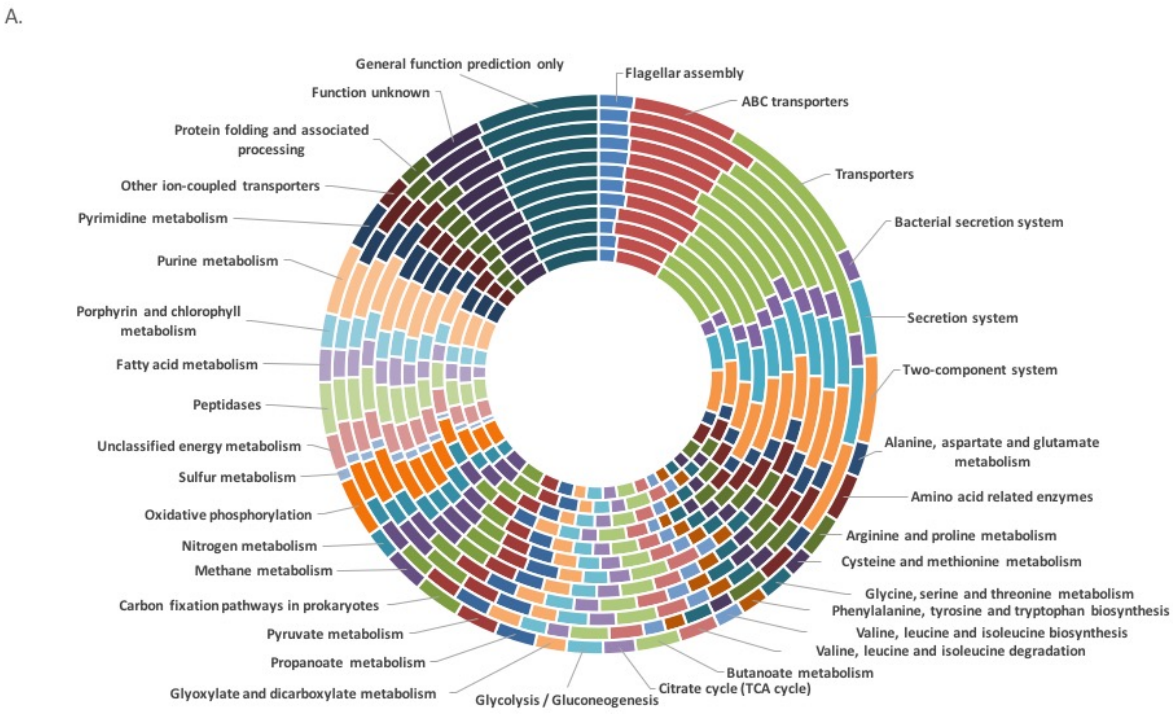


Figure 7. The nearest sequenced taxon index (NSTI) values calculated by PICRUSt for the bacterial (blue) and archaeal (red) connumities. The NSTI value describes the sum of phylogenetic distances of each OTU to its nearest relative with a sequenced reference genome, and measures substitutions per site in the 16S rRNA gene and the weighted the frequency of the each OTU in a sample dataset. A higher NSTI value indicates greater distance to the closest sequenced relatives of the OTUs in each sample.



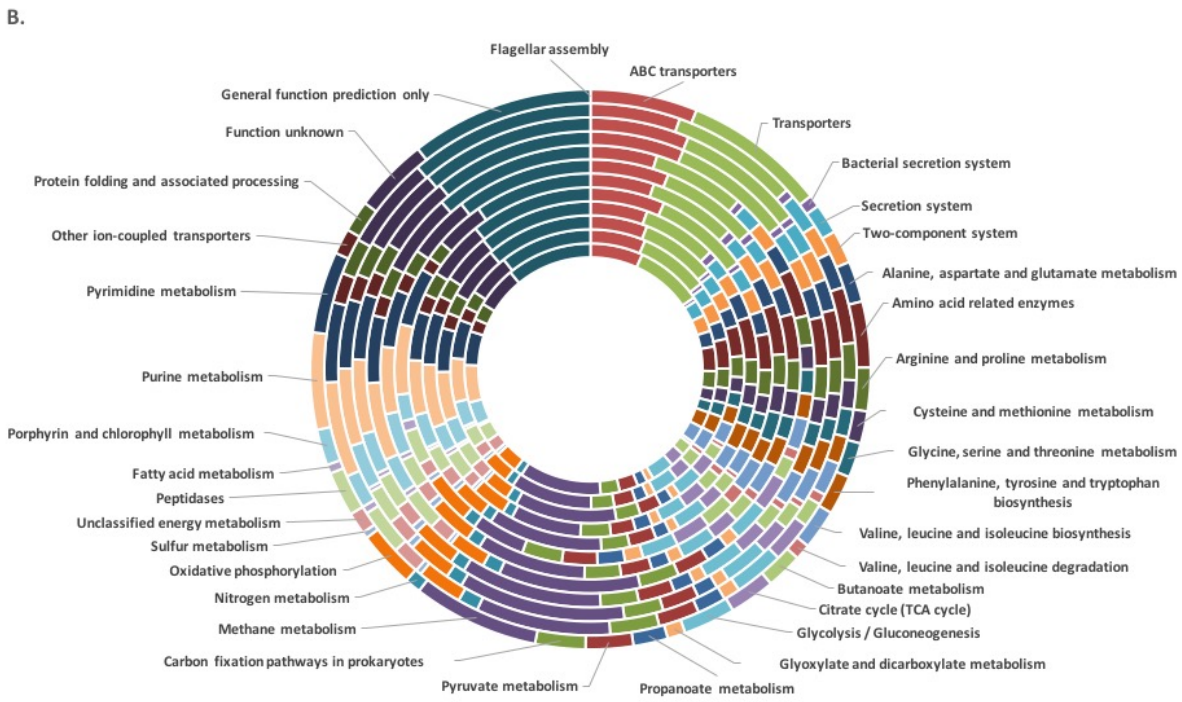


Figure 8. The relative abundance of predicted genes of the most abundant pathways identified in the A) bacterial and B) archaeal populations in the PICRUSt analysis. The pathways are presented according to KEGG. The samples are ordered according to depth, with OL-KR13/296m as innermost and OL-KR29/798m as the outermost sample.

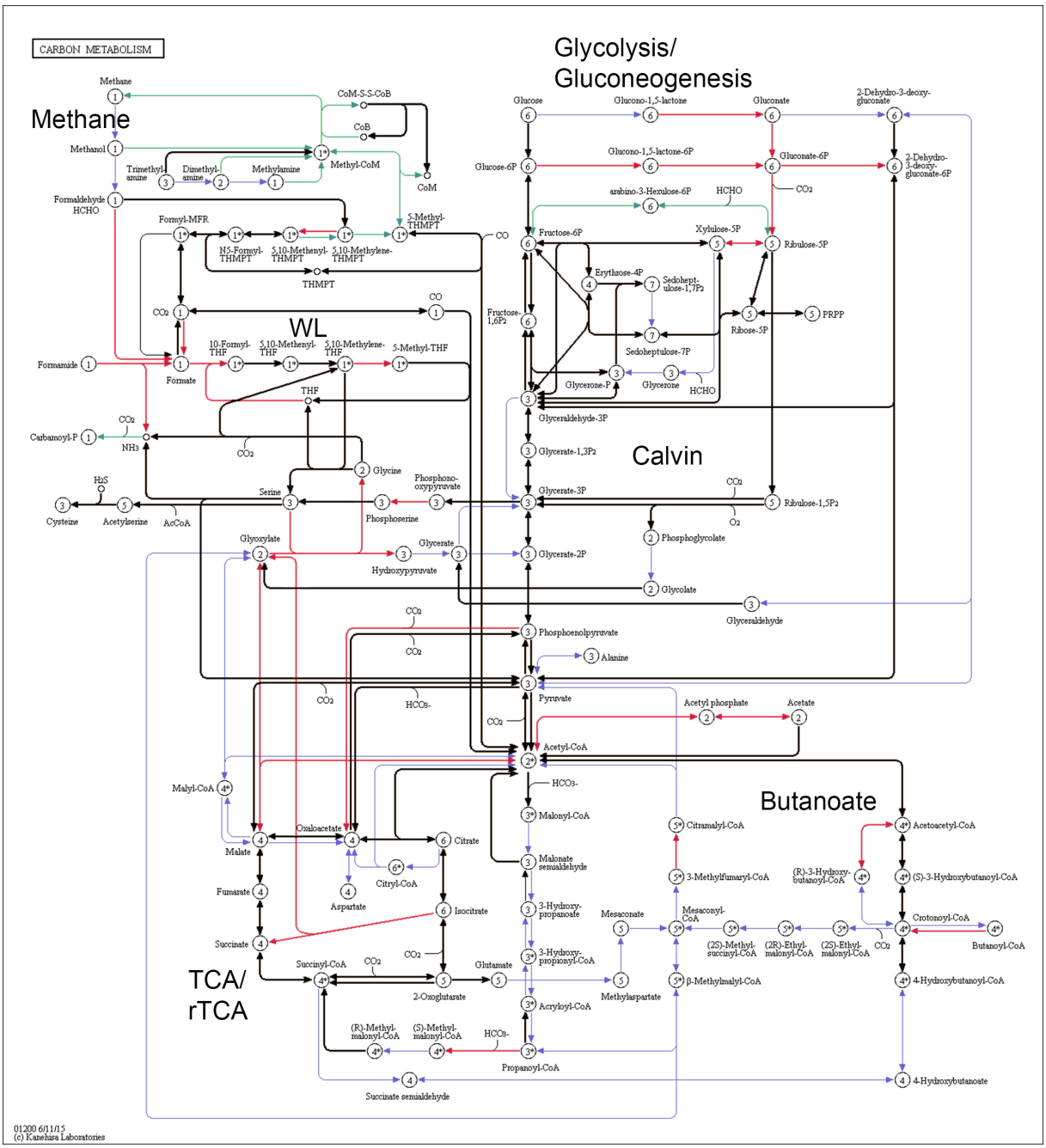


Figure 9. The microbial carbon metabolism pathway according to KEGG. The predicted genes combined from all samples were plotted on the map. Green arrows indicate enzymes predicted only in the archaeal communities, red arrows indicate genes predicted only in the bacterial communities, black arrows show enzymes predicted in both the archaeal and bacterial communities and blue arrows show enzymes that were not predicted in any of the communities.

Supplementary figures

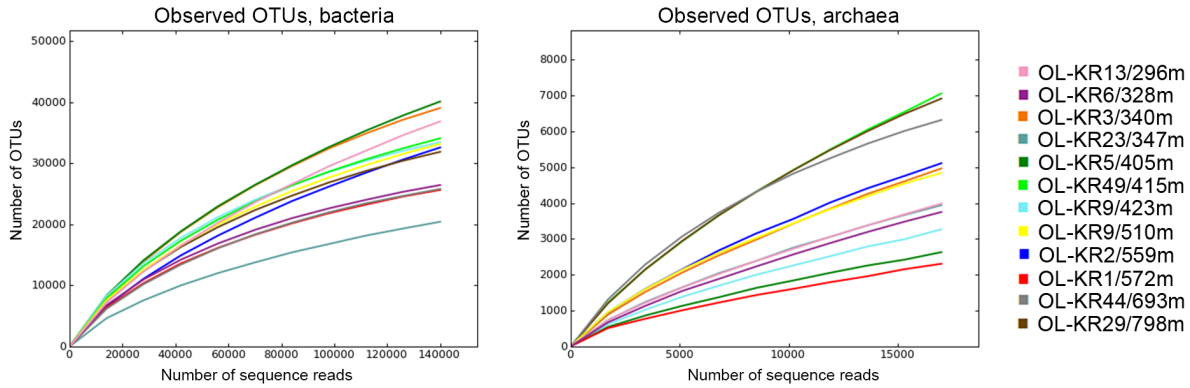


Figure S1. The rarefaction curves of observed bacterial (left pane) and archaeal (right pane) OTUs in each sample generated on sequence data normalized to 140,000 reads for bacteria and 17,000 reads for archaea.

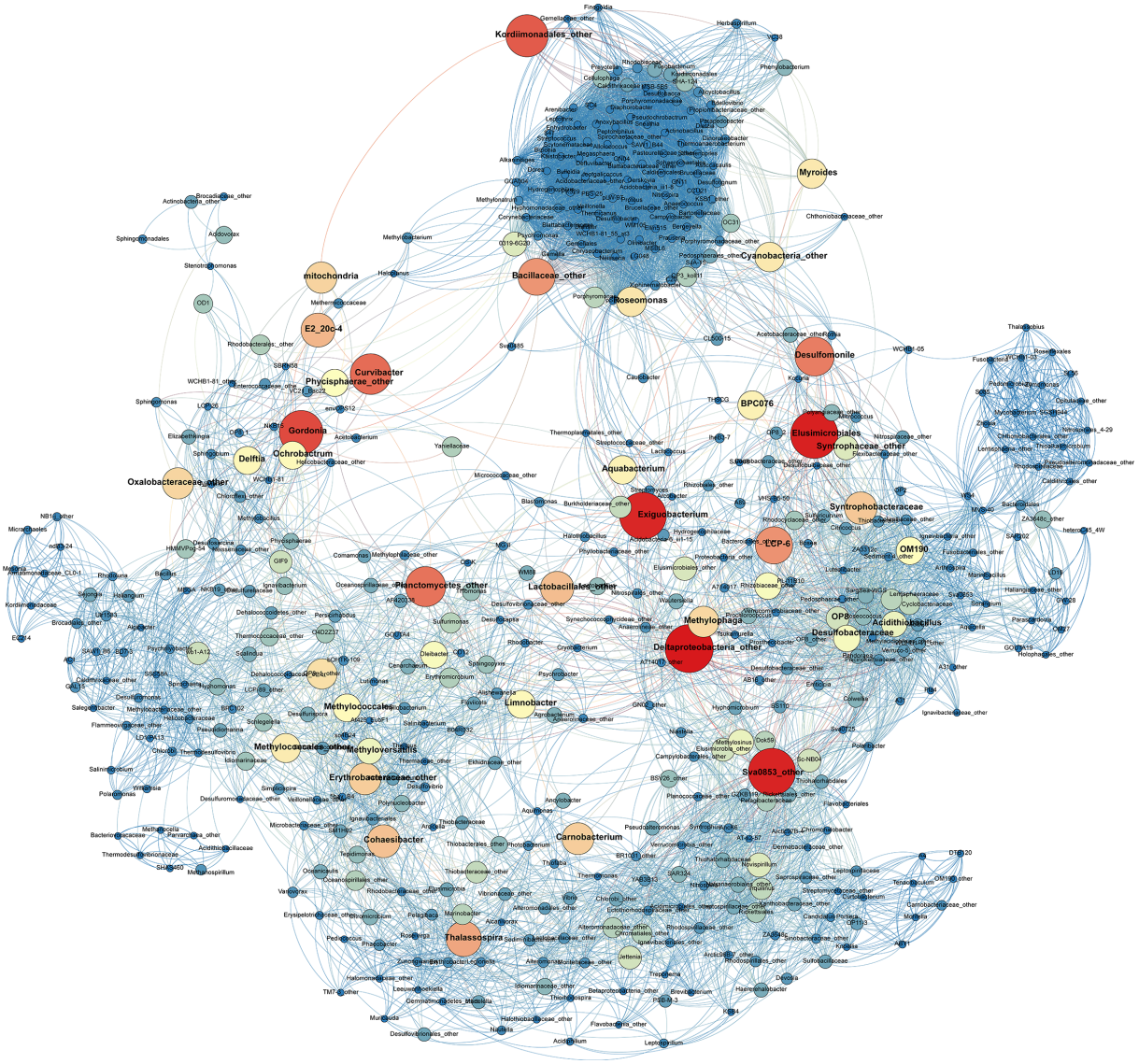


Figure S2. Network of co-occurring microbial taxa based on Spearman's rank correlation values between pairs of taxa. correlation (R>0.7, p<0.01) between different taxa. Each circle (node) represents a taxon and the size of the node is proportional to the number of connections (Spearman correlation value) of the node. The colour of the nodes indicates degree of centrality of the taxon, with low centrality shown as blue, increasing centrality by green to yellow to orange and highest centrality as red. Taxa with less than 10% centrality range (<2 connections) were filtered out. The names for all taxa included in the analysis are shown.

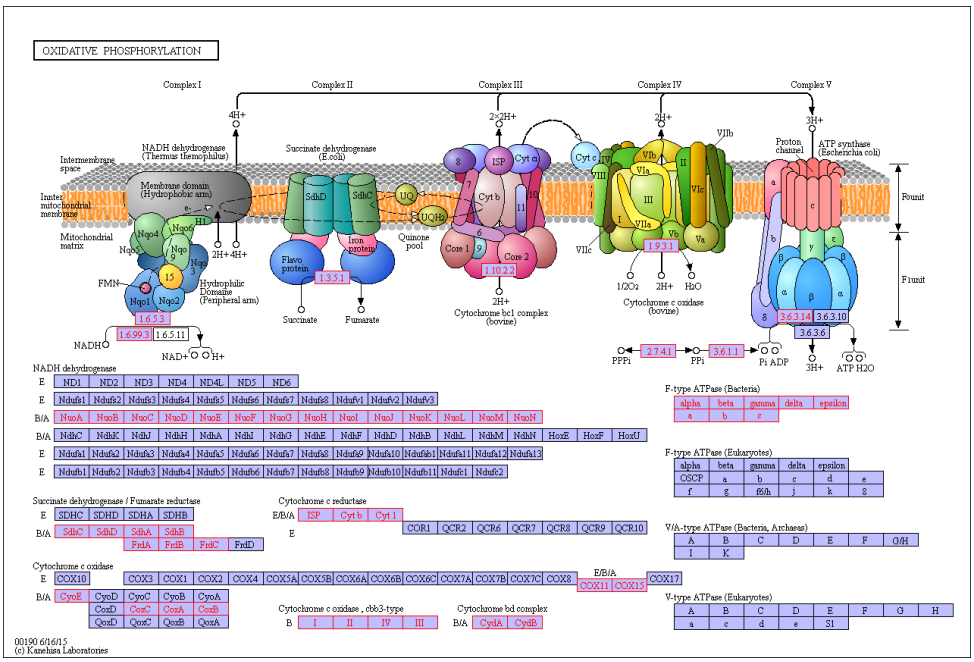
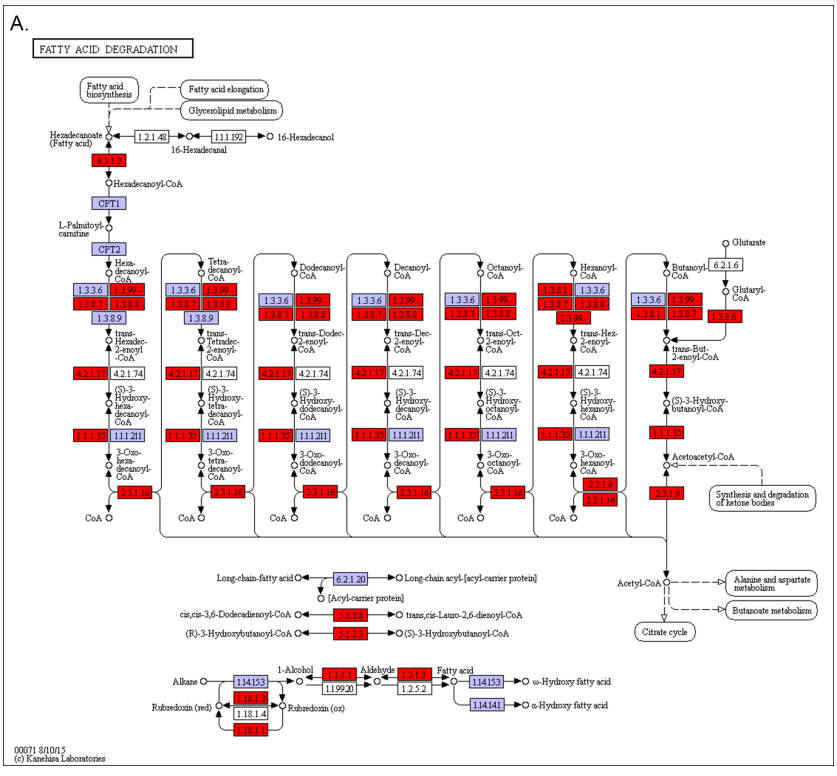


Figure S3. Bacterial oxidative phosphorylation according to KEGG. The predicted genes from the bacterial communities belonging to the oxidative phosphorylation are shown in pink.



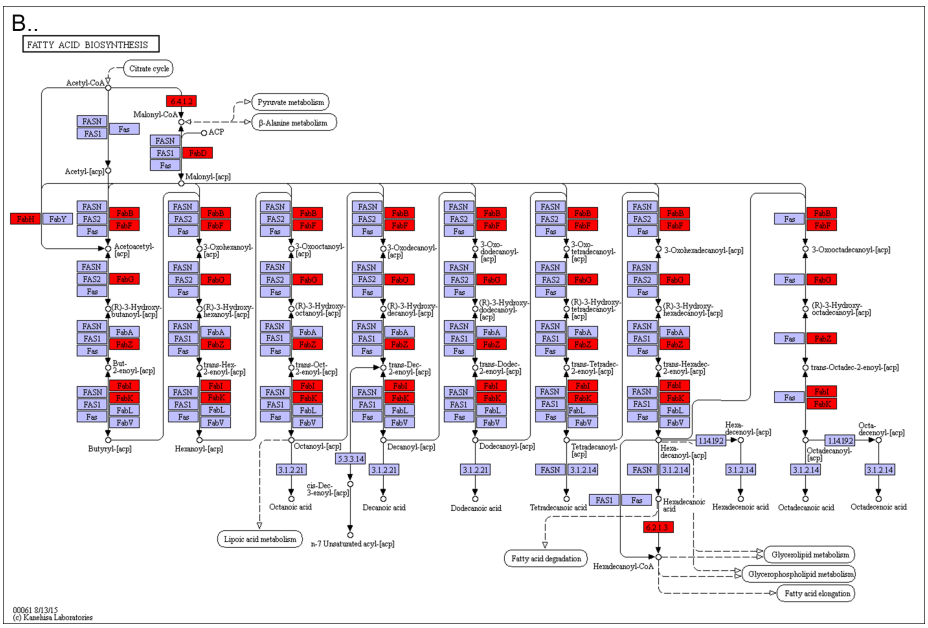


Figure S4. Predicted genes shown in red of the bacterial A) fatty acid degradation and B) fatty acid biosynthesis pathways, combined from all samples.

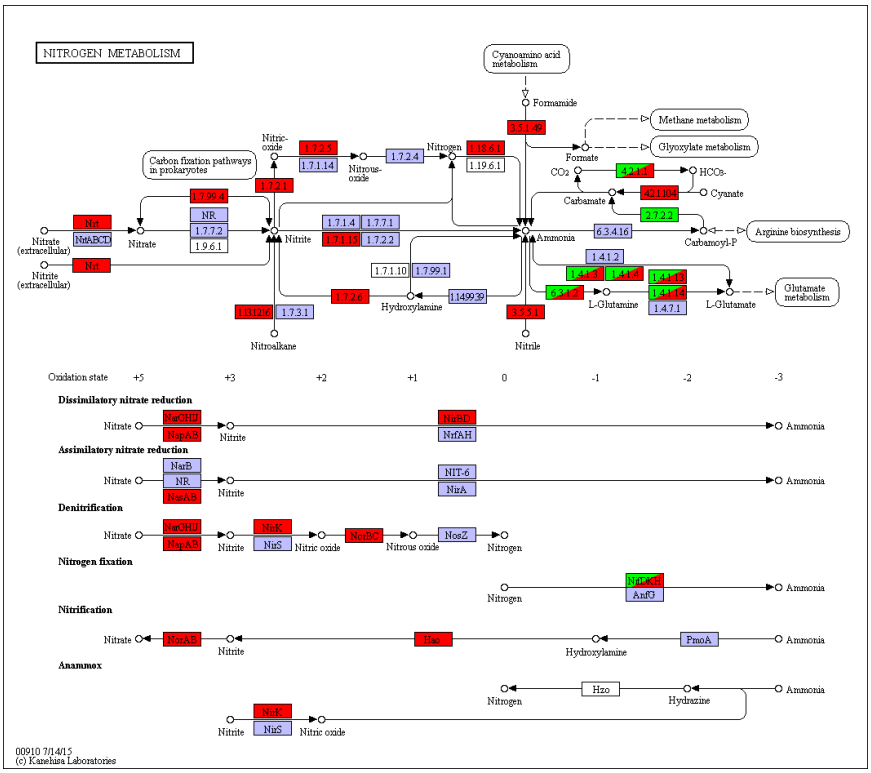


Figure S5. The predicted genes of enzymes included in the microbial nitrogen metabolism according to KEGG. Enzymes predicted from the bacterial communities are shown in red, archaeal communities in green and enzymes predicted from both archaeal and bacterial

communities in green/red. Enzymes not predicted from either community are shown in blue or white.

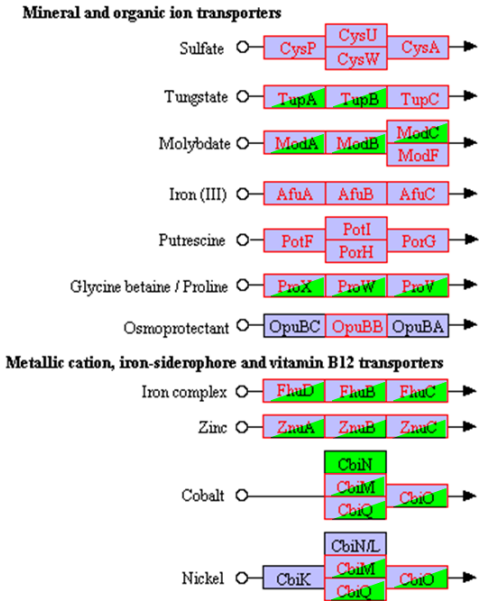


Figure S6. The genes of ABC transporters predicted from the bacterial (pink), archaeal (green) or both (pink/green) communities. Genes not predicted in any of the communities are shown in blue.

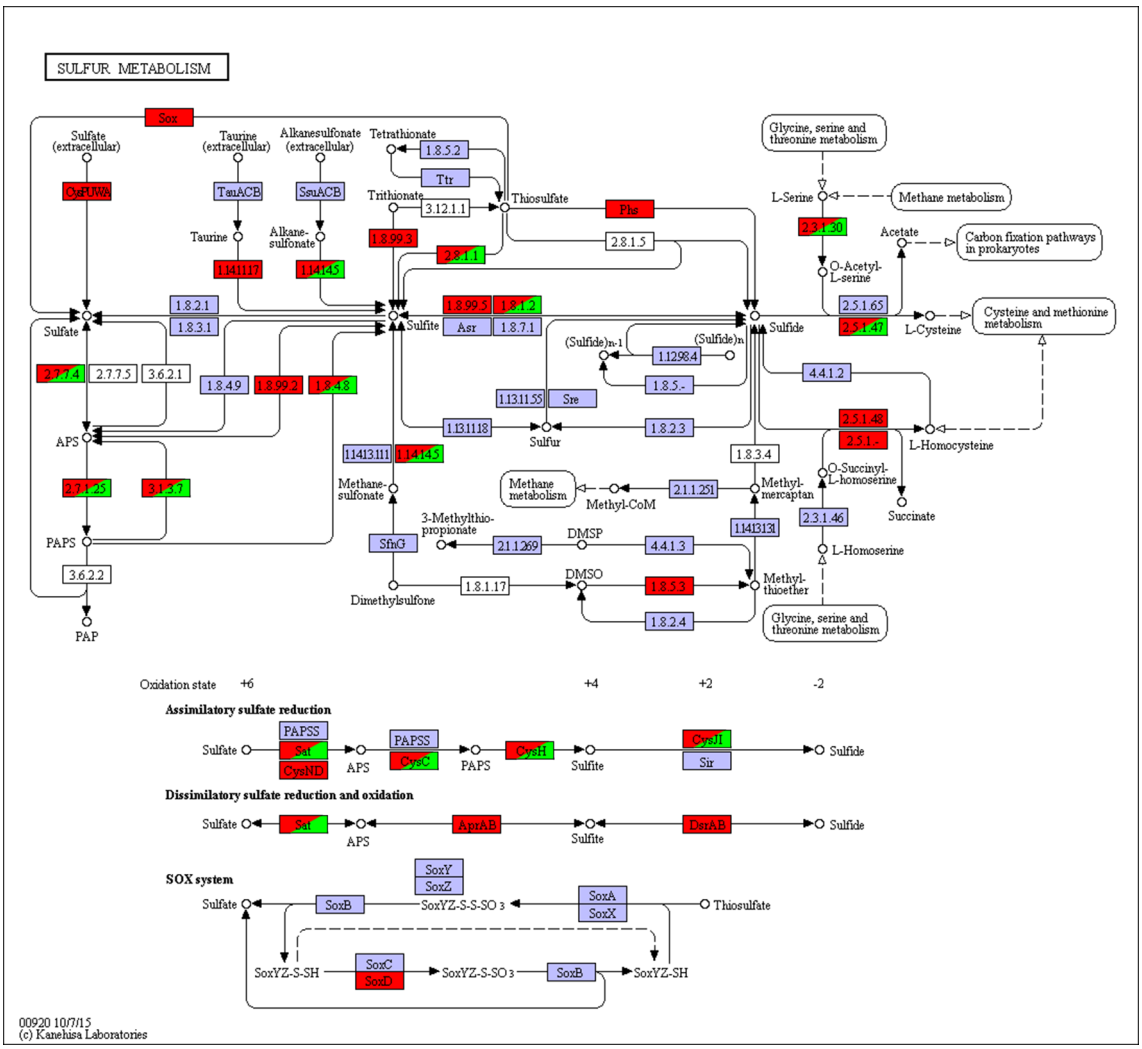


Figure S7. The predicted genes of enzymes included in the microbial sulphur metabolism according to KEGG. Enzymes predicted from the bacterial communities are shown in red, archaeal communities in green and enzymes predicted from both archaeal and bacterial communities in green/red. Enzymes not predicted from either community are shown in blue or white.

Author responses to Reviewer 1

A: We would like to thank the reviewer for critically reviewing our manuscript. Our detailed responses to the reviewer's comments are marked below with 'A:' and the reviewer's comments are marked with 'R:'

R: General comments.
Broadly the interest in subsurface life has grown in recent years, and the linkage of 16S rRNA gene identified taxa distribution and geochemical parameters would be of broad interest to journal readers from a variety of backgrounds. Overall, the manuscript reads cleanly and is easy to follow. The authors carefully inferred from the data- however additional details on how certain analyses were conducted, or in the case of piecrust, more information on the potential error is necessary. The 4 figures are largely overshadowed by the massive information contained in tables, in general I don't think tables are as effective for visually reporting data, and think the authors could include more visual representation of the data. For instance rather than the table or in addition to the tables, some visual representation of community structure and change would be appreciated. Also the figures and tables in the text are very small and needs to be increased throughout.

A: These comments have been addressed in the Specific comments below.

Specific comments.

R: Abstract:
line 6: IN general avoid "great" in sequencing depth- as it is qualitative. Report number of reads and depth of sampling and let it stand for itself. Depth is dependent on structure of community and amount of reads.

A: L6 – The sentence has been changed to indicate the number of sequences obtained: 'a sequencing depth of up to 1.2×10^6 reads per sample' (P1,L15)

R: I appreciated the "background" information being included in this manuscript so readers do not have to look up information in another paper to understand the system.

A: Thank you.

R: Why was V6 selected as the primer region? Might be nice to include a reference for this region from recent studies done on a similar sequencer (read length). Also please confirm sequencer and model- a HiSeq and include model, 2000 or GAII?

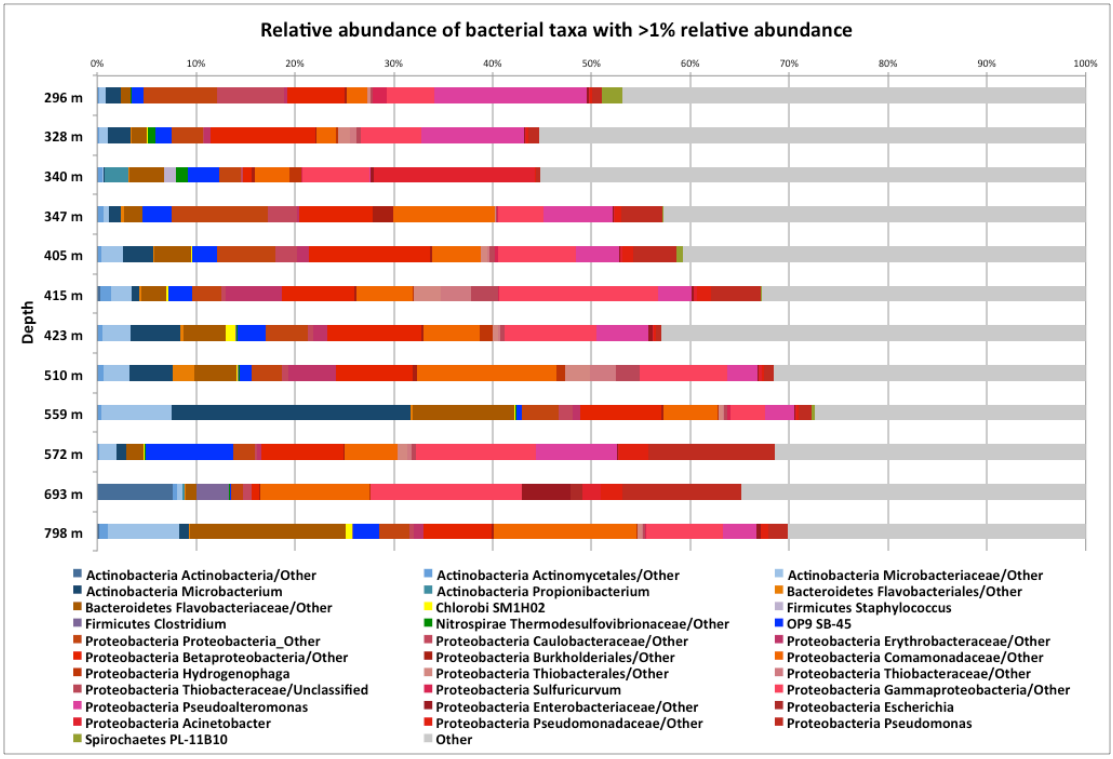
A: The v6 region was the one provided by the Census of Deep Life (CoDL) sequencing program. This region has been rigorously tested by the CoDL and for maximizing capture of all known groups of bacteria and archaea the V6 region was targeted with a mixture of similar primers for each primer site. The sequence quality was also maximized by using a completely overlapping paired-end approach, for which the V6 region was perfect (described in Eren et al., 2013, reference added to the text). More information about the bacterial and archaeal 16S rRNA gene v6 primers can be found at https://vamps.mbl.edu/resources/primers.php. This link has been added to the paper. The sequencer was a HiSeq1000.

R: Results:
Line 15, in a few words define "occurring". Does this mean a single read, or a certain level of abundance (at least 1%) in all samples? Also define rare.

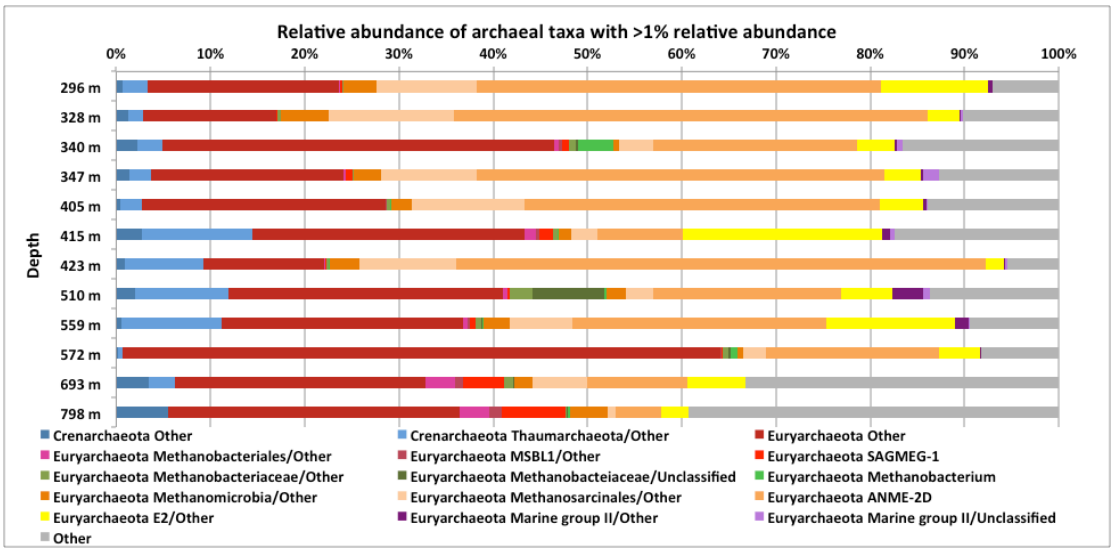
A: ‘occurring’ was changed to ‘detected’.

In the previous section on p11 (L7-14) we present the most common bacterial and archaeal groups where the relative abundance of the reported genera (or equivalent group) was at least 1% of the sequence reads in any sample. 31 bacterial genera and 15 archaeal genera belonged to these ‘most common’ taxa. In the core community we have not set a threshold, but report the taxa that were detected in all samples, no matter how few the sequence reads were per taxon. We did not look at this data on OTU level, but only on genus level so we did not have any single sequence groups. In section 3.5, L4, the rare biosphere is considered the microbial taxa that were sporadically detected in only some of the samples, but not in all samples. This will be added to the text as; ‘These groups represented the rare biosphere, i.e. taxa that were only detected at low relative abundance in some of the samples, but not in all’. Two different representations of these results are shown in the figures below for Bacteria and Archaea separately. Now all the ‘not common’ taxa are presented as ‘Other’. These may be easier to interpret than Tables 3 and 4.

Relative abundance of the most common bacterial taxa:



Relative abundance of the most common archaeal taxa:



R: Figure 3. Can the authors provide some more information on the image- perhaps color and shape to represent the sample scores (dots) by depth and location more easily than text? As a reader I am trying to understand what is unique about each of the samples and the geochemistry. For instance is OL-KR44 unusually deep or location wise distinct from the others.

A: **Figure 3** – The figure has been changed to separate NMDS plots for the archaea and bacteria (Figure 5). The depth of each sample is given in the sample names in this figure and all the chemistry that the calculations are based on is given in Table 1. The OL-KR44 sample is chemically very different from the other deep samples in this study. There is a peak in the concentration of organic carbon (NPOC) and sulphate, which are more typical for the samples from lesser depth, while at the same time the salinity of the sample is high, which is typical for the deeper samples. The font size of the sample names has been increased for clarity. A

map of the Olkiluoto site showing the positions of all the boreholes we sampled for this study has also been included in the paper (Figure 1).

R: Were all samples collected at the same time? Sorry if I missed this.

A: The sampling times for all samples are provided in Table 1 and a note 'The collection of samples occurred between December 2009 and January 2013 as described previously…' has been added to section 2.2.

R: Please check language on top of 13831, I wasn't quite sure what was being said here. An introductory sentence could also help. *"Of the 651 bacterial and 81 archaeal genera (or equivalent groups) identified in this study 42 bacteria and 59 archaeal genera showed any significant correlation with other genera."* In general I am having a difficult time tracking this analyses- additional details would help simplify the reading of the manuscript. How was rare calculated and determined? Not clear.

A: P13, This text has been omitted.

R: From this section and figure 4.
R: 1) Please in the text provide how are the "communities" 1-7 defined (analogous to figure 4- different clusters of microbial taxa)? I find use of "communities" somewhat confusing, and think it is sufficient to call them co-occuring clusters. For instance the piecrust was done on the level of the individual samples (also communities) from different or the co-occuring cluster communities that were just defined two sentences before? Please refrain from using name community for the in silica identified clusters.

R: 2) Figure 4.

- R: How was the chemistry overlayed on the groups, was this included into the analyses or done manually based on another analyses (I assume strong= statistically significant correlations, and if so state rather than strong).
- R: Do the circles represent OTU level designations- if so why is there differences in taxonomy- some are family level IDs others are genus? If different, why wasn't the same taxonomic unit selected for this analyses.
- R: Also please make figure 4 larger, it was difficult to read when printed out.

A: The figure has been recalculated and completely changed (Figure 6)

R: Abstract and again in results on Line 24. Is rough an euphemism for inaccurate? Can the authors give a scale for NSTI scale- I know 1 means no match, but for instance is 0.282 for Archaea considered too far diverged that the data is error prone "or rough"? I personally do not use Piecrust for environmental systems, for the reasons the authors allude to (why not do metagenomics, there is a danger in inferring function from divergent 16S), but I am open to entertaining its use if necessary precautions are taken and quantified. So I think the readers would benefit from some authors providing some additional data here. For instance, typically for human microbiome samples the NSTI ranges from XX-XX, while other NSTI reported from environmental datasets have had a range of XX-XX. Provide precedence please for including seemingly high numbers, and thus more inaccuracy, in the analyses, as this will be good to incorporate in future studies/comparisons.

A: We mean a not very fine-scaled estimation, not inaccurate. Meant more as an approximation. 'Rough prediction' will be changed to 'approximate estimation'. Langille et al. (2013) describes the NSTI as 'the sum of phylogenetic distances for each organism in the OTU table to its nearest relative with a sequenced reference genome, measured in terms of substitutions per site in the 16S rRNA gene and weighted by the frequency of that organism in the OTU table'. If the NSTI value is given as 0.282 it means that the test subject shares about 78% 16S rRNA gene similarity to its nearest sequenced match, i.e. not very close. It is close enough, however, to be able to say that this uncultured putative methanogen has methane metabolism and that it probably uses, say, methanol for its methanogenesis, because all the closest relatives do so and it falls within a bigger archaeal cluster that all use methanol. Whether this is the case in reality is not sure, of course, but here we are trying to link taxonomical data from uncultured communities to what is known for cultured and tested species. I agree that metagenomic analysis would be better, and we have done a few. The metagenomes show quite the same as the PICRUSt analyses, but the metagenomes also suffer from uncertainty, since we get good matches only to properly annotated and well-known species. Agreed, the sequence data and predicted gene data etc would be obtained. Nevertheless, the biomass obtained from the collected samples is very low and they were not originally collected for metagenomic analysis. The DNA yield is low, which means that the DNA needs to be amplified before sequencing (or at least had to be, then). These samples were also a part of the Census of Deep Life sequencing effort and the data is here published if the name of this sequencing project and we tried to get out as much as possible from the data. Now we can continue to prove these estimations right or wrong.

R: I presume inferred community metabolism change did not also with geochemistry if not depth? Table 9b is very difficult to read. After reading the piecrust analyses in the results, I am really not sure what level of information it adds-The discussion was more clear and contained many details not included in the results.

"However, at specific depths (328, 423 m) the archaea may contribute with over 50 % of the estimated 16S rRNA gene pool (Table 1). The major archaeal group present at these depths were the ANME- 2D archaea indicating that nitrate-mediated anaerobic oxidation of methane may be 25 especially common (Haroon et al., 2013)." **Is this consistent with geochemistry from the site?**

A: The NSTI of environmental samples is higher than that of the human microbiome, which is stated in Langille et al. (2013). This has been discussed in more detail. Table 9 (which is now Figure 8) presents the percentage of predicted genes from each sample that is connected to a specific metabolic pathway. Of course, some genes (or enzymes) function in many pathways, but the ones presented in Table 9 were the best covered ones. The table shows the distribution of predicted genes. From these 'whole pathways' were extracted simply by checking if a gene (or actually KO number) was present in a sample and how abundant that gene was in the sample. In the discussion only whole pathways are included. We have also included KEGG maps (Figure 9, Figures S3-S7) to show what parts of the different pathways are covered.

ANME-2D – well, this is a problem, because the nitrate level is below the detection limit of the assay. Ntot and ammonia could be measured, but not nitrate or nitrite. This

could mean that nitrate is a rate limiting factor for the ANME-2D or that the ANME-2D are using the nitrate released from a possible ammonia oxidation process immediately. Or, the AMNE-2D are either doing something else or could be using sulphate as electron acceptor. However, sulphate for ANME-2D has not yet been shown to function as TEA, but it is possible. The geochemistry might support this theory, but it is too speculative to say in the paper.

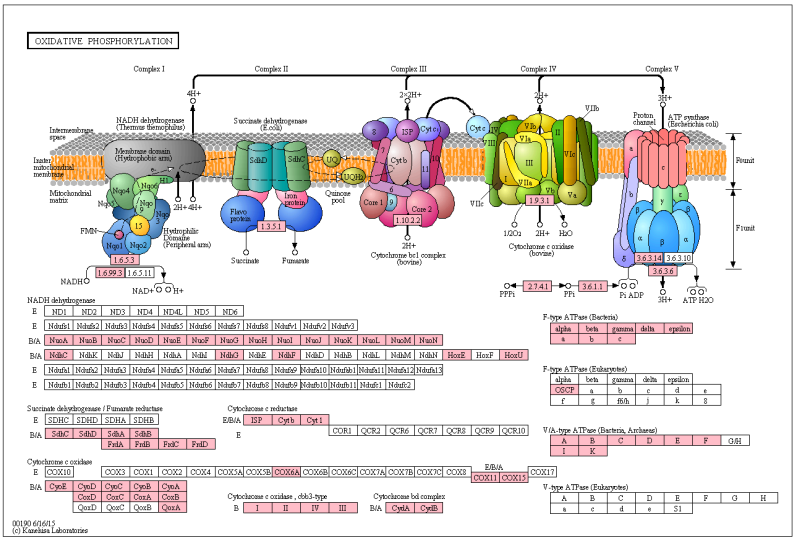
R: The NSTI values for both the bacterial and well as the archaeal communities were great indicating that no closely related species have yet been sequenced
The values were high not great.

A: The text has been changed to say that the NSTI values were high.

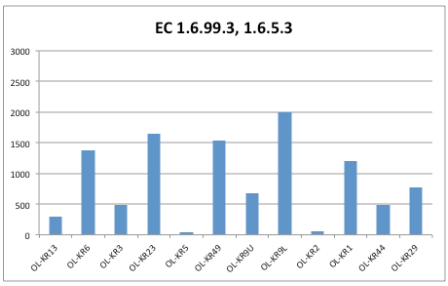
R: This statement may not be correct, as many obligate fermenters are known to have and use ATP synthase using a variety of alternative proton pumping mechanisms outside of NADH dehydrogenase. What criteria used to determined oxidative phosphorylation? The presence of NADH dehydrogenase? A full ETC? cytochrome oxidase. Please qualify by noting what genes were detected in this category.

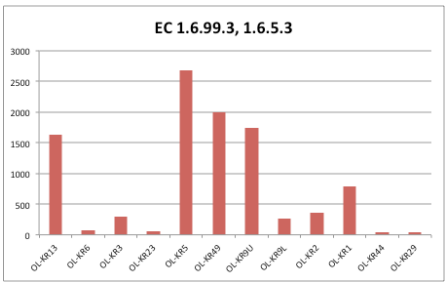
"Oxidative phosphorylation was one of the most prominent energy generating 15 metabolic pathways in the bacterial community. This indicates that ATP is generated by electron transfer to a terminal electron acceptor, such as oxygen, nitrate or sulphate."

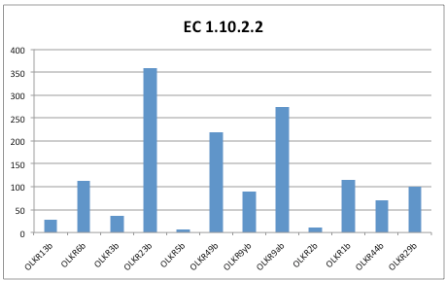
A: Oxidative phosphorylation was determined based on 114 KO numbers detected in the predicted genes, which occur in this pathway. In the figures below the predicted KO numbers were plotted on the Kegg map for oxidative phosphorylation, pink means hit, and the number of hits belonging to the EC numbers of the different enzyme complexes are displayed separately for the bacteria and the archaea. This is presented in Figure S3.

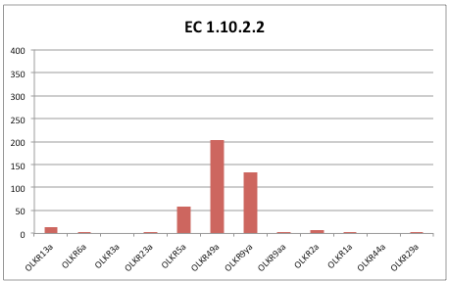


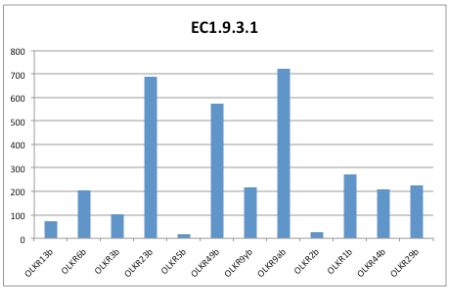
Bacteria Archaea

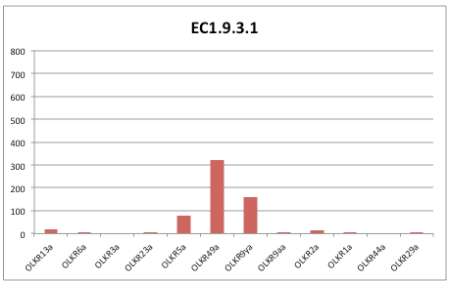


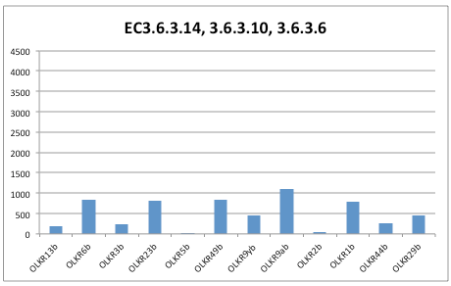


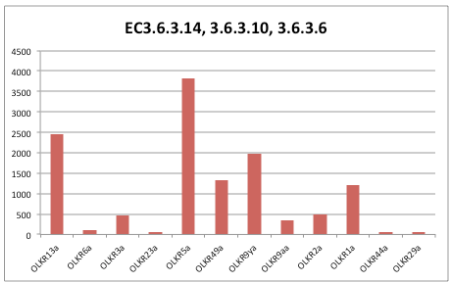












Autors' responses to Reviewer 2

A: We would like to thank the reviewer for critically reviewing our manuscript. Our detailed responses to the reviewer's comments are marked below with 'A:' and the reviewer's comments are marked with 'R:'

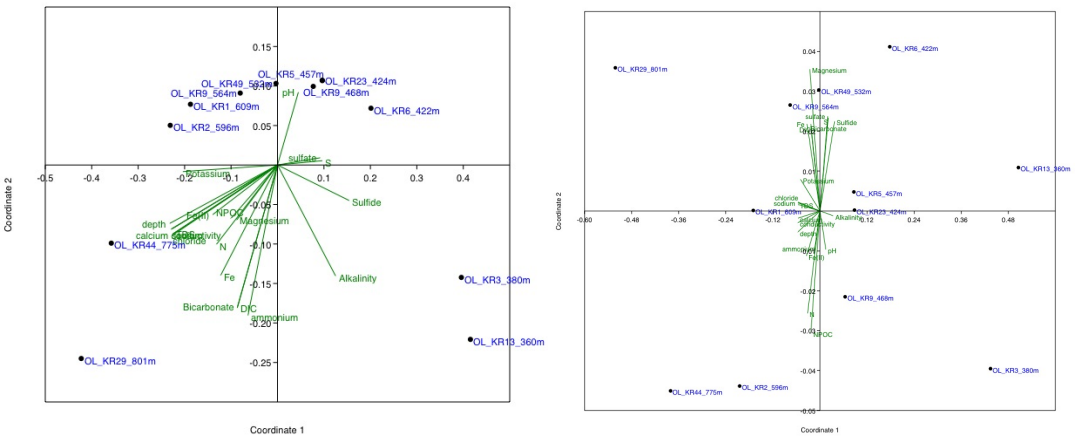
R: From the data presented, their main results include (1) the correlation of rare taxa with geophysical and geochemical settings → discussion on rare biosphere and (2) predicted metabolisms derived from PICRUSt. My main concerns with the analysis are as follows:

1. The description of the approach taken to perform the correlation analysis and subsequent significance are not clearly stated. For example it is not stated whether or not the data was normalized in any manner prior to analysis (this is also omitted when describing their implementation of CCA on the data). More importantly, the authors do not seem to correct for the high false discovery rate when testing multiple hypotheses. If I understood correctly, the authors tested the pairwise correlation of 732 genera (651 bacterial and 81 archael). Under this scenario there would be $(731 * 732)/2 = 267, 546$ comparisons made. Thus, a p-value threshold of 0.01 (as denoted in the methods section, I noticed figure 4 uses $p < 0.001$) would, in expectation, yield 2, 675 $(= 0.01 * 267, 546)$ tested hypotheses that would appear significant just by random chance. *Note, that in lines 2 and 3 of page 13831, the authors report the number of genera (101) that were found to be significantly correlated to other genera not the number of significant correlations identified.* To correct for the false discovery rate, the authors need to use some sort of correction for multiple hypothesis testing such as the q-value (Storey, John D. "The positive false discovery rate: a Bayesian interpretation and the q-value." Annals of statistics (2003): 2013-2035.) or the Bonferroni. Until an appropriate test for significance is conducted, the results and discussion based on the correlation analyses, in my opinion, are not yet in publishable form.

A: 1. The data was tested again. Normality tests (Shapiro-Wilkins, Anderson-Darling) were run on the chemical and physical parameters as well as on the taxonomical data obtained from the samples. Only parameters and taxa for which the null hypothesis could be rejected with both the S-W and A-D tests when p(normal) and p(Montecarlo)< 0.05 were included in the subsequent test. One-way ANOVA (Levine's test for homogeneity of variance from means and from medians) and Kruskal-Wallis test indicated significant differences between samples (p=0). Correlation between physicochemical parameters and bacterial and archaeal taxa was tested with the Mann-Whitney pairwise test and the p-values were corrected using the Bonferroni method. As the reviewer well predicted we ended up with no significant correlations. After discussions with several knowledgeable statisticians we have come to the

conclusion that we cannot apply Pearson correlations to out data matrix. Thus we are forced to remove these analyses from the paper.

Instead of these tests we have performed a non-metric multidimensional scaling test on the archaeal and bacterial communities vs. the environmental parameters. The archaeal data is presented in the left plot and the bacterial in the right plot. These are presented in the manuscript as Figures 5a and b.



R:

2. Although the authors note (in lines 22 to 27 of page 13822 and elsewhere) that the method of functional inference from 16S data using PICRUSt has limitations, almost the entire discussion of the submitted paper is based on the predicted functional metabolisms identified through this method. My main concern is that they are too focused on reporting the PICRUSt results rather than how the metabolisms (and 16S data) may relate to the larger context of the paper, environment type, and field. This is especially concerning when the PICRUSt results may contradict other observations that the authors report based on the 16S data such the statement about the low abundance of sulfur metabolisms (PICRUSt) on page 13825 lines 10-12 and the results of their CCA (as wells as their correlations that need correcting – see above – in lines 14 to 17 page 13833) that show communities that correspond to increased sulfur and sulfate concentrations. What is changing between these sites if not the relative abundance of sulfate/sulfur reducers? What other thoughts might you have on this? Another anomaly that concerns me is the presence of methanogens and absence of the Wood-Ljungdahl (acetyl-CoA) pathway in archaea – a pathway that is a feature of methanogens. In my opinion, there is too much dependence on reporting these results that may not be entirely accurate and are subject to interpretation (two of my major concerns are listed above). The discussion and presentation of PICRUSt results can be enhanced by performing a deeper investigation and

interpretation of the results – i.e. have the same trends (as far as predicted metabolisms) been observed before in the Fennoscandian Shield? How do they compare to metagenomes from the area or other subsurface sites? Other questions that seem important but appear to be largely ignored: Is there a difference in sulfur metabolism in the sites OL-KR5,6,9,13,23 than the rest as, taxonomically, they seem to correspond to increase in S and sulfate concentrations. What are the taxa? Are these taxa the same in the sites? Also the discussion of rare versus core is interesting but not well developed. Some things I would like to see more of are: Do the predicted metabolisms vary within the core set vs the rare? What does that observation potentially say about the theory of rare biosphere?

A: 2. PICRUSt provides an estimation based on the data present in the PICRUSt database. Only well-characterized and whole-genome sequenced microbial species are present. For example, the ANME-2D representative Candidatus Methanoperedens nitroreducens might not yet be included. However, representatives of most methanogenic clusters are present. At least the M. nitroreducens has been shown to have the genes for the Wood-Ljungdal pathway. The archaeal WL pathway appears to differ a bit from the bacterial one, so this was a misinterpretation of the results on our part. In Fig 10 the carbon metabolism of both bacteria and archaea is shown and it is clear that both versions are represented. This has now been changed in the text. The carbon monoxide dehydrogenase and acetyl-CoA decarboxylase/synthase in the archaeal community are shown in Figure 9.

The discussion about the correspondence of the microbial groups to environmental factors, such as sulphate and sulfur, has been heavily revised. The correspondence analyses have been omitted, since they can't after correction of the p-values be considered significant anymore. Some comparison between other Fennoscandian deep biosphere stydies have been made. However, there are no published metagenomes from the Olkiluoto site, only from Outokumpu (Nyyssönen et al., 2014).

Since a lot of space will be freed when the correlation tables are removed we can show more pathway maps. For example, the sulfur metabolism (as brought up by the reviewer). The taxa in all the sites are presented in the supplementary tables. We have more focused on the functions of the core groups as they represent the biggest part of the community.

R: **3 Minor Comments:**

3.1 Abstract

- Lines 6-10 are confusing and need clarification. For example, I think you are referring to 95 and 99% of the alpha diversity but it is unclear

A: Abstract – this has revised. We mean 95 and 99% of the total number of sequences obtained from the bacterial and archaeal communities, respectively. The significance statement will be revised. The discussion about the rare biosphere will be revised as suggested.

R: Significance needs to be reexamined as described in **Major Comment (1)**

A: This has been omitted from the manuscript.

R:

"It may consist of remnants of microbial communities prevailing in earlier con- ditions on Earth" is a bit misleading in the context of the rest of the paper. In my opinion, as written and throughout the paper, the discussion on the rare bio- sphere is rather ambiguous and subject to misinterpretation by the reader and need clarification. For example, the rare biosphere itself is not necessarily ancient (as stated in line 21 of page 13821) – it is a feature that is present in the microbial community observed today. However, the introduction of these taxa into the Fennoscandian Shield may have happened a long time ago and, over time, the taxa have persisted in the environment at low abundance. This is a feature not just of the Fennoscandian site but something that relates to all microbial communities. Also, it is important to note that in Sogin et al. (2008), the aforementioned mechanism is not the only avenue by which the rare biosphere may appear. Sogin et al. (2008) note: *"The large number of highly diverse, low-abundance OTUs constitutes a 'rare biosphere' that is largely unexplored. Some of its members might serve as keystone species within complex consortia; others might simply be the products of historical ecological change with the potential to become dominant in response to shifts in environmental conditions (e.g., when local or global change favors their growth). Because we know so little about the global distribution of members of the rare biosphere, it is not yet possible to know whether they represent specific biogeographical*

distributions of bacterial taxa, functional selection by particular marine environments, or cosmopolitan distribution of all microbial taxa (the 'everything is everywhere' hypothesis)"

A: This has been toned down to concern more the Olkiluoto environment than the whole earth and the 'ancient' part has been omitted.

R: 3.2 Introduction

Lines 4 and 5 of page 13821, I think it would be important to note that a core microbiome in the South African subsurface has been reported *(Magnabosco, Cara, Memory Tekere, Maggie CY Lau, Borja Linage, Olukayode Kuloyo, Mariana Erasmus, Errol Cason et al. "Comparisons of the composition and biogeographic distribution of the bacterial communities occupying South African thermal springs with those inhabiting deep subsurface fracture water." Frontiers in microbiology 5 (2014))*. This may also serve as an interesting paper for further comparison as the same region (V6) of the 16S was sequenced

A: The Magnabosco et al paper has been included in the discussion

R: Line 21 of page 13821 again, as written it is slightly misleading. See section Abstract, last comment

A: the sentence was altered

R: 3.3 Methods

2.4: I think the use cell counts and qPCR were a nice addition to the paper. I'm curious as to why primers were not V6 for the qPCR as it was the primer used in amplification

A: we have used the 'qPCR-primers' previously in our work and used for sequencing on the 454 platform. Based on these older results the primers have been deemed quite specific and to detect bacteria and archaea broadly. In addition, the archaeal qPCR was a bit tricky and we tested several different primer pairs in order to find the one that worked most reliably. The sequencing for this work was not done by us, but by the Census of Deep Life collaboration and in the CoDL the method has been standardized for the v6 region. We also wanted a slightly longer fragment that that produced by the v6 primers. The v6 primers used also consisted of a mix of primers, and we wanted to use only one primer/direction.

R: 2.6: What quality filtering method was used? When talking about the rare bio- sphere it is important to not that the QC step can greatly influence the number of taxa and size of the rare biosphere. See *(Huse, Susan M., David Mark Welch, Hilary G. Morrison, and Mitchell L. Sogin. "Ironing out the wrinkles in the rare bio- sphere through improved OTU clustering." Environmental microbiology 12, no. 7 (2010): 1889-1898.)* and *(Eren, A. Murat, Joseph H. Vineis, Hilary G. Morrison, and Mitchell L. Sogin. "A filtering method to generate high quality short reads using Illumina paired-end technology." (2013): e66643.)* for more information. Typically, is suggested to use a 100% overlap when working with sequences form the V6 region (Eren et al. 2013).

A: 2.6: The fastq files were combined in mothur using default parameters and the resulting fasta files were screened with QIIME allowing for no errors in barcodes (primers were removed by mothur).

R: 2.7: Concerns are listed in **Major Comment (1)**. Please also specify if any nor- malization was performed and correct for multiple hypothesis testing

A: 2.7: these concerns are addressed above.

R: Results

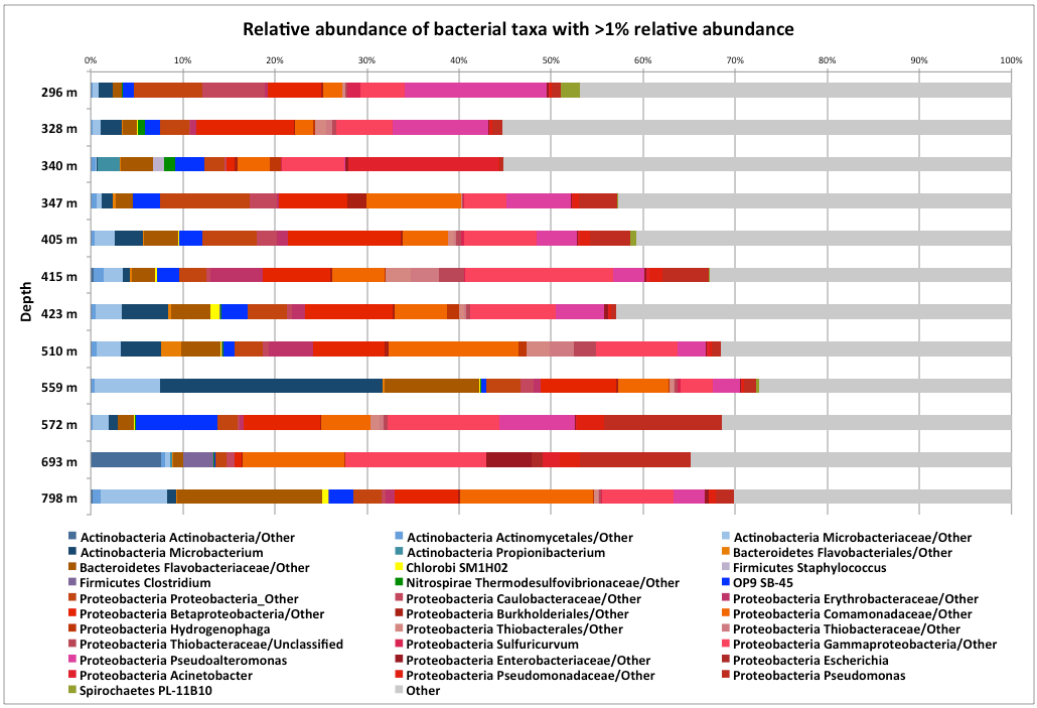
3.2: Sequences statistics; Lines 14-20 on page 13828 (Chao and ACE) are difficult to follow, please clarify

A: **3.2:** Chao1 and ACE results were simplified and the reader is referred to the table for more details.

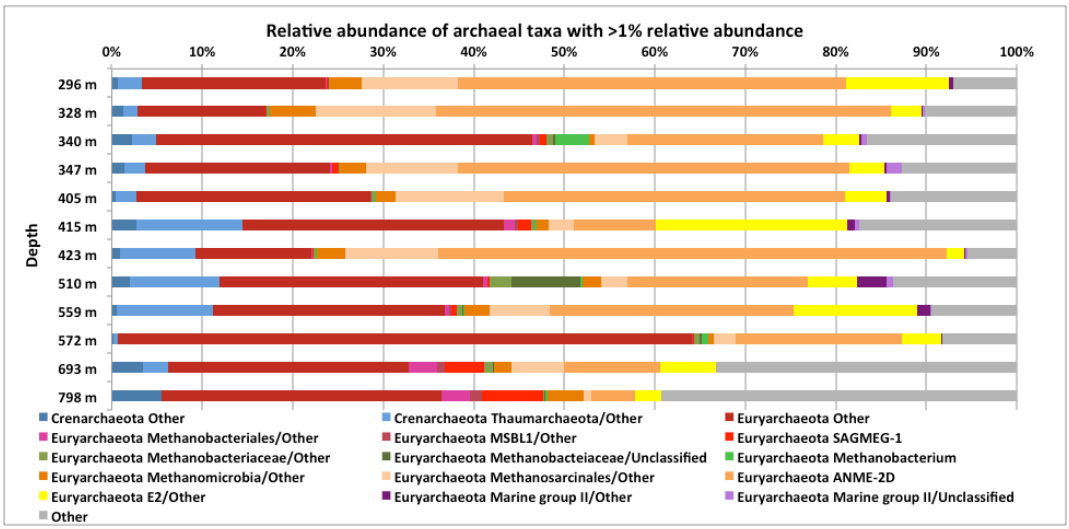
R: A similarity index between samples would be helpful. It is somewhat illustrated in figure 3 but it would be interesting to get a sense of how many taxa are shared vs how many taxa are present. A visualization of tables 3 and 4 would achieve a similar objective.

A: The similarity between the samples is presented in a Bray-Curtis UPGMA tree (Figure 5). I tried to show the data in a heat map like plot, but there were just too many taxa to be able to show them well. The tables 3 and 4 have been visualized as presented below.

Relative abundance of the most common bacterial taxa:



Relative abundance of the most common archaeal taxa:



R: Can add a citation about the difficulty in assigning taxonomy to short se- quences (lines 5-6 page 13829). GAST (Huse, Susan M., Les Dethlefsen, Julie A. Huber, D. Mark Welch, David A. Relman, and Mitchell L. Sogin. "Ex- ploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing." PLoS Genet 4, no. 11 (2008): e1000255.) is

a tool that has been used minimize this problem and may serve as a useful citation

A: The proposed reference to Huse et al. (2008) has been added and discussed.

R: The abstract is misleading, there is in fact a core community that makes up an extremely high proportion of the dataset. Please address

A: 3.3: The text has been changed

R: Several of the genera are "TaxaX, Other" which is not very informative and may inflate the number of "shared". What about the number of shared OTUs vs not shared? This is where a similarity index or a visualization of the taxonomic data may be useful

A: The TaxaX, Other is a problem. However, these 'Others' is what the GG database gives for many of the more unknown groups. This has been discussed in accordance to the Huse et al., 2018 paper. For example, the OTUs falling with the Proteobacteria;Other are between 900-3000 OTUs/sample, so visualization for these is difficult. Similarities have been presented as a UPGMA tree (Figure 4).

R: 3.4: Impact of

Again see **Major Comment (1)**

It would be helpful to have a better sense of the taxonomic composition and environmental parameters of the samples before getting into the discussion of the CCA. Sections describing these 2 data types would be helpful earlier in the results section

A: The section has been changed.

R: As in 3.3: Core Communities, you report a lot of Actinobacteria/Other, Gammaproteobacteria/Other, etc which I, as a reader interpret as unclassified species within the aforementioned phylum or class. This encompasses quite a lot of diversity. It would be helpful for you to describe "Other" category more completely and state exactly what it means. Points to think about: How might this larger group of Gammaproteobacteria/Other (i.e. how many OTUs fit this classification) influence your interpretations when compared to Gammaproteobacteria/Shewanella

A: 3.4: We have addressed the 'Other' in the discussion. This is probably due to the problem with the short sequence reads that may get many similarity good hits to the 16S rRNA sequence database.

R: 3.5: Co-occurrence network – see **Major Comment (1)**

A: 3.5: The co-occurrence network has been recalculated and is now loosely based on Spearman's rank correlations. The idea is not to study absolute correlations, but to obtain some value for interactions between taxa that can be used to visualize these interactions in a network.

3.6: PICRUSt is rather new and has not been used for many environmental studies yet. However, we tried in on a bog community where Acidobacteria were prevailing and the results were different from the ones we got here. Staley et al found that the PICRUSt performed quite well on riverine microbiomes when 16S rRNA gene data was compared with metagenomic data, but they did call for caution in the interpretation of the PICRUSt results.

The listed features are those that were the most common. Table 9 has been changed in to a Figure (Figure 8). We have added metabolic pathway maps. The core vs rare biosphere comparisons are not shown, because the rare biosphere PICRUSt results were not as robust as the ones we are presenting. This is probably due to the fact than the rare biosphere is not well known.

3.5 – Discussion

R: Lines 1-6 on page 13833 was difficult to follow

A: L1-6 have been revised

R: Line 10 on page 13833 "genera, respectively seem low". Have you considered the number of genera within the GreenGenes reference dataset? This may deflate the number of observed genera.

A: L10 – we used the GG reference for the taxonomic assignments, so this is certainly a possibility. The GG was used, because PICRUSt is not compatible with other reference databases.

R: Tables 5-8 could be in the supplement

A: Tables 5-8 were removed.

R: Which sulfur and sulfate reducers are present? Who are the archaea? (lines 13-17 page 13833)

A: L13-17 – Sulphate reducers were not detected in the major groups, but sulfur, sulfide and thiosulphate oxidizers were. These have been added to the text. The archaeal communities consisted mainly of undetermined Euryarchaeota, ANME-2D and Thermoplasmatales_E2 groups. This has been added to the text.

R: 4.1: Energy Metabolism

– line 14 and 25 page 13834: It is important to note that mixotrophy (ability to shift between autotrophy and heterotrophy) has also been suggested to be an important option for low-energy, subsurface systems. See:

1. Moser, Duane P., Thomas M. Gihring, Fred J. Brockman, James K. Fredrickson, David L. Balkwill, Michael E. Dollhopf, Barbara Sherwood Lollar et al. "Desulfotomaculum and Methanobacterium spp. dominate a 4-to 5-kilometer-deep fault." Applied and Environmental Microbiology 71, no. 12 (2005): 8773-8783.

2. Magnabosco, Cara, Kathleen Ryan, Maggie CY Lau, Olukayode Ku- loyo, Barbara Sherwood Lollar, Thomas L. Kieft, Esta van Heerden, and Tullis C. Onstott."A metagenomic window into carbon metabolism at 3 km depth in Precambrian continental crust." The ISME journal (2015).

3. Osburn, Magdalena R., Douglas E. LaRowe, Lily M. Momper, and Jan P. Amend. "Chemolithotrophy in the continental deep subsurface: Sanford Underground Research Facility (SURF), USA." Frontiers in microbiology 5 (2014). –a potential for both

chemolithotrophy and heterotrophy (not necessarily within the same organisms) in the Sanford Underground Research Facility is reported here

A: 4.1 – mixotrophy, or more precisely the possibility that the microorganisms couled change from one metabolic strategy to another depending on the environmental conditions, has been included in the discussion, and Magnabosco et al. (2015) and Osburn et al. (2014) have been included as references.

R: Line 4 page 13835, my concern about PICRUSt, methanogens, and the Wood-Ljungdahl pathway are summarized in (Marjor Comment 2)

A: L4, P13835 – A chart on the carbon metabolism in bacteria and archaea hand the identified enzymes has been included (Figure 9). This figure also shows the archaeal WL pathway and how it differes from the bacterial one. The discussion has been altered accordingly.

R: I think it would be helpful for the discussion to include more about how their results compare to Purkamo et al. (2015) and other subsurface sites. For example, a study by Itävaara et al sampled the Fennoscandian Shield at various depths saw a marked change in community composition with depth. It would be interesting to see how this study compares since they are from what seem to be a similar locality (Itävaara, Merja, Mari Nyyssönen, Anu Kapanen, Aura Nousiainen, Lasse Ahonen, and Ilmo Kukkonen. "Characterization of bacterial diversity to a depth of 1500 m in the Outokumpu deep borehole, Fennoscandian Shield." FEMS microbiology ecology 77, no. 2 (2011): 295-309.)

A: Outokumpu is very different from Olkiluoto and only one of the metagenomes from Outokumpu is from a depth close to those examined in out paper. The mentioned paper was based on DGGE and clone libraries and the samples were from the borehole water column and not the fracture zones, as in our study. The geology and water chemistry is different between these sites and we have seen major differences in the bacterial and archaeal communities between these two sites. Some comparison between the Olkiluoto and

Outokumpu deep biosphere communities has been added to the discussion referring to Purkamo et al. (2015).

R: AnotherpointofcomparisonarethewholegenomemetagenomesfromOutokumpu by Nyyssönen et al 2014. Are the same metabolisms predicted by PICRUSt as identified in the metagenomes? (Nyyssönen, Mari, Jenni Hultman, Lasse Ahonen, Ilmo Kukkonen, Lars Paulin, Pia Laine, Merja Itävaara, and Petri Auvinen. "Taxonomically and functionally diverse microbial com- munities in deep crystalline rocks of the Fennoscandian shield." The ISME journal 8, no. 1 (2014): 126-138.)

A: Discussion has been added and the results compared. We find similar trends in our PICRUSt estimations as in the metagenomes.

R: And there are several other useful and interesting citations for comparison on page 13832 lines 16-18 as well as those listed in the first and second comments of this section and

1. Dong, Yiran, Charu Gupta Kumar, Nicholas Chia, PanâA Ř˘Jun Kim, Philip A. Miller, Nathan D. Price, Isaac KO Cann et al.“Halomonas sul- fidaerisâA Ř˘dominatedmicrobialcommunityinhabitsa1.8kmâA Ř˘deep subsurface Cambrian Sandstone reservoir.” Environmental microbiol- ogy 16, no. 6 (2014): 1695-1708.

2. Fukuda, Akari, Hiroki Hagiwara, Toyoho Ishimura, Mariko Kouduka, Sei- ichiro Ioka, Yuki Amano, Urumu Tsunogai, Yohey Suzuki, and Takashi Mizuno. “Geomicrobiological properties of ultra-deep granitic ground- water from the Mizunami Underground Research Laboratory (MIU), central Japan.” Microbial ecology 60, no. 1 (2010): 214-225.

A: Dong et al reference included in the discussion. Fukuda et al reference not included, because the microbial community observed in this reference is more similar to the Outokumpu deep biosphere than the Olkiluoto deep biosphere. But a really nice reference. Thanks for pointing it out.

A: 4.2-4.5: the text will be revised based on reviewer's comments.

Table3-4; presented as figures above.

Figure 2 – the legends have been reordered and the figure placed in supplements

Figure 3 – the figure has been revised and presented as NMDS plots for bacteria and archaea separately

Figure 4: the network has been redone.