



RESEARCH ARTICLE

Investigation of viable taxa in the deep terrestrial biosphere suggests high rates of nutrient recycling

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One sentence summary: qPCR and 16S rRNA gene sequencing of total and viable cells (intact cellular membrane) from three groundwaters with different ages and chemical composition.

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ABSTRACT

The deep biosphere is the largest 'bioreactor' on earth, and microbes inhabiting this biome profoundly influence global nutrient and energy cycles. An important question for deep biosphere microbiology is whether or not specific populations are viable. To address this, we used quantitative PCR and high throughput 16S rRNA gene sequencing of total and viable cells (i.e. with an intact cellular membrane) from three groundwaters with different ages and chemical constituents. There were no statistically significant differences in 16S rRNA gene abundances and microbial diversity between total and viable communities. This suggests that populations were adapted to prevailing oligotrophic conditions and that non-viable cells are rapidly degraded and recycled into new biomass. With higher concentrations of organic carbon, the modern marine and undefined mixed waters hosted a community with a larger range of predicted growth strategies than the ultra-oligotrophic old saline water. These strategies included fermentative and potentially symbiotic lifestyles by candidate phyla that typically have streamlined genomes. In contrast, the old saline waters had more 16S rRNA gene sequences in previously cultured lineages able to oxidize hydrogen and fix carbon dioxide. This matches the paradigm of a hydrogen and carbon dioxide-fed chemolithoautotrophic deep biosphere.

Keywords: 16S rRNA gene; deep subsurface; fracture groundwaters; propidium monoazide; viable cells; candidate phyla radiation

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INTRODUCTION

The continental subsurface is estimated to contain 2% to 19% of the earth's total biomass (McMahon and Parnell 2014). However, due to the difficulty of sampling in the deep subsurface, this environment is one of the least understood ecosystems on earth. In terrestrial groundwaters, microbial abundance and activity are strongly positively related to the proximity of the photosynthesis-fueled surface (Magnabosco *et al.* 2016) and thus, water-bearing deep fracture systems are extremely oligotrophic (Lever *et al.* 2015). Terrestrial groundwaters in granitic bedrock fractures have been studied by means of laboratory culture, 16S rRNA gene sequencing and metagenomics, uncovering a resident mixed community of anaerobic microbes (Pedersen 2013; Nyssonson *et al.* 2014; Wu *et al.* 2015; Hubalek *et al.* 2016; Ino *et al.* 2016; Ino *et al.* 2017; Wu *et al.* 2017). This community contains up to 50% of ultra-small cells passing through a 0.2 μm pore-size filter (Wu *et al.* 2015) with extensive representation from archaea (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota and Nanohaloarchaeota (DPANN)) and bacteria (Candidate Phyla Radiation (CPR)) (Rinke *et al.* 2013; Castelle *et al.* 2015; Anantharaman *et al.* 2016; Baker *et al.* 2016; Hug *et al.* 2016; Ortiz-Alvarez and Casamayor 2016). The CPR along with several DPANN phyla typically have streamlined genomes and a fermentative metabolism. However, a critical question in the deep biosphere is whether all the resident populations are viable. Successful culturing efforts combined with detection of ATP (Pedersen 2013), presence of phages (Kyle *et al.* 2008; Eydal *et al.* 2009), and capacity for biofilm formation on rock surfaces (Momper *et al.* 2017; Wu *et al.* 2017) supports that at least a portion of the microbial community is viable. In addition, a portion of the dormant microbial cells in the deep biosphere have been found to be activated when exposed to methanol or methane (Rajala *et al.* 2015; Rajala and Bomberg 2017) as well as CO_2 or carbonate (Bomberg *et al.* 2017). However, very few cultivation-independent investigations of the viable taxa have been published for the deep terrestrial biosphere.

Fluorescein diacetate and propidium iodide stains for microscopy and flow cytometry have been used to distinguish between viable and non-viable cells (Gerber *et al.* 2016; Lopez-Fernandez *et al.* 2018). However, it was not the optimal solution for this study due to the low cell density in the deep biosphere environment (Wu *et al.* 2015). Instead, investigation of viable versus total cells was carried out using propidium monoazide (PMA) that can be used as a pre-PCR DNA inactivation method. If applied to cells, it is a more conservative marker of microbial viability than staining with propidium iodide (Nocker *et al.* 2010). Briefly, PMA intercalates with DNA in dead cells with compromised membranes. Upon light exposure, DNA is then irreversibly modified rendering it unavailable for amplification (Nocker and Camper 2009). Several studies have demonstrated that PMA binds to DNA from damaged cells or extracellular DNA, while having a minimal effect on genomic DNA from cells with intact membranes (Nocker, Cheung and Camper 2006; Nocker *et al.* 2010; Fittipaldi, Nocker and Codony 2012; Carini *et al.* 2016). Light exposure also inactivates excess PMA that has not entered the cells. Hence, residual PMA will not modify DNA from live cells (with intact cell membranes) after their DNA is retrieved in the extraction procedure (Nocker *et al.* 2010). The PMA method has previously been utilized to investigate the frequency, diversity and distribution of viable bacteria in spacecraft assembly cleanrooms (Vaishampayan *et al.* 2013).

This study was carried out at the Swedish Nuclear Fuel and Waste Management Company owned and operated Äspö Hard

Rock Laboratory (Äspö HRL), Sweden. This underground tunnel excavated in Proterozoic crystalline bedrock of the Fennoscandian shield extends to a depth of 460 m below sea level (mbsl) (Hallbeck and Pedersen 2008) and provides access to investigate the microbial life in the deep continental subsurface. Three different waters were collected from boreholes connected to water bearing bedrock fractures in Äspö HRL: SA1229A-1 containing a 'modern marine' (MM) water type; KA3105A-4 holding an 'undefined mixed' water type; and KA3385A-1 with 'old saline' water type. Since the investigated fracture waters are all oligotrophic (Wu *et al.* 2015), we hypothesized that recycling of biomass of non-viable cells would be rapid and that this would be reflected in high similarity between the total and the viable portion of the community. To test this hypothesis, we used PMA treatments to enable a cultivation-independent comparison of the total and viable portions of the microbial community in the terrestrial deep subsurface.

MATERIALS AND METHODS

Water types

Along the tunnel, boreholes are drilled into the bedrock, providing direct access to water flowing by gravity in bedrock fractures. Three boreholes were sampled: borehole SA1229A-1 (171 mbsl), KA3105A-4 (415 mbsl) and KA3385A-1 (448 mbsl). These groundwaters have been observed to contain ferrous iron, dissolved sulfide (HS^-), temporally stable chemistry and $\delta^{18}\text{O}$, and neutral pH (Wu *et al.* 2015). However, both chemical composition and $\delta^{18}\text{O}$ differ between water masses, enabling these groundwaters to be characterized according to origin and age (Laaksoharju *et al.* 2008). SA1229A-1 had tracers of marine waters (Gimeno *et al.* 2014) and also similar chemical values as modern Baltic Sea water (Mathurin *et al.* 2012). This groundwater was thus composed of infiltrated brackish marine (Baltic Sea) water and was termed MM. The age of this groundwater was estimated to be <20 years or even less (Mathurin *et al.* 2014). The origin and age of the KA3105A-4 groundwater could not be exactly defined since it is influenced by different water sources, including freshwaters, Baltic Sea or Littorina Sea water, and probably also older water. Accordingly, it was termed 'undefined mixed' (UM). The deepest borehole, KA3385A-1 has features typical for saline groundwaters with a residence time of thousands of years or more (Louvat, Michelot and Aranyossy 1999). However, its chloride concentration was considerably lower than for pure old saline water at the site (Mathurin *et al.* 2012). Therefore, this groundwater had been diluted by waters with lower salinity a few thousands of years ago (Mathurin *et al.* 2012) and it was termed 'old saline' (OS).

Sampling of the groundwaters

The three boreholes were sampled in February 2017. Borehole water was flushed for three to five section volumes before collecting cells from between 100 and 604 L of water. Triplicate samples of planktonic cells were collected on hydrophilic polyvinylidene fluoride (PVDF) membranes with 0.1 μm poresize (47 mm Durapore, Merck Millipore, USA) under *in situ* conditions by connecting a High-Pressure Stainless Steel Filter Holder (Merck Millipore, USA) with a downstream needle valve and pressure gauge directly to the borehole. After processing of an appropriate volume of water (Data S1, Supporting Information), each filter was aseptically cut in half and the respective pieces were placed in separate sterile cryogenic tubes (Thermo Scientific, USA). One tube was placed on ice, while the second cryogenic tube was

immediately frozen in liquid nitrogen. The samples were transported to the laboratory and DNA extracted with and without PMA treatment (described below) within 6 h of sampling. This sampling regime allowed us to test if freezing in liquid nitrogen induced damage of the cell membranes.

PMA treatment and DNA extraction

For the viability assessment, the filters already halved for freezing in liquid nitrogen or stored on ice were once again divided into two further halves and each quarter was separately suspended in 1 mL of sterile phosphate buffered saline. For each ice and flash frozen sample, the first filter-suspension was treated with 12.5 μL of 2 mM PMA (Biotium, Inc., USA) to a final concentration of 25 μM for the viability assessment, whereas the second filter-suspension was untreated for detection of total cells (viable plus non-viable). Both samples were thoroughly mixed and incubated in the dark for 5 min at room temperature according to (Vaishampayan et al. 2013). All samples (PMA-treated and non-treated) were then exposed to the PhAST blue-Photo activation system for tubes (GenIUL, S.L., Spain) for 15 min. In the case of the PMA-treated samples, this light exposure neutralized the DNA of dead cells and thus, enabled the detection of DNA from only the viable cells. On the other hand, the light exposure of the PMA-untreated samples did not affect the DNA, allowing the detection of DNA from total cells, i.e. both viable and non-viable.

DNA of all samples was extracted using the MO BIO PowerWater DNA isolation kit by following the manufacturer's instructions except that the final DNA was re-suspended in 50 μL of eluent. The quality and quantity of extracted DNA was analyzed by gel electrophoresis and a Qubit 2.0 Fluorometer (Life Technologies, USA), respectively. Extracted DNA was stored at -20°C . A negative control was included for each borehole by extracting DNA from an unused filter rolled and collected at each borehole site. DNA concentrations for these negative controls were below the Qubit detection limit (0.348 ng/ μL ; Data S1, Supporting Information).

Quality control of the PMA reaction was carried out using *Desulfovibrio aespoensis* (DSM 10631), a bacterial strain isolated from the Äspö HRL groundwater (Motamedi and Pedersen 1998), that was recently renamed as *Pseudodesulfovibrio aespoensis* (Cao et al. 2016). Cultures were grown using *Desulfobacter* medium (DSMZ 193) with strain-specific modifications (2.5 g/L sodium lactate and 1 g/L yeast extract as substrates, incubation at 30°C under anaerobic conditions). Growth was confirmed via microscopy. Bacterial cells were collected after 4 weeks of incubation on sterile hydrophilic PVDF membranes with 0.1 μm poresize (47 mm Durapore, Merck Millipore, USA). Then, PMA treatment and DNA extraction were performed as described above. 16S rRNA genes of PMA-treated and non-treated subsamples were quantified via quantitative real-time PCR (qPCR; see below).

Quantitative PCR

Quantification of prokaryotic 16S rRNA genes was performed by qPCR on a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, USA). The qPCR reaction mixture contained 5 μL ABsolute QPCR SYBR Green ROX Mix (Thermo Scientific, USA), 1 μL BSA (3 g L^{-1}), 0.5 μM of forward primer 515FB (GTGYCAGCMGCCGCGGTAA; Parada, Needham and Fuhrman 2015) and reverse primer 806RB (GGACTACNVGGGTWTCTAAT; Apprill et al. 2015) that both target the V4 region of the 16S rRNA gene, 1 μL DNA template, and filled to the final volume of 10 μL

with DNase free water. The thermocycling profile consisted of an initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 45 s; 50°C 45 s; 72°C 45 s and 80°C 15 s. Standard curves were prepared from dilutions of purified PCR products from genomic *Escherichia coli* DNA. Efficiency ranged from 94.4% to 95.3%. Product specificity of template DNA was confirmed by melt curve analysis and agarose gel electrophoresis (product size). All reactions were run in triplicates. Paired t-tests were used to test if the 16S rRNA gene abundance was significantly higher in non-treated samples compared to PMA-treated samples.

Sequencing and bioinformatics

The 16S rRNA gene tag sequencing was carried out as previously described (Wu et al. 2015). Briefly, region V3-V4 of the bacterial and archaeal 16S rRNA gene was amplified utilizing primers 341F and 805R (Herlemann et al. 2011) according to published procedures (Hugerth et al. 2014). Sequencing was carried out at the Science for Life Laboratory, Sweden (www.scilifelab.se) on the Illumina MiSeq platform as previously described (Lindh et al. 2015). The UPARSE pipeline was used to process the sequences and cluster operational taxonomic units (OTUs) at a 97% similarity threshold (Edgar 2013). OTUs were then annotated against the SINA/SILVA database (SILVA 132; (Quast et al. 2013)) and finally analyzed in Explicet 2.10.5 (Robertson et al. 2013). The amount of pair-end reads received from the sequencing facility, merged and quality trimmed reads, and amount of OTUs clustered can be found in Data S2, Supporting Information. Final count values were normalized by relative abundance (i.e. % of total sample size) as this has been found to be more accurate than rarefying (McMurdie and Holmes 2013). After normalization of counts, one-way ANOVA Tukey posthoc test was used to test for significant differences in relative phyla abundance ($P < 0.05$) between the three water types for the total and viable communities. In addition, Principal Coordinate Analysis (PCoA) was performed with the normalized 16S rRNA gene counts in PAST 3.17 (Hammer, Harper and Ryan 2001) to visualize the differences between the three water types and between the total and viable population within the three groundwaters. Phylogenetic analysis was conducted in MEGA 7 (Kumar, Stecher and Tamura 2016) by first aligning sequences with MUSCLE (Edgar 2004) using two iterations, and then creating an un-rooted Tamura-Nei distance maximum likelihood tree. Alpha diversity (Shannon H index) was calculated based on rarefied data to the lowest sample size (30494 counts) and bootstrapped 100 times. One-Way ANOVA test was used to test for significant differences in alpha diversity and number of OTUs between total and viable communities as well as between the different waters for total communities.

Data accessibility

16S rRNA sequences are available at NCBI database with the Bio-project accession number PRJNA431355.

RESULTS AND DISCUSSION

Pseudodesulfovibrio aespoensis was chosen for the quality control as it was isolated from the Äspö HRL groundwater (Motamedi and Pedersen 1998) and is therefore, representative of the slow growing and difficult to culture microbes in the deep biosphere. The results confirmed that the PMA method worked, i.e. resulting in significant lower 16S rRNA gene copy numbers for PMA-treated cells compared to non-treated cells (rejecting the null

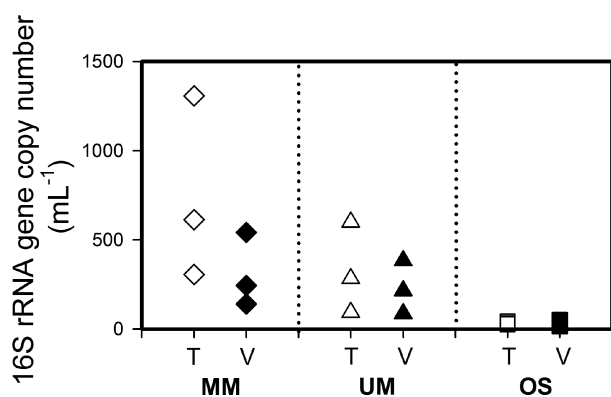


Figure 1. Total (T; open symbols) and viable (V; closed symbols) 16S rRNA gene abundances of the three water types MM (diamonds), UM (triangles) and OS (squares). Abundances are shown as 16S rRNA gene copies per mL fracture water.

hypothesis: no difference between methods; paired t-test, $P = 0.001$; Data S3, Supporting Information). However, the PMA methodology has some limitations in that not all non-viable cells have compromised membranes (Nocker and Camper 2009). Consequently, we define viable cells as having an intact membrane, preventing the PMA to penetrate and intercalate with the DNA. A second issue is that low light transparency in the samples can cause the viable cell population to be underestimated (Wagner et al. 2008). This should not be a problem in this study as the cells were captured from pristine water. The final drawback is that damage to cell membranes can occur due to secondary effects and/or other inactivation mechanisms (Nocker and Camper 2009) that was an issue for filters flash frozen in liquid nitrogen. These filters showed lower 16S rRNA gene abundances than filters stored on ice (Data S3, Supporting Information) and had differences between the total and viable populations (e.g. Deltaproteobacteria decreased in the MM water) that were not seen for filters kept on ice prior to PMA treatment and DNA extraction (Data S4, Supporting Information). Consequently, only data from filters stored on ice are presented below and we conclude that flash freezing of filters for viability assays with PMA should be avoided.

Total and viable prokaryotic 16S rRNA gene abundances

Prokaryotic 16S rRNA gene abundances did not significantly differ between the total and viable populations in the MM, UM and OS fracture waters (keeping the null hypothesis: no difference in gene abundance between total and viable samples, paired t-test, $P = 0.07$, $P = 0.29$ and $P = 0.35$, respectively; Fig. 1). This suggests that the main proportion of cells are viable and that any non-viable cells are rapidly degraded. Considering the highly oligotrophic conditions with a very low carbon availability in these groundwaters (Wu et al. 2015), carbon and nutrients of dead microbial biomass are likely recycled into the viable biomass. The number of 16S rRNA gene copies were higher in the MM, lower in the UM, and much lower in the OS water type (means of 743, 326 and 34 16S rRNA gene copies mL⁻¹, respectively; Fig. 1); suggesting a decrease of 16S rRNA gene copies with depth. Considering that a single bacterial cell can have 1–15 copies of the 16S rRNA gene within its genome (Větrovský and Baldrian,

2013), the gene copies measured in this study are in the same range as the cell counts reported by Wu et al. (2015) in the same water types.

Total and viable diversity

The only statistically significant difference in the total and viable alpha diversities (Shannon H index) was a higher variety in the viable MM versus total MM communities (rejecting the null hypothesis: no difference in alpha diversity between total and viable samples, One-Way ANOVA, $P = 0.04$; Fig. 2A). Although the differences in alpha diversity between the three water types were statistically insignificant, there was a trend that the UM water had a higher diversity and the OS water a lower diversity (Fig. 2A). This was also in accordance with the higher number of total community OTUs in the UM (2268 ± 386) and lower amount of OTUs in the OS water (1425 ± 230) compared to MM (2038 ± 285) (rejecting the null hypothesis: no difference in OTU numbers between the water types; One-Way ANOVA test, $P < 0.05$; $n = 3$ per site, $SD = 1$; Data S2, Supporting Information). This may have been due to mixing of microbial communities from several water types in the undefined mixed sample that included glacial meltwater, modern meteoric water, Baltic Sea or Littorina Sea water, and older high saline waters. There was also a trend that the MM and UM waters with higher organic carbon contents (Wu et al. 2015) had a higher diversity and a greater number of OTUs than the ultra-oligotrophic OS water, the latter suggested to be fueled by hydrogen and carbon dioxide of geological origin (Pedersen 2013; Wu et al. 2017). Beta diversity tests (Bray–Curtis dissimilarity) showed that the three water types contained different microbial communities, while total and viable populations for each water type were similar (Fig. 2B). This is in contrast to other environments tested with PMA such as soils where binding of relic DNA results in large differences between the total and viable communities (Carini et al. 2016).

16S rRNA gene-based communities in the fracture waters

In the three fracture waters, $31.7 \pm 10.2\%$ (number of replicates ($n = 18$)) of the total and viable 16S rRNA gene amplicon OTUs could not be classified taxonomically, suggesting that a significant portion of the deep biosphere community is poorly defined (Fig. 3A). Of these unclassified OTUs, many were most closely affiliated with Patescibacteria and Archaea including Nanoarchaea (Data S5, Supporting Information). This further highlights the importance of typically ultra-small cells in the deep subsurface (Wu et al. 2015).

PCoA based on the 16S rRNA gene OTUs supported that there were differences in microbial communities between MM, UM and OS waters (Fig. 4). The largest difference between the three water types was the significantly higher number of OTUs for MM and UM compared to OS water (see above). However, this did not translate into a statistically significant increase in the diversity as a whole (e.g. alpha diversity; Fig. 2A). The increased number of OTUs was likely due to higher dissolved organic carbon concentrations in the MM and UM boreholes, supporting a wider range of growth strategies (Wu et al. 2015; Hubalek et al. 2016). The archaeal phyla Crenarchaeota and Diapherotrites had a significantly higher relative abundance in the UM compared to the MM and OS water (Fig. 3B; Data S6, Supporting Information), while Nanoarchaeota had a higher relative abundance in MM and UM compared to OS water (Fig. 3B). Little is known about some of these candidate archaeal phyla, although the Nanoarchaeota and Diapherotrites have small genomes and have been suggested to be potential symbionts (Randau et al. 2005; Youssef et al. 2015).

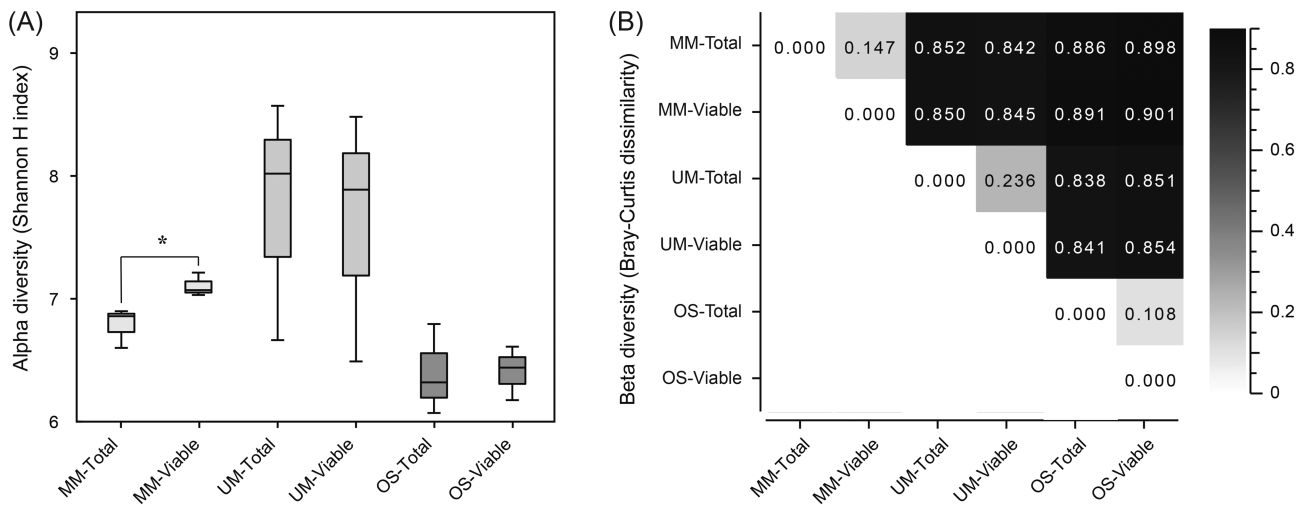


Figure 2. Shannon H alpha (A) and Bray–Curtis (B) diversities based on all OTUs from the total and viable samples. The statistically different Shannon H alpha diversity ($P < 0.05$) is marked with a star.

The relative abundance of candidate phyla was significantly higher in the MM and UM compared to the OS water. The most abundant Patescibacteria phylum was ABY1 (Fig. 3C; Data S6, Supporting Information) that has previously been found in ultra-oligotrophic water (Urbach et al. 2001). Additional identified Patescibacteria phyla included e.g. Gracilibacteria (GN02), Microgenomates (OP11), Parcubacteria (OD1) and Saccharibacteria (TM7) (Rinke et al. 2013; Anantharaman et al. 2016; Hug et al. 2016). Parcubacteria, typically have reduced genome size, are suggested to ferment sugars to organic acids and are predicted to exist in a syntrophic relationship with other populations (Nelson and Stegen 2015). Gracilibacteria lack electron transport pathways suggesting a syntrophic or fermentative lifestyle (Rinke et al. 2013). Further candidate phyla with small, streamlined genomes that had significantly increased relative abundances in the MM compared to OS water were the Aegeribacteria and Dependientiae (Fig. 3C; Data S6, Supporting Information). Aegeribacteria are potential syntrophs living with hydrogen oxidizers (Hamilton et al. 2016), while Dependientiae are suggested to require a Eukaryotic host (Yeoh et al. 2016). Reduced genomes and streamlined metabolic potential coupled to a small cell size are likely adaptations to the oligotrophic conditions in Äspö HRL groundwaters (Wu et al. 2015). The relative abundance of Latescibacteria (WS3) was also higher in both, the MM and UM waters compared to OS water where they potentially recycle nutrients from cell walls (Fig. 3C; Data S6, Supporting Information) (Farag, Youssef and Elshahed 2017). In addition, Margulisbacteria had a higher relative abundance in the MM and UM compared to the OS water (Data S6, Supporting Information). Bacteria from this phylum have the genetic potential to oxidize hydrogen during sulfur cycling (Anantharaman et al. 2016). The relative abundance of Poribacteria was higher in the UM water compared to OS (Data S6, Supporting Information) where it has the genetic potential to fix carbon via the Wood–Ljungdahl pathway (Siegl et al. 2010). Finally, the Rokubacteria had statistically higher relative abundances in the UM compared to the OS water (Data S6, Supporting Information). Despite being previously identified from aquifers, Rokubacteria have extra-large genomes providing the potential for a versatile, mixotrophic metabolism (Becraft et al. 2017). Taxa with cultured representatives were also present in the MM and UM fracture waters

where the Deltaproteobacteria class had statistically higher relative abundance compared to the OS water (Fig. 3D; Data S6, Supporting Information). These Deltaproteobacteria might include heterotrophic and autotrophic sulfur and sulfate reducing bacteria (Wu et al. 2015). Other phyla that had a significantly higher relative abundance in MM or UM compared to OS water were Actinobacteria ($P = 0.045$), Tenericutes ($P = 0.000$) and Verruimicrobia ($P = 0.004–0.038$) (Data S6 and S7, Supporting Information).

There were a lower number of phyla with increased relative abundances in the OS water compared to the MM and UM waters, which support a more diverse population with e.g. heterotrophic or fermentative metabolisms. In the case of the OS water, the majority of the increased microorganisms belonged to phyla with cultured representatives (Data S6, Supporting Information). Within the Archaea, Hadesarchaeota and Hydrothermarchaeota had a higher relative abundance in the OS water compared to either the MM or UM waters (Fig. 3B; Data S6, Supporting Information). Despite being known to have streamlined genomes of approximately 1.5 Mbp, the Hadesarchaeota have the genetic potential to oxidize carbon monoxide and hydrogen oxidation coupled to nitrite reduction (Baker et al. 2016). In addition, Hydrothermarchaeota are suggested to couple hydrogen oxidation to sulfate reduction (Anantharaman et al. 2017). The only candidate bacterial phylum to have a higher relative abundance in the OS water compared to the MM and UM waters was Zixibacteria that is suggested to respire organic carbon (Fig. 3C; Data S6, Supporting Information) (Castelle et al. 2013). Several taxa with cultured representatives also had higher relative abundances in the OS water including the Alpha- and Gammaproteobacteria (Fig. 3D; Data S6, Supporting Information). The Gammaproteobacteria may be organic carbon oxidizing, nitrate reducing Pseudomonadaceae that have been previously identified in Fennoscandian fracture waters (Bomberg et al. 2015; Purkamo et al. 2016). Finally, Chloroflexi had a significantly higher relative abundance in OS compared to MM water (Data S7, Supporting Information).

CONCLUSIONS

Similar 16S rRNA gene abundances in total and viable populations pointed to a fast degradation of non-viable microbes and

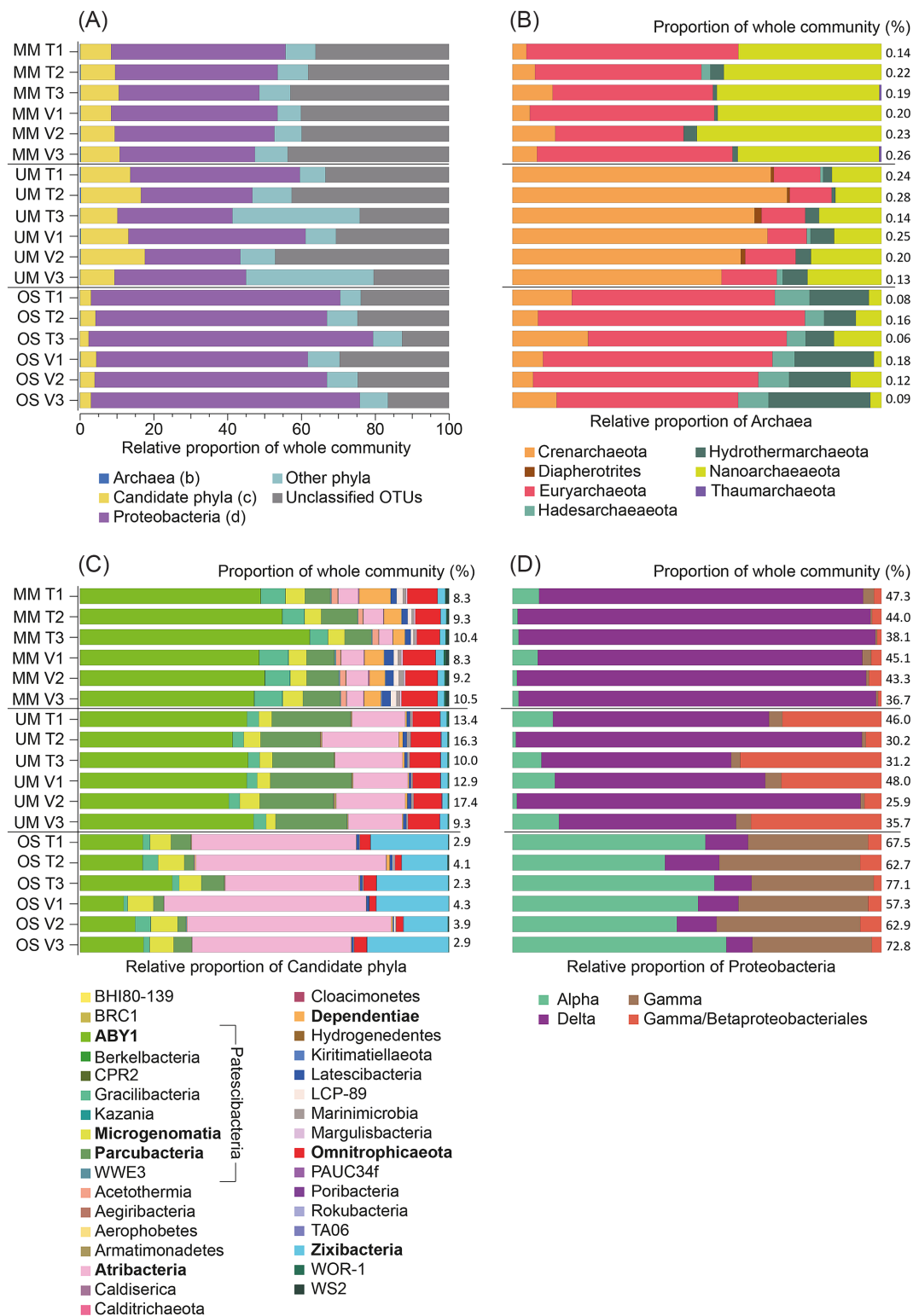


Figure 3. Total and viable 16S rRNA gene OTUs amplified from the filters used to harvest the total (T) or viable (V) microbial community in the three water types. The relative abundance (%) of OTUs is presented as: (A) the whole microbial community, while (B) shows the relative proportion of phyla (or proteobacteria classes plus betaproteobacteriales) aligned to Archaea, (C) candidate phyla, (D) Proteobacteria. Bolded text indicates phyla with a high relative abundance in the stacked bars.

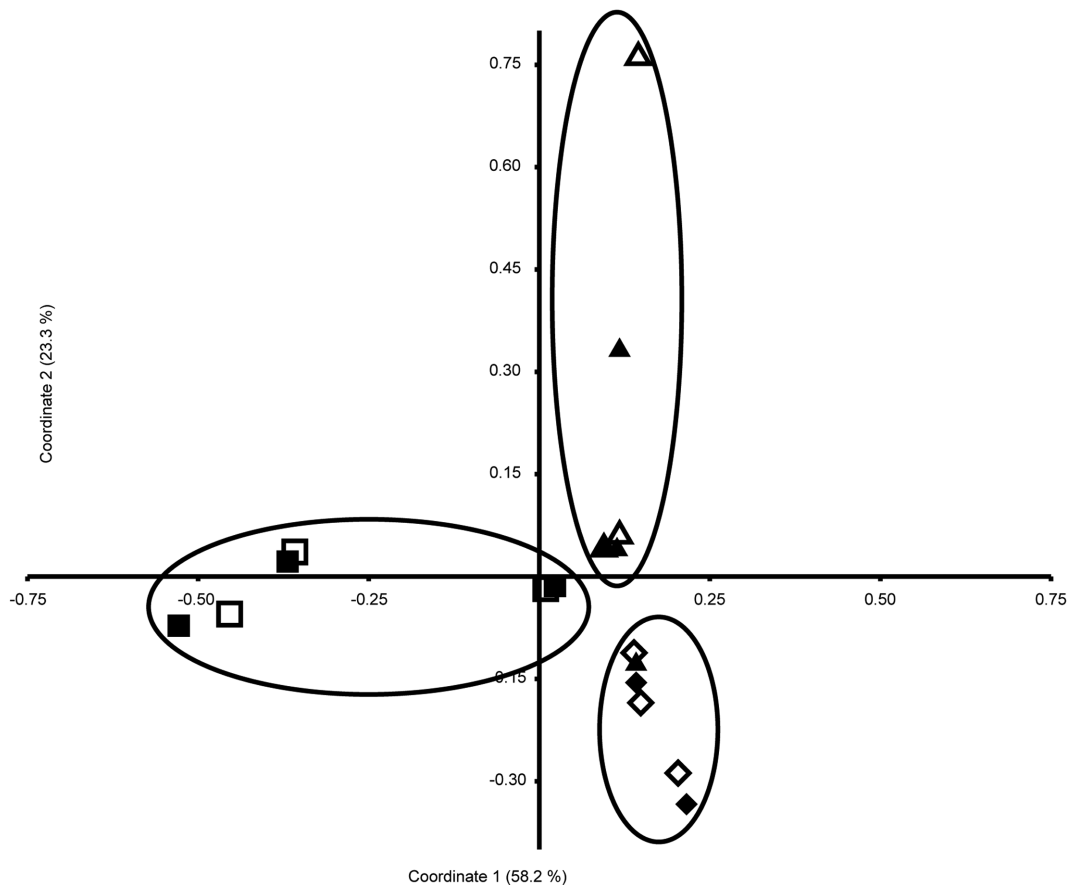


Figure 4. PCoA of the total (open symbols) and viable (closed symbols) communities for the MM (diamonds), UM (triangles), and OS (squares) waters.

thus, a recycling of nutrients (such as DNA from lysed cells) in the oligotrophic fracture waters. The similar alpha and beta diversities between the total and viable communities for each respective water type suggested that the three communities were well adapted to the oligotrophic conditions. As described by Purkamo et al. (2015) deep biosphere heterotrophic microorganisms can assimilate carbon from different sources. In our study, populations likely to utilize the organic matter from dead cells include fermenters that aligned most closely with ultra-small archaea (DPANN) and bacteria (CPR) that constituted a substantial proportion of the deep terrestrial microbiome. However, compared to the MM and UM waters, DPANN and CPR were not as prevalent in the deepest OS groundwater potentially due to the extremely low concentration of organic carbon selecting for autotrophic nitrate and sulfate reducers. This is in line with the paradigm of a hydrogen and carbon dioxide fed deep biosphere (Hallbeck and Pedersen 2008).

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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Authors' contributions. ML-F and XW collected samples, ML-F carried out laboratory work and analyzed data; ST carried out qPCR analyses; EB carried out statistical analyses; ML-F, MD and SB conceived the study; ML-F drafted the manuscript. All authors gave final approval for publication.

Conflict of interest. None declared.

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