Geomicrobiology Journal

ISSN: 0149-0451 (Print) 1521-0529 (Online) Journal homepage: https://www.tandfonline.com/loi/ugmb20

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To cite this article: Susanne Sjöberg, Nolwenn Callac, Bert Allard, Rienk H. Smittenberg & Christophe Dupraz (2018) Microbial Communities Inhabiting a Rare Earth Element Enriched Birnessite-Type Manganese Deposit in the Ytterby Mine, Sweden, Geomicrobiology Journal, 35:8, 657-674, DOI: 10.1080/01490451.2018.1444690

To link to this article: https://doi.org/10.1080/01490451.2018.1444690

Published online: 03 Apr 2018.

GEOMICROBIOLOGY JOURNAL, 2018
VOL. 35, NO. 8, 657–674
https://doi.org/10.1080/01490451.2018.1444690

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Microbial Communities Inhabiting a Rare Earth Element Enriched Birnessite-Type Manganese Deposit in the Ytterby Mine, Sweden

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ABSTRACT

The dominant initial phase formed during microbially mediated manganese oxidation is a poorly crystalline birnessite-type phyllomanganate. The occurrence of manganese deposits containing this mineral is of interest for increased understanding of microbial involvement in the manganese cycle. A culture independent molecular approach is used as a first step to investigate the role of microorganisms in forming rare earth element enriched birnessite-type manganese oxides, associated with water bearing rock fractures in a tunnel of the Ytterby mine, Sweden. 16S rRNA gene results show that the chemotrophic bacterial communities are diverse and include a high percentage of uncultured unclassified bacteria while archaeal diversity is low with *Thaumarchaeota* almost exclusively dominating the population. Ytterby clones are frequently most similar to clones isolated from subsurface environments, low temperature milieus and/or settings rich in metals. Overall, bacteria are dominant compared to archaea. Both bacterial and archaeal abundances are up to four orders of magnitude higher in manganese samples than in fracture water. Potential players in the manganese cycling are mainly found within the ferromanganese genera *Hyphomicrobium* and *Pedomicrobium,* and a group of *Bacteroidetes* sequences that cluster within an uncultured novel genus most closely related to the *Terrimonas*. This study strongly suggest that the production of the YBS deposit is microbially mediated.

ARTICLE HISTORY
Received 16 December 2017
Accepted 20 February 2018

KEYWORDS
Birnessite; microbial diversity; manganese oxidizing bacteria; organomineralization; subsurface microbiology

Introduction

Microbially mediated and abiotic processes involved in manganese (Mn) cycling strongly interact at the biosphere-lithosphere interface, allowing the investigation of cryptic cross-linkages within the biogeochemical cycles (e.g., Hansel et al. 2015). Oxidation of reduced species of iron (Fe) and Mn may result in the precipitation and accumulation of brown to black insoluble oxides, often associated with seepages of reduced water into aerobic environments such as water-bearing rock fractures that crop out in caves or tunnels (Frierdich et al. 2011; Nealson 2006; Pedersen 1997). This solid-liquid and oxic-anoxic interface provides favorable conditions for biofilm development and strong microbe-mineral interactions (Donlan 2002; Pedersen 1997). Although thermodynamically favorable under oxic conditions (Ehrlich 1978; Stumm and Morgan 1981), oxidation of Mn is a slow process, which can take years to complete in environments at circumneutral pH (Krauskopf 1957; Tebo et al. 2004). The catalytic role that microbial communities and processes have in the Mn redox cycle is well documented (Hansel and Learman 2016). Nevertheless, the microbial mechanisms that are driving these processes remain to some extent unknown, but Mn oxidation involves direct (enzymatic) or indirect antioxidative (interactive with Reactive Oxygen Species (ROS)) processes and possibly also lithotrophy (Hansel and Learman 2016; Nealson 2006; Tebo et al. 2004). Only a few molecular 16S rRNA phylogenetic studies have been conducted on Mn cave or tunnel deposits (Carmichael et al. 2013; Carmichael and Bräuer 2015, and references therein; Northup et al. 2003; Saiz-Jimenez et al. 2012; Santelli et al. 2010; Spilde et al. 2005).

The Ytterby mine, once a quartz and feldspar mine, known for the discovery of scandium, yttrium, tantalum and five of the rare earth elements (REE), was recently found to host a REE+Y enriched Mn deposit denoted YBS, Ytterby black substance (Sjöberg et al. 2017). Elemental analysis and phase analysis by XRD indicate that the dominant phase is a birnessite-type Mn oxide with low Fe content but with traces of organics. Poorly crystalline birnessite-like phyllomanganates often represent the initial phase precipitated by bacteria and fungi during microbially mediated Mn oxidation (Hansel and Learman 2016). Electron paramagnetic resonance (EPR) spectroscopy indicates a microbial origin of a large fraction of the manganese oxide precipitates (Sjöberg et al. 2017).

The Mn precipitates in the Ytterby mine provide a window into the complex Mn cycle and on the development of microbial communities in special conditions, such as low constant temperature (8°C), absence of light, low nutrient and carbon sources, and high metal content. Here a culture independent molecular phylogenetic approach is used to characterize this newly observed underground ecosystem. The objectives are to provide further insight into the putative biogenic origin of the YBS and to identify and characterize any associated microbial community.

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Materials and methods

Study site and geochemical data on the Ytterby black substance (YBS)

The Ytterby mine is located on Resarö, about 25 km NE of Stockholm, Sweden (Figure 1). The Mn accumulations (YBS) are associated with water-bearing rock fractures in a subterranean tunnel leading to the main shaft of the mine (Figure 1). The tunnel is located at shallow depth, 29 m below ground surface and 5 m above the Baltic Sea mean sea level, and was built to convert the former mine into a fuel deposit for the Swedish Armed Forces. The 400 m long tunnel links the old mine shaft with a quay located to the NE of the quarry along the Baltic Sea coastline. In this stretch the tunnel passes through granitic and mafic rocks of varying chemical composition and metamorphic grade. The YBS occur as rock wall deposits in association with a 2–3 mm thick underlying blanket of mineralized calcium carbonate precipitate (Figure 1).

The YBS deposit is located in the unsaturated zone of the tunnel section where water bearing fractures provide a continuous supply of water to the fully oxidized tunnel environment which holds a nearly constant temperature of 8°C year round. Artificial lighting is used for purposes of mine maintenance, in average 2–3 hrs/month in the otherwise completely dark tunnel. The maximum age of the YBS is 60–70 years, assuming that accumulation started when the tunnel was constructed. The elemental composition of the YBS (excluding oxygen, carbon and silicon) was 82% Mn, 13.5% Ca and 2±0.5% REE +Y, with all other metals being less than 2% in total (Sjöberg et al. 2017). The dominant mineral phase was a birnessite-type phyllomanganate, as evidenced by XRD, with minor fractions of quartz, plagioclase and calcium carbonate (likely arising from the underlying bedrock and underlying mineralized calcium carbonate precipitate).

Characterization of the YBS by IR- and EPR-spectroscopy, analysis of concentrations and isotopic signatures of carbon and nitrogen, sequential extraction procedures and lipid analysis indicated the presence of about 1.8% carbon, one third of which was organic (Sjöberg et al, 2017). In a previous study by Sjöberg et al. (2017), a microbial origin of a major fraction of the manganese oxide precipitation was suggested by the EPR-spectroscopy: none of the metals appeared to be present as metal-organic or humic bound species. The organic matter was predominantly hopanoids, and the presence of C_{31} to C_{35} extended side chain species was a distinct indication of bacterial presence. Environmental scanning electron microscopy (ESEM-EDS) confirmed the elemental analysis and showed three dominant microstructures in the YBS: (1) Dendritic/shrub-like, (2) microspherolitic/botryoidal, and (3) filaments of various thicknesses. Cross-sections of the dendritic and microspherolitic Mn oxide microstructures show signs of iterative growth in the form of alternating light and dark laminae. These laminae mainly express variation in Mn and Ca concentrations but also in the Mn/Ca ratio. Figure 2 shows cell-like structures

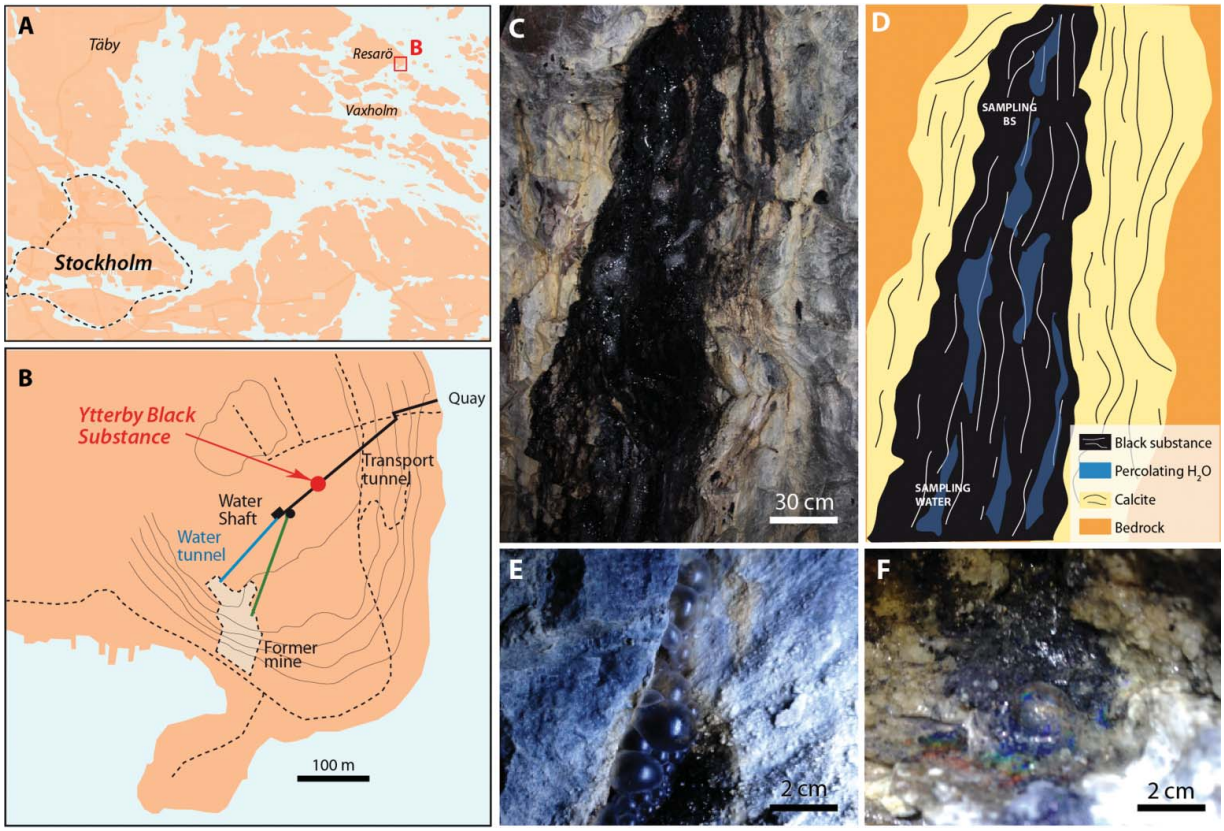

Figure 1. Maps showing the location of the Ytterby mine area and the Ytterby black substance (A) Map of the Stockholm area with location of Resarö indicated. Modified from free Vector Maps.com (B) Map of underground tunnels linking the Ytterby mine shaft with a more recently constructed quay to the NE. The black substance precipitates from water provided by water conducting rock fractures that crop out in these transport tunnels. Modified from the Swedish Fortifications Agency, (2012). (C) Photograph of the substance (D) Sketch showing sampling locations and underlying lithified CaCO3. Water percolating through the YBS provides favorable conditions for growth of microorganisms. (E and F) Occasionally bubbles of various sizes sometimes with a metallic luster are observed on the surface of the YBS.

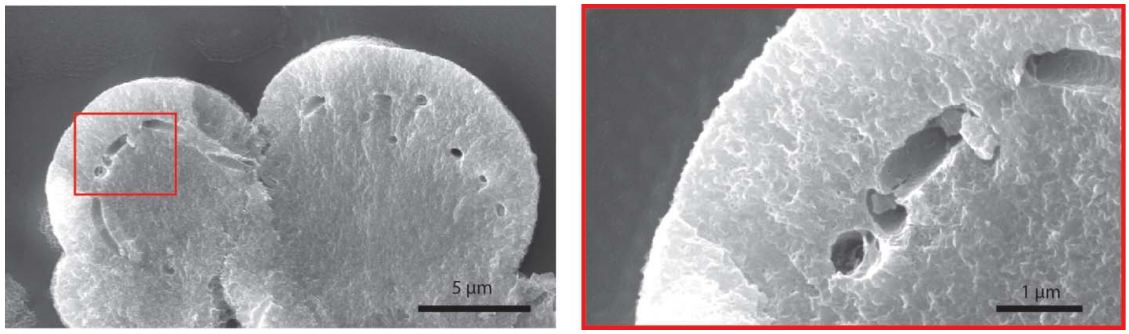

Figure 2. Cryo-SEM images showing cell-like structures embedded in microsperolitic/botryoidal Mn oxide microstructures suggesting that the cell-like shapes follow internal laminae (A and B).

embedded in these microstructures and suggest that the cell-like shapes occur within specific laminae (Sjöberg 2017; Sjöberg et al. 2017).).

Sample collection

YBS samples used for geochemical analyses were collected during the winter season in 2014. Samples for the microbial diversity survey and quantitative analyses, the YBS and the water seeping through it, were collected during winter and spring seasons in 2015.

DNA extraction

DNA was extracted from the YBS using the Mo Bio PowerSoil DNA kit (Carlsbad, CA), following the manufacturer's instructions. Around 0.25 g of the YBS was used for each extraction, taken with care not to include the supernatant water. DNA from the water was extracted using the DNeasy Blood and Tissues Kit (QIAGEN) following the manufacturer's instructions. For each extraction, 4 ml were used.

Quantitative PCR (Q-PCR)

The quantitative PCR analyses targeted the bacterial and archaeal 16S rRNA genes and were determined using specific primers' couples BACT1369f/BACT1492r (Suzuki et al. 2000) at an annealing temperature of 60°C for the bacteria and ARC787f/ARC1059r (Yu et al. 2005) at an annealing temperature of 58°C for the archaea. All quantifications, samples and ten-fold dilution series of standard curves, were done in triplicates, in 35 cycles, along with negative controls to rule out laboratory contamination. Q-PCR conditions were: 500 nM of each primer, 5μL of DNA template, 10μL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) following the manufacturer's recommendations, and the sterile deionized water was added to a final volume of 25μL. The standard curves were calibrated using ten-fold dilutions from pure cultures of *Citreicella thiooxidans* (Sorokin et al. 2005) for the bacteria and *Methanoculleus marisnigri* (Maestrojuan et al. 1990) for the archaea. All reactions were realized in 96 well Q-PCR plates using CFX96 Touch™ Real-Time PCR Detection System (C1000 Touch™ Thermal, Cycler, Bio-Rad) Instrument and associated software. The total gene copy numbers per gram of black substance or per mL of water were calculated from the triplicate sample averages as previously described (Sylvan et al. 2013) and by estimating 1.86 copy of the 16S rRNA gene for the archaea and 4.1 copies for the bacteria. The R^2 (coefficient of determination) of the Q-PCR was up to 0.997 and the efficiency of the reactions was up to 92%.

PCR, cloning, sequencing and phylogenetic analyses

PCR targeting the bacterial and archaeal 16S rRNA gene was conducted using the primers' combination: E8F/U907R for bacteria (Lane et al. 1985; Lane 1991) and A8F/ARC915R for archaea (Casamayor et al. 2000; Kolganova et al. 2002). Both bacterial and archaeal 16S rRNA gene amplification reactions were performed in 50μL reaction mixture containing: 25μL of the GoTaq® G2 Colorless Master Mix (Promega), and 0.25μL of each primer at 100μM and 24μL of nuclease free sterile deionized water. All amplifications were realized in 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min 30 s at 55°C for bacterial 16S rRNA gene and at 58°C for the archaeal 16S rRNA gene, extension at 72°C for 2 min followed by a final extension at 72°C for 7 min. All PCR reactions were carried out using an Eppendorf thermal cycler (Mastercycler, nexus gradient), and PCR products were visualized using gel electrophoresis.

PCR products were excised from agarose gel and gel purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel) prior to cloning, according to the manufacturer instructions. Clone libraries were carried out with pGEM®-T cloning kit (Promega) following manufacturer recommendations. Clones were cultured and treated for sequencing with Macrogen (Korea) using M13 primers. Sequences were compared with those available on NCBI BLAST network service (NCBI website: http://www.ncbi.nlm.nih.gov/BLAST) to determine their phylogenetic affiliations and aligned, edited and analyzed using Bioedit version 7.2.5 software. Sequences were checked manually for chimera. Phylogenetic trees were constructed using the MEGA7 program (Kumar et al. 2008). The robustness of inferred topologies was tested using 1000 bootstrap resampling of the tree calculated on the basis of neighbor-joining algorithm (Saitou and Nei 1987) with Kimura two-parameter correction matrix (Kimura 1980). All sequences presenting more than 97% similarity were considered to belong to the same phylotype and were clustered together as operational taxonomic units (OTUs) in the alignment (Schloss and Handelsman 2004). The bacterial and archaeal sequences reported in this study have been deposited

to GenBank nucleotide sequence databases under accession number MG657047 to MG657237.

Results

Q-PCR data on the YBS and the fracture water

Q-PCR data indicated that both bacterial and archeal abundance were up to four orders of magnitude higher in the YBS samples compared to the fracture water samples, independent of the season (Figure 3). Bacteria were dominant with respect to archaea in both the YBS and the fracture water, winter and spring samples. The estimation of the total amount of bacteria in the Ytterby substance was in the range 2×10^{10} to 7×10^{10} cells per g YBS depending on season, while the water feeding the fracture was on the order of 10^6 cells per mL groundwater. The corresponding numbers for archaea were in the range 6×10^8 to 1×10^9 cells per g substance depending on season, and 7×10^4 to 2×10^5 cells per mL groundwater. Notably, the water samples constitute a very small percentage of total prokaryote abundance compared to the Mn samples. Bacterial quantification in both the substance and water samples were lower in the spring compared to the winter samples, whereas the opposite was valid for archaeal cell quantification (Figure 3).

Archaeal and bacterial compositions: General trends and statistics

A total of 184 archaeal and 384 bacterial partial 16S rRNA gene sequences were sequenced in this study. Among the sequenced clones, 131 archaeal 16S rRNA gene sequences, grouped into 14 operational taxonomic units (OTU, defined at 97% sequence similarity), were obtained from the four samples: YBS winter, YBS spring, Water winter and Water spring. YBS and Water winter, as well as YBS spring showed a diversity profile, which was almost exclusively dominated by *Thaumarchaeota* (87–100% of the relative abundance determined from the clone libraries). The remaining 0–13% was represented by methanogenic *Methanomicrobiales* and clones belonging to an undetermined OTU represented by Ytterby clone 4 A07 within the *Euryarchaeota* phylum (Figure 4 and Figure 6). The Water spring sample was also dominated by *Thaumarchaeota* (61% of the relative abundance) but had a higher relative abundance of *Euryarchaeota* associated to OTUs Ytterby clone 5 B09 and Ytterby clone 5 E09 within the *Methanomicrobiales*. Regarding the bacteria, 359 partial 16S rRNA gene sequences were obtained from the four samples representing 117 OTUs. *Bacteroidetes* dominated the bacterial 16S rRNA gene clone libraries in the YBS samples and *Alphaproteobacteria* in the fracture water (Figure 5). The five dominant phyla in each sample sum up to 83, 63, 62 and 77% of the relative abundance among the total number of clones. Considering each sample separately, the five dominant phyla (shown in detail in Figure 5) were for:

- YBS winter: Bacteroidetes (29%), Nitrospirae (17%), Alphaproteobacteria (14%), Acidobacteria (13%) and Betaproteobacteria (10%),
- YBS spring: Bacteroidetes (18%), Alphaproteobacteria (16%), Acidobacteria (11%), Candidatus Methylomirabilis (10%) and Gammaproteobacteria (8%),
- Water winter: Alphaproteobacteria (22%), Deltaproteobacteria (12%), Chloroflexi (10%), Firmicutes (10%) and Planctomycetes (8%)
- Water spring: Alphaproteobacteria (32%), Chloroflexi (14%), Bacteroidetes (11%), Actinobacteria (11%) and Deltaproteobacteria (9%).

Among bacteria, only five OTUs contained 10 or more sequences. These five OTUs represented 108 out of the 359 bacterial clones and belonged to the *Bacteroidetes* (with 47 sequences in the bacterial clone library), *Nitrospirae* (23), *Candidatus Methylomirabilis* (15), *Acidobacteria* (13), and *Betaproteobacteria* (10). The most abundant OTU, Ytterby clone 1 A02 within the *Bacteroidetes*, is unique for the YBS and was not detected in the water samples. This is also the case for the fourth and fifth largest OTUs representing the *Acidobacteria* and the *Betaproteobacteria*. For the remaining two OTUs the majority of clones

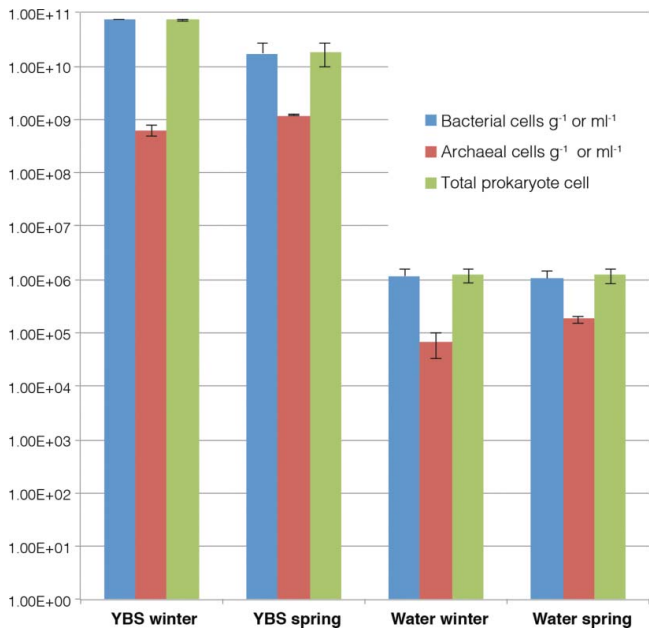

Figure 3. Distribution of total number of bacteria and archaea in the Mn deposit (YBS) and fracture water samples for winter and spring seasons. Triplicate Q-PCR reactions were run on each sample and error bars show the standard error of the mean.

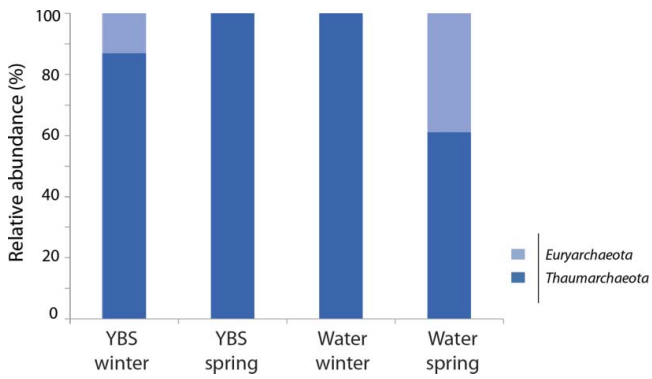

Figure 4. Archaeal community composition based on 16S rRNA gene analyses of 119 archaeal clones. Percentages show the relative abundances of the archaeal community based on the frequency of archaeal 16S rRNA in each sample.

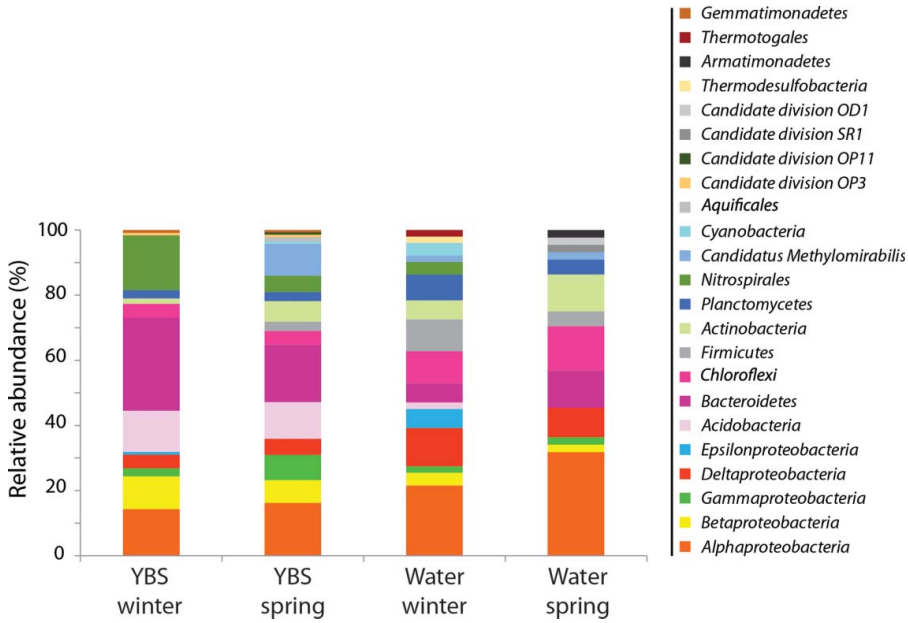

Figure 5. Bacterial community composition based on 16S rRNA gene analyses of 359 bacterial clones. Percentages show the relative abundances of the bacterial community based on the frequency of bacterial 16S rRNA in each sample.

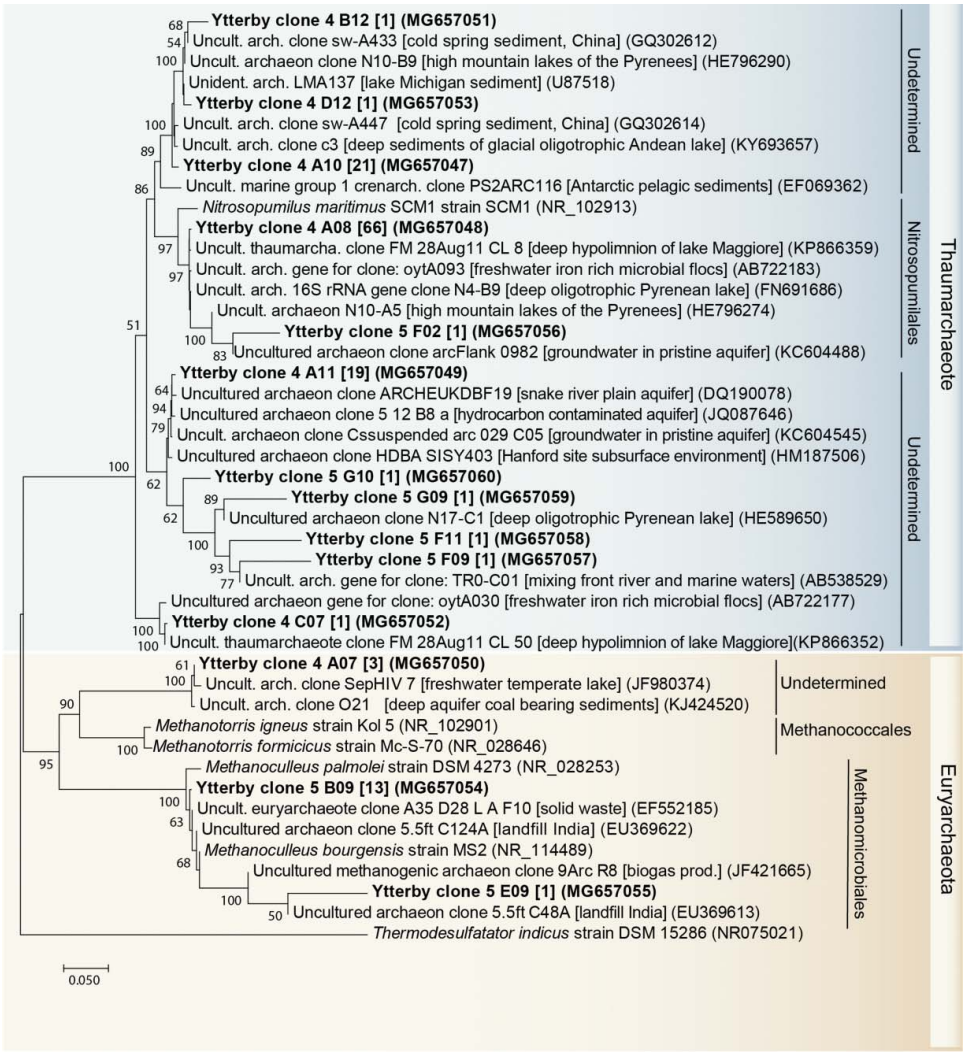

Figure 6. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of archaea. Sequences obtained in this work are shown in bold text and the number of sequences represented by each OTU are shown in square brackets and accession numbers are in parentheses. Only bootstrap values above 50% are given at branch nodes. The scale bar represents the number of substitutions per unit branch length.

were detected in the YBS, but a small proportion of clones were also found in the water samples. Among the archaea 4 out of the total 14 OTUs contained 10 or more sequences. These four OTUs represented 119 out of the total 131 sequences and belonged to the *Thaumarchaetota* (106) and to *Methanomicrobiales* within the *Euryarchaeota* (13). The clones that grouped with *Methanomicrobiales* all belonged to the same OTU (Ytterby clone 5 B09) and were all collected in the Water spring sample.

Good's coverage estimator indicated 70–94% coverage in the archaeal clone libraries thus exhibiting a reasonable capture of the archaeal diversity within the YBS microbial community. Bacterial sampling on the other hand, did not show any signs of reaching the plateau phase, as is highlighted with the Good's coverage values (42–51% coverage in the YBS bacterial libraries and 24–26% for the fracture water), indicating that the full extent of bacterial diversity was not captured and that these data should be interpreted with caution.

Phylogenetic analyses

Many sequences recovered in this study grouped with uncultured environmental clones, and a substantial number of these clones were retrieved from subsurface environments, cold climates, or localities associated with high metal concentrations. There was also a certain overlap with clones collected from calcium carbonate precipitates such as cold freshwater lake microbialites, cave karst aquifers and cave moonmilk. A selection of taxa/lineages that are either the most abundant or particularly relevant to the challenging Ytterby environment are proposed below. The phylogenetic trees shows selected lineages recovered from the YBS and fracture water.

Archaea

The archaeal population was almost exclusively dominated by *Thaumarchaea* (87% of the relative abundance determined from the clones libraries) and included the three most abundant OTUs, associated with Ytterby clone 4 A08, 4 A10 and 4 A11. The most abundant OTU, represented by Ytterby clone 4 A08 (66 related clones), showed 97% similarity to *Nitrosopumilus maritimus* strain SCM1 (NR_102913), an autotrophic aerobic marine ammonia oxidizing archaea (Walker et al. 2010) and was detected in all samples. Ytterby clone 4 A10 (21 clones) was 99% similar to an environmental clone isolated from deep sediments of a glacial oligotrophic Andean lake (KY693657, Parro et al. NCBI GenBank 2017). The third most abundant OTU, Ytterby clone 4 A11 (19 clones), mostly detected in the Water spring sample, showed 99% similarity to a subsurface microbial community at the former US nuclear production at the Hanford site (clone HDBA_SISY403, HM187506, Lin et al. 2012). The remaining OTUs were only represented by one sequence and mainly clustered with uncultured environmental clones. The Euryarchaeota consisted of 3 OTUs. The fourth most abundant in our clone library, Ytterby clone 5 B09 (13 related clones), was exclusively found in the Water spring sample and showed 98% similarity to *Methanoculleus palmolei* strain DSM 4273, a methanogenic archaea (Zellner et al. 1998). Figure 6 shows archaeal lineages recovered from the YBS and fracture water.

Bacteria

Bacteroidetes phylum

A group of *Bacteroidetes* sequences that cluster within an uncultured novel genus most closely related to *Terrimonas* was identified. This cluster is composed of Ytterby clone 1 A02, 3 G07, 3 B06, 2 H04, 2 A04 and 3 H06. Ytterby clone 1 A02 within this cluster represented the most abundant OTU (47 related clones) and showed 98% similarity to an uncultured *Terrimonas* clone isolated from a cold desert rhizosphere (HE861150, Mapelli et al. 2012) but only 94% similarity to the closest cultivated strain *Terrimonas ferruginea* DSM 30193 (NR_042494, formerly named *Flavobacterium ferrugineum* (Xie and Yokota 2006)) and *Terrimonas arctica* strain R9-86 (NR_134213, Jiang et al. 2014). OTU 1 A02 also showed 99% similarity to clone B061 from alpine grasslands on the Tibetan plateau (JX967634, Yuan et al. 2014), 98% similarity to clone SL-AD1–12 from soil associated with different vegetation types on the Tibetan plateau (JQ978624, Zhu and Ma, NCBI GenBank 2017) and 99% to a clone Amb collected from a study on changes in atmospheric CO_2 on soil microbiota associated with trembling aspen (EF018587, Lesaulnier et al. 2008) (Figure 7). The remaining five OTUs in the novel *Bacteroidetes* genus were more distantly related to the closest environmental sequence (JX967634, Yuan et al. 2014) with Ytterby OTU 2 A04 only being 88% similar.

Proteobacteria phylum

Proteobacteria were prevalent in all samples. Family *Hyphomicrobiaceae* belonging to the subdivision *Alphaproteobacteria* was frequently represented (19 clones) and clones belonging to *Hyphomicrobium, Pedomicrobium* and *Filomicrobium* were retrieved (Figure 8).

A large number of clones (19) within the *Alphaproteobacteria* remained undetermined. These undetermined clones formed 12 OTUs containing a total of 20 clones and 10 of these OTUs were most similar to clones from subterranean environments associated with either metals or calcium carbonates (overlapping each other): e.g. Ytterby clone 3 F12, was most similar (99%) to clone c4–4 from an ancient gold and arsenic mine (FN594648, Tomczyk-Zak et al. 2013), Ytterby clone 3 B12 most similar (98%) to clone MACA-CC31 from karst cave aquifer sediments (GQ500722, Fowler, NCBI GenBank 2017), Ytterby clone 2 C06 was most similar (99%) to clone cv81 from karst cave wall biofilms (EF530680, Macalady et al. 2007) and Ytterby clone 1 D03 was most similar to clone GRF1041c05 from white microbial mats in a lava tube (JF266208, Riquelme et al. NCBI GenBank 2017) and ytterby clone 6 A10 was most similar (99%) to clone J71 from Fe affected drinking water systems (GQ389024, Li et al. 2010). One of the OTUs (Ytterby clone 1 C05) also showed high similarity (99%) to a clone from permafrost soil from Kunlun mountains on the Tibetan plateau (JQ684271, Hu and Feng, NCBI GenBank 2017). The remaining OTU, Ytterby clone 6 A09 (5 related clones), was most similar to a clone from a chlorinated drinking water system (EU809306, Noguera et al. 2009).

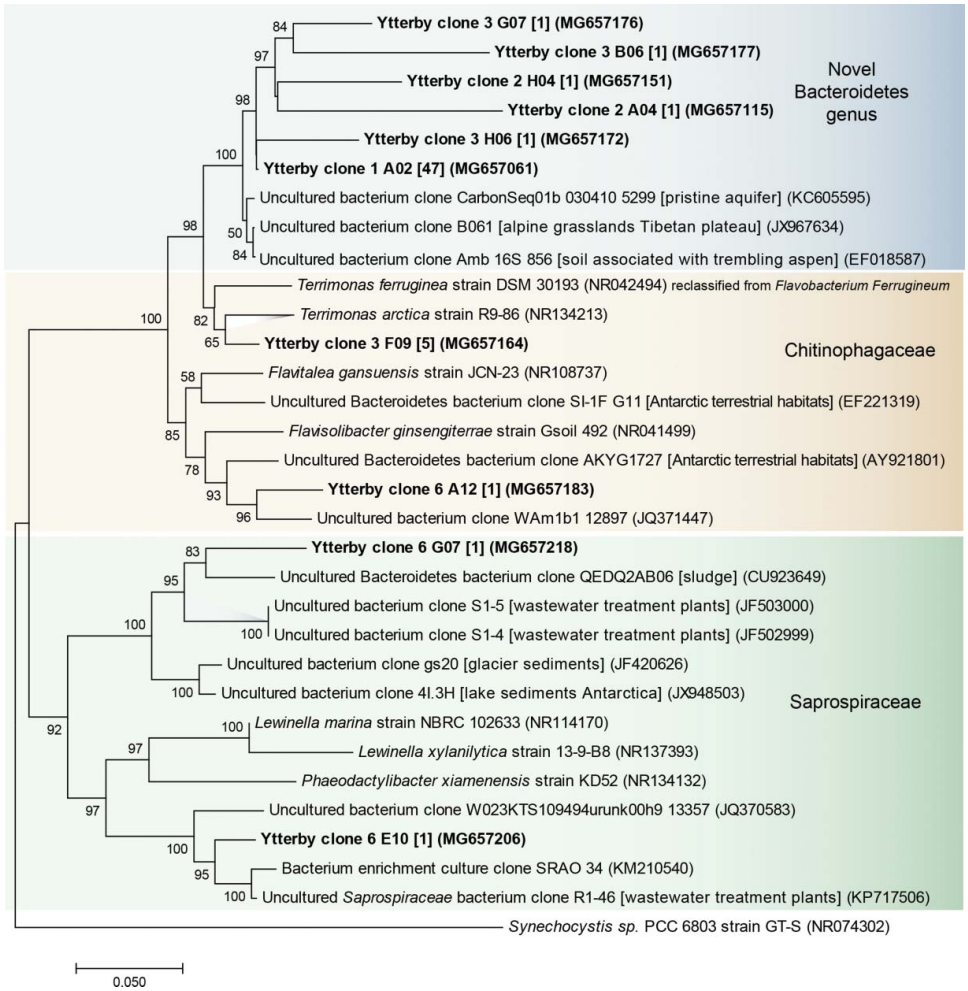

Figure 7. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of the Ytterby Bacteroidetes cluster and representatives of the Chitinophagaceae and Saprospiraceae families within the order Sphingobacteriales of the Bacteroidetes phylum. Sequences obtained in this work are shown in bold text and the number of sequences represented by each OTU are shown in square brackets and accession numbers are in parentheses. Only bootstrap values above 50% are given at branch nodes. The scale bar represents the number of substitutions per unit branch length.

Clone 1 A07, which represents a group of 10 clones within the *Betaproteobacteria*, was 97% similar to a nitrite oxidizer active at low temperatures, '*Candidatus Nitrotoga arctica*' (Alawi et al. 2007) and 96% similar to an Fe(II) oxidizing species *Sideroxydans lithotrophicus* (He et al. 2016), both within the *Gallionellaceae* family. The remaining 13 clones within *Betaproteobacteria* were represented by 9 OTUs. Clone 1 D09 (3 clones) was 99% similar to a clone recovered from a bacterial population of a polyaromatic hydrocarbon contaminated soil (FQ660388, Martin et al. 2012) and 96% similar to the closest cultivated strain, *Rugosibacter aromaticivorans* strain Ca6, a member of the family *Rhodocyclaceae,* capable of degrading polycyclic aromatic hydrocarbons (CP010554, Singleton et al. 2015). Among the remaining 10 clones, Ytterby clone 6 B01 (2 clones) was 99% similar to *Undibacterium Oligocarboniphilum* strain EM 1 within the *Oxalobacteracea* family, commonly found in substrates low in carbon (NR_117348, Eder et al. 2011) and also 99% similar to a clone from a uranium contaminated subsurface sediment (DQ316806, Akob et al. 2007). Three OTUs were 99% similar to clones from a study on metal retention in Fe rich microbial mats: Ytterby clone 2 E05 (1 clone) to LN870736, Ytterby clone 6 B10 (1 clone) to LN870654 and Ytterby clone 1 H10 (2 clones) to LN870812 (Zeitvogel et al. NCBI GenBank 2017). The closest cultivated strain (98% similar) to Ytterby clone 6 B10 was *Methylibium petrolephilum* PM1, a methylotroph able to metabolize different compounds in petroleum products (CP000555, Kane et al. 2007). Two clones were 99% similar to clones collected from the study on changes in atmospheric CO_2 on soil microbiota associated with trembling aspen (Ytterby clone 1 D05 to EF020061 and Ytterby clone 2 B10 to EF019329, Lesaulnier et al. 2008). Ytterby clone 1 D05 was also 99% similar to a clone from the Tibetan plateau (JX967677, Yuan et al. 2014). Ytterby clone 1 A04 (1 clone) was 99% similar to 3 different studies on the Tibetan plateau (HQ863981, Duan and Ma, NCBI GenBank 2017; HQ864114, Shang and Ma, NCBI GenBank 2017 and JQ825172, Liu and Zhang, NCBI GenBank 2017). Ytterby clone 2 E06 (1 clone) was most closely related to a clone from a biofilm associated with phragmites (AB240262, Nakamura et al. NCBI GenBank 2017).

Clones within the *Gamma-* and *Deltaproteobacteria* (31 in total) whose closest relatives were from subsurface environments associated with metals and/or radionuclides: e.g. Ytterby clone 1 A06 and 6 A06 were most similar (98% and 98% respectively) to clones from the former US nuclear production at the Hanford site (HM185957, HM186019, Liu et al. 2012);

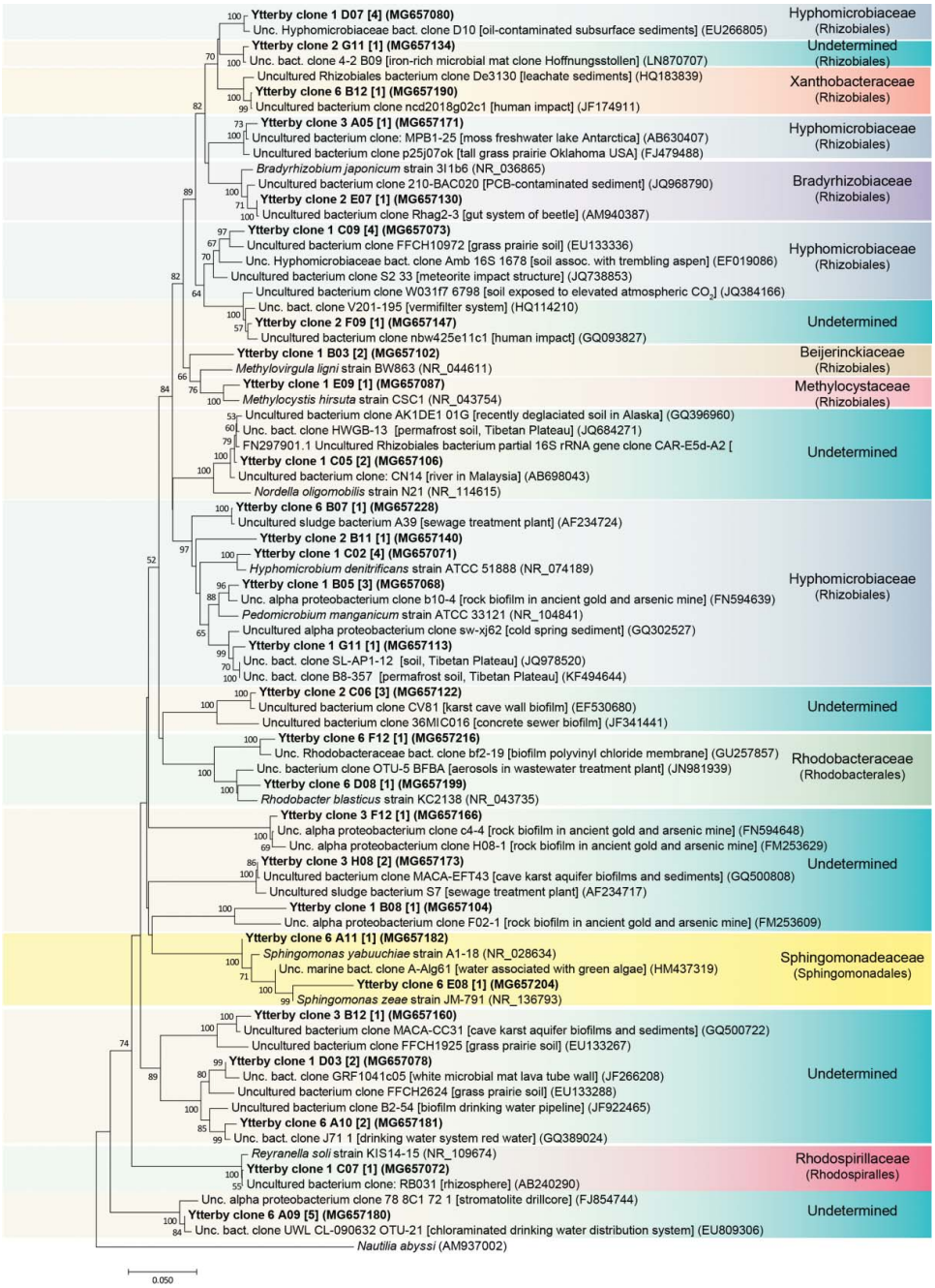

Figure 8. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing all identified operational taxonomic units (OTU) within the Alphaproteobacteria. Sequences obtained in this work are shown in bold text and the number of sequences represented by each OTU are shown in square brackets and accession numbers are in parentheses. Only bootstrap values above 50% are given at branch nodes. The scale bar represents the number of substitutions per unit branch length.

Ytterby clone 1 E05 was 98% similar to a clone from rock biofilm in an old gold and arsenic mine (HE614740, Zielenkiewicz et al. NCBI GenBank 2017) and Ytterby clone 2 H12 was most closely related (99% similar) to a study on metal retention in Fe rich microbial mats (LN870782, Zeitvogel et al. NCBI GenBank 2017) were most abundant. Cold environments and microbial communities associated with calcium carbonate precipitates were also recurrent (Ytterby clone 2 C07 was most similar (99%) to a clone from cave moonmilk precipitates, KC255343, Engel et al. NCBI GenBank 2017) and Ytterby clone 2 B02 (4 clones) was most closely related (98%) to a clone from the Tibetan plateau (JQ978628, Zhu and Ma, NCBI GenBank 2017).

Nitrospirae, Candidatus Methylomirabilis, Cyanobacteria and Planctomycetes phyla

Phylogenetic relationship of the detected *Nitrospirae, Candidatus Methylomirabilis, Cyanobacteria and Planctomycetes* retrieved sequences are presented in Figure 9. *Nitrospirae* contributed 17% of total bacterial clones in the YBS winter sample and 5% in the YBS spring. The Water spring sample contributed 4% while the Water winter lacked representatives of this phyla. Within the *Nitrospirae* phylum there was a newly identified Ytterby *Nitrospira* cluster (composed of Ytterby clone 2 G04, 3 B07 and 1 B02 representing a total of 25 clones) for which there do not exist any described strains to date. Ytterby clone 1 B02 within this cluster was most similar (99%) to a

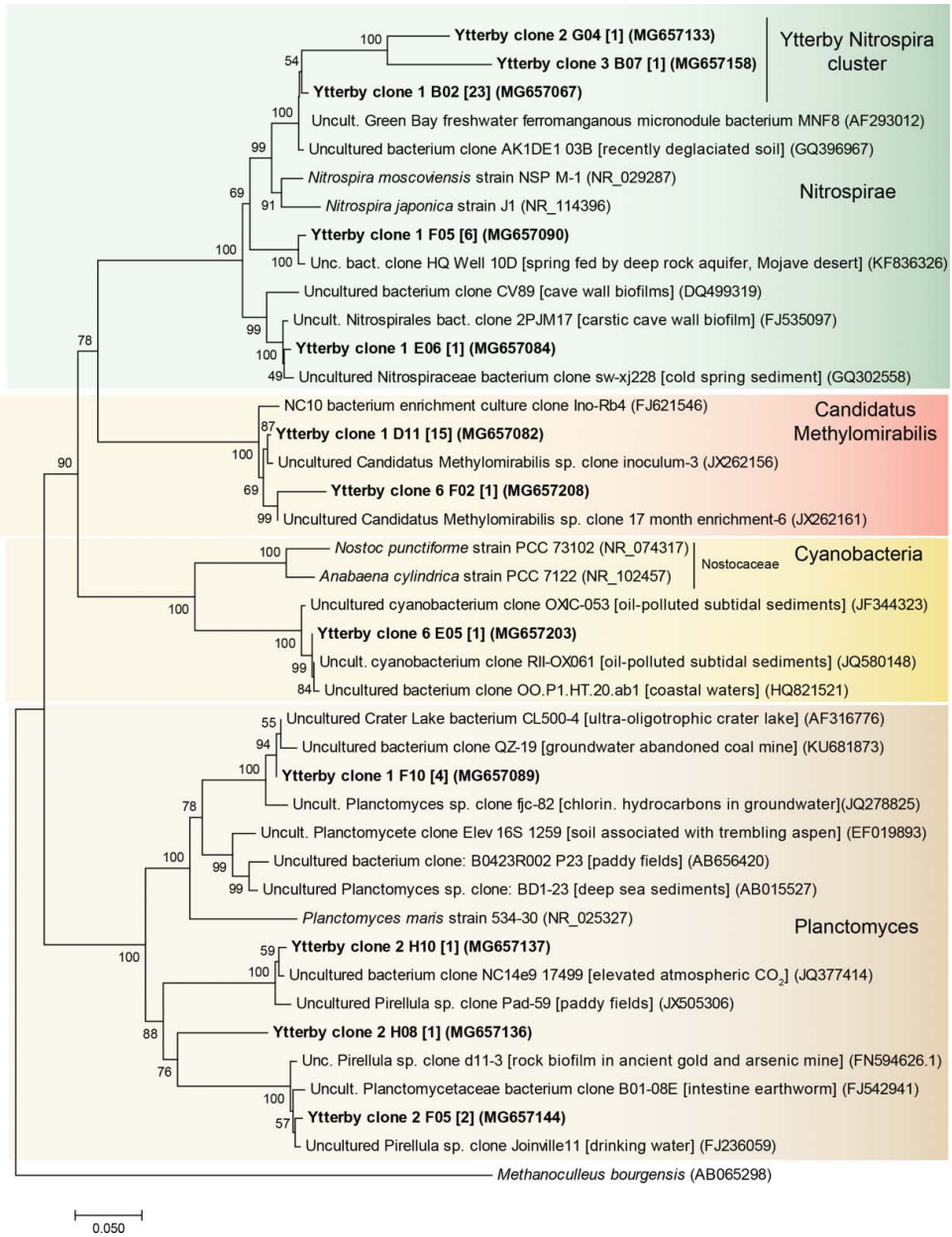

Figure 9. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of Nitrospirae, Candidatus Methylomirabilis, Cyanobacteria and Planctomyces. Sequences obtained in this work are shown in bold text and the number of sequences represented by each OTU are shown in square brackets and accession numbers are in parentheses. Nucleotide accession numbers used to construct the dendrogram are given in brackets. Bootstrap values above 50% (from 1000 bootstrap samples) are indicated near their corresponding nodes. The scale bar represents the number of substitutions per unit branch length.

clone detected in an Fe rich microbial mat covering a tunnel wall (LN870976, Zeitvogel et al. NCBI GenBank 2017), 99% similar to a clone from a biofilter removing Mn and ammonia from groundwater (KC900078, Cai, NCBI GenBank 2017) and 98% similar to a Green Bay ferromanganese micronodule bacterium MNF8 (AF293012, Stein et al. 2001). The closest cultivated strain (95% similar) was *Nitrospira moscoviensis NSP M-1* (CP011801, Koch et al. 2015).

Candidatus Methylomirabilis, a denitrifying methanotroph, contributed 10% of total bacterial clones in the YBS spring sample but was absent in the winter sample. *Cyanobacteria* were recovered from the YBS spring and the Water winter sample. A total of 14 clones grouped within the *Planctomycetes* and the most abundant OTU (4 related clones) was most closely related to an ultra-oligotrophic crater lake bacteria (Urbach et al. 2001) and 97% similar to an uncultured *Plantomyces* clone.

Acidobacteria, Actinobacteria and Firmicutes phyla

Acidobacteria were abundant in the YBS but almost absent in the water samples (in total 30 clones grouped in 11 OTUs). Ytterby clone 1 C10, which represents a group of 13 clones unique for the YBS, were 96% similar to the most closely related cultivated strain, an aerobic chemoorganoheterotroph, *Stenotrophobacter terrae* strain Ac_28_D10, isolated from an old flood plain in Namibia (NR_146023, Pascual et al. 2015) and 98% similar to an uncultured clone from cave moonmilk. Acidobacterial clones, whose closest relative were from cold climates (Antarctica, glacier sediments and permafrost core profiles from both Tibetan plateau and Northeast Greenland),

were abundant: e.g. Ytterby clone 2 F07 was 97% similar to a clone from permafrost affected soil in Northeast Greenland (KF973990, Ganzert et al. 2014), Ytterby clone 1 C11 was 99% similar to a clone from glacier sediments (JF420784, Simon et al. NCBI GenBank 2017) and Ytterby clone 2 D11 99% similar to a clone from soil associated with accidental diesel spill in Antarctic coastal areas (KY190490, Vazquez et al. NCBI GenBank 2017) and 99% similar to a clone from a permafrost core on the Tibetan plateau (KF494531, Hu, NCBI GenBank 2017). OTU 2 C08 (representing 2 clones) was most closely related to a clone from a study on metal retention in Fe rich microbial mats (LN870702, Zeitvogel et al. NCBI GenBank 2017) and yet another OTU (Ytterby clone 1 D03 representing 2 clones) was most closely affiliated with a clone from a white microbial mat in a lava tube (JF266208, Riquelme et al. NCBI GenBank 2017).

Actinobacteria were present in all samples grouped as 18 OTUs including 20 clones. Clone 6G10 which is affiliated within the *Microbacterium* genus shows 96% similarity to a Mn(II)-oxidizing soil isolate B150, HQ877782 (Yang et al. 2013). Four OTUs were most similar to clones from glacier sediments or from permafrost core profiles at the Tibetan plateau and another four OTUs to clones from heavy metal associated sites (the Hanford site, a rock biofilm in ancient gold and arsenic mine and high arsenic concentrations in aquifer in inner Mongolia). Three OTUs unique for the water samples were most similar to clones from hypersaline lakes collected in two different studies: Ytterby clone 6 H09 grouped with *Nocardioides aquaticus* strain EL-17K, isolated from Ekho lake in Antarctica (NR_044903, Lawson et al. 2000) and Ytterby clone 6 F10 and 6 G03 with a clone from Ebinur lake sediments in China (KT893270, Lv et al. NCBI GenBank 2017).

Firmicutes were represented in all but the YBS winter sample and the fourth most abundant phyla in the Water winter sample (10%). Ytterby clone 6 E02 (3 clones) grouped with the low growth temperature *Psychrobacillus psychrotolerans* (Krishnamurthi et al. 2010) formerly named *Bacillus psychrotolerans* (El-Rahman et al. 2002). Two OTUs were most closely related to clones from the Hanford site (Ytterby clone 6 B03 and 6 C03 were 96% and 95% similar respectively to clone HM186620, Lin et al. 2012) and one OTU, Ytterby clone 1 B06, was 98% similar to a clone from rock biofilm in an ancient gold and arsenic mine (HE614740, Zielenkiewicz et al. NCBI GenBank 2017).

Discussion

Microbial communities associated with the Mn deposit

Influence of physicochemical parameters

The chemotrophic microbial communities studied in the Ytterby mine are diverse and reveal a high percentage of uncultured unclassified bacteria that repeatedly show close similarity to clones isolated from subsurface environments, low temperature milieus, and/or settings rich in metals. The results also indicate limited, but recurring similarities with clones isolated from environments associated with hydrocarbons (stored in the mine shaft 200 m away from the studied Mn deposit, Figure 1b), or calcium carbonates (mineralized layer of calcium carbonate underlying the YBS, Figure 1c-d). The YBS communities show considerable overlap when combining these environmental conditions together, e.g., an Ytterby sequence similar to a clone from permafrost cores on the Tibetan plateau will often be closely related to clones from sites characterized by one or more of the other external factors.

The low stable temperature (an average of 8°C all year long) seems to be a controlling factor for the studied microbial population composition. Sequences affiliated to psychrotolerant bacterial species or species from very cold environments (both archaea and bacteria) are numerous and recurrent throughout the samples. Recognized species include *Psychrobacillus psychrotolerans* (Krishnamurthi et al. 2010) within the *Firmicutes, Filomicrobium sp*., within the *Alphaproteobacteria*, isolated from the Siberian permafrost (JN25 1893, Kudryashova et al. 2013) and uncultured environmental clones belonging to the *Acidobacteria, Actinobacteria, Bacteroidetes, Betaproteobacteria, Chloroflexi or Planctomycetes* isolated from glacier sediments, permafrost core profiles from northeast Greenland and the Tibetan plateau, the Andes, Antarctica and Arctic regions.

Many Ytterby clones are closely related to organisms reported in other subsurface environments containing heavy metals, e.g., an ancient gold and arsenic mine in Zloty Stok, Poland (Tomczyk-Zak et al. 2013; Zielenkiewicz, NCBI GenBank 2017) and the metal contaminated Hanford site in Washington state, USA (Lin et al. 2012). The metal concentrations in the waters (groundwaters, fracture water) are however representative of the regional groundwaters, possibly with the exception of the REE and Cu, which are at the high end of regional values (Sjöberg et al. 2017). Although the Ytterby mine water cannot be considered as a 'high metal content' environment, the YBS (which host a highly diverse community) can. Whether the bacterial communities are actually adapting to a local physicochemical oversaturation and precipitation at water-air redox interface or indirectly creating their own metal rich-environment through their metabolic activities (see below 'Constraining the prime suspects for Mn cycling'), the YBS communities are indeed developing in metal-rich environment.

The ability of Mn oxides to accumulate potentially harmful heavy metals could protect microbial communities (Ghiorse 1984). The sequestering of metals in the Ytterby birnessite-type Mn oxides may therefore help reducing the potential bio-availability and thus toxicity of metals to the microbial community. Also, phenotypic diversification in microbial biofilms compared to planctonic cells, is argued to aid biofilm populations to cope with environmental stressors such as metal toxicity (Harrison et al. 2007 and references therein). However, REE+Y are firmly incorporated in the birnessite structure, not merely adsorbed or associated with organics or biomass, and the most toxic metal in the birnessite-microbe system may in fact be Mn, or possibly Cu, and not the REE. Present day concentrations of Ca, Na and REE in the fracture water where the latest YBS sample was recovered are 68, 34 and 0.0027 mg/L, respectively, and of Mn 0.003 mg/L (Sjöberg et al 2017). The high affinity for REE, in comparison with Ca and Na, with similar effective ionic radii (coordination number 6) is demonstrated by the (concentration in solid)/(concentration in solution)-

ratio, which is about three and five orders of magnitude higher for REE than for Ca and Na, respectively.

Although Ytterby microbial populations show clear affinity with communities developing under high Fe-rich metal content, the mine deposit is dominated by Mn, Ca and REE+Y; Fe and other metals being less than 2% in total. Mn oxidizing bacteria are hypothesized to also be capable of Fe oxidation under the right circumstances (Ghiorse 1984). These two elements are therefore sometimes grouped and treated in a similar way in the geomicrobiology literature, but their specific microbially-mediated redox cycles are rather different. Although the kinetics of both Fe and Mn oxidation is greatly increased by metabolic activity, Fe-related metabolisms generally involve lithotrophy (mostly using oxygen as electron acceptor) and/or anoxygenic photosynthesis (e.g., Konhauser et al. 2011), metabolic activities that are not observed in Mn cycling. The fact that similar clones are observed in Mn deposits where Fe is only present in trace amounts (i.e. the studied YBS) and Fe-dominated environments may indicate that the reactive intermediate and the associated cryptic geomicrobiological cycles may play a larger role than anticipated in Fe-rich ecosystems.

Despite the low organic content in the YBS (0.6 wt%; Sjöberg et al. 2017) and the long distance to the location of the former storage of petroleum products, a few sequences detected in this study are closely affiliated with clones from environments associated with hydrocarbon contaminated sites: e.g. Ytterby 1 D09 within *Betaproteobacteria* is 99% related to a clone from a bacterial population of a polyaromatic hydrocarbon contaminated soil (FQ660388, Martin et al. 2012), Ytterby clone 6 B10 is 98% similar to *Methylibium petrolephilum* PM1 (CP000555, Kane et al. 2007) and Ytterby clone 2 D11 within *Acidobacteria* is 99% similar to a clone from soil associated with accidental diesel spill in Antarctic coastal areas (KY190490, Vazquez et al. NCBI GenBank 2017). The latter is also 99% similar to a clone from a permafrost core on the Tibetan plateau (KF494531, Hu, NCBI GenBank 2017) and it is therefore difficult to say whether it is the cold climate or the diesel spill, or possibly both, that are related to the detected clone. Ytterby clone 6 B10, i.e. the clone affiliated with *Methylibium petrolephilum* was most closely related (99%) to a clone from a study on metal retention in Fe rich microbial mats (LN870654, Zeitvogel et al., NCBI GenBank 2017). A fair number of sequences showing high similarity to clones from hydrocarbon contaminated environments are also observed in a study of Mn oxidizing bacteria in caves of the upper Tennessee River basin, without evident association to contamination by hydrocarbon products (Carmichael et al. 2013). Whether the similarities with clones from hydrocarbon contaminated sites are specific for the Ytterby mine or associated with subsurface, possibly Mn, Fe and/or heavy metal rich environments, are thus difficult to say from these results. The mineralized layer of calcium carbonate is most likely physicochemically precipitated through CO_2 degassing (travertine, e.g. Pentecost 2005) and could have an important physiological role as an environmental pH buffer.

General trends

Results show an unequivocal predominance of bacteria over archaea for both the YBS and the fracture water independent of the seasons. The bacterial dominance is in accordance with other subterranean ferromanganese deposits (Carmichael et al. 2013). Comparisons with other systems should be made with caution because of the inherent bias associated with the different methods applied to estimate cell density, e.g., different DNA extraction methods and qPCR primers, different FISH fixation protocols, nucleic acid stain (DAPI, SYBR Green), oligonucleotides probes used for cell counting (Kepner and Pratt 1994; Lloyd et al. 2010). Nevertheless it is important to initiate such comparisons and address this gap in knowledge, since very little data are available on cell abundance in mines and caves. When compared to other cave systems, the total number of 16S rRNA bacterial genes in the YBS (2×10^{10} to 7×10^{10} cells/g substance) is similar to a Mn oxide rich sample (1×10^{10} cells/g wet sample) and one to three orders of magnitude higher than cell densities reported for other ferromanganese biofilms found in shallow cave systems (7×10^{7} to 9×10^{9} cells/g wet sample) also estimated by Q-PCR (Carmichael et al. 2013). The total number of bacteria in groundwater from granitic subterranean environments, similar to the studied Ytterby mine, ranges from 10^{3} to 10^{7} cells/mL groundwater (Pedersen 1997). The measured abundance in Ytterby fracture water, on the order of 10^{6} cells per mL groundwater, is within this range. The corresponding values for archaeal cells in the YBS (6×10^{8} to 1×10^{9} cells per g substance depending on season) are comparable to, but still about an order of magnitude higher than, cave values (5×10^{6} to 1×10^{8} cells per g wet sample) reported by Carmichael et al. (2013).

Bacterial community composition is far more diverse than archaeal community composition which is in accordance with other ferromanganese deposits (Carmichael et al. 2013; Shiraishi et al., 2016). The Archaeal diversity is remarkably low in both the Mn accumulations and the fracture water. The archaeal population in the Ytterby samples is almost exclusively dominated by *Thaumarchaea* (87–100%); similar to the archaeal diversity retrieved in the ferromanganese nodules (Shiraishi et al. 2016), but differ from the study by Carmichael et al. (2013) where the *Euryarchaeota* represent 60% of the archaeal 16S rRNA gene library.

Constraining the prime suspects for Mn cycling

Linking populations to processes using 16S rRNA sequencing is always a risky task as this approach is providing information about who is there, but not really about who is doing what. However, the parameters of the Ytterby mine ecosystem have been well defined and demonstrate strong constraints of high heavy metal content and strong REE+Y enrichment in the studied Mn precipitates with a main mineral product being the birnessite variety (Sjöberg et al. 2017). Previous studies have shown that poorly crystalline birnessite-like phyllomanganates often represent the initial phase precipitated by bacteria and fungi during microbially mediated Mn oxidation (Bargar et al. 2005; Santelli et al. 2011; Tebo et al. 2004; Villalobos et al. 2003; Villalobos et al. 2006). Catalysis of Mn oxidation is thermodynamically favorable compared to the physicochemical process and is carried out by phylogenetically diverse microbes (Tebo et al. 2004). It is therefore a matter of interest to look into the

presence or absence of known Mn oxidizing bacteria in the YBS.

Why bacteria oxidize Mn remains a subject of discussion (see the comprehensive review in Tebo et al. 2004; Tebo et al. 2010). Figure 10 presents a simplification of the redox Mn cycle with the main biotic and abiotic pathways. The benefit of Mn reduction is understandable in anoxic environments rich in oxidized Mn, where Mn(III) and Mn(IV) are serving as final electron acceptors in homogene and/or heterogene reduction. Thamdrup et al. (2000) demonstrated that a large part of the organic matter in the Black Sea is mineralized through Mn respiration. In certain systems Mn can go through multiple redox cycles before getting immobilized in Mn oxides (Canfield et al. 1993; Tebo et al. 2005). Although Mn reduction cannot be excluded in the YBS at the oxic-anoxic interface, the final product is found under the form of Mn oxides of the birnessite-type. A vast array of processes are involved in the oxidation of Mn (Figure 10). Although this process is thermodynamically favorable and can occur physicochemically, direct or indirect microbial catalyses drastically increase the kinetics of oxidation.

No examples of modern Mn-based anoxygenic photosynthesis has been documented so far, and photosynthesis is difficult to invoke in the YBS cave environment. Microbial Mn oxidation involves two one electron transfers: Mn(II) to Mn(III) to Mn(IV) (Tebo et al. 2010). The first oxidation step, from Mn (II) to Mn(III) corresponds to a potential difference too large for a an oxygen-based respiratory chain (Ehrlich and Newman 2008; Hansel and Learman 2016). What is indeed working for other elements such as Fe^{2+} or H_2S seems difficult for Mn, which makes chemolithoautotrophic Mn oxidation rather unlikely using the enzymes known today (Carmichael and Bräuer 2015). However, the formation of Mn oxides containing various Mn oxidation states, such as birnessite, may potentially allow to bypass the theoretical barrier of the large cell potential associated with the first oxidation step (Carmichael and Bräuer 2015). The storage of Mn(III) within a metastable crystal will allow bacteria to use the second step of oxidation (between Mn (III) and Mn(IV), which is enzymatically very favorable for possible lithotrophy. It is thus possible that various homogeneous and heterogeneous oxidation pathways (direct and indirect) are combined, creating metabolic synergy between various microbial communities, the mineral product, and the environment.

The first one electron oxidation step, from Mn(II) to Mn (III) is strongly favorable using reactive intermediates such as superoxide (O_2^-) or hydroxyl (OH^-) and favorable at pH above 4 using hydrogen peroxide (H_2O_2) (Carmichael and Bräuer 2015; Luther 2010). The reactive intermediates are increasingly regarded as important elements in biogeochemical cycles, despite their low-concentration and their short life in natural environments, because of their potential ability to create 'cryptic linkage' between major element cycling (Hansel et al. 2015). As an example, ROS, mainly peroxide (H_2O_2) and superoxides (O_2^-), can be coupled to the Mn-cycle (Figure 10). Microbes can use enzymatic pathway in which they utilize a multicopper oxidase (MCO), a peroxidase or a combination of both to directly oxidize Mn (e.g., Geszvain et al. 2013). Bacterial species can also indirectly oxidize Mn through superoxide production during heterotrophic growth or reproduction (Learman et al. 2011; Carmichael and Bräuer 2015). Superoxides are reacting with Mn(II) to produce Mn(III) and H_2O_2. Mn(III) is also an unstable reactive intermediate which can disproportionate and produce both reduced and oxidized Mn species (Figure 10). Mn oxidation is thus acting as an important antioxidant, removing potentially dangerous highly reactive chemical intermediates produced during respiratory metabolism (Hansel and Learman 2016).

Up to now all known Mn oxidizers are heterotrophs that do not oxidize Mn for generation of energy (Hansel and Learman 2016). Potential players involved in the Mn cycling clustered mainly

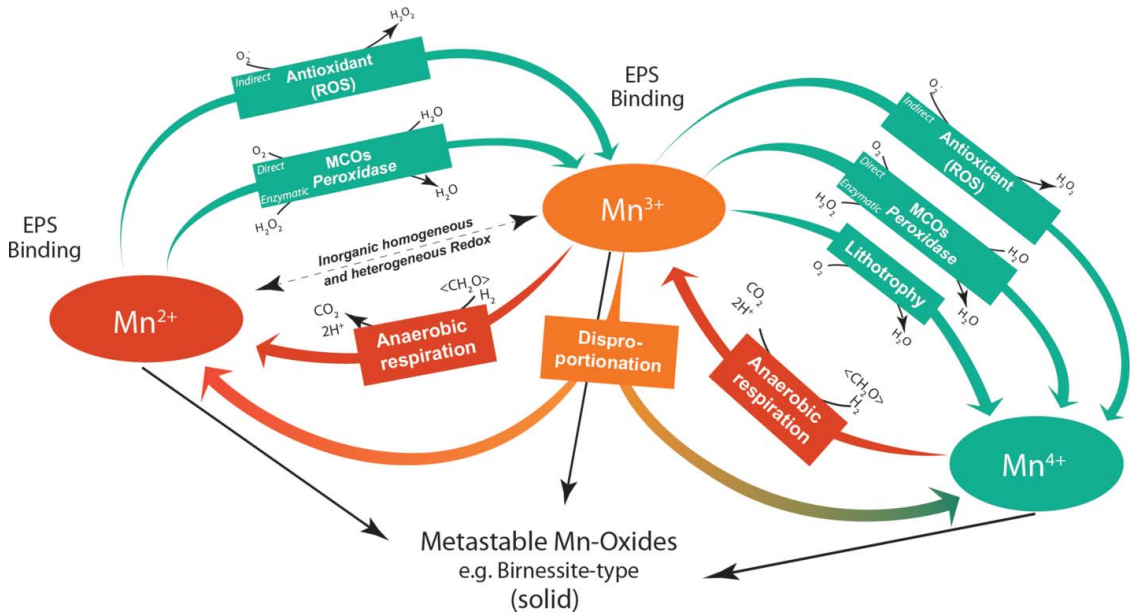

Figure 10. Simplified Mn cycle showing the main biologically-induced and physicochemical processes involved in the oxidation and reduction of Mn. Green and red colors indicate oxidation and reduction, respectively. These processes can lead to the precipitation of various types of Mn oxide minerals, with a birnessite-type being the dominant form in the Ytterby system. See text for details.

within the hyphae budding, ferromanganese genera *Hyphomicrobium* and *Pedomicrobium* (notably *P. mangancium*) in the *Alphaproteobacteria* and the *Bacteroidetes* group most closely related to *Terrimonas* (Table 1). Within the *Alphaproteobacteria*, three clones (1 C07, 1 E07 and 1 E10) show 98% similarity to a newly discovered Mn oxidizing strain, *Reyranella* T26AA, KU713087 (Marcus et al. 2017). In addition, clones belonging to known Mn oxidizing genera *Microbacterium* within the *Actinobacteria* and the *Bacillus* within the *Firmicutes* are also detected (Table 1).

Hyphal budding bacteria have been found in Fe- and Mn-rich environments including sea floor lavas (Santelli et al. 2009), caves (Northup et al. 2003; Spilde et al. 2005), podzolic soils (Geber 1981), hydroelectric pipelines (Tyler and Marshall 1967; Tyler 1970) and Baltic Sea ferromanganese nodules (Ghiorse and Hirsch 1982). This group of budding bacteria are not using Mn(II) as electron donor for possible lithotrophy, as they are enzymatically oxidizing Mn through chemoorganotrophy (Santelli et al. 2009). The metabolic *moxA* gene which encodes a multicopper oxidase (MCO) homolog is responsible for Mn oxidation and laccase-like activity in *Pedomicrobium* sp. ACM 3067 (Larsen et al. 1999; Ridge et al. 2007). The oxidation of Mn is here supposedly linked to the reduction of O_2 or H_2O_2 in a direct enzymatic pathway. Ghiorse and Hirch (1979, 1982) have shown that *P. manganicum* and a group of hyphal budding bacteria identified in Baltic Sea ferromanganese nodules oxidize and deposit Mn on a matrix of extracellular polymeric substances (EPS). The mechanism of Mn oxidation associated with these *Pedomicrobium*-like bacteria involves a two-step process, in which negatively charged EPS scavenge reduced Mn that is then oxidized by Mn oxidizing bacteria (Ghiorse 1980). Assuming that *P. manganicum* and *Hyphomicrobium* sp. are involved in the Mn oxidation in the YBS, this

Table 1. Potential Mn oxidizers detected in the Ytterby Mn deposit.

Clone category	Accession no.	Most similar sequences (accession no., % similarity)	Abun.	Reference to related Mn-oxidizing bacterial isolates
Alphaproteobacteria				
1 B05	MG657068	*Pedomicrobium manganicum* ATCC 33121 (NR_104841.1, 98)	3	Gebers, 1981; Northup et al., 2003
1 G11	MG657113	*Pedomicrobium manganicum* ATCC 33121 (NR_104841.1, 96)	1	Gebers, 1981; Northup et al., 2003
2 B11	MG657140	*Pedomicrobium manganicum* ATCC 33121 (NR_104841.1, 93)	1	Gebers, 1981; Northup et al., 2003
2 D12	MG657128	*Pedomicrobium manganicum* ATCC 33121 (NR_104841.1, 94)	1	Gebers, 1981; Northup et al., 2003
1 C02	MG657071	*Hyphomicrobium facile subsp. tolerans* strain JJ-89 (KX682017.1, 98)	4	Tyler and Marshall, 1967; Ghiorse, 1984; Stein et al., 2001
2 C10	MG657124	*Hyphomicrobium* KC-IT-W2 (FJ711209.1, 95)	3	Tyler and Marshall, 1967; Ghiorse, 1984; Stein et al., 2001
1 C05	MG657106	*Hyphomicrobium* sp 16–60 (HM124367.1, 96)	2	Tyler and Marshall, 1967; Ghiorse, 1984; Stein et al., 2001
6 B07	MG657228	*Hyphomicrobium* KC-IT-W2 (FJ711209.1, 100)	1	Tyler and Marshall, 1967; Ghiorse, 1984; Stein et al., 2001
1 C07	MG657072	*Reyranella soli* strain KIS14–15 (NR_109674.1, 99)	1	Marcus et al., 2017
1 E07	MG657085	*Reyranella soli* strain KIS14–15 (NR_109674.1, 99)	1	Marcus et al., 2017
1 E10	MG657088	*Reyranella soli* strain KIS14–15 (NR_109674.1, 99)	1	Marcus et al., 2017
Bacteroidetes				
1 A02	MG657061	*Terrimonas* sp. env. clone (HE861150, 98); *T. ferruginea* strain DSM 30193 (NR_042494.1, 94)	47	Northup et al., 2010; Carmichael and Bräuer, 2015
2 A04	MG657115	*Terrimonas* sp. env. clone (HE861150, 87); *T. ferruginea* strain DSM 30193 (NR_042494.1, 84)	1	Northup et al., 2010; Carmichael and Bräuer, 2015
2 H04	MG657151	*Terrimonas* sp. env. clone (HE861203, 90); *T. ferruginea* strain DSM 30193 (NR_042494.1, 88)	1	Northup et al., 2010; Carmichael and Bräuer, 2015
3 F09	MG657164	*Terrimonas* sp strain C3–5(KY060007.1, 96)	1	Northup et al., 2010; Carmichael and Bräuer, 2015
3 H06	MG657172	*Terrimonas* sp. env. clone (HE861150, 94); *T. ferruginea* strain DSM 30193 (NR_042494.1, 89)	3	Northup et al., 2010; Carmichael and Bräuer, 2015
3 G07	MG657176	*Terrimonas* sp. env. clone (HE861150, 93); *T. ferruginea* strain DSM 30193 (NR_042494.1, 89)	1	Northup et al., 2010; Carmichael and Bräuer, 2015
6 C10	MG657195	*Terrimonas* sp .env.clone (HE860936, 98); *T. arctica* strain R9–86 (NR_134213.1, 95)	3	Northup et al., 2010; Carmichael and Bräuer, 2015
Actinobacteria				
6 G10	MG657219	*Microbacterium pumilum* strain HPG1 (JQ291594.1, 99)	1	Yang et al., 2013
Firmicutes				
6 E02	MG657201	*Psychrobacillus psychrodurans* strain 68E3 (NR_025409.1, 98)	3	De Vrind et al., 1986; Francis and Tebo, 2002
6 B09	MG657187	*Aeribacillus pallidus* strain DSM 3670 (NR_026515.1, 99)	1	De Vrind et al., 1986; Francis and Tebo, 2002

suggests that microbial exudates serve as nucleation sites for the formation of the birnessite-type Mn oxides. The precipitated bacterogenic Mn oxides are negatively charged as a result of cation vacancy sites (Spiro et al. 2008) and thus attract more reduced Mn (and other available trace elements). The bacterogenic Mn oxides then serve as catalysts for further Mn(II) oxidation (Bargar et al. 2005). This second step of Mn oxidation, which occur at the surface of already formed deposits, could theoretically also be accelerated by Mn oxidizing bacteria (Ghiorse 1980; Ehrlich 1980). Sly et al. (1990) also showed that EPS produced by *P. Manganicum* can attract pre-formed MnO_2 colloids, implying that EPS have the ability to bind both reduced and oxidized forms of Mn.

Clones related to *Terrimonas ferruginea* represent other plausible candidates of Mn oxidation. Bacterial clones belonging to the Ytterby *Bacteroidetes* cluster and closely related (94%) to *T. ferruginea,* dominate both the spring and winter samples of YBS. *T. ferruginea* is strictly aerobic, gram-negative, non-motile single rods (Xie and Yokota 2006). Members of the *Terrimonas* genus isolated from rock varnish have been reported to oxidize Mn(II) (Carmichael and Bräuer 2015; Northup et al. 2010). Also members of the *Flavobacterium* genus have been identified as Mn-oxidizers (Carmichael et al. 2013; Ford and Mitchell 1990; Nealson 1978; Santelli et al. 2010) and members of the *Bacteroidetes* phylum were predominant in a Mn rich biofilm in a shallow cave system in the Appalachians (Carmichael et al. 2013). Sanchez-Moreno et al. (1989) showed that the superoxide scavenging enzyme (SOD), essential during Mn oxidation and concomitant to O_2^- reduction, was present as both Fe-SOD and Mn-SOD in 11 different strains of *Flavobacterium*, with the highest Mn-SOD activity detected in *F. ferrugineum* ATCC 13523. Thus, the oxidation of Mn is here supposedly linked to the interaction of reduced Mn with ROS produced during heterotrophic respiration. In view of the mine's past as a fuel deposit, it is interesting that *T. ferruginea* (formerly *F. ferrugineum)* was isolated from an oil brine in a study on hydrocarbon utilizing bacteria and the closest relative to bacteria involved in hydrocarbon degradation (IIzuka and Komagata 1964; Nishikawa et al. 2006). This stands in contrast to the low organic carbon concentration in the YBS (0.6%; Sjöberg et al. 2017), which is in accordance with concentrations reported for other subterranean ferromanganese deposits ($\leq$ 0.09% in Lechuguilla and Spider caves, United States; Northup et al. 2003).

The dominant archaeal OTU, represented by Ytterby clone 4 A08 (66 related clones), showed 97% similarity to *Nitrosopumilus maritimus* strain SCM1 (NR_102913), a marine ammonia oxidizer which belong to the Marine group 1 (MG1) *Thaumarchaeota*. This group of chemoautotrophs were found in large numbers on the surface of deep sea ferrromanganese nodules and are suggested as potential candidates for Mn oxidation due to their possession of a multicopper oxidase (MCO) enzyme (Shiraishi et al. 2016).

Conclusions

An underground microbially driven production of REE+Y enriched birnessite-type Mn oxides is investigated in this study. 16S rRNA gene results from samples of the Mn deposit and associated fracture water show a high diversity of bacteria and a high percentage of unknown bacteria. For archaea however, all samples show a low diversity profile with *Thaumarchaeota* almost exclusively dominating the population. Ytterby clones are frequently most similar to clones isolated from milieus characterized by similar environmental constraints (i.e. subsurface environments, low temperature milieus and/or settings/precipitates rich in metals), which indicates that these external factors may strongly influence the studied microbial community composition. The sequestering of metals in the Ytterby birnessite-type Mn oxides (Sjöberg et al. 2017) may reduce the potential toxicity of the metals to the microbial community. Taken together, these results indicate that microbial populations are able to respond and adapt to local conditions. The high enrichment of REE in the birnessite lattice may, however, reflect a physico/chemical reaction rather than a microbial process.

Favorable conditions for the formation of microbially-induced Mn deposits are provided by (1) the continuous supply of reduced Mn by the fracture water, (2) the well buffered system keeping the pH stable slightly above circumneutral, and (3) the sharp redox boundary between the anoxic environment of the water bearing bedrock fractures and the oxygenated tunnel. Although the 16S rRNA molecular data do not allow determination of which microorganisms are responsible for a particular metabolism, the findings of bacterial clones related to known species capable of Mn oxidation show the potential of microbial mediation in the deposition of the birnessite-type Mn oxides. Potential players involved in the Mn cycling are mainly affiliated to the hyphae budding, ferromanganese genera *Hyphomicrobium* and *Pedomicrobium* in the *Alphaproteobacteria*. Additionally, 16S rRNA sequences formed two new clusters: one among the *Bacteroidetes*, named Ytterby *Bacteroidetes* cluster, closely related to the *Terrimonas* and one among the Nitrospirales, identified as Ytterby *Nistrospira* cluster, closely affiliated to clones detected in Fe and Mn rich environments. It is probable that Ytterby *Bacteroidetes* cluster is involved in the Mn cycling and the Ytterby *Nistrospira* cluster in the N cycle. All together the 16S rRNA gene data associated to the Ytterby manganese deposit support the hypothesis of having Mn oxidizers involved in the formation of the birnessite-type manganese oxides.

Thus, this study in combination with previous results by Sjöberg et al. (2017) indicates that the production of the YBS deposit is microbially mediated. Further analyses of the Ytterby birnessite-type Mn oxides are in progress as well as cultivation experiments of the microorganisms involved in the birnessite formation process and their REE enriched ecosystem.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank the Swedish Fortifications Authority for allowing access to the Ytterby mine and the supervisors of the mine, P-O Lindgren (The Swedish Defense), Martin Lundmark and Annika Agnesson (The Swedish Fortifications Agency) for help during sampling campaigns and for sharing data on this locality. Helpful suggestions from Hildred Crill improved the final version of the manuscript.

Funding

The working hours of Nolwenn Callac, the use of the CFX96 Touch™ Real-Time PCR Detection System Instrument and its software, the use of

the Mastercycler nexus gradient and the DNA of the pure strain for the Q-PCR standard curves were funded by grant number 336092 to E Chi Fru from the European Research Council. As for the rest, this research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sector.

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Appendix
Statistical analyses

The alpha diversity of each sample was calculated in EstimateS (Version 9.1.0) (Colwell 2013). The species richness was estimated using both the ACE (Abundance based coverage), and the Chao 1 (non-parametric estimator) estimators. The species evenness was evaluated using the Shannon index and the inverse Simpson indices (estimator non affected by sampling, Campbell et al. 2013). The rarefaction curves from the 16S rRNA gene libraries for both bacteria and archaea collected during winter and spring were calculated and made on R (software environment for statistical computing and graphics) using the vegan package within the R package version 1.15–1 (Oksanen et al. 2008; Team RDC 2011).

Table A1. Analysis of the archaeal and bacterial diversity, from the 16S rRNA gene libraries.

Sample	No. OTUs[a]	Good's coverage	ACE	Chao1 (95% ci)[b]	Shannon	Inverse Simpson
Archaea						
YBS winter	7	0.70	9.67	7.16 (5.23–20.27)	0.99	2.46
YBS spring	2	0.91	28.14	20.72 (11.4–60.39)	1.31	3.16
Water winter	3	0.94	47.2	34.41 (17.63–98.87)	1.5	3.44
Water spring	8	0.78	73.45	49.73 (24.21–139)	1.57	3.21
Bacteria						
YBS winter	58	0.51	223.18	212.42 (122.02–429.53)	3.54	21.39
YBS spring	83	0.42	354.67	387.02 (244.28–674.31)	4.10	30.56
Water winter	39	0.24	437.78	516.10 (343.84–835.21)	4.36	34.07
Water spring	35	0.26	513.21	623.90 (434.06–953.95)	4.53	34.30

[a]Operational taxonomic units (OTUs) defined at 97% sequence similarity.
[b]ci, confidence interval.

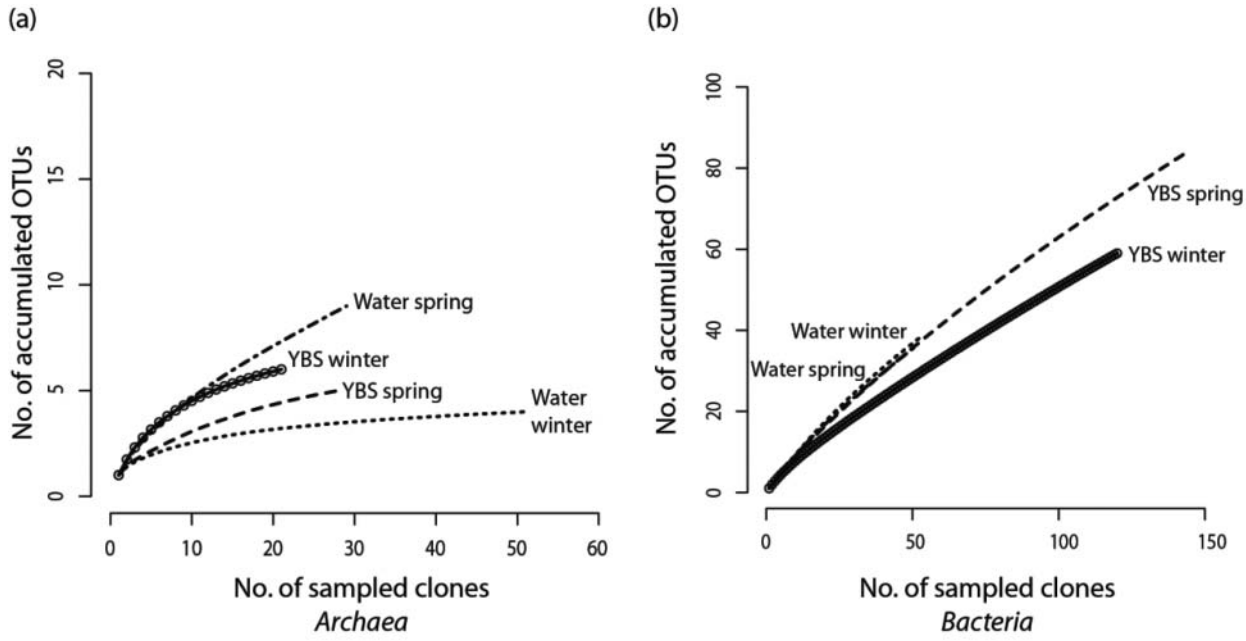

Figure A1. Rarefaction analysis for (a) archaeal clones and (b) bacterial clones.

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