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Microbial diversity in bentonite buffer of aged bentonite buffer experiment

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Publishable Summary

This report describes the results obtained from the long-term experiment with bentonite buffer. The MX-80 Na-bentonite was compacted inside a copper cylinder, which was set inside a plastic bottle containing non-saline groundwater simulant. The aim of this experiment was to evaluate changes of chemical, mineralogical and microstructural parameters of bentonite in both oxic and anoxic conditions. Microbiological analyses performed at the end of the experiment included evaluation of bacterial and fungal communities by sequencing and visual evaluation microscopically.

Detected changes in the bentonite mineralogy included the observation of secondary copper minerals formation in the middle part of the bentonite matrix in oxic conditions. Copper content in pyrite increased when moving from the middle of the bentonite towards the copper cylinder surface in both oxic and anoxic experiments. Microstructural studies on bentonite did not show any significant differences in bentonite structure between samples taken from anoxic and oxic experiments. Chemical changes were typical dissolutions of gypsum and calcite, which released sodium, sulphate and carbonates into external water, while calcium exchanged sodium in the interlamellar spaces of bentonite.

The presence of living microbes on bentonite and on copper surface could not be demonstrated in this study but microscopical studies revealed living microbial cells in the external water surrounding copper cylinders. According to the IonTorrent sequencing, sulphate (SRB) and iron reducing bacteria (IRB) were detected in bentonite matrix, water and copper surface. SRBs can produce corrosive sulphide and IRBs can have role in processes that could be linked to the loss of swelling properties in bentonite. Fungal conidia and hyphae were detected by SEM in water and several groups of Ascomycetes and Basidiomycetes were identified by sequencing from bentonite samples. Many of the fungal genera detected are able to produce organic or inorganic acids that help fungi in solubilization of minerals from rock substrates.

Contents

1 Introduction

Geological disposal is considered at present as the best method to dispose nuclear wastes. In Olkiluoto, Finland high level nuclear waste (HLW) will be stored inside Olkiluoto bedrock, in the depth of about 400-450 meters. The KBS-concept is used in Sweden and Finland, and is based on the multiple overlapping protective barriers principle. Multibarrier systems includes engineered barriers containing cast iron- copper canister, tunnel backfill and bentonite buffer, and bedrock is used as natural barrier. According to the Olin et al. [1] the main functions of the bentonite buffer as barrier element in nuclear waste management are: minimize the water flow in the buffer near copper capsule, maintain suitable chemical environment, limit the mass transfer in water (e.g. release of radionuclides), limit the mechanical load to copper capsule (swelling pressure, elastoplastic properties) and limit microbiological activity.

Minerological composition and structure of bentonites is complex, and it consists mainly of montmorillonite that is a member of the smectite group. Montmorillonite swells and absorbs water and can form very high swelling pressure. Bentonite is extremely sorptive and thixotropic and it is mainly used in industrial applications in two forms, Ca-bentonite and Na-bentonite [2]. Montmorillonite carries a negative charge that is balanced by cations, which exchange readily with other cations in the external solution. In the worst case, the exchange of cations can cause the montmorillonite structure to collapse and the hydraulic conductivity to increase markedly.

Corrosion of steel and copper canisters surrounding the HLW is considered as one of the risks in the KBS-concept. Corrosion can result in the release of radionuclides and their migration into the groundwater and finally to the biosphere. Bentonites are natural clay minerals and their source of origin will affect the mineralogical properties, buffering capacity and protective actions. Mineralogical composition of bentonite has also influence on corrosion processes [3] and Fe-rich bentonites are considered to be more corrosive [4]. Moreover, corrosion products may alter bentonite properties such as swelling and adsorption properties desired from bentonite in the repositories.

Physicochemical properties of compacted bentonite and small pore size of the mineral structures in compacted bentonite have been expected to prevent the growth of microorganisms [5]. Microbiological research in case of bentonite has concentrated on sulphate reducing bacteria SRBs, which may form corrosive hydrogen sulphides [6,7]. Sulphate is available in most bentonites, and sulphate reduction is usually limited by the availability of electron donors, e.g. organic carbon or molecular hydrogen [8]. Bacteria have also been shown to influence several properties of bentonite including cation exchange capacity (CEC), exchangeable cations, swelling and the rheological properties of clay minerals (reviewed by Mueller [9]). The composition of organic carbon in bentonite can also influence microbial activity. Organic compounds in bentonite originate mainly from plant-derived waxes and lignin derived phenols containing very low amounts of molecules, which would be suitable sources of nutrients for microorganisms [10,11].

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The long-term experiment of compacted bentonite with ground-water simulant and copper canister was initiated already in 1997 [12,13]. The aim of the experiment was to evaluate changes in chemical, mineralogical and microstructural parameters of bentonite in oxic and anoxic conditions and as a function of time. Although the experimental setup was not initially designed for microbiological studies, they were performed at the end of the experiment. Microbiological analyses included evaluation of bacterial and fungal communities by sequencing and visual evaluation microscopically. Detailed results of experiment has been published recently by Vikman et al. [14].

2 Long-term experiment with compacted bentonite

2.1 Experimental set-up

An experimental set up was designed to study long-term effects on compacted bentonite in simulated repository conditions [12,13]. The bentonite was compacted inside a copper cylinder, which was set inside a plastic bottle containing 100 mL of non-saline groundwater simulant. Steel sinters were assembled at the ends of the copper cylinder to enable interaction between bentonite and external solution. The experiment was started in 1997, and was planned to be finished after ten months. Two samples (both one anoxic and one oxic) were stored for later analysis, which was carried out in 2013 after dismantling in 2012. Ion Torrent sequencing and analysis of the results were performed in 2017-2018. Test conditions are shown at the Table 1.

Table 1. Test conditions in long-term experiment with compacted bentonite.

Parameter	**Test conditions**
Type of bentonite	Na-bentonite MX-80, consists mainly of Na-montmorillonite
Dry density of the bentonite in the beginning	1.5 g/cm^3
Groundwater simulant	Allard water [15]
Copper cylinder	Diameter 24 mm, thickness 44 mm
Pre-saturation	With deionized water
Incubation temperature	Room temperature
Incubation conditions	Anoxic under the nitrogen atmosphere Oxic under normal atmospheric conditions

The anoxic experiment was dismantled inside a glove box under argon atmosphere and the oxic experiment under ambient atmosphere (Figure 1). The cylindrical bentonite samples were cut into four 10 mm thick disks. The chemical composition of the bentonite and external water, microstructure of the bentonite, microbial diversity of bentonite, external solution and on copper surfaces and mineralogy of the bentonite were studied. Microbiological studies were not originally planned but were analysed at the end of the experiment.

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Figure 1. Dismantling of the experiment.

2.2 Chemical and structural analyses

Chemical, mineralogical and structural analyses made from bentonite and water samples are presented at the Table 2.

Table 2. Analyses made after dismantling. More detailed analyses can be found from Vikman et al. [14].

Target	**Analysis**
Bentonite microstructure	Small-angle X-ray scattering (SAXS) Nuclear magnetic resonance (NMR) Anion exclusion analysis
Sodium, potassium, calcium, magnesium, copper and iron in water	ICP-OES (Inductively coupled Plasma- Optical emission spectrometry)
Sulphate and chloride in water	IC (Ion chromatography)
Bicarbonate	Titration
Cation exchange capacity (CEC)	Cu(II)-triethylenetetramine method [16,17]
Minerology	Scanning electron microscope (SEM) + energy dispersive spectrometer (EDS) Electron probe micro-analyzer (EPMA)

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2.3 Microbiological analyses

2.3.1 Microscopical analyses

Water samples were stained with Live/dead -staining kit (Molecular Probes, Leiden, The Netherlands) and analysed with epifluorescence microscope. For SEM analysis samples were filtered on membrane and fixed in phosphate (0.1 M, pH 7.2) buffered with 2.5% glutaraldehyde. Dehydration was carried out with an ethanol series from 30% to absolute, followed by treatment with hexamethyldisilazane. The samples were coated with Au/Pd and examined with Hitachi S-4800 FESEM (Tokyo, Japan) operated at 1 kV.

2.3.2 Microbial diversity

The composition of bacterial community was assessed by sequencing the universal small subunit ribosomal RNA gene, which is also known as the 16S rRNA gene in bacteria. The fungal internal transcribed spacer (ITS) gene marker was used to examine the total fungal diversity in samples.

Water samples were filtrated on 0.2 µm polyethersulfone filters (Corning, MA USA) to concentrate biomass. DNA extractions from bentonite were performed by using 0.9 g sample, which was diluted into water. Microbial biofilm on the surface of copper was scraped with sterile scalpels and diluted in sterile PBS and subsequently filtered on polyethersulfone-filters of 0.2 µm pore size. The DNA was extracted using ZR Soil Microbe DNA MidiPrep in accordance with the manufacturer's instructions. Negative DNA extraction controls were included in the DNA extractions.

The amplification libraries for high throughput sequencing on the Ion Torrent PGM platform were prepared by PCR from the DNA samples. Bacterial 16S genes were amplified with primers S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 [18] targeting the variable region V3-V4 of the 16S rDNA gene, and fungal internal transcribed spacer (ITS) gene markers with primer pair ITS1 and 58A2R targeting the fungal ITS1 region [19].

PCR amplification was performed in parallel reactions for every sample containing 1× MyTaqTM Red Mix (Bioline, London, U.K.), 20 pmol of each primer, 2 µL of template and nuclease-free water (Sigma, St. Louis, MI, USA). Parallel amplicon libraries were combined and sent to Bioser, University of Oulu (Finland) for sequencing on the Ion Torrent PGM sequencer (Thermo Fisher Scientific) using the 314 and 316 Chip Kit v2 with the Ion PGM Template IA 500 and Ion PGM Hi-Q sequencing kits.

The sequence reads obtained from Ion Torrent sequencing were subjected to sequence analysis using the Mothur software version 1.39.5 [20] using the standard operating protocol by Kozich et al. [21] with some modifications. Adapters, barcodes, and primers were removed from the sequence reads, and chimeric sequence reads were removed from the data set with the VSEARCH algorithm [22] by de novo detection and through similarity searches against the SILVA reference dataset (version 128) [23] with bacterial sequences, and UNITE reference dataset (version 7.2 2017-06-28) [24] for fungal sequences.

Differing from the SOP by Kozich et al. [21] no alignment for the fungal sequences was done instead, a column-formatted distance matrix was calculated with pairwise.seqs command with cutoff 0.10. The sequences were grouped into Operational Taxonomic Units (OTUs), following Mothur OTU-picking protocol using average neighbour algorithm to cluster sequence reads at 97% sequence similarity. Taxonomy from the domain- to species-level was assigned to OTUs via representative OTU sequences with the Wang algorithm at minimum confidence threshold of 80% [25] using SILVA database for the bacterial sequences and UNITE for the fungal sequences. Sequence reads obtaining no taxonomical assignments in the analyses and OTUs that were also found from the negative control sample were excluded from the datasets.

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3 Results from long-term experiment

3.1 Structural, mineralogical and chemical changes

Structural and mineralogical changes in bentonite were studied after dismantling and the results are reported in detail by Vikman et al. [14]. Microstructural studies with SAXS, NMR and anion exclusion did not show significant differences between anoxic and oxic samples (Figure 2). Despite of the high porosity, the bentonite had very fine pore structure, with most of its pore volume being one nanometer pores.

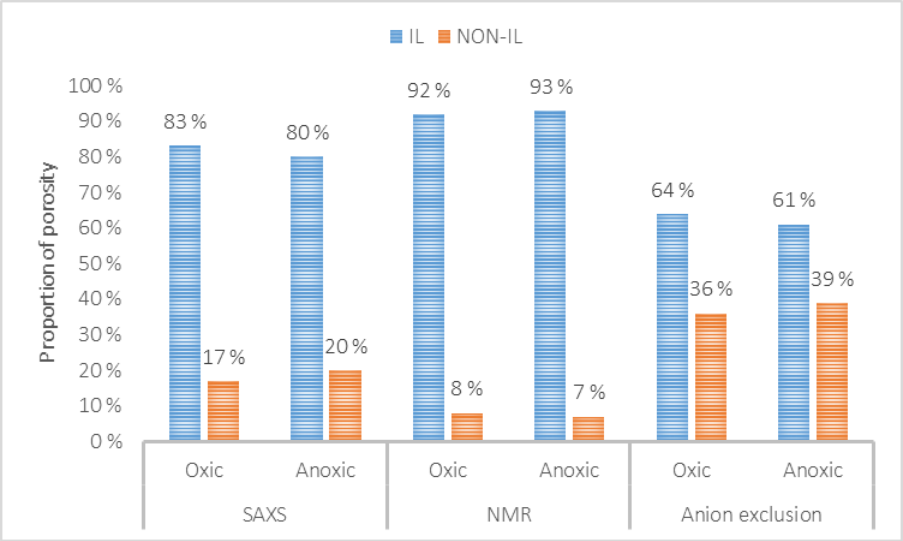

Figure 2. Results of the microstructural investigation: proportion of interlamellar and non-interlamellar porosity (partly from Matusewicz et al, [26]). IL =interlamellar and non-IL = non-interlamellar.

Smectite (montmorillonite) content was over 95 weight-% in all bentonite samples. Most common accessories were quartz and feldspars together with carbonates and sulphides. In addition, also iron oxides and sulphate minerals were detected. Mica in the bentonite samples was biotite and no transformation of smectite into illite was observed.

There were some differences in mineralogical composition in the bentonite taken from oxic and anoxic experiments. Samples kept in oxic environment contained newborn secondary mineral phases that were not observed in anoxic experiments. Especially bentonite samples taken from the middle part of the copper cylinder showed secondary copper minerals, especially cuprite (Cu_2O) and malachite ($Cu_2(CO_3)(OH)_2$). The copper mobilization was also detected in the bentonite matrix.

The availability of copper was also seen in pyrite composition, and copper had replaced part of iron in pyrite lattice. All the bentonite samples had pure pyrites (FeS_2), but copper-rich pyrites were also analysed. In some cases new Cu-Fe-S-phases were formed. In addition, pyrite with bornite (Cu_5FeS_4) rims was observed.

Anoxic bentonite samples did not contain cuprite or malachite. Copper-rich pyrites were not identified in the bentonite samples taken from the middle part of the cylinder, but their amount increased towards the copper cylinder surface. In addition, calcite, goethite and apatite were more

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common near the copper cylinder wall. It is likely that part of these minerals had leached out from the middle part of the cylinder.

The most prominent composition change in external water was due to increase of Na_2SO_4, which is typically observed with MX-80 bentonite. Gypsum was dissolved and calcium exchanged sodium as charge compensating cation, and sodium and sulphate started to diffuse out of the bentonite. These processes had mainly taken place during the first 10 months; the concentration of the sulphate was slightly greater in the oxic experiments. Similar processes were caused by dissolution of calcite, which caused the increase of bicarbonate concentration.

In anoxic and oxic experiments CEC of the bentonite were 0.924 and 0.925 eq/kg (dw), respectively. The exchangeable cation was mostly sodium. The amount of sodium, copper and magnesium in the bentonite matrix where greater in oxic experiments, whereas amount of calcium were greater in anoxic experiments in the cation exchange places. Iron was not found as an exchangeable cation in the bentonite.

3.2 Evaluation of microbes by microscopy

Epifluorescence microscopy revealed living cells in the water samples, and fungal conidia and hyphae were detected in oxic water samples. In the anoxic water bacterial spore forming microorganisms and filamentous bacteria were observed. However, the number of microorganisms in anoxic sample was low and the putative bacteria were showing signal only on green channel suggesting that these microorganisms had intact plasma membrane. The bacteria in water sample from oxic experiment had poor fluorescence, which might suggest that these organisms were not in active stage. In both oxic and anoxic water samples fluorescing inorganic substances were observed.

FESEM analysis revealed that water samples contained substances resembling bentonite as well as other substances with inorganic appearance. However, microorganisms were observed in waters from both oxic and anoxic experiment. In the water collected from the capsule incubated in oxic conditions, number of microorganisms with appearance of mould conidia were observed. In addition, some fungal hyphae were present. In the water collected from the capsule incubated in anoxic conditions some bacterial spore looking microorganisms as well as filamentous bacteria were observed. Epifluorescence and scanning electron micrographs are presented in the report written by Vikman et al. [14].

3.3 Bacterial community

According to the sequencing results, the bacterial community in the water, bentonite and copper surface consisted mainly of Alphaproteobacteria (13-29 % of the community), Betaproteobacteria (5-16 % of the community), Gammaproteobacteria (14-42 % of the community), Flavobacteria (2-15% of the community) and Actinobacteria (3-25% of the community) (Figure 3).

No big differences between oxic and anoxic conditions were detected. In addition, bacterial community structure in water and copper surface was quite similar when assessed in class-level. The bacterial community in bentonite differed to some extent from bacterial community in water and copper surface. Because the experiment was not originally planned for microbiological studies, no samples were taken in the beginning of the experiment. Therefore, information of the bacterial community changes as a function of time could not be achieved.

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Sulphate reducing bacteria (SRB) are one of the main concerns for the safety case of the geological disposal because sulphide formed by SRBs is a corrosive agent for copper cylinders. SRBs consist of a diverse, distantly related assembly of Bacteria and Archaea characterized by the use of sulphate as a terminal electron acceptor during anoxic respiration [27]. SRBs have been shown to grow in various extreme environmental conditions including high pressure [28] and they contain several spore-forming gram-positive species [29]. Sequencing analysis revealed several potential SRBs in bentonite samples including families of Desulfobacteraceae and Desulfofurellaceae. Copper and water samples contained SRB-bacteria belonging to family Desulfobulbaceae.

Another interesting microbial group in deep geological environment are iron-reducing bacteria (IRB) because the reduction of Fe (III) to Fe (II) can influence swelling properties of clays [30,31]. The ability to reduce Fe (III) is widely available within the domains of both Bacteria and Archaea. For example, Shewanellaceae belonging Gammaproteobacteria are shown to be potential iron reducing bacteria IRB [32]. Interestingly this microbial group formed 0.2-5.7% of the sequences in studied samples. Shewanella spieces (genus of the family of Shewanellaceae) are gram-negative, facultatively anaerobic bacteria that have also been isolated from high-pressure marine environments [33].

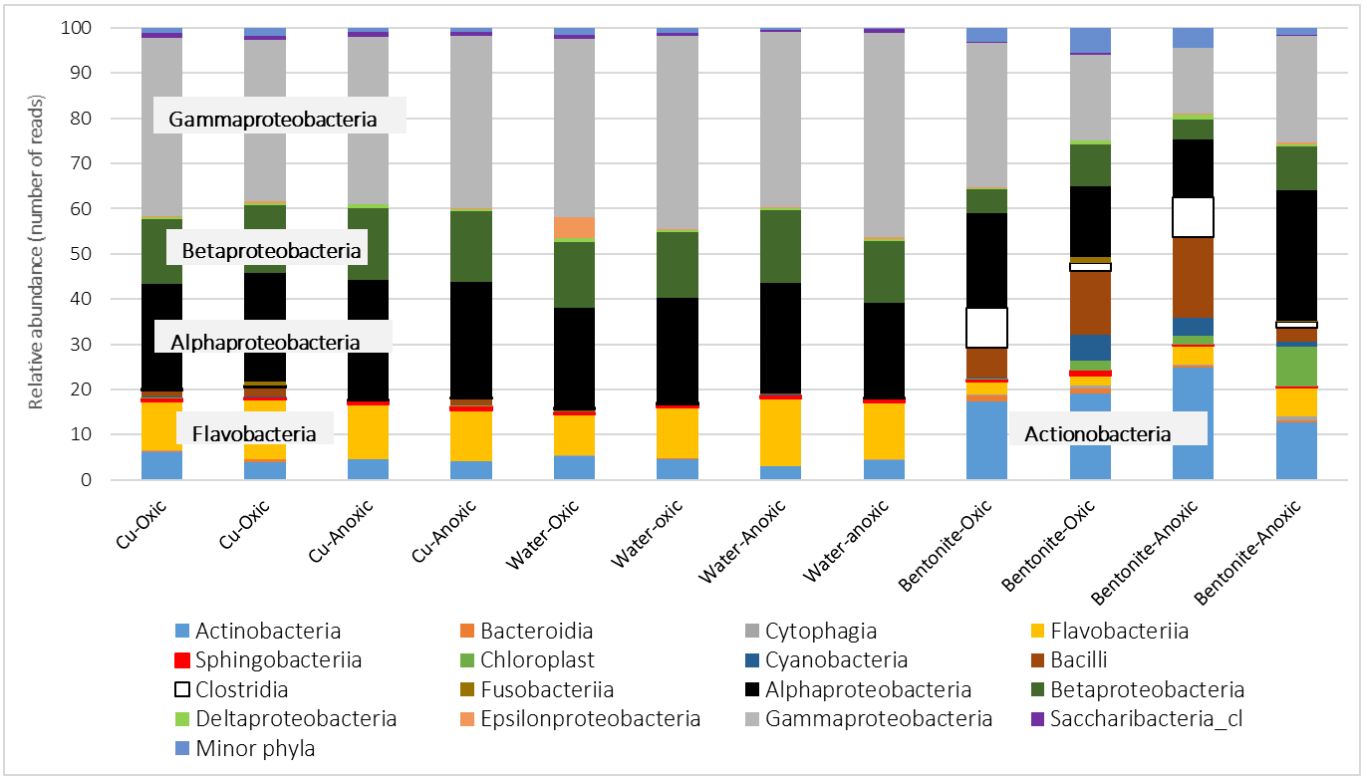

Figure 3. Relative abundance of bacterial communities in bentonite, copper-surface and in external water presented as taxonomic class-level.

3.4 Fungal community

According to the sequencing, fungi were identified to belonging several groups of Ascomycetes and Basidiomycetes. Ascomycetes and Basidomycetes formed 74-99 % and 0.7-21.4 % of the fungal community, respectively. Dothideomyces, Eurotiomyces and Sordariomyces belonging Ascomycetes were most dominant phyla in class-level (Figure 11).

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The biggest fungal genera that were detected in all samples were Cladosporium (5-93% of sequence reads) and Alternaria (7-28% of sequence reads) belonging class Dothideomycetes. Most of the species detected have been encountered in rock substrates according to literature [34,35]. Many of the fungal genera detected for example Penicillium (class Eurotiomycetes), Trichoderma (Ascomycota), Cladosporium (class Dothideomycetes) and Exophiala (Ascomycota) produce organic or inorganic acids that help fungi in solubilization of minerals from rock substrates [36]. Because pH was maintained alkaline during 15 years of experiment, it indicated very low fungal activity in compacted bentonite. In addition, Alternaria, Cladosporium and Penicillium strains that were detected in copper samples are able to oxidase iron and manganese [36]. DNA-based analyses do not give information about the viability of the microbes but active fungi have been detected in deep biosphere environment indicating that fungi are able to maintain cellular activity also in oligotrophic conditions [37].

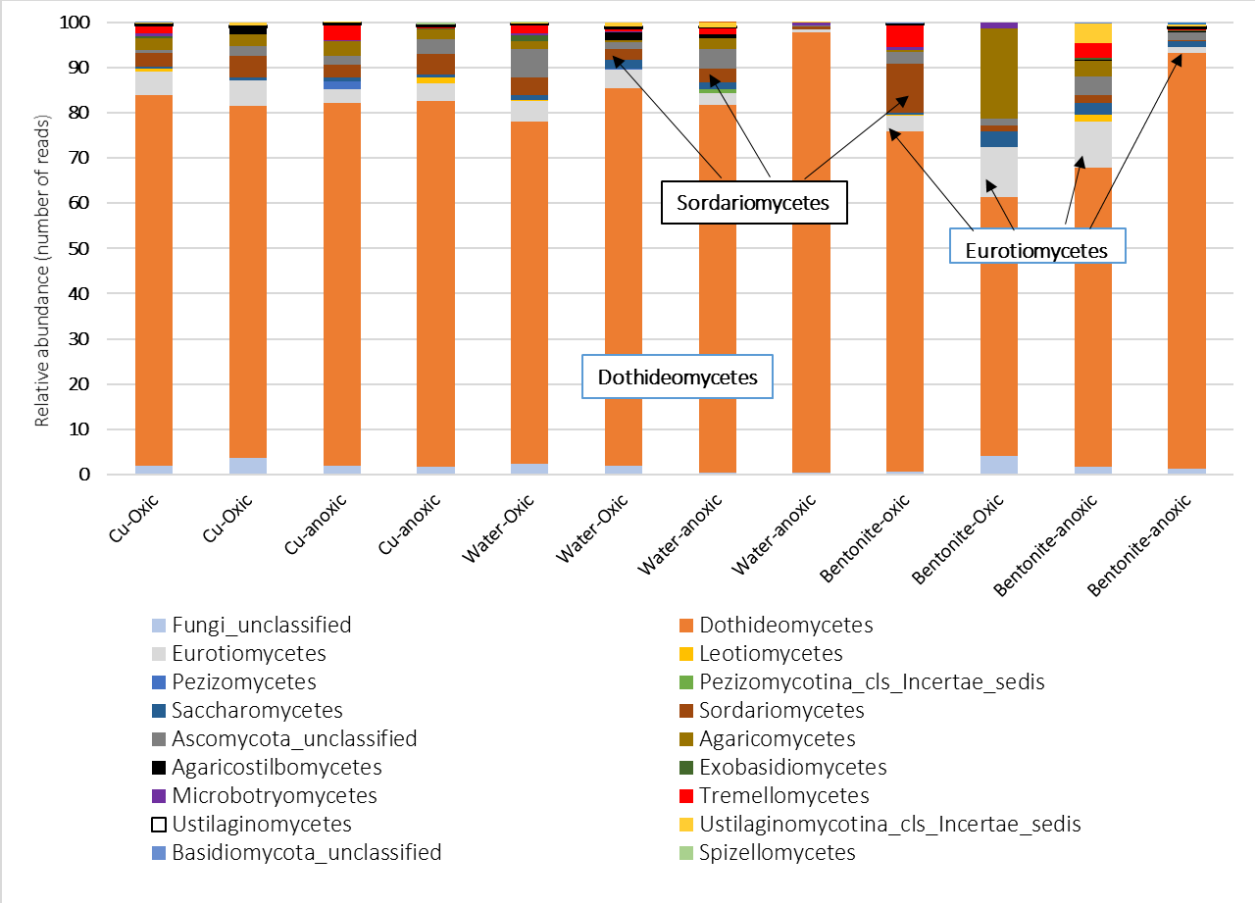

Figure 4. Relative abundance of fungal communities presented as taxonomic class-level.

4 Conclusions

Living microbial cells were detected in the water samples by epifluorescent microscopy but the presence of living microbes on bentonite matrix and on copper surface could not be demonstrated. Microbial DNA was extracted from water, bentonite and copper surface of the capsule and used for analysis of bacterial and fungal community. Because DNA represents total microbial biomass

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including living, dormant and dead microbial cells, it does not give any information about the viability of the microbes.

Sequencing revealed the presence of sulphate and iron reducing bacteria in bentonite, water and copper surface. SRBs can produce corrosive sulphide and IRBs can be involved in processes, which could be linked to the loss of swelling properties in bentonite. In addition, species typically found in high-pressure environment were identified.

Fungal conidia and hyphae were detected by SEM in water and several groups of Ascomycetes and Basidiomycetes were identified by sequencing from bentonite samples. Many of the fungal genera detected in this study are able to produce organic or inorganic acids that help fungi in solubilization of minerals from rock substrates.

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