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# Long-term stability of bentonite in the presence of microorganisms

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# Publishable Summary

Due to high swelling capacity and a low hydraulic conductivity, bentonites are used as geotechnical barriers and in sealing and buffering functions in the nuclear waste repositories. The aim of this report was to study if microbes influence these beneficial properties of bentonites by either changing the solubility and/or composition of minerals and the reduction of important ions (e.g. ferric iron) of the studied bentonites. In order to elucidate the microbial potential within different bentonites, we performed two types of microcosm-experiments using two Bavarian bentonites and MX-80 bentonite.

The experiment with Bavarian bentonite consisted of an industrial/processed bentonite (B25) and a natural one (N01) and were performed at HZDR. The set-ups contained the respective bentonite and an anaerobic synthetic Opalinus-clay-pore water solution under an $N_2/CO_2$-gas-atmosphere. Acetate, lactate or hydrogen gas were used to stimulate potential microbial activity and geochemical effects. The set-ups were incubated in the dark at 30 °C and 60 °C for one year. Bio-geochemical parameters were monitored and the microbial diversity as well as mineralogical analysis were analysed. Only B25 set-ups at 30 °C supplemented with lactate or hydrogen gas showed notable effects regarding microbial diversity, changes in bio-geochemical parameters as well as structural changes. In contrast, the raw material of B25 (powder) revealed a very diverse microbial community. Microcosm set-ups, containing hydrogen or lactate, where dominated by spore-forming, sulphate-reducing *Desulfosporosinus* spp. In the respective lactate-containing batches of B25, lactate and sulphate concentrations dropped. Furthermore, the simultaneous formation of acetate, as well as an increase of ferrous iron and a concomitant decrease of ferric iron was observed in lactate-supplemented batches. Moreover, the colour of the bentonite material changed to different shades of grey with sporadic black spots when lactate was added. Mineralogical analysis with Scanning Electron Microscopy indicated a significantly higher part of iron-sulphur accumulations in these samples, showing that microbial activity under the applied conditions led to the formation of new mineral phases when the conditions were favourable. Similar observations were made when hydrogen gas served as an electron donor. Again, sulphate concentration decreased with a simultaneous increase of ferrous iron and a decrease of ferric iron with a concomitant formation of black precipitates.

The aim of the laboratory scale MX-80 bentonite storage experiment (VTT) was to simulate bentonite behaviour in circumstances that can take place in the interfaces of bentonite, host rock fractures and water flow in nuclear waste geological disposal. The bentonite was studied as a slurry in which water, gases, nutrients and microorganisms were able to move freely at the temperature hospitable for microorganisms. The objective was, to find out if microorganisms and the metabolites they produce are able to change the bentonite structure and if these changes could be significant for the bentonite stability in long-term. MX-80 bentonite microcosms after one year of storage initiated both, at aerobic and anaerobic conditions, showed no essential changes in bentonite mineralogy compared to the initiation of experiment. However, clear microbial activity in terms of ongoing sulphate reduction and sulphide formation as well as high number of sulphite reductase genes (*dsr*B) were detected in anaerobic samples. Microbial activity also affected bentonite water-phase chemistry and bentonite cation exchange capacity. These effects were not detected in sterile controls, demonstrating the microbial origin of these changes. In aerobic microcosm, oxygen was used steadily and after half a year, only trace of oxygen was left. Overall, microbial activity was lower in aerobic than in anaerobic microcosms and neither sulphate reduction, nor sulphite reductase genes were detected in aerobic microcosms after one year. The experiment is planned to continue for some years further as potential changes in bentonite mineralogy caused by microbial activity happen slowly in the studied conditions.

# Contents

# 1 Introduction

For the long-term storage of spent fuel, a deep geological disposal using a multi-barrier concept is favoured, consisting of the canister, containing the high-level radioactive waste (technical barrier), which is surrounded by a sealing/buffering material (e.g. bentonite, geo-technical barrier). This system is then placed into the host rock (geological barrier; Sellin and Leupin, 2013).

Bentonites are clay minerals, which consist mostly of the smectite Montmorillonite. Due to their mineral composition, they are characterized by a high swelling capacity and a very low hydraulic conductivity (Pusch, 1992; Pusch *et al.*, 2012). These favourable properties give reasons for its application as buffer, seal or backfill in almost every program for high-level radioactive waste (HLW) disposal worldwide (Sellin and Leupin, 2013). It has already been shown, that prokaryotes are present in bentonites and that they may influence the properties of this clay mineral (Lopez-Fernandez *et al.*, 2015; Kim *et al.*, 2004; Pedersen, 2002; West *et al.*, 2002). These microorganisms have the potential to influence the mineralogy and the pore-water composition, thereby changing the chemical composition of the sedimentary environment (Konhauser *et al.*, 2002; Weber *et al.*, 2006; Lloyd, 2003). Regarding the disposal of highly radioactive waste, prokaryotes can affect actinide- and metal- chemistry *via* a number of processes (Gadd, 2002; Konhauser *et al.*, 2002; Morris and Raiswell, 2002; Lloyd and Macaskie, 2002). A prerequisite in understanding the microbial impact on bentonite is the understanding of metabolic activities, which could influence the desired properties. For this purpose, it is important to identify microorganisms within the bentonite in order to deduce potential metabolic activities and to look for specific metabolites and the respective geochemical effects. Although bentonites are known for a low-biomass environment, the potential for metabolically active microbes is feasible, as it has been demonstrated before for other low-biomass environments (Lollar *et al.*, 2014; Kotelnikova, 2002; Kietäväinen and Purkamo, 2015).

This report address the question whether microbial activity could influence the beneficial properties of the bentonite by either changing the solubility or composition of minerals and/or important ions like ferric and ferrous iron. Metabolism – life – is a continuous input and output of matter and energy. The presence of an electron source – the driving force of live – as well as an energy source are mandatory. Each living cell is endowed with a system that transforms chemical and physical energy taken up into biologically useful energy that can be used for "metabolic work" (Thauer *et al.*, 1977a). With respect to the used bentonite, they all provide many suitable electron donors and acceptors, enabling microbial life. The microbial activity near waste canisters and in bentonite is planned to be restricted with high bentonite density and swelling pressure, which reduce water activity. However, in geological disposal, host rock fractures, groundwater flow and bentonite interfaces provide conditions where these restricting conditions do not always apply, especially not for spores.

In order to analyse these issues different bentonites were analysed regarding to their microbial diversity and metabolic activity under selected conditions as well as to their potential geochemical and mineralogical effects. For this purpose, a Bavarian bentonite- and a commercial MX-80 bentonite - experiments were initiated and studied. Bavarian industrial bentonite (B25) was compared with a natural Bavarian bentonite (N01). To stimulate potential microbial activity and possible geochemical and mineralogical effects within the two bentonites, substrates were added to microcosm setups. The aim of the MX-80 bentonite storage experiment was to simulate bentonite behaviour in circumstances that can take place in the interfaces of bentonite and host rock fractures in nuclear waste geological disposal. The bentonite was studied as a slurry in which water, gases, nutrients and microorganisms were able to move freely at the temperature hospitable for microbes that originated from bentonite and disposal site water and rock. The objective was to find out, if microbes and the metabolites they produce are able to change the bentonite structure and if these changes could be significant for the bentonite stability in long-term.



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# 2 Experimental set up with Bavarian bentonites (HZDR)

## 2.1 Materials and Methods

### 2.1.1 Bentonites

The industrial and processed Bavarian bentonite B25 was provided by Steffen Kaufhold (BGR, Hannover, Germany). The natural bentonite N01 was sampled at a pit mining near Landshut (EMERYS, pit mining "Bergschapfl", Bavaria, Germany) under sterile and anaerobic conditions. In order to avoid contamination of exogenous microorganisms, both bentonites were handled in a sterile manner all the time.

### 2.1.2 Microcosms experiments

Microcosm-experiments were set up by supplementing 20 g bentonite (processed Bavarian bentonite B25 or natural Bavarian bentonite N01) with 40 ml sterile synthetic Opalinus-clay-pore water solution (212 mM NaCl, 26 mM $CaCl_2$, 14 mM $Na_2SO_4$, 1.6 mM KCl, 17 mM $MgCl_2$, 0.51 mM $SrCl_2$ and 0.47 mM $NaHCO_3$, degassed with a $N_2/CO_2$ gas mixture (80/20) while stirring). Selected microcosms were supplemented with 50 mM acetate, 50 mM lactate, 10 mM lactate or 50 kPa $H_2$. Microcosms were incubated for approximately one year at 30 °C and 60 °C in the dark without shaking. During this incubation, samples were taken at 5 different time-points and analysed regarding bio-geochemical parameters and microbial diversity.

### 2.1.3 Sampling of microcosms

All procedures were carried out under strictly anaerobic conditions in an anaerobic glove box (Braun) containing an $N_2$-atmosphere. Microcosms were introduced into the glove box, well mixed and a portion of the suspension (10 ml) was filled into sterile 50 ml tubes (Greiner) in order to do all bio-geochemical analyses. The bottle with the remaining suspension was sealed again and stored at -70 °C until DNA was extracted to determine the microbial diversity. For iron-determination 300 µl of the suspension were filled up in 1.5 ml tubes and treated as described in 2.1.4. Sensory measurement of $O_2$-concentration, redox potential and pH-value were performed by using the respective, calibrated sensors. The centrifuged and filtrated (0.2 µm filter) supernatant of the suspension was used for determining organic acids (2.1.5) and sulphate-concentration (2.1.6).

### 2.1.4 Determination of ferric and ferrous iron

Ferric and ferrous iron was determined by using a modified protocol of Voillier *et al.*(Viollier *et al.*, 2000). Approximately 300 µl of well-mixed microcosm suspension were supplied with the same volume of 12 M hydrochloric acid (HCl; 37 %). Samples were incubated over night at room temperature, in the dark in an anaerobic glove box ($N_2$-atmosphere). Afterwards, the HCl-suspension-mix was centrifuged and 200 µl of the cleared supernatant (or appropriate dilutions) were applied to the Ferrozine-assay. The colorimetric test was conducted as described in Viollier et al. (2000) and relative values of ferric and ferrous iron were calculated.

### 2.1.5 Determination of organic acids

The filtrated supernatant of microcosm-suspensions was analysed for organic acids *via* HPLC analysis with an "Agilent 1200" (Degasser G1322A, diode array detector G1315B, quaternary pump G1354A). For separation of organic acids, a Nucleogel Ion 300 OA column was used. The releasing agent was 5 mM $H_2SO_4$ with a flow of 0.4 ml/min at 70 °C.



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### 2.1.6 Determination of sulphate

Sulphate-concentration was determined from the cleared and filtrated microcosms-supernatant by ion chromatography using a Dionex Integrion HPIC (Thermo Scientific). For calibration, a $K_2SO_4$-standard solution was used (0.05 mg - 10.0 mg $SO_4^{2-}$) and treated like the samples separated by using a AS23-column (Thermo Scientific). A defined $Na_2CO_3$-$H_2CO_3$ mixture served as eluent with a flowrate of 250 µl/min.

### 2.1.7 DNA-isolation, -purification and -sequencing

For analysing the microbial diversity within the respective sample, DNA was extracted using a modified protocol from Selenska-Pobell (Selenska-Pobell, 1995). The extracted DNA was used for amplification of the V4-region of the 16S rRNA gene by using Polymerase chain reaction (Mullis K *et al.*, 1986). PCR reactions contained 5 µl PCR water, 1 µl $MgCl_2$ (25 mM), 2 µl 5x buffer (Promega), 0.4 µl each of oligonucleotides 515f and 806r (0.4 µM final concentration; Caporaso *et al.*, 2011), 0.1 µl dNTPs (125 µM final concentration), 0.1 µl Taq-Polymerase (Promega, 5U/µl) and 1 µl genomic DNA. Reactions were held at 95 °C for 2 min to denature the DNA, with amplification proceeding for 30 cycles at 95 °C for 30 s, 50 °C for 60 s and 72 °C for 60 s; a final extension of 10 min at 72 °C was added to ensure complete amplification. The successful amplification of DNA was controlled *via* gel electrophoresis. Successfully amplified DNA was purified with MSB Spin PCRapace (Stratec molecular) according to manufacturer`s protocol. Cleaned amplicons were quantified using Qubit (Thermo Scientific) and quality control was done by using NanoDrop (Thermo Scientific) according to manufacturer`s protocol. Samples were sequenced with MiSeq Illumina at RTL genomics (Texas, USA). Data analysis was done according to RTL genomics (http://rtlgenomics.com/).

### 2.1.8 SEM-analysis

SEM-analysis were carried out at the institute for Geography and Geology at the University of Greifswald. Measurements were conducted at a ZEISS EVO MA 10. Images were taken by using a secondary electron detector. For element-mapping, a Team EDAX EDS detector was used. Accelerating voltage was 15 kV. The working distance was in between 8 and 8.5 mm. Mapping of overview images was done at 300- to 500-fold enlargement with 32 frames and a dwell time of 500 µs.

## 2.2 Results and Discussion

The addition of substrates has a big influence on the evolution of microbial diversity and activity as well as on – connected – changes of geochemical parameters and even the mineral structure. The most striking effects showed the industrial and processed bentonite B25 at 30 °C, a bentonite that could be used for the potential repository of high-level radioactive waste. In microcosm-experiments including the natural bentonite N01 no striking effects were observed during the respective time frame of one year. Reasons for this could be that the microorganisms in bentonite B25 are already adapted to harsh conditions due to the several processing steps of bentonite B25 and/or that a different microbial population exists in the bentonites. Same is true for set ups of both bentonites at 60 °C, showing no significant changes with respect to the analysed parameters, regardless of the bentonite or the added substrate. In the following, some of the obtained results regarding B25 at 30 °C are depicted and discussed.

### 2.2.1 Hydrogen drives intrinsic microbial metabolic activity

The presence of hydrogen clearly affected microbial diversity and activity as well as geochemical parameters. Whereas the pH-value remained more or less stable, the redox potential decreased from 100 mV to -300 mV within 181 days of incubation (**Error! Reference source not found.**, A). Within the same period an increase of ferrous iron with a concomitantly decrease of ferric iron was observed, as well as a decrease of sulphate concentration, relatively to the original conditions (**Error! Reference source not found.**, B). Due to microbial sulphate-reduction, sulphides were formed: hydrogen gas was



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an electron donor, sulphate an electron acceptor and carbon dioxide served as a carbon source, as already described by Deshmane (Deshmane *et al.*, 1993). Hence, grey precipitates were formed in the respective batches, sometimes with black spots, indicating the formation of iron sulphides. The control batches did not show these changes regarding geochemical parameters within the considered period (data not shown).

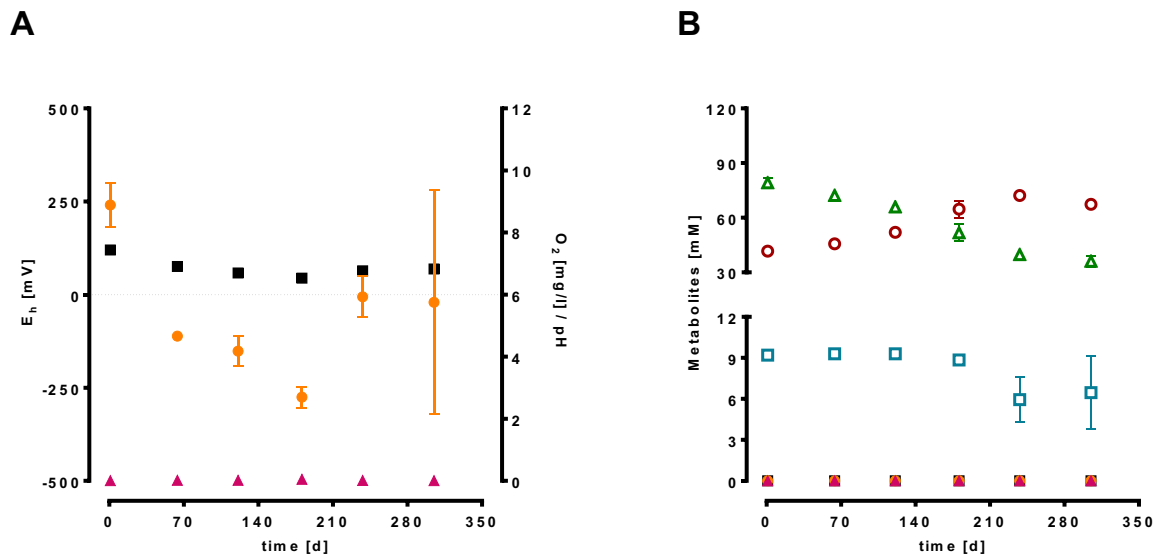


*Figure 2- 1: Evolution of geochemical parameters and metabolites in the presence of 50 kPa hydrogen gas in bentonite B25 at 30 °C. Geochemical parameters (A): redox potential ($E_h$; orange dots), oxygen ($O_2$; red triangles), pH (black squares). Metabolites (B): ferrous iron (open red circles), ferric iron (open green triangles), sulphate (open blue squares), pyruvate (red triangles), acetate (orange dots), lactate (black squares). Values are averages and standard deviations from at least two independent microcosms.*

The analysis of 16S rRNA gene sequences showed that *Desulfosporosinus* spp. dominated the respective batches, ranging from 50 % up to 80 % (data not shown). Members of this genus were not detected in significant amounts in the raw bentonite material or in control batches (batches containing no substrate as well as sterile batches), which incubated for the same time or even longer. A reason for this could be the formation of spores by *Desulfosporosinus* spp. (Hippe and Stackebrandt, 2015), which prevent the isolation of DNA by the applied extraction-protocol. Spores become vegetative, metabolically active cells, when environmental conditions become more favourable (reviewed in Checinska *et al.*, 2015). Thus, microbial diversity developed in a completely different way, controlled by the presence of hydrogen, setting the environment for microorganisms best adapted to the respective conditions. Members of the genus *Desulfosporosinus* are known to be strictly anaerobic, sulphate-reducing bacteria, which can use a broad spectra of substrates (Hippe and Stackebrandt, 2015). In the respective microcosms hydrogen served as electron donor for the reduction of sulphate with carbon dioxide as carbon source, already described by Klemps et *al.* (Klemps *et al.*, 1985). The produced sulphides lead to the visible formation of grey and black iron sulphides (Rickard and Morse, 2005; Hunger and Benning, 2007), resulting in the observed changes of ferric and ferrous iron. However, mineralogical analysis using scanning electron microscopy (SEM) revealed no significant changes regarding iron-sulphur compounds compared to the raw material or the control batches (data not shown). Either the microbial effect was not that distinct under the tested conditions, or observed changes affected other parameters, not considered so far. The reason for this needs to be further investigated.

Although for some *Desulfosporosinus* strains iron transformations have been described (Bertel *et al.*, 2012), the obtained results regarding the evolution of ferric and ferrous iron, do not give any information about a direct iron reduction due to microbial activity and common iron-reducing



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microorganisms were not detected with the used methods. Furthermore, the secretion of organic acids, that could have been formed due to autotrophic growth of *Desulfosporosinus* (Fuchs, 2011), were not detected (**Error! Reference source not found.**, B). Either the amount was below the detection limit or they served as substrates for further organisms within these microcosms, creating a kind of food web as already described (Bagnoud *et al.*, 2016).

Anyway, the obtained results clearly show, that the presence of hydrogen leads to presence of metabolically active sulphate-reducing organisms, being at least responsible for the formation of sulphides. This is of importance because the formation of hydrogen sulphide due to sulphate reduction by bacteria, could promote the corrosion process (Sellin and Leupin, 2013). Microbial influenced corrosion (MIC) can lead to a mechanical failure of metals and alloys and, thus, failure of potential materials used in a HLW repository (Kim *et al.*, 2004). Additionally, the generated sulphides could react with minerals and ions, thereby changing their properties (e.g. solubility, complexation behaviour).

### 2.2.2 Lactate drives microbial activity and striking effects in mineralogy

Likewise, to the observations made in the hydrogen gas containing batches, the redox potential decreases with proceeding time, regardless of the supplied concentration of lactate (compare **Error! Reference source not found.**-2, A and C). Again, an increase of ferrous iron and a simultaneous decrease of ferric iron as well as a decrease of sulphate concentration was observed with a concomitant formation of iron-sulphides that precipitated. SEM analysis revealed in the respective batches a significant accumulation of iron-sulfur compounds compared and in contrast to the control batches (data not shown), showing that the microbial formation of sulphides indeed effects the mineral composition of bentonites.


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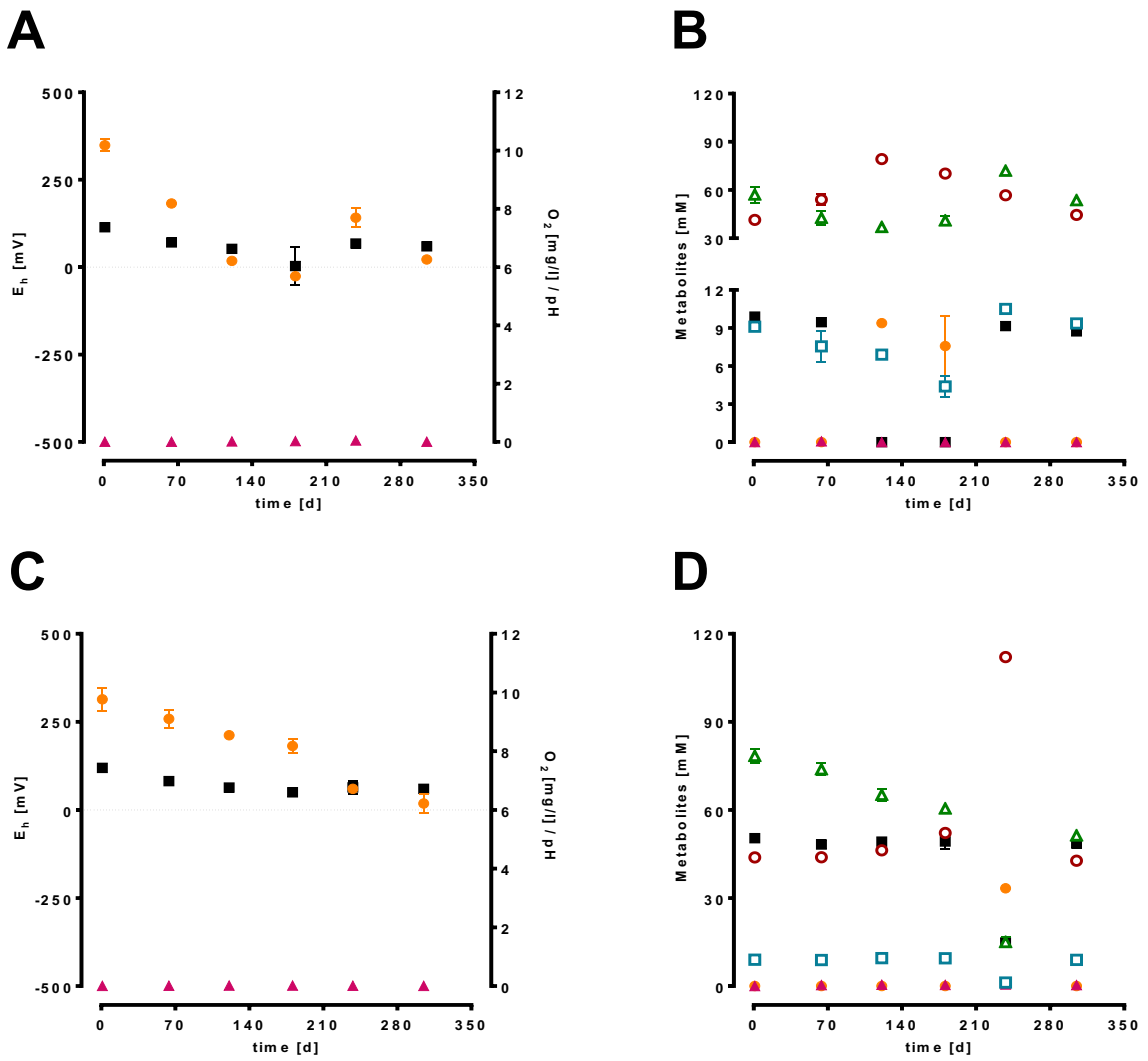


*Figure 2- 2: Evolution of geochemical parameters and metabolites in the presence of 10 mM and 50 mM lactate at 30 °C. Geochemical parameters in the presence of 10 mM lactate (A) and 50 mM lactate (C): redox potential ($E_h$; orange dots), oxygen ($O_2$; red triangles), pH (black squares). Evolution of metabolites in the presence of 10 mM lactate (B) and 50 mM lactate (D): ferrous iron (open red circles), ferric iron (open green triangles), sulphate (open blue squares), pyruvate (red triangles), acetate (orange dots), lactate (black squares). Values are averages and standard deviations from at least two independent microcosms.*

Additionally, the formation of cavities and gas bubbles within the respective batches were observed, strongly arguing for the formation of gases, very likely also hydrogen sulphide gas, which was formed due to sulphate-reduction.

Furthermore, the respective batches showed the consumption of lactate and a simultaneous formation of acetate in approximately equimolar amounts (**Error! Reference source not found.**, B and D). In the respective set ups members of *Desulfosporosinus* were dominant again and used the lactate as energy source. *Desulfosporosinus* species are known to grow homoacetogenic, which means an incomplete oxidation of organic acids, in this case lactate, *via* pyruvate to acetate, that is secreted (Newman *et al.*, 1997). Additionally, in set ups containing 50 mM lactate, pyruvate was secreted. Microbial secretion of pyruvate has been observed before (Speck and Freese, 1973; Ruby and Nealson, 1977; Chubukov and Sauer, 2014). Since pyruvate is a very valuable metabolite for every organism, excretion is here very likely due to a stress reaction, caused by the high lactate concentration within the microcosms compared to starved microorganisms within the raw bentonite. Similar observations regarding the pyruvate formation have been made for starved *E. coli* cells, showing excretion of pyruvate due to inactive pyruvate dehydrogenase (Chubukov and Sauer, 2014). The observation that



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the pyruvate concentration remained stable and was not metabolized over time within the respective batches, favours this hypothesis. In general, secreted metabolites are potential energy sources for other microorganisms. In this respect, syntrophic interactions for example with hydrogen- , pyruvate- and/or acetate-consuming microorganisms cannot be ruled out (Zhou *et al.*, 2011).

### 2.2.3 Microbial metabolic potential within bentonite B25

All batches where *Desulfosporosinus* spp. represented the dominating genus, showed effects regarding geochemical parameters and mineralogy. Because of this, *Desulfosporosinus* was the key player under the applied conditions. This genus is known for its substantial degree of intraspecific physiological diversity (Hippe and Stackebrandt, 2015), shown here by the usage of hydrogen/carbon dioxide or lactate with the concomitant reduction of sulphate and the consequent formation of hydrogen sulphide gas, forming visible gas bubbles and cavities in the respective batches as well as insoluble complexes with iron. Furthermore, the formation of spores enables members of this genus to be resistant to heat, UV and γ-radiation treatments even if the respective exposure lasts years (Checinska *et al.*, 2015). Sulphate-reducing organisms in general can cope with stress very well by using complex stress responses (Zhou *et al.*, 2011). So to say, best suitable to outlast in a HLW-repository, until environmental conditions are favourable – namely the presence of (pore-) water, suitable electron donors and acceptors as well as a carbon source. The electrons for the reduction of sulphate can be provided by a broad range of organic substances or hydrogen gas, potentially formed during radiolysis of water (Barr and Allen, 1959), fermentation pathways of organic matter (Nealson *et al.*, 2005) or anaerobic corrosion of the steel container when exposed to water (Libert *et al.*, 2011). Vice versa it has been described, that enhanced sulphide concentrations can be toxic to cells of *Desulfosporosinus*, as described for *Desulfosporosinus orientis* (Klemps *et al.*, 1985). Since we did not analyse the gas phase, except for methane, we cannot comment on the hydrogen sulphide formation rate or the general metabolic potential regarding the formation of further gases formed/consumed. Gases like carbon dioxide, carbon monoxide, hydrogen, hydrogen sulphide and methane could play a crucial role when talking about anaerobic, microbial metabolism.

That microbes are able to use oxidized metals as terminal electron acceptor, has already been shown (Lovley and Blunt-harris, 1999; Weber *et al.*, 2006). For solid-phase Fe(III) bio-reduction, many factors control kinetics and equilibrium of Fe(III) bio-reduction. Since we did not detect conventional iron-reducing microorganisms, the observed changes in ferric and ferrous iron concentration are very likely due to the formation of sulphides, forming the already mentioned iron complexes. Anyway, Liu *et al*. also showed, that sulphate-reducing organisms are able to reduce structural Fe(III) in clay minerals (Liu *et al.*, 2012). Thus, we cannot rule out, that these processes took place and contribute to the evolution of ferric and ferrous iron.

The 16S rRNA gene is the most widely used marker for the performance of phylogenetic analysis, allowing the classification of many taxa. It is therefore an excellent phylogenetic marker (Pace, 1997). Depending on the used oligonucleotides, it is possible to map the microbial population of a given environment. It has been shown, that the oligonucleotides used in this study are very well suited for this purpose (Caporaso *et al.*, 2011). There are many factors influencing the sequencing results (Gohl *et al.*, 2016). A very important one is the DNA-extraction method. As already mentioned, with the here used protocol, DNA from spores cannot be isolated. Since many microorganisms, especially in soils, form spores, it is very likely that we missed a large portion of these organisms when analysing the isolated DNA for their sequences. Furthermore, structural effects on the bentonites themselves could not be demonstrated or verified so far, as well as the microbial influence on the swelling pressure and the cation exchange capacity. Setups without addition of substrates didn´t show any significant changes regarding geochemical or mineralogical changes. Although lactate- and hydrogen gas-containing batches showed clear effects on the geochemical and microbial point of view, significant structural effects on the bentonites were not observed, especially with respect to the process of



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illitization. It has to be elucidated, if the formation of gas bubbles and fractures, observed in this study, do have an influence on the stability and further parameters.

# 3 Experiment with MX-80 bentonite (VTT)

The aim of the laboratory scale microbial storage experiment was to simulate the bentonite behaviour in nuclear waste geological disposal in a way which could happen in some interfaces of the final repository, especially in Finnish conditons but also in general to simulate the interactions of bentonite and microorganisam. The bentonite was studied as a slurry in which water, gases, nutrients and microorganisms were able to move freely at a temperature, hospitable for microorganisms. These circumstances can take place in the interfaces of bentonite and host rock fractures. The objective was to find out, if microorganisms and the metabolites they produce in hospitable conditions, are able to change the bentonite structure and if these changes could be significant for the bentonite stability in long-term scale.

## 3.1 Experimental set up

The experiment was initiated in spring 2016 with MX-80 bentonite (Colloid environmental technologies company) from the same batch which mineralogy has been earlier characterised (Kiviranta and Kumpulainen, 2011). MX-80 was determined being predominantly Na-bentonite with approximately 88 wt. ±2 % of smectite. The amount of organic carbon was low ranging between 0.14 and 0.17 wt. %. There were some exchangeable Ca and Mg cations and iron (4.4 wt. %) was mostly in ferric form (3.9 wt.%) (Kiviranta and Kumpulainen, 2011). The overall experiment design is shown in Table 3-1. Experiment was started in both anaerobic and aerobic conditions simulating long-term storage and the beginning phase of the long-term storage. Both experiments were supplemented with low amount of nutrients (0.05 to 0.1 mM final concentration) including acetate, formate and methanol and rock crush (2 to 6 mm diameter) from Olkiluoto. The rock crush added, was assumed to contain graphite as a potential carbon source for microbes, based on original information of the rock mineralogy. The gas composition for anaerobic part of the experiment was designed based on the gases potentially available in the Olkiluoto repository. Two type of control samples were included in the experiment. In "sterile" controls, water was sterile filtered with 0.2 µm filters (Whatman, GE Healthcare), bentonite was heat treated at 180°C for 16 hours, and rock crush was autoclaved (121 °C, 15 min). These heat treatments and sterile filtration of water were not absolute methods to get rid of all microbial life and it was assumed that in some point at least spores could activate. In the so called "half-sterilised" controls only the used water was sterile filtered and no nutrients were added. This treatment only decreased the microbial activity compared to microbial samples in the beginning of the experiment.

All bentonite microcosms were prepared in 100 mL sterile glass bottles closed with butyl rubber stoppers and aluminium crimps (Figure 3-1). At first 5 g of air-dry bentonite (dry weight 91.5%,) and rock crush were weighted in the glass bottles. Anaerobic experiment microcosms were transferred into anaerobic glove box containing $N_2$:$CO_2$:$H_2$ (80:10:10) gas atmosphere. Anaerobic microcosms were incubated in the glove box covered with sterile foils for at least 2.5 weeks to minimise the oxygen contamination arising from bentonite and rock crush before the anaerobic water and nutrients were added. The surface water used to provide some additional nutrients for anaerobic experiments, was bubbled with sterile filtered nitrogen before use. After water and nutrient addition, the bottles were closed and sterile filtered methane was added with syringe and needle. Aerobic microcosms were filled with water and nutrients in laminar flow hood and rubber stoppers were closed. Anaerobic microcosms were incubated at 30 °C and aerobic microcosms at 37 °C in dark, stationary.


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*Figure 3-1: MX-80 bentonite storage experiment aerobic microcosms.*

*Table 3-1: MX-80 bentonite storage experiment parameters.*

| Parameters | Anaerobic | Aerobic |
|---|---|---|
| **Water mixture** | 3 anaerobic groundwater and 1 surface water from Olkiluoto (25:25:25:5 in all 80 mL), Finland | Surface water and 3 anaerobic groundwater from Olkiluoto (65:5:5:5 in all 80 mL), Finland |
| **Gas mixture** | $N_2$:$CO_2$:$H_2$ 80:10:10 + $CH_4$ 15 ml | Air |
| **Temperature** | 30 °C | 37 °C |
| **Nutrients** | Na-acetate and Na-formate 0.1 mM, methanol 0.05 mM, final concentrations | |
| **Additions** | 5 g of Olkiluoto rock crush (2-6 mm) | |
| **Controls** | 1) Heat sterilized bentonite (180°C, 16 h), sterile filtered water, autoclaved rock "Sterile"<br>2) Only the water mixture sterilised, no nutrients "Half sterilised" | |

## 3.2 Analyses

Samples from bentonite experiments were analysed at the beginning of the experiment and after one year of storage. In addition, some measurements were performed for the water mixtures used in starting the experiments and for the original bentonite.

### 3.2.1 Chemistry and mineralogy

After a year of storage aluminium, silicon and total iron were analysed from the water phase of all sample types and frozen samples from the beginning of the experiment (Eurofins Environment Testing Finland Oy). In addition, sulphate was analysed from the water phase with LCK153 kit (DR 2800 Spectrophotometer, Hach company). pH and conductivity were measured with PHC301 and CDC401-electrodes (HQ30d, Hach Company).

Cation exchange capacity was measured from the original bentonite that was heat treated and from one year stored samples with Cu(II)-triethylenetetramine method (Meier and Kahr, 1999; Amman et al., 2005). The gas phase of the bentonite microcosms was studied by taking 1 mL sample with needle from three parallel microcosms. Gases were studied with gas chromatography (Agilent 6890).



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Mineralogy of the rock crush added to the experiment microcosms was studied with x-ray diffraction, XRD (Bruker D8 Discover A25) from angular space 2Θ 2-70° CuKα (GTK).

### 3.2.2 Microbiology

Water-bentonite-rock mixture samples for the adenosine triphosphate (ATP) measurement were prepared first by mixing the samples to release microbes from the particles and then centrifuging particles down for 3 min at 1300 x g as they inhibit the light measurement. In addition, aerobic samples that were less saline and the particle sedimentation was weaker, were additionally centrifuged for the second time for 3 min at 1800 x g. Supernatant was then analysed with ATP Biomass Kit HS (BioThema).

Sulphate reduction rate of one year stored microcosms were analysed by studying 20 ml of water. $^{35}SO_4$ –label was added to the samples and they were incubated for 7 days at 30/37 °C in dark. Formed sulphide was precipitated with zinc acetate (ZnAc) as zinc sulphide. Radiolabelled sulphide was measured with scintillation counter (Tri-Carb 2810TR). Negative controls, where $^{35}SO_4$ –label was added after termination of the incubation with ZnAc, were included.

For DNA extraction from bentonite samples several commercial kits including PowerSoil DNA Isolation Kit (Mobio Laboratories), NucleoSpin Soil (Macherey-Nagel) with different combinations of kit lysis buffers and enhancer and ZR Soil Microbe DNA (Zymo Research) were tested. Because DNA yield in these commercial kits was low, three additional DNA extraction protocols were tested. These protocols were modified from methods already used for some bentonites or soil materials and are described in more detail by Miettinen et al. (2018). Differences between the extraction methods were based on different lysis buffers and DNA purification steps. DNAs extracted with the modified method of Lever et al. (2015) were further analysed with quantitative PCR (qPCR). The abundance of bacterial 16S rRNA gene was determined as described by Tsitko et al. (2014) and the abundance of dissimilatory sulphite reductase gene (*dsr*B) was measured as described by Purkamo et al. (2016).

### 3.2.3 Microscopy

Bentonite samples were studied at the beginning of the experiment and after one year with atomic force microscopy (MultiMode 8 atomic force microscope equipped with a NanoScope V controller, Bruker Corporation). AFM imaging was used to calculate particle size and shape distribution of montmorillonite layers. Collected shapes were used to calculate the equivalent diameter defined as the diameter of a circle having an area equal to the area of the shape, and the aspect ratio defined as the ratio of the particle length in the broadest point to its width in perpendicular direction. Those simple geometrical parameters were chosen to check if the average size and shape of montmorillonite layers were changed during the duration of the experiment.

In addition, in cooperation with the University of Granada bentonite samples were studied with High-angle annular dark-field scanning transmission electron microscopy (HAADF‐STEM, FEI TITAN G2 80–300). TEM specimen's holders were cleaned by plasma prior to STEM analysis to minimize contamination.


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## 3.3 Results and discussion

### 3.3.1 Chemical results

The gas composition in the headspace of MX-80 experiment microcosms was followed as a function of time. Figures 3-2 and 3-3 show the gas composition development in the headspace of both anaerobic and aerobic experiment microcosms, respectively. In anaerobic experiment the gas phase was very stable except of the disappearance hydrogen. Hydrogen was only detectable in the sterile control microcosms at day 12. It seems that hydrogen acts as an electron donor and was used effectively by microbes, which was supported by the fact that hydrogen was detected only in sterile controls where the microbial activity was the lowest. Oxygen was detected in all anaerobic samples and was around 0.5 % in every sampling. This results probably from air contamination during sample injection. The gas-phase in the aerobic microcosms changed rapidly at the beginning of the experiment as the share of oxygen decreased from around 21 % to less than 18 % in 12 days and to 8 % in four months after which it stabilised to around 2 % after 7 months. In half sterilised control microcosms, the oxygen decrease was a little slower and in sterile control microcosms the oxygen consumption was the slowest, however stabilising also to around 2 % in one year. As the oxygen share diminished the share of nitrogen increased in all aerobic microcosms.

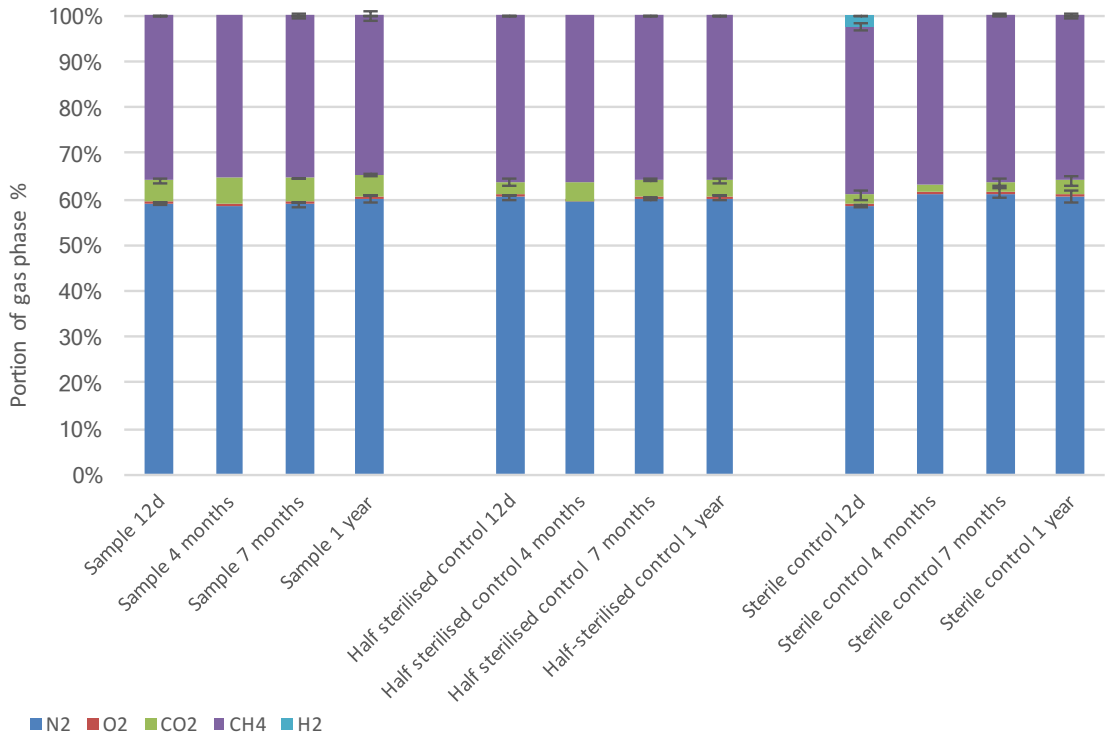


*Figure 3-2. The composition of the gas-phase as a function of time in the anaerobic MX-80 bentonite storage experiment microbial samples and control microcosms.*



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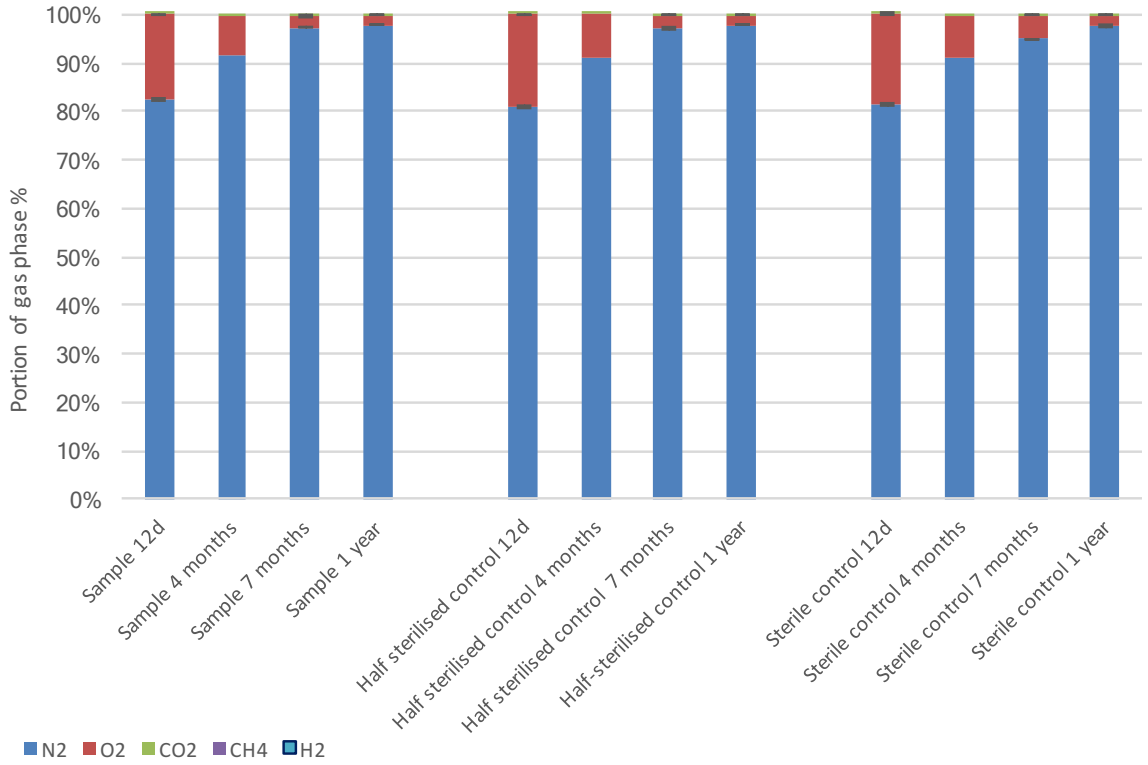


*Figure 3-3. The composition of the gas-phase as a function of time in the aerobic MX-80 bentonite storage experiment microbial samples and control microcosms.*

The rock crush added to the microcosms was analysed with XRD for mineralogical composition. Originally it was assumed that the rock contained graphite to offer microbes an additional carbon source. However, in XRD analysis no graphite was detected. The rock consisted mostly of plagioclase (40 %), quartz (35%) and mica (10 %). In addition, feldspar, chlorite and pyrrhotite were detected. Graphite may be present in rock crush but its content is small.

Initially the pH of the added water was 7.7 in both aerobic and anaerobic water mixtures. pH of the anaerobic water-bentonite slurry was 7.0 to 7.2 in microbial microcosms and in half sterilised controls after 8 months and one year. In sterile control microcosms pH was a little higher 7.4 to 7.7 which may be affected by the heat treatment of the bentonite and rock crush added. In aerobic microcosm bentonite-water slurries pH was higher than in anaerobic experiments, 8.3 to 8.6 in all microcosm types after one year. Conductivity was lower in aerobic microcosms (3.4 mS $m^{-1}$) than in anaerobic microcosms (11.3 to 11.9 mS $m^{-1}$) due to the anaerobic water mixture consisting mainly of groundwaters that were saline compared to the surface water.

In general, the concentrations of aluminium, silicon and iron in the water phase were increased after one year compared to the beginning of the experiment as well as to original water mixtures added to the experiments (Table 3-2). There were two exceptions to this for in the anaerobic experiment, the microbial microcosms contained less aluminium than measured in the beginning. In addition, iron level was under detection limit in half sterilised anaerobic microcosms even though in both sterile and microbial microcosms iron concentrations were higher than in the beginning. Sulphate concentration was at the same level as in the beginning in the aerobic experiment in all microcosms types. However, in the anaerobic microcosms the sulphate concentration decreased especially in the microbial samples and also in half sterilised controls. In sterile controls the sulphate concentration was at the same level as in the beginning. This may be related to microbial sulphate reduction in microbial microcosms and half sterilised control microcosms.



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Cation exchange capacity was stable in aerobic experiment bentonites but there was a slight increase in CEC in anaerobic microbial samples and half sterilised control bentonites. The increase of exchangeable cations may be due to dissolution or precipitation of accessory minerals or alteration of montmorillonite. The effect of heat treatment on the bentonite CEC value was studied in the original bentonite used for the experiments, and the heating effect seemed to be minor (Table 3-1).

*Table 3-2. Aluminium, silicon, iron and sulphate concentrations from water phase of anaerobic and aerobic MX-80 bentonite storage experiment and cation exchange capacities (CEC) from the bentonite phase after one year of storage. As a comparison water phase results from the beginning of the experiments and from the original water mixtures added to the experiments.*

| | | Water | | | | Bentonite |
|---|---|---|---|---|---|---|
| | | Aluminium | Silicon | Iron(tot) | Sulphate | CEC |
| | | ug $L^{-1}$ | mg $L^{-1}$ | ug $L^{-1}$ | mg $L^{-1}$ | meq $g^{-1}$ |
| Anaerobic | Added water mixture | 24 | 8.8 | 29 | 120 | |
| | Sample at the beginning | 180 | 6.2 | 87 | 309 | |
| | Sample 1 year | <50 | 11 | 260 | 138 | 0.89 |
| | Half sterilised 1 year | <50 | 11 | <50 | 237 | 0.87 |
| | Sterile 1 year | <50 | 26 | 120 | 291 | 0.84 |
| Aerobic | Added water mixture | 23 | 1.6 | 28 | 57 | |
| | Sample at the beginning | <50 | 7.6 | <50 | 282 | |
| | Sample 1 year | 580 | 25 | 150 | 334 | 0.84 |
| | Half sterilised 1 year | 500 | 21 | 130 | 287 | 0.84 |
| | Sterile 1 year | 1600 | 34 | 410 | 222 | 0.84 |
| Heat sterilised bentonite | | | | | | 0.85 |

### 3.3.2 Microbial results

Microbial activity during experiments was evaluated by measuring ATP, sulphate reduction by $^{35}SO_4$–label method and DNA-based quantitative PCR. ATP-measurements from the bentonite experiment demanded sample treatment as the bentonite particles disturbed the light detection during the measurements. Short centrifugation removed microorganisms attached to the bentonite particles but also part of the microorganisms moving freely in the water phase. For this reason, ATP results give only indication about microbial activity. In part of the samples standard deviations were relatively high, which describes the sensitivity of the method (Figure 3-4). In general, ATP-amounts were a little higher in anaerobic than in aerobic experiment microcosms. This was unpredictable as aerobic microorganisms in general are considered to be more active and having more efficient energy metabolism than anaerobic microorganisms. As the ATP amounts were low already 14 days after beginning of the aerobic experiment, there probably were other more significant factors that inactivated aerobic microorganisms before the oxygen level dropped substantially. Another explanation for the lower ATP results from the aerobic than anaerobic experiment may be that the aerobic samples were centrifuged an additional time to get rid of disturbing bentonite particles. Anaerobic and aerobic water mixtures differed in terms of salinity which caused clearly slower sedimentation of bentonite particles in less saline aerobic microcosms. Sterile and half sterilised bentonite controls at the beginning did not differ from the microbial microcosms. However, after 8 months in anaerobic microcosms it seemed that microbial samples had a higher ATP-amount than in the measurements for the half sterilised and sterile control microcosms.



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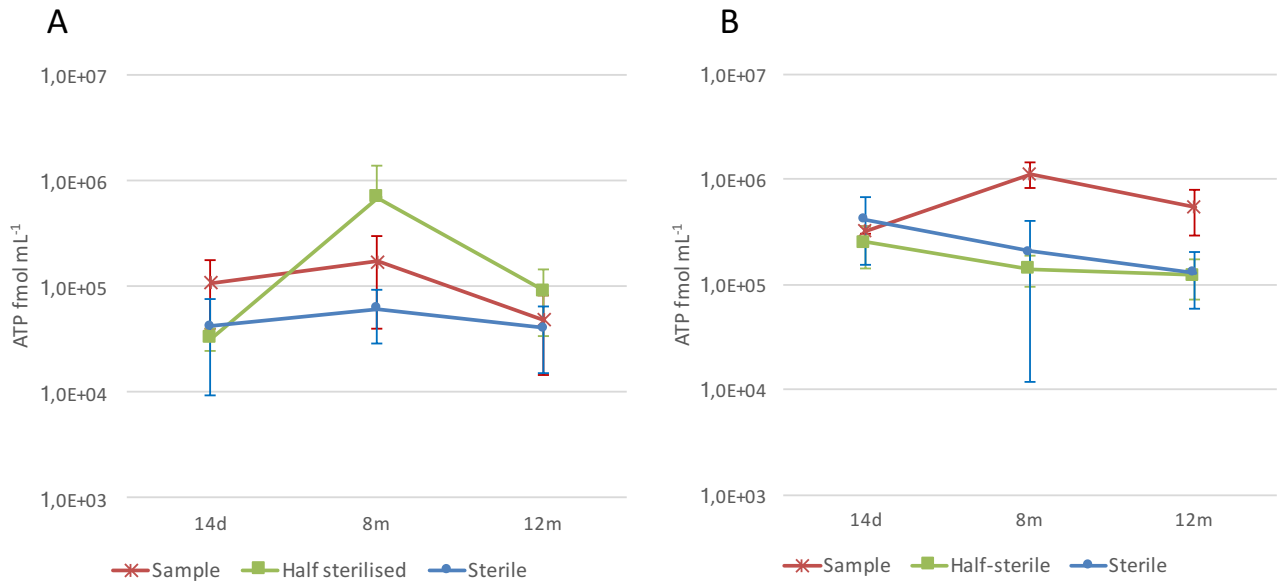


*Figure 3-4. ATP amounts from MX-80 bentonite experiment water phase A) aerobic experiment, B) anaerobic experiment as a function of time (14 days, 8 months and 12 months).*

Presence of sulphate reducing bacteria in commercial bentonite clays and their ability to form sulphide is well known (Masurat et al., 2010; Bengtsson and Pedersen, 2017). Sulphide as corrosive compound is an unwanted product near nuclear waste metal containers. In addition, microbiologically formed sulphide may reduce ferric iron in bentonite and increase the ferrous iron content (Pedersen et al., 2017) which may affect the bentonite stability in long-term. Based on sulphate reduction rate measurement with $^{35}SO_4$–label method no sulphate reduction was detected in any of the controls nor in aerobic microbial microcosms after one year of storage. However, in anaerobic microbial microcosms sulphate reduction was ongoing, being around 6 nmol $mL^{-1}$ per day. This was relatively high rate as typical sulphate reduction rate in marine coastal surface sediments is in the order of 20 nmol $mL^{-1}$ per day (Jørgenssen, 1982). Sulphate reduction method results were consistent with the sulphate concentration results as the sulphate concentration was clearly lower in anaerobic microbial sample than in sterile control. It is possible that also in the anaerobic half sterilised control the sulphate reduction had started as the sulphate concentration was higher than in sterile control, though lower than in microbial sample. After a year of storage in anaerobic microbial microcosm, black layer was discovered on top of bentonite (Figure 3-5). Later also in half sterilised microcosm the black layer was visible. This phenomenon was probably a result from iron precipitation with sulphide as black iron sulphide. However, the black layer disappeared after a few months.

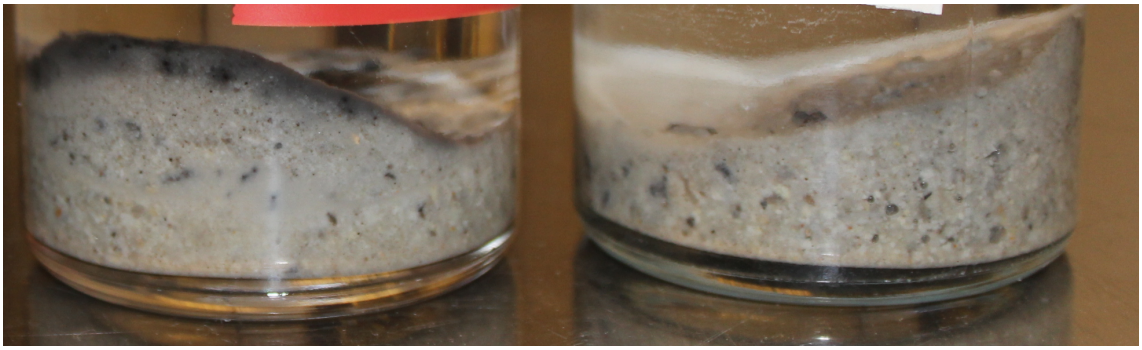

*Figure 3-5. On the left black layer on anaerobic microbial MX-80 bentonite microcosm and on the right a half sterilised microcosm after one year of storage.*



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Moreover, the reported cation exchange capacity increases also point to direction that something had happened microbiologically in anaerobic microbial microcosms and in half sterilised control microcosms. In addition, the preliminary results from the extracted DNA of the microbial samples analysed with qPCR (Figure 3-6) show that the bacterial copy numbers were almost a log unit higher in anaerobic samples than in aerobic samples at the beginning and after one year. In addition, the copy numbers of sulphate reducers (*dsr*B) were high ($10^8$) in anaerobic samples both at the beginning and after one year of storage. In aerobic microbial samples, the *dsr*B copy numbers were 4 log unites lower in the beginning of the experiment and not detected after one year of storage. However, there was clear inhibition in all samples analysed and it may affect the results. In the anaerobic experiment microbial microcosms, *dsr*B copy numbers were only one log unit lower than the 16S rRNA gene copy numbers in both studied time points. The ratio of *dsr*B and 16S RNA genes in Olkiluoto deep groundwaters is rarely that high and usually ten to thousand times lower (Miettinen *et al.*, 2015). All these results indicate high sulphate reducer activity in the anaerobic microbial microcosms. Later on, also microbial diversity will be examined with sequencing. This provides data on possible iron reducers present in the microcosms as they have been detected in MX-80 bentonite previously (Vikman *et al.*, 2018).

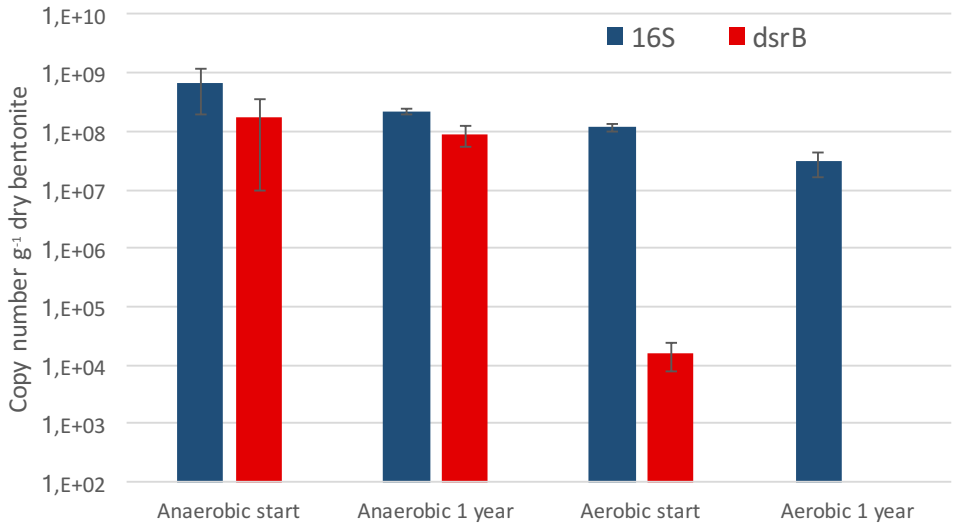


*Figure 3-6. Number of bacterial 16S rRNA and* dsr*B gene copies determined by qPCR from anaerobic and aerobic MX-80 bentonite experiment microbial microcosms.*

### 3.3.3 Microscopical results

Atomic force microscopy showed that the particle size distribution overall was decreased over one year as was also the aspect ratio (Figure 3-7). Particle roundness by contras increased (Figure 3-8). Unfortunately, not yet, enough images were received to statistically analyse differences between separate sample and control types, additional images will be taken later.



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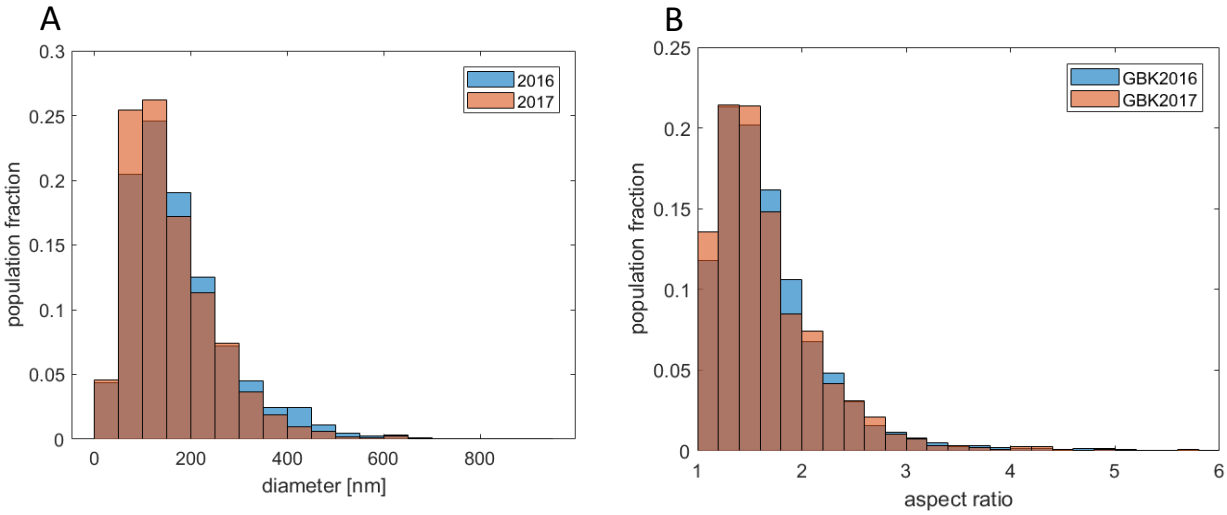


*Figure 3-7. Particle A) size distribution B) aspect ratio of MX-80 bentonite experiment after one month and one year.*

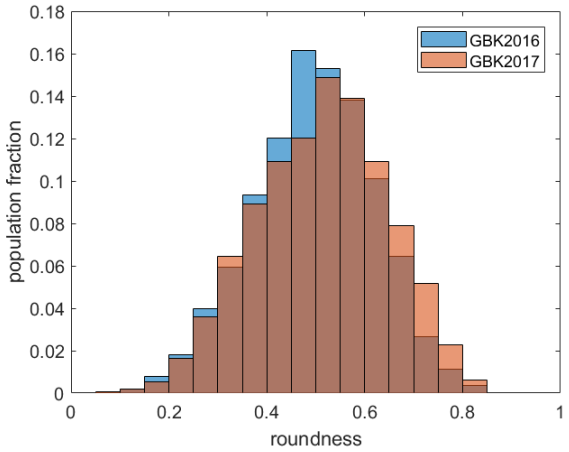


*Figure 3-8. Particle roundness of MX-80 bentonite experiment after one month and one year. As roundness approaches one the more round the particles are.*

In cooperation with the University of Granada the HAADF-STEM microscopy identified plenty of different minerals like smectite, quartz, feldspar, chlorite, calcite, iron oxide, iron sulphide, gypsum, apatite, titanium oxide, paragonite, rutile, biotite etc. Anaerobic microbial samples contained atypical iron sulphide that was not initially found from sterile anaerobic controls but after additional search some were also detected in sterile controls. Thus, this atypical iron sulphide was not a result of microbiological sulphate reduction. Montmorillonite interlamellar space in anaerobic sample and in original bentonite were the same studied by high-resolution TEM.

The MX-80 bentonite mineralogy after one year of storage starting from aerobic and anaerobic conditions showed no essential changes in bentonite mineralogy compared to the beginning. However, clear microbial activity in terms of sulphate reduction and high number of sulphite reductase genes (*dsr*B) was detected in anaerobic samples, that also affected bentonite water phase chemistry and cation exchange capacity. These effects were not detected in sterile controls demonstrating the microbial origin of the changes.



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# 4 Conclusion

Prokaryotes are an essential component of soil (Whitman *et al.*, 1998). Although bentonites are not rich of carbon, many microbes are used to live in oligotrophic environments and gases like $CO_2$ and methane provide an additional source of carbon in geological disposal (Posiva, 2013). In addition, carbon-rich sources may be transferred into the repository (e.g. processing steps of used materials). The results in this report imply that sulphate-reducing and/or spore-forming microorganisms play an important role in bentonite, especially with respect to sulphate reduction and the consequent production of corrosive sulphide compounds. Sulphides may react directly with phyllosilicate Fe(III) and reduce it to ferrous iron (Pedersen *et al.*, 2017). This ferric iron reduction may destabilise the bentonite structure in long-term if lots of sulphide is formed. Sulphide formation leads also to the risk of its diffusion through bentonite which causes corrosion or other unwanted reactions.

Our results clearly show the presence of metabolically active microbes and ongoing sulphate reduction and sulphide formation in bentonites in near natural conditions and in conditions stimulated with excess nutrients. Low concentration of organic acids and mesophilic temperatures were favourable for the metabolic activity of indigenous microbes with both Bavarian (B25) and MX-80 bentonites. Hydrogen had clear effect on $E_h$, which decreased significantly, in batches containing B25 bentonite. Low redox potential, favours the activity of strictly anaerobic, gas-consuming and -forming microbes (Thauer *et al.*, 1977b). On the other hand, in MX-80 experiments the added hydrogen gas was spent already in few days, which showed the preference of anaerobic community for the hydrogen as an electron donor. In the experiments started form atmospheric conditions, oxygen was used in about half a year by the aerobic community. After one year, this community was still adapting to the anoxic condition and they were not performing sulphate reduction nor were as active as were the initially anaerobic communities. However, as many of the sulphate reducers are spore forming microbes they probably turn out later. Issues like swelling pressure, space and water activity that are often addressed when talking about unwanted microbial activity in bentonite are not a challenge for spore-forming organisms.

# 5 Acknowledgement

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