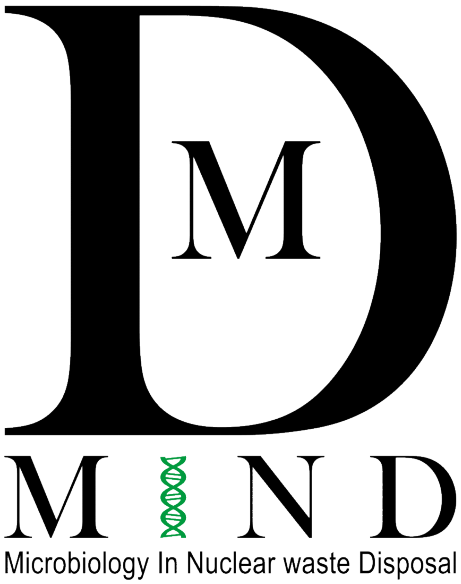

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Microbial diversity in aged bentonites

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Publishable Summary

Bentonite is an integral part of the engineered barriers in geological repositories for low-, intermediate- and high-level radioactive wastes and hence it is crucial that its functionality is maintained for extremely long periods of time. However, the swelling ability, the most important feature of the bentonite barrier, can be affected by many factors including the presence of microorganisms.

Bacteria occurring in bentonites originate from two different sources. First, the microorganisms come from surrounding environment (underground water, host rock) and they can, under certain conditions, penetrate the bentonite barrier. Second source of microorganisms is the bentonite itself. All bentonite materials tested to be used as engineered barriers in nuclear waste repositories contained indigenous bacteria. Density of bacterial cells in the bentonite is generally decreasing with the level of the compactness of the bentonite, but it has been reported that microorganisms remain present even in densities above 2000kg/m^3 (probably mainly in form of spores characterized by unbelievable durability).

In this work, we studied a diversity of the microbial communities present in the compacted bentonites originating from two independent long-term in situ experiments ("Mock-Up experiment" and "Bentonite95 experiment") performed under near field conditions. The compacted bentonite buffer was exposed under conditions similar to a high-level radioactive waste repository in crystalline host rock. We employed 16S rDNA amplicon sequencing to determine the microbial profiles in different parts of bentonite samples.

The results of our analyses showed that there were bacteria present in all of the samples studied. Surprisingly, the microbial communities detected in the bentonite samples showed a high level of similarity. The bacterial profiles were characterised by the dominance of the heterotrophic, aerobic or facultatively anaerobic capable of respiring oxygen or nitrates. Almost no strict anaerobic or autotrophic bacteria were detected. The majority of detected bacteria belong to common soil or ubiquitous microorganisms with wide ecological amplitude enabling them to survive under various conditions. The commonest bacteria present in the bentonites were the representatives of genera *Nocardia*, *Pseudomonas*, *Pseudonocardia*, *Saccharopolyspora* and *Streptomyces*. Our results suggest that microorganisms found in the bentonite samples in this study were most probably present in the bentonite already before the start of the experiment. Further study is needed to determine whether the uncovered microbial diversity represents a metabolically active microbial community able to survive under harsh conditions present in the compacted bentonite rather than a DNA originating from dead cells, trapped and preserved between the layers of the clay minerals in the bentonite.

Contents

1 Introduction

Nowadays, the most appropriate method for the disposal of spent nuclear fuel and high-level and long-lived nuclear waste consisted of its placement in deep rock formations. It was considered that this option would fulfil the various safety, technical feasibility and economic requirements. Safety analysis demonstrated that certain rock formations are able to maintain the integrity of the storage system for hundreds of thousands or even millions of years.

The primary concern for the implementation of a proposed technical design is to demonstrate both operational and long-term safety which, owing to the nature of the waste stored, is on the order of hundreds of thousands of years. When evaluating the long-term safety (following closure) even extremely unlikely processes and eventualities should be considered as well as events or accidents that might occur within the next hundreds of thousands of years. Typically, the spent nuclear fuel and other radioactive waste will be handled and stored at a depth of several hundred meters below the Earth's surface.

The typical disposal concept envisages crystalline rock environments and steel- or copper-based disposal canisters with bentonite as the engineered barrier system material. When designing a storage section for spent nuclear fuel, the spacing of the individual storage wells must be designed so as to make the most effective use of the rock mass available and must also respect the geological characteristics of the rock massif (rock channeling, massif fracturing, the direction of the impact of stress, etc.). Moreover, the residual heat output of the stored fuel will influence the positioning of the storage containers in the deep repository. The extent of heat output will depend on both the design concept applied and the materials employed. Evidently, a temperature of 100°C represents the most restrictive condition of the entire storage system and could lead to the degradation of its main component, montmorillonite, which features most of the safety characteristics of bentonite, i.e. especially its ability to absorb stress in case of seismic-tectonic events, to prevent advective water flow into the storage containers, to slow down the migration of any radionuclides released from damaged waste packages and activity of microorganisms present in bentonite or the surrounding environment.

1.1 Bentonite barrier

Bentonite is a geological term for soil materials with a high content of a swelling mineral, most often montmorillonite. The montmorillonite belongs to the smectite group of minerals and is responsible for the most important feature of bentonite – the swelling ability (Karnland et al., 2006). Montmorillonite is an ion exchanger consisting of stationary negatively charged silicate layer and interlayer of mobile counter cations and water molecules. The two often dominating counter ions are Na^{+} and Ca^{2+}, giving Na- and Ca-bentonite respectively (Svensson et al., 2011), although the content of other ions may be

quite large (Karnland et al., 2006). In bentonite, water molecules can be intercalated between the individual montmorillonite layers to create an interlayer ionic solution. Because there is demand for electrical neutrality, the cations cannot freely diffuse away from the mineral surface. As a result, water will consequently be transported into the inter-layer space, if water with a higher chemical potential is available, and the interlayer distance will increase resulting in the swelling (Karnland et al., 2006). In a fixed total volume, the water uptake into the interlayer space will reduce the volume of initially larger bentonite pores. The full water saturation is reached at the moment when the uptake is forced to stop by the complete filling of the pore volume with the introduced water. The remaining difference in ion concentration, between the high concentration interlayer solution and the water supplying solution, leads to an osmotic pressure build-up in the clay (swelling pressure) (Karnland et al., 2006).

High-quality commercial bentonites contain over 80% of montmorillonite. However, the amount of montmorillonite is rather different in various commercial bentonites ranging from 60% to more than 80% (Pedersen, 2017; Svensson et al., 2011). Moreover, the elemental composition of particular montmorillonites varies a lot among different bentonites and there is also variable amount of other accessory minerals in bentonite such as feldspars, quartz, cristobalite, gypsum, calcite and pyrite (Karnland et al., 2006). As a result, different commercial bentonites vary a lot in their quality and potential sealing properties. The detail characteristics of various commercially available bentonites have been described by (Bengtsson et al., 2017; Karnland et al., 2006; Karnland, 2010; Pedersen, 2017; Svensson et al., 2011).

The swelling ability was the most important feature of bentonite in its use to construct engineered barriers in geological repositories for low-, intermediate- and high-level radioactive wastes (Pedersen et al., 2017). Although bentonite definitely represents unusual and extreme environment, bentonite deposits are not sterile. Bacterial growth in swelling bentonite clay is generally expected to be limited due to the small pore size and to the scarcity of nutrients and electron acceptors necessary to maintain the bacterial population. Presence of bentonite in solutions decreased cell transport in porous media regardless of cell types (Gram-negative or Gram-positive) and solution chemistry (ionic strength and ion valence) (Yang et al., 2012). Furthermore, it was long expected, that the organic carbon naturally present in the buffer and backfill clays may be difficult to degrade (Stroes-Gascoyne and West, 1996). However, recent research showed, that bentonite clay contains various organic materials, such as alcohols, esters, ketones, aldehydes, fatty acids or alkanes (Pedersen, 2017), as well as molecular hydrogen or carbon dioxide, which are common energy sources used by various prokaryotes (Svensson et al., 2011).

1.2 Microbial interactions with bentonite

Microbial interactions with minerals can affect biogeochemical process and subsequently, formation and dissolution of minerals (Dai et al., 2014). There are many possible interactions between bentonite and microbes. One of the most discussed processes is illitization - transformation of montmorillonite into illite, which would have great impact on the porosity of the buffer by altering the buffer's properties in term of hydraulic conductivity (Mulligan et al., 2009). In absence of microbial activity, the process of conversion may take longer time, but it is accelerated in the presence of microorganisms especially iron reducing bacteria (Meleshyn, 2014).

It has been reported that microorganisms can enhance degradation of bentonite (pressure 101 kPa) within 2 weeks by reducing structural Fe(III) to Fe(II) under room temperature. This process otherwise would need much higher temperature from 300 to 350°C and pressure of 100 MPa and time period of 4 to 5 months (Kim et al., 2004). On the same hand, (Perdrial et al., 2009) studied an interaction between bentonites (MX80 and nontronite) and the facultative anaerobic bacteria (*Shewanella putrefaciens*) under anaerobic condition and at dry density of 1.3 kg/m. The results highlighted that the presence of bacteria in MX-80 bentonite leaded to noticeable increase in water content and available pore space while dissolution of minerals was noticed in nontronite owing to bacterial activity.

Alteration of mineral caused by microbial activities is a process of biomineralization (Figure 1). Besides illitization, decreased solid content or coagulation of pores owing to precipitation can be influenced by bacteria. For instance, pores size of buffer material may increase under anaerobic environment by the reduction of Mn. Alike, carbonate one of the constituent of commercial bentonite, can be either dissolved or precipitated as a function of microbial process (Mulligan et al., 2009).

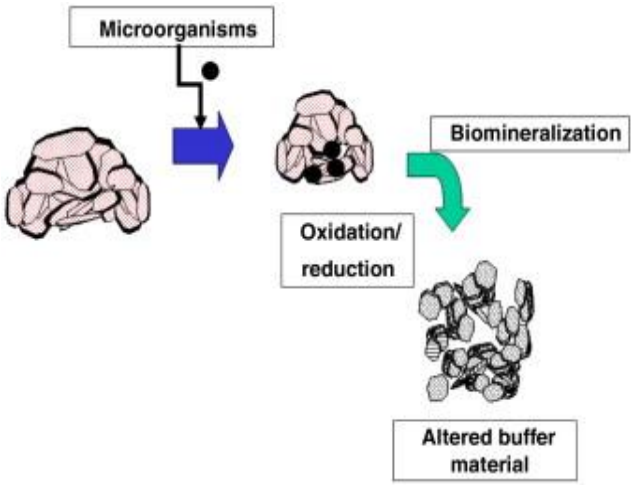

Figure 1. Biomineralization of bentonite buffer (Mulligan et al., 2009)

Dai et al., (2014) studied Gram-negative *Bacillus litoralis* isolated from soil in bentonite of different content. In the presence of bacteria, higher release of Ca^{2+} and Mg^{2+} was reported.

As a result of active microbial metabolism, the interlayer space of bentonite was found to be increased approximately by 0.283 – 0.534 nm correspondingly to the decrease of mineral content.

1.3 Factors influencing the microbial abundance in bentonites

It is important to understand factors that affect the activity of different microbes and accordingly the microbial diversity in bentonites. Wet density is the most commonly studied factor that influences the microbial content of compacted bentonites. The laboratory experiments with compacted bentonites of variable mineralogical composition show that the cultivable, sulfate reducing microorganisms are present in bentonites of any wet density that was studied (1500-2000 kg/m^3). However, in the bentonite type MX-80 it is possible to observe a pattern, where the count of cultivable microorganisms is negatively correlated to wet density after 4 months of storing in simulated deposit conditions. The measurements of precipitated $Cu_x{}^{35}S$ on the copper container also show that the sulfide precipitation steeply decreases (up to below the detection limit) in range of wet densities between 1740 and 1880 kg/m^3. Above the 1880 kg/m^3 threshold there is practically no sulfide precipitation (Bengtsson et al., 2017). Despite the fact that wet density of compacted bentonites (and so the swelling pressure) has obviously an influence on sulfate reduction, it is not possible to say based on this knowledge how this factor controls the structure and diversity of the whole microbial community in long-term stored bentonites. The diversity can be driven by many other factors. Apart from redox conditions in the deposit it might be pH, temperature, water content, pore size and the pore water composition (Pedersen, 2017). The explanation of the relationship between microbial diversity (or metabolic diversity) and these factors would considerably contribute to the knowledge of the microbial processes and their influence on long-term stored bentonite barriers.

Long-lived high level radioactive waste will be buried several hundred meters below ground in geological formation in canisters made of corrosion resistant materials such as copper or stainless steel surrounded by a buffer of compacted bentonite. Bentonite buffer consists of bentonite blocks with low water content (10-17%) at deposition of the canisters. There will be slots between the bentonite and the canister, between the different bentonite blocks and between the bentonite and the rock, which will enable smooth lowering of the canisters and blocks into the deposition holes. These slots will be filled with groundwater from fractures in the rock or, alternatively, with groundwater or surface water added at deposition. The bentonite will start to swell and will eventually reach the planned full compaction density (2 kg/m^3) and a water content of around 26% (Pedersen et al., 2000a).

The repository conditions in the near-field following high radioactive waste (HLW) deposition generally evolves from initially warm and oxidising to cool and anoxic in the long term and consists of four phases summarized by (King et al., 2017): (i) immediate post-placement, when the environment will be aerobic and the γ-radiation dose rate will be at its highest

level. At this stage, the conditions will be extreme. The temperature at the canister surface will reach 95°C, the radiation dose rate will be about 52 Gy/h and the pressure in the buffer and backfill materials can rise up to 12 MPa (Simmons and Baumgartner, 1994). The second stage is (ii) dry-out, whose duration will depend on the initial compacted bentonite moisture content and can take from several years (in case of crystalline host rock) to several hundreds of years (in case of sedimentary host rock). Third stage, (iii) container re-wetting and buffer saturation is characterized by transition from a dry to a saturated near-field environment, and consumption of residual oxygen The last stage (iv) is long-term anoxic phase, which begins once the near-field reaches full saturation. It is the period of the continued cooling and anoxic conditions.

Because of the described extreme conditions, most early analyses considered repository to be either a completely sterile environment, or at least not seriously threatened by bacterial activity (Stroes-Gascoyne and West, 1997). However, many microorganisms are able to tolerate really extreme environments (Humphreys et al., 2010; Ratto and Itavaara, 2012) and citations therein) implying that the conditions in early stages post deposition does not need to be as devastating as expected for all microorganisms. (Stroes-Gascoyne and West, 1997) reviewed microbial effects on ILW repository and concluded that combination of radiation and high temperature effects in the close proximity of ILW container could result in the creation of zone with severely reduced microbial activity, which should however be relatively narrow (25-50 cm). The potential diffusion of corrosive microbial metabolic products to the container surface thus must be considered. The other important question is possible bacterial repopulation after the amelioration of conditions within the repository. Further we discuss some of the most important factors influencing microbial activity in compacted bentonites under repository conditions.

1.3.1 Effect of pressure

The deposited HLW canisters will be surrounded by compacted bentonite as one of the multiple barriers. Upon contact with groundwater, the bentonite will expand and develop a mechanical pressure when constrained by the surrounding rock walls leading to the sealing of the repository (Pedersen, 2002). Depending on the minerology of the specific bentonite type and salinity of the groundwater, different swelling pressures are produced at the same wet density (Karnland et al., 2006).

A high density is believed to have an inhibiting influence on bacterial activity of the natural bacterial populations in the bentonite clays. In the various waste disposal concepts, the desired swelling pressure is at least 5 MPa, which requires a clay dry density of >1600 kg/m^3 (Pedersen, 2017). Even in low-density bentonites (wet density 1500 kg/m^3), a pore size in the nm range would theoretically not allow for bacterial existence unless the bacteria could withstand the mechanical pressure from the separating flocs (0.09 MPa at 1500 kg/m^3) (Pedersen, 2017). Prokaryotic cells can compensate for the mechanical pressure (as in compacted bentonite) by turgor pressure. Published data on turgor pressure in prokaryotic

cells mention pressures between 0.08 MPa and 2 MPa in most species, which means that cell integrity is generally possible at bentonite swelling pressures below 2 MPa (Pedersen, 2017; Potts, 1994). However, there are many species of prokaryotes specialized to survive in high pressure environments, such as deep ocean. These organisms are known as piezophiles and were reviewed by (Ratto and Itavaara, 2012). Furthermore, endospores of any spore-forming bacteria can survive high pressure for reasonably long time. Spore-forming bacteria were not completely eliminated after 15 months at the repository conditions, although their numbers were significantly reduced (Pedersen et al., 2000b).

When Choi and Oscarson (1996) compacted two types of bentonite to dry density 1300 kg/m^3, Na-bentonite saturated with water developed pore sizes of around 0,01 μm with only very few larger pores present, which is about 100 times smaller than size of an average bacterium (Stroes-Gascoyne and West, 1997). The tested Ca-bentonite on the other hand tended to have more large pores than Na-bentonite at the same density, which was ascribed to the generally larger quasicrystal size of the Ca-bentonite clay. This feature is further responsible for higher diffusion coefficients of Ca-bentonite compared to Na-bentonite (Choi and Oscarson, 1996). Transport of bacterial nutrients and metabolic products in compacted bentonites is diffusion limited due to the low porosity of buffers and backfill. Bacterial activity will be consequently diffusion limited, because the rates of diffusion are probably very slow as was demonstrated for sulfide (Pedersen, 2017; Pedersen et al., 2017). As a result bacterial growth will stop and their metabolic activity will cease. However, the bacterial viability may be (and often is) preserved (Pedersen, 2017).

There is strong experimental evidence, that bacterial activity is negatively affected by compactness of bentonite. Various kinds of non-spore-forming bacteria inoculated in compacted MX-80 bentonite (wet density 2 kg/m^3, swelling pressure about 5 MPa) were significantly eliminated in 15 months under repository conditions (Pedersen et al., 2000b). Microbial sulphide-producing activity has been found to decrease with increasing density of MX-80 bentonite (Masurat et al., 2010; Pedersen et al., 2000a; Pedersen, 2010). Similarly, the sulphide-production results (by infested SRB) for the three types of bentonite (MX-80 and Asha belonging to Na bentonites) and Calcigel (Ca bentonite) indicated intervals between 1740 and 1880 kg/m^3 in wet densities within which sulphide-producing activity dropped from high to very low or below detection (Bengtsson and Pedersen, 2017). On the other hand, Bengtsson and Pedersen (2016) reported no clear cut-off in Boom clay density with respect to presence and activity of cultivable SRB, but it appeared as if sulphide production was increasingly possible at or below 1800 kg/m^3. At higher densities (2000 kg/m^3), numbers were lower, but the SRB were still cultivable and active. Although the effect of swelling pressure in bentonite is definitely limiting for various bacteria, cultivable bacteria were further reported in compacted bentonite of different density both in case of indigenous bacteria (Aoki et al., 2010; Motamedi, 1996; Pedersen et al., 2000b) or cultivable bacteria introduced to bentonite prior compaction (Bengtsson and Pedersen, 2017; Pedersen et al., 2000b). Sulphide-producing bacteria could be cultivated from following

compacted clay samples: Wyoming Volclay MX-80 (USA), Asha (India), Calcigel (Germany), GMZ (Gaomiaozi, China, Rokle (Czech Republic) and FEBEX clay (Switzerland). Their numbers decreased over wet density (1400 to 2000 kg/m^3) for some but not all tested clays. Acetate formation from natural organic matter was observed at all wet densities indicating presence of bacterial activity at the higher bentonite densities, when sulfate production could not be detected (Pedersen, 2017). These results indicate, that density alone does not control bacterial activity in clays, but the other variables such as clay type, temperature, water content, presence of organic matter and other energy sources, pore space or pore water composition must be also taken in consideration and need further attention (Bengtsson and Pedersen, 2017; Pedersen, 2017). We will shortly review the effect of some of these factors below.

1.3.2 Effect of temperature

Except from the high pressure, high temperature is other important feature of near-field environment in the HLW repository. The detrimental effect of temperature on microbial activity was demonstrated by several studies. The bacterial heat tolerance is however remarkable. Thermophilic bacteria can live in temperatures over 60°C, in some extreme environments even above 100°C. Typically, moderate thermophiles are Eubacteria with optimal growth temperature between 60 and 80°C, while hyperthermophiles are Archaea growing in temperatures higher than 80°C.

The surface temperature of the waste canisters is expected to be about 90-95°C and high temperature is expected to be one of the important protections from microbial life. In accordance with this, (Pedersen et al., 2000b) reported, that at 80°C, the introduced spore-forming SRB were the only surviving bacteria in 28 weeks' time of the experiment in compacted MX-80 bentonite. Similarly, Lydmark and Pedersen (2011) described that the higher temperatures (up to 85°C) led to decrease in the bacterial abundance and activity in repository experiment. Results of other experiments were however less encouraging. Heat treatment of the bentonite at 110°C for 170 h failed to eradicate SPB in the bentonite and intensive sulphide-producing activity and large numbers of cultivable SPB were observed in the heat-treated MX-80 bentonite samples (Bengtsson and Pedersen, 2017). Masurat et al. (2010) in similar experiment detected loss of SRB viability after treatment of the bentonite at 120°C for 20 h, but (Bengtsson and Pedersen, 2016) reported the presence of indigenous SRB, which survived heat treatment of 120°C for 48h. Unifying element of all of these studies is presence and survival of spore-forming microorganisms, which are capable of remarkable endurance (see below).

Moreover, the elevated temperature can also have negative effect on bentonite sealing capability. Svensson et al. (2011) detected a significant difference in swelling pressure between bentonite exposed to high temperatures and reference untreated material for two of the investigated bentonite materials, Asha 505 and Deponit CAN. The bentonite samples which were closer to the heater were affected more by the swelling pressure decrease.

These results show the need for the further detailed studies concerning the effect of temperature on bacterial survival (with the special emphasis to spore-forming microorganisms) while taking into account the physical features of particular bentonite clays.

1.3.3 Effect of dessication

In contrast to nutrient, energy, pressure, temperature and radioactivity constraints, few bacteria can tolerate removal of water from the cell (Motamedi, 1996). The availability of water for microbial processes can be represented by the water activity (aw). Most bacteria grow well at aw around 0.98 but relatively few species can grow at aw of 0.96 or lower (Motamedi, 1996). The most tolerant bacteria can grow at aw of 0.85 and halophilic archaea even at aw of 0.75 (Grant, 2004).

In non-saline soils, water availability is determined by the thickness of the water film around soil particles. In the soils with low water content, substrate diffusion and cell motility reduces microbial activity and these conditions are more detrimental to microbes than osmotic stress in saline soils (Chowdhury et al., 2011). Reducing the water activity of buffer material thus may be a potential approach to limiting microbial activity in the vicinity of containers (Stroes-Gascoyne and West, 1997). In accordance with this assumption, (Motamedi, 1996) evaluated the effect of water activity on survival of introduced SRB under repository conditions and found that water activity lower than 0.96 was lethal for the studied microbial species. Similarly, (Aoki et al., 2010) demonstrated that the number of viable aerobic heterotrophs in compacted bentonite well correlated with the water content, which was affected by the temperature (presence of the heater).

However, many bacteria can withstand desiccation (and other unfavourable environmental conditions) by spore formation. Bacterial endospores are survival forms with reduced water content and undetectable metabolic activities that can tolerate adverse environmental conditions such as extreme drying, wet and dry heat, and UV and gamma irradiation (Nicholson et al., 2000). Dormant spores exhibit remarkable longevity in the environment. They can survive extreme conditions under repository conditions at least 15 months (Pedersen et al., 2000b) see below. The recovery and revival of spores from deep-sea bed samples as old as million years has been reported (Jørgensen and Boetius, 2007). Spore-forming bacteria are found among various bacteria including aerobic heterotrophs, anaerobe acetogens or iron and sulphate reducers (Nicholson et al. 2000). Vegetative cells of some desiccation-tolerant bacteria can also maintain viability in the dry state. The maximum time of survival in this dormant state is however unknown (Billi and Potts 2002).

Montmorillonite with its high affinity for water could efficiently extract water from the bacterial cells, when surrounded by bentonite, leaving them in a desiccated (and possibly well preserved) state, because the bacterial cell membrane is water permeable (Pedersen, 2017). Slow desiccation is a common process for bacterial preserving in microbiology. Interestingly, there is possible connection between the resistance to desiccation and radiation tolerance. The increased ability to survive radiation observed in some indigenous

microorganism in bentonite was ascribed to their increased tolerance to desiccation. The desiccation resistance requires extensive DNA repair similarly to the repair of DNA damage from ionizing radiation. Microbial cells thus seem to use similar mechanism to reduce detrimental effect of both factors (Lucht and Stroes-Gascoyne, 1996; Stroes-Gascoyne and West, 1997). Although (Stroes-Gascoyne and West, 1997) concluded, that the presence of SRB might not pose a high risk to waste containers, if aw of the saturated bentonite is kept low, the evaluation of the behaviour of microorganism and especially the spore-forming bacteria under repository conditions seems to be crucial.

1.4 The origin of microorganisms in bentonites

Microorganisms building up the pool from which a new, in local conditions prospering microbial community arises are of two origins. The first source of microbes is the bentonite itself – its natural microbial content from the place where it was quarried together with microbes that come from the mining process, bentonite post-processing or storing. The second source is the ground water and rock in the place of planned deposit (or water used for bentonite saturation). The presence of cultivable microorganisms was well proven in bentonite quarries previously (Fukunaga et al., 2005, López-Fernández et al., 2014). Further, high diversity of prokaryotes on the phylum level was observed there too (López-Fernández et al., 2015). Also in ground water obtained from clay mineral layers dwell microbial communities (Wouters et al., 2013). The second potential source of microbes is the deep underground environments (the planned waste deposits). They also host a significant number of microorganisms. It is well known, that in granites microbial cell counts reach concentrations 10^3 - 10^7 ml^{-1} of ground water (Pedersen, 1997). When the rocks are amply fractured or they lie closely to surface the cell concentrations can be even higher (Gohn et al., 2008). Also the metabolic diversity of microbial communities in deep ground water is very high (Ino et al., 2018, Hubalek et al., 2016, Pedersen, 1997). Common metabolic abilities of abundant microorganisms in deep aquifers include fermentation, anaerobic oxidation of methane, oxidation of hydrogen, reduction of sulfate or oxidation of sulfide (Hubalek et al., 2016, Wu et al., 2016). All these metabolic processes can endanger the safety of isolation barriers in high radioactive waste deposits. Moreover, if the ground water is in contact with atmosphere (the case of still open deposit), it is enriched in aerobic microorganisms which can produce more biomass, because aerobic oxidation is the energetically most favourable type of metabolism. It is thus necessary to count with increased concentrations of microbes at the beginning of radioactive waste storing into underground deposits compared to undisturbed rocks. It is essential that microorganisms of both origins (from bentonite and deep underground) represent a sufficiently large and heterogenic (phylogenetically and metabolically) pool. The diversity of microorganisms in long-term stored bentonite barriers is therefore not driven by that whether appropriate microbes will get there but it is dependent on that which conditions will be set up after deposit closure. That is why it is so important to understand how different conditions influence the microbial diversity in bentonites. Only the

simulation of conditions after the deposit closure and subsequent bentonite and groundwater microbial community analysis has a potential to approach the real composition of microbial communities present in bentonites after deposit closure (Chi Fru and Athar 2008, Smart et al., 2014).

1.4.1 Identification of microorganisms in bentonites

There are several possible approaches, how to study the bacterial occurrence in clay soil samples. The simple bacterial presence (or viability), recently most often detected by means of DNA based methods, does not imply that the bacteria must be alive or active in vivo or in situ, it only states that they were present and sometimes are able to become activated when a suitable environment presents itself (Figure 2)(Pedersen, 2017). In soil generally, the active microorganisms compose only about 0.1-2% of the total microbial biomass and very seldom exceed 5% in soils without input of easily available substrates. Usually (in water environments for example) dead cells and free DNA are quickly degraded as a potential source of energy or nutrients for active microbes, however, in clay minerals the free DNA ("dead DNA") can be protected against degrading enzymes, remain conserved for long time and cause bias during data analysis obtained from DNA sequencing. Furthermore, the fraction of potentially active microorganisms (ready to start utilization of available substrates within few hours) is much higher, contributing between 10 and 40% (up to 60%) of the total microbial biomass (Blagodatskaya and Kuzyakov, 2013). Furthermore, dormant bacteria can survive for a very long time, sometimes for millions of years as was described in subseafloor sediments (Jørgensen and Boetius, 2007). Bacterial activity on the other hand is a clear measure of living cell presence and can be generally measured either directly via staining (acridine orange direct count method, DAPI, Live/Dead fluorescence staining) and observing, viable microorganisms can be further enumerated using the most probable number (MPN) technique, or by the turn-over of one or several metabolic products such as acetate, sulphide or ATP (Eydal and Pedersen, 2007; Pedersen, 2017).

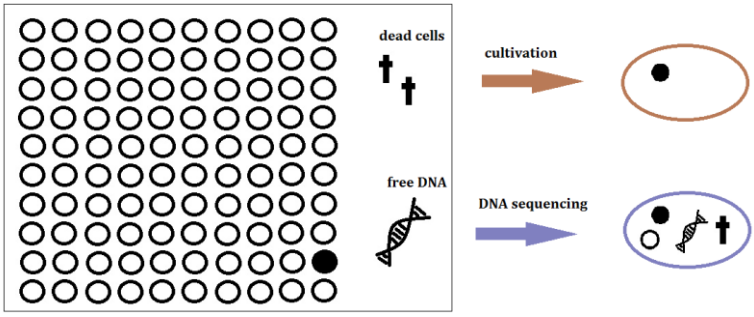

Figure 2. The microbiological content of bentonites detectable by cultivation or DNA sequencing is shown in the black frame. Circles represent the viable cells (active and dormant cells). Filled and empty circles represent the cultivable and uncultivable cells respectively. The crosses depict dead cells and the DNA molecule represents free DNA. Using cultivation methods only few to less than one percent of viable cells can be analysed. Using DNA sequencing information about cultivable, uncultivable microbes, dead cells and free DNA can be gained.

The widely used methods for bentonite (and other clay minerals) microbial diversity description are at present time cultivation-based methods complemented sometimes by colonies 16S rDNA sequencing (Lopez-Fernenadez et al., 2014, Jalique et al., 2016). Enrichment cultures and following prokaryotic DNA sequencing is also common (Poulain et al., 2008). However sequencing of DNA isolated directly from bentonite is rather rare despite its great potential to depict the microbial diversity (López-Fernández et al., 2015.) The reason why scientist put the cultivation-based methods before the molecular genetic approaches is apparently caused by the fact that isolation of nucleic acids from clay minerals is a challenging procedure requiring special protocols and experienced laboratory technicians. The DNA isolation from some types of clay minerals was unsuccessful despite using different isolation procedures. Important is that the studied minerals were not sterile, because microbial presence was proven by other methods (Poulain et al., 2008, Stroes-Gascoyne et al., 2007). Today it is believed that the reason of these difficulties is an easy adsorption of DNA to clay minerals which hinders its release performed by common isolation kits. The adsorption is driven by different interaction mechanisms: electrostatic interactions, cation and hydrogen bonds or the ligand exchange. These mechanisms act at different strength degrees depending on the mineralogical composition, chemical features of water held in the clay mineral and on the properties of DNA. (Yu et al., 2013) This means that the isolation of DNA from bentonite requires special isolation protocols tuned for a particular bentonite type.

Some studies focus on detection and quantification of phospholipid-derived fatty acids (PLFA) in clay minerals (Mauclaire et al., 2006). Since PLFA are after cell death quickly degraded, this approach allows assessing the presence of viable cells. However, for diversity investigation has this method lower distinguishing efficiency than the 16S rDNA amplicon sequencing.

Both, cultivation-based methods and the 16S rDNA sequencing produce more or less serious bias which needs to be considered when interpreting the obtained diversity results. In general, the cultivation-based methods represent not a good tool for total diversity assessment in oligotrophic environments like compacted bentonites or naturally occurring clay minerals in deep underground environments. There are two reasons for that: The cultivability of microorganisms from underground environments is very low, usually less than one percent (Haveman and Pedersen, 2002). It is necessary therefore to consider that the cultivation-based analysis of diversity is based only on a little fragment of the real community and thus most of the microbiological information about the studied environment remains hidden. This is a very serious limitation of cultivation-based approaches for diversity analysis. The second reason is that the cultivation media generate a selective pressure on the original microbial community and shift the composition of microbial community - usually eutrophic microorganisms benefit from the cultivation media despite they might have played only a minor role in the original community (one cell is enough for colony growth!).

The cultivable fraction definitely doesn´t represent the ecological requirements of the whole community. It has been shown that the cultivable microorganisms are repeatedly from the same phyla – typically Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Bianchi et al., 1995, Jalique et al., 2016, López-Fernández et al., 2014, Chi Fru and Athar 2008). The diversity bias caused by cultivation can be that strong, that it completely erases the differences between samples from environments as different as seawater, various types of sediments and ice (Lozupone and Knight, 2005).

The sequencing of microbial 16S rDNA provides more complete picture of microbial diversity in bentonites than cultivation-based methods. Anyway, it is important to count with some degree of unreliability. The problem is that all DNA is sequenced from both, viable or metabolically active cells and dormant, dead or already lysed cells. This problem is worth attention especially in environments with clay minerals, which have a great ability to conserve DNA molecules. For that reason, the “dead” DNA could possibly form a significant part of total DNA isolated. The research quantifying the proportion of “dead” DNA from the total DNA isolated from different types of bentonites is still missing hence some progress in this area would be useful.

In Urios et al. (2013) it was shown how significantly different the results of molecular genetic approaches from cultivation-based methods can be. The samples collected from compacted argillite (clay rock) in the contact area with carbon steel after 10 years of interaction in an underground tunnel were treated by both methods. The result was that only two phylotypes were detected by both methods (*Pseudomonas stutzeri*, *Pseudomonas frederiksbergensis*). Another example could be two studies focusing on diversity of microbes in surface bentonite quarries in Spain. The cultivation-based approach revealed three phyla Actinobacteria, Proteobacteria, Firmicutes (López-Fernández et al., 2014). Whereas the Illumina sequencing and the 16S rRNA amplicon cloning detected 14 different prokaryotic phyla in samples from the same bentonites (López-Fernández et al., 2015).

2 Objectives

The objective of DL 2.07 is to present a detailed characterization of microbial communities in long-term exposed bentonites under near field conditions in two in situ experiments held in the Josef URL, Czechia in order to describe microbial communities in different parts of the compacted bentonite buffer exposed under conditions similar to a high-level radioactive waste repository in crystalline host rock. Final objective of this deliverable is to evaluate the applicability of the outcomes for the safety assessment of deep radioactive waste repository.

3 Mock-Up experiment in Josef URL

Physical model Mock-Up Josef was designed to provide information on the long-term behaviour of bentonite sealing barriers in a realistic underground environment in Josef URL. It was designed to evaluate effect of temperature and swelling pressure on the stability of bentonite buffer. The physical model was started in January 2013 and was dismantled in 2017. This experiment was originally not planned to include microbial analyses, but it was possible to obtain bentonite samples after its dismantling.

3.1 Study site

The experiment was held in Josef URL in the Psí Hory locality in Czech Republic (Figure 3), where the host rock for the model comprises volcanic rock types forming the Slapy spur of the Central Bohemian magmatic complex, namely amphibolite-biotite granodiorites of the Sázava-River type. The granodiorites are of the Variscan (Hercynian) age. Fissure systems appear to be tight, without crushing.

Figure 3. Location of Josef URL in the Czech Republic.

3.2 B75 Bentonite

Commercially produced natural bentonite B75 from the Černý Vrch deposit in Northern Bohemia was tested in the experiment. It is a calcium-magnesium bentonite with a montmorillonite content of around 60%. The plastic limit, liquid limit and specific gravity of solid soils are 65%, 229%, and 2.87, respectively (Šťástka and Smutek, 2015).

Bentonite segments used for the construction of the model were compacted/compressed to dry density of ρ_d =1.75 Mg/m^3. Bentonite compacted to ρ_d =1.75 Mg/m^3 rate displays low permeability (hydraulic conductivity k=10^{-13}m/s) and is highly swelling when receiving water (swelling pressure σ_{sw}= 10 MPa).

3.3 Experimental design

The model was designed by Centre of Experimental Geotechnics (CEG, Faculty of Civil Engineering, CTU Prague). It was formed by 33 layers of bentonite segments, the height of individual layers was 67 mm and the height of the model was 2170 mm (Figure 4. Physical model consisted of heating element, compacted bentonite and monitoring profiles (temperature and pressure sensors). Source: Centre of Experimental Geotechnics CTU Prague and SÚRAO.Figure 4). The diameter of the circle constructed from the segments was 700 mm.

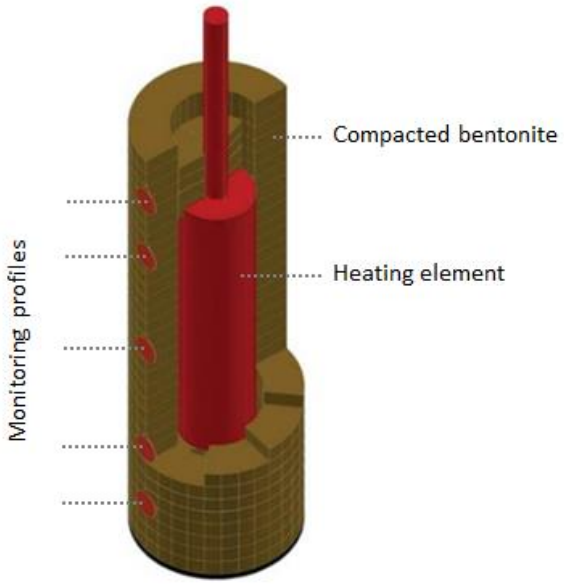

Figure 4. Physical model consisted of heating element, compacted bentonite and monitoring profiles (temperature and pressure sensors). Source: Centre of Experimental Geotechnics CTU Prague and SÚRAO.

In December 2012, the model was transported to the experimental site and placed into bore hole (2.8 m deep and 0.75 m in diameter). The neighbourhood of the bore hole was improved by casting concrete slabs on the gallery stub bottom. The upper edge of the concrete slabs was above the surface of water in the stub, preventing the direct flowing of water into the wells over the concrete slab edges (Figure 5).

Figure 5. Gallery stub with the drilled wells. Source: Centre of Experimental Geotechnics CTU Prague and SÚRAO.

All monitoring sensors (pressure, moisture), including data transfer, were tested before starting the model. After launching in the borehole, the model was closed with a lid and fixed with bracing tubes (Figure 6).

Figure 6. Installation of the model in the gallery stub and the monitoring system. Source: Centre of Experimental Geotechnics CTU Prague and SÚRAO.

The heating element was switched on at 90 °C on 7th January 2013, approximately two weeks after starting the model in the bore hole. Due to the loading of bentonite at only 80 °C, the heating system performance was increased to 100 °C on January 23, to reach the surface temperature of the heating element to 90 °C.

Samples for microbial analyses were obtained from three different depth (corresponding to different temperatures) and stored at room temperature in anaerobic conditions before use. We included samples from depth 20 mm (corresponding to 25 °C; marked B25), 480 mm (approximately 50 °C; B50) and 735 mm (approximately 65 °C, B65).

3.4 Methods

3.4.1 Physical parameters

The majority of tests performed by CEG Prague, were focused on the observation of the development of the temperature inside the bentonite body. Temperature sensors were also installed in the rock surrounding the model. In addition, hydraulic cells measuring the values of pressure were installed inside the bentonite body. These measuring devices were installed in every third profile being measured. In total, 45 hydraulic pressure cells were installed in the model. Relative moisture sensors providing information on the course of the bentonite saturation process were also installed.

3.4.2 Molecular-biology analysis

DNA extraction

Approximately, 14 g of bentonite was used from each sample to extract DNA. DNA was extracted using a commercial DNA isolation kit, Power max soil DNA isolation kit, catalogue number 12900-10 from MoBio was used according to manufacturer's instruction where the chemical cell lysis was further assisted by mechanical technique using a bioruptor. The bioruptor was set for high power mode (sonication cycle: 30 sec ON, 90 sec OFF) for 20 minutes. The purification of DNA was performed by Zymo research kit. The extracted DNA was quantified by using a Qubit 2.0 fluorometer.

Library preparation and sequencing

For the library preparation, two consecutive PCR reactions per sample were performed with the use of normal and bar code fusion primers. Moreover, variable V4 region of 16S rDNA gene was amplified with primers 515F (Dowd et al., 2008) and 802R (Claesson et al., 2010) for amplicon sequencing. Most importantly, the size of the amplicon was kept below 400 bp to cover as much microbial diversity as possible by performing In silico analysis of primers (Němeček et al., 2017).

The PCR conditions for the first PCR were as follows: 95°C for 3 min; 10 cycles at 98 °C for 20 s, 50 °C for 15 s and 72 °C for 45 s; and a final extension at 72 °C for 1 min. The second PCR was performed as follow: 95 °C for 3 min; 35 cycles at 98 °C for 20 s, 50 °C for 15 s and 72 °C for 45 s; with a final extension at 72 °C for 1 min. The quality of the library product was checked by gel-electrophoresis. Additionally, the PCR products were purified using the Agencourt Ampure XP system (Beckman Coulter, Brea, CA, USA), and the concentration of the purified PCR products was measured with a Qubit 2.0 fluorometer (Life Technologies, MA, USA). Then, the barcode-tagged amplicons from different samples were mixed in equimolar concentrations. Sequencing of the amplicons was performed on an Ion Torrent Personal Genome Machine using the Ion PGM Hi-Q Sequencing Kit with the Ion 314 Chip following the manufacturer's instructions (Thermo Fisher Scientific).

Sequencing data evaluation

The raw Ion Torrent reads were processed with Mothur software (Schloss et al., 2009). Low quality reads were removed and sequences were assigned to each sample. Chimeric sequences were identified using UCHIME (Edgar et al., 2011) and subsequently removed. Sequences exceeding 400 bases were trimmed and sequences shorter than 170 bases were removed. Sequences were classified against the Silva database version 128 with a bootstrap value set at 80% and the NCBI database. A cut-off value of 97% was used for clustering of sequences into operational taxonomic units (OTUs). Sequence data were normalized to

sample with the least sequences by randomly selecting a number of sequences from each sample. Cluster analysis was performed using the Vegan package in the R statistical package (Oksanen et al., 2012).

The microbial community characterisation is based on the 30 most common OTUs from each sample. There is a rationale for eliminating the little abundant microorganisms from the analysis. Those microorganisms that are abundant in the microbial community characterise the environment best because they are able to propagate in that environment (occur in their ecological optimum). Rare microorganisms, on the contrary, are likely to be metabolically inactive or to occur in the dormant phases and their ties to the environment are looser. Moreover, the vast majority of the sequences isolated fall among the few dozen of most abundant OTUs. The taxonomically classified OTUs (with a ≥96% sequence similarity based on the hit) were characterized metabolically based on literature, and at the same time, the environment in which their phylogenetically nearest relatives had been detected was considered (hits with the highest sequence similarity in the GenBank database, even if taxonomically unnamed). The nature of the environment in which their nearest relatives live can be used for quite a good assessment of the nature of the environment of the microorganisms analysed. We focused on the following parameters: the oxygen requirements (strictly anaerobic; facultatively anaerobic; aerobic or facultatively anaerobic; aerobic), the terminal electron acceptor (O_2 or NO_3; O_2 only; O_2 or anaerobic respiration; anaerobic respiration; fermentation or AsO_{3-} or SeO_{4-} or O_3Se_{2-} or NO_{3-}), the electron donor (organic compounds; NH4 or reduced forms of sulphur or organic compounds) and the trophy type (heterotrophic, mixo- or heterotrophic, hetero- or autotrophic).

3.5 Results

3.5.1 Temperature monitoring

Temperature was monitored at different levels of the model. An example shows temperature profile at level 16 near the heating body (Figure 7). The closest sensor to the heater measured temperatures approximately 75°C, while the sensor that was closest to the host rock measured temperatures around 45°C. The vicinity of the model was also monitored, the temperature increased as well, above 30°C.

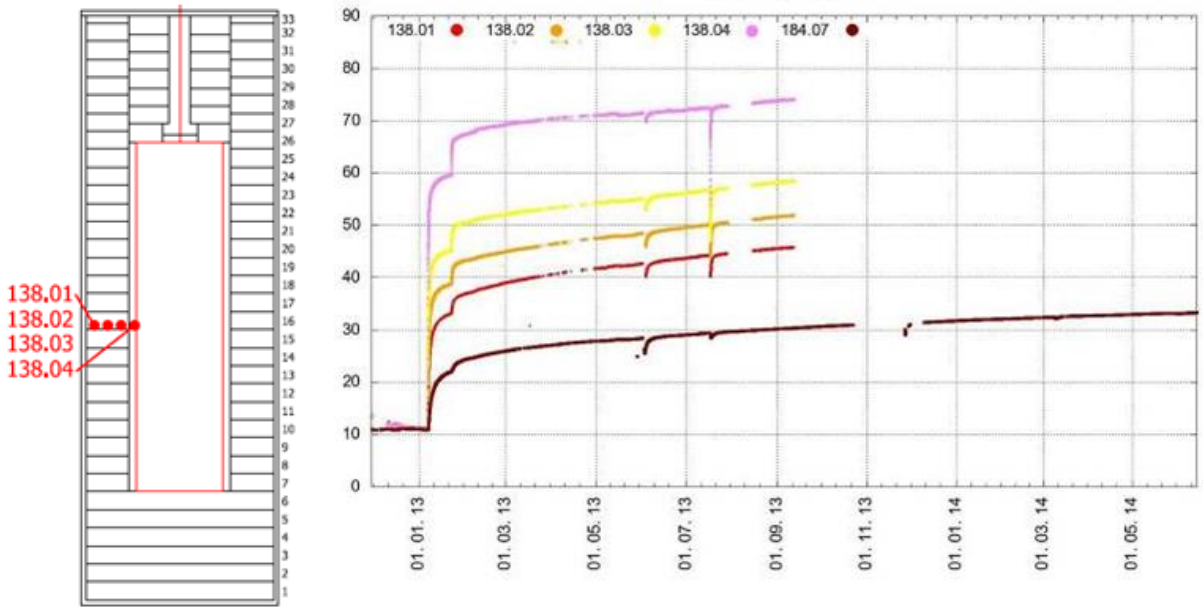

Figure 7. Temperature monitoring (°C) in layer 16 (left); magenta line - closest sensor to the heater; yellow, and orange– sensors in the middle of bentonite barrier; red – sensor closest to the host rock. Data provided by Centre of Experimental Geotechnics CTU Prague and SÚRAO.

3.5.2 Relative humidity and pressure

Relative humidity values rapidly increased during the first months of the experiment (Figure 8). Middle part was saturated already after five months, while the 80% saturation of the bentonite closest to heater body was reached after eight months.

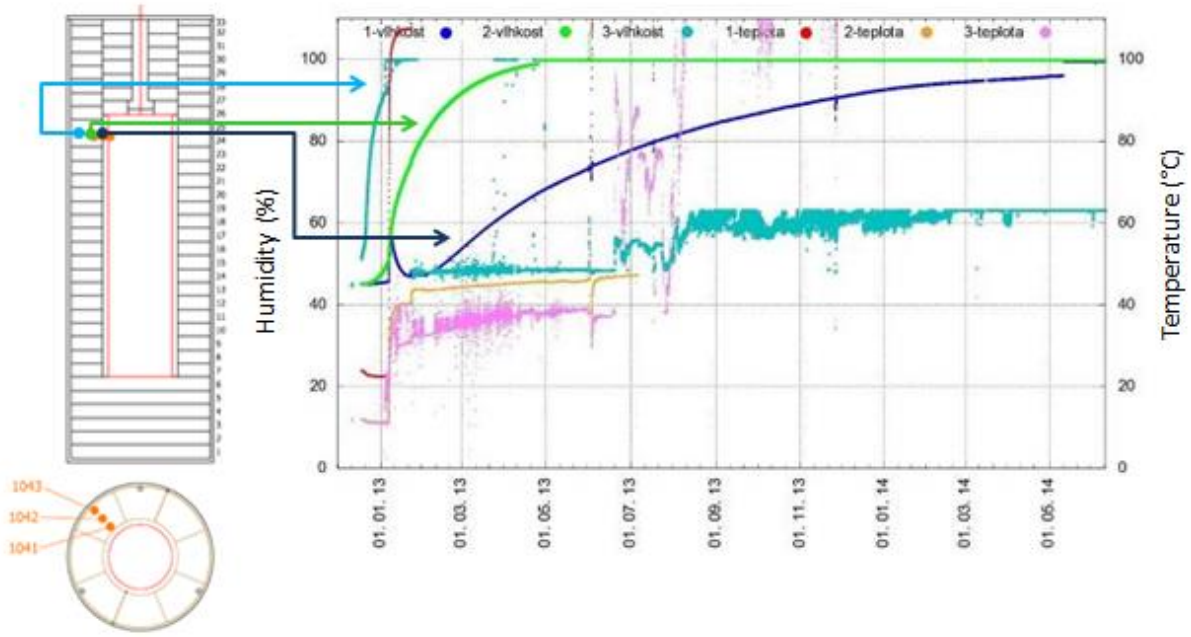

Figure 8. Relative humidity in bentonite. Left – position of the sensors; Right – development of humidity from the beginning till month 17. Light blue line – humidity closest to the host rock, green line – humidity in the middle of the bentonite buffer, and dark blue – humidity near the heater. The temperature values cannot be considered because the sensors for humidity

were not suitable for the temperature measurement. Data provided by Centre of Experimental Geotechnics CTU Prague and SÚRAO.

3.5.3 Microbial diversity

The number of sequences for each of samples was in the range of 18,783-24,802. 6,791 OTUs (2,957 excluding singleton and doubleton OTUs) were retrieved from our dataset. The microbial community characterisation results are visualised on Figures 9, 10, 11 and 12.

The microbial community detected in the bentonite samples is characterised by the dominance of the aerobic or facultatively anaerobic soil or ubiquitous microorganisms capable of respiring mainly nitrate or oxygen (Figure 9, Figure 10). The most abundant OTUs detected in the samples cannot use any energy source other than organic mass (Figure 11, Figure 12). The samples collected from different parts of bentonite did not significantly differ in terms of oxygen requirements, terminal electron acceptor or electron donor.

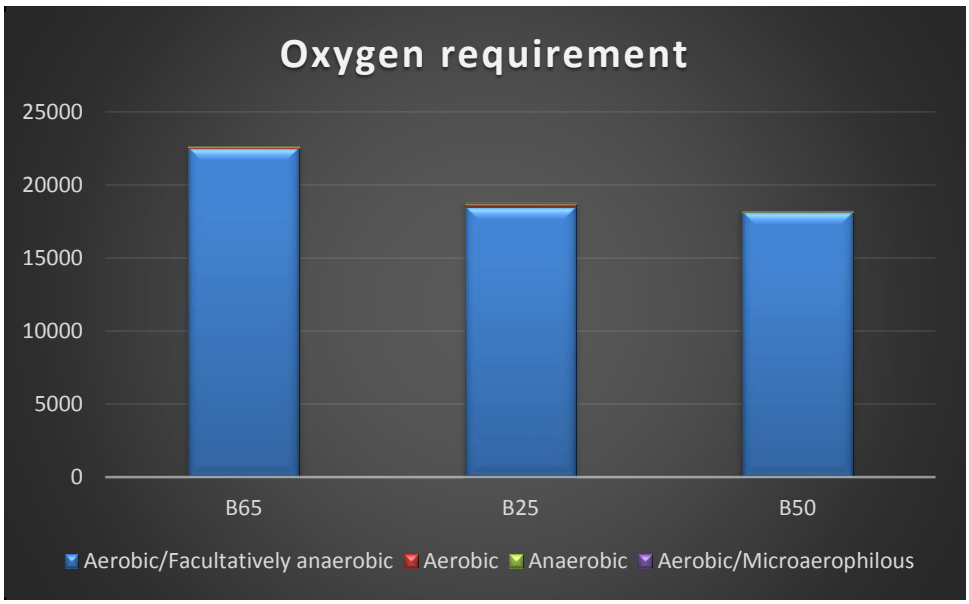

Figure 9 The evaluation of oxygen requirements in different parts of bentonite.

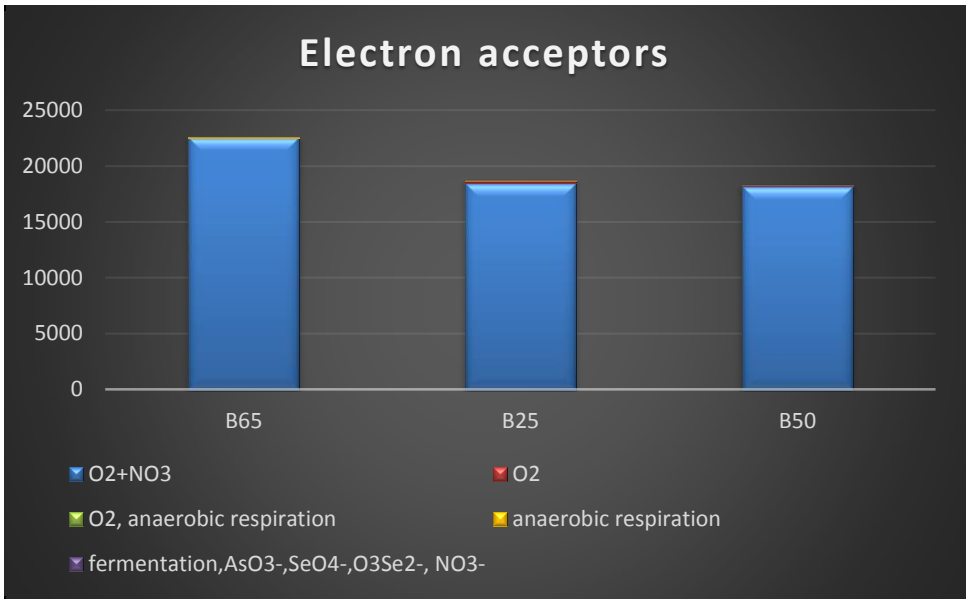

Figure 10 The comparison of terminal electron acceptors in different parts of bentonite.

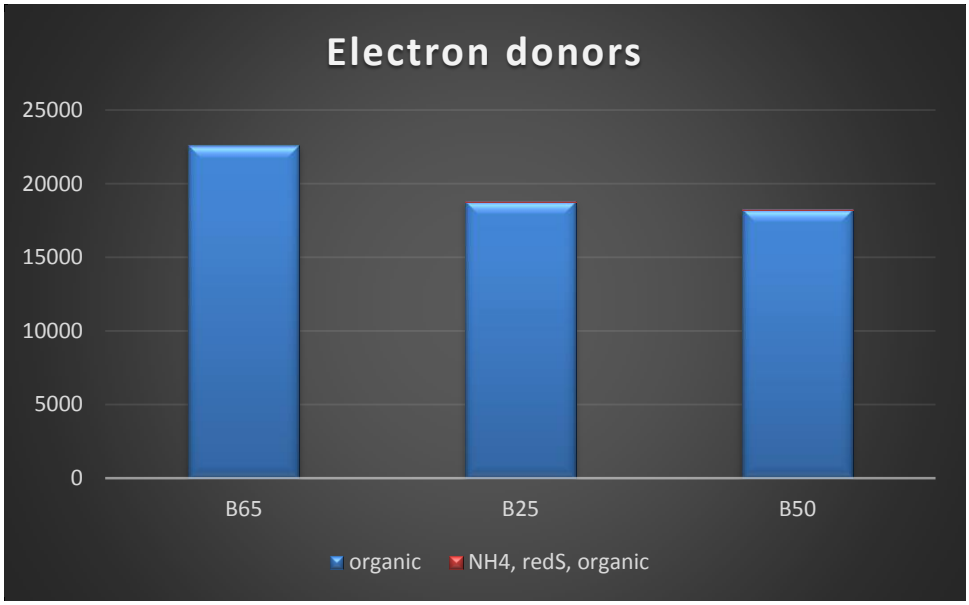

Figure 11 The comparison of electron donors in the somples from different parts of bentonite.

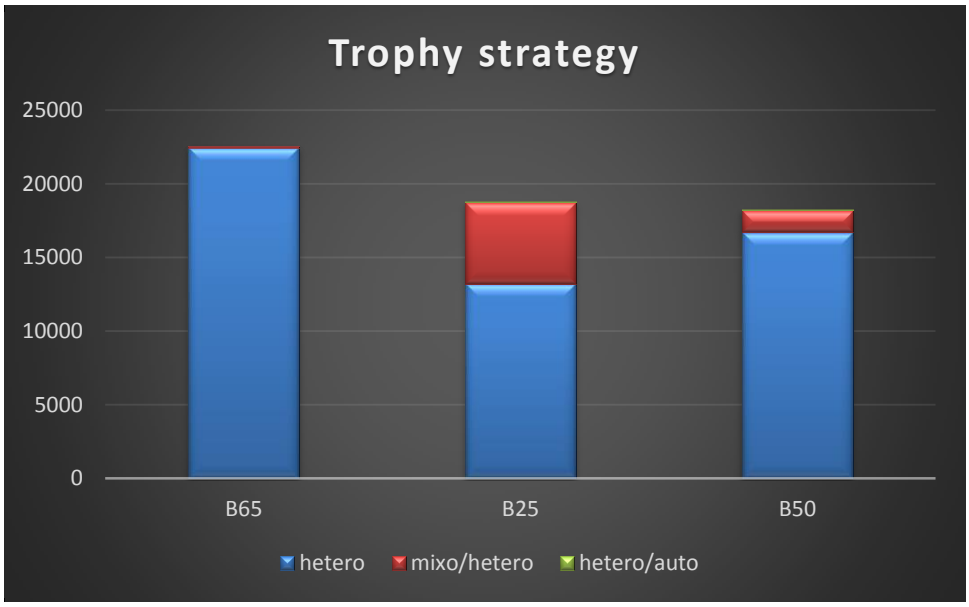

Figure 12 The comparison of different trophy strategies in the somples from different parts of bentonite ("hetero" - heterotrophic,"mixo/hetero" - mixo- or heterotrophic; "hetero/auto" - hetero- or autotrophic).

Nocardia sp. and *Pseudonocardia* sp. were the commonest bacteria present in the samples exposed to 25 °C and 50 °C. In contrast, sample exposed to 65 °C was dominated by *Saccharopolyspora* sp and other detected microorganisms were detected only in low abundancies. Other common bacteria that were detected in the bentonite samples include *Acinetobacter* sp., *Brevibacterium* sp., *Janibacter* sp., *Pseudarthrobacter* sp., *Rhodococcus* sp. or *Streptomyces* spp (see Table 1). Most of detected bacteria belong to ubiquitous soil microorganisms with relatively wide ecological amplitude. Many of them share the versatile metabolism (meaning they can switch from using oxygen to nitrates as terminal electron acceptors) which may enable them to survive (and possibly even propagate) under anaerobic conditions. Another explanation of the above-mentioned results is that the DNA from dead cells might have been trapped and preserved between the layers of the clay minerals in the bentonite.

Table 1 The list of thirty commonest OTUs with their taxonomical classification (to class level) detected in bentonite samples.

	Genus	Family	Order	Class
1	*Saccharopolyspora*	Pseudonocardiaceae	Pseudonocardiales	Actinobacteria
2	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria
3	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria
4	*Streptomyces*	Streptomycetaceae	Streptomycetales	Actinobacteria
5	*Schlegelella*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
6	*Pseudonocardia*	Pseudonocardiaceae	Pseudonocardiales	Actinobacteria
7	*Ottowia*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
8	*Acidovorax*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
9	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria
10	*Hydrogenophaga*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
11	*Flavobacterium*	Flavobacteriaceae	Flavobacteriales	Bacteroidia
12	*Limnobacter*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
13	*Acinetobacter*	Moraxellaceae	Pseudomonadales	Gammaproteobacteria
14	*Nocardioides*	Nocardioidaceae	Propionibacteriales	Actinobacteria
15	*Rhodoferax*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
16	*Vulcaniibacterium*	Xanthomonadaceae	Xanthomonadales	Gammaproteobacteria
17	*Nocardioides*	Nocardioidaceae	Propionibacteriales	Actinobacteria
18	*Azoarcus*	Rhodocyclaceae	Betaproteobacteriales	Gammaproteobacteria
19	*Paracoccus*	Rhodobacteraceae	Rhodobacterales	Alphaproteobacteria
20	*Janibacter*	Intrasporangiaceae	Micrococcales	Actinobacteria
21	*Conexibacter*	Solirubrobacteraceae	Solirubrobacterales	Thermoleophilia
22	*Tepidimonas*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
23	*Arthrobacter/Pseudarthrobacter*	Micrococcaceae	Micrococcales	Actinobacteria
24	*Acinetobacter*	Moraxellaceae	Pseudomonadales	Gammaproteobacteria
25	*Micromonosphora*	Micromonosporaceae	Micromonosporales	Actinobacteria
26	*Tepidiphilus*	Hydrogenophilaceae	Betaproteobacteriales	Gammaproteobacteria
27	*Polaromonas*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
28	*Azonexus*	Rhodocyclaceae	Betaproteobacteriales	Gammaproteobacteria
29	*Zoogloea*	Rhodocyclaceae	Betaproteobacteriales	Gammaproteobacteria
30	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria

Surprisingly high microbial similarity of studied samples might be as well caused by the way of storage of bentonite after the end of the experiment. Samples of bentonites could not be processed imediatelly after dismantling of the experiment and they were stored under anaerobic conditions in room temperature. This could possibly lead to homogenisation of microbial profiles.

4 Bentonite95 experiment in Josef URL

The two-year experiment was focused on the research of chemical and physical interactive processes of an experimental bentonite body placed into the aquifer of the host rock in Josef URL (details in chapter 3.2). These processes were accelerated by heating element in the

middle of the model. The experiment evaluated the interaction of stainless steel, bentonite, heat and rock environments.

4.1 BaM bentonite

BaM bentonite was obtained from Keramost, Obrnice, Czechia. The calcium-magnesium bentonite consisted of particles from 0.5 to 50 µm, median 6.4 µm (Figure 13).

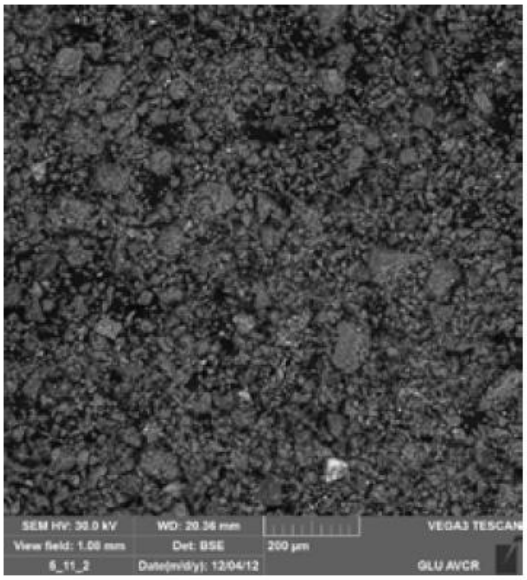

Figure 13. Surface morphology of homogenized BaM bentonite from Černý vrch, Czechia. Images taken by SEM, scale bar – 200 um. Source: Watrad, Prague.

The XRD analysis revealed 78% of montmorillonite, 9% of illite, 9% of SiO_2, 3% of kaolinite, 1% of TiO_2 (anatase) and amorphous phase (Matal 2018).

Bentonite segments used for the construction of the model were compacted/compressed to dry density of ρd =1.75 Mg/m^3. Bentonite compacted to ρd =1.75 Mg/m^3 rate displays low permeability (hydraulic conductivity k=10-13m/s) and is highly swelling when receiving water (swelling pressure σsw= 10 MPa). The moisture content was about 7%.

4.2 Experimental design

A model built of bentonite blocks in the CEG, Prague (similar to model in Mock Up Josef experiment described in Chapter 3.3) was installed in a granitic host rock in 2013 (Figure 14). It was presumed that heat significantly affects the physico-chemical parameters. The heat source was centred in the model and the temperature was set to 95 °C. Stainless steel and carbon steel test bodies and monitoring sensors were placed into different layers of bentonite. Bentonite was analysed in detail both in the stage before activation of the heat

source and after the completion of the tempering stage. In-situ physical parameters were monitored at all stages of the experiment.

Figure 14. Model installation in a drilled well in the Josef URL Czechia. Photo by CEG.

After installing the model, a technological gap (2.5 cm) between the model and the borehole wall was filled with water.

The dismantling began by turning off the heating system in February 2015. The model was open in April, water was drained and the bentonite blocks were carefully removed layer after layer. Each block was photographed, weighted, sealed in vacuumed package and the orientation to heater was indicated by an arrow. Vacuumed samples were then stored in open boxes in the gallery stub in the Josef URL before further analysis.

4.3 Methods

4.3.1 Bentonite sample

Compacted bentonite block (2 kg) from layer 24 (Figure 15) was stored in vacuumed package in Josef URL under *in situ* temperature until May 2018. The bentonite block was then transported in a cooled box to TUL for molecular analysis.

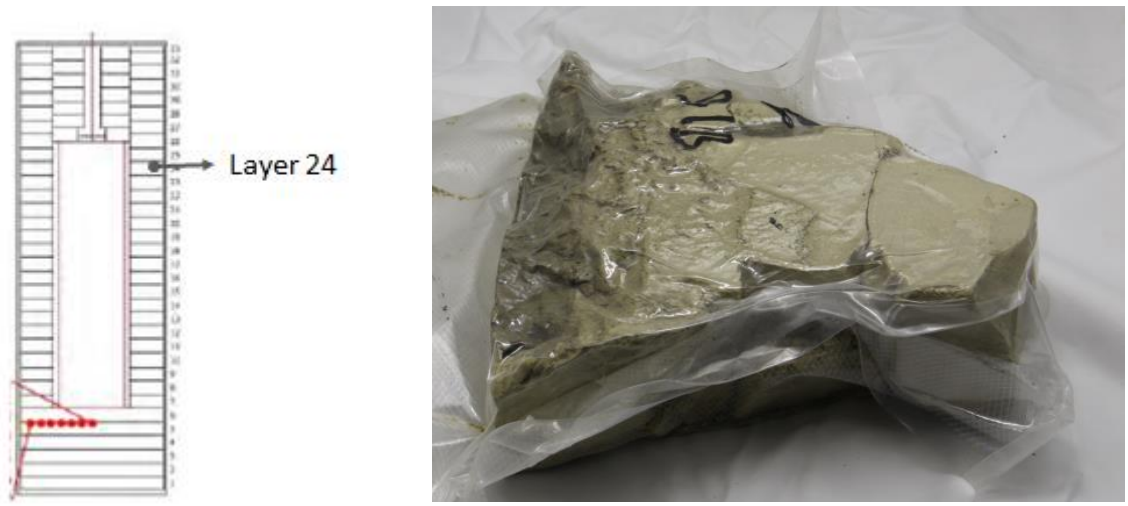

Figure 15. Bentonite block from the layer 24 of the Bentonites95 model in Josef URL. Darker colour – part in contact with host rock; lighter colour – part close to heater. Photo by Petr Novák, Watrad.

4.3.1 Physical-chemical parameters

Hydrochemical analysis of pore water

Hydrochemical analysis including pH, temperature and oxidative-reductive potential (ORP) of pore water samples from five monitoring wells in the vicinity of the main model was performed in the Czech Geological Survey certified laboratories in Prague.

Temperature and pressure

Two types of thermometers were installed to continuously monitor temperature in the bentonite model: thermometer with sensors LM35DZ and digital thermometers. Thermometers sensors were installed with 3 cm-distance of each other to monitor temperature profile. Pressure sensors were also installed in different layers with 3-cm distance of each other.

Saturation of bentonite

Saturation level of bentonite samples was calculated after dismantling the experiment from measured humidity (wet/dry weight ratio) and bentonite density.

4.3.2 Molecular-biology analysis

DNA extraction

DNA was extracted from 7 different parts of the bentonite block (front part originally closest to the heater, top and bottom surface originally in contact with other bentonite layers, central part, right side, middle and left side originally closest to the host rock).

Approximately, 10 g of bentonite from each part was used for DNA extraction. A commercial DNA extraction kit, DNAeasy® Power Max® Soil kit from QIAGEN was used according to manufacturer's instruction. This kit was found to be the best in the MIND round robin study on the optimisation of DNA extraction from the Opalinus Clay rock. The DNA yield was quantified by using a Qubit 2.0 fluorometer.

Library preparation and sequencing

The procedure of library preparation and Ion Torrent sequencing followed the same process as described in section 3.4.1.

Sequencing data evaluation

The raw Ion Torrent reads were processed with Mothur software (Schloss et al., 2009). Low quality reads were removed and sequences were assigned to each sample. Chimeric sequences were identified using UCHIME (Edgar et al., 2011) and subsequently removed. Sequences exceeding 400 bases were trimmed and sequences shorter than 170 bases were removed. Sequences were classified against the Silva database version 128 with a bootstrap value set at 80% and the NCBI database. A cut-off value of 97% was used for clustering of sequences into operational taxonomic units (OTUs). Sequence data were normalized to sample with the least sequences by randomly selecting a number of sequences from each sample. Cluster analysis was performed using the Vegan package in the R statistical package (Oksanen et al., 2012).

Thirty dominant OTUs of each sample were evaluated (as described in the section 3.4.2.) in terms of their oxygen requirements (strictly anaerobic; facultatively anaerobic; aerobic or facultatively anaerobic; aerobic), terminal electron acceptor (O_2 or NO_3; O_2 only; Fe_3 or Mn_4 or O_2 or NO_3; chlorinated substances or O_2 or NO_3; O_2 or NO_2; sulphur compounds), electron donor (organic; H_2 or organic compounds; NH_4 or reduced forms of sulphur or organic compounds; H_2 or sulphur compounds or organic compounds) and trophy type (heterotrophic, mixotrophic; mixo- or heterotrophic, hetero- or autotrophic).

4.4 Results

4.4.1 Hydrochemical analysis of pore water

The pore water can be characterised as Ca-Na-HCO_3 type based on main elements present. Arsenic concentration ranged from 77 to 445 µg/l and pH values ranged from 7.2 to 8.1 in five monitored wells. Temperature was approximately 12°C. ORP indicated slightly reducing conditions during most of the time.

4.4.1 Temperature and pressure

Temperature and pressure were monitored during whole experiment using numerous sensors situated in different levels of the compacted bentonite blocks. One data course example is temperature measured in the bentonite blocks near the heater and in three depth profiles (Figure 16). Temperature was almost constant during the experiment, around 68°C until turning the heater off in February 2015 and then the temperature dropped to 12°C. Pressure was measured in three profiles, the sensors in the upper and bottom profiles were operating only until October and July 2014, respectively, due to corrosion of the sensors. The sensor in the middle part was operating during the whole experiment. The pressure reached 400 kPa in the beginning of the experiment and was decreasing to 220 kPa till the end.

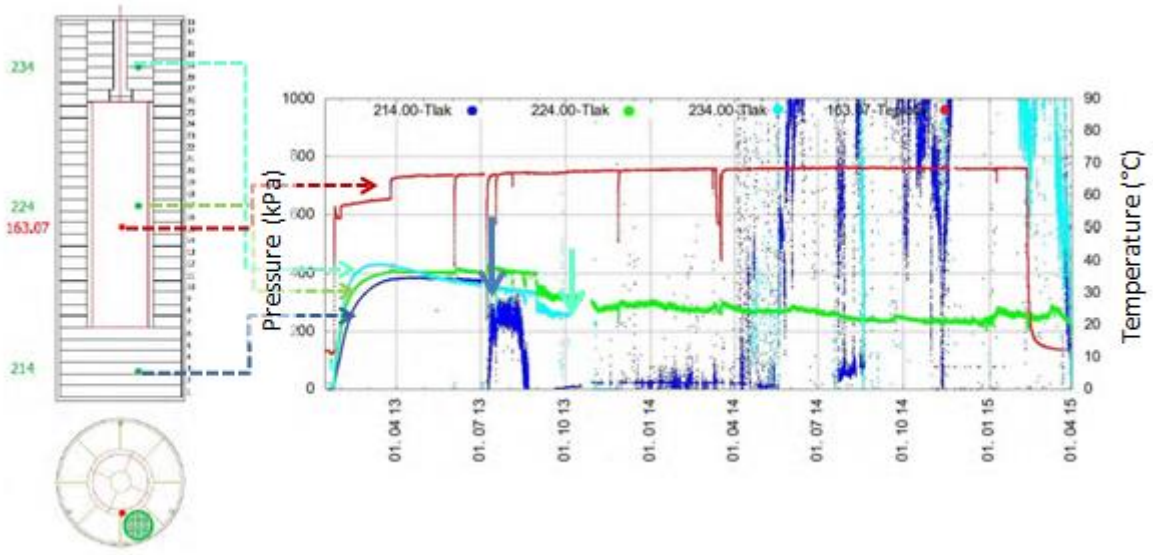

Figure 16. Pressure and temperature data measured from January 2013 to April 2015 in different layers of the model. Red line: temperature; turquoise - pressure in the upper part; green - pressure in the middle part; blue – pressure in the bottom part. Arrows indicate corrosion of the pressure sensors, data after that could not be used. Data were provided by Watrad.

An example of temperature in horizontal profile 148 shows almost constant temperature conditions in different distances from the heater, the highest being closest to the heater (around 75°C) and the lowest being closer to the host rock (around 28°C) (Figure 17).

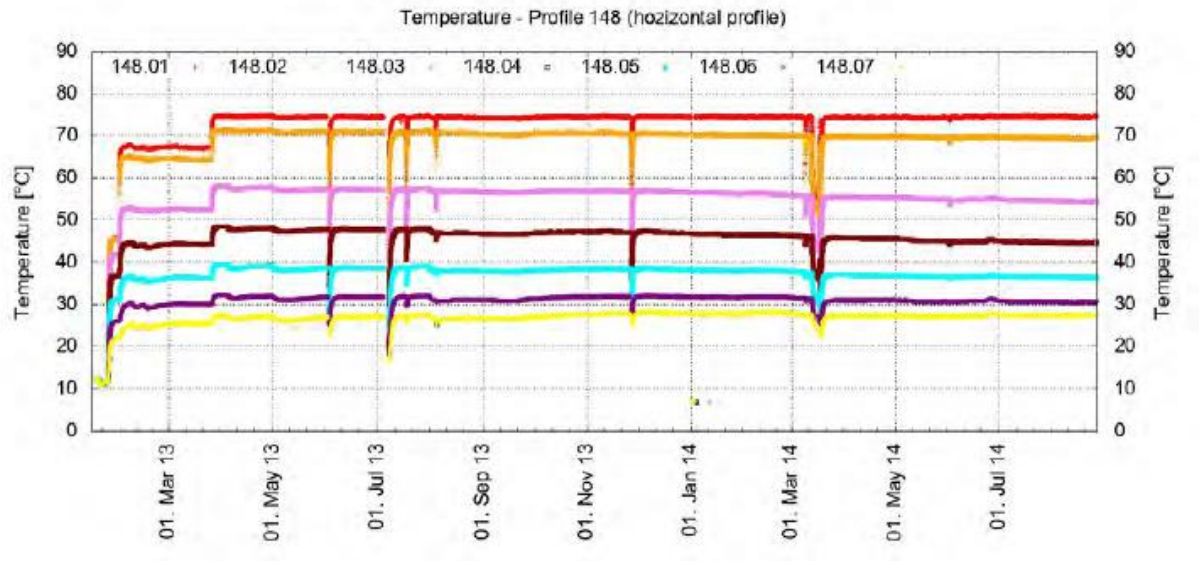

Figure 17. Temperature in horizontal profile 148. Data were provided by Watrad.

Bentonite saturation was calculated after dismantling the experiment. Almost full saturation was reached only in parts that were in direct contact with host rock (Figure 18). Bentonite samples that were closest to the heater and in the heater vicinity were saturated to 40-50% after two years.

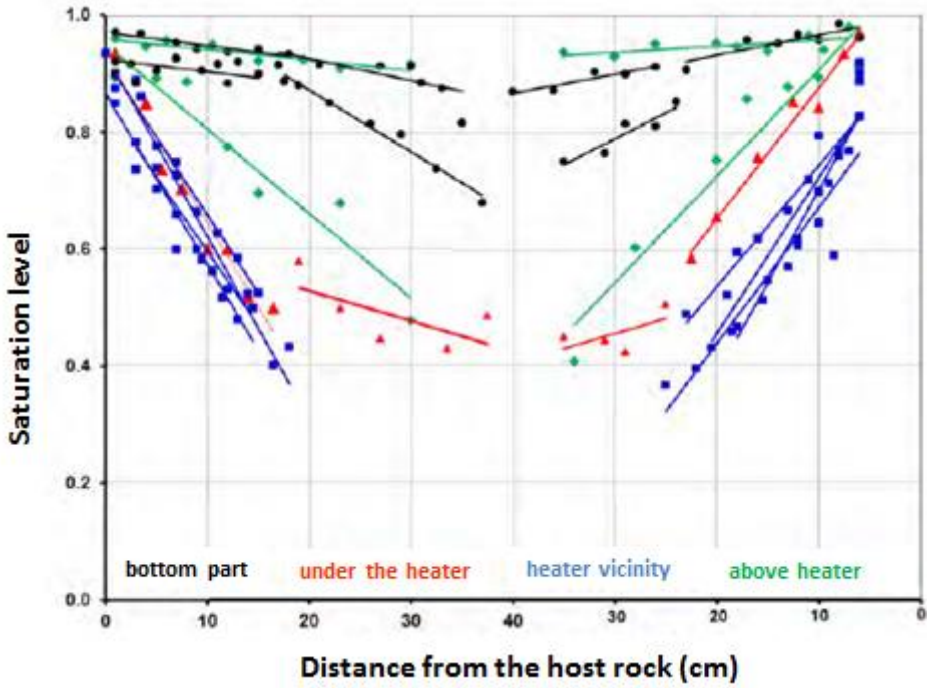

Figure 18. Saturation of bentonite body in the end of the experiment. Black circle - the bottom part, red triangles – directly under the heater body, blue squares – heater vicinity at different depths and green squares – saturation above the heater. Each line represents one horizontal profile. Data were provided by Watrad.

4.4.1 Microbial diversity

The number of sequences for each of samples was in the range of 4,596-18,086. Compared to previous experiment, the number of OTUs retrieved from our dataset was considerably lower: 176 OTUs (90 excluding singleton and doubleton OTUs).

Almost no strict anaerobic bacteria were found in the bentonite samples (Figure 19). Surprisingly, all four strictly anaerobic OTUs detected were retrieved from the top surface of the bentonite. Most of OTUs detected in our dataset can switch from aerobic to facultatively anaerobic metabolism. No clear pattern correlating oxygen requirements with the bentonite sample position was observed.

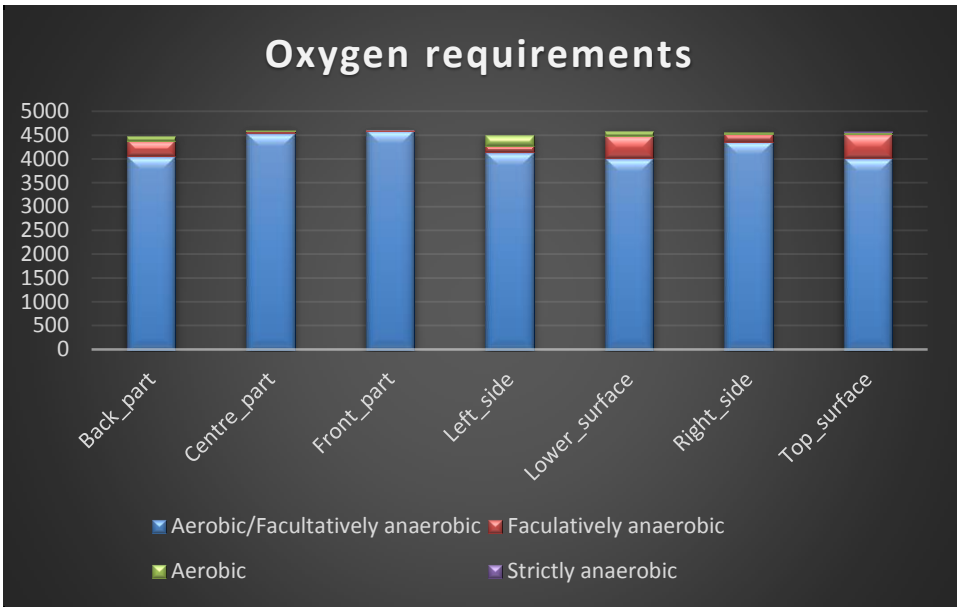

Figure 19 The evaluation of oxygen requirements in different parts of bentonite.

Oxygen and/or nitrates represented the most common terminal electron acceptor detected in all bentonite samples studied (see Figure 20). Other terminal electrons were very rare.

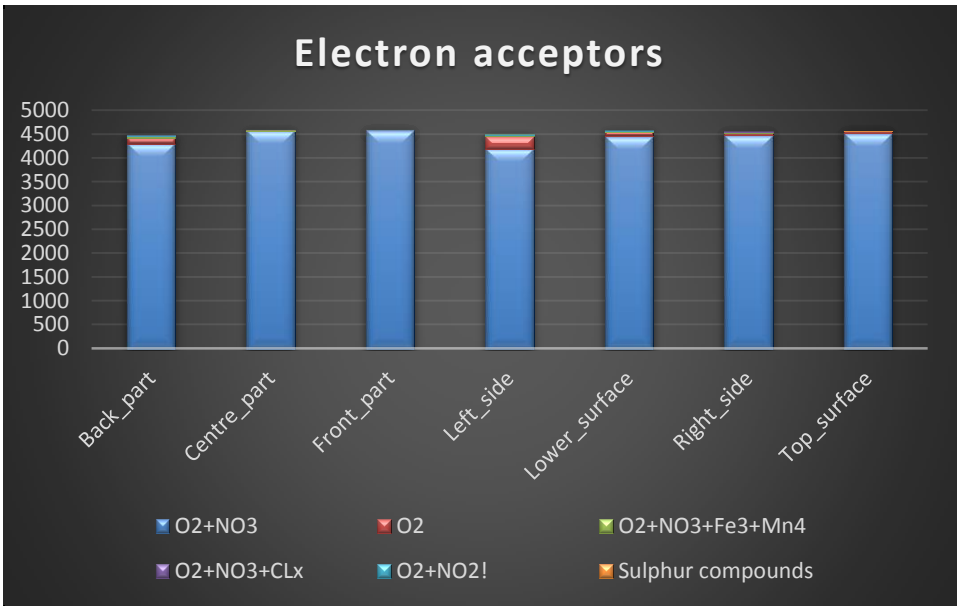

Figure 20 The comparison of terminal electron acceptors in different parts of bentonite.

Electron sources for energy metabolism in all samples are rather uniform – they mainly induce organic mass, occasionally accompanied with hydrogen or thiosulfate.

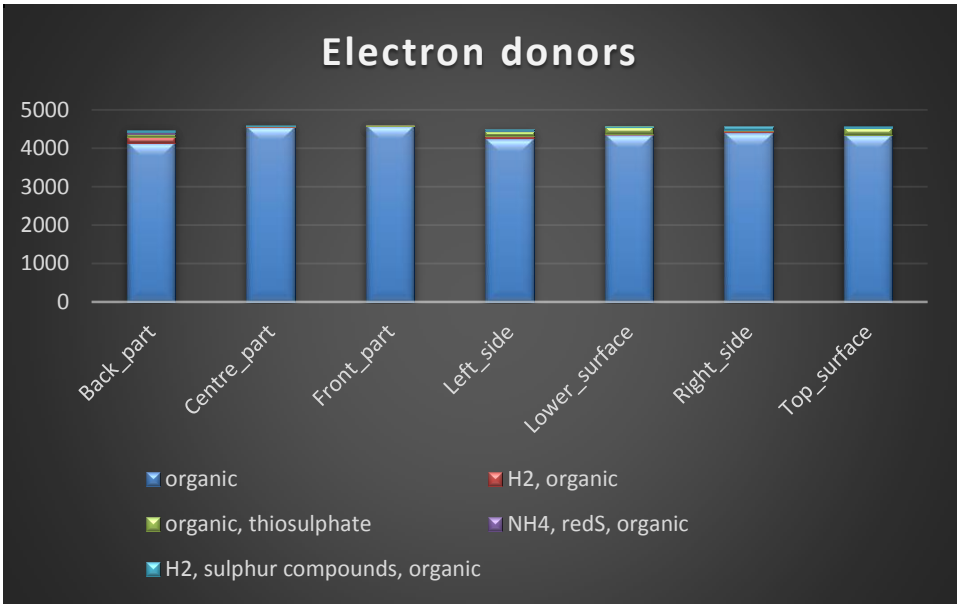

Figure 21 The comparison of electron donors in the somples from different parts of bentonite.

Figure 18 summarizes comparison of trophy strategies among different samples. Considering the above-mentioned results, not surprisingly heterotrophy is the commonest trophy strategy.

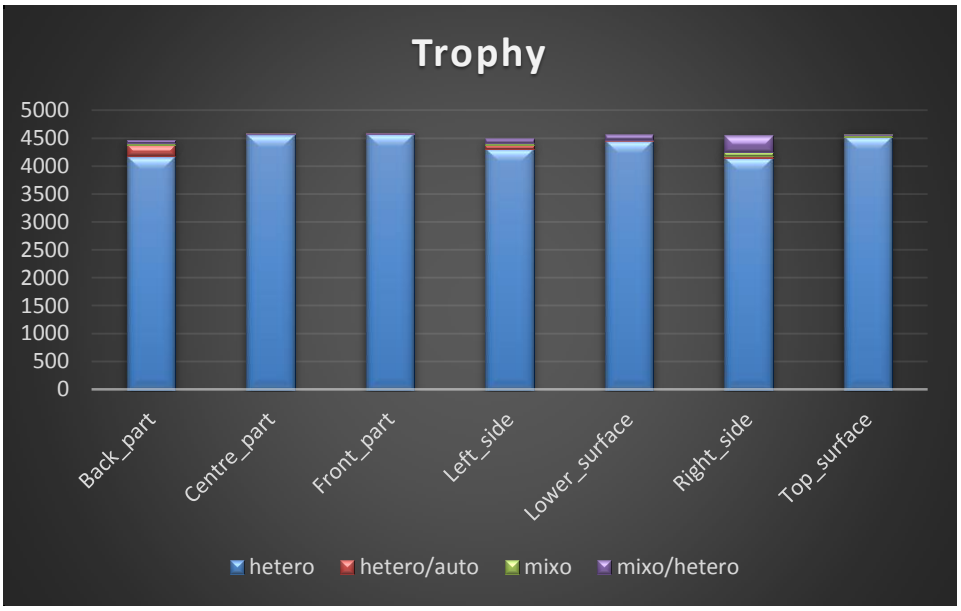

Figure 22 The comparison of different trophy strategies in the somples from different parts of bentonite ("hetero" - heterotrophic, "hetero/auto" - hetero- or autotrophic; "mixo" - mixotrophic; "mixo/hetero" - mixo- or heterotrophic).

All analysed samples showed a surprisingly high similarity with respect to the OTU composition. Ubiquitous aerobic soil bacteria belong to the commonest taxa. Five out of seven samples were dominated by *Saccharopolyspora* sp. Representatives of genus *Pseudomonas* dominated in other two samples and were common in all studied samples. Genera *Streptomyces*, *Schlegelella*, *Pseudonocardia*, *Ottovia* or *Acidovorax* belong to other typical representatives characterising the bacterial communities in all studied samples. Thirty commonest OTUs are shown in **Fel! Hittar inte referenskälla.**.

Table 2 The list of thirty commonest OTUs with their taxonomical classification (to class level).

	Genus	Family	Order	Class
1	*Saccharopolyspora*	Pseudonocardiaceae	Pseudonocardiales	Actinobacteria
2	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria
3	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria
4	*Streptomyces*	Streptomycetaceae	Streptomycetales	Actinobacteria
5	*Schlegelella*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
6	*Pseudonocardia*	Pseudonocardiaceae	Pseudonocardiales	Actinobacteria
7	*Ottowia*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
8	*Acidovorax*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
9	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria
10	*Hydrogenophaga*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
11	*Flavobacterium*	Flavobacteriaceae	Flavobacteriales	Bacteroidia
12	*Limnobacter*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
13	*Acinetobacter*	Moraxellaceae	Pseudomonadales	Gammaproteobacteria
14	*Nocardioides*	Nocardioidaceae	Propionibacteriales	Actinobacteria
15	*Rhodoferax*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
16	*Vulcaniibacterium*	Xanthomonadaceae	Xanthomonadales	Gammaproteobacteria
17	*Nocardioides*	Nocardioidaceae	Propionibacteriales	Actinobacteria
18	*Azoarcus*	Rhodocyclaceae	Betaproteobacteriales	Gammaproteobacteria
19	*Paracoccus*	Rhodobacteraceae	Rhodobacterales	Alphaproteobacteria
20	*Janibacter*	Intrasporangiaceae	Micrococcales	Actinobacteria
21	*Conexibacter*	Solirubrobacteraceae	Solirubrobacterales	Thermoleophilia
22	*Tepidimonas*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
23	*Arthrobacter/Pseudarthrobacter*	Micrococcaceae	Micrococcales	Actinobacteria
24	*Acinetobacter*	Moraxellaceae	Pseudomonadales	Gammaproteobacteria
25	*Micromonosphora*	Micromonosporaceae	Micromonosporales	Actinobacteria
26	*Tepidiphilus*	Hydrogenophilaceae	Betaproteobacteriales	Gammaproteobacteria
27	*Polaromonas*	Burkholderiaceae	Betaproteobacteriales	Gammaproteobacteria
28	*Azonexus*	Rhodocyclaceae	Betaproteobacteriales	Gammaproteobacteria
29	*Zoogloea*	Rhodocyclaceae	Betaproteobacteriales	Gammaproteobacteria
30	*Pseudomonas*	Pseudomonadaceae	Pseudomonadales	Gammaproteobacteria

The microbial communities present in different parts of bentonite were characterised by the metabolism which uses oxygen or nitrates as terminal electron acceptors. The typical soil bacteria that were detected by the microbiological analyses are characterisef by the ability to switch from the aerobic metabolism (using oxygen as terminal electron acceptor) to the anaerobic metabolism in which nitrates represent the terminal electron acceptor. These bacteria were hence probably able to survive also in the unaerobic environment of the in-situ experiment. Another possible explanation is that a high portion of sequences retrieved from the bentonite may belong to spores or dead biomass. Further analyses employing RNA or live/dead staining should be used to test these hypotheses in the future.

Similarly to the previous experiment, the bentonite samples could not be microbiologocally analysed immediately after the end of the experiment. The way of storage (under anaerobic conditions at

approximately 12 °C) could have possibly affected the results of microbial analyses. Possible differences in the microbial profiles obtained from different parts of bentonites (exposed originally to different temperatures and water content) might have been replaced by more homogeneous microbial structure due to the fact that different parts of the compacted bentonite were exposed to the similar conditions during the time of storage.

5 Conclusions

In this study, microbial diversity present in the compacted long-term exposed bentonites from two in situ experiments ("Mock-Up experiment" and "Bentonite95 experiment") performed under near field conditions has been studied. The compacted bentonite buffer was exposed under conditions similar to a high-level radioactive waste repository in crystalline host rock. We used 16S rDNA amplicon sequencing to determine the microbial profiles in different parts of bentonite samples.

The microbial communities detected in the bentonite samples were surprisingly similar and were characterised by the dominance of the heterotrophic, aerobic or facultatively anaerobic capable of respiring oxygen or nitrates. Almost no strict anaerobic or autotrophic bacteria were detected. Most of detected bacteria represent typical soil or ubiquitous microorganisms (e. g., *Acinetobacter* sp., *Blastococcus* sp., *Brevundimonas* sp., *Flavobacterium* sp., *Halomonas* sp., *Nocardia* spp., *Pseudomonas* spp., *Pseudonocardia* sp., *Saccharopolyspora* sp., *Streptomyces* spp.) with wide ecological amplitude enabling them to survive under various conditions. These results suggest that microorganisms found in the bentonite samples in this study were most probably present in the bentonite already before the start of the experiment. Further study is needed to determine whether the uncovered microbial diversity represents a metabolically active microbial community able to survive under harsh conditions present in the compacted bentonite rather than a DNA originating from dead cells, trapped and preserved between the layers of the clay minerals in the bentonite. Due to a high microbial similarity among the samples we were not able to correlate differences in microbial community structure from different parts of bentonites exposed to different levels of physical parameters (temperature, humidity). This might be, however, caused also by the fact that analysed samples could not be processed immediately after the end of the experiments and were stored in the conditions that might have led to the homogenisation of the microbial communities among the samples.

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