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Microbial mobility in saturated bentonites of different density

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Publishable Summary

Indigenous bacteria are naturally present in bentonite materials to be used as an engineered barrier in deep geological repositories of nuclear waste. Their density is generally decreasing with the compactness of the bentonite, but they remain present even in densities above 2000 kg/m^3 presumably in the form of spores characterized by unbelievable durability. Other factors, such as temperature or water availability do not limit the Gram-positive spore-forming bacteria predominating among indigenous bacteria as much as they do in the case of the non-spore-forming bacteria. Studies specifically focused on the spore-forming bacteria are thus highly needed.

The second source of bacteria that may affect bentonite in deep geological repository is contamination from the surrounding environment, mostly pore water with predominating Gram-negative non-spore-forming bacteria. Moreover, bacteria will be inevitably introduced from the surface during construction and operation of the repository, before its final closure. The speed of penetration of allochthonous bacteria into the bentonite barrier is strongly dependent on the swelling pressure and composition of the clay material, on hydrogeological conditions and on the stability of the bentonite/host rock interface.

Our study fulfilled two most important goals. First, we developed a reliable method for direct detection of bacterial presence (both viable and dead cells) in the bentonite, which has been missing. Our method is based on the extraction of bacteria from bentonite using density gradient centrifugation and their subsequent Live/Dead fluorescence staining. Although our method needs further optimization and testing of its general functionality on different bentonite types, we believe it will be very useful for future research of bacterial presence in various clay materials.

Our second goal was to study microbial mobility within compacted BaM bentonite from Czechia of two different dry densities – 1400 kg/m^3 and 1600 kg/m^3. Fourteen sections of saturated bentonite samples differing in their distance from the source of natural bacterial community (Josef URL, Czechia) were inspected for the presence of bacteria. Viable cells were observed in each section of both bentonite densities tested. This finding indicates that bacteria are able to move through saturated bentonite of even higher dry density (1600 kg/m^3) and thus higher swelling pressure of about 5 MPa. Czech BaM bentonite belongs to Ca-bentonites that might generally allow for better microbial mobility due to its physical properties, different from Na-bentonites. Based on previous studies, bacterial mobility in highly compacted Na-bentonite (≥ 1800 kg/m^3) is very limited. Therefore, further tests are needed to reveal whether the surprisingly high bacterial mobility detected in our experiment was caused solely by the lower bentonite density used, or by the unique properties of BaM bentonite (or Ca-bentonites in general), or if our novel method is more sensitive than previously used methods.

Contents

1 Introduction

1.1 Microbial ecology and occurrence in bentonite

1.1.1 Microorganisms in groundwater

The knowledge about the composition of microbial communities in groundwater is crucial, as it represents one of the two major sources of possible microorganism contamination of underground waste repositories and can also influence the indigenous biota naturally present in bentonite, which is second source of repository microbial contamination.

Microbes can grow in any environment in the presence of liquid water if their nutrient and energy requirements are met and if they can tolerate the environment physiologically (Stroes-Gascoyne and West, 1997). Not surprisingly, microbes (including groups of specific interest such as sulphate-reducing prokaryotes, acetogens or iron reducers discussed below) have been shown to be present in many relevant geological formations, both in groundwater and in the host rock (Fru and Athar, 2008; Hallbeck and Pedersen, 2012; Humphreys et al., 2010; Pedersen et al., 2008, 2014). The particular characteristics of the active microbial population is always specific to the particular environment (Humphreys et al., 2010) and microbial communities and cell densities also varies as a function of sampling depth (Eydal and Pedersen, 2007; Pedersen et al., 2008).

Granitic aquifers, which are matter of choice in various waste disposal concepts in Europe (Humphreys et al., 2010) contain a relatively diverse, and mostly Gram-negative non-spore-forming mesophilic microbial communities (Fru and Athar, 2008; Pedersen, 1997). The sampling of groundwater from various depths (3-1000 m) in nearly 100 sites across Fennoscandian Shield was used to estimate the overall microbial density present in groundwater. The observed viable cell numbers ranged from 10 to 10^5 cells ml^{-1}, while the observed total cell number ranged from 10^3 to 2×10^6 cells ml^{-1}. Shallow groundwater (depths of 3–15 m) generally contained more cells than deep groundwater from depths of 20–1000 m (Eydal and Pedersen, 2007). Bacterial load also seems to be positively correlated to the concentration of dissolved organic carbon (Pedersen et al., 2008).

1.1.2 Microorganisms in bentonite deposits

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, 2010; Karnland et al., 2006; 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). 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 (Lima and Sleep 2007). 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).

Although bentonite definitely represents unusual and extreme environment, bentonite deposits are not sterile. Fukunaga et al. (2005) detected microbial activity of mostly mesophilic bacteria at four sites in two different bentonite deposits in Japan and reported that the activity is lower than in typical soils and aquatic sediments. Similar results were reported by (Boivin-Jahns et al., 1996) in Belgian Boom clay. Fru and Athar (2008) and Pedersen et al. (2000a) reported natural occurrence of the spore-forming Gram-positive

bacteria in MX-80 bentonite. Svensson et al. (2011) searched various natural bentonite samples for the presence of microorganisms with special attention to aerobic heterotrophs, anaerobic sulphate and iron reducers and autotrophic acetogens, because of their abilities to affect the storages for nuclear waste in different ways. Various mesophilic and thermophilic microorganism belonging to these groups were detected in all eleven studied types of bentonite, although the differences in microbial abundances and compositions were generally large.

Aerobic heterotrophs respire oxygen and thus could be of importance especially at the oxidative phase of the repository development following the waste disposal (see below). Aerobic microorganisms reduce the oxygen in the buffer environment much faster than it happens by abiotic processes, which could be beneficial for the repository, since oxygen itself is corrosive to metal canisters (Bennett and Gens, 2008). However, many aerobic microorganisms produce compounds not favourable for a HLW repository, such as organic acids and bioligands that can mobilize several radionuclides (Johnsson et al., 2006).

Anaerobic iron reducers may represent a threat mostly to the bentonite buffer itself. These microorganisms are capable to rapidly reduce iron present in iron-rich smectites changing the swelling montmorillonite to non-swelling illite (Kim et al. 2004), which may dramatically reduce swelling and sealing capacity of the buffer material (Stucki et al., 2009). Iron reducing ability was reported also in the genus Bacillus, which was reported as member of indigenous bentonite fauna (Matschiavelli et al., 2017).

Sulphate-reducing bacteria (SRB) are obligate anaerobic organisms mostly belonging to Deltaproteobacteria, which utilize a wide range of carbon substrates or grow autotrophically on molecular hydrogen and carbon dioxide (Muyzer and Stams, 2008). SRB are of particular interest because of their prominent role in anaerobic metal corrosion either indirectly due to their ability to dissimilatory reduce sulphate to hydrogen sulphide, which corrodes canister materials (chemical microbially influenced corrosion), or directly via direct withdrawal of metal electrons (electrical microbially influenced corrosion) (Enning and Garrelfs, 2014). Independent studies proved, that SRB, although they are not easy to detect by means of cultivation approaches (Pedersen et al., 2000b) represents one of the most frequent and important metabolic groups of bacteria occurring in natural bentonite samples (Masurat et al., 2010a; Svensson et al., 2011). Some of them show extreme tolerance to desiccation, high temperatures and salinity (Masurat et al., 2010b). Notably, the sulphide is produced not only by the dissimilatory reduction of sulphate by SRB, by also thiosulphate and sulphur by sulphide-producing bacteria (SPB), which are also commonly present in bentonite deposits (Pedersen, 2017). Except for the direct corrosion effect, microbially produced sulphide also reduces ferric iron naturally occurring in most commercial bentonites to ferrous one. This chemical process is responsible for relatively high bentonite immobilisation capacity for diffused sulphide, which can significantly reduce the mass of sulphide that corrode metal canisters (Pedersen et al., 2017), but on the other hand the reduction of ferric iron to ferrous one may have destabilizing effect on montmorillonite similar to the effect described for iron-reducers (Kim et al., 2004; Lantenois et al., 2005).

Except for presence of SRB, many of which are heterotrophic and thus require presence of organic carbon source, studies also confirmed the natural occurrence of autotrophic acetogens producing acetone from inorganic H_2 and CO_2 in natural bentonite (Fru and Athar, 2008; Svensson et al., 2011). Interestingly, a correlation between produced acetate and sulphide was repeatedly observed, suggesting that acetogenesis boosted the sulphate-reducing populations in the clay mineral systems (Bengtsson et al., 2017; Svensson et al., 2011). The other physiological group of bacteria such as nitrate-reducing bacteria that have been implicated in metal corrosion (Enning and Garrelfs, 2014) are commonly present in bentonite deposits (Jalique et al., 2016; Svensson et al., 2011).

1.1.3 Microorganisms under repository conditions

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 or cement (Bennett and Gens, 2008). 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 (2000 kg/m^3) and 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 oxidizing 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, which is the main scope of this review, 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.

1.1.4 Effect of pressure

In most of the waste disposal concepts, the deposited HLW canisters will be surrounded by compacted bentonite as one of the multiple barriers (Bennett and Gens, 2008). 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 mineralogy 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 0,01um 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 sulphide (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).

Although cultivable bacteria were 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), 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 2000 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., 2010b; 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). 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 with the density threshold for metabolic activity (detected by the production of radioactively marked Cu_2S) varying between 1690 kg/m^3 (Rokle) and over 2000 kg/m^3 (Boom Clay). Moreover, acetate formation from natural organic matter was observed at all wet densities indicating presence of bacterial activity at the higher bentonite densities, when sulphate production could not be detected (Pedersen, 2017). Similarly, Bengtsson and Pedersen (2016) reported no clear cut-off in Boom clay density with respect to presence and activity of cultivable SRB, sulphide production was increasingly possible at or below 1800 kg/m^3.

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 shortly review the effect of some of these factors below.

1.1.5 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 mentioned below.

The surface temperature of the waste canisters is expected to be about 90-95°C, in some concepts will even exceed 100°C, and high temperature is expected to be one of the important protections from microbial life (Bennett and Gens, 2008). 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. (2010b) 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 48 h. 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.

1.1.6 Effect of desiccation

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 H_2O for microbial processes can be represented by the water activity (a_w). Most bacteria grow well at a_w around 0.98 but relatively few species can grow at a_w 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. Many subsurface microorganisms have mean generation times of up to thousands of years (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). Not only dormant-state spores, but also vegetative cells of some desiccation-tolerant bacteria can maintain viability in the dry state. The maximum time of survival in this dormant state is however unknown (Billi & 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 (Stamp, 1947). 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 a_w 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.

1.1.7 Studies under repository conditions

Up to date and for obvious reasons, there is no study performed under all the extreme conditions present within a real HLW repository and in the reasonably long time scale, so the evaluation of possible microbial effect on it is always challenging. In most cases, we have to

rely on investigation performed under realistic repository conditions without radiation performed in underground labs e.g. (Arlinger et al., 2013; Jalique et al., 2016; Lydmark and Pedersen, 2011) or on mathematical modelling approaches e.g. (King et al., 1999; Zhao et al., 2014).

Further we mention studies evaluating the effect bacterial survival in the bentonite at different compaction densities under repository conditions without radiation. Pedersen et al. (2000b) tested viability of various introduced aerobic and anaerobic bacteria in compacted bentonite under high-level radioactive waste repository conditions in the time frame of 15 months. Their results confirmed the detrimental effect of swelling pressure on bacterial survival in the compacted bentonite with the temperature being further limiting factor in bacterial growth and proliferation. The spore-forming microorganisms were the only one, which survived high pressure and high temperature conditions in repository for 15 months but in much lower numbers, than inoculated.

Stroes-Gascoyne et al. (2002) studied the response of buffer to resaturation by groundwater over a 6.5-year period by placing 2.4 m^3 of clay-based buffer (18% moisture content, density not stated, developed total pressure 1.2–2.1 MPa at the end of the test) in a borehole at the 240 m level of AECL's Underground Research Laboratory. Results suggested presence of the viable population of cells in the buffer. The constrictive and nutrient-poor conditions in buffer environment resulted in the loss of microbial activity and cultivability but not viability. The interfaces between buffer and surrounding environment may be preferred sites for microbial activity and transport, the microbial populations at interfaces were an order of magnitude larger than inside the bulk buffer. Authors further detected the presence of cultivable SO_4-reducing bacteria, although SO_4 reduction was not a dominant process, and they detected slight reduction in oxygen level near the surface but not deeper inside the buffer. The overall results suggested that microbial processes were depressed in the buffer but may have been more active near the concrete/buffer interface. No significant evolution towards reducing conditions occurred during the duration of the experiment.

Aoki et al. (2010) performed 440 day long experiment resembling repository conditions using bentonite, OT-9607, with mineral composition similar to Kunigel V1. The underground compacted bentonite block included internal heater. The bentonite was heated for 260 days (to 100°C) followed by the cooling phase of 180 days. The sampling of bentonite at various depths and distances from the heater was performed at the end of each phase and authors searched for the presence of microbes. Although, they were able to detect only the aerobic heterotrophs due to methodical obstacles in the work with bentonite samples, they found strong correlation between the presence of living heterotrophs and water content (influenced by heat and desiccation). No living heterotrophs were detected in places with the water content below 12%. These results confirms the hypothesis that microbial activity might be severely limited near waste container in the vault for some time after disposal, due to desiccation as a result of the heat output of the waste container.

Lydmark and Pedersen (2011) performed analysis of microbial diversity in compacted MX-80 bentonite (density over 2000 kg/m^3) after five years under authentic KBS-3 storage

conditions in the rock. The only source of microbial life in bentonite could be indigenous bentonite microorganisms or bacteria naturally present in groundwater. They detected presence of viable and active sulphate-reducing bacteria, acetogenic bacteria and heterotrophic aerobic bacteria in collected bentonite samples by means of cultivation experiments, measurements of sulphide and acetate production and ATP measurements. Detected numbers were lower than in underground water surrounding repository. Most of the bacteria were detected in the bentonite buffer close to the rock but in a few samples also in bentonite close to the copper canister. Bacteria from various distances from the rock and the inside of the bentonite were viable and active, but the higher temperatures in some parts of the repository (up to 85°C), obviously made it more difficult for the bacteria to survive.

In the subsequent experiment Arlinger et al. (2013) reported low numbers of bacteria in buffer and on canister surfaces, but they detected presence of anaerobic thermophilic bacteria and other thermophilic aerobic bacteria with the potential to form spores in buffer areas with high water saturation and low density and traces of (probably unviable) SRB on canister surface. Authors hypothesized that these bacteria must have been dormant since the formation of the bentonite blocks, but viable when given appropriate growing conditions.

Finally, Stroes-Gascoyne et al., (2010) short term study and Jalique et al. (2016) eight years long study with highly-compacted Wyoming MX-80 bentonite under repository conditions revealed that a dry density ≥ 1.600 kg/m^3 (resulting in a swelling pressure above 2000 kPa, aw < 0.96 and an average pore size < 0.02 µm) suppressed cultivability of bacteria (and presumably activity and growth). Viable cell counts were similar to those in the original non-compacted bentonite and most of the detected bacteria belonged to Gram-positive spore-formers.

1.1.8 Microbial migration in compacted bentonite

Size distribution of pores and pore throats is an important factor controlling microbial activity in soils. Small pore throats limit the transport of microbes and nutrients (Itävaara et al., 2011). Although there is huge number of bacteria living in various types of soils, it is largely unknown how bacteria live and move through pores of very small size (Männik et al., 2009). Filtering through the pores of 0.1 µm size is generally considered to be sufficient to sterilize the solution, but microbial ability to pass through 0.1 µm pore size filters and grow thereafter was reported (Wang et al., 2007).

Männik et al. (2009) performed detailed study on bacterial growth and motility in cavities smaller than one micrometre. These authors showed that *Escherichia coli* and *Bacillus subtilis* are motile in channels with the width exceeding their diameters only by 30%. For smaller widths, the motility vanishes but bacteria can penetrate even narrower channels by initiating growth into the channels, when elongation and division pushes bacteria forward until they fill the whole channel. While *B. subtilis* exhibits less morphological plasticity than

E. coli and is able to growth through channels of similar width as its diameter, *E. coli* are even able to penetrate channels twice smaller than their diameter (penetration threshold is 0,3-0,4 µm channel diameter). Such growth results in drastic but reversible morphological change of *E. coli* cell shape and reversible loss of motility. Interestingly, repeated transitions from aberrant to regular cells occur as the bacterial population repeatedly advances from one chamber to the next through the narrow tunnels. The difference in motility of both species was ascribed to the difference in the cell wall thickness and composition, which is caused by a clear trade-off between the motility (and morphological variability) and pressure tolerance. Sub-micrometre-size pores and cavities thus can be much more prolific bacterial habitats than previously assumed.

Natural bentonite and other clay types are typical for their compact structure and small pore sizes, with gradual decrease of pore sizes in bentonite compacted for use as the repository buffer (Appelo, 2013). However, as we demonstrated above, bacterial survival and activity is possible even is such extreme conditions and presence of microorganisms belonging to wide range of species and metabolic groups was repeatedly reported from various clay types under different environmental conditions. Nevertheless, the key question remains, how exactly these microorganisms managed to inhabit the clays and how fast process such bacterial penetration is.

The information about the motility of bacteria in compacted bentonite is rather scarce. The conducted laboratory experiments almost identically reported existence of bacterial motility in compacted bentonite but in a very limited form. Stroes-Gascoyne and West (1997) studied mobility of introduced *Pseudomonas stutzeri* with cell size 1-3 x 0.5-0.8 µm (Niel and Allen, 1952) in the MX-80 bentonite compacted to the densities of 1680 and 1800 kg/ m^3. Results showed that, although the bentonite plugs were slowly saturating and water activities were suitable for microbial growth (aw > 0.96), no evidence for *P. stutzeri* could be found in the plugs except for the first 0.5 cm of the plugs in 16 week period of the experiment. Similarly, Fukunaga et al. (2000) studied transport of *E. coli* with mean diameter of 0.76 µm (Männik et al., 2009) through compacted buffer material consisting of bentonite and silica sand with a density of 1200, 1600 or 1800 kg/m^3. The bacteria freely diffused when the proportion of Na-bentonite was 20 wt % or that of Ca-bentonite was 50 wt %. Bacterial movement was less than 5 mm in three weeks when the proportion of Na-bentonite was 70-100 wt % or that of Ca-bentonite was 100 wt %. Lastly, Pedersen et al. (2000a) studied the process of bacterial migration into compacted MX-80 bentonite clay (1900 kg/ m^3 dry density) during swelling phase using various aerobic and anaerobic bacterial species in four different experimental temperatures. Bentonite infested with bacteria was sampled for the presence and activity of bacteria at three depth zones 0-1, 1-3, 3-6 mm in four times - 8 h, 2, 12 and 28 weeks. In general, fewer cultivable bacteria were detected with increasing depth and experimental duration. The highest numbers of cultivable bacteria were observed after 8 h and the aerobic bacteria were detected in higher depths, than anaerobes regardless of time. Some bacterial species remained cultivable at all sampled depths during the whole

course of the experiment at lower temperatures, but their numbers significantly decreased. None of the species remained cultivable after 28 weeks in 80°C.

Although above mentioned laboratory studies showed, that bacterial motility in compacted bentonite is principally possible, they operated on a very short time scale of a few weeks or mostly months. This is definitely not sufficient, when reported generation times in subsurface microorganisms could be several thousands of years (Jørgensen and Boetius, 2007) and the assumed HLW repository cool-down time can be up to several hundreds of years (King et al., 2017). Indirect evidence of the possible bacterial mobility can give us studies on microbial compositions of clay deposits. Boivin-Jahns et al. (1996) and Fukunaga et al. (2005) studied the presence of indigenous microorganism in natural undisturbed clay deposits and both identically reported decrease in bacterial densities as a function of sampling depths. Such results may imply that the bacterial movement through natural bentonite is a slow process. Similarly, Lydmark and Pedersen (2011) in their 5 years underground experiment reported, that most of the detected bacteria were found in the bentonite buffer close to the rock and could thus represent the microorganism originating from the surrounding groundwater.

The most relevant study concerning microbial mobility in situ conditions was performed by (Fru and Athar, 2008). These authors studied how indigenous groundwater bacteria interact with MX-80 bentonite buffer materials in the long term HLW repository simulating experiments conducted in the aquifers. They concluded that the repository simulating conditions appeared to retard the overall success of the predominantly Gram-negative mesophilic groundwater microorganisms. The spore-forming Gram-positive bacteria commonly found in the clay predominated, suggesting that the non-spore-forming microorganisms and Gram-negative microorganisms predominant in granitic ground-waters might not form a prominent portion of the bacterial community that will develop in bentonite under repository conditions. Furthermore, the complete absence of the Gram-negative microorganisms in the bentonite samples that incubated at ≥ 67°C in contrast to their presence in the lower temperature blocks implied that their survival was most likely controlled by temperature and not by compaction density or the porosity of the bentonite. Authors described some bacterial lineages unique for bentonite or groundwater. Interestingly, some bacterial lineages showed a potential to coexist with both the bentonite and groundwater (e.g. *Sedimentibacter* and *Desulfosporosinus*). Although SRB appeared not to predominate in the clay, they were sufficiently active to mediate sulphate reduction with the resultant production of hydrogen sulphide. Authors further specified the microbial lineages that might survive the longest in repository such as *Desulfosporosinus*, *Pseudomonas stutzeri*, *Bacillus megaterium*, and *Sedimentibacter hydroxybenzoicus*.

1.2 Methods for detection of bacteria in bentonite samples

Here, we shortly discuss some of the most important methods used to detect and distinguish bacteria in the sample with the special emphasis on the methods applicable for bentonite samples. We will show that bentonite is very specific medium, which often disables the

usage of common microbiology methods or requires at least significant optimization of traditional working protocols.

Microbial cells can be either viable (are able to reproduce) or non-viable (damaged or dead), viable cells can further be active or non-active (Olsen et al., 2016). The simple bacterial presence in the studied sample thus does not necessarily imply that the bacteria must be active, it only states that bacteria are able to become activated when a suitable environment presents itself (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. Nonetheless, 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 in form of endospores can survive for a very long time, sometimes for millions of years as was described in subseafloor sediments (Jørgensen and Boetius, 2007).

There are several possible attitudes, how to study the bacterial presence, viability and activity in bentonite samples. The easiest way, how to detect the presence of bacteria in studied sample and enumerate their density is direct fluorescent staining followed by epifluorescence microscopy or flow cytometry (Olsen et al., 2016). There are various commercially available dyes, such as frequently used acridine orange or DAPI which indiscriminately dyes all present cells. Other dyes selectively stain either viable cells (e.g. carboxyfluorescein diacetate acetoxymethyl ester - CFDA-AM) or dead (damaged) cells (e.g. propidium iodide). It is possible to distinguish between viable cells with intact cell walls and dead (damaged) cells in the sample (live/dead staining) by combining dyes of various properties (Itavaara et al., 2008). Although these staining methods work well when studying water samples, electronegatively charged bentonite (for details see 2.1.2) negatively interferes with often electrically charged dyes, which excludes most of the commonly used dyes (such as DAPI or acridine orange) from use on bentonite samples (Aoki et al., 2010; Stroes-Gascoyne et al., 1996). The CFDA-AM belongs to dyes used for detection of viable cells and has uncharged molecule that can permeate cell membranes. Because of electroneutral state of CFDA-AM, this dye was successfully used to detect bacteria in bentonite samples (Fukunaga et al., 2005). We further successfully used LIVE/DEAD™ BacLight™ Bacterial Viability Kit for microscopy (Invitrogen) for monitoring the viability of bacterial populations in bentonite samples. This live/dead staining kit consists of two dyes – SYTO 9 generally labels all bacteria in a population (those with intact membranes and those with damaged membranes) while propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present (kit manual).

Viability of bacteria can be further measured by means of various cultivation methods using either liquid or solid media. Plate counts are commonly used to enumerate aerobic heterotrophic microorganism in bentonite (Svensson et al., 2011). Although the cultivation of anaerobic microorganisms is generally more challenging and requires very accurate and

complex media, various anaerobic bacterial groups of interest present in bentonite, such as acetogens, iron reducers or sulphate reducers, can be cultivated and enumerated by means of most probable number method (Haveman and Pedersen, 2002). The major drawback of all cultivation methods is that only a minor fraction of the bacteria present in the sample is generally cultivable. Furthermore, as we will demonstrate below, the indigenous bacterial composition of bentonite deposits and deep groundwater, which represent two most important sources of bacterial contamination of bentonite seals in high level waste repositories, is very specific and not easy to cultivate. As a result, cultivability of bacteria in deep granitic aquifers measured as total number of cells percentage ranged from 0.01 to 35.9 %, averaging 5.12 % (Hallbeck and Pedersen, 2012) and similar results are reported in other studies (Eydal and Pedersen, 2007; Haveman and Pedersen, 2002). The profitability of cultivation methods in bentonite studies is thus rather limited.

Problems related to cultivation methodology can be circumvented by studying microbial activity, a clear measure of presence of viable cells. Bacterial activity can be generally measured by the amount and/or turn-over of one or several metabolic products such as acetate, sulphide, oxygen or ATP (Eydal and Pedersen, 2007; Pedersen, 2017). These methods are especially useful for simple bacterial detection in the sample, but are not very suitable for precise estimation of bacterial density, because such estimation requires knowledge of the metabolite rate per cell. In the case of ATP measurements, although it is possible to use a simple ATP-per-cell conversion rate published in previous studies e.g. (Eydal and Pedersen, 2007) to estimate cell concentrations from detected ATP concentration in the sample, particular care should be exercised with this approach, because three main factors can influence the amount of ATP-per-cell (Hammes et al., 2010): firstly, different bacterial species can have different ATP contents. Secondly, physiological differences can account for differences in microbial ATP concentrations. The third factor is biomass or biovolume. Larger cells generally have more ATP per cell than smaller cells. ATP measurements are thus specifically relevant when calibrated e.g. to total number of cells detected by direct staining and flow cytometry (Hammes et al., 2010). The other problem of ATP analysis (and also DNA analysis described below) is the presence of extracellular ATP resulting from damaged or lysed cells in the sample. Although ATP hydrolyses easily in the natural environment with a half-life on the order of a few hours at 2-4°C (Cowan and Casanueva, 2007), Hammes et al. (2010) reported 3 - 97% of total sample ATP being extracellular in various water samples. These results indicate that ATP analysis is not a clear measure of bacterial activity unless the source of ATP in the sample is carefully distinguished. Unsurprisingly, presence of bentonite in the sample further hinders the ATP analyses, because clays very effectively bind ATP, similarly to the situation with fluorescent dyes. Barnett (2010) showed that ATP recovery was less than 10% in high clay samples, which were spiked by the known amount of ATP. The ATP recovery rate was unfortunately not stated in case of the only one published ATP analysis performed in compacted bentonite (Lydmark and Pedersen, 2011).

The last common microbiological approach we want to shortly mention is DNA sequencing. This method is based on existence of nucleic acid sequence variability enabling accurate discrimination of present taxa. Although DNA sequencing might give us a good idea about microbial composition in studied matrix, results can be severely biased by the presence of extracellular DNA resulting from cell death and subsequent cell lysis caused by various factors (Alawi et al., 2014). Traditional DNA extraction protocols do not distinguish between intracellular (microbial) DNA and extracellular DNA although it is known, that extracellular DNA represent a major part of the total DNA pool in marine sediments or various soils (Ceccherini et al., 2009; Corinaldesi et al., 2011, 2008). Furthermore, DNA attached to mineral surfaces was shown to be well protected from degradation (Yu et al., 2013) and is commonly reported as ancient or fossil DNA, although there are concerns about credibility of experimental setups and authentication of results in many studies reporting the oldest (geologically ancient) DNA (Hebsgaard et al., 2005). The yields of DNA extracted from bentonite are indeed often very low, which promotes the development of optimized extraction protocols. If we want to take advantage of the DNA sequencing in bentonite studies to describe present bacterial composition, we should take care to eliminate the extracellular DNA as described by (Alawi et al., 2014) and try to maximize the DNA recovery from bentonite by means of specialized protocols targeted on bentonite extraction.

1.3 Aims of the study

The general aim of DL 2.10 was to enhance the knowledge on the ability of bacteria to migrate through the compacted bentonite.

We specifically aim:

1) To develop a reliable method for detection of bacteria in the bentonite (both viable and dead cells)
2) To monitor bacterial mobility in the compacted BaM bentonite with a focus on:
   - the differences in bacterial mobility among bentonites compacted to two different dry densities (1400 kg/m^3 and 1600 kg/m^3),
   - detection of distances that are bacteria able to reach from the source (VITA water) through the compacted bentonite of both densities, and
   - comparison of the bacterial abundance (viable cells) in various zones within the compacted bentonite samples.

2 Methods

2.1 Clay type characteristics

Bentonite BaM is a Ca-Mg bentonite exploited in the northern part of the Czech Republic and produced by the Keramost company, Obrnice plant as Bentonite-B. It consists of 78.2% of montmorillonite and its natural water content is up to 8 % by weight. The elemental composition analysis showed, that BaM bentonite includes 7.59% of Fe, 2.48% of K, 2.46% of Mg and 1.22% of Ca by weight (Matal et al., 2018). The bentonite compacted to dry density of approximately 1400 kg/m^3 results in the wet density of about 1900 kg/m^3 after full saturation with water content 38% and swelling pressure 1.1 MPa. The bentonite compacted to dry density of approximately 1600 kg/m^3 results in the wet density over 2000 kg/m^3 after full saturation with water content 25% and swelling pressure above 5 MPa (Ilona Pospíšková, SÚRAO, personal communication).

2.2 Preparation of compacted bentonite samples

A set of experimental cells with accessories was designed and constructed in order to provide a simple tool to study the bacterial migration in saturated bentonite. The method of preparation compacted bentonite on required density and following saturation inside the test cell was based on previous experimental applications (Bengtsson et al., 2015; Bengtsson and Pedersen, 2016).

2.2.1 Test cell

Test cell was made of a chemically inert material black Polyamide 6. The cylindrical space for sample has a diameter of 40 mm and thickness of 20 mm. The construction of the cell is described in Figure 2-1. The cell body can be attached to the pressing equipment Exceed E45 and the equipment used for the water saturation.

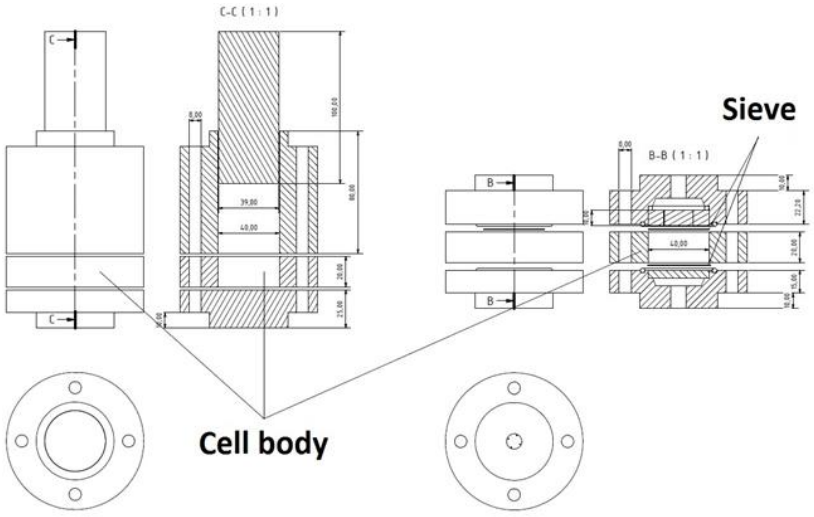

Figure 2-1. The scheme of arrangement for bentonite pressing into the cell (left) and complete cell design (right)

2.2.2 Pressing of bentonite on required density

The pressing equipment consists of three main parts – the bottom, the cell body and the piston holder with a piston. These parts are screwed together, piston is movable (Figure 2-1, Figure 2-2). The calculated mass of bentonite (converted to bentonite with residual moisture) is loaded into the cell and consequently pressed to required density by the device Exceed E45, MTS System Corporation (force capacity 300 kN, velocity movement min. 0.001 $mm.s^{-1}$, min. 0.01 $kN.s^{-1}$) (Figure 2-3, Figure 2-4). The very precise position and shift of the piston is controlled by the software TW Elite. For the migration experiment we prepared two cells with compacted bentonite of 1400 kg/m^3 and 1600 kg/m^3 dry densities.

Figure 2-2. Dismantled assembly for bentonite pressing (cell body in the middle)

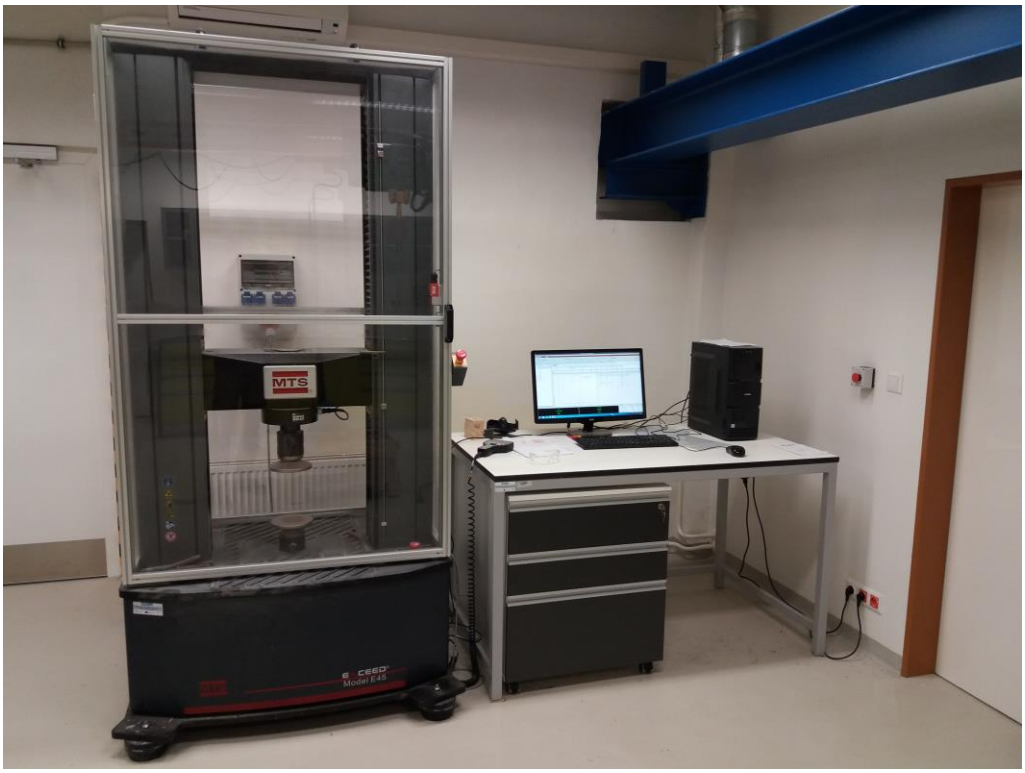

Figure 2-3. Laboratory with pressure source MTS Exceed E45

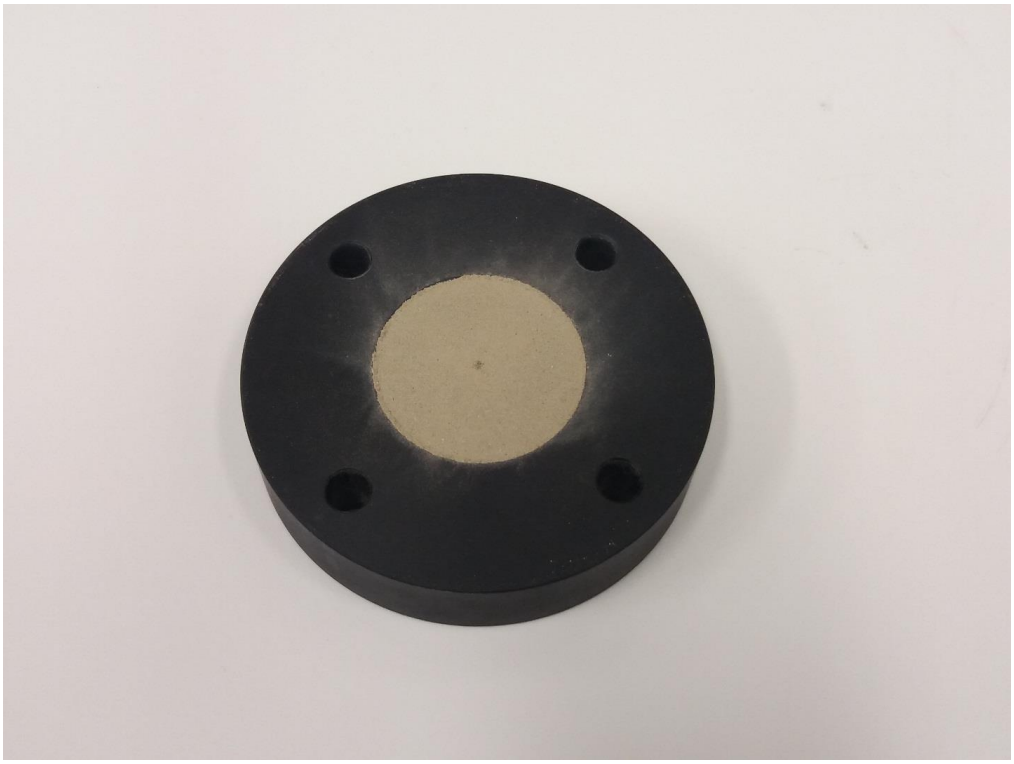

Figure 2-4. Cell body with compressed BaM bentonite, diameter 40 mm

2.2.3 Preparation of saturated samples

The compressed bentonite is covered by stainless sieves on the top and bottom (Figure 2-5). Sieves have therefore a direct contact with a bentonite. O-rings serve as a seal in the cell. First we tested sieves with diameters of 10, 20 and 50 µm. The 50 µm sieves were found to be most suitable and thus further used in the migration experiments. After completing the cell, the pressed bentonite sample remained 24h in an anaerobic environment for degassing oxygen prior beginning of water saturation.

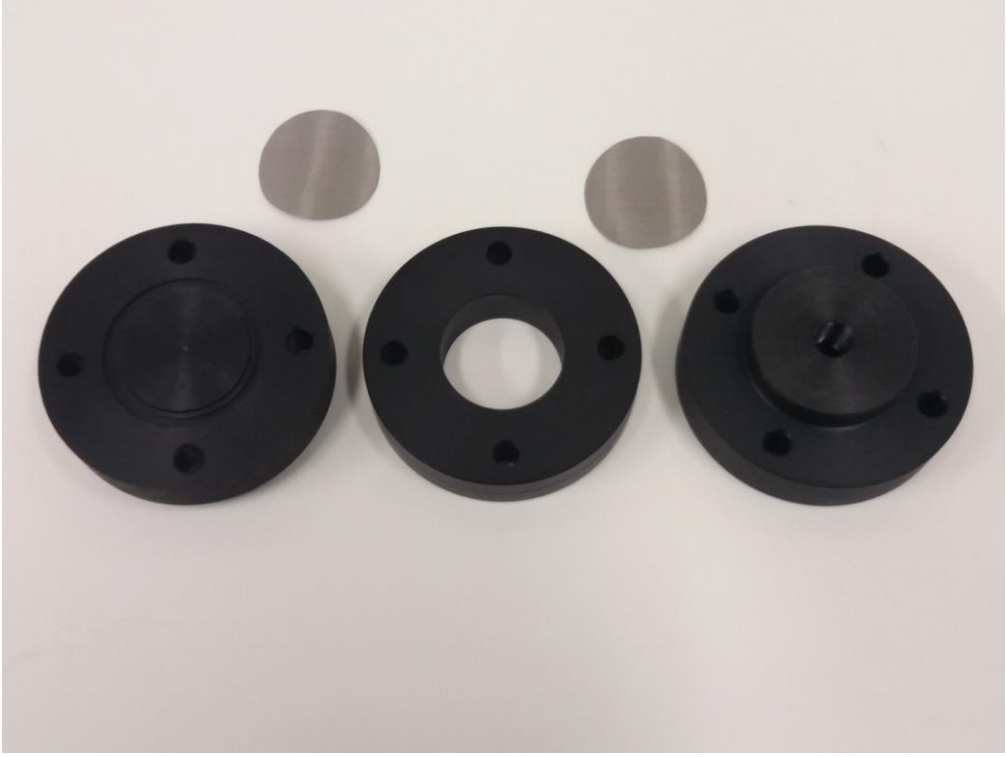

Figure 2-5. Dismantled complete cell consist of top lid, cell body, bottom lid and two stainless steel sieves

2.2.4 Saturation

The water was pushed into the cell using high pressure pump (LCP 5020 Ingos) with a double acting piston and a sensitive system of pressure control. The pressure used for pumping was limited by the value of 2 MPa . When a higher pressure was measured, the pump stopped to pump the water into the cell, until the lower value was reached. The saturation process took place in the glove box under strictly anaerobic conditions ($C_{(O2)} < 1$ ppm) (Figure 2-7) and full saturation was reached in 11 days in the case of 1400 kg/m^3 sample and in 55 days in the case of 1600 kg/m^3 sample. Duration of the whole experiment was 94 days.

Figure 2-6. Assembled complete cell with bentonite prepared for saturation

Figure 2-7. Saturation of bentonite sample in the anaerobic glove box

2.3 Detection of bacteria in bentonite samples

2.3.1 Processing of the samples

The cells were disconnected from the source of VITA water in the glove box, immediately closed into the air-proof plastic bags and transported to the lab for the subsequent microbiological analyses. The samples were processed within two working days, each cell in one day.

The cell was opened by means of two spanners. Cell consists of three metal parts (Figure 2-8). We removed the top and the bottom lid of the cell, we took off the upper in-flow stainless sieve and bottom out-flow sieve from the surface of the compacted bentonite stuck in the body of the cell and prepared samples of them by washing each by 1 ml of 0.1 M NaOH in a vial (sample 1 and 2). Further, we either directly cut the ring one from the cell body by the ring cutter (Figure 2-8) in the case of less dense 1400 kg/m^3 bentonite sample or we gently pushed the whole bentonite plug from the cell body and cut it with the ring cutter as described below (in the case of 1600 kg/m^3 sample). In each case we took the sample from the inner surface of the cell body (sample 3) and also scratched the upper surface of the compacted bentonite by razor blade (sample 4). We used metal ring cutters with the diameter varying by 5 mm to slice the bentonite into concentric rings, each one of 5 mm thickness. We used the largest ring cutter to slice off the most outer ring (ring 1). We cut the ring 1 into three horizontal layers and took separate sample from each layer (samples 5, 6, 7). Further, we sliced the residual bentonite column by the second largest ring cutter to get the second ring (ring 2), which we also sliced into three layers and took samples (8, 9, 10). The residual bentonite was finally sliced by the smallest ring cutter to get the third ring (ring 3), which was again divided into three layers and sampled (samples 11, 12, 13). Finally we took one more sample from the very innermost part of the residual bentonite column (sample 14). The schema of bentonite slicing is shown in (Figure 2-9) and samples are listed in (Table 2-1).

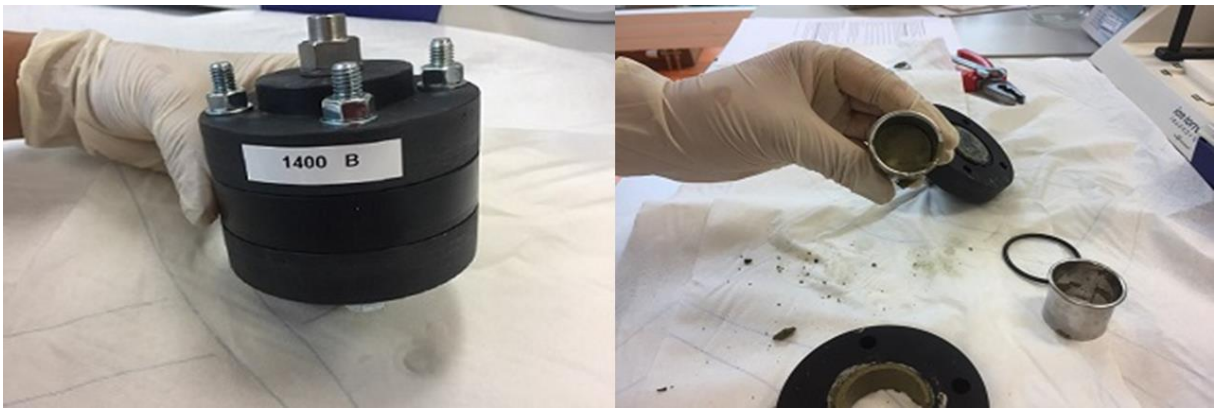

Figure 2-8. Cell filled with bentonite of density 1400 kg/m^3 (left) and using the ring cutter for the bentonite sampling (right)

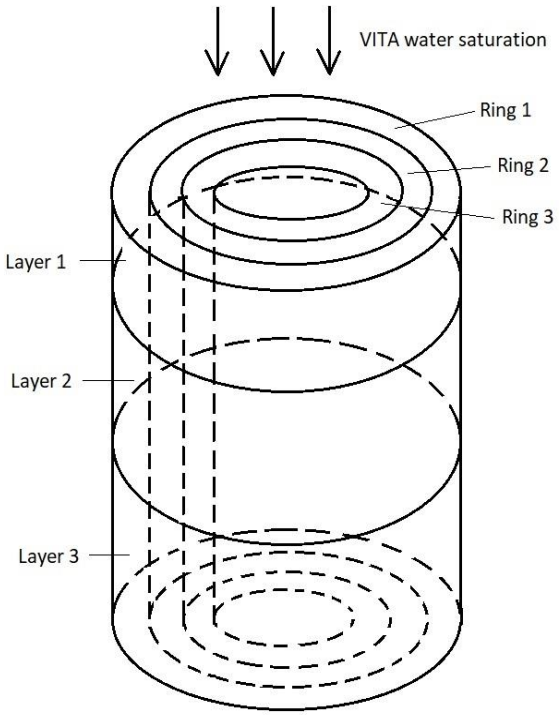

Figure 2-9. Scheme of bentonite slicing

Table 2-1. List of the samples (same for both densities)

Sample number	Description
1	In-flow sieve
2	Out-flow sieve
3	Inner surface of the cell body
4	Upper surface of the compacted bentonite
5	Ring 1, layer 1
6	Ring 1, layer 2
7	Ring 1, layer 3
8	Ring 2, layer 1
9	Ring 2, layer 2
10	Ring 2, layer 3
11	Ring 3, layer 1
12	Ring 3, layer 2
13	Ring 3, layer 3
14	The innermost part of the residual bentonite

All 14 samples from each cell were divided into two parts – one half of each sample was used for the extraction protocol and live/dead staining, the other half was used for the cultivation purposes described below.

2.3.2 Extraction protocol and staining of bacteria

Direct microscopic count was impossible due to the presence of bentonite in the sample, which is a problem also known from the literature (Aoki et al., 2010; Stroes-Gascoyne et al., 1996). Therefore, we had to develop a special method for the extraction of bacteria from the bentonite based on the protocols used for various soil samples prior to cell staining (Alawi et al., 2014; Liu et al., 2010; Vasiliadou and Chrysikopoulos, 2011; Yang et al., 2012).

The cell samples (except for the samples 1 and 2 - sieves) were weighted on the laboratory scales. We added 3 parts of 0.1 M NaOH by weight to 1 part of the sample by weight and mixed it properly to get smooth suspension. We took 1ml of each suspension and mixed it with 4ml 0.1 M NaOH. Sodium hydroxide was used to help the detachment of bacteria from the mineral matrix (Yang et al., 2012). After shaking the sample properly to wash the bacteria to the solution, we used a density gradient centrifugation with Percoll (Sigma-Aldrich). We gently pipetted 1 ml of Percoll underneath each sample and centrifuged it for 5 min at 1000 x *g* (Figure 2-1). Such a treatment results in the sedimentation of bentonite particles to the bottom, while bacteria stay in the solution due to the presence of Percoll. We carefully transferred the complete supernatant into a new tube without disturbing the sediment and centrifuged the supernatant at 6000 x *g* for 15 min. We discarded the supernatant and resuspended the pellet in 50 - 100 µl of sterile water (the amount of water was amended based on the density of bacteria in the supernatant) (Figure 2-11).

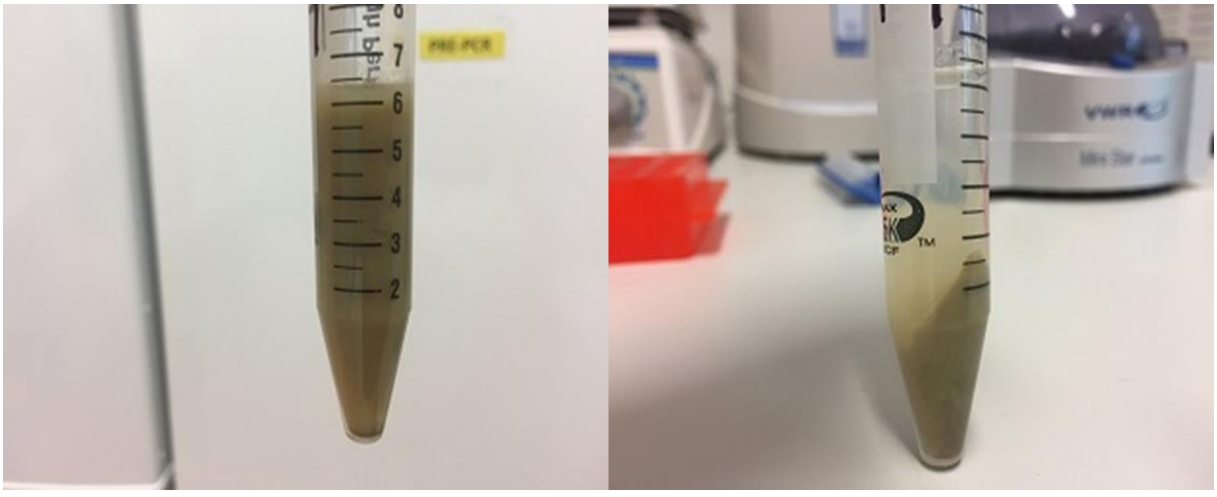

Figure 2-10. Homogenized bentonite suspension with Percoll (left), centrifuged tube at 1000g for 5 min (right)

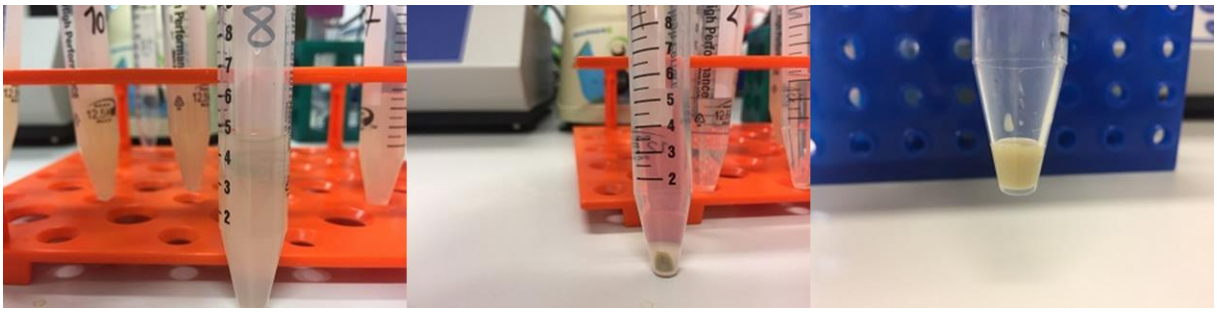

Figure 2-11. Supernatants (left), tube centrifuged at 6000 x g for 15 min with a pellet (middle), resuspended pellet in 75 µl of sterile water (right) - sample ready for Live/Dead staining.

For the subsequent visualization of bacteria under the fluorescence microscope we used Live/Dead staining by means of LIVE/DEAD™ BacLight™ Bacterial Viability Kit, for microscopy (Invitrogen). This kit includes two dyes which differ in their spectral characteristics and ability to penetrate the bacterial cells. SYTO 9, green-fluorescent nucleic acid stain, generally labels all bacteria in a population and the red-fluorescent nucleic acid stain, propidium iodide, penetrates only bacteria with damaged membranes and causes the reduction in the SYTO 9 stain fluorescence when both dyes are present. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide (kits manual).

We prepared the 30 x diluted (in water) working mixture of SYTO 9 and propidium iodide from the stock solutions of both stains. For each sample, we took 13 µl of homogenized resuspended pellet and mixed it with 8 µl 30x diluted dye mixture in a small tube and incubate in dark for 15 min. After this time we transferred 10 µl of sample-dye mixture on the clean slide and covered it with the cover slide. The amount of sample was determined in order to completely fill the space between both slides and no liquid leaks out. The slide was observed under fluorescence microscope Axio Imager M2 (Carl Zeiss) by software Axiovision and using 63 x objective. Typically 20 representative images of the various slide parts were taken for each slide.

To compare the results from the compacted bentonite to original BaM bentonite powder, we further prepared the suspension of 3 g of dry bentonite powder in 10 ml of deionised water and processed it according the protocol described above.

2.3.3 Cultivation methods

One gram of each bentonite sample (samples 3-14, samples from sieves were not included) was diluted in 10 ml of physiological saline and resulting suspension was shaken on a shaker for one hour. Afterwards, we prepared the dilution series. One ml of prepared suspension was diluted in 9 ml of physiological saline (dilution 10^{-1}) and the procedure was repeated five more times (up to dilution 10^{-6}).

We performed both aerobic and anaerobic cultivation on agar plates using PCA medium. We prepared samples in dilutions from 10^{-1} to 10^{-6} for aerobic cultivation (six plates per sample) and from 10^{0} to 10^{-3} for anaerobic cultivation (four plates per sample). Samples

for the anaerobic cultivations were sealed in the air-proof plastic bags or anaerobic jars (Figure 2-12) with oxygen binding medium and oxygen indicator inside. We cultivated the plates under aerobic conditions for six days and plates in anaerobic conditions for 10 days. We expressed the resulting number of cultivable bacteria as colony forming units (CFU) per 1 g of sampled saturated bentonite.

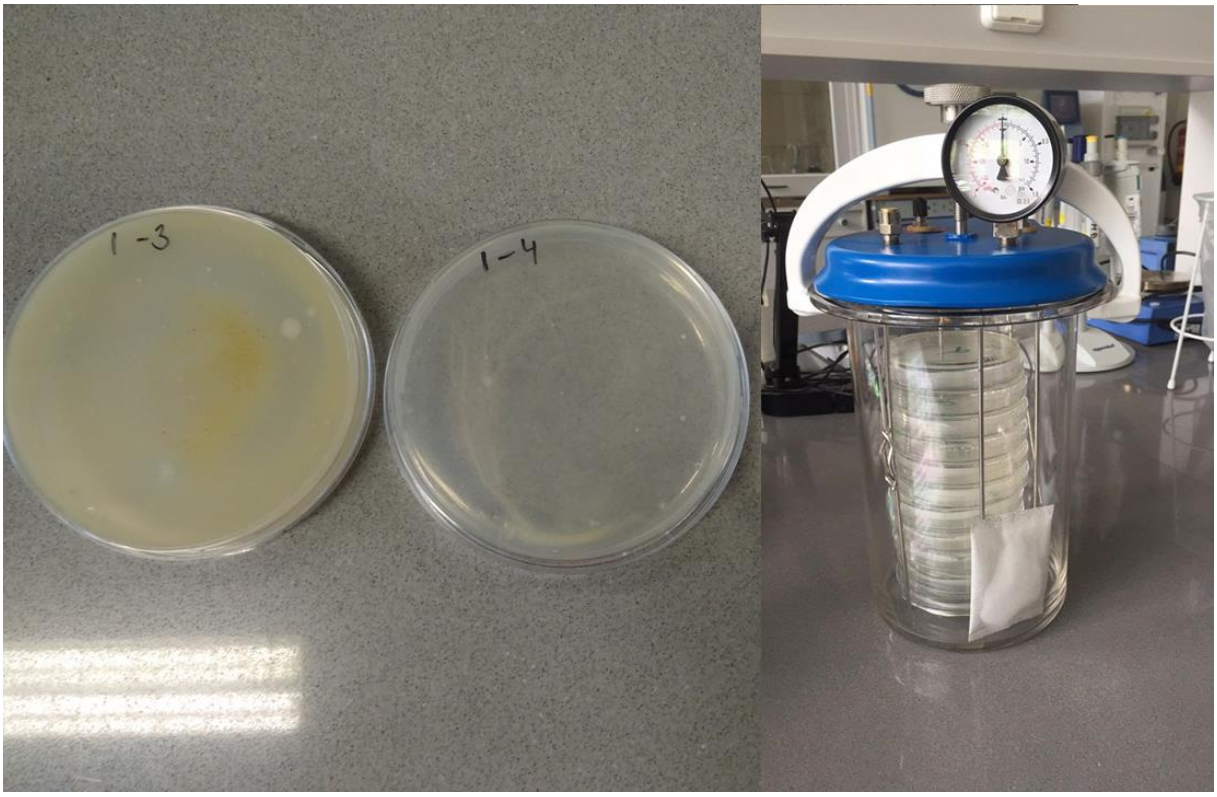

Figure 2-12. Examples of agar plates cultivated aerobically (10^{-3} and 10^{-4} dilution) on the left, the plates in anaerobic jar on the right

3 Results and discussion

Our study was divided into three important tasks. Firstly we needed to design and construct an experimental cell for bentonite compaction and saturation, second, we had to develop a reliable method for direct detection of bacterial cells (both viable and dead) in bentonite and final task was to study mobility of natural bacteria in bentonite compacted to 1400 and 1600 kg/m^3.

Reliable method for detection of bacteria in bentonite is still missing although it would represent very important tool for future bentonite studies. The direct count of bacteria by means of cell staining is not possible, because bentonite severely interferes with the analysis. Some of the dyes commonly used to visualize the bacteria, such as acridine orange or DAPI, bind to the bentonite particles resulting in strong fluorescence of the background. The others (such as CFDA-AM, or SYTO 9) do not bind so strongly to the bentonite particles, but still the mere presence of large amount of bentonite particles together with limited number of bacterial cells in the sample makes the counting impossible. We thus decided to

develop a protocol, which would extract the bacteria from the bentonite prior to their staining.

Our newly developed protocol for the bacterial extraction followed by their Live/Dead staining was successful. The method enabled us to detect the presence of bacteria in various zones within compacted bentonite samples differing in their distance from the source of bacteria (VITA water) and we could also compare both bentonite samples of different densities in this matter. However, the method needs further optimization (problems are discussed in separate section below). For this reason, we decided not to perform direct counting of the cells, which could be partially misleading, but rather to evaluate the samples only visually. The images chosen for the purposes of this report were specifically chosen to demonstrate the presence of bacteria in the particular samples. The density of cells on the images thus does not correspond to their true density in the particular sample.

3.1 Presence of bacteria in bentonite samples

Firstly, we searched for the presence of bacteria within the dry BaM bentonite powder and we detected both viable and dead bacterial cells here. BaM bentonite thus contains indigenous microbiota similarly to other bentonite studied before (Fru and Athar, 2008; Pedersen et al., 2000a; Svensson et al., 2011). However, most of the cells were dead and only a few viable bacteria were visible within the dry BaM bentonite powder (Figure 3-1). This result suggests that the major source of bacteria observed in the compacted bentonite samples is thus VITA water used for the bentonite saturation.

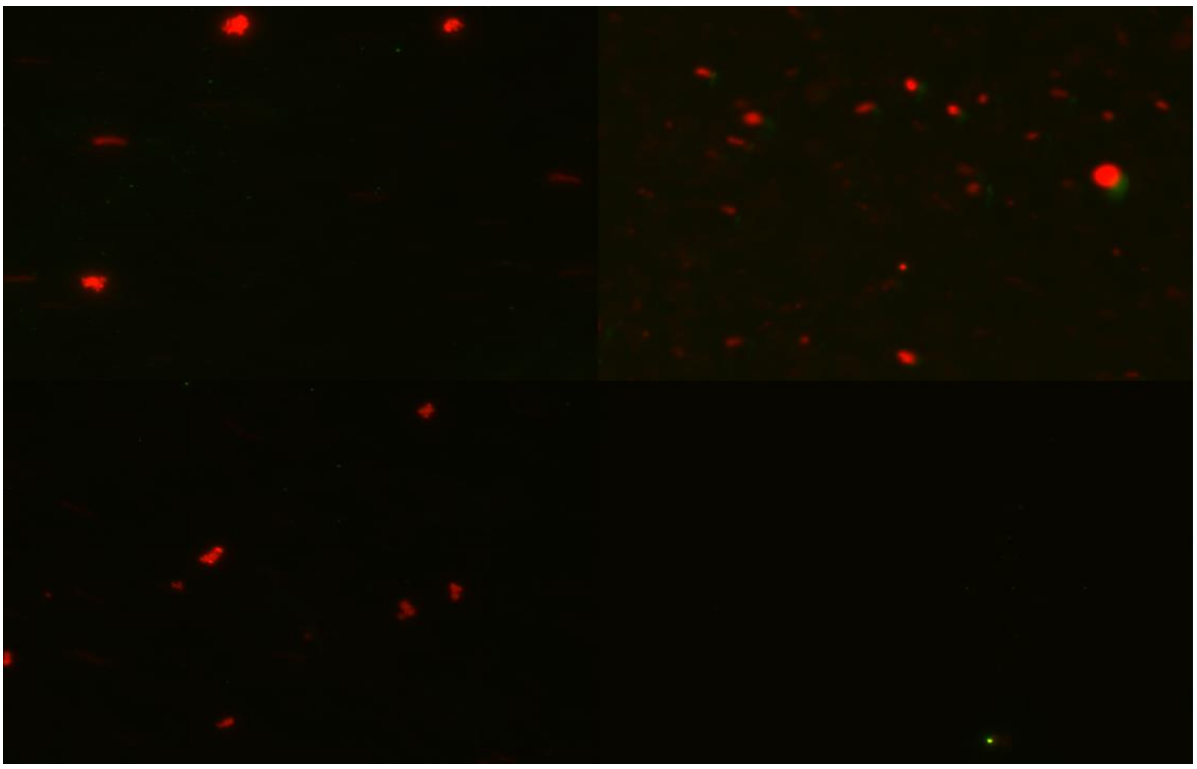

Figure 3-1 Live/Dead stained bacteria from dry BAM bentonite powder in fluorescence microscope. Red – dead cells, green – viable cells

We detected the presence of viable bacteria in all 28 studied samples in the compacted bentonite samples of density 1400 kg/m^3 and 1600 kg/m^3 (see Figure 3-2 - Figure 3-23).

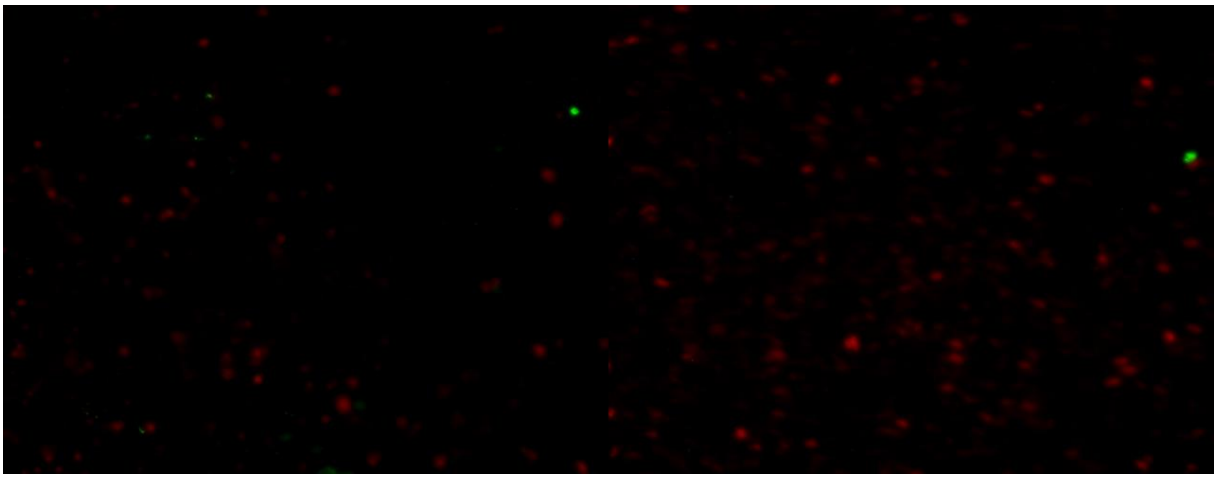

Figure 3-2. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 5 (ring 1, layer 1) of 1400 kg/m^3 bentonite

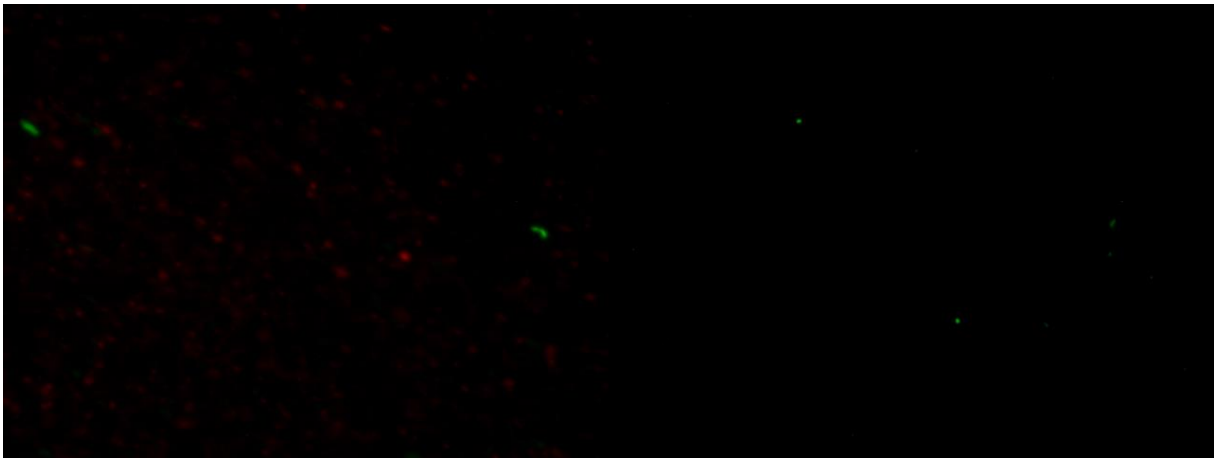

Figure 3-3. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 6 (ring 1, layer 2) of 1400 kg/m^3 bentonite.

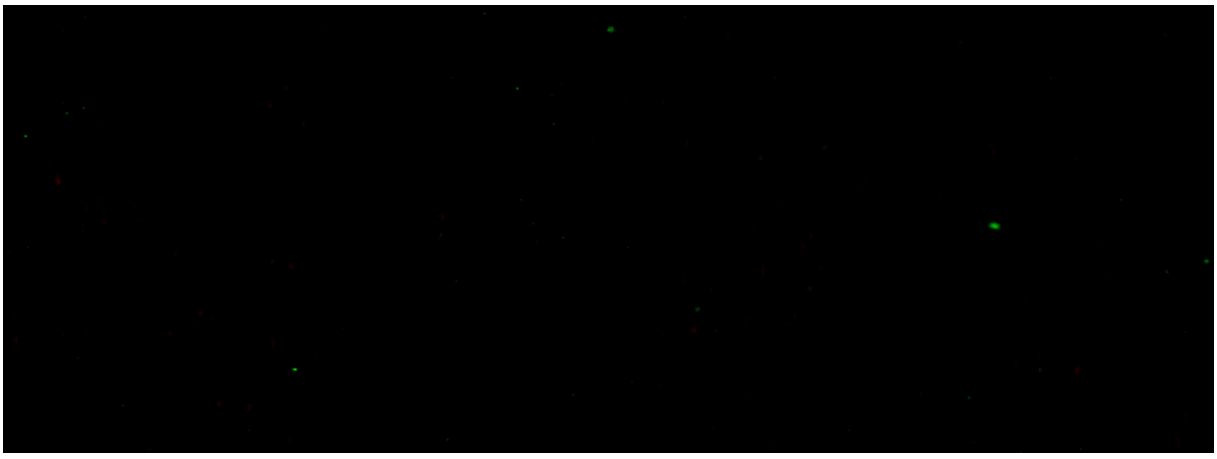

Figure 3-4. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 7 (ring 1, layer 3) of 1400 kg/m^3 bentonite.

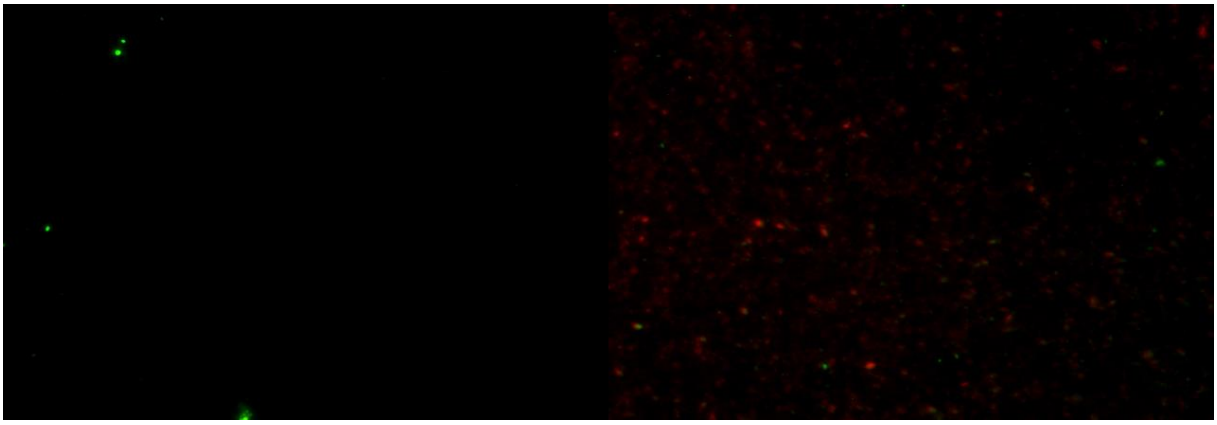

Figure 3-5. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 8 (ring 2, layer 1) of 1400 kg/m^3 bentonite.

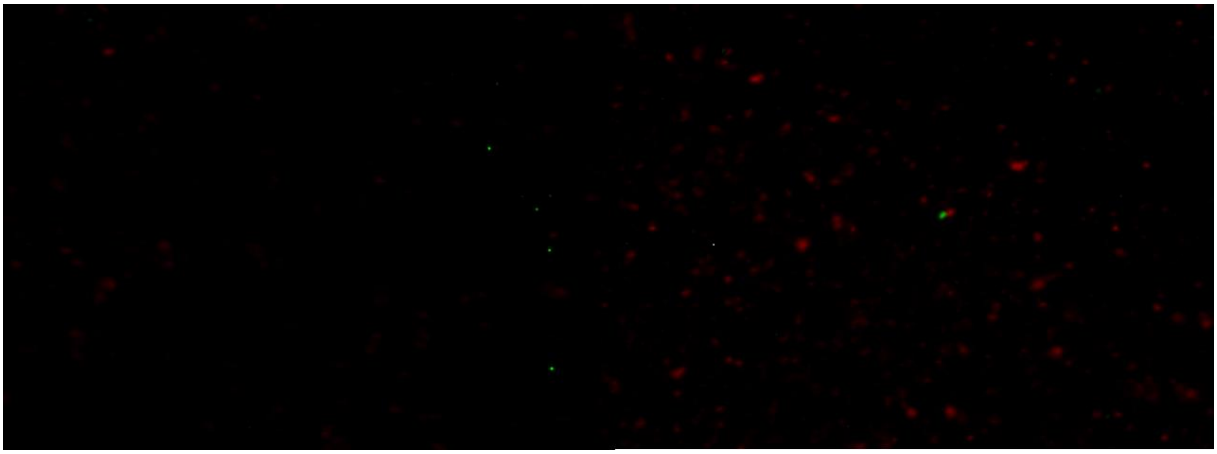

Figure 3-6. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 9 (ring 2, layer 2) of 1400 kg/m^3 bentonite.

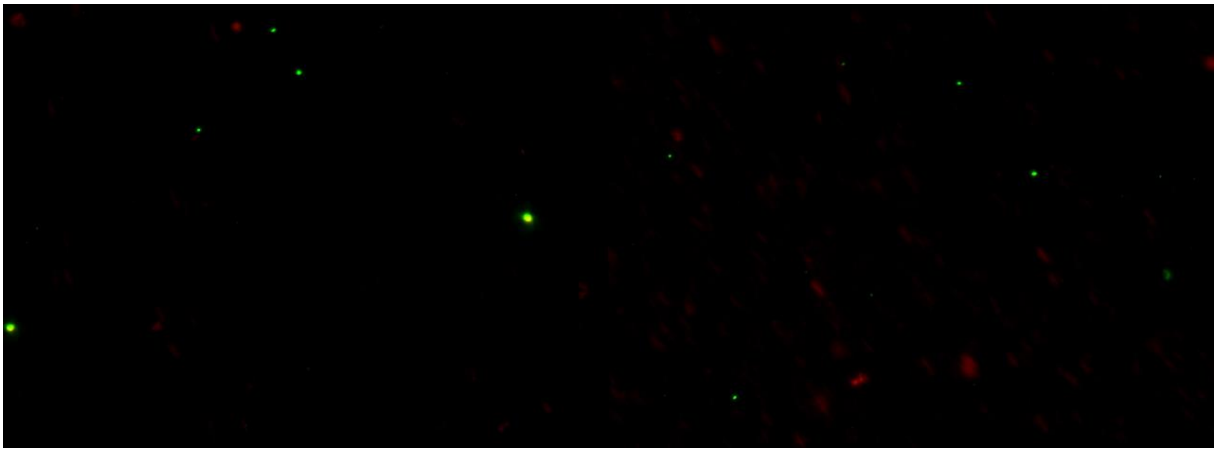

Figure 3-7. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 10 (ring 2, layer 3) of 1400 kg/m^3 bentonite

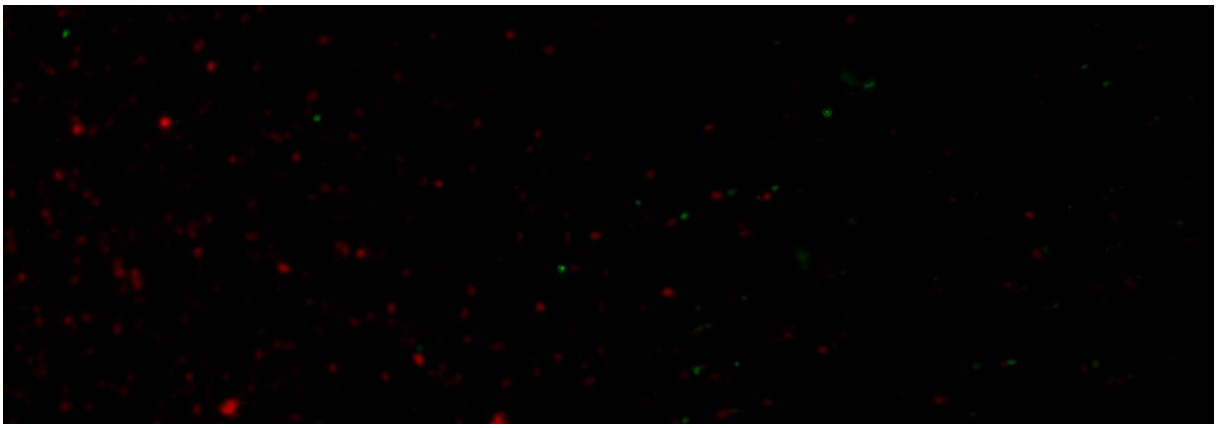

Figure 3-8. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 11 (ring 3, layer 1) of 1400 kg/m^3 bentonite

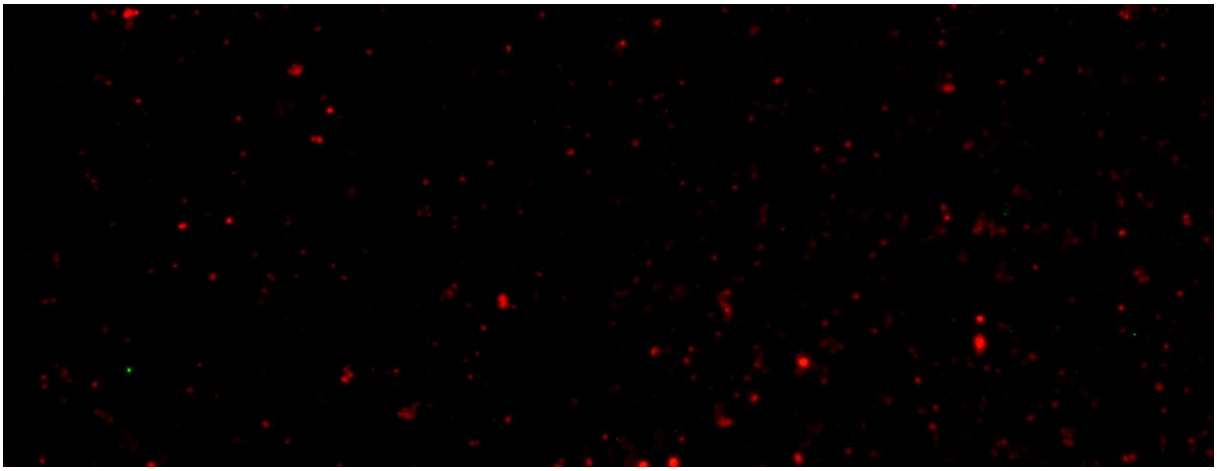

Figure 3-9. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 12 (ring 3, layer 2) of 1400 kg/m^3 bentonite.

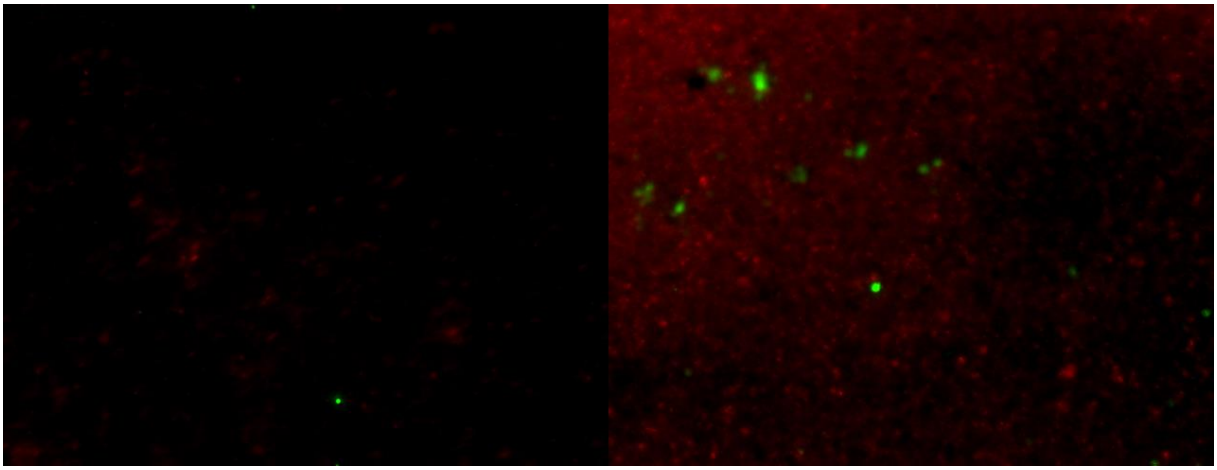

Figure 3-10. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 13 (ring 3, layer 3) of 1400 kg/m^3 bentonite.

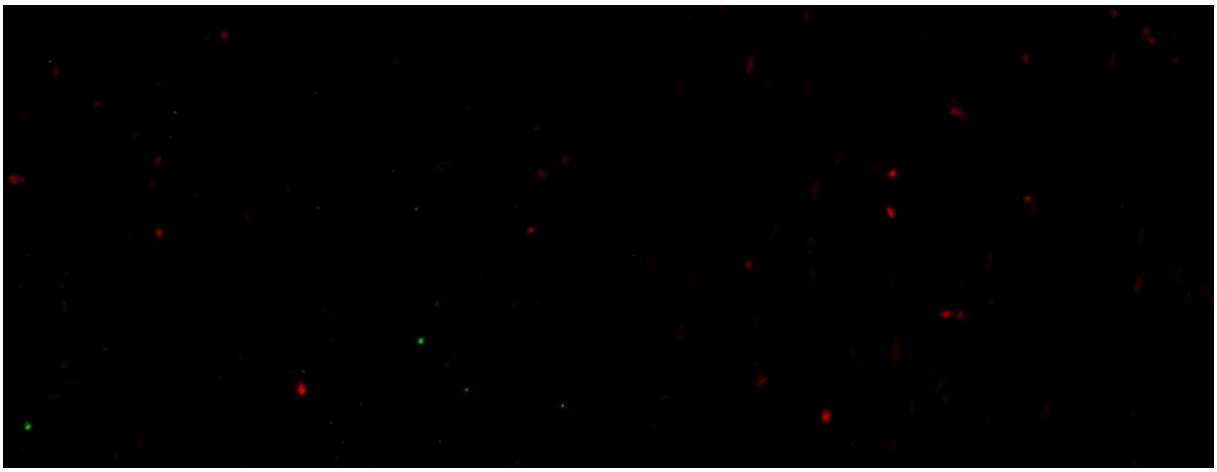

Figure 3-11. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 14 (the innermost sample) of 1400 kg/m^3 bentonite.

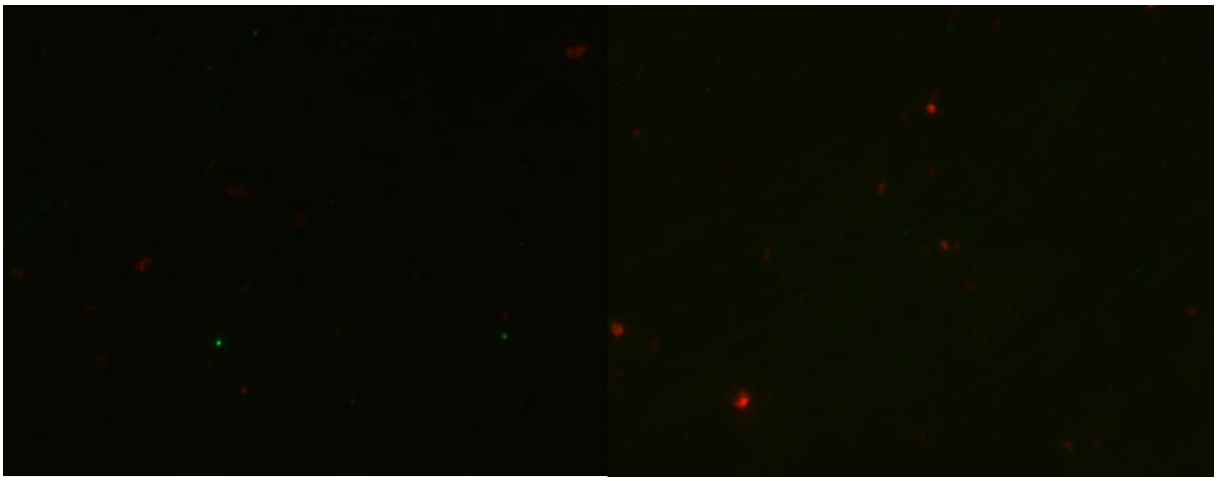

Figure 3-12. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 5 (ring 1, layer 1) of 1600 kg/m^3 bentonite.

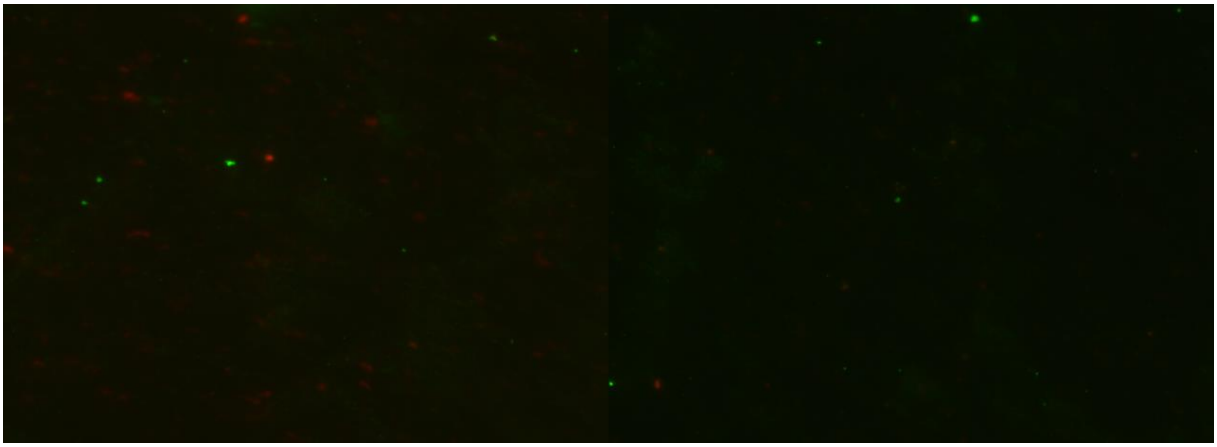

Figure 3-13. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 6 (ring 1, layer 2) of 1600 kg/m^3 bentonite.

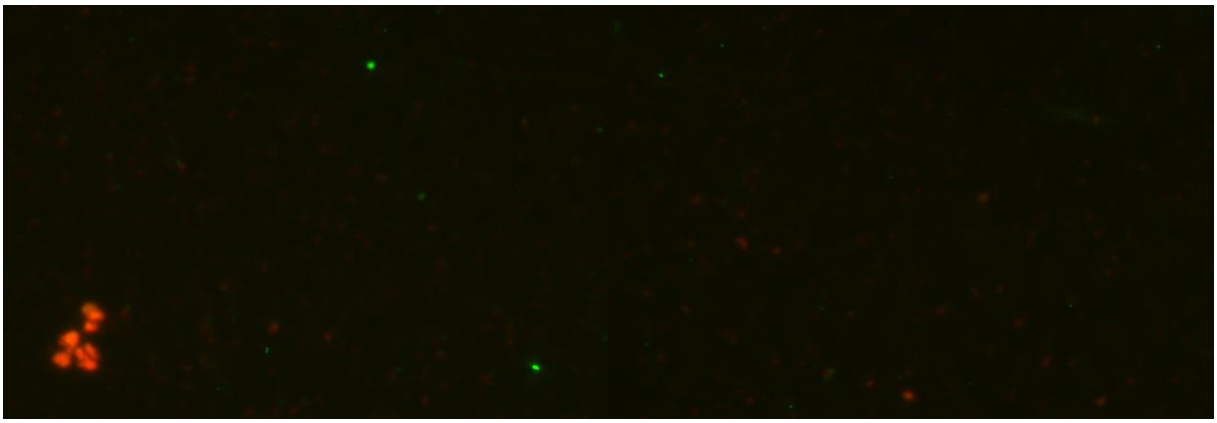

Figure 3-14. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 7 (ring 1, layer 3) of 1600 kg/m^3 bentonite.

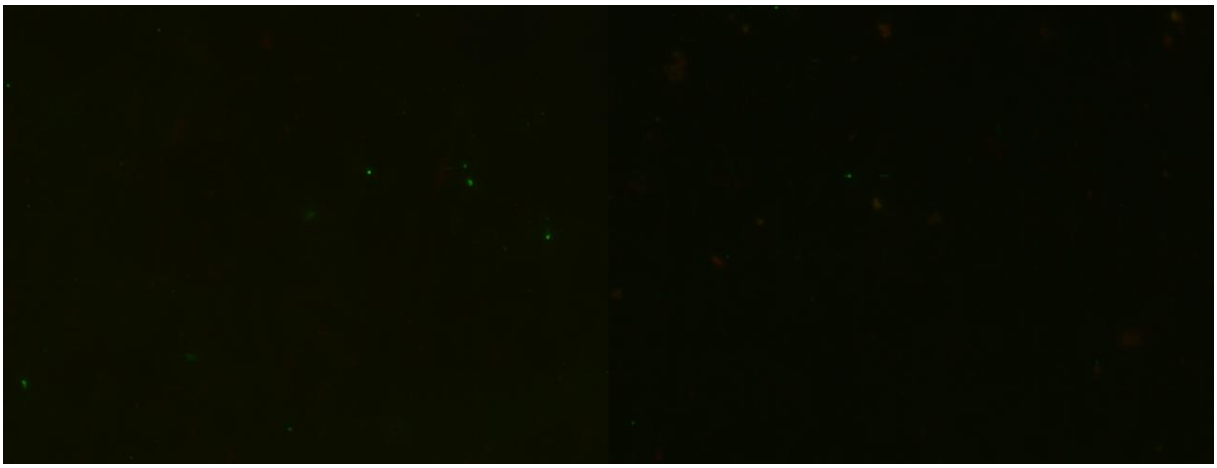

Figure 3-15. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 8 (ring 2, layer 1) of 1600 kg/m^3 bentonite.

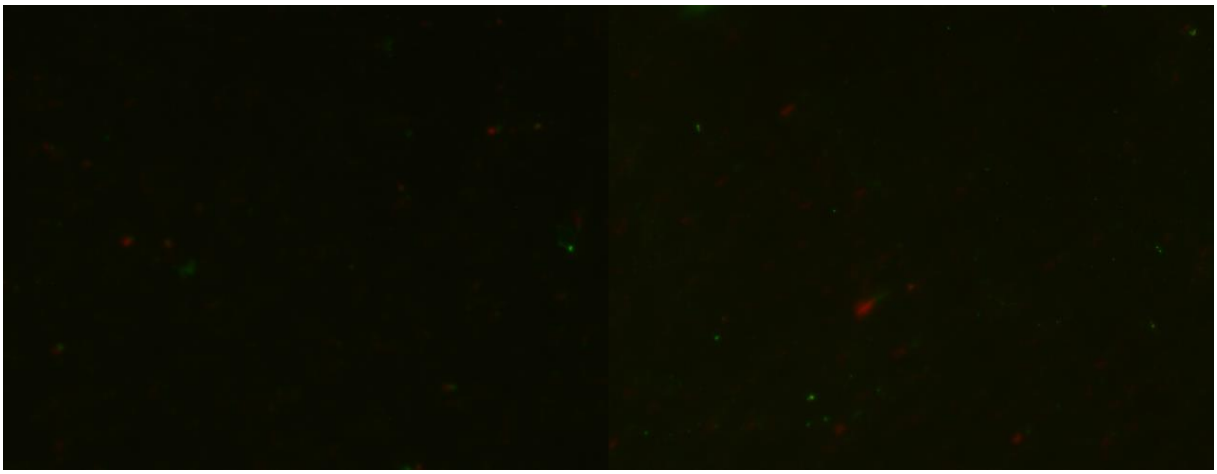

Figure 3-16. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 9 (ring 2, layer 2) of 1600kg/m^3 bentonite.

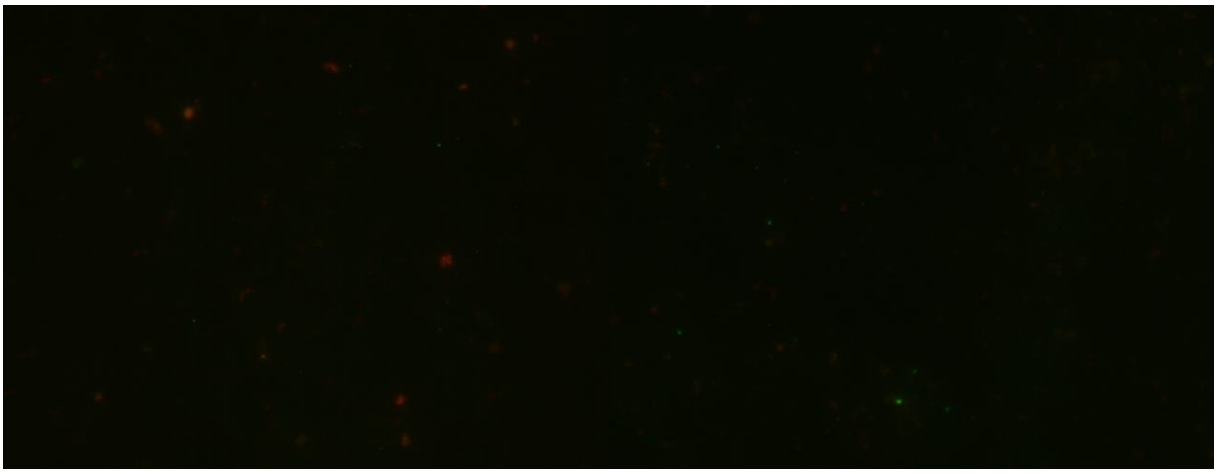

Figure 3-17. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 10 (ring 2, layer 3) of 1600kg/m^3 bentonite.

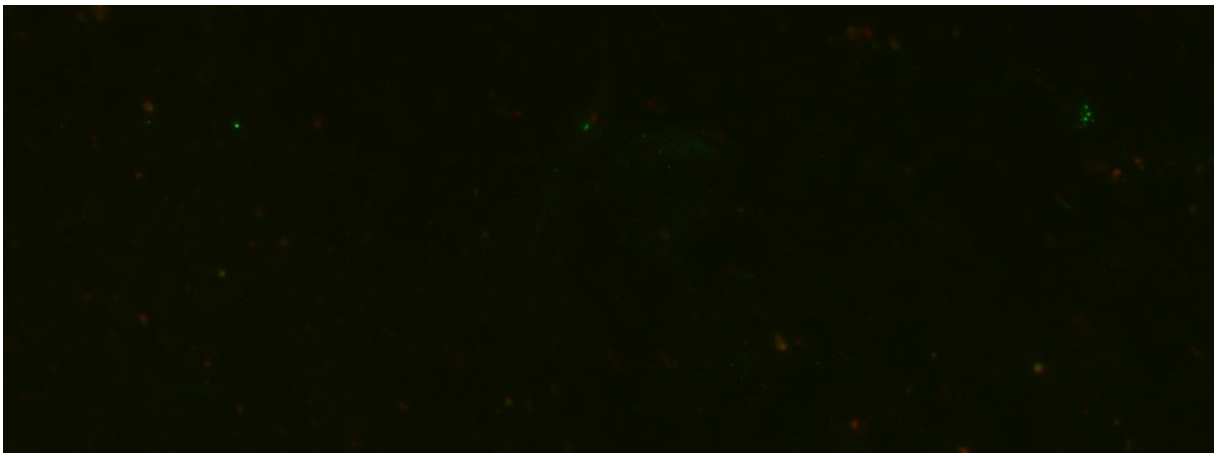

Figure 3-18. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 11 (ring 3, layer 1) of 1600kg/m^3 bentonite.

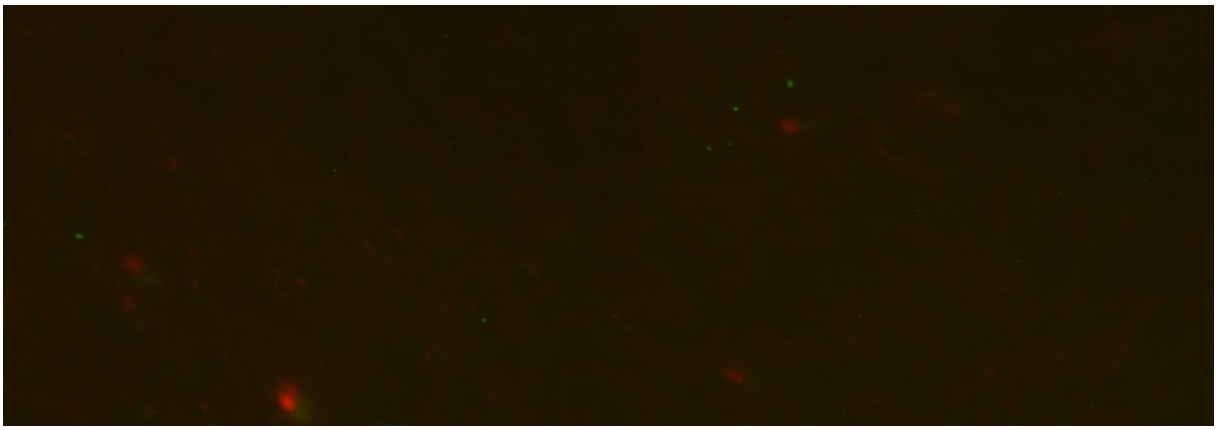

Figure 3-19. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 12 (ring 3, layer 2) of 1600kg/m^3 bentonite.

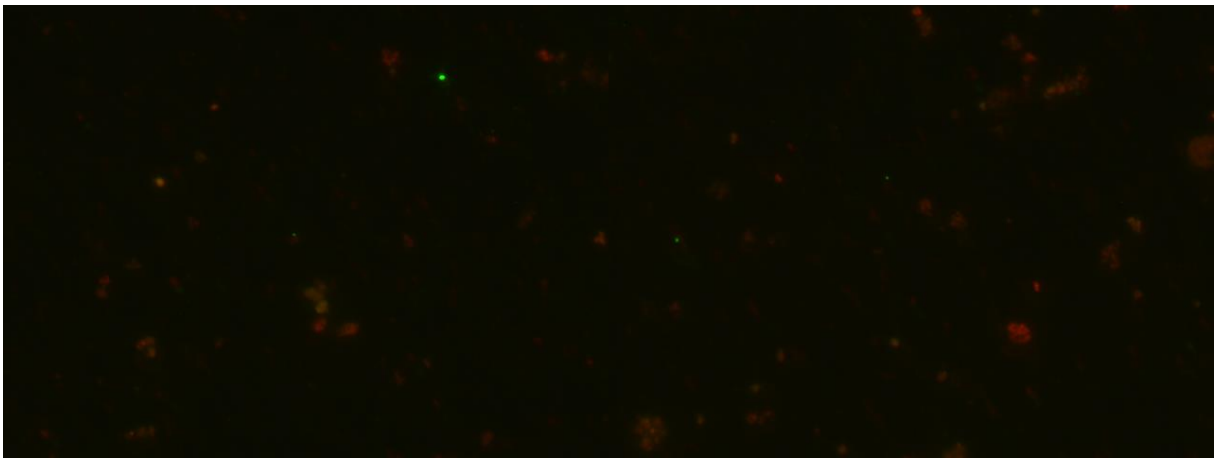

Figure 3-20. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 13 (ring 3, layer 3) of 1600kg/m^3 bentonite.

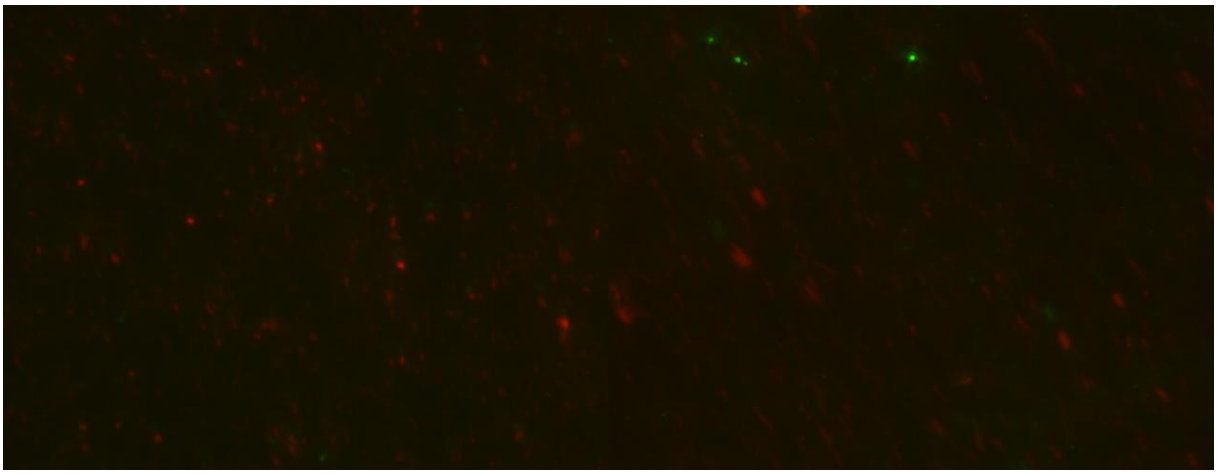

Figure 3-21. Viable (green fluorescence) and dead (red fluorescence) cells detected in the sample 14 (the innermost sample) of 1600kg/m^3 bentonite.

Detection of both live and dead bacteria across the whole bentonite samples of both densities indicates that bacteria are able to move through compacted bentonite of both dry densities (1400 and 1600 kg/m^3). Previous studies showed that bacteria were able to penetrate only to a very short distance (0,5 cm) from the surface during similar short term experiments in highly compacted (1900 kg/m^3 and 1800 kg/m^3 dry density respectively) MX-80 Na-bentonite (Pedersen et al., 2000b; Stroes-Gascoyne and West, 1997). 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 of wet density 2000 kg/m^3 were significantly eliminated in 15 months under repository conditions (Pedersen et al., 2000b). Similarly, the sulphide-production results indicated intervals between 1690 kg/m^3 and over 2000 kg/m^3 in wet densities within which bacterial sulphide-producing activity was significantly reduced in various tested bentonite types (Bengtsson and Pedersen, 2017; Pedersen, 2017), but acetate formation from natural organic matter was observed in all samples even at high wet densities (Pedersen, 2017).

Similarly, Bengtsson and Pedersen (2016) reported no clear cut-off in Boom clay density with respect to presence and activity of cultivable SRB. The largest dry density of compacted BaM bentonite we used (1600g/cm3) equals to the wet density of over 2000 kg/m^3 and swelling pressure exceeding 5 MPa after full saturation (Ilona Pospíšková, SÚRAO, personal communication), which resembles the values discussed above and also recommended cut-off in bentonite density (dry density of >1600 kg/m^3, swelling pressure at least 5 MPa) in various waste disposal concepts (Pedersen, 2017). The detected presence of living bacteria in bentonite compacted to density of 1600 kg/m^3 is thus in accordance with expectation. The future experiments with the BaM bentonite of higher density are needed.

The other factor important for the bacterial mobility is the composition of bentonite. It was shown, that bacterial mobility was slightly better in the presence of either Ca-bentonite or silica sand in the mixtures packed to similarly high densities (Fukunaga et al., 2000). Each bentonite has specific physical and chemical properties and various bentonites also differ in their native microbial composition (Svensson et al., 2011). The BaM bentonite used in our experiment is Ca-Mg-bentonite and for this reason it probably has different properties than MX-80 bentonite used in most previous studies. Choi and Oscarson (1996) demonstrated, that Ca-bentonite developed more large pores than Na-bentonite at the same density and the diffusion coefficients of Ca-bentonite was thus higher than detected in Na-bentonite. Both of these results can be ascribed to the generally larger quasicrystal size of the Ca-bentonite clay. The higher permeability of microorganisms could thus be a typical feature of Ca-bentonites, but this hypothesis needs further investigation.

The last relevant point is that no one has ever tried to detect bacteria by extracting them from bentonite as we did, which could also played significant role in the detected higher bacterial mobility. It is possible that our method provides more sensitive tool to detect the bacteria in compacted bentonite than methods used previously (direct count or cultivation based methods). The sensitivity of our novel method in various bentonites of different densities thus also needs further investigation.

3.2 Comments on bacterial density

Although we were not able to count the cells as we planned, we observed some general patterns in the bacterial presence and density within the compacted bentonite samples.

Firstly, there were fewer cells visible as we got deeper inside the compacted bentonite samples, but even in the very innermost part of the compacted bentonite, the viable cells were present in low density in both compacted bentonite samples. The density of dead cells was generally difficult to estimate, because the dye tended to non-specifically bind to the background especially in 1600 kg/m^3 density, which is a problem discussed below. The well visible dead cells were detected especially in 1400 kg/m^3 samples. In 1600 kg/m^3 samples bacteria were visible mostly in the first ring, and their distinguishing tended to be more difficult deeper inside the bentonite sample. The viable cells were generally observed more easily across all the samples.

The overall density of cells was noticeably lower in the bentonite of higher density (1600 kg/m^3) than in the bentonite of lower density (1400 kg/m^3), which is also partially visible in the figures above. Further, the cells in the higher density samples tended to be noticeably smaller (also visible in the Figure 3-12- Figure 3-20). This finding is not surprising as the selection for the decreasing body size must be severe in the harsh conditions of densely compacted bentonite. We detected only a few live bacteria inside the very innermost part of the 1600 kg/m^3 bentonite.

The more accurate evaluation of bacterial density across the compacted bentonite will be performed after the optimization of the extraction protocol.

3.3 Bacteria in outer-flow sieve

Interestingly, we detected presence of some aberrantly shaped viable cells in the bottom outer-flow sieve of the 1600 kg/m^3 bentonite sample (Figure 3-23), which should be contaminated only by the cells naturally occurring in the bentonite or cells from VITA water which passed through the complete bentonite sample. Their shape very much resembles the shapes of bacteria detected by Männik et al. (2009) after they grew through very narrow channels. This finding indirectly indicates that the bacteria are most probably able to grow through the compacted bentonite. Similar aberrantly shaped bacteria were not detected in 1400 kg/m^3 bentonite sample.

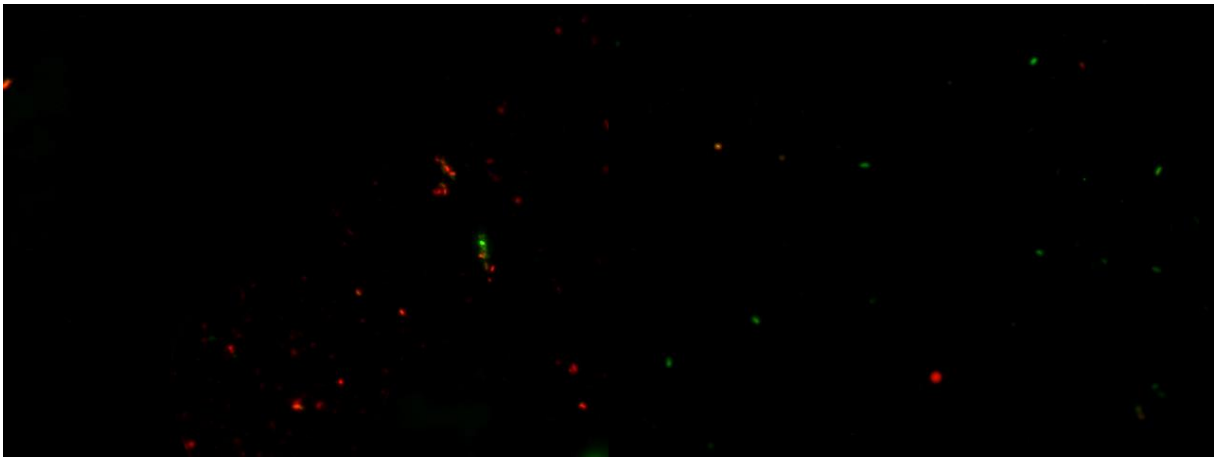

Figure 3-22. Viable (green fluorescence) and dead (red fluorescence) cells detected in the bottom outer-flow sieve (sample 2) of the 1400 kg/m^3 bentonite.

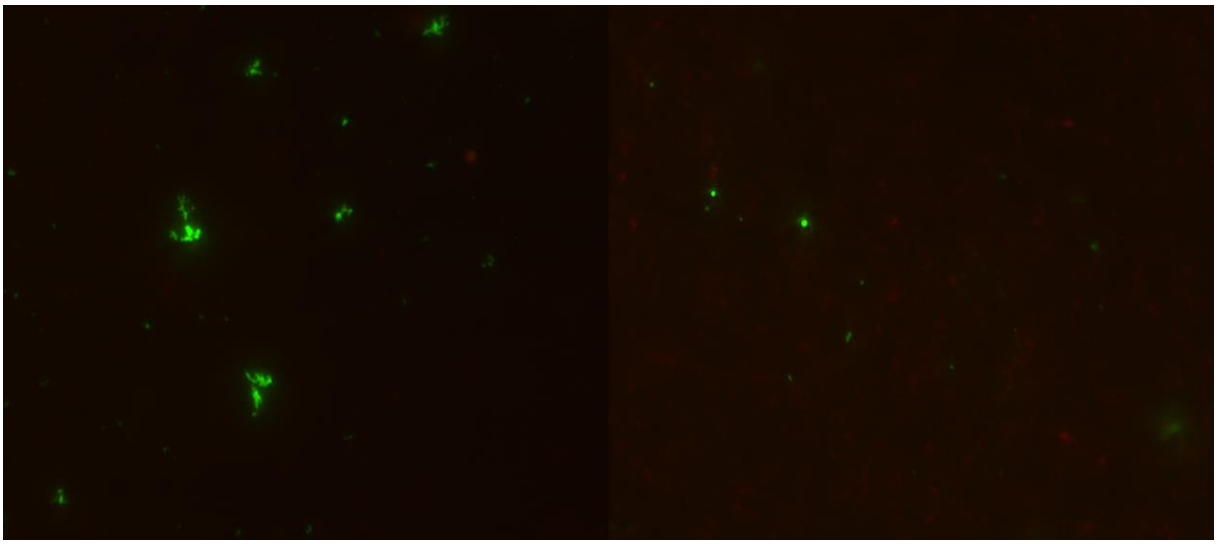

Figure 3-23. Aberrantly shaped live cells (on the left) and normally shaped live cells (on the right) detected in the bottom outer-flow sieve (sample 2) of the 1600 kg/m^3 bentonite.

3.4 Optimization of the extraction protocol and bacterial staining

The estimation of the fluorescence microscopy bacterial counts in the samples was not possible for the following reasons at the moment, therefore we continue to optimize this very promising protocol.

The major problems and proposed improvements are:

1) The presence of Percoll in the samples sometimes resulted in presence of some kinds of artefacts that devaluated the images. Thus we will work on the removing the Percoll from the samples prior staining.
2) The density of bacteria was too high in some parts of the microscopy glass slide. Thus we will work on better homogenization procedure to achieve more even distribution of bacteria.
3) The usage of dead stain (propidium iodide) does not seem to be very helpful after all. It worked relatively well in the BaM powder or in the compressed bentonite of lower density (1400 kg/m^3), where we could observe both the live and dead cells. Deeper inside the bentonite (of both densities) and in the 1600 kg/m^3 density, we observed significant increase in red (dead) stained undefinable artefacts disabling the evaluation of images. The stain probably bound non-specifically to the matrix itself. There are two possible explanations. Either the physical or chemical conditions deeper inside the bentonite and in higher density are somehow different, which could influence the behaviour of the dye, or it might be caused by the presence of large amount of smashed and destroyed bacterial material in these samples. The DNA from the destroyed cells could easily bind to the matrix and cause the non-specific binding of the dye. However, this hypothesis needs further investigation. For this reason we will focus on testing of viable cells stains. We have already performed trial experiments using stain CFDA-AM, based on esterase activity, which was successfully used in study of Fukunaga et al. (2005).

3.5 Cultivable bacteria

The cultivation experiments confirmed the results obtained by direct cell observations in the fluorescence microscope. Both aerobic and anaerobic bacteria were detected in all samples in the two bentonite densities. The concentration of cultivable bacteria was however rather uniform among all tested samples without any clear pattern among various depths (sections). Higher number of aerobic bacteria was found in bentonite of lower density, while number of anaerobic bacteria was similar in both densities. We presume that majority of the cultivable bacteria were spores already present in bentonite, which will be subjected to further investigation.

In similar experiment with 1900 kg/m^3 dry density MX-80 bentonite Pedersen et al. (2000a) detected fewer cultivable bacteria with increasing depth and duration of the experiment. The highest numbers of cultivable bacteria were observed shortly after the inoculation and the aerobic bacteria were detected in higher depths, than anaerobes regardless of time.

Table 3-1. Cultivation results of aerobic and anaerobic bacteria in colony forming units (CFU) per g of saturated bentonite, calculated from duplicate samples. Description of the samples is in Table 2-1 (same sample numbers)

Sample No.	Aerobic bacteria (CFU/g)		Anaerobic bacteria (CFU/g)	
	1400 kg/m^3	1600 kg/m^3	1400 kg/m^3	1600 kg/m^3
3	2.27×10^5	2.09×10^4	2.63×10^3	1.527×10^3
4	7.2×10^5	2.72×10^4	1.181×10^4	6.818×10^3
5	7.02×10^5	6.10×10^3	3.454×10^3	1.18×10^3
6	6.09×10^4	5.45×10^4	2.454×10^3	1.636×10^3
7	7.63×10^5	1.836×10^5	1.909×10^3	2.909×10^3
8	4.09×10^5	3.45×10^4	1.981×10^3	2.363×10^3
9	9.27×10^5	5×10^4	9.09×10^2	3.636×10^3
10	3×10^5	7.27×10^4	1.554×10^3	5.454×10^2
11	1.272×10^5	3.1×10^4	3.09×10^3	1.563×10^3
12	1.454×10^5	3.81×10^4	1.181×10^3	2.636×10^3
13	1.436×10^6	5.09×10^5	4.909×10^3	4.363×10^3
14	5.27×10^5	1.5×10^4	1.836×10^3	2.063×10^3

4 Conclusions on microbial mobility in saturated bentonites

Our study fulfilled two most important goals. Firstly we developed reliable method for direct detection of bacterial presence (both viable and dead cells) in compacted and saturated bentonite, which has been missing. Our method is based on the extraction of bacteria from bentonite using density gradient centrifugation and their subsequent Live/Dead staining. Although our method needs further optimization and testing of its general functionality on

other bentonite types, we believe it will be very useful for the future research of bacterial presence in various bentonite samples.

Our second goal was to use this newly developed method to study microbial mobility within compacted BaM bentonite of two different dry densities – 1400 kg/m^3 and 1600 kg/m^3. Our method enabled us to detect the presence of live and dead bacteria across the compacted bentonite samples. Fourteen samples differing in their distance from the source of bacteria (VITA water) was searched for the presence of bacteria in each of both compacted bentonite samples and we detected live cells in all tested samples including the very innermost part and the bottom part of the bentonite samples of both densities. This finding indicates that bacteria are able to move through compacted bentonite of relatively high dry density (1600 kg/m^3). The high bacterial mobility is further supported by the finding of aberrantly shaped live cells in the sample from outer-flow sieve, which are known from experiments studying the space limitations effects on bacterial proliferation. Previously published studies showed that the bacteria were generally able to survive and activate in bentonite compacted to similar dry density (1600 kg/m^3). Studies focused specifically on bacterial motility further demonstrated, that bacteria were able to move only to a very short distance (0.5 cm) from the surface in the highly compacted Na-bentonite (1800 kg/m^3 and higher). However, we used lower dry density and BaM bentonite belongs to Ca-bentonites that might generally have better microbial permeability. Further experiments are needed to test, whether the surprisingly high bacterial mobility detected in our experiment is caused by the lower bentonite density, or by unique features of BaM bentonite (or Ca-bentonites in general), or if our novel method is more sensitive than previously used methods and thus enabled us to detect the bacterial mobility more accurately than previous experiments.

Both aerobic and anaerobic cultivations confirmed the fluorescence microscopy results as we detected the presence of anaerobic and aerobic bacteria in all samples. Their number was rather consistent across all sections of the compacted bentonite of both densities without clear pattern. Higher number of aerobic bacteria was recorded in lower-density bentonite as could be expected.

5 Acknowledgement

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