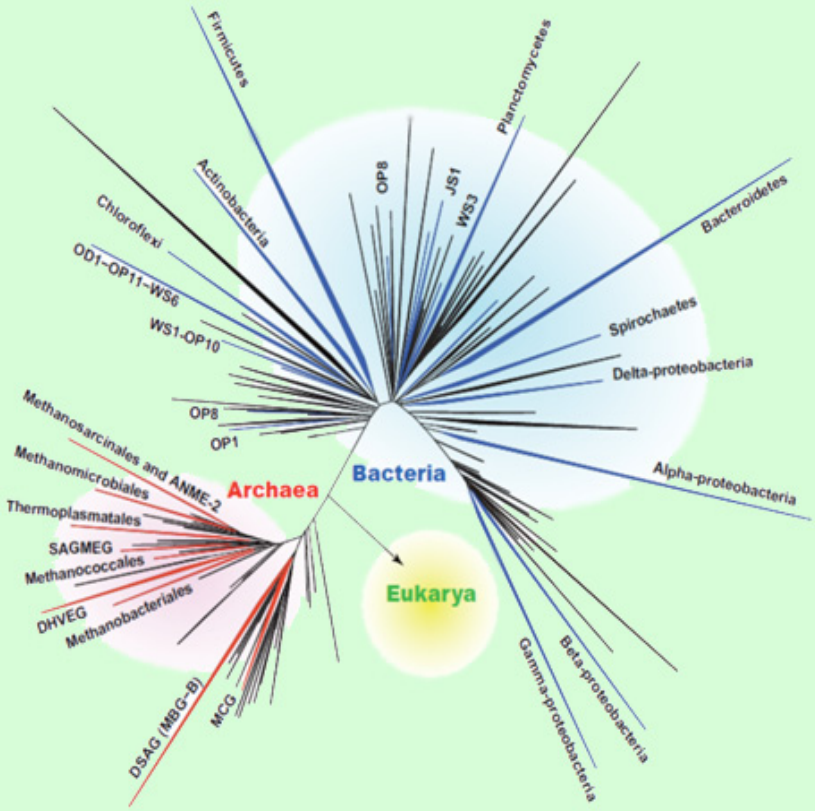

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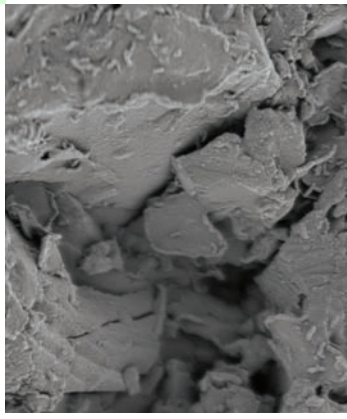
Microbial activity in bentonite buffers

Literature study

Marjaana Rättö | Merja Itävaara



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Microbial activity in bentonite buffers
Literature study

Marjaana Rättö & Merja Itävaara. Espoo 2012. VTT Technology 20. 30 p.

Abstract

The proposed disposal concept for high-level radioactive wastes involves storing the wastes underground in copper-iron containers embedded in buffer material of compacted bentonite. Hydrogen sulphide production by sulphate-reducing prokaryotes is a potential mechanism that could cause corrosion of waste containers in repository conditions.

The prevailing conditions in compacted bentonite buffer will be harsh. The swelling pressure is 7–8 MPa, the amount of free water is low and the average pore and pore throat diameters are small. This literature study aims to assess the potential of microbial activity in bentonite buffers. Literature on the environmental limits of microbial life in extreme conditions and the occurrence of sulphate-reducing prokaryotes in extreme environments is reviewed briefly and the results of published studies characterizing microbes and microbial processes in repository conditions or in relevant subsurface environments are presented

The presence of bacteria, including SRBs, has been confirmed in deep groundwater and bentonite-based materials. Sulphate reducers have been detected in various high-pressure environments, and sulphate-reduction based on hydrogen as an energy source is considered a major microbial process in deep subsurface environments. In bentonite, microbial activity is strongly suppressed, mainly due to the low amount of free water and small pores, which limit the transport of microbes and nutrients. Spore-forming bacteria have been shown to survive in compacted bentonite as dormant spores, and they are able to resume a metabolically active state after decompaction. Thus, microbial sulphide production may increase in repository conditions if the dry density of the bentonite buffer is locally reduced.

Keywords bentonite, nuclear waste, microorganisms, piezophiles, sulphate reducers

Contents

1. Introduction

The proposed disposal concept for high-level radioactive wastes involves storing the wastes in copper-iron containers with an outer copper shell and cast iron inset. The containers will be placed in disposal holes in a granitic deposition tunnel and embedded in buffer material of compacted bentonite blocks and pellets. The tunnels will be backfilled and sealed. At deposition, the water content of bentonite blocks is low (10–17%). By absorbing groundwater from rock fractures, added groundwater or surface water, the bentonite buffer will swell and thus fill the voids in the disposal holes and create an impermeable zone around the containers. After repository enclosure, oxygen is rapidly consumed and the conditions become anaerobic (Komine & Ogata 2004, Pedersen et al. 2000b).

Hydrogen sulphide production by sulphate-reducing prokaryotes is a potential mechanism that could cause corrosion of waste containers in repository conditions. Sulphate is available in most bentonites, and sulphate reduction is limited by the availability of organic carbon or molecular hydrogen, which act as electron donors (King et al. 2011).

The prevailing conditions in the compacted bentonite buffer will be harsh. The swelling pressure of bentonite is 7–8 MPa at full water saturation, and the amount of free water in the buffer is low ($a_w < 0.96$). The average pore and pore throat diameters in fully compacted bentonite are small. Stroes-Gascoyne et al. (2011) report micropores in the range of 0.005–0.1 µm with most of the pore diameters around 0.02 µm and a small fraction of macropores in the range 5–100 µm. Due to radiation, the temperature around the containers will reach 50–80ºC leading to very low water content (5% or less) in the bentonite directly adjacent to the containers. After the first 1000 years, the temperature will slowly cool down (Motamedi et al. 1996, Aoki et al. 2010).

This literature study aims to assess the potential of microbial activity in bentonite buffers in repository conditions. The literature on the environmental limits of microbial life in extreme conditions and the occurrence of sulphate-reducing prokaryotes on conditions resembling those in repositories is reviewed briefly, and the results of published studies characterizing microbes and microbial processes in repository conditions or in relevant subsurface environments are presented.

2. Microbes in extreme environments

The known diversity of prokaryotes includes approximately 6000 species based on cultivation while the actual microbial diversity is estimated to be 10^6-10^9 species (Dong & Yu 2007). Microbes have developed mechanisms to survive in environments with extremes of temperature, pH, pressure, salinity or radiation (Table 1) and are present in environments like crystalline and sedimentary rocks, hypersaline lakes, dry deserts and deep ocean hydrothermal vents. The metabolic diversity among microbes is great. Light, inorganic (hydrogen, reduced sulphur or nitrogen compounds, ferrous iron) or organic chemicals can be used as an energy source, and organic carbon or carbon dioxide as the sole carbon source. Besides aerobic respiration with molecular oxygen as a terminal electron acceptor, anaerobic respiration with ferric iron, nitrate, sulphate, carbonate or sulphur as an electron acceptor, or fermentation with internally balanced oxidation-reduction reactions can also be carried out.

Table 1. Examples of extremophiles and their environmental limits (from Seckbach & Oren 2005).

Environmental factor	Organism	Habitat	Phylogenetic affiliation	Tolerance to stress
High temperature	*Pyrolobus fumarii*	Hot undersea hydrothermal vents	Archaea - Crenarchaeota	Maximum 113°C, Optimum 106°C, Minimum 90°C
Low temperature	*Polaromonas vacuolata*	Sea-ice	Bacteria	Minimum 0°C Optimum 4°C, Maximum 12°C
Hydrostatic pressure	Strain MT41	Mariana Trench	Bacteria	Maximum >100 MPa, Optimum 70 MPa, Minimum 50 MPa
Low pH	*Picrophilus oshimae*	Acidic hot springs	Archaea - Euryarchaeota	Minimum pH -0.06, Optimum pH 0.7, Maximum pH 4 (is also thermophilic)
High pH	*Natronobacterium gregoryi*	Soda lakes	Archaea - Euryarchaeota	Maximum pH 12, Optimum pH 10, Minimum pH 8.5 (is also halophilic)
High salt concentration	*Halobacterium salinarum*	Salt lakes, salted hides, salted fish	Archaea - Euryarchaeota	Maximum NaCl saturation, Optimum 250 g/l salt, Minimum 150 g/l salt
Ultraviolet and ionizing radiation	*Deinococcus radiodurans*	Isolated from ground meat; true habitat unknown	Bacteria	Resistant to 1.5 kGy gamma radiation and to 1500 J/m^2 of ultraviolet radiation

2.1 Piezophiles

Piezophiles (formerly called barophiles) are microorganisms that reproduce preferentially or exclusively at pressures greater than atmospheric pressure. The pressure required for optimal growth varies from < 10 MPa for piezotolerants to > 50 MPa for hyperpiezophiles (Table 2). Suggested mechanisms enhancing pressure-resistance of cells include alteration of the membrane lipid composition to increase membrane fluidity, the presence of intracellular stabilizers (salts, sugars) and the presence of an S-layer around the cell membrane. The pressure tolerance of non-piezophilic prokaryotes can be increased by adaptation to other environmental stresses like high temperature, high salt concentration or decreased water activity (Kish 2012). Pressure-tolerant mutants (up to 100 MPa) of non-piezophilic bacteria have been obtained by selection (Bartlett 2002).

Table 2. Classification scheme of piezophiles based on optimal growth temperature and pressure (from Fang et al. 2010).

P_{kmax}/T_{kmax}	< 15°C	15–45°C	45–80°C	> 80°C
Piezotolerant (< 10 MPa)	Psychro-piezotolerant	Meso-piezotolerant	Thermo-piezotolerant	Hyperthermo-piezotolerant
Piezophilic (10–50 MPa)	Psychro-piezophile	Meso-piezophile	Thermo-piezophile	Hyperthermo-piezophile
Hyperpiezophilic (> 50 MPa)	Psychro-hyperpiezophile	Meso-hyperpiezophile	Thermo-hyperpiezophile	Hyperthermo-hyperpiezophile

The oceans cover 70% of the earth's surface. The average depth of the oceans is 3800 m and the average pressure is 380 times the pressure at the earth's surface. In oceans below the depth of 1000 m, the hydrostatic pressure is over 10 MPa and rises to over 100 MPa in the deepest marine subsurface sediments (Table 3). The study of piezophiles has focused, for a great part, on deep-sea environments. The majority of the marine piezophilic microorganisms described so far are Gram-negative, facultatively anaerobic bacteria with *Shewanella* spp. most frequently isolated as a pure culture, but the actual diversity of marine piezophiles is unknown (Fang et al. 2010). In deep marine sediments, a variety of *Bacteria* and *Archaea* containing mainly uncultured phylotypes has been detected by molecular methods (Fry et al. 2008, Orcut et al. 2011). The first hyperthermophilic obligate piezophilic archaeon Pyrococcus CH1 was isolated from a hydrothermal site in the mid-Atlantic ridge at a depth of 4100 m (Zeng et al. 2009).

Dense microbial communities are often found on the seafloor around geologically active areas (cold seeps, mud volcanoes) where methane- and hydrogen-sulphide-rich fluid release occurs. Sulphate reduction rates of 3.8–66.5 mmol m-2 d-1 and anaerobic oxidation of methane rates of 0.1–5.8 5 mmol m^{-2} d^{-1} were measured in mud volcanoes in the eastern Mediterranean Sea at water depths of 992–3022 m. Molecular methods revealed the presence of methane-oxidizing *Archaea* as well as some methane-oxidizing *Bacteria* while a large fraction of bacterial 16S rRNA sequences were related to SRP of the genera *Desulfosarcina, Desulfococcus, Desulfocapsa* and *Desulfobulbus* (Omoregie et al. 2009).

Table 3. High-pressure microbiological environments and their documented approximate upper pressures (from Allen & Bartlett 2002)

Environment	Approximate pressure
Deep-sea water column/surface sediments	112 MPa
Deep-sea invertebrates	108 MPa
Deep-sea fish	63 MPa
Deep-sea brines	15 MPa
Hydrothermal vents	41 MPa
Whale falls	41 MPa
Lake Baikal, Siberia[a]	16 MPa
Lake Vostok, Antarctica[a]	41 MPa
Deep marine sediments	14 MPa
Deep basaltic rock	67 MPa
Deep granitic rock	55 MPa
Deep oil reservoirs	31 MPa

[a] No microbiological studies have yet been done on samples from these deep freshwater environments

2.2 Sampling and cultivation methods of phiezophiles

Deep-sea piezophiles have been isolated from deep-sea animals (amphipods, fish), water and sediments using animal traps and various types of water or sediment samplers, e.g. multi-bottle water sampler, sterile bag sampler and multicoring sediment sampler. After surfacing, the samples can be maintained and cultivated in pressurized vessels, while short-time manipulation in atmospheric pressure is often reported (Alain et al. 2002, Lauro et al. 2007, Zeng et al. 2009). Samplers equipped with a deep-sea camera, and sediment or core samplers controlled by manipulators of manned or unmanned submersibles have also been constructed (Kim & Kato 2010, Orcutt et al. 2011). Various vehicles used for deep-sea sampling and their maximal diving depths are presented in Table 4.

Deep-sea piezophiles are extremely sensitive to UV irradiation and large temperature fluctuations. The degree of decompression sensitivity varies. Most of the strains isolated so far are facultative piezophiles, and many of them were originally isolated from deep-sea environments using conventional, non-piezophilic cultivation (Takai et al. 2009). Community changes in a deep-sea microbial community due to decompression and rupture of the cell envelope of the deep-sea methanogen *Methanococcus jannascii* by rapid decompression have been reported (Yanagibayashi et al. 1999, Park & Clark 2002) but the spectrum of decompression sensitivity of piezophilic prokaryotes is still not fully understood.

Table 4. Deep-sea vehicles (from Kim & Kato 2010).

Country	Manned submersible (maximum diving depth; m)	Unmanned submersible (maximum diving depth; m)
USA	Turtle (3,000) Alvin (4,500) Seacliff (6,000)	Magellan 725/825 (7,620) Trieste-I, II (12,000)
France	Deepstar (1,000) Cyana (3,000) Nautile (6,000)	Victor 6000 (6,000) Archimedes (11,000)
Japan	Shinkai 600 (600) Shinkai 2000 (2,000) Shinkai 6500 (6,500)	Hyper dolphin (2,000) Kaiko 7K (7,000) Kaiko 11K (11,000)
Russia	Mir I & II (6,000)	RTM 6000 (6,000)
Korea	Ocean 250 (250)	Haemirae (6,000) Okpo 6000[a] (6,000)

[a]AUV – Autonomous Underwater Vehicle

Special instruments for isolation and cultivation of piezophilic microbes without decompression have been constructed in various laboratories. The DEEP BATH system of JAMSTEC, originally described by Kyo et al. (1991), consists of a pressure-retaining sampling device, a dilution device under pressure conditions, an isolation device and a cultivation device with maximal capacity of 3 litres. The pressure and temperature ranges for the system are 0.1–65 MPa and 0–150ºC. The samples are mixed with a liquid medium and cultivated in a high-pressure vessel. From this liquid culture, moderate piezophiles and piezotolerants can be isolated by plating, obligatory piezophiles in low-melting-point agar by the pressure bag method and thermophilic piezophiles by the dilution-to-extinction method (Kim & Kato 2010). The isolation of obligate piezophiles in agar in the DEEP BATH is presented in Figure 1.

Parkes et al. (2009) describe another system for sampling and handling subsurface sediments for the recovery of depressurization-sensitive anaerobes. The system consists of HYACINTH pressure-retaining drilling and core storage system, PRESS core cutting and processing system, and DeepIsoBUG for enrichment, growth and isolation at elevated pressure. The system enables sediment handling without depressurization at up to 25 MPa and anaerobic enrichment and isolations at up to 100 MPa.

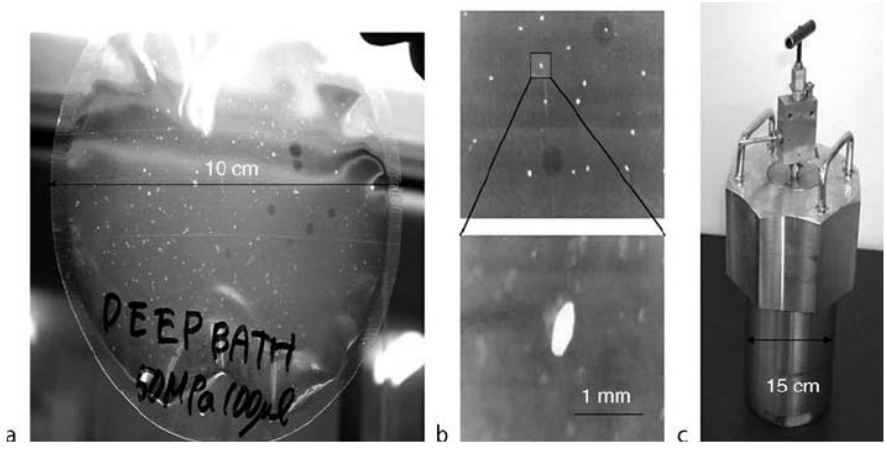


Figure 1. Isolation of obligate piezophiles in agar by the pressure bag method: a) plastic bag after high-pressure cultivation, b) colonies on agar surface and c) the pressure vessel (from Kim & Kato 2010).

Takai et al. (2008) have developed a technique for cultivating chemolithoautotrophs in gas-rich fluid under high hydrostatic pressures. Cultivation is carried out in glass syringes in a pressure vessel. The method has been used for characterization of thermophilic methanogens under high-pressure conditions. Houghton et al. (2007) constructed a continuous culture bioreactor for cultivation at 25 MPa, which allows sampling for liquid and microbiological analysis during the cultivation.

2.3 Desiccation-tolerant microbes

Water is essential to life. Water availability in a system can be limited by the concentration of solutes (osmotic effects) or by capillary and surface binding (matric effects). Most literature on microbial water stress tolerance relates to environments in which water availability is limited by the presence of solutes. In preserved foods, the growth of most bacteria is inhibited at a water activity (a_w) of 0.97–0.95, while the most tolerant bacteria can grow at a_w of 0.85 and halophilic archaea at a_w of 0.75 (Grant 2004). In non-saline soils, water availability is determined by the matric potential. At low matric potential, the thickness of the water film around particles decreases. Limited substrate diffusion and cell motility in soils at low matric potential reduce microbial activity and these conditions have been found to be more detrimental to microbes than a corresponding low osmotic potential at optimal soil water content (Chowdhury et al. 2011).

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. Spore formation is triggered by nutrient depletion. Dormant spores exhibit remarkable

longevity in the environment, and the recovery and revival of spores from environmental samples as old as 10^5 years has been reported. Spore-forming bacteria are found among, e.g., aerobic heterotrophs, anaerobes 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 unknown (Billi & Potts 2002).

2.4 Pore-size effects on microbial activity

The size of the prokaryotic cells varies from a 0.1–0.2 µm diameter to a diameter of more than 50 µm, and a typical prokaryotic cell is a rod with dimensions of 1 x 3 µm. The estimates for the minimum diameter of microbial cells needed to contain the necessary macromolecules calculated by various authors are in the range 0.34–0.1 µm (Kieft 2000).

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. The cells of most bacteria in soils are small. Some microscopic studies have shown the majority to be coccoid cells with a diameter of less than 0.3 µm. The small size can be induced by starvation, but a fraction of the soil bacteria is proposed to be intrinsically small (Kieft 2000). In an investigation of microbial activities in shale and sandstone cores collected in New Mexico, no metabolic activity (carbon mineralization, sulphate reduction) was detected in samples with pore throat diameters < 0.2 µm whereas in samples with pore throats > 0.2 µm, microbial activity was commonly detectable. After 14 d enrichment cultivation, sulphate-reducing bacteria and sulphate-reducing activity were detected in some of the small pore throat samples (Fredrickson et al. 1997). Living bacteria, including sulphate-reducing bacteria, have been recovered from sediments 2800 m underground with 0.5–4 µm size pores and < 0.04 µm pore throats (Onstott et al. 1998).

3. Sulphate-reducing prokaryotes

Sulphate-reducing bacteria (SRB) or more correctly sulphate-reducing prokaryotes (SRP) consist of a diverse, distantly-related assembly of *Bacteria* and *Archaea* characterized by the use of sulphate as a terminal electron acceptor during anaerobic respiration. The functional gene *dsrAB* coding dissimilatory sulphite-reductase, an enzyme that catalyses the final step in sulphate respiration, can be used as a marker when studying the abundance of SRPs in the environment (Leloup et al. 2004).

SRPs are a physiologically complex group. Heterotrophic SRPs use various low molecular weight organic compounds as substrates while autotrophic SRPs can use CO_2 as a carbon source and hydrogen as the sole energy source (Liamleam & Annachhatre 2007). Based on the rRNA sequence analysis, SRPs have been classified in four distinctive groups (Castro et al. 2000):

- Gram negative mesophilic SRP
- Gram positive spore-forming SRP
- Thermophilic bacterial SRP
- Thermophilic archaeal SRP.

SRPs are widely distributed in anaerobic ecosystems. They may grow in various extreme environments, e.g. at low or high temperatures, and at high alkalinity, salinity or pressure (Olliver et al. 2007). The thermophilic bacterial SRPs grow at temperatures of up to about 70–80ºC (Kaksonen et al. 2007) whereas the thermophilic archaeal species exhibit optimal growth temperature above 80ºC (Castro et al. 2000). The gram positive spore-formers (*Desulfotomaculum* spp., *Desulfosporosinus* spp.) can be mesophilic or thermophilic, and the endospores produced have been reported to resist temperatures of up to 131ºC (Rosnes et al. 1991). *Desulfotomaculum*-related environmental clones have been detected from several subsurface environments (Lin et al. 2006).

SRPs have been detected in high pressure environments including deep marine sediments and deep continental sedimentary rocks (Muyzer & Stams 2008). *Desulfovibrio piezophilus* isolated from wood falls at a depth of 1693 m in the Mediterranean Sea grows at 0–30 MPa pressures with the optimum at 10 MPa (Khelaifia et al. 2011). *Desulfovibrio hydrothermalis* isolated from deep-sea hydrothermal chimney at a depth of 2600 m grows faster at 26 MPa pressure than at

1 atm and can use hydrogen or low molecular weight organic compounds as electron donors (Alazard et al. 2003). Sulphate reduction based on hydrogen as an energy source is considered a major microbial process in the deep subsurface environments devoid of sedimented organic carbon (Swanner & Templeton 2011). A single phylotype related to spore-forming SRPs was found to be dominant in a microbial community in high-pressure fracture water at a depth of 2.8 kilometres (Lin et al. 2006).

4. Indigenous Microbes in bentonite and other clay minerals

The numbers of aerobic and anaerobic heterotrophs detected by Stroes-Gascoyne et al. (2010) in commercial Wyoming MX-80 bentonite were 10^5 cfu/g and 10^2 cfu/g, respectively. Masurat et al. (2010a) isolated *Desulfovibrio africanus* from Wyoming bentonite MX-80 using medium selective for SRBs. The strain could grow in temperatures of up to 40ºC and in salt concentrations of 0.7%–4.0%, and it was shown to remain viable in bentonite after 20 h of heat treatment at 100ºC.

Fukunaga et al. (2005) studied the microbiology of bentonite deposits as a natural analogue of bentonite-based buffer material using culture-based methods and microscopic counting of viable cells. CFDA-AM was used as viable stain to avoid interference of clay particles that can affect methods using DAPI or acridine orange. Dry densities of the samples at a distance of 1 m from the drilling mouth varied between 1.1 and 1.5 g cm^{-3}. Aerobic and anaerobic plate counts on R2A decreased with increasing depth and were below 10^2 cfu/g DW at a distance of 1 m from the drilling mouth. At the same time, viable cell counts decreased from 10^9-10^7 /g DW to 10^5-10^6 /g DW.

Studies of microbial occurrence in Opalinus clay resulted in the detection of a low number of viable microbes by culture-based methods whereas attempts to extract PCR-amplifiable DNA from the clay were not successful, most probably due to the low numbers of microbes present and the strong binding of DNA into clay minerals (Stroes-Gascoyne et al. 2007b, Poulain et al. 2008). On the other hand, Boivin-Jahns et al. (1996) detected bacteria by a PCR-based method at all distances along the 20-m-long core in Bloom clay formation at a depth of 224 m, though culturable bacteria were not detected below 80 cm.

Clay minerals inhibit sulphate-reducing activity in natural sediments. By using pure cultures of *Desulfovibrio vulgaris,* Wong et al. (2004) showed that the inhibition is correlated to Al_2O_3 content in clays.

5. Groundwater microbes

In an investigation of 16 groundwater samples from the Fennoscandian Shield in Finland and Sweden, the average total planktonic cell number was 3.7 x 10^5 cells/ml and the average cell number remained constant at depths between 65 and 1390 m. Sulphate-reducing bacteria were cultured from groundwaters sampled at 65–586 m (Haveman & Pedersen 2002). A microbial community dominated by *Desulfotomaculum* spp. and *Methanobacterium* spp. has been detected in alkaline, 54–60ºC water at 4–5 km below the land surface (Moser et al. 2005).

Microbial density measurements of the Outokumpu deep borehole water revealed that microbial cell density varied from 10^5 cells ml^{-1} at the surface to 10^3 cells ml^{-1} at a depth of 2350 m (Itävaara et al. 2011a). Molecular biological analyses demonstrated that microbial communities varied as a function of sampling depth. Sulphate reducers were detected at all depths and an increasing trend in the number of species related to *Desulphotomaculum* sp. and *Desulphosporosinus* sp. was observed at the deeper depths (Itävaara et al. 2011b). SEM micrographs of microorganisms in the Outokumpu deep borehole water are presented in Figure 2.

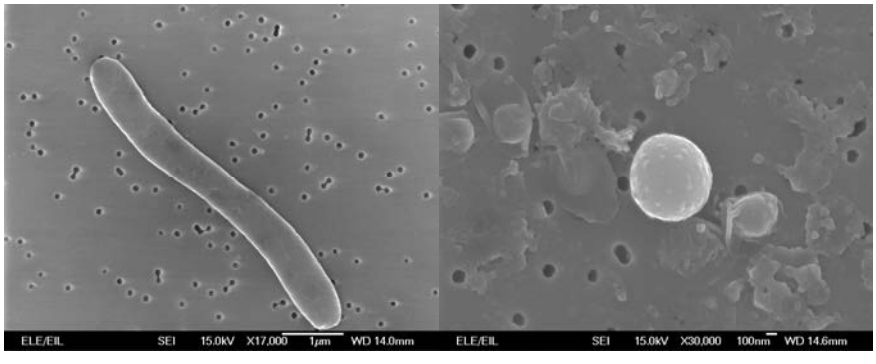


Figure 2. SEM micrographs of microorganisms in the Outokumpu deep borehole water at a depth of 1000 m.

6. Laboratory-scale studies on bacteria/bentonite interactions

6.1 Culturability of introduced bacteria

Laboratory-scale studies on the survival of introduced bacteria in swelling bentonite have been carried out with the main focus on sulphate-reducing bacteria. Motamedi et al. (1996) inoculated sodium bentonite with *Desulfomicrobium baculatum* and *Desulfovibrio* sp., originally isolated from deep groundwater. The bentonite was compacted to three different densities (1.5, 1.8 and 2 g cm^{-3}) corresponding to a_w values of 1.0, 0.99 and 0.96, respectively. After incubation anaerobically at 30ºC for 1 and 60 days, the number of viable cells was estimated by the MPN method. According to the authors, the survival of SRBs in bentonite was dependent on the availability of water. Both strains lost viability in one day at a_w 0.96 or in 60 days at a_w 0.99.

Pedersen et al. (2000b) compacted Wyoming bentonite MX-80 in swelling pressure oedomers to 1.90 g cm^{-3} and introduced bacteria (SRPs *Desulfovibrio salaxigens*, *Desulphomicrobium baculatum* isolated from Äspö groundwater, *Desulfotomaculum nigrificans* and *Thermodesulfobacterium commune* as well as aerobic bacteria, including spore-forming *Bacillus* spp. and radiation- and desiccation-resistant *Deinococcus radiodurans*) on top on the compacted bentonite. After 28 weeks, only two of the introduced bacteria (*Deinococcus radiodurans* and *Bacillus subtilis*) could be cultivated from the deepest bentonite layer (3–6 mm) studied. *Desulfotomaculum nigrificans* was cultivated from the surface layer (0–1 mm).

6.2 Culturability of indigenous bacteria

Microbial activity is suppressed in compacted bentonite. The effect of the physical properties of bentonite on the culturability of indigenous microbes was studied in Wyoming MX-80 bentonite compacted to dry densities from 0.8 to 2.0 g cm^{-3}. Pore water salinities varied from 0 to 200 g L^{-1}. After 40–90 days of stabilization, the effects of dry density and porewater salinity on swelling pressure and a_w were evaluated. The range of bentonite pore diameters measured at 1.63 g cm^{-3} was 0.005–0.1 µm, which is below the size range of the vegetative cells of most bacteria. The microbiological analysis performed included enumeration of indigenous aerobic

and anaerobic heterotrophs by plating and indigenous SRBs by the MPN method. In addition, the number of viable cells was calculated based on phospholipid fatty acid (PLFA) analysis. The numbers of anaerobes and SRBs recovered were low in all the samples. The numbers of culturable aerobes decreased as the dry density increased and was below the detection limit at a dry density of 1.6 g cm^{-3}, but the PLFA analysis indicated that bacteria were present as spores or dormant cells (Stroes-Gascoyne et al. 2010). Upon reduction of the dry density of compacted bentonite from 1.6 g cm^{-3} to 1 g cm^{-3}, the culturability of microbes was shown to be restored (Stroes-Gascoyne et al. 2011).

6.3 Sulphide production by introduced bacteria

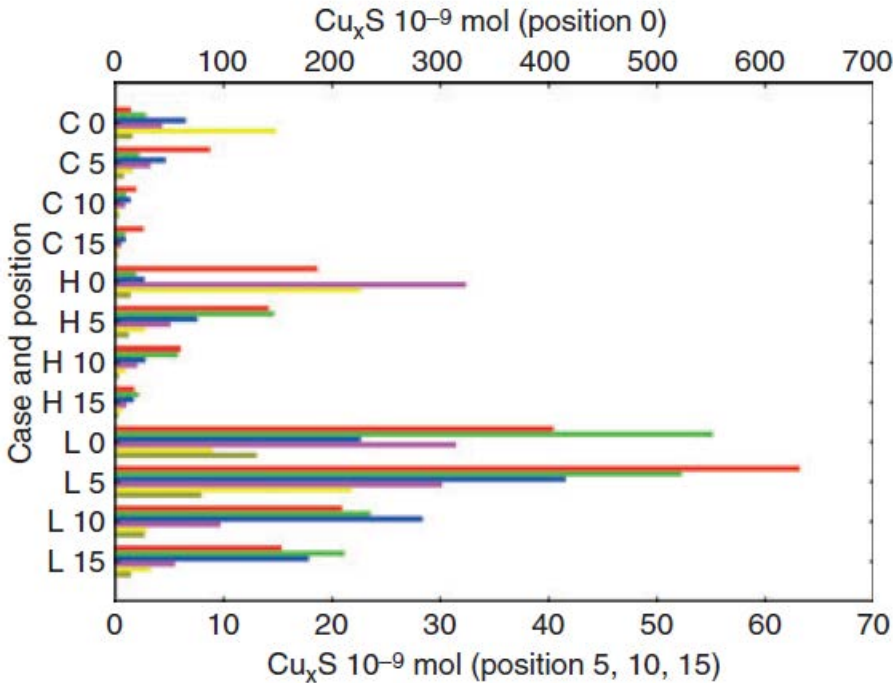
Pedersen et al. (2000b) studied the sulphate-reducing activity of *Desulphomicrobium baculatum* (*isolated* from Äspö groundwater) and *Desulfotomaculum nigrificans* in Wyoming bentonite MX-80 compacted to 1.5, 1.8 and 2.0 g cm^{-3}. The bacteria were introduced on top of the compacted bentonite, covered with a copper disc and incubated for 4 weeks. Sulphate reduction was detectable by radioisotope imaging at the lowest density studied (1.5 g cm^{-3}).

6.4 Sulphide production by bacteria originating from bentonite and deep groundwater

Masurat et al. (2010b) studied in situ sulphide production in compacted bentonite by indigenous SRPs occurring in deep groundwater or bentonite. The experiments were carried out in anaerobic conditions at the Äspö Hard Rock Laboratory. Copper plates were placed in Wyoming bentonite MX-80, lactate was added to the bentonite as a source of energy and organic carbon and the bentonite was compacted in swelling pressure oedometer cells to densities of 1.5, 1.8 and 2.0 g cm^{-3} (swelling pressure 0.1, 1.5 and 7.8 MPa). Groundwater from a borehole was circulated through the experimental system, and the production of copper sulphide on the copper plates was monitored using labelled sulphur. The results demonstrated that both bentonite and groundwater act as a source of SRPs and that increasing bentonite density correlated with decreasing copper sulphide production rates. A low level of sulphide production was detected in bentonite compacted to 2.0 g cm^{-3}, but the authors concluded that sulphide production rates in highly compacted bentonite are not high enough to corrode through the copper capsule in 100 000 years.

Pedersen (2010) analysed sulphate reduction rates by indigenous SRPs in deep groundwater circulated over bentonite compacted into different densities (1750–2000 kg m^{-3}) in oedometer chambers and studied the diffusion of sulphide into the bentonite. Sulphide was mainly produced in groundwater and at the water/bentonite boundary, and dissolved sulphide concentrations were dependent on SRP growth conditions (availability of sulphate, carbon source and electron donors) as well as geochemical conditions (presence of ferrous iron to precipitate

sulphide). The amounts of copper sulphide on the copper plates embedded in the bentonite increased with the SRP activity in the water and decreased with increasing depth and bentonite density (Figure 3).



Case: C= control, H= hydrogen and carbon dioxide added, L= lactate added
Position (from surface): 0 = 0–5 mm, 5 = 5–10 mm, 10 = 10–15mm, 15 = 15–20 mm
Density (Kg m^{-3}): ,1750; ,1800; , 1850; , 1900; , 1950 and , 2000

Figure 3. Graphical representation of the amounts of copper sulphide analysed on copper plates embedded in bentonite compacted into different densities (from Pedersen 2010).

7. Studies on bentonite/bacteria interactions in repository conditions

7.1 Viability of introduced bacteria

The viability of sulphate-reducing bacteria introduced into compacted bentonite clay under repository conditions was studied by Pedersen et al. (2000a). In addition to three SRPs (*Desulfovibrio aespoeensis* isolated from deep Äspö groundwater, thermophilic spore-forming *Desulfotomaculum nigrificans* and moderately halophilic *Desulfovibrio salexigens*), three aerobic non-spore-forming and two aerobic spore-forming bacteria were used. The bacteria were mixed into bentonite and the inoculated bentonite samples were compacted using a laboratory compaction device to form plugs at a density of 2 g cm^{-3}. The plugs were placed in holes drilled into bentonite blocks and the blocks were installed into boreholes at 450 m underground and exposed to low (20–30ºC) and high (50–70ºC) temperatures. After 15 months, no viable non-spore-forming bacteria were detected (plating or MPN). The numbers of spore-forming bacteria had decreased, but all three spore-formers could be cultivated from blocks exposed to the low temperature, and *D. nigrificans* and *B. subtilis* also survived at the high temperature.

7.2 Bacterial colonization of bentonite-based materials

A full-scale waste disposal container experiment carried out in Canada 1991–1994 consisted of an electric heater surrounded by buffer material of sand and bentonite (50%/50%). During the experiment, a moisture content gradient developed, ranging from 13% closest to the heater to 23% at the rock wall. Upon decommissioning after 2.5 years, the samples were analysed using viable counts and API identification, lipid analysis and the 16S rRNA se quencing (Stroes-Gascoyne et al. 1997). Microorganisms could be cultured from all the samples with a moisture content above 15% but not from samples with a moisture content below 15%. Heterotrophs were found ranging from 10^1 to 10^6 cells/g DW. SRBs were also detected at 10^2 cells/g or less. Furthermore, 79 isolates representing beta, gamma and delta *Proteobacteria* and Gram-positive bacteria were identified using API, and 67 16S rRNA clones obtained from buffer material were classified into 21 clone groups representing alpha and gamma *Proteobacteria*, Gram-positive bacteria and a yeast.

Culture-based methods were used for the analysis of bacterial diversity of clay-based sealing materials from a tunnel sealing experiment carried out in Canada 1996–2004 (Stroes-Gascoyne et al. 2007a). The bulkhead material used was a mixture of 70% Kunigel VI bentonite and 30% sand, pressed into blocks with a dry density of 1.9 Mg m^{-3}. Plate cultivation and MPN methods revealed culturable populations of heterotrophic aerobes and anaerobes, SRBs and facultative anaerobic nitrate-respiring and nitrate-reducing bacteria in the clay bulkhead. Culturable populations of bacteria were detected in all samples (Figure 4). The samples from interfaces (clay block-clay block, clay block-rock and clay-geotextile) had clearly higher populations of heterotrophic aerobes than the bulk clay whereas the populations of heterotrophic anaerobes and SRBs were constant at all locations, suggesting that the SRBs survived in a metabolically almost inactive form. The water activity a_w values were > 0.96 in all samples.

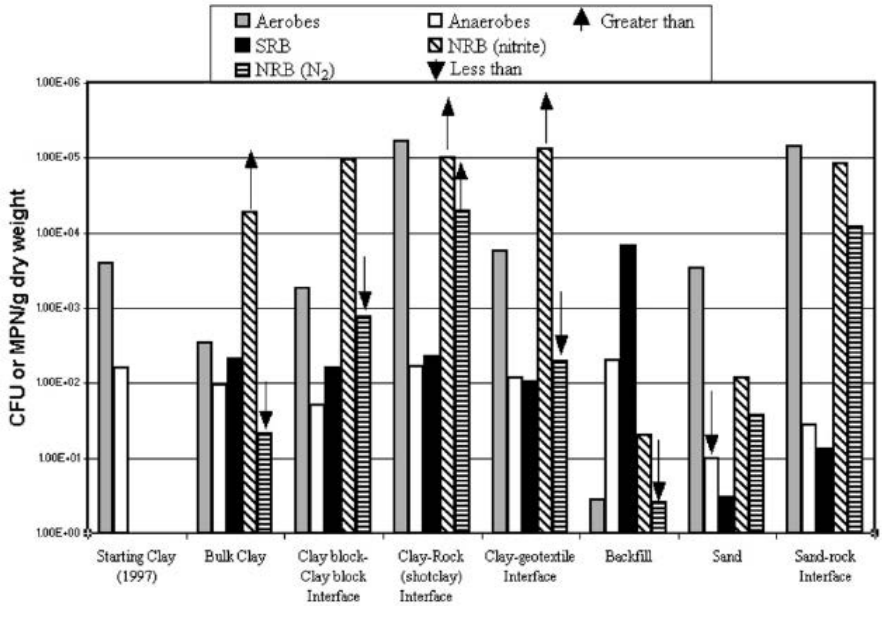


Figure 4. Comparison of average microbial populations in various types of clay samples from a Canadian tunnel sealing experiment (from Stroes-Gascoyne et al. 2007a).

Culture-based and molecular biology methods were applied to study the presence of groundwater microbes in the bentonite buffer in Äspö Hard Rock Laboratory's long-term and canister retrieval tests (Fru & Athar 2008). The tests were carried out under in situ conditions using Wyoming MX-80 bentonite blocks compacted to a density of 2 t m^{-3} placed around copper tubes supplied with a central electric heater. After 5 years of equilibration with deep groundwater at three different tem-

peratures, bentonite samples were collected for microbiological analysis. The analysis included enrichment of sulphate-reducing bacteria and acetogens and plate cultivation of heterotrophic bacteria. In addition, genomic DNA was extracted from groundwater, from the enrichment cultures and directly from the bentonite and the 16S rRNA and αβ subunits of dissimilatory bisulphite reductase genes (DsrAB) were PCR amplified. The 16S rRNA gene clone library revealed that 44% of the sequences retrieved belonged to the bacilli and only bacilli were discovered in the DNA amplified directly from the clay (Table 5). Production of hydrogen sulphide was detected in the enrichment cultures, but the DsrAB gene markers were not detected. In DGGE fingerprinting, *Desulfovibrio aespoeensis* was prominent in the groundwater but could not be detected in clay, whereas bands identical to *Desulfosporosinus* were common in both clay and groundwater.

Table 5. GenBank analysis of representative 16S rRNA gene sequences identified in compacted bentonite in Äspö Hard Rock Laboratory Long term and canister retrieval tests (from Fru & Athar 2008).

Detection method	Identified relative	Sequence identity (%)	Classification	Temperature (°C)
Clones—direct DNA extraction				
LOTb	*Bacillus firmus* (1)	98	Firmicutes	19
LOTd	*Paenibacillus wynnii* (1)	97	Firmicutes	19
LOTa, c, e_f	*Bacillus benzoevorans* (6)	100	Firmicutes	19
LOTg-h	*Bacillus benzoev orans* (2)	100	Firmicutes	67–110
LOTi	*Bacillus litoralis* (1)	98	Firmicutes	19
LOTj–k	*Bacillus koguryoae* (2)	100	Firmicutes	19 and 67–110
LOTl	*Bacillus pichinotyi* (1)	94	Firmicutes	19
CRT1–2	*Bacillus pichinotyi* (2)	93	Firmicutes	35–55
CRT3	*Bacillus koguryoae* (1)	99	Firmicutes	35–55
CRT25	*Paenibacillus polymyxa* strain GBR_1 (1)	99	Firmicutes	35–55
CRT18	*Bacillus megaterium* (1)	99	Firmicutes	35–55
CRT4r	*Bacillus borophilicus* (1)	99	Firmicutes	35–55
Heterotrophic isolates				
LOT1, 1a_d	*Pseudomonas Stutzeri* (5)	99	γ-Proteobacteria 19	
LOT3	*Devosia riboflavina* (1)	95	α-Proteobacteria	19
LOT2	*Ornithinimicrobium* sp. (1)	99	Actinobacteria	19
LOT4	*Dietzia daqingensis* (1)	99	Actinobacteria	19
CRT11	*Microbacterium phyllosphaerae* (1)	98	Actinobacteria	35–55
CRT11a	*Streptomyces albidoflavus* (1)	100	Actinobacteria	35–55
CRT12	*Arthrobacter* sp. KT1115 (1)	99	Actinobacteria	35–55
CRT13, 17	*Pseudomonas stutzeri* (2)	100	γ-Proteobacteria	35–55
SRB and acetogens enrichment				
Lace1_2 (LOT)	*Clostridium* sp. EBR.02E_0599 (2)	92	Firmicutes	19
Lace3_5 (LOT)	*Clostridium* sp. (BN II) (3)	96	Firmicutes	19
Lace6 (LOT)	*Clostridium hydrobenzoicum* (1)	97	Firmicutes	19
Lace7 (LOT)	*Bacillus megaterium* strain GP S10 (1)	99	Firmicutes	19
Lace8 (LOT)	*Sedimentibacter* sp. JN18_A14_H (1)	97	Firmicutes	19
2Ben5 (LOT)	*Clostridium hydroxybenzoicum* (1)	98	Firmicutes	19
3Ben5 (LOT)	*Clostridium* sp. EBR-02E-0599 (1)	94	Firmicutes	19
4Ben5, 5Ben5 (LOT)	*Desulfospo rosinus* sp. (2)	99	Firmicutes	19
7Ben5 (CRT)	*Desulfospo rosinus* sp. (1)	99	Firmicutes	35–55

Numbers in parenthesis represent the number of similar clones or isolates

8. Summary

The presence of bacteria, including SRBs, has been confirmed in deep groundwaters and in bentonite-based materials. Sulphate reducers have been detected in various high-pressure environments, and sulphate reduction based on hydrogen as an energy source is considered a major microbial process in deep subsurface environments. In compacted bentonite, microbial activity is strongly suppressed, mainly due to the low amount of free water present and the small size of pores and pore throats, which limit the transport of microbes and nutrients. A very low amount of sulphide production has been measured, however, even in fully compacted bentonite. Spore-forming bacteria have been shown to survive in compacted bentonite as dormant spores and are able to resume a metabolically active state after decompaction. Thus, microbial sulphide production may increase in repository conditions if the dry density of the bentonite buffer is locally reduced.

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VTT Technology 20

Title	**Microbial activity in bentonite buffers** **Literature study**
Author(s)	Marjaana Rättö & Merja Itävaara
Abstract	The proposed disposal concept for high-level radioactive wastes involves storing the wastes underground in copper-iron containers embedded in buffer material of compacted bentonite. Hydrogen sulphide production by sulphate-reducing prokaryotes is a potential mechanism that could cause corrosion of waste containers in repository conditions. The prevailing conditions in compacted bentonite buffer will be harsh. The swelling pressure is 7–8 MPa, the amount of free water is low and the average pore and pore throat diameters are small. This literature study aims to assess the potential of microbial activity in bentonite buffers. Literature on the environmental limits of microbial life in extreme conditions and the occurrence of sulphate-reducing prokaryotes in extreme environments is reviewed briefly and the results of published studies characterizing microbes and microbial processes in repository conditions or in relevant subsurface environments are presented. The presence of bacteria, including SRBs, has been confirmed in deep groundwater and bentonite-based materials. Sulphate reducers have been detected in various high-pressure environments, and sulphate-reduction based on hydrogen as an energy source is considered a major microbial process in deep subsurface environments. In bentonite, microbial activity is strongly suppressed, mainly due to the low amount of free water and small pores, which limit the transport of microbes and nutrients. Spore-forming bacteria have been shown to survive in compacted bentonite as dormant spores, and they are able to resume a metabolically active state after decompaction. Thus, microbial sulphide production may increase in repository conditions if the dry density of the bentonite buffer is locally reduced.
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Microbial activity in bentonite buffers. Literature study

"Assessment of bentonite properties" (BOA) one of the coordinated projects in Finnish Research Programme on Nuclear Waste Management KYT2014. The Programme is based on the Nuclear Energy Act (990/1987) according to which the aim of research is "ensuring that the authorities have such sufficient and comprehensive nuclear engineering expertise and other facilities at their disposal that are needed for comparisons of the various ways and methods of carrying out nuclear waste management". This literature study aims to assess the potential of microbial activity in bentonite buffers in waste repository conditions.

The proposed disposal concept for spent nuclear waste in granitic deposition tunnels involves storing the wastes in copper-iron containers embedded in buffer material of compacted bentonite. Hydrogen sulphide production by sulphate reducing microbes is a potential mechanism which could cause corrosion of waste containers in repository conditions. In this review published data on adaptation of microorganisms to different environments and on occurrence of sulphate reducing microbes in extreme environments is shortly reviewed and results of published studies characterising microbes and microbial processes in repository conditions or in relevant subsurface environments are presented. Literature survey revealed that bentonite may contain variable number of micoorganisms. Sulphate reducing microorganisms may stand high pressures after adapting to such conditions. In compacted bentonite microbial activity is strongly suppressed mainly due to the absence of free water and the small size of pores which limit the transport of microbes and nutrients. Nevertheless, very low amount of sulphide production has been detected even in fully compacted bentonite. In addition dormant sporeforming microorganisms may survive in bentonite for long periods and may be metabolically activated after decompaction. The microbial sulphide production may increase in repository conditions if dry density of bentonite buffer is locally reduced.

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