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TECHNICAL REPORT 95-27

Survival of bacteria in nuclear waste buffer materials

The influence of nutrients, temperature and water activity

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December 1995

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SURVIVAL OF BACTERIA IN NUCLEAR WASTE BUFFER MATERIALS

THE INFLUENCE OF NUTRIENTS, TEMPERATURE AND WATER ACTIVITY

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by

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Key words: bentonite, corrosion, nuclear waste, sulphate-reducing bacteria, water activity.

Abstract

The concept of deep geological disposal of spent fuel is common to many national nuclear waste programs. Long-lived radioactive waste will be encapsulated in canisters made of corrosion resistant materials e.g. copper and buried several hundred meters below ground in a geological formation. Different types of compacted bentonite clay, or mixtures with sand, will be placed as a buffer around the waste canisters. A major concern for the performance of the canisters is that sulphate-reducing bacteria (SRB) may be present in the clay and induce corrosion by production of hydrogen sulphide. This report presents data on viable counts of SRB in the bedrock of Äspö hard rock laboratory. A theoretical background on the concept water activity is given, together with basic information about SRB. Some results on microbial populations from a full scale buffer test in Canada is presented. These results suggested water activity to be a strong limiting factor for survival of bacteria in compacted bentonite. As a consequence, experiments were set up to investigate the effect from water activity on survival of SRB in bentonite. Here we show that survival of SRB in bentonite depends on the availability of water and that compacting a high quality bentonite to a density of 2.0 g/cm^3, corresponding to a water activity (a_w) of 0.96, prevented SRB from surviving in the clay.

Sammanfattning

Slutförvar av utbränt kärnbränsle kommer i de flesta länder att ske på stort djup i berggrund eller andra geologiska formationer. Avfallet skall kapslas in korrosionsbeständiga behållare innan det deponeras. Olika typer av bentonitlera, eller bentonitlera blandad med sand, skall placeras runt behållarna som en buffert mot grundvatten- och bergrörelser. Från olika håll har uttryckts oro över att sulfatreducerande bakterier kan förekomma i bentoniten och där inducera korrosion av behållarna genom sin produktion av vätesulfid. Rapporten redovisar förekomst av sulfatreducerande bakterier i berget kring Äspö-laboratoriet. Vidare ges en teoretisk genomgång av konceptet vattenaktivitet samt om sulfatreducerande bakterier. Relevanta delar av ett fullskaligt bufferförsök i Kanada presenteras också. En av slutsatserna därifrån var att tillgång på vatten tycks vara en absolut begränsade faktor för bakterier i kompakterad bentonit. Till följd av dessa resultat planerades och genomfördes en serie experiment för att studera relationen mellan överlevnad av bakterier och vattenaktivitet under kontrollerade förhållanden på laboratoriet. Våra resultat visar att överlevnad av sulfatreducerande bakterier i bentonit beror av tillgängligheten på vatten och att om en bentonit av hög kvalitet kompakteras till 2 g/cm^3, motsvarande en vattenaktivitet på 0.96, så överlever inte sulfatreducerande bakterier - de dör av uttorkning.

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Summary

The concept of deep geological disposal of spent fuel is common to most international nuclear waste programs. Assessments of the performance of disposal systems are often done covering periods of up 10,000 years or more. Corrosion is an important process to consider in such an assessment. Sulphate-reducing bacteria (SRB) may cause corrosion of canister materials due to their dissimilatory reduction of sulphate to hydrogen sulphide. Consequently, it is very important to reveal if SRB can survive and produce hydrogen sulphide in bentonite buffers.

A summary of data on viable numbers of SRB in the Äspö Hard Rock Laboratory shows that the anticipated risk for presence of SRB at disposal depths is real and must be accounted for in the safety performance assessment. Two scenarios must be assessed: In the first scenario, SRB will grow in the surrounding geological formation and produce hydrogen sulphide that must diffuse through the buffer to corrode the copper canister. This case has been thoroughly discussed elsewhere. The second scenario, were SRB will grow in the buffer material around the canister and produce hydrogen sulphide that is corrosive to copper, is dealt with here.

This report first gives a theoretical background on solutes and water activity (chapter 2) and it summarises present knowledge about SRB (chapter 3). Thereafter, the buffer as a habitat for bacteria is discussed and some relevant microbiology data from a full scale buffer test at the AECL underground facility URL is summarised (chapter 4). Finally, an investigation of the survival of SRB in bentonite at three different densities is presented (chapter 5).

The results from a statistical analysis of variance of viable bacteria in a full scale buffer test in Canada indicated water content to have a large impact on the viability of bacteria in the buffer mass. The effect from the *in situ* buffer mass temperature was not significant. At a low water content, there where few cultivable bacteria, but high temperature was not limiting. The only limitation for the viability of bacteria in the buffer masses seems to have been the water activity.

In the last study reported here, sodium bentonite (MX-80) was inoculated with two species of SRB and compacted to three different densities, 1.5, 1.8 and 2.0 g/cm^3 by means of a hydraulic press and incubated at 30°C. These densities correspond to a_w of 1.0, 0.99 and 0.96, respectively. All samples were incubated at 30°C for 1 or 60 days. The amount of water available in the bentonite significantly influenced the survival of the studied SRB. Both strains were 100% non-viable after 1 d at the lowest a_w studied, 0.96. The dry conditions at this density of 2 g/cm^3 effectively killed 100 million SRB per g bentonite in less than 24 h. The best survival was observed in the bentonite with an a_w of 1.0, but the survival differed markedly between the

species. About 10% of the initial population of *D. baculatum* survived for 60 days, but *Desulfovibrio sp* did not survive at all after this time. Limitation in nutrients and energy sources, accumulation of hydrogen sulphide and interference of the redox potential may add constrains to a closed batch system like the one used here. A better survival may be expected in an open system at non-limiting a_w values, i.e. at a_w close to 1.

The pore size of highly compacted bentonite is in the nanometer range which makes contamination of a compacted buffer with SRB, migrating into the buffer from groundwater, impossible. The only way with which bacteria can be seeded in nuclear waste buffers is during mixing of the bentonite and water (sometimes groundwater) before compaction. Here, we used a compaction technique similar to what will be used for production of buffer at an industrial scale, and we deliberately introduced very high levels of viable SRB to simulate a "worst case scenario" in such a production. The results show that survival of these SRB depended on the amount of water that was available (a_w). When a_w approached 0.96 in the bentonite, SRB were killed by desiccation. In conclusion, there is no rapid mechanism of microbiologically induced sulphide corrosion inside a nuclear waste bentonite buffer if an a_w of 0.96, or lower, is maintained.

1 INTRODUCTION

The concept of deep geological disposal of spent fuel is common to most international nuclear waste programs. Long-lived radioactive waste will be encapsulated in canisters made of corrosion resistant materials, e.g. copper (McCright, 1994), and buried several hundred meters below ground in a geological formation (Pedersen and Karlsson, 1995). Different types of compacted bentonite clay, or mixtures with sand, will be placed as a buffer around the waste canisters (Atabek et al, 1985, Werme et al, 1992). A common demand to waste disposal concepts developed in countries concerned, is that canisters and buffers must remain intact for a very long time. Assessments of the performance of disposal systems are often done covering periods of up 10,000 years or more (Pedersen and Karlsson, 1995, Werme et al, 1992). Corrosion is an important process to consider in such an assessment for at least two reasons. The first is obvious: the canisters is an absolute barrier to radionuclide dispersal as long as they remain intact. A second reason is that a separate gas phase may form at a sufficiently high corrosion rate, which may exert a pressure on the system and add to the dispersion of radionuclides by gas bubble transport. Sulphate-reducing bacteria (SRB) may cause corrosion of canister materials due to their dissimilatory reduction of sulphate to hydrogen sulphide (Hamilton, 1985, Philp et al, 1991, Pedersen, 1996). Consequently, it is very important to reveal if SRB can survive and produce hydrogen sulphide in bentonite buffers.

Direct and indirect evidence for the presence and activity of SRB in deep geological formations have been reported elsewhere (Laaksoharju et al, 1995, Pedersen and Karlsson, 1995) A summary of data on viable numbers of SRB in the Äspö Hard Rock Laboratory area is shown in Figure 1.1. Obviously, the anticipated risk for presence of SRB at disposal depths is real and must be accounted for in the safety performance assessment. Two scenarios must be assessed: In the first scenario, SRB will grow in the surrounding geological formation and produce hydrogen sulphide that must diffuse through the buffer to corrode the copper canister. This case has been thoroughly discussed elsewhere (Pedersen and Karlsson, 1995). The second scenario, were SRB will grow in the buffer material around the canister and produce hydrogen sulphide that is corrosive to copper, is dealt with here.

This report first summarises present knowledge about SRB (chapter 2) and it gives a theoretical background on solutes and water activity (chapter 3). Thereafter, the buffer as a habitat for bacteria is discussed and some relevant microbiology data from a full scale buffer test at the AECL underground facility URL is summarised (chapter 4). Finally, an investigation of the survival of SRB in bentonite at three different water activities presented (chapter 5).

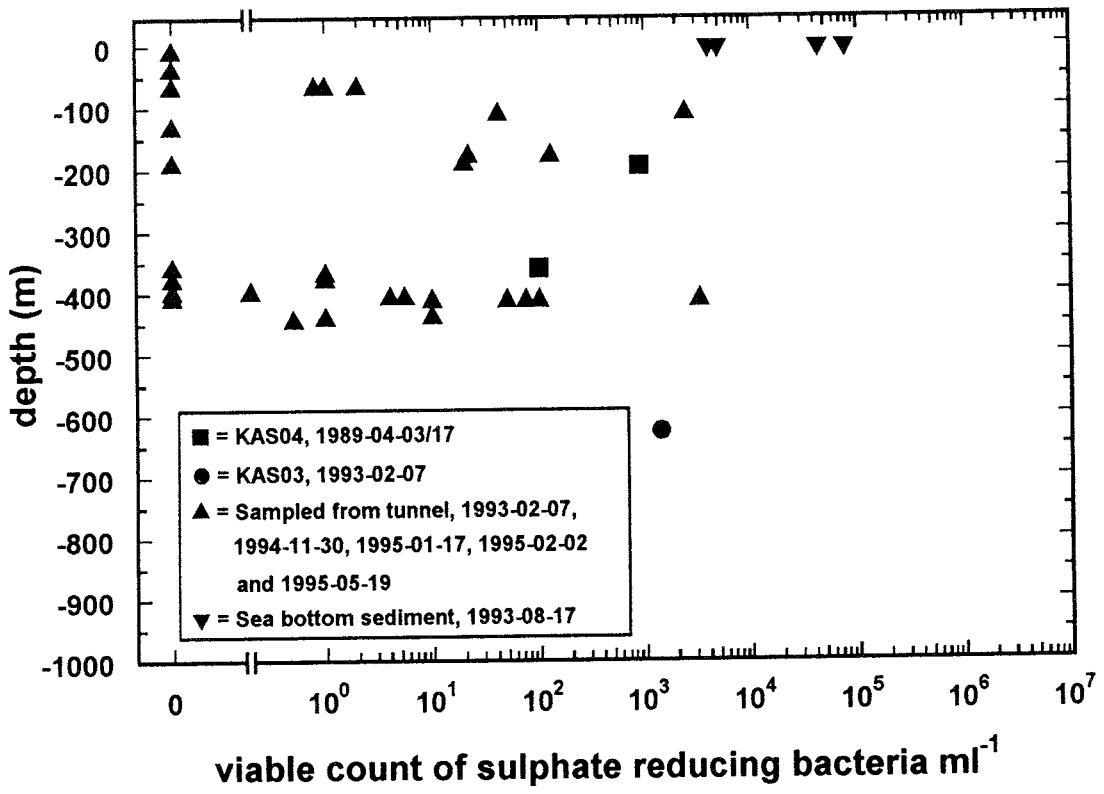


Figure 1.1 A compilation of results obtained on the viable numbers of sulphate reducing bacteria in the Äspö hard rock laboratory area. The KAS03-04 data are from Pedersen and Ekendahl, (1990). Methods and background information about the remaining data are reported elsewhere (Laaksoharju et al, 1995, Pedersen and Karlsson, 1995).

2 BACTERIA AND THE SULPHUR CYCLE

Sulphur is among the ten most abundant elements on earth. It occurs in a large number of chemical compounds of which sulphate and sulphides are the quantitatively dominating forms. Microorganisms contribute considerably to the sulphur cycle as shown in figure 2.1. The most important process relating to nuclear waste is sulphate reduction with sulphide as end product. This is because sulphide is corrosive for the copper canisters. Special attention is therefore directed towards this process and the bacteria involved.

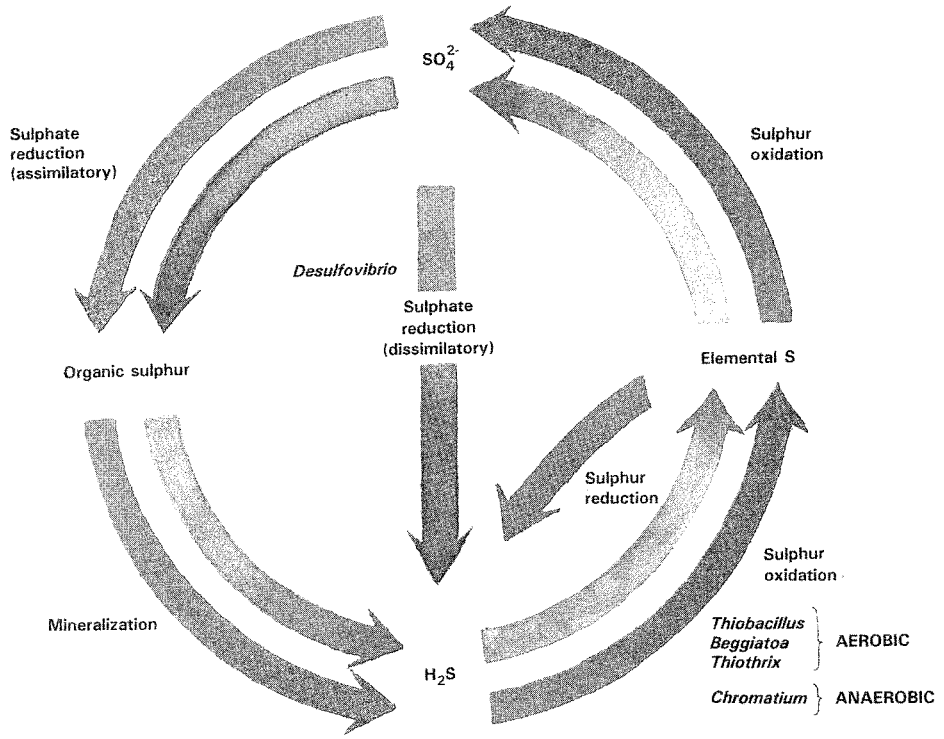


Figure 2.1 The sulphur cycle. Photosynthetic (not present in subterranean environments) and chemosyntethic microorganisms contribute to the environmental sulphur cycle. Anaerobic sulphate reduction by sulphate reducing bacteria, a dissimilatory process, is noted with a purple arrow. Sulphate reduction also can occur in assimilatory reactions. Elemental sulphur reduction to sulphide is carried out by both eubacteria and archaea. Sulphur oxidation can be carried out by a wide variety of chemotrophs and phototrophs.

Table 2.1 Some properties of selected classified sulphate reducing bacteria from Widdel (1988).

Species	Electron donors	Autotrophic growth (CO_2 as carbon source)	Temperature optimum (°C)	Oxidation of lactate
Desulfotomaculum				
nigrificans	hydrogen, formate, lactate, ethanol		55 (max 70)	incomplete
antarcticum	lactate		20-30	incomplete
orientis	hydrogen, formate, lactate, ethanol	yes	37	incomplete
Desulfovibrio				
desulfuricans	hydrogen, formate, lactate, ethanol		30-36	incomplete
vulgaris	hydrogen, formate, lactate, ethanol		30-36	incomplete
salexigens	hydrogen, formate, lactate, ethanol		30-36	incomplete
thermophilus	hydrogen, formate, lactate		65 (max 85)	incomplete
Desulfomicrobium				
baculatum	(hydrogen), formate, lactate		30-36	incomplete
Thermodesulfo-bacterium				
commune	hydrogen, lactate		70 (max 85)	incomplete
Desulfobulbus				
propionicus	hydrogen, lactate, ethanol		28-39	incomplete
Desulfobacter				
hydrogenophilus	hydrogen, acetate	yes	28-32	complete
Desulfobacterium				
autotrophicum	hydrogen, formate, lactate, ethanol	yes	20-26	complete
Desulfococcus				
niacini	hydrogen, formate, ethanol, acetate	yes	29	complete
Desulfosarcina				
variabilis	hydrogen, formate, lactate, ethanol	yes	33	complete
Desulfonema				
limicola	hydrogen, formate, lactate, acetate	yes	30	complete

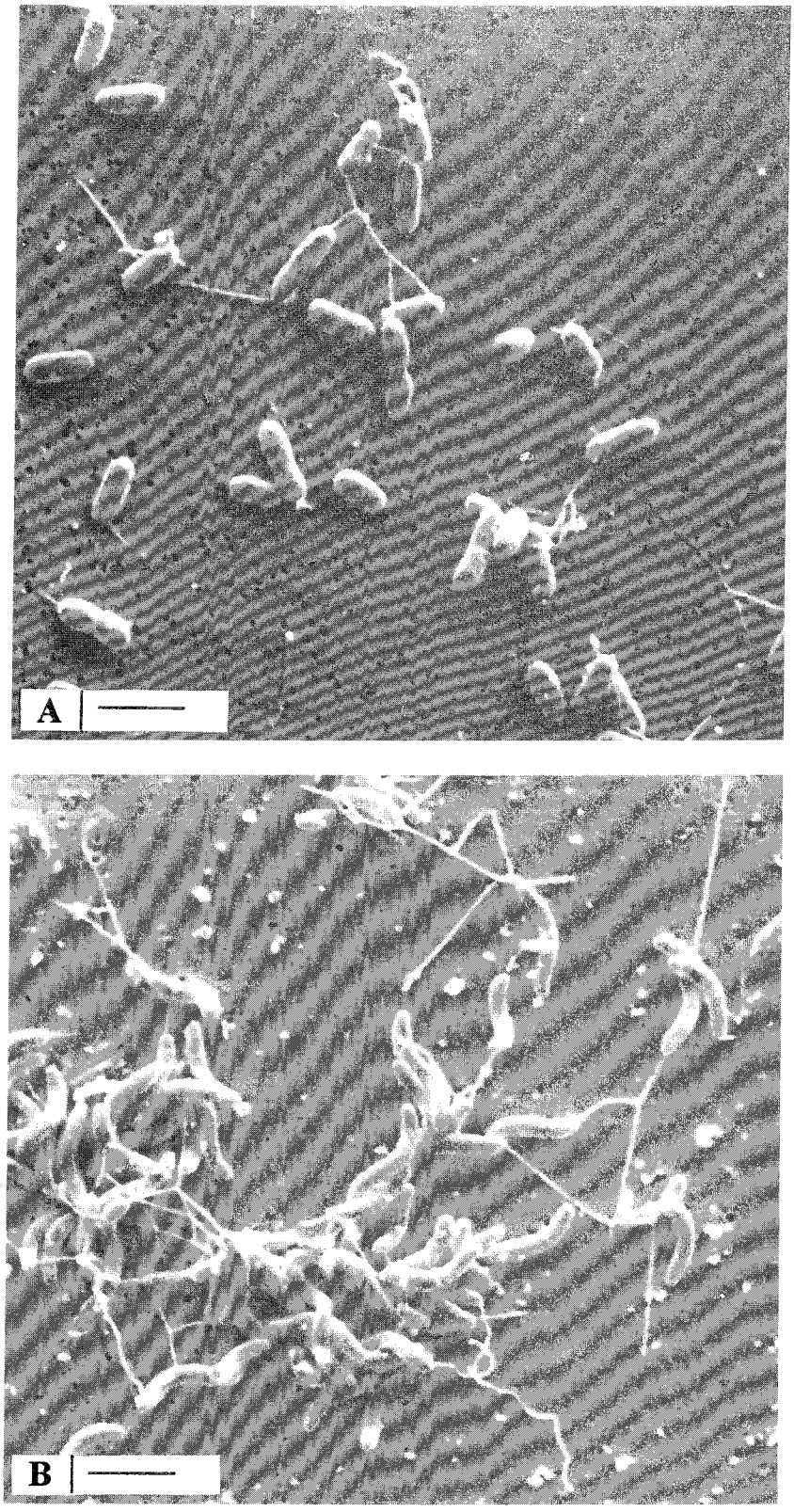


Figure 2.2 Scanning electron microscopy image of sulphate reducing bacteria that have been enriched and isolated from the Äspö HRL environment. DNA sequences have demonstrated the bacterium in Figure A to be Desulfomicrobium baculatum *(see table 2.1), while the bacterium on Figure B was found to be related to the genus* Desulfovibrio. *Bar denotes 2 μm.*

2.1 SULPHATE REDUCING BACTERIA

Sulphate-reducing bacteria are notable for their end product, hydrogen sulphide (often briefly termed sulphide) which starts to dissociate to HS^{-1} at pH above 6 and to S^{-2} at pH above 10. The chemical properties and physiological effects of sulphide are far more significant than the substrate sulphate. Sulphate is a chemically rather inert, non-volatile, and non-toxic compound. It is widespread in rocks, soils and waters. In contrast, sulphide is chemically reactive. In aqueous habitats where it is formed, it often blackens the sediments due to the production of ferrous sulphide from iron-containing materials. As a reductant, dissolved sulphide traps oxygen and may be converted to sulphur. Hydrogen sulphide, which even at low concentration in the atmosphere (> 0.2 ppm) is recognised by its smell, is toxic to plants, animals and humans. Thus, sulphide appears as an unusual form of sulphur in the part of the biosphere with which we are most familiar. Indeed, sulphate-reducing bacteria thrive outside the aerobic environment, in niches where oxygen has no access. Organically bound reduced sulphur is, however, an indispensable constituent of every living organism.

2.2 CLASSIFICATION OF SULPHATE REDUCING BACTERIA

With the isolation of a bacterium reducing sulphate to sulphide, which he called *Spirillum desulfuricans,* Beijerinck clearly demonstrated the correlation of the observed process to a special kind of bacteria (Beijerinck, 1885). The isolated type of sulphate reducer, which had curved motile cells, was known for a while as *Vibrio desulfuricans* and then finally named *Desulfovibrio desulfuricans*. The genus designation *Desulfovibrio* was maintained for non-sporing sulphate reducers usually having curved motile cells growing on a relatively limited range of organic substrates; preferred substrates are lactate or pyruvate, which are incompletely oxidised to acetate. *Desulfovibrio* species are still the best studied sulphate reducers. Sporing sulphate reducing bacteria with a similar metabolism were classified within the genus *Desulfotomaculum*. Later, additional types of sulphate reducers were described, several of which differed markedly physiologically and morphologically from the known *Desulfovibrio* and *Desulfotomaculum* species. Hence, the term sulphate reducing bacteria describes a rather heterogeneous assemblage of bacteria having in common merely dissimilatory sulphate reducing metabolism and obligate anaerobism (table 2.1).

Not all types of sulphate reducers have been classified so far. Some of the species isolated by us from Äspö groundwater are indicated by 16S-rRNA gene comparisons to be new species (e.g. *Desulfovibrio* in Fig. 2.1B). Very little is known about the range of thermophilic sulphate reducers, or about types that grow very slowly and yield low cell densities under laboratory conditions.

2.3 ELECTRON DONORS AND AUTOTROPHIC CAPACITY

For a long time lactate (or pyruvate) has been used as an excellent organic substrate for enrichment, isolation and cultivation of incompletely lactate oxidising *Desulfovibrio* and *Desulfotomaculum* species. Lactate is also oxidised by several completely lactate oxidising sulphate reducers; *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfobacterium* and *Desulfonema.*

Incomplete oxidation of lactate to acetate, hydrogen sulphide and carbon dioxide:

$$2CH_3CHOHCOO^- + SO_4^{2-} \Rightarrow 2CH_3COO^- + 2\,HCO_3^- + HS^- + H^+ \qquad (1)$$

$$\Delta G^{\circ\prime} = -160.1 \text{ kJ}$$

Complete oxidation of lactate to hydrogen sulphide and carbon dioxide:

$$2CH_3CHOHCOO^- + 3SO_4^{2-} \Rightarrow 6\,HCO_3^- + 3HS^- + H^+ \qquad (2)$$

$$\Delta G^{\circ\prime} = -255.3 \text{ kJ}$$

Incomplete oxidising sulphate reducers using lactate are usually able to grow just as well with hydrogen as electron donor. *Desulfovibrio* species may grow rather fast on hydrogen, which is, therefore, an almost unfailing electron donor for their enrichment. The utilisation of hydrogen by *Desulfovibrio* species was the first hint from nutrition physiology that sulphate reducers can conserve energy solely by electron transport phosphorylation.

$$4H_2 + SO_4^{2-} + H^+ \Rightarrow 4H_2O + HS^- \qquad (3)$$

$$\Delta G^{\circ\prime} = -152.2 \text{ kJ}$$

Hydrogen is also used, with relative slow growth, by several complete oxidising sulphate reducing bacteria that may grow autotrophically (CO_2 is used as single carbon source). After the classical *Desulfovibrio* species had been shown to be chemolithoheterotrophic (growth with hydrogen as electron donor and organic carbon as carbon source), autotrophic growth of hydrogen-using sulphate reducers was reported for *Desulfosarcina variabilis, Desulfonema limicola,* and *Desulfococcus niacini*; however, growth was rather slow. Later when studied, many other sulphate reducers have been shown to grow autotrophically (table 2.1).

2.4 POSSIBLE ENVIRONMENTAL LIMITATIONS FOR SURVIVAL OF SRB IN NUCLEAR WASTE BUFFERS

SRB are obligate anaerobic organisms and they can generally utilise a wide range of carbon substrates. Several species can survive using carbon dioxide and hydrogen as carbon and energy sources (Widdel, 1988). Bentonite clay

contains organic material, and hydrogen and carbon dioxide can also be found at repository depths (Pedersen, 1993b, Stevens and McKinley, 1995). It has, therefore, been considered plausible that SRB can become established in a repository bentonite environment, as it will be anaerobic, having reducing conditions together with nutrients and energy available for propagation. The temperature in bentonite surrounding the waste canisters will reach between 50°C and 80°C during the first 1000 years, but that does not pose any conceptual hindrance for growth of SRB as some are thermopiles that grow at an optimum temperature around 65°C, e.g. *Desulfotomaculum* and *Thermodesulfobacterium* (Widdel and Hansen, 1992). A corrosive effect on steel from *Desulfotomaculum nigrificans* in bentonite at 50°C has recently been demonstrated (Philp et al, 1991). Nor will the high pressure at repository depth constitute any limitation for bacteria as many can withstand among the highest hydrostatic pressures on the planet - those found in the deepest parts of the ocean (Kato et al, 1994).

3 SOLUTES AND WATER ACTIVITY - SALINITY LIMITS

3.1 SOLUTES

Because a selectively permeable plasma membrane separates bacteria from their environment, they can be affected by changes in the osmotic concentration of their surroundings. If a bacterium is placed in a hypotonic solution, water will enter the cell and cause it to burst unless something is done to prevent the influx. Most bacteria have rigid cell walls that maintain the shape and integrity of the cell. Indeed, many bacteria keep the osmotic concentration of the protoplasm above that of the habitat by use of compatible solutes, so that the plasma membrane is always pressed firmly against their cell wall. Compatible solutes are solutes that are compatible with cell metabolism and growth when at high concentrations. The bacteria may synthesise choline, betanine, proline, glutamic acid and other aminoacids; elevated levels of potassium ions are also involved to some extent. A few bacteria like *Halobacterium salinarium* raise their osmotic concentration with potassium ions and have enzymes that require high salt concentrations for normal activity.

3.2 WATER ACTIVITY

The amount of water available to bacteria can be reduced by interactions with solute molecules (the osmotic effect) and by adsorption to the surface of solids (the matrices effect). Because the osmotic concentration of a habitat has such profound effects on bacteria, it is useful to be able to express quantitatively the degree of water availability. Microbiologists generally use *water activity* (a_w) for this purpose, which is a thermodynamic parameter. The water activity of a solution is 1/100 the relative humidity of air in equilibrium with the solution (when expressed as percent). It is also equivalent to the ratio of the solution's vapour pressure (P_{soln}) to that of pure water (P_{water}).

$$a_w = \frac{P_{soln}}{P_{water}} \qquad (4)$$

The water activity of a solution or solid can be determined by sealing it in a chamber and measuring the relative humidity after the system has come to equilibrium. Curiously, the most limiting condition for microbial growth seems to be the availability of water. As far as is known nothing can grow within solid ice, nor in steam. In solutions, or on surfaces, a substantial amount of water is needed. A limiting a_w value of 0.6-0.7 seems to hold so

that only about 40% of available water can be removed before growth of even the most desiccation resistant microorganism stops. The reason is far from obvious. Future workers on extreme environments must look further into the structure and functions of water itself, especially intracellular water. We may find that some microorganisms can grow in almost any environment, provided that temperature is not high enough to disrupt essential molecules and that the cells are able to maintain a "comfortable" form of intracellular water.

Table 3.1 Water activity of several materials with some microorganisms growing at that water activity in comparison with Na-bentonite, MX-80 with different water contents.

Water activity	**Material**	**Some organisms growing at stated water activity**
1.000	Pure water	*Caulobacter, Spirillum*
0.995	Human blood	*Streptococcus, Escherichia*
0.990	Groundwater (500 m)	*Bacillus, SRB, Pseudomonas*
0.980	Sea water	*Pseudomonas,* SRB, *Vibrio*
0.960	**MX-80**, 25% water	
0.950	Bread	Most gram-positive rods
0.920	**MX-80**, 20% water	
0.900	Maple syrup, ham	Gram-positive cocci
0.850	Salami	*Saccharomyces rouxii* (yeast)
0.800	Fruit cake, jams	*Saccharomyces bailii Penicillium* (fungi)
0.780	**MX-80**, 15% water (start)	
0.750	Salt lake, salt fish	*Halobacterium, Halococcus*
0.700	Cereals, dried fruit	Xerophilic fungi

Bacteria differ greatly in their ability to adapt to habitats with low water activity (Table 3.1). A bacterium must expend extra efforts to grow in a habitat with a low a_w value because it must maintain a high internal solute concentration to retain water. Some bacteria do this and are osmotolerant; they will grow over wide ranges of water activities or osmotic concentrations. For example, *Staphylococcus aureus* can be cultured in media containing any sodium chloride concentration up to about 3 M. Although a few bacteria are truly osmotolerant, most only grow well at water activities around 0.98, (the approximate a_w for sea water) or higher. *Extreme halophiles* have adapted so completely to saline conditions that they require high levels of sodium chloride to grow, concentrations between about 2.8 M and saturation, about 6.8 M. Extreme halophilic bacteria have adapted successfully to environmental conditions that would destroy most organisms. In the evolutionary process, they have become so specialised that they have lost ecological flexibility and can prosper only in a few extreme habitats.

3.3 CHEMICAL POTENTIAL

The activity of water (a_w) is related to its concentration through an activity coefficient (γ_w), where $a_w = \gamma_w N_w$ (N_w is the mole fraction of water in the system) (Potts, 1994). The chemical potential of water (μ_w) in a system is expressed according to the following equation:

$$\mu_w = \mu_w^* + RT \ln a_w + V_w P + z_w FE + m_w gh \qquad (5)$$

In this equation, the term $RT \ln a_w$ - the activity term (where R is the gas constant) - gives the water activity term in the units of energy per mole. V_w is also the partial molal volume of water, i.e., in a bacterial cell, in contrast to μ_w which is the partial molal Gibbs free energy ($\delta G/\delta n_w$). V_w is differential increase or decrease in the volume of a bacterial cell when a differential amount of water is added or removed, respectively, and it is expressed as the volume per mole. Pure water, or a very dilute solution, has a value of V_w equal to 18×10^{-6} m^3 mol^{-1}. P is the hydrostatic pressure in excess of the atmospheric pressure, so that the $V_w P$ term in the equation reflects the effects of pressure on the chemical potential of water and is expressed, therefore, in energy per mole. $z_w FE$ is the electrochemical potential, and because water is uncharged ($z_w = 0$), the electrical term $z_w FE$ can be ignored. The gravitational term $m_w gh$, represents the work needed to move a given mass per mole of water, m_w (18.016 g mol-1), to a given height (h) under gravitational acceleration (g). Only under circumstances when cells are distributed at high altitudes throughout a water vapour can this term contribute significantly to the overall potential energy, μ_w^* is an additive constant and represents the chemical potential of water in a standard (ideal) reference state where $RT \ln a_w = 0$, $V_w P = 0$, $z_w FE = 0$, and $m_w gh = 0$. For practical purposes, one compares the chemical potentials of cells with different intermediate water contents, say those of a dried bacterial cell (μ_w^D) and a cell at some stage of rehydration (μ_w^R). During comparison of these two chemical potentials, the two μ_w^* terms cancel out.

3.4 OSMOTIC PRESSURE

The addition of solute to a pool of water cause a net displacement of the water molecules. The decrease in the partial molal volume depends on the amounts of solute that go into solution and on the extent to which they do so. The lowering of μ_w cause a_w to decrease, and the $RT \ln a_w$ term in equation 5 becomes more negative. Concomitant with this decrease in μw, there is also an increase in the osmotic pressure (II), which is attributable to the addition of a species of solute. As bacterial cells undergo changes in the amounts of water (and thus in the net concentration of solutes) that they contain, the equilibrium is continuously shifted to one of higher or lower osmotic pressure with concomitant changes in a_w. Equation 6 relates water activity to osmotic pressure:

$$RT \ln a_w = V_w \Pi \qquad (6)$$

3.5 WATER POTENTIAL

The water potential of a system (Ψ) is proportional to μ_w-μ_w*(see section 3.3 for an explanation of the symbols), so that the term μ_w - μ_w* has considerable utility when the water relations of bacterial cells are compared. The term represents the work involved in moving 1 mole of water from some point in a system (at constant pressure and temperature) to a pool of pure water at atmospheric pressure and at the same temperature as the system under consideration (the gravitational term is ignored for reasons describe above). A difference between two locations in the values of μ_w - μ_w* indicates that water is not in equilibrium, so there will be a net tendency for water to move toward a region where μ_w - μ_w* is lower.

3.6 WATER ACTIVITIES IN REPOSITORY AND ROCK ENVIRONMENTS

The groundwater in the repository rock and the surrounding rock will have a relatively high water activity, usually not lower than that of sea water (0.98) and will, therefore, not constitute any environmental limitation for most bacteria adapted to aquatic ecosystems (Table 3.1). In addition, the backfill of bentonite/sand (15/85%) will have a fairly high water activity. Turning to the bentonite, MX-80, that will be used around the canisters, the picture changes dramatically. At start, the bentonite may have a water activity of approximately 0.75 which is low enough to exclude the absolute majority of microorganisms. Endospores formed by many *Bacillaceae* species may in fact be the only life form that possibly will survive. Therefore, keeping the water activity low, at for instance 0.96 (water content 25%) will probably be a very potent environmental limitation for most bacteria trying to invade the canister clay environment.

4 INVESTIGATION OF SURVIVAL OF BACTERIA IN A FULL SCALE BUFFER/TEST AT URL IN CANADA

Early in 1994, we were invited to participate in the decommissioning of a full scale buffer heater (BMC) test at the Underground Research Laboratory (URL) close to Whiteshell laboratories, AECL, north of Winnipeg in Canada. The test was set up to study temperature effects on the water distribution in the buffer as a function of temperature. In addition, it was decided that valuable information about the survival of bacteria during 2.5 years under realistic circumstances in a buffer clay could be obtained. Microbiologists from AECL, ANDRA in France, and Göteborg University Sweden, participated. The full report will be available during spring 1996. Here, some important key conclusions from this experiment are presented.

4.1 VIABLE COUNTS OF AEROBIC, ANAEROBIC AND TERMOPHILIC BMC-BACTERIA

The buffer masses were mixed aerobically (Bentonite/sand, 50%/50%) and laid down in layers aerobically. There was not much reducing capacity in the buffer per se, anaerobic and reduced conditions would only occur as a result of exchange processes with surrounding reduced groundwater, a process expected to take years. Therefore, it can be anticipated that oxidised conditions and probably also at least microaerophilic conditions prevailed in the buffer mass. SRB and metanogenic bacteria will not develop under such conditions, and should, therefore, not be expected. This was also the case, neither the Swedish nor the French lab could demonstrate any significant presence of such bacteria. Instead, relatively large numbers of mesophilic aerobic bacteria were detected.

4.2 STATISTIC EVALUATION OF THE CFU COUNTS

The results from a statistical analysis of variance strongly indicated water content to have a large impact on the viability of bacteria in the buffer mass. The effect from the *in situ* buffer mass temperature was not significant. Figures 4.1 and 4.2 show the CFU results. At a low water content, there where few cultivable bacteria (Figure 4.1) but high temperature was not limiting (4.2). The reduction in CFU at high temperatures in Figure 4.2 is in fact due to the concomitantly low water activity. The effect from different culturing conditions was, as expected, significant. The only limitation for the viability of bacteria in the buffer masses seems to have been the water activity.

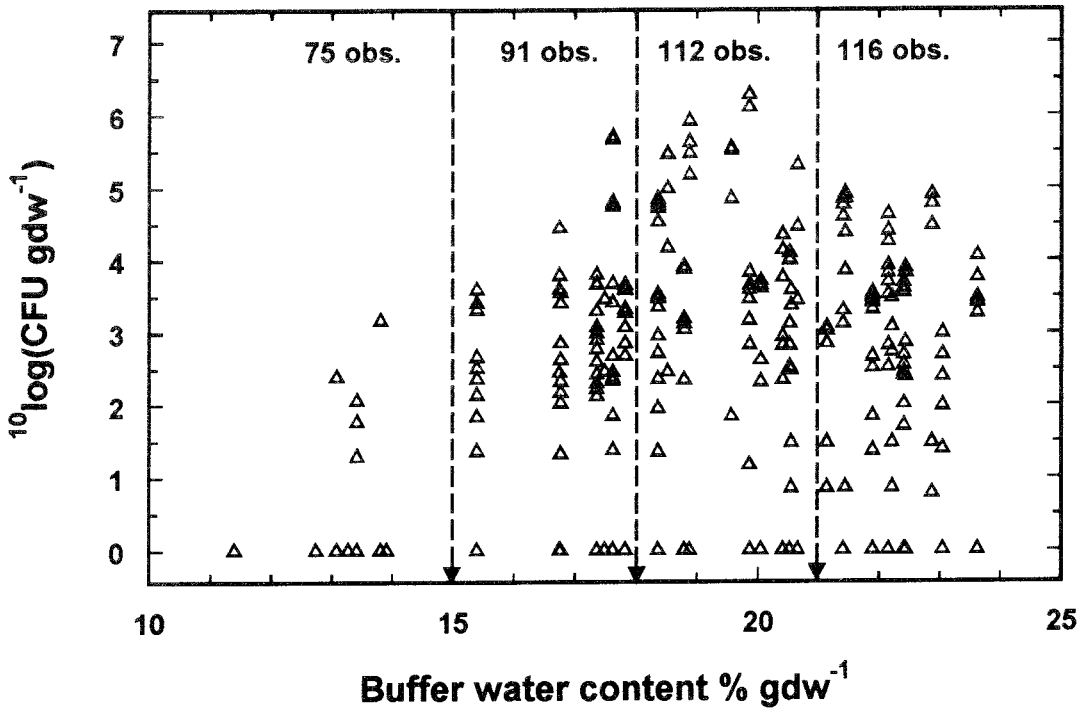


Figure 4.1 The distribution of all colony forming unit (CFU) data obtained (394 observations) over the in situ water contents for the buffer samples investigated. The four water content classes used for the analysis of variance are indicated and the number of observations within each class level are presented.

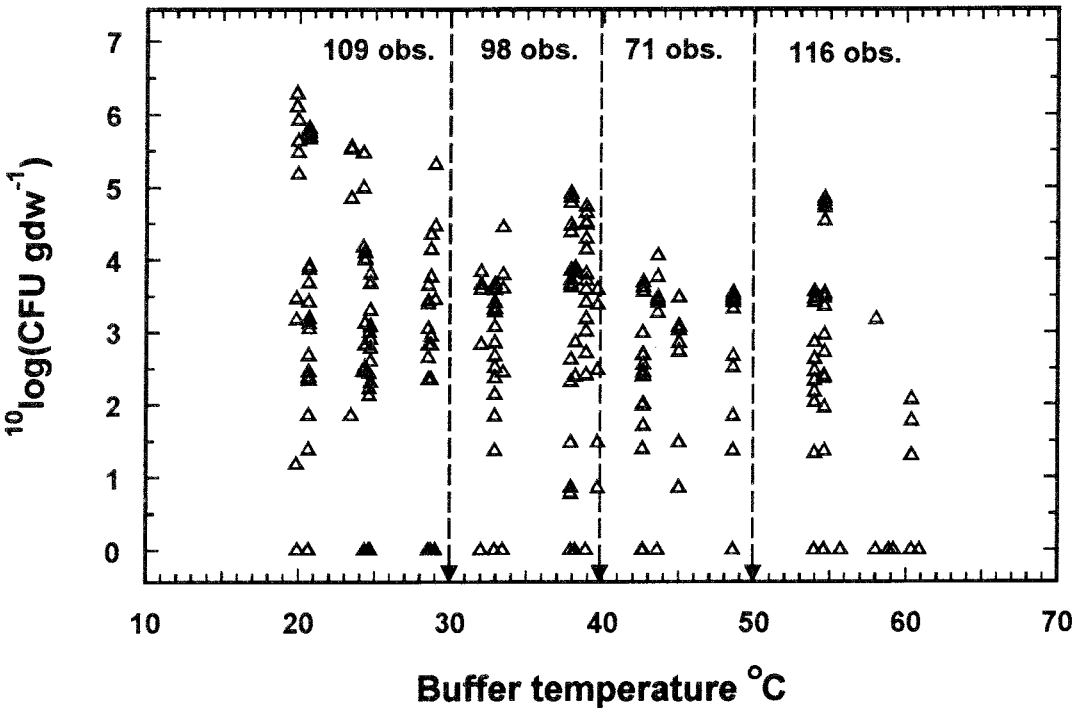


Figure 4.2 The distribution of all colony forming unit (CFU) data obtained (394 observations) over the in situ temperatures for the buffer samples investigated. The four temperature classes used for the analysis of variance are indicated and the number of observations within each class level are presented.

4.3 WATER AVAILABLE FOR BACTERIA IN THE BMC-TEST BUFFER MASSES

When the buffer masses were laid down at the start of the experiment, the water content was homogenous and averaged 18%. During the experiment, the heat from the heater caused a mass transport of water out from the vicinity of the heater to the peripheral parts of the buffer. Gradients of water contents developed. Approximately 50% of the buffer mass is sand and this part will not influence the amount of free water by a matrix adsorption effect more than marginally compared to the bentonite part. The clay will sorb water until it is saturated, and only water in excess of what is sorbed by the clay will be available for the bacteria. The water content at which water becomes available in a 50/50% sand-bentonite mixture is about 15%, which correlates well with the values at which viable bacteria could not be demonstrated (Figure 4.1).

4.4 ACTIVITY OF THE BMC-BUFFER BACTERIA

The activity of the found bacteria was measured as in situ assimilation of the tritiated amino acid leucine. The assimilation measured in the layers around the top of the heater was significant, indicating viable bacteria. It can be compared with data from groundwater and surfaces exposed to flowing ground water in Swedish granitic rock at the Stripa research mine (Pedersen and Ekendahl, 1992a) and the Äspö hard rock laboratory environments (Pedersen and Ekendahl, 1992b) (Table 4.1). The percentage of bacteria active in leucine uptake in Stripa and Äspö was from 9-99% and it was possible to calculate the assimilation per bacterium for these results. As there were only negative results for a microautoradiography experiment performed on the buffer samples, such calculations can not be done for the BMC experiment. Briefly, the BMC leucine assimilation activity was similar to what has been registered for the deep groundwater of the Stripa research mine, harbouring between 10^4 to 10^5 cells per ml of groundwater.

Table 4.1 The amount of leucine assimilated by bacteria in the BMC buffer mass test and in groundwater and on surfaces exposed to flowing groundwater in Swedish granitic rock at the Stripa research mine (Pedersen and Ekendahl, 1992a) and the Äspö Hard Rock Laboratory environments (Pedersen and Ekendahl, 1992b).

Environment	10^{14} mole leucine/gdw	10^{14} mole leucine/ml	10^{14} mole leucine/cm^2
BMC	1-7	-	-
Stripa	-	1-5	160-280
Äspö	-	3-66	16-150

4.5 THE IMPLICATIONS OF THE BMC-RESULTS FOR PERFORMANCE ASSESSMENT OF THE SWEDISH SFL 2 HIGH LEVEL RADIOACTIVE WASTE REPOSITORY

The Swedish buffer will consist of 100% bentonite and will have a swelling pressure corresponding to a water activity around 0.96 (see table 3.1). This is low enough to reduce the viability of many bacteria including sulphur and sulphate reducing bacteria (SRB). The production of sulphide by SRB constitutes one of the very few chemical circumstances under which copper will corrode anaerobically (Pedersen and Karlsson, 1995). If it can be shown that the water activity of the bentonite is low enough to kill any viable sulphur and sulphate reducing bacteria through desiccation, then copper corrosion induced by sulphide-producing bacteria will totally depend on the production of sulphide in the rock outside the bentonite and the diffusivity of sulphide in bentonite. There are spore-forming SRB belonging to the genus *Desulfotomaculum*, but spores are inactive and do not produce sulphide. Such production may occur after germination, but then the spore-forming SRB species are as intolerant to desiccation as most other SRB.

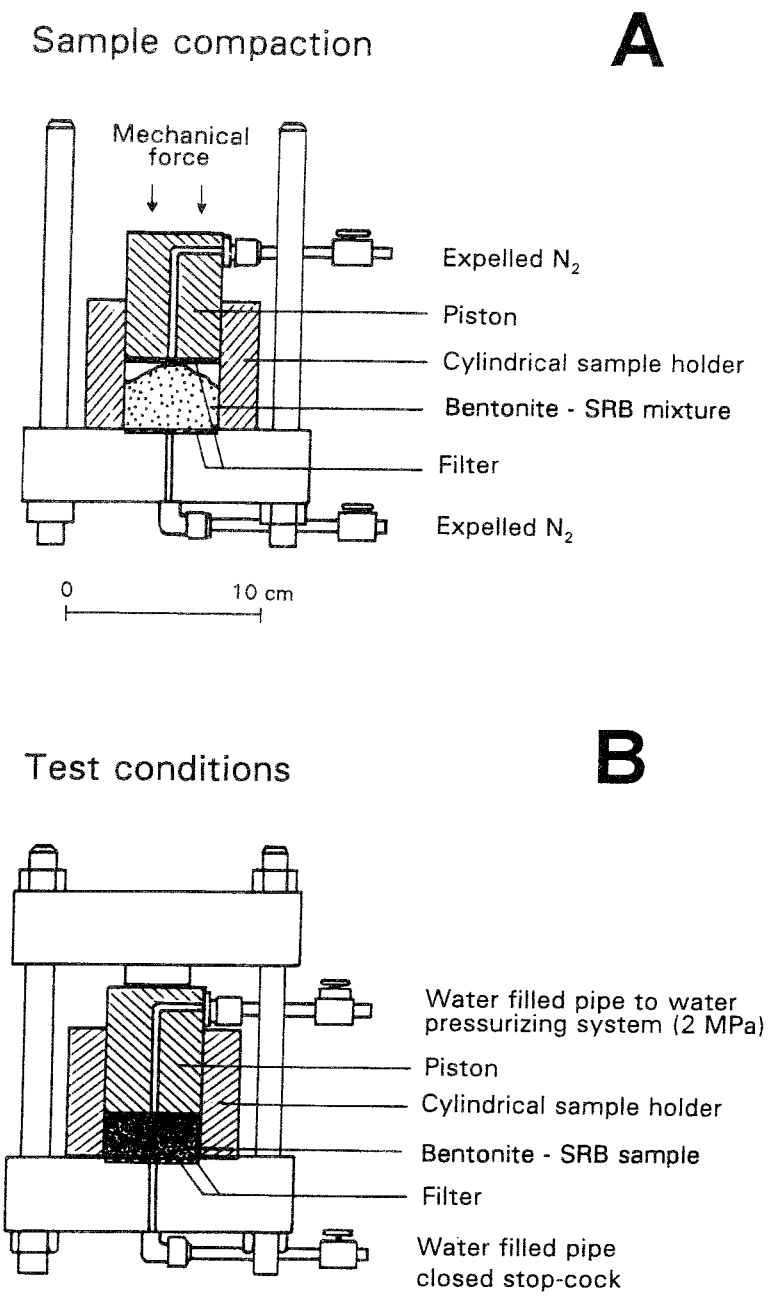
As a consequence of the hypothesis discussed above, a series of experiments were performed studying the survival of SRB in bentonite at different water activities to determine the limit at which SRB no longer survive. The project was executed as a collaborative effort between Clay technology AB, Lund, Sweden, Department of General and Marine Microbiology, Göteborg and SKB AB.

5 SURVIVAL OF SRB IN BENTONITE AT DIFFERENT WATER ACTIVITIES

In this study, sodium bentonite (MX-80) was inoculated with two species of SRB and compacted to three different densities, 1.5, 1.8 and 2.0 g/cm^3 by means of a hydraulic press as depicted in Figure 5.1, and incubated at 30°C. These densities correspond to a_w of 1.0, 0.99 and 0.96, respectively. All samples were incubated at 30°C for 1 or 60 days. The parts of the odometer that were in contact with the bentonite samples (sample containers and piston) and the MX-80 sodium bentonite were heat sterilised at 160°C for 2 h. Two fresh cell-suspensions of SRB from late exponential growth phase, *Desulfomicrobium baculatum* and *Desulfovibrio sp.*, were used at a cell density of approximately 10^8 cells/g as determined by acridine orange direct count (AODC Pedersen and Ekendahl, 1990). The bacteria were isolated earlier from deep crystalline bed-rock groundwater (Pedersen and Ekendahl, 1990). An anoxic medium, containing lactate as a carbon and energy source and sodium sulphate as electron acceptor, was used for cultivation (Ollivier et al, 1988, Widdel and Bak, 1992). The quantities of dry bentonite and SRB suspensions used in the mixtures (Figure 5.1) were: 31.2 g bentonite and 27.8 g SRB suspension, 50 g bentonite and 20.8 g SRB suspension and 62.4 g bentonite and 15.2 g SRB suspension corresponding to sample densities of 1.5, 1.8 and 2 g/cm^3 respectively. The number of viable SRB was estimated by a MPN method (Koch, 1994) after incubation at 1 and 60 days (Table 5.1).

The amount of water available in the bentonite significantly influenced the survival of the studied SRB. Both strains were 100% non-viable after 1 d at the lowest a_w studied, 0.96. The dry conditions at this density of 2 g/cm^3 effectively killed 100 million SRB per g bentonite in less than 24 h. The best survival was observed in the bentonite with an a_w of 1.0, but the survival differed markedly between the species. About 10% of the initial population of *D. baculatum* survived for 60 days, but *Desulfovibrio sp* did not survive at all after this time (Table 5.1). Limitation in nutrients and energy sources, accumulation of hydrogen sulphide and interference of the redox potential may add constrains to a closed batch system like the one used here (Figure 5.1). A better survival may be expected in an open system at non-limiting a_w values, i.e. at a_w close to 1.

Bentonite clay has been proposed as buffer material since it reduces the effects on the canister of a possible rock displacement, an it minimises water flow over the deposition holes (Pusch, 1983). The transport through the buffer will thereby be reduced principally to diffusion both with respect to corrosive components in the ground water and to escaping radionuclides in case of a canister failure. The bentonite material is a natural mixture of smectite and several common minerals like quartz and feldspar. The composition varies considerably depending on the mining site but the smectite component, which normally is montmorillonite, dominates the



*Figur 5.1 Schematic drawing of the swelling pressure oedometer that was used for preparation of the bentonite samples. All procedures, except compacting, were performed under nitrogen atmosphere in a glove box. **A.** The bentonite-SRB suspension mixture was placed in the central cylindrical sample holder and compacted to test density by forcing the piston down. **B.** The nitrogen gas in the sample was evacuated by the confining filters, and pressurized water (2 MPa) was contacted to the sample by the upper three way stop cock to simulate hydrostatic ground water pressure.*

Table 5.1 The number of viable cells of Desulfomicrobium baculatum *and* Desulfovibrio sp. *in bentonite samples with different densities and water activities (a_w) for 1 and 60 days incubation at 30°C. The term of a_w quantitatively expresses the degree of water available to bacteria in the samples.*

Sample density	Water activity	No. of viable bacteria per g bentonite			
		Desulfomicrobium baculatum		*Desulfovibrio sp.*	
g/cm^3	a_w	Incubation time (days)		Incubation time (days)	
		1	60	1	60
1.5	1.0	8.0×10^7	9.0×10^6	2.1×10^4	0
1.8	0.99	6.0×10^6	0.0	7.0×10^2	0
2.0	0.96	0.0	0.0	0.0	0

material (Grim and Guven, 1978). The smectite is characterised by a high water affinity which yields swelling when contacted with water. If swelling is restricted due to mechanical hindrance from the surrounding rock, the smectite will give rise to a swelling pressure and to a reduced water activity ($a_w<1$). Both effects are sensitive to the ratio between water and smectite (w). This ratio will be controlled in the deposition holes by compacting the bentonite to a relatively high density, approaching 2.0 g/cm^3 (a_w=0.96), either by compaction in situ or by use of pre-compacted bentonite blocks. Bentonite with lower densities will likely be used in other constructions in the repository, e.g. in the tunnel backfill material.

The pore size of highly compacted bentonite is in the nanometer range (Pusch, 1983) which makes contamination of a compacted buffer with SRB, migrating into the buffer from groundwater, impossible. The only way with which bacteria can be seeded in nuclear waste buffers is during mixing of the bentonite and water (sometimes groundwater) before compaction (Figure 5.1). A similar inoculation process has been suggested for viable bacteria that are found in subsurface confined clay layers. These bacteria were probably mixed into the clay when it was laid down during sedimentation and they have remained viable since then (Pedersen, 1993a). Here, we used a compaction technique similar to what will be used for production of buffer at an industrial scale, and we deliberately introduced very high levels of viable SRB to simulate a "worst case scenario" in such a production. The results show that survival of these SRB depended on the amount of water that was available (a_w). When a_w approached 0.96 in the bentonite, SRB were killed by desiccation. In conclusion, there is no rapid mechanism of microbiologically induced sulphide corrosion inside a nuclear waste bentonite buffer if an a_w of 0.96, or lower, is maintained.

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