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Microbial processes in radioactive waste disposal

Karsten Pedersen
Department of Cell and Molecular Biology, Microbiology
Göteborg University

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Svensk Kärnbränslehantering AB
Swedish Nuclear Fuel
and Waste Management Co
Box 5864
SE-102 40 Stockholm Sweden
Tel 08-459 84 00
+46 8 459 84 00
Fax 08-661 57 19
+46 8 661 57 19

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Abstract

Independent scientific work has unambiguously demonstrated life to be present in most deep geological formations investigated, down to depths of several kilometres. Microbial processes have consequently become an integral part of the performance safety assessment of high-level radioactive waste (HLW) repositories. This report presents the research record from the last decade of the microbiology research programme of the Swedish Nuclear Fuel and Waste Management Company (SKB) and gives current perspectives of microbial processes in HLW disposal. The goal of the microbiology programme is to understand how microbes may interact with the performance of a future HLW repository. First, for those who are not so familiar with microbes and their ways of living, the concept of "microbe" is briefly defined. Then, the main characteristics of recognised microbial assemblage and microbial growth, activity and survival are given. The main part of the report summarises data collected during the research period of 1987–1999 and interpretations of these data. Short summaries introduce the research tasks, followed by reviews of the results and insight gained.

Sulphate-reducing bacteria (SRB) produce sulphide and have commonly been observed in groundwater environments typical of Swedish HLW repositories. Consequently, the potential for sulphide corrosion of the copper canisters surrounding the HLW must be considered. The interface between the copper canister and the buffer is of special concern. Despite the fact that nowhere are the environmental constraints for life as strong as here, it has been suggested that SRB could survive and locally produce sulphide in concentrations large enough to cause damage to the canister. Experiments conducted thus far have indicated the opposite.

Early studies in the research programme revealed previously unknown microbial ecosystems in igneous rock aquifers at depths exceeding 1000 m. This discovery triggered a thorough exploration of the subterranean biosphere in the aquifers of the Fennoscandian Shield. Microbial processes at repository depths will have several important influences on repository performance. Some identified processes are the production of sulphide, carbon dioxide, organic carbon and methane, and the reduction of oxygen. The repository performance must be predicted for very long times. Natural analogues therefore become invaluable. Time-related processes of radionuclide migration have been studied at analogue sites that have been evolving over very long time periods. High pH conditions occur in Maqarin, Jordan; fuel-related processes have evolved at the natural reactors in Oklo, Gabon, and uranium migration processes have developed around the uranium body of Palmottu, in Finland.

The majority of the radionuclides are metals. The transport, chemical speciation, and ultimate fate of dissolved metals in aqueous systems are controlled to a large extent by reactions that occur at solid surfaces. Bacteria are at least as widely distributed and probably as reactive as many inorganic solids in aqueous systems. The behaviour of bacteria as geochemically reactive solids can be inferred from extensive research documenting their performance as sorbents of dissolved metals, and as nucleation templates for a wide range of authigenic minerals. They may consequently play a significant role in radionuclide retention and transport processes.

Summary

This report presents the research record from the last decade of the Swedish Nuclear Fuel and Waste Management Company (SKB)'s microbiology research programme and gives current perspectives of microbial processes in HLW disposal. First, however, for those who are not so familiar with microbes and their ways of living, the concept of "microbe" is defined. Also, some main characteristics of recognised microbial assemblages are listed and an introduction to microbial growth, activity and survival is given.

Buffer and canister corrosion research

The conclusions of research results on the survival and activity of microbes in compacted bentonite can be summarised as follows: at the start of the deposition, there will be a canister, some bentonite blocks and a hole in the rock. The next step will be to allow water to fill up all void volume. This will be either groundwater from the rock, or possibly groundwater or technical water added from above at the time of deposition. Irrespective of the source, water will contain microbes and these microbes will mix with the buffer, according to the results. The swelling of the clay will seed groundwater microbes to depths possibly within a couple of centimetres from the canister and the rock surfaces. The microbes indigenous to the bentonite will be present inside the bentonite and also, in the mixing zone. Results on the survival and activity of microbes in bentonite suggest that the number of viable microbes will decrease rapidly during swelling and that very few viable cells will be present at full compaction. Possible sulphate-reducing activity will also approach zero when full compaction is achieved. The only survivors will be microbes that have formed spores. Our results indicate that viable cell activity will be impossible at full compaction, as spores are inactive. Although spores are generally very resistant to difficult environmental conditions, they do die off. All our experiments so far indicate a decrease in the number of viable spores at full compaction. A slow but significant death rate of spores would eventually lead to the complete eradication of life in the buffer. It is not yet clarified whether this will occur within the lifetime of a radioactive repository. Once the bentonite becomes sterile, it will probably not be reinfected. The pore size of the clay is 100–1000 times smaller the average-sized microbe, meaning that after the microbes have died out, no new microbes can enter into the buffer. The model presented is based on current data, obtained with laboratory cultures. It can be argued that naturally occurring microbes are more tolerant; however, the working hypothesis will still be a total eradication of all life in the buffer.

Backfill research

The backfill will have a large diversity of microbes in relatively abundant numbers. A main concern about the backfill is its content of oxygen at the outset. This oxygen has a corrosive effect on the copper canisters. Performed experiments all indicated that microbes would be very efficient in removing oxygen from groundwater, if introduced. It can be hypothesised that this would also occur in the backfill. In addition, active sulphate-reducing bacteria (SRB) and iron-reducing bacteria (IRB) would produce sulphide and ferrous iron, both of which lower the redox potential of the groundwater in the backfill. A low redox potential is important for achieving low radionuclide mobility in the case of an accidental release of radionuclides. In such a scenario, the microbes

would also guard the environment from the products of radiolysis of water. They would efficiently recombine the oxygen and hydrogen produced by water, thereby buffering the redox downwards.

Geosphere research

Several projects, using experimentally independent methods, have pointed out the oxygen reduction capacity of microbes in hard rock aquifers and tunnels. All indicate that a large benefit of geosphere microbes for repository performance is their enormous capacity to protect the host rock and repository from oxygen, and their production of groundwater components which lower the redox potential. Oxygen will flow into the basement rock along with recharging groundwater and will diffuse from the tunnel air into the rock matrix. The recharging groundwater will contain organic matter and microbes will continuously reduce the oxygen by oxidising organic carbon. Anaerobic microbes in the hard rock aquifers in the host rock reduce ferric iron, manganese(IV) and sulphate to ferrous iron, manganese(II) and sulphide with organic carbon. These metals and the sulphur will react with oxygen when the water reaches a tunnel. Mats of microbes develop on the tunnels walls where groundwater seeps out. They produce organic carbon with the energy from these inorganic groundwater components. Other microbes can later use the organic matter produced for additional oxygen reduction. Thus the microbes close biogeochemical cycles.

A special case concerns periods of glaciation. During such events, the input of organic carbon with recharging groundwater will be low because photosynthetic production of organic carbon will cease during a period of glaciation. The REX projects showed significant activity of methane-oxidising bacteria. Methane is produced in deep mantle rocks and migrates upward. The continuous flow of methane from deep mantle rocks will not depend on glaciation events.

Natural analogue research

Bangombé. The Bangombé reactor zone in Oklo, Gabon, was visited in March 1993, July 1994, September 1996 and February 1998 and a field analysis programme was designed with the main aim of investigating distribution and numbers of bacteria, special attention being given to IRB and SRB. These two groups of microorganisms oxidise organic matter with ferric iron and $SO4^{2-}$ -producing ferrous iron, and S^{2-}, respectively. Electron microscopy investigations have revealed structures similar to microorganisms on fracture material but the results are difficult to interpret owing to difficulties in obtaining material that conclusively is from open fractures with a flow of groundwater. Water is a requirement for bacterial activity. The main effects of aerobic and anaerobic microbial activity on the Bangombé groundwater chemistry are a consumption of dissolved O_2 and solid iron(III) oxides and the production of CO_2. A lowering of the redox potential will occur concomitantly with the production of the reduced electron acceptor iron(II) from iron(III). This conclusion is in agreement with multivariate mixing and the mass balance calculation (M3) of the reactor zone groundwater that showed an increase in alkalinity in the reactor zone as a result of microbial degradation of organic matter.

Palmottu. The total number of microorganisms at Palmottu, in Finland, has been shown to decrease with depth and this relation has also been observed at the Äspö Hard Rock Laboratory (HRL). The number of anaerobic reducing microorganisms increased with depth, which finding was in agreement with results from other Fennoscandian groundwater sites. There was a direct correlation between the number of IRB and SRB detected with the concentrations of total iron and sulphate. Generally, a lower redox

correlated with more IRB, SRB, heterotrophic acetogens (HAs) and autotrophic acetogens (AAs). These relations can be expected. It is not obvious from the data which of the correlation variables depend on which. Typically, microbial activity decreases the redox potential but it is premature to conclude whether the redox of the sampled Palmottu groundwater is coupled with the reduction activities of the found microorganisms. In borehole R387, the distribution of SRB and IRB showed an inverse correlation with dissolved uranium. An attempt to mimic the groundwater situation in culture tubes inoculated with enrichment cultures of IRB and SRB was only partly successful. Some uranium reduction was detected with cultures enriching IRB. It is consequently possible that microorganisms contribute to keeping the Palmottu groundwater system reduced and that they may also be directly involved in reducing uranium(VI) to U(IV).

Maqarin. The Maqarin site in northern Jordan is unique, situated in bituminous marls which have been thermally altered by natural in situ combustion. As a result, the groundwater discharging at Maqarin is hyperalkaline and geochemically similar to Portland cement pore water. The site is therefore considered to be an excellent natural analogue for the high pH environments that will dominate around and in low- and intermediate-level waste repositories, and in SFL 3-5 repositories. Among the questions to be answered with respect to microbial processes is whether microorganisms can survive and be active at the extreme pH values typical of the Maqarin groundwater. Molecular methods, microscopy, culturing techniques and chemical analysis were used in an attempt to find an answer to this question. Microorganisms were found in all of the Maqarin groundwater but it could not be conclusively demonstrated that they are viable and growing in situ, rather than just being transported there from neutral groundwater. The diversity of the microorganisms found was similar to what has been detected with the 16S rRNA gene-sequencing method used previously, but none of the sequences found was typical of known alkaliphilic organisms. A possible hypothesis based on the obtained results is that the majority of the investigated Maqarin springs may be a little too extreme for active life, even for the most adaptable microbe; however, this remains to be demonstrated. A new field research campaign was started in November 1999 with the goal of further evaluating the upper pH limit for the survival and activity of microbes.

Radionuclide transport and retardation research

Bacteriogenic iron oxides (BIOS) accumulate various metals. The accumulation of strontium, cesium, lead, uranium, sodium, cobalt, copper, chromium and zink was studied in BIOS from underground mine and tunnel sites. The BIOS samples were found to contain only poorly ordered (amorphous) hydrous ferric oxide, as determined by X-ray diffraction. Inductively coupled plasma mass spectroscopy revealed hydroxylamine-reducible iron and manganese oxide contents ranging from 55% to 90% on a dry weight basis. Distribution coefficients (K_d values), calculated as the ratio between BIOS and dissolved heavy metal concentrations, revealed solid-phase enrichments which, depending on the metal and iron oxide content of the sample, extended from 10^0 to 10^5. At the same time, however, a strong inverse linear relationship was found between log K_d values and the corresponding mass fraction of reducible oxide in the samples, suggesting that metal uptake was strongly influenced by the relative proportion of bacterial organic matter in the composite solids. Based on the metal accumulation properties of the BIOS, an important role can be inferred for intermixed iron oxides and bacterial organic matter in the transport and fate of dissolved metals in groundwater systems.

Sammanfattning

Oberoende vetenskapliga undersökningar har otvetydigt visat att liv förekommer på kilometerstora djup i de flesta underjordiska geologiska formationer som studerats. Mikrobiella processer har därför kommit att bli en integrerad del i säkerhetsanalyserna av underjordiska förvar med högaktivt radioaktivt avfall. Denna rapport presenterar forskningsresultaten från över tio års forskning inom Svensk Kärnbränslehantering AB's mikrobiologiska forskningsprogram. Programmets långsiktiga mål är undersöka hur mikrobiella processer kommer att samverka med olika förvarsfunktioner. Första delen av rapporten presenterar begreppet mikrober för läsare som inte är så hemma i den vetenskapliga disciplinen mikrobiologi. (En god populärvetenskaplig översikt av ämnesområdet återfinns också i Nationalencyklopedin.) Generella kännetecken för mikrobiella ekosystem presenteras tillsammans med en genomgång av karakteristiska drag i mikrobernas tillväxt, aktivitet och överlevnad. Den resterande, huvuddelen av rapporten bekantar läsaren med de vetenskapliga data och tolkningar som erhållits under perioden 1987 till 1999. Korta sammanfattningar introducerar forskningsfrågorna och följs av översikter av de resultat och den kunskap som erhållits. Varje större forskningsområde sammanfattas i konklusiva avsnitt.

Sulfatreducerande bakterier (SRB) bildar sulfid och de förekommer allmänt i grundvatten på förvarsdjup. Därför är det viktigt att risken för sulfidkorrosion av kopparkapslarna undersöks. Ett specialfall rör gränsytan mellan kopparkapseln och bufferten av bentonit. Ingenstans i ett förvar är begränsningarna för möjligheten till liv så stora som här. Ändå har det framförts oro för att SRB skall växa där och bilda sulfid i sådana mängder att kopparkapseln skadas allvarligt. De experiment som hittills genomförts tyder dock klart på motsatsen.

Tidiga studier i forskningsprogrammet uppenbarade förut okända mikrobiella ekosystem i akvifererna i magmatiskt berg på djup överstigande 1000 meter. Den upptäckten satte igång noggranna undersökningar av den nyupptäckta så kallade djupa biosfären i det Fennoskandiska urberget. Mikrobiella processer på förvarsdjup kommer att inverka på funktionen hos ett förvar på flera viktiga punkter. Några av dessa innebär bildning av sulfid, koldioxid, organiskt kol och metan samt reduktion av syre. Förvarsfunktioner måste kunna prognostiseras för en lång tid framöver. Till vår hjälp har vi de så kallade naturliga analogerna. Tidsrelaterade processer som rör mikrober och transport av radionuklider har studerats på platser där processerna utvecklats under mycket långa tidsrymder. Miljöer med mycket basiska pH värden studeras i Maqarin, Jordanien, bränslerelaterade processer har man kunnat följa vid de naturliga kärnreaktorerna i Oklo, Gabon och transportprocesser som rör uran har undersökts kring den naturligt förekommande uran-mineraliseringen i Palmottu, Finland.

Huvuddelen av de radionuklider som skall lagras i ett förvar är metaller. Transport, speciering och den slutliga destinationen för lösta metaller i grundvatten avgörs till stor del av reaktioner som sker vid fasta ytor. Mikrober är minst lika allmänt förekommande i grundvatten, och reaktiva, som de flesta oorganiska fasta partiklar i naturliga vatten. Mikrobers förmåga att reagera som geokemiskt aktiva material bekrivs i en mycket omfattande vetenskaplig litteratur där forskningsresultat dokumenterar mikrobers goda förmåga att uppträda som sorbenter för metaller. De kan också fungera som groddkorn för nybildning av en rad olika mineral. Mikrober kan således komma att spela en väsentlig roll i retentions- och transportprocesser av radionuklider.

Contents

1 Background to the report

Independent scientific work has unambiguously demonstrated life to be present in most deep geological formations investigated, down to depths of several kilometres (Pedersen 1993a; Pedersen 2000). Sedimentary rocks (Fredrickson and Onstott 1996), igneous rocks (Pedersen 1997a) and sub-sea floor environments (Fisk et al 1998; Wellsbury et al 1997) all harbour life. The distribution of underground life is conceptually restricted only by temperature. At present, the known temperature limit for life is 113°C (Stetter 1996). This temperature is reached at very different depths around our planet, from the seafloor surface at marine hot springs to 10 km or deeper in massive sedimentary rock formations. In the Fennoscandian Shield, the temperature typically increases by 1–2°C per 100 m, which suggests that microbial life may extend as far down as 6–7 km at any igneous rock site chosen for the future Swedish high level radioactive waste (HLW) repository. Microbial processes have, consequently, become an integral part of the performance safety assessment of the Swedish HLW repository (SKB AB 1999a).

This report presents the research record from the last decade of the SKB microbiology research programme and gives current perspectives of microbial processes in HLW disposal, with SR-97 as a template (SKB AB 1999a). However, for those who are not so familiar with microbes and their ways of living, I will start by defining the concept of "microbe", briefly list the main characteristics of recognised microbial assemblages and give an introduction to microbial growth, activity and survival.

2 The microbes

A microbe is a living entity which contains all that it needs in order to perform a life cycle, including feeding, growth and reproduction, in one, single cell.

The size of a microbe varies significantly, from the smallest bacterium with a diameter of about 0.2 μm to some unicellular animals and plants which may reach 1 mm or more in diameter. The largest known bacterium is the sulphur-oxidising microbe *Thiomargarita namibiensis* which reaches a maximum diameter of 0.75 mm (Schulz et al 1999).

2.1 Microbes – what are they?

The tree of life, based on the gene 16/18S rRNA, is depicted in Figure 2-1. It shows the phylogenetic relationship between all known and characterised organisms on earth. The organisms cluster in three major domains, viz. Bacteria, Archaea and Eukarya. All organisms in the domains Bacteria and Archaea are microbes. Most of the branches of the domain Eukarya are microbial as well. In fact, multi-cellular organisms are only represented in the three branches comprising animals, plants and fungi. Thus, microbes can be found virtually everywhere in the tree of life. They constitute the absolute dominating diversity of life on our planet. Biochemically, much of this diversity is contradictory to multi-cellular life whose diversity is largely morphological. The enormous biochemical diversity among the microbes explains their huge adaptability to almost any environment on the planet, where temperature allows life. The microbes are commonly divided into five different groups, based mainly on a mix of morphological, biochemical and molecular criteria. The most important criteria for each of the five groups, and their relevance to a HLW repository, are given below.

2.1.1 Bacteria

A typical bacterium is a very robust creature which generally survives extremely well in the niche it is adapted to live in. Different bacteria are adapted to different conditions and as a group, the bacteria cover all possible combinations of environmental circumstances. This is reflected in the species diversity of the domain Bacteria (Fig 2-1). The domain comprises many millions of species or more, as reflected by environmental ribosomal rDNA sequencing (Pace 1997). Approximately 10 000–15 000 of these microbes have been characterised (see, e.g., Fig 2-2); the rest remain molecular imprints on the environment of organisms that await exploration and characterisation. This vast diversity of unknown species represents an uncertainty with respect to possibly unknown microbial processes of importance for nuclear waste disposal. One obviously unwanted species would, for instance, be a species that would, under repository conditions, produce large quantities of radionuclide-chelating agents. Anaerobic methane oxidisers would, by contrast, be very beneficial as they would contribute to keeping groundwater redox potential levels low.

There appear to be some overriding characteristics which unify many of the main branches of the domain Bacteria. The capability to photosynthesise is a typical characteristic of green bacteria, cyanobacteria and some of the proteobacteria. Because of their need for light, these groups are not naturally represented in groundwater. Some other groups are also naturally absent, such as the pathogenic microbes (e.g. Chlamydia)

and all obligate parasitic microbes (mostly among the proteobacteria) that generally require a multi-cellular host. Representatives of the remaining branches have been reported from various underground environments. Fennoscandian Shield rocks are generally cold to moderately warm for the first 2 km. The rock temperature at repository depth (500m) is some 15–20°C and thermophilic (i.e. heat-loving) organisms will not be common before disposal. It is uncertain to what extent thermophilic Bacteria (and also Archaea) will invade and/or multiply in a repository area, with a temperature falling from 80°C to 50°C during the first 3000 years. They certainly can be found active in all naturally occurring high-temperature groundwater. The consensus today is that thermophiles will appear in significant numbers in a warm repository. This question will be further addressed in Sections 3.1–3.2.

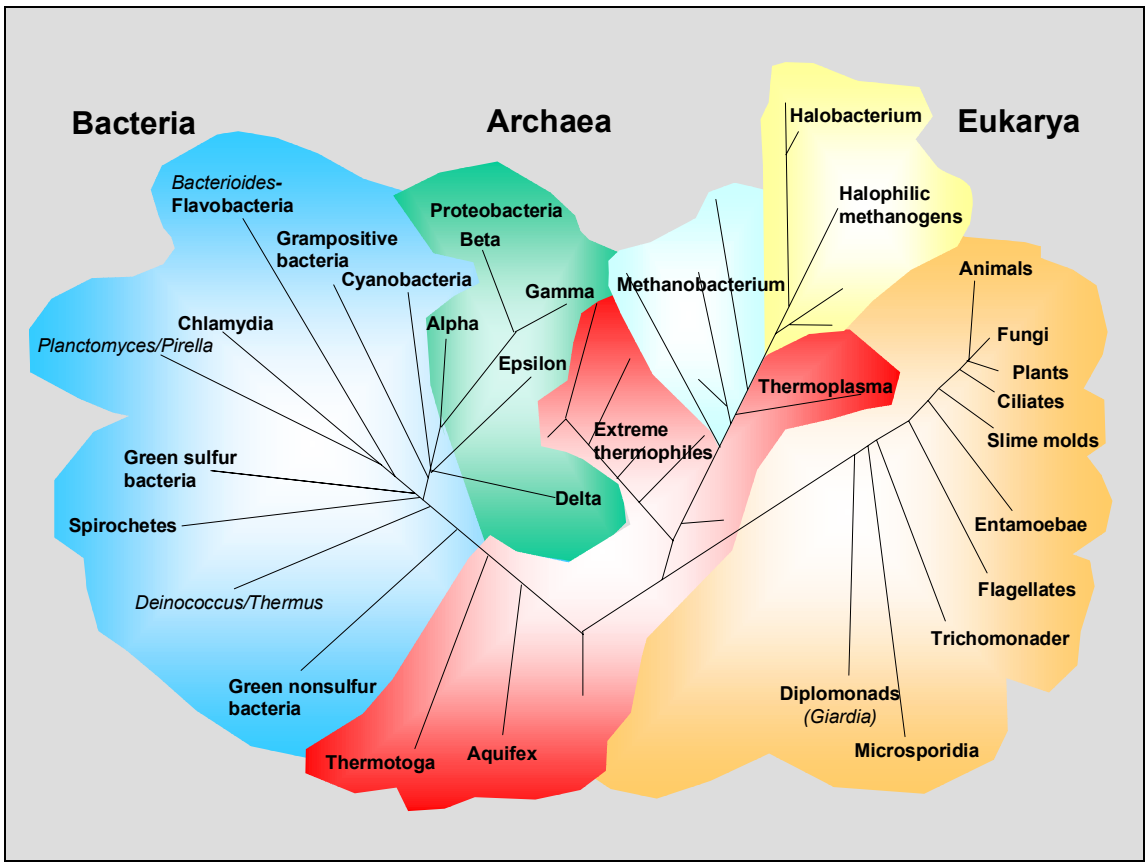


Figure 2-1. *The phylogenetic relationship between all main organism groups on the planet can be revealed by comparing their 16S rDNA and 18S rDNA genes, coding for the ribosomes which are the protein factories of the cell (Woese et al 1990). The red colour represents microbes that are adapted to high temperatures (60°C –113°C), many of which utilise hydrogen as a source of energy. Yellow depicts microbes that can live in saturated salt solutions (25–30% NaCl). Green shows the proteobacteria, which group many of the microbes found in the Fennoscandian Shield aquifers belong to. Methanogens living at low or intermediate temperatures (0°C –60°C) appear light blue. They constitute an important group in most underground environments. The bulk of the domain Bacteria is shown in blue and the domain Eukarya is shown in light brown.*

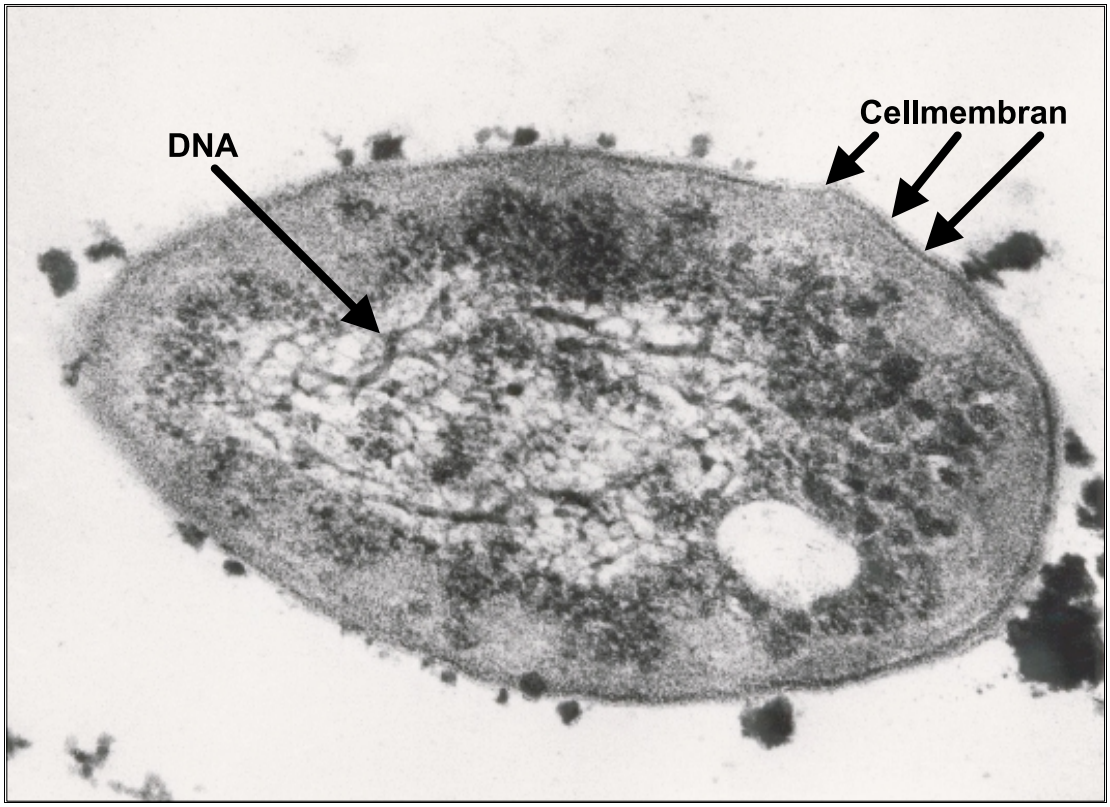


Figure 2-2. *A cross-section of the bacterium Gallionella ferruginea produced with a transmission electron microscope (TEM). This microbe is very common in groundwater seeps on the walls, the floor and in ponds in the Äspö Hard Rock Laboratory (HRL) tunnel. The cell wall gives the microbe its form and rigidity. The cell membrane controls the transport of nutrients in, and wastes out, of the cell. The nucleic acid DNA constitutes a large part of the interior of the cell. This organism is a chemolithotroph. It uses ferrous iron (Fe^{2+}) as a source of energy. The energy is used to reduce carbon dioxide to cell carbon constituents, just as plants do, but with iron energy instead of solar energy. The visible structures in this cell do not look very different from those of a bacterium that uses organic carbon as a source of energy and a carbon source for cell constituents. The differences are almost completely on the molecular scale, a scale which is not resolved by the TEM. The diameter of the cell is approximately 1 μm. (Photograph: Lena Bågenholm and Lotta Hallbeck.)*

2.1.2 Archaea

Microorganisms in the domain Archaea (Fig 2-1) were regarded as bacteria until molecular data revealed that they belong to a domain which is totally different from all bacteria as well as all plants, animals and fungi. A unifying characteristic of organisms in this domain is their ability to adapt to what is called “extreme conditions”. Different species of Archaea are active under different conditions. Some Archaea like it very hot. For instance, the optimum temperature for growth of the genus Pyrolobus is 105°C and it survives in temperatures of up to 113°C. Many other genera of Archaea grow best at about 100°C. The temperature of the HLW repository will consequently not exceed the temperature range within which life can exist. Some genera of Archaea are adapted to extreme pH levels, as low as 1 or up to 12, and some may even survive under conditions of lower/higher pH.

A group that are important for a HLW repository are the methanogens (Fig 2-3). They produce methane gas from hydrogen and carbon dioxide, or from short organic carbon compounds such as formate, methanol, or acetate. The importance of these processes for HLW disposal will be addressed in more detail in Sections 3.1–3.2.

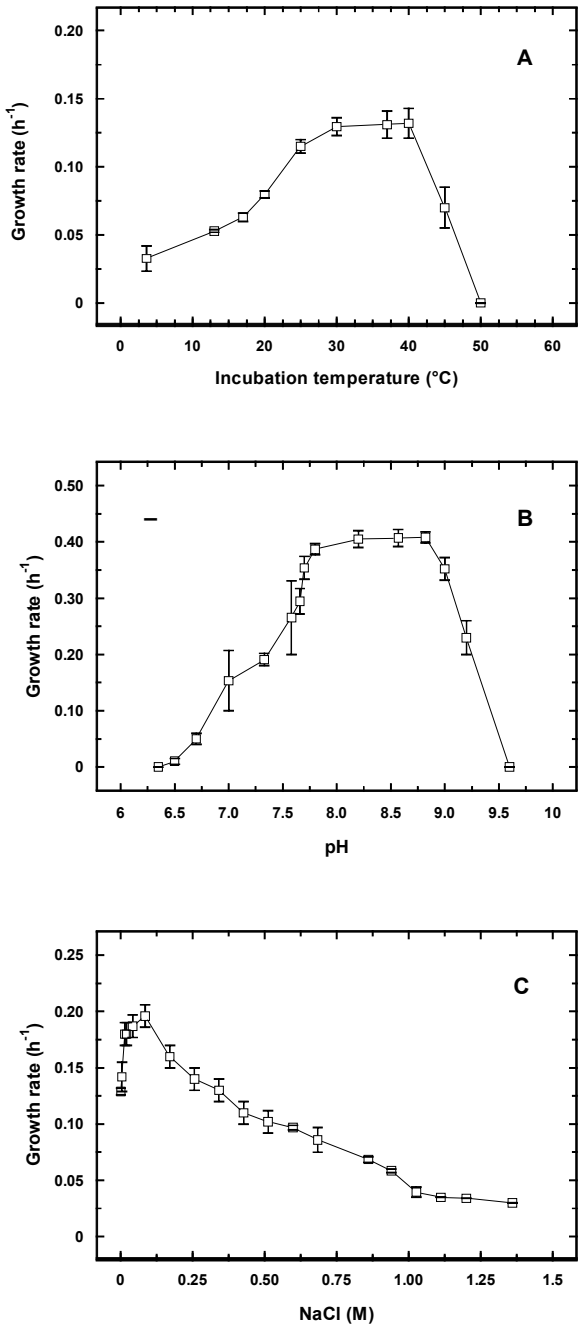


Figure 2-3. *Methanobacterium subterraneum is a genus of Archaea that has been isolated from the Äspö hard rock aquifers and characterised (Kotelnikova et al 1998). It has temperature (**A**), pH (**B**) and salt (**C**) requirements that include typical values of these parameters in the repository. This species is therefore likely to be an important inhabitant of the repository when the temperature is below 50°C.*

2.1.3 Unicellular fungi

The fungi belong to the domain Eukarya (Fig 2-1) and represent a large morphological and biochemical diversity. Yeast belongs to the unicellular fungi, commonly with the abilities to live without oxygen and to ferment many different organic compounds to carbon dioxide, organic acids, alcohols and hydrogen. Some of these organic acids, for example citric acid, are excellent chelating agents and are therefore unwanted in a repository in the case of a canister failure. Mould is another group of fungi that are regarded as unicellular, despite their ability to grow as multi-cellular mycelia (i.e. networks of treads). Each cell in a mycelium is capable of a complete life cycle independent of other cells and therefore falls into the category of microbes. There are as yet no data in the scientific literature that demonstrate fungi to be natural inhabitants of deep rock aquifers; however, there is no conceptual argument against their dwelling underground. Fungi are capable of performing anaerobic metabolism (i.e. of living without oxygen) and are small, typically no bigger than a couple of μm or more, which makes them suitable for life in the narrow aquifers of hard rock. Recent investigations of groundwater from the Äspö HRL strongly suggest that fungi are a natural part of the subterranean biosphere in Fennoscandian Shield igneous rock aquifers (Fig 2-4). This finding introduces an uncertainty with respect to the performance of a repository and fungal chelating agents and radionuclide migration.

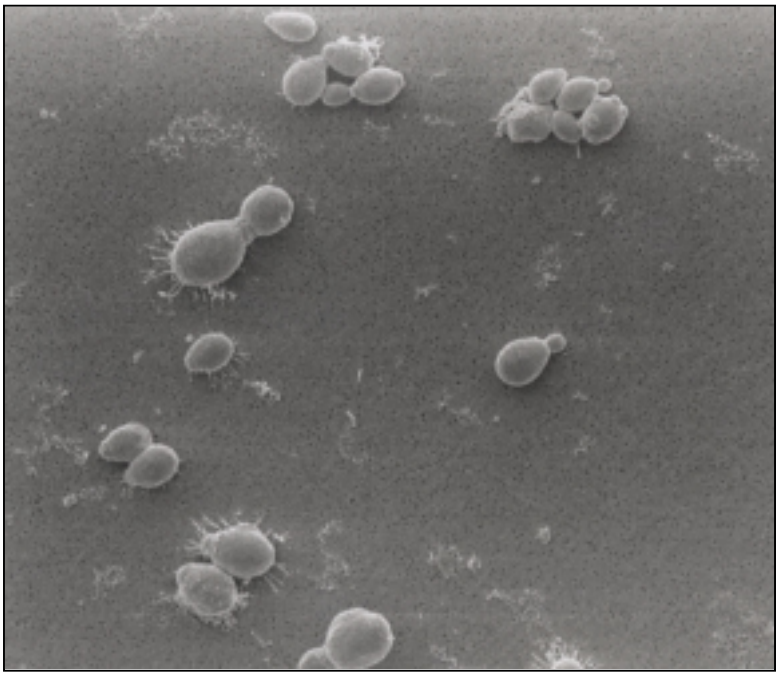


Figure 2-4. *Using sterile syringes and needles, groundwater was sampled direct from fractures and boreholes and placed in appropriate culturing media. Growth of yeast and fungi occurred frequently. The isolated yeasts depicted are unique and represent new species with growth demands that correlate with the environment in groundwater at repository depth. The diameter of the yeast cells on the image is approximately 2 μm. (Photograph: Susanne Ekendahl and Margit Fredrickson.)*

2.1.4 Unicellular animals

Unicellular animals belong to the domain Eukarya. They are found in all taxonomic branches except the fungi and plant branches (Fig 2-1). Their natural presence in deep groundwater remains to be established. Some unicellular animals, particularly the

flagellates, are so small (a few μm) that they are difficult to distinguish from large bacteria and yeast. Their obvious function in deep groundwater ecosystems would be as grazers of other microbes. Many of the unicellular animals feed on organisms of the genera Bacteria and Archaea.

2.1.5 Unicellular photosynthetic organisms

Unicellular photosynthetic microbes are found in several of the branches in the domain Bacteria and also in the plant branch of the domain Eukarya (Fig 2-1). The domain Archaea does not have any known true photosynthetic organisms. The process of photosynthesis requires light, which requirement is not fulfilled underground, except in illuminated underground vaults and tunnels. Mosses, cyanobacteria and some other photosynthetic organisms have been observed in the Äspö HRL tunnel and will certainly occur where there is light in a repository during the open phase. They fix carbon dioxide to organic carbon and will therefore add some organic constituents to the repository environment. Their activity is, however, not foreseen to interfere with the performance of the HLW repository.

2.2 Microbial processes in closed systems – the batch culture situation

The common way to culture microorganisms in the laboratory is by using a batch culture. A culture vessel is supplied with all constituents necessary for growth, and inoculated with the microbe of interest. A typical batch growth curve can be registered (Fig 2-5). First, there is an adaptation phase during which the cells adjust to the conditions in the culture vessel. Then the cells start to divide and grow exponentially to high counts, doubling their number at even time intervals. Finally, growth is arrested when some limiting component is used up, or when a toxic component forms at too high a concentration (e.g. alcohol, in fermentation cultures). Figure 2-5 shows that the cells, basically, are active only during the exponential growth phase. The batch culture represents a closed system with no input or output of components from the system. It is a superb tool for many research purposes in the laboratory but it does not mimic the life of microbes in natural environments. The environment generally consists of a huge number of open systems with continuous input and output of matter in between. Models of microbial processes in the repository should therefore be based on continuous culture situations, as described below, rather than on batch culture situations.

2.3 Microbial processes in open systems – the continuous culture situation

Hard rock aquifers can be considered as open systems. A particular fracture will have a water composition that reflects the origin of the water and various reactions between solid and liquid phases occur along the flow path. A new composition may be the result of two fractures meeting and of their water mixing. These processes may be slow but there is a continuum of varying geochemical conditions in hard rock aquifers at repository depth, and the repository with all its alien construction components will add variance to these conditions. Microbes are experts on utilising any energy in the environment that becomes thermodynamically available for biochemical reactions. A slow but steady flow of organic carbon from the surface or a flow of reduced gases such as hydrogen and methane from the interior of our planet will ultimately be the driving force of active life of deep aquifer microbes.

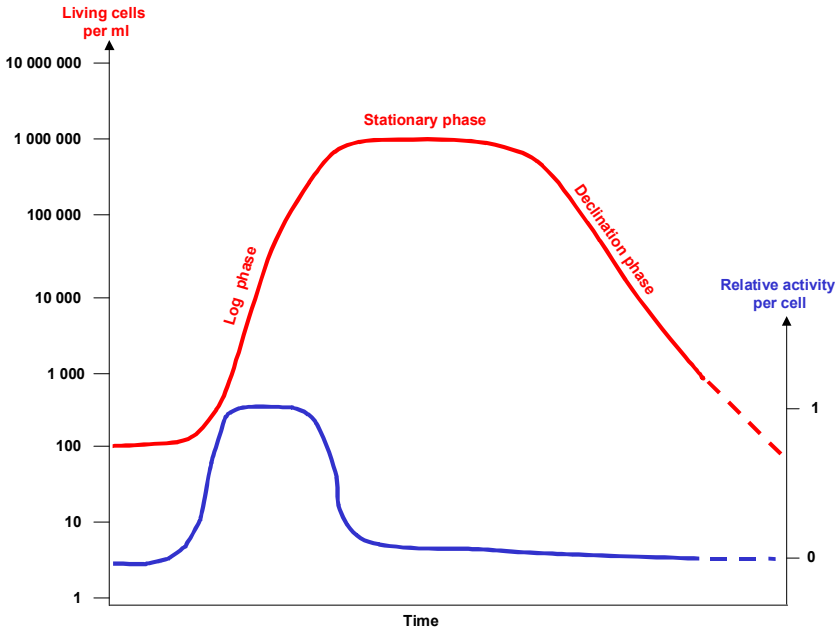


Figure 2-5. *A schematic representation of microbial growth in a closed batch culture. The microbes are basically active only during the exponential growth phase, when they double their number during a specific time period. The doubling time can be as short as 15 minutes for some easily culturable microbes and it may be many hours for microbes more difficult to work with.*

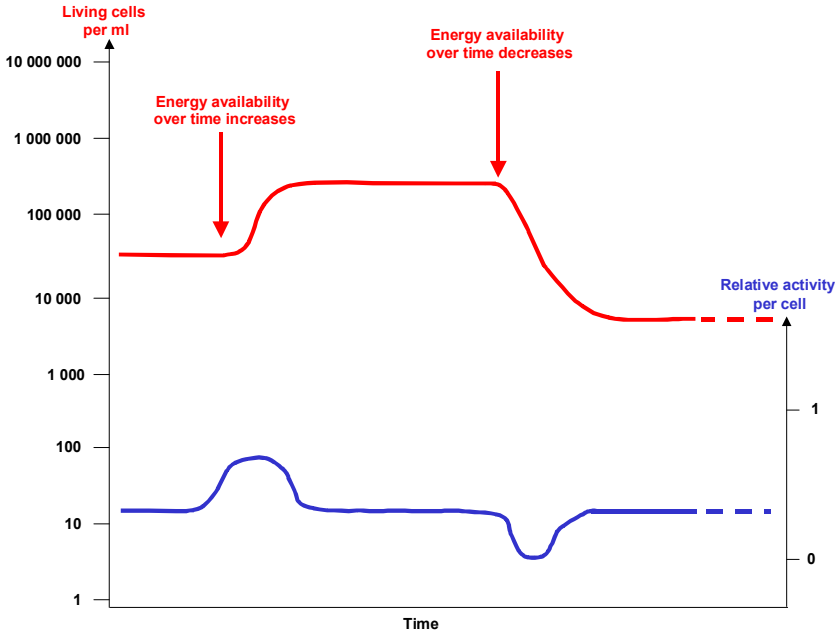


Figure 2-6. *The graph is a schematic representation of microbial growth in an open, continuous culture system. The microbes are continuously active except for periods when there is a decrease in availability of energy over time. The doubling time of the population can be very long. More than a month has been registered for deep groundwater biofilms (Ekendahl and Pedersen 1994).*

Continuous growth of microbes can be studied in the laboratory using a chemostat. The culture vessel is continuously supplied with energy by a slow inflow of nutrients. The inflow is balanced by an outflow that removes waste products and some cells. The number of cells will therefore remain constant in the chemostat. The microbes will, however, be active (Fig 2-6). A chemostat system is, unlike the batch system, open as it has an influx and outflow of matter. The continuous culture situation of the chemostat is applicable to any hard rock aquifer experiencing flux of matter through a continuous mixing of groundwater of varying compositions. The flows may be very slow, but over geological time scales, they will be significant.

The open, continuous culture system concept can be used for the interpretation of microbiology data from groundwater, such as the number of cells in arbitrarily chosen groundwater measured at various times (see, e.g., Pedersen et al 1996b). If we apply the batch concept (Fig 2-5), we would conclude that the microbes are not growing and are inactive because we do not register any increase in cell numbers over time. By contrast, with the continuous culture concept, it can be predicted that the microbes are active and that they grow slowly at constant environmental conditions over the time period studied. This prediction requires processes that balance an increase in cell numbers due to growth. There are several possibilities for growth of cell populations in deep aquifers. Phages (i.e. viruses that attack microbes) may balance cell growth. Their activity results in lysis of the infected cells, and in the production of new phages. This process has as yet not been demonstrated in deep aquifers, but occurs in most surface environments. Many unicellular animals graze on other microbes and their possible presence and activity in deep aquifers would also counterbalance cell growth.

A special case is the possible occurrence of microbes that grow attached to aquifer surfaces, a phenomenon which has repeatedly been observed in groundwater from deep hard rock aquifers (Ekendahl and Pedersen 1994; Pedersen 1997a; Pedersen et al 1996b). Such biofilms will increase their cell numbers until they reach steady state, as previously described for continuous growth of unattached microbes.

2.4 The microbes enigma – death or survival

In periods of inactivity due to lack of energy and necessary nutrients, or other environmental constraints such as desiccation or slowly decreasing water activity, microbes can do one of two things. They die or enter one of many different possible states of survival. Different species have different ways of addressing the problem of unfavourable conditions for active life. The most resistant form of survival is the endospore formed by certain gram-positive bacteria (see Fig 2-1) and SRB. There is no measurable sign of life in an endospore, yet after many years of inactivity, it can germinate to an actively growing cell within hours. It resists desiccation, radiation, heat and aggressive chemicals far better than does the living cell.

The endospore is the most resistant state of survival of any known life form but there are many more survival strategies among the microbes, which are more or less resistant to environmental constraints. Transforming into morphologically specific survival states is an advantage when the environment changes. However, in response to mere nutrient and energy deficiency, many microbes just shut down their metabolism to an absolute minimum level, at which they may survive for many years. Most such responses result in shrinkage of the cell to a fraction of its volume at optimal growth conditions. All these survival strategies have in common that the cell is active at an absolutely minimal level, or shows no activity at all. It is consequently possible that certain microbes may

survive initially harsh conditions in a repository, radiation, desiccation, heat, high pH, and so on until the conditions for growth again become favourable. However, if the conditions are so difficult that all survival forms die off, and if the pore size of the environment does not allow for transport of microbes, as in high-density bentonite, then it is possible that specific environments in the repository stay free of microbes once the original microbe population have disappeared. It is at present uncertain whether this will be the case.

3 The research record

In 1987, microbiology became an operative part of the SKB's scientific programme for the safe disposal of HLW in igneous rock and research is ongoing. The goal of the microbiology programme is to understand how microbes would interact with the performance of a future HLW repository. In this Section, we summarise data collected during the research period of 1987–1999 and interpretations of these data. A short summary below may serve as an introduction to the research tasks; this is followed by a review of the results and knowledge obtained.

Sulphate-reducing bacteria produce sulphide and have commonly been observed in groundwater environments typical of a Swedish HLW repository. The potential for sulphide corrosion of the copper canisters used in HLW storage must consequently be considered. The bentonite buffer around the copper canisters will be a hostile environment for most microbes, owing to the combination of radiation, temperature and low availability of water. Discrete microbial species can overcome each of these constraints and it is theoretically possible that sulphide-producing microbes may be active inside a buffer, although the experiments conducted thus far have indicated the opposite (Motamedi 1999; Motamedi et al 1996), as discussed under "Buffer Research" and "Backfill Research". A special concern is the interface between the copper canister and the buffer. Nowhere are the environmental constraints for life as strong as in this area. Still, it has been suggested that SRB could survive and locally produce sulphide in concentrations large enough to cause damage to a canister. This possibility is further discussed under 3.1, "Copper Canister Research".

In the early stages of the research programme, previously unknown microbial ecosystems were revealed in igneous rock aquifers at depths exceeding 1000 m (Pedersen and Ekendahl 1990). This discovery triggered a thorough exploration of the subterranean biosphere in the aquifers of the Fennoscandian Shield (Pedersen 1997b). Similarly, the Canadian radioactive waste disposal programme has stimulated investigations of microorganisms in deep igneous rock aquifers of the Canadian Shield (Stroes-Gascoyne and Sargent 1998). Early investigations examined the potential risk of radionuclide migration caused by microorganisms able to survive in the deep groundwater systems (Birch and Bachofen 1990) (see also 3.6, "Retention and Transport of Radionuclides"). It soon became apparent that microbial communities exist in most, if not all, deep aquifers (Pedersen and Ekendahl 1990). Attention was then shifted to examine the activity potential of these microorganisms using radiotracer methods (Ekendahl and Pedersen 1994; Kotelnikova and Pedersen 1998; Pedersen and Ekendahl 1992b, 1992b). The activity results suggested remarkable metabolic and species diversity, which led to DNA extraction and 16/18S rDNA cloning and sequencing for assessment of subterranean microbial diversity (Ekendahl et al 1994; Pedersen et al 1996b, 1997c). The work revealed several previously unknown microbial species adapted to life in igneous rock aquifers (Kalyuzhnaya et al 1999; Kotelnikova et al 1998; Motamedi and Pedersen 1998).

The repeated observations of autotrophic, hydrogen-dependent microorganisms in the deep aquifers suggest that hydrogen may be an important electron and energy source, and carbon dioxide an important carbon source in deep subsurface ecosystems. Hydrogen, methane and carbon dioxide have been found in μM concentrations at all

sites that have been tested for these gases. Methane is a major product of autotrophic methanogens, which have been shown to be present at the Äspö HRL. Therefore, a model has been proposed of a hydrogen-driven biosphere in deep Fennoscandian Shield igneous rock aquifers. A similar model has been suggested for deep basalt aquifers (Stevens and McKinley 1995). The organisms at the base of these ecosystems are assumed to be autotrophic acetogens (AAs) capable of reacting hydrogen with carbon dioxide to produce acetate, autotrophic methanogens (AMs) that produce methane from hydrogen and carbon dioxide, and acetoclastic methanogens that produce methane from the acetate product of the AAs. Microbial processes at repository depths will have several important influences on repository performance. Some identified processes are the production of sulphide, carbon dioxide, organic carbon and methane, and the consumption of oxygen. These processes will be further discussed under 3.4, "Geosphere Research".

The repository performance must be predicted far into the future and natural analogues therefore become invaluable. Time-related processes of radionuclide migration have been studied at analogue sites that have been evolving over very long periods of time. High pH conditions occur in Maqarin, in Jordan. Fuel-related processes evolved at the natural reactors in Oklo, Gabon, and uranium migration processes developed around the uranium body of Palmottu, in Finland. The microbiology research performed on these sites is discussed under 3.5, "Natural Analogues".

3.1 Copper canister research

3.1.1 Background

The worst case scenario in copper canister corrosion would be if SRB formed biofilms on the canisters or grew intensively in the buffer close to the canister. The corrosion process would be controlled by the transport of sulphate to the canister, if enough hydrogen or degradable organic carbon would be available for such growth. This could lead to considerably accelerated corrosion since the transport of sulphate is expected to be much faster than the transport of sulphide, due to the fact that the sulphate concentrations in the bentonite can be up to tens of mmol/dm^{-3}.

During the initial phase, the temperature in the repository will be elevated, with a maximum temperature of 90°C on the copper surface. As was discussed for Archaea and Bacteria under 2, "The Microbes", this is not an absolute constraint. Sulphate-reducing bacteria may survive. The radiation will also be very high at the canister surface, which will add to the effect of an elevated temperature on the survival of microbes. Finally, low availability of water in the buffer (i.e. the water content relative to groundwater) will also add a constraint on the likelihood of long-term survival. Altogether, conditions for survival will be very difficult for microbes existing close to the canister. This can be investigated with techniques in microbial ecology and some preliminary investigations have been performed.

3.1.2 Methods

Swelling pressure odometers (Motamedi et al 1996; Pedersen et al 1995) were loaded with bentonite at different densities corresponding to different water activity values. A copper disc was placed between the bottom lid and the bentonite that was compacted to various densities. Different SRB were added to the clay and the discs, together with $^{35}SO_4^{2-}$. The species used were from laboratory cultures and were Desulfomicrobium baculatum (with an optimum temperature for growth of 30°C) and Desulfotomaculum

nigrificans (the optimum temperature for growth being 55°C). Finally, oxidised silver foil was placed between the disc and the clay. The odometers were reassembled and incubated for 4 weeks at the respective optimum temperatures and three different densities: 1500, 1800 and 2000 kg bentonite m^{-3}, corresponding to the water activities 0.999, 0.994, and 0.964, respectively. After incubation, ^{35}S-silver-sulphide was localised on the silver foils and quantified by electronic autoradiographic imaging (Packard instant imager electronic autoradiography system, Meriden, USA). The amount of silver-sulphide found corresponded to the sulphate-reducing activity of the SRB.

3.1.3 Preliminary results and conclusions

Figure 3-1 shows that the SRB used were active, producing sulphide at a density of 1500 kg m^{-3}, and that the sulphide production was virtually absent at the higher densities tested. This experiment indicates that SRB cannot be active at the canister surface at a repository density of 2000 kg m^{-3}. However, the experiment was run with two laboratory species and it can be argued that other species which were not tested may survive better. Therefore, new experiments should be executed with natural groundwater that commonly contains many hundreds of different microbial species and several naturally occurring species of SRB.

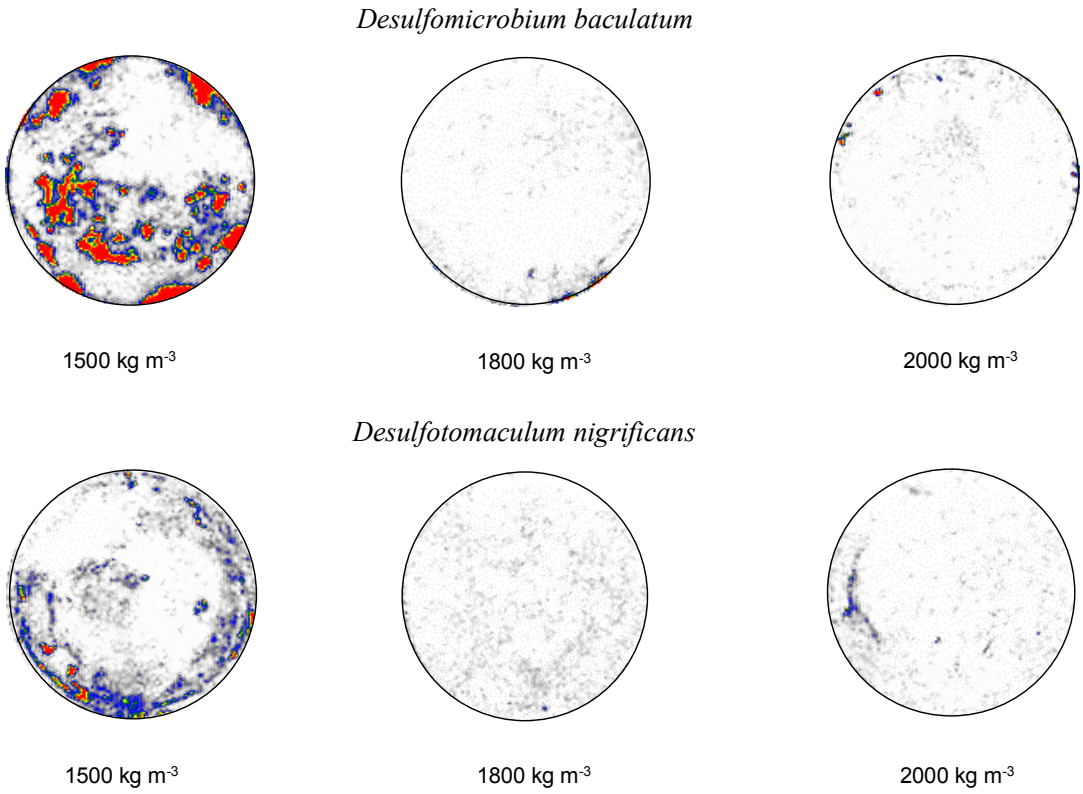


Figure 3-1. *Radioisotope (^{35}S-sulphide) images of copper discs are shown after incubation with SRB and $^{35}SO_4^{2-}$. The discs were incubated with bentonite at three different densities and with two different species. Coloured areas indicate ^{35}S-sulphide,with red indicating higher concentrations than blue. See Section 3.1.3 for details. The diameter of the images is 45 mm.*

3.2 Buffer research

Microbial processes in anaerobic environments commonly result in the formation of gas and sulphide. Gas formation may give rise to disrupting mechanical effects on the buffer, and sulphide can corrode the canister. As discussed above, sulphide production must occur close to the canister and must be vigorous for damaging corrosion to be possible. This is mainly due to the fact that sulphide has a low solubility in groundwater and, therefore, that its diffusive transport capacity is very low. Many bacteria consume oxygen during their degradation of organic carbon. Such a process would be beneficial anywhere in the buffer and particularly in backfill where the oxygen content in pores is expected to be significant.

Research has been ongoing regarding the effects of microbial processes in buffer and backfill. Some processes have been studied for a long time; others are now becoming available for study, with the new research facilities at the Äspö HRL. Obtained results and interpretations from buffer and backfill research, as well as ongoing and planned experiments, are presented below.

3.2.1 The Buffer Mass Container experiment

A full-scale experiment with buffer material consisting of 50/50% bentonite/sand was performed at Atomic Energy of Canada Limited´s (AECL) underground laboratory in Canada. The results showed that microbes, with a few exceptions, could only be cultured from buffer samples with a water content of 15% or more, which is approximately equivalent to a 100% bentonite density of 2000 kg m^{-3} (Stroes-Gascoyne et al 1996, 1997). Elevated temperatures had no effect on the microbes. These results were interpreted as an effect of limited availability of water. The cell wall of most microbes (except for some fungi) is freely permeable to water. Microbes cannot keep more free water inside than outside the cell. They have therefore learned to compensate for a low water content in the environment by intake of salt ions to adjust the inner osmotic pressure to the outside level. Some microbes can, alternatively, produce polyalcohols or other osmotically active organic molecules. This production requires energy and organic carbon, which in buffer and backfill is available at limited concentrations. The result of the Buffer Mass Container (BMC) experiment invoked questions about the survival of microbes, and especially SRB, in buffer materials in 100% bentonite and led to detailed laboratory experiments, as described in the next Section.

3.2.2 Survival under laboratory conditions

Two species of SRB were mixed with MX-80 bentonite at varying densities, from 1500 kg m^{-3} to 2000 kg m^{-3} (Motamedi et al 1996; Pedersen et al 1995). The species were Desulfovibrio aespoeensis and Desulfomicrobium baculatum, both isolated from deep groundwater at the Äspö HRL. None of the species survived 60 days at densities above 1800 kg m^{-3}. Desulfomicrobium baculatum survived the better of the two, remaining culturable for 60 days at 1500 kg m^{-3}. It can be argued that the laboratory conditions during this experiment may have added some extra constraints to the ones found in the field situation. The laboratory experiment represents a closed situation, while field conditions would be of the open system type. The differences between these two situations are discussed above, in Sections 2.2 and 2.3. A long-term field experiment was therefore initiated and the results have recently become available. The following Section discusses this experiment.

3.2.3 Survival under field conditions

The long-term test (LOT) of buffer performance aims to study models and hypotheses of the physical properties of a bentonite buffer (SKB AB 1999b). Processes related to microbiology, radionuclide transport, copper corrosion and gas transport under conditions similar to those found in a KBS-3 repository are also investigated. The project is ongoing and some of the experiments have been analysed. The long-term test offers the possibility to expose strains of bacteria to conditions realistic of those found in a repository, with the exception of a high radiation.

For the LOT, several species of bacteria with different relevant characteristics were chosen (Motamedi 1999; Motamedi et al 2000). The SRB included were Desulfovibrio aespoeensis isolated from deep Äspö groundwater, the moderately halophilic bacterium Desulfovibrio salexigens, and the thermophilic, spore-forming Desulfotomaculum nigrificans. Aerobic bacteria included Deinococcus radiophilus, which can tolerate high doses of radiation, the chemoheterotrophic bacterium Pseudomonas aeruginosa that frequently occurs in soil, the chemoorganotrophic and chemolithotrophic (hydrogen-utilising) organism Alcaligenes eutrophus, the chemoheterotrophic, spore-forming bacterium Bacillus subtilis, and the thermophilic, spore-forming bacterium Bacillus stearothermophilus.

Suspensions of the SRB (anaerobic) and aerobic bacteria were mixed with bentonite clay to approximately 100 million bacteria per gram of dry weight clay. The clay with bacteria was subsequently formed into cylindrical plugs with a 20 mm length and diameter, and installed in bentonite blocks (Fig 3-2) exposed to low (20–30°C) and high (50–70°C) temperatures. The blocks were installed in the LOT boreholes immediately after the bacteria plugs were introduced (Motamedi 1999; Motamedi et al 2000). The experiment was terminated after 15 months. The major outcome was elimination below the detection limits of all bacteria except the spore-forming ones (Fig 3-3).

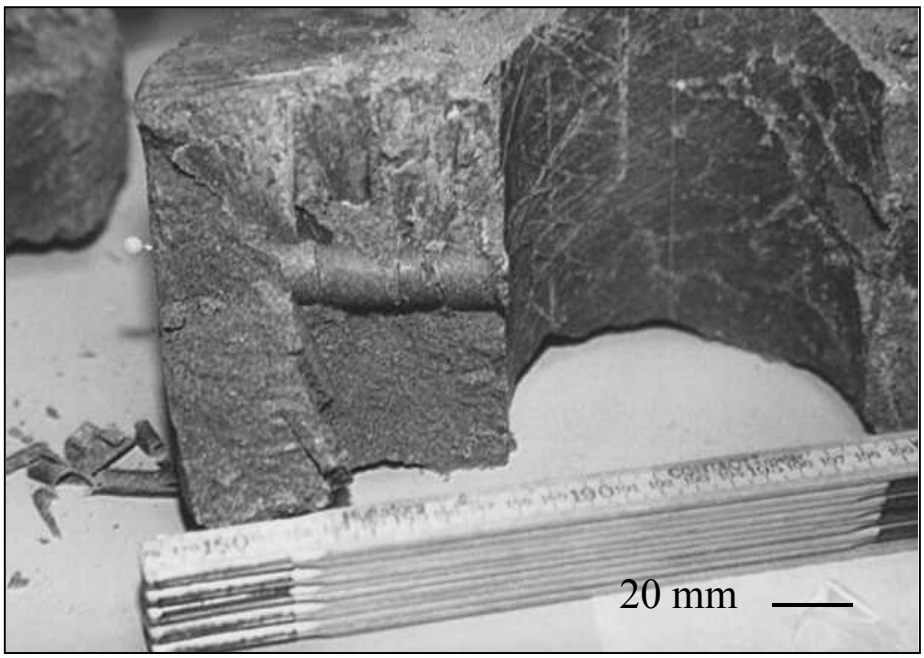
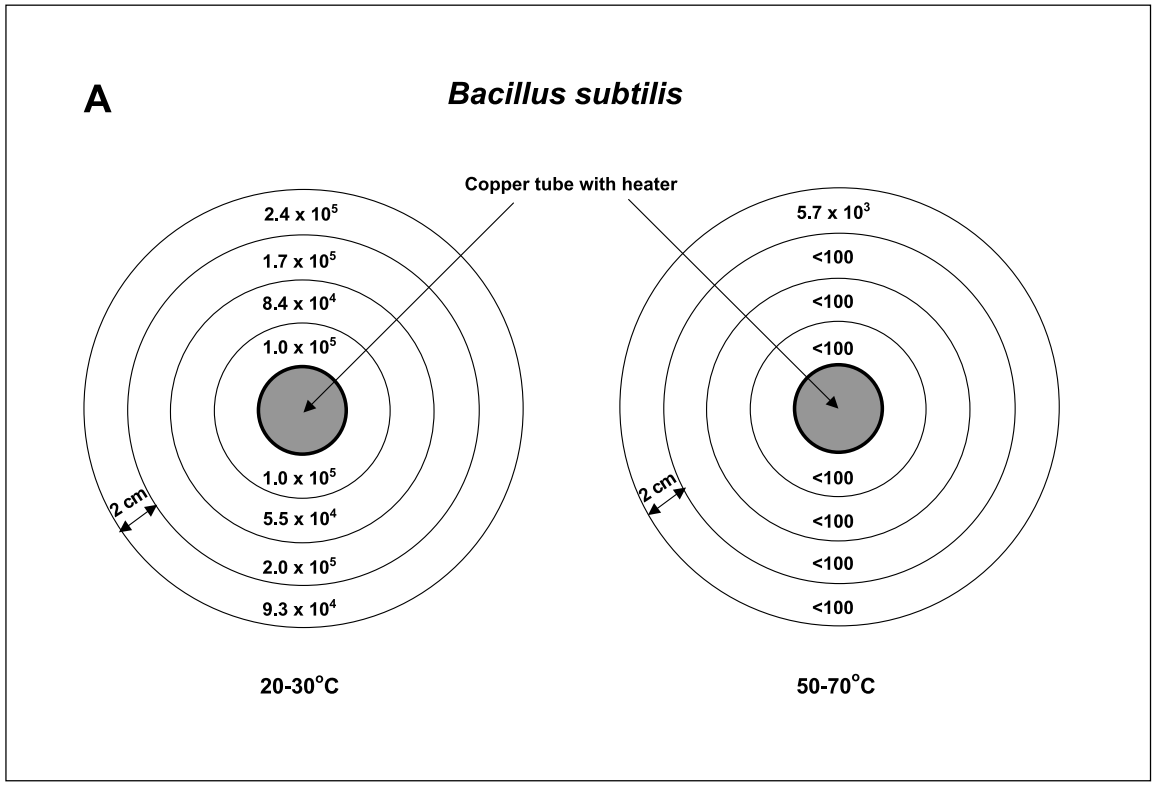
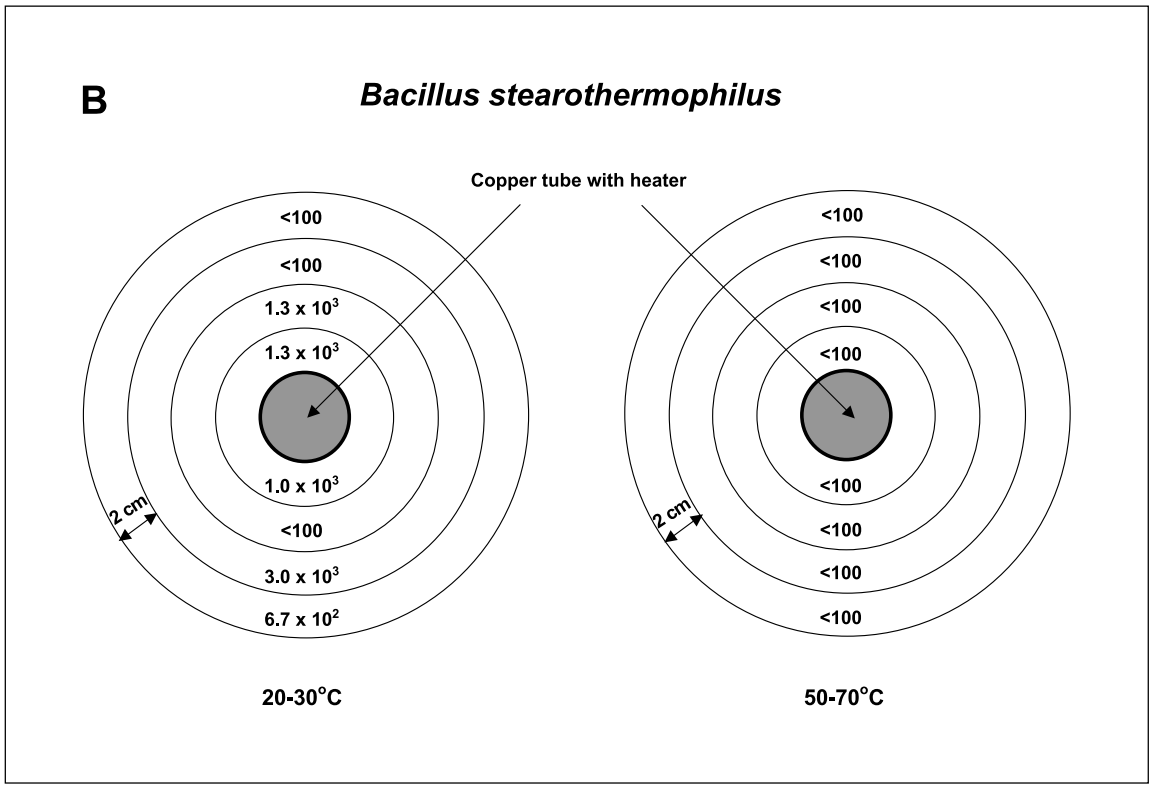


Figure 3-2. *Block No. 29 from one of the LOT experiments was ruptured and a set of plugs that were inoculated with bacteria became visible. The plugs were observed and sampled after 15 months' exposure. (Photograph: M Motamedi.)*


A
Bacillus subtilis
Copper tube with heater
2.4 x 10^5
1.7 x 10^5
8.4 x 10^4
1.0 x 10^5
1.0 x 10^5
5.5 x 10^4
2.0 x 10^5
9.3 x 10^4
2 cm
20-30°C
5.7 x 10^3
<100
<100
<100
<100
<100
<100
<100
<100
2 cm
50-70°C


B
Bacillus stearothermophilus
Copper tube with heater
<100
<100
1.3 x 10^3
1.3 x 10^3
1.0 x 10^3
<100
3.0 x 10^3
6.7 x 10^2
2 cm
20-30°C
<100
<100
<100
<100
<100
<100
<100
<100
2 cm
50-70°C

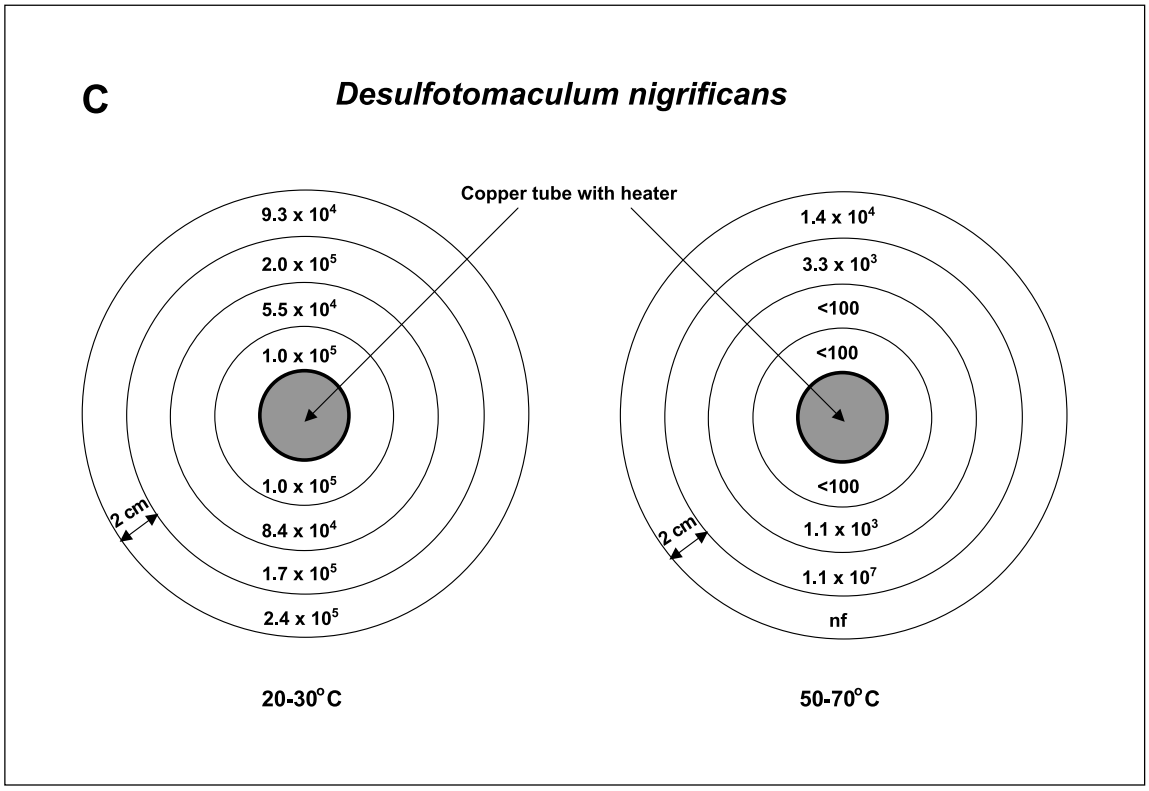


***Figure 3-3 A; B; C**. Three different spore-forming bacteria survived after 15 months' exposure to different temperatures in the LOT experiment. They were exposed to gradients of the temperatures indicated, with the highest temperature being closest to the heater. The numbers given are per gram dry weight bentonite clay. (nf = plug not found.)*

All of the three spore formers survived at the low temperature. The numbers remaining were, however, much lower than those initially introduced. The approximately 100 million spore-forming bacteria per gram of dry weight clay were reduced 100- to 10 000-fold. This can be interpreted as cell death rate being higher than the growth rate, which may have been zero, or close to zero. The spore-forming SRB, D nigrificans, was the only of the three, which survived at high temperature. This species obviously was best suited, possibly genetically, to the conditions in the clay. It can be concluded that the spore formers most probably survived as inactive spores, and that spores do not produce sulphide. Survival is, as pointed out previously (See 2.4, "The Microbes Enigma – Death or Survival"), not equivalent to activity. Since the methods used in this experiment did not reveal activity, new experiments were set up to include measurement of sulphide production at relevant repository conditions (see below).

3.2.4 Microbial mixing and survival during the buffer swelling process

Bentonite blocks have a low water content (approximately 10%) at the time of deposition of the canisters. There is a thin gap between the bentonite and the canister, and between the bentonite and the rock, to enable smooth lowering of the canister and the blocks into the deposition hole. These gaps are filled with groundwater from the rock, or alternatively, with water added at the time of deposition. The bentonite begins to swell and eventually, it will reach the planned full compaction density (2000 kg m^{-3})

and water content (approximately 30%). It was expected that microbes could be mixed with part of the clay during the swelling process. It was also of interest to examine whether microbes could migrate into the bentonite from the groundwater. An experiment series was therefore set up to investigate the course of these events.

Swelling pressure odometers were installed with compacted bentonite with 10% water content, and a gap was left between the bentonite and the filter lid of the odometer, to mimic the gap in a deposition hole. Mixtures of bacteria were added to the gap and the odometers were left for sampling at different times between 8 hours and 28 weeks. The following anaerobic bacteria were used: Desulfomicrobium baculatum, which has been isolated from the deep groundwater of the Äspö HRL, the moderately halophilic bacterium Desulfovibrio salexigens, the thermophilic, spore-forming bacterium Desulfotomaculum nigrificans and the thermophilic bacterium Thermodesulfobacterium commune which has an optimal temperature for growth of 70°C. Aerobic bacteria included Deinococcus radiophilus, which bacterium can tolerate high doses of radiation and desiccation, Pseudomonas aeruginosa, a chemoheterotrophic bacterium that frequently occurs in soil, the chemoorganotrophic, chemolithotrophic bacterium Alcaligenes eutrophus, the chemoheterotrophic, spore-forming bacterium Bacillus subtilis and the thermophilic spore-forming bacterium Bacillus stearothermophilus. The incubation temperatures varied from 30°C to 80°C, depending on the respective optimum temperature for the bacteria used.

The part of the bentonite that came in contact with the microbes was sliced in layers perpendicular to the gap and the different microbes were analysed. The survival varied significantly from species to species and between different depths (Table 3-1). The radiation and desiccation-resistant bacterium Deinococcus radiophilus and the spore-forming bacterium Bacillus subtilis showed the best survival rates. They also could be found in the deepest layer analysed, 3–6 mm, meaning that they mixed with the clay to a depth of at least 3 mm. The other bacteria tested also survived, but for shorter times and they did not survive at depth in the clay.

3.2.5 Microbes occurring naturally in MX-80 bentonite

The bentonite is not sterile at the outset. Inoculation of dry bentonite samples (with a water content of 10%) in our different culture media revealed many different species to be present. Typically, we found spore-forming genera and species such as Bacillus subtilis, Bacillus cereus and Brevibacillus brevis, and desiccation-resistant species such as Pseudomonas stutzeri and the actinomycete Thermoactinomyces. The culturing experiments also had a limited range. Many more species would most probably have been discovered had the experiments been more extensive. It has, however, become apparent that there will be two sources of microbes in the buffer, viz. (1) those naturally present in the commercially available MX-80 bentonite, and (2) those introduced via the groundwater.

3.2.6 The current model of microbial survival in compacted bentonite

The results obtained on the survival and activity of microbes in compacted bentonite can be summarised in a conceptual model, as depicted in Figure 3-4. At the time of deposition, there will be a canister, bentonite blocks and a hole in the rock. The next step will be to allow water to fill up all void volume. This water can be groundwater from the rock or alternatively, groundwater or technical water added from above at deposition. Irrespective of the source, microbes will be present in the water and these microbes will mix with the buffer, as described above.

Table 3-1. The number of viable cells of bacteria in swelling, compacted bentonite, analysed at different times of exposure.

Species introduced	Viable cells ml^{-1} suspension at the start of the experiment	Viable cells per gram dry weight bentonite			
		8 hours	2 weeks	12 weeks	28 weeks
Deinococcus radiophilus	5.7×10^8				
A[a] (0–1 mm)		2.5×10^7	2.7×10^5	2.0×10^4	1.4×10^4
B (1–3 mm)		1.6×10^4	1.0×10^5	1.6×10^4	2.5×10^4
C (3–6 mm)		1.4×10^4	1.5×10^5	8.3×10^3	2.4×10^4
Pseudomonas aeruginosa	5.0×10^9				
A		2.8×10^8	1.7×10^5	9.0×10^3	1.4×10^4
B		7.7×10^4	4.0×10^4	–[b]	–
C		–	–	–	–
Alcaligenes eutrophus	2.4×10^9				
A		3.1×10^6	1.0×10^4	–	–
B		N.d.	3.0×10^3	–	–
C		1.3×10^4	6.3×10^3	–	–
Bacillus subtilis	N.d.[c]				
A		4.3×10^5	1.0×10^8	4.9×10^4	5.9×10^4
B		N.d.	1.9×10^6	5.5×10^4	1.7×10^3
C		1.3×10^4	1.6×10^5	1.6×10^4	1.9×10^4
Bacillus stearothermophilus	N.d.				
A		1.2×10^5	2.3×10^3	–	–
B		–	–	–	–
C		–	–	–	–
Desulfovibrio salexigens	1.7×10^8				
A		1.7×10^5	7.3×10^3	–	–
B		2.4×10^3	4.9×10^2	–	–
C		–	–	–	–
Desulfovibrio baculatum	1.3×10^8				
A		2.8×10^7	1.3×10^5	2.4×10^2	–
B		3.3×10^4	3.3×10^2	–	–
C		–	–	–	–
Desulfotomaculum nigrificans	1.7×10^7				
A		9.4×10^5	3.5×10^5	7.9×10^4	1.4×10^2
B		2.4×10^4	2.3×10^2	–	–
C		–	–	–	–
Thermodesulfobacterium commune	7.9×10^7				
A		3.3×10^2	–	–	–
B		–	–	–	–
C		–	–	–	–

a = A, B and C gives the positions of the samples, measured from the surface
b = <100
c = Not determined

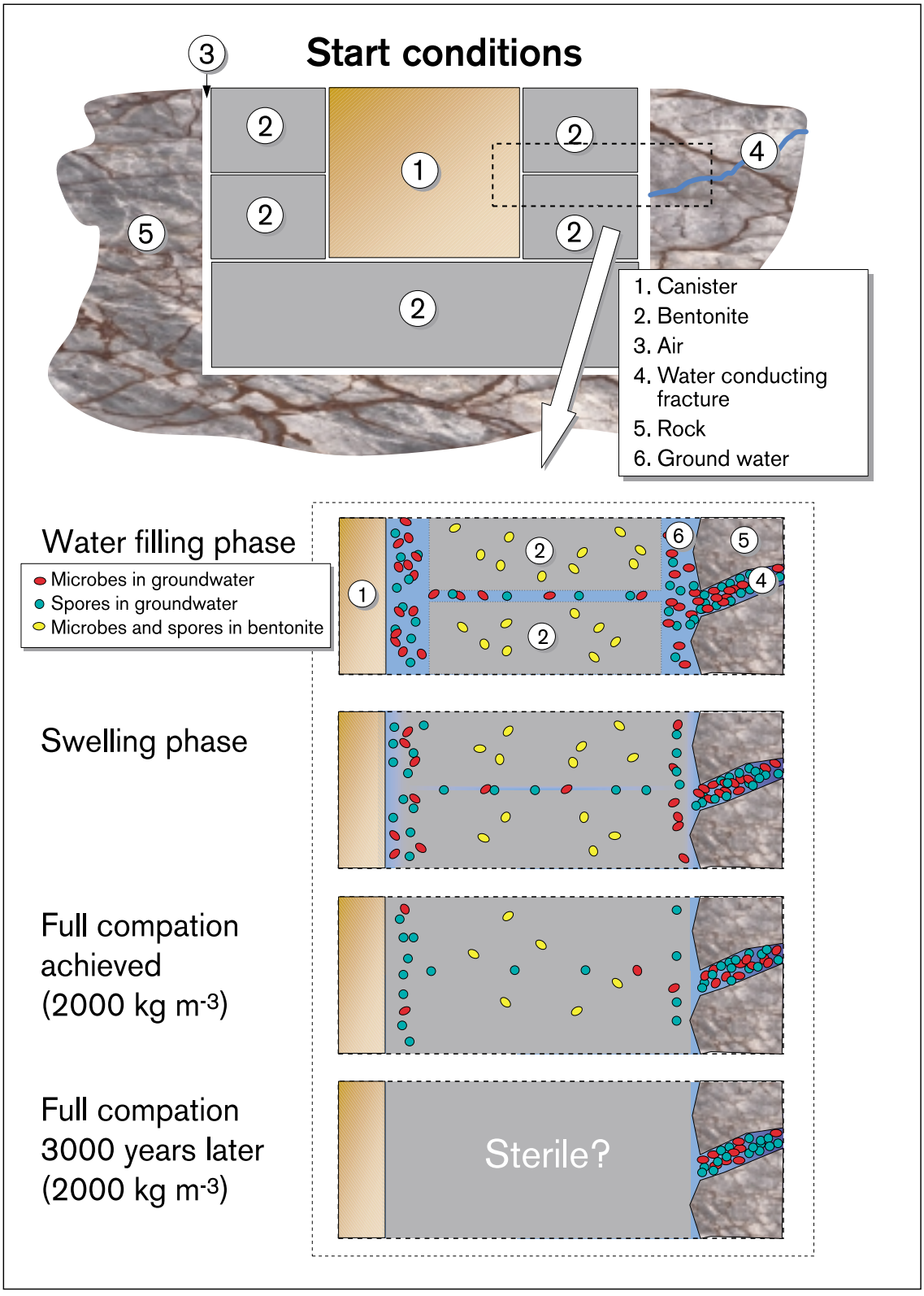


Figure 3-4. *A schematic model of how microbial populations will alter their presence in the buffer. See Section 3.2.6 for an explanation.*

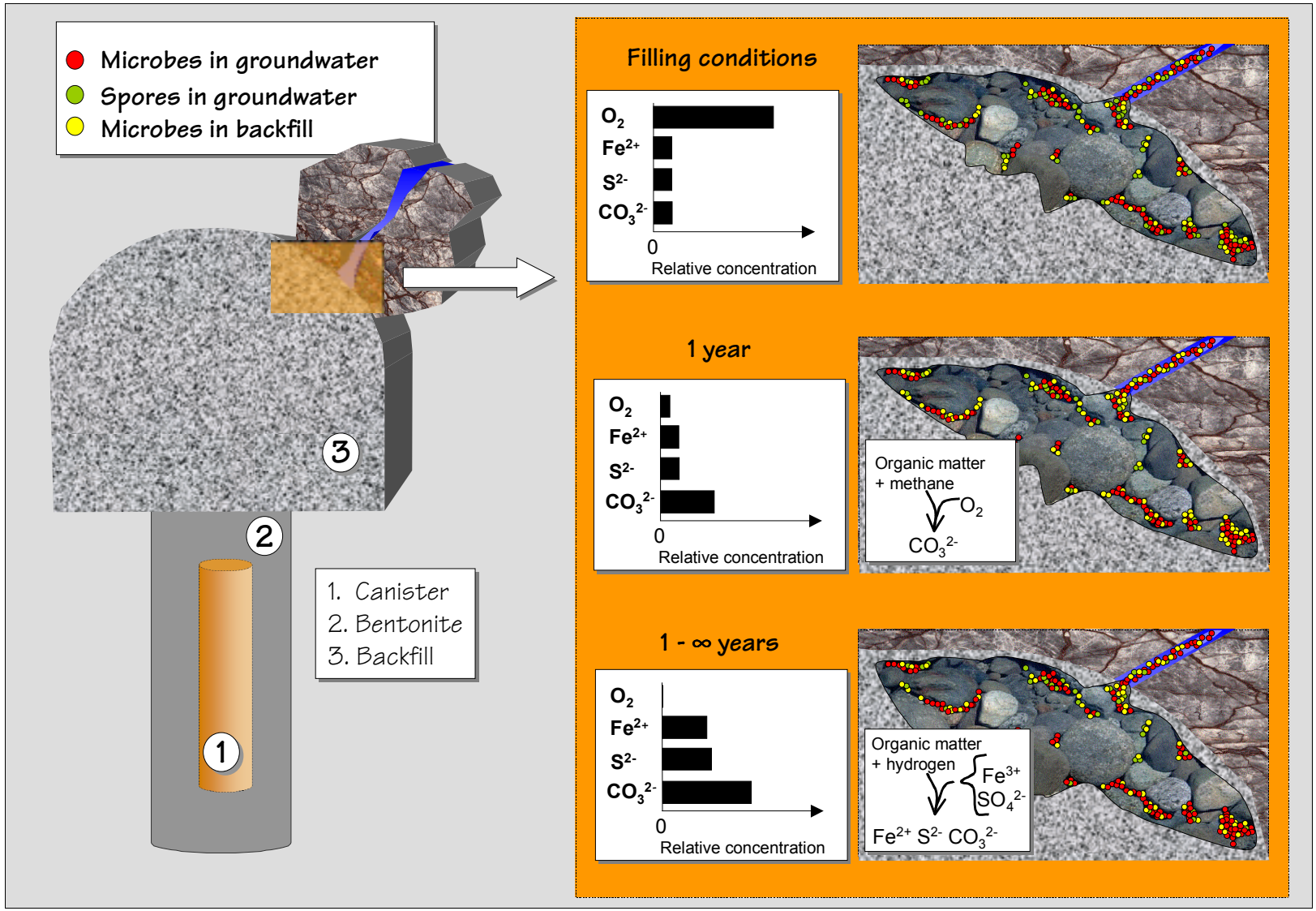


Figure 3-5.
A schematic model of how microbial populations will inhabit and change the geochemistry of the backfill. See Section 3.3.1 for an explanation.

The swelling of the clay will seed groundwater microbes to depths possibly reaching a couple of centimetres from the canister and rock surfaces. The microbes indigenous to the bentonite will be present inside the bentonite and also in the mixing zone. The abovementioned findings on the survival and activity of microbes in bentonite suggest that the number of viable microbes will decrease rapidly during swelling and that very few viable cells will be present at full compaction. The sulphate-reducing activity will also approach zero when full compaction is achieved. The only survivors will be microbes that have formed spores. Our results indicate that viable cell activity will be impossible at full compaction, as spores are inactive. Although spores generally are very resistant to difficult environmental conditions, they do still die off. All our experiments so far indicate a decrease in the number of viable spores at full compaction. A slow but significant death rate of spores would eventually lead to the complete eradication of life in the buffer. It has not yet been clarified whether this will occur in the lifetime of a radioactive repository. Once the bentonite becomes sterile, it will probably not be reinfected. The pore size of the clay is 100–1000 times smaller than the average-sized microbe, meaning that no new microbes can enter into the buffer.

The model presented is based on current data, obtained with laboratory cultures. It could be argued that naturally occurring microbes are more tolerant, although the working hypothesis remains to be a total eradication of all life in the buffer. Ongoing and planned experiments will continue to test this hypothesis under increasingly relevant field conditions.

3.3 Backfill research

Thus far, there have been few results from backfill research. A full-scale backfill and plug test has been started at the Äspö HRL (SKB AB 1999b). Laboratory cultures of various bacteria were introduced at specific positions in the middle of the backfill and the microbes in the backfill were analysed. The results showed a significant diversity of culturable bacteria in the backfill material at the outset. We found SRB that were culturable at 30°C and 60°C. Pseudomonas stuzeri and Stenotrophomonas maltophilia appeared in the rock/bentonite (70/30%) backfill. Aeromonas encheleia appeared in 100% crushed rock. Bacillus thermophilica (cultured at 60°C) was found in both backfill mixtures. Other species also appeared, but were not identified. Obviously, many different microbes are present in the backfill, and more will travel via the groundwater into the backfill pores. There will be a relatively dense population of microbes in the backfill, which may be beneficial for the repository, as outlined below.

3.3.1 The current model of microbial activity in the backfill environment

As previously mentioned, backfill has a large diversity of microbes in relatively abundant numbers. A main concern about backfill is its oxygen content at the outset (see, e.g., Wersin et al 1994). This oxygen has a corrosive effect on the copper canisters. The REDOX, Microbe REX, and REX experiments discussed below all indicated that microbes would be very efficient in removing oxygen from groundwater, if introduced. It can be hypothesised that this also will occur in the backfill. Also, active SRB and IRB would produce sulphide and ferrous iron, both of which reduce oxygen and lower the redox potential of the groundwater in the backfill. A low redox potential is important for achieving low radionuclide mobility in the case of an accidental release of radionuclides. In such a scenario, the microbes would protect the environment from the products from radiolysis of water by efficiently recombining the oxygen and hydrogen produced to water, and thus, buffering the redox downwards.

3.4 Geosphere research

The geosphere research has comprised, since 1987, activities at eleven subterranean sites in Sweden and Finland (Fig 3-6), exploring depths down to 1700 m (Table 3-2). We have also investigated, and are still investigating, a number of other subterranean sites. They are: (1) the natural nuclear reactors of Oklo, in Gabon (Crozier et al 1999; Pedersen et al 1996a,c), (2) the natural hyperalkaline springs of Jordan (Pedersen et al 1997a), (3) the underground research laboratory at Pinawa, in Canada (Stroes-Gascoyne et al 1997), (4) the ultra-deep gold mines of Witwatersrand, in South Africa (Barnicoat et al 1997), and (5) sub-sea floor basement rock environments of the Pacific Ocean The Ocean Drilling Programme, http:www.oceandrilling.org/. This Section will, however, mainly deal with findings obtained during 12 years of investigation of microorganisms in Finnish and Swedish igneous rock aquifers and the implications of the results on HLW disposal. A total of 75 specific borehole positions in 55 different boreholes (Table 3-2) have been investigated for geology, chemistry, numbers of microorganisms, and microbial diversity and activity.

Early geosphere investigations aimed to understand the potential risk of radionuclide migration by microorganisms that were thought able to possibly survive in deep groundwater (Birch and Bachofen 1990; Pedersen and Albinsson 1991, 1992). It soon became apparent that microbial populations can be obtained from any deep aquifer studied (Pedersen and Ekendahl 1990), and more attention was paid to assaying the activity of these microorganisms with radiotracer methods (Ekendahl and Pedersen 1994; Kotelnikova and Pedersen 1998; Pedersen and Ekendahl 1992a, 1992b). The activity results indicated a notable metabolic and species diversity and motivated the introduction of 16/18S rDNA sequencing for assessment of subterranean microbial diversity (Ekendahl et al 1994; Pedersen et al 1996b, 1997c). Nucleic acid probes for 16/18S rRNA are at present being applied for in situ identification (Pedersen 1997a). Work has also been performed to describe new species adapted to life in igneous rock aquifers (Kalyuzhnaya et al 1999; Kotelnikova et al 1998; Motamedi and Pedersen 1998). The finding of many hydrogen-utilising chemolithotrophs invoked the model of a subterranean, hydrogen-driven biosphere in igneous rock aquifers (Pedersen 1993b, 1997a; Pedersen and Albinsson 1992). All vital necessary components have now been proved to exist at depth, but quantitative data remain to be obtained.

3.4.1 Drilling in the exploration of microorganisms in deep igneous rock aquifers

3.4.1.1 Drilling and sampling of aquifer rock surfaces

All sampling of igneous hard rock aquifer material and groundwater requires penetration of the rock to reach the aquifers in target. There is only one principal way of achieving this goal and that is via drilling of holes (Fig 3-7). The detailed procedure can be modified in many ways, but there are two main drilling procedures, namely from the ground surface or from an underground tunnel. Drilling in hard rock can be done with either percussion or core drilling. The percussion drill does not recover any rock material and it may introduce air into intersected aquifers. The debris created during drilling, and groundwater, once an open fracture has been intersected, are forced to the surface by the air. Drilling is done using compressed air with a pressure higher than the groundwater pressure. This method has a limit at about 100–150 m, after which the pressure needed will be too high for normal percussion drilling machines. Deeper boreholes must be core drilled, a procedure which produces a core that can be used for mapping of the geological strata penetrated. The retrieval of rock aquifer material

during drilling in igneous hard rock always requires core drilling. Triple-tube drilling (Fig 3-8) is the best available method for obtaining cores with the smallest possible disturbance. This type of drilling has been used with good results in the Äspö HRL tunnel

3.4.1.2 Evaluation of the contamination risk during drilling and excavation

The risk of contamination of intersected aquifers during drilling is obvious. Drill water may penetrate aquifers. Control of drill water contamination is done by addition of one or more tracers to the drill water. Fluorescent stains have been employed on a routine basis for the drilling of the boreholes listed in Table 3-2. The stains used were fluorescein and uranin (Aldrich, Germany). Drill water contamination of aquifers penetrated by surface-drilled boreholes decreased from some 2.6–13% in KAV01 to 0.06–0.8% in KAS02, KAS04 and KLX01 (Pedersen and Ekendahl 1990). This decrease was due to a new drilling technique used on KAS02 and KAS04 and since then, but not for KAV01, so-called "telescope-type drilling" (Almén and Zellman 1991; Pedersen 1993a). Drilling in tunnels reduces the risk of drill water contamination of aquifers when the drilling is done at tunnel depths deeper than about 60–70 m. The risk decreases with depth because of an increase in aquifer pressure which will be higher than that used for the drill water, at least in water-conducting fractures. Pumping or flushing of boreholes to measure the maximum hydraulic water capacity will concurrently clean aquifers and the borehole from drill water, mud and cuttings, provided there is enough water inflow for intense pumping.

The potential risk of microbial contamination of hard rock aquifers was examined, using 16/18S rDNA sequencing and culturing methods, during the drilling of the Äspö HRL SELECT boreholes (Pedersen et al 1997c). It was found that the tubing used for the drill water supply constituted a source of bacterial contamination to the aquifers via the drilling equipment. The sequencing results showed that although large numbers of contaminating bacteria were introduced to the boreholes during drilling, they did not establish at detectable levels in the aquifers. The number of microorganisms varied from 10^5 to 10^6 cells per ml^{-1} in the drill water introduced into the boreholes. The drill water contamination of the studied boreholes was below one part per 1000; in other words, fewer than 10^3 cells ml^{-1} in the aquifers of the drilled boreholes could be expected to originate from contamination during the drilling operation. This number was more than one order of magnitude smaller than the number found directly after drilling, and could therefore not explain the origin of the observed total numbers in the new boreholes. Microorganisms must therefore have been present in the aquifers before the drilling operation was undertaken.

Pumping from the surface and all withdrawal of groundwater via tubes may likewise introduce unwanted effects, including degassing due to a pressure decrease, and the possibility of microbial biofilm formation on tube walls during prolonged pumping through more than 1000 m long tubing in very deep boreholes. Most such effects can be avoided by the application of down-hole samplers. We have tested the suitability of two types of down-hole samplers in addition to sampling via pumping. The samplers were the BAT (Torstensson 1984) and PAVE (Haveman et al 1999) samplers (Fig 3-7, B and C). The BAT sampler was constructed with gas sampling as a major aim and consisted of two sterile cylindrical tubes (or one larger) made of stainless steel. The tubes were supplied with nitrile rubber stoppers and evacuated. They were opened and closed at sampling depths by penetration of the stoppers with hypodermic needles with a

mechanical device controlled from the ground level. For several reasons, however, this sampler was not a reliable microbiology sampling tool. The piston pump, which was very difficult to clean, was placed before the sample containers and the sudden decrease in pressure when the sample containers opened seemed to rip off biofilms from the pump. Additionally, the pressure difference between the evacuated sample cylinders and the groundwater at depth (up to 100 atmospheres) may have caused a "French press" effect on cells in the groundwater, disrupting some by a sudden drop in pressure when they passed out of the narrow hypodermic needle orifice penetrating the sampling containers. The BAT sampler (Fig 3-7 B) never came into routine use for microbiology sampling. The boreholes at ground surface at Hålö, Laxemar, Palmottu, Ävrö and Äspö (Table 3-2) were sampled with the borehole pump technique described under "A" in Figure 3-7. A mobile chemistry laboratory was used on the ground for sample retrieval and preparations (Grenthe et al 1992).

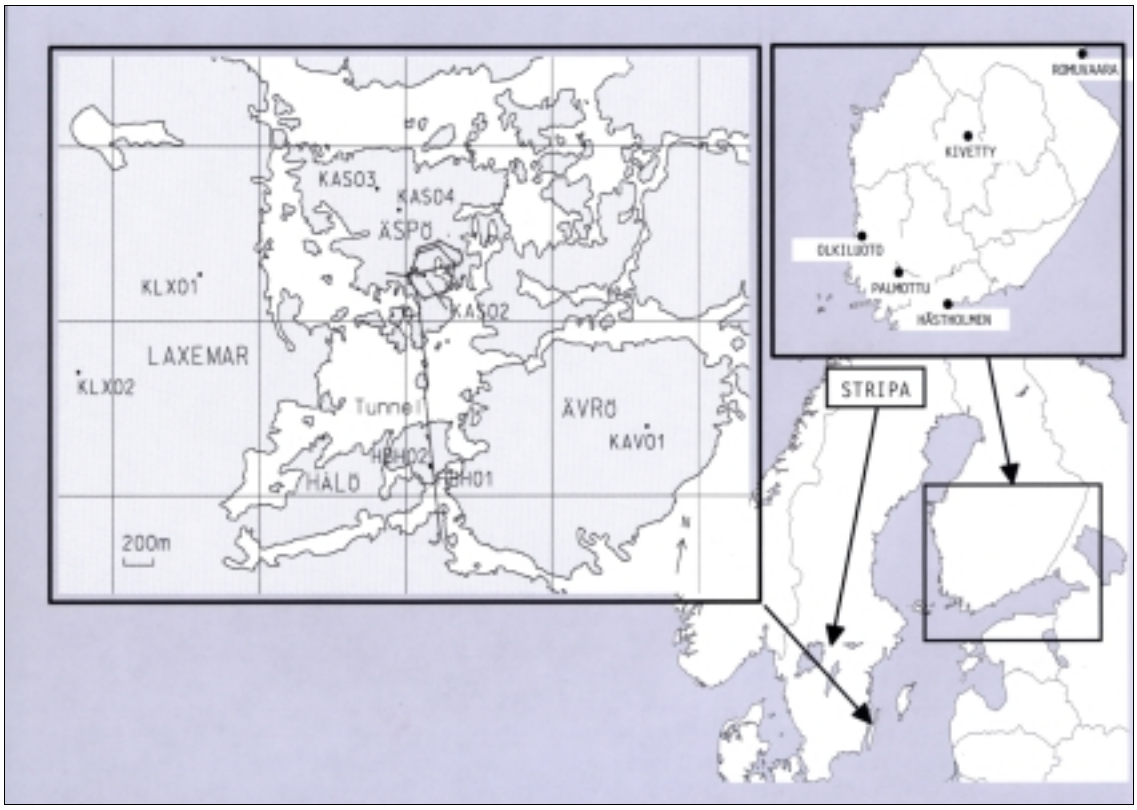


Figure 3-6. *Locations of subterranean igneous rock sites investigated by the Deep Biosphere Laboratory at Göteborg University, Göteborg, Sweden. Detailed information about the sites is given in Table 3-2.*

The PAVE system was constructed with both gas and microbiological sampling as major objectives. The system consists of a rubber membrane pump placed above a sample container with two sterile, evacuated and closed pressure vessels filled with argon gas so that the movable piston can move to the top of the pressure vessel (Fig 3-7 C). The argon pressure is set just below the hydrostatic pressure at the sample depth, which makes the drop in pressure during sampling negligible. Prior to sampling, the complete PAVE system is disinfected by rinsing for 30 minutes with a 10 mg l^{-1} chlorine dioxide water solution (Freebact, Wecantech AB, Märsta, Sweden), then flushed with sterile water for 10 minutes. To ensure the efficiency of the sterilisation, control samples were analysed for growth (Haveman et al 1999). The section of the

borehole to be sampled with PAVE is packed off with inflatable rubber packers, as was done with the BAT system (Fig 3-7 B). Groundwater is pumped from the packed-off zone past the closed pressure vessels and out of the borehole. Groundwater parameters (i.e. pH, E_h, conductivity, and temperature) are monitored in a N_2-shielded flow through cells in the field laboratory at the surface until they have stabilised. At this point, samples for field and laboratory analysis for hydrogeochemical characterisation are collected. After this phase, the pressure valve of the PAVE is opened. Groundwater pressure pushes down the piston in the sampler to fill the sampler with groundwater. The valve is left open for several hours to allow water to pump through the sampler, and then the PAVE sampler is closed again and raised out of the borehole. This system was employed for sampling of the Hästholmen, Kivetty, Olkiluoto and Romuvaara sites listed in Table 3-2.

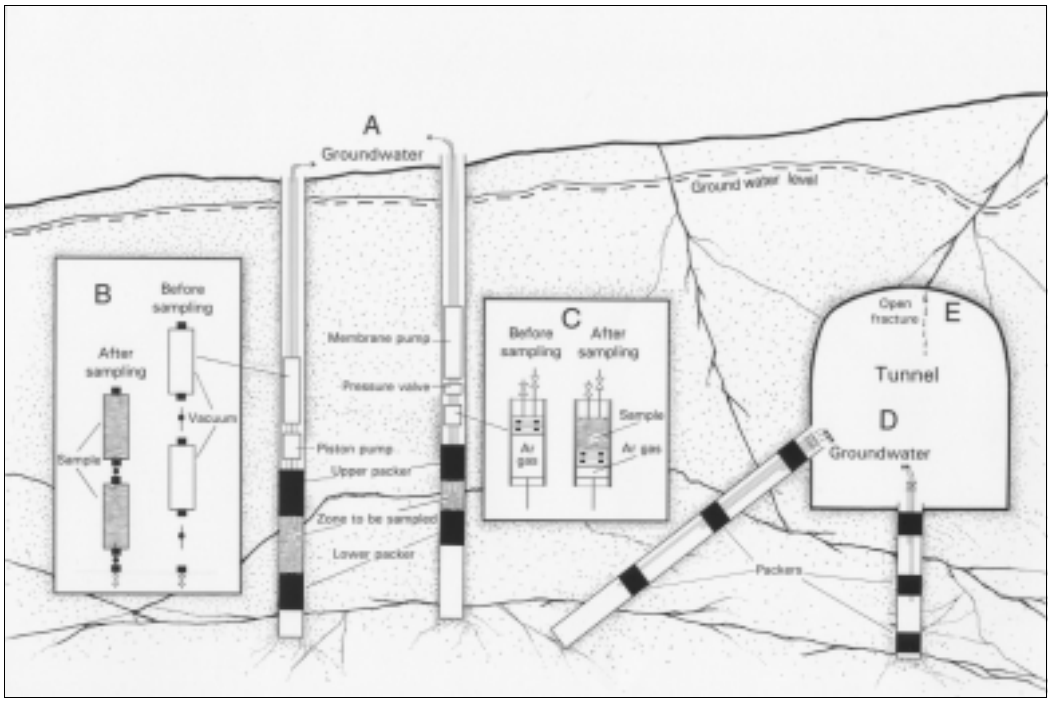


Figure 3-7. *Access to aquifer material and groundwater occurs via drilling of boreholes from ground surface or tunnels. After retrieval of drill core material, the boreholes are packed off in one or several sections which each isolate one or more specific aquifers.* ***A****. Down hole pumps of various types force groundwater from the aquifer to the ground surface for subsampling.* ***B.*** *Borehole BAT sampler that can be opened and closed from the surface and is designed for gas sampling (Torstensson 1984).* ***C.*** *The PAVE borehole sampler which can be opened and closed from the surface and is designed for gas and microbiological sampling (Haveman et al 1999). One or more sample vessels can be used simultaneously.* ***D.*** *Tunnel boreholes do not require pumps when the tunnel is under the groundwater table. Aquifers can be packed off and connected to sampling devices in the tunnel with pressure-resistant tubes. It is important, however, to understand the potential danger and technical problems connected with the high hydrostatic pressure occurring at depth. For example, there is a pressure of 30–40 atmospheres in the boreholes drilled at the bottom of the Äspö HRL.* ***E.*** *Open fractures in tunnels can be sampled direct and represent groundwater with minimal disturbance, except for the pressure decrease due to entering the tunnel.*

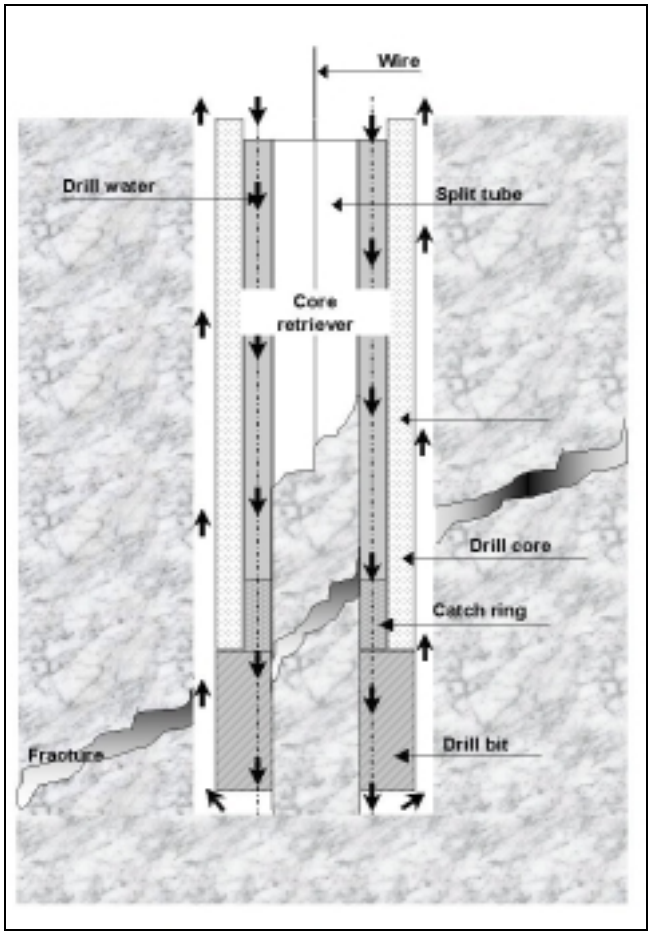


Figure 3-8. *The triple-tube drilling principle. The use of a core retriever minimises the exposure of the core to drill water and delivers the core intact to the surface, also when multi-fractured rock is penetrated. The drill tube protects the drill core from contact with aquifer systems intersected during drilling. This is of great importance when layers with contaminants, such as hydrocarbons, are penetrated. The split tube keeps intersected fractures intact, with small pieces of rock in their original place.*

Sampling from tunnels under the groundwater table (Fig 3-7 D) significantly reduces sampling difficulties. Boreholes become artesian, and packers and pressure-resistant tubes with valves are all that is needed for successful sampling. The high pressure encountered as the tunnel has gone deep requires very robust anchoring and packer systems but the establishment of such equipment is routine. This sampling method was used for all the Äspö HRL tunnel boreholes described in Table 3-2.

A final possibility is to sample open fractures that enter tunnels underground (Fig 3-7 E). These are free of drilling effects, but may have encountered some disturbance from the blasting operations used for tunnel construction. Parts of the Äspö tunnel were drilled with a tunnel-boring machine (diameter of the drill = 5.5 m) and the disturbance on the surrounding rock mass is minimal in those (lower) parts of the tunnel.

Table 3-2. Site data comprising all boreholes in igneous rock investigated for microbiology by the Deep Biosphere Laboratory at Göteborg University, Göteborg, Sweden, during 1987–1999.

Site[a]	Year	Borehole	Depth (m)	Site characteristics	Original scientific publications
Hålö (S[b])	1992–1996	HBH01	45	Hålö is an island above the Äspö HRL tunnel (Fig 3-6). The rock is mainly a red to grey porphyritic monzogranite belonging to the vast Transscandinavian granite-porphyry belt (Gàal and Gorbatschev 1987) with intrusion ages (Uranium-Lead) of 1760–1840 million years. Major fractures and fracture zones control recharge, discharge and groundwater flow through the island (Banwart et al 1996). The groundwater is dilute shallow groundwater, brackish Baltic Sea water and saline native groundwater (11–4890 mg Cl L^{-1}). (See Äspö below for details.)	(Banwart et al 1996) (Pedersen et al 1996a) (Kotelnikova and Pedersen 1998)
	1992–1996	HBH02	10		
Hästholmen (F[c])	1997	HH-KR1	938–948	The rock consists of rapakivi-type granite. The main fracture minerals include calcite, dolomite, iron hydroxides and clay minerals. Iron sulphides occur only sporadically. Iron and iron hydroxides are very common, and therefore, iron is important in controlling redox processes. The groundwater ranges from fresh to brackish to saline, and is composed of several different end members (Haveman et al 1998).	(Haveman et al 1999)
	1997	HH-KR2	907–912		
	1997	HH-KR3	211–216		
	1998	HH-KR4	683–688		
	1998	HH-KR5	382–387		
	1998	HH-KR6	62–66		
Kivetty (F)	1997	KI-KR5	717–725	The main rock types are granodiorite and granite. Major fracture minerals include calcite, iron sulphides, iron oxyhydroxides and clay minerals. Iron hydroxides (goethite and limonite, or iron oxyhydroxides and quartz are also present in some places. The iron oxyhydroxides are mostly found at shallow depths, down to about 130–170 m. Calcites and iron sulphides are found below 50–100 m. All groundwater is fresh water, with a regional maximum of total dissolved solids of <200 mg L^{-1}. Sulphur compounds are thought to control redox processes, buffering the redox to below –300 mV at depth (Haveman et al 1998).	(Haveman et al 1999)
	1998	KI-KR13	494–499		

Site[a]	Year	Borehole	Depth (m)	Site characteristics	Original scientific publications
Laxemar (S)	1988	KLX01	272–277 456–461 680–702	Laxemar is part of the Precambrian bedrock where Smål and graites predominate over the older, Sveocarelian complexes. The borehole KLX01 is situated in the centre of a major block. The groundwater is brackish, with a redox at or below –220 mV. A second borehole, KLX02, was drilled to 1700 m depth. The groundwater (approximately 10%) ranges from fresh to brackish to very saline at depth, and is composed of several different end members (Gustafsson et al 1988).	(Pedersen and Ekendahl 1990) (Pedersen and Ekendahl 1992b)
	1990	KLX01	830–841 910–921 999–1078		
	1998–1999	KLX02	0–1700		
Olkiluoto (F)	1998–1999	OL-KR3	243–253 438–443	The main rock types at this site include gneisses, schists, granodiorites and granites. The major fracture minerals include calcite ($CaCO_3$), pyrite, chlorite and other clay minerals. Sulphur species have an important role in controlling redox processes. The groundwater ranges from fresh to brackish to saline, and is composed of several different end members (Haveman et al 1998).	(Haveman et al 1999)
	1998–1999	OL-KR4			
	1998	OL-KR8	302–310 861–866		
	1998–1999	OL-KR9	563–571 324–332 470–475		
Palmottu (F)	1998	R302	80–132	The Palmottu area lies in a zone of metamorphosed supracrustal volcanic and sedimentary rocks that extends from south-western Finland into central Sweden (Blomqvist et al 1995). The site has a Uranium-Thorium mineralisation with a total length of about 400 m, which is related to the latest stages of orogenic events in Finland of about 1800–1700 million years ago. The area surrounding Palmottu is characterised by granites and highly metamorphosed migmatitic rocks. The main types of groundwater found are fresh Ca-HCO_3 water, dilute Ca-HCO_3-SO_4 water, and slightly saline water of the Na-Cl or Na-SO_4 type.	
	1998	R337	80–100		
	1998–1999	R387	32–38 119–127 304–309		
Romuvaara (F)	1998	RO-KR10	561–566	The main rock types at Romuvaara include different	(Haveman et al 1999)

Site[a]	Year	Borehole	Depth (m)	Site characteristics	Original scientific publications
	1999	RO-KR11	540–545	types of gneisses, intersected by granodiorite and metadiabase dykes. The major fracture minerals are calcite, iron sulphides, iron oxyhydroxides (goethite and limonite), and clay minerals. The iron oxyhydroxides are found at shallow depths down to 150 m. Iron sulphides, including pyrite, are found at depths of > 100 m. All groundwater is fresh, with maximum Cl^- concentrations of <300 mg L^{-1} at depth. No effects of glacial meltwaters have been detected. Sulphur compounds are thought to control redox processes, buffering redox to below –200 mV (Haveman et al 1998).	
Stripa (S)	1987–1991	V1 V2	865–866 799–807 812–820 970–1240	Between 1976 and 1991, the Stripa mine served as an underground research facility. The ore consists of a quartz-banded haematite and occurs in a lepatite formation. Adjacent to the lepatite formation is a large body of medium-grained granite through which the boreholes run from drifts in the mine at 360 m (V1) and 410 m (V2). Most fractures are partly or fully sealed with chlorite, epidote or calcite. Because of silica weathering, the pH of the groundwater has approached 10. All groundwater is fresh to slightly brackish, with maximum Cl^- concentrations of <700 mg L^{-1} at depth (Nordstrom et al 1985).	(Pedersen and Ekendahl 1992a) (Ekendahl et al 1994) (Ekendahl and Pedersen 1994)
Ävrö (S)	1987	KAV01	420–425 522–531 558–563 635–924	See Äspö for geology, hydrogeology and geochemistry.	(Pedersen and Ekendahl 1990)
Äspö (S)	1988–1989	KAS02	202–214 314–319 463–468 860–924	The geology of Äspö is characterised by a red to grey porphyritic granite-granodiorite (known regionally as the “Småland type”) dated to 1760–1850 million years ago. The rock area belongs to the vast Transscandinavian granite-porphyry belt (Gàal and Gorbatschev 1987). The	(Pedersen and Ekendahl 1990) (Pedersen et al 1997b) (Pedersen et al 1996a)

Site[a]	Year	Borehole	Depth (m)	Site characteristics	Original scientific publications
	1996	KAS02	207–208	main fracture-filling minerals at Äspö are, in decreasing order, chlorite, calcite, epidote, fluorite, quartz, haematite, Fe-oxyhydroxides, pyrite, and clay minerals (Smellie et al 1995). The hydrological situation at Äspö is characterised by a low hydraulic gradient. The recharge/discharge is mainly controlled by tectonic zones and major fractures. The Äspö groundwaters are shown to be mainly reducing (with available redox data record values of –250 to –350 mV), nearly all total iron is in the ferrous state, and sulphide is detectable in small quantities up to approximately 1 mg L^{-1}. The water is moderately alkaline, generally between 7.3 and 8.3. Approximate depth trends show a change from a Na-Ca(Mg)HCO_3-Cl type groundwater near the surface (0–150 m), through a Na-Ca(Mg)Cl-SO_4 type at depths of 300–800 m, to, finally, a Ca-Na(Mg)Cl-SO_4 type for the deepest, most saline waters occurring below approximately 800 m. There is an increase with depth of Cl, Br, Na, Ca, SO_4, Sr and Li; while HCO_3, Mn, Mg, $Fe_{(tot)}$, $Fe_{(+II)}$ and total organic carbon (TOC) decrease with depth (Smellie et al 1995).	
	1989	KAS03	129–134 860–1002		
	1992	KAS03	533–626		
	1987–1989	KAS04	195–205 290–300 380–415		
Äspö HRL tunnel (S)	1992–1996	KR0012	68	See Äspö above for geology, hydrogeology and geochemistry. The Äspö HRL has been constructed as part of the development of the Swedish concept of deep geological disposal of spent nuclear fuel and the work has been divided into three phases, the pre-investigation (1986–1990), construction (1990–1995), and operating (1995–) phases. The Äspö HRL is situated on the island of Äspö, adjacent to the Baltic coast of Sweden approximately 400 km south of Stockholm. The access tunnel to the HRL descends with a declination of 14% from the Baltic shoreline for a distance of approximately	(Pedersen et al 1996a) (Pedersen et al 1997c) (Kotelnikova et al 1998) (Kotelnikova and Pedersen 1998) (Motamedi and Pedersen 1998) (Banwart et al 1996)
	1992–1996	KR0013	68		
	1992–1996	KR0015	68		
	1992–1996	SA813B	112		
	1992–1996	SA923A	134		
	1992–1996	SA1062A	143		
	1992–1996	HA1327B	179		
	1992–1996	SA1420A	192		

Site[a]	Year	Borehole	Depth (m)	Site characteristics	Original scientific publications
	1994–1996	KA2511A	345	1700 m under the ocean floor, where it spirals down and terminates 460 m below the island of Äspö (Fig 3.6). An extensive geoscientific evaluation and a detailed site characterisation have been executed during all three work phases mentioned above and the work is published in a series of reports, summarised by Stanfors et al (1997) and Rhén et al (1997).	
	1994–1996	KA2512A	345		
	1995–1996	KA2858A	380		
	1994–1996	KA2862A	380		
	1994–1996	KA3005A	400		
	1994–1996	KA3010A	400		
	1994–1996	KA3067A	409		
	1996–1996	KA3105A	414		
	1994–1996	KA3110A	414		
	1994–1996	HD0025A	420		
	1994–1996	KA3385A	446		
	1999–*d	KA3539G	446		
	1999–*	KA3548A01	446		
	1999–*	KA3600F	446		
	1999–*	KJ0050F01	448		
	1999–*	KJ0052F02	448		
	1999–*	KJ0052F03	448		

a = See Figure 3-6 for the location of each site
b = Sweden
c = Finland
d = experiments are ongoing

3.4.2 Environmental parameters of importance for microbial life in groundwater

The Fennoscandian Shield (also called the "Baltic Shield") is the slightly vaulted Precambrian rock area comprising parts of Norway, most of Sweden, all of Finland and most of the Karelian and Kola peninsulas. The Fennoscandian Shield mostly consists of granite and gneiss rocks. Apart from these, it is covered with platforms of sedimentary rock in the north, east and south, and the shield borders to the younger Scandinavian mountains to the west (Norway). The age of the Fennoscandian Shield rock spans from 3100 million years in north to 1700 million years in the south and west (Gàal and Gorbatschev 1987). The rock composition varies significantly between the sites discussed here, but still, most of the rock types found (Table 3-2) could be characterised as "igneous". When formed, igneous rocks are too hot to host life of any kind. Therefore, observed life in these hard rocks must have entered after cooling and fracturing of the rock mass. The rock considered has generally been of granitic composition, with quartz, feldspars and mica as the bulk rock minerals. In addition to these, there are accessory minerals that influence the hydrochemical conditions (Table 3-2). Calcite may influence pH and HCO_3^-, pyrite dissolution and precipitation affect redox, apatite may act as a source of HPO_4^{2-} to the groundwater, and fluorite is involved in F^- exchange between dissolved and solid phases. Clay minerals may act as ion exchangers. Many of these occur as fracture-filling minerals and some of them have been formed in fractures because of weathering reactions elsewhere in the aquifer system. Minor amounts of iron oxyhydroxide minerals (e.g. goethite, limonite and FeOOH) are found in the fractures, especially in the shallow (i.e. <100 m) parts of the rock. Old fractures and vaults in igneous rock commonly contain ferric iron because of an historical oxidising action of deep, very hot water. Disintegration of this water to oxygen and hydrogen occurs when the water comes under the very high pressure and temperature which prevail in contact with magmatic layers under the crust (Apps and Van de Kamp 1993). During its flow, the oxygen in this hydrothermally altered water oxidises ferrous iron in the rock closest to the transport paths to ferric iron, giving the rock bordering to the fractures a red colour up to a distance of several tens of cm from the aquifer.

3.4.2.1 Groundwater flow in igneous rock aquifers

The distribution of flow in hard rock aquifers has an influence on groundwater composition. The hydraulic conductivity varies considerably, depending on location in the rock, and structures such as fracture zones may act as conductors and have a dominating influence. Vertical conductive zones are important for groundwater recharge at depth. Horizontal zones may act as hydraulic shields and separate groundwaters with a different composition. Especially deep groundwater with a relatively high salinity will have a higher density, which helps to stabilise the layering (Smellie and Wikberg 1991). Openings in rock fractures are potential channels for groundwater. Model studies done on flow and transport in fractures (Moreno et al 1985) suggest that considerable channelling is to be expected in such fractures and that there is a tendency for some pathways to carry much more water than others. In a limited mass of rock, one or more channels will dominate the flow and transport of nutrients and microorganisms. Hydraulic conductivities have been measured in boreholes at different depths and this information, together with the groundwater surface topography, which in Sweden approximately corresponds to the ground surface topography, is used to calculate the groundwater flow field. Groundwater flow at about 500 m depth is calculated to be in the range of 0.01–1 L m^{-2} $year^{-1}$ (Pedersen and Karlsson 1995). Near the surface, there

is an increase of hydraulic conductivity and flow. At or below sea level, the hydraulic gradient evens out and because of this, the flow rate is very small here. The hydraulic gradients increase considerably in the vicinity of a tunnel because the flow pattern is different from what it was before tunnelling. Note that a significant part of the inflow comes from aquifers situated deeper than the tunnel position concerned. This situation is taken into consideration when data from the Äspö HRL are interpreted.

3.4.2.2 Geochemistry of igneous rock groundwater

In general, groundwater under land in Sweden is of meteoric origin. The infiltrated water is almost "pure water" derived from rain or melting snow, with dissolved air as an important component. The processes in the biologically active soil zone are therefore very important for the composition of recharge water. Oxygen will be consumed and carbon dioxide produced. The carbonic acid will react with minerals such as calcite and feldspars, form carbonate ions and release calcium and alkali ions to the water. Ion exchange with clay minerals may affect the proportion of cations. Organic materials such as humic and fulvic acids and other substances are added to the water from the soil.

Biological processes will have an influence on groundwater also if seawater infiltrates through organically rich sea sediments. At great depths or under the ocean floor, saline water is found where chloride is the dominating anion (Table 3-3). The most common cation in saline groundwater is either sodium or calcium. The saline water may be of marine origin but other end members are also possible, depending on location and other conditions. Very deep, at depths of 1000–1500 m or more, the salinity can be very high, 8% or more, reaching well above seawater and even approaching brine composition (e.g. KLX02, Table 3-3). It is also common that in coastal regions, saline groundwater is found at shallower depth than further inland. This may of course be relict seawater that infiltrated several thousands of years ago, when land near the Swedish coast was covered by the sea due to the glacial depression. The infiltration of seawater continued until land was reclaimed by the land uplift, which is continuing in Sweden. However, an alternative explanation can be found in the lack of a driving hydraulic force under the "flat" surface of the sea. With no, or a very low, hydraulic gradient in the groundwater beneath the bottom of the sea, fossil saline conditions can be preserved for very long time periods and need not always be the result of a relatively recent infiltration of seawater. In other words, deep saline water may have originated even far before the last glaciation some 10 000 years ago.

Table 3-3. Selected chemical parameters of the Stripa, Laxemar and Äspö HRL sites (Pedersen 1997b) and four sites in Finland (Haveman et al 1999).

Borehole	Depth (m)	Ph	Temp (°C)	E_h (mV)	HCO_3^- (μM)	DOC (μM)	Fe_{tot} (μM)	Fe^{2+} (μM)	S^{2-} (μM)	SO_4^{2-} (mM)	Na^+ (mM)	Ca^{2+} (mM)	Cl^- (mM)	TDS (g l^{-1})
Hästholmen														
HH-KR1	937–947	7.0	13.8	–46	380	310	28.6	25	<0.3	1.44	213	92.3	433	24.7
HH-KR2	907–912	7.3	10.0	–214	450	300	23.3	17	<0.3	0.35	231	75.9	420	23.9
HH-KR3	211–216	7.8	19.6	.[a]	2220	340	21.5	12	1.9	6.70	109	16.2	149	9.54
Kivetty														
KI-KR5	717–725	8.1	5.6	.	1440	180	1.8	1.6	0.3	0.02	0.43	0.40	0.1	0.144
Laxemar														
KLX01	830–841	8.2	19.5	–270	104	.	3.9	3.8	2.3	7.10	120	77	259	15.7
KLX01	910–921	8.4	21.2	.	98	.	0.94	0.92	11	8.10	135	97	315	19.0
KLX01	999–1078	8.5	22.9	–220	190	.	6.5	6.4	5.6	7.20	146	115	355	22.1
KLX02	1420–1705	7.9	35.0	–334	230	75	6.1	6.1	0.3	11.30	370	495	1330	76
Olkiluoto														
OL-KR3	243–253	8.3	.	–180	380	140	1.8	1.1	11	0.01	63	7.0	77.8	4.57
OL-KR8	302–310	7.8	.	.	730	130	0.3	0.5	0.6	4.90	88	25.5	135	8.51
OL-KR10	324–332	7.9	10.4	.	360	120	6.6	6.1	0.5	0.09	84	30.9	152	8.73
OL-KR9	563–571	8.2	.	.	220	130	0.2	02	<03	0.01	183	81.1	324	19.2
Romuvaara														
RO-KR10	561–566	8.4	14.4	–418	1800	1050	0.9	0.2	0.3	0.03	1.09	0.26	0.1	0.172
Stripa														
V2	799–807	9.5	18	+205	158	.	0.3	0.27	<0.3	0.052	3.78	0.80	5.07	0.30
V2	812–821	9.4	18	+199	50	.	0.14	0.09	4.4	0.86	9.10	5.60	19.7	1.23
V2	970–1240	10.2	26	–3	57	.	0.07	0.07	100	0.38	9.10	2.82	14.4	0.88
Äspö HRL														
KR0012	68	7.7	9.3	.	4980	920	3.5	3.5	.	1.18	17.7	3.2	23.7	1.75
KR0013	68	7.5	8.9	.	4900	920	6.4	6.0	.	1.20	33.4	8.9	50.5	3.38
KR0015	68	7.5	8.9	.	4960	1500	6.6	6.3	.	1.15	23.0	4.8	22.3	2.07
KA3005/2[b]	400	7.6	14.3	.	1300	170	11	10.5	.	3.16	75	33.6	152	8.96
KA3010/2	400	7.6	14.3	.	910	210	13.9	12.7	.	3.49	82	46.8	186	10.9
KA3110/1	414	7.6	13.4	.	2700	340	19.8	18.9	.	2.84	69	15.0	108	6.63

a = not examined. b = number after slash denotes sampled borehole section.

Table 3-4. The content of nitrogen, hydrogen, helium, argon and carbon-containing gases and the total volumes of gas extracted from groundwater samples from the Stripa, Laxemar and Äspö HRL sites (Pedersen 1997b) and four sites in Finland (Haveman et al 1999).

Borehole	Depth	µM gas												Total volume of gas
	m	N_2	H_2	He	Ar	CO	CO_2	CH_4	C_2H_2	C_2H_4	C_2H_6	C_3H_6	C_3H_8	ml
Hästholmen														
HH-KR1	937–947	4800	78	600	400	<0.3	30	4	0.002	0.009	0.14	0.010	0.01	158
HH-KR2	907–912	6500	13	900	900	<0.5	10	9	0.004	0.009	0.12	<0.009	0.02	228
HH-KR3	211–216	2800	0.6	9	800	<0.2	20	2	<0.002	0.003	0.02	<0.003	<0.003	77
Kivetty														
KI-KR5	717–725	4900	0.06	0.7	400	<0.2	50	6	<0.02	<0.02	0.2	<0.05	0.02	147
Laxemar														
KLX01	830–841	2074	.[a]	205	.	0.02	21	1.2	<0.004	<0.004	<0.004	.	.	52
KLX01	910–921	1650	.	156	.	0.004	22	1.2	<0.004	<0.004	<0.004	.	.	41
KLX01	999–1078	803	.	109	.	0.03	71	1.4	<0.004	<0.004	<0.004	.	.	22
Olkiluoto														
OL-KR3	243–253	1870	2.9	49	140	<0.2	2	1170	<0.02	0.002	4.1	<0.04	<0.04	82
OL-KR8	302–310	1660	<0.08	40	30	<0.08	6	2	0.002	0.004	0.008	<0.02	<0.02	47
OL-KR10	324–332	2240	0.2	180	50	<0.2	6	3330	<0.02	<0.02	16	<0.05	0.3	145
OL-KR9	563–571	2290	15	480	40	<0.6	6	10500	<0.08	0.007	69.4	0.01	0.5	340
Romu-vaara														
RO-KR10	561–566	1100	0.08	0.2	140	<0.06	4	3	<0.006	0.001	0.07	<0.01	0.01	33
Stripa														
V2	799–807	1115	.	<10	.	<0.04	1.4	11	<0.004	<0.004	0.013	.	.	25
V2	812–821	1383	.	<10	.	<0.04	0.49	7.6	<0.004	<0.004	0.027	.	.	31
V2	970–1240	1093	.	<10	.	<0.04	0.5	13	<0.004	<0.004	0.129	.	.	25
Äspö HRL														
KR0012	68	981	.	1.8	.	0.004	270	46	<0.004	<0.004	<0.004	.	.	29
KR0013	68	1115	.	4.9	.	0.008	430	88	<0.004	<0.004	<0.004	.	.	37
KR0015	68	981	.	2.9	.	0.004	670	182	<0.004	<0.004	<0.004	.	.	41
KA3005/2[b]	400	1157	0.075	78	.	<0.04	48	77	<0.004	<0.004	<0.004	.	.	32
KA3005/4	400	1190	0.005	170	.	<0.04	94	82	<0.004	<0.004	<0.004	.	.	34
KA3010/2	400	1812	1.4	354	.	0.06	6.3	2.5	<0.004	<0.004	<0.004	.	.	49
KA3110/1	414	663	0.65	20	.	<0.04	82	41	<0.004	<0.004	<0.004	.	.	18

a = not examined. b = number after slash denotes sampled borehole section.

Typical groundwater compositions at different depths and locations encountered in the course of the research and exploration of the sites described in this Section are given in Tables 3-3 and 3-4. It is obvious that concentrations of major constituents, such as the cations sodium and calcium, and the anions bicarbonate and chloride, may vary considerably, depending on where and at which depth the samples are taken. Chloride behaves conservatively but many other ions obviously interact with the minerals. This is particularly evident in groundwater of marine origin. An example is the ion exchange of calcium for sodium, and vice versa. A further observation is that ions such as potassium and magnesium, which are common in seawater, are evidently suppressed in groundwater, presumably by reactions with the minerals. In addition, sulphate is partly consumed, probably by SRB. Carbonate is less common at depth, possibly because slow reactions with the rock minerals cause precipitation of carbonate as calcite and an autotrophic microbial organic carbon and methane formation.

The pH of granitic groundwater in Sweden is buffered by the carbonate system and is slightly alkaline. Calcite is abundant as mineral in the rocks and can, together with available feldspars, react with acids. Therefore, "acid rain" or any similar disturbance of pH does not propagate very far down under the ground. Measurements of redox potential with E_h-electrodes commonly give values of between –100 mV and –400 mV. There is a dependence of E_h on pH and on the ferrous iron concentration but the low concentrations of redox-active species in groundwater make the measurement of E_h a delicate operation. In situ measurement has been found to offer the most accurate results (Grenthe et al 1992).

3.4.2.3 Gases dissolved in igneous rock groundwater

The studied groundwater contains dissolved gases such as nitrogen, hydrogen, and carbon dioxide, methane, as well as some ethane, propane and the noble gases helium, neon, argon, krypton and radon (Table 3-4). If found at all, oxygen is only present at very shallow depths. The amount of dissolved gas varies from 18 ml to 340 ml gas L^{-1} groundwater, with the Finnish site groundwaters generally containing more gas than the Swedish groundwater. For many of the observed gases, local variations and depth variations are common within and between the studied sites. Nitrogen is the dominating gas in most samples examined. Some of the nitrogen may have been dissolved from air in rain and surface waters that infiltrate as groundwater with time. However, the solubility of nitrogen at 10°C and atmospheric pressure is 870 μM. Most of the nitrogen values in Table 3-4 exceed this solubility limit. Other sources of dissolved nitrogen to groundwater must exist as well. Nitrogen gas is used by nitrogen-fixing bacteria as a source of nitrogen; during the anaerobic respiration process called "denitrification", many of these bacteria produce nitrogen gas from nitrate. Microbial processes could, then, contribute to the pool of dissolved nitrogen in groundwater through denitrification processes of nitrogen dissolved from deep mantle rocks (Apps and Van de Kamp 1993), but it is not known whether this process occurs at the studied sites.

The trend of a carbon dioxide concentration generally decreases with depth. Active organisms expel carbon dioxide from degradation of organic material and many autotrophic microorganisms transform carbon dioxide to organic carbon. The concentration of this gas may therefore be influenced by microorganisms with the pertaining effects it may have on the carbonate system, on pH and on mineral precipitation and dissolution. One obvious example of this process was recorded when intrusion of shallow groundwater to the Äspö HRL tunnel was followed. Microorganisms degraded organic carbon to carbon dioxide, which gave a significant

increase in alkalinity of the groundwater and presumably also influenced mineral precipitation on the aquifer surfaces (Banwart et al 1996).

The content of hydrogen and methane varies considerably between the studied sites. Hästholmen had the highest hydrogen values and Olkiluoto showed some very high methane values. Data on hydrogen in hard rock have been published previously (Sherwood Lollar et al 1993a, 1993b). Between 2 μM and 1600 μM of hydrogen in groundwater from Canadian Shield and Fennoscandian Shield rocks were found. Methane occurs frequently in subterranean environments all over the globe, not only in hard rock environments. Evidence of an ongoing methane-generating process in deep Swedish granite has been published (Flodén and Söderberg 1994; Söderberg and Flodén 1991, 1992). Pockmarks in Baltic Sea sediments were found, indicating gas eruption, mainly of methane, from fracture systems in the underlying granite. Between 1 μM and 18 600 μM of methane in groundwater from Canadian Shield and Fennoscandian Shield rocks have been registered (Sherwood Lollar et al 1993a, 1993b). Data indicate that levels of up to 720 μM methane exist at 440 m depth at the Äspö HRL (Kotelnikova and Pedersen 1997). The origin of methane at the sites listed in Table 3-4 has not been well researched. The significant content of $C_{2-3}H_{2-8}$ suggests that most of the methane found at Finnish sites is of inorganic origin. The lack of these compounds at Äspö and Laxemar would suggest a biological origin (Des Marais 1999). Some results on the $^{13}C/^{12}C$ signatures indicate a biogenic origin for the Äspö methane (Banwart et al 1996).

3.4.3 Fossils of microorganisms in a fracture calcite mineral

Many old fractures in hard rock are no longer open, because they have been filled with precipitating minerals such as calcite, dolomite, pyrite, epidote and chlorite, to mention some of the more common ones (Table 3-2). This fact presents interesting possibilities for the search for microbial fossils, which have been captured in fluid inclusions in hard rock aquifers, as has been reported for halophilic bacteria (Vreeland et al 1999).
Two boreholes in the Äspö HRL tunnel (SA813B and SA923A, Table 3-2) carried groundwater that was oversaturated with carbonate and calcium. They were explored for attaching and biofilm-forming microorganisms (Pedersen et al 1996b). Glass surfaces exposed to flowing groundwater at flow rates of 8–14 mm s^{-1} for 67 days were subsequently observed through light and scanning electron microscopes. Some 1.2 x 10^6 cells per cm^{-2} were found more or less buried in dense precipitates of calcite (Fig 3-9 A). Although partly artificial, this experiment supports the idea that microbes attached to fracture minerals in deep hard rock may become buried or trapped in fluid inclusions during growth of a fracture mineral.

An unusually long fracture core sample from 207 m borehole length (200 m depth) in KAS02 (Table 3-2) was extensively investigated with respect to stable isotopes (Tullborg et al 1999). Obvious signs of biological activity in the form of light $\delta^{13}C$ signatures in the calcite carbon were found. It was therefore assumed that fossil microorganisms may be detectable in the fracture mineral. Investigating the fracture with electron microscopy did indeed reveal bacterium-like structures in parts of it (Fig 3-9 B) and X-ray analysis demonstrated these structures to be enriched with carbon (Pedersen et al 1997b). The found fossil microorganisms in the deep igneous rock fracture minerals were a good indication that microbial life was present deep under the island of Äspö long before drilling and excavation of the Äspö HRL. This observation and the biofilm example discussed above seem to reflect a past and present situation of attached subterranean microorganisms that are neatly linked. The isotope and electron microscopy results strongly suggest that microbial activity has been ongoing in deep

granitic aquifers of Äspö. The presence of modern autotrophic and heterotrophic microbial life in aquifers in this rock volume has likewise been repeatedly demonstrated (Kotelnikova and Pedersen 1998). Modelling historical and present geochemical processes in deep granitic aquifers, of importance for HLW disposal, should, then, include biologically catalysed reactions to be correct. However, it remains to be determined at which rates subterranean microorganisms shuttle carbon between various dissolved and solid phases in hard rock aquifers.

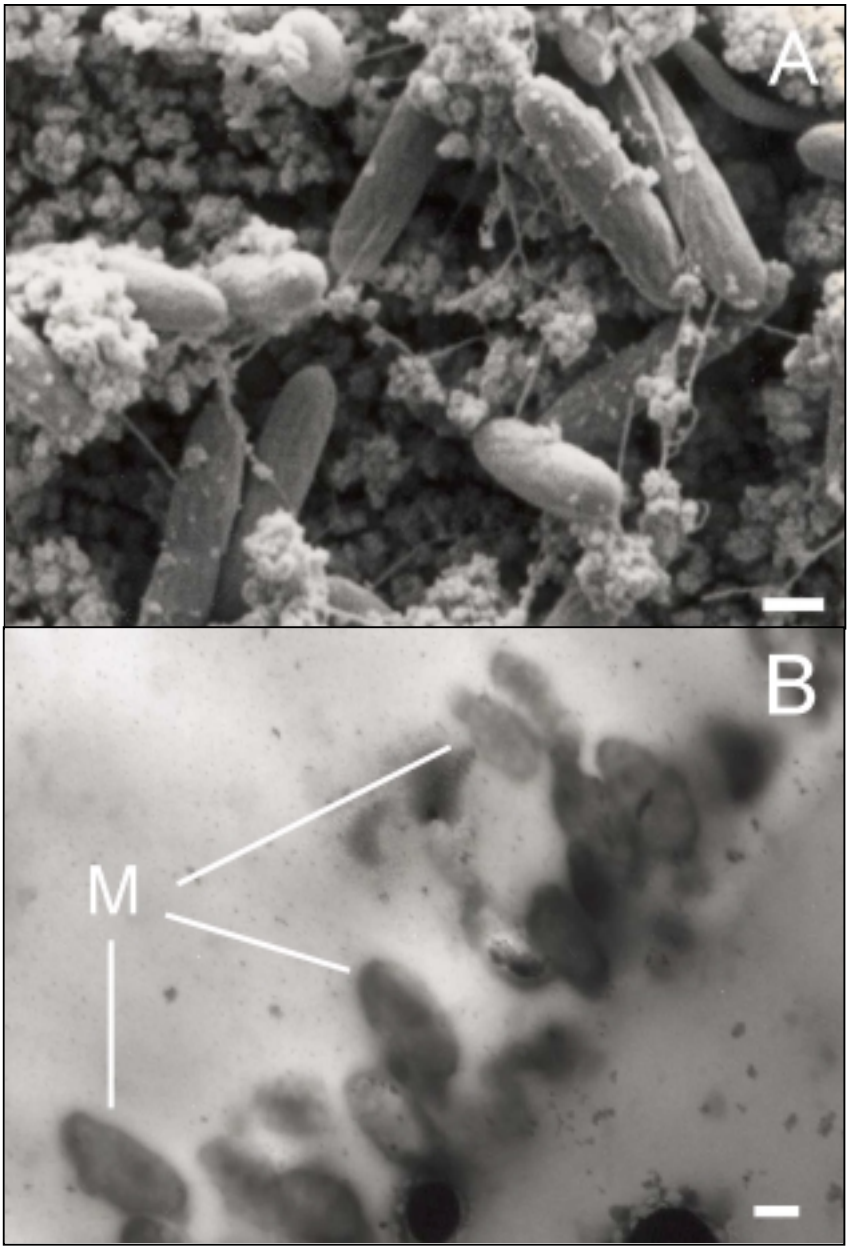


Figure 3-9. ***A.*** *Bacterial biofilm mixed with calcite precipitates on a surface exposed to slowly flowing groundwater from the borehole SA813B in the Äspö HRL tunnel (Table 3-2). Culturing results and 16S rDNA analysis demonstrated this biofilm to be predominated by an acetogenic bacterium and a SRB (Pedersen et al 1996a) (scale bar = 1 μm).* ***B.*** *Thin section transmission electron microscopy of a calcite precipitate which coated a fracture in crystalline rock from 207 m below sea level in south-eastern Sweden. Fossil microorganisms (M) are arranged in a typical biofilm formation (see Pedersen et al 1997b for details) (scale bar = 1 μm).*

3.4.4 Numbers of microorganisms in deep groundwater

3.4.4.1 Total number of microorganisms

Total numbers of subsurface microorganisms vary notably, depending on the site studied. Values in the range of 10^3–10^8 per ml groundwater or gram of sediment have been reported for deep environments (Fredrickson and Onstott 1996; Ghiorse and Balkwill 1983; Ghiorse and Wilson 1988; Pedersen 1993a). The total numbers of microorganisms in igneous rock groundwater samples have been examined ever since the first boreholes were visited back in 1987 (Table 3-2). Unattached microorganisms have been counted with epifluorescent microscopy after filtration using 0.2 μm filters and staining with acridine orange (AO) and/or 4',6-diamidino-2-phenylindole (DAPI) (Fry 1990; Herbert 1990). The average total number of cells registered in the Fennoscandian igneous rock aquifers is generally within the range of 10^5–10^6 cells ml^{-1}, although the range of single observations is from 1 x 10^3 to 5 x 10^6 cells ml^{-1} (Fig 3-10). A large set of boreholes examined at a site results in a larger range of total numbers than a small set of boreholes. This correlation can be expected if there are large local variations between the aquifers examined at one site, as seems to be the case with Laxemar, Stripa and the Äspö HRL. All of these sites show ranges in total numbers of almost three orders of degree.

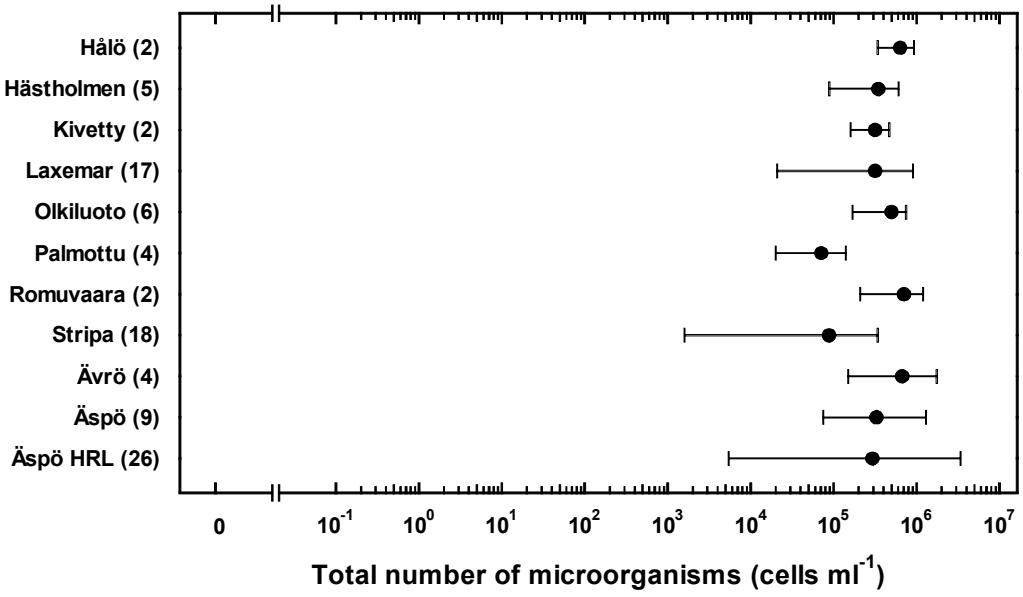


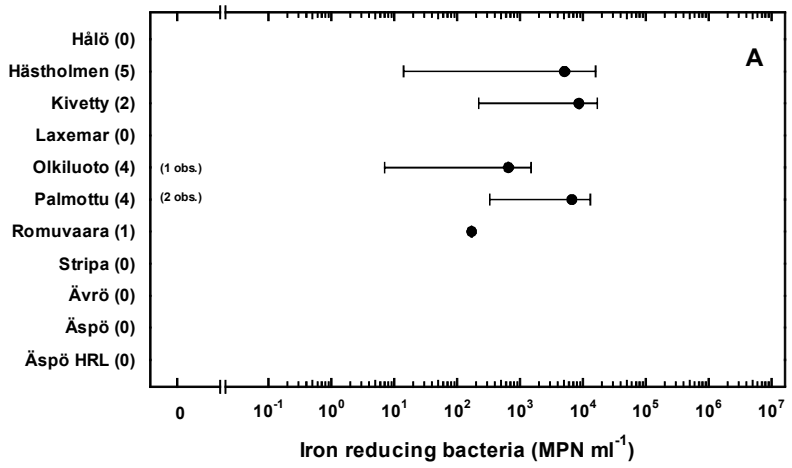
Figure 3-10. *The total number of microorganisms observed at the eleven sites investigated. The data are extracted from publications listed in Table 3-2, except for some recent, unpublished data obtained during the spring of 1999. The figures shows the average total number of microorganisms per site (●); the bar gives the range of data used to calculate the average number. The number in parenthesis following the site name gives the sum of observations for the site and sums of observations are sets of mean values based on between two and six repetitions.*

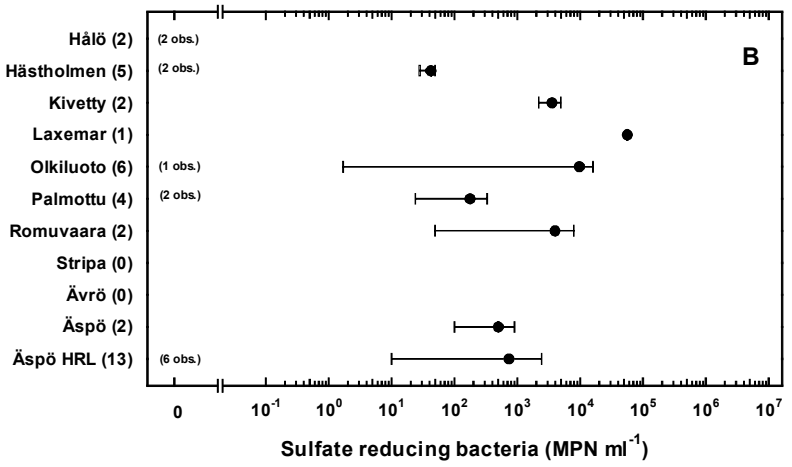
The variability of the total number in specific boreholes has been studied extensively at Stripa and Äspö and it has been found to be remarkably small. The Stripa borehole V2 delivered reproducible and non-variable numbers for the whole period studied, which was 4 years (Ekendahl and Pedersen 1994; Pedersen and Ekendahl 1992a). Four new drilled boreholes in the Äspö HRL tunnel were revisited three times during one year and showed matching total numbers over this period (Pedersen et al 1997c), as did seven other boreholes at the Äspö HRL revisited three times during a period of 6 months (Pedersen et al 1996b). The variability in total numbers between boreholes and the non-variability in total number within specific boreholes are indicative of stable environments with little or no changes in the conditions for microbial life. Such conditions may, however, vary considerably between sites and boreholes intersecting the Fennoscandian Shield igneous rock aquifers. This observation compares well with data on the groundwater chemistry in boreholes, which may vary significantly between boreholes, depths and sites (Table 3-3), but which is non-variable within specific boreholes over time (Nilsson 1995).

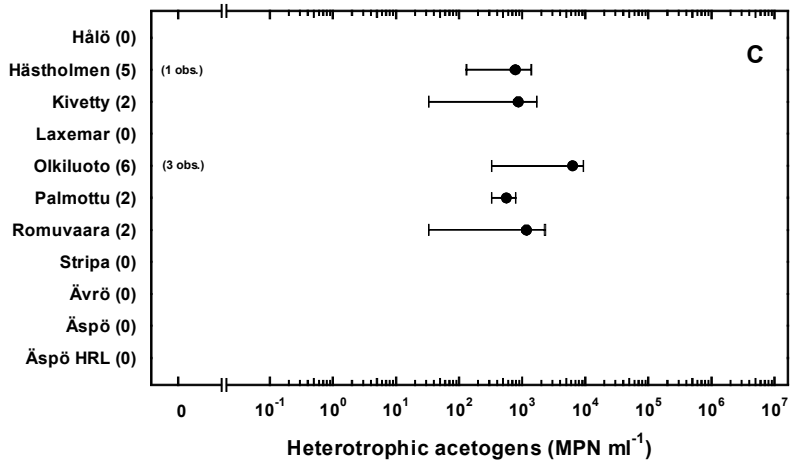
Correlation analyses of the obtained total number with several other groundwater parameters were performed early on during the programme (Pedersen and Ekendahl 1990) and showed that the only measured parameter which correlated with the total number was the amount of total organic carbon (TOC). The same relation was later found for the shallow (0–105 m) groundwater of Bangombé, in Oklo, Gabon (Pedersen et al 1996c). It appears that the total number of microorganisms in groundwater does not correlate with any measured parameter other than TOC. Repeated attempts (not published) to find a significant correlation between individual groundwater parameters other that TOC, and total numbers have failed.

3.4.4.2 Viable counts of microorganisms in igneous rock groundwater

The plate count technique has been employed for determinations of the number of colony-forming units (CFU) in deep igneous rock groundwater. The percentages of total numbers that could be cultured from Äspö borehole groundwater with this method ranged from <0.1% to 10%, with an average of 1.7% (Pedersen and Ekendahl 1990). The media used were general purpose types for heterotrophic bacteria and further characterisation was required for information about the kinds of CFU obtained. Typically, Pseudomonas and Shewanella were found. More recent use of this method for Äspö HRL tunnel borehole groundwater resulted in very low CFU percentages, of <0.1%, of the total numbers (Pedersen et al 1997c). These low viable count numbers and the inability of the plate count method to reveal information about the metabolic diversity of the investigated microorganisms motivated adaptation of more selective media for different predominant physiological groups of microorganisms. A range of anaerobic culturing media for physiological microbial groups were, therefore, developed and applied on Äspö HRL tunnel groundwater and at five groundwater sites in Finland (Table 3-2). Anaerobic Hungate tubes (Hungates 1969) and serum bottles with aluminum crimp-sealed butyl rubber stoppers (Bellco, USA) were used for most probable number (MPN) determinations with a set of various media (Haveman et al 1999; Kotelnikova and Pedersen 1998). Figure 3-11 summarises the MPN results of IRB and SRB, heterotrophic acetogens (HAs) and AAs and heterotrophic methanogens (HMs) and autotrophic methanogens (AMs).


A
Hålö (0)
Hästholmen (5)
Kivetty (2)
Laxemar (0)
Olkiluoto (4)
(1 obs.)
Palmottu (4)
(2 obs.)
Romuvaara (1)
Stripa (0)
Ävrö (0)
Äspö (0)
Äspö HRL (0)
0
10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7}
Iron reducing bacteria (MPN ml^{-1})


B
Hålö (2)
(2 obs.)
Hästholmen (5)
(2 obs.)
Kivetty (2)
Laxemar (1)
Olkiluoto (6)
(1 obs.)
Palmottu (4)
(2 obs.)
Romuvaara (2)
Stripa (0)
Ävrö (0)
Äspö (2)
Äspö HRL (13)
(6 obs.)
0
10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7}
Sulfate reducing bacteria (MPN ml^{-1})


C
Hålö (0)
Hästholmen (5)
(1 obs.)
Kivetty (2)
Laxemar (0)
Olkiluoto (6)
(3 obs.)
Palmottu (2)
Romuvaara (2)
Stripa (0)
Ävrö (0)
Äspö (0)
Äspö HRL (0)
0
10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5} 10^{6} 10^{7}
Heterotrophic acetogens (MPN ml^{-1})

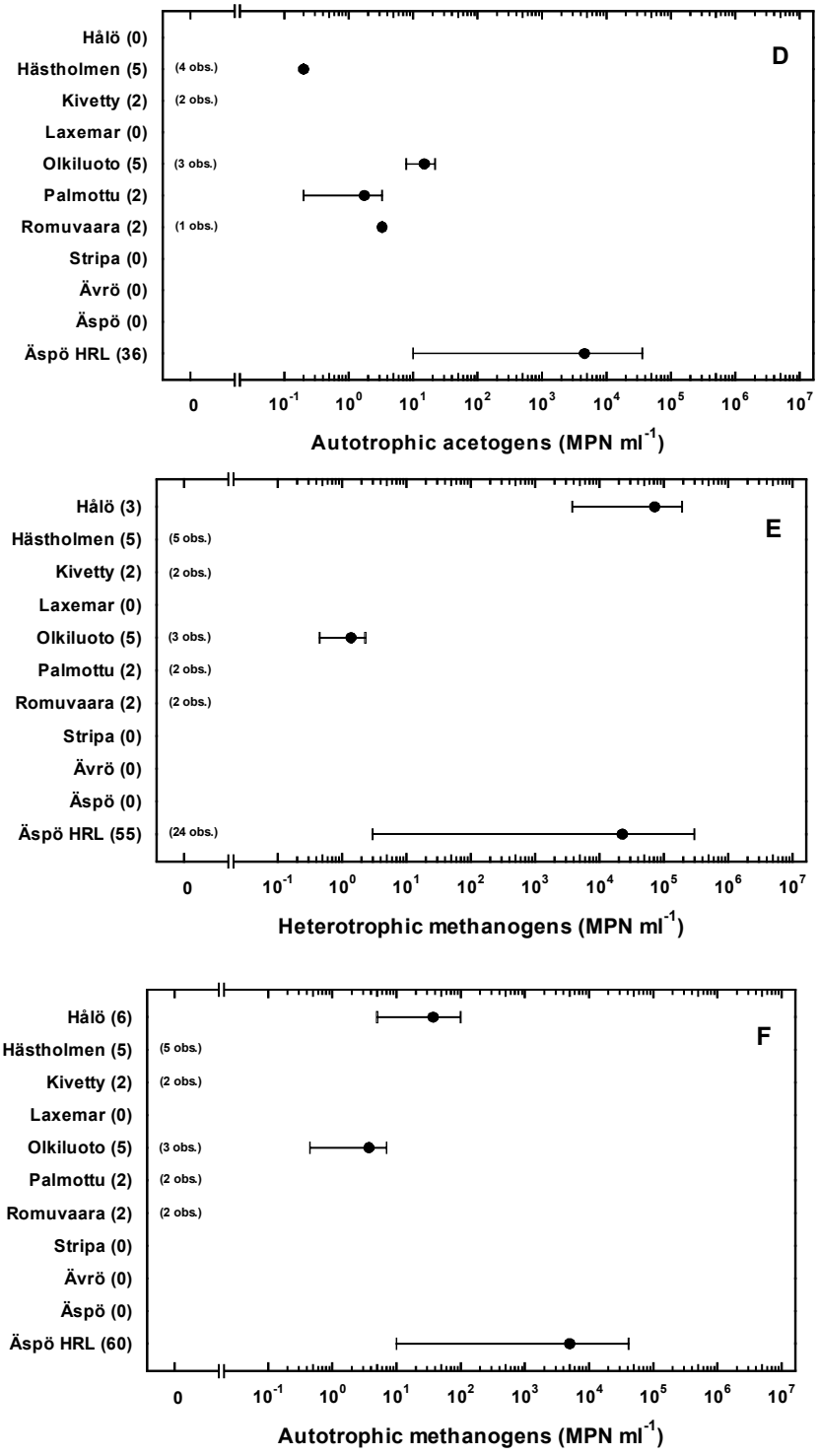


Figure 3-11. A–F. *Most probable numbers of physiological groups of microorganisms observed at the eleven sites investigated. The data are extracted from publications listed in Table 3-2, except for recent, unpublished data obtained during the spring of 1999. The figures show the average total number of microorganisms per site (●), and the bar indicates the range of data used to calculate the average number. Numbers in parenthesis following the site name give the sum of observations for the site and each observation consists of an MPN determination with three or five parallel tubes in the dilution series.*

Iron-reducing bacteria and SRB were detected at all sites in Finland at most depths investigated in the range of 10^0–10^4 cells ml^{-1} (Fig 3-11 A–B). An inverse relationship between IRB and SRB, which correlated with the predominating types of fracture minerals at the sites, became evident when the Hästholmen and Olkiluoto sites were compared (Haveman et al 1999). Olkiluoto had a relatively high average number of SRB and a low number of IRB, while the opposite was true for Hästholmen. Hästholmen groundwater is rich in iron, containing up to two orders of magnitude more total iron than what is found in Olkiluoto (Table 3-3). Fracture minerals at Hästholmen include iron oxhydroxides, but pyrites are only sporadically present, while in the Olkiluoto fracture minerals, pyrite is one of the major components. This indicates that the presence or absence of pyrite as a fracture mineral correlates well with the presence or absence of SRB at the compared sites. Pyrite formation on fractures in these cold aquifers may reflect long-term SRB activity, which is not apparent from groundwater chemistry data. Examination of the sulphate and sulphide concentrations was much less informative in predicting the presence or absence of SRB than was work with the MPN of SRB. No correlation could be found between sulphate or sulphide concentrations and SRB. The largest numbers of SRB in the samples from Finland were found in boreholes with either very low or very high sulphate and generally, low sulphide concentrations. This example demonstrates that it is important to search for conservative indicators of microbial activity that are insensible to transport processes, because processes in hard rock may span millions of years at steady-state conditions. Even at the slowest transport rate, sulphate may be replenished to SRB during such long periods, resulting in a steady-state concentration of sulphate and a buildup of pyrite precipitates. Long-term and very slow processes should be in focus when searching for evidence of subterranean microbial activity. Signatures in fracture minerals therefore seem to be reliable indicators of past and present microbial activity, especially if stable isotope ratios are added to the analysis protocol (Des Marais 1999; Pedersen et al 1997b).

In most hydrothermally altered fractures (see above), IRB have access to an almost unlimited source of ferric iron, provided they can reach it. Humic and fulvic acids are common in most deep groundwater and these complex compounds have been demonstrated to act as electron shuttles between ferric iron sources and IRB (Coates et al 1998). The molecule size of these compounds is small enough to allow penetration of the rock matrix which then enables iron reduction of parts in the rock that are not directly accessible to the IRB. Attempts to correlate numbers of IRB with amounts of ferric and ferrous iron have not been successful because the iron redox couple is sensitive to inorganic processes, much more than are sulphur redox couples, at least where reduction is concerned. Therefore, it is not possible to discriminate between biological and chemical iron redox reactions. The adaptation of mixing models has been demonstrated to be more fruitful (Banwart et al 1996). The effect of IRB on carbon dioxide and ferrous iron production has been demonstrated in a shallow groundwater intrusion system at the Äspö HRL tunnel. Organic carbon in the groundwater that reached the studied fracture zone was oxidised with ferric iron as the electron acceptor. This process rapidly reached a steady state that has been sustainable since the start of measurements in March 1991. A similar approach was taken at the Äspö HRL for determination of sulphate reduction along the tunnel. It was found that the MPN of SRB correlated well with geological, hydrological and groundwater isotope data indicative of ongoing sulphate reduction (Laaksoharju et al 1995; Pedersen 1997a).

Repeatedly obtained pure cultures and 16S rDNA sequences of acetogenic bacteria from Äspö HRL groundwater indicated that this physiological group of bacteria are important to the subterranean environment (Pedersen et al 1996b). Later application of MPN

media for HAs and AAs supported this hypothesis. Autotrophic acetogens form acetate from hydrogen and carbon dioxide, and the carbon may then be further transformed by the acetoclastic methanogens to methane. Heterotrophic acetogens were found at all sites in Finland (Fig 3-11 C); AAs frequently occurred in the Äspö HRL tunnel and at several of the Finnish sites (Fig 3-11 D). The numbers of acetate-producing bacteria in the Äspö HRL environment correlated well with the numbers of HMs (Fig 3-11 E), including acetoclastic ones (Kotelnikova and Pedersen 1998).

The presence of hydrogen and carbon dioxide in most deep groundwater examined (Table 3-4) indicates that autotrophic methanogenesis should be possible and the MPN analyses indeed report significant numbers of organisms responsible for this process at Hålö, the Äspö HRL, and Olkiluoto (Fig 3-11 F). We have no obvious explanation for the lack of positive indications of methanogens in most Finnish samples, other than that the media used were developed and adjusted at Äspö during many repeated sampling occasions in the tunnel. On the other hand, during the investigations in Finland, we only had one opportunity of sampling and media preparation at each borehole level. All samples from Finland were collected from the ground surface in boreholes with the PAVE method (Fig 3-7 C) which offers one sample of 300 ml groundwater per level. Generally, it is not financially possible to repeat such sampling campaigns. However, it cannot be excluded that the MPN determinations were correct and that there were no, or very few, methanogens at most studied Finland sites.

3.4.5 Carbon transformation activities

3.4.5.1 Methodology

Radioactive compounds for the estimation of microbial activity have been in use in microbial ecology for decades (see, e.g., Grigorova and Norris 1990). With this technique, samples are incubated with the radiotracer of interest and then examined. Cells, or products, can be separated and examined for radioactivity using standard liquid scintillation techniques. This method will give average activity results for the whole sample. The activity of individual cells can be examined using a microautoradiography (MARG) technique (Tabor and Neihof 1982, 1984).

Both the liquid scintillation and the MARG technique were applied to microorganisms from the Laxemar, Stripa and Äspö HRL sites, with varying radiotracers and incubation times (Table 3-5). The strong advantage of the MARG technique is that individual cells can be examined and the method can be successfully combined with nucleic acid probing offering specific information of selected metabolic activities (Amann et al 1995). However, at very low metabolic rates, the MARG method is less applicable than liquid scintillation. In an aquatic sample supplemented with a radio-labelled substrate for a short period, an individual cell must have a minimum uptake rate to become sufficiently labelled to produce a positive microradiogram. The lowest radioactivity that resulted in cells radioactive enough to expose the film faster than the background radiation was found to be 10^{-3} disintegrations per minute (Pedersen and Ekendahl 1992a). This level corresponds to 0.1–1 x 10^{-16} mole ^{14}C per bacterium, and 2.1 x 10^{-19} mole ^{3}H per bacterium for the method used to generate the MARG data in Table 3-5. A prolonged time of incubation, of more than a couple of hours, will lower the detection limits, but will concurrently increase the background and also allow for growth on the added substrates. The data in Tables 3-5 and 3-6 were generated with short incubation times for Laxemar and Stripa, and an extended incubation time for the Äspö HRL samples.

Table 3-5. In vitro radiotracer and radiographic estimations of carbon transformations by unattached microorganisms in deep groundwater. The radiotracer data have been normalised to mole per litre of groundwater per hour to enable comparisons, although the time for incubation varies from 6–9.5 hours for Laxemar and Stripa 4–10 days for the Äspö HRL. Acetate and leucine were labelled with ^{3}H and all other compounds were labelled with ^{14}C. For further details on the technique, see Ekendahl and Pedersen 1994; Kotelnikova and Pedersen 1998; and Pedersen and Ekendahl 1990, 1992a, 1992b.

Borehole	Depth (m)	Transformation of carbon compounds (nM h^{-1})/(% active cells)								
		CO_2 to cells	CO_2 to CH_4	CO_2 to acetate	Formate to cells	Acetate to cells	Acetate to CH_4	Lactate to cells	Glucose to cells	Leucine to cells
Laxemar										
KLX01	831–841	–[a] / –	.[b]	.	– / –	0.001 / 29	.	0.230 / 21	0.072 / 2	0.005 / 56
KLX01	910–921	0.028 / –	.	.	– / –	0.001 / 27	.	0.700 / 16	0.016 / –	0.023 / 87
KLX01	999–1078	0.160 / 3	.	.	0.085 / 51	0.001 / 21	.	3.160 / 83	0.048 / –	0.048 / 98
Stripa										
V2	799–807	0.520 / 5	.	.	0.018 / 4	. / .	.	0.043 / 16	0.031 / 5	0.002 / 55
V2	812–820	0.110 / 5	.	.	0.005 / 6	. / .	.	0.016 / 34	0.031 / 8	0.008 / 23
V2	970–1240	0.150 / –	.	.	– / –	. / .	.	0.110 / 6	0.110 / .–	0.008 / 9
Äspö HRL										
KR0012B	68	.	1170 / .	9630 / .	.	.	12460 / .	.	.	.
KR0013B	68	.	0 / .	4620 / .	.	.	3100 / .	.	.	.
KR0015B	68	.	1470 / .	15300 / .	.	.	12370 / .	.	.	.
SA813B	112	.	0 / .	33230 / .	.	.	6300 / .	.	.	.
SA1420A	192	.	0 / .	3 / .	.	.	120 / .	.	.	.
KA2511A	345	.	530 / .	2220 / .	.	.	300 / .	.	.	.
KA2512A	345	.	0 / .	0 / .	.	.	1290 / .	.	.	.
KA2862A	380	.	7 / .	.0 / .	.	.	0 / .	.	.	.
KA3005A	400	.	150 / .	30 / .	.	.	30 / .	.	.	.
KA3010A	400	.	59 / .	2 / .	.	.	0 / .	.	.	.
KA3067A	409	.	980 / .	2 / .	.	.	110 / .	.	.	.
KA3105A	414	.	70 / .	7 / .	.	.	130 / .	.	.	.
KA3110A	414	.	510 / .	65 / .	.	.	0 / .	.	.	.
HB0025A	420	.	25 / .	90 / .	.	.	130 / .	.	.	.
KA3385A	446	.	20 / .	0 / .	.	.	0 / .	.	.	.

a = not detected; b = not examined.

3.4.5.2 In vitro activity of unattached cells

Both the Laxemar and the Stripa populations transformed all added compounds at varying rates. Carbon dioxide was assimilated at relatively low rates, as were formate, acetate and glucose. The fastest uptake was found to be with lactate. Generally, ten times more lactate than other ^{14}C-labelled compounds was transformed, and up to 83% of the deepest population at Laxemar were active in lactate transformation. Lactate seemed to be the preferred carbon source. Lactate can be utilised by SRB and HAs at the anaerobic conditions prevailing in deep groundwater. A look at Figure 3-11 B and D will confirm that these two physiological groups of bacteria are common. The incorporation of acetate was not high but as ^{3}H was used, the sensitivity to the MARG technique was higher than to ^{14}C. The MARG technique revealed that a large proportion of the cells took up this compound (see Ekendahl et al 1992 for a detailed discussion of method sensitivity). Acetate is used by many SRB and HMs, both of which groups are frequently represented in the MPN data (Fig 3-11 B and E). Likewise, acetoclastic methanogens have been found active in many of the studied Äspö HRL boreholes (Table 3-5). The uptake of carbon dioxide by the Stripa and Laxemar populations points to the presence of autotrophic organisms. Later studies confirmed the assumptions and AAs and AMs have been enriched, enumerated (Fig 3-11 D and F) and isolated at many of the studied sites. Autotrophic methane production is common at the Äspö HRL, as shown by the radiotracer scintillation technique (Table 3-5). Consequently, a relatively large data set describing carbon transformations has been obtained over a 12-year investigation period. When these data are compared with MPN data, it appears that most of the observed radio-labelled carbon transformations can be correlated with the detected physiological groups of microorganisms capable of the observed transformations.

Significant formation rates of methane and acetate were obtained in vitro from Äspö groundwater at a temperature (17°C) close to the in situ temperature (10–17°C) (Table 3-5). Heterotrophic methane formation and acetate formation followed the trends observed with the MPN and with the enrichments (Kotelnikova and Pedersen 1998). The highest activity was found in the shallow boreholes (45–68 m) which also had the highest numbers of HMs and homoacetogens. Autotrophic methane formation did, however, not follow the culturability trend, which may have been owing to the increasing difficulty, with increasing depth, to mimic in situ conditions in vitro for parameters such as pressure and dissolved gases. With this exception, three independent methods, MPN (Fig 3-11), enrichments (Kotelnikova and Pedersen 1998) and radiotracer assays (Table 3-5), have all established the presence of active HMs and homoacetogens in the examined groundwater.

3.4.5.3 In vitro activity of attached cells

An aquifer in hard rock is made of two surfaces that are wavy and rough. They are in contact with each other at some points and are distanced from each other at others. Aquifers generally expose a large surface to the contained groundwater and the surface/volume ratio increases as the aquifer width decreases. Microorganisms commonly have a strong tendency to attach to surfaces in aquatic environments (Marshall 1984) and to form biofilms (Characklis and Marshall 1990). The ability of deep aquifer microorganisms to form biofilms on artificial glass and rock surfaces introduced in aquifers has been studied with slowly flowing groundwater at three depths in Stripa (Ekendahl and Pedersen 1994; Pedersen and Ekendahl 1992a), four depths at Laxemar (Pedersen and Ekendahl 1990, 1992b), and four depths in the Äspö HRL

tunnel (Pedersen 1997a; Pedersen et al 1996b). The time of exposure ranged from 20 days to 161 days at flow rates of 1–31 mm sec^{-1}. All surfaces exhibited attached cells. In cases of prolonged exposure (> 25 days), growing colonies could be found, showing that the microorganisms in the aquifers do not only attach passively; they are able both to attach and to grow on the surfaces.

A hypothetical comparison of cell numbers and activities of attached and unattached bacteria in a 0.1mm wide fracture is shown in Table 3-6. It demonstrates the potential importance of attached microorganisms versus unattached microorganisms in subsurface environments. The attached bacteria have generally exhibited a higher activity per cell than have the unattached bacteria (not shown) and they would be up to five orders of magnitude more active than the unattached microorganisms suspended in the assumed 0.1 mm fracture. The natural flow rate of groundwater in deep aquifers depends on the existing hydraulic gradients, but it is generally very slow, much slower than the flow rate used in the experiments described above. It is still an open question whether attached microorganisms are common and active on aquifer rock surfaces at pristine conditions. It would be necessary to drill and directly examine undisturbed aquifers to answer this question. The very high drilling cost and the relatively low probability of intersecting an open fracture during a drilling operation tie up this question with extreme experimental costs before a statistically significant observation series would be obtained.

3.4.5.4 In vitro viability of attached and unattached cells

Leucine assimilation is virtually specific to bacteria if low (nM) concentrations are applied. This amino acid is used for protein synthesis by many bacteria during growth (Kirchman et al 1985). It can also be used as a carbon and energy source and can be fermented by proteolytic clostridia via the Strickland reaction. High percentages, up to 98%, of most populations described in Tables 3-5 and 3-6 assimilated leucine. This assimilation indicates that major portions of the studied communities were viable.

3.4.6 Diversity and phylogeny of microbes

3.4.6.1 Molecular investigations

The MPN assays and activity measurements described above have supplied extensive information about present and active physiological groups in the examined deep aquifers, but these methods do not reveal species diversity and phylogeny. Classical microbiology involves characterisation and species affiliation based on large sets of phenotypic and genotypic data, which is a very time-consuming procedure not suitable for the screening of environmental samples for species diversity. The concept of microbial diversity has been changed by the availability of sequence data from ribosomal 16/18S rRNA. The cloning and sequencing of RNA from microbes living in their natural environments has revealed a genetic diversity beyond the dreams of researchers whose tools were limited not so long ago to culturing and microscopy (Pace 1997). The strategy became to add 16/18S rDNA sequencing of environmental DNA to the investigations. The first site thus examined was the Stripa borehole V2 (Ekendahl et al 1994). Attached microorganisms were studied and all sequences found among the 72 clones investigated belonged to the Proteobacteria.

Table 3-6. The total number of bacteria in groundwater and on surfaces exposed to flowing groundwater, and the amounts of carbon dioxide and lactate transformed by these microorganisms. Source: Pedersen and Ekendahl 1992a; Pedersen and Ekendahl 1992b.

Borehole	Depth (m)	Unattached bacteria			Attached bacteria			Hypothetical volume/surface ratios in a 0.1 mm wide fracture		
								Cells m^{-2} x 10^{10}	µm CO^2 m^{-2} day^{-1}	µm lactate m^{-2} day^{-1}
		Cells m^{-3} x 10^{10}	µm CO^2 m^{-3} day^{-1}	µm lactate m^{-3} day^{-1}	Cells m^{-2} x 10^{10}	µm CO^2 m^{-2} day^{-1}	µm lactate m^{-2} day^{-1}	Cells m^{-3} x 10^{10}	µm CO^2 m^{-3} day^{-1}	µm lactate m^{-3} day^{-1}
Laxemar										
KLX01	831–841	1.5	– [a]	5.6	0.09 [b]	0.9	2.6	1200	…	9200
KLX01	910–921	2.1	0.68	17	0.12 [b]	1.1	6.0	1100	32 000	7000
KLX01	999–1078	6.8	4	76	0.10 [b]	1.5	0.14	300	7500	36
Stripa										
V2	799–807	0.54	12.5	1	1.2 [c]	–	2.0	44 000	…	40 000
V2	812–820	0.18	2.6	0.4	7.1 [c]	0.48	5.5	790 000	3600	280 000
V2	970–1240	12	3.5	2.7	5.9 [c]	1.8	37	9800	10 000	270 000

a = not detected
b = 20 days' exposure to flowing groundwater
c = 120 days' exposure to flowing groundwater

Two of the major groups fell into the beta group and the third into the gamma group. The next site to be investigated for 16/18S rDNA studies was the Äspö HRL tunnel. In a first campaign, 155 clones of unattached and attached bacteria from nine boreholes were sequenced (Pedersen et al 1996b). A comparison of the predominating 16S rRNA gene sequences with the international sequence data bases revealed three clone groups that could be identified as bacteria on the genus level, the Bacillus, Desulfovibrio and Acinetobacter genera. One of the clone groups could be identified as being fungi. A second campaign was executed during a contamination control investigation while drilling boreholes in the Äspö HRL tunnel (Pedersen et al 1997c), and 158 clones were sequenced. Several clones showed a high similarity with 16S rRNA genes from known and sequenced bacteria such as Bacillus, Desulfovibrio, Desulfomicrobium, Methylophilus, Acinetobacter, Shewanella, and an yeast.

The diversity size of the bacterial community detected in the Äspö HRL groundwater is not large compared with the numbers expected in surface soils, i.e. 4000 species in one gram of soil (Torsvik et al 1990). Of the total of 385 sequenced clones from Stripa and the Äspö HRL, 122 constituted unique sequences, each representing a possible species not entered into the data base at the time the comparison was done. On average, approximately one-third of the sequenced clones represented unique species. In other investigations similar to the Äspö HRL study, a matching molecular biodiversity per total number of sequenced clones was observed, namely 44 specific clone groups out of 130 sequenced clones from five boreholes at the natural nuclear reactor in Bangombé, Gabon (Pedersen et al 1996c), 20 specific clone groups out of 67 sequenced clones from nuclear waste buffer material (Stroes-Gascoyne et al 1997), and 23 specific clone groups out of 87 sequenced clones from alkaline spring water in Maqarin, Jordan (Pedersen et al 1997a). These investigations and the Äspö HRL and Stripa studies clearly have not exhausted the sequences because new sequences were found in nearly every additional sample repetition. The 16/18S rDNA sequence data collection effort therefore clearly has to be scaled up significantly for the study of most groundwater sites inasmuch as it requires automated procedures. Bacteria are likely to be the first group of organisms for which such automated biodiversity assessment is practicable (although other soil microbiota may also be surveyed via this technique). As a major constituent of the community, they deserve assessment in their own right, in addition to their value as indicators (Crozier et al 1999).

3.4.6.2 Characterisation and description of new species

The molecular work described above has provided good insight into the phylogenetic diversity of igneous rock aquifer microorganisms, but it does not reveal species-specific information unless 100% identity of the 16S rDNA gene with a known and described microorganism is obtained. The huge diversity of the microbial world makes the probability of such a hit very small. None of the 122 specific sequences mentioned above had 100% identities with described species. Still, if a 100% identity is obtained, there may yet be strain-specific differences in some characters unravelled by the 16S rDNA information (Fuhrman and Campbell 1998). If species information is required, time-consuming methods in systematic microbiology must be applied to a pure culture. Known genera or species can be identified through these methods. Several isolates from the Laxemar, Äspö and Äspö HRL sites have been identified as Shewanella putrefaciens, Pseudomonas vesicularis and Pseudomonas fluorescens (Pedersen and Ekendahl 1990; Pedersen et al 1996b). An isolate that does not match a known genus or species obviously provides the opportunity to describe a new species.

Three new subterranean species from deep igneous rock aquifers have been described and reported. Sulphate-reducing bacteria are common in the deep aquifers studied (Fig 3-11 B) and three SRB species, based on their different 16S rDNA sequences, were repeatedly isolated from different boreholes in the Äspö HRL tunnel (KAS03, at 533–626 m, and KR0013, SA1062 and HA1327) (Pedersen et al 1996b). One of them, Aspo-1, had a 16S rDNA identity of above 99% with Desulfomicrobium baculatum and was not studied further. This genus seems to be very common at Äspö since its 16S rDNA sequence was repeatedly retrieved from other tunnel boreholes at Äspö (KA2858 and KA3105) (Pedersen et al 1997c). The isolate Aspo-2 was characterised in detail and was described as a new species, Desulfovibrio aespoeensis (Motamedi and Pedersen 1998). It is a mesophilic species with growth characteristics that appear well adapted to life in the aquifers, from where it was isolated.

Three autotrophic, methane-producing strains of Archaea were isolated from the Äspö HRL tunnel boreholes at depths of 68 m, 409 m and 420 m. These organisms were non-motile small, thin rods, 0.1–0.15 μm in diameter, and able to utilise H_2+CO_2 or formate as substrates for growth and methanogenesis. One of the isolates, denoted A8p, was studied in detail. Phylogenetic characterisation based upon 16S rRNA gene sequence comparisons placed this isolate in the genus Methanobacterium. Phenotypic and phylogenetic characters indicate that the alkaliphilic, halotolerant strain A8p represents a new species and we proposed the name Methanobacterium subterraneum. It grew with a doubling time of 2.5 hours under optimal conditions (20– 40°C, pH 7.8–8.8, and 0.2–1.2 M NaCl). Methanobacterium subterraneum is eurythermic since it can grow at a wide range of temperatures, from 3.6°C to 45°C.

Methane is common in most groundwater studied (Table 3-4). There has been a growing interest in methanotrophs. Their consumption of oxygen, with methane as electron donor, is beneficial for HLW repositories and their activities have therefore been studied in detail (Kotelnikova and Pedersen 1999). During the investigations of microbial methane oxidation in the Äspö HRL tunnel, several oxygen-dependent methanotrophic isolates were obtained and a first isolate, SR5, was successfully described in close collaboration with Russian experts on methylotrophic bacteria (Kalyuzhnaya et al 1999). Methane-utilising bacteria were first enriched from deep granitic rock environments and affiliated by amplification of the functional and phylogenetic gene probes. Type I methanotrophs belonging to the genera Methylomonas and Methylobacter dominated in the enrichment cultures from depths below 400 m. A pure culture of an obligate methanotroph, strain SR5, was isolated and characterised. Based on phenotypical and genotypic characteristics, we proposed to refer to the strain SR5 as a new species Methylomonas scandinavica. Pigmented motile rods of the new organism contained intracytoplasmic membranes as stacks of vesicles, assimilated methane via the ribulose monophosphate pathway and had an incomplete tricarboxylic acid cycle. Methylomonas scandinavica grows at temperatures of 5–30^{o}C, with an optimum of 15^{o}C, close to the in situ temperature. Whole cell protein, and enzyme and physiological analyses of the M scandinavica revealed significant differences between this and the other representatives of Type I methanotrophs. The prospect of anaerobic methane oxidation is an intriguing possibility which has been approached in different environments (Hindrichs et al 1999). However, absolute evidence in the form of a laboratory culture of an anaerobic methane-consuming species or a consortium is still lacking.

3.4.7 Hydrogen dependency in deep microbial ecosystems

The repeated observations of autotrophic, hydrogen-dependent microorganisms in the deep aquifers studied (Fig 3-11 D and F, Table 3-5) suggest that hydrogen is an important electron and energy source and that carbon dioxide is a important carbon source for the subsurface biosphere. Hydrogen and carbon dioxide have been found in μM concentrations at all sites investigated for these gases (Table 3-4), together with methane which is a major product of HMs and AMs, and which has been shown to be very active in vitro at the Äspö HRL (Table 3-5). A model of a hydrogen-driven biosphere in deep Fennoscandian Shield igneous rock aquifers has therefore been suggested in various shapes during the course of the subsurface microbiology programme (Kotelnikova and Pedersen 1998; Pedersen 1993b, 1997a, 1999; Pedersen and Albinsson 1992). The organism base for this biosphere is suggested to be composed of AAs which have the capability of reacting hydrogen with carbon dioxide to produce acetate and AMs. These AMs in turn yield methane from hydrogen and carbon dioxide or from acetate produced by AAs (or acetoclastic methanogens) (Fig 3-12). All components needed for the life cycle in Figure 3-12 have been shown in deep igneous rock aquifers and the microbial activities expected have been demonstrated at significant rates in vitro. The model consequently has convincing support from the qualitative data obtained. The in situ rates remain to be examined, which process will require meticulous experimental conditions, because of the expected very slow metabolic rates under undisturbed conditions. The central question to be addressed during such an experimental endeavour is whether hydrogen-driven microbial chemolithotrophic in situ activities at depth are in balance with the renewal rates of hydrogen. Such balance is crucial for the unequivocal confirmation of a deep hydrogen-driven biosphere in the deep igneous rock aquifers of the Fennoscandian Shield, where a HLW repository will be placed.

The theory of a deep biosphere driven by hydrogen generated in deep geological strata (Fig 3-12) requires more research. There are at least six possible processes by which crustal hydrogen is generated: (1) reaction between dissolved gases in the system C-H-O-S in magmas, especially in those with basaltic affinities, (2) decomposition of methane to carbon (graphite) and hydrogen at temperatures above 600°C, (3) reaction between CO_2, H_2O and CH_4 at elevated temperatures in vapours, (4) radiolysis of water by radioactive isotopes of uranium, thorium and their daughters, and potassium, (5) cataclasis of silicates under stress in the presence of water, and (6) hydrolysis by ferrous minerals in mafic and ultramafic rocks (Apps and Van de Kamp 1993). It is important to explore the scale of these processes and the rates at which the produced hydrogen is becoming available for deep microbial ecosystems.

3.4.8 Microbial oxygen reduction

The accurate performance of a HLW repository requires an oxygen-free, reduced environment. Oxygen is corrosive for the copper canisters and some radionuclides, such as Np, Pu, Tc and U, are more soluble and mobile under oxygenated conditions. Oxygen is introduced with air to the repository during the open phase. At closure, some of this air will be captured in voids of the repository. Other ways of oxygen intrusion have also been suggested, such as transport with groundwater from the ground surface, penetration of oxygen into the rock in the tunnels, which will create an oxidised rock environment and finally, radiolysis of water to oxygen and hydrogen if radionuclides escape owing to a canister failure. Periods of glaciation provide a special scenario, when the transport of surface water from melting ice deep into the ground can be significant.

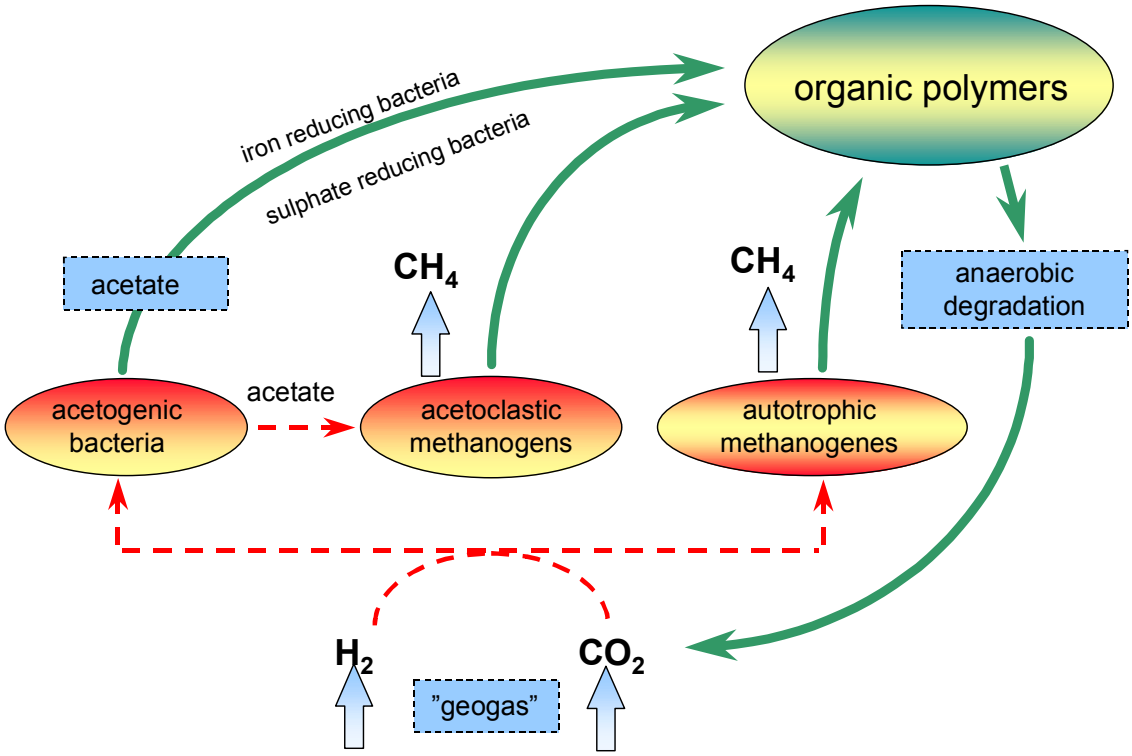


Figure 3-12. *The deep hydrogen-driven biosphere hypothesis, illustrated by the carbon cycle. At relevant temperature and water availability conditions, subterranean microorganisms are theoretically capable of performing a life cycle that is independent of sun-driven ecosystems. Hydrogen and carbon dioxide from the deep crust of Earth, or organic carbon from sedimentary deposits can be used as energy and carbon sources. Phosphorus is available in minerals such as apatite and nitrogen for proteins; nucleic acids, and so on can be obtained via nitrogen fixation; and nitrogen predominates in most groundwaters (Table 3-4).*

The possibility that microorganisms may be able to buffer against an oxidising disturbance in bentonite, backfill and the deep host rock environment had previously been overlooked. Microbial decomposition and the production of organic material depend on the sources of energy and on the electron acceptors present. Hydrogen, organic carbon, methane and reduced inorganic molecules are possible energy sources in subterranean environments. During microbial oxidation of these energy sources, the microbes use electron acceptors in a certain order (Fig 3-13). First, oxygen is used, followed by nitrate, manganese, iron, sulphate, sulphur and carbon dioxide. Simultaneously, fermentative processes supply the respiring microbes with hydrogen and short organic acids. The solubility of oxygen in water is low and for many microbes, oxygen is the preferred electron acceptor. This is because the microbes get much more energy per organic molecule if the molecule has been oxidised with oxygen, than they do with other electron acceptors. The presence of an active and diversified microbiota at repository depths is well documented in this report, as is the reducing capacity of microorganisms in any environment. The major oxygen buffers that can be used are methane and organic carbon. Hydrogen, sulphide and ferrous iron can also contribute but these compounds generally appear in much lower concentrations than do methane and organic carbon. However, locally, they may have a significant effect.

During the past decade, a series of different projects have been launched, aiming at understanding the fate of oxygen in a repository as well as the redox buffer capacity of rock and groundwater. The general conclusion from these projects is that microbes will dominate the oxygen removal and redox control processes. The projects are reviewed below.

3.4.8.1 The Äspö redox investigations in block scale – the "REDOX" project

The first project studied the induction of organic carbon oxidation during large-scale shallow (0–70 m) water intrusion into a vertical fracture zone at the Äspö HRL (Banwart et al 1996). The initial models suggested that oxygenated groundwater should reach the tunnel at 70 m depth, 3–4 weeks after the fracture zone is intersected by the tunnel (Banwart 1995). Oxygen never appeared. Instead, an increased ferrous iron concentration and an increase in alkalinity were observed. The conclusion was that microbes degrading organic carbon rapidly consume intruding oxygen. The degradation continues after all oxygen has been consumed, but now with ferrous iron as the dominating electron acceptor (Fig 3-13).

3.4.8.2 Microbial oxygen reduction in the Äspö tunnel – the "Microbe-REX" project

A variety of bacteria, the methanotrophs, readily oxidise methane with oxygen. They utilise oxygen as an electron donor for energy generation and as a sole source of carbon. Most of these bacteria are aerobes and are widespread in nature soils and water. They also present a morphological diversity, and appear related solely through their ability to oxidise methane. Methanotrophs are found wherever stable sources of methane are present. There is some evidence that although methane oxidisers are obligate aerobes, they are sensitive to oxygen and prefer microaerophilic habitats for development. Recently published data, however, indicate that methane oxidation can occur in some anaerobic environments (Hindrichs et al 1999). The methane oxidisers are often concentrated in a narrow band between anaerobic and aerobic zones were methane meets an oxygenated system. Such environments will be common in future repositories during the open phase and for some time after closure. Once established, this group of bacteria will be active for as long as oxygen is present for the oxidation of methane. After closure, they will most probably react all available methane with the remaining oxygen.

The project "Microbe-REX" established that methane oxidisers are very common in the Äspö HRL tunnel (Kotelnikova and Pedersen 1999). Consequently, a deep repository will rapidly become anoxic after closure if methane is in excess. One molecule of methane (CH_4) contains eight electrons that can be used to reduce two molecules of oxygen (O_2). In the worst case scenario, there will be approximately 250 μM dissolved oxygen in groundwater close to the repository, which can be balanced by 125 μM methane. The concentration of methane in many deep groundwaters is so high (Table 3-4) that for microbial removal of all oxygen, all that is needed is one to five volumes of methane-containing groundwater to mix with one volume of oxygen-containing groundwater. The time required for this process depends on the bacterial activity, but it will probably take much less than a year, as most microbes work very fast when given the chance to proliferate. The "Microbe-REX" project also documented a significant microbial oxygen consumption with the organic carbon naturally present in groundwater (Table 3-3). A model was developed to predict the microbial oxygen reduction in a repository based on chemical groundwater data, microbial groups present, and specific

kinetic properties of these microbes (Km, V_{max}, K_{mO2}, V_{maxO2} and E) (Kotelnikova and Pedersen 1999).

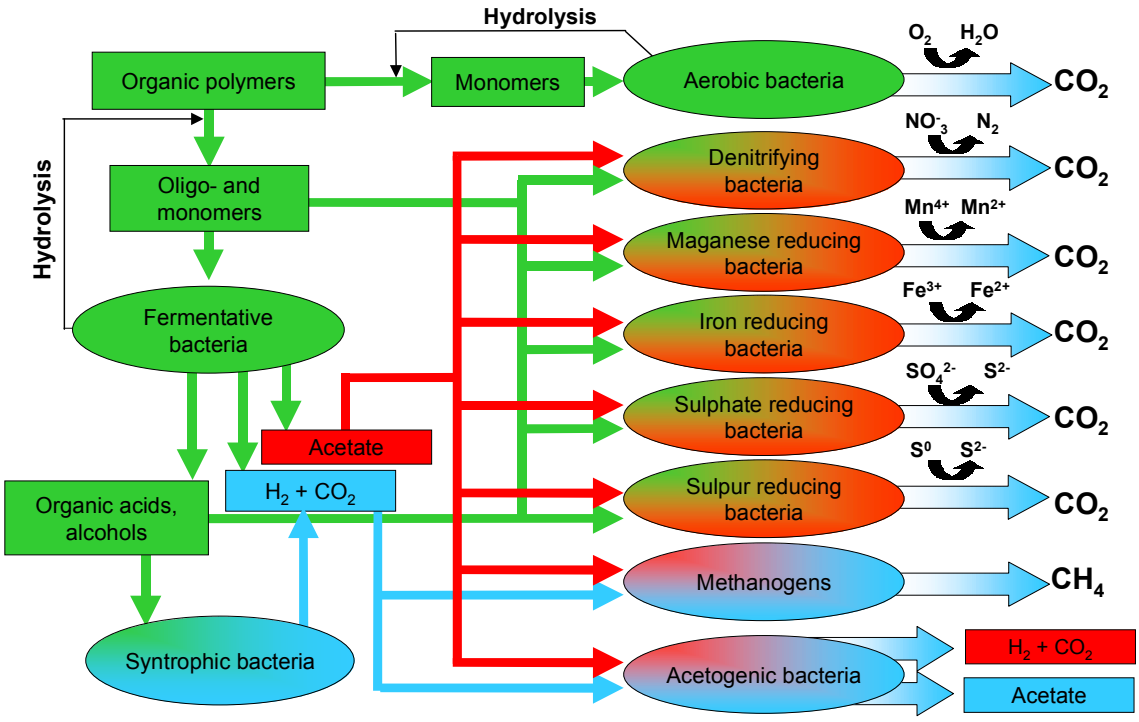


Figure 3-13. *The degradation of organic carbon can occur via a number of different metabolic pathways, characterised by the principal electron acceptor in the carbon oxidation reaction. A range of significant groundwater compounds are formed or consumed during this process. Of great importance for HLW disposal is the production of hydrogen sulphide, a potential copper corrodant, and the turnover of gases such as carbon dioxide, hydrogen and methane.*

3.4.8.3 Redox experiment on a detailed scale – the "REX" project

The main objective of the "REX" project was to investigate dissolved molecular oxygen consumption by creating a controlled oxidising perturbation in a deep rock environment at the Äspö HRL. The fieldwork of this project was completed in the summer of 1999 and the preparation of final reports is currently in progress. Briefly, the results demonstrated that introduction of oxygen to an anaerobic hard rock aquifer induces growth of aerobic attached and unattached microbial populations. During the experiments, the proportion of culturable aerobic microbes increased continuously, while the number of culturable strict anaerobic microbes decreased. The aerobic microbes were demonstrated to have a dominating role in the oxygen reduction during a series of oxygen injection experiments. Laboratory work with a replica rock core confirmed the dominating influence of microbes on oxygen reduction in a HLW host rock environment.

3.4.8.4 Tunnel microbes reduce oxygen with ferrous iron, sulphide or manganese

Tunnels in hard rock commonly develop brown, black or white precipitates on walls, in ponds and in ditches (Pedersen and Karlsson 1995). The majority of these masses are

built up of iron oxides and sulphur grains, mixed with tremendous numbers of microorganisms. The microbes take advantage of the energy available in ferrous iron manganese and sulphur where the tunnel wall interface provides an aerobic environment. They use oxygen to oxidise the metals and the sulphur, and this gives them energy for carbon dioxide fixation in organic molecules. These microbes are so-called "gradient organisms" living in redox gradients between anoxic and oxic environments. Their activity stops oxygen from migrating into the rock and they produce organic carbon from carbon dioxide.

3.4.9 Model for how microbial activity interacts with the geochemistry of groundwater

The three projects described above, with experimentally independent methods, all conclude that microbes in hard rock aquifers and tunnels are capable of reducing oxygen. The results indicate that a large benefit of geosphere microbes for repository performance is their massive capacity to protect the host rock and repository from oxygen, and their production of groundwater components that lower the redox potential. Figure 3-14 illustrates a possible geosphere scenario. Oxygen will move with recharging groundwater into the basement rock and will diffuse from the tunnel air into the rock matrix. However, the recharging groundwater will contain organic matter and microbes will continuously reduce this oxygen by oxidising organic carbon. Anaerobic microbes in the hard rock aquifers in the host rock are known to reduce ferric iron, manganese(IV) and sulphate to ferrous iron, manganese(II) and sulphide with organic carbon. These metals and the sulphur will react with oxygen when the water reaches a tunnel. Mats of microbes develop on the tunnel walls where groundwater seeps out and produce organic carbon with the energy derived from these groundwater components. Other microbes can later use the organic matter for additional oxygen reduction. Thus the microbes close biogeochemical cycles (Pedersen and Karlsson 1995).

Periods of glaciation present a special case (Fig 3-14). During such events, the input of organic carbon with recharging groundwater will be low because during a glaciation, photosynthetic production of organic carbon will cease. The REX projects demonstrated a significant activity of methane-oxidising bacteria. Methane is produced in deep magmatic rocks and migrates upwards (Apps and Van de Kamp 1993). The continuous flow of methane from deep mantle rocks will not depend on glaciation events. Hydrogen is an even better oxygen reducer for microbes than is methane, but this gas appears in lower concentrations (Table 3-4).

3.5 Natural analogues

Natural analogues can be used for comparisons between predicted events in the evolution of a repository and similar events in nature that have occurred within a geological period. Research on microbial processes in the geosphere has shown that they are important for repository performance and also for our conceptual understanding of biogeochemical processes. The research into microbial processes in natural analogues has thus been motivated, and the natural nuclear reactors of Oklo, in Gabon, the uranium body of Palmutto, Finland, and the alkaline spring water of Maqarin, in Jordan, were investigated. The results and interpretations are summarised below.

3.5.1 Bangombé

Molecular investigations during Phase I of the OKLO natural analogue project demonstrated that the reactor zone at Bangombé, Gabon, is inhabited by a diverse microbial population (Crozier et al 1999; Pedersen et al 1996a; Pedersen et al 1996c).

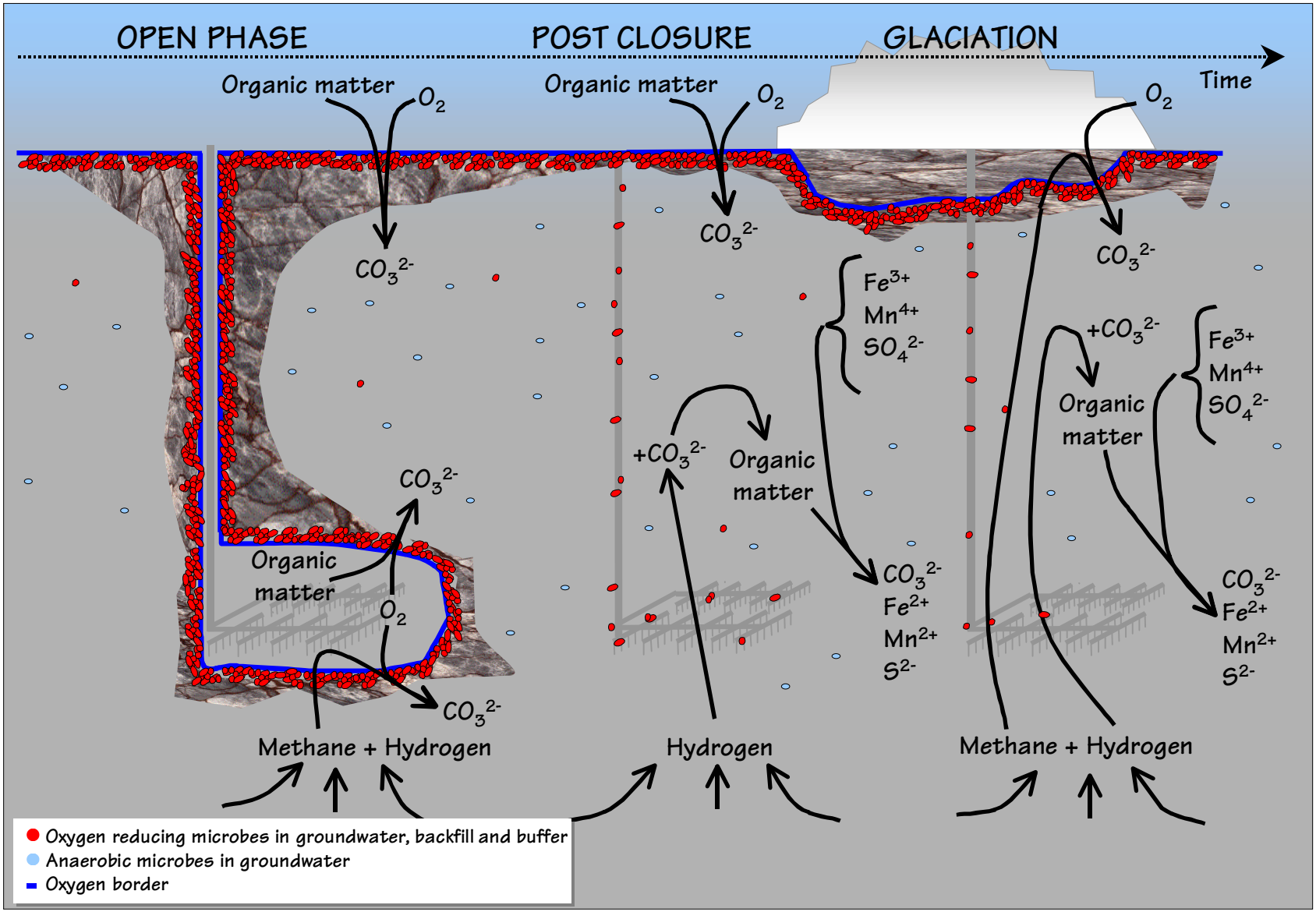


Figure 3-14. *A schematic model of how microbes in the geosphere would stop oxygen from reaching a HLW repository and keep the groundwater redox potential at low levels. See Section 3.4.9 for an explanation.*

It was concluded that the next step in the microbiological investigations should be to gather information about predominating groups and the influence of their activity on the groundwater environment. Important parameters to study, which relate to radionuclide mobility, are the effects of the microorganisms on the redox potential and on the mobility of metals. The Bangombé reactor zone was revisited in September 1996 and February 1998 and a field analysis programme was designed with the main aim of investigating distribution and numbers of bacteria, paying special attention to IRB and SRB. These two groups of microorganisms oxidise organic matter with iron(III) and $SO4^{2-}$, producing iron (II), and S^{2-}, respectively. Geosphere research at the Äspö HRL has demonstrated IRB to be very important for oxygen reduction and the redox control (see 3.4.9 "Model for how Microbial Activity Interacts with the Geochemistry of Groundwater"). The Bangombé reactor offered an excellent opportunity to investigate the long-term effect of microbes on redox stability.

Four expeditions, with microbiology as part of the aim, were undertaken. The first two, in 1993 and 1994, aimed to study the microbial diversity of the analogue. During the third, in September 1996, new boreholes were drilled. Fracture surfaces were collected for microscopic investigations. The fourth expedition, in February 1998, aimed to collect samples to determine number and diversity of microorganisms in the reactor zone. Results of the two first expeditions have been published in depth (Crozier et al 1999; Pedersen, Allard et al 1996; Pedersen et al 1996b) and are therefore only briefly mentioned here.

3.5.1.1 The 1996 expedition

The objective of the 1996 expedition was to sample fracture surfaces for microscopy investigations. Before the expedition, on-site personnel were instructed on sampling techniques and the handling of samples. Samples were successfully obtained and delivered in Göteborg, Sweden, on two occasions during autumn in 1996. The aim was to search for attached microbes on fracture surfaces in water-conducting parts of the Bangombé aquifer system and to use a nucleic acid probe method to study the diversity of possibly attached microbes. Drill core samples were obtained during drilling from the BAX08 (960911), BAX11 (961001) and BAX14 (961007-09) boreholes, preserved in formaldehyde/alcohol, and transported to Sweden. Several different microscopy investigations were performed. The experimental approach was that scanning electron microscopy (SEM) would reveal surface structures that could possibly be microbes, while transmission electron microsopy (TEM) and confocal laser scanning microscopy (CLSM) were supposed to confirm the SEM observations.

SEM analysis. Three fracture samples from BAX08, 10.88 m, coloured red were observed through a scanning electron microscope equipped with an energy dispersive X-ray sond (EDS). The pieces of rock were placed directly onto aluminium stubs, with the untreated fracture in a sideward or upward position, using a two-component epoxy resin, and sputtered with gold-palladium for 2 minutes. The specimens were observed under a JSM 6400 scanning electron microscope.

TEM analysis. Fracture samples similar to those used for SEM analysis were cut into 0.5 cm thick 0.5 x 1 cm coupons with a rock-cutting device, with the fracture coating and the rock immediately under it left preserved. The coating side of the rock coupons was ground flat using an aluminium-oxide grinding tool and glued to plastic slides with a two-component epoxy glue. Subsequently, coupons were ground to 30 µm sections consisting mainly of coatings and partly of granite. Several subsamples were collected from each such section using a razor blade, put into gelatine capsules, dehydrated with

99.5% ethanol for 30 minutes, and embedded in epoxy plastic (LR White Resin, Hard Grade Acrylic Resin, London Resin Company Ltd, Reading, UK). After 30 minutes, the plastic was replaced with a new batch to remove residual alcohol, and left overnight at room temperature, before a final replacement of plastic was performed and hardening at 60°C for 24 hours allowed. Thin sections were made with a diamond knife on carbon-formvar-coated copper grids, and stained with uranyl acetate. A Jeol JEM 100S transmission electron microscope (at 60kV) was used for viewing and photographing the samples. Microanalysis of possible microfossils was done with a Philips CM200 TEMscan microscope (120 kV) equipped with an EDAX energy dispersive X-ray system. For each X-ray analysis of the microfossils, one analysis of the epoxy/grid film background was made.

Confocal laser microscopy. Fracture surfaces from BAX11, 9.95–10.33 m, were studied by in situ hybridisation with a Cy-5-labelled probe for the domain Bacteria (EUB-338), revealing attached bacteria on the surface. The Cy-dyes are based on the cyanine fluor and all seven different flours offer intense colours with narrow emission spectra (Amersham LIFE SCIENCE). A Molecular Dynamics 2010 confocal laser scanning microscope equipped with a Kr/Ar laser was used for observation with the software Image Space running on a Silicon Graphics UNIX-based computer. In situ hybridisation with a Cy-5-labelled probe for the domain Archaea (ARC-915) was also done.

3.5.1.2 The 1998 expedition

Table 3-8 shows the boreholes investigated. It is of importance that sampling of the "dead volume" water, with a long residence time in the borehole or in tubes, is avoided. Each borehole was therefore pumped for a period of time ensuring that the water sampled originated from aquifers in the packed-off sections. The number of dead volumes pumped before sampling varied from 2.5 for BAX05 to eleven for BAX07. Specified volumes for the different microbiological and chemical investigations were collected from the orifice of the tube from the pumped sections.

Table 3-7. Bangombé reactor zone field sampling data, February 1998.

Borehole	Section (high-low packer) (m)	Sampling date (Y-M-D)	Dead volume (litre)	Renewed volume before sampling (litre)	Borehole volume turnover
BAX02	27.2–33.9	98-02-16	101	407	4.0
BAX03	11.9–12.5	98-02-14	14	80	5.7
BAX04	8.9–10.2	98-02-14	22	147	6.7
BAX05	23.8–31.0	98-02-16	73	180	2.5
BAX07	4.5–6.5	98-02-15	8	90	11

Groundwater chemistry. Samples for groundwater chemistry were collected in 1-litre plastic bottles and shipped to an analytical laboratory in Sweden for analysis (KM Laboratory, Borås). Selected groundwater data of importance for interpretation of the microbiological data are given in Table 3-8.

Table 3-8. Chemical parameters of the investigated groundwater.

Borehole	Temp. (°C)	pH	TOC (mg l^{-1})	NO_2^- (mg l^{-1})	NH_4^+ (mg l^{-1})	Fe (mg l^{-1})	SO_4^{2-} (mg l^{-1})
BAX02	29	5.48	1.5	<0.002	<0.02	0.56	3
BAX03	31	5.54	1.2	<0.002	<0.02	1.3	4
BAX04	25	5.37	1.3	<0.002	<0.02	1.5	3
BAX05	25	5.82	1.4	0.003	<0.02	3.6	2
BAX07	25	3.99	1.4	0.014	0.29	0.06	<1

Sampling and determination of the total number of bacteria. Samples of 40 ml from BAX02, BAX03, BAX04, BAX05 and BAX07 were preserved with formaldehyde (2% final concentration) and transported to the laboratory in Göteborg. Total cell numbers were determined according to the direct count method described previously (Pedersen and Ekendahl 1990), except that filters were rinsed twice with 1.0 ml of 0.2 μm filtered (DynaGard filters, Microgon Inc, Laguna Hills, CA, USA), double-distilled water to dissolve salt crystals prior to staining for 10 minutes with 10 μg/ml AO or DAPI. Cells were counted under an Olympus BH-2 microscope, with blue filters for AO and UV filters for DAPI. Results were calculated as an average of two filters prepared for each stain, with sample standard deviation (SD) as the error.

Media for and culturing of IRB and SRB. Media were designed for each groundwater sample based on chemical data measured during an earlier field campaign. This allowed synthetic media to be designed which came close to the groundwater chemistry, with the goal of providing the microbes with a medium similar to their natural environment. All media were prepared anaerobically, according to the Hungate method (Hungate 1969). Salt and buffer concentrations varied according to borehole parameters analysed earlier (Pedersen et al 1996c). In addition, all media contained 0.01 mg l^{-1} KH_2PO_4, 0.002 g l^{-1} Na_2SO_4, 0.001 g l^{-1} $FeCl_2 \cdot 4H_2O$, 0.25 g l^{-1} cysteine $HCl \cdot H_2O$, 0.25 g l^{-1} $Na_2S \cdot 9H_2O$, 10 ml element solution, 5 ml vitamin solution (Wolin et al 1963), and 0.2 mg resazurin. The element solution contained 12.8 g l^{-1} nitrilotriacetic acid, 0.1 g l^{-1} $FeSO_4 \cdot 7H_2O$, 0.1 g l^{-1} $MnCl_2 \cdot 4H_2O$, 0.17 g l^{-1} $CoCl_2 \cdot 6H_2O$, 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 g l^{-1} $ZnCl_2$, 0.02 g l^{-1} $CuCl_2$, 0.01 g l^{-1} H_3BO_2, 0.01 g l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 1 g l^{-1} NaCl, and 0.01 g l^{-1} Na_2SeO_3. The pH of the media was adjusted to borehole pH analysed earlier (Pedersen et al 1996c) after autoclaving. Substrates were added to separate aliquots of media for the different physiological groups of microorganisms investigated. The medium for SRB contained 14 mM Na_2SO_4 and 6 mM lactate. The medium for IRB contained 7 g l^{-1} amorphous iron and 11 mM lactate. The different types of media were dispensed anaerobically in 9 ml aliquots in sterile Hungate test tubes (Bellco) with N_2 in the gas phase, and stoppered with sterile blue rubber stoppers (Bellco).

The MPN of each physiological group of IRB or SRB in each sample was determined (Koch 1994) using the media prepared for the various groups. After inoculation, a 1 ml aliquot of 0.2 μm filter-sterilised groundwater (DynaGard filters, Microgon Inc) was added to each MPN dilution to provide any growth factors present in the groundwater but not in the synthetic medium. Negative controls were prepared with medium only, with 1 ml 0.2 μm filter-sterilised groundwater added, inoculated with 1 ml groundwater and immediately killed with 2% formaldehyde. Thereafter, MPN tubes were incubated

on their sides in the dark at 25°C for 2 months. The MPN tubes were analysed for products of metabolism. The SRB tubes were analysed for sulphide using the $CuSO_4$ method (Widdel and Bak 1992). The IRB tubes were analysed for both total iron and ferrous iron using a spectrophotometric ferrozine method (Stookey 1970). Tubes were graded "positive" or "negative" with negative controls as comparison, and the MPN was calculated with a computer program from Yamanashi University/Ishikawajima-Harima Heavy Industries, Ltd, Japan (Hurley and Roscoe 1983). The detection limit for MPN was 0.2 cells/ml.

3.5.1.3 Drill core investigations

Energy dispersive X-ray sond analysis showed the presence of typical clay minerals, mainly consisting of Al, Si and Fe (Fig 3-15 A). Some of the material on the fracture surfaces from BAX08, at 10.88 m depth, showed large similarities with colonies of attached microbes. They had the correct size and shape, but that alone is not enough to conclusively demonstrate bacteria with SEM, unless very typical shapes and forms are observed. This was not the case here. Figure 3-15 B–D shows what could be a microcolony of coccoid bacteria but the EDS analysis indicated a strong iron signal, suggesting that these could be iron minerals and not bacteria. The SEM observations did, however, reveal some structures typical of fungi or filament-forming bacteria (Fig 3-15 E and F). The samples were also studied with TEM and some structures that could be microbes were observed (Fig 3-16 A–D). The aim of the TEM observations was to search for fossilised bacteria that have been successfully demonstrated in calcite minerals on deep hard rock aquifer walls at the Äspö HRL (Pedersen et al 1997b). Despite a large number of samples, we were unsuccessful in finding similar structures in the Bangombé reactor material. One major reason for this was the difficulty in identifying which parts of the obtained fracture material had been in contact with groundwater and which had not. Each investigation spot in the SEM and TEM investigations only covered a tiny part of a square mm, which meant chances of finding microbes in a blind search, as was the case here, were very small. This problem continued during the CLSM investigations and we were not successful in obtaining good observations that could be related to open fracture surfaces. Drilling with orientation of the drill cores, logging of boreholes for inflowing groundwater, and subsequent analysis of selected open fractures will greatly increase the likelihood of finding fracture material significant for microscopic investigations, but these are all techniques that are difficult to apply under the field conditions that prevailed at Bangombé.

Earlier investigations indicated lower total numbers of bacteria during summer (July 1994) than during spring (March 1993) (Pedersen et al 1996c). Table 3-9 compares earlier observations with the February 1998 expedition data. The February 1998 data correlate with March 1993 data, indicating a seasonal variation in the number of bacteria. Variations in number of bacteria generally reflect variations in the activity status of a habitat, with higher microbial activity during and/or after periods with an input of organic matter to the system, as during spring and the rainy seasons. An inspection of the amount of available organic carbon in relation to the total number of bacteria for each of the three sampling occasions (Fig 3-17) revealed an inverse relationship between TOC and the total number of bacteria.

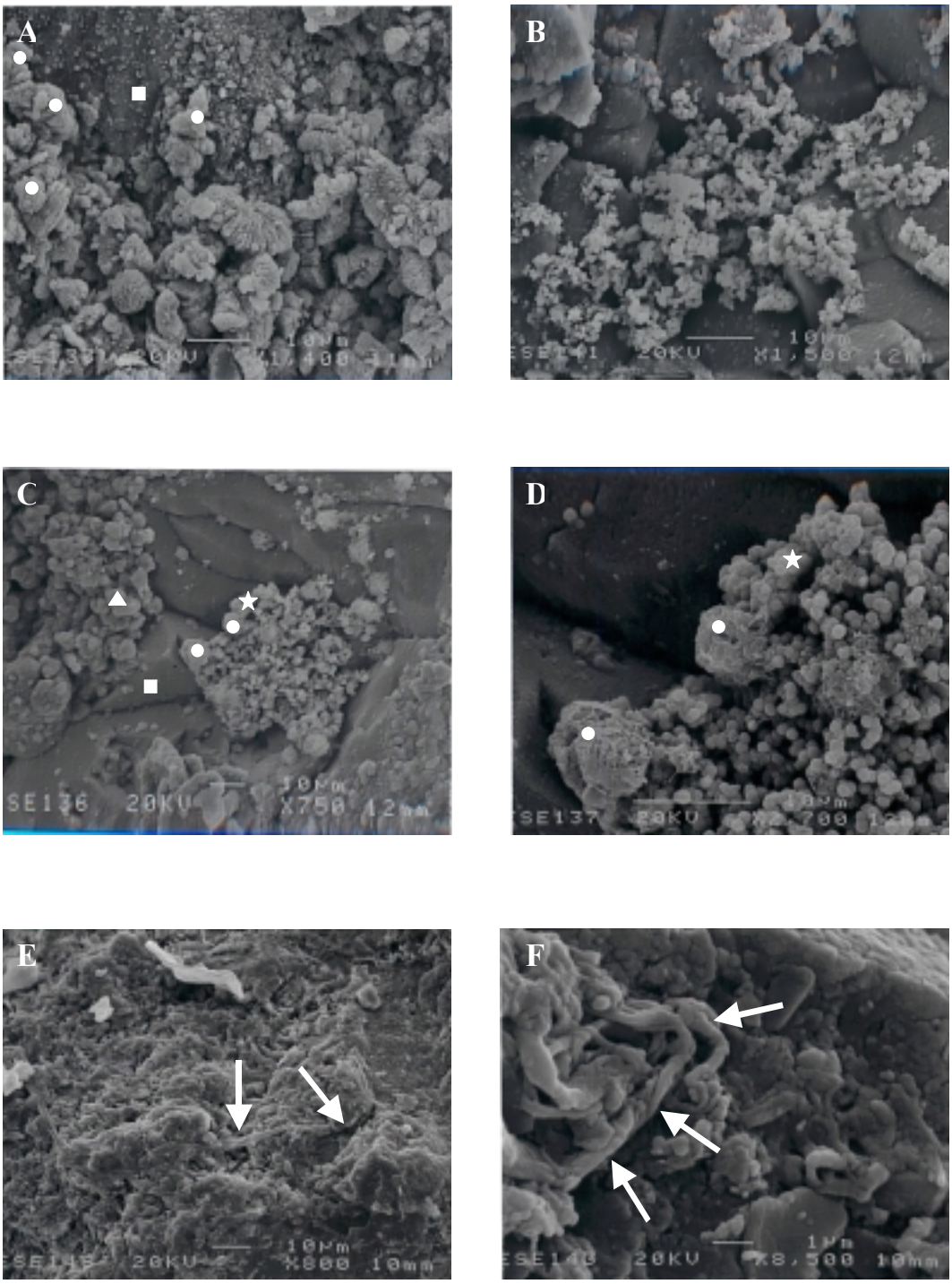


Figure 3-15. *Scanning electron microscopy images of fracture material from the BAX08 drill core at a depth of 10.88 m, approximately 18 cm below the reactor.* ***A.*** *Clay minerals on a quartz mineral. Circles show EDS probe points with an aluminium, silica and iron signal. The square shows a point with a pure silica signal.* ***B, C*** *and* ***D****. Bacterium-like structures on a quartz mineral mixed with aluminium, manganese and iron. The circles show EDS probe points with manganese, some iron and tiny amounts of aluminium. The square shows a point with a pure silica signal, the triangle shows a point with iron and aluminium, and the star shows a point with only iron.* ***E*** *and* ***F.*** *Bacterium-like filamentous structures (arrow).*

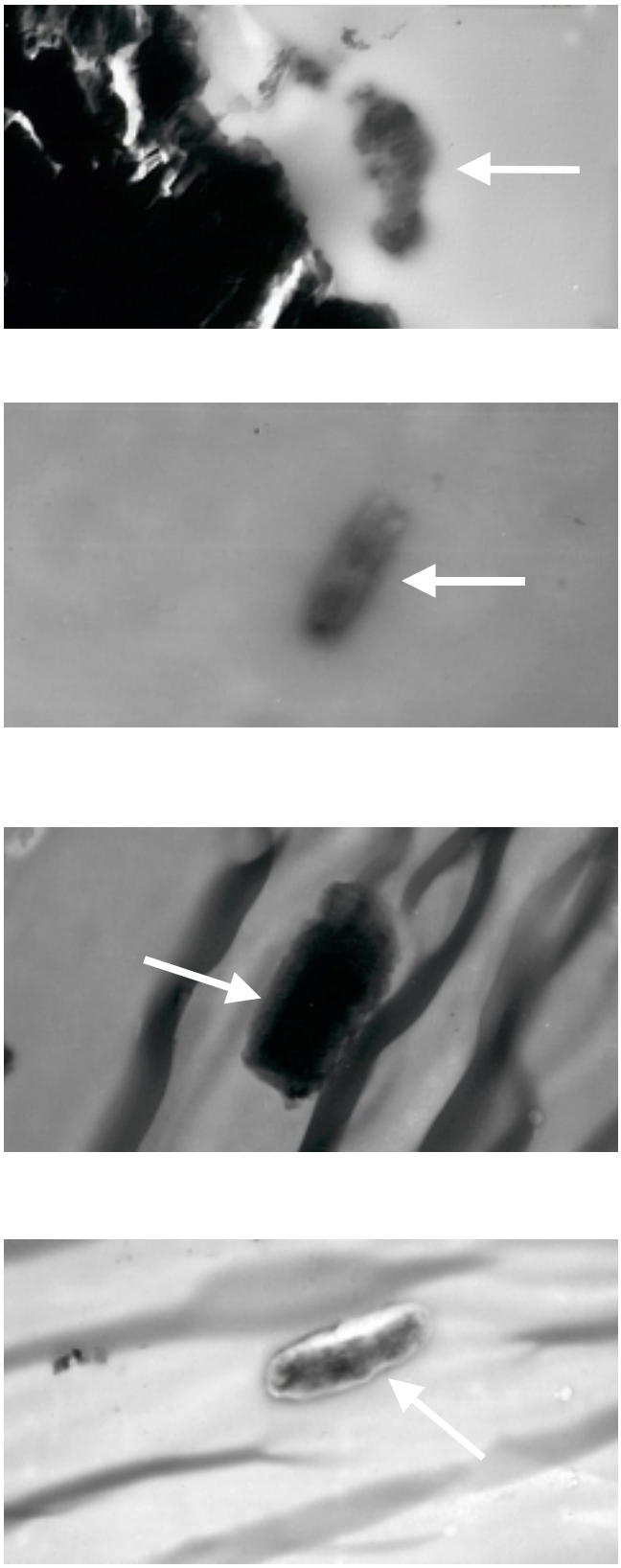


Figure 3-16. *Transmission electron microscopy images of fracture material with bacterium-shaped structures (arrow) from BAX08, 10.70 m in the reactor (scale 1 cm = 0.5 μm).*

Table 3-9. Total number of bacteria data obtained during three expeditions to the Bangombé reactor zone.

	Cells ml^{-1} x 10^5 groundwater		
Borehole	**March 1993**	**July 1994**	**February 1998**
BAX01	5.8	4.0	n.a.
BAX02	2.9	0.8	5.8
BAX03	5.5	1.3	4.9
BAX04	2.4	0.45	1.7
BAX05	1.7	n.a.[a]	1.0
BAX07	n.c.[b]	n.a.	n.a.

a = not analysed
b = not counted owing to high background.

If available, most bacteria in shallow groundwater will degrade organic carbon with O_2 as electron acceptor and employ other acceptors such as iron and sulphur when anaerobic conditions occur. As demonstrated in Figure 3-17, the reactor zone groundwater contains organic carbon, which is degradable by bacteria. Aerobic bacteria will therefore constitute a barrier for O_2 in groundwater, recharging to the reactor zone as long as organic carbon is available. The microorganisms will reduce the O_2 to water and oxidise the carbon to CO_2. The organic carbon oxidation continues with alternative electron acceptors once the O_2 is depleted, with nitrate, ferric iron and sulphate commonly used, depending on the availability of respective electron acceptors. The results from the February 1998 expedition clearly show that IRB predominate among the anaerobic organic carbon degraders studied (Table 3-10). Very few SRB could be detected. No nitrate reducers were analysed, but their activity is indirectly inferred in BAX05 and BAX07. During their respiration of nitrate, nitrate reducers produce nitrite and the lack of detectable nitrate, together with some nitrite in two boreholes, suggests that nitrate reduction has been ongoing. Once a groundwater system is depleted of nitrate, IRB and/or SRB will continue the anaerobic oxidation of organic carbon.

The main effects of aerobic and anaerobic microbial activity on the Bangombé groundwater chemistry are a consumption of dissolved O_2 and solid iron(III) oxides, and the production of CO_2. A lowering of the redox potential will occur concomitant with the production of the reduced electron acceptor iron(II). The results of multivariate mixing and mass balance calculations (M3) of the reactor zone groundwater (Gurban et al 1998) showed an increase in the alkalinity of the reactor zone, which was suggested to be a result of microbial degradation of organic matter, in accordance with what is suggested here. The report by Gurban et al (1998) did not find indications of sulphate reduction (i.e. a negative deviation in sulphate concentration relative to the predicted concentration). This model result is in agreement with the microbiology result, with very few or no detectable SRB in the sampled boreholes.

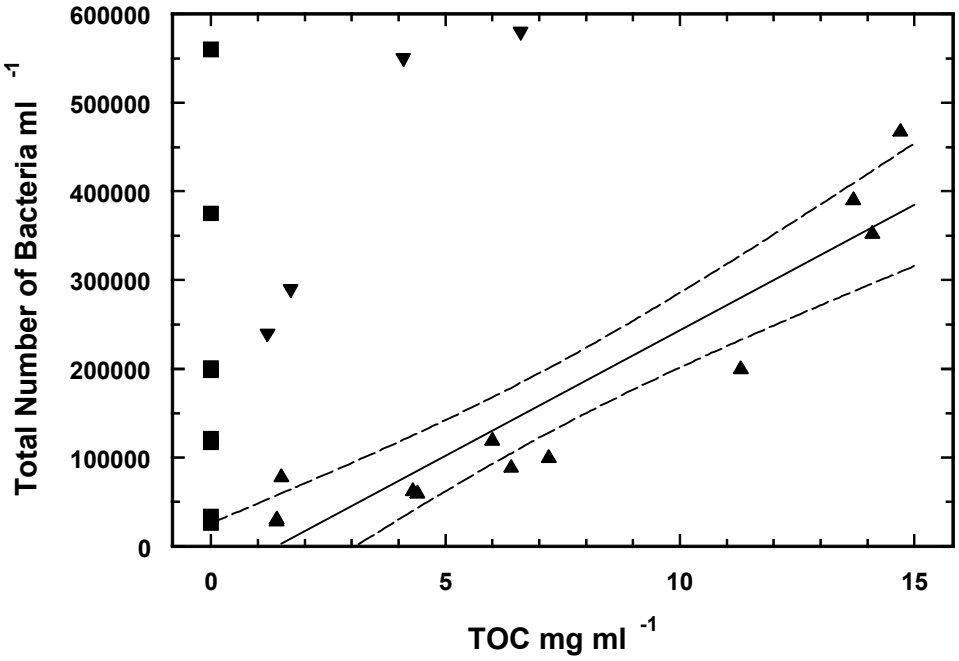


Figure 3-17. *Correlation between the total number of bacteria and the content of TOC in flowing (pumped) groundwater from the Bangombé boreholes BAX01 to BAX07, sampled in March 1993, July 1994 and February 1998.* ▼ *= March 1993;* ▲*= July 1994; and* ■ *=February 1998. The dashed lines show the 95% confidence interval; the correlation coefficient, r = 0.94.*

The distribution of IRB and SRB did not correlate with the measured concentrations of total iron and sulphate. The only relationship found between groundwater chemistry and the obtained microorganism numbers was a weak to moderate correlation with available nitrite and ammonium (Fig 3-18). That microorganisms require nitrogen for growth is well known (Pedersen and Karlsson 1995) and not surprising. Access to nitrogen in the shallow BAX07 groundwater attests favourable conditions for microbial activity, including O_2 reduction during the oxidation of organic matter.

Table 3-10. Microbiology results obtained during the February 1998 expedition.

	Cells ml^{-1} groundwater		
Borehole	**Total number of bacteria**	**Iron-reducing bacteria**	**Sulphate-reducing bacteria**
BAX02	5.8×10^5	5.4×10^3	1.7
BAX03	4.9×10^5	0.33×10^3	n.d.[a]
BAX04	1.7×10^5	0.013×10^3	n.d.
BAX05	1.0×10^5	0.17×10^3	1.7
BAX07	n.c.[b]	1.3×10^4	33

a = not detected; b = not counted owing to high background.

3.5.1.4 Main conclusions from the investigations of microbial processes in Bangombé

The Bangombé reactor zone was visited in March 1993, July 1994, September 1996 and February 1998 and a field analysis programme was designed with the main aim of investigating the distribution and size of the bacterial population, paying special attention to IRB and SRB. These two groups of microorganisms oxidise organic matter with ferric iron and SO_4^{2-}, producing iron ferrous, and S^{2-}, respectively. Electron microscopy investigations revealed structures similar to microorganisms. However, the results are difficult to interpret owing to difficulties in obtaining fracture material which conclusively is from open fractures with a flow of groundwater, water being an obligate need for bacterial activity. The main effects of aerobic and anaerobic microbial activity on the Bangombé groundwater chemistry are a consumption of dissolved O_2 and solid iron oxides, and the production of CO_2. A lowering of the redox potential appears to occur concomitantly with the production of the reduced electron acceptor iron ferrous from iron ferric. This conclusion is in agreement with multivariate mixing and the mass balance calculation (M3) of the reactor zone groundwater that showed an increase of the alkalinity in the reactor zone as a result of microbial degradation of organic matter.

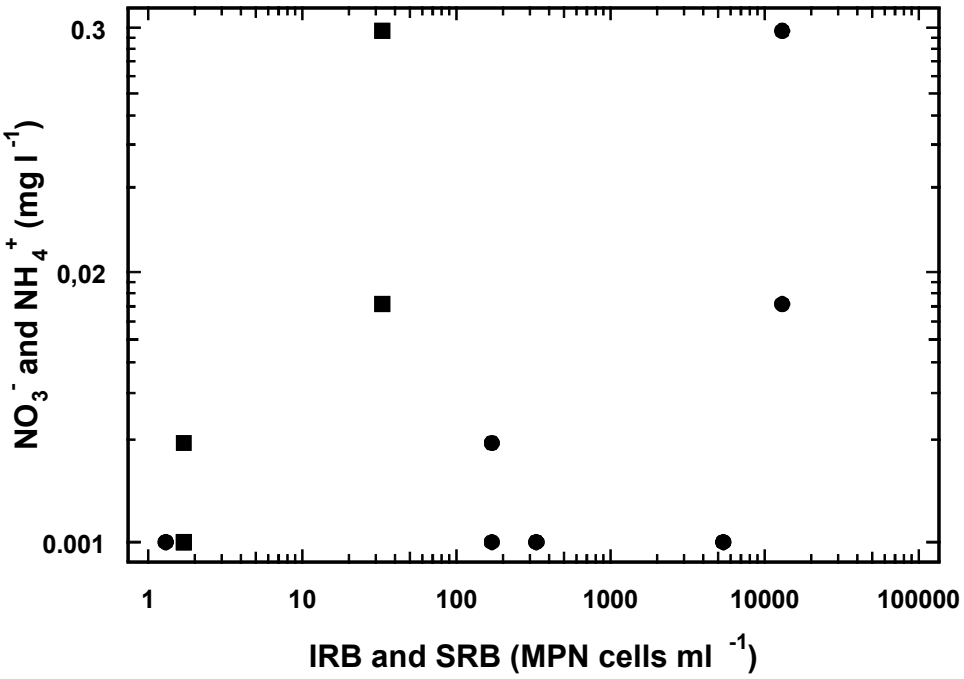


Figure 3-18. *The relationship between the MPN of IRB (●) and SRB (■), versus the concentration of nitrogen that can be used as a source of energy in the metabolism of cells during the growth and degradation of organic matter.*

3.5.2 Palmottu

The investigations of Finnish and Swedish deep groundwater described in this report have revealed that numerous populations of microorganisms dwell deep below the ground (Figs 3-10 and 3-11). The possibility of microbially driven redox processes in the Palmottu area motivated a search for several of the physiological groups of microorganisms indicated in Figure 3-11. Iron-reducing bacteria, SRB, HAs, AAs,

HMs and AMs were analysed in three Palmottu boreholes. Several investigations have demonstrated that microorganisms can reduce U(VI) to U(IV) (Lovley and Phillips 1992a, 1992b; Lovley et al 1991). The potential for uranium reduction of enrichment cultures of IRB and SRB was therefore also studied. Groundwater was sampled from the Palmottu site in south-western Finland. The boreholes and depths sampled are outlined in Table 3-11.

Table 3-11. Groundwater samples analysed.

Borehole	Depth (m)	Date sampled (Y-M-D)
R337	80–100	98-07-21
R302	80–132	98-07-21
R387	119–127	98-11-03
R387	304–309	98-09-15
R387	32–36.8	99-05-16

3.5.2.1 Methods of sampling and analysis used for Palmottu groundwater

The SKB mobile laboratory wagon (Grenthe et al 1992) was used for sampling of the borehole R387. Borehole R302 and 337 were sampled with the GTK method. Each borehole section was pumped until readings stabilised. A groundwater sample was collected in a 5-litre polycarbonate bottle and shipped on ice to the laboratory in Göteborg within 12 hours of sample collection. This groundwater was used to prepare media, as described below. Microbiologists travelled to Palmottu to collect the sample and inoculate the MPN tubes. Samples were collected inside the mobile laboratory wagon into sterile glass bottles under a stream of sterile N_2. Inoculation of media was started immediately after collection of the groundwater, and work with each sample was complete within 6 hours of sample collection. The MPN tubes were transported back to the laboratory overnight and incubated at 17^{o}C on their sides. Upon return to the laboratory, H_2 was added to AM and AA tubes at 2-bar overpressure.

Samples for geochemistry were collected during the same sampling period as the microbial samples. Analysis methods and results are reported elsewhere. Table 3-12 gives data important for microbial processes.

In previous sampling of groundwater from Finland, viable microbes were cultured using media designed for each groundwater sample based on groundwater chemical data (Haveman et al 1999). After inoculation, 10% 0.2 μm filter-sterilised groundwater (DynaGard filters, Microgon Inc) was added to provide any growth factors present in the groundwater but not in the media.

For sampling in Palmottu, media were prepared with filter-sterilised groundwater as a base. A sterile, 5-litre polycarbonate bottle was filled with groundwater and shipped to the laboratory at Göteborg University on ice. Samples arrived at the laboratory the same day. Upon receipt of groundwater, it was brought into an anaerobic chamber (Coy Laboratory Products Inc, USA.) with an atmosphere of approximately 4% H_2, 5% CO_2 and balance N_2. To sterilise the groundwater, it was filtered with 0.22 μm nitrocellulose filters. Thereafter, it was kept in the anaerobic chamber overnight and then refiltered prior to use in preparing anaerobic media.

The groundwater-based media were prepared anaerobically, according to the Hungate method (Hungate 1969). Media components were added from sterile, anaerobic stock solutions. Media contained 0.0002 g l^{-1} resazurin, 0.4 g l^{-1} NH_4Cl, 0.01 g l^{-1} KH_2PO_4, 0.002 g l^{-1} Na_2SO_4, 0.25 g l^{-1} cysteine $HCl{\cdot}H_2O$, and 0.25 g l^{-1} $Na_2S{\cdot}9H_2O$, as well as 10 ml element solution (Haveman et al 1999), and 5 ml vitamin solution (Wolin et al 1963). Buffers were added from sterile, anaerobic stock solutions, depending on borehole pH. Media for groundwater with a pH of 7.0–8.0 contained 1.72 g l^{-1} $NaHCO_3$. Media for groundwater with a pH of 8.0–9.0 contained 0.86 g l^{-1} $NaHCO_3$ and 1.21 g l^{-1} Tris HCl. The pH of the media was checked and adjusted to borehole pH, if necessary, with sterile, anaerobic HCl and NaOH solutions.

Table 3-12. Groundwater geochemistry data.

	Borehole				
Measurement	**R302 80–95 (m)**	**R337 80–100 (m)**	**R387 32–36.8 (m)**	**R387 119–127 (m)**	**R387 304–309 (m)**
Ph	8.47	7.7	7.0	8.9	8.7
E_h (mV)	–40	+20	+250	–300	–300
HCO_3 (mg $l^{-1)}$	103.7	103.7	115.9	134.2	54.9
NO_3 (mg $l^{-1)}$	<0.2	<0.2	<0.2	<0.2	<0.2
Mn (mg $l^{-1)}$	0.034	0.291	0.016	0.0253	0.0254
Fe_{tot} (mg $l^{-1)}$	0.12	0.2	<0.03	0.7	0.08
SO_4^{2-} (mg $l^{-1)}$	14.4	17.3	14	28.2	747
U (µg l^{-1})	369	172	87.8	8.45	1.56
Na (mg $l^{-1)}$	17.5	17.4	1.89	57.5	506
Ca (mg $l^{-1)}$	17.4	20.5	35.9	6.99	39.4
Mg (mg $l^{-1)}$	4.4	5.43	1.43	2.14	14.9
Cl (mg $l^{-1)}$	1.5	2.6	1.2	14	315

For the R387/304–309m sample, synthetic medium was prepared as a comparison with the groundwater-based media. This synthetic medium contained 0.0002 g l^{-1} resazurin, 0.4 g l^{-1} NH_4Cl, 0.01 g l^{-1} KH_2PO_4, 0.002 g l^{-1} Na_2SO_4, 0.1 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0.05 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0.001 g l^{-1} $FeCl_2{\cdot}4H_2O$, 1.29 g l^{-1} $NaHCO_3$, 1.82 g l^{-1} Tris HCl, 0.25 g l^{-1} cysteine $HCl{\cdot}H_2O$, and 0.25 g l^{-1} $Na_2S{\cdot}9H_2O$, as well as 10 ml element solution (Haveman et al 1999), and 5 ml vitamin solution (Wolin et al 1963). The pH of the media was checked and adjusted to a pH of 8.7 with sterile, anaerobic HCl and NaOH solutions.

Substrates were added to separate aliquots of media for the different physiological groups of microorganisms investigated. The medium for AMs contained no additions. The medium for HMs contained 10 mM acetate, 10 mM trimethylamine (TMA), 50 mM methanol and 74 mM formate. The medium for AAs contained 50 mM 2-bromethanesulphonic acid (BESA) as an inhibitor of methanogenesis. The medium for HAs contained 50 mM BESA, 10 mM TMA, 74 mM formate, and 2 g l^{-1} yeast extract. The medium for SRB contained 14 mM Na_2SO_4 and 6 mM lactate. The medium for IRB contained 7 g l^{-1} amorphous iron and 11 mM lactate. For the HH-KR6 sample, IRB were tested with a combination of acetate and lactate. This IRB+acetate medium

contained 7 g l^{-1} amorphous iron, 11 mM lactate, and 10 mM acetate. The different types of media were dispensed anaerobically in 9 ml aliquots in sterile Hungate test tubes (Bellco) with N_2 in the gas phase, and stoppered with sterile blue rubber stoppers (Bellco).

Total cell numbers were determined according to the direct count method previously described for Bangombé (3.5.1). The MPN of each physiological group of Bacteria or Archaea in each sample was determined using the media prepared for the various groups. For the groundwater-based media, negative controls were prepared with medium only, inoculated with 1 ml groundwater and immediately killed with 2% formaldehyde. In the case of synthetic media, a 1 ml aliquot of 0.2 μm filter-sterilised groundwater (DynaGard filters, Microgon Inc) was added to each dilution to provide any growth factors present in the groundwater, but not in the media. Three types of negative controls were prepared for the synthetic media: with medium only, with the addition of 1 ml 0.2 μm filter-sterilised groundwater, and inoculated with 1 ml groundwater and killed immediately with 2% formaldehyde. The AM and AA tubes were gassed with 2-bar overpressure oxygen-free H_2. The MPN tubes were incubated on their sides in the dark at 17°C for 6–8 weeks.

The MPN tubes were analysed for products of metabolism. The methanogenic (i.e. AM and HM) tubes were analysed for presence of CH_4 by gas chromatography, as described previously (Kotelnikova and Pedersen 1998). The acetogenic (i.e. AA and HA) tubes were also analysed for CH_4 as negative controls for methanogenesis. Acetate was analysed in the acetogenic tubes by an enzymatic and UV method (Boehringer Mannheim, Mannheim, Germany). The SRB tubes were analysed for sulphide using the $CuSO_4$ method (Widdel and Bak 1992), and the IRB tubes were analysed for both total and ferrous iron using spectrophotometric ferrozine (Stookey 1970). Tubes were graded "positive" or "negative" with negative controls as comparison and MPN was calculated with a computer program from Yamanashi University/Ishikawajima-Harima Heavy Industries, Ltd, Japan (Hurley and Roscoe 1983). The detection limit for MPN was 0.2 cells/ml.

The uranium reduction capability of the sulphate reducers and iron reducers grown in the MPN tubes from R387, at 304–309 m, was next tested. The synthetic media described above were used, in 9 ml aliquots in anaerobic tubes. All media contained 11 mM lactate as carbon source. Uranium was added from a stock solution of 0.01 M UO_2^{2+} in 0.1 M HCl to a final concentration of 100 μM. Three variants were tested, namely with uranium only, with uranium plus 14 μM Na_2SO_4, and with uranium plus 9 μM amorphous ferric iron. Several different types of controls that were prepared were uninoculated, without uranium, with antibiotics (0.1 g l^{-1} streptomycin and 0.1 g l^{-1} ampicillin) to inhibit microbial activity, and with 20 mM molybdate to inhibit sulphate reduction. Tubes were inoculated with 0.1 ml of a mixture of the first row of MPN tubes. Uranium only tubes were inoculated with a mixture of SRB and IRB. Uranium plus sulphate tubes were inoculated with SRB, and uranium plus iron tubes were inoculated with IRB. Tubes were incubated on their sides at 17°C for 15 weeks. Tubes were analysed for sulphide and ferrous iron and samples were preserved for total counting. Remaining media were centrifuged at 20 000 rpm for 30 minutes. The supernatant was analysed for uranium by GTK.

3.5.2.2 Numbers of microorganisms

Results of total counting and MPN are presented in Table 3-14 and Figure 3-19. These numbers represent the highest results of synthetic or groundwater-based media where both were analysed. Comparisons between synthetic and groundwater-based media for the R387 borehole at 304–309 m are presented in Table 3-14.

Table 3-13. Results of total counting and MPN analyses of Palmottu samples.

	Borehole				
Cells counted	**R337 80–100 (m)**	**R302 80–132 (m)**	**R387 32–36.8 (m)**	**R387 119–127 (m)**	**R387 304–309 (m)**
Total number of bacteria (± SD) x 10^5	0.59	0.65	2.1	1.4	0.23
Autotrophic methanogens	n.t.[a]	n.t.	n.t.	<0.2	<0.2
Heterotrophic methanogens	n.t.	n.t.	<0.2	<0.2	<0.2
Autotrophic acetogens	n.t.	n.t.	n.t.	<0.2	3.3
Heterotrophic acetogens	n.t.	n.t.	<0.2	790	330
Sulphate-reducing bacteria	<0.1	<0.1	0.8	24	170
Iron-reducing bacteria	<0.1	<0.1	54	13 000	3300
% of total cells cultured	0	0	0.026	9.9	30.1

a = not tested.

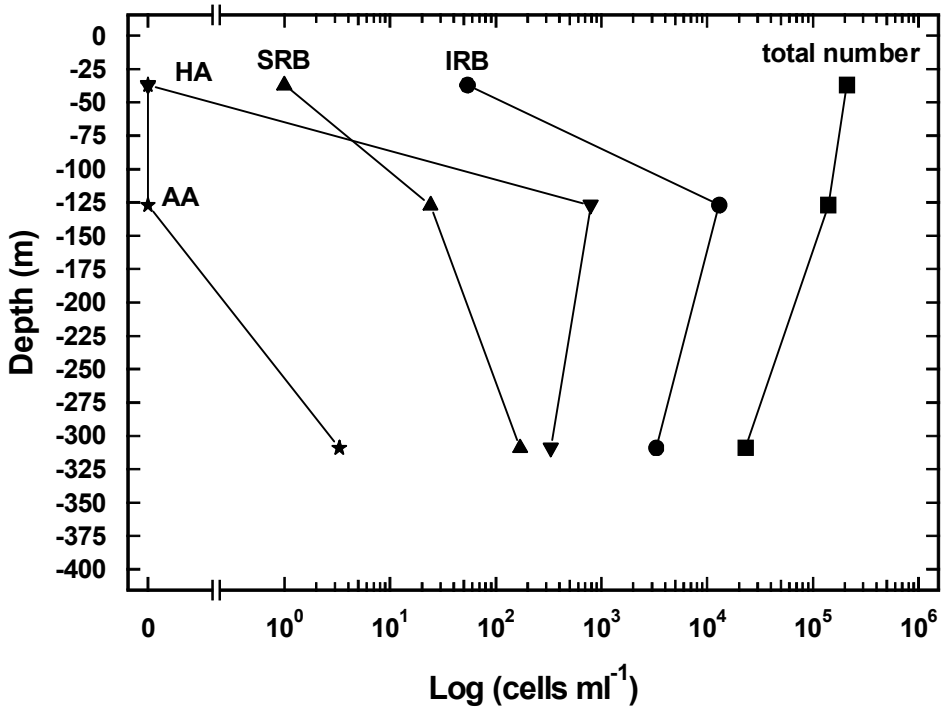


Figure 3-19. *The number of physiological groups of microorganisms in borehole R387. See Table 3.14 for symbol descriptions.*

Table 3-14. Comparison of groundwater-based and synthetic growth media.

	Borehole	
	R387 (304–309 m)	
Media type	**Gw**[a]	**Syn**[a]
Autotrophic methanogens	< 0.2	n.t.[b]
Heterotrophic methanogens	< 0.2	n.t.
Autotrophic acetogens	3.3	n.t.
Heterotrophic acetogens	330	n.t.
Sulphate-reducing bacteria	170	3300
Iron-reducing bacteria	3300	3300
% of total cells cultured	19.0	33.0

[a] = Gw = groundwater-based; Syn = synthetic. [b] = not tested.

3.5.2.3 Bacterial reduction of uranium

The data from the uranium reduction test are shown in Table 3-15. No difference was observed for uranium alone or uranium plus sulphate. With uranium plus iron, the presence of bacteria resulted in greater precipitation of uranium compared with the sterile control.

Table 3-15. Summary of results from uranium reduction experiments with enrichment cultures.

Electron acceptor	**Average concentration dissolved U of triplicate samples (µg l^{-1})**	
	Palmottu microbes	**Uninoculated control**
U	20 400	21 800
U+SO_4^{2-}	25 700	23 600
U+Fe^{3+}	644	1787

3.5.2.4 Main conclusions from the investigations of microbial processes in Palmottu

The protocols used for enumeration of physiological groups of microorganisms have been in use at the Deep Biosphere Laboratory at Göteborg University in Sweden for several years. The data obtained generally appear robust and reproducible. More than 30 discrete sample analysis procedures have been run with no problem, and always reveal some results for IRB and SRB. Therefore, it appears that there has been a technical problem with the samples from R302 and R327. These two boreholes had <0.1 SRB and IRB per ml of groundwater, which is unusually low for groundwater from such depths. The boreholes were sampled with a GTK method, which differs significantly from the equipment used for R387. It is possible that the GTK sampling procedure is inappropriate for MPN determinations of anaerobic microorganisms. Because of this difficulty, the boreholes R302 and R327 have been excluded from further discussion. The remaining discussion will deal with results obtained from R387.

The test of synthetic and groundwater-based media (Table 3-15) showed good correlation between the obtained data. This result also demonstrates the reproducibility of the culturing technique applied. Two different media types inoculated with discrete samples from the same borehole level gave results showing similar trends for IRB and SRB.

The total numbers of microorganisms decreased with depth, a relation which has also been observed at the Äspö HRL (Pedersen et al 1996b). The numbers of anaerobic reducing microorganisms increased with depth (Fig 3-19), which finding is in agreement with results from other Baltic Shield groundwater sites (Fig 3-10).
There was a direct correlation between the number of IRB and SRB detected and the concentration of total iron and sulphate. Generally, a lower redox correlated with more IRB, SRB, HA and AA. These findings were to be expected. It is not obvious from the data which of the correlation variables depend on which. Typically, microbial activity decreases the redox potential but it is premature to conclude whether the redox of the sampled Palmottu groundwater is coupled to the reduction activities of the found microorganisms. Such information would require a much more extensive programme in microbiology, including measurements of in situ activities with radiotracer techniques.

The distribution of SRB and IRB showed an inverse correlation with dissolved uranium at R387. The attempt to mimic the groundwater situation in culture tubes inoculated with enrichment cultures of IRB and SRB was only partly successful. Some uranium reduction was detected with cultures enriching IRB. It is consequently possible that microorganisms contribute to keeping the Palmottu groundwater system reduced and also, that they may be directly involved in reducing uranium(VI) to U(IV).

3.5.3 Maqarin

The Maqarin site in northern Jordan is unique, situated as it is in bituminous marls that have been thermally altered by natural in situ combustion. As a result of this, the groundwaters discharging at Maqarin are hyperalkaline and geochemically similar to Portland cement pore water. The site is therefore considered to be an excellent natural analogue for the high pH environments that will dominate around and in low-level and intermediate-level waste repositories, and in SFL 3-5 repositories (Pedersen and Karlsson 1995, page 3). Among the questions to be answered with respect to microbial processes is whether microorganisms can survive and be active at the extreme pH values typical of the Maqarin groundwater. Molecular methods, microscopy, culturing techniques and chemical analysis were used in an attempt to study this question. The results are reported in detail by Pedersen (1997b). Microorganisms were found in all of the Maqarin groundwater but could not be conclusively demonstrated viable and growing in situ, since they may have been just transported there via neutral groundwater. The diversity of the found microorganisms was similar to what had previously been detected with the 16S rRNA gene sequencing method, but none of the sequences found was typical of known alkaliphilic organisms. A possible hypothesis based on the obtained results is that the majority of the investigated Maqarin springs may be a little too extreme for active life even for the most adaptable microbe; however, this remains to be demonstrated. A new field research campaign was started in November 1999, with the goal to further evaluate the upper pH limit for the survival and activity of microbes.

3.6 Retention and transport of radionuclides

3.6.1 Bacteria and metals

The majority of the radionuclides are metals. The transport, chemical speciation, and ultimate fate of dissolved metals in aqueous systems are largely controlled by reactions that occur at solid surfaces (Stumm and Morgan 1996). Recognition of the importance of solid-phase reactivity in aqueous geochemistry has fostered the development of the surface complexation-precipitation theory (SCPT) as the leading model for

understanding the behaviour of dissolved metals in pristine and contaminated waters (Dzombak and Morel 1990; Stumm and Morgan 1996). This concept embraces the principles of thermodynamics and chemical equilibria to predict when solid-phase partitioning of metal ions is likely to occur in response to sorption, and quantify subsequent surface precipitation reactions. The SCPT approach has thus far been applied almost exclusively to minerals, particularly to hydrous iron oxides (Dzombak and Morel 1990); however, Warren and Ferris (1998) recently demonstrated that a continuum exists between ferric iron sorption and precipitation reactions on bacterial surfaces, as anticipated with SCPT. Pedersen and Albinsson (1991) report a similar process with the iron-reducing bacterium Shewanella putrefaciens. This is an important step forward as bacteria are at least as widely distributed in aqueous systems and probably as reactive as many inorganic solids. Moreover, if SCPT is to emerge as a true guiding paradigm for aqueous geochemistry, it must be firmly established to be applicable to both organic and inorganic solids.

The behaviour of bacteria as geochemically reactive solids can be inferred from extensive research documenting their performance as sorbents of dissolved metals, and nucleation templates for a wide range of authigenic minerals (Konhauser and Ferris 1996; Konhauser et al 1994; Pedersen and Albinsson 1991, 1992). This reactivity stems directly from the presence of amphoteric surface functional groups (i.e. carboxyl, phosphoryl, and amino constituents), which are associated with structural polymers in the cell walls and external sheaths or capsules of individual cells (McLean and Beveridge 1990). Direct interaction between these surface functional groups and dissolved metals accounts for the sorptive properties of bacteria, while surficially sorbed metals provide discrete sites for subsequent mineral nucleation and precipitation reactions (Pedersen and Albinsson 1991).

Because of their ubiquitous distribution and reactive surface properties, hydrous iron oxides are considered to be dominant sorbents of dissolved metals in aquatic environments (Stumm and Morgan 1996). This perception is somewhat tempered by work which shows that natural iron oxides often contain significant amounts of silica (e.g. siliceous ferrihydrite) and sulphate (e.g. jarosite and schwertmannite), as well as organic matter, including intact bacterial cells (Konhauser and Ferris 1996). This intermixing of compositionally variable iron oxides and organic matter produces composite multiple sorbent solids with highly variable metal retention properties, so-called “bacteriogenic iron oxides” (BIOS).

3.6.1.1 Accumulation of metals by bacteriogenic iron oxides

Bacteriogenic iron oxides and groundwater samples were collected underground at the Stråssa mine in central Sweden and from the Äspö HRL tunnel. Ferrous iron-oxidising bacteria, including stalked Gallionella ferruginea and filamentous Leptothrix sp, were prominent in the BIOS samples from Stråssa, while Gallionella ferruginea dominated in the Äspö HRL samples. The goal of these investigations was to understand the accumulation of various metals by BIOS. Strontium, cesium, lead, and uranium were studied in the Stråssa BIOS (Ferris et al 2000), and sodium, cobalt, copper, chromium and zink were studied in Äspö HRL BIOS (Ferris et al 1999).

The BIOS samples were found to contain only amorphous hydrous ferric oxide, as determined by X-ray diffraction. Inductively coupled plasma mass spectroscopy revealed hydroxylamine-reducible iron and manganese oxide contents ranging from 55% to 90% on a dry weight basis. Distribution coefficients (K_d values), calculated as

the ratio between BIOS and dissolved heavy metal concentrations, revealed solid-phase enrichments of 10^0–10^5, depending on the metal and iron oxide content of the sample (Ferris et al 1999, 2000). At the same time, however, a strong inverse linear relationship was found between log K_d values and the corresponding mass fraction of reducible oxide in the samples, implying that metal uptake was strongly influenced by the relative proportion of bacterial organic matter in the composite solids. Based on the metal accumulation properties of the BIOS, an important role can be inferred for intermixed iron oxides and bacterial organic matter in the transport and fate of dissolved metals in groundwater systems.

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