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INVESTIGATIONS OF SUBTERRANEAN MICROORGANISMS

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THEIR IMPORTANCE FOR PERFORMANCE ASSESSMENT OF RADIOACTIVE WASTE DISPOSAL

Karsten Pedersen[1] *and Fred Karlsson*[2]

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[1] Göteborg University
General and Marine Microbiology
The Lundberg institute
Medicinaregatan 9C
413 90 Göteborg, Sweden
tel. +46-31-773 25 78; fax. +46-31-773 25 99
E-mail pedersen@gmm.gu.se

[2] Swedish Nuclear Fuel and Waste Management Co.
Box 5864
102 40 Stockholm
tel +46-8-665 28 00; fax +46-8-661 57 19
E-mail skbfk@skb.se

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Karsten Pedersen[1], Fred Karlsson[2]

1 Göteborg University, General and Marine Microbiology, The Lundberg Institute, Göteborg, Sweden
2 Swedish Nuclear Fuel and Waste Management Company, Stockholm, Sweden

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ABSTRACT

The main actors in this report are the microorganisms (microbes is a synonymous designation). Let us therefore first define this concept. The following description can be read in the Swedish National Encyclopaedia: A microorganism is an organism that is invisible for the naked eye (smaller than some tenths of a millimetre), and requires adapted techniques for a successful study, such as sterilisation, sterile techniques, methods in molecular biology, microscope, and particular culturing devices and chemicals. Bacteria, unicellular algae, yeast, microscopic fungi, and protozoa (unicellular animals) are all microorganisms; sometimes, also viruses are improperly included (they are not true living entities). A unifying characteristic is that all micororganisms have the ability to grow as unicellular organisms in nature. The intention with this report is, with present knowledge as a starting-point, to give a broad and thorough description of how microorganisms may influence performance safety assessment of repositories for radioactive waste. Potential positive, neutral and negative effects have been identified. To the best of our knowledge, bacteria are the totally dominating microorganisms in the deep subterranean environment. Therefore, the report mainly deals with this group of microorganisms. Most of the microbiological concepts discussed in the repost are discussed in relation to the disposal concepts for radioactive waste in specific concluding sections in the text and also in summaries ending each chapter.

The first chapter gives an overview of the Swedish concepts for disposal of low-, intermediate- and highlevel radioactive waste. Further, the geological, chemical and hydrological conditions in repositories, that can be related to microorganisms are discussed. The following two chapters are intended to give the reader knowledge of the requirements for life of microorganisms and possible limiting or stimulating growth factors. Their relations to oxygen, temperature, pH, radiation, pressure, availability of water and nutrients, and their need for energy sources are discussed in detail. More, a basic knowledge about bacterial processes is presented. The participation of bacteria in the cycling of carbon, nitrogen, sulphur, iron, manganese and hydrogen is dealt with.

Chapter four is mainly a literature review of the knowledge about subterranean bacteria. Chiefly, we aim at a current description of the far-field environment in which repositories are or will be placed. The problems connected with sampling without disturbing the samples is reviewed. Also, a large number of techniques are presented for the determination of the numbers of bacteria in natural environments, their spatial distribution and species diversity, and methods for determination of microbial activity. Thereafter follows a summary of results about the microbiology in various

subterranean environments such as hydrothermal groundwater, deep sediments (mainly in North America), the hard rock laboratories of Stripa and Äspö, and from a number of different natural analogues, among them are Cigar Lake, Canada; Oklo, Gabon; Maqarin, Jordan; Poços de Caldas, Brazil and Palmottu in Finland. Chapter five treats investigations concerning microorganisms in repository(like) environments, the near-field. At first, a literature overview is given and then the conditions for life in the near-field are discussed. Particularly, the low water availability in bentonite at the intended swelling pressure is hypothesised to be to low for an active life of most microorganisms - they will simply be desiccated. Finally, the chapter five treats microbial corrosion and redox processes as they are of significance for the performance of construction materials such as canisters and concrete, and for the mobility of radionuclides.

Chapter six brings up the possibilities to predict presence and activity of microorganisms in the far-field as well as in the near-field by mathematical models. An important conclusion drawn is that successful modelling demands a multi-disciplinary approach. Microbiology is an integrated part of the whole, dealt with also in other disciplines such as geology, chemistry, hydrology, and additionally in the near-field, technology and engineering. At last, chapter seven summarises the conclusions made in the report and identifies research needs and how microorganisms may influence performance safety assessment of radioactive waste disposal.

The report consists of 43 figures, 36 tables, it has approximately 200 pages of text and refers to 293 articles, book titles and reports. Separate lists of figures and tables are included after table of contents. Further, an index is included at the end of the report to facilitate for the reader to where in the report central concepts, names etc. can be found.

SAMMANFATTNING

Huvudaktörerna i denna rapport är de så kallade mikroorganismerna (mikrober är en synonym benämning). Låt oss därför allra först definiera begreppet mikroorganism. I den Svenska Nationalencyklopedin står bl.a. följande att läsa (för ytterligare information rekommenderas även uppslagsordet mikrobiologi i samma bokverk): En mikroorganism är en organism som är osynlig för blotta ögat, dvs. mindre än någon tiondels millimeter, och som för att kunna studeras kräver speciellt anpassad teknik, såsom sterilisering, sterilteknik, molekylärbiologiska tekniker, mikroskop och särskilda odlingsmaterial. Till mikroorganismerna räknas bakterier, encelliga alger, jästsvampar, mikroskopiskt små svampar och protozoer (encelliga djur); oegentligt inkluderas ofta även virus (de har inte förmåga till eget liv). Alla mikroorganismer har förmågan att i naturen växa som enskilda celler. Denna rapport avser att, med utgångspunkt från dagens kunskapsläge, ge en heltäckande bild av hur mikroorganismer kan inverka på funktion och säkerhet hos slutförvar för radioaktivt avfall. Såväl positiva, neutrala som negativa effekter har kunnat utskiljas. I den djupa underjord där förvaren placeras, förekommer, vad vi idag känner till, främst bakterier. Därför behandlar rapporten huvudsakligen denna grupp av mikroorganismer. De flesta mikrobiologiska begreppsområden som tas upp i rapporten relateras till lagringskoncepten för radioaktivt avfall i sammanfattande avsnitt, både i den löpande texten och i slutet av varje kapitel.

I rapportens första kapitel ges en översiktlig bild av de svenska lagringskoncepten för låg-, medel- och högaktivt avfall. Vidare diskuteras de geologiska, kemiska och hydrologiska förvarsförutsättningar som är av betydelse för mikroorganismer. De två följande kapitlen avser att ge läsaren en god kunskap om mikroorganismernas behov och vad som kan begränsa eller gynna deras tillväxt och aktivitet. Deras förhållande till syre, temperatur, pH, strålning, tryck, tillgänglighet på vatten och närningsämnen samt behovet av energikällor diskuteras ingående. Mikroorganismernas, särskilt bakteriernas, förmåga att fästa och växa på ytor tas också upp. Vidare presenteras grundläggande kunskap om bakteriella processer. Bakteriernas medverkan in de naturliga kretsloppen av kol, kväve, svavel, järn, mangan och väte behandlas.

Kapitel fyra är till största delen en litteraturöversikt om kunskapsläget gällande bakterier i miljöer djupt ned under jordytan samt i vissa fall i djup under havs- och sjöbottnar. Främst avses att ge en god beskrivning av det så kallade fjärrområde i vilket förvaren kommer att placeras. Först diskuteras svårigheterna när det gäller att provta dessa djupa miljöer utan att förstöra proven. Vidare gås ett stort antal metoder igenom för bestämning av antalet mikroorganismer i naturliga miljöer, deras spridning och artförekomst samt

för mätning av eventuellt pågående mikrobiell aktivitet. Kapitel fyra innehåller även redovisning av resultat från olika miljöer; hydrotermala grundvatten, djupa sediment (främst i Nordamerika), Stripa och Äspö bergforskningslaboratorier och från ett antal olika så kallade naturliga analoger, däribland Cigar Lake, Kanada; Oklo, Gabon; Maqarin, Jordanien; Poços de Caldas, Brasilien och Palmottu i Finland. Kapitel fem behandlar undersökningar av mikroorganismer i förvarsmiljön, det så kallade närområdet. Först ges en litteraturöversikt och sedan diskuteras de mikrobiella livsförutsättningarna i förvarsmiljöer. Särskilt noteras att bentonitens vattenhalt vid projekterat svälltryck är så låg att de flesta mikroorganismer inte kan överleva - de torkar helt enkelt ut. Mikrobiella korrosionsprocesser och redoxprocesser i förvarsmiljöer diskuteras sedan eftersom de är av betydelse för livslängden hos konstruktionsmaterial och radionukliders rörlighet.

Kapitel sex tar upp möjligheterna att med matematiska modeller beskriva och förutsäga mikroorganismernas förekomst och aktiviteter i såväl fjärr- som närområdet. En viktig slutsats i detta kapitel är att framgångsrik modellering kräver ett så kallat multi-disciplinärt angreppssätt. Mikrobiologi är en del i den helhet som i fjärrområdet utgörs av bland annat geologi, kemi, hydrologi, och i närområdet också av teknik och ingenjörskonst. Kapitel sju slutligen sammanfattar de viktigaste slutsatserna i rapporten och identifierar de viktigaste forskningsbehoven om hur mikroorganismer kan inverka på funktion och säkerhet vid slutförvar för radioaktivt avfall.

Rapporten innehåller 43 figurer, 36 tabeller, den omfattar cirka 200 sidor text och refererar till 293 olika artiklar, böcker eller rapporter. En översikt av förekommande figurer och tabeller ges i separata förteckningar direkt efter innehållsförteckningen. Vidare finns i slutet av rapporten ett index som avser underlätta för läsaren att finna var i rapporten vissa centrala begrepp, namn, etc. behandlas.

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SUMMARY AND CONCLUSIONS

This report comprises current knowledge about subterranean bacteria and their potential influence on the disposal of nuclear waste summarised as follows:

Viable bacteria can be found in most, if not all subterranean environments investigated, that has a temperature below 110 °C and enough water available. They appear in numbers from some hundred bacteria up to several millions of bacteria per ml groundwater, gram sediment or cm^2 solid surface. The species diversity is usually large and they appear unevenly distributed with respect to species affiliation. Direct as well as indirect measurements show that the subterranean bacteria found are alive and active, although their activity usually proceeds at rates much lower than can be found in terrestrial environments. The only true environmental limitations for subterranean bacteria seem to be temperature, water availability and the supply of utilisable energy sources.

There are two principally different ways in which bacteria may influence radionuclide migration. First, uptake of a radionuclide to a free-living bacterium will mobilise the radionuclide while uptake to an attached bacterium tend to immobilise it. Modelling of this process has indicated radionuclide migration by bacteria to be negligible mechanism. Second, many bacteria produce chelating agents, unspecified such as organic acids, but also chelators with very high affinity for specific metals.

Trapped oxygen in bentonite clays etc. is calculated to have disappeared in 300 years time due to inorganic reactions. Bacteria consume oxygen and may decrease this time. Iron reducing bacteria and also other bacteria have been demonstrated to consume oxygen in infiltrating surface groundwater. This will probably protect the repository far-field from extensive oxidation during the time period of surface groundwater draw-down and enforced inflow due to drainage (construction and operation phases). Iron reducing bacteria and sulphate reducing bacteria will produce ferrous iron and sulphide, respectively, which will further improve the redox situation by decreasing the redox potential in deep rock environments. Sulphate reducing bacteria have been shown to reduce dissolved uranium(VI) to uranium(IV) which will precipitate.

Bacterial enzymes act as catalyst for many different inorganic reactions, and the strong potential of such reactions for recombination of radiolysis

products demonstrated. At conditions satisfactory for bacterial life, enzymatic recombination may out-compete inorganic catalysts.

Gas production by bacteria may occur as a result of several different processes . The processes of aerobic degradation, fermentation and anaerobic respiration of organic matter all produce carbon dioxide in relation to the amount of organic carbon available for degradation. Hydrogen evolve during fermentative processes and di-nitrogen, hydrogen sulphide and methane may form in anaerobic environments. At saturation, or when the pressure drops, gas bubbles may form. A gas produced by one type of bacteria, may be simultaneously or later be consumed by other bacteria, for instance when the growth conditions changes. This will result in cyclic flows. For example, methane is produced under anaerobic conditions and when oxygen is introduced, other bacteria will oxidise the methane to carbon dioxide and water.

The canister is an important barrier to radionuclide release. Sulphide reacts with copper and therefore sources of sulphide must be evaluated. Sulphate reducing bacteria produce sulphide and factors that governs their activity must be studied. We know that sulphate reducing bacteria occur in many subterranean environments.

Concrete can be inhabited by alkalophilic bacteria but the actual pH of fresh cement is in the absolute upper region of the pH range where life has been shown possible. Data from the alkaline analogue in Maqarin indicate that bacteria survive pH at 12.5, which is the pH of leached concrete. Acid producing bacteria, such as nitrification bacteria that produce nitric acid, may cause concrete degradation.

The degradation of bitumen has been demonstrated to occur with several different microorganisms. Although the process may be significant at aerobic conditions, independent investigations indicate microbial degradation of bitumen to be a very slow process at anaerobic conditions.

This report has identified needs of research regarding the influence of microorganisms on performance assessment of nuclear waste disposal. The different fields of research recognised, deal with effects from microorganisms that may have different *effect values* for repository performance: Effects that will have a positive influence on repository performance (P), effects that will have a negative influence on repository performance (N), and effects that must be studied for a general understanding of the influence of bacteria on repository performance (U). Below, the research needs are summarised and respective effect value on performance assessment is suggested as well.

Now, very convincing evidence exists for a subterranean biosphere that reaches as deep as temperature and water availability allow (Chapter 4).

These parameters, temperature and water availability, can easily be measured once boreholes etc. have made access possible. It is more problematic to get correct measurements of available energy sources and the flux of them. It will not be possible to correctly model subterranean bacterial activity until we have data on fluxes of organic carbon from the terrestrial sun-driven ecosystems downwards including geological deposits and fluxes of gases from the inner of the earth such as hydrogen and methane. ***Research need: What energy sources and fluxes of energy will be available for bacteria at repository conditions? Effect value: U.***

The direct uptake and migration of radionuclides by bacteria seem to be negligible components for the modelling of migration. Dissolution of precipitated or in other ways immobilised radionuclides and the production of complexing agents remains to be elucidated. The production of complexing agents is nested within the question of fluxes of energy, as such organic molecules cannot be produced by bacteria without an energy source. ***Research need: To what extent, if any, can bacterial dissolution of immobilised radionuclides and production of complexing agents increase radionuclide migration rates? Effect value: N.***

The radioactive waste disposal concepts may benefit from bacterial redox processes such as consumption of oxygen, production of reducing compounds (e.g. ferrous iron and sulphide) and reduction of uranium(IV). Today we have convincing results indicating that iron reducing bacteria contributed to (and still do) keeping groundwater, which infiltrates to the access tunnel of the Äspö hard rock laboratory, reducing for soon 4 years. Still, there is a lack of data for modelling the accurate participation of bacteria in subterranean redox processes. Correct modelling of repository redox processes preferably should include reactions catalysed by bacteria. ***Research need: Will bacterial oxygen consumption significantly add to the inorganic oxygen consumption system, and to what extent may bacterial production of reducing compounds such as sulphide and ferrous iron contribute to keeping the repository host rock reduced? Effect value: P.***

This report suggests that bacteria significantly may contribute to the recombination of radiolysis products. The calculations indicate bacterial recombination to be a process that will compete with inorganic reactions. This theory needs to be validated, preferably at a suitable analogue site where radiolysis has been going on for a long time. ***Research need: Will bacterial recombination of radiolysis products significantly contribute to the removal of such unwanted oxidising molecules? If so, how can it be validated? Effect value: P.***

Bacteria produce and consume many different gases, such as carbon dioxide, hydrogen, oxygen and methane. Gas production is generally an unwanted process, while gas consumption may be beneficial for a waste repository. Corrosion of steel in a penetrated copper canister may produce hydrogen gas

that in a worst case may form bubbles that has to be released through the buffer. Hydrogen oxidising bacteria may consume the hydrogen - or at least a part of it. Some results exist that indicate gas production in low level waste with high content of organic material to be possible. The relation between gases and bacteria in deep repositories is unknown - beneficial as well as negative effects can be anticipated. ***Research need: Will bacterial production and consumption of gases like carbon dioxide, hydrogen and methane influence the performance of repositories? Effect values: N and P.***

Corrosion of copper canisters due to the production of sulphide is a well known and unwanted scenario. It is important to study if sulphide producing bacteria in bentonite will survive at actual swelling pressures. If bacterial sulphide production in the bentonite can be excluded, it remains to study factors that govern sulphide production in the surrounding rock. Wee need to know the conditions for sulphide production, extent and distribution of sources in the subterranean environment. ***Research need: Bacterial corrosion of the copper canisters, if any, will be a result of sulphide production. Two important questions arise: Can sulphide producing bacteria survive and produce sulphide in the bentonite around the canisters? Can bacterial sulphide production in the surrounding rock exceed a performance safety limit? Effect value: N.***

Concrete has been used during the construction of SFR and will possibly be used also during SFL 2 construction. ***Research need: Do relevant bacteria survive at pH equivalent to that of repository concrete and can they possibly influence repository performance by concrete degrading activities such as acid production? Effect value: N.***

1 INTRODUCTION

1.1 WASTE DISPOSAL CONCEPTS

1.1.1 Repository for spent fuel - SFL 2

Radioactive waste in Sweden arises mainly from the production of nuclear power. Some waste also comes from research, hospitals and industry. The bulk part of radionuclides produced in a nuclear power reactor remains in the spent fuel elements. After a period of intermediate storage in the facility CLAB at Oskarshamn (about 40 years), the spent fuel elements will become encapsulated and later sent for final disposal to a deep geologic repository. This is referred to as the KBS-3 concept (KBS-3, 1983). Recently, a performance assessment of spent fuel disposal according to the KBS-3 concept has been made, which proves the importance of the near-field barriers such as the copper canister and the bentonite clay backfill. It was also demonstrated that the main role of the bedrock is to provide stable mechanical and chemical conditions in the repository for a long period of time so that the functions of the engineered barriers are not jeopardised (SKB-91, 1992).

Spent fuel elements may be characterised as high level radioactive waste. The elements are emitting heat, even after a period of about 40 years in the interim pool storage CLAB. Radioactivity will decay with time, but some long-lived radionuclides will make the waste hazardous for a very long time. Compared to uranium ore, for example, it will remain more radiotoxic for over 100 000 years. The KBS-3 concept takes this into account. The spent fuel elements will be encapsulated in copper canisters and placed in deposition holes in tunnels at an envisaged depth of about 500 m. The deposition holes will be backfilled with compacted bentonite clay in order to protect the canisters from mechanical damage and excessive water flow. The tunnels will be backfilled with a mixture of sand and bentonite. The amount of spent fuel in a canister and the distances between the canisters in the repository are chosen so that the peak temperature is only reaching about 80 °C at the warmest location at the canister surface. The restriction in temperature is mainly there to guarantee the long time performance of the bentonite backfill (figure 1.1). The low solubility of the spent fuel matrix, the copper canister, the bentonite buffer and the depth of emplacement in stable host rock are the main barriers to protect man from the radionuclides.

It is anticipated that a repository for spent fuel, SFL 2, can be taken into operation at the earliest towards the end of the next decade. Before that, a facility for encapsulation of spent fuel elements in copper canisters should have been taken into operation (RD & D Programme 92, 1992). The operation of all the Swedish power producing reactors until 2010 will yield a total of 7700 tonnes (uranium weight) of spent fuel, and about 4500 canisters will be needed for the final disposal.

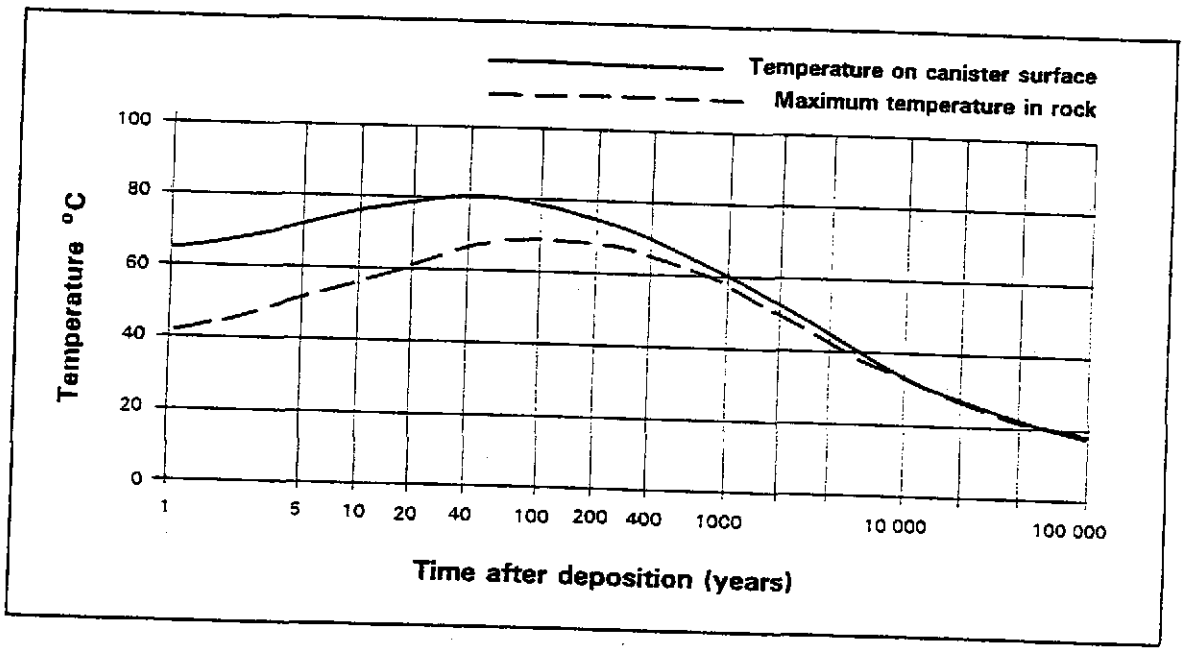

Figure 1.1 Calculated temperature in the central part of a repository built in one level (from KBS-3, 1983).

1.1.2 Repository for low and intermediate level waste - SFR

Operation of the nuclear power plants and the CLAB facility in Sweden also generates low level waste (LLW) and intermediate level radioactive waste (ILW), mainly in the form of spent ion exchange resins used for water purification. Also included in this category are contaminated trash and scrap, and ash from incineration of combustible radioactive waste. Operational waste is packed into containers of steel (drums) or concrete, in some cases first solidified (conditioned) with concrete or bitumen. The LLW and ILW operational waste contains only very small amounts of long-lived radionuclides. It needs to be disposed of in a repository, although it will decay to harmless levels in a relatively short time. Roughly, its radioactivity is comparable to background in 500-1000 years. In contrast to high-level waste, it does not emit heat that needs to be dissipated. Consequently, LLW and ILW waste will not need the same number and levels of protective barriers as spent fuel.

A repository for LLW and ILW, SFR, has been in operation since 1988. SFR is situated underground at a distance of 1 km off the coast in Forsmark. The rock coverage is more than 50 m and the depth of the sea above is about 6 m. SFR consists of an underground concrete silo and caverns where the waste is emplaced. Waste from industry, research and hospitals are also being disposed of in SFR. The completed construction, SFR 1, is scheduled for a total of about 90 000 m^3 of waste. In a later stage an expanded SFR will also receive waste from the decommissioning of nuclear power plants that have reached the end of their operational life-time. This waste will consist of scrap-metal, concrete debris etc.

1.1.3 Repository for remaining nuclear waste - SFL 3, 4 and 5

Some relatively small volumes of LLW and ILW from nuclear power plants, research etc. contain too much of long-lived nuclides to be suitable for disposal in SFR. Otherwise, the waste is similar in character to the SFR waste. A third repository SFL 3-5, is being planned for this kind of long-lived LLW and ILW. The construction will in many aspects be similar to the SFR facility, but the repository will be placed at about the same depth as the spent fuel in order to provide a better barrier for the long-lived nuclides. According to current planning, SFL 3-5 will be a separate part of the deep repository for long-lived waste of which SFL 2, the repository for spent fuel, is the main part.

Strictly taken, not all of the waste intended for SFL 3-5 falls into the category of long-lived waste, because this repository will also be there to receive waste from the operation of the CLAB and the encapsulation facility after SFR has finished operation. Decommissioning waste from the facilities that are in operation after SFR like CLAB, the encapsulation facility etc. will also go to SFL 3-5. The estimated total volume of waste for SFL 3-5 is 25 000 m^3.

1.2 GEOLOGY, HYDROGEOLOGY AND GEOCHEMISTRY

1.2.1 Host rock

Sweden is part of the Fenoscandian Shield and crystalline bedrock has been used for the construction of the underground repository SFR. The deep underground repositories consisting of SFL 2 (spent fuel) and SFL 3-5 (other long lived waste) will also be constructed in crystalline bedrock. A number of places in Sweden have been investigated as study sites for the deep disposal of spent fuel. Up to 15 core-drilled holes, down to depths between 500 and 1000 m, were typically drilled at a study site and in addition to that a number

of percussion drilled holes were sunk to about 100 m. The drill cores and the boreholes were investigated. Among other things hydraulic tests were carried out and groundwater was sampled and analyzed. Site investigations including drilling operations were performed prior to the construction of the SFR facility in Forsmark. Test holes were also made and investigated along with the construction work. The data gathered were used in the performance assessment for the Final Safety Report. Two underground hard rock laboratories have been constructed. The underground research facility at Stripa was in operation between 1977 and 1992 (SKB, 1993). Stripa was mined for iron until 1976. The research facility was constructed in a granite body adjacent to the mine area. Several experiments were carried out, such as bentonite backfilling and sealing, fracture zone detection and characterisation, groundwater flow, nuclide migration, and hydrogeochemical investigations. Starting in 1990 a new underground hard rock laboratory is being constructed at Äspö, near the coast at Simpevarp. Before and along with the construction work test holes have been drilled and used for sampling and investigations of the rock and groundwater (SKB, 1993).

The crystalline rock considered so far has generally been of granitic composition with quartz, feldspars and mica as the bulk rock minerals. In addition to that, there are accessory minerals which are important to consider from a geochemical point of view. Many of these occur as fracture filling minerals and some of them have been formed as a result of weathering reactions. Important geochemical aspects of repository host rock are redox properties, pH-buffering, ion exchange capacity, radionuclide sorption and diffusivity. Feldspars are easily weathered which can influence the pH and composition of groundwater (calcium, sodium and potassium). Clay minerals are formed by the weathering. Calcite is a frequent fracture filling mineral. Which participates in the buffering of pH and it acts as a source of carbonate ions to the groundwater. Pyrite and magnetite can be redox active and for example react with oxygen and precipitate iron(III) hydroxide. Complete oxidation of pyrite will also generate sulphate. Fluorite will control the fluoride concentration in groundwater and apatite is a source of phosphate. Clay minerals such as chlorite, kaolinite, illit, montmorillonite etc have ion exchange capacities and can influence the water composition or bind released radionuclides.

The mechanical stability of the rock, the occurrence of fracture zones as potential locations for rock movements etc., are important for the outline and construction of a repository. However, that is outside the scope of this report, which is more oriented towards the chemical conditions and the importance of microbes, and will therefore not be discussed here.Some geochemical points that are of interest in the context of this report are summarised below:

- The repository host rock will mainly be of granitic composition with quartz, feldspars and mica.

- Accessory minerals which influence the hydrochemical conditions are calcite (*pH and* HCO_3^-), *pyrite (redox), apatite* (HPO_4^{2-}), *fluorite* (F^-) and clay minerals (ion exchange).

- Minor amounts of iron(III) oxy-hydroxy minerals are found in the fractures, especially in the shallow part of the rock.

1.2.2 Hydraulic conditions

Hydraulic conditions are of great importance for the long time safety of an underground repository. Water flow will influence the geochemical conditions, transport dissolved species into the repository near-field (corrodants etc.) and disperse the radionuclides, if they manage to penetrate the overpack (canister and backfill). Hydraulic conductivities are measured in boreholes at different depths and this information together with the groundwater surface topography, which in Sweden is approximately the ground surface topography, is used to calculate the groundwater flow field. Groundwater flow at repository depth is an important parameter. The flow calculated for the investigated study sites is in the range 0.01-1 L/m^2a, and the value 0.1 L/m^2a is frequently used for assessment of the importance of flow at 500 m depth (KBS-3, 1983). Hydraulic conductivity and flow increase near the surface. The SFR facility is a special case, where the conductivity at about 50 m is higher than anticipated for a deep repository, but the sea above evens out the gradient and therefore the flow will be very small after the repository have been closed and come to a state of equilibrium pressure.

The distribution of flow has an influence on groundwater composition. The hydraulic conductivities varies considerably between different locations in the rock, and structures like fracture zones may act as conductors and have a dominating influence. Vertical conductive zones will be important for groundwater recharge at depth. Horizontal zones may act as hydraulic shields and separate groundwaters with different compositions. Especially deep groundwaters with a relatively high salinity will have a higher density which helps to stabilise the layering. An example of that has been studied in Finnsjön (Smellie and Wikberg, 1991).

Radionuclide transport will depend very much on the flow distribution. If we imagine a situation where radionuclides have been released to the groundwater and are transported to the surface it will be very important if a fraction of the nuclides are following a relatively fast pathway. This may in fact dominate the release to the biosphere, even if the fraction is small

because an early arrival of migrating radionuclides gives less time for radioactive decay. It has also been pointed out that a restriction to isolated pathways, if this is indeed the case, will reduce the contact surface available for sorption. Less sorption means less retardation. The existence of an interconnected system of microfractures has been demonstrated even for seemingly intact rock. Radionuclides that diffuse into these microfractures and sorb onto their mineral surfaces will become very strongly retarded. However, even in this case the contact surface between the contaminant in the flowing water and the surface of the fracture that carry the flow is important. Less contact surface means less area through which to reach the underlying microfracture system, filled with stagnant water.

Some hydraulic points of importance for this report are:

- Hydraulic conductivity and gradient decrease at depth and consequently the flow is very low at the depth suggested for SFL 2 and SFL 3-5. A figure often used for assessment of the importance of flow at a depth of 500 m is 0.1 L/m^2a.

- Water flow occurs in rock fractures. Fractured zones can act as hydraulic conductors.

- Dissolved species in the groundwater can be transported by the flow in the fractures. By diffusion they can also reach an interconnected system of microfractures in the seemingly intact rock where the water is stagnant.

- The transport of dissolved species by groundwater flow and the retention due to sorption and diffusion are of importance for the assessment of the rock as a barrier to radionuclide dispersion.

1.2.3 Groundwater chemistry

The chemical composition of groundwater will affect the near-field components such as bentonite and canister material, and will also influence the solubility of the waste form. It will also interact with concrete if this is present in a repository (eg repositories for low- and intermediate-level waste). The redox state and speciation of radionuclides in the groundwater are important for the retention due to sorption on the backfill and rock mineral surfaces. Several natural processes are active in the creation of a certain groundwater composition, such as recharge of meteoric water, in some cases input of marine water, weathering reactions etc. Near-field components such as concrete and bentonite can further influence the chemis-

try. Not least due to the fact that groundwater recharge in general occurs through the biologically active soil layer, makes microorganisms and their life processes utterly important. Inversely, the microbes will be very much dependent on the composition of the groundwater.

Groundwater has been carefully sampled and analyzed at all study sites and underground rock laboratory locations (Stripa and Äspö). Groundwater samples were also taken from investigation drillholes in the SFR-repository facility. The samples are collected from conductive zones isolated by packer sleeves. If boreholes from the ground surface are used for sampling it is generally necessary to use a downhole pump to bring up the water. There are also a number of ways in which the groundwater may have become contaminated, such as drilling water infiltration, mixing due to short circuiting of different aquifers by the open borehole etc. (Karlsson and Wikberg, 1987). Excessive pumping may also cause water from outside the isolated section to circumvent the packers and mix with the sample. In underground facilities, however, the groundwater conditions are artesian due to the position below the water table, and therefore no pumping is required and good quality samples are generally obtained. Particularly sensitive samples such as natural groundwater colloids, are best taken from packed off sections of artesian boreholes drilled from underground facilities. In such a case, pumping would agitate the water in the isolated section and in the adjacent fractures and thereby release particulate matter to the samples.

Groundwater under land in Sweden has in general a meteoric origin. The infiltrating water is almost "pure water" 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 added. The carbonic acid will react with minerals such as calcite and feldspars and form carbonate ions and release calcium and alkali ions to the water. Ion exchange with clay minerals may affect the proportions between cations. Organic materials such as humic- and fulvic acids and other substances will be added to the water from the soil. The biological processes will also have a marked influence if seawater infiltrates through organic rich sea sediments. Also in this case carbon dioxide will be generated, oxygen consumed, etc.

At great depths or under the sea bottom saline water is found where chloride is the dominating anion. The most common cation in saline groundwater is either sodium or calcium. The saline water may have a marine origin but other end members are also possible, depending on location and other conditions. Very deep, the salinity can be high and reach well above ocean sea water and even approach brine composition. It is also common that saline groundwater are found at shallower depth in coastal regions than further inland. This may of course be relict seawater that infiltrated several thousands of years ago, when land near the coast in Sweden was covered by

the sea due to the glacial depression (land pressed down by the ice cover). The infiltration of seawater continued until land was reclaimed by the land-uplift, which is still continuing in Sweden. However, an alternative explanation can be found in the lack of driving hydraulic force under the "flat" surface of the sea. With no, or very low, hydraulic gradient in the groundwater beneath the sea bottom, fossil saline conditions can be preserved for very long time periods and it must not always be the result of a relatively recent infiltration of seawater that we are observing. In other words, saline water may have originated even before the glaciation.

Some typical groundwater compositions to be expected at different depths and locations possible for a nuclear waste repository and encountered in the course of research and exploration within the Swedish radioactive waste management program are given in Table 1.1. It is obvious that major constituents such as the cations sodium and calcium, and the anions bicarbonate and chloride can vary considerably in concentration depending on where and at which depth the samples have been taken. Chloride behaves conservatively but many other ions obviously interact with the minerals. This is particularly evident in groundwater with a marine origin. An example of that is the ion exchange of calcium for sodium and vice versa. A further observation is that ions like potassium and magnesium, which are common in seawater, are evidently suppressed in groundwater - presumably by reactions with the minerals. Even sulphate is partly consumed. Carbonate is less common at depth. A possible explanation is that slow reactions with the rock minerals cause precipitation of carbonate as calcite.

Table 1.1 Chemical parameters of groundwater from boreholes in Finnsjön, Klipperås and Äspö.

Area		Finnsjön [a]		Klipperås [b]		Äspö [c]	
Borehole		KFI 09	KFI 09	KKl 01	KKl 09	KAS 03	KAS 03
Depth	m	94	360	404	581	129	860
pH		7.3	7.6	8.3	7.6	8.0	8.0
E_h	mV	-245	-	-300	-270	-270	-250
Na^+	mg/L	415	1500	47	15	600	3050
K^+	mg/L	6	7	1	1	2	7
Ca^{2+}	mg/L	115	1700	14	29	162	4400
Mg^{2+}	mg/L	16	84	2	3	20	50
Sr^{2+}	mg/L	-	-	-	-	3	75
Fe^{2+}	mg/L	0.56	0.34	0.01	0.09	0.12	0.08
Mn^{2+}	mg/L	0.19	0.36	-	-	0.10	0.20
HCO_3^-	mg/L	285	32	80	120	61	11
F^-	mg/L	3	9	4	3	2	2
Cl^-	mg/L	680	5200	45	6	1230	12300
Br^-	mg/L	2	27	0.4	0.05	5	85
I^-	mg/L	0.01	0.07	0.008	0.002	0.10	0.70
SO_4^{2-}	mg/L	175	340	1.5	4.3	32	720
HS^-	mg/L	0.22	0.03	0.10	0.01	0.70	1.10
NO_2^-(N)	mg/L	-	-	-	-	0.001	0.001
NO_3^-(N)	mg/L	0.02	0.01	-	-	0.01	0.01
NH_4^+(N)	mg/L	-	-	-	-	0.04	0.01
HPO_4^{2-}(P)	mg/L	0.001	0.004	0.001	0.003	0.002	0.002
SiO_2(Si)	mg/L	7.6	7.6	4.4	9.9	4.8	4.2
TOC	mg/L	18	1.0	3.7	1.2	2.0	0.5
U	(µg/L)	2.1	8.2	0.01	0.04	0.15	0.13

a) (Smellie and Ahlbom, 1989)
b) (Smellie et al, 1987)
c) (Smellie and Laaksoharju, 1992)

The pH of granitic groundwater in Sweden is buffered by the carbonate system. Calcite is abundant as mineral and feldspars can also react with acids. Therefore "acid rain" or any similar disturbance of pH does not propagate very far down under the ground surface (Wersin et al, 1995).

Deep groundwater does not contain any oxygen. Measurements of redox potential with E_h-electrodes give values between -100 and -400 mV. There is a dependence of E_h on pH and Fe^{2+}-concentration which has been used to explain and model the redox potential (Grenthe et al, 1992). This model has been found to be applicable to a wide variety of groundwater chemical conditions that have been measured in the course of the so-called natural analogue investigations (Brandberg et al, 1993). 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 best quality. The low content of for example Fe^{2+} gives the water only a low redox buffer capacity. However, a considerable capacity is contained in the rock and its content of iron(II) minerals and pyrite.

Uranium and manganese are indirect redox indicators. Manganese is soluble in the divalent state and measurable amounts of manganese can be used as a test that E_h is at least below 340 mV (Allard et al, 1983). The total uranium concentration is mostly below 1 μg/L and the main solubility limiting phases are probably crystalline uraninite (UO_2) and the more amorphous U_4O_9 phase (Smellie and Laaksoharju, 1992). Deep groundwater pH and E_h are within the stability field of U(IV) and the reducing character of the water is further confirmed by the measurements of the activity ratio of uranium isotopes $^{234}U/^{238}U$. The activity ratio is well above unity which indicates low uranium solubility (Smellie and Laaksoharju, 1992).

Nitrogen and phosphorus compounds are scarce in deep groundwater and generally less than what is found in water near the ground surface. In fact the occurrence of these "nutrient salts" in the groundwater samples is often a sign of contamination with surface or near-surface water. The nitrogen compounds analyzed for are nitrate, nitrite and ammonium. Phosphorus occurs as phosphate.

Groundwater contains dissolved gases such as nitrogen, carbon dioxide, methane, hydrogen, helium, neon, argon, krypton and radon. Oxygen is only found at relatively shallow depths. Carbon dioxide is part of the carbonate system (Table 1.2). Methane and hydrogen can have a biological origin. Helium and radon are generated by the radioactive decay of nuclides in the rock and the other noble gases may come from the atmosphere. Groundwater samples from depth may be de-gassed if brought up to the surface and to normal atmosphere pressure. However, all the samples taken so far show that there is not enough of dissolved gas to form a separate gas phase at 500 m.

Table 1.2 The content of nitrogen, hydrogen and carbon-containing gases, and the total volumes of gas extracted from the samples of the groundwaters of the Stripa boreholes V2, the Laxemar boreholes KLX 01 and Äspö boreholes KR0012, 13 and 15 (Pedersen and Ekendahl, 1992a, b, Pedersen, 1993b).

Boreholes	Sampling depth (m)	N_2 ($\mu L/L^{-1}$)	H_2 + He ($\mu L/L^{-1}$)	CO ($\mu L/L^{-1}$)	CO_2 ($\mu L/L^{-1}$)	CH_4 ($\mu L/L^{-1}$)	C_2H_6 ($\mu L/L^{-1}$)	[a] C_2H_{2-4} ($\mu L/L^{-1}$)	Volume of extracted gas (%)
Stripa									
V2	799-807	25000	<10	<1	32	245	0.3	<0.1	2.4
V2	812-821	31000	<10	<1	11	170	0.6	<0.1	3.4
V2	970-1240	24500	<10	<1	10	290	2.9	<0.1	2.7
Laxemar									
KLX01	830-841	46500	4600	0.5	460	26	<0.1	<0.1	5.7
KLX01	910-921	37000	3500	0.1	500	27	<0.1	<0.1	4.4
KLX01	999-1078	18000	2450	0.7	1600	31	<0.1	<0.1	3.5
Äspö									
KR0012	68	22000	40	0.1	6050	1030	<0.1	0.1	2.9
KR0013	68	25000	110	0.2	9640	1970	<0.1	0.1	3.7
KR0015	68	22000	64	0.1	15037	4070	<0.1	0.1	4.0

[a] The content of C_2H_2 + C_2H_4

Colloids are sampled, but this is a difficult operation and there is a tendency to generate particles by the sampling procedure (Laaksoharju et al, 1993). The particles are composed of minerals such as calcite, iron hydroxide, gibbsite, iron sulphides and quartz, and the total concentration is expected to be below 0.4 mg/L (Allard et al, 1991).

The groundwater composition in general and the composition of natural stable and radioactive isotopes in particular can give important information about groundwater origin, transit times, mixing etc. Stable isotopes such as ^{18}O and ^{2}H are regularly sampled for and analyzed, and so are the radioactive isotopes ^{3}H and ^{14}C. The evaluation of conditions related to hydrogeology is outside the scope of this report, but isotope methods have also been used to trace the influence of microbial processes on geochemical conditions. In this context stable isotopes such as ^{34}S in sulphide and sulphate, ^{18}O in sulphate and ^{13}C in methane, are of interest (see 4.2.3).

Groundwater conditions of importance for this report are as follows:

- pH is normally in the range 6.5-9.5 and the carbonate system is the most important to control pH.
- The alkalinity (HCO_3^- and CO_3^{2-}) of groundwater is in general below 600 mg/L. There is a tendency for alkalinity to decrease with depth and values above 300 mg/L are unusual at 500 m depth.

- The measured redox potential at 100 m depth and below is in general between -100 and -400 mV and there is a dependence on pH which suggests that the iron system (Fe^{2+} and goethite) controls the E_h.

- The concentration of dissolved iron first increases with depth and has generally a maximum of between 0.1-1 mg/L at a depth of roughly 100 m. Deeper down the Fe^{2+} concentration is generally in the µg/L-range. The concentration of Fe^{3+} (ferric iron) is always extremely low under ambient groundwater pH-conditions. The activity of ferric iron ions can consequently only be judged from the presence of iron(III) minerals such as for example goethite (FeOOH).

- Sulphide is more frequent below 100 m. The concentration is rarely above 1 mg/L and generally in the µg/L-range. Iron concentrations are low where sulphide is found which indicates that some of the iron may have been precipitated by sulphide.

- There is in general no traces of oxygen found in the groundwater below 100 m. This is clearly demonstrated by the low negative E_h-values measured and the presence of Fe^{2+}-ions and sometimes also sulphide ions.

- Groundwater contains organic material and part of it is humic and fulvic acids. The DOC (Dissolved Organic Content) of relatively shallow groundwater is generally higher than deeper down. It can be 10-20 mg/L near the surface and 1-2 mg/L at depth. Fulvic acids with relatively low molecular weights dominate over humic acids at depth.

- Chloride concentration varies considerably with location and depth. It is therefore practical to divide between "normal granitic groundwater" with HCO_3^- as the dominating anion, and "saline groundwater" with Cl^- as the most abundant negative ion. The chloride concentration in saline water at 500 m depth is generally below 6000 mg/L, but considerably higher concentrations have been encountered deeper down.

- Sulphate concentration is highest in saline water but not correlated to the chloride concentration. Solubility of gypsum is generally not a limiting factor. Where saline groundwater has a marine origin sulphate concentrations have evidently been reduced by bacteria. The concentration of SO_4^{2-} at 500 m is generally below 500 mg/L, even in saline groundwater. In normal granitic groundwater the sulphate concentration is in general below 15 mg/L (Allard et al, 1983).

- Sodium and calcium are the most common cations in granitic groundwater. The proportions between these ions can vary. Very dilute groundwater is unusual and it is important for performance assessment to have an enough high concentration of cations in general and divalent ions like Ca^{2+} in particular in order to destabilise colloids. The concentration of sodium or calcium is usually in the range 10-2000 mg/L at 500 m depth.

- Magnesium and potassium are generally present but their concentrations are kept relatively low by geochemical reactions. Magnesium is generally below 150 mg/L and potassium below 12 mg/L at 500 m, even in saline groundwater.

- Deep groundwater contains very little dissolved P- and N-compounds. The concentration of total phosphate and total nitrate-nitrite is in general below 0.1 mg/L (Allard et al, 1983). The concentration of ammonium, NH_4^+, is generally below 0.4 mg/L (Smellie and Laaksoharju, 1992).

- Groundwater contains dissolved gases such as carbon dioxide, nitrogen, methane, noble gases and presumably also hydrogen (hydrogen has been difficult to analyze). The dominating component is nitrogen, and the total concentration of dissolved gas is less than 100 ml NTP/L (Normal temperature and pressure), which implies that in groundwater below 100 m, a separate gas phase will not easily form (SKB-91, 1992).

- Inorganic particles may occur as colloids. The concentration is expected to be below 0.4 mg/L (Allard et al, 1991).

- Isotope analyses have indicated transit times for deep groundwater of several thousand years.

1.3 MATERIALS AND SUBSTANCES IN A REPOSITORY

1.3.1 Spent fuel

Spent fuel consists of uranium dioxide with fission products and actinides embedded in the uranium dioxide matrix. There is a certain inhomogeneity in the distribution of the radionuclides in the spent fuel. For example, radionuclides of the elements cesium and iodine are enriched to grain boundaries etc. Small metallic particles containing for example palladium are also present in the burnt out fuel. There are also bubbles of gas generated by fission. Some elements, like the actinides, are expected to be well integrated in the uranium oxide phase, but not necessarily at the same concentration everywhere.

Uranium dioxide has a very low solubility in oxygen free groundwater. Except for a fraction of some elements such as cesium and iodine, which is enriched at the fuel surfaces, the radionuclides are well protected by the spent fuel matrix and there is a considerable resistance to leaching. The low solubility of the spent fuel uranium oxide matrix is therefore a barrier to radionuclide dispersal if groundwater should come in contact with spent fuel.

Spent fuel will generate heat due to the decay of the radionuclides. The emission of heat will decrease with time. The emission of heat from fuel, the day it is sent for final disposal, varies depending on the burnup and how long it has been stored after use in the reactor. As an example, fuel with a burnup of 38 GWd/tU and a storage time of 30 years will yield a heat output of roughly 0.8 kW/tU (SKB-91, 1992). The temperature in the repository will depend on the dissipation of heat. To a large extent that can be controlled by the distribution of waste and the materials used (backfill).

The decay of the radionuclides in the spent fuel will also yield radiation. Alpha and beta particles will dominate in the short range close to the surface of the fuel, and the dose rate will decrease with time. As an example, the mixed alpha and beta radiation will give dose rates of about 0.4, 0.1 and 0.001 Gy/s at the fuel surface after 40, 1000 and 100 000 years of storage respectively. Alpha will dominate over beta. The time given is after release from the reactor and a BWR-fuel with a burnup of 33 GWd/tU is used in the example (Christensen and Bjergbakke, 1982b).

Deep groundwater is oxygen-free and reducing in character. The bentonite backfill contains oxygen consuming compounds, such as for example pyrite, and the canister materials copper and iron also react with oxygen. Consequently, the spent fuel is well protected from oxidation by external sources, such as free oxygen originating from the atmosphere above ground.

This is important, because oxidation can influence spent fuel leaching by either increasing the solubility of uranium or by transforming the crystal structures of the uranium dioxide.The last mentioned phenomena may occur if uranium dioxide is oxidised to higher oxidation states (beyond U_3O_7) and thereby alter the crystal lattice structure. Radionuclides embedded in the uranium oxide matrix may then become accessible to leaching. Radioactivity will decay but the radiation level will remain relatively high at the spent fuel surface for a long time due to for example the alpha emitting actinides. Radiolysis of water generates hydrogen and oxidising species such as oxygen and hydrogen peroxide. Radiolysis is therefore an important process. Since hydrogen is relatively inactive at ambient temperatures, there will be a net oxidising capacity. The oxidants are produced at the exposed surface if fuel comes into contact with the groundwater. Consequently it has been important to calculate the rate of radiolysis and the reactions of the oxidants when assessing the performance of the spent fuel as a barrier to radionuclide release (KBS-3, 1983, SKB-91, 1992).

The properties of spent fuel which are of interest in the context of this report can be summarised as follows:

- The spent fuel matrix consists of uranium oxide with a composition close to UO_2, that is the tetravalent oxidation state of uranium. Uranium dioxide has a very low solubility in groundwater and almost all of the fission products, actinides and other radionuclides are embedded in the solid spent fuel matrix.

- The decay of the radionuclides in the spent fuel generates heat and radiation. Heat emission and radiation will decline with time. The radiation consists of gamma radiation, neutrons, alpha- and beta-particles. All of the alpha and beta, and most of the gamma radiation and neutrons are shielded by the canister (see below). However, if water penetrates to the fuel, there will be a relatively strong radiation field at the fuel surface mainly due to the short range alpha particles.

- The fission products cesium and iodine will to a certain fraction be enriched to the surfaces of the fuel. They are soluble in water so these fractions of cesium and iodine can be leached out if water penetrates to the fuel.

- Many of the radionuclides, for example the actinides, will have a very low solubility in deep reduced groundwater. Their low solubility will limit their release even if they should escape the fuel matrix.

1.3.2 Canister materials

The canister is an important barrier to radionuclide release. For example, a copper/steel canister has been suggested, which consists of a 5 cm thick inner steel canister inside a 5 cm thick welded copper canister (SKB, 1993). The outer copper canister is the main barrier to penetration by corrosion. The outer dimensions are 5 m in length and 0.88 m in diameter, and one canister can take up to 12 fuel elements from a BWR (Boiling Water Reactor) reactor.

In comparison to most other metals used for construction, copper has a broad range in the E_h - pH field where it is stable (Werme et al, 1992). In fact, oxygen and copper are the only normal groundwater constituents that will corrode copper. Oxygen is not present in deep groundwater and the concentration of sulphide is generally low. The possibility of stress corrosion cracking has been discussed. This process can be promoted by nitrogen containing compounds. However, to be of any concern it is necessary to have a high concentration of nitrogen compounds (for example 1 mM of nitrite) and high redox potentials, neither of which is relevant for groundwater (Werme et al, 1992).

Steel, on the other hand, will corrode in water. If the water contains oxygen, iron(III) oxyhydroxides will be generated but no hydrogen. However, under anaerobic conditions, which are more likely in a repository, hydrogen will be generated along with ferrous corrosion products. The rate of anaerobic steel corrosion is probably less than 1 μm/a. If water penetrates the outer copper canister and comes into contact with the inner steel canister, anaerobic corrosion is expected. The rate of hydrogen generation can be estimated from the rate of corrosion and an estimate of the total steel surface available. Such calculations show that it may be necessary to anticipate the creation of a separate gas phase. Lead has been suggested as an alternative for using steel as a construction material for the inner canister. A separate gas phase was then not considered for such a lead filled canister. That was because the maximum rate of hydrogen production with a lead filled canister is low enough to be transported out by diffusion. All possible sources of hydrogen generation including radiolysis were considered, but it was demonstrated that the concentration of hydrogen could not surpass the solubility limit in water and consequently no separate gas phase could form (KBS-3, 1983).

The canister will shield most of the radiation coming from the fuel. Only some gamma radiation and very few neutrons will penetrate the canister. As an example, the dose rate outside a 6 cm (presently suggested to be 5 cm) copper canister according to the KBS-3 concept (copper filled) with 40 year old spent BWR-fuel with a burnup of 33 GWd/tU, would be of the order 10^{-4} Gy/s (Christensen and Bjergbakke, 1982a, Lundgren, 1982). The neutron contribution is negligible. The radiolysis due to radiation outside an intact

canister is of no importance for corrosion, bentonite stability etc. because the radiation is simply too weak (Christensen and Bjergbakke, 1982a).

Conclusions on the corrosion aspects can be summarised as follows:

- Oxygen is a copper corrodant. Reactions and conditions which prevent oxygen to reach a canister are therefore of interest. Pitting corrosion by oxygen has been observed which underlines the relative importance of oxygen.

- Sulphide reacts with copper. Therefore sources of sulphide in the repository have to be evaluated. Examples of that are concentration of sulphide in deep groundwater, sulphides in the backfill and reactions that can possibly generate sulphide from sulphate.

- Anaerobic corrosion generates hydrogen. In particular corrosion of an inner steel container may occur at a rate high enough (of the order 1 μm/a) to generate a separate hydrogen gas phase. However, this would first require a failure of the surrounding copper canister.

- Redox conditions, pH-conditions, salinity and trace constituents such as nitrogen compounds are of general interest to assess the copper stability.

1.3.3 Bentonite

Bentonite clay will be used as backfill material (for composition see Table 1.3). In the individual deposition holes, which will be vertical, about 8 m deep and have a diameter of 1.6 m, blocks of compacted bentonite will be emplaced between the canister and the rock (KBS-3, 1983). The bentonite blocks will take up groundwater, swell and form a plastic homogenous mass around the canisters that completely fills all voids. The bentonite clay protects the canister from rock movements, it can buffer pH and redox changes in the environment and it will also act as an important "resistor" to mass transport. Hydraulic flow is prevented by the clay and all transport will have to occur by diffusion, which is a very slow process. This prevents corrosive substances from reaching the canister and retards any release of radionuclides in the event of a canister failure.

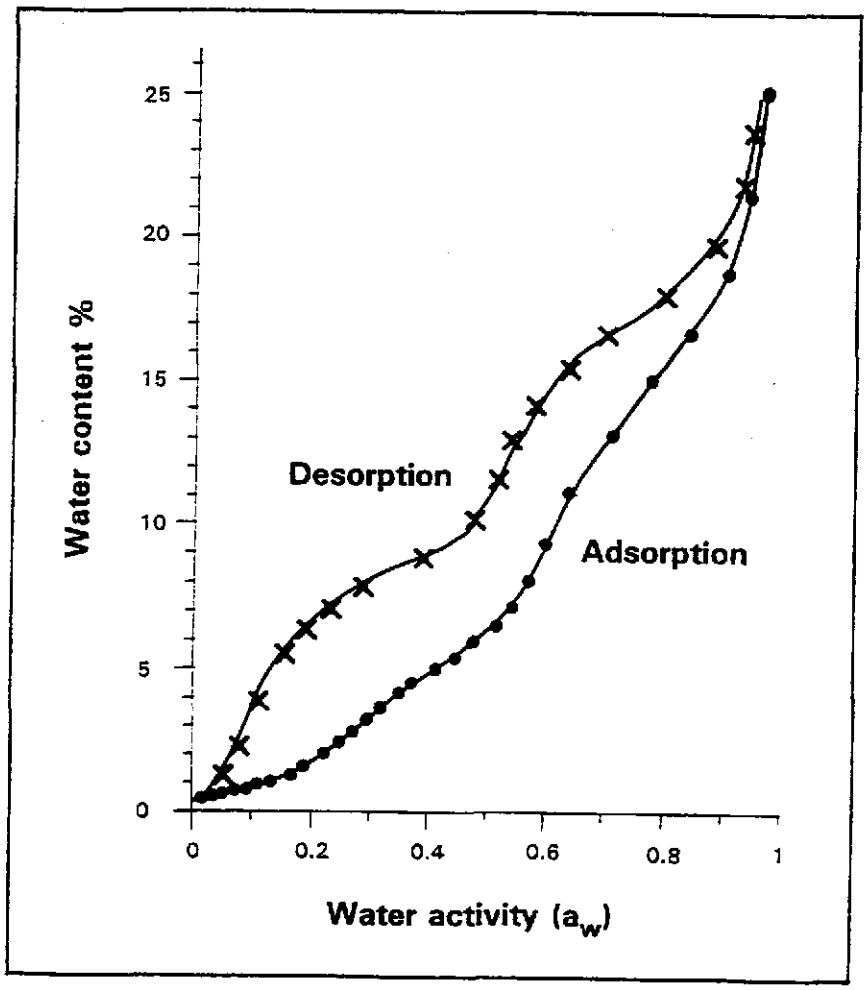

***Figure 1.2** Adsorption and desorption isotherms of water steam at 20°C in MX-80 bentonite (Kahr et al, 1986).*

The upper part of the deposition holes, and the tunnels and shafts are to be backfilled with a mixture of sand and bentonite. The proportions being presently considered are 15 % bentonite and 85 % sand.

Sodium bentonite marked MX-80 from Wyoming or South Dakota, USA, is the main candidate, but other qualities are possible. Bentonite is a natural clay containing 65-80 % of the smectite mineral montmorillonite. Other mineral components of bentonite are quarz (about 15 %) and feldspar (about 5-8 %) (Müller-Vonmoos and Kahr, 1983). Due to the smectite content, bentonite has a strong tendency to swell when taking up water. At a density of 2 tonnes/m^3 (water saturated) the swelling pressure is 5 MPa and the hydraulic conductivity is about 10^{-13} m/s (SKB-91, 1992). Bentonite contains small amounts of other minerals and impurities. Chemically important

components, other than clay minerals, are carbonates (eg calcite), sulphates, fluorides, sulphides (e.g. pyrite), ferrous iron and organic matter. Typical values reported are: carbonates (as CO_3) 1 % (Müller-Vonmoos and Kahr, 1983) sulphates (as SO_4) up to 0.8 % (Mattsson, 1983), phosphate (as PO_4) 0.1 %, fluorides (as F) 0.1 %, sulphides (as S) between 0.1 and 0.2 % (Jacobsson and Pusch, 1978) and organic matter up to 0.3 % (SKB-91, 1992). The organic matter has initially been characterised as non-humic and non-fulvic, probably kerogenic matter (Hallberg, 1978). The content of organic matter can be lowered by heat treatment to less than 0.02 % (Hallberg, 1978). The heat treatment will also bring down the sulphide content to the same figure (< 0.02 %). Nitrogen compounds have been determined as total-N to 0.01 % (Hallberg, 1978). All of the percent values are by weight and referring to the dry bentonite. The composition of bentonite clay is summarised in Table 1.3.

Table 1.3 The composition of the bentonite clay MX-80 from Wyoming.

Bentonite components	Content weight %
Montmorillonite	65-80
Quarz	15
Feldspar	5-8
Carbonate (CO_3)	1
Sulphate (SO_4)	0.8
Phosphate (PO_4)	0.1
Fluoride (F)	0.1
Sulphide (S)	0.1-0.2
Organic matter (C)	0.3
N-Compounds (N)	0.01

Bentonite will interact chemically with the groundwater. An example of that is given by experiments where porewater was pressed from sodium bentonite (MX-80) after equilibration with synthetic groundwater, so called Allard water. The porewater composition was characterised by a relatively high pH-value of about 9 and an increase in the concentration of carbonate and sulphate ions (Wanner et al, 1992). The concentration of carbonate (as HCO_3) was about 400 mg/L and the concentration of sulphate (as SO_4) was roughly 3000 mg/L as compared to the concentration of these constituents in the Allard water of 123 and 9.6 mg/L respectively. The porewater composition is a result of the ion exchange properties of the bentonite, the groundwater composition and the minerals contained in the clay. This was confirmed by successful simulations by model calculations (Wanner et al,

1992). Further calculation by Wanner has revealed the influences of other soluble bentonite components (o g Ca SO_4) on the pH (Wanner, 1995). The pH in the porewater of compacted bentonite was calculated to be in the range 6.8 to 9.3. Sodium dominated bentonite has a higher pH than calcium bentonite. Leaching of soluble components from bentonite will lend to increase the pH (towards 9). Experiments with interactions between Allard water and bentonite MX-80 have also been performed by (Snellman et al, 1986). From these and later studies they concluded that bentonite reacts with fresh groundwater and significantly rises the pH and alkalinity, increases the sodium content at the expense of calcium, magnesium and potassium which decreases (Pitkänen et al, 1992). Sulphate dissolution from the bentonite was noted and the values 60, 0.4 and 7.5 mg/L were given as typical values for sulphate (as SO_4), phosphate (as HPO_4) and fluoride, respectively. This was referred to as "bentonite water" in the Finnish safety analysis of spent fuel disposal, TVO-92 (Vieno et al, 1992). Due to the content of sulphides and ferrous iron, for example in the form of pyrite, the bentonite will also have a certain capacity to react with oxygen and control the redox conditions.

It is important that bentonite retains its physical properties and remains swelling, plastic and with a low hydraulic conductivity. Potential difficult transformations that have been discussed are illitisation, cementation and calcium/sodium exchange. Illitisation, the transformation to illite, may be the most important effect to be considered in this context. Supply of potassium and temperature are controlling parameters in this case and the repository has been set a temperature limit to avoid the reaction. Temperatures below 100 °C should be safe and 80 °C is being discussed as a target temperature. This is the maximum temperature in the bentonite, which will occur at the waste canister surface.

The chemical aspects of bentonite, which have been discussed and which are of relevance for objectives of this report can be summarised as follows:

- Bentonite is a swelling clay with a water content of at least 20 % at a wet density of 2 tonnes/m^3 in a deposition hole. The backfill in the tunnels and shafts is a mixture of 15 % bentonite and 85 % sand. The reference clay material is the natural sodium rich bentonite clay MX-80 from Wyoming or South Dakota.

- The most important component of bentonite is the clay mineral montmorillonite which gives the backfill its important swelling properties and ion exchange capacity.

- The mineral montmorillonite is an ampholyte that will buffer the pH in the pore water of bentonite clay in the range 7-9. Soluble salts, present

in the clay or added by the groundwater, will influence the pH through ion exchange reactions. Leaching of the buffer will increase pH (towards 9) and additions of saline water will tend to decrease the pH (towards 7).

- The total carbonate content in bentonite MX-80 (as CO_3) is roughly 1 % of the bentonite weight. Calcite in the clay will contribute to the pH buffering capacity.

- Bentonite MX-80 contains sulphate (0.8 % as SO_4), phosphate (0.1 % as PO_4) and fluoride (0.1 % as F). Ion exchange of calcium for sodium in the clay can to some extent increase the solubility of sulphate, phosphate and fluoride if they are initially present as calcium salts.

- Bentonite contains sulphides (between 0.1 and 0.2 % as S) and ferrous iron. Part of this is in the form of pyrite. Therefore wet bentonite will react with oxygen and to some extent buffer the redox conditions in the backfill. The sulphide will be reduced to less than 0.02 %, if it is decided to let the bentonite undergo heat treatment.

- Bentonite contains organic matter (up to 0.3 %), which have been initially characterised as nonhumic, nonfulvic and probably kerogenic. The organic content can be reduced to less than 0.02 % by heat treatment of the bentonite.

- Traces of nitrogen compounds have been reported (0.01 % as total-N)

1.3.4 Concrete

Concrete is used for underground construction, for manufacturing of waste containers and for conditioning of waste. The SFR-silo and caverns, shotcrete and rock sealing are examples of the use of concrete for underground construction. Spent ion exchange resins from nuclear power plants are to a large extent being conditioned with concrete in reinforced concrete containers, which are disposed in SFR. Conditioning in bitumen is an alternative method. The steel drums with bitumen are also emplaced in a concrete construction in the cavern BMA in SFR. Concrete for certain parts of the underground constructions of a spent fuel repository is being anticipated and concrete is also to be used for the underground disposal of long-lived LLW and ILW (construction and containers conditioning).

Concrete is a mixture of cement, ballast and water. The ballast constitutes about 65-70 % of the concrete volume and consists of sand or gravel and the

particles should not be less than 0.125 mm. The cement paste forms a continuous phase between the ballast grains. The total porosity of concrete is about 15 %. The porewater of fresh concrete made of standard Ordinary Portland Cement, OPC, can have a pH above 13.5 due to the content of alkali hydroxides (NaOH and KOH). An example of a typical porewater of fresh concrete is sodium, 207 mM; potassium, 435 mM; calcium, (saturated); chloride, 142 mM; sulphate, 4 mM and hydroxide 500 mM. This formula have been used for simulation of concrete conditions and the pH is 13.5 (activity). Examples of analyzed concrete porewater compositions are given in Table.1.4.

Table 1.4 Analysed concrete porewater. Ion concentrations in mg/L.

	Fresh cement		Leached cement	
	OPC[a]	OPC[b]	Sample 1[c]	Sample 2[c]
Age	3 months	10 months	70 years	70 years
w/c[d]	0.4	0.5	-	-
Na	644	1500	1500	260
K	3245	6300	1530	180
Ca	36	90	92	570
Mg	-	0.2	-	-
Al	1.1	<5	-	-
Si	22	<6	-	-
Fe	-	0.5	-	-
I, calc[e]	-	0.23		
pH	13.1	13.4	12.9	12.6

a (Lagerblad and Trägårdh, 1995)
b (Andersson, 1983)
c two different samples of 70 years old cement (Gjörv and Havdal, 1982)
d w/c = water/cement ration
e ionic strength, calculated according to $I = \frac{1}{2} \Sigma c_i z_i^2$

The relative content of alkali hydroxides in concrete is low, so if the concrete is leached by water the pH will soon drop to about 12.5. The resulting pH-value is due to the content of portlandite ($Ca(OH)_2$). One m^3 of concrete contains about 210 kg of lime, CaO, in the cement paste and a large portion of that is hydrated to portlandite. According to experiments, it may need as much as 900 renewals of the porewater in order to dissolve and remove the portlandite. However, even if all the portlandite is leached out, the remaining calcium silica hydrate (CSH) phases will keep the pH well above 10. Organic substances like lignosulphonate, cellulose derivatives etc are often added to the concrete in order to regulate its properties. The kind and quantity of these substances can vary, but a total amount of additives of about 0.1 % of the concrete weight is common.

The steel reinforcement is important component of concrete constructions. Steel will corrode but very slowly if it is protected by concrete. At oxic

conditions iron will corrode to "rust" (iron(III)-hydroxy-oxides) and no hydrogen will be generated in that process. However, in a closed and sealed underground repository, the oxygen will sooner or later be consumed and anaerobic corrosion will dominate, which yields hydrogen. Anaerobic corrosion of steel at high pH conditions typical for concrete porewater have been investigated. It was concluded that the average rate of corrosion will be lower than 0.1 μm/a (Kreis, 1993).

The chemical properties of concrete which are important for this report are:

- Fresh concrete (OPC) has a pH above 13 due to its content of alkali hydroxides (NaOH and KOH). The total amount of these hydroxides is low.

- One m^3 of concrete contains about 210 kg of lime which can form portlandite and thereby control the pH at a level of 12.5 for a very long time (roughly 900 porewater exchanges). After that, the calcium silica hydrate phases will control the pH (pH>10).

- Additives to concrete are used which contain organic substances such as lignosulphonate and cellulose derivatives. The total amount of additives can be about 0.1 % of the concrete weight.

- The steel reinforcement is protected by the concrete, but a slow corrosion is feasible. Oxic corrosion will consume oxygen and anaerobic corrosion will generate hydrogen. The average rate of anaerobic corrosion is well below 0.1 μm/a according to measurements.

1.4 PERFORMANCE SAFETY ASSESSMENT

In the performance assessment of radioactive waste repositories both operational safety and long term safety is evaluated. The assessment of operational safety of underground repositories is dealing with the radiation hazards and also the hazards usually encountered in underground works. The operational hazards are minimized by the fact that only packaged waste is being handled in the repository. The assessment of the long time safety of a closed repository for radioactive waste is almost entirely devoted to the radiotoxic effects. A leading principle is to have several different barriers in order to prevent radionuclide dispersion in the surrounding groundwater. By isolating the radionuclides long enough, they will decay to insignificant levels before they can ever reach man and his environment.

1.4.1 Barriers to radionuclide dispersal

Sometimes a distinction is made between so called engineered barriers and natural barriers. Engineered barriers are waste, canisters and backfill, and a natural barrier is the surrounding rock. The distinction is not entirely unambiguous, because the selection of disposal depth is of course part of the engineering. However, it is interesting from the point of safety philosophy. The construction and quality of the engineered barriers, or rather technical barriers, can be controlled by man in great detail compared to the geo-barrier. On the other hand, the rock has proven its long time performance as a stable environment. The consequences of slow processes and traces of past events and changes in the geosphere are there for us to study. A sensible combination of the two approaches will increase the safety of a disposal concept.

A good example of the application of barriers, is the spent fuel disposal concept. The basic safety principle is to completely enclose and isolate the spent fuel in tight canisters at a depth of about 500 m. The isolation shall be provided and remain long enough for the radionuclides to decay and not become released. The most important role of the rock barrier is to provide long time stable chemical and mechanical conditions for the technical barriers. However, the performance safety assessment of spent fuel disposal rests on the multi barrier principle. This implies that the safety should not be entirely dependent on the technical barriers and their anticipated functions. An important safety function of host rock is to retain radionuclides or retard their migration if the technical barriers are damaged.

The Swedish disposal concepts for spent fuel, LLW and ILW have been briefly described in previous sections. Barriers effective in these concepts for different waste forms are listed in Table 1.5 No distinction have been made between operational and long-lived low level waste, due to the similarity between SFR and SFL 3-5 (see Section 1.1):

Table 1.5 Barriers effective in concepts for different waste forms.

Barrier	Spent fuel SFL 2	LLW and ILW SFR and SFL 3-5
Waste form	Uranium dioxide matrix	Concrete or bitumen used for conditioning
Containers and constructions	Copper/steel canister	Concrete containers and constructions
Backfill	Bentonite clay buffer	Bentonite clay used around the SFR silo
Host rock	About 500 m deep in granitic rock	More than 50 m of granitic rock

The barriers are dependent on chemical and hydrogeological conditions in order to remain stable and function properly. This has been highlighted in the preceding Section 1.3. Another important aspect of barrier integrity is mechanical stability. The emplacement of the repository, in the host rock and the construction details of a spent fuel repository such as clay buffer and canister, are made with regard to possible external forces, rock movements etc. However, these aspects are outside the scope of this report.

1.4.2 Retention barrier functions

Barrier functions associated with radionuclide retention are generally of chemical and hydrogeological nature. Important retention barrier functions of this kind are:

- **Radionuclide solubility**. The low solubility of many important radionuclides is efficient to prevent radionuclide release. Examples of elements with low solubilities in groundwater and bentonite porewater are Th, Pu, Am and, provided the conditions are reducing, U, Np and Tc. This will lower any release of these nuclides, if spent fuel becomes exposed to groundwater (SKB-91, 1992). Solubilities will also limit any release from LLW and ILW, but it is more difficult to assess its importance, due to the relatively low concentrations of radionuclides in this kind of waste and the more complicated composition. However, nickel solubility in concrete is one example where solubility limitations have been evaluated in the performance assessment of LLW and ILW. The nickel isotopes appears in scrap metal with induced activity (Wiborgh, 1995).

- **Radionuclide diffusion**. The slow diffusion of radionuclides through a bentonite buffer or a concrete overpack is an important barrier function. Some radionuclides have a relatively short half-life. This, in

combination with slow diffusion and these nuclides may become either entirely retained or at least considerably reduced by the diffusion barrier. Examples of that such radionuclides are ^{137}Cs, ^{241}Am and ^{239}Pu which are considerably retarded by the bentonite buffer (Karlsson, 1990). Long-lived nuclides like ^{129}I will not decay in the buffer, but their rate of escape will be reduced by diffusion. Important in this context is the diffusive transport to the slowly flowing groundwater outside the buffer. This is dependent on the groundwater flow rate (square root dependence). In the case of a penetrated waste canister, the diffusive transport through the hole is another important resistance in the chain of resistors to material transport which are included in the near-field release process.

- **Reduction of flow**. A canister is of course an absolute barrier to flow, for as long as it remains intact. However, even a penetrated canister is a hinderance to flow, at least if there is only a small hole in it. The bentonite buffer will also prevent water flow and this is one of its most important functions. Concrete overpacks and concrete constructions used for LLW and ILW will also prevent flow. Although in the performance assessment it is necessary to consider that, in contrast to bentonite clay, cracks may develop in concrete. The selection of "good host rock" for the repository is another way of reducing the potential flow.

- **Radionuclide sorption**. The uptake of radionuclides on the overpack materials in the near-field and on the rock mineral surfaces of the far-field are important retention processes. Sorption of radionuclides in concrete is an efficient barrier function in repositories for LLW and ILW. Sorption of radionuclides on rock fracture surfaces is important for all disposal concepts in hard crystalline rock.

- **Matrix diffusion**. This term is used to describe the combined process of sorption and diffusion, when dissolved radionuclides are retained by escaping from the slowly flowing groundwater in the rock fractures into the interconnected system of microfissures of the seemingly intact rock. This is in fact the most efficient mechanism for retention of radionuclide migration in the far-field.

A complication in the assessment of far-field retention of radionuclide transport, is the need to have reliable information on the dispersion of groundwater flow. Even a small fraction of the radionuclides can dominate the release, if they travel fast compared to their decay. Another important flow related factor is the fracture surface available for contact with dissolved radionuclides in the fractures. This contact surface, referred to as "flow wetted surface", is an important parameter. A large flow wetted surface means a large surface for radionuclide sorption and, even more important, a large area through which the radionuclides can reach the stagnant water in the interconnected microfracture system by diffusive transport (matrix diffusion).

The retention barrier functions which have been presented here are not the only ones. The important retention of radionuclides in the waste matrix has not been elucidated here, but it was mentioned in the previous section on material properties.

Some other barrier functions exist but it is difficult to use them in performance assessment. A good example of that is co-precipitation. The solubility of an element can be lowered by formation of a mixed solid pH face with another compound. This will happen when for example radionuclides are in contact with corroding metals. The uptake of cations in iron hydroxides is a well known phenomenon. Similar fixations will occur if for example calcite precipitates. However, so far there is a hesitation to use this information. One reason for that is the difficulty to assess the stability of the precipitate. A phase transformation could in principle cause the release of the co-precipitated nuclide. So, either one has to demonstrate that the first solid phase formed is stable, or that the new phase does also contain the nuclide.

A last barrier function is dilution. Strictly taken it is not a retention barrier, but it serves well to protect man and the environment from toxic substances released to the groundwater, if they should ever reach a well, a stream, a lake etc. The potential dilution barrier is of course a considerable advantage when the repository is situated under the sea floor as SFR. That is by the way, not the only advantage with under sea emplacement. The driving hydraulic pressure in groundwater under the sea bottom is generally very low due to the flat surface of the sea above, as there is not any horisontal components in the gradient of hydraulic pressure.

1.4.3 Influences on retention barriers

In the process of performance assessment, all barriers and barrier functions are critically examined. Anything that can have an adverse influence is carefully examined and assessed. Potential influences on the stability of different barriers have already been mentioned in preceding sections on materials. Chemical conditions that have been scrutinized for their possible influences on retention barrier functions are as follows:

- **Colloids**. Colloidal particles are potentially important for different reasons. Radionuclides can in principle exist in groundwater above their solubility limit, if they occur as colloids. Colloids can have different sorption and different diffusion properties than the ions. Radionuclides as colloids can be generated differently; precipitate as true elementary colloids or become attached to already existing inactive colloids and form "pseudocolloids". Based on this knowledge, different scenarios can be imagined in a repository. The radionuclides can form colloids such as: 1) directly leached out as true elementary colloids, 2) or attached to colloidal particles released from waste matrix, the canister, the backfill etc, or leached out as ions to the near-field and subsequently, 3) taken up on natural colloids in the groundwater. Bentonite clay backfill is, in these scenarios, an important filter for particular matters released from the waste. The uptake of radionuclides on natural colloids is limited by the concentration of colloids and their surface properties (Allard et al, 1991).

- **Complexing agents**. Inorganic components in groundwater such as carbonate ions will form complexes with for example the actinide ions. Humic and fulvic acids, which are also present in natural groundwater, will form strong complexes with for example threevalent actinide ions such as Am^{3+}. Low and intermediate level waste contains organic materials that can in principle generate products with complexing properties. Radionuclide complexes can behave differently as compared to the "free, naked" ion. They may exhibit higher solubility and lower sorption. It is particulary important to assess the influence of possible complexing agents on three- and tetravalent cationic radionuclides such as many of the actinides.

- **Gas phase**. Gas present, or generated at such a rate that it can form a separate gas phase in the repository, has to be anticipated in the performance assessment of the barriers. The content of dissolved gaseous compounds is generally low in groundwater and not enough to form a gas phase at repository depth. Gaseous substances can be generated in a repository by for example radiolysis of water (H_2, O_2), anaerobic corrosion of steel (H_2) and decomposition of organic matter (CO_2, CH_4, H_2). Radiolysis is too slow to generate a separate gas phase. The gas-forming species can remain dissolved and escape by diffusion in the water phase. However, it is possible that corrosion and perhaps also decomposition can generate enough gas for a separate phase to form (bubbles). It is important that the gas is allowed to escape without harming the construction and without pressing too much contaminated water out into the fractures of the near-field rock (Moreno and Neretnieks, 1988). Other more remote possibilities of gas influence on radionuclide dispersion have been discussed, such as effects on the hydraulic flow, and radionuclides being carried by gas bubbles. Recent publications have discussed the combined transport of colloids and gas (Wan and Wilson, 1994 a,b).

These are not the only adverse effects considered in a full performance safety assessment, but of most relevance for the continued discussion in this report. Readers, who are interested in a more complete account, are referred to recent safety reports, for example SKB-91 (SKB-91, 1992).

2 SUBTERRANEAN BACTERIA AND ENVIRONMENTAL LIMITATIONS

Normal conditions have been used to understand the foundations of bacterial cell functions and to describe the origin of physiological biochemistry. Normal conditions are neutral pH, a temperature at 37 °C, aerobic atmosphere with no over pressure, 1% salinity and with glucose as the main energy and carbon source. The preceding chapter shows that conditions for life in waste repository environments in most respects will be extreme, i.e. very different from what is understood as normal conditions. Modelling bacterial processes in nuclear waste disposal therefore must concentrate on bacteria adapted to such environments. Detailed understanding of these bacteria is unfortunately limited in comparison with what is known for bacteria living at normal life conditions, especially when it comes to predicting bacterial processes in future waste repositories. This obvious lack of knowledge has motivated us to initiate field and laboratory investigations on subterranean bacteria and their importance to performance assessment.

Bacteria are much more diverse in comparison with plants and animals. Among the huge diversity of bacteria there are microorganisms capable to grow at or adapt to extreme conditions from our point of view. Some bacteria grow at temperatures above 100 °C, other thrive in high salinity such as 20-30% NaCl, still others can live at pH lower than 2 or pH higher than 10. Bacteria differ in part from plants and animals because they can adapt and grow under very extreme conditions. There have been many different phases in the evolution of life which started for more than 4 billion years ago. It is generally agreed that the earth was cooling and occupied by numerous volcanic features at that time. The early atmosphere was anaerob and therefore free of oxygen. Slowly, about 1-2 billion years ago, photosynthetic life became powerful enough to change the environment totally on the surface of earth from anaerobic and reducing to aerobic and oxidizing. The adaptation to extreme environments is believed to have taken place during this sequence of events which led to the biosphere and the kinds of bacteria we have today. The repository environments are constructed by man and therefore not previously exposed to microbes. Any adaptation features needed in addition to what is already present among the bacteria will probably, at least for the initial life time of a repository, act as limiting factors. This chapter reviews what is known about environmental limitations for bacteria and compare them with the conditions for the Swedish nuclear waste concept described in *Chapter 1*.

2.1 PRINCIPLES OF BACTERIAL CATALYSIS

A key feature of a living bacterium is its ability to organise molecules and chemical reactions into specific structures and systematic sequences. The ultimate expression of this organisation is the ability of a living organism to replicate itself. The term *metabolism* is used to refer to all chemical processes taking place within a cell. The word metabolism is derived from the Greek word metabole which means change, and we think of a bacterial cell as continually changing as it carries out its life processes. Although the bacterium appears to be a fixed and stable structure under the microscope, it is actually a dynamic entity, continually undergoing change, as a result of the chemical reactions which are constantly taking place.

Bacteria are built of a wide variety of chemical substances and when a cell grows all of these chemical constituents increase in amount. The basic chemical elements of a cell all come from outside the cell, the environment, but these chemical elements are transformed by the cell into characteristic constituents of which the cell is composed.

2.1.1 *Activation energy, catalysis and enzymes*

Free-energy calculations of various reactions tell us only what conditions will prevail when the reaction or system is in equilibrium. They do not tell us how long it will take for equilibrium to be reached. The formation of water from hydrogen and oxygen is a good example. This reaction is very exergonic, but just mixing the two gases will not start the reaction even if we wait for a lifetime. A certain amount of activation energy (e.g. a burning match) has to be added before the reaction will start. The idea of activation energy leads us to the concept of catalysis. Hydrogen oxidizing bacteria are able to utilise the energy in this reaction, not by adding fire, but by the use of a catalyst. A catalyst is a substance which serves to lower the activation energy of a reaction and to increase the reaction rate even though it itself is not changed.

Most reactions in living organisms will not occur at appreciable rates without catalysis. The catalysts of biological reactions are proteins called enzymes. Enzymes are highly specific for the reactions which they catalyse. That is, each enzyme catalyses only a single type of chemical reaction or in the case of certain enzymes, a class of closely related reactions. This specificity is related to the precise three-dimensional structure of the enzyme molecule. In an enzyme-catalysed reaction the enzyme temporarily combines with the reactant, which is termed a substrate (S) of the enzyme. Then, after the reaction occurs, the product (P) is released and the enzyme (E) is returned to its original state:

$$E + S \Leftrightarrow E\text{-}S \Leftrightarrow E + P$$

The enzyme is generally much larger than the substrate. The small portion of the enzyme to which the substrates bind is referred to as the *active site* of the enzyme. The catalytic power of enzymes is impressive. Enzymes typically increase the rate of chemical reactions some 10^8 to 10^{20} times the rate that would occur spontaneously. Note that enzymes can catalyse both endergonic and exergonic reactions.

The chemistry of biosynthesis has one marvelously simple aspect: virtually all biosynthetic pathways, polymerizations and assembly processes, are fundamentally the same in all bacteria. Even the co-ordination of biosynthetic pathways is conceptually simple, because it involves adjustments in flow through essentially unidirectional pathways with few alternative routes to a given building block.

2.1.2 The kinetic properties of enzymes

For many enzymes, the rate of catalysis, V, varies with the substrate concentration, [S], in a manner shown in Figure 2.1. At a fixed concentration of enzyme, V is almost linearly proportional to [S], when [S] is small. At high [S], V is nearly independent of [S]. In 1913, Leonor Michaelis and Maud Menten proposed a simple model to account for these kinetic characteristics. The critical feature in their treatment is that a specific ES complex is a necessary intermediate in catalysis. The model proposed, which is the simplest one that accounts for the kinetic properties of many enzymes, is

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P \qquad (1)$$

An enzyme, E, combines with S to form an ES complex, with a rate constant k_1. The ES complex has two possible fates. It can dissociate to E and S, with a rate constant k_2, or it can proceed to form product, P, with a rate constant k_3. It is assumed that nothing of the product reverts to the initial substrate, a condition that holds in the initial stage of a reaction before the concentration of product is appreciable.

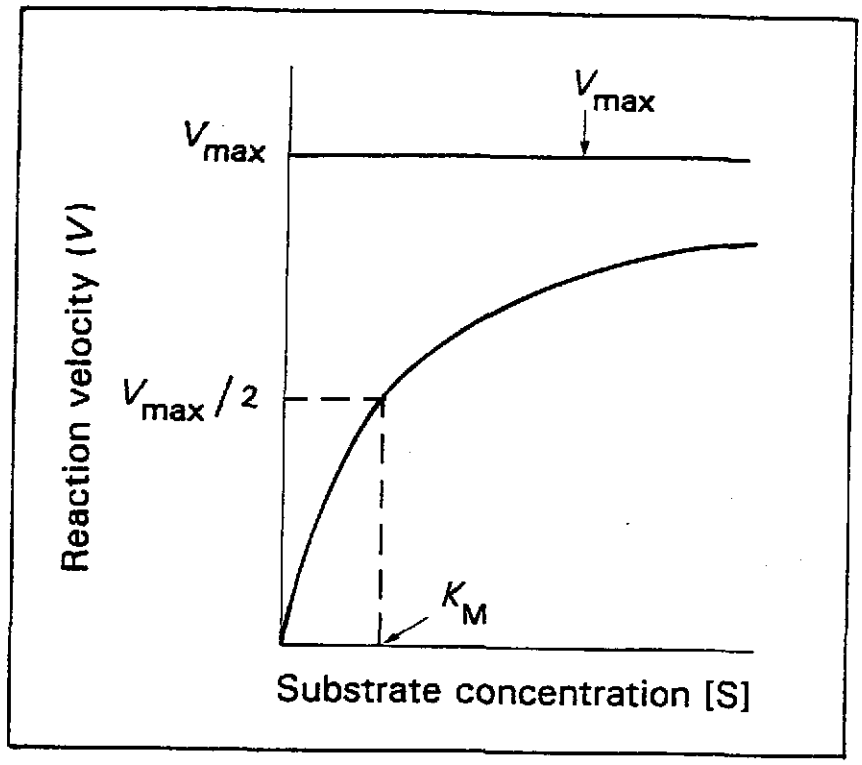

Figure 2.1 *A plot of the reaction velocity, v, as a function of the substrate concentration, [S], for an enzyme that obeys Michaelis-Menten kinetics (V_{max} is the maximal velocity and K_M is the Michaelis constant).*

We want an expression that relates the rate of catalysis to the concentrations of substrate and enzyme and the rates of the individual steps. The starting point is that the catalytic rate is equal to the product of the concentration of the ES complex and k_3:

$$V = k_3[ES] \tag{2}$$

From this expression the Michaelis - Menten equation can be deduced:

$$V = V_{max} \frac{[S]}{[S] + K_M} \tag{3}$$

This equation, 3, accounts for the kinetic data given in Figure 2.1 At low substrate concentration, when [S] is much less than K_M, $V = [S]\ V_{max}/K_M$; that is, the rate is directly proportional to the substrate concentration. At high substrate concentration, when [S] is much greater than K_M, $V=V_{max}$; that is, the rate is maximal, independent of substrate concentration.

The meaning of K_M is evident from equation 3. When [S] = K_M, then $V = V_{max}/2$. Thus, K_M, *is equal to the substrate concentration at which the reaction rate is half of its maximal value.*

The maximal rate, V_{max}, reveals the turnover number of an enzyme if the concentration of active sites [E_T] is known because

$$V_{max} = k_3[E_T] \tag{4}$$

For example, a 10^{-6} M solution of the enzyme catalase catalyzes the formation of 0.2 M $H_2O + O_2$ from H_2O_2 per second when it is fully saturated with substrate. Hence, k_3 is 2×10^5 sec^{-1}. The kinetic constant k_3 is called the *turnover number.* The turnover number of an enzyme is *the number of substrate molecules converted into product per unit time when the enzyme is fully saturated with substrate.* The turnover number of 200,000 sec^{-1} for catalase is one of the largest known. Each round of catalysis occurs in a time equal to $1/k_3$, which is 5.1 microseconds for catalase.

When the substrate concentration is much greater than K_M, the rate of catalyzis is equal to k_3, the turnover number, as described in the preceding section. However, most enzymes are not saturated with substrate under physiological conditions. The [S]/K_M ratio is typically between 0.01 and 1.0. When [S] << K_M, the enzymatic rate is much less than k_3 because most of the active sites are unoccupied. Is there a suitable parameter to characterise the kinetics of an enzyme under these conditions? Indeed there is V, the reaction rate when S is >> K_M which is describen by following reaction.

$$V = \frac{k_3}{K_M}[E][S] \tag{5}$$

When [S] << K_M, the concentration of free enzymes, E, is nearly equal to the total concentration of enzyme [E_T], and so

$$V = \frac{k_3}{K_M}[S]\ [E_T] \tag{6}$$

Thus, when [S] <<K_M, the enzymatic velocity depends on the value of k_3/K_M and on [S]. Are there any physical limits on the value of k_3/K_M? Note that this ratio depends on k_1,k_2 and k_3, as can be shown by substituting for K_M

$$k_3/K_M = \frac{k_3\ k_1}{k_2 + k_3} \tag{7}$$

The ultimate limit on the value of k_3/K_M is set by k_1, the rate of formation of the ES complex. *This rate cannot be faster than the diffusion-controlled encounter of an enzyme and its substrate.* Diffusion limits the value of k_1 so that it cannot be higher than between 10^8 and 10^9 M^{-1} sec^{-1}. Hence, the upper limit on k_3/K_M is between 10^8 and 10^9 M^{-1} sec^{-1}.

This restriction also pertains to enzymes having more complex reaction pathways than that of equation 1. Their maximal catalytic rate when substrate is saturating, denoted by k_{cat}, depends on several rate constants rather than on k_3 alone. The pertinent parameter for these enzymes is k_{cat}/K_M. In fact, *the k_{cat}/K_M ratios of a number of enzymes, such as acetylcholinesterase, carbonic anhydrase, catalase, and triosephosphate isomerase, are between 10^8 and 10^9 M^{-1} sec^{-1}, which shows that they have attained kinetic perfection. Their catalytic velocity is restricted only by the rate at which they encounter substrate in the solution.* Any further gain in catalytic rate can come only by decreasing the time for diffusion.

2.1.3 Enzymes transforming reactive oxygen species to molecular oxygen

Highly reactive and thereby toxic forms of oxygen are produced in aerobic cells. For example, the reduction of O_2 to $2H_2O$ which occurs during respiration requires addition of four electrons (Table 2.1 A) This reduction usually occurs by single electron steps, and the first product formed in the reduction of O_2 is the **superoxide** anion, O_2^-, a potentially toxic form of oxygen. Superoxide is probably formed transiently in small amounts during normal respiratory processes, and it is also produced in the presence of light following one-electron transfer to oxygen. Flavins, flavoproteins, quinones, thiols, and iron-sulphur proteins all carry out one-electron reductions of oxygen to superoxide. Superoxide is highly reactive and can cause oxidative destruction of lipids and other biochemical components. It has the longest life of the various oxygen intermediates and may even pass from one cell to another.

The next product in the stepwise reduction of oxygen is **peroxide**, O_2^{2-}. The peroxide anion is best known in the form of hydrogen peroxide, H_2O_2, which is sufficiently stable that it can be used as an item of chemical commerce. Peroxide is commonly formed biochemically during respiratory processes by a two-electron reduction of O_2, generally mediated by flavoproteins. Hydrogen peroxide is produced in small amounts by almost all organisms growing aerobically.

The **hydroxyl radical**, $OH^{\bullet}$, is the most reactive of the various oxygen intermediates. Although it may have a half-life in the cell of only 10^{-9} seconds, the hydroxyl radical is the most potent oxidizing agent known and is capable of attacking any of the organic substances presents in cells. It most frequently exerts damage by destroying enzymes and membranes, nicking

DNA, and ultimately leading to cell lysis. Hydroxyl radicals is formed as a result of the action of ionising radiation, and is probably one of main agents in the killing of cells by X-rays and gamma-rays. Hydroxyl radicals can also be produced from H_2O as shown in Table 2.1 A.

With such an array of toxic oxygen derivatives, it is perhaps not surprising that the organisms have developed enzymes that destroy certain oxygen species (Table 2.1 B). The most common enzyme in this category is **catalase**, which acts on hydrogen peroxide. Another enzyme that acts on hydrogen peroxide is **peroxidase,** which requires the presence of a reductant, usually NADH. Superoxide is destroyed by the enzyme **superoxide dismutase** (Table 2.1 B), which combines two molecules of superoxide to form one molecule of hydrogen peroxide and one molecule of oxygen. Superoxide dismutase and catalase working together can thus bring about the conversion of superoxide back to oxygen. No enzymatic system exists to deal with hydroxyl radicals, probably because of the transient nature in the cell. However, removal of H_2O_2 from cells probably protects the cells in part by preventing the formation of hydroxyl radicals.

Table 2.1 A. Four-electron reduction of O_2 to water by stepwise addition of electrons. All of the intermediates formed are reactive and toxic to cells.

B. Enzymes acting on toxic oxygen species. a) Catalases and b) peroxidases are generally porphyrin-containing proteins, although some flavoproteins may act in this manner. c) Superoxide dismutases are metal-containing proteins, the metal being either copper, zinc, manganese, or iron.

A	B
$O_2 + e^- \rightarrow O_2^-$ Superoxide $O_2^- + e^- + 2H^+ \rightarrow H_2O_2$ Hydrogen peroxide $H_2O_2 + e^- + H^+ \rightarrow H_2O + OH^\bullet$ Hydroxyl radical $OH^\bullet + e^- + H^+ \rightarrow H_2O$ Water Overall: $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$	*a)* Catalase: $H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$ *b)* Peroxidase: $H_2O_2 + NADH + H^+ \rightarrow 2H_2O + NAD^+$ *c)* Superoxidismutase: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

2.2 OXYGEN AND REDOX CONDITIONS

2.2.1 Oxygen concentration and redox potential

A bacterium able to grow in the presence of molecular oxygen, either in gaseous or dissolved form, is an *aerobe*, whereas one that can grow without oxygen is an *anaerobe* (Table 2.2). Bacteria that are completely dependent of oxygen for growth are termed *obligate aerobes.* Oxygen serves as the terminal electron acceptor for the electron transport chain in aerobic

respiration. *Facultative anaerobes* do not require oxygen for growth but do grow better in its presence. If not available, they can use alternative electron acceptors such as NO_3^-, Mn^{4+} or Fe^{3+}. In addition, many facultative anaerobic bacteria switch between respiration and fermentation, of which the latter process does not require any external electron acceptor. *Aerotolerant anaerobes* simply ignore oxygen and grow equally well whether it is present or not. In contrast, *strict* or *obligate anaerobes* as the sulphate reducing and metanogenic bacteria, do not tolerate oxygen at all and will not grow in its presence. Finally, there are a few aerobes, called *microaerophiles*, that are damaged by the normal atmospheric level of oxygen and require oxygen levels in the range of 2 to 10% for growth.

Table 2.2 Terms used to describe O_2 relations of bacteria.

Group	O_2 relation
Aerobes	
Oligate	O_2 is required
Facultative	O_2 is not required but growth is better with O_2
Microaerophilic	O_2 is required but at levels lower than atmospheric
Anaerobes	
Aerotolerant	O_2 is not required and growth is not better with O_2
Obligate (strict)	O_2 is harmful or lethal

These different relationships to oxygen appear due to several factors, including the inactivation of proteins and the effect of toxic oxygen species. Enzymes can be inactivated when sensitive groups like sulpphydryls are oxidised. A notable example is the nitrogen-fixation enzyme nitrogenase, which is very oxygen sensitive. Oxygen accepts electrons and is readily reduced because its two outer orbital electrons are unpaired. In aerobic cells, flavoproteins, cytochromes and several other cell constituents and radiation promote oxygen reduction. The result is usually some combination of the reaction products *superoxide radical, hydrogen peroxide* and *hydroxyl radical*. These products are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly. Obligate aerobes and facultative anaerobes possess enzymes that afford protection against such oxygen products and therefore are not killed when growing in the presence of oxygen. All strict anaerobes lack one or more of these enzymes or have them in very low concentrations and can therefore not tolerate oxygen (see section 2.1.3 for details.)

Oxygenated environments have a high redox potential which decreases as oxygen disappears, which is often due to bacterial oxygen respiration. Most aerobes therefore are adapted to oxidizing environments with a high redox

potential while the life patterns of strict anaerobes demand reduced conditions. Bacteria frequently change the redox potential of their own habitat by producing redox active metabolic waste products. For example, the production of ferrous iron by iron reducing bacteria (IRB), hydrogen sulphide by sulphate reducing bacteria (SRB), hydrogen by fermenting bacteria and methane by methanogenic bacteria all force the redox potential of the system towards more negative values.

2.2.2 Oxygen and redox conditions in repository and rock environments

Deep groundwater does not contain any oxygen and measurements of redox potential using electrodes give values between -100 to -400 mV (Table 1.1). It is expected, except for the operational and closure phases, that the repository oxygen and redox environment will be equal to that of the surrounding groundwater - anaerobic with a low redox potential. Therefore, investigations must include facultative and strict anaerobes adapted to different long term repository environments. Aerobes must be considered important during the operational phase, the transitional state and in scenarios when radiolysis of water produces oxygen enough to stimulate aerobic growth.

2.3 TEMPERATURE

Environmental temperature profoundly affects bacteria, like all other organisms (Table 2.3). Indeed bacteria are particularly susceptible because they are unicellular and poikilotherm - their temperature varies with that of the external environment. A most important factor influencing the effect of temperature upon growth is the temperature sensitivity of enzyme catalysed reactions. At low temperatures, a temperature rise increases the growth rate because the velocity of an enzyme-catalysed reaction, like that of any chemical reaction, will roughly double for every 10°C rise in temperature. The rate of each reaction increases at higher temperature and the bacteria grow faster. Beyond a certain point, further increase in temperature damage bacteria by denaturing enzymes, transport carriers and other proteins and finally growth is inhibited because the damage cannot be repaired.

Because of these opposing temperature influences, bacterial growth has fairly characteristic temperature dependence with distinct cardinal temperatures - minimum, optimum and maximum growth temperatures. The cardinal temperatures for a specific species are not rigidly fixed, but often depend to some extent on other environmental factors such as pH and the available nutrients.

Table 2.3 The upper temperature limits (°C) for growth.

Organism		Temperature
Animals		
	Fish	38
	Insects	45-50
Plants		
	Vascular plants	45
	Mosses	50
Eukaryotic microorganisms		
	Protozoa	56
	Algae	55-60
	Fungi	60-62
Photosynthetic bacteria		
	Cyanobacteria	55-70
	Purple bacteria	55-60
	Green bacteria	70-73
Other eubacteria		
	Gram-positive bacteria including *Bacillus, Clostridium* and actinomycetes	50-75
	Gram-negative aerobes and anaerobes	50-75
	Thermus	85
	Thermotoga maritima	90
Archaea		
	Thermoplasma	65
	Methanobacterium	75
	Methanothermus	97
	Sulfolobus	87
	Acidianus	95
	Pyrococcus	103
	Pyrodictum	110
	Methanopyrus	110

The cardinal temperatures vary greatly between bacteria. Optima normally range from 0°C to as high as 75°C, while bacterial growth occurs at temperatures extending from - 5°C to over 100°C. The growth temperature range for a particular species usually spans about 30°C. Some species have a small range and are called *stenothermal*; others will grow over a wide range of temperatures and are called *eurythermal*. Generally, temperatures below the minimum value act dormant while temperatures above the maximum value are lethal. Spore-forming bacteria may survive much higher temperatures resting as spores than as active bacteria.

Bacteria can be placed in five classes based on their temperature range for growth:

1. *Psychrophiles* grow well at 0°C and have an optimum growth temperature of 15°C or lower; the maximum is around 20°C. They are readily isolated from Arctic and Antarctic habitats, and because 90% of the ocean is 5°C or colder, it constitutes an enormous habitat for psychrophiles. Most psychrophilic bacteria are members of the genera *Pseudomonas, Flavobacterium, Achromobacter* and *Alcaligenes.*

2. Many species can grow at 0°C even though they have optima between 20 and 30°C, and maxima around 35°C. These are called *psychrotrophs* or *facultative psychrophiles.*

3. *Mesophiles* are bacteria with growth optima around 20 to 45°C and a temperature minima of 15 to 20°C. Their maximum is about 45°C or lower. Most known bacteria probably falls within this category.

4. Some bacteria are *thermophile s*;they can grow at temperatures of 45-70°C. Their growth minimum is usually around 45°C and they often have optima between 55 and 65°C. Thermophiles differ from mesophiles in having much more heat-stable enzymes and protein synthesis systems able to function at high temperatures (Table 2.4).

5. *Hyperthermophilic* eubacteria and archaea are unable to grow below 60°C, some not even below 80°C. They have optimal growth between 80 and 100°C. During the last years, more than 50 hyperthermophilic strains have been isolated. Most hyperthermophiles are obligate anaerobes and use sulphur, sulphate or carbon dioxide as electron acceptor. Among them are some autotrophs (Table 2.9).

Table 2.4 Major groups of thermophilic bacteria.

Genus		Number of species	Temperature range °C
EUBACTERIA			
Phototrophic bacteria			
	Cyanobacteria	16	55-70
	Purple bacteria	1	55-60
	Green bacteria	2	70-73
Gram-positive bacteria			
	Bacillus	15	50-70
	Clostridium	11	50-75
	Lactic acid bacteria	5	50-65
	Actinomycetes	23	55-75
Other eubacteria			
	Thiobacillus	3	50-60
	Spirochete	1	54
	Desulfotomaculum	7	37-55
	Gram-negative aerobes	7	50-75
	Gram-negative anaerobes	4	50-75
	Thermotoga	1	55-90
ARCHAEA			
	Methanogens	4	55-110
	Extreme thermophiles	18	55-110
	Thermoplasma	1	37-55

2.3.1 Temperature conditions in repository and rock environments

The SFL 2 repository concept (1.1.1) for spent fuel is designed to have an initial peak temperature of 80°C at the canister surface and the temperature will decrease with the distance from the repository to some 60°C in the repository rock and to the ambient 17°C in the far-field environment. The repository temperature during the first 3000 years will favour thermophilic bacteria. As a temperature above the maximum temperature for a bacterial species is lethal, (except for sporeformers) most bacteria other than thermophiles will be killed. With time, the repository temperature slowly decreases towards the temperature of the surrounding rock (17°C) appropriate for first mesophiles and then psychrotrophs (Figure 1.3). These bacterial groups then may reinvade the repository area. Therefore, investigations for the SFL 2 repository must include both thermophilic, mesophilic and probably also psychrotrophic bacteria. The SFR (1.1.2) and SFL 3-5 (1.1.3) concepts assume psychrotrophic to mesophilic conditions and are consequently included within the SFL 2 concept, when it has cooled to appropriate temperatures, with respect to bacteria and temperature.

2.4 THE INFLUENCE FROM pH

It is not surprising that pH dramatically affects bacterial growth (Table 2.5). Each species has a definite pH growth range and pH growth optimum. *Acidophiles* have their growth optimum between 1.0 and 5.5; *neutrophiles* between pH 5.5 and 8.5; and *alkalophiles* prefer the pH range of 8.5 to 11.5. *Extreme alkalophiles* have growth optima at pH 10 or higher. In general, different bacterial groups have characteristic pH preferences. Most bacteria and protozoa are neutrophiles, while most fungi prefer slightly acid surroundings, about pH 4 to 6.

Despite wide variations in habitat pH, the internal pH of most bacteria is close to neutral. This may result from plasma membrane impermeability to protons. Possibly, protons and hydroxyl ions are pumped out to maintain the proper internal pH. Extreme alkalophiles maintain their internal pH close to neutrality by exchanging internal sodium ions for external protons.

Although bacteria often grow over wide ranges of pH, there are limits to their tolerance. Drastic variation in pH can harm bacteria by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Changes in external pH might alter the ionisation of nutrient molecules and thus reduce their availability to the organism.

Bacteria frequently change the pH of their own habitat by producing acidic or basic metabolic waste products. Fermenting bacteria may form organic acids from carbohydrates while chemolithotrophs like *Thiobacillus* oxidise sulphide ores to sulphuric acid. Other bacteria make their environment more alkaline by generating ammonia through amino acid degradation.

Table 2.5 pH and temperature ranges for bacterial growth for some different bacteria.

Organisms		Range for growth pH	Range for growth temperature
Eubacteria			
	Thiobacillus thiooxidans	0.9-4.5	20-45
	Thiobacillus ferrooxidans	1.5-4.0	20-45
	Bacillus acidocaldarius	2.0-6.0	55-65
	Bacillus alcalophilus	8.5-11.6	30-40
Archaea			
	Thermoplasma acidophilum	1.0-4.0	30-80
	Sulfolobus acidocaldarius	0.9-5.8	55-87
	Natronobacterium	9.0-10.0	20-50
	Natronococcus	8.5-11.0	35-40

2.4.1 pH conditions in repository and rock environments

The pH of deep groundwater is normally in the range of 6.5 to 9.5 and will select for neutrophiles and also for alkalophiles (Table 1.1). However, the pH measurements made in groundwater probably represent the gross pH and do not necessarily take into account microniches, where for example acid producing bacteria will lower the pH and methane-forming organisms will increase the pH. Therefore, acidophiles can not be totally ruled out as being present, but they will most certainly not dominate any present bacterial population.

The bentonite clay has a pH around 9 (1.3.3), which is a pH environment that can be defined as alkaline and therefore alkalophiles can be expected to dominate among present bacteria. The elevated repository temperatures will, at least for an initial repository life time of approximately 3000 years (se Figure 1.1), select for thermophilic (temperature >55°C) alkalophiles (pH>9) in the repository clays and for thermophilic neutrophiles in the surrounding repository rock. Thereafter psychro- and mesophiles will reinvade the repository area.

Concrete is used for various underground constructions in all different waste disposal concepts (1.3.4). The pH ranges from that of fresh Ordinary Portland Cement (OPC) at 13.5 down to just above 10 to 12.5 in more or less leached concrete. In other words, the concrete environment will select for extreme alkalophiles. Repository concepts other than SFL 2, may therefore eventually be enriched with psychro- and mesophilic extreme alkalophiles, while there may be a further restriction with respect to temperature (thermophiles) for the SFL 2 concept depending on the location of concrete constructions.

2.5 SOLUTES AND WATER ACTIVITY - SALINITY LIMITS

2.5.1 Solutes

Because a selectively permeable plasma membrane separates bacteria from their environment, they can be affected by changes in the osmotic concentration of their surroundings. If a bacterium is placed in a hypotonic solution, water will enter the cell and cause it to burst unless something is done to prevent the influx. Most bacteria have rigid cell walls that maintain the shape and integrity of the cell. Indeed, many bacteria keep the osmotic concentration of the protoplasm above that of the habitat by use of compatible solutes, so that the plasma membrane is always pressed firmly against their cell wall. Compatible solutes are solutes that are compatible with cell metabolism and growth when at high concentrations. It can be synthesis of choline, betanine, proline, glutamic acid and other aminoacids;

elevated levels of potassium ions are also involved to some extent. A few bacteria like *Halobacterium salinarium* raise their osmotic concentration with potassium ions and have enzymes that require high salt concentrations for normal activity.

2.5.2 Water activity

The amount of water available to bacteria can be reduced by interactions with solute molecules (the osmotic effect) and by adsorption to the surface of solids (the matrice effect). Because the osmotic concentration of a habitat has such profound effects on bacteria, it is useful to be able to express quantitatively the degree of water availability. Microbiologists generally use *water activity* (a_w) for this purpose, which is a thermodynamic parameter (see Section 2.5.4). The water activity of a solution is 1/100 the relative humidity of air in equilibrium with the solution (when expressed as percent). It is also equivalent to the ratio of the solution's vapour pressure ($\boldsymbol{P_{soln}}$) to that of pure water ($\boldsymbol{P_{water}}$).

$$a_W = \frac{\boldsymbol{P_{soln}}}{\boldsymbol{P_{water}}}$$

The water activity of a solution or solid can be determined by sealing it in a chamber and measuring the relative humidity after the system has come to an equilibrium. Water activity is inversely related to osmotic pressure Π; if a solution has a high osmotic pressure, its a_w is low.

$$\Pi = \frac{RT}{V_w} ln \frac{I}{a_w}$$

Curiously, the most limiting conditions for microbial growth seems to be the availability of water. As far as is known nothing can grow within solid ice, nor in steam. In solutions, or on surfaces a substantial amount of water is needed. A limiting a_w value of 0.6-0.7 seems to hold so that only about 40% of available water can be removed before growth of even the most desiccation resistant microorganism stops. The reason is far from obvious. Future workers on extreme environments must look further into the structure and functions of water itself, especially intracellular water. We may find that some microorganisms can grow in almost any environment, provided that temperature is not high enough to disrupt essential molecules and that the cells are able to maintain a "comfortable" form of intracellular water.

Table 2.6 Water activity of several materials with some microorganisms growing at that water activity in comparison with Na-bentonite, MX-80 with different water contents.

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

Bacteria differ greatly in their ability to adapt to habitats with low water activity (Table 2.6). A bacterium must expend extra efforts to grow in a habitat with a low a_w value because it must maintain a high internal solute concentration to retain water. Some bacteria do this and are osmotolerant; they will grow over wide ranges of water activities or osmotic concentrations. For example, *Staphylococcus aureus* can be cultured in media containing any sodium chloride concentration up to about 3 M. Although a few bacteria are truly osmotolerant, most only grow well at water activities around 0.98, (the approximate a_w for sea water) or higher. *Extreme halophiles* have adapted so completely to saline conditions that they require high levels of sodium chloride to grow, concentrations between about 2.8 M and saturation, about 6.8 M. Extreme halophilic bacteria have adapted successfully to environmental conditions that would destroy most organisms. In the process, they have become so specialised that they have lost ecological flexibility and can prosper only in a few extreme habitats.

2.5.3 Chemical potential

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

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

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

2.5.4 Water potential

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

2.5.5 Water activities in repository and rock environments

The groundwater in the repository rock and the surrounding rock will have a relatively high water activity, usually not lower than that of sea water (0.98) and will therefore not constitute any environmental limitation for most bacteria adapted to aquatic ecosystems (Table 2.6). In addition, the backfill of bentonite/sand (15/85%) will have a fairly high water activity. Turning to the bentonite, MX-80, that will be used around the canisters, the picture changes dramatically. At start, the bentonite will have a water activity of approximately 0.75 which is low enough to exclude the absolute majority of

microorganisms. Endospores formed by many *Bacillaceae* species may in fact be the only life form that possibly will survive. Therefore, keeping the water activity low, at for instance 0.96 (water content 25%) will definitely be a very potent environmental limitation for most bacteria trying to invade the canister clay environment.

2.6 RADIATION

Many forms of electromagnetic radiation are very harmful to bacteria. This is particularly true for ionising radiation which can cause atoms to lose electrons or ionise. Among the potential chemical species formed by ionising radiation are chemical free radicals, of which the most important is the hydroxyl radical, $OH^{\bullet}$. Free radicals react with inactive macromolecules in the cell, of which DNA is the most important. At low doses of ionising radiation only a few hits on DNA occur, but since each DNA molecule contains one copy of most genes, inactivation can lead to permanent mutations. Low doses of ionising radiation may indirectly lead to death while higher levels are directly lethal. Although bacteria are much more resistant to radiation than larger organisms, they will still be destroyed by a sufficiently large dose. The acute lethal dosage for many bacteria are approximately some 10 Gy or more (Figure 2.2). Some bacteria and bacterial endospores can survive large doses. For example, *Deinococcus radiodurans* survive doses as high as 10,000 Gy!

Unlike inanimate things, unicellular and multicellular organisms evolve. This implies that hereditary changes, which occur at low but regular rates in all cells, and can influence in a positive or negative way the overall fitness of the cell or higher organism. The result of evolution is selection for those organisms best suited for life in a particular environment. Low doses of radiation will cause mutations of which the absolute majority will be quiet or lethal and only very few will lead to expressed hereditary changes. High doses will be lethal. The possibility of rapid evolution of new species, even bacteria pathogenic to man, as a result of elevated radiation levels in repositories has been suggested. There will however not exist any selective pressure for pathogens or other bacteria harmful to man in subterranean repositories and consequently such bacteria will not evolve.

2.6.1 Radiation in a SFL 2 repository

All the alpha and beta and most of the gamma radiation are shielded by the canister and the dose-rate at the canister surface will be of the order of 10^{-4} Gy/s [8.6 Gy/d]. This will be enough to kill radiation sensitive bacteria, especially if they are inactive. Consequently there may be a noticeable selection towards radiation resistant species of bacteria eventually colonising the canister surface or the buffer. In the case of water penetrating to the fuel,

the most significant effect on bacteria probably will be that radiolysis of water introduces oxygen and raises the redox potential in the vicinity of the canister.

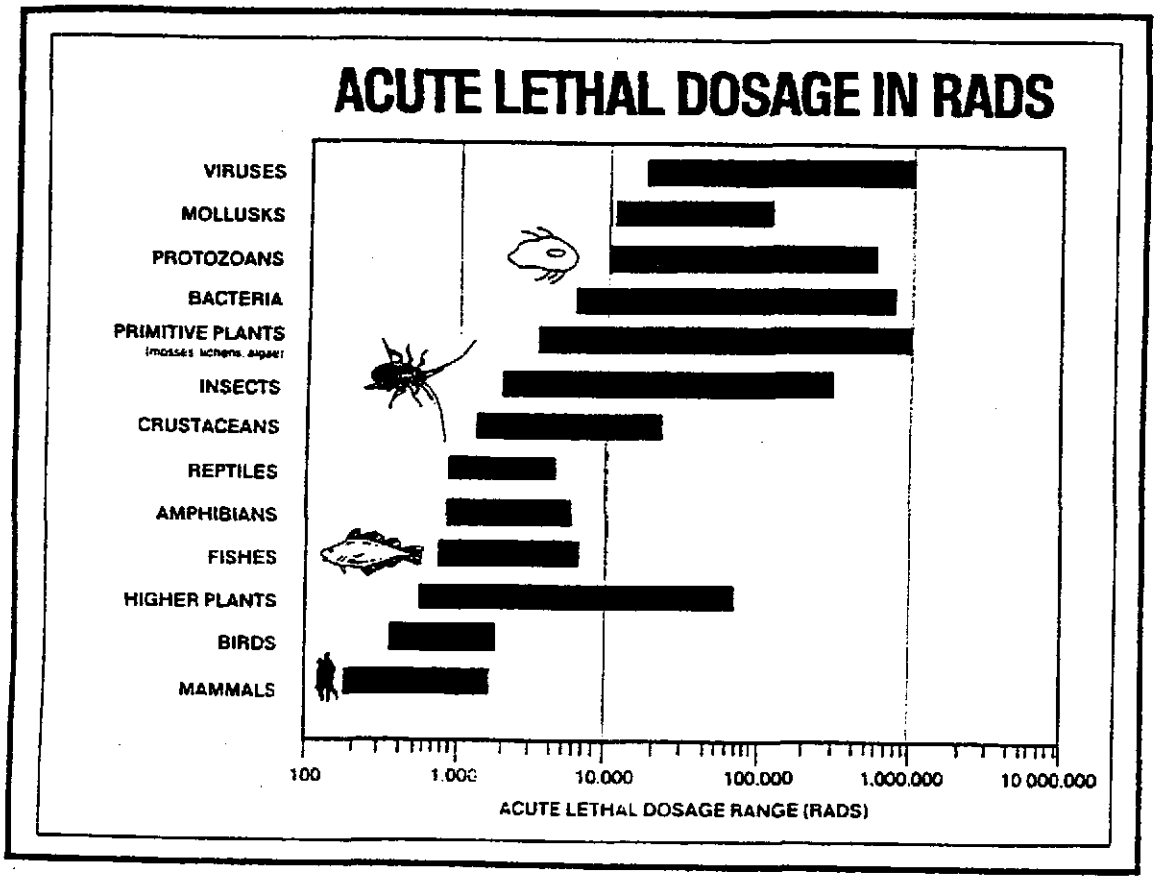

Figure 2.2 Approximate acute lethal dose ranges for various taxonomic groups. 1 rad = 0.01 Gy. (Whicker and Schultz, 1982).

2.6.2 Bacterial recombination of hydrogen and oxygen from radiolysis

Inorganic, catalytic decomposition of radiolysis reactants like H_2O_2, $OH^\bullet$ and O_2^- has been suggested (Christensen, 1994). Ferrous iron oxidation by such reactive oxygen species is believed to take part in this process. A repository enviroment will however not be sterile. It will harbour many aerobic bacteria, which usually posses enzymes according to Table 2.1, B. It may therefore be interesting to calculate if bacterial enzymes, like catalase and hydrogenase, can constitute an additional mechanism for the oxidation of radiolysis oxidants. The rate constant for oxidation of ferrous iron to ferric iron and $OH^\bullet$ with H_2O_2 is 60 M^{-1} s^{-1}.

$$\left[Fe^{2+}\right] \left[H_2O_2\right] \; 60 \; = \; V \qquad (8)$$

Combining equations 5 and 8 for Fe^{2+}, catalase and H_2O_2 gives:

$$[Fe^{2+}][H_2O_2]60 = \frac{2\cdot 10^5}{0.025}\cdot[E_T][H_2O_2] \quad (9)$$

simplifying gives:

$$[E_T] = \frac{60\cdot 0.025}{2\cdot 10^5}\cdot[Fe^{2+}] \quad (10)$$

A Fe^{2+} concentration of 10^{-7} M requires a concentration of 7,5 x 10^{-14} catalase, to balance the oxidation at the same rate. Not more than 0.1g catalase can do the work of 1 ton of Fe^{2+}. One aerobic bacterium contains approximately 1% catalse. With a molecular weight of catalase of 250000 u and an average bacterium weight of 10^{-13} g gives that one bacterium contains $4x10^{-21}$ M catalase. To balance the ferrous iron + H_2O_2 reaction the following amount of bacteria is needed:

$$\frac{7\cdot 5\cdot 10^{-14}}{4\cdot 10^{-21}} \approx 2\cdot 10^7 \quad (11)$$

That is 2 x 10^7 bacteria per 1itre or 2 x 10^4 bacteria per ml would theoretically would balance the oxidation power of 10^{-7} M Fe^{2+}. This is within the range of bacteria observed in deep groundwater. Results from the Stripa mine and Äspö tunnel area indicate that there may be many more bacteria attached to surfaces, i.e. in rock fractures, than unattached (Pedersen and Albinsson, 1992, Pedersen and Ekendahl, 1992a, Pedersen and Ekendahl, 1992b). Assuming a mean channel width of 0.1 mm, the results imply that there may be up to 7.9 x 10^5 attached bacteria per unattached bacteria in fractured rock. Then, taking maximal possible numbers of bacteria (7.9 x 10^5 x 5.4 10^7 bact./ml) we get 4.2 x 10^{13} bacteria per ml including attached ones. As 2 x 10^4 bacteria per ml were needed to counteract chemical oxidation by ferrous iron of the radiolysis product H_2O_2, there would be 2.1 x 10^9 bacteria in excess. In conclusion, even much more conservative calculations will confirm that bacteria can constitute a powerful oxidation agent for radiolysis products.

2.6.3 Enzymes recombining oxygen with hydrogen

Next step in the bacterial decomposition of radiolysis products is the removal of oxygen. In addition to respiration of organic material with oxygen, the recombination of oxygen with hydrogen is a very energy rich bacterial process. The background for bacterial oxidation of hydrogen is as follows.

Although the first hydrogen oxidising bacteria were isolated many years ago, in the past 30 years many new genera, species, and strains of hydrogen

oxidizing bacteria have been isolated (Table 2.7). With more extensive knowledge of the physiology, biochemistry, and genetics of bacteria and with a wealth of information on the physical and chemical parameters of various ecosystems, we may ask basic questions about the habitats (i.e., the dwelling places) and ecological niches (i.e., the functions) of these microorganisms.

Table 2.7 Genera and species of hydrogen oxidizing bacteria (knallgas bacteria).

Gram-negative species:		**Gram-positive species:**
Alcaligenes eutrophus	*Pseudomonas facilis*	*Arthrobacter* strain 11/X
A. hydrogenophilus	*P. flava*	*Bacillus schlegelii*
A. ruhlandii	*P. pseudoflava*	*B. tusciae*
A. latus	*P. hydrogenovora*	*Mycobacterium gordonae*
A. paradoxus	*P. hydrogenothermophila*	*Nocardia autotrophica*
A. eutrophus CH34	*P. palleronii*	*N. opaca*
Aquaspirillum autotrophium	*P. thermophila*	
Azospirillum lipoferum	*P. saccharophila*	
Calderobacterium hydrogenophilum	*Renobacter vacuolatum*	
Derxia gummosa	*Rhizobium japonicum*	
Flavobacterium autothermophilum	*Xanthobacter autotrophicus*	
Hydrogenobacter thermiphilus	*X. flavus*	
Microcyclus aquatis	*Most carboxydobacteria*	
Paracoccus denitrificans		

The ratio in which H_2, O_2, and CO_2 are consumed by a growing culture of hydrogen-oxidizing bacteria is about the following:

$$4H_2 + 2O_2 \rightarrow 4H_2O \qquad (12)$$

$$2H_2 + CO_2 \rightarrow (CH_2O) + H_2O \qquad (13)$$

$$6H_2 + 2O_2 + CO_2 \rightarrow (CH_2O) + 5H_2O \qquad (14)$$

Thus, the oxidation of $4H_2$ to water yields enough ATP to allow the synthesis to cell material (CH_2O) from CO_2 and H_2.

ATP synthesis proceeds by a chemiosmotic mechanism as in aerobic respiration. Cytochromes, ubiquinone, and metaquinone have been found in membrane fractions of hydrogen-oxidizing bacteria. Differences between hydrogen-oxidizing bacteria have been encountered as to the transfer of electrons from H_2 to the respiratory chain. *Nocardia opaca* contains a soluble hydrogenase which catalyzes the reduction of NAD^+ by H_2. The product NADH, then serves as H-donor for the respiratory chain. This is the

exception. All other hydrogen-oxidizing bacteria studied contain a particulate (membrane-bound) hydrogenase which feeds electrons directly into the respiratory chain. This enzyme does not react with NAD^+. Very few hydrogen-oxidizing bacterium (e.g. *Alcaligenes eutrophus*) contain, in addition to the particulate enzyme a soluble hydrogenase with reduces NAD^+ and which is primarily responsible for the provision of NADH for CO_2 reduction.

The uptake-hydrogenases discussed here catalyze the oxidation of H_2 to H^+ according to the equation

$$H_2 \rightarrow 2H^+ + 2e^- \qquad (15)$$

and the transfer of the electrons to the appropriate acceptors. The enzymes of the H_2 oxidizers have been shown to be nickel-proteins. In addition they contain iron sulphur centers. The K_M values for hydrogenases are less than 1 μM, so these enzymes are very effective in oxidizing H_2 (Table 2.8). The turnover for hydrogenases lies between 10^3 and 10^5 sec^{-1}. As K_M for hydrogenases is below 1 μM, this reaction will not be rate limiting in comparison with reaction (6) for catalase.

Table 2.8 Apparent K_M values for hydrogen and threshold concentrations of H_2 (nM) of representative bacteria.

Strain or species	K_M (μM)	Threshold dissolved H_2 (nM)	Threshold of H_2 in gas phase (ppmv)
Alcaligenes eutrophus H16	> 1	6.2	8.3
Alcaligenes paradoxus SA29	> 1	4.2	5.5
Xanthobacter ausotrophicus /C	> 1	5.0	6.7
Xanthobacter SA35	> 1	0.9	1.3
Aquaspirillum autotrophicum SA32	> 1	1.9	2.5
Strain PP5	> 1	2.1	2.8
Strain PP18	> 1	24	33
Strain PP19	> 1	133	178

2.6.4 Radiolysis products in repositories

Reactive radiolysis products may very efficiently be converted to oxygen by aerobic and facultatively anaerobic bacteria. The hydrogen produced during radiolysis together with the oxygen produced from reactive oxygen species can subsequently be recombined by bacteria under the formation of organic material, heat and water. Energy from radioactive decay may in fact be partly conserved as organic material in the form of bacteria. This is an alternative or complementary model for the fate of radiolysis products. Results from investigations of the Cigar Lake uranium ore have shown that bacteria are present and that there are also measurable amounts of organic material

ranging between 0.9 up to 7 mg TOC per litre. The presence of this bacterial process will contribute to the stability of uranium(IV) in the ore. The same process occuring in a spent fuel repository would in fact add to the stability of the waste matrix (uranium dioxide) by consuming oxygen from the radiolysis.

2.7 HYDROSTATIC PRESSURE AND BACTERIAL GROWTH

Bacteria are able to withstand and flourish at the highest hydrostatic pressure on the planet - those found in the deepest parts of the ocean (Kato et al, 1994a). Because water flows readily into bacteria, high hydrostatic pressure cannot crush them; but it can inhibit certain chemical reactions by preventing the formation of the activated state, the obligatory intermediate of any enzyme reaction. If the specific molecular volume of the activated state exceeds that of the reactants, high hydrostatic pressure will slow or even stop the reaction. In contrast, if the specific molecular volume of the activated state is less than that of the reactants, the reverse result obtains: the reaction is speeded up. Natural selection is able to affect the molecular volume of the activated state of an enzyme-catalysed reaction, so one might anticipate that various bacteria would respond differently to hydrostatic pressure, depending on the environment in which they evolved. Such is indeed the case. A pressure dependent enzyme expression, more sensitive than the mechanism described above, has recently been demonstrated (Kato et al, 1994b). In the deep ocean, one class of bacteria, called *barophiles,* can grow better at elevated hydrostatic pressure than at atmospheric pressure. There are even bacteria, called *obligate barophiles* that can grow only under hydrostatic pressure that exceeds 1 atm.

Ordinary bacteria that probably have not been challenged by high hydrostatic pressure during their evolution are, nevertheless, remarkably tolerant to such pressure. *Escherichia coli* grows well at hydrostatic pressures as high as 300 atm. In strong contrast, most yeasts cannot grow at pressures that exceeds 8 atm, a fact that makes bottled champagne possible. Further fermentation activity of the yeast will be blocked at this pressure.

2.7.1 Hydrostatic pressure in repository and rock environments

The SFL 2 repository will be situated at a depth with approximately 50 atm of hydrostatic pressure. This is a pressure that most bacteria can tolerate and pressure therefore will not constitute any important environmental limitation for bacteria.

2.8 BACTERIAL NUTRITION

Analysis of bacterial cell composition shows that over 95% of cell dry weight is made up of a few major elements: carbon, oxygen, hydrogen, nitrogen, sulphur, phosphorus, potassium, calcium, magnesium and iron. These are called *macroelements* because they are required by bacteria in relatively large amounts. The first six are components of carbohydrates, lipids, proteins, and nucleic acids. The remaining four macroelements exist in the cell as cations and play a variety of roles. All bacteria require several *trace elements* besides the macroelements. The trace elements manganese, zink, cobalt, molybdenum, nickel and copper are needed by most cells and are needed in so low concentrations that contaminants in water, glass ware and chemicals often are adequate for dense growth on lab. Trace elements are normally important parts of enzymes and cofactors, and they aid in the catalysis of reactions and maintenance of protein structure. Besides the common macroelements and trace elements, bacteria have particular requirements that reflect the special nature of their physiology and the environment where they live.

2.8.1 The requirement for carbon, hydrogen and oxygen

The requirement for carbon, hydrogen and oxygen is often satisfied together. Carbon is required for the skeleton or backbone of all organic molecules, and molecules serving as carbon sources usually also provide both oxygen and hydrogen atoms. One carbon source for which this is not true is carbon dioxide (CO_2). Probably all bacteria can fix CO_2; that is, reduce it and incorporate it into organic molecules. However, by definition, only *autotrophs* (Table 2.9) can use CO_2 as their sole or principal source of carbon. The reduction of CO_2 is a very energy expensive process. Thus, many bacteria can not use it as their sole carbon source but must rely on the presence of more reduced, complex molecules for supply of carbon. Organisms that use reduced, preformed organic molecules as carbon source are *heterotrophs* These preformed molecules normally come from other organisms, often recently produced but many bacteria can also use fossil organic carbon sources such as oil. Most heterotrophs use organic nutrients as a source of both energy and carbon.

A most remarkable nutritional characteristic of bacteria is their extraordinary flexibility with respect to carbon sources. All naturally occurring organic molecules can be used by different bacteria. Actinomycetes degrade amyl alcohol, paraffin and even rubber. Other bacteria are able to employ almost anything as carbon source; for example, *Pseudomonas capacia* can use over 100 different carbon compounds. In contrast to bacterial omnivores, some bacteria are exceedingly fastidious and catabolize only few carbon compounds. Methylotrophic bacteria catabolize only methane, methanol, carbon monoxide, formic acid and a few other related one carbon molecules.

2.8.2 The requirement for nitrogen, phosphorus and sulphur

To grow, a bacterium must be able to incorporate nitrogen, phosphorus and sulphur. Bacteria usually employ inorganic sources of these elements. Nitrogen is needed for the synthesis of aminoacids, purines, pyrimedines, some carbohydrates and lipids, enzyme cofactors and other substances. Many bacteria can reduce nitrate to ammonia which is incorporated via biosynthetic pathways. Other are able to reduce and assimilate atmospheric nitrogen, by *nitrogen fixation*, using a specific enzyme called *nitrogenase*. This enzyme is extremely sensitive to oxygen which irreversibly inhibits its function. Nitrogen gas usually is the dominating gas found in granitic groundwater (Table 1.2) and nitrogen limitation will only occur as a function of energy limitation. This is because nitrogen fixation is a very energy expensive metabolic process.

Phosphorus is present in nucleic acids, phospholipids, nucleotides like ATP, several cofactors, some proteins and other cell components. Almost all bacteria use inorganic phosphate as their phosphorus source and incorporate it directly. Although the concentration of phosphorus usually is low in granitic groundwater (Table 1.1) it is enough to support the standing crop of bacteria observed. A key question is, what is the source of phosphate to subterranean bacteria? Most granites contains a small portion of apatite [$Ca_5(PO_4)_3(F,Cl,OH)$], enough to support a microbial population. For example, the P_2O_5 content in the Äspö hard rock varies between 0.03% up to 0.4% - we find the low values in fine grained granites and larger values in the gouge material of the fracture zones (Wikman and Kornfält, 1995). Locally produced acid conditions by biofilm bacteria will mobilise such phosphate into the bacterial pool. This has been neatly shown for epilithic microbial biofilms in Arctic Canada (Konhauser et al, 1994). As long as there exists a pool of phosphorus, in equilibrium with solid phosphorus minerals, it will be the flux of energy that limit the activity of subterranean bacteria, not the concentration of phosphorus.

Sulphur is needed for the synthesis of substances like the amino acids cystein and methionine, some carbohydrates, biotin and thiamine. Most bacteria use sulphate as a source of sulphur and reduce it by assimilatory reduction. Sulphate is readily available in groundwater and will not be limiting (Table 1.1).

2.8.3 Nutritional types of bacteria

All organisms require sources of energy, carbon and electrons for growth. Organisms often are grouped into classes based on how they satisfy these requirements (Table 2.9). *Autotrophs* use CO_2 as their sole or principal source of carbon. Organisms that use reduced, organic molecules as carbon source are *heterotrophs*. There are only two sources of energy available, (1) light used during photosynthesis and (2) the energy derived from oxidation of

inorganic or organic molecules. *Phototrophs* use light as their energy source; *chemotrophs* obtain energy from oxidation of chemical compounds (either inorganic or organic). Bacteria also have only two sources of electrons (reducing power). *Lithotrophs* (that is, "rock-eaters") use inorganic substances as their electron source whereas *organotrophs* extract electrons from organic compounds.

Table 2.9 Sources of carbon, energy and hydrogen/electrons for different nutritional types of organisms.

Nutritional type	Carbon sources	Energy sources	Electron sources (reducing power)	Examples of organisms
Photolihtotrophic autotrophy	CO_2	Light	H_2O	Green plants, algae cyanobacteria
			H_2S, S, S_2O_3, H_2	Purple and green sulphur bacteria
Photoorganotrophic heterotrophy	Organic compound	Light	Organic compound	Purple and green nonsulphur bacteria
Chemolihtotrophic autotrophy	CO_2	Chemical	NH_4^+, NO_2^-, Mn^{2+}, Fe^{2+}, H_2S, S, H_2	Ammonium, nitrite, manganese, iron, sulphur and hydrogen oxidizing bacteria methanogenic bacteria
Chemoorganotrophic heterotrophy	Organic compound	Chemical	Organic compound	Most bacteria fungi, animals

Despite the great metabolic diversity seen in bacteria, most of them may be placed in one of four nutritional classes based on their primary source of energy, carbon and electrons. The large majority of bacteria thus far studied are either photolithotrophic autotrophs (often called photoautotrophs) or chemoorganotrophic heterotrophs (often called chemoheterotrophs). Frequently, the same organic nutrient will satisfy the chemoorganotrophic heterotroph with all its energy, carbon and electron requirements. Lithotrophs on the other hand have different carbon and energy sources. For chemolithotrophic autotrophs, energy and electron sources usually are the same. When it comes to energy sources available to subterranean bacteria, light is not available, except for the light used during construction and operational phases of repositories. For long term modelling, all bacterial processes having light as the main energy source can be excluded.

2.8.4 Bacterial life at low nutrient availability

Heterotrophic bacteria appear to dominate the microflora of most subsurface ecosystems (se chapter 4). Thus, organic carbon should be expected to be the most limiting nutrient for growth and activity of subterranean bacteria. Autotrophs on the other hand will be limited by the availability of inorganic chemical energy like hydrogen.

Bacterial cells, deprived of exogenous carbon and energy sources, have to exploit endogenous resources to meet their maintenance energy requirements. The endogenous metabolism has been defined as the total metabolic reactions that occur when the bacterial cell is deprived of compounds or elements that may serve specifically as exogenous substrates. Studies on some bacteria have indicated that starvation survival may be dependent upon the regulation of endogenous metabolism in accordance with energy maintenance requirements. No universal patterns or predictable sequences of utilisation of intracellular constituents have, however, been found. The degradation of endogenous material seems to result in a continuous size reduction that, in addition to a reductive cell division, contributes to the formation of ultra-microcells. For example growing *Vibrio* cells may have a cell volume of 6 μm^3 while starved cells are as small as 0.05 μm^3.

In deeper aquifers, the supply of readily utilizable carbon and energy sources may be extremely small. In theory, only the most refractory organic compounds will survive long and complex pathways through biologically active subsoil and subsurface sediments. Thus, as indicated above, organic carbon probably is the most limiting nutrient in deep pristine environments. However, inorganic compounds must also be considered. The possibility of unknown geological sources of organic carbon and hydrogen and methane can presently not be excluded.

2.8.5 Bacterial nutrition in repository and rock environments

Bacterial heterotrophic metabolism in SFL 2 repository and rock environments will be more or less limited by the availability of organic material possible to use for growth and maintenance. Chemolithotrophic metabolism may in theory be possible, provided there exist inorganic energy sources and suitable electron acceptors (confer Table 2.9). The activity of present bacterial populations probably will be low but conclusive data is lacking and this topic must be further studied. Geothermally produced hydrogen and sedimentary organic material may act as energy sources for subterranean ecosystems. This is a major task, because, if this is true, these subterranean ecosystems would be much more independent of terranean systems than anticipated today. The SFR and eventually also SFL 3-5 will have waste that are organic and therefore, these environments will not be energy and carbon limited.

2.9 ATTACHED VERSUS UNATTACHED BACTERIA

Bacteria adapt and orient toward environments that favour their survival and growth. Most solid surfaces in aquatic ecosystems harbour attached bacteria that often appear in microcolonies or even in multi-layered biofilms revealing growth to occur. There is yet not any universal understanding of the advantages bacteria achieve from being attached. It is usually suggested that the nutrient availability may be larger at a surface than in the surrounding water phase due to physical sorption processes. The development of attached bacterial populations generally follows a sequence of processes:

- *Conditioning.* There is considerable evidence that organic material dissolved in water is adsorbed to clean surfaces exposed to the water. The process follows adsorption isotherms and starts instantaneously at exposure. This is in accordance with the view that surface absorption of organic molecules, at least at relatively uncharged surfaces, is governed by the lowering of free energy resulting from reduction of interactions between non-polar groups and water molecules. The adsorbed films that form on surfaces in biologically active systems are believed to lower the surface free energies and always to proceed bacterial attachment. Biological macromolecules such as glycoproteins, proteoglucans or their end product humic and fulvic residues are examples of molecules readily adsorbed to interfaces. The conditioning film may alter the surface properties; the wettability is affected and the surface charge may also change.

- *Bacterial colonisation and growth:* Conditioning films may offer bacteria both a nutrient and an energy source of better status than what the surrounding water does, at least in low nutrient habitats as groundwater. A positive correlation between bacterial growth and the available surface area was found long ago. There are several possible ways for bacteria to accumulate at interfaces. In a macro-scale, water movement and gravitation may act as transport mechanisms. In the immediate vicinity of the surface, other mechanisms can be suggested. Bacterial motility via chemotaxis, electrostatic and hydrophobic interactions are discussed. Once established at the surface, the bacteria may start to grow depending on the availability of nutrients and energy. Their original adhesion, thought to be a reversible process, is changed to an irreversible attachment by production of extracellular polymers. The bacterium becomes anchored to the surface.

- *Steady state:* At some stage, controlled by environmental factors, the attached population reaches a steady state where the increase in cell numbers due to growth and attachment is balanced by detachment and other processes decreasing the number of cells. Predation by protozoa is common in surface aquatic ecosystems but to what is known, not

common in the deep subterranean biosphere. Lysis due to bacteriophages is a plausible decreasing process but remains to be demonstrated for this environment.

Figures 2.3 - 2.6 shows different attached bacteria on surfaces exposed to slowly flowing groundwater from Äspö tunnel.

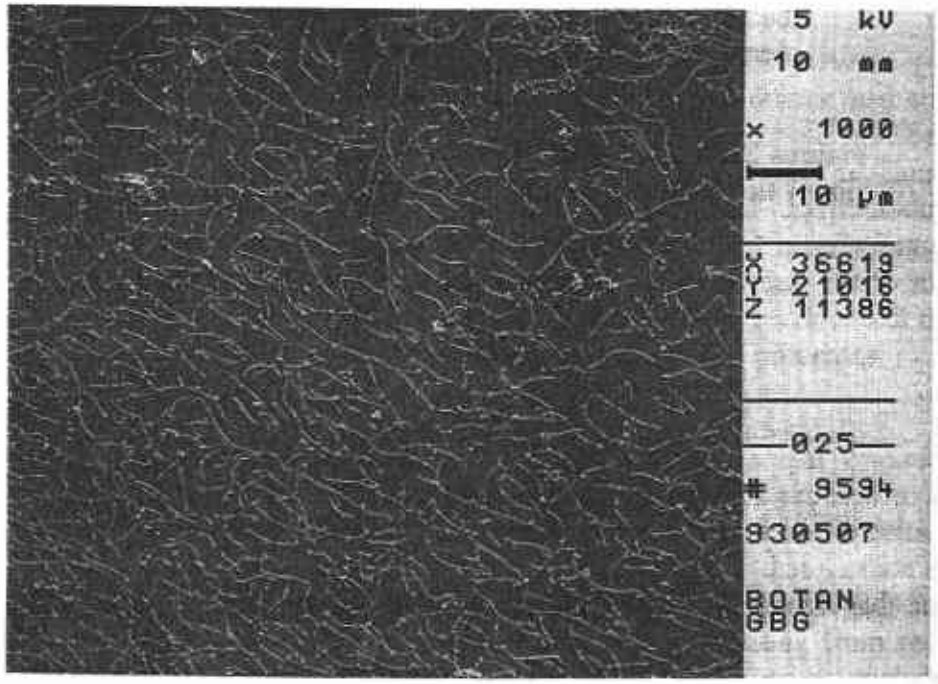

Figure 2.3 Scanning electron microscopy image of attached bacteria that grew on a surface exposed to slowly flowing groundwater from the Äspö tunnel borehole KR0013 for 70 days (70 m below ground). DNA sequencing (Section 4.2.2) indicates the large bacterium to be a gram positive species closely related to Bacillus megaterium. *Bar indicates 10 µm.*

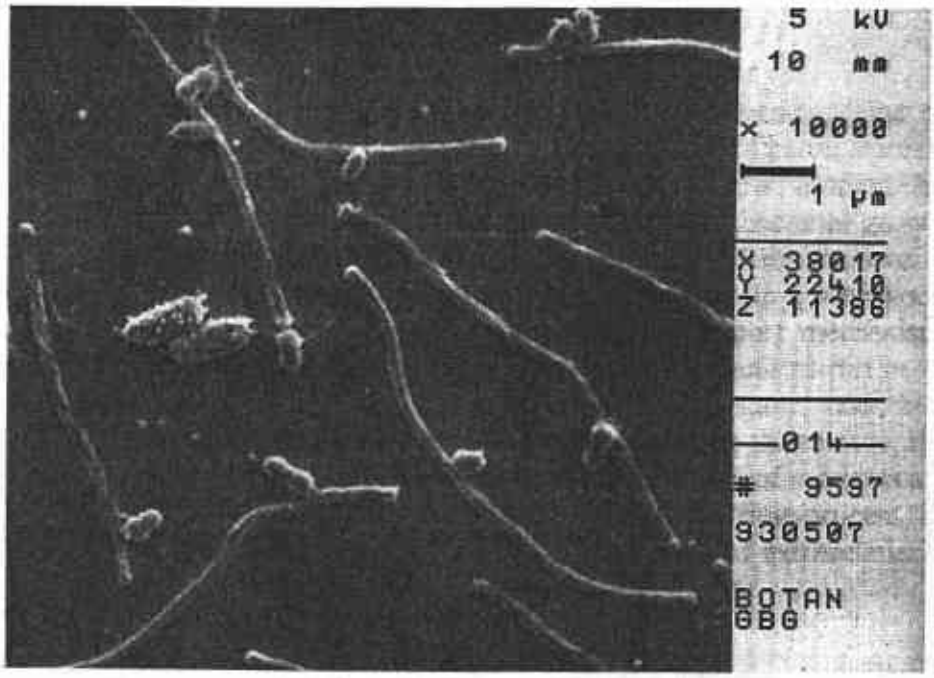

Figure 2.4 The same preparation as in Figure 2.3, but at a greater enlargement. Bar indicates 1 µm.

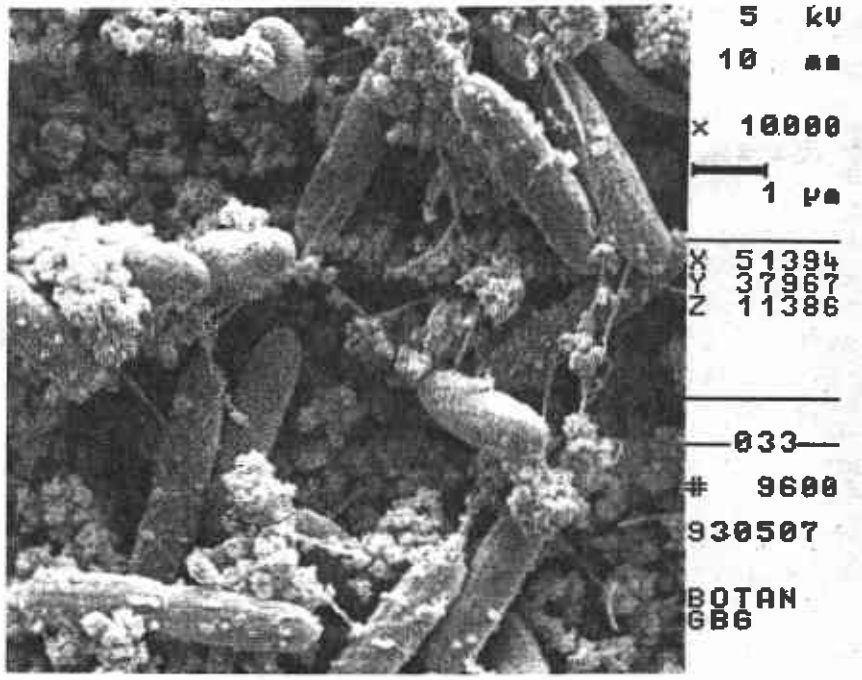

Figure 2.5 Scanning electron microscopy image of attached bacteria that grew on a surface exposed to slowly flowing groundwater from the Äspö tunnel borehole SA813 for 70 days (112 m below ground). DNA sequencing (Section 4.2.2) indicates this to be a sulphate reducing bacterium. Bar indicates 1 µm.

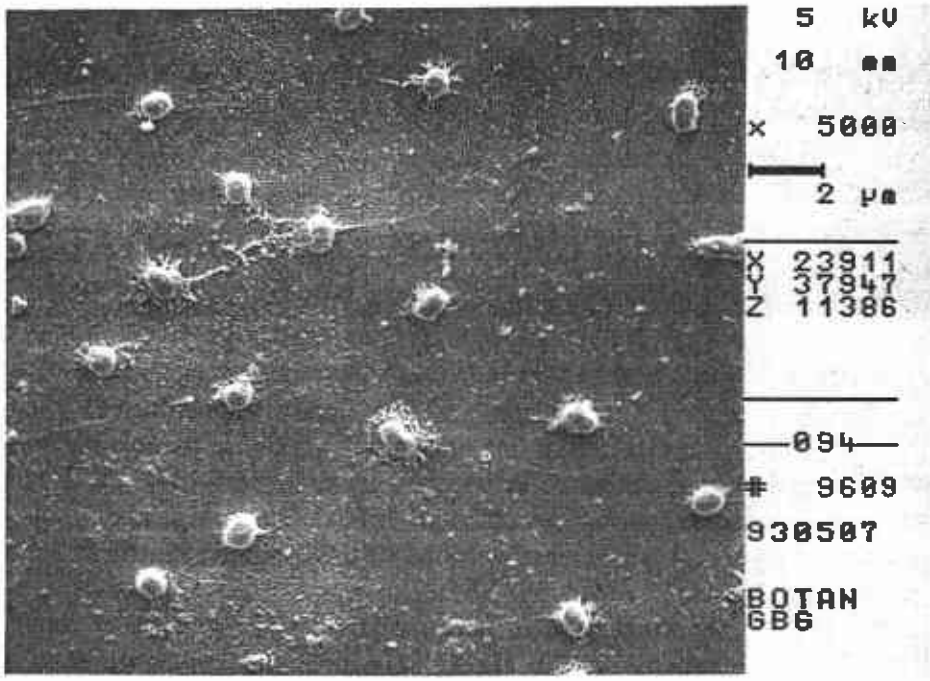

Figure 2.6 Scanning electron microscopy image of attached bacteria that grew on a surface exposed to slowly flowing groundwater from the Äspö tunnel borehole HA1327 for 70 days (179 m below ground). DNA sequencing indicates this to be new and unknown bacterial species. Bar indicates 2 µm.

2.9.1 Diffusion may limit attachment and growth in very slowly flowing groundwater

The transport of nutrients to, and wastes from bacteria on surfaces in laminar flow water systems will be diffusion limited, and if the flow is very slow, the mass transport due to flow to and from the surface will also be limiting. There may therefore exist a slowest flow rate where bacteria gain a nutritional advantage of being attached. Motility may then be a better alternative, but it cannot be excluded that at such slow flow rates, there will not be any nutrients present in the water, because they have already been consumed. The overall growth limiting step will then be transport from the biosphere or from other, still unknown, subterranean sources (confer 2.8.4). As mentioned above, conclusive data is lacking and this topic must be further studied.

2.10 SIZE OF BACTERIA

The size of a bacterium varies considerably depending on species and nutritional status. Growing bacteria may be several μm long with a volume of several μm^3 while the same species in a non-growing state in low nutrient environments may be as small as 0.2-0.3 μm with a volume of not more than 0.05 μm^3, which is about the smallest possible size of a living organism. This is because all organisms need a certain amount of essential molecules such as DNA, RNA, enzymes and also a cell membrane and usually a cell wall, all which will occupy a certain space. Therefore, there is an absolute minimum size of bacteria and also a minimum pore size through which a bacterium can be transported.

2.11 SUMMARY OF ENVIRONMENTAL LIMITATIONS

A number of limitations for life in subterranean environments can be identified, but none alone is enough to retain bacteria from living, possibly with exception of water activity and temperatures above 110°C. Combination of different limitations will make life harder, but in most cases, it is not enough for a conceptual dismissal of bacterial life in different repository constructions (Table 2.10). Some of the most important environmental limitations for bacterial subterranean life are as follows:

- **Oxygen and redox potential.** It is expected that repository environments will be anaerobic with a low redox potential. Therefore, investigations should include facultative and strict anaerobes adapted to different long term repository environments. Aerobes must be considered important during the operational phase, the first years after closure and sealing of the repository and in scenarios when radiolysis of water produces oxygen enough to stimulate aerobic growth.

- **Temperature.** The temperature at start of a spent fuel repository will favour thermophilic bacteria and with time slowly change towards the temperature of the surrounding rock which is appropriate for mesophiles and also psychrotrophs. Therefore, investigations must include both thermophilic, mesophilic and psychrotrophic bacteria. The low and intermediate concepts assume psychrotrophic to mesophilic conditions and are consequently included under the spent fuel concept with respect to bacteria and temperature.

- **pH.** The pH of deep groundwater is normally in the range of 6.5 to 9.5 and will select for neutrophiles and also for alkalophiles. The bentonite clay has a pH around 9, which is a pH environment that can be defined as alkaline and therefore alkalophiles can be expected to dominate. The elevated repository temperatures will, at least for an initial repository life time of approximately 3000 years (se Figure 1.3), select for thermophilic (temperature >55°C) alkalophiles (pH>9) in the repository clays and for thermophilic neutrophiles in the surrounding repository rock. Concrete is being used for various underground constructions in all different waste disposal concepts and will select for extreme alkalophiles.

- **Water activity.** The groundwater in the repository rock and the surrounding rock will have a relatively high water activity, usually not above sea water (0.98) and will therefore not execute any environmental limitation for most bacteria adapted to aquatic ecosystems. In addition, the backfill of bentonite/sand (15/85%) will have a fairly high water activity. Turning to the bentonite, MX-80, that will be used around the canisters, the picture changes dramatically. At start, the bentonite will have a water activity of approximately 0.75 which is low enough to exclude the absolute majority of microorganisms. The endospores formed by many *Bacillaceae* species may in fact be the only life form that possibly can survive. Therefore, keeping the water activity low, at for instance 0.92 (water content 20%) will definitely be a very potent environmental limitation for most bacteria trying to invade the canister environment.

- **Radiation.** There may be a noticeable selection towards radiation resistant species of bacteria colonising nearfield environments with radiation. In the case of water penetrating to the fuel, the most significant effect on bacteria probably will be that radiolysis of water introduces oxygen and raises the redox potential in the vicinity of the canister.

- **Radiolysis.** Reactive radiolysis products may very efficiently be converted to oxygen by aerobic and facultatively anaerobic bacteria. The hydrogen produced during radiolysis together with the oxygen produced from reactive oxygen species can subsequently be recombined by bacteria under the formation of organic material, heat and water. Energy from

radioactive decay may in fact be partly conserved as organic material in the form of bacteria. This is an alternative or complementary model for the fate of radiolysis products. Results from investigations of the Cigar Lake uranium ore have shown that bacteria are present and that there are also measurable amounts of organic material ranging between 0.9 up to 7 mg TOC per litre. The same process occuring in a spen fuel repository would in fact add to the stability of the waste matrix (uranium dioxide) by consuming oxygen from the radiolysis.

- **Hydrostatic pressure.** The SFL 2 repository will be situated at a depth with approximately 50 atm of hydrostatic pressure. This is a pressure that most bacteria can tolerate and pressure therefore will not constitute any important environmental limitation for bacteria.

- **Nutrients and bacterial metabolism.** Bacterial heterotrophic metabolism in repository and rock environments will be more or less limited by the availability of organic material possible to use for growth and maintenance. Chemolithotrophic metabolism may in theory be possible provided there exist inorganic energy sources and suitable electron acceptors. The activity of present bacterial populations probably will be low, but conclusive data is lacking and this topic must be further studied.

- **Size.** The absolute minimum size of bacteria is approximately 0.2 μm with a volume of 0.05 μm^3 which then defines a minimum pore size through which a bacterium can be transported in groundwater.

2.12 FURTHER READING

Most of what is written about bacteria in this chapter can be extracted from textbooks on microbiology. Two good such books are:

- **Prescott, L M. Harley, J P. and Klein, D A. (1993)** Microbiology. 2 ed. WCB Brown Publishers, Oxford.

- **Brock, T D. Madigan, M T. Martinko, J M. Parker, J. (1994)** Biology of microorganisms. 7 ed. Prentice Hall International Editions.

The detailed knowledge about bacteria, systematics, identification, culturing etc. can be found in either, one of the two following sources:

- **Holt JG (1984-1988)** Bergey's manual of systematic bacteriology, vol 1-4. *Williams & Wilkins (Baltimore).*

- **Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H. (1991)** The prokaryotes. A handbook on the biology of bacteria: Ecophysiology, isolation, identification, applications. Springer-Verlag, New York.

A good reference on anaerobes is:

- **Zehnder AJB (1988)** Biology of anaerobic microorganisms. John Wiley & sons, New York.

Table 2.10 Summary of environmental limitations for bacteria in different repositories and in crystalline bed-rock groundwater.

Type of environment	Oxygen and redox	Temperature	pH	Water activity	Energy	Nutrient	Radiation	Bacterial diversity on a relative scale
Fractures in bed-rock	anaerobic and reduced	psychro- and mesophilic	neutro- and alkalophiles	0.98-1	organic material and possibly H_2	organic material	negligible or local from failed canister	+++++
SFL 2 bentonite, 0 -3000 years	anaerobic and reduced	thermophilic	alkalophiles	0.96-0.92	organic material < 0.3 %	organic material 0.3%	8.6 Gy/d	(+)
SFL 2 bentonite, more than 3000 years	anaerobic and reduced/aerobic and oxidised	psychro- and mesophilic	alkalophiles	0.96-0.92	organic material < 0.3 %	organic material < 0.3 %	< 8.6 Gy/d	+
SFL 2 backfill and host rock, 0 - 3000 years	anaerobic and reduced	thermophilic	neutro- and alkalophiles	0.98-1	organic material < 0.3 %	organic material < 0.3 %	negligible or local from failed canister	+++
SFL 2 backfill and host rock, 0 - 3000 years	anaerobic and reduced	psychro- and mesophilic	neutro- and alkalophiles	0.98-1	organic material < 0.3 %	organic material < 0.3 %	negligible or local from failed canister	++++
SFR	anaerobic and reduced	psychro- and mesophilic	alkalophiles (local acido-, neutrophiles)	low - 1	(organic material)	(organic material)	negligible or local from waste	++++++
SFL 3, 4, 5	anaerobic and reduced	psychro- and mesophilic	alkalophiles (local acido-, neutrophiles)	low - 1	organic material	organic material	negligible or local from waste	++++++

3 SUBTERRANEAN BACTERIAL PROCESSES

A full understanding of the environments of nuclear waste repositories cannot be achieved until bacterial processes are included in models, theories, interpretation of results etc. This is because bacteria catalyse many reactions that otherwise would be very slow or not possible at all for kinetic reasons. One obvious example of the latter is the bacterial reduction of sulphate to sulphide which is the only source of low temperature sulphide to anoxic waters. This reaction does not occur at normal pressure and temperature. Another example is that bacterial activity usually influences the redox potential in the environment. If, for instance, the environment is rich in Fe(III) and organic matter, iron reducing bacteria will dominate, produce Fe(II) and carbon dioxide in large quantities and the resulting redox will be controlled by Fe(II) at or below approximately -100 mV. A third example is methane producing bacteria that typically coexist syntropically with hydrogen producing bacteria that ferment and respire organic material. If the hydrogen concentration increases too much, the decomposition of organic material by these bacteria stops. Because methane bacteria produce methane from hydrogen and carbon dioxide, they remove hydrogen from the environment and the bacterial decomposition of organic material can continue.

The bacteria act as catalysts in geochemical processes through their metabolism (see Section 2.1). This metabolism has two aspects. One, *catabolism* is the portion which provides the cell with needed energy and with some compounds that can serve as building blocks for polymers. It generally involves the oxidation of a suitable nutrient or metabolite (a compound metabolically derived from a nutrient). The other, *anabolism*, is the portion of metabolism that deals with assimilation and leads to the formation of organic polymers such as nucleic acids, proteins, polysaccharides, lipids and other cell components.

Bacterial decomposition and production of organic material depend on the sources of energy and electron-acceptors present. Organic carbon, reduced inorganic molecules (see Table 3.1) or hydrogen are possible energy sources in subterranean environments. During bacterial oxidation of these energy sources the bacteria use electron acceptors in a certain order according to figure 3.1. First oxygen is used, thereafter follows the utilisation of nitrate, manganese, iron, sulphate, sulphur and carbon dioxide. Simultaneously, fermentative processes supply the respiring organisms with hydrogen, short organic acids etc. As the solubility of oxygen in water is low, and because

oxygen is the preferred electron acceptor by many bacteria utilising organic compounds in shallow groundwater, anaerobic environments and processes usually dominate at depth in the subterranean environment.

Important questions about geobacterial processes in subterranean environments are:

- What types of bacterial processes, if any, dominate in different subterranean environments?
- What influences do these bacterial processes have on the geochemical environment?
- Which bacterial processes are of importance for performance assessment of nuclear waste disposal?
- Can unwanted processes be avoided or manipulated?

This chapter reviews bacterial processes possibly occurring to some extent in subterranean environments and evaluates their importance for the Swedish nuclear waste disposal concept described in chapter 1.

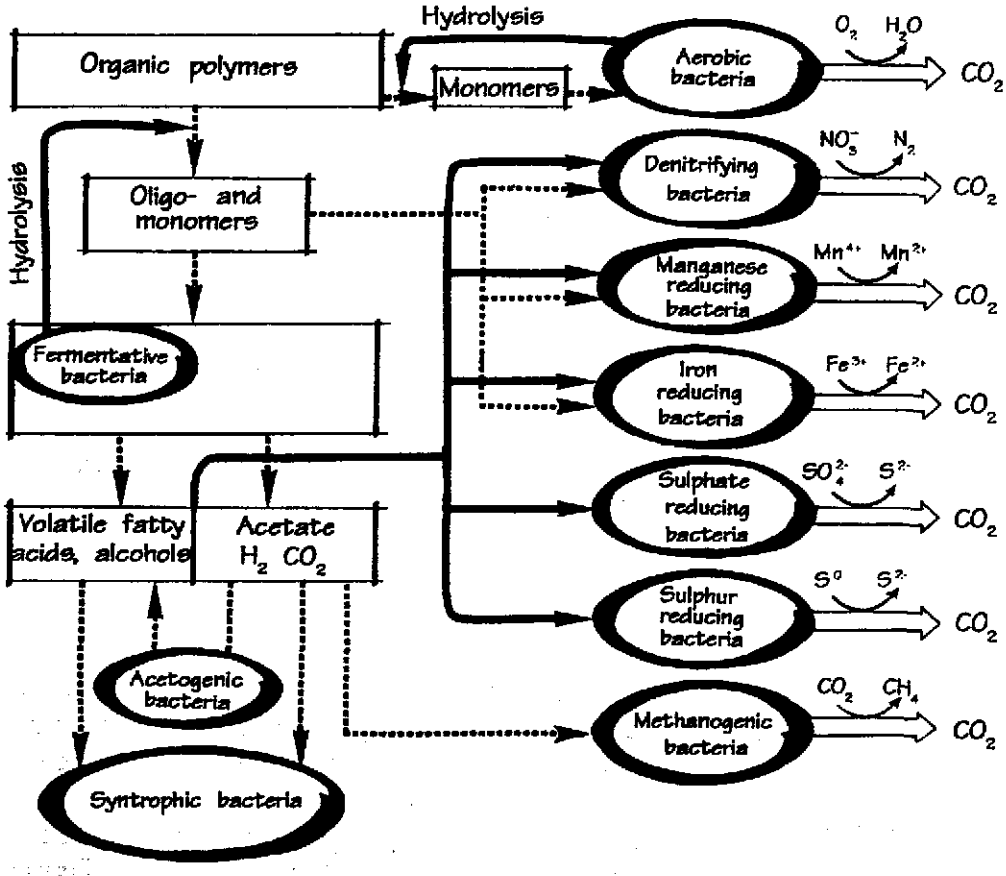

Figure 3.1 *Possible pathways for the flow of carbon in the subterranean environment. Organic carbon is respired with oxygen, if present, or else fermentation and anaerobic respiration with an array of different electron acceptors occur.*

3.1 BACTERIA AND THE CARBON CYCLE

Carbon is the central atom of life. The property of carbon to form covalent bonds as well as to share electron pairs with oxygen, hydrogen, nitrogen and sulphur is necessary for life. The cycles of all other important elements tie in with that of carbon. The only major way in which new organic carbon is synthesised is via autotrophic processes of which photolithotrophy dominate (Table 2.9). Autotrophs are organisms able to synthesise their cell substance from inorganic carbonate as their main source of carbon. It should be noted that this definition refers to carbon dioxide as the main source of carbon; it is of minor importance that many autotrophs require organic growth factors, or that some are facultative and can grow heterotrophically when appropriate organic energy sources are present. As there is no light in subterranean environments, production of new organic carbon can only occur via chemolitho(auto)trophic pathways which all are bacterial. The reverse process, carbon dioxide production from organic material, so called mineralisation, is executed by a large variety of heterotrophic organisms of which bacteria by far is the most versatile group when it comes to the range of different types of organic compounds used. With almost no exception, any naturally occurring organic compound can be utilised as energy and/or carbon source by some or several bacterial species or strains. The carbon cycle is illustrated in Figure 3.2.

As bacteria can consume or produce carbon dioxide in the subterranean environments, they will also affect the equilibria between carbon dioxide, hydrogen carbonate and other carbonates thereby influencing the formation and degradation of various precipitated carbonates (Fig 3.9).

3.1.1 Chemolitho(auto)trophic bacteria

Nature harbours a large variety of bacteria which can derive energy from the oxidation of inorganic compounds (Table 3.1). Many use oxygen as electron acceptor but a number of them can also survive anaerobically using nitrate and ferric iron (Ehrlich, 1990b). Some obligate anaerobic chemolithotrophs are found among sulphate reducers and the methanogenic bacteria. Recently, a bacterial species has been discovered which can derive energy from the disproportionation reaction of sulphur and thiosulphate (Bak and Pfennig, 1987, Kuenen and Bos, 1989). Most of the chemolithotrophic bacteria are able to grow autotrophically transforming carbon dioxide to organic carbon.

Table 3.1 Inorganic electron donors for chemolithotrophs (see table 2.9).

Hydrogen
Carbon monoxide
S^0 and other reduced inorganic sulphur compounds
Reduced inorganic nitrogen compounds
Iron (Fe^{2+})
Manganese (Mn^{2+})

In the following sections, cycling of nitrogen, sulphur, iron, manganese, hydrogen and methane will be reviewed. The implications for performance assessment will be discussed under respective cycle section. All of these cycles have steps that more or less heavily depend on the activity of chemolithotrophs. With respect to the carbon cycle, they contribute to the production of organic carbon from carbon dioxide with inorganic compounds as energy- and electron sources. However, the actual occurrence of autotrophic processes in deep groundwater has not been well documented and will be restricted by the availability of inorganic energy sources.

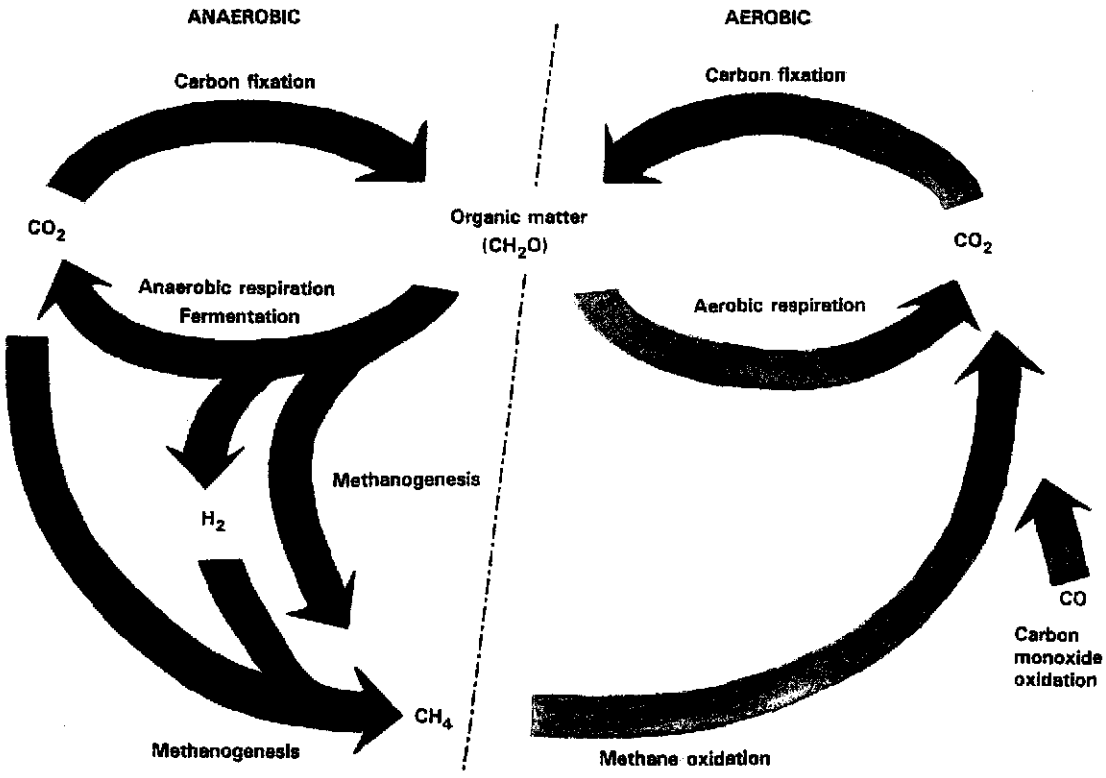

Figure 3.2 *The carbon cycle in the environment. Microorganisms play important roles in the environmental carbon cycle. Carbon fixation in the subterranean environment can occur only through chemoautotrophic processes. Methane can be produced from inorganic substrates, hydrogen and carbon dioxide, or from one carbon compounds and the two carbon compound acetate. Carbon monoxide produced by cars, industry etc. is returned to the carbon cycle via the methylotrophic carbon monoxide oxidizing bacteria. Aerobic processes are noted with blue arrows, while anaerobic processes are shown with red arrows.*

3.1.2 Heterotrophic bacteria

Depending upon the available amount of easily degradable carbon, heterotrophic bacteria will mineralise such carbon to inorganic nutrients and carbon dioxide. Oxygen consumption will exceed the oxygen diffusion and transportation rates at depths from a few millimetres in waterlogged soils and sediments and usually also at depth in fractured rock. This will result in a shift in the microbial metabolism from aerobic to anaerobic. Under aerobic conditions, the degrading community consists of bacteria, fungi and protozoa together with larger meio- and macrofauna in the surface layers and root zone. A common feature of all these aerobic organisms is their ability to mineralise organic molecules completely to carbon dioxide. Deeper, at anaerobic conditions, mainly bacteria are responsible for biological mineralization. The anaerobic mineralization requires interactions of a complex bacterial food web, where the product of one microbial group serves as substrate for the subsequent group, and were the consumption of a product regulates its type and formation rate. Finally, organic carbon can be mineralised either completely to carbon dioxide by combined oxidative processes or to methane by oxidation-reduction processes, depending on the availability of inorganic electron acceptors in the system. Figure 3.1 shows the main features of anaerobic metabolism and the microbial groups responsible for the degradation of organic matter, when various electron acceptors are present.

The contents of dissolved organic carbon (DOC) in deep groundwater have been measured and mean values at depths usually are around 1 mg per litre, although large local variations occur (Table 1.1). Such concentrations may however be underestimates since many organic compounds may sorb onto rock surfaces. Typically, about 50 % of the DOC found is composed of complex humic and fulvic acids, and a smaller part is organic substances with shorter carbon chains than glucose. The sources of this organic carbon is unexplored. It may be organic material percolating down from the ground surface, but dating with ^{14}C analysis often indicates groundwater ages of 5000 - 20,000 years. Thus the DOC may consist of recalcitrant fractions of organic substances derived from surface plant material grown long ago, delivered through hydrological recharge and groundwater flow. The other possible source would be organic deposits in the rock or organic compounds migrating up from very deep layers of the earth (Gold, 1992). It will be an important task to investigate the sources and fluxes of organic carbon in the subterranean environment. This is because such carbon may be the main fuel for bacterial processes in the far field of importance for nuclear waste disposal and the fluxes will then determine the process rates. Investigations of bacterial carbon transformations and the ecology of important groups of deep granitic groundwater bacteria should therefore be continued.

3.1.3 Formation and degradation of carbonates

Some of the CO_2 generated in biological respiration can be fixed in insoluble carbonates. Indeed, much of the insoluble carbonate at the earth's surface is of biogenic origin, although some is the result of magmatic and metamorphic activity. There are many ways in which carbonates can be formed by a large variety of microbes. Carbonates likely to form as a result of bacterial activity in groundwater are calcium, sodium, manganese and iron carbonates (Figure 3.9). This is dealt with in detail by (Ehrlich, 1990b). Some of the processes discussed and relevant to the subterranean environment are:

- Aerobic and anaerobic oxidation of carbon compounds consisting of carbon and hydrogen, with or without oxygen, for example carbohydrates, organic acids and hydrocarbons. If such oxidations occur in well buffered, neutral or alkaline environment containing adequate amounts of calcium or other appropriate cations, at least some of the carbon dioxide that is generated will be transformed into carbonate which will then precipitate with appropriate cations.

- Aerobic or anaerobic oxidation of organic nitrogen compounds with the release of ammonia and carbon dioxide in unbuffered environments containing sufficient amounts of calcium, magnesium or other appropriate cations. The ammonia is produced especially during microbial degradation of compounds containing amino groups like proteins and nucleic acids and will raise the pH. Some of the carbon dioxide will transform into carbonates that may precipitate.

- The anaerobic reduction of $CaSO_4$ to CaS by sulphate reducing bacteria. The CaS is readily hydrolysed to H_2S and the Ca^{2+} will react with carbon dioxide produced when the SRB oxidise short organic acids like lactate or acetate.

Carbonates in nature may also be readily dissolved as a direct or indirect result of biological activity. A chemical basis for this decomposition is the instability of carbonates in acid solutions. Even mere metabolic generation of carbon dioxide during respiration may have this effect, because:

$$CO_2 + H_2O + CaCO_3 \rightarrow Ca^{2+} + 2HCO_3^{2-}.$$

Therefore, any organism that generates acid metabolites or carbon dioxide is capable of dissolving insoluble carbonates. Many of the fermenting bacteria produce weak acids like lactic, acetic and formic acid during their metabolism, while some of the chemolithotrophic bacteria generate strong acids, sulphuric

acids by sulphur oxidisers and nitric acid by nitrifying bacteria. Such activity may of course have undesirable effects on concrete constructions and is responsible for the decay of many limestone buildings, monuments etc.

3.1.4 The carbon cycle in repository and rock environments

Most deep rock environments have a relatively low content of organic carbon and measurable amounts of carbon dioxide and carbonates. The organic carbon content is usually measured only in water samples and may therefore be underestimates since many organic compounds are sorbed onto rock surfaces. Also, the contents measured reveal levels but eventual fluxes can not be extracted from existing data. It will therefore be an important task to investigate the *sources* and *fluxes* of organic carbon in the subterranean environment. This is because such carbon may be the main fuel for bacterial processes of importance for nuclear waste disposal at least in the far-field environment where no other sources are available. The fluxes will then determine the process rates. Fluxes of inorganic energy- and electron sources will be important for the understanding of possible new formation of organic carbon in the subterranean environment. The participation of bacteria in the carbon cycle also implies an impact on fracture mineral formation and degeneration which may affect sorption and desorption of radionuclides. Further, bacterial production and consumption of carbon dioxide may have an influence on pH and alkalinity of groundwater.

The SFR repository and the planned SFL 3-5 repositories contain many different types of organic carbon compounds with the potential for production of gasses like carbon dioxide, hydrogen and methane and of acids due to fermentative processes.

3.2 BACTERIA AND THE NITROGEN CYCLE

The main components of the nitrogen cycle are outlined in Fig 3.3. The major flow of nitrogen is from organic nitrogen to mineral products and back again, and small losses from nitrate to nitrogen are compensated for by the process of nitrogen fixation to ammonia and subsequently to organic nitrogen. Important aspects of the nitrogen cycle are the processes of nitrification, denitrification and nitrogen fixation.

3.2.1 Nitrification

Nitrification is the chemolithotrophic, aerobic process of ammonium ion oxidation to nitrite and subsequently nitrite oxidation to nitrate. Nitrifying organisms are a physiologically defined group of bacteria which consists of phylogenetically heterogeneous genera. Most nitrifying microorganisms are highly specialized obligately lithotrophic bacteria, growing only in the

presence of an inorganic energy source. The most comprehensive investigations on physiology and biochemistry have been conducted with members of the genera *Nitrosomonas* and *Nitrobacter*. Nitrification acts acidifying due to the production of nitric acid. Recently, soil acidification and nitrogen saturation from weathering of ammonium-bearing rock was reported due to nitrification (Vogels et al, 1988). Geological nitrogen is particularly common in sedimentary and metasedimentary rocks, the former being the dominant rock type on the earth's surface. The results demonstrate that geological nitrogen, commonly overlooked in biogeochemical cycling, but known to be present in appreciable quantities in certain rocks, may represent a large and reactive pool which can have significant biogeochemical effects.

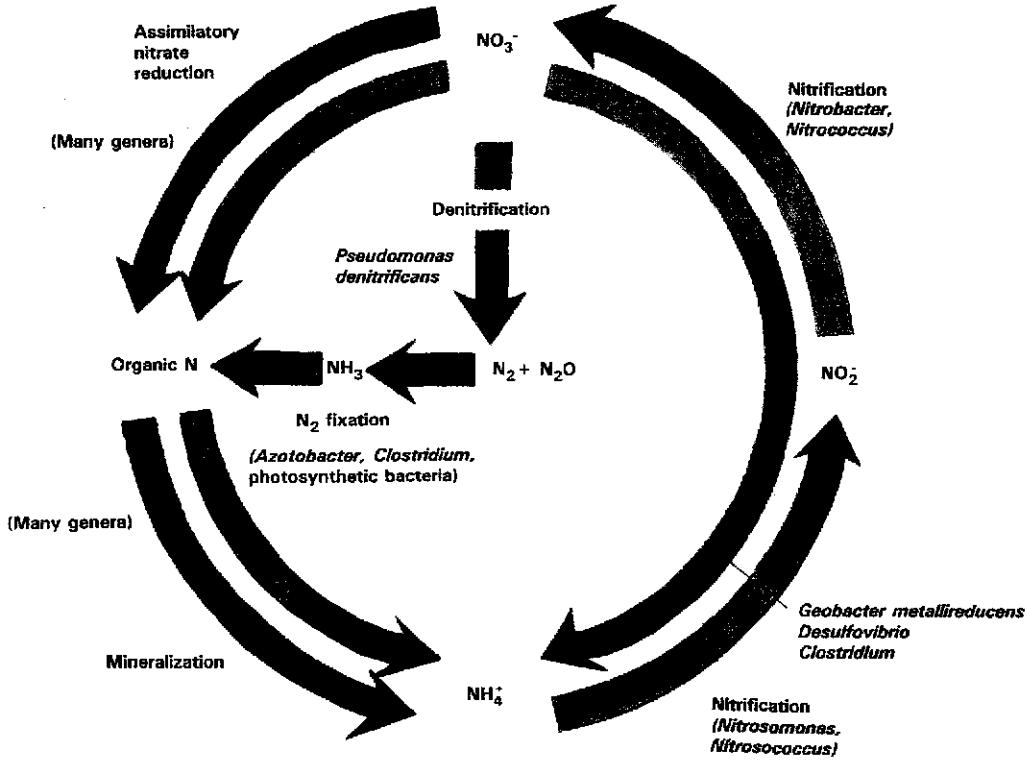

Figure 3.3 *The environmental nitrogen cycle. Flows that occur predominantly under aerobic conditions are noted red. Anaerobic dissimilatory processes are noted with purple arrows. Processes occurring under both aerobic and anaerobic conditions are marked with blue and red arrows.*

3.2.2 Denitrification

The process of denitrification requires a set of environmental conditions different from nitrification. The dissimilatory process, in which nitrate is used as an oxidant in anaerobic respiration (compare with the carbon cycle), usually involves heterotrophic bacteria. The major products of denitrification include nitrogen gas and nitrous oxide although nitrite also can accumulate (Figure 3.3).

Recently, a microbiologically mediated anaerobic ammonium oxidation to nitrogen with nitrate has been demonstrated . The following reaction was taking place at fully anoxic conditions:

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9\,H_2O + 2\,H^+ \quad \Delta G^{\circ\prime} = 1\,483.5\ KJ/5\ mole\ NH_4^+$$

This process may take place in the subterranean environment if ammonium and nitrate are present.

3.2.3 Nitrogen fixation

Nitrogen fixation can be carried out by aerobic or anaerobic bacteria (Figure 3.3). The reductive process is extremely sensitive to oxygen and must occur under anaerobic conditions even in aerobic organisms. It is the key enzyme nitrogenas that becomes irreversibly blocked by oxygen. Under aerobic conditions, the enzyme is protected by various mechanisms, including physical barriers, as occurs with heterocysts (specialized nitrogen fixing cells) in some cyanobacteria, oxygen scavenging molecules and high rates of metabolic activity. Such mechanisms are not necessary under anaerobic conditions. An important free-living anaerobic nitrogen fixing genus is *Clostridium.*

3.2.4 The nitrogen cycle in repository and rock environments

Deep groundwater usually is depleted in nitrate, nitrite, ammonia and other nitrogen compounds. This is however not any principal obstacle for bacterial growth because the main gas present is nitrogen and many bacteria fix such nitrogen to ammonium and further to organic material. Nitrification processes usually are aerobic and will be limited by the anaerobic character of repository and rock environments together with the limited availability of ammonium. Under aerobic conditions or if anaerobic nitrification occurs, the nitrification process produces nitric acid which pose a (theoretical?) acid threat to concrete material etc. The very low concentrations of nitrate in most deep groundwater indicate electron acceptors other that nitrate to be important for respiration of organic carbon in the subterranean environment such as for instance iron(III).

3.3 BACTERIA AND THE IRON CYCLE

3.3.1 Iron

Iron is the fourth most abundant element in the Earth's crust and it is a very reactive compound. Its common oxidation states are +2 and +3. In most environments exposed to air or in aerated solution at pH values greater than 5, its ferrous form (+2) readily autooxidises to the ferric form (+3). In the presence of an appropriate reducing agent, i.e. under reducing conditions, ferric iron is readily reduced to the ferrous state. Ferric iron precipitates as hydroxide or oxide in neutral to slightly alkaline solution, but is soluble as Fe^{3+} in acid solution and dissolves in strongly alkaline solution because of the amphoteric nature of $Fe(OH)_3$. Due to the prevailing oxidizing conditions on earth, the distribution of iron is not uniform and is primarily confined to oxidised phases in the lithosphere.

Microbial transformations of iron play an important role in the cycling of iron in nature. Weathering of iron-containing minerals in rocks, soils and sediments introduces iron into the cycle (Figure 3.4). This weathering action is partly promoted by bacterial action and partly by chemical activity (Ehrlich, 1990c).

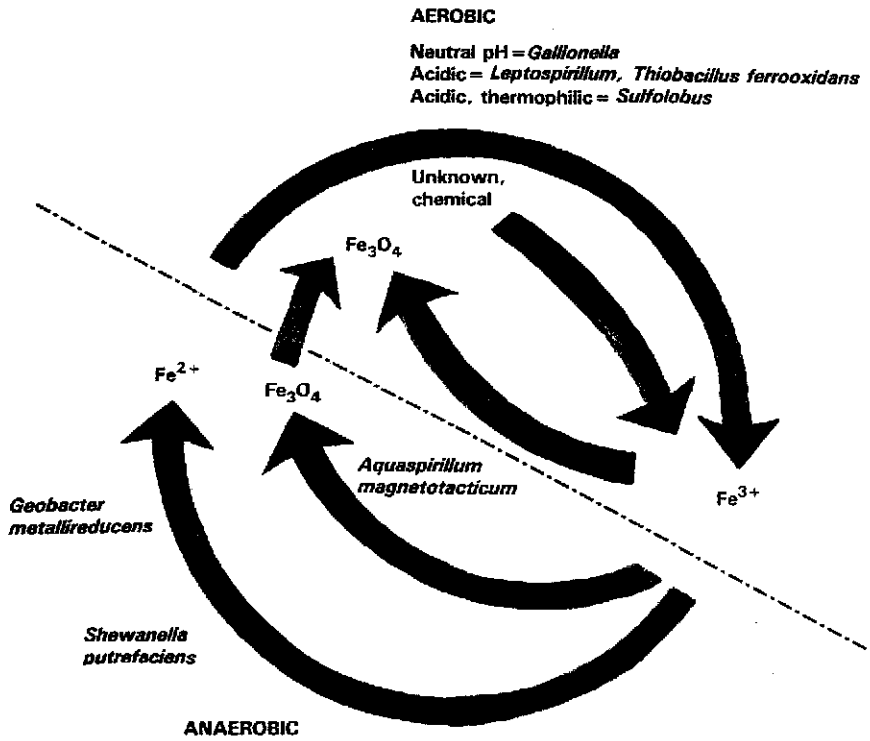

Figure 3.4 *The iron cycle. A simplified iron cycle with examples of bacteria contribution to these oxidation and reduction processes. In addition to ferrous ion and ferric ion reduction, magnetite (Fe_3O_4), a mixed valence iron compound formed by many iron reducing bacteria is important in the iron cycle. Different bacterial groups carry out the oxidation of ferrous iron, depending on environmental conditions.*

All organisms require iron nutritionally. A small group of homolactic fermenting bacteria consisting of the lactic streptococci is the only exception. The other organisms need iron in enzymatic processes involving transfer of electrons. Ferrous iron may also serve as a major energy source to certain bacteria and ferric iron may serve as a terminal electron acceptor. These bacteria can greatly increase the velocity and rate of the oxidation and reduction of iron in nature. There are environments in which iron cycles rapidly. A prerequisite for such environments is the presence of a reduced or semi-anaerobic zone, where iron reduction and mobilization can occur.

3.3.2 Iron reducing bacteria

Microbial Fe(III) reduction is considered to play an important role in a variety of processes of environmental concern, such as: the oxidation of natural and contaminant organic matter, the release of phosphate and trace metals into water supplies, the release of undesirable high concentrations of dissolved iron into groundwater, soil gleying, the magnetisation of aquatic sediments and the inhibition of methane production in shallow freshwater environments (Lovley, 1991). It has also been suggested that microbial Fe(III) reduction may have been the first globally significant mechanism for the oxidation of organic matter to carbon dioxide.

The importance of the iron-reducing bacteria has only been subject for more thorough investigations in the last decade. These bacteria are anaerobic or facultative anaerobic heterotrophs and utilize a wide variety of different carbon sources, including mono- and disaccharides, amino acids, fermentation acids, and some can completely oxidize aromatic compounds. Other can utilize H_2 as electron donor and formate as carbon source. Iron reducing bacteria have been isolated from marine and freshwater sediments, anoxic basins such as fjords and the Black Sea and from deep groundwater (Nealson and Myers 1992). At present there are only a few species isolated and identified of which the two most well known are:

- *Geobacter metallireducens*, a strict anaerobic bacterium that can utilize a wide range of organic substrates, including several short chain fatty acids, alcohols and monoaromatic compounds, with Fe(III) as electron acceptor. It can also oxidize acetate with the reduction of Mn(IV), U(VI) and nitrate. *G. metallireducens* was isolated from surficial bottom sediment collected from a freshwater site (Potomac River, Maryland, USA). It is shown to produce magnetite during growth in a defined medium in the laboratory (Lovley and Lonergan, 1990, Lovley et al, 1993).

- *Shewanella putrefaciens*. The most thoroughly investigated strain is *S. putrefaciens* MR-1 (former *Alteromonas putrefaciens* MR-1). This strain was isolated from the anaerobic sediment of Oneida Lake, New York and is reported to be able to use, besides Fe(III), also MnO_2, oxygen, nitrate, nitrite, sulphite, thiosulphate, tetrathionate,

trimetylamine n-oxide, fumarate and glycine as electron acceptors. It has also been shown that MR-1 couples the reduction of Fe(III) to proton translocation (Lovley et al, 1989, Myers and Nealson, 1988, Myers and Nealson, 1990). Another relatively well characterised strain of *S. putrefaciens* is strain 200 (7963 of Woods Hole Oceanographic Institutes culture collection) formerly called *A. putrefaciens* 200 and *Pseudomonas* sp. strain 200 (Arnold et al, 1986, DiChristina et al, 1988, DiChristina, 1992, Semple and Westlake, 1987). This strain has been used for inhibitor studies of the iron reductase, traditional microbial characterisation and studies of the effect of nitrate and nitrite on the dissimilatory iron-reduction.

Fe(III) reduction has also been reported for bacteria involved in the sulphur cycle. The marine, sulphur reducing bacterium *Desulfuromonas acetooxidans* was found to grew on a defined anoxic bicarbonate-buffered medium with acetate as sole electron donor and poorly crystalline Fe(III) or Mn(IV) as the sole electron acceptor (Roden and Lovley, 1993). Ethanol, propanol, pyruvate and butanol could also serve as electron donors for Fe(III) reduction. It has also been suggested that sulphate-reducing bacteria may act as an agent for Fe(III) reduction in aquatic sediments and groundwater (Coleman et al, 1993). In addition, Fe(III) reduction has been reported for many unidentified strains of bacteria.

Beside these studies with pure cultures of bacteria there are many reports describing the existence of iron-reducing bacteria, indicated by a measurable iron reduction in samples of sediments from both fresh-water and marine sites. As controls were used sediments, with killed bacteria (Jones et al, 1984, Jones, 1983, Ottow and Glathe, 1971, Ottow, 1979, Sörensen and Jörgensen, 1987, Sörensen, 1982, Sörensen, 1987).

The first Fe(III) and Mn(IV) reducing bacteria found to effectively couple the oxidation of organic compounds to the reduction of these metals were the organic acid oxidisers. The following reactions may occur:

Geobacter metallireducens:

$$CH_3COOH + 8Fe(OH)_3(s) + 14H^+ \rightarrow 2HCO_3^- + 8Fe^{2+} + 20H_2O$$

Shewanella putrefaciens

$$CHOOH + 2Fe(OH)_3(s) + 2H^+ \rightarrow HCO_3^- + 3Fe^{2+} + 5H_2O$$

$$CHOOH + MnO_2(s) + H^+ \rightarrow HCO_3^- + Mn^{2+} + H_2O$$

$$CH_3CHOHCOOH + 4Fe(OH)_3(s) + 7H^+ \rightarrow CH_3COOH + HCO_3^- + 4Fe^{2+} + 10H_2O$$

$$CH_3CHOHCOOH + 2MnO_2(s) + 3H^+ \rightarrow CH_3COOH + HCO_3^- + 2Mn^{2+} + 2H_2O$$

Other types of iron and manganese reducing bacteria are fermenting, sulphur-

oxidizing and aromatic compound oxidizing ones (Lovley, 1991). In growth experiments with pure cultures of iron reducing bacteria, the resulting Fe(II) has been reported to form $FeCO_3$, $FePO_4$ and Fe_3O_4. The products formed are dependent of the chemical composition of the growth medium and in natural systems the composition of the groundwater and other bacterial activity.

3.3.3 Availability of Fe(III) for microbial iron-reduction.

The ease with which ferric iron is reduced by bacteria depends in part on the form in which it appears. Bacteria have been reported to preferentially reduce amorphous Fe(III) oxyhydroxides (Munch and Ottow, 1980, Sörensen and Jörgensen, 1987). In case of insoluble forms, the order of decreasing solubility in one study was $FePO_4$ > $Fe(OH)_3$ > lepidochrocite (γ-FeOOH) > goethite (α-FeOOH) > hematite (α-Fe_2O_3) (Ottow, 1969). In a study of Munch and Ottow (Munch and Ottow, 1980) it was found that amorphous iron oxide was more readily attacked then crystalline oxides. It has to be considered that the availability of Fe(III) is dependent of the chemical composition of the groundwater since the iron(III) oxides chemically can be dissolved by acids, adsorbed by chelate ligands or by an adsorbed reductant (reducing organic ligands or reducing metal complexes). The presence of chelating agents probably increases the availability of Fe(III) to bacteria.

3.3.4 Iron-oxidising bacteria.

The iron-oxidising bacteria oxidize the ferrous iron under partly or fully aerobic conditions. The oxidation may be followed by immediate precipitation of the iron as a hydroxide, oxide, phosphate or sulphate. It can also be converted to soluble complexes and dispersed from its site of formation, if complexing agents such as fulvic or humic substances are present. The presence of iron-oxidizing bacteria often increases the precipitation rate of the ferric iron and many of these bacteria have extracellular structures like stalks or sheets on which the oxidised iron adhere. These structures are made of organic material and they increase the volume of the ochre masses produced. Stalks, sheets, bacteria and precipitated iron therefore often cause severe clogging problems in field drains, wells, drainage systems and distribution nets for drinking water. There are no adopted method to avoid clogging problems caused by iron-oxidizing bacteria. A problem that arise from the precipitation of iron, is the concurrent accumulation of other heavy metal ions by coprecipitation and adsorption to hydrous iron. The iron-oxidizing bacteria can be divided in two groups: the acidophilic (acid tolerant forms) and the neutrophilic bacteria.

3.3.5 The acidophilic iron-oxidizing bacteria

The acidophilic bacteria are often found in environments with low pH such as acidic drain waters and acidic hot springs. The most well-known species is *Thiobacillus ferrooxidans,* which is industrially exploited in bio-leaching of metal sulphide and uraninite ores. It is a gram negative, motile rod (0.5 x 1.0 μm) and is strictly aerobic. *T. ferrooxidans* is able to derive energy and reducing power from the oxidation of ferrous iron. It can also utilize reduced forms of sulphur such as H_2S, S^0, $S_2O_3^{2-}$ and metal sulphides. The carbon source used is carbon dioxide and it derives its nitrogen preferentially from ammonium but also from nitrate. Some strains are reported to fix N_2. The pH optimum for *T. ferrooxidans* range from 2.0 to 3.5.

Other acidophilic bacteria capable of oxidizing ferrous iron enzymatically have been discovered in more recent years (Harrison, 1984). Some of them are mesophilic and others thermophilic. *Leptospirillum ferrooxidans*, an strain of *Metallogenium* is an example of a mesophile. A representative thermophile is *Sulfobacillus thermosulfidooxidans*, that has an optimum temperature of 50 °C and that can grow autotrophically with Fe^{2+} or S^0 or metal sulphides as energy sources. Its pH range for growth is 1.9-3.0, with optimum at 1.9-2.4 (Golovacheva and Karavaiko, 1978).

Well-known extreme thermophiles among acidophilic iron-oxidizing microorganisms are *Sulfolobus acidocaldarius* and *Acidianus*, (Formerly *Sulfolobus brierleyi*). Both belong to Archaea. These organisms can grow autotrophically with Fe^{2+} or S^0 as energy sources.

3.3.6 The neutrophilic iron-oxidizing bacteria

The only bacterium known to gain energy from iron-oxidation at neutral pH is the stalk-forming bacterium *Gallionella ferruginea* (Hallbeck et al, 1993). This bacterium is autotrophic and produces a twisted stalk composed of carbon-containing material on which oxidized iron adhere (Hallbeck and Pedersen, 1990b). Besides the CO_2 fixation, *G. ferruginea* is able to utilize glucose, fructose and sucrose as carbon source together with iron(II) as energy source (Hallbeck and Pedersen, 1991). In anoxic or near anoxic environment, *G. ferruginea* is free-living without stalk. The stalk production starts when the cells reach an oxidized milieu. The stalk is a protection against toxic oxygen species, which are formed through the reduction of oxygen by ferrous iron in slightly acidic environment in the presence of ferrous iron (Stumm and Lee, 1960). *G. ferruginea* is found in ground-waters with low contents of organic carbon, and relatively high concentrations of hydrogen carbonate and ferrous iron. Heavy stalk formation is found at places where anoxic iron(II)bearing groundwater comes in contact with air.

Other bacteria that are associated with iron oxidation include sheathed bacteria such as *Sphaerotilus, Leptothrix* spp., *Crenothrix polyspora, Clonothrix* spp. and *Lieskiella bifida*, and some encapsulated bacteria like the Siderocapseae-group. It has been argued that these bacteria could perform energy-yielding iron-oxidation, but most probably they accumulate oxidized iron on the sheaths or similar structures without gaining any energy. There is evidence for enzymatic oxidation of iron and manganese (Emerson and Ghiorse, 1992). The iron-oxidation by these bacteria may be a protection mechanism similar to that of *Gallionella* against toxic oxygen species. Many of the described iron-oxidzing bacteria are easily identified by their extracellular structures, such as sheaths, capsules or stalks. There are probably also uni-cellular iron-oxidizing bacteria without visible extracellular structures involved in the iron oxidation processes.

Ferric ions can be stabilized in solution by chelation. Naturally produced chelators that may solubilize extensive amounts of ferric ions include microbially produced oxalate, citrate, humic acids and tannins. Subsequent precipitation of ferric hydroxides may result from microbial destruction (mineralization) of these chelators.

The microbiological iron cycle

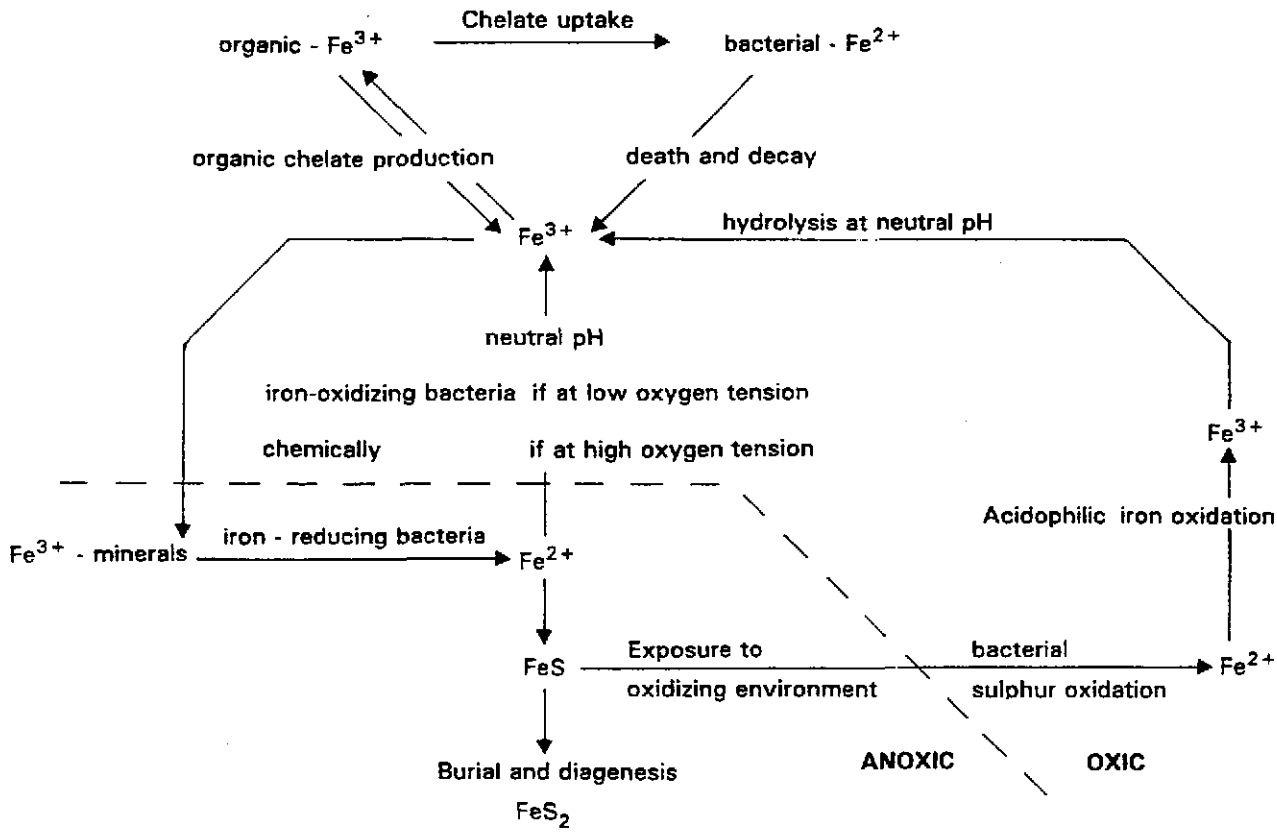

Figure 3.5 *The microbial iron cycle showing the processes that are caused or enhanced by microorganisms. The dashed line indicates the interface between anaerobic and aerobic environments.*

3.3.7 The iron cycle in repository and rock environments

Most deep rock environments are anaerobic and this is an advantage for the long-term safety of a deep repository. Ambient reducing groundwater conditions at depth will protect the waste canisters from aerobic corrosion and in case of a canister failure, important redox sensitive radionuclides like U, Np and Tc have low solubilities and low mobilities (high sorption retention) in a reducing environment. It is also important that reducing capacity is left, after the open phase of construction and operation of a repository, so that trapped air after sealing and closure will become consumed and not cause corrosion or any other adverse effects.

Infiltrating groundwater normally loses its oxygen during the recharge process. At anaerobic conditions iron reduction may be expected, at least in the upper 100 m or so. Results will be presented later in this report showing that iron reducing bacteria in fact constitute a major redox moderator system by their reduction of ferric iron during the oxidation of organic material in the recharging groundwater.

In the open repository, oxygen will be introduced through ventilation air etc. When groundwater, rich in ferrous iron, reaches this atmosphere, gradients suitable for iron oxidizing bacteria will develop. These bacteria precipitate iron in various forms together with other metals like manganese. As autotrophs are among the iron oxidizers, a net build up of organic carbon can be expected on rock walls, drainage ditches etc. The importance of such organic material for performance assessment must be evaluated.

3.4 BACTERIA AND THE MANGANESE CYCLE

The abundance of manganese in the Earth crust is 1.5% and this is 1/50 as plentiful as iron. The distribution of manganese in nature is not uniform. In soil its concentration ranges from 0.002% to 10%, the average concentration in fresh water is reported to be 8 $\mu g\ kg^{-1}$ and in sea water an average concentration has been reported to be 0.2 $\mu g\ kg^{-1}$.

Major accumulations of manganese occur in the form of oxides, carbonates and silicates. Manganese can exist in the oxidation states 0, +2, +3, +4, +6 and +7. In nature, the +2, +3 and +4 oxidation states are commonly found. The +2 oxidation state is the only one that can occur as a free ion in solution or as a soluble inorganic or organic complex. The +3 state can occur in solution only when it is complexed and tends to dismutate into the +2 and +4 states. Theoretically Mn^{2+} should autooxidize at pH above 4 when exposed to air, but it usually does not do so until the pH exceeds 8. Possible explanations for this could be the high energy of activation requirement of

the reaction or that Mn^{2+} may be extensively complexed and thereby stabilized by inorganic ions such as Cl^-, SO_4^{2-} and HCO_3^- or by organic compounds such as amino acids, fulvic and humic acids and others. Manganese tends to be transformed toward the solid-phase oxidized forms of manganate minerals represented as $Mn(IV)O_2$ in high Eh and pH environments, while in low pH-Eh environments, it tends toward soluble Mn(II). This distribution is controlled by diffusion and mixing processes.

There are many similarities between the manganese and the iron cycles (Figure 3.4 and 3.6), but one important difference lies in their relative tendency to form insoluble sulphide precipitates. Iron can be continuously removed from the environment via the formation of iron sulphides while manganese very seldom forms precipitates in the sulphide form.

Manganese is an essential trace element of nearly all organisms, but the nature of the requirements is not well understood. Some specific involvements of manganese with enzyme systems are known, but in most cases magnesium or other divalent cations can substitute for manganese. In addition to the basic requirements of manganese by organisms, some accelerate the cycling of manganese in the environment by oxidation and others are able to reduce the oxidized state of manganese (fig 3.6). These organisms are often found at the interface between highly oxidized and reduced environments.

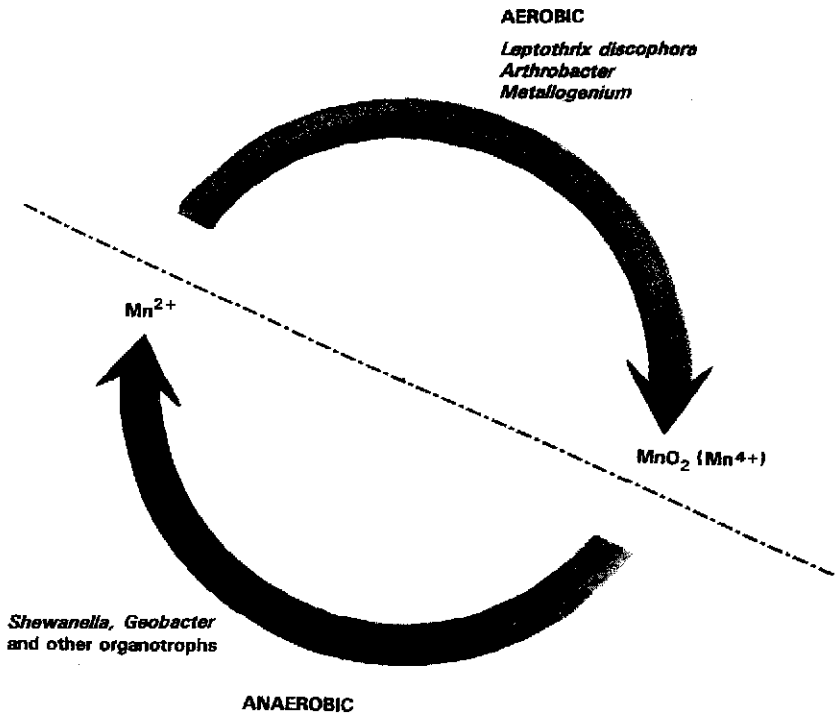

Figure 3.6 *The manganese cycle. Microorganisms make important contributions to the manganese cycle. Manganous ion (+2) is oxidized to manganic oxide (+4). The manganese cycle has many bacteria in common with the iron cycle.*

3.4.1 The manganese-reducing bacteria

It was early suggested that manganese oxide could be an alternative electron acceptor, analogous to nitrate (Trimble and Ehrlich, 1968), but it was not until 1988 that three reports described organisms that could obtain energy for anaerobic growth using oxidized manganese (and iron) as electron acceptor (DiChristina et al, 1988, Lovley and Phillips, 1986, Myers and Nealson, 1988). Microbial manganese reduction often occurs in stratified environments with high inputs of organic material. In such environments oxygen depletion is rather rapid, and anaerobic organisms, both fermenting and respiring ones, degrade the remaining organic material (see figure 3.1). The manganese reducing bacteria are suggested to be important in the mineralization of organic material in environments rich in Mn, such as fresh-water sediments.

Most of the manganese reducing bacteria have been isolated from fresh-water sediments. The bacteria that have been identified are similar to those reported to reduce Fe(III), *S. putrefaciens* and *G. metallireducens*. The organisms are versatile with regard to the electron donors used in manganese reduction. *S. putrefaciens* can utilize lactate and succinate but oxidizes these only to acetate. *G. metallireducens*, can at least utilize butyrate, propionate, lactate, succinate and acetate, which are completely oxidized to CO_2. On the other hand, since it is a facultative organism, *S. putrefaciens* is not restricted to anaerobic environment as *G. metallireducens*.

3.4.2 The manganese oxidizing bacteria

Jackson (1901) was the first to describe the existence of manganese-oxidizing bacteria., Since then, a number of bacteria, many which are taxonomically unrelated, have been reported to oxidize manganese. This manganese oxidation can be enzymatic or non-enzymatic. All recognized bacteria so far are aerobes and autotrophs or heterotrophs. Manganese oxidizing bacteria have been reported from very diverse environments, such as "desert varnish", on rock surfaces, in soil, in the water column and in sediments of fresh-water lakes and streams, in ocean water and sediments, and on and in ferro-manganese concretions (nodules) from the ocean floor. The manganese oxidation occurs preferentially at neutral pH values since the free energy of this reaction decreases steadily until it assumes a positive value near pH 1.0. Some of the bacteria involved in manganese oxidation are:

- *Leptothrix* spp., sheathed bacteria thought to be heterotrophs that deposit Mn-oxides on their sheaths. *L. discophora* has been shown to produce a Mn-oxidizing protein, excreted to the growth medium in pure cultures (Emerson and Ghiorse, 1992).

- *Siderocapsa* spp., encapsulated unicellular Mn-depositing bacteria.

- *Hyphomicrobium*, *Pedomicrobium*, *Tetramicrobium*, which are all hyphal budding bacteria (thread forming bacteria).

The nature of the manganese oxidation still is relatively unknown, but the manganese-oxidizing bacteria can cause problems with clogging in accordance with the iron-oxidizing bacteria (see section 3.3).

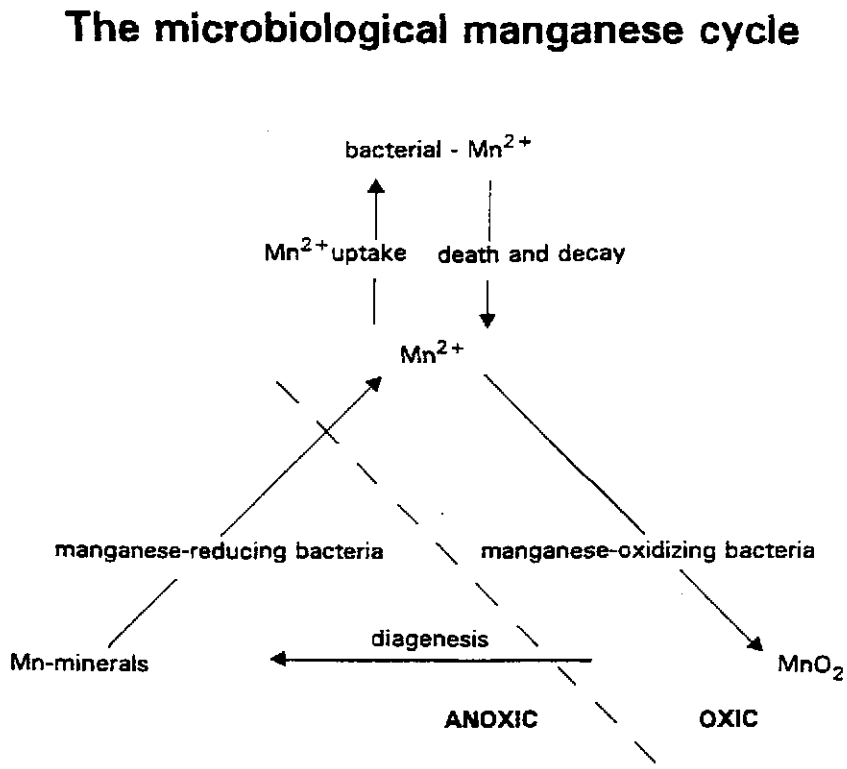

Figure 3.7 *The microbial manganese cycle. A simplified picture of the processes with microorganisms involved. The dashed line denotes the interface between anaerobic and aerobic environments.*

3.4.3 The manganese cycle in repository and rock environments

Manganese behaves very much like iron and the general comments made for iron in section 3.3.7 are also valid for manganese.

3.5 BACTERIA AND THE SULPHUR CYCLE

Sulphur is among the ten most abundant elements on earth. It occurs in a large number of chemical compounds of which sulphate and sulphides are the quantitatively dominating forms. Microorganisms contribute considerably to the sulphur cycle as shown in figure 3.8. The most important process relating

to nuclear waste is sulphate reduction with sulphide as end product. This is because sulphide is corrosive for the copper canisters. Special attention is therefore directed towards this process and the bacteria involved.

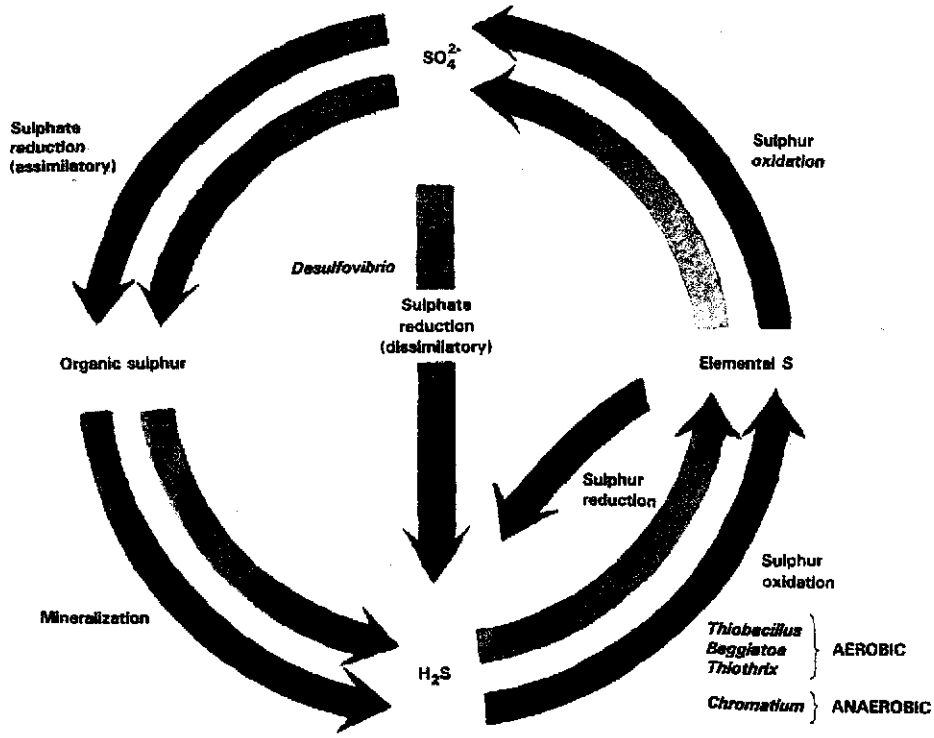

Figure 3.8 *The sulphur cycle. Photosynthetic (not present in subterranean environments) and chemosyntethic microorganisms contribute to the environmental sulphur cycle. Anaerobic sulphate reduction by sulphate reducing bacteria, a dissimilatory process, is noted with a purple arrow. Sulphate reduction also can occur in assimilatory reactions. Elemental sulphur reduction to sulphide is carried out by both eubacteria and archaea. Sulphur oxidation can be carried out by a wide variety of chemotrophs and phototrophs.*

Table 3.2 Some properties of selected classified sulphate reducing bacteria from (Widdel, 1988).

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

3.5.1 Sulphate reducing bacteria

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

3.5.2 Classification of sulphate reducing bacteria

With the isolation of a bacterium reducing sulphate to sulphide, which he called *Spirillum desulfuricans*, Beijerinck clearly demonstrated the correlation of the observed process to a special kind of bacteria (Beijerinck, 1885). The isolated type of sulphate reducer, which had curved motile cells, was known for a while as *Vibrio desulfuricans* and then finally named *Desulfovibrio desulfuricans*. The genus designation *Desulfovibrio* was maintained for non-sporing sulphate reducers usually having curved motile cells growing on a relatively limited range of organic substrates; preferred substrates are lactate or pyruvate, which are incompletely oxidized to acetate. *Desulfovibrio* species are still the best studied sulphate reducers. Sporing sulphate reducing bacteria with a similar metabolism were classified within the genus *Desulfotomaculum*. Later, additional types of sulphate reducers were described, several of which differed markedly physiologically and morphologically from the known *Desulfovibrio* and *Desulfotomaculum* species. Hence, the term sulphate reducing bacteria describes a rather heterogeneous assemblage of bacteria having in common merely dissimilatory sulphate reducing metabolism and obligate anaerobism (table 3.2).

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

3.5.3 Electron donors and autotrophic capacity

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

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

$$2CH_3CHOHCOO^- + SO_4^{2-} \Rightarrow 2CH_3COO^- + 2\,HCO_3^- + HS^- + H^+$$
$$\Delta G^{\circ\prime} = -160.1 \text{ kJ}$$

Complete oxidation of lactate to hydrogen sulphide and carbon dioxide:

$$2CH_3CHOHCOO^- + 3SO_4^{2-} \Rightarrow 6\,HCO_3^- + 3HS^- + H^+$$
$$\Delta G^{\circ\prime} = -255.3 \text{ kJ}$$

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

$$4H_2 + SO_4^{2-} + H^+ \Rightarrow 4\,H_2O + HS^-$$
$$\Delta G^{\circ\prime} = -152.2 \text{ kJ}$$

Hydrogen is also used, with relative slow growth, by several completely oxidizing sulphate reducing bacteria that may grow autotrophically (CO_2 is used as single carbon source). After the classical *Desulfovibrio* species had been shown to be chemolithoheterotrophic (growth with hydrogen as electron donor and organic carbon as carbon source), autotrophic growth of hydrogen-using sulphate reducers was reported for *Desulfosarcina variabilis*, *Desulfonema limicola*, and *Desulfococcus niacini*; however, growth was rather slow. Later when studied, many other sulphate reducers have been shown to grow autotrophically (table 3.2).

3.5.4 Sulphide and sulphur oxidation

There are many processes leading to the oxidation of sulphide and sulphur. Phototrophic anaerobic bacteria may use reduced sulphur compounds as electron donors but they are of course not to be present in the subterranean environment. The chemotrophic sulphur oxidizers on the other hand may very well be active in subterranean environments. This is a heterogeneous assortment of bacteria, the most studied are the colourless sulphur bacteria belonging to the families Thiobacteriaceae, Beggiatoaceae and Achromatiaceae. The genus *Thiobacillus* of the Thiobacteriaceae has been best studied and contains chemolithotrophic autotrophs deriving energy from the oxidation of reduced sulphur compounds and coupling this energy to the reduction of carbon dioxide. Oxygen is not the only oxidant for sulphur compounds. Nitrate can also act as a terminal electron acceptor for the oxidation of many reduced sulphur compounds.

3.5.5 The sulphur cycle in repository and rock environments

Sulphate reducing bacteria pose a potential threat to the copper canister concept due to their anaerobic sulphide production. It is necessary to show the extent of this process in the repository environment.

A positive effect of sulphate reducing bacteria from the point at view of waste disposal can be found in their ability to generate sulphide which reacts with oxygen and therefore contributes to the maintenance of reducing conditions in the underground environment. The action of the sulphate reducing bacteria may in that sense be seen as a way to make hydrogen or organic compounds participate in the redox reactions.

3.6 HYDROGEN RELATED BACTERIA

Many bacteria are able to oxidize hydrogen either under aerobic or anaerobic conditions (Table 2.7). Some of them are autotrophs with hydrogen as energy source while other oxidize hydrogen without autotrophic carbon dioxide fixation. The "true" hydrogen-oxidizing bacteria are a physiologically defined group that comprises bacteria from different taxonomic units. This group is defined by the ability to utilize gaseous hydrogen as electron donor with oxygen as electron acceptor and to fix carbon dioxide; i.e., to grow chemolithoautotrophically (Table 3.3). Their need for oxygen restrict them to surface environments and they will not be present in the closed repository environment.

Another physiologically defined group of hydrogen related bacteria comprises bacteria that are able to utilize hydrogen under anaerobic

conditions (Table 3.3). They are the sulphate reducing bacteria, discussed above, and the methanogenic and acetogenic bacteria discussed below. These groups have very different phylogeny; the methanogens belong to archaea while the other two groups belong to eubacteria. Finally, many bacteria utilize hydrogen as an extra source of energy when available without carbon dioxide fixation and usually under aerobic conditions.

3.6.1 Sources of hydrogen in the environment

Hydrogen is a product of anaerobic fermentations and an important intermediate in anaerobic food chains. Most of this hydrogen is converted in *situ* by interspecies hydrogen transfer to methane, acetic acid and hydrogen sulphide by anaerobic bacteria. Hydrogen is also a product of various chemical reactions going on due to the conversion of methane, carbon monoxide and other trace gases. In very deep areas of the crust production of hydrogen may be ongoing very much like what can be observed in geothermal areas on the ocean bottom and elsewhere in geothermal areas on land where very hot water dissolves salts, produces ions (HS^-, NH_4^+ Fe^{2+}, Mn^{2+}) and releasing volcanic gases (H_2, CO, CH_4). These elements are potential substrates for chemolithotrophic bacteria. Another source of hydrogen that may be significant is the integrated production of hydrogen through radiolysis of water due to disintergration of naturally occurring radionuclides (2.6.4).

Table 3.3 The basic metabolic processes of hydrogen utilization by various groups of bacteria in the presence of different hydrogen acceptors.

Electron and hydrogen acceptor	Metabolic process	Resulting compound
H_2 + oxygen	aerobic respiration	water
H_2 + carbon dioxide	carbon reduction cycle	organic compounds
H_2 + nitrate	nitrate respiration	nitrogen, nitrite
H_2 + sulphate	sulphate respiration	sulphide
H_2 + carbon dioxide	acetate formation	acetate
H_2 + carbon dioxide	methane formation	methane
H_2 + organic acids, light, carbon dioxide	anoxygenic photo- synthesis	organic compounds
H_2 + fumarate	fumarate reduction	succinate

3.6.2 Hydrogen in repository and rock environments

Evidence for presence of hydrogen in Fennoscandian shield rock environments was published recently (Sherwood Lollar et al, 1993a, b). Low concentrations of hydrogen in many environments may be due to that it is consumed by different bacteria and transformed e.g. to methane. It becomes

important to assay any naturally occurring fluxes of hydrogen because such fluxes will determine much of the anaerobic chemolithoautotrophic activity of bacteria like sulphate reducers, methanogens and acetogens.

Possible sources for hydrogen in a waste repository are corrosion of metals, for example steel, and radiolysis. Radiolysis production in low level waste or outside high level waste canisters will be relatively unimportant. Alpha and beta radiolysis will be of some importance if water enters a damaged canister and comes into direct contact with for example spent fuel. However, corrosion is likely to dominate if for example steel has been used in the construction of the canister.

3.7 METHANE RELATED BACTERIA

Methanogenic bacteria belong to the primary kingdom Archaea and share this property with some other organisms living in extreme environments as the salt-loving halobacteria and the the thermoacidophiles. The methanogenic bacteria are extremely oxygen-sensitive. This is not a great disadvantage for them in nature. In habitats rich in degradable organic compounds and in most subterranean environments oxygen is trapped by organisms in the surface layers. Thus methanogenic bacteria are particularly abundant in all sorts of mud and sediments. Other important habitats of these organisms are rumen and the (man-made) anaerobic digesters of sewage plants. The oxygen sensitivity of the methanogens creates problems when pure cultures are to be carried out. An appropriate method, the so called Hungate technique (Hungate 1969), has been worked out for this purpose.

3.7.1 Methane production

The common physiological property of methanogenic bacteria is the production of methane from a limited number of one-carbon compounds and the two-carbon compound acetate according to the reactions in table 3.4:

Table 3.4 Various reactions by which methane producing bacteria form methane.

1.	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	$\Delta G^{o'}$ = -139.2 kJ/mol CH_4	(Ferry et al, 1992)
2.	$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O$	$\Delta G^{o'}$ = -126.8 kJ/mol CH_4	(Ferry et al, 1992)
3.	$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	$\Delta G^{o'}$ = -185.1 kJ/mol CH_4	(Ferry et al, 1992)
4.	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	$\Delta G^{o'}$ = -103 kJ/mol CH_4	(Ferry et al, 1992)
5	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	$\Delta G^{o'}$ = -121.1 kJ/mol CH_4	(Zehnder et al 1982)
6.	$4CH_3NH_3^+ + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$	$\Delta G^{o'}$ = -101.6 kJ/mol CH_4	(Zehnder et al 1982)
7.	$2(4CH_3)_2NH_2^+ \rightarrow 3CH_4 + 3CO_2 + 2NH_4^+$	$\Delta G^{o'}$ = -86.3 kJ/mol CH_4	(Zehnder et al 1982)
8.	$4(4CH_3)_3NH^+ \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$	$\Delta G^{o'}$ = -80.2 kJ/mol CH_4	(Zehnder et al 1982)
9.	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	$\Delta G^{o'}$ = -36.0 kJ/mol CH_4	(Ferry et al, 1992)
10.	$(CH_3)_2S + H_2O \rightarrow 1/2\ CO_2 + CH_4 + H_2S$		(Ni and Boone, 1991)
11.	$4MMPA^* + 2H_2O \rightarrow + CO_2 + 4\ MPA^* + 3CH_4$		(van der Maarel et al, 1995)
12.	$4CH_4CHOHCH_3 + HCO_3 + H^+ \rightarrow 4CH_3\ CHOHCH_3 + CH_4 + 3H_2O$	$\Delta G^{o'}$ = -36.5 kJ/mol CH_4	(Widdel, 1986)
13.	$2CH_3CH_2OH + 2CH_3HCOO^- \rightarrow + CH_3 + H_2O + H^+$	$\Delta G^{o'}$ = -116.3 kJ/mol CH_4	(Frimmer and Widdel, 1989)

*) MMPA - 3-S methylmercaptopropionate
MPA - mercaptopropionate

The different species are more or less specialized with respect to the number of substrates for conversion into methane. For some species the substrate serves as both energy and the sole carbon source. Other organisms can only grow when media are supplemented with additional carbon compounds. Such and other characteristics of the metanogenic bacteria are enlisted by Vogels et al. (1988).

Methanogens are entirely dependent on the metabolic activities of other anaerobes for providing their growth substrates. The breakdown of organic matter in anaerobic ecosystems proceeds sequentially from the complex to the simple (Figure 3.1). Thus, biopolymers such as cellulose undergo initial attack to biomonomers (e.g. sugars) which are eventually degraded to the level of methanogenic substrates. Methanogens therefore occupy the terminal position in a complex anaerobic food web, although they can be displaced from this position by bacteria that use other electron acceptors. Only when methanogenic substrates are channelled into anoxic environments from nonbiological sources are methanogens released from their substrate dependence of anaerobic food webs. Examples of such situations are thought to occur when geothermally produced hydrogen enters crystalline bedrock, hot springs, geological rift zones, and perhaps ocean floor hydrothermal vents.

Organic compounds formed in the biosphere are stored in living organisms, decomposed and transferred to the oceans and atmosphere, or buried in sediments. Sedimentary organics are attacked by bacteria including methanogens and if environmental conditions are suitable, methane may be entrapped within the sediment matrix. Therefore, methanogenic activity occurring during the diagenesis of recent sediments can ultimately result in deep deposits of natural methane gas.

3.7.2 Anaerobic methane oxidizing bacteria

Concentration profiles of methane in anoxic seawaters and sediments frequently have "concave upward" appearances. Kinetic models of such profile data indicate that there is less methane in these regions than would be predicted by simple diffusion. The models achieve a better fit to the data when a term for methane consumption is included (Ormeland, 1988). Because sulphate is the only apparent oxidant present in sufficient quantity to cause a significant removal of methane, this process is suggested to be mediated by sulphate reducing bacteria (Figure 3.9). Thermodynamic calculations indicate that methane oxidation is favourable but inadequate for bacteria growth. It remains to gather conclusive data on this issue.

3.7.3 Methane in repository and rock environments

Many deep groundwater samples contain methane of biological origin, indicating ongoing methanogenic activity (Sherwood Lollar et al, 1993a). Still, much of the evidence for such activity depends on stable carbon isotope values for methane (confer 4.2.3). It remains to prove the occurrence of active methanogens in Swedish crystalline bed-rock. The SFR and SFL 3-5 repositories may experience methane production from decomposition of organic wastes.

3.8 ACETOGENIC BACTERIA

Both methanogenic and acetogenic bacteria are able to gain energy from the reduction of carbon dioxide by molecular hydrogen:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^{\circ\prime} = -130.4 \text{ kJ/mol } CH_4$$

$$4H_2 + CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \qquad \Delta G^{\circ\prime} = -95 \text{ kJ/mol } CH_4$$

However, the organisms carrying out these two reactions are very different. Acetogens are typical eubacteria whereas methanogens represent the largest

and best known group of the archaea. Chemolithotrophic acetogens are found in various genera of anaerobes (Fuchs, 1986). Among them are mesophiles and thermophiles, sporeformers and non-sporeformes. The first acetogenic bacterium, *Clostridium aceticum*, was described 1936.

The production of acetate from hydrogen and carbon dioxide by acetogenic bacteria for the generation of energy may appear confusing as we keep learning that the breakdown of organic matter continues from complex biopolymers such as cellulose to biomonomers and further to the level of simple methanogenic substrates such as carbon dioxide and hydrogen. The acetogens reverse this electron flow back to a two carbon organic compound. Acetate still may end up as methane because some metanogens can use this carbon source (confer section 3.7.1 reaction 9 and figure 3.1). In addition, many acetogenic bacteria produce acetate and hydrogen from sugars. They are thereby a vital link between hydrolysis/acidogenesis and methanogenesis in anaerobic ecosystems.

3.8.1 Acetogenic bacteria in repository and rock environments

Not long ago very little was known about this group of bacteria. The scientific community has improved the knowledge enormously during the last 10 years but the presence and activity of microorganisms in deep subterranean environments and their eventual influence on nuclear waste disposal is still unknown.

3.9 GAS FORMATION FROM BACTERIAL ACTIVITY

Gas production by bacteria may occur as a result of several different processes (Bachofen, 1991). The processes of aerobic degradation, fermentation and anaerobic respiration of organic matter all produce carbon dioxide (Fig 3.1) in relation to the amount of organic carbon available for degradation. Hydrogen evolves during fermentative processes and dinitrogen, hydrogen sulphide and methane may form in anaerobic environments. Gas may also be produced by inorganic processes such as radiolysis and thermal and chemical corrosion. At saturation, or when the pressure drops, gas bubbles may form.

Wan and Wilson (1994b) describe how colloids (including radionuclide containing ones) may sorb irreversible to the gas-water interface of a gas bubble. Both hydrophobic and hydrophilic colloids sorb. The authors conclude that this preferential sorption of colloid particles onto gas-water interfaces suggests a mechanism in vadose zone transport. A stationary gas-water interface in porous or fractured media can retard the transport of

particle contaminants. Moving interfaces may enhance colloid mobility (Dahlbäck et al, 1981). The enrichment of bacteria to the gas-water interface is well-known (Ehrlich, 1990a, Norkrans, 1980) and not only non-living colloids but also living bacteria may be transported by bubbles in the vadose zone.

3.9.1 Gas formation in repository and rock environments

There is groving evidence for significant methane production by bacteria in *deep subterranean rock environments* (Sherwood Lollar et al, 1993a, b). It remains to be determined whether this bacterial gas is produced by on-going processes of methanogenesis or whether it originates in geologically old reservoires stored in the crystalline rock. Direct evidence of such bacterially mediated methanogenesis is in the focus of our present research activities.

Gross biological gas production is not probable in SFL 2 but may occur in the SFR and SFL 3-5 repositories. Roffey and Nordqvist (1990) showed that there will be enough organic material, especially bitumen, in SFR for significant production of carbon dioxide (Roffey and Nordqvist, 1991).

3.10 GEOCHEMICAL IMPLICATIONS OF BIOLOGICAL ACTIVITIES IN THE SOIL ROOT ZONE

Soils are dominated by a solid phase and provide a unique environment for microorganisms. The conditions for microbial life vary widely with different soil characteristics and environmental conditions. Soil is also the habitat for organisms other than bacteria including, soil-dwelling protozoa, insects, nematodes, and other animals. These organisms together with plants contribute to the formation and maintenance of soils.

Plants assist soil evolution by adding organic matter through excretions from their root systems and as dead organic matter. The plant excretions may react directly with some soil mineral constituents or they may be modified together with the dead matter by microbial activity forming products that react with soil mineral constituents. The root systems of plants help to prevent destruction of the soil through wind and water erosion by anchoring it. Microorganisms contribute to soil evolution by mineralizing some or all of the added organic matter during the decay process. Some of the metabolic products of this decay, such as organic and inorganic acids, carbon dioxide or ammonium interact slowly with soil minerals and cause their alteration or dissolution, an important step in soil profile formation. Many bacteria may interact enzymatically with certain inorganic soil constituents (N, Hg, Fe,

Mn, and S containing compounds) by oxidizing or reducing them (Bossier et al, 1988). Microorganisms also play an important role in humus formation. Humus consists of partially degraded and stabilized organic matter, consisting of humic and fulvic acids as well as amino acids, lignin, amino sugars, and other compounds of biological origin. Humus is therefore an important constituent in soil, attracting and binding a variety of organic and inorganic substances.

From the viewpoint of the microorganism, the soil contains of a variety of surfaces and pores, distributed in heterogeneous aggregates of various size. Because of limited gas diffusion in and out of such aggregates and the possibility of spaces between aggregates being completely flooded, major changes in dissolved salts and gases can occur in these small pore environments.

Soils have markedly higher concentrations of carbon dioxide, carbon monoxide and other gases in comparison with the atmosphere, and a corresponding decrease in the oxygen concentration. When it rains, soil may quickly change from an aerobic environment with many separate anaerobic microsites to a predominantly anaerobic soil. Going deeper, in wet soil and further down in the saturated zone, oxygen depletion will be more or less permanent and the environment steadily anaerobic.

Rain water percolating soils will extract soluble compounds and gasses and bring them down to the groundwater. Microbial activity in the soil and root zone may therefore be important for the groundwater formation process. They reduce the organic content of the groundwater, consume oxygen and simultaneously enrich it with metabolic gasses, like carbon dioxide, which in turn affect alkalinity and pH. Finally, exchange reactions between water and inorganic phases of the soil and rock depend on pH. Such series of events, starting with biological processes, demonstrate how microbial activity in top soils influence the formation of groundwaters.

3.11 SUMMARY OF BACTERIAL PROCESSES

A number of microbially related processes in subterranean environments can be identified. Most of them influence geochemical processes and some would not occur at all unless bacteria catalysed the reactions.

- **The cycling of carbon.** Most deep rock environments have a relatively low content of organic carbon but measurable amounts of carbon dioxide. The organic carbon contents are usually measured in water

and may therefore be underestimates since many organic compounds are sorbed onto rock surfaces. Also, the contents measured reveal levels, not fluxes. It will therefore be an important task to investigate the sources and fluxes of organic carbon in the subterranean environment. This is because such carbon may be the main fuel for bacterial processes of importance for nuclear waste disposal (e.g. sulphate reduction in a SFL 2 repository) and the fluxes will determine the process rates. Fluxes of inorganic energy- and electron-sources will be important for the understanding of possible new formation of organic carbon in the subterranean environment. The participation of bacteria in the carbon cycle also implies their interaction with fracture mineral formation and degeneration which may influence sorption and desorption of radionuclides. Further, bacterial production and consumption of carbon dioxide may execute an effect on pH and alkalinity of groundwaters. The respositores for low and intermediate level waste like SFR and SFL 3-5 will contain many different types of organic carbon compounds with the potential for production of gasses like carbon dioxide, hydrogen and methane and of acids due to fermentative processes.

- **The cycling of nitrogen.** Deep groundwater is usually depleted in nitrate, nitrite, ammonia and other nitrogen compounds except for dinitrogen gas. This is however not any principal obstacle for bacterial growth because many bacteria fix dinitrogen gas to ammonium and further incorporate it into organic material. Nitrification processes are usually aerobic and will be limited by the anaerobic character of repository and rock environments and the limited availability of ammonium. Possible deposits of geological nitrogen in rock or clays may however offset this limitation. Under aerobic conditions or if anaerobic nitrification occurs, the nitrification process generates acid. The very low concentrations of nitrate in most deep groundwater indicate electron acceptors other that nitrate to be important for respiration of organic carbon in the subterranean environment.

- **The cycling of iron and manganese.** Most deep rock environments are anaerobic and it is important that the repository environment resumes anaerobic conditions after closure. Remaining oxygen can attack the canisters and the ability of radionuclides to sorb in the rock matrix may change. The activity of iron reducing bacteria may help to bring down the redox of infiltrating water by reducing ferric iron to ferrous iron during the oxidation of organic material. When groundwater, rich in ferrous iron, reaches an oxygenated atmosphere during the operational phase, gradients suitable for iron and manganese oxidizing bacteria develop. They precipitate iron or manganese in various forms together with other metals. As autotrophs are among these oxidizers, a netto build up of organic carbon can be expected on

rock walls, drainage ditches etc. The importance of such organic material for performance assessment must be evaluated.

- **The cycling of sulphur.** Sulphate reducing bacteria pose a potential threat to the copper canister concept due to their anaerobic sulphide production. It remains to show the extent of this process in the repository environment.

- **Hydrogen related bacteria.** Evidence for the presence of hydrogen in fennoscandian shield rock environments was published recently. Low concentrations of hydrogen in many environments may be due to the fact that it is consumed by different bacteria and transformed e.g. to methane. It becomes important to assay any naturally occurring fluxes of hydrogen because such fluxes will determine much of the anaerobic chemolithoautotrophic activity of bacteria like sulphate reducers, methanogens and acetogens.

- **Methane related bacteria.** Many deep groundwater samples contain, often in large quantites, methane of biological origin, indicating on-going methanogenic activity. Still, much of the evidence for deep biological methanogenic activity depend on stable carbon isotope values for methane. It remains to be determined whether this bacterial gas is produced by on-going processes of methanogenesis or whether it originates in geologically old reservoires stored in the crystalline rock. Direct evidence of such bacterially mediated methanogenesis is in the focus of our present research activities. Repositories for low and intermediate level waste may experience methane production from organic materials in the waste.

- **Acetogenic bacteria.** Acetogenic bacteria are able to gain energy from the reduction of carbon dioxide by molecular hydrogen. In addition, many acetogenic bacteria produce acetate and hydrogen from sugars. They are thereby a vital link between hydrolysis/acidogenesis and methanogenesis in anaerobic ecosystems. It remains to evaluate their presence and activity in deep subterranean environments and their possible influence on nuclear waste disposal.

- **Gas production.** Gross biological gas production is not probable in a high level waste repository due to the general lack of organic materials there but may occur in the a repository for low and intermediate level waste. Roffey and Nordqvist (1991) demonstrated for example, bitumen can produce carbon dioxide.

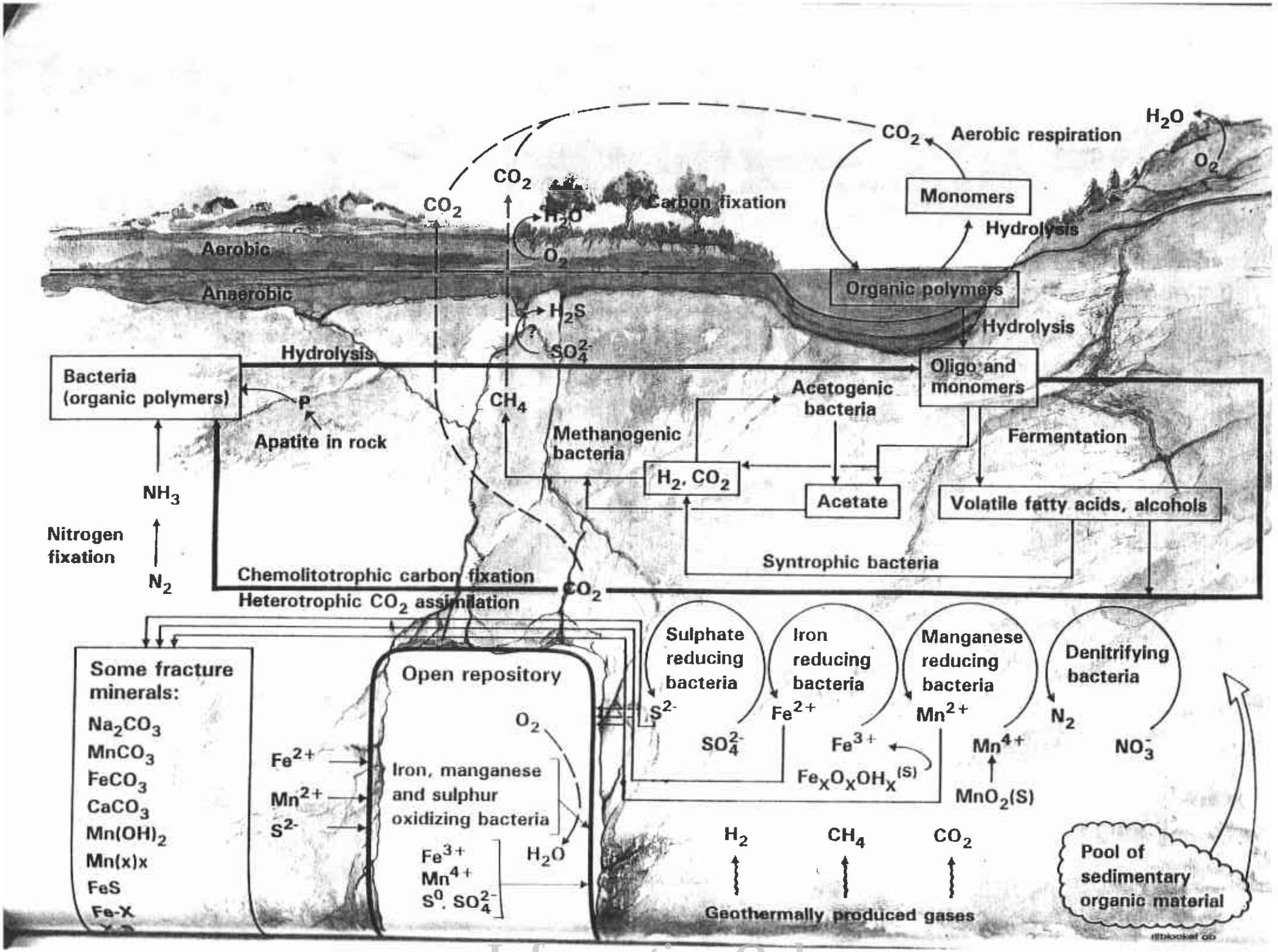

Figure 3.9 Summary of subterranean bacterial processes. Terranean photosynthetic primary production processes result in organic polymers that enter various food chains. As living entities die, they will enter degradation chains. Aerobic organisms have the ability to mineralise organic molecules completely to carbon dioxide. Deeper, at anaerobic conditions, bacteria are mainly responsible for biological mineralization. The anaerobic mineralization requires interactions of a complex bacterial food web, where the product of one microbial group serves as substrate for the subsequent group, and were the consumption of a product regulates its type and formation rate. Finally, organic carbon can be mineralised either completely to carbon dioxide by combined oxidative processes or to methane by oxidation-reduction processes, depending on the availability of inorganic electron acceptors in the system. When groundwater rich in ferrous iron, manganese(II) and reduced sulphur compounds reaches an oxygenated atmosphere during the operational phase, gradients suitable for chemolithotrophic and metal oxidizing bacteria develop. They precipitate metals and elemental sulphur in various forms together with organic material. Bacteria may be involved in many subterranean geochemical processes, such as diagenesis, weathering, precipitation, and in oxidation/reduction reactions of metals, carbon, nitrogen and sulphur- just as they are in most terranean environments.

4 INVESTIGATIONS OF SUBTERRANEAN BACTERIA

In recent years, published papers on various aspects regarding the microbiology of the subterranean environment have increased in numbers. There has been a significant expansion in the understanding of the bacterial ecology of shallow groundwater systems down to some 50-100 m, accurately reviewed by Ghiorse and Wilson (1988) and Matthess et al (1992), but our knowledge rapidly diminishes as we go deeper down into the crust of earth. The aim of deep drilling has, until recently, with few exceptions been restricted to hot water and oil extraction.

The main purpose of this chapter is to review the present research on the microbiology of subterranean environments, with emphasis on the Swedish research program on bacteria and their influence on performance assessment of radioactive waste disposal. Included are mainly studies where drilling, excavation, core sampling and groundwater sampling have been made for research. Studies done in environments penetrated for industrial purposes, such as water wells, mining, oil recovery etc., have usually been dismissed because of the obvious risk for contamination of the environment during the penetration. Such contamination makes the unambiguous interpretation of achieved microbiological data very difficult or impossible.

4.1 GETTING ACCESS TO THE SUBTERRANEAN ENVIRONMENT

All sampling of subterranean environments requires substantial efforts in drilling or tunnelling. The possibility of bacterial contamination of the sampled specimens by access operations is indisputable and must be considered when interpreting the obtained results. Conditions like the geological formation, the history of a borehole or a tunnel, available equipment and the type of sample considered are variables that will influence the prospect to get non-contaminated samples. Depending on these prevailing conditions, precautions realizable against contamination vary from virtually none to specific devices aimed for sterile sampling.

4.1.1 The risk for contamination of aquifers during drilling

The risk for bacterial contamination of the aquifers with the drilling water used to transport the drill cuttings to the surface during drilling is obvious. Some different measures can be applied to reduce such contamination. A clean drilling water as free from microbes as possible is an essential prerequisite. Pumping of a borehole to measure its maximum hydraulic water capacity is often made and will concurrently clean the aquifers and the borehole from drilling water, mud and cuttings. In addition, a control of the mixing of drilling water in the groundwater can be made by introducing different tracers in the drilling water that subsequently can be analysed for in the groundwater samples (Russell et al, 1992). The necessity of clean drilling equipment free from contamination is evident, but not always achievable.

Coring sedimentary rocks

No drilling operation to day allows sterile collection of deep subterranean sediment or rock samples. A goal of the Microbiology of the deep subsurface environments program, a subprogram of the U.S. Department of Energy (DOE) drilling project (US, 1991), has been to develop procedures that minimise the level of contamination. Extensive precautions were taken, such as steam cleaning of the bore equipment and using chlorinated water in the drilling mud. To minimise potential organic carbon contamination from fuel, lubricants, hydraulic fluids etc., thick concrete pads were constructed that sloped away from the boreholes. A key component in minimising on-site contamination was an enclosed drilling fluid circulation system (Russell et al, 1992). A main method developed for sampling sedimentary geological formations was core sampling by rotary drilling with bentonite viscosifier drilling fluids (Phelps et al, 1989a). The success of sampling was a function of the type of geological formation. For instance, Chapelle et al (1987) sampled sediments in Maryland down to 182 m, driving 46 cm plastic sleeves into the sediment with a hammer apparatus. Sand tended to fall out of the samplers unless a retaining basket was used.

Coring crystalline bed-rock

As the geological formation becomes harder, more drilling force is needed with increasing risk for contamination. Coring crystalline bed-rock requires vigorous drilling action with high drilling fluid pressures. The risk for intrusion of drilling fluids and drill cuttings in fractures is obvious. A new drilling technique was developed during the Swedish subsurface program, the so called telescope-type drilling (Almén and Zellman, 1991). Briefly, a 155 mm borehole was percussion drilled down to approximately 100 m.

The borehole was then continued by 56 mm core drilling after temporary casing of the 155 mm hole. The bottom ten meters of the casing was

perforated to allow water to pass into the annulus between the casing and the borehole wall. Two one-inch air tubes were mounted in the annulus. Compressed air was blown through the tubes during the drilling operation. When the air bubbles entered the borehole, the density of the water column decreased, lowering the hydrostatic pressure in the drill pipe. A mixture of water, drill cuttings and air was then forced upwards. Typical drill water contents obtained in groundwater samples from boreholes drilled with this technique were 0.06-0.8% compared to 2.6-13.7% without telescope-type drilling (Pedersen and Ekendahl, 1990).

Sampling from tunnels, mines etc. is possible in geological formations that allow such constructions. Breaking out of rock from tunnels without explosives may be one way to achieve fracture surfaces without drilling as an alternative to core sampling. This was done in Rainer Mesa at the Nevada test site in USA by Amy et al. (1992a). Water and rock samples were taken from a mined tunnel system 400 m below ground. Microbial viable counts ranged from 10^2 up to 10^4 cells g^{-1} dry weight of rock sample and 10^2 cells ml^{-1} groundwater sample. Discovered bacteria were aerobic heterotrophs that could grow on complex media and *Pseudomonas* was the most commonly identified genus.

4.1.2 Clean sampling and rapid processing of samples

All groundwater sampling for microbiological investigations needs sterile containment of the sample followed by fast transport from the sampling depth to the lab for analysis. It can be sampled at the surface by pumping (Pedersen and Ekendahl, 1990, 1992b) or driven by an artesian head (Denman et al, 1991, Olson et al, 1981, Pedersen and Ekendahl, 1992a) or at the actual depth in sterilised containers enclosed in a borehole sampler (Pedersen and Ekendahl, 1990, Torstensson, 1984). Specific geological formations or aquifer systems usually are screened off and connected with tubes to the surface. If not under an artesian head, pumps have to be installed in the screened sections. Pumps, tubing and screening equipment are all subjects of contamination and should if possible be sterilised before use, which for different practical reasons not always is possible. Flushing of the installed equipment with the groundwater to be sampled is one way to reduce the degree of contamination. It is very important that all materials used are non toxic and that they do not leach organic substances that may induce growth of bacteria on tube walls and in the water. When a borehole sampler is used, the critical point of contamination is the orifice of the sampler. This is a problem very much alike what has been discussed for sampling in the marine environment (Oppenheimer, 1968). An advantage with borehole samplers is that they can be constructed to maintain the pressure at the sample depth, keeping gases under pressure in equilibrium with the water table (Pedersen and Ekendahl, 1990, Torstensson, 1984). There is often a very high content of dissolved gases in deep groundwater (Table 1.2), (Sherwood Lollar et al, 1993a, b). They participate in the groundwater

chemical equilibria, thereby influencing bacterial activity. Keeping ambient pressure may therefore be an important condition when performing *in situ* experiments.

4.1.3 Aquifers in fractured rocks and sediments

The aquifer structure is different in sediments compared to fractured rock. The sediments typically are horizontally stratified with aquifers in sands, sandy clays and clayey sand separated by non transmissive, clay confining layers that retard the flow between the aquifers; an analogue to the bentonite barrier. Fractured rocks have aquifers that run through faults and multiple or single fracture systems. They can orient any way, vertically or horizontally, very different from aquifers in sediments. Drilling horizontally in sediments then usually gives a core representing the profile of the present aquifers, while a single borehole in rock usually not is enough to guarantee the true picture of the aquifer system in a rock mass. Several boreholes, complemented with borehole radar (Almén and Zellman, 1991, Andersson et al, 1989b) may be needed.

The openings in rock fractures are potential channels for groundwater. Several model studies have been made on flow and transport in fractures with variable apertures (Moreno et al, 1988, Tsang et al, 1988). The results suggested 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 a few channels will dominate flow and transport of nutrients and bacteria. The questions of how much of a fracture is wetted and water conducting, how much is containing enclosed water and to what degree the fracture is filled with precipitation, still are waiting for thorough answers.

Most investigations of subterranean bacteria have been performed in sedimentary environments. Sediment data may give information about the microbiology of the backfill material and survival in bentonite clays, eventually mixed with sand. Although such results definitely are of a general interest, they are not conclusive when it comes to model bacterial life in hard rock.

4.1.4 Underground laboratories

There are currently large national and international research programs in Canada, Finland, France, Great Britain, Japan, Spain, Sweden, and the United States of America for the study of different questions concerning the safety of future underground repositories for nuclear waste. The international Stripa research project of which all of the countries arrayed above have taken part, was performed in an old iron mine in Sweden (Olsson, 1992, Pusch, 1992, SKB, 1986, 1989) for an overview of the Stripa project). However,

the construction of subsurface vaults like the Stripa mine disturbs the geological environment, especially when no precautions are taken to assure a good research environment. Therefore, several rock laboratories are being constructed with research as the only purpose, and the Stripa project and the mine closed. In Sweden, the construction of the subterranean rock-lab was finished in summer 1995 at 460 m below the surface of the island Äspö, situated on the South-east coast of Sweden (Gustafsson et al, 1988, 1989, 1991). In Canada, an underground research laboratory (URL, Whitshell, Pinawa) has been built on 240 and 420 m depths. In France, a new law by 30 December 1991 says that two subterranean laboratories shall be constructed. In Switzerland, a research station in crystalline bed-rock has been constructed in Grimsel in the Alps. These and other subterranean laboratories may offer the *in situ* research environments judged fundamental in the following text.

4.2 THE DETERMINATION OF BIOMASS, DIVERSITY AND ACTIVITY OF SUBTERRANEAN MICROBIAL COMMUNITIES

The requirement for reliable estimations of the present biomass (i.e. numbers of bacteria), its activity and diversity in subterranean microbial ecosystems, is fundamental. There is an array of different methods to achieve this goal in terranean environments (Grigorova and Norris, 1990). The applicability of them depends on the subterranean system of interest. The methods range from the more than a century old technique of plating microbes on solid media for their cultivation and enumeration to new methods in molecular biology where DNA can be extracted from the environment, analysed for the diversity of the community studied and finally *in situ* probed with labelled oligonucleotides (Stackebrandt and Goodfellow, 1991).

4.2.1 Methods for the enumeration of microorganisms

Methods for the enumeration of microorganisms and the determination of their biomass in natural environments have recently been reviewed and discussed (Fry, 1990, Herbert, 1990, Kepner et al, 1994). Enumeration of bacteria encompasses either viable or total counts or both. Viable counts depend strongly on the ability of the investigator to develop growth media that suits all present bacteria. This has turned out to be impossible and viable count usually underestimate the number of viable bacteria present in a sample. Total count on the other hand gives a better understanding of the actual number of bacteria present, but no information is given about the viability and diversity of the community. It is also a well-known fact that attached microbial populations often dominate over the bacteria in suspension in microbial aquatic ecosystems (Kölbel-Boelke and Hirsch, 1989, Lappin-Scott and Costerton, 1989). It therefore is important to include enumeration methods that work on bacteria attached on fracture fillings such as gravel, sand, clay or silt as well as on fracture surfaces in hard rock. This

is in practice very difficult and has not been done successfully yet. We tried to collect rock directly after blasting sections of the Äspö tunnel. It was however dangerous due to the risk of falling rocks and the fracture surfaces obtained were disturbed by the drilling fluids, explosives and the shock wave from blasting. Gentle, mechanical sampling of fracture systems with a known water flow is needed. Such sampling may possibly be done during future research phases at the Äspö lab.

4.2.2 Methods for the determination of bacterial diversity

Culturing techniques

The classical way to study the diversity of bacterial communities is to inoculate different solid and liquid media with environmental samples and subsequently incubate at different temperatures and gas compositions. For instance Balkwill et al (1989) Balkwill (1989) and Fredrickson et al (1991) studied colony morphology and physiological diversity of subterranean bacteria with this technique. Godsy (1980) reports successful isolation and cultivation of *Methanobactertium bryanti* from a deep artesian aquifer in Florida. Growing bacteria can then be enumerated and classified according to their morphological, chemical, and physiological properties. The Bergey's manual of determinative bacteriology (Holt, 1984-89) describes an array of such procedures.

Biochemical and molecular techniques

An obvious drawback with all culturing methods is that only so-called cultivable bacteria can be studied. As mentioned above, there are large numbers of bacterial species in different environments that we presently are not able to culture and study with traditional methods. Microbial biomass, community structure and nutritional status of groundwater aquifer microbes can instead be studied with biochemical methods as were done by Balkwill et al (1988) and White et al (1983) or with methods in molecular biology (Stackebrandt and Goodfellow, 1991). DNA can be isolated from environmental samples (Johnson, 1991), amplified with the Polymeras Chain Reaction (PCR), sequenced (Ludwig, 1991) and compared with DNA-sequences of known bacterial species. Most such work this far has been performed on the 16S-rRNA subunit of the bacterial ribosome (Amann et al, 1991, Giovannoni et al, 1990, Weller and Ward, 1989, Woese, 1987). These methods are now rapidly becoming standard protocols for studies of microbial ecosystems including subterranean environments.

The 16S-rRNA technique

In our investigations of subterranean bacteria we use today the information available in the ribosomal 16S-rRNA gene to map diversity and distribution

of subterranean bacterial populations. Principles of the method we use are presented in Ekendahl et al (1994) but we use a blunt end cloning kit today (Stratagene pCR-Script SK(+)) instead of the published cloning method. This molecular method can reveal both cultivable as well as uncultivable bacteria in deep groundwater. We regard our results as an approximate true reflection of the actual distribution of different bacterial cells in the original samples, based on the following discussion.

Grampositive bacteria and archaea are generally more resistant to cell lysis than gramnegatives (Stackebrandt and Goodfellow, 1991) because of stronger and different cell walls. Since the majority of the bacteria in our samples were uncultivable with the media used (compare Figures 4.1 and 4.3 and 4.5), it was not possible to use for instance penicillin to weaken the cells during growth before lysis. We used both SDS, lysozyme and proteinase in the lysis step, and there was no problem to extract the bacterial DNA. Control experiments showed that the lysis of cells in the samples was complete or near complete. Additional control experiments with lysis of *Escherichia coli*, *Desulfomicrobium baculatum* and *Bacillus megaterium* in cell suspensions showed that 90-95% of both gram negative and gram positive bacterial cells were lysed with the method used.

The PCR method is fast, technically simple, and, important in our case, sensitive to small amounts of DNA. It has become a major tool in studies of genomic relatedness. One drawback is the sensitivity to contamination. The PCR conditions must be very strictly controlled and have a negative control. The PCR method may not amplify all rRNA gene sequences to the same extent. Small differences in rRNA gene sequences from pure cultures and also in universally conserved regions of some small rRNAs have been reported (Ward et al, 1992). Differential or imperfect annealing of primers due to these differences in universal regions or to inappropriate hybridisation stringency may cause errors which are amplified in the PCR reaction. An archaea having an intron within its 16S rRNA gene has been found (Ward et al, 1992). If such bacteria were present in our samples and had the intron where the primer should anneal, it would not be amplified. Likewise, microheterogeneity within 5S rDNA has been found (Davis and Nomura, 1972). These small differences are not thought to have any significance in ribosome function.

We used the PCR for amplifying rRNA genes from mixed natural populations instead of rRNA itself, thereby avoiding the sometimes great variation in transcription activity between bacteria at different growth conditions. There may be up to 71 000 rRNA copies in actively growing cells (Ward et al, 1992). The number of 16S-rRNA genes also vary somewhat among different species. Archaea usually have single copies of the rRNA gene, while eubacteria commonly have 5-10 copies per haploid genome (Davis and Nomura, 1972, Ward et al, 1992). For example, *E. coli* has 7 rRNA operons in its' genome, each containing one 16S-rRNA gene. A control experiment for quantitative PCR (Ekendahl et al, 1994) indicated

that the used PCR technique was quantitative for populations with equally sized bacteria in addition to its qualitative nature, but biased when bacteria of different sizes were investigated. This result indicates that bacteria may have rRNA gene copies in relation to their size, which seems reasonable. The transcription activity per rRNA gene locus at defined growth conditions will then be approximately constant and independent of large differences in size for bacteria with similar growth kinetics and comparable growth rates.

The organisation of these multi-copy rRNA genes is remarkably stable, but recombination and mutations have been reported to occur between these operons (Ingraham *et al.*, 1987). Although differences in the *E. coli* operons exist, they are virtually (>99%) identical. The possibility thus exists that a single cell could have different 16S-rRNA genes. An example is *Haloarctula marismortui* which expresses different rRNA sequences simultaneously (Ward et al, 1992). Although too few species seem to have been investigated for gene copy differences, it seems unlikely that great differences should be common, since rRNA genes are so important for the cell. Transfer of rRNA genes between species has not been reported.

To get the gene abundance for a particular species, one should multiply the number of cells of the studied species with the number of rRNA gene copies per cell. The specific/total rRNA gene ratios achieved in this study is thus an approximate and not an absolute estimate of cell numbers, but are more accurate for estimating organism abundance than the rRNA ratios. In our study we have only detected one archaea yet and the size distribution of the investigated bacteria was equal in the different samples judged from microscopic investigations, which indicates the clone distribution results and frequencies of detected species (Table 4.5) to be an accurate approximation of the relative species abundance.

The cloning of PCR fragments is efficient for creating clone libraries and - as in our investigations - for separating the different 16S-rRNA genes in mixed natural populations. The cloning step is a random process, since each piece of foreign DNA is inserted into any of the available plasmids, and any of the plasmids is transformed into any of the competent *E. coli* cells. This process does not affect the final result in clone distribution more than by random distribution. Considering the 5-10 16S-rRNA genes present in each eubacterium as opposed to the number of cells and the enormous amount of DNA created in the PCR reaction, there is only a small probability that the clones selected for further studies have inserts from the same cell. It is therefore reasonably certain that sequence differences reflect the input of equal sized different cells. We select all clones randomly and choose to study 10-12 clones from each DNA-extraction. This allow us to detect the most frequently occurring bacteria in the population, and we considered this to be enough for the purpose of performance assessment of nuclear waste disposal.

The method of sequencing genes, the 16S-rRNA gene in particular, has become a major breakthrough in evolutionary and phylogenetic studies of prokaryotes and has now become a standard atomised technique. The 16S-rRNA molecule is universally distributed in nature, functionally homologous and has highly conserved regions suitable for such studies. Sequencing small but informative domains of the gene - as we do with our clones - gives a relatively quick answer to the diversity and distribution of bacteria in mixed populations, and to where in the systematic they belong.

4.2.3 Methods for estimating bacterial activity

Observation of bacterial populations in any environment usually invokes the question whether they are active or not. This can be studied with many different methods. They are usually based on the registration of uptake and transformation of various compounds (Hall et al, 1990, Moriarty, 1990). As for the enumeration techniques, it is crucial that the methods for finding microbial activities include attached microbial populations (Characklis and Marshall, 1990, Ladd and Costerton, 1990). The most accurate way to do this is by *in situ* experiments, but obviously such experimental conditions are very hard to achieve in deep subterranean environments. Samples have been brought up on top of the boreholes and transported to laboratories, often far away, before the experiments could start. This will definitely modify the environmental conditions in different ways. Flow rates, pressure, redox potential, possibly temperature and different dissolved chemical and gas equilibria will change. During the Swedish investigation program for a future high level waste repository, it was found that 14 days were needed to re-establish a stable redox potential in deep bore holes (Grenthe et al, 1992a, Wikberg, 1987). Since the *in situ* environment can be that redox sensitive it will be much more difficult to establish *in situ* conditions in a lab away from the borehole. This is nevertheless the conditions used by many investigators, when there was a lack of *in situ* conditions. As a compromise, the effect of possible deviations from the actual deep subterranean conditions has to be accounted for when interpreting our results. For instance, it can be made in relation to theoretical calculations as was done by Chapelle and Lovley (1990) who performed geochemical modelling of groundwater chemistry changes along aquifer flow paths in the coastal plain of South Carolina. They reported that laboratory incubations may greatly over-estimate the *in situ* rates of microbial metabolism in deep subsurface sediments. The rate estimations from their geochemical modelling suggested the deep coastal plain aquifers to be among the most oligotrophic aquatic environments in which there is ongoing microbial metabolism.

Brockman et al (1992a) recently contributed to the knowledge about the deep subterranean environment with a study on the unsaturated, vadose zone paleosols of Washington state, USA. Among the results was the demonstration that storage at 4°C and small increases in water potential resulted in large increases in microbial growth and activity in the laboratory. These data further show the importance of *in situ* experimental conditions

for the study of the deep subterranean environment. Therefore, in the Swedish program, we usually have done and still do all inoculations and incubations on site. A portable lab was set up in the Stripa mine, 410 m below ground, for this purpose. At Äspö, we used the mobile field lab on top of the sampled borehole and later standing first in the tunnel, then at the entrance of the Äspö tunnel, an now in the research village and Äspö. The on site work was done to minimise any disturbance occurring during transport and storage of samples.

Assimilation studies

The assimilation of different introduced compounds is often used to assay the metabolic activity of bacterial populations (Hall et al, 1990). The choice of compound may have a profound influence on the results as was shown by Pedersen and Ekendahl (1992a, b). We measured assimilation of formate (one carbon organic acid), acetate (two carbon organic acid), lactate (three carbon organic acid), glucose (six carbon sugar) and leucine (amino acid) that proved the presence of heterotrophic bacteria. The assimilation of lactate by attached bacteria dominated over acetate and glucose at all depths by a factor of approximately 50. Leucine was assimilated by from 9 up to 99% of the populations and showed that major portions of the populations were viable. The results show that the use of several different compounds reduces the risk for false conclusions about the viability and the metabolic activity of the deep groundwater populations.

Stable isotopes

Bacteria express a kinetic isotope effect by favouring lighter isotopes over heavier isotopes. The study of stable isotope fractionation therefore reflects the diversity and activity of bacteria in different environments. Methanogenic bacteria favour ^{12}C over ^{13}C so the methane produced will be enriched in ^{12}C compared with ^{13}C and the CO_2 used will be enriched in ^{13}C compared with ^{12}C (Ormeland, 1988). Belyaev and Ivanov (Belyaev and Ivanov, 1983, Belyaev et al, 1983) obtained negative ^{13}C-values (-37.9 to -94.1‰) of methane from oil-bearing sedimentary rocks and formation waters, sampled from old oil fields. Their results suggested that much of the methane from the oil deposits studied was of bacterial origin and they could obtain enrichment cultures of methanogenic bacteria. Barker and Fritz (1982), and Fritz et al (1989) used data on carbon isotope fractionation to draw conclusions about the presence of methane producing and sulphate reducing bacteria in some North American and Swedish groundwaters, respectively. The sulphate reducers can be predicted by elevated values of $^{34}SO_4$ over $^{32}SO_4$ compared with standard values (Widdel, 1988). Dockins et al (1980) made stable sulphur isotope studies in Montana groundwaters that suggested a biological role in sulphate reduction and they could enrich sulphate reducers. Fontes et al. (1989) got isotope data that proved bacterially enhanced redox processes to play an important role in the control of the concentration and heavy

isotope contents of aqueous sulphate in the Stripa groundwaters. As most activities of subterranean bacteria probably are slow processes, assimilation studies may not be sensitive enough to detect such activities. That is where the stable isotope technique is has its major benefits. It will reveal very slow and also past activities of microbes in investigated systems by studying the bacterial effect on the environment (geochemical record) rather the bacterial transformation (reaction) itself.

4.3 MICROBIAL LIFE IN THE CRYSTALLINE BED-ROCK OF STRIPA AND ÄSPÖ, SWEDEN

The Swedish research program on subterranean microbiology has been performed on two sites, the Stripa research mine in the middle of Sweden and the Äspö area, next to the Baltic sea in the South Eastern part of Sweden.

The Stripa mine is situated 250 km west of Stockholm and was an iron mine until 1976. A total of 16.5×10^6 tons of iron ore has been mined out since 1448. Since 1976 the mine has been used as a deep underground research facility. The ore consisted of a quartz-banded hematite and occurred in a lepatite formation. Adjacent to the lepatite is a large body of 1.7 billion year old medium-grained granite, in which the Stripa project experiments have been performed (Gustafsson et al, 1988, 1989, 1991).

The Äspö investigation area, situated on the South-east coast of Sweden, is a part of the Precambrian bed-rock in SE Sweden where the Småland granites predominate the older, Sveocokarelian complexes. This is where the Swedish hard rock laboratory is under construction, at 460 m below the surface of the island Äspö (Gustafsson et al, 1988, 1989, 1991). Presently, we are also investigating groundwater sampled from the natural nuclear reactor analogue in Bangombé, Gabon, Africa, the high pH cement analogue Maqarin in Jordan and the uranium ore body in Palmottu, Finland. The labwork on buffer masses from a full scale heated waste canister experiment at Whiteshell laboratories, Pinnawa, Canada is also finished (Atomic Energy Canada Ltd; AECL). Summaries of the results obtained this far are presented in this and the following chapters. More detailed descriptions of materials, results etc. are documented in the following SKB Technical Reports and in the publications cited in the text:

- Pedersen, K. (**1987**) Preliminary investigations of deep groundwater microbiology in Swedish granite rocks. 22 p. SKB Technical Report **88-01**.

- Pedersen, K. (**1989**) Deep groundwater microbiology in Swedish granitic rock and its relevance for radionuclide migration from a Swedish high level nuclear waste repository. 13 p. SKB Technical Report **89-23**.

- Pedersen, K. (1990) Potential effects of bacteria on radionuclide transport from a Swedish high level nuclear waste repository. 55 p. SKB Technical Report **90-05**.

- Pedersen, K. Ekendahl, S. & Arlinger, J. (1991) Microbes in crystalline bedrock: Assimilation of CO_2 and introduced organic compounds by bacterial populations in groundwater from deep crystalline bedrock at Laxemar and Stripa. SKB Technical Report **91-56**.

- Ekendahl, S. Arlinger, J. Ståhl, F. & Pedersen K. (1993) Carbon transformations in deep granitic groundwater by attached bacterial populations characterised with 16S-rRNA gene sequencing technique and scanning electron microscopy. SKB Technical Report **93-22**.

4.3.1 Total numbers of bacteria in granitic groundwater

Unattached bacteria

We use the fluorochrome acridine orange for total numbers. Details about the staining technique can be found in Pedersen and Ekendahl (1992b). Figure 4.1 summarizes most of our data achieved this far on total numbers, including some analogue studies (4.8). To avoid individual investigator bias (Kepner et al, 1994), our collected data on total numbers, presented in the figure, have been counted by two "calibrated" persons, the second taking over from the first 2.5 years ago. The total numbers ranges from 10^3 up to 10^7 cells per ml groundwater. Pedersen (1993a) reviewed the deep subterranean biosphere and reported from 10^3 up to 10^8 bacteria per ml groundwater or gram sediment. Ghiorse and Wilson (1988) compiled total numbers of bacteria from many different, mostly shallow, pristine groundwater sites and reported a range from 10^3 up to 10^8 microbes per ml. Most of their values are groundwater from sedimentary environments or samples of sediments, sands etc. Van Es and Meyer-Reil (1982) have compiled 44 reports of total numbers of bacteria in marine aquatic environments over the world. They report a range between 10^4 up to 10^7 bacteria per ml of sea water. The ranges from groundwaters, sediments and marine waters coincide rather well.

Generally the total number of bacteria in natural systems can not be used to indicate bacterial activity. An ecosystem with a large number of bacteria may be inactive while an ecosystem with fewer bacteria can be very active but also under a predation pressure from protozoa (or bacteriophages) that continuously graze growing bacterial populations down to a low total number (Pedersen, 1982). Methods for the registration of activity must therefore be applied as we have done in several investigations, reviewed

below (4.3.4). In addition, as discussed in next paragraph, attached bacteria must also be included when assessing a microbial ecosystem and for that fraction of the subterranean microbes, appropriate techniques must be used, other than what is used for unattached bacteria.

We have observed differences in total numbers before and after start of pumping of a borehole. Usually, there is a decrease in the total numbers as the flow is started. Therefore, we always try to sample a borehole after allowing a couple of borehole volumes or more to flood. Data obtained and presented below show that this procedure gives results with low unexplained variability. Flushing of boreholes has also been shown to be of importance for the concentration of bacteria in boreholes drilled in granitic rock at the AECL underground research laboratory (URL), 100 km Northeast of Winnipeg, Canada (Stroes-Gascoyne et al, 1993).

The stability of our total number of bacteria for each borehole level has been assayed after different sampling times and amounts of water withdrawn and at different occasions. The borehole V2 in Stripa is a subvertical shaft with a diameter of 76 mm and it runs from one of the deepest drifts of the mine, 410 m, down to a depth of 1240 m. Three sampling depths of this artesian borehole were closed off with packers made of inflatable 76 mm rubber tubes and connected to the drift with 6 mm Teflon tubing. The three different levels of this borehole showed different chemistry (Pedersen and Ekendahl, 1992a) and different total numbers of bacteria. Tab. 4.1 shows the significant, 20 times higher number of bacteria at the lowest level (see also Figure 4.1) compared with the two upper levels and that the numbers did not change much over a period of five years. There is a striking analogy between a chemostate, producing a constant number of bacteria as a function of the nutrient status in the culture chamber, and the bed-rock aquifers crossing the borehole studied. The bacteria seem to grow to the maximal number allowed by the borehole environment and stay at that number and growth rate as long as the growth conditions are maintained. Sampling over a short period of time gives an even more stable picture. Figure 4.4 shows total numbers of bacteria in 4 different boreholes at the Bangombé site, Gabon, Africa. With one exception, the data are stable. The field working conditions are rather primitive in this area. Still, reliable data could be obtained, demonstrating that skilled personnel will probably get reliable data on total numbers of bacteria anywhere on earth. (Pedersen, 1987) report stability of total number data collected every 5:th minute for 30 minutes, similar to what is reported above. Recently, we have started an analysis series of the "aging" of new drilled boreholes at the 390-440 m level of the Äspö lab. We presently follow total and viable numbers, and diversity of bacteria in drilling waters and formation waters after drilling in eight boreholes. We will continue to follow these boreholes for years ahead.

Table 4.1 The total number of attached and unattached bacteria (ml^{-1}) in groundwater from three sampling depths of the Stripa borehole V2, 799-807 m, 812-820 m and 970-1240 m, measured at different occasions. (From Pedersen and Ekendahl, 1992a, Ekendahl and Pedersen, 1994).

Sampling date			899-807 m Bacteria[b] x 10^5		812-820 m Bacteria x 10^5		970-1240 m Bacteria x 10^5	
		N[a]		SD%		SD%		SD%
Groundwater sampling								
17 September	1987	1	0.097	...	0.061	...	2.3	...
18 April	1990	1	0.036	...	0.016	...	1.6	...
8 June	1990	1	0.240	...	0.047	...	2.3	...
1 October	1990	6	0.054	26	0.018	45	1.2	12
15 April	1991	3	0.225	33	0.129	59	3.4	21
29 May	1991	2	0.102	10	0.232	14	2.5	11
26 June	1991	2	0.143	20	0.222	49	1.4	79
Mean value (n=7)			0.128	-	0.104	-	2.1	-

Sampling date		Exposure time	N		SD %		SD %		SD%
Surfaces exposed to flowing groundwater									
1 October	1990	117 days	6	12	30	71	38	59	31
April	1990	56 days	3	10	6	72	20	22	42
June	1991	161 days	1	...	...	100	...	...	...
June	1991	90 days	1	11	...	81	...	90	...
Mean value (n=2-4)				11	-	81	-	57	-

a) N is the number of independent samples
b) populations are per ml or per cm^2

Attached bacteria

In all microbial ecosystems that harbour microbes in the water phase, there will also develop microbial biofilms (Characklis and Marshall, 1990, Characklis and Wilderer, 1989, Marshall, 1984). Even pure and disinfected drinking water shows this phenomenon (Pedersen, 1990, Schoenen and Schöler, 1985). Such a biofilm forming potential is proposed by Hazen et al (1991) for the South Carolina coastal plain sediments and is present in the deep subterranean environments of Stripa and Äspö (Figures 2.1, 4.2). Assuming a mean channel width of 0.1 mm (Moreno et al, 1985) the results from Stripa imply that there could be from 4000 up to 800 000 more attached than unattached bacteria in a channel after four months of contact with groundwaters flowing at 1 mm/sec (Pedersen and Ekendahl, 1992a). The bacterial enrichment to fracture surfaces may be considerably larger, as the surface available for attachment and growth in fractured rock often is wavy and rough with larger surface area than the flat surfaces used in the Stripa and Äspö experiments.

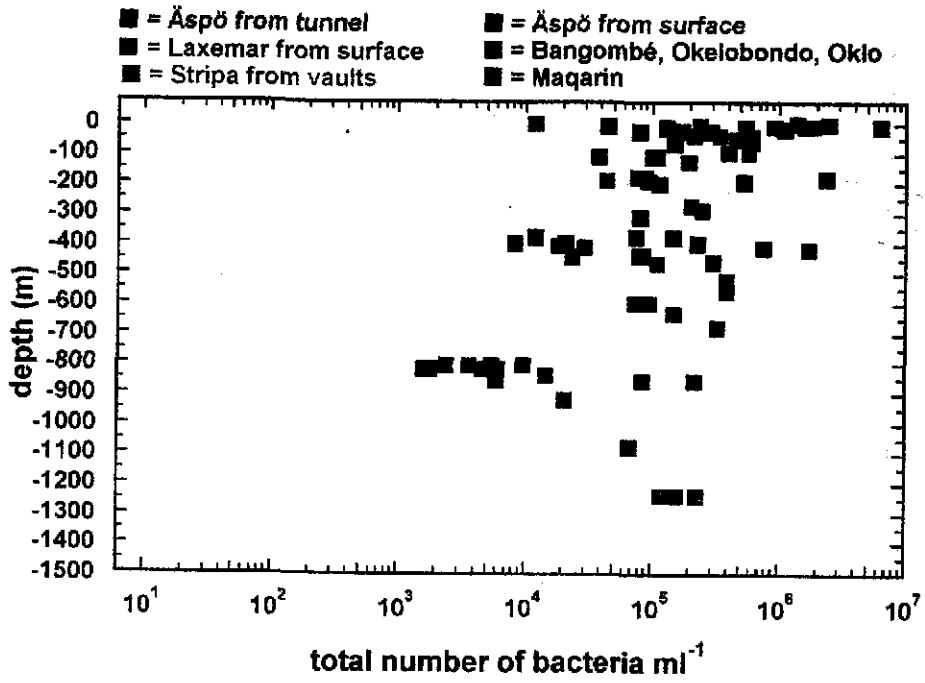

Figure 4.1 The total numbers of bacteria in groundwater systems down to 1240 m, determined with the acridine orange counting technique. Data have been collected over a period of 9 years from 30 boreholes, 49 different levels, and up to four repeated samplings have been performed during this time. Äspö surface data and background information can be found in Pedersen and Ekendahl (1990) Laxemardata: Pedersen and Ekendahl (1992a), Stripadata: Pedersen and Ekendahl (1992b), Pedersen (1987). The rest of the data are presently in preparation for publication.

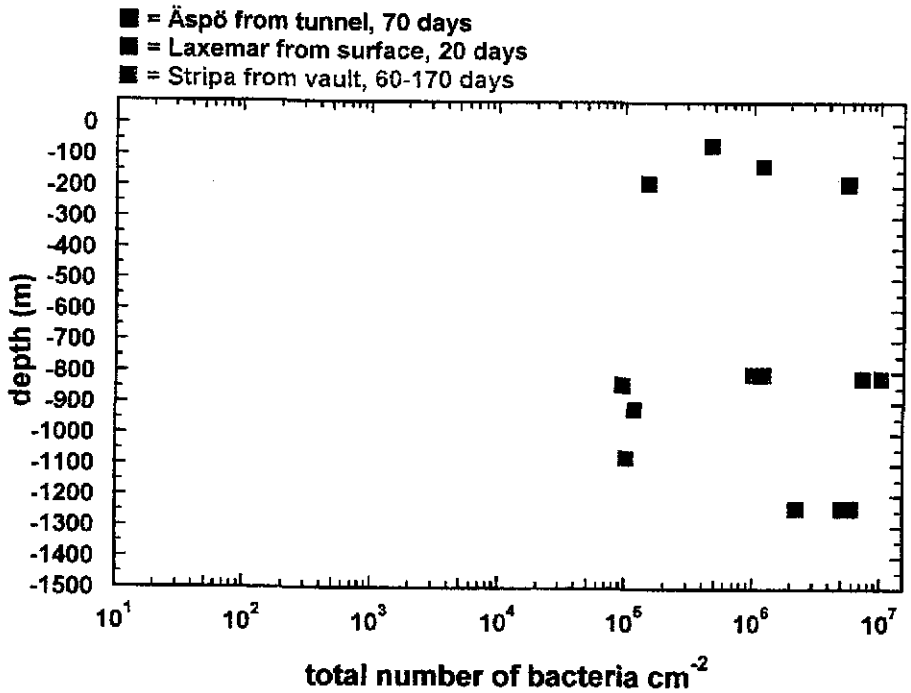

Figure 4.2 The total numbers of attached bacteria on surfaces exposed to slowly flowing groundwater. Laxemar data and background information can be found in Pedersen and Ekendahl (1992a), Stripa: Pedersen and Ekendahl (1992b).

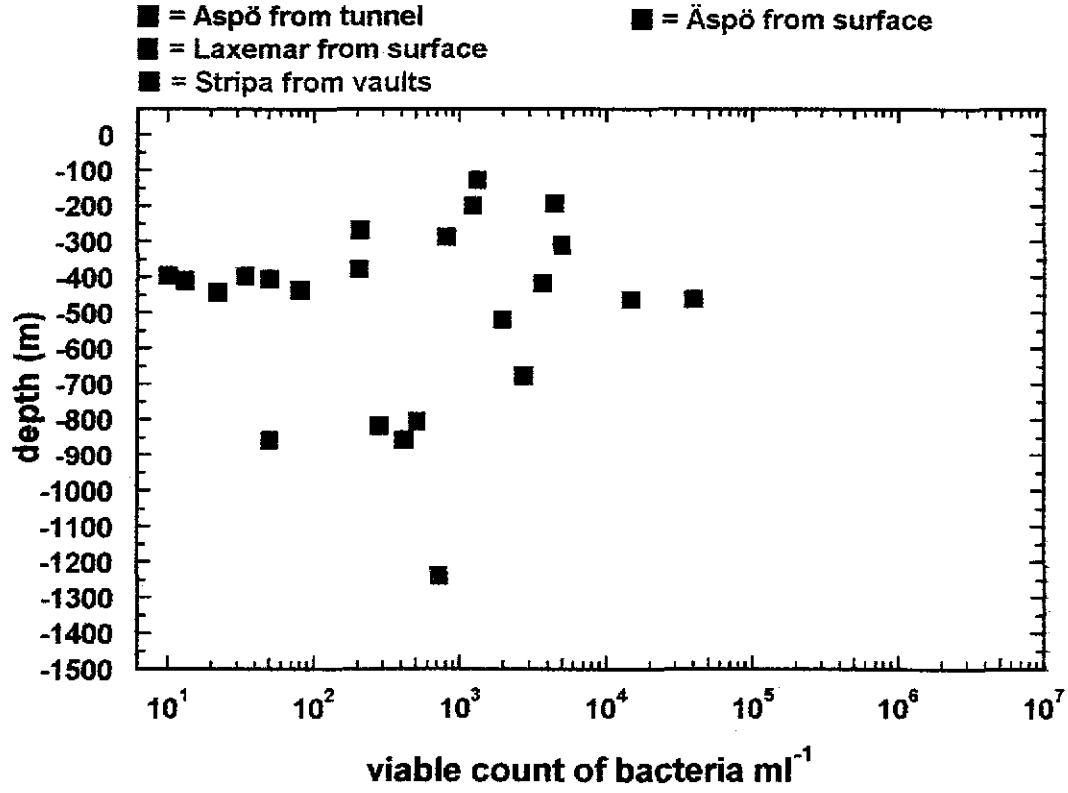

Figure 4.3 *The viable counts of bacteria in groundwater systems down to 1240 m, determined with plate count on a medium for heterotrophic aerobic and facultative aerobic bacteria. Data have been collected over a period of 9 years from 7 boreholes, 18 different levels. Äspö and Laxemar surface data and background information can be found in Pedersen and Ekendahl (1990), Stripadata: Pedersen (1987).*

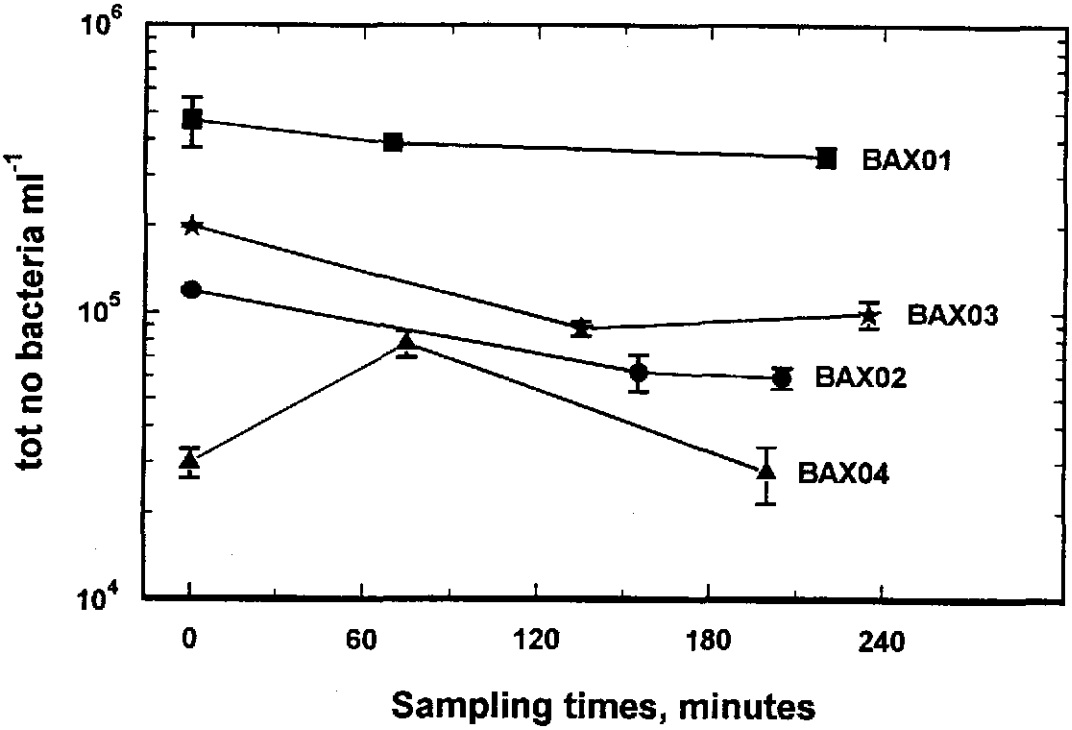

Figure 4.4 *The total number of bacteria in flowing (pumped) groundwater from the Bangombé boreholes in Gabon, Africa, sampled at three different times. BAX01, 96 m; BAX02, 27 m; BAX03, 12 m; BAX04, 9 m.*

The availability of energy and nutrients over time for a biofilm is transport dependant and will decide whether a biofilm will develop *in situ* and how many bacteria can be maintained. The slower the transport is, the slower the development rate of a biofilm down to when the attached bacteria becomes dependent on transport mediated only by diffusion and not by flow. Such diffusion transport is probably driven by very low concentration differences in an oligotrophic environment like deep groundwater, and selects for bacteria with advanced morphological and physiological mechanisms to survive a very limited availability of nutrients (Kjelleberg et al, 1987). However, the age of subterranean environments is millions up to some billion years, thus there is practically no time limit for even the slowest developing biofilm to reach a steady state. The average hydraulic conductivity, K, has been found to be 10^{-6} m sec^{-1} or less in the fractured rock of Stripa (Carlsson et al, 1983), but it will be considerably higher in individual channels (Carlsson et al, 1983). K is a function of the injection flow rate, the injection excess head, the length of the injection interval and the radius of the borehole (Andersson et al, 1989a). The flows used during the Stripa and Äspö experiments were probably even higher than in a channel with high conductivity; instead the experiment time was very short in relation to the time a channel will be open for flowing groundwater and bacteria.

There is not any positive correlation between the numbers of attached and unattached bacteria. This becomes very clear when comparing Stripa data in Table 4.1 and Figure 4.1, with Figure 4.2. Although there were 20 times more bacteria in the 970-1240 m than in the 812-820 m groundwater there were more attached bacteria on the surfaces exposed to the 812-820 m groundwater. Instead, a negative correlation can be anticipated, because the higher tendency a bacterial population has to attach, the less percentage of the population will be unattached. Therefore, a low total number of unattached bacteria is not a conclusive indication for a low total attached plus unattached number of bacteria and vice versa.

4.3.2 Viable counts of bacteria in granitic groundwater

The viable counts of bacteria on a medium called MB (see Pedersen and Ekendahl, 1990) are presented in Figure 4.3. The number of bacteria possible to culture on this medium ranged from 0.1% up to 10 % of the total number of bacteria. The result is typical and has invoked the question whether the non-cultivable bacteria are dead or non-viable. Comparing our data on activity with different numbers of bacteria shows that most bacteria found are viable, (i.e. they take up leucine) as can be seen in the example in Table 4.2. The results on activity are reviewed in section 4.3.4.

Table 4.2 A comparison between different numbers of bacteria in the Äspö borehole KAS03 from (Pedersen and Ekendahl, 1990). The total number of bacteria was determined with acridine orange staining, viable counts as colony forming units on MB-agar plates and the viable number was determined as the percentage of the total number of bacteria that assimilated leucine (81 %) using an autoradiography method.

Depth (m)	Total number of bacteria per ml	Cultivable bacteria per ml	Viable bacteria per ml	Viable but not cultivable bacteria per ml
860	223 000	420	181 100	180 680

The inability of heterotrophic media plate counts to reveal all viable bacteria has urged microbiologists to develop media adapted to different bacteria. In addition to the MB-medium, we have also used a counting technique for viable SRB. Figure 4.5 summarizes data obtained. The technique is very time consuming and was therefore used less frequently than other culturing methods. We find SRB at depth, down to 680 m, and also in the sea bottom sediments above the tunnel.

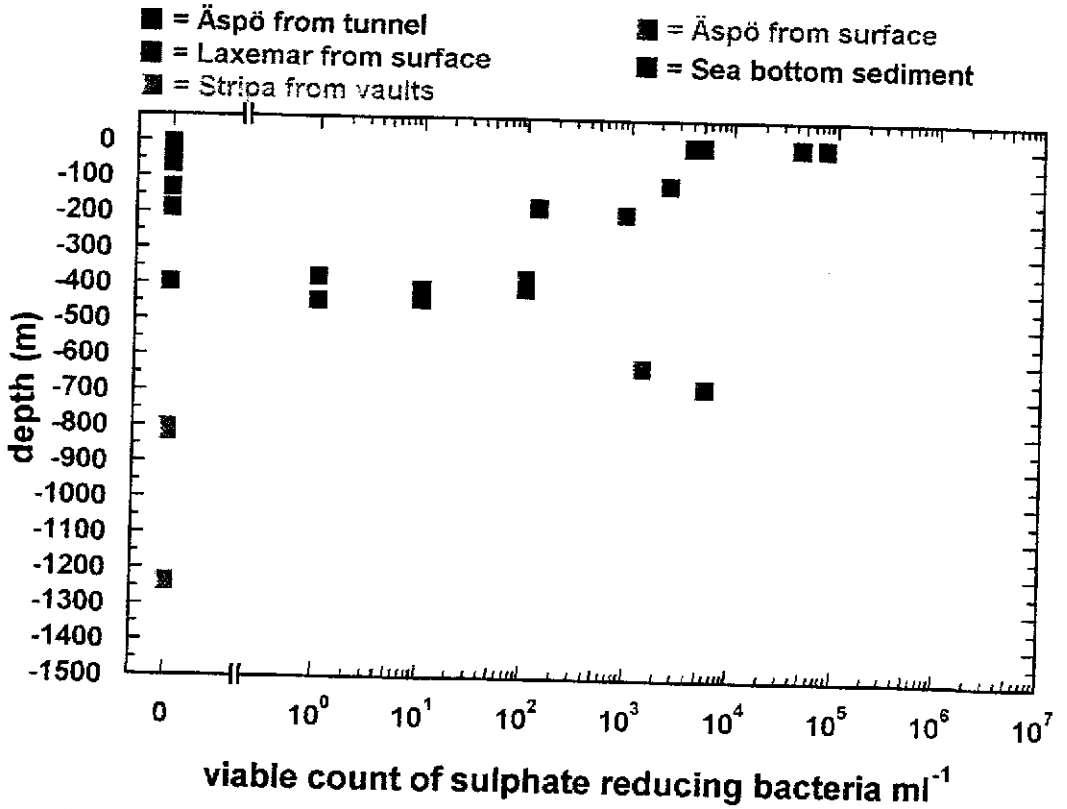

Figure 4.5 *Viable counts of sulphate reducing bacteria as determined with an agar roller tube technique. All cultures were done on sampled groundwater except for the sea bottom sediments which were done on dilution series of sediments from sediment cores.*

4.3.3 Diversity and distribution of bacteria in granitic groundwater

Diversity and distribution examined with traditional culturing techniques

We have used traditional culturing techniques with numeric taxonomy for the phenotypic characterisation and the 16S-rRNA technique for genotype characterisation to determine bacterial diversity. Table 4.3 shows bacterial groups that we have enriched for with different types of media. All groups of bacteria expected were also found. Some have subsequently been isolated, characterised and eventually identified according to a number of different techniques, including the sequencing of the 16S-rRNA gene. Facultative anaerobic, heterotrophic bacteria were identified from the boreholes KAS02-03 as being *Pseudomonas* and *Shewanella* species (Pedersen and Ekendahl, 1990). Later, identifications of heterotrophic facultative bacteria from the Äspö tunnel demonstrated that also *Serratia* and *Bacillus* species are present. Many other obtained isolates were not possible to identify and therefore may represent new species. We have concentrated our identification efforts on physiological groups of bacteria that are important for performance assessment. Iron reducing bacteria is one such group and SRB is another. The iron reducing bacterium *Shewanella putrefaciens* could repeatedly be enriched from the block scale redox zone and from surface boreholes as well (Pedersen and Ekendahl, 1990). SRB are also frequently occurring and more than 20 randomly chosen isolates turned out to be three different species, one known and described, *Desulfomicrobium baculatum* and two new species belonging to the genus *Desulfovibrio*. These two species are presently being characterised and will be published separately. The 16S-rRNA results have shown that additional SRB species are present in the Äspö environment, although we presently have not succeeded to isolate them.

Table 4.3 Different types of subterranean bacteria that could be enriched with various enrichment procedures from the investigated subterranean environments.

	Investigated environment		
Types of bacteria	**Stripa**	**Laxemar**	**Äspö**
Aerobes	no	yes	yes
Facultative anaerobes	no	yes	yes
Obligate anaerobes	no	yes	yes
Iron oxidizing bacteria	not investigated	not investigated	yes
Iron reducing bacteria	not investigated	not investigated	yes
Sulphate reducing bacteria	no	yes	yes
Sulphur reducing bacteria	not investigated	not investigated	yes
Methane producing bacteria	no	not investigated	yes

Diversity and distribution examined with DNA techniques

Phenotypic characterisation methods can not be applied on so called "viable but not cultivable" bacteria. Therefore, and for reasons discussed in section 4.2.2, genotype methods must be practised instead. For different reasons relating to the safe disposal of radioactive waste and performance assessment, we have investigated or are presently investigating the microbial diversity and distribution of five very different microbial ecosystems. The borehole V2 extending to 1240 m below ground in the Stripa research mine is published (Ekendahl and Pedersen, 1994a, Ekendahl et al, 1994) or (Ekendahl et al, 1993). The labwork comprising a total of 10 boreholes down to 600 m in the Äspö area is finished. Presently, we are investigating groundwater sampled from the natural nuclear reactor analogue in Bangombé, Gabon, Africa, the high pH cement analogue Maqarin in Jordan and the uranium ore body in Palmottu, Finland. The labwork on buffer masses from a full scale heated waste canister experiment at Whiteshell laboratories, Pinnawa, Canada is also finished (Atomic Energy Canada Ltd; AECL). Summaries of the results obtained this far are presented in this and the following chapters.

The Stripa mine

Our first attempt to describe the diversity and distribution of bacteria in a subterranean environment with molecular biology tools was done on surfaces exposed to slowly flowing groundwater from two levels of the borehole V2 in Stripa. Microscope observations of the attached bacteria in Stripa had revealed morphotypes that suggested one unparalleled dominating bacterium at each level (Pedersen et al, 1991). Although up to 74% of these attached bacteria could be shown to be active (Pedersen and Ekendahl, 1992a), metabolising lactate, anaerobic enrichment cultures for bacteria in the biofilms from the Stripa mine were not successful (Ekendahl et al, 1994). Therefore, extraction of DNA from the attached populations was performed, followed by PCR amplification of the 16S rRNA gene, cloning, sequencing and analysis of the population structure and identification of the present phylogenetic groups of bacteria. The 16S rRNA analysis confirmed the microscope observations. Three major groups of bacteria were found, called clone group I, II and III. They were not identical to anything in the Genebank and EMBL DNA-databases, but all belonged to the proteobacteria and were gram negative. *Pseudomonas* and *Acinetobacter* were among the species closest related to the clones found. The two levels investigated had very different distribution of the found clones as can be judged from Table 4.4. Clone group I could only be detected at the upper level while the two others appeared on both levels and the group II dominated at the lower level.

Table 4.4 Groups of bacterial clones screened from two levels of the Stripa borehole V2 and their distribution. DNA from bacteria attached to glass surfaces has been extracted, the 16S-rRNA genes amplified with PCR, cloned and sequenced using primer 907-926. Each of the groups I, II and III contains identical clones. Single observations were not identical. (From Ekendahl et al, 1994).

	812-820 m		970-1240 m	
Clone group	Number of clones	% of total	Number of clones	% of total
I	15 (6+9)[a]	63	0 (0+0)	0
II	2 (2+0)	8	20 (10+10)	83
III	4 (2+2)	17	1 (1+0)	4
Single observations	3 (2+1)	12	3 (1+2)	12

The Äspö hard rock environment

Next, we applied the 16S-rRNA technique on a much larger number of samples from Äspö, than was examined at Stripa. A total of 9 different boreholes and 4 surfaces exposed to flowing groundwater from boreholes in and above the Äspö tunnel was investigated. We now could investigate the microbial diversity and distribution between boreholes, and differences between the microbial populations colonising surfaces and the populations present in the flowing groundwaters. A total of 155 clones from 13 different samples distributed over 9 boreholes were sequenced between bases 520 and 900 (E.coli Brosius numbering, [Brosius et al, 1978]). Four samples were taken from surfaces exposed to flowing groundwater and 9 were filtered out from the groundwater. The results can be summarised and interpreted in relation to section 4.2.3 as follows.

There was a large overall diversity of bacterial species as demonstrated by the total of 58 different clones, i.e. species found in the groundwater and on the surfaces. In the different samples, the species diversity varied from 3 up to 10 different species as can be seen in table 4.5. The frequency column shows that on all surfaces, and in three of the groundwater samples, one single species constitutes 50% or more of the obtained sequences. That implies these boreholes to have been populated by one dominating species, just as was found in the Stripa borehole V2, while other boreholes show more differentiated populations without dominating species.

Comparing bacteria that colonised the surfaces with the bacterial populations in the groundwater shows a high similarity in clone composition in case, SA813 (Table 4.6). The other comparisons show different populations in the groundwater and on the surfaces, with only one clone appearing in both the water and on the surface in the HA1327 samples. This is one of the first molecular comparisons done between microbial populations on solid and

liquid phases in the same ecosystem. The surface populations are much less susceptible to sampling variance than water populations which obviously is inherent with the attached lifestyle of these microbes. The differences in populations registered in the other cases are probably due to that the surfaces select for other bacteria than those dominating the groundwater populations. The similarity in the surface populations of the boreholes HA1327 and 1420 supports this assumption. These boreholes are relatively close to each other (93 m) and intersect a major water transporting fracture zone at Äspö called NE1.

Most species found appear in only one or two boreholes with exception for the clone named A24otpmn that was found in 5 boreholes and represented 10% of the sequenced material. This species has a 16S-rRNA sequence that shows a high similarity (98%) with *Acinetobacter calcoaceticus* which is a frequently reported groundwater bacterium. The sequencing results show that there seems to exist a large spatial distribution of bacterial species between different boreholes. Subterranean microbial ecosystems are not homogeneously inhabited. Rather, local conditions determine which bacteria will dominate a specific environment. This fact makes predictions about microbial activities in the subterranean environment complicated.

Table 4.5 The number of different species detected in each examined borehole, in groundwater and on surfaces exposed to flowing groundwater for 70 days. The total number of randomly choosen clones, sequenced from each borehole was 12. The frequency with which each sequence appeared is also indicated.

Borehole	Depth(m)	Number of different species	Frequency of detected species
Groundwater			
HBH02	10	8	(4,2,1,1,1,1,1,1)
HBH01	40	4	(6,3,2,1)
KR0012	68	10	(3,1,1,1,1,1,1,1,1,1)
KR0013	68	7	(4,3,1,1,1,1,1)
KR0015	68	9	(2,2,1,1,1,1,1,1,1,-)
SA813	112	3	(6,4,2)
HA1327	179	3	(10,1,1)
SA1420	192	8	(4,2,1,1,1,1,1,1)
KAS03	626	7	(4,2,2,1,1,1,1)
Surfaces			
KR0013	68	4	(9,1,1,1)
SA813	112	3	(7,4,1)
HA1327	179	5	(6,2,2,1,1)
SA1420	192	4	(8,2,1,1)

Table 4.6 The numbers of identical sequences shared between surface biofilms and groundwater from 4 boreholes in the Äspö tunnel (- = no common sequences).

		Water (w)			Surfaces (s)			
	KR0013w	SA0813w	HA1327w	SA1420w	KR0013s	SA0813s	HA1327s	SA1420s
KR0013w	12	-	-	4	-	-	-	-
SA0813w		12	-	-	1	9	-	-
HA1327w			12	-	-	-	1	-
SA1420w				12	-	-	-	-
KR0013s					12	1	-	-
SA0813s						12	-	-
HA1327s							12	9
SA1420s								12

Many new and unknown species are indicated in the subterranean environment

Table 4.7 presents the identity of 135 unique 16S-rRNA sequences with known and sequenced bacterial 16S-rRNA. A total of 429 clones has been sequenced from 38 independent samples this far and of them, 135 were unique sequences. In the Stripa, Äspö and Bangombé environments few sequences, 25%, 18% and 30% respectively) were significantly related (>95 % identity) to know species and 33%, 54% and 36 % showed an identity of less than 90 %. This confirms the presence of large new groups of bacteria in the subterranean environments investigated and is an argument against the idea that bacteria found in granitic groundwater should be contaminants introduced during drilling. Instead, it shows that diverse populations of bacteria have adapted to a subterranean life for a long time. In comparison, data from our buffer mass investigations in Canada show that 52 % of the sequences had a high identity with known species (>95%) and only 14% of the sequences had less than 90% identity. Such a result should be expected in a system contaminated by man as was the case with the AECL buffer masses. Making phylogenetic trees from these data shows phylogenetic relationship (true evolutionary relationship) between found species and confirms these conclusions. Comparing figures 4.6 and 5.4 shows that the Äspö hard rock tree has large clusters of bacterial species only very distantly related to sequenced bacteria while the AECL buffer tree has most clones close to known species.

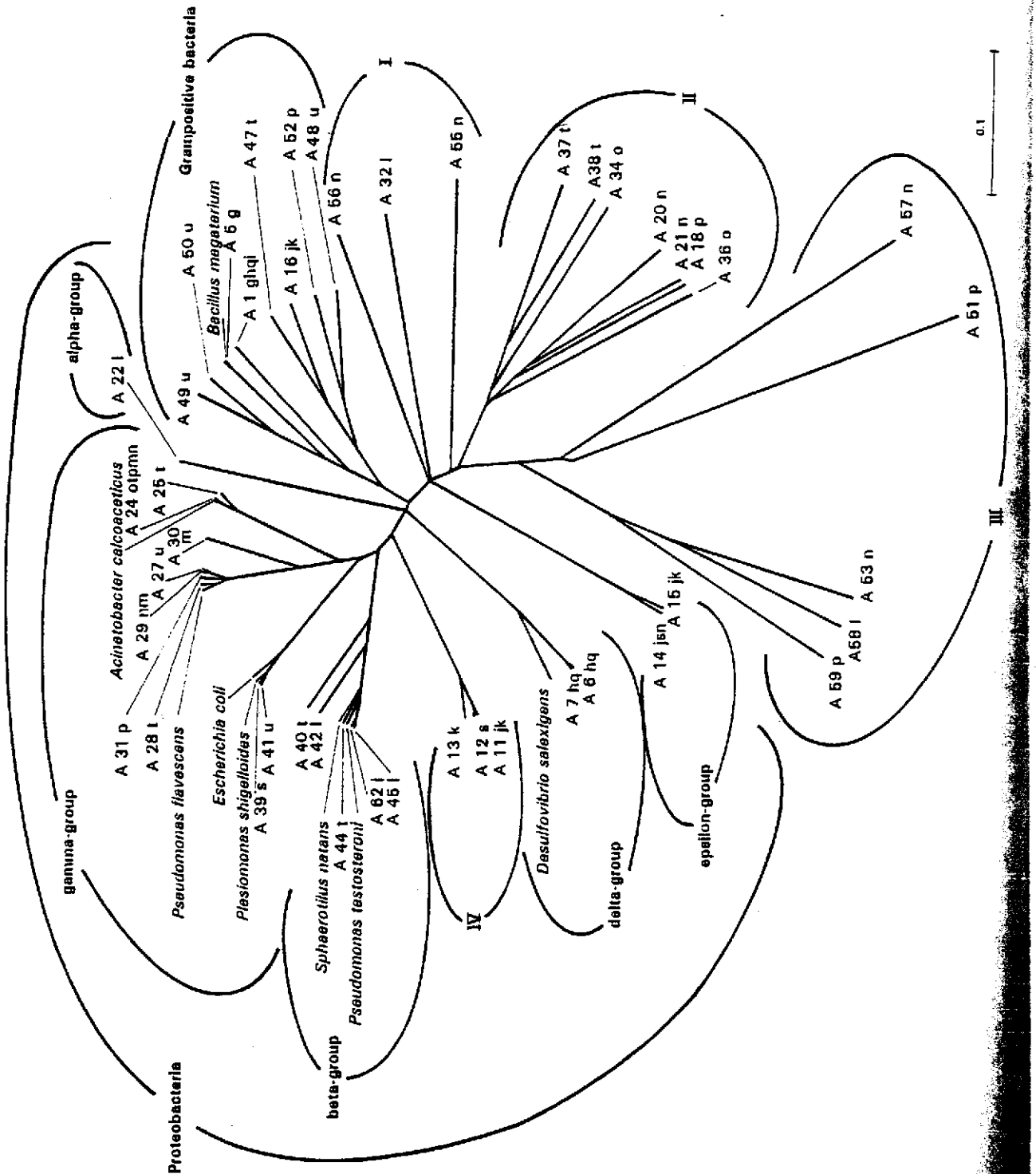

Figure 4.6 *Evolutionary distance tree based on the 16S rRNA gene sequences of clones from boreholes in the Äspö tunnel. Only sequences for species with an identity >95% with respective clones were added to the tree.*

Table 4.7 Comparing obtained unique 16S-rRNA sequences with sequences available in EMBL and Genbank DNA databases render closest identity of the unknown 16S-rRNA sequence with known sequenced bacteria. The table shows the identity distribution as percentage of the obtained sequences grouping within five different, arbitrarily chosen, classes of identity.

	% of the obtained sequences belonging to each identity class			
Class of identity with EMBL DNA-database	**Stripa**	**Äspö**	**Buffer masses**	**Bangombé**
100%	0%	0%	0%	0%
95-99.9%	25%	18%	53%	30%
90-94.9%	42%	26%	3%	34%
80-89.9%	25%	28%	14%	27%
70-79.9%	8%	26%	0%	9%
Sum of clones	12	58	21	44

4.3.4 Activity of bacteria in granitic groundwater

Carbon cycling

Radiolabelled CO_2 and several organic compounds were introduced to assay the activity of the observed unattached and attached Stripa and Äspö populations. The assimilation of $^{14}CO_2$, ^{14}C-formate, ^{3}H-acetate, ^{14}C-lactate, ^{14}C-glucose and ^{3}H-leucine were studied with microautoradiographic and liquid scintillation counting techniques (Pedersen and Ekendahl, 1992a, b). The results showed that the attached populations were in more metabolically active states than the unattached bacteria and that lactate was the preferred carbon source of the introduced compounds. A significant autotrophic activity and lactate respiration to CO_2 was also shown for the Stripa populations (Ekendahl and Pedersen, 1994b).

Comparing the amount of carbon dioxide assimilated during a certain time and the amount of carbon needed to build up a bacterium growing during that time, the capability for "autotrophy" can be evaluated. The quotients calculated for carbon utilization of the Stripa biofilms were between 0.07 and 0.25 (Ekendahl and Pedersen, 1994b), indicating that autotrophy could not support the levels of growth observed and that heterotrophy was the dominating carbon transformation process for growth of the studied populations. The CO_2 assimilation did not decrease consistently when lactate

was added, suggesting that there were no autotrophs changing to a heterotrophic metabolism (Hallbeck and Pedersen, 1991) when organic carbon appeared. The measured CO_2 assimilation may thus have been due to heterotrophs using CO_2 for anapleurotic reactions to replace intermediates in *i.e.* the tricarboxylic acid cycle. It is important to use several different carbon sources for assimilation experiments, as shown in previous Stripa V2 studies (Pedersen and Ekendahl, 1992a). One reason is that different carbon sources are utilised at varying degrees by the same species. The earlier observed utilisation of added lactate and also glucose, leucine and formate by some parts of the populations support that heterotrophs were present. The Stripa bacteria could further be seen not only to assimilate but also to degrade lactate and release CO_2, which adds to the indications of a heterotrophic dominance in the Stripa environment.

There were typically between 30 and 300 µmole/L of dissolved organic carbon (DOC) in the Stripa groundwaters studied and also methane. About 50 % of this DOC was composed of complex humic and fulvic acids (Pettersson et al, 1990) and a smaller part was organic substances with shorter carbon chains than glucose. The sources of this organic carbon is unexplored. It may be organic material percolating down from the ground surface, but as dating with ^{14}C analysis indicates groundwater ages of 5000 - 20,000 years (Nordström et al, 1985) it seems to be a less plausible explanation. The other possible source would be organic deposits in the rock or organic compounds migrating up from very deep layers of the earth (Gold, 1992). It will be an important task to investigate the sources and fluxes of organic carbon in the subterranean environment (confer Figure 3.10). The results presented here indicate that organic carbon was the main fuel for bacterial processes in the Stripa V2 borehole and the fluxes will then determine the process rates. The general applicability of this conclusion remains to be proven. Investigations of bacterial carbon transformations and the ecology of important groups of deep granitic groundwater bacteria are now continued in the environment around the Äspö Hard Rock Laboratory.

If most bacteria are attached, as indicated by our results (4.3.1), then a large pool of organic material in the subterranean environment will be attached in the form of bacteria. Organic material tends to sorb to surfaces and this will further increase the distribution of organics towards solid subterrestrial phases. Measurements of organic carbon in groundwater only, will therefore give a misleading picture of the nutrient situation in deep rock. During this year (1995) we will start to analyse the content of organic carbon on fracture surfaces, fillings etc. and on crushed rock in stainless steel canisters exposed for flowing deep groundwater for more than two years. These canisters were set up in February 1993 to mimic the conditions in fracture zones with crushed rock that intersect the Äspö tunnel.

Iron reducing bacteria

We have discovered two biogeochemical processes in granitic rock where bacteria seem to be of major importance. The first process was revealed during a block scale redox experiment at the Äspö hard rock laboratory (ÄHRL) (Banwart et al, 1994). The unexpected redox stability of the studied system could only be explained by the mobilization of solid phase ferric iron oxi-hydroxides to liquid phase ferrous iron by iron reducing bacteria (IRB) with organic carbon as electron donor. We have isolated more than 60 different bacteria from this habitat able to reduce ferric iron to ferrous iron, including one of the few known and described IRB from literature, *Shewanella putrefaciens*. Our 16S-rRNA gene results (see above) show that several of the dominating species sampled from this fracture zone have a 95% or more identity with known IRB like *Pseudomonas medosina*. The presence of IRB keeping the rock environment around a radioactive waste repository anoxic during the operational phase is good news because copper canisters are stable (no oxygen corrosion) in an anoxic environment and some important radionuclides are immobile at reduced conditions but mobile at oxic conditions. We presently work on the hypothesis that much of the ferrous iron in anoxic groundwater is a result of IRB and not due to pure inorganic redox-reactions as generally anticipated. The work on IRB constitutes a significant part of our performance assessment oriented research program.

Sulphate reducing bacteria

The second bacterial geochemical process discovered to be important is the reduction of sulphate and sulphur to sulphide by sulphate and sulphur reducing bacteria (SRB). They frequently appear in the ÄHRL environments at depths greater than approximately 100 m (see Figure 4.5). A "lack of sulphate" and an oversaturation of carbonate was indicated by geochemical and hydrological modelling, and stable isotope data (S^{34}) indicated ongoing biological sulphate reduction as the tunnel was excavated under the Baltic sea (Pedersen et al, 1995). Our sequencing results, enrichment and viable count results all showed significant amounts of SRB in the groundwater of this tunnel section and also deeper. However, these signs of ongoing sulphate reduction have more or less disappeared as the tunnel has aged with time. For instance, a borehole (SA813, 112 m) with a dominating SRB population in December 1992 and January 1993 had lost all SRB when it was sampled again January 1994 (viable counts and 16S-rRNA sequencing). Obviously, the construction of the tunnel with shallow groundwater draw-down etc. has disturbed the SRB-system. These results suggest that before tunnel construction, the Äspö hard rock environment was in a state with ongoing sulphate reduction and that this bacterial activity decreased when the tunnel was made. Recently, new results indicate that sulphate reducing bacteria

again are becoming active as a result of the in-mixing of Baltic seawater in the groundwater that reaches the tunnel. The results raise some very important questions and implications. If sulphate reduction is the long term natural situation in deep bedrock as indicated, the extent of this process must be modelled carefully. A very central performance assessment question concerns what regulates the SRB activity; why did they alter their activity after the tunnel was excavated to explain the decrease in activity? Is the decrease in salinity due to initial shallow groundwater intrusion enough or are there other regulating factors as well? SRB produce sulphide that may corrode the radioactive waste copper canisters and is therefore of great concern for to explain the decrease in activity. We aim at understanding how such a choice must be made. We presently develop *in situ* hybridisation techniques for rapid detection of SRB to be used as one of the molecular tools to reach this aim.

Hydrogen and methane related bacteria

Most deep groundwaters contain measurable contents of methane and hydrogen. The methane usually has a biogenic stable isotope signature indicating bacterial methane production, but there are also nonbiogenic sources (Sherwood Lollar et al, 1993a, b). Therefore, we are now working with enrichments, identification and activity of anaerobic bacteria in the ÄHRL environment including methanogenic bacteria. Hydrogen is an energy source used by many different bacteria including methanogens. We presently speculate that geothermally produced hydrogen and sedimentary organic material may act as energy sources for subterranean ecosystems (confer Figure 3.10). This is a major task for our program because if it is true, subterranean ecosystems would be much more independent of terranean ecosystems than anticipated today. With access to fracture systems, 400 m below ground at ÄHRL, it should be possible to inject water with known composition through a known fracture system and collect what is coming out downstream the fracture. Any continuous output of hydrogen or organic carbon indicates a continuous supply of these components to the injected water. If so, it should be possible to study rates and activities of bacterial populations postulated to utilize hydrogen or organic carbon as energy sources.

4.4 MICROBIAL LIFE IN DEEP THERMAL GROUNDWATERS

Bacterial life has been found existing in all locations on earth that can supply the bacteria with energy and that have temperatures below the maximum of which bacteria can adapt. Many subterranean environments fulfil the simple requirements for bacterial life and it can therefore be argued that bacteria probably inhabited such environments far before boreholes and tunnels were made. A population of bacteria, slowly migrating vertically at a rate of a

decimetre to a meter a year in present physical and chemical gradients and with the groundwater flow and tidal movements, would need 1,000 to 10,000 years to reach a depth of 1,000 m. This is rapid in relation to the geological age of many million up to some billion years of deep rocks and sediments.

What is then the depth limit for subterranean life? Probably, the limit is set by temperature and water activity, if there is energy available for microbial life. In some extreme surface environments, halophilic bacteria thriving in alkaline, neutral or acidic salt brines (20-30 %) can be observed (Grant and Larsen, 1989). Therefore, increasing salinity would not be any principal obstacle to bacteria. Instead, temperature probably is what will hold back the penetration of bacteria into large depths of the earth's crust. The upper presently known temperature limit for thermophilic bacteria is 110 °C (Staley et al, 1989). This temperature then defines the approximate depth limit for subterranean life - different in different geological formations. If subterranean life extends down to depths at 110 °C, it should be possible to enrich thermophilic bacteria from deep hot groundwater - which also has been done.

A borehole was drilled in granitic rock approximately 100 km north of the Stripa mine at Gravberg, Sweden, to a maximum depth of 6779 m. Fermenting, thermophilic bacteria could be enriched and isolated from 3900-4000 m (Szewzyk et al. personal communication). The morphological and physiological characteristics of some isolates suggested a relationship to the genus *Thermoanaerobium* that has been isolated from hot springs and similar environments. They also enriched for sulphate reducing bacteria, with negative result. (Olson et al, 1981) reported the presence of thermophilic sulphate reducing and methanogenic bacteria in groundwater from the dolomitic limestone Madison formation, which underlies a large portion of the USA northern Great plain. Attempts to detect aerobic and other anaerobic bacteria were unsuccessful. In a bacteriological study of geothermal spring waters of the Paris basin, Daumas et al (1985) found a predominance of anaerobes over aerobes and thermophiles over mesophiles, sulphate reducing bacteria, methanogens and heterotrophic bacteria could be enriched. The main microbial activity seemed to proceed via chemolithotrophic metabolism. Assimilation of CO_2 was detected, and there was a measurable assimilation and respiration of glucose. (Denman et al, 1991) were able to isolate many *Thermus aquaticus* strains from flowing Australian artesian boreholes, which extends the known ecological habitat of this group of organisms.

The SFL 2 repository will have a temperature selecting for thermophilic bacteria for a long time. The adaptation of many archaea and some eubacteria to high temperatures is well known from hot water wells, mud

pots, deep sea trenches etc. In fact, our common ancestor is believed to have been a thermophile. The short review presented above demonstrates the presence of thermophiles also in very deep environments. The adaptation of thermophiles also to SFL 2 should therefore be expected. Their influence on performance assessment remain to be investigated; a good natural analogue may be difficult to find.

4.5 MICROBIAL LIFE IN THE DEEP SEDIMENTARY ENVIRONMENTS AND CLAYS

There are only a few sites where the subterranean microbiology has been studied in multi-disciplinary programs including chemistry and geology. The two most extensively published sites are the sediments of the Atlantic coastal plain of South Carolina, USA, studied in a subsurface program, initiated and sponsored by the U.S. Department of Energy (DOE) and crystalline bed-rock in Sweden studied in programs concerning the safety of future underground repositories for nuclear waste. The U.S.-program has recently been extended to the Hanford reservation in Washington (Brockman et al, 1992b) the Nevada test site (Amy et al, 1992b) and the Idaho National Engineering Laboratory in Idaho (Fredrickson, 1992). This program is almost exclusively studying sedimentary subterranean environments. The bentonite and the sand-bentonite backfill material will have minerals and hydraulic properties resembling such sediments. The most important difference is probably the way these environments are made, naturally over geological time periods and man made over a period of decades. Still, if bacteria survive and proliferate in natural sedimentary environments, they can be expected to do the same in man-made sediments.

The Atlantic coastal plain of South Carolina consists of a series of fluvial, deltaic, and marine sediments deposited since the late Cretaceous period 70 - 80 million years ago. These sediments were deposited on the eroded surface of a crystalline basement complex consisting of metamorphic gneisses and schists with intrusive end extrusive igneous rocks (Sargent and Fliermans, 1989). Within the DOE program, a number of boreholes were drilled in these sediments. Most of the coastal plain studies have been done with samples from the recharge area at the Savannah River Plant on the upper plain where flow rates are high and the groundwater is relatively young and aerobic. More recently, work has been published on the lower plain where there are low flow rates and anaerobic old groundwater (e.g. Chapelle and Lovley, 1990, Murphy et al, 1992). Core and water samples have been collected aseptically under nitrogen atmosphere from different depths with designed core samplers, shipped on ice to different laboratories in USA within 16 h and stored at 4°C until they were examined (never more than 72 h) (Phelps et al, 1989a, Russell et al, 1992).

4.5.1 Total numbers and viable counts of bacteria in deep sedimentary environments

Samples of groundwater and the enclosing sediments were compared for densities of bacteria using acridine orange direct staining (AODC) and viable count methodology (Hazen et al, 1991). Bacterial densities in sediment down to depths of 550 m ranged from less than 10^6 bacteria/g dry weight (gdw) up to 5.01×10^8 bacteria/gdw for total counts while viable counts were less than 10^3 colony forming units (CFU)/gdw up to 4.1×10^7 CFU/gdw. Bacterial densities in groundwater were less than 10^3 - 6.3×10^4 bacteria/ml and 5.8 - 460 CFU/ml for total and viable counts, respectively. The authors conclude that oligotrophic aquifer sediments have unique and dense bacterial communities that are attached and not reflected in groundwater found in the strata. This observation is in agreement for what was found for the Äspö biofilms (see Table 4.6). (Chapelle et al, 1987) sampled sediments of Maryland, USA, down to 182 m and observed numbers of bacteria that agreed with the South Carolina plain sediments as well as sulphate reducing bacteria and methanogenic bacteria.

Sinclair and Ghiorse (1987) reported bacterial numbers in the same order as above and in addition they, surprisingly, observed protozoa, algae and fungi. Protozoa and fungi have earlier been reported from the uppermost soil and groundwater layers, 0-10 m, (Beloin et al, 1988, Hirsch et al, 1992) but not from deep undisturbed environments. The algae were observed in two of three Savannah River Plant boreholes drilled and sampled with the same technique (Phelps et al, 1989a) which possibly excludes contamination during drilling. It can be argued that the sediment aquifers of the Savannah River Plant with algae recently must have been in connection with surface environments. As the Savannah River Plant site has been penetrated by many boreholes before these were drilled, cross-contamination from old boreholes is the most probable explanation. Lithotrophic H_2-oxidizers, microaerophilic N_2-fixating bacteria, nitrifiers, sulphur-oxidizers, sulphate reducing bacteria and methanogenic bacteria have also been found (Francis et al, 1989, Fredrickson et al, 1989, Jones et al, 1989).

4.5.2 Diversity of bacteria in deep sedimentary environments

Balkwill et al (1989), Balkwill (1989) and Fredrickson et al (1991) investigated the physiological diversity of many isolates from the Savannah River Plant sediments. Initially, three boreholes were studied, P24, P28 and P29. (Balkwill, 1989) found a total of 576 different morphologies at depths from 0 m to 265 m. Fredrickson et al (1991) isolated a total of 198 different morphologies at depths from 365 m to 467 m. There was also a physiological diversity, (Balkwill et al, 1989) found 626 distinct physiological types at depths from 0 m to 265 m. (Fredrickson et al, 1991) used 108 distinct physiological measurements that revealed 21 different biotypes. Hazen et al (1991) found that isolates from sediment cores assimilated a wider variety of

carbon compounds than groundwater bacteria. Sedimentary subterranean environments seem to be as diverse with respect to bacteria as the granitic hard rock.

4.5.3 Activity of bacteria in deep sedimentary environments

South Carolina coastal plain samples have been brought to different laboratories for investigations of microbial activities. The upper plain subsurface sands exhibited three to four orders of magnitude greater activity and cultivable microorganisms than clay zones that had low permeability (Phelps et al, 1989b). This is in agreement with the results of Hicks and Fredrickson (1989) who found mineralization of acetate, phenol or 4-methoxybenzoate to be negatively correlated with the clay content. Other factors that appeared to influence were the pH and the heterotrophic abundance as measured by plate counts. In addition, Jones et al (1989) report small or negligible anaerobic activity associated with thick clay layers. Denitrifying microorganisms where present in all samples below the water table (Francis et al, 1989).

Although all samples were collected aseptically under nitrogen atmosphere, they usually contained oxygen. These results show that the deep sediments of the upper coastal plain are mostly aerobic. The plain has been considered laterally, exploring the age, the evolution and the flow paths of the groundwater. Murphy et al (1992) studied how microorganisms affect the Middendorf aquifer groundwaters as it flow from the recharge area to the major discharge, the Savannah river. The upper well, denoted P29, was modern with respect to ^{14}C but contained no tritium, suggesting the groundwater age to be greater than 50 years but less than 500 years. This was one of the wells where Sinclair and Ghiorse (1989) found algae - their suggestion that the well relatively recently must have been in connection with surface environments appears correct. Murphy et al (1992) speculate that the observed fermentation of organic matter, anaerobic iron reduction and sulphate reduction probably occurs in the many anaerobic zones associated with lignite in the otherwise aerobic groundwater system of heterogeneous Middendorf sediments. The microbial diversity and activity decrease as the groundwater reaches the lower plain, where the groundwater age approaches 11,500 years. The presence of bacteria may affect the groundwater environment in many different ways and it can be speculated that they execute significant control on the groundwater conditions. For instance, Chapelle and Lovley (1992) proposed a model for how iron reducing bacteria increase the Fe^{2+} content of many groundwaters by respiring organic carbon with Fe^{3+}. Such reduction probably competitively excludes sulphate reduction and may be a very important process in the development of anaerobic reduced subterranean environments (Lovley, 1991).

The next chapter (5) reports some microbiological results from a full scale heated canister experiment at URL, AECL, Canada. It confirms that bacteria can survive in the bentonite-sand mixture around the canister, also at high temperatures, as indicated by investigations of natural sedimentary subterranean environments.

4.6 BACTERIA IN OPEN ROCK GALLERIES

Excavation for tunnels, mining etc. introduces several changes in the subterranean environment that will induce activities in the tunnel by bacteria other than present in the fractured rock. These resulting bacterial populations are not always obvious for the layman, but as there are implications for performance assessment, a brief background is given here, with some illustrative examples.

4.6.1 The gradients between reduced and oxygenated environments

Oxygen is normally introduced in tunnels by ventilation which makes growth of aerobic bacteria possible. The groundwater at depth usually is anoxic with a low redox potential, and marked redox and oxygen gradients develop when such groundwater reaches the oxygenated tunnel atmosphere. Typical redoxpairs participating in these gradients are manganese(II) oxidizing to manganese(IV), ferrous iron to ferric iron and sulphide to sulphate. Such gradients are the habitats for many different lithotrophic and also heterotrophic bacteria. Among them are the iron (3.3.4), manganese (3.4.2) and sulphur (3.5.4) oxidizing bacteria that generate chemical energy for anabolic reactions through the oxidation of reduced inorganic compounds with oxygen. The energy gained is used to reduce carbon from CO_2 to organic carbon and this is the first step in an environmental succession that eventually ends as a reduced environment again.

4.6.2 Observations of bacterial growth in open rock galleries

Commonly, seeps of groundwater from fractures intersected by tunnels or flows of groundwater from boreholes in the Äspötunnel (Figure 4.7) turn light brown to dark brown of precipitates (Figure 4.8) that sometimes can be very voluminous. They usually appear within some weeks after excavation/drilling and may in some cases reach a thickness of 10 cm or more (Figure 4.9). A frequently observed inhabitant in these precipitates is the lithotrophic iron-oxidizing bacterium *Gallionella ferruginea* (Hallbeck and Pedersen, 1990a, 1991, Hallbeck et al, 1993). It forms moss-like covers on rocks and sediments in ponds in tunnels (Figure 4.12) and is very abundant close to the outflow of groundwater from rock wall fractures.

Close to the outflows, white, threadlike precipitates can be observed (Figure 4.10, 4.11). These brown and white precipitates seem lifeless at a first look in the microscope although twisted stalks (Figure 4.13) and light refractile bodies (Figure 4.15 and 4.18) usually can be observed. The picture changes dramatically if a nucleic acid specific fluorochrome like acridine orange is added to the samples. What looked dead is in fact full of life, the green fluorescent particles are bacteria that was obscured by the stalks and precipitates (Figure 4.14, 4.16). The precipitates described above have also been observed in the Swedish SFR repository in Forsmark and in the uranium mine of Oklo, Gabon in Africa and many other places as reviewed by Ghiorse and Ehrlich (1992).

Figure 4.7 The Äspö access tunnel from 205 m and downwards. The height and width are 5.5 m x 5.5 m respectively and the inclination is 7 %. The large tube under the tunnel roof is the ventilation. The picture was taken the 17 of August 1993.

Figure 4.8 A water transporting fracture with a slimy "curtain" of iron oxidizing bacteria. The light brown colour indicates a young precipitate with an active oxidizing population. As such precipitates ages, it turns dark brown and brittle. The precipitates will be dark brown to black if manganese oxidation is ongoing. The picture is from the Äspö tunnel, the A side, length 1993 m. And was taken the 6 of June 1993.

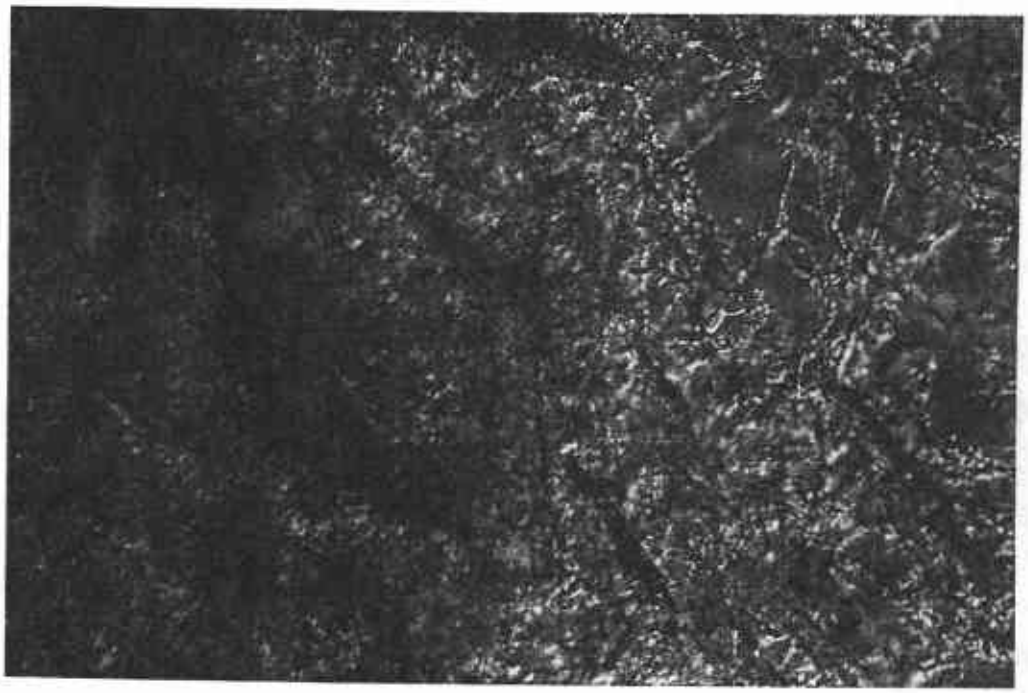

Figure 4.13 *An interference light microscopy image of a wet mount of the precipitates from Figure 4.12. A net-work of* Gallionella ferruginea *stalks with precipitated iron can be seen. Scale: 1,5 cm = 25 μm.*

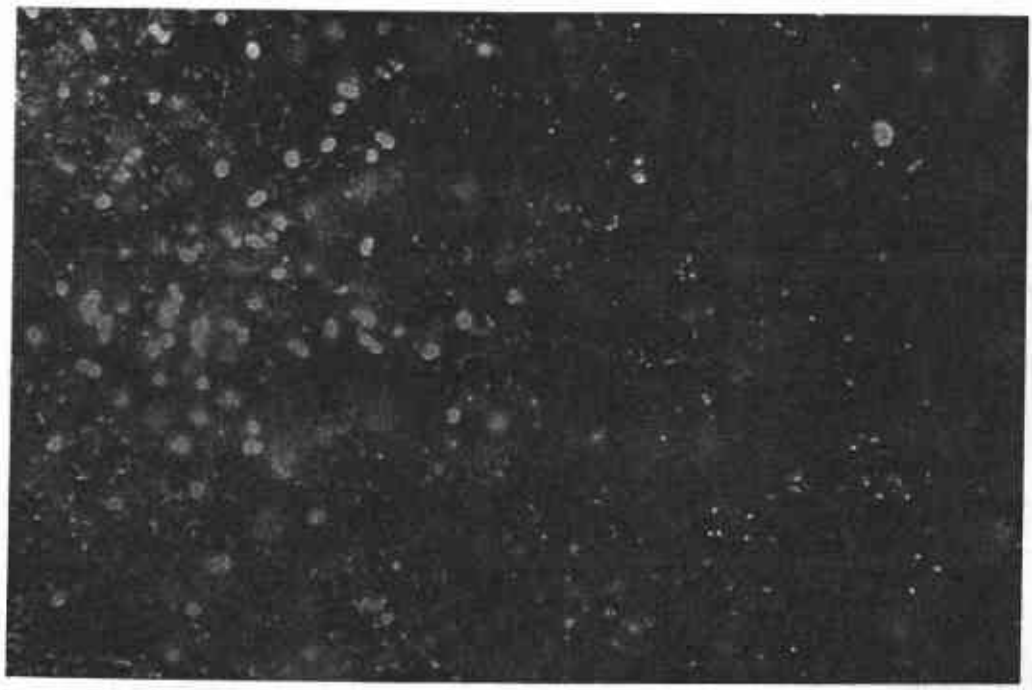

Figure 4.14 *An epifluorescence microscope image of exactly the same microscopic wet mount as shown in Figure 4.13 stained with acridine orange. Cells that were obscured by the stalks and precipitates can now clearly be seen as green bodies with different volumes. Scale: 1,5 cm = 25 μm.*

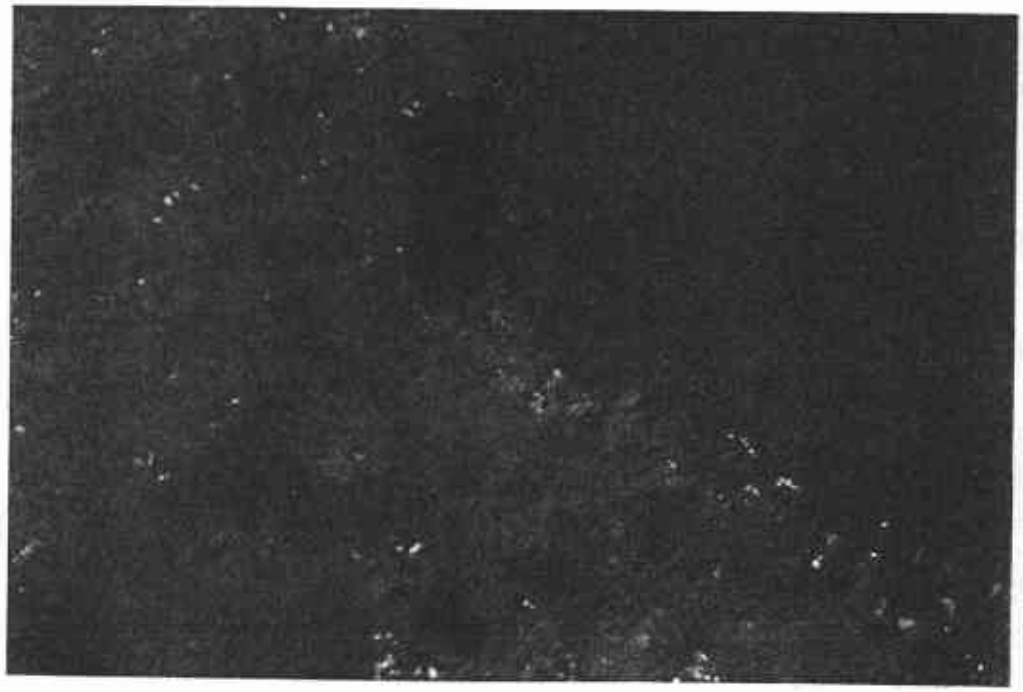

Figure 4.15 *An interference light microscopy image of a wet mount of the white treads in Figure 4.11. A large amount of refractile bodies, presumably elementar sulphur can be seen, mixed with a matrix of unknown kind. Scale: 1,5 cm = 25 µm.*

Figure 4.16 *An epifluorescence microscope image of exactly the same microscopic wet mount as shown in Figure 4.15 stained with acridine orange. Cells that were obscured can now clearly be seen as green bodies of different sizes. Note cells that contrast well against the dark background of the black particle. Scale: 1,5 cm = 25 µm.*

Figure 4.17 *Outflow of anoxic groundwater that is rich in ferrous iron and sulphide at Äspö tunnel length 1130 m, B side. Lithotrophic iron oxidising bacteria and sulphide oxidising bacteria thrive in this water. The sulphide oxidising bacteria is shown in Figure 4.18. It has the ability to slowy move and will therefore migrate up on the top of iron precipitates. the picture was taken 28 of April 1995.*

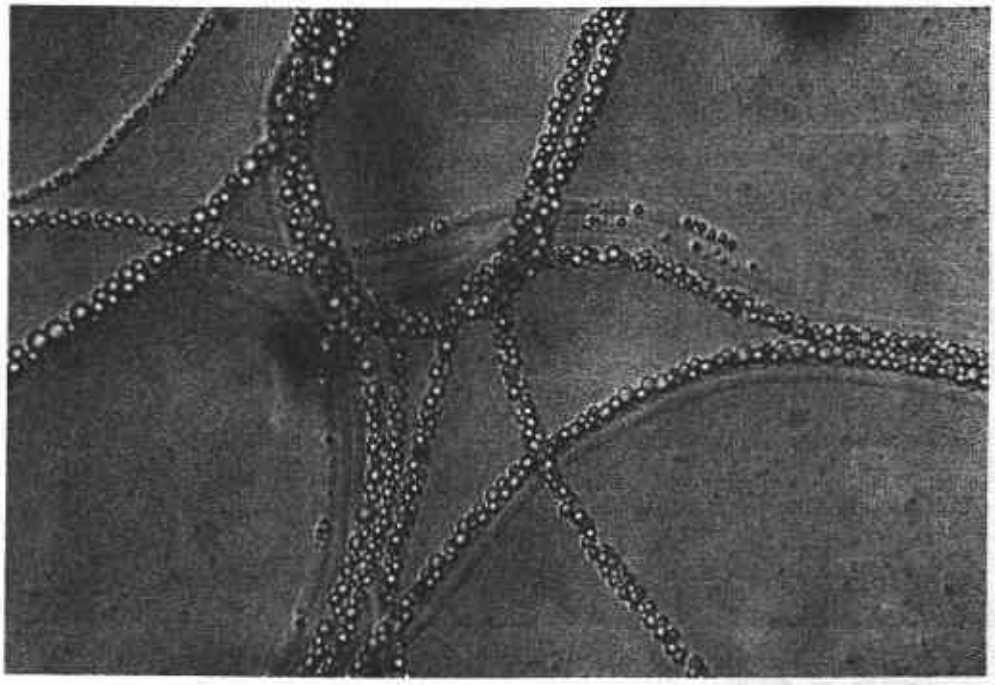

Figure 4.18 *An interference light microscopy image of a wet mount ot the sulphide oxidising bacterium growing as white threads in Figure 4.17. Intracellular deposits of elementar sulphur are clearly visible and are what render the bacterial colonies in Figure 4.17 its white colour. Size: 1.5 cm = 10 μm.*

Figure 4.19 *A pond with reducing conditions some cm below the surface, as indicated by black iron sulphide precipitates. In the redox cline between anoxic and oxic conditions, white brittle clouds can be seen. Figure 4.20 reveal a dense growth of a sulphide oxidising bacterium that deposit the resulting sulphur as extracellular sulpur threadlike needles, giving the white colour to the clouds. The pond was situated in a side vault at the B side of the Äspö tunnel at a length 1130 m. The picture was taken 28 of April 1995.*

Figure 4.20 *An epifluorescence microscopy image of a wet mount ot the sulphide oxidising bacterium (green fluorescence colour) growing as white clouds in Figure 4.19. Extracellular threadlike deposits of elementar sulphur are clearly visible and are what render the bacterial colonies in Figure 4.19 its white colour. Size: 1.5 cm = 10 µm.*

4.6.3 Succession of populations after the opening of a shaft

The precipitates in discrete parts of a tunnel system represent different steps in a succession of bacterial populations. Changes in water flow due to changed draw-down conditions and clogging of fractures by precipitates often reduces the flow as the vault gets older. This will create a series of conditions that will favour a succession of different bacteria colonising the rock walls and ponds.

1. The first step comprises lithotrophic oxidation of reduced inorganic compounds as energy sources by bacterial populations that fix carbon dioxide to organic carbon. This step will build up a matrix full of inorganic precipitates and organic material.

2. As the precipitate ages and grow thicker, anaerobic conditions may develop due to oxygen diffusion limitations and as a function of reduced groundwater flow.

3. The aerobic and microaerophilic parts of the bacterial populations will eventually die and start to be degraded by anaerobic bacteria.

4. Anaerobic heterotrophs can now grow using the organic material produced by the lithotrophs. If sulphate reducing bacteria colonise the precipitates, hydrogen sulphide will be produced that will give a black colour and a smell of hydrogen sulphide to the bottom of precipitates, ponds etc (Figure 4.19).

4.6.4 Implications for performance assessment

The growth of lithotrophic autotrophs results in a build-up of organic carbon and finally in sulphide production. Organic compounds may complex and migrate radionuclides and sulphide is a potential corrosion threat for the copper canisters. Therefore, the amount of organic carbon in a repository environment should be kept as low as possible. Removal of precipitates before backfilling will contribute to this requirement.

4.7 INVESTIGATIONS OF THE MICROBIOLOGY OF NATURAL ANALOGUES

The term "analogue" is usually loosely defined, referring to both natural and man-made materials. Natural analogues have been defined as: the occurrence, in the natural environment, of materials and processes that are

the same, or related to, those which are predicted to occur in some parts of the repository, the surrounding rock formation and the biosphere' (IAEA 1987, (Brandberg et al, 1993). The essential feature is that they are phenomena which are not controlled by man and, as such, include archaeological evidence. Good overviews and reviews about natural analogue studies in the geological disposal of radioactive wastes can be found in Miller et al (1994) and Brandberg et al (1993).

Long term predictions of the effect from microbial activity on repository performance assessment are as difficult as most other predictions of the behaviour of the natural environment. Therefore, natural analogues have been investigated for presence of bacteria and signs of bacterial activity over geological times. Important questions on which clues to answers may be obtained from natural analogues, can be:

- **Redox control**. Bacterial heterotrophic activity usually force the redox of a system towards more negative voltages and is a bacterial process of major interest as copper (canister) is more stable and the mobility of some important radionuclides decrease considerably at low enough redox potential.

- **Gas generation.** Hydrogen, methane, nitrogen and carbon dioxide are gases commonly produced by bacteria, in volumes that can be significant in systems rich in degradable organic matter.

- **Corrosion** of man-made or naturally occurring metals in repository like environments, i.e. anaerobic corrosion of copper by sulphide produced by SRB.

- Production of **complexing agents** by microbes may influence radionuclide migration and the search for such agents can be made at analogue sites.

- **Migration** of radionuclides by unattached and motile bacteria can be studied at analogue sites with naturally occurring radionuclides.

- **Recombination of radiolysis products** can in theory, be mediated by bacteria. This has not yet been studied *in situ*.

Understanding the microbial part of biogeochemical processes influencing radionuclide behaviour requires information not only about activities but also about the diversity and distribution of present bacteria. Traditional studies of the diversity of microbial analogue communities have been incomplete

because of the inability to identify and quantify all contributing populations. New methods in molecular biology are however rapidly setting a better scene as described in section 4.3.3. Today we study the information available in the ribosomal 16S-rRNA gene to map diversity and distribution of subterranean bacterial populations in the analogues of Gabon, Maqarin and Palmottu.

Below follows a review on available information about bacteria in some important analogues. Summaries on recent data collected by the Swedish research team on subterranean microbiology and performance assessment tasks have also been included.

4.7.1 Cigar Lake uranium ore, Canada

Important questions about geobacterial processes in a deep high level nuclear waste repository that can be addressed for this analogue are:

- What are the bacterial processes, if any, dominate in the Cigar Lake analogue?
- What influences do these bacterial processes have on the geochemical situation in the Cigar Lake analogue?
- Which bacterial processes in the Cigar Lake analogue relate to performance assessment?

Bacteria may influence the stability of UO_2

Processes that govern the stability of uranium(IV) and the reduction of soluble, oxidised forms of uranium(VI) to insoluble uranium(IV) are of great importance for the long term stability of the UO_2 matrix under disposal conditions. The preservation of vast amounts of UO_2 and $USiO_4$ in the Cigar Lake deposit for ~ 1 Ga, despite potential effects of radiolysis, was judged to necessitate a detailed study of the mineralogy, geochemistry and the surface composition of the uraninite phases (Cramer, 1994).

In addition to inorganic redox reactions, microbial processes may influence the uraninite stability in several ways. It can be bacterial reduction of uranyl and bacterial recombination of radiolysis products. Indirectly, bacteria may also control the redox situation by redox reactions between organic material and sulphur and iron compounds.

Direct microscopy, viable counts, biomass determinations, and most probable number (MPN) analyses have confirmed the presence of bacteria in the ore zone of the Cigar Lake deposit, holes # 79, 198 and 220, and in all other

samples analysed. They include aerobes and anaerobes like sulphate reducers and methanogens. (table 3.54 in Stroes-Gascoyne et al (1994a) and table 3 in Francis et al (1994)). The total numbers of bacteria in borehole number 220 ranged between 5.1×10^2 up to 5.4×10^7 bacteria per ml. Overall, the range of bacterial numbers ranged from 5.1×10^2 up to 8.8×10^7 bacteria per ml, depending on sample site and method used. Results from analysis of organic material indicate significant amounts of colloids, >450 nm, in boreholes 67, 122, 220 and surface lake waters, i.e. the size of most bacteria (Cramer, 1994). Consequently, the potential effect from bacteria must be assessed.

Bacterial reduction of uranium(VI)

The results from the microbial investigations demonstrate the presence of sulphate reducing bacteria in groundwater from the ore and also in surrounding groundwaters (Francis et al, 1994, Marques et al, 1990). Such bacteria have been reported to be able to reduce uranium(VI) (Lovley and Phillips, 1992, Lovley et al, 1991). If uranium(IV) was oxidised by radiolysis products in the ore, bacterial reduction of uranium(VI) during oxidation of organic matter or hydrogen would be possible. Since there is no mineralogical evidence for recent groundwater alteration of uraninite in samples from drill cores 220 and FH-18, this process may not be significant here (Janeczek and Ewing, 1994). Bacterial recombination of radiolysis products, as described in sections 2.6.3 - 2.6.5, may in fact reduce the potential effects of radiolysis, thereby reducing the oxidative effect from such compounds.

Bacterial recombination of radiolysis products

In the study of the Cigar Lake natural analogue, analysis of the effect from radiolysis phenomenon forms part of the main study objectives. The analogue offers the possibility to study the effect of low level radiation at very long time periods and at ambient temperatures. Assuming radiolysis of water at the ore/clay contact, oxidants and hydrogen will be generated. Hydrogen is not very reactive at ambient ***inorganic*** conditions but is utilised by bacteria at threshold dissolved H_2 levels around 1 nM (table 2.8). Oxidants are expected to react in the geochemical environment in and around the ore. Including bacteria, the recombination of oxygen with hydrogen, or the oxidation of ferrous iron and reduced sulphur compounds with oxygen (and nitrate), as well as the oxidation of organic material with oxygen to carbon dioxide and water is plausible.

Reactive radiolysis products may very efficiently be converted to oxygen by aerobic and facultative, anaerobic bacteria. The hydrogen produced during radiolysis together with the oxygen produced from reactive oxygen species can subsequently be recombined by bacteria under the formation of organic

material, heat and water. Energy from radioactive decay may in fact be partly conserved as organic material in the form of bacteria. This is an alternative or complementary model for the fate of radiolysis products. Results from investigations of the Cigar Lake ore have shown that the bacteria are present and that there are also measurable amounts of organic material ranging between 0.9 up to 7 mg TOC per litre. The presence of this bacterial process will of course be good news for safety assessments since it will contribute to the stability of uranium(IV).

The observed ferric iron halo around the Cigar Lake ore may have an additional explanation to inorganic processes if iron-oxidizing bacteria are present. As microbiological data indicate iron-related bacteria to be present in most sampled locations (Stroes-Gascoyne et al, 1994a), iron-oxidiser may very well be active. They would then catalyse the oxidation of ferrous species from outside of the ore with oxygen from radiolysis inside the ore. Such activity would be present in the gradient between reduced and oxidised conditions as a diffuse zone of ferric precipitates rather than a sharp redox front.

4.7.2 Oklo, Okelobondo and Bangombé, Gabon

The Oklo groundwater has about the same total numbers of bacteria as all other groundwater sampled by us; from some 100 million up to 10 billion bacteria per litre of groundwater (Figure 4.1 and 4.4). The DNA sequencing technique has been applied on samples from Bangombé, from the boreholes BAX01, 02, 03 and 04, and analysis is presently ongoing. Sampling wall growth from the Okelobondo uranium mine demonstrated a diverse microbial flora in all samples. Many relate to iron- and sulphur-oxidizing bacteria. Among them, the well known iron oxidising bacterium, *Gallionella ferruginea*, was very frequent.

The main task for this study is to characterise dominant bacteria and investigate if their activity has influenced the Oklo groundwater and sediments and thereby the radionuclide distribution and speciation. The achieved knowledge may be used to complete present models of radionuclide behaviour in such environments.

4.7.3 Maqarin

The highly alkaline springs of the Maqarin area of NW Jordan are currently investigated as part of an international project testing models used in the performance assessment of low to intermediate radioactive waste repositories. The Maqarin area provides a rock-groundwater system which is an ideal natural analogue of a cementitious repository in a sedimentary host rock (Alexander et al, 1992).

The microbiology of this analogue has been studied by (Coombs et al, 1994), and recently the Swedish research team on subterranean microbiology and performance assessment tasks has performed total numbers and DNA analysis of this site.

Coombs et al (1994) report diverse populations of alkaline tolerant microbes at the M1 - M6 sites sampled at Maqarin during this investigation. pH values up to 12.9 have been measured on these sites. SRB were concluded to be of more geochemical significance than the aerobes. A code for modelling purposes was applied on the results and the outcome is discussed in chapter 6.

Recently, samples from the alkaline sites M5, M8 and from the neutral sites M17 and M18 were analysed for total numbers and 16S-rRNA gene diversity. The M5, M17 and M18 all had high total numbers of bacteria, above 10^6 bacteria per ml, while M8 was lower, 10^4 bacteria per ml (Figure 4.17). Sampling M17 and M18 over time for total numbers of bacteria showed that the numbers decreased at start and then stabilized. The sampling for DNA analysis was done at the end of the sampling intervals. Analysis is presently ongoing.

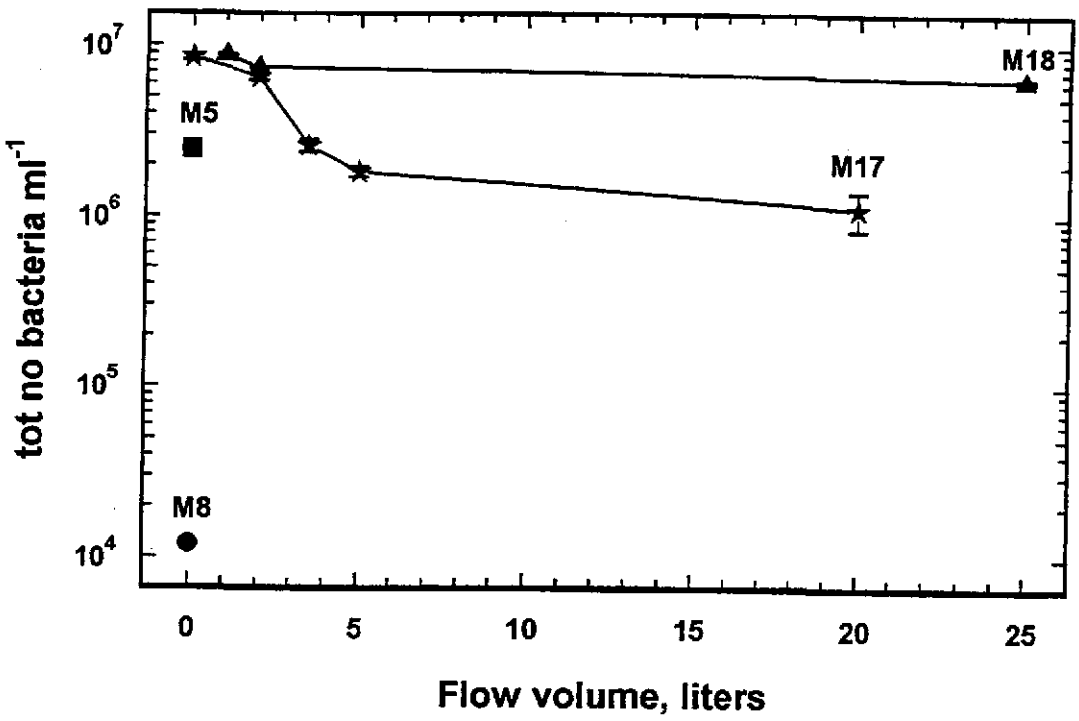

Figure 4.21 *The total number of bacteria in flowing groundwater from the Maqarin site in Jordan. M5: hyperalkaline seeps through basalt-chert-limestone colluvium at Western Springs, pH 12.9; M8: hyperalkaline seeps through organic-rich clay biomicritic limestone, pH 12.5; M17: a 16 m deep borehole above western Springs discharge, pH 7.2; M18: probably a mixture of hyperalkaline groundwater and neutral groundwater, pH 8.2.*

4.7.4 Poços de Caldas, Brazil

Part of the Poços de Caldas project has involved characterising microbial populations with traditional culturing techniques and epifluorescence microscopy counting (West et al, 1992). Microbial populations were found in rock and groundwater samples to the maximum depth sampled. The influence of populations found on the various processes was discussed. It was concluded that there was little evidence of trace element mobilisation by organic by-products and that the main role of microbes in this system seems to be catalysis of specific redox reactions.

4.7.5 Palmottu, Finland

We have so far only performed a pilot microbiology investigation of the Palmottu analogue, during a first expedition that took place in March 1995.

Hydrogeochemical investigations have shown that dissolved uranium concentrations correlate with changes in redox which in turn reflects

variations in depth. There were indications that the Fe^{2+}/Fe^{3+} redox couple and pyrite may play important roles for redox control. Our results from Äspö hard rock laboratory demonstrate that iron reducing bacteria obviously are very important for in the control of groundwater redox. It is also known that sulphate reducing bacteria may reduce U(VI) to U(IV) using the uranium as an electron acceptor. At oxidizing conditions, some iron and sulphide oxidizing bacteria produce organic carbon from carbon dioxide using pyrite as an energy source. Influence from microbial activity on the hydrogeochmical situation in Palmottu and in a future repository can therefore be expected.

Goals of the coming microbiology study at Palmottu.

The microbial study is one task among others at the Palmottu analogue project. The investigations of microbes will be done in close co-operation with other members of this project. The main task for this study is to characterise dominant bacteria and investigate if their activity has influenced the Palmottu groundwater and rock formations and thereby the radionuclide distribution and speciation. The achieved knowledge may be used to complete present models of radionuclide behaviour in such environments. The following sub-tasks have been identified:

- We will apply the molecular 16S-rRNA technique for the investigation of dominating bacterial groups present and distribution of species. We make enrichments and/or viable counts, MPN etc. Of indicated groups of bacteria, such as iron reducing and iron oxidising bacteria, sulphate and sulphur reducers, methane bacteria and aerobic and facultative anaerobic heterotrophs.

- The distribution of bacteria can be studied with confocal laser fluorescent microscopy using species and group specific fluorescent nucleic acid probes. The results will be compared with the radionuclide distribution and speciation in corresponding samples.

- Having species information from molecular and culturing analysis and distribution from the confocal laser microscopy we now can enter the last step, the study of microbial activities that may have influenced the radionuclide behaviour in the source term. This part of the study will be concentrated on groups of bacteria, indicated to dominate in the Palmottu environment.

4.8 SUMMARY OF MICROBIAL INVESTIGATIONS OF THE SUBTERRANEAN BIOSPHERE

Some 50 years ago it was generally anticipated that microbes in the sea only could survive in the uppermost meters. Now we know that the microbial ecosystems of the seas extend down into the deepest sea. Approximately 10 years ago, it was generally proposed that microbes only could thrive in the uppermost meters of the ground. It now seems as we have to update our knowledge about the subsurface in the same way as for the sea. This chapter presents an array of independent reports suggesting that microbial life is widespread at depth in the crust of earth - the deep subterranean biosphere. The obvious conclusion is that microbes may be involved in many subterranean geochemical processes, such as diagenesis, weathering, precipitation, and in oxidation/reduction reactions of metals, carbon, nitrogen and sulphur - just as they are in most terranean environments.

- **Total numbers of bacteria**. The total numbers of bacteria in subterranean hard rock environments ranges from 10^3 up to 10^7 cells/ ml groundwater. Different reviews report from 10^3 up to 10^8 bacteria/groundwater or gram sediment. Most reviews concern mostly shallow, pristine groundwater sites but they also report a range from 10^3 up to 10^8 microbes per ml. A compilation of the total numbers of bacteria in marine aquatic environments over the world reports a range between 10^4 up to 10^7 bacteria per ml of sea water. The ranges from groundwaters, sediments and marine waters coincide rather well.

- **The relation between attached and unattached bacteria in crystalline bed-rock.** There is no positive correlation between the numbers of attached and unattached bacteria. Instead, a negative correlation can be anticipated, because the higher the tendency of a bacterial population to attach, the lesser percentage of the population will be unattached. Therefore, a low total number of unattached bacteria is not a conclusive indication for a low total attached plus unattached number of bacteria and vice versa. Comparing bacteria that colonised the surfaces with the bacterial populations in the groundwater shows a high similarity in clone composition in case, the other comparisons show very different populations in the groundwater and on the surfaces. The differences in populations registered are probably due to influence of the surfaces which select for bacteria other than those dominating the groundwater populations.

- **Diversity of bacteria in crystalline bed-rock.** The presence of large new groups of bacteria in the subterranean environments has been demonstrated with molecular biology tools. More than 99% of the species sequences found are new and not earlier described. The result

is an argument against the idea that bacteria found in granitic groundwater should be contaminants introduced during drilling. Instead, it shows that diverse populations of bacteria have adapted to a subterranean life for a long time.

- **Distribution of bacteria in crystalline bed-rock.** Most species found seems locally unique as they appear in only one or two boreholes. The sequencing results confirm that there exists a large spatial distribution of bacterial species between different boreholes exists. Subterranean microbial ecosystems are not homogeneously distributed. Rather, local conditions determine which bacteria will dominate a specific environment. This fact makes predictions about microbial activities in the subterranean environment complicated.

- **Activity of bacteria in crystalline bed-rock.** We have discovered two biogeochemical processes in granitic rock where bacteria seem to be of major importance. The first process is the mobilization of solid phase ferric iron oxi-hydroxides to liquid phase ferrous iron by iron reducing bacteria (IRB) with organic carbon as electron donor. The presence of IRB keeping the rock environment around a radioactive waste repository anoxic during the operational phase is good news because some important radionuclides are immobile at reduced conditions but mobile at oxic conditionsand anoxic conditions are favourable for the stability of the canisters made of copper. The second bacterial geochemical process discovered to be important is the reduction of sulphate and sulphur to sulphide by sulphate and sulphur reducing bacteria (SRB). They frequently appear in the ÄHRL environments at depths greater than approximately 100 m (see Figure 4.5). Most deep groundwaters contain measurable contents of methane and hydrogen. The methane usually has a biogenic stable isotope signature indicating bacterial methane production, but there are also nonbiogenic sources. Hydrogen is an energy source used by many different bacteria including methanogens. We presently speculate that geothermally produced hydrogen and sedimentary organic material may act as energy sources for subterranean ecosystems (confer Figure 3.10). This is a major task for our program because if it is true, subterranean ecosystems would be much more independent of terranean ecosystems than anticipated today.

- **Microbial life in thermal environments.** The SFL 2 repository will have a temperature selecting for thermophilic bacteria for a long time. The adaptation of many archaea and some eubacteria to high temperatures is well known from hot water wells, mud pots, deep sea threnches etc. In fact, our common ancestor is believed to have been a thermophile. The short review presented above demonstrates the presence of thermophiles also in very deep environments. The adaptation of thermophiles also to SFL 2 should therefore be expected.

Their influence on performance assessment remain to be investigated, a good natural analogue may be difficult to find.

- **Microbial life in sedimentary environments.** The bentonite and the sand-bentonite backfill material will have a structure resembling natural sediments. The most important difference is probably the way these environments are made, naturally over geological time periods and man made over a period of decades. Still, bacteria survive and proliferate in natural sedimentary environments and they can be expected to do the same in man-made sediments. A full scale heated canister experiment at URL, AECL, Canada has now confirmed that bacteria can survive in the bentonite-sand mixture around the canister, also at high temperatures (section 5.3).

- **Bacteria in open rock galleries.** The growth of lithotrophic autotrophs result in a build-up of organic carbon and finally in sulphide production. Organic compounds may complex and migrate radionuclides and sulphide is a potential corrosion threat for the copper canisters. Therefore, the amount of organic carbon in a repository environment should be kept as low as possible. Removal of precipitates before backfilling will contribute to this requirement.

5 THE MICROBIOLOGY OF RADIOACTIVE WASTE DISPOSAL - THE REPOSITORY ENVIRONMENTS

Chapter 4 in this report was devoted to the so called "far-field" environment. The far-field usually is defined as the surrounding rock formations not altered by man and all investigations reported in the preceding chapter were done on the undisturbed environments in which repositories will be placed. Some data on, mostly undisturbed, natural analogues were also reported. Undisturbed in this case is of course relative, as drilling, excavation etc. have introduced some disturbance. These effects are however small relative to the disturbance from the excavation of the tunnel and the shafts, the drained and ventilated period of operation (40-50 years), the new materials such as metals (copper, steel), bentonite, concrete and bitumen (LLW) and the waste itself (heat, radiation, radiolysis etc.). However, an understanding of the far-field environment is a first step towards the understanding and assessment of the changes taking place in the near-field. Investigations of the undisturbed far-field is also an important step in the investigations of a potential site for waste disposal which, among other things, is necessary to describe the geochemical system of importance.

Most of the literature on subjects concerning radioactive waste disposal has been published as reports of various national or international agencies dealing with the disposal of radioactive waste, and such reports are not always easily accessible. A group of microbiologists working on different topics concerning the microbiology of radioactive waste disposal therefore initiated a multi-authored review. The goal was to exchange information with a larger audience than that having the reports available. This review was published in Experientia, volumes 46 (1990) and 47 (1991). A brief summary of this review is given in section 5.1.

In June 1991, A. Rosevear published a report review on national research programmes on the microbiology of radioactive waste (Rosevear, 1991) and West (1994) recently compiled literature on the progress in geomicrobiology of nuclear waste disposal. These reviews are also commented below.

There has been a lot of good work done on the microbiology in the past, with the methods available at the time. There are, however, very convincing reasons to continue these studies. The main reason is that our knowledge about microbial ecology continues to increase and is very much strengthened by the use of new powerful tools in molecular biology. Research in new

underground labs provide an understanding about subterranean microbiology not available some years ago. Another strong reason not to stop studying microbiology of radioactive waste disposal is that the technical arrangements of repositories change as the concepts and performance assessment conclusions are updated. New situations to be evaluated in the light of microbiology continue to appear. One good example could be the use of glass as a containing material, thought to be resistant to microbes. Recently, several papers now describe how bacteria alter natural basaltic glass (Thorseth et al, 1992a, 1995). It may not be important for high-level vitrified waste with its intense field of radiation, but worth considering if there are other applications of glass as a containing material.

The Swedish research team on subterranean microbiology has included near-field tasks in their program. A major concern for the engineered barrier in SFL 2 is the potential for corrosion of the copper canisters. We joined an international microbiology task force with Canadian, French and Swedish representation that analysed the bacterial situation of the buffer during the decommissioning of a full scale heated waste canister experiment at URL, Whiteshell Laboratory (AECL) in Canada. The full report will appear in fall 1995. Here, a summary of the findings are given, with important conclusions for performance assessment.

5.1 MICROORGANISMS IN NUCLEAR WASTE DISPOSAL, A MULTI-AUTHOR REVIEW APPEARING IN EXPERIENTIA 1990/91

The review is structured in 5 blocks and some of the block topics coincide with the chapters in this report. The main conclusions for each paper and author in the Experientia review are given below.

5.1.1 Introduction, problems and principles of waste repositories. Types of repositories and of waste.

In an introductory paper McCabe (1990) concludes that microorganisms can exist in any proposed environment, if the basic requirements for life are satisfied (see chapter 2). At non-favourable conditions, many microorganisms can survive in a dormant state until some change allows them to be active again. The possibility of local pockets with microenvironments suitable for growth is discussed. Microbial activity in such pockets may lead to changes in the near field followed by microbially induced degradation and breakdown of barriers, gas generation and/or uptake and transport of radionuclides.

Next, basic aspects of microbial effects on the disposal of low and intermediate level waste in Switzerland is presented by Knecht et al (1990). A presentation of the low and intermediate disposal concept is given and potentially important microbial effects on the long term performance of the system are added. They comprise microbial degradation of barrier materials and organics, the effect of microorganisms on sorption and their role as catalysts. The Swedish repository for low and intermediate waste is described by Roffey (1990) and some of the possible problems caused by microbial activity during storage are discussed. Microbial degradation of bitumen accompanied by gas production has been assessed in connection of research programs in this area. The major gas produced by the microbes would be carbon dioxide which may, at least locally, enhance steel corrosion which generate hydrogen gas. The author reaches the general conclusion that a safety assessment for the repository should take into account the various activities of microbes. Finally, Francis (1990a) gives a characterisation of nuclear and fossil wastes, their content of toxic metals and organics. Some of the organic compounds may support indigenous microbial activity resulting in solubilisation or stabilisation of toxic metals and radionuclides.

The Swedish waste disposal concepts are presented in chapter 1. Environmental limitations and microbial processes are presented in chapters 2 and 3 respectively.

5.1.2 Microbiology of the subsurface

This section of the Experientia review is opened by Kaiser and Bollag (1990) presenting microbial activity in terrestrial sedimentary subsurface environments of North America. The major part of the paper discusses how xenobiotic substances can be degraded by subsurface soil or groundwater bacteria. The starvation and penetration of bacteria in soils and sedimentary rocks are overviewed (Lappin-Scott and Costerton, 1990). The ability of bacteria to survive with reduced size at low nutrient concentrations is judged to increase opportunities for bacteria to reach deep waste disposal sites. Next, Morita (1990) reviews the starvation-survival state of microrganisms and concludes that the starvation state of microorganisms in ecosystems is real and should be considered as the normal state of most microorganisms in nature. The energetic of bacterial adhesion is described by Loosdrecht and Zehnder (1990), followed by a discussion about the effect of adhesion on survival and growth of microorganisms (Bar-Or, 1990).

Investigations of subterranean bacteria, with emphasis on Swedish hard rock conditions, are presented in chapter 4.

5.1.3 Metabolism of microbes and consequences for a repository

The microbial production of and influence from complexing agents is discussed in section 5.7. Birch and Bachofen (1990) extensively reviewed complexing agents from microorganisms in this block of the multiauthored review. They express complexes as species formed by the association of two more simpler species, each capable of independent existence. It is evident from their paper that although detailed information exists on the many individual metal/microorganism interactions, information from mixed communities in heterogeneous systems such as soils and rock is sparse. The behaviour of such a heterogeneous system as a waste site is considered to be as yet too complex to allow the prediction of mobilization effects but certainly merits further research.

Bacteria, and other microorganisms, exhibit a number of metabolism-dependant and -independent processes for uptake and accumulation of heavy metals and radionuclides. Such processes are discussed by Gadd (1990). The dissolution and stabilisation of toxic metals and radionuclides in mixed wastes is reviewed by Francis (1990b). Fundamental information on microbial effects, especially for actinides and fission products in radioactive waste, under various conditions was very limited. Information about metal waste components from existing waste disposal sites is suggested to be useful in the development of predictive models on the fate and long term transport of radionuclides and toxic metals from waste disposal sites.

Microorganisms produce or consume various gases, all found in the atmosphere. Environmental conditions favouring gas production and consumption are discussed in general and also with respect to repository conditions by Bachofen (1991). Only a few studies were found that had tried to estimate gas production in actual repositories for radioactive waste. Microbial corrosion of concrete was reviewed by Diercks et al (1991). In their conclusions, they state that microorganisms may contribute substantially to the degradation of ceramic materials and that this process only is important if the microbes are supplied with substrate. The metabolism of thiobacilli (3.5.4) and nitrifying bacteria (3.2.1) results in the excretion of sulphuric of nitric acid, which may change the resistance of inorganic materials. The substrate may originate from exogenous or endogenous sources. To ensure the stability of radioactive wastes, it must be avoided that the microorganisms are supplied with substrate.

5.1.4 Microbiological processes in repositories

First, Bolliger et al (1991) evaluate the suitability of the deep sea as a repository for radioactive waste material with respect to the potential

microbial activity in such environments. They find a large dormant potential for such activity in the abyssal sediments of North Atlantic Ocean which may cause a release of radionuclides when deposition changes the environmental conditions at the sea floor. A number of groundwater environments in Europe were examined by Christofi and Philip (1991). They found bacteria but think it still remains to demonstrate whether waste and waste isolation material such as cellulose and bitumen will provide an exogenous nutrient source for microbial activity.

Cement degradation by two acid-producing alkalophilic fungi was demonstrated by Prefettini et al (1991). Their experiment indicates a potential threat to constructions made of cement, at least in aerobic environments and without organic carbon starvation conditions.

The degradation of bitumen with a high content of saturated hydrocarbons was demonstrated to occur with several different microorganisms (Ait-Langomazino et al, 1991). The microbial activity concerned predominately the oxidation of saturated hydrocarbons and bitumen with low content of saturated hydrocarbons, and a high content of aromatic hydrocarbons and resins was more resistant to biodegradation. Biodegradation of bitumen was also demonstrated under aerobic and anaerobic conditions simulating those in the silo part of the SFR repository (Roffey and Nordqvist, 1991). A gas production of 0.6-1.5 µmoles carbon dioxide per month and mg bitumen is reported. Based on linear extrapolation of experimentally determined degradation rates, 25-70% deterioration of the bitumen matrix under aerobic and 0.3-0.8 % under anaerobic conditions were calculated for a time period of 1000 years by Wolf and Bachofen (1991). This paper demonstrates the importance of having anaerobic repository conditions and that aerobic conditions may at least in theory result in a rapid deterioration of a bitumen matrix.

West et al (1991b) found that SRB could impair the sorption of the radionuclide ^{137}Cs on the host rock matrix. An important conclusion was that unless sterile conditions are employed doing radionuclide migration experiments, conclusions about the effects from inorganic parameters on radionuclide migration may unconsciously be biased by the activity of bacterial contamination.

Finally, Philip et al (1991) describe a corrosion effect from SRB on carbon steel in bentonite clay. They found a three times higher rate of corrosion with SRB compared to the control without bacteria.

5.1.5 Management of repositories and modelling of processes in radioactive waste repositories.

The last block comprises one paper on the microbiology program of UK Nirex (Colasanti et al, 1991). Two other papers discuss modelling approaches (Arter et al, 1991, McKinley and Grogan, 1991) and will be commented in chapter 6.

5.2 RESEARCH PROGRAMMES ON THE MICROBIOLOGY OF RADIOACTIVE WASTE DISPOSAL

Research programmes on microbial activity of relevance to radioactive waste disposal were reviewed by Rosevear (1991) and updated by West (1994). Individual programmes in Belgium, Canada (Stroes-Gascoyne and West, 1994), Finland, France, Germany, Italy, Japan, Sweden (this report), Switzerland, United Kingdom (Colasanti et al, 1991) and United States of America are discussed to a varying extent. Generally, the major concern of these programmes is the influence from microorganisms on the safety of low and intermediate waste repositories and much experimental data is available for this type of repositories. Another very significant reason for producing such data is the continuing chemical contamination of groundwater. Toxic waste dumps and municipal landfills, industrial chemicals, pesticides and fertilisers and the mobilisation of toxic metals by acid rains have created serious environmental problems that have warranted a large number of research programs in this area. Much of these results can directly be, and have been (confer 5.1), applied on low and intermediate deposition of radioactive waste. The knowledge about what microbiological conditions and activities will prevail in deep, high level waste repositories is much more limited for most repository concepts. This is possibly due to previous lack of deep, underground laboratories. As new such labs are constructed (see Section 4.1.4) the knowledge about the deep subterranean biosphere will hopefully increase. Another common characteristic of most programmes is that they regard microbial activity, with few exceptions, as being very negative for the waste concepts. The demonstration of a bacterial contribution to the redox control of groundwater at Äspö (see Section 4.3.4, (Banwart et al, 1994)) shows that in reality, microbial activity will not generally be a nuisance for waste repositories.

5.3 THE BUFFER MATERIALS AS A HABITAT FOR MICROORGANISMS

In section 2.4 it was suggested that microbial life in bentonite would be restricted by water activity (table 2.5). A high temperature was concluded not to be limiting (section 2.2). Early in the year 1994, we were invited to participate in the decommission of a full scale buffer heater test at the Underground Research Laboratory (URL) close to Whiteshell laboratories, AECL, north of Winnipeg in Canada. The test was set up to study temperature effects on the water distribution in the buffer as a function of temperature. In addition, it was decided that valuable information about the survival of bacteria during 2.5 years under realistic circumstances in a buffer clay could be achieved. Microbiologists from AECL, ANDRA in France, and Göteborg University Sweden, participated. The full report will be available during fall 1995. Here, some important key conclusions from this experiment are presented.

5.3.1 Viable counts of aerobic, anaerobic and termophilic bacteria

The buffer masses were mixed aerobically and laid down in layers aerobically. There is not much reducing capacity in the buffer per se, anaerobic and reduced conditions will only occur as a result of exchange processes with surrounding reduced groundwater, a process expected to take centuries. Therefore, it can be anticipated that oxidised conditions and probably also at least microaerophilic conditions prevailed in the buffer mass. Sulphate reducing bacteria and metanogenic bacteria will not develop under such conditions, and should therefore not be expected. This was also the case, neither the Swedish or the French lab could demonstrate any significant presence of such bacteria. Instead, relatively large numbers of mesophilic aerobic bacteria were detected.

5.3.2 Statistic evaluation of the CFU counts

The results from a statistical analysis of variance strongly indicated water content to have a large impact on the viability of bacteria in the buffer mass. The effect from the *in situ* buffer mass temperature was not significant. Figures 5.1 and 5.2 show the CFU results. At a low water content, there where few cultivable bacteria (Figure 5.1) but high temperature was not limiting (5.2). The reduction in CFU at high temperatures in Figure 5.2 is in fact due to the concomitantly low water activity. The effect from different culturing conditions was as expected significant. The only limitation for the viability of bacteria in the buffer masses seems to have been the water availability.

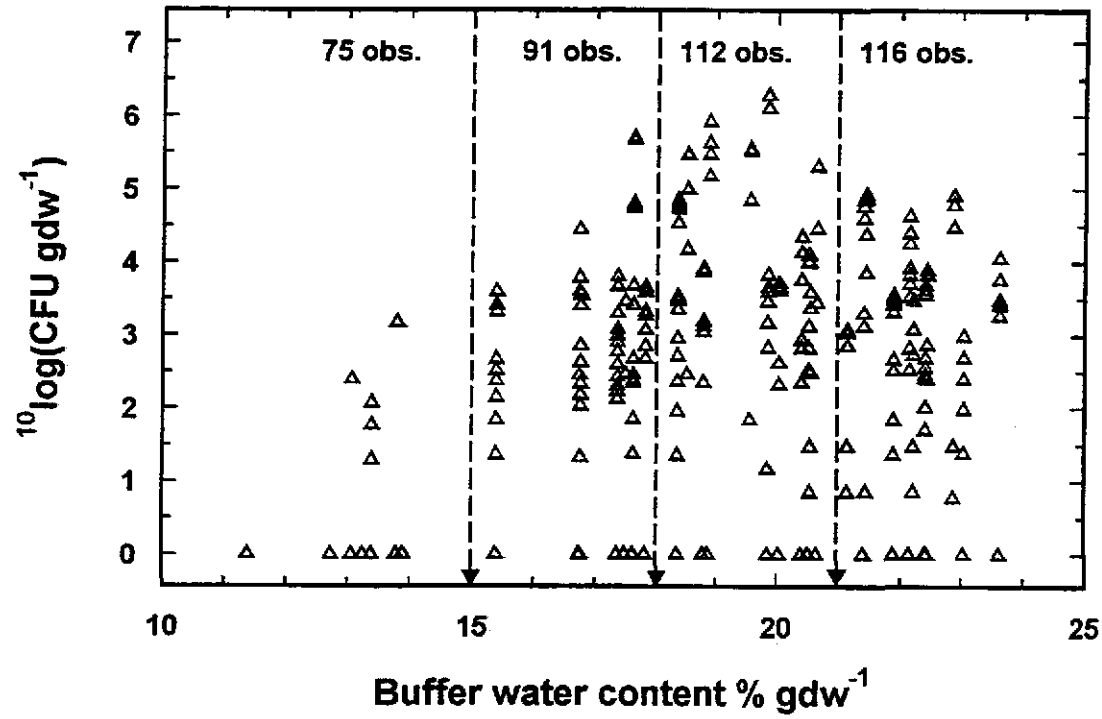

Figure 5.1 The distribution of all colony forming unit (CFU) data obtained (394 observations) over the in situ water contents for the buffer samples investigated. The four water content classes used for the analysis of variance are indicated and the number of observations within each class level are presented.

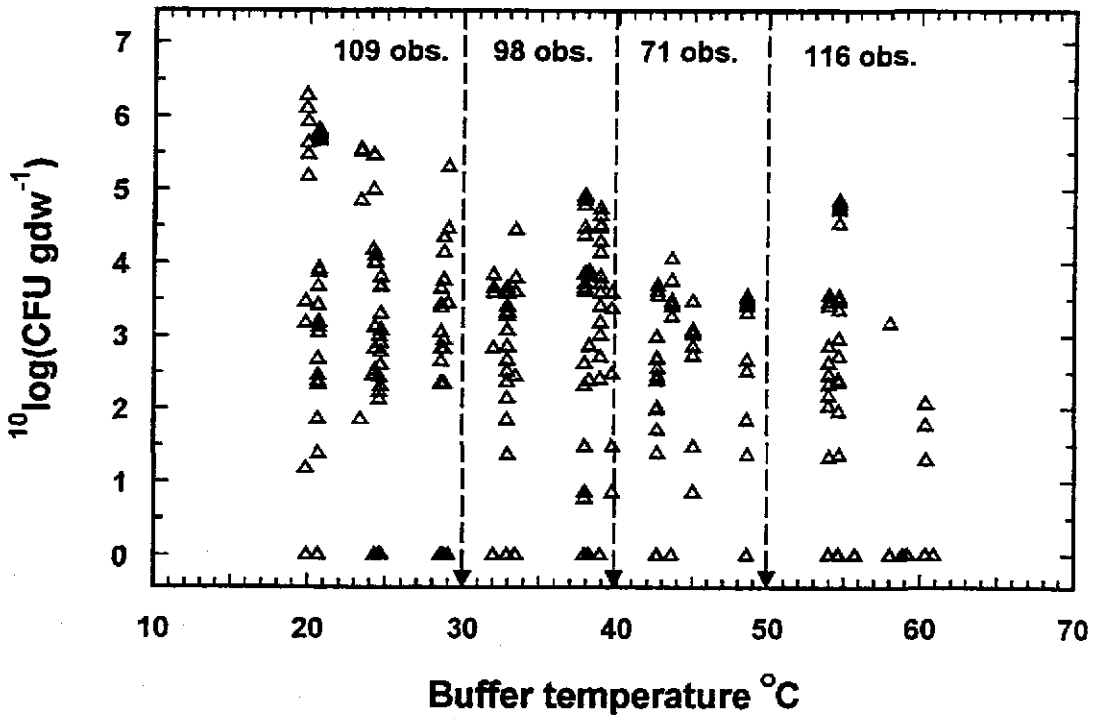

Figure 5.2 The distribution of all colony forming unit (CFU) data obtained (394 observations) over the in situ temperatures for the buffer samples investigated. The four temperature classes used for the analysis of variance are indicated and the number of observations within each class level are presented.

5.3.3 Water available for bacteria in the BMC-test buffer masses

When the buffer masses were laid down at start of the experiment, the water content was homogenous and averaged 18%. During the experiment, the heat from the heater caused a mass transport of water out from the vicinity of the heater to the peripheral parts of the buffer. Gradients of water contents developed. Approximately 50% of the buffer mass is sand and this part will not influence the amount of free water by a matrix adsorption effect more than marginally compared to the bentonite part. The clay will sorb water until it is saturated, and only water in excess of what is sorbed by the clay will be available for the bacteria. The water content at which water becomes available in a 50/50% sand-bentonite mixture is about 15%, which correlates well with the values below where viable bacteria could not be demonstrated (Figure 5.1).

5.3.4 Activity of bacteria

The activity of the found bacteria was measured as in situ assimilation of the tritiated amino acid leucine. The assimilation measured in the layers around the top of the heater was significant, indicating viable bacteria. It can be compared with data from groundwater and surfaces exposed to flowing ground water in Swedish granitic rock at the Stripa research mine (Pedersen and Ekendahl, 1992a) and the Äspö hard rock laboratory environments (Pedersen and Ekendahl, 1992b) (Table 5.1). The percentage of bacteria active in leucine uptake in Stripa and Äspö was from 9-99% and it is possible to calculate the assimilation per bacterium for these results. However, as there were only negative results for the microautoradiography performed on the buffer samples, such calculations can not be done for the BMC experiment. Briefly, the BMC leucine assimilation activity was similar to what has been registered for the deep groundwater of the Stripa research mine, harbouring between 10^4 to 10^5 cells per ml of groundwater.

Table 5.1 The amount of leucine assimilated by bacteria in the BMC buffer mass test and in groundwater and on surfaces exposed to flowing groundwater in Swedish granitic rock at the Stripa research mine (Pedersen and Ekendahl, 1992a) and the Äspö Hard Rock Laboratory environments (Pedersen and Ekendahl, 1992b).

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

5.3.5 Distribution of bacteria in the buffer analysed by 16S-rRNA gene analysis

A total of 21 clones could be identified (Figure 5.3) using the 16S-rRNA technique (see section 4.2.2). The three levels investigated (H97 M18, P66) indicated a homogenous system with three identical and dominating bacteria shared between each level. Also, each level shared about 50 % of its clones identities with each of the other two levels. This result was expected as the buffer mass was mixed from common sources of groundwater, clay and sand. The temperature differences did not correlate with any drastically changes in the distribution of the dominating species, although the frequencies of single clones differ between the sampled layers. One such minor difference noticed was the presence of eukaryotic DNA. The closest species in the database was the yeast *Saccharomyces cerevisiae*. This sequence, clone K20, appeared only at the two lower layers analysed, M18 and P66. This observation coincides with a phospholipid fatty acid analysis indicating eucaryotic fungi in these layers but not at the top layer H97.

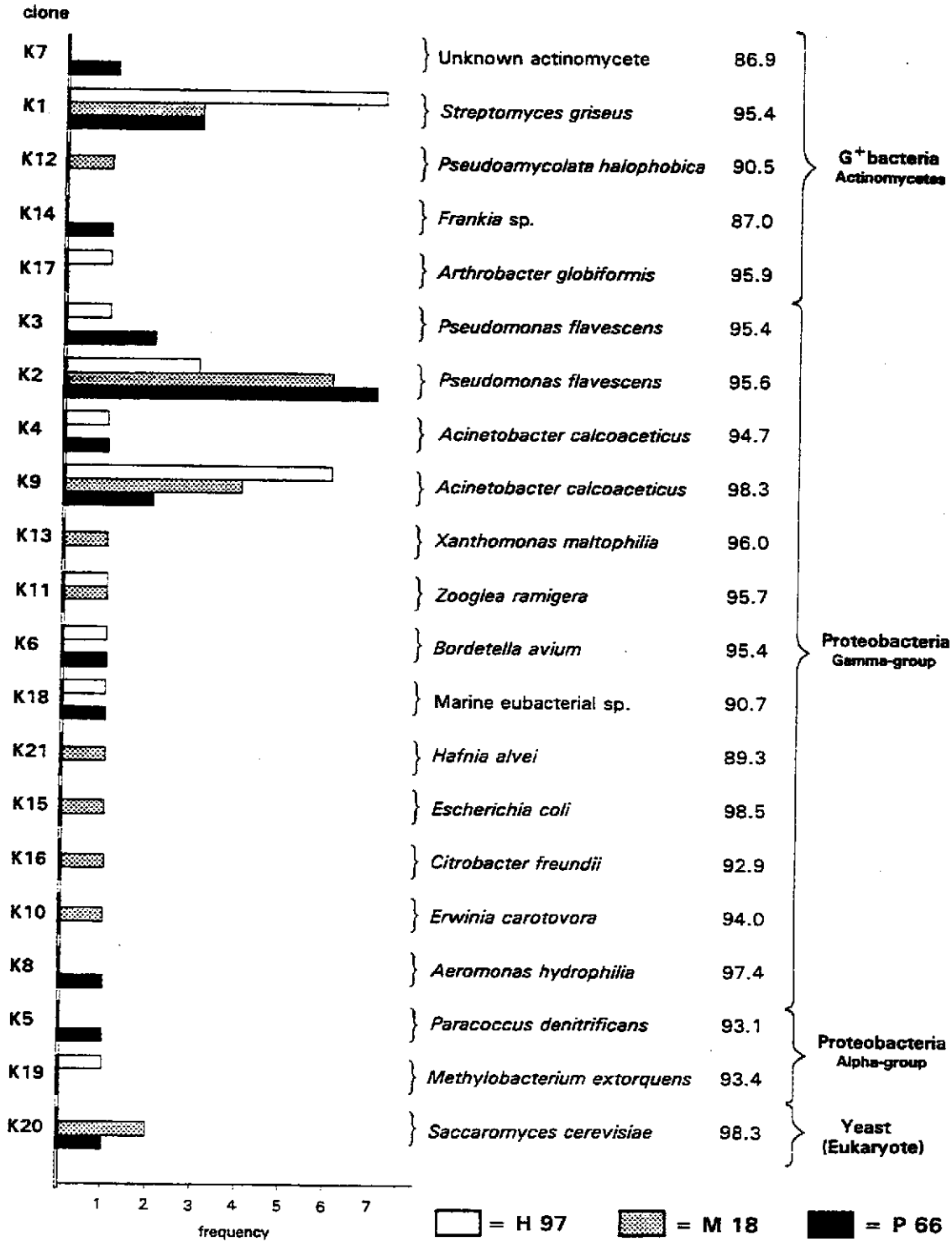

Figure 5.3 The distribution of the 21 different 16S rRNA gene clones, K1 - K21, obtained from three sampling levels of the buffer masses (H97, M18 and P66), and the identity values for the closest organism available in the database. The database source used for comparison is the EMBL database. The phylogenetic affiliation of the clones is depicted to the right. The total number of clones from each sampling level was: H 97, 23; M 18, 22; and P 66, 22.

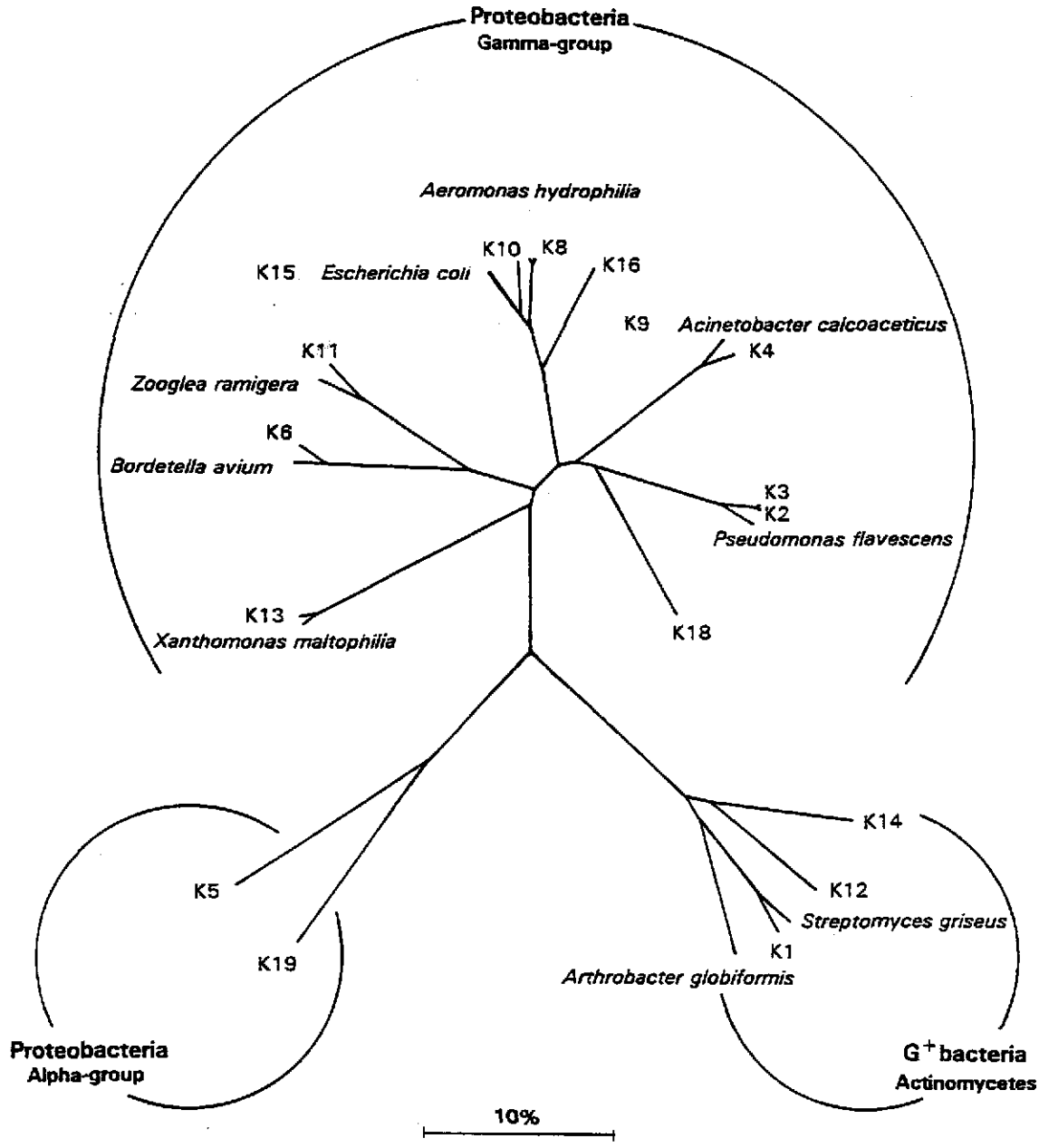

Figure 5.4 *Evolutionary distance tree based on the 16S rRNA gene sequences of clones from three buffer mass samples, H97, M18 and P66. Only sequences for species with an identity >95 % with respective clones are added to the tree.*

5.3.6 Diversity of bacteria in the buffer analysed by 16S-rRNA gene analysis

Three distinct phylogenetic groups of bacteria were found, proteobacteria alpha and gamma groups and grampositive bacteria belonging to the actinomycetes (Figure 5.4). Most clones belonged to the gamma group of the proteobacteria and this is a group were many bacteria living close to man can be found. Clones indicating anaerobic sulphate reducing bacteria (proteobacteria delta group) were not found and methanogenic sequences (archaea) could not be found either. This result supports the negative or low viable counts obtained for such bacteria.

Two of the three dominating species found had high identity with the typical groundwater bacteria *Pseudomonas flavecens* (95.6%) and *Acinetobacter calcoaceticus* (98.3%). This is not surprising because the buffer was made from clay, sand and URL groundwater. *Pseudomonas* appears to be the most dominant species in URL groundwater, and also *A. calcoacetius* has been observed (Stroes-Gascoyne et al, 1994b). The third one was an actinomycete related to *Streptomyces*. Many actinomycets are adapted to life in soils and their mycelia-like morphology make them well adapted to desiccation. The finding of these groups of bacteria in the buffer masses was expected.

5.3.7 The implications of the results for performance assessment of the Swedish SFL 2 high level radioactive waste repository

The Swedish buffer will consist of 100% Wyoming bentonite and will have a swelling pressure corresponding to a water activity between 0.92-0.96 (confer table 2.5). This is low enough to reduce the viability of many bacteria including sulphur and sulphate reducing bacteria (SRB). The production of sulphide by SRB constitutes one of the very few chemical circumstances under which copper will corrode anaerobically. If it can be shown that the density of the bentonite is high enough to kill any viable sulphur and sulphate reducing bacteria through desiccation, then copper corrosion induced by sulphide producing bacteria will totally depend on the production of sulphide in the rock outside the bentonite and the diffusivity of sulphide in bentonite. There are spore forming SRB belonging to the genus *Desulfotomaculum* but spores are inactive and do not produce sulphide. Such production may occur after germination, but then the sporeforming SRB species are as intolerant to desiccation as most other SRB. In other words, sporeforming SRB species will not constitute a larger problem with respect to sulphide production than any other SRB.

As a consequence of the conclusions arrayed above, a series of experiments studying the survival of SRB at different swelling pressures to determine the water activity at which SRB no longer survive are presently being launched in Sweden. It is executed as a collaborative project between Clay technology AB, Lund, Sweden, Department of General and Marine Microbiology, Göteborg and SKB AB.

5.4 CORROSION

Corrosion is an important process to consider in the performance assessment of a radioactive waste repository for at least two reasons. The first is obvious; if canisters are used, they are an absolute barrier to radionuclide dispersal, for as long as they remain intact. Copper/steel canisters are considered in the present Swedish spent fuel concept and especially the outer copper canister is an important protective barrier. A second reason for an interest in corrosion is gas generation. This will be discussed here in connection with corrosion, but is also treated in Sections 3.9, 5.1.3 and 5.1.4. Gaseous compounds are mainly of interest in performance assessment because, if generated at a enough high rate they may form a separate gas phase, exert a pressure on the construction and add on to the dispersion of contaminants.

5.4.1 Bacterial corrosion of steel

Sulphate reducing bacteria have been identified as the main cause of the widespread observation of steel casing failure in oil wells. This has been referred to in an overview of possible consequences of microbial activities in a Swiss HLW repository (McKinley et al, 1985). The HLW in the Swiss concept consists of vitrified reprocessing waste, and thick steel canisters are to be used for encapsulation. Both aerobic and anaerobic corrosion was considered in the Swiss study, but bacterially induced corrosion was not regarded as critical. The scarcity of nutrients and lack of energy sources were expected to limit the growth of microbes.

The effect of bacteria on steel corrosion rates has also been discussed in connection with the performance assessment of SFR in Sweden. The main focus was on hydrogen gas production as a result of corrosion (Moreno et al, 1985). The main source would be anaerobic corrosion of steel components in the repository such as concrete armouring, steel containers etc. However, in the course of this evaluation, indirect gas generation resulting from a bacterial increase of corrosion rate was also assessed. Biodegradation of bitumen could in principle generate carbon dioxide which in turn enhances steel corrosion. This would only be possible inside steel drums containing a bitumen waste matrix, because normally carbon dioxide is consumed by reactions with concrete in the repository. However, inside the drums it is possible for bacteria to generate carbon dioxide and lower pH without the buffering effect of concrete.

A combined copper/steel canister is presently being considered as the main concept for the Swedish spent fuel repository SFL 2. The main purpose of the inner steel canister is to facilitate the filling operation and to support mechanically the outer copper canister after it has been emplaced in the repository. Although the main barrier to penetrating corrosion is the outer

copper hull, some credit can probably also be given to the inner steel canister. In a scenario with a defect outer canister, it is an advantage to have an intact inner steel canister to allow relatively short-lived nuclides like ^{137}Cs to decay before any contact with groundwater. However, corrosion of the inner steel canister is not only being evaluated from the point of being a containment barrier. Important is also the generation of hydrogen gas due to anaerobic corrosion of steel. Diffusion of dissolved hydrogen in water saturated bentonite clay is a slow process. So if the rate of hydrogen generation is too high it may cause the formation of a separate gas phase. This is a scenario that is being further evaluated for its influences on the technical barriers (buffer) and radionuclide retention. So far a relatively high corrosion rate has been anticipated and it may not be necessary to specifically consider microbial processes.

Generation of corrosion products from steel is another performance assessment related issue. It can be a potential advantage by forming an appropriate substrate for radionuclide sorption and by filling narrow passages, but it may also be a disadvantage by expanding and exerting a mechanical pressure on the surrounding.

Steel corrosion will generate a chemically reducing environment by first consuming any oxygen and, in the anaerobic phase, generating ferrous iron in the form of dissolved ions and solid corrosion products. Reducing conditions are generally favourable for the stability of the waste form (i.e. spent fuel) and they keep down the solubility of redox sensitive radionuclides.

This section may be concluded by stating that steel corrosion is of interest in performance assessment of spent fuel disposal in copper/steel canisters for at least two reasons: penetration of the inner steel canister and hydrogen gas generation. The last point; gas production, is also of interest for underground repositories with low and intermediate level waste. Other effects that have been discussed to some extent are generation of corrosion products and influences on the redox chemistry. The importance of bacterial influence on steel corrosion should not be overestimated, because steel will corrode anyway in contact with water. However, two processes of some relevance have been identified: enhancement of anaerobic corrosion by sulphate reducing bacteria and influence on steel corrosion rate of carbon dioxide from bacterial degradation of organic matter.

5.4.2 Bacterial corrosion of copper

The only components of groundwater that will corrode copper are oxygen and sulphide ions. Oxygen reacts with copper forming copper oxides. Sulphide ions reacts forming copper sulphides and hydrogen.

Oxygen

Groundwater at a depth of about 500 m will be anoxic. However, oxygen trapped in the repository after sealing can result in corrosion of the copper canisters. Even if the supply of oxygen is limited, it needs to be considered because it can cause pitting corrosion. Microbiologically induced oxic pitting corrosion has been observed in copper pipes for municipal waters (Bremer and Geesey, 1991). The bacteria causing the trouble have in some cases been isolated and further tested in laboratory experiments with copper corrosion. An important factor in the corrosion process was the formation of a biofilm on the copper surface. From a performance assessment point of view it is important to note that pitting corrosion can not be excluded under these circumstances. However, it is also important to remember that oxygen is the necessary reactant. This is the key to the assessment of the potential influence of trapped oxygen. No penetrating pitting will occur if there is not enough oxygen reaching the canister. A certain minimum amount of oxygen will be needed to form a pit large enough to penetrate the copper hull. The transport of oxygen by diffusion through the clay buffer will limit the rate of supply, but more important it can be demonstrated that oxygen will be consumed in a sealed and water saturated repository. This may again involve bacterial action and will be further discussed in Section 5.6.

Sulphide

Sulphide corrosion has been carefully evaluated in connection with performance assessment of spent fuel disposal (Mattsson, 1983). However, the concentration of sulphide in groundwater is very low and sulphides in the backfill and surrounding rock have a low solubility. Therefore the supply rate of sulphide from the groundwater or sulphide minerals in the near-field will be too slow to be of any significance. In addition, sulphide is not prone to cause pitting on copper.

Sulphate

The situation would be different if sulphate could act as a source of sulphide. Groundwater sometimes contains significant concentrations of sulphate ions and bentonite clay also contains soluble sulphate. Reduction of sulphate is inhibited (steric kinetic hindrance), so, for example, sulphate does not react with copper metal at ambient temperatures. However, sulphate reducing bacteria can overcome the kinetic inhibitions and generate sulphide, if they are supplied with adequate organic substrates, and sulphide will react with copper. This has been regarded in the assessment of copper canister performance (Mattsson, 1983). The low concentration of organic matter in groundwater and bentonite was identified as important limitations.

It is also interesting to note that analogues in the form of old lightning plates were used to demonstrate sulphide corrosion. One of these plates which was buried beneath the groundwater table exhibited sulphide corrosion generated by sulphide reducing bacteria. This plate was in fact less affected than two others that had been subject to oxic corrosion and it did not show any signs of pitting (Mattsson, 1983).

Limiting factors

This section may be concluded by stating that the corrosion of copper is an important issue and in principle two corrodents have to be anticipated: oxygen and sulphide. Bacteria may be involved in both cases and of particular relevance are the sulphate reducing bacteria due to the abundance of sulphate. At least two factors have to be considered: the supply of substrate and the question whether the reaction of sulphate reduction can be local or not. Considering the first factor, recent studies have pointed to the possibility that other substances such as methane and hydrogen may act as electron donors. The activity of sulphate reducing bacteria will be energy limited as discussed in Section 2.8.

5.4.3 Hydrogen and sulphate reduction

Hydrogen can in principle be used as an electron donor by sulphate reducing bacteria. Produced sulphide can react with copper ions and thereby lower the free energy of reaction enough for copper metal to become oxidised by hydrogen ions in the water. From a stoichiometric point of view the following redox reactions would occur;

$$SO_4^{2-} + 4H_2 \rightarrow HS^- + OH^- + 3H_2O \text{ (bacterially mediated)}$$

$$2Cu + HS^- + H_2O \rightarrow Cu_2S + H_2 + OH^- \text{ (spontaneous)}$$

Consequently, the stoichiometric relation between copper and hydrogen would be 1.5 mol of H_2 for one mol of Cu.

Ultimately such reactions would be limited by the supply of hydrogen which is only found in relatively low concentration in deep groundwater.

Transport of hydrogen

The location of the individual deposition holes and the bentonite clay buffer are selected so that mass transport by dissolved substances will be resisted.

This will limit the transport of corrodents to the intact canister and retain the escape of radionuclides in case a canister fails. These precautions will also create a considerable transport resistance, if we imagine a scenario where hydrogen dissolved in groundwater is being supplied to microbes at a an individual canister. Firstly the dissolved hydrogen will have to be transported by the very slow flow of groundwater at this depth. Secondly it will have to be transported by diffusion from the flowing water in the rock fractures to the outside of the bentonite buffer and finally the hydrogen will have to diffuse through the bentonite to the microbes. In addition to that the concentration of hydrogen in groundwater is limited by the solubility of hydrogen in water according to Henry's law

$$c_0=HP$$

where c_0 is the saturation concentration at the pressure P. The Henry's law coefficient at 20 °C is H=0.166 L hydrogen (STP) per L water and MPa (Rodwell and Nash, 1992). According to Henry's law the maximum concentration of hydrogen dissolved in groundwater at 50 bar, which is the hydrostatic pressure at a repository depth of 500 m, is about 37 mmol/L.

The transport of dissolved groundwater components to the outer surface of a waste canister can be expressed as

$$N=Q_{eq}c_0$$

The parameter Q_{eq} which is referred to as the equivalent flow of water has been calculated for a deposition hole at different rates of groundwater flow in the near-field (KBS-3, 1983a). At a flow rate of 0.1 L/m^{2}a which is typical for repository conditions in Swedish crystalline bedrock, we obtain the calculated value Q_{eq}=0.57 L/a. This would yield a flux of 21 mmol/a of hydrogen to the canister provided that the groundwater is saturated with hydrogen according to Henry's law which is a conservative assumption.

However, we can imagine a less favourable situation. For example, if hydrogen is generated by corrosion at some location in the repository at such a rate that saturation is reached and a separate gas phase is formed. Gas filling the voids in tunnels and fractures can be transported at a relatively high rate. Gas would not penetrate the bentonite buffer due to the high pressure in excess of the hydrostatic pressure needed to open the flow path for the gas (Wikramaratna et al, 1993). Therefore the last part of the transport will still have to be by diffusion of dissolved hydrogen in water saturated bentonite clay.

Diffusion of dissolved groundwater components through a bentonite clay buffer to the surface of a waste canister is described by Fick's first law

$$N=-D_eA\cdot dc/dx$$

Where D_e is the effective diffusivity, A the area perpendicular to the consentration gradient and c the concentration field. For a slab of bentonite clay with the thickness l, concentration c_0 on the outside and 0 on the inside we obtain

$$N=D_eAc_0/l$$

Diffusion of hydrogen in water saturated compacted bentonite is surprisingly slow with $D_e=2 \cdot 10^{-11}$ m^2/s (Neretnieks and Skagius, 1978). For a 1 m thick slab with the area of 1 m^2, and input concentration of hydrogen equal to saturation $c_0=37$ mmol/L and $3.1536 \cdot 10^7$ s/a we obtain the mass flux

$$N=D_ec_0/l=23 \text{ mmol } m^{-2} a^{-1}$$

We can now calculate the maximum flux of hydrogen diffusion from a hydrogen gas filled tunnel to the top of the canister. This is a distance of 2.75 m and consequently

$$n_l=23/2.75=8.5 \text{ mmol } m^{-2} a^{-1}$$

Another scenario would be hydrogen gas in the fractures of the rock around the deposition hole and radial diffusion through the mantel of the bentonite buffer. The calculated diffusion flux would then be about an order of magnitude higher due to the smaller thickness 0.35 m of the buffer mantel. On the other hand the low porosity of the rock would create a restriction at the outside of the buffer.

Hydrogen induced corrosion of copper

Oxic corrosion of copper is limited by the amount of oxygen that may become trapped in a closed and sealed repository, and the rate of consumption of that oxygen by different buffering reactions in the repository and surrounding rock

Sulphide corrosion of copper is limited by the very low concentration of sulphide in groundwater unless microbes can reduce sulphate. Bacteria then need a substance which can act as electron donor (reductant). It has already been demonstrated that organic material in groundwater and bentonite backfill is too scarce to be of any concern (Mattsson, 1983). However, a

scenario which has not previously been considered is if bacteria could use hydrogen, generated by for example steel corrosion in some part of the repository, as an electron donor for sulphate reduction.

If we make the very conservative assumption that hydrogen reaching the canister surface is used by bacteria to reduce sulphate and thereby reacts in stoichiometric proportions with copper, there will be one mole of copper reacted for every 1.5 mole of hydrogen. With hydrogen arriving at a flux of, for example $n(H_2)$=1 mmol/am^2, there will be a copper consumption of

$$n(Cu)=63.5/1.5=42.3 \text{ mg m}^{-2}\text{a}^{-1}$$

where 63.5 g/mol is the elemental weight of copper. This corresponds to a copper corrosion rate of

$$s=42.3/8.94=0.0047 \text{ μm/a}$$

where 8.94 g/cm^3 is the density of copper metal. In the above described scenario of hydrogen diffusing vertically from the tunnel down to the canister through 2.75 m of bentonite buffer we calculate the corrosion rate

$$s_1=8.5\cdot0.0047=0.04 \text{ μm/a}$$

As indicated above, the diffusive flux of hydrogen could be higher through the mantel of the buffer due to the smaller thickness there. However, even with a ten time higher corrosion rate it would take 2500 years to corrode one mm of the canister.

Therefore we conclude, that if hydrogen is generated and if it is used by bacteria in the buffer to reduce sulphate to sulphide this will constitute a route to copper corrosion. However, in such a case the slow diffusion of hydrogen in compacted bentonite is an important barrier which will considerably limit the corrosion.

Generation of sulphide by the same microbial process outside the buffer, somewhere in the near-field, is another possibility. However, in such a case the produced sulphide will have to be transported to the buffer surface and diffuse to the canister. This implies that the transport resistance at the interface between buffer and flowing water in rock fractures will be efficient again and the supply will be restricted by the term Q_{eq}. In addition to that, the diffusivity D_e of sulphide ions in bentonite is much less that of hydrogen (Eriksen and Jacobsson, 1982). Therefore less corrosion will be obtained if the sulphide production occurs away from the canister surface.

5.5 REDOX BARRIER

The fact that the environment is reducing at depth was well established during the early investigations of Swedish study sites (Karlsson and Wikberg, 1987). Measurement of the groundwater redox potential was always negative below 100 m depth. Analysis revealed the reducing components Fe^{2+} or sulphide ions, but generally in very low concentrations (trace levels). However, the redox buffering capacity of the rock itself is considerable due to its content of ferrous iron and sulphide minerals. Recently it has been demonstrated that dissolved organic material is playing an important role in the reduction of infiltrating oxygenated groundwater (Banwart et al, 1994).

Reducing conditions are of great advantage for many of the barrier functions and it is therefore important that the natural rock-groundwater system shields of the atmosphere, consumes all infiltrating oxygen and buffers the redox potential to low values. This is discussed in section 4.3.4.

5.6 REDOX PROCESSES IN THE REPOSITORY

Redox conditions at depth are stable and protected from atmospheric influence by the geochemical reactions in the groundwater and overlying rock. However, the construction and operation of a deep repository will inevitably break the isolation and introduce oxygen by the ventilation. Draw-down of near surface water due to drainage of the repository can also introduce oxygen into the overlying rock. The total time needed for construction and operation will probably be about 40 years. Hydraulic conditions will be restored after the repository has been closed and sealed, but air will remain trapped in the porosities of backfill and buffer materials.

Later perturbations of the ambient reducing conditions can also be envisaged. Two scenarios for redox disturbances in the long-time perspective have been discussed; radiolysis and later intrusion of oxidising surface water.

As demonstrated by groundwater analysis and specific in-situ and laboratory experiments, the rock-groundwater system itself has a capacity to buffer and control the redox conditions at depth. However, in addition, we have also the possibility to improve the chemical environment in the repository by engineering measures. Natural bentonite clay has, in addition to its many favourable material properties as buffer and backfill, also a capacity to consume oxygen. Crushed granitic rock has also been considered as backfill and even this material has a certain capacity to react with oxygen.

Bacteria, if suitable strains are present, can be expected to participate in the redox reactions.

5.6.1 Consumption of oxidants

Trapped oxygen

The consumption of trapped oxygen in a closed and sealed HLW repository by inorganic reactions has been evaluated by Wersin et al (1994). In the later report it was demonstrated by calculations that the pyrite component of the bentonite (0.2 % by weight) was sufficient to account for consumption of all the trapped oxygen. The rate the of inorganic reaction was high enough to dominate over diffusion and the canister will therefore be well protected. It was calculated that all oxygen would be consumed in less than 300 years.

Radiolysis

Later disturbances due to radiolysis, if high level waste (spent fuel) becomes exposed to groundwater, have been discussed by Neretnieks and Åslund (1983). Ionizing radiation, for example alpha particles emanating from exposed HLW, can split the water molecules and thereby produce hydrogen and oxidizing species (oxygen and hydrogen peroxide). Even with very conservative assumptions of high radiolysis yields, it can be demonstrated by calculations that the extension of the oxidised zone was limited. The reductant envisaged in the calculations was the ferrous iron in the granitic rock minerals (KBS-3, 1983).

Less conservative approaches to radiolysis yields, including the bentonite redox buffering capacity in the calculations, would limit the expected extension of the oxidised zone even further (Romero et al, 1995). Attempts are also being made to include the reducing capacity of the waste form, the canister materials and their corrosion products. Anyway, it may be concluded that the important chemical agents involved in the redox reactions are iron(II) minerals (and iron(II) corrosion products), pyrite and uranium oxides.

Glacial melt water

Another reason to evaluate the consequences of redox disturbances is the glaciation scenario. It has been argued that melt water under the ice sheet and mainly at the marginal ice or at the front might infiltrate the open rock fracture system and reach great depths due to the large differences in groundwater heads (Ahlbom et al, 1991). Likely or not, it is worth considering, because it could possibly increase flow at repository depth and change the groundwater composition.

The melt water would presumably have a low ionic strength and it might also escape reduction of its oxygen content. Reasons for that would be the absence of soil and sediments, and the low temperatures at the recharge which inhibit biological processes. This has been evaluated by Ahonen and Vieno (1994). A relatively high oxygen content of 45 mg/L was assumed for the infiltrating glacial melt water in the calculations, but despite that the redox capacity of the rock and the backfill turned out to be efficient barriers which protected the copper canisters of the Finnish spent fuel disposal concept.

Experiments and analogue studies

Oxidation of iron(II), oxidation of sulphide minerals and reduction of uranium(VI) are important redox reactions that can be catalysed by bacteria. As already mentioned, these reactions are expected to occur also without bacteria and their participation will only improve the situation. However, it is very important to consider redox active bacteria, when experiments are performed *in situ* and in the laboratory. It would be awkward to conclude that reactions are purely inorganic in an experimental set-up where microbes are not controlled.

Natural analogues are valuable study objects of redox reactions. Redox fronts can be found in nature where oxidizing groundwater infiltrates reducing minerals. Uranium and many other trace metals have a tendency to precipitate at redox fronts. Many of these fronts are active and microbes can be sampled and searched for. A redox front in the uranium mine Osamu Utsumi in Brazil was sampled for microbes (West et al, 1990). The front was sharp which proves that the reaction of pyrite oxidation was fast, at least compared to diffusion. No were there any sulphide oxidizing bacteria, as analysed with culturing techniques, at the front which indicates that the reaction proceeds inorganically.

Uranium precipitated at the front, but a sluggish reaction was indicated by the fact that the uranium nodules (pitchblende) were formed a few centimetres ahead of the front on the reducing side. It is interesting to note that sulphur reducing bacteria were found around the front and this was accepted as a possible explanation for the nodular form of pitchblende concretions and the presence of secondary pyrite.

Based on these observations alone, one cannot exclude the possibility that sulphide reducing bacteria are essential for the trapping of uranium at the redox front.

Summary on redox conditions

Ferrous and sulphide minerals in the bentonite of the buffer and backfill, in the granitic host rock and in the crushed granitic rock used as backfill will have a redox buffering capacity and protect the near-field from disturbing oxidants from such sources as trapped air, radiolysis and glacial melt water. Components in the waste, such as UO_2 in spent fuel and organic materials and metals in LLW can act as additional sinks for oxidants. Metal, and metal corrosion products from waste canisters (copper and iron) and construction materials (steel reinforcement in repositories for LLW and ILW) can also be consumers of oxidants. Reducing conditions generally improve the performance of the repository and bacteria can act as catalysts for the redox reactions. However, if the reactions are important in the assessment of barrier functions, it would be preferable if they were spontaneous and did not require a catalyst of any kind. They would be more reliable then. Therefore it is important to know if bacteria are essential or not and control their interference in any experiment in-situ or in the laboratory.

5.7 DISSOLUTION AND TRANSPORT OF TRACE METALS BY BACTERIA

Dissolution and transport with the groundwater is by far the most important migration mechanism for radionuclides, if released from an underground nuclear waste repository (Francis, 1990b). Deep groundwater in Swedish bed-rock are usually anoxic and reduced with a pH around 7. These factors are critical for a safe function of a repository. This is because the mobility of many radionuclides depends on the pH and redox potential of the system and many of them take very insoluble forms at high pH and low redox potentials. Figure 3.9 gives an overview of potential ways of uptake and exchange of the radionuclides in groundwater. The bed-rock surrounding a repository is expected to sorb escaping radionuclides in its porous matrix and thereby retarding the migration from the repository. The retardation may be negatively affected if there are particles or compounds in the groundwater that sorb radionuclides stronger than the rock. This effect will increase with the concentration of particles and their ability to attract and bind the radionuclides. Consequently, the content of bacteria and bacterial complexing agents constitute important factors in the evaluation of how radionuclides may travel to the terranean biosphere.

The presence of bacteria can influence the groundwater transport of radionuclides in different ways. Free-living bacteria constitute mobile suspended particles which may have a radionuclide sorbing capacity higher than that of the surrounding rock (Pedersen and Albinsson, 1991). Radionuclide transport will then proceed faster with, than without bacteria. On the other hand, if the majority of the bacteria are growing in biofilms on fracture surfaces, transport of radionuclides may be reduced. Finally,

bacterial production of complexing agents and other metabolites can affect speciation and thus mobility of radionuclides independent of whether the bacteria are attached or not.

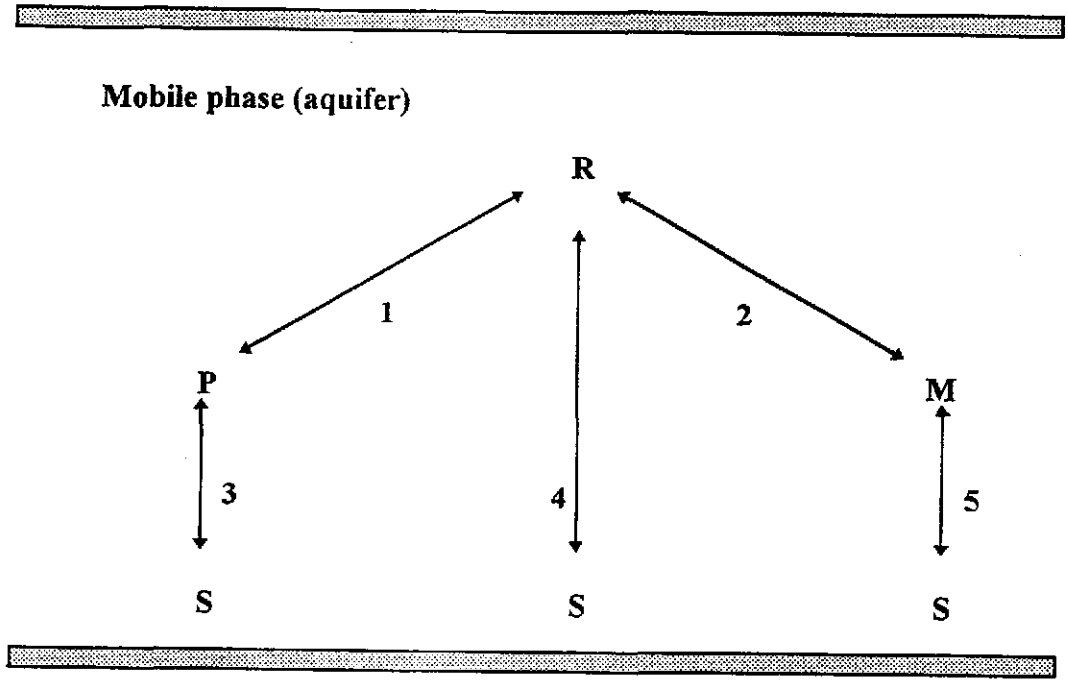

Figure 5.5 *Distribution of a radionuclide between mobile and immobile phases in groundwater (from McCabe, 1989). R: Radionuclide in solution, inorganic or organic complex related to the hydrochemical conditions. P: Solid mobile phase, precipitate or coprecipitate that can be formed or dissolved when the chemical conditions are changed, possibly by bacteria, in nearly saturated systems e.g. $Fe(OH)_3$, $Al(OH)_3$, $CaCO_3$, macromolecular organics. M: Solid mobile phase, natural colloids, e.g. bacteria, clay minerals, silica. S: Solid stationary phase, rock surface with or without bacterial biofilms. 1: Precipitation, coprecipitation - dissolution. 2,4: Sorption, uptake - desorption, related to chemical speciation. 3,5: Attachment, filtration, mineralisation, sedimentation - detachment, sloughing, resuspension, weathering.*

5.7.1 Migration of radionuclides

Microbes have been sampled and analysed in groundwater at Stripa, Ävrö, Äspö and Laxemar. Samples were collected by down hole equipment as deep as 1 km. The concentrations of bacteria generally ranges between 10^5 and 10^6 bacteria/ml. The concentrations at depth are typically around 10^5 bacteria/ml (4.5.1).

An assessment of the potential influence of bacteria on the migration of radionuclides from a deep spent fuel repository was made for the safety study SKB-91 (Allard et al, 1991). It was assumed that radionuclides were released to the near-field of a spent fuel canister and taken up by bacteria in the groundwater. It can be demonstrated that reversible uptake is of no concern, because the nuclides will then be successively lost from the microbes and become retained by sorption on the mineral surfaces as the flow continues away from the point of release. Irreversible uptake could, in principle, be more of a problem. A conservative assessment of the potential effect of irreversible sorption of radionuclides on mobile particles can be made in the following way: The concentration of particle bound radionuclides in the near-field is expressed as

$$c_s = c \cdot c_b \cdot K_{part}$$

where c_s is the concentration of radionuclides attached to the particles, c the concentration of radionuclides in water, c_b the concentration of particles and K_{part} the sorption coefficient of radionuclides on the particles. The fraction of release that is particle bound is then

$$c_b \cdot K_{part} / (1 + c_b \cdot K_{part})$$

By this factor we can judge the importance of radionuclide uptake on particles. The study by Allard et al. (1991) selected 10^5 bacteria/ml as a central value and 10^6 bacteria/ml as a maximum value for groundwater at repository level (about 500 m depth). It was conservatively assumed that the bacteria would have an average weight of 10^{-16} kg. This gave the bacteria concentration 10^{-5} kg/m^3 for the central case and a maximum value of 5 10^{-5} kg/m^3. Sorption coefficients for radionuclides on bacteria have been measured in the laboratory, for example promethium on gramnegative bacterium (Pedersen and Albinsson, 1990). Promethium in solution is a three-valent lanthanide and the value of K_{part} varied with pH between 0.002 to 0.1 m^3/kg in the pH-range of interest (pH=7 to 9). Taking the higher value of K_{part}=0.1 m^3/kg and the central case of c_b=10^{-5} kg bacteria per m^3 as an example we can calculate the fraction of bacteria bound release to 10^{-6}, which can be neglected in this case (Allard et al, 1991).

5.7.2 Formation of complexing agents

There are two groups of microbial compounds which can be considered as complexing agents.

1. *By-products of microbial metabolism* e.g. simple organic compounds or macromolecular humic and fulvic acids such as those released as a result of the degradation of higher plant material.

2. *Microbial exudates induced by the presence or absence of specific metal ions,* e.g. iron binding siderophores and metal binding proteins, and including those many bacterial exudates as yet uncharacterised. (section 3.11.3)

Birch and Bachofen (1990) extensively have reviewed complexing agents from microorganisms. They express complexes as species formed by the association of two more simpler species, each capable of independent existence.

5.7.3 Bacteria releasing specific complexing agents

Specific chelating agents are produced by microorganisms that require iron or other essential metals for growth. Among them, the iron-chelating compounds, siderophores, have received considerable attention. They are metabolites with a very high chelating affinity for Fe^{3+} and a low affinity for Fe^{2+}. Typical stability constants log K ($K=[Fe^{3-n}]/[Fe^{3+}][L^{n-}]$ are around 30 (Bossier et al, 1988). After the uptake of the ferric siderophore, with the help of a high-affinity siderophore uptake system installed in the cell envelop, the iron is released by reduction to Fe^{2+}. Although these siderophores are normally specific for iron they also have relatively high affinities for radionuclides such as plutonium and facilitate their uptake into cells. The occurrence of other specific metal complexing agents has been recorded, e.g. gallium(III), chromium(III), aluminium(III), scandium and indium as well as several metal ions essential for cells e.g. Mg, Mn and Ca (Birch and Bachofen, 1990).

Siderophores are produced by many different groups of organisms including bacteria, actinomycetes, yeast, fungi and dinoflagellates. The major factor influencing siderophore production is the availability of organic substrates. This has been shown under field conditions as well as in laboratory experiments (Bossier et al, 1988). In soils, the availability of substrates seems to be the driving force behind siderophore production. The ultimate concentrations found in soils may depend upon the water activity in the soil, the adsorption capacity for siderophores, the humus content and the iron extractability.

5.7.4 Radionuclide transport and bacteria

The relevance of these different mechanisms for radionuclide transport can only be evaluated with knowledge about sorption and uptake properties, and the ecology of the microorganisms that might inhabit a repository and its surroundings. This leads to fundamental questions about subterranean microbiology and the interaction between microbes and radionuclides. What are the numbers, species and activities of subterranean bacterial populations

in Swedish granitic rock? Which interactions will develop between a nuclear waste repository, its surroundings, migrating radionuclides and indigenous and introduced bacterial populations? The preceding chapters review our present knowledge on these questions.

6 MODELLING BACTERIAL PROCESSES IN RADIOACTIVE WASTE DISPOSAL

Few would agree on using a common model for feeding, growth and reproduction of very different, visible organisms such as for instance elephants and mosquitoes. Still, a corresponding approach is frequently applied on bacteria. Compared to sulphate reducing bacteria (SRB) and iron oxidizing bacteria (IOB), the elephant and the mosquito are much more closely related (Woese, 1987). They also have a similar cell physiology, as both are being chemoheterotrophs. The SRB are usually chemoorganotropic heterotrophs, while the IOB are chemolithotrophic autotrophs; compared with each other, they have very different cell physiology (2.8.3). Any model that includes bacteria therefore clearly must state what types of bacterial activities it is assumed to predict.

The finding of living bacteria in the glass rim of pillow lava 432 m below the sea bottom (Furnes et al, 1995) has demonstrated a volcanic subterranean biosphere not earlier known. These bacteria seem to dissolve the glass, thereby having a significant bearing on the mechanism for the chemical exchange between oceanic crust and ocean water. In surface volcanic environments, bacteria have been shown to increase the rate of palagonite formation from basaltic glass (Thorseth et al, 1992a, b). The palagonite formation as such is not new, it is the fact that bacteria may speed up the process that is current. These are two good examples where bacterial activity accelerate the alteration of inorganic mineral systems. The bacteria may act as catalysts for many very different reactions and the goal of any model of bacterial processes in a nuclear waste repository is to predict rates of bacterial transformations i.e. enzyme reactions. It can be production of acid or complexing agents, sulphate reduction, gas production - all are reactions catalysed by bacterial enzymes.

The kinetics of an enzyme depends on the maximal turnover rate of the enzyme, K_m and the substrate concentration (confer section 2.1). In section 2.6.2, it was shown that not more than 0.1g catalase can do the work of 1 metric ton of Fe^{2+}. Therefore, although the amount of bacteria in a subsurface repository environment may be low on a weight basis, they should not be ignored until their catalytic power has been evaluated for the processes of interest. This is however not always easily done as many reactions mediated by bacteria are catalysed in series of reaction steps each very difficult to model.

Some published models on bacteria and nuclear waste only account for potentially negative effects from microbes and argue that potentially positive effects from bacteria can be conservatively ignored (McKinley and Grogan, 1991). This is in our opinion a too narrow approch because geochemistry nowadays is becoming more aware of the variety and importance of influences from bacteria. An increasing number of geochemical processes has been shown to be inseparable from bacterial processes, as discussed in chapter 3 (biogeochemistry). A full understanding of repository environments, approached via modelling, must therefore include all possible processes, or wrong conclusions may be achieved.

6.1 MODELS IN THE LITERATURE

6.1.1 Low and intermediate waste repositories

Most models published on microbial processes in nuclear waste disposal deal with repositories for low and intermediate level waste (Arter et al, 1991, Colasanti et al, 1991, McKinley and Grogan, 1991). This is because such repositories will contain a lot of potentially degradable organic carbon. The possibilities of containment degradation due to microbial corrosion, radionuclide mobilization due to microbial production of organic complexant or direct uptake of radionuclides in mobile microorganisms and gas production with the organic carbon as energy and carbon source are obvious and must be considered. (McKinley and Grogan, 1991) present a model for a Swiss low and intermediate repository based on considerations earlier published (Grogan and McKinley, 1990, Grogan, 1987, West et al, 1991a). They suggest three basic approaches, a) mass balance, b) thermodynamic, c) kinetic.

The mass balance approach

The mass balance approach models the maximum biomass in a repository and concludes that if the biomass is low, then the microbial processes can be neglected. Recalling the discussion on enzyme kinetics in section 2.1 and on last page this is not necessarily true. It is not the biomass *per se* that determines the rate of a process. The catalytical efficiency of the enzymes involved and the substrate concentration are also important components. A small standing crop of bacteria may very well rapidly catalyse reactions, as for instance the oxidation of radiolysis products, without being in a state of active growth.

The mass balance approach assumes a number of nutrients (C, N P S) to be limiting for biomass accumulation and especially the availability of phosphorus is argued to be limited. This statement may however be

questioned, at least for granitic environments, because most granites contain a small portion of apatite [$Ca_5 (PO_4)_3 (F,Cl,OH)$] enough to support a microbial population. Locally produced acid conditions by biofilm bacteria can mobilise such phosphate into the bacterial pool. This has been clearly shown for epilithic microbial biofilms in oligotrophic environments of the Arctic Canada (Konhauser et al, 1994b). As long as some dissolved phosphorus exists, in equilibrium with solid phosphorus minerals, it will be enough to support bacterial life. Most bacteria can store phosphorus as intracellular polyphosphate. This will enable a bacterial population to build up a pool of phosphorus that can be circulated within the population. Once there is enough phosphorus to support the population need, additional phosphorus is not crucial for its catalytic activities. Nitrogen is not limiting at all in groundwater with dissolved nitrogen gas (se table 1.2), because many bacteria, especially anaerobic ones, are capable of nitrogen fixation (see section 3.2.3). Section 2.8 discusses this issue in more detail.

The thermodynamic approach

The thermodynamic approach assumes available energy sources to be limiting which is correct. An uncertainty exists about which energy sources are available in deep environments as discussed in 2.8.3. The basaltic glass bacteria mentioned in the introduction of this chapter may illustrate this fact. These bacteria seem to proliferate slowly in the 6 million years old volcanic rock. To be able to survive they must have an energy source and at present the most probable candidate is hydrogen that slowly diffuses up from the deep interior of the earth. Addition of such hydrogen to models of bacterial processes in deep subterranean environments changes the picture from a system with microbial processes limited by a certain amount of energy available, to a system where microbial processes may be ongoing for ever, certainly slow, but definitely not reaching a dead end due to energy depletion.

The kinetic approach

The kinetic approach models microbial processes using empirical or mechanistically derived data. This approach was earlier regarded as undeveloped by (McKinley and Grogan, 1991) due to the lack of data. As will be discussed below, data has now become available for some microbial processes at the Äspö hard rock laboratory and these data has been used in multi-disciplinary approaches for the modelling of the redox buffer capacity of bed rock environments (Banwart, 1994, Gustafsson, 1994, Pedersen, 1994) and the activity of sulphate reducing bacteria (Pedersen et al, 1995).

The computer code EMMA for calculation of microbiological biomass was used on data from the natural analogue Maqarin (section 4.6.3) thought to be an analogue for high pH repository environments (Coombs et al, 1994).

EMMA predicted biomasses in the 10^8 cells per ml range but the viable counts only indicated 10^4 cells per ml. Recently, some boreholes were sampled for total numbers (see section 4.6.3) and the total numbers were rather high (figure 4.17). 5 x 10^6 bacteria were registered in borehole M5. This is still far below the predicted EMMA numbers although 100 times higher than the viable counts. However, to be able to check EMMA correctly, attached bacteria must be counted as well - which is difficult for different technical reasons. Recalling section 4.3.1, data from Stripa indicated that there were from 4000 up to 800 000 more attached than unattached bacteria. Assuming a similar situation in Maqarin, EMMA have under-estimated the biomass considerably.

6.1.2 High level waste repositories

There are some reports dealing with models on high level waste environments (McKinley et al, 1985a, Stroes-Gascoyne, 1989, West et al, 1985). The approach used is very much the same as for low and intermediate repositories described above.

6.2 THE MULTI-DISCIPLINARY APPROACH TO MODEL BACTERIAL PROCESSES IN NUCLEAR WASTE DISPOSAL

A number of different approaches to the modelling of bacterial processes in nuclear waste disposal been executed within the Swedish program, often with a multi-disciplinary approach. In the following text, the main characteristics, results and conclusions from these experiments are presented. Most of the items below have been discussed earlier in this report, or have been, or will be published elsewhere. For full descriptions, consultation of these other sources is recommended.

6.2.1 Carbon cycling in the Stripa research mine environment

Data obtained from the Stripa mine were used to model possible carbon sources. It was concluded that the observed attached bacterial populations in groundwater from 800-1240 m depth were using organic carbon sources (Ekendahl and Pedersen, 1994b) (see section 4.3.4).

6.2.2 Can bacterial DNA signatures be used as tracers for modelling groundwater flow, mixing and origin?

In a multi-disciplinary experiment (Pedersen, 1994), we compared the DNA sequences obtained from different boreholes with tracer test data and found a

correlation between the number of identical sequences shared between boreholes and the flow time calculated between corresponding boreholes. The shorter the flow time, the more sequences were shared.

Groundwater was sampled from five different boreholes through the fracture zone intersected by the Äspö tunnel at 70 m below ground. The sampling was performed in December 1992. Boreholes HBH02 (10 m) and HBH01 (45 m) were drilled from the surface while the three vault boreholes KR0012, 13 and 15 were drilled from a vault 70 m below ground perpendicular through the fracture zone. Present bacteria were filtered on 0.2 µm polycarbonate filters, the DNA was extracted, cloned and sequenced.

Table 6.1 The numbers of identical sequences shared between the different boreholes sampled in the redox zone (- = no common sequences).

Borehole	HBH02	HBH01	KR0012	KR0013	KR0015
HBH02	12	-	-	-	-
HBH01		12	2	4	3
KR0012			12	1	1
KR0013				12	1
KR0015					11

The results in table 6.1 were compared with hydrochemistry tracer test data. The table shows that all clones in HBH02 were unique and did not appear in any of the other boreholes sampled. Further, it can be seen that HBH01 shares between 2 to 4 clones with the vault boreholes KR0012 to KR0015. Finally, the vault boreholes (12, 13 and 15) share one identical clone each. In the hydrochemical tracer test, it was found that water from the HBH01 reached the vault boreholes KR0012 and KR0013 after three days while it took 13 days for the HBH02 tracer to reach the HBH01 borehole. Gustafsson (1994). Obviously, the shallow (15 m) borehole HBH02 seems to have a relatively poor hydraulic connection to the deeper part of the fracture zone. The DNA data in table 6.1 are agreement with the tracer test data.

Making a comparison between the tracer test result and the DNA analysis, it must be remembered that the bacterial composition in a borehole reflects a complex situation. In addition to act as a particle, specific species of bacteria grow and divide at appropriate conditions but are reduced at other conditions. The DNA results therefore reveal a combination of environmental and transport conditions. Still, a considerable agreement was achieved, and as this probably was the first time such a comparison has been made, the outcome must be regarded as good indeed.

The possibility of using DNA analysis as a tracer tool for groundwaters is

reliable only if bacterial populations in the groundwater environments are stable. Results from the Stripa mine indicate this to be the case. There, we found very stable populations of bacteria that also differed in composition between sampled boreholes (Ekendahl et al, 1994). In the Äspö tunnel, we have returned to one of the boreholes sampled one year earlier and we found approximately the same bacterial DNA representation as when sampled the first time. This indicates the DNA analysis to be a very strong tool for tracing groundwater origin and mixing. Different groundwaters must be expected to have different and specific DNA signatures, not only making it possibly to identify the groundwater but also to reveal information about its character as different bacteria thrive in species specific water environments.

6.2.3 Microbial sulphate reduction in the Äspö HRL tunnel

Sulphate reducing bacteria (SRB) were discussed in section 3.5. Figure 4.5 showed that cultivable SRB are common in the Äspö hard rock environment. Since sulphide is a corroding agent to a copper canister placed in an anaerobic and reduced environment, a process generating sulphide has to be known in such a detail that a repository can be located without risk of massive sulphide generation. Therefore, a multi-disciplinary approach was launched with the goal to evaluate present results from Äspö with relevance for sulphate reduction. Some of the more important observations and present conclusions are given in section 4.3.4. See (Pedersen et al, 1995) for all details.

6.2.4 The redox experiment in block scale

Section 4.3.4 discusses another multi-disciplinary approach where microbiology was integrated as a natural part of a multi-disciplinary project. It was concluded that a large part of the redox buffering capacity of the studied rock was due to microbial iron reduction activity. The final model includes the microbial activity (Banwart et al, 1993, Banwart, 1994, Gustafsson, 1994, Pedersen, 1994).

6.2.5 Modelling the survival of bacteria in buffer masses

Earlier in this report, it has been suggested that water availability in the buffer clay will act as the overall limiting factor for microbial growth (section 2.4). The survival of bacteria in bentonite was therefore studied in co-operation with AECL, Canada as described in section 5.3.3. The results obtained support the idea that water activity is a strong viability regulator. To confirm this, we are presently running experiments where sulphate reducing bacteria are exposed to different bentonite densities, thereby exposing them to different water potentials (see Section 2.5.2). The results are expected to show the density limit where SRB no longer can survive. The

present hypothesis is that the water potential constitutes a viability regulating factor that over-rides all other environmental factors of importance for the survival of vegetative bacteria in bentonite.

6.2.6 Modelling bacterial processes in the Cigar lake analogue

This analogue has been discussed in section 4.6.1. By modelling the efficiency of specific enzymes it could be shown that bacteria successfully may compete with inorganic reactions in the neutralisation of radiolysis products, also discussed in section 4.6.

6.2.7 Modelling radionuclide transport by bacteria

An assessment of the potential influence of bacteria on the migration of radionuclides from a deep spent fuel repository was made for the safety study SKB-91 (Allard et al, 1991). The calculations showed that, taking actual numbers of bacteria in groundwater (Figure 4.1) and sorption coefficients for *Shewanella putrefaciens* and promethium (Pedersen and Albinsson, 1990), radionuclide transport by bacteria can be ignored.

6.3 THE MODELLING APPROACH - CONCLUSIONS

Recalling the introductory paragraphs in this chapter it can be concluded that bacterial processes in the subterranean environment are catalysed by enzymes and that they do not stand alone. They are instead integrated parts of the inorganic and organic geological system - biogeochemistry. We therefore recommend that modelling of bacterial processes must be performed in close co-operation with scientists in other disciplines dealing with processes that may be involved. Preferably, first identify the "problem" that is important for repository performance, then ask: is it possible that bacteria will interact? If bacterial interaction can be anticipated, include microbes in the theoretical and practical investigations judged necessary to penetrate the problem. Such an approach will be process oriented and will study a limited part of the microbial world at the time, e.g. sulphate reduction, radiolysis product neutralisation or redox control. Progress within the Swedish program on bacteria and nuclear waste disposal, as arrayed above, shows that this approach is fruitful.

7 CURRENT KNOWLEDGE AND RESEARCH NEEDS OF IMPORTANCE FOR NUCLEAR WASTE DISPOSAL

This report comprises current knowledge about subterranean bacteria and their potential influence on the disposal of nuclear waste. In the present chapter, central questions are summarised and references are given to major relevant sections, tables and figures elsewhere in the report. Section 7.1 is intended to help the reader with a special interest for some specific question to find appropriate information, while section 7.2 is intended to summarise issues for future research.

The easiest way to find a section, figure or a table is to consult the tables of contents at the beginning of the report which will reveal respective page numbers. There are separate tables for sections, figures and tables.

7.1 CURRENT KNOWLEDGE

7.1.1 Subterranean bacteria

Viable bacteria can be found in most, if not all subterranean environments investigated, that has a temperature below 110 °C (2.3) and enough water available (2.5). They appear in numbers from some hundred bacteria up to several millions of bacteria per ml groundwater, gram sediment or cm^2 solid surface. The species diversity is usually large and they appear unevenly distributed with respect to species affiliation. Direct as well as indirect measurements show that the subterranean bacteria found are alive and active, although their activity usually proceeds at rates much lower than can be found in terrestrial environments. The only true environmental limitations for subterranean bacteria seem to be temperature, water availability and the supply of utilisable energy sources.

Table 7.1 Cross references to parts of this report where some important subjects on subterranean bacteria are presented and discussed.

Subject	Section/s	Figure/s	Table/s
Numbers of bacteria	4.2.1, 4.3.1, 4.3.2, 4.5.1	4.1, 4.2, 4.3, 4.4, 4.5, 4.21	4.1
Species distribution	4.3.3		4.4, 4.5, 4.6
Diversity of species	4.2.2, 4.3.3	4.6	4.6, 4.7
Activity of bacteria	4.2.3, 4.3.4, 4.5.3		4.3
Environmental limitations	2	2.2, 2.5	2.10

7.1.2 Migration of radionuclides, induced by bacteria

There are two principally different ways in which bacteria may influence radionuclide migration. First, uptake of a radionuclide to a free-living bacterium (2.9) will mobilise the radionuclide while uptake to an attached bacterium (2.9) tend to immobilise it. Modelling of this process (5.7.1) has indicated radionuclide migration by bacteria to be negligible mechanism. Second, many bacteria produce chelating agents, unspecified such as organic acids, but also chelators with very high affinity for specific metals.

Table 7.2 Cross references to parts of this report where some important issues on bacterial influence on radionuclide migration are discussed.

Subject	Section/s	Figure/s	Table/s
Sorption of radionuclides	5.7.1	5.5	
Complexing agents	5.1.3, 5.7.2, 5.7.3		

7.1.3 Redox processes catalysed by bacteria, consumption of oxidants.

Trapped oxygen in bentonite clays etc. is calculated to have disappeared in 300 years time due to inorganic reactions. Bacteria consume oxygen and may decrease this time. Iron reducing bacteria and also other bacteria have been demonstrated to consume oxygen in infiltrating surface groundwater. This will probably protect the repository far-field from extensive oxidation during the time period of surface groundwater draw-down and enforced inflow due to drainage (construction and operation phases). Iron reducing bacteria and

sulphate reducing bacteria will produce ferrous iron and sulphide, respectively, which will further improve the redox situation by decreasing the redox potential in deep rock environments. Sulphate reducing bacteria have been shown to reduce dissolved uranium(VI) to uranium(IV) which will precipitate.

Table 7.3 Cross references to parts of this report where some important redox processes that involve bacterial activity are discussed.

Subject	Section/s	Figure/s	Table/s
Oxygen consumption	2.1, 5.6.1		2.1
Iron reduction	3.3.2, 4.3.4	3.4, 3.5	
Sulphate and uranium reduction	3.5.1, 4.3.4, 4.7.1	3.8	

7.1.4 Bacterial recombination of radiolysis products

Bacterial enzymes (2.1) act as catalyst for many different inorganic reactions. In sections 2.6.2 - 2.6.4, the strong potential of such reactions for recombination of radiolysis products is demonstrated. At conditions satisfactory for bacterial life, enzymatic recombination may out-compete inorganic catalysts.

Table 7.4 Cross references to parts of this report where bacterial recombination of adiolysis products is discussed.

Subject	Section/s	Figure/s	Table/s
Recombination of oxygen and hydrogen	2.6.3, 2.6.4, 2.6.5, 4.7.1		2.6, 2.7, 2.8

7.1.5 Bacterial gas production and consumption

Gas production by bacteria may occur as a result of several different processes . The processes of aerobic degradation, fermentation and anaerobic respiration of organic matter all produce carbon dioxide in relation to the amount of organic carbon available for degradation. Hydrogen evolve during fermentative processes and di-nitrogen, hydrogen sulphide and methane may form in anaerobic environments. At saturation, or when the pressure drops, gas bubbles may form. A gas produced by one type of bacteria, may be simultaneously or later be consumed by other bacteria, for instance when the

growth conditions changes. This will result in cyclic flows as depicted in figure 3.10. For example, methane is produced under anaerobic conditions and when oxygen is introduced, other bacteria will oxidise the methane to carbon dioxide and water.

Table 7.5 Cross references to parts of this report where bacterial gas production and consumption is discussed.

Subject	Section/s	Figure/s	Table/s
Carbon dioxide	3.1	3.1, 3.2, 3.10	
Hydrogen	3.6.1	3.1, 3.10	3.3
Methane	3.7	3.1, 3.10	3.4
Nitrogen	3.2.2	3.1, 3.10	

7.1.6 Bacterial induction of corrosion

The canister is an important barrier to radionuclide release. Sulphide reacts with copper and therefore sources of sulphide must be evaluated. Sulphate reducing bacteria produce sulphide and factors that governs their activity must be studied. We know that sulphate reducing bacteria occur in many subterranean environments.

Table 7.6 Cross references to parts of this report where bacterial induction of corrosion is discussed.

Subject	Section/s	Figure/s	Table/s
Canister corrosion	1.2.2		
Bacterial corrosion	5.4		
Sulphide producing bacteria	3.5.1, 4.3.3	3.8	

7.1.7 Concrete degradation

Concrete (1.3.4) can be inhabited by alkalophilic bacteria but the actual pH of fresh cement (13.5) is in the absolute upper region of the pH range where life has been shown possible. Data from the alkaline analogue in Maqarin (4.7.3) indicate that bacteria survive pH at 12.5, which is the pH of leached concrete. Acid producing bacteria, such as nitrification bacteria that produce nitric acid, may cause concrete degradation.

Table 7.7 Cross references to parts of this report where bacterial characteristics of importance for degradation of concrete is discussed.

Subject	Section/s	Figure/s	Table/s
Alkalophilic bacteria	2.3		2.4
Alkaline analogue, Maqarin	4.7.3	4.21	
Concrete degradation	3.2.4, 5.1.4		

7.1.8 Degradation of bitumen

The degradation of bitumen has been demonstrated to occur with several different microorganisms. Although the process may be significant at aerobic conditions, independent investigations indicate microbial degradation of bitumen to be a very slow process at anaerobic conditions.

Table 7.8 Cross references to parts of this report where bacterial degradation of bitumen is discussed.

Subject	Section/s	Figure/s	Table/s
Degradation of bitumen	5.4.1		

7.2 RESEARCH NEEDS

This report has identified needs of research regarding the influence of microorganisms on performance assessment of nuclear waste disposal. The different fields of research recognised deal with effects from microorganisms that may have different *effect values* for repository performance: Effects that will have a positive influence on repository performance (P), effects that will have a negative influence on repository performance (N), and effects that must be studied for a general understanding of the influence of bacteria on repository performance (U). Below, the research needs are summarised and respective effect value on performance assessment is suggested as well.

7.2.1 Subterranean bacteria

Now, very convincing evidence exists for a subterranean biosphere that reaches as deep as temperature and water availability allow (Chapter 4). These parameters, temperature and water availability, can easily be measured once boreholes etc. have made access possible. It is more problematic to get correct measurements of available energy sources and the flux of them. It will not be possible to correctly model subterranean bacterial activity until we

have data on fluxes of organic carbon from the terrestrial sun-driven ecosystems downwards including geological deposits and fluxes of gases from the inner of the earth such as hydrogen and methane.

Research need:

What energy sources and fluxes of energy will be available for bacteria at repository conditions?

Effect value: U

7.2.2 Dissolution and transport of radionuclides by bacteria

The direct uptake and migration of radionuclides by bacteria seem to be negligible components for the modelling of migration. Dissolution of precipitated or in other ways immobilised radionuclides and the production of complexing agents remains to be elucidated. The production of complexing agents is nested within the question of fluxes of energy, as such organic molecules cannot be produced by bacteria without an energy source.

Research need:

To what extent, if any, can bacterial dissolution of immobilised radionuclides and production of complexing agents increase radionuclide migration rates?

Effect value: N

7.2.3 Redox processes catalysed by bacteria

The radioactive waste disposal concepts may benefit from bacterial redox processes such as consumption of oxygen, production of reducing compounds (e.g. ferrous iron and sulphide) and reduction of uranium(IV). Today we have convincing results indicating that iron reducing bacteria contributed to (and still do) keeping groundwater, which infiltrates to the access tunnel of the Äspö hard rock laboratory, reducing for soon 4 years. Still, there is a lack of data for modelling the accurate participation of bacteria in subterranean redox processes. Correct modelling of repository redox processes preferably should include reactions catalysed by bacteria.

Research need:

Will bacterial oxygen consumption significantly add to the inorganic oxygen consumption system, and to what extent may bacterial production of reducing compounds such as sulphide and ferrous iron contribute to keeping the repository host rock reduced?

Effect value: P

7.2.4 Bacterial recombination of radiolysis products

This report suggests that bacteria significantly may contribute to the recombination of radiolysis products. The calculations indicate bacterial recombination to be a process that will compete with inorganic reactions. This theory needs to be validated, preferably at a suitable analogue site where radiolysis has been going on for a long time.

Research need:

Will bacterial recombination of radiolysis products significantly contribute to the removal of such unwanted oxidising molecules? If so, how can it be validated?

Effect value: P

7.2.5 Bacterial gas production and consumption

Bacteria produce and consume many different gases, such as carbon dioxide, hydrogen, oxygen and methane. Gas production is generally an unwanted process, while gas consumption may be beneficial for a waste repository. Corrosion of steel in a penetrated copper canister may produce hydrogen gas that in a worst case may form bubbles that has to be released through the buffer. Hydrogen oxidising bacteria may consume the hydrogen - or at least a part of it. Some results exist that indicate gas production in low level waste with high content of organic material to be possible. The relation between gases and bacteria in deep repositories is unknown - beneficial as well as negative effects can be anticipated.

Research need:

Will bacterial production and consumption of gases like carbon dioxide, hydrogen and methane influence the performance of repositories?

Effect values: N and P

7.2.6 Bacterial induction of corrosion

Corrosion of copper canisters due to the production of sulphide is a well known and unwanted scenario. It is important to study if sulphide producing bacteria in bentonite will survive at actual swelling pressures. If bacterial sulphide production in the bentonite can be excluded, it remains to study factors that govern sulphide production in the surrounding rock. Wee need to know the conditions for sulphide production, extent and distribution of sources in the subterranean environment.

Research need:

Bacterial corrosion of the copper canisters, if any, will be a result of sulphide production. Two important questions arise: Can sulphide producing bacteria survive and produce sulphide in the bentonite around the canisters? Can bacterial sulphide production in the surrounding rock exceed a performance safety limit?

Effect value: N

7.2.7 Concrete degradation

Concrete has been used during the construction of SFR and will possibly be used also during SFL 2 construction.

Research need:

Do relevant bacteria survive at pH equivalent to that of repository concrete and can they possibly influence repository performance by concrete degrading activities such as acid production?

Effect value: N

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Our growing co-operations concerning microbes and nuclear waste disposal with British Geological Survey on the Maqarin analogue, CEA on the OKLO analogue and Finland Geological Survey on the Palmottu analogue, and also numerous Swedish researchers involved in the Stripa and Äspö projects are, in our opinion, very stimulating. The growing pool of new results all demonstrate the power of multi-disciplinary work.

Stockholm and Göteborg

Fred Karlsson and Karsten Pedersen

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