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Investigations of subterranean micro-organisms and their importance for performance assessment of radioactive waste disposal

Results and conclusions achieved during the period 1995 to 1997

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November 1997

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INVESTIGATIONS OF SUBTERRANEAN MICROORGANISMS AND THEIR IMPORTANCE FOR PERFORMANCE ASSESSMENT OF RADIOACTIVE WASTE DISPOSAL

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ABSTRACT

In 1987, microbiology became a part of the Swedish scientific program for the safe disposal of high level nuclear waste (HLW). The goal of the microbiology sub-program is to understand how subterranean microorganisms will interact with the performance of a future HLW repository. The Swedish research program on subterranean microbiology has mainly been performed at two sites in granitic rock aquifers at depths ranging from 70 m down to 1240 m; the Stripa research mine in the middle of Sweden and the Äspö hard rock laboratory (HRL) situated on the south eastern coast of Sweden. Some work has also been performed in co-operation with other national or international research groups in Sweden, Canada and at the natural analogue sites in Oklo in Gabon and Maqarin in Jordan. The most recent report in the SKB technical report series on microbiology and performance assessment, SKB TR 95-10, gave the state of the art regarding microorganisms and their importance for performance assessment. That report is recommended as a source of knowledge about basic microbiology, microbial ecology of subterranean environments and the nuclear waste disposal concept in a microbiological perspective. The present report summarises results and conclusions achieved during the period 1995 to 1997 and is a continuation of SKB TR 95-10. The report is structured as summary which explains and analyses the obtained results and conclusions in a performance assessment perspective. The scientific basis for the summary is an enclosed series of eleven papers of which eight have gone through an international peer review process for publication in international scientific journalsand reports and papers published earlier.

SAMMANFATTNING

Mikrobiologiska undersökningar blev 1987 en del av det svenska forskningsprogrammet för slutförvar av högaktivt, använt kärnbränsle. Målet för detta delprogram är att förstå hur mikroorganismer i och kring ett förvar kan interagera med funktionerna hos ett framtida förvar. Forskning inom det mikrobiologiska programmet har huvudsakligen utförts på två platser i granitiskt berg på djup mellan 70 och 1240 m; Stripa forskningsgruva i Bergslagen och Äspö berglaboratorium strax utanför Oskarshamn. En del forskningsarbete har också utförts i samarbete med andra nationella och internationella forskningsgrupper i Sverige, Kanada och vid den naturliga analogierna Oklo i Gabon och Maqarin i Jordanien. Den senast utgivna tekniska rapporten om mikrobiologisk forskning i SKB:s rapport serie, SKB TR 95-10, redogjorde ingående för kunskapsläget om mikroorganismer och deras betydelse för utvärdering av olika förvarsfunktioner. Den rapporten rekommenderas som en kunskapsbas om grundläggande mikrobiologi, den djupa biosfärens ekologi och slutförvarskonceptet i ett mikrobiologiskt perspektiv. Den nu publicerade rapporten summerar resultat och konklusioner som har uppnåtts under perioden 1995 till 1997 och utgör en fortsättning på SKB TR 95-10. Rapporten är strukturerad som en sammanfattning vilken förklarar och analyserar uppnådda resultat och konklusioner ur ett förvarsperspektiv. Den vetenskapliga basen för rapporten utgörs av elva bifogade artiklar av vilka 8 har genomgått granskning av internationella experter före publicering i internationella vetenskapliga tidskrifter samt rapporter och artiklar publicerade tidigare.

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SUMMARY AND CONCLUSIONS

The present report summarises results and conclusions achieved during 1995 to 1997 and is a continuation of SKB TR 95-10. The report is structured as summary which explains and analyses the obtained results and conclusions in a performance assessment perspective. The scientific basis for the summary is enclosed as a series of eleven papers of which eight have gone through an international peer review process for publication in international scientific journals. The other three papers (papers 4, 8 and 11) are pending a similar quality assurance process. Each paper is referred to in the summary with a number ranging from 1 to 11.

The following conclusions are made:

- The inability of foreign microorganisms to establish in granitic aquifers and the presence of fossil microorganisms in aquifers at 207 m below ground have been observed at Äspö HRL. These observations suggest that granitic rock aquifers are inhabited by microorganisms before drilling and excavation operations. It can consequently be concluded that there is a very high probability for the existence of a deep subterranean biosphere in granitic rock.

- Microorganisms have the capability to reduce important groundwater components such as sulphate to sulphide and to produce and consume gases. The documented presence of a deep biosphere implies that relevant microbial reactions should be included in the performance assessment for a high level nuclear waste (HLW) repository.

- Microorganisms that use hydrogen as an energy source and carbon dioxide as their sole carbon source have been found to be active in deep granitic groundwater.

- A HLW repository will be situated in a subterranean biosphere that is independent of solar energy and photosyntetically produced organic carbon. The ultimate limitation will be the availability of hydrogen over time.

- Hydrogen is necessary for an active deep biosphere and it can be found in most deep groundwaters.

- Major gas components in deep groundwater are helium, nitrogen, methane and carbon dioxide. All these, except helium, can be produced or consumed by microorganisms.

- Recently obtained data now conclusively demonstrate that significant microbial oxygen consumption will occur in a HLW repository.

- Sulphide producing microorganisms are active in environments typical for a Swedish HLW repository and the potential for microbial corrosion of the copper canisters must be considered.

- The bentonite buffer around the copper canisters will be a hostile environment for most microbes due to the combination of radiation, heat and low water availability. Discrete microbial species can coup with each of these constrains and it is theoretically possible that sulphide producing microbes may be active inside a buffer, although the experiments conducted this far have shown the opposite.

- Microorganisms have the capability to enzymatically recombine radiolysis oxidants formed by radiation of water.

- The relevance of microbial effects on 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 has lead to fundamental questions about subterranean microbiology and the interaction between microbes and radionuclides. Numbers, species and activities of subterranean microbial populations in Swedish granitic rock have been determined. It has earlier been concluded that the migration of radionuclides due to sorption on microorganisms can be neglected.

- The influence from microbially produced complexing agents remains to be studied at realistic conditions in deep groundwater. This research task would require a designated site for microbiology at the Äspö HRL.

- Microorganisms have been found in natural alkaline groundwaters but it could not be conclusively demonstrated that they were *in situ* viable and growing, rather than just transported there from neutral groundwater.

- The diversity of microorganisms in the natural alkaline Maqarin groundwater was similar to what has been detected elsewhere with the 16S rRNA gene sequencing method, but none of the sequences found were typical for known alkaliphilic organisms.

- A possible hypothesis based on the obtained results from investigations of natural alkaline groundwaters is that fresh concrete may be a bit too extreme for active life even for the most adaptable microbe - but this remains to be demonstrated.

1 INTRODUCTION

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, characterised as high level radioactive waste (HLW). Radioactivity will decay with time, but some long-lived radionuclides will make the HLW hazardous for a very long time. It will for example remain more radiotoxic than uranium ore for over 100 000 years. The spent fuel elements will be encapsulated in copper-steel canisters and placed in deposition holes in tunnels at an envisaged depth of about 500 m (Fig. 1.1). The deposition holes will be filled with compacted bentonite clay to protect the canisters from mechanical damage and excessive water flow. The tunnels will be backfilled with a mixture of sand and bentonite or with crushed granitic rock. 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 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. 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.

Nuclear power, research, hospitals and industry 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 HLW, 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 HLW. A repository for LLW and ILW, called SFR, has been in operation since 1988 in Sweden. SFR is situated underground at a distance of 1 km off the coast in Forsmark north of Stockholm.

In 1987, microbiology became a part of the Swedish scientific program for the safe disposal of HLW. The goal of the microbiology sub-program is to understand how subterranean microorganisms will interact with the performance of a future HLW repository. The Swedish research program on subterranean microbiology (Pedersen 1996, 1997b) has mainly been performed at two sites in granitic rock aquifers at depths ranging from 70 m

down to 1240 m; the Stripa research mine in the middle of Sweden (Ekendahl and Pedersen 1994, Ekendahl et al 1994, Pedersen and Ekendahl 1992a) and the Äspö hard rock laboratory (HRL) situated on the South-eastern coast of Sweden (Pedersen 1997a, Pedersen and Ekendahl 1990, 1992b, Pedersen et al 1996b paper 3, 1997b paper 2, 1997c paper 1). The Äspö HRL has been constructed as a part of the development of the Swedish concept for deep geological disposal of spent nuclear fuel and the work has been divided into three phases; the pre-investigation (1986-1990), the construction (1990-1995) and the operating (1995-) phases. The Äspö HRL related work presented in this report was performed during the construction and operating phases. Some work has also been performed in co-operation with other national or international research groups in Canada (Stroes-Gascoyne et al 1996, paper 10) and at the natural analogue sites in Oklo in Gabon (Pedersen et al 1996a, c) and Maqarin in Jordan (Pedersen et al 1997a, paper 11). The reports and publications produced this far are listed in appendix 2 and 3. The most recent report in the SKB technical report series on microbiology and performance assessment, SKB TR 95-10 by Pedersen and Karlsson (1995) gave the state of the art regarding microorganisms and their importance for performance assessment. That report is recommended as a source of knowledge about basic microbiology, microbial ecology of subterranean environments and the nuclear waste disposal concept in a microbiological perspective. The following items were discussed (Numbers in bracket refer to the respective chapter in SKB TR 95-10):

- Nuclear waste disposal concepts (1)
- Subterranean bacteria and environmental limitations (2)
- Subterranean bacterial processes (3)
- Investigations of subterranean bacteria (4)
- The microbiology of radioactive waste disposal - the repository environments (5)
- Modelling microbial processes in radioactive waste disposal (6)
- Current knowledge and research needs of importance for radioactive waste disposal (7)

The present report summarises results and conclusions achieved during 1995 to 1997 and is a continuation of SKB TR 95-10. The report is structured as summary which explains and analyses the obtained results and conclusions in a performance assessment perspective. The scientific basis for the summary is enclosed as a series of eleven papers of which eight have gone through an international peer review process for publication in international scientific journals. The other three papers (papers 4, 8 and 11) are pending a similar quality assurance process. Each paper is referred to in the summary with a number ranging from 1 to 11.

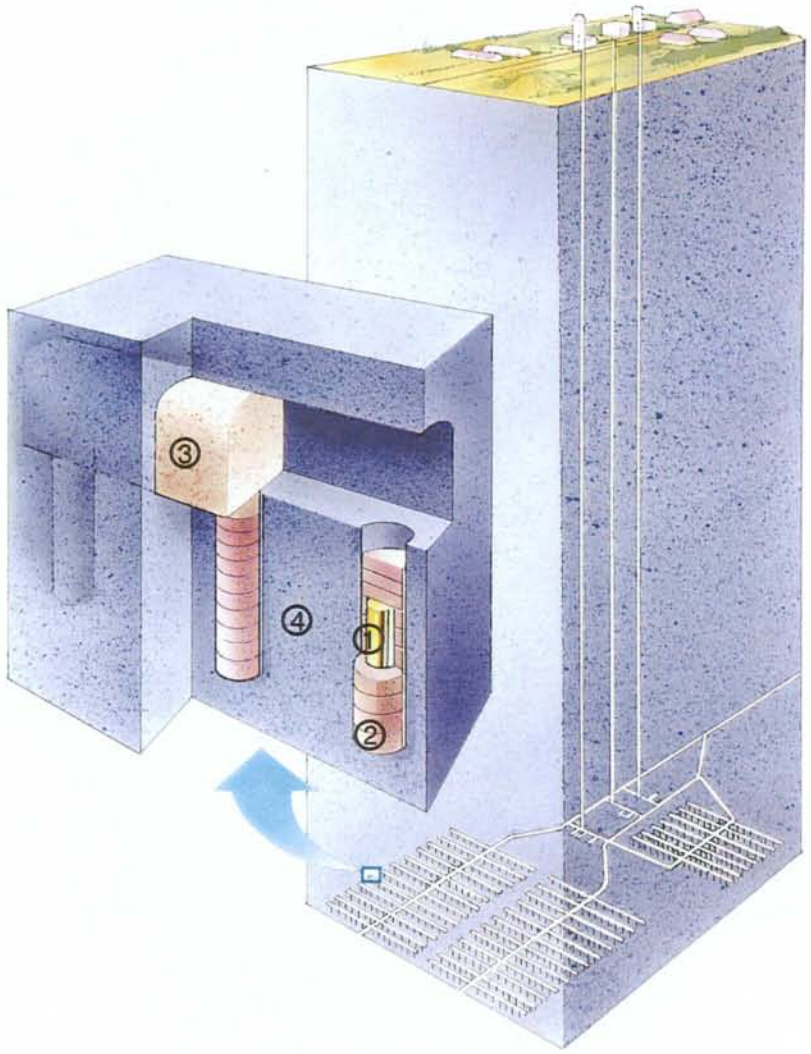


Figure 1.1 *Schematic drawing of a deep repository. A system of tunnels with vertical deposition holes is built at a depth of about 500 metres. The spent fuel assemblies are encapsulated in copper canisters. The canisters are placed in the holes, where they are embedded in bentonite clay. Multiple barriers will protect the spent fuel in the deep repository. 1.* ***Copper canister****. The canister isolates the fuel from groundwater contact. The fuel itself is in solid form and has very low solubility. 2* ***Blocks of bentonite clay****. The clay prevents groundwater flow around the canister and protect it against minor movements in the rock. The diffusivity of radionuclides is very low in bentonite, which will prevent radionuclide migration in case of a failing canister. 3.* ***A tunnel backfill****. A mixture of sand and bentonite, or crushed rock, fills up the tunnels. 4.* ***A rock barrier.*** *The rock offers a durable environment, both mechanically and chemically. It also acts as a filter for radionuclides possibly released to the groundwater.*

2 RESEARCH TASKS

Seven research tasks were identified by Pedersen and Karlsson (1995, chapter 7) that are of importance for the performance assessment of microorganisms in radioactive waste disposal. They are:

1. **The subterranean biosphere**
   Is there a deep subterranean biosphere and how does it sustain its life processes? What energy sources and fluxes of energy will be available for microorganisms in and around a HLW repository?

2. **Microbial production and consumption of gases**
   Will bacterial production and consumption of gases like carbon dioxide, hydrogen, nitrogen and methane influence the performance of repositories?

3. **Microbial reducing activity**
   Will bacterial oxygen consumption significantly contribute to oxygen removal from a HLW and to what extent may bacterial production of reduced compounds such as organic material, methane, sulphide and ferrous iron contribute to keeping the repository host rock reduced?

4. **Microbial corrosion of copper**
   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 surrounding the canisters? Can bacterial sulphide production in the surrounding rock exceed a performance safety limit?

5. **Microbial recombination of radiolysis products**
   Will bacterial recombination of radiolysis products significantly contribute to the removal of unwanted oxidised molecules such as oxygen?

6. **Microbial influence on radionuclide migration**
   To what extent, if any, can bacterial dissolution of immobilised radionuclides and production of complexing agents increase radionuclide migration rates?

7. **Alkaliphilic microbes and concrete**
   Do relevant microorganisms survive at pH equivalent to that of repository concrete and can they possibly influence repository performance by concrete degrading activities such as acid production?

The following section summarises the state of art for the above arrayed research tasks.

3 CURRENTLY ACHIEVED RESULTS AND RESEARCH IN PROGRESS

3.1 THE SUBTERRANEAN BIOSPHERE

3.1.1 Evidence for the existence of a deep subterranean biosphere

Diverse and active populations of microorganisms have been observed in most subterranean and sub-seafloor environments investigated (Bachofen 1997), including granitic rock aquifers at depths ranging from 70 m down to 1240 m (Pedersen 1997a, Pedersen and Karlsson 1995). The drilling and excavation to access these microbial ecosystems are vigorous operations and it can be argued that the observed life is an artefact of the access operations (Pedersen 1993a) and not a true deep biosphere. The risk for microbial contamination of the aquifers by the drill water used to cool the drill and transport the drill cuttings out of the borehole during drilling is obvious and investigations were, therefore, undertaken to study this risk. During the end of the construction phase of the Äspö HRL, samples were collected from boreholes in the Äspö HRL tunnel concomitant with geological, hydrological and hydrogeochemical characterisation of designated experimental rock volumes (Winberg et al 1996). 16S rRNA gene sequencing and culturing methods were used to investigate whether a lasting microbial contamination due to the drilling occurred. Samples were collected from the drill water source, the drilling equipment and from the drilled boreholes. This work is described in detail in paper 1.

The results in paper 1 show that total and viable counts of bacteria in drilled boreholes were several orders of magnitude lower than in samples from the drilling equipment, except for sulphate reducing bacteria. A total of 158 bacterial 16S rRNA genes that were cloned from the drill water source, the drilling equipment and the drilled boreholes were partially sequenced. The drilled boreholes generally had a 16S rRNA diversity that differed from what was found in samples from the drilling equipment. Several of the sequences obtained could be identified on genus level as one of the genera *Acinetobacter, Methylophilus, Pseudomonas* and *Shewanella.*

Proving that certain species of microorganisms found in the drilled boreholes are intrinsic and not introduced during drilling is extremely difficult. Instead, as was shown in paper 1, the opposite situation was easier to investigate, i.e. testing if a known contaminating microbial population establishes or not in deep granitic aquifers during drilling. The 600 m long tubing used for drill water supply constituted a source of bacterial contamination to the rest of the drilling equipment and the boreholes. Nevertheless, using molecular and culturing methods, it was shown that although large numbers of contaminating bacteria were introduced in the boreholes during drilling, they did not become established in the aquifers at

detectable levels. Therefore, is seems reasonable to conclude that we find no evidences for lasting microbial contamination of boreholes drilled in granitic rock. The reason for this is the inability of foreign microbes to adapt to the prevailing oligotrophic, reducing, anaerobic and low temperature environmental conditions in deep granitic aquifers. Earlier documentation of in situ activity of microbial populations in deep granitic rock environments suggests that the microbes present are active at low but significant levels (Ekendahl and Pedersen 1994, Pedersen and Ekendahl 1990, 1992a-b). It can, therefore, be speculated that most of the microorganisms found in the new boreholes were present in the intersected aquifers before drilling. Recent findings of bacteria-like fossils in a granitic aquifer 207 m below ground at Äspö (Pedersen et al 1997b) is described in paper 2 and support the hypothesis of a deep and intrinsic subterranean biosphere.

- *The inability of foreign microorganisms to establish in granitic aquifers and the presence of fossil microorganisms in aquifers at 207 m below ground have been observed at Äspö HRL. These observations suggest that granitic rock aquifers are inhabited by microorganisms before drilling and excavation operations. It can consequently be concluded that there is a very high probability for the existence of a deep subterranean biosphere in granitic rock.*

3.1.2 Subterranean microbes and biogeochemical processes

The subterranean biosphere, concluded above to exist, may influence the geochemical situation in many different ways. A full understanding of deep subterranean environments can, therefore, not be achieved until microbial processes are included in models, theories and interpretation of results. This is because microbes catalyse many reactions that, for kinetic reasons, are very slow or not possible at low temperature and pressure. One obvious example is the bacterial reduction of sulphate to sulphide in anoxic waters. 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 potential will be controlled by Fe(II) at or below approximately -100 mV. A third example is methane producing archaea which typically coexist syntrophically with hydrogen producing bacteria that ferment organic material. If the hydrogen concentration increases too much, the decomposition of organic material by these bacteria stops. As methanogens produce methane from hydrogen and carbon dioxide, they remove hydrogen from the environment and the bacterial decomposition of organic material can continue.

Microbial decomposition and production of organic material depend on the sources of energy and electron-acceptors present. Organic carbon, reduced inorganic molecules or hydrogen are possible energy sources in subterranean environments. During microbial oxidation of these energy sources the microbes 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 microbes with hydrogen and short organic acids. As the solubility of oxygen in water is low and because oxygen is the preferred electron acceptor by many microbes utilising organic compounds in shallow groundwater, anaerobic reduced environments and processes usually dominate at depth in the subterranean environment.

- *Microorganisms have the capability to reduce important groundwater components such as sulphate to sulphide and to produce and consume gases. The documented presence of a deep biosphere implies that relevant microbial reactions should be included in the performance assessment for a HLW repository.*

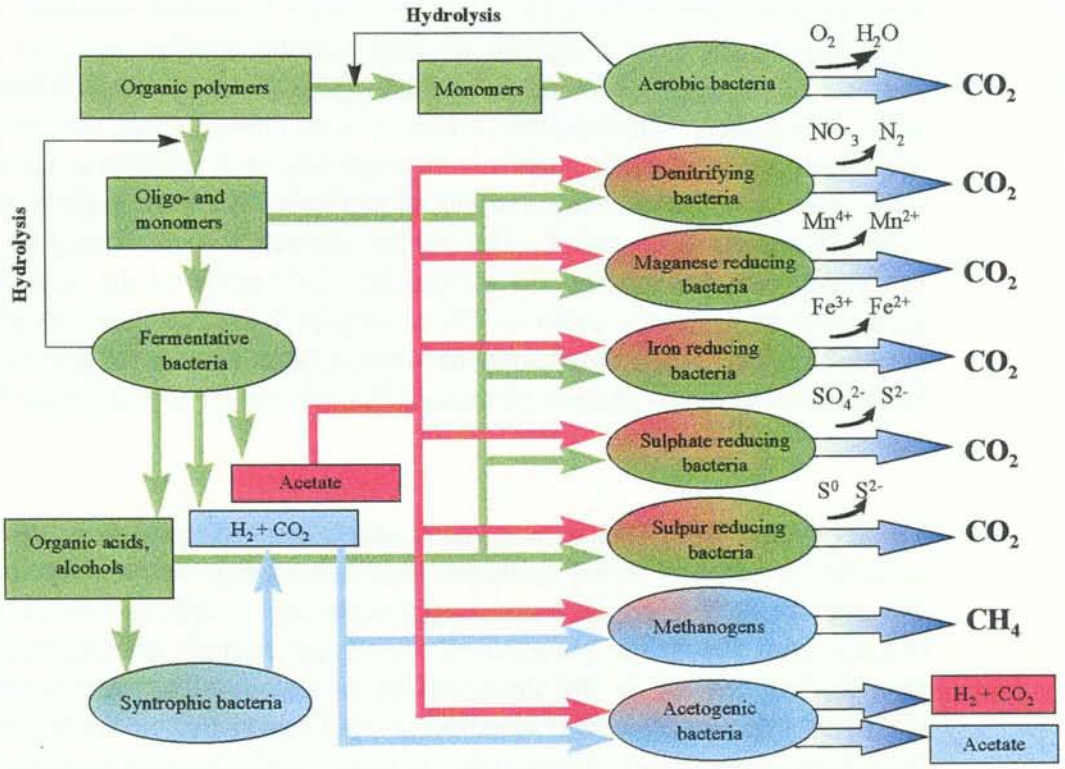


Figure 3.1 *The degradation of organic carbon can occur via a number of different metabolic pathways, characterised by the principal electron acceptor in the carbon oxidation reaction. A range of significant groundwater compounds are formed or consumed during this process. Of great importance for HLW disposal is the production of hydrogen sulphide, a potential copper corrodant and the turnover of gases such as carbon dioxide, hydrogen and methane.*

3.1.3 The hydrogen dependent subterranean biosphere hypothesis

Groundwater at depths of 500 m can be very old and ages of 10,000 years is not unusual. This pose a conceptual problem for the deep subterranean biosphere: What ultimate energy source is it using? Organic carbon from sun driven surface ecosystem would not last for so long time. Typical values at depth are one or a couple of mg dissolved organic carbon per litre

groundwater (Pedersen and Karlsson 1995, Table 1.1) Any energy source at this depth must be renewable. Throughout the work arrayed in Appendixes 2 and 3, results have indicated the presence of autotrophic microorganisms in the studied deep granitic rock environments that utilise hydrogen as a source of energy. Therefore, a hypothesis of a hydrogen driven biosphere in deep granitic aquifers has been suggested (Pedersen 1993b, 1997a, Pedersen and Albinsson 1992). The organism base for this biosphere is suggested to constitute of acetogenic bacteria that have the capability of reacting hydrogen with carbon dioxide to acetate (homoacetogens) and methanogens that yield methane from hydrogen and carbon dioxide (autotrophic methanogens) or from acetate produced by homoacetogens (acetoclastic methanogens) (Fig. 3.3). A similar hypothesis has recently been published for deep basaltic rock aquifers (Stevens and McKinley 1995). One of the aims of our recent studies has, therefore, been to collect evidence for a hydrogen driven deep biosphere in deep granitic aquifers and has been focused on acetogenic bacteria and methanogens as the autotrophic base for such a biosphere. Distribution, numbers and physiological diversity of homoacetogens and methanogens in deep granitic rock aquifers at the Äspö HRL were investigated using a variety of methods. This work is presented in detail in papers 6, 7 and 8. The results showed that methanogens and homoacetogens are present and are metabolically active in the Äspö HRL groundwaters at depths down to 450 m (papers 7 and 8). Pure cultures of autotrophic, rod-shaped methanogens were isolated and one of them could be described as a new species *Methanobacterium subterraneum* (paper 6).

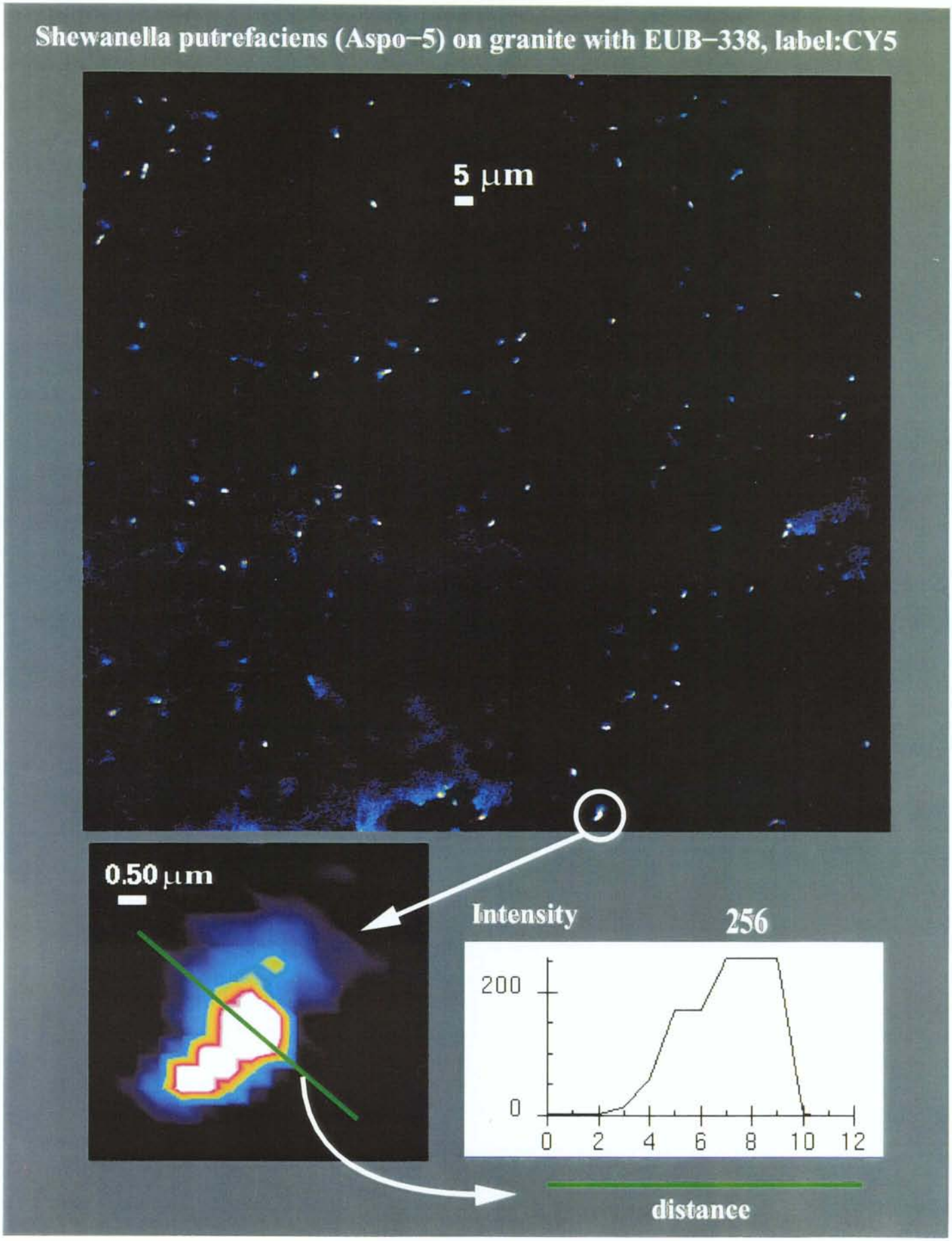
Viable counts using plates and tubes under various general or specific culturing conditions reveal cultivable microorganisms, but usually only a few percent or less of what is detected with total counts can be cultured (Amann et al 1995). The plate versus total count anomaly of environmental samples has led us to the development of non-culturing techniques to overcome this problem, such as extraction and sequencing of the ribosomal 16S rRNA genes (papers 3 and 4) and in situ hybridisation with nucleic acid probes (Fig. 3.2 a). The inability to reveal the large majority of microorganisms present with culturing techniques is of course a major drawback, but presently culturing is required for description of new species (confer papers 5 and 6). This merits the continued use of culturing in microbial ecology. Using the 16S rRNA approach, we found evidence for acetogenic bacteria as being common in Äspö HRL groundwater (paper 1 and 3) and the nucleic acid technique has demonstrated attached methanogens of rock exposed to flowing groundwater from 70 m below ground (Fig. 3.2 b)

- *Microorganisms that can use hydrogen as an energy source and carbon dioxide as their sole carbon source have been found to be active in deep granitic groundwater.*

Figure 3.2 a) *Granite rock coupons were exposed over night to a growing culture of* Shewanella putrefaciens *and washed with a buffer. Subsequent in situ hybridisation with a Cy-5 labelled probe for the domain* Bacteria *(EUB-338), revealed attached bacteria on the surface. The Cy dyes are based on the cyanine fluor and all seven different flours offer intense colours with narrow emission spectra (Amersham LIFE SCIENCE). A Molecular Dynamics 2010 confocal laser scanning microscope equipped with a Kr/Ar laser was used for observation with the software Image Space running on Silicon Graphics UNIX based computers. The hybridisation signal obtained was maximal with virtually no background at all, as can be seen from the intensity diagram reflecting a section over an attached bacterium.*

b) *Small stones of granite were exposed to flowing ground water for 3.5 years from 70 m depth (KR0013A, paper 3, Fig. 1) and in situ hybridised with a Cy-5 labelled probe for the domain* Archaea *(ARC-915). A chain of growing archaeal microorganisms is displayed from top (0 degrees relative to the light path in the microscope), side (90 degrees relative to the light path in the microscope) and "upside down" (180 degrees) views using the image processing program. The scanned depth was 21.6 µm. The depth resolution of the microscope is about three times less than the side resolution which gives the microorganisms a three times too thick appearance in the side view. Considering this artefact, it can be concluded that the observed signal is emitted from a threadlike structure with the size of typical prokaryotic cells growing in chains, presumably a methanogenic species.*

a


Shewanella putrefaciens (Aspo−5) on granite with EUB−338, label:CY5
5 µm
0.50 µm
Intensity
256
200
0
0
2
4
6
8
10
12
distance

b

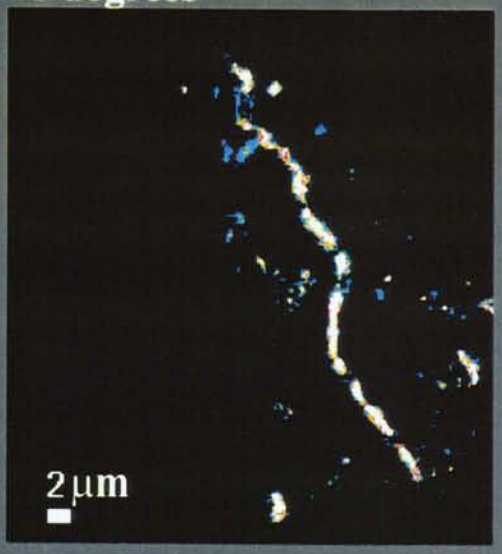
Probe: ARC−915, label:CY5

Sample: Granite rock exposed to flowing ground water from KR0013B during 3.5 years.

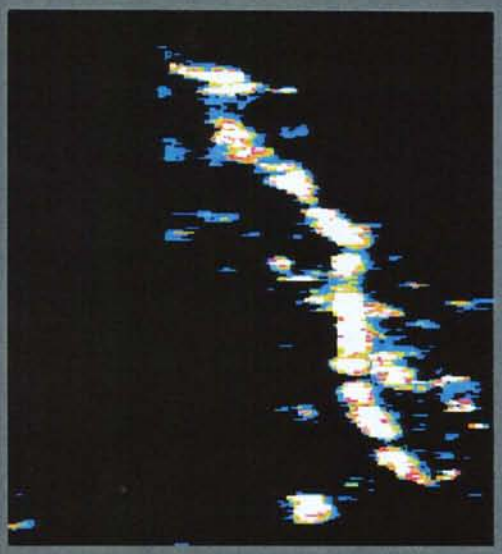
Laser: Attenuation filter = 3% power = 5 mW

Scanned depth: 27 x 0.8 = 21.6 μm

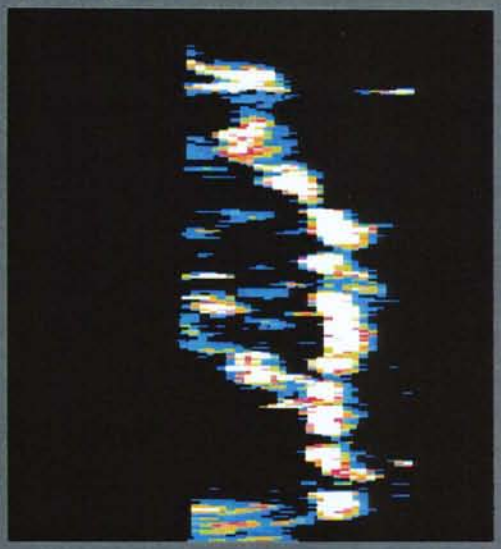
0 degrees



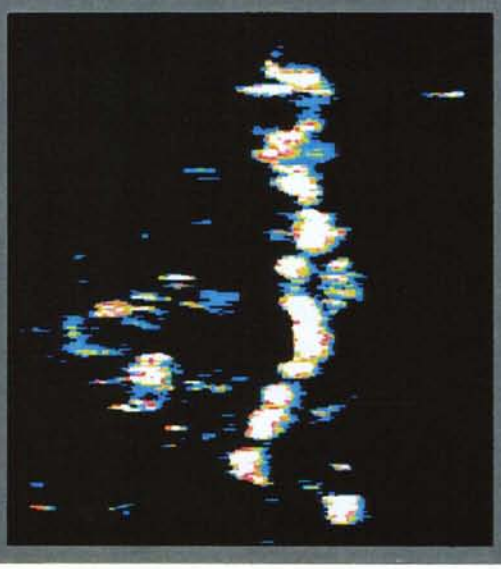
45 degrees



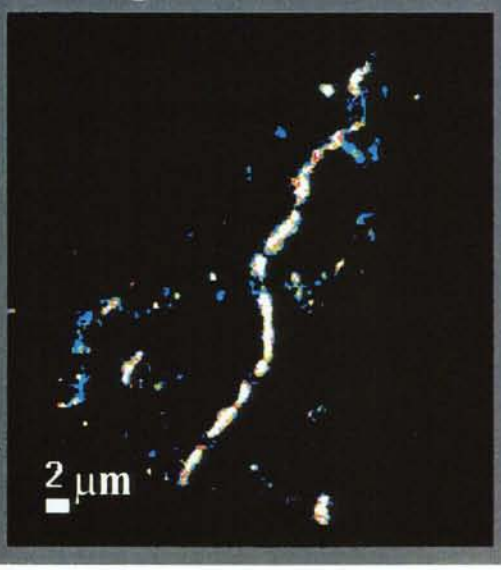
90 degrees



135 degrees



180 degrees



3.1.4 The deep hydrogen driven biosphere

Until recently, it has been a general concept that all life on Earth depends on the sun via photosynthesis; including most of the geothermal life forms found in deep sea trenches as they use oxygen for the oxidation of reduced inorganic compounds (almost all oxygen on earth is produced via photosynthesis). Our result now suggest that a deep subterranean granitic biosphere exists, driven by the energy available in hydrogen formed through radiolysis, mineral reactions or by volcanic activity (Fig. 3.3). Knowledge about this biosphere has just begun to emerge and is expanding the spatial borders for life from a thin layer on the surface of the planet Earth and in the seas to a several kilometre thick biosphere reaching deep below the ground surface and the sea floor. If this theory holds, life may have been present and active deep down in Earth for a very long time and it cannot be excluded that the place for the origin of life was a deep subterranean igneous rock aquifer environment (probably hot with a high pressure) rather than a surface environment.

- *A HLW repository will be situated in a subterranean biosphere that is independent of solar energy and photosyntetically produced organic carbon. The only ultimate limitation will be the availability of hydrogen over time.*

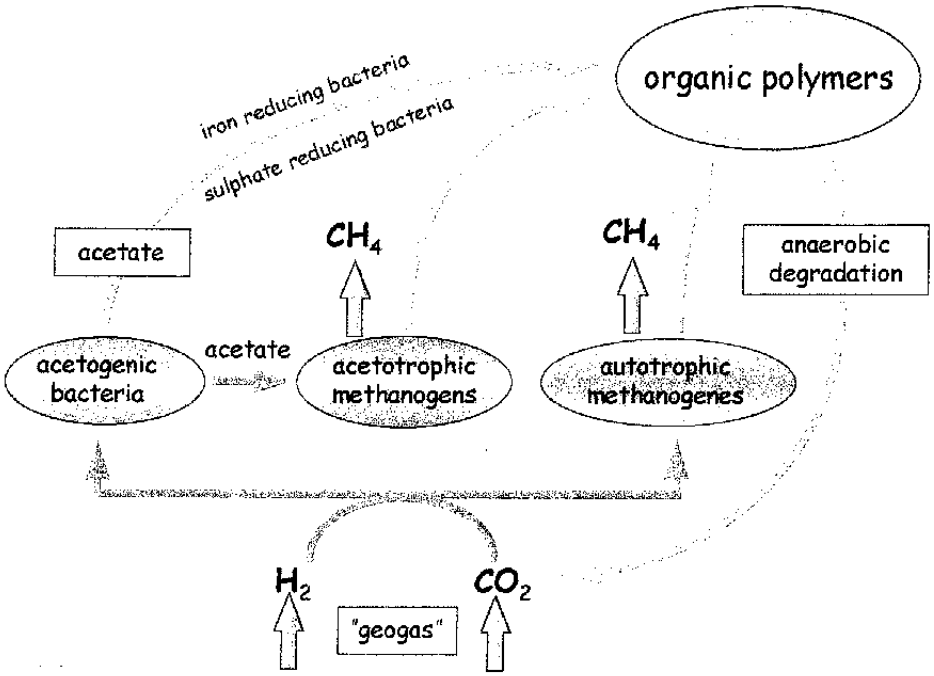


Figure 3.3 *The deep hydrogen driven biosphere hypothesis, illustrated by its carbon cycle. At relevant temperature and water availability conditions, subterranean microorganisms theoretically are capable to perform a life cycle that is independent of sun-driven ecosystems. Hydrogen and carbon dioxide from the deep crust of earth or organic carbon from sedimentary deposits can be used as energy and carbon sources. Phosphorus is available in minerals like apatite and nitrogen for proteins, nucleic acids etc. can be obtained via nitrogen fixation; this gas is predominating in most ground waters (Table 3.1).*

3.1.5 Research task, conclusions and performance assessment relation

The research task:

Is there a deep subterranean biosphere and how does it sustain its life processes? What energy sources and fluxes of energy will be available for microorganisms in and around a HLW repository?

Current conclusions:

- The inability of foreign microorganisms to establish in granitic aquifers and the presence of fossil microorganisms in aquifers at 207 m below ground have been observed at Äspö HRL. These observations suggest that granitic rock aquifers are inhabited by microorganisms before drilling and excavation operations. It can consequently be concluded that there is a very high probability for the existence of a deep subterranean biosphere in granitic rock.

- Microorganisms have the capability to reduce important groundwater components such as sulphate to sulphide and to produce and consume gases. The documented presence of a deep biosphere implies that relevant microbial reactions should be included in the performance assessment for a HLW repository.

- Microorganisms that use hydrogen as an energy source and carbon dioxide as their sole carbon source have been found to be active in deep granitic groundwater.

- A HLW repository will be placed in a subterranean biosphere that is independent of solar energy and photosyntetically produced organic carbon. The only ultimate limitation will be the availability of hydrogen over time.

Current research:

Work is ongoing at Äspö HRL and elsewhere with the aim to collect additional evidence for a deep biosphere. The *in situ* rates of microbial activities are of particular interest, presently, because data on the turnover of substrates to products are necessary for correct modelling of various microbial effects on repository performance.

Performance assessment relation for the deep subterranean biosphere:

The HLW environment and the surrounding rock will not be sterile. Microorganisms will, at various rates, be active in biogeochemical processes of which several do not occur without them. They may influence the performance of a HLW repository in negative, neutral and positive ways. Six major influence areas are discussed below.

3.2 MICROBIAL PRODUCTION AND CONSUMPTION OF GASES

3.2.1 Dissolved gas in groundwater

The content of gases in deep groundwater that can be produced or consumed by microorganisms have been analysed earlier, but the method used did not separate hydrogen from helium (Table 3.1). The deep biosphere hypothesis depend totally on the presence of hydrogen in deep groundwater. We have, therefore, recently invested significant work in the measurement of dissolved gases in deep granitic rock environments, now including hydrogen. The technique developed is described in detail in appendix 1. Recent result on dissolved gas, analysed with this method, has been done for the boreholes denoted KA3005, KA3010 and KA3110 (paper 3, Fig. 1) and the results are presented in Table 3.1. It can be seen that the total amount of gas found differs at most 3 times between boreholes and sites. The sensitivity and reproducibility of the new gas extraction and analysis methods are good and hydrogen could be detected. Each of the found gases are discussed in more detail below, from a microbiological perspective.

Table 3.1 *The content of nitrogen, hydrogen, helium and carbon-containing gasesand the total volumes of gas extracted from groundwater samples of the Stripa borehole V2, the Laxemar borehole KLX01 and the Äspö boreholes KR0012, 13 and 15 (Pedersen 1993b, Pedersen and Ekendahl 1992a-b). The boreholes KA3005, KA3010 and KA3110 was sampled during 1997 and the data has not been published yet, see appendix 1 for details.*

Boreholes	Sampling depth (m)	N_2 (µL L^{-1})	H_2 (µL L^{-1})	He (µL L^{-1})	CO (µL L^{-1})	CO_2 (µL L^{-1})	CH_4 (µL L^{-1})	C_2H_6 (µL L^{-1})	[a] C_2H_{2-4} (µL L^{-1})	Total gas (µL L^{-1})
Stripa										
V2	799-807	25000	n.a[b].	<10	<1	32	245	0.3	<0.1	25277
V2	812-821	31000	n.a	<10	<1	11	170	0.6	<0.1	31181
V2	970-1240	24500	n.a	<10	<1	10	290	2.9	<0.1	24803
Laxemar										
KLX01	830-841	46500	n.a	4600	0.5	460	26	<0.1	<0.1	51586
KLX01	910-921	37000	n.a	3500	0.1	500	27	<0.1	<0.1	41027
KLX01	999-1078	18000	n.a	2450	0.7	1600	31	<0.1	<0.1	22082
Äspö HRL										
KR0012	68	22000	n.a	40	0.1	6050	1030	<0.1	0.1	29120
KR0013	68	25000	n.a	110	0.2	9640	1970	<0.1	0.1	36720
KR0015	68	22000	n.a	64	0.1	15037	4070	<0.1	0.1	41171
KA3005/2[c]	400	25930	1.68	1757	<1	1082	1715	<0.1	<0.1	32300
KA3005/4	400	26661	0.11	3809	<1	2100	1849	<0.1	<0.1	34419
KA3010/2	400	40626	30.96	7946	1.4	142	55	<0.1	<0.1	48801
KA3110/1	414	14861	14.50	448	<1	1832	925	<0.1	<0.1	18080

[a] The content of $C_2H_2 + C_2H_4$

[b] not analysed

[c] number after slash denotes sampled borehole section

Nitrogen

Nitrogen is the by far most dominating gas in all samples analysed (Table 3.1). Some of the nitrogen may have been dissolved from air in rain and surface waters that become groundwater with time, but the solubility of nitrogen at 10°C and atmospheric pressure is 19.6 ml l^{-1}. Most of the nitrogen values in Table 3.1 exceeds this solubility limit and other sources of dissolved nitrogen to groundwater must exist as well. Nitrogen can be used by nitrogen fixing bacteria as a source of nitrogen and many produce nitrogen from nitrate during an anaerobic respiration process called denitrification (Pedersen and Karlsson 1995, Fig. 3.3). Microbial processes could contribute to the pool of dissolved nitrogen in groundwater through denitrification processes, but it is unknown if this occurs in enough amounts to explain the excess of dissolved nitrogen.

Helium

Helium is a noble gas and it is not produced or consumed by microorganisms.

Carbon dioxide

All living and active organisms expels carbon dioxide from their degradation of organic material and many microorganisms and all plants and algae can transform carbon dioxide to organic carbon (Pedersen and Karlsson 1995, Fig. 2.6). The concentration of this gas may, therefore, be influenced by microorganisms with the pertaining effects it may have on the carbonate system, pH and on mineral precipitation and dissolution.

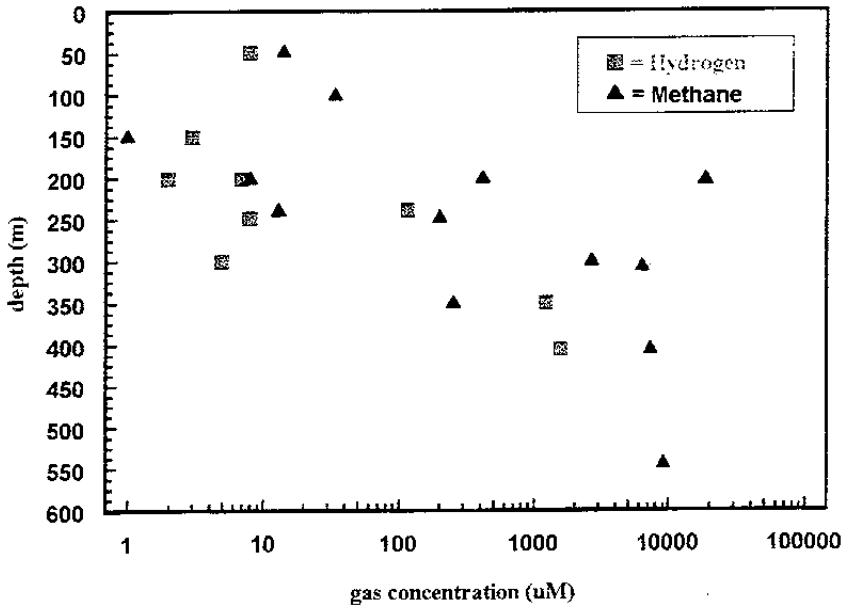


Figure 3.4 *The concentrations of hydrogen and methane in groundwater from the Fennoscandian shield in Finland. Data are from Sherwood-Lollar et al (1993).*

Hydrogen

The suggested deep biosphere hypothesis requires hydrogen as its energy base. Hydrogen is expected to act as an inert gas in most geochemical systems and it is therefore usually overlooked and not analysed for. Some data on hydrogen in hard rock were published earlier (Sherwood Lollar et al 1993a-b). From 2.2 up to 1574 μM hydrogen in groundwater from Canadian shield and Fennoscandian shield rocks were found as shown in Figure 3.4. The origin of such hydrogen can vary. Most granitic rocks show a low but significant radioactivity which can generate hydrogen by radiolysis of water. Anaerobic mineral reactions (e.g. anaerobic corrosion of iron) will also create hydrogen (Stevens and McKinley 1995). Finally, deep volcanic gases contain hydrogen. Screening the Äspö HRL groundwater for hydrogen with a simple "closed bottle head space" method revealed significant amounts of hydrogen in most samples analysed (Fig. 3.5 and paper 8). The sampling and extraction method described in appendix 1 confirmed that hydrogen is present (Table 3.1) and consequently, there is an energy base available for the deep biosphere.

Methane

Methane occurs frequently in subterranean environments all over the globe. Evidence for an ongoing methane generating process in deep Swedish granite has been published (Flodén and Söderberg 1994, Söderberg and Flodén 1991, 1992). Pockmarks in Baltic sea sediments were found, indicating gas eruption from fracture systems in the underlying granite, mainly of methane. From 1.3 up to 18576 μM of methane in groundwater from Canadian shield and Fennoscandian shield rocks have been published (Fig. 3.4). Concentrations from 1 up to 181 μM of methane in Swedish groundwater have been published previously (Table 3.1). Recent data indicate up to 720 μM of methane down to 440 m depth at Äspö HRL (Fig. 3.5). The stable carbon isotope profile is commonly used as an indication of a biogenic origin of the methane. Some results on the $^{13}C/^{12}C$ signatures indicate biogenic origin of the Äspö methane (Banwart et al 1996).

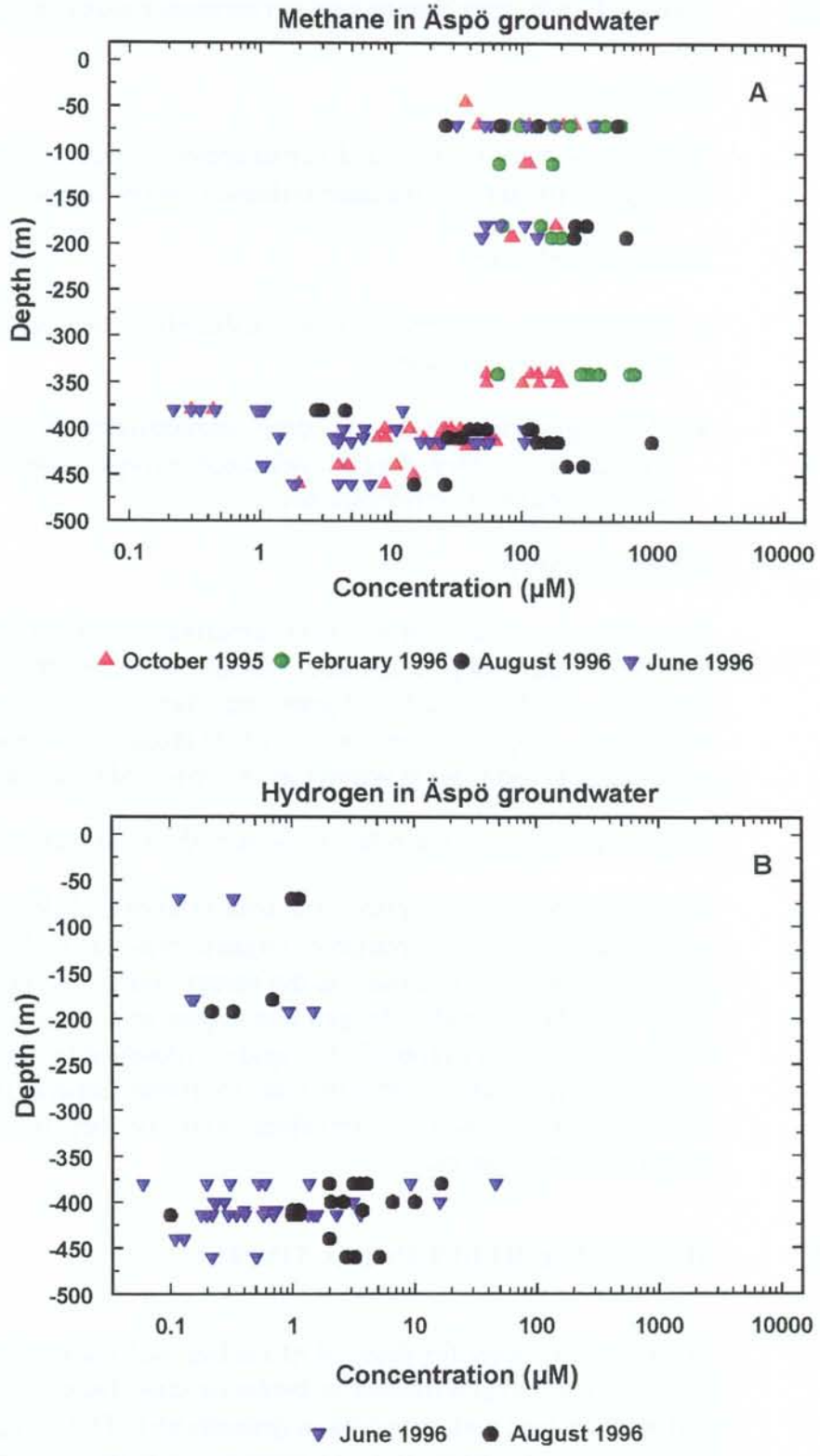


Figure 3.5 *Data from repeated sampling expeditions on the concentration of hydrogen and methane in Äspö groundwater, measured in the head space of closed water samples and re-calculated to dissolved hydrogen and methane.*

3.2.2 Research task, conclusions and performance assessment relation

The research task:

Will bacterial production and consumption of gases like carbon dioxide, hydrogen, nitrogen and methane influence the performance of repositories?

Current conclusions:

- Hydrogen is necessary for an active deep biosphere and it can be found in most deep groundwaters.
- Major gas components in deep groundwater are helium, nitrogen, methane and carbon dioxide. All these, except helium, can be produced or consumed by microorganisms.

Current research:

More data is needed on the concentrations of microbially transformable gasses and gas samples are continuously collected from Äspö HRL and analysed. Another question of great importance is to determine if there is a continuous supply or production of hydrogen in various subterranean environments and if so, at which rate is consumed hydrogen replenished?

Performance assessment relation for microbial gas transformations:

The deep biosphere organisms will tend to keep a HLW repository reduced as long as hydrogen is available. Organic material will be produced from carbon dioxide with hydrogen as the energy source and eventually degraded again to carbon dioxide. Oxygen that appear in a repository during closure or via radiolysis will probably be rapidly consumed by microorganisms that oxidise methane and hydrogen (and available organic material as well). Such microbial gas transformations may be beneficial for the HLW repository performance.

3.3 MICROBIAL REDUCING ACTIVITY

The present concept for disposal of nuclear fuel waste (Fig. 1.1) includes a scenario with oxygen trapped in bentonite clay, backfills, in pockets of the surrounding rock and dissolved in groundwater. This oxygen is detrimental for the copper canisters and will increase the mobility of several long-lived radionuclides. It has been calculated that oxygen will have disappeared within 300 years time due to inorganic reactions (Wersin et al 1994). Several anaerobic microorganisms utilise hydrogen efficiently and many more can use hydrogen and methane if oxygen is available. Adding the catalytic abilities of microorganisms to the modelling of oxygen disappearance may therefore reduce the inorganic 300 years scenario to an organic scenario lasting not more than months or a couple of years.

- *Adding the catalytic abilities of microorganisms to the modelling of oxygen disappearance may reduce the inorganic 300 years scenario to an organic scenario lasting not more than months or a couple of years.*

The ability of microorganisms to buffer against an oxidising disturbance in bentonite, backfill and the deep environment is a rather overlooked possibility until now. The presence of an active and diversified microbiota at repository depths is well documented, as is the reducing capacity of microorganisms in surface environments. The major redox buffers possible to use are methane and organic carbon. Additionally can hydrogen, sulphide and ferrous iron contribute but these compounds generally appear in much lower concentrations than methane and organic carbon.

A variety of bacteria, the methanotrophs, oxidise methane readily with oxygen, utilising it as an electron donor for energy generation and as a sole source of carbon. Most of these bacteria are aerobes and they are widespread in nature soils and water. They are also of a diversity of morphological types, seemingly related only in their ability to oxidise methane. They are found wherever stable sources of methane are present. There is some evidence that although methane oxidisers are obligate aerobes, they are sensitive to oxygen and prefer microaerophilic habitats for development. They are therefore often found concentrated in a narrow band between anaerobic and aerobic zones were methane meets an oxygenated system. Such environments will be common in future repositories during the open phase and for some time after closure. Once established, this group of bacteria will be active as long as there is oxygen present for the oxidation of methane and they will most probably react all available methane with remaining oxygen after closure. Consequently, a deep repository will rapidly go anoxic after closure if methane is in excess. Recent DNA results on the microbial diversity during drilling of boreholes in the Äspö HRL tunnel revealed 16S rRNA sequences closely related to methanotrophs (paper 1). One molecule of methane (CH_4) contains 8 electrons that can be used to reduce 2 molecules of oxygen (O_2). In the worst case scenario, there will be approximately 250 μM dissolved oxygen in groundwater close to the repository which can be balanced by 125 μM methane. Consulting Fig. 3.5 reveals that the standing concentration of methane in many groundwaters can reduce all oxygen assuming a 1:1 mixing ratio. The time needed for this process depends on the bacterial activity, but it will probably take much less than a year as most microbes work very fast when given a possibility to proliferate.

It has been demonstrated that microbial organic carbon oxidation is responsible for keeping shallow groundwater reduced (Banwart et al 1996). It has also been shown (preliminary data by Linköping Univ, not published) that the content of TOC decreases with depth at Äspö from some 10-20 mg l^{-1} down to about 1 mg l^{-1} at 400 m depth. The percent humic and fulvic acids of TOC decreased from 90% to 30-40% and the percent of hydrophilic acids increased with depth. It was also found that the organic concentration correlated with the concentration of chloride. The traditional model on the

fate of organic carbon suggests degradation of humic and fulvic acids with depth, but the deep biosphere hypothesis suggests that the organic carbon is produced at depth with hydrogen as the energy source. Most present organic carbon, except humic substances, will be readily oxidised if oxygen is present.

A project denoted MICROBE-REX is ongoing and will be closed in August 1998. The aim is to establish *in situ* microbial oxygen removal at various conditions relevant to a HLW repository. Figure 3.6 shows that the potential for microbial oxygen removal is significant indeed.

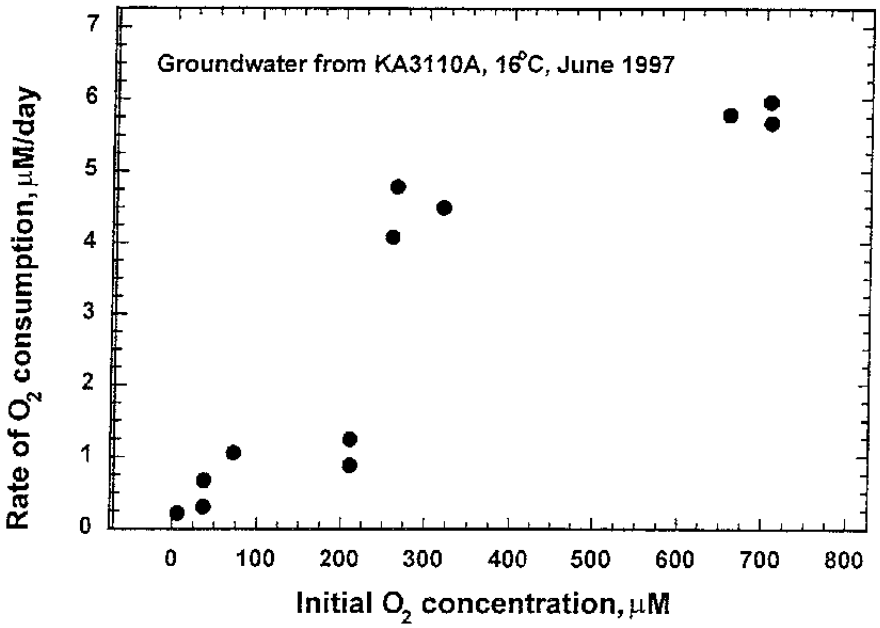


Figure 3.6 *Preliminary data on rates of microbial oxygen removal at various start concentrations of oxygen. The data have been generated during a first field campaign summer 1997. Although provisional, the data imply that there is a very significant potential for microbial oxygen removal in the Äspö HRL tunnel environment and thereby also in a HLW repository.*

3.3.1 Research task, conclusions and performance assessment relation

The research task:

Will bacterial oxygen consumption significantly contribute to oxygen removal from a HLW and to what extent may bacterial production of reduced compounds such as organic material, methane, sulphide and ferrous iron contribute to keeping the repository host rock reduced?

Current conclusion:

- Recently obtained data now conclusively demonstrate that significant microbial oxygen consumption will occur in a HLW repository.

Current research:

A project denoted MICROBE-REX is ongoing and will be closed in August 1998. The aim is to establish in situ microbial oxygen removal rates at various conditions relevant to a HLW repository.

The Oklo Phase II and the Palmottu Phase I European Commission projects (Pedersen and Karlsson 1995, section 4.7) include investigations of the effect from microbes on the behaviour of radionuclides in natural environments. In the field, the microbial reducing capacity will be evaluated and compared to observations of the mobility of radionuclides.

Performance assessment relation for microbial reducing activity:

The presence of active microorganisms in a HLW repository cannot be avoid and may be very beneficial for obtaining reducing conditions rapidly there. Microbial activity will thereby have a positive influence on the performance of a HLW repository and reduce the risk for oxygenic copper corrosion of the canisters.

3.4 MICROBIAL CORROSION OF COPPER

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. Gaseous compounds are mainly of interest in performance assessment because, if generated at a enough high rate, they may form a separate gas phase that exerts a pressure on the construction and add on to the dispersion of contaminants.

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. Microbial corrosion was treated extensively in SKB TR 95-10 (Pedersen and Karlsson 1995, section 5.4, 168-174) and will not be discussed here. It was concluded that at least two limiting factors have to be considered: 1) the supply of substrate and 2) the question whether the reaction of sulphate reduction can occur in the bentonite or only outside. Considering the first factor, recent studies have pointed to the possibility that other substances such as methane and hydrogen may act as electron donors in addition to organic material.

Direct and indirect evidence for the presence and activity of sulphate reducing bacteria in deep geological formations are reported in papers 1, 3 and 5 and by Laaksoharju et al (1995). Obviously, the anticipated risk for sulphide production at disposal depths is relevant and must be accounted for in the safety performance assessment. Two scenarios must be assessed: In

the first scenario, sulphate reducing bacteria will grow in the surrounding geological formation and produce hydrogen sulphide that must diffuse through the buffer to corrode the copper canister. This case has been thoroughly discussed elsewhere (Pedersen and Karlsson 1995, section 5.4, 168-174). It was concluded that if sulphide is generated by microbes outside the buffer, somewhere in the near-field, it will have to be transported to the buffer surface and diffuse to the canister. The transport resistance at the interface between buffer and flowing water in rock fractures was concluded to be efficient and less corrosion will be obtained if the sulphide production occurs away from the canister surface compared to if it occurs in the buffer. In the second scenario, sulphate reducing activity will occur inside the bentonite and although the slow diffusion of hydrogen in compacted bentonite is an important barrier which will considerably limit sulphide production and corrosion, the actual sulphide production rate must be assessed.

In a first approach, a full scale nuclear fuel waste disposal container experiment was carried out 240 m below ground in the underground granitic rock research laboratory in Canada (paper 10). An electric heater was surrounded by buffer material composed of sand and bentonite clay and provided heat equivalent to what is anticipated in a Canadian type nuclear fuel waste repository. During the experiment, the heat caused a mass transport of water and gradients of moisture content developed in the buffer ranging from 13 % closest to the heater to 24 % at the rock wall of the deposition hole. Upon decommissioning after 2.5 years, microorganisms could be cultured from all samples having a moisture content above 15% but not in samples with a moisture content below 15% corresponding to a water activity (a_w) of approximately 0.96. The results suggested that a nuclear fuel waste buffer will be populated by active microorganisms only if the moisture content is above a value where free water is available for active life, i.e. $a_w \geq 0.96$.

- *A nuclear fuel waste buffer will be populated by active microorganisms only if the moisture content is above a value where free water is available for active life, i.e.* $a_w \geq 0.96$.

In a second approach, sodium bentonite (MX-80) was inoculated with two species of sulphate reducing bacteria and compacted to three different densities, 1.5, 1.8 and 2.0 g cm^{-3} by means of a hydraulic press and incubated at 30°C (paper 9). These densities correspond to water activities of 1.0, 0.99 and 0.96, respectively. All samples were incubated at 30°C for 1 or 60 days. The amount of water available in the bentonite significantly influenced the survival of the studied sulphate reducing bacteria. Both strains were 100% non-viable after 1 d at the lowest a_w studied, 0.96. The dry conditions at this density of 2 g cm^{-3} effectively killed 100 million sulphate reducing bacteria per g bentonite in less than 24 h. The best survival was observed in the bentonite with an a_w of 1.0, but the survival differed markedly between the species. About 10% of the initial population of *D. baculatum* survived for 60 days, but *Desulfovibrio sp* did not survive

at all after this time. Limitation in nutrients and energy sources, accumulation of hydrogen sulphide and interference of the redox potential may add constrains to a closed batch system like the one used here. Therefore, this work has been followed by field experiments and additional laboratory tests that are in progress and will be in reported towards the end of 1998.

3.4.1 Research task, conclusions and performance assessment relation

The research task:

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 surrounding the canisters? Can bacterial sulphide production in the surrounding rock exceed a performance safety limit?

Current conclusions:

- Sulphide producing microorganisms are active in environments typical for a Swedish HLW repository and the potential for microbial corrosion of the copper canisters must be considered.
- The bentonite buffer around the copper canisters will be a hostile environment for most microbes due to the combination of radiation, heat and low water availability. Discrete microbial species can coup with each of these constrains and it is theoretically possible that sulphide producing microbes may be active inside a buffer, although the experiments conducted this far have shown the opposite.

Current research:

Long term tests of buffer materials is ongoing at Äspö HRL. As part of these investigations, microbes have been added to bentonite that is exposed to relevant repository conditions. A series of laboratory experiments have been designed to follow the survival and activity of different microorganisms in compacted bentonite at conservative conditions. The effect from varying water availability on microbial activity and survival in bentonite will be studied as well as the ability of microorganisms to migrate through a compacted buffer.

Performance assessment relation for microbial corrosion:

Microbial corrosion is an important process to consider in the performance assessment of a radioactive waste repository because the canisters used 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. Sulphide producing bacteria are present and active at HLW repository depth and possible places and extent of their production must be considered.

3.5 MICROBIAL RECOMBINATION OF RADIOLYSIS PRODUCTS

In the event of high level waste (spent fuel) becomes exposed to groundwater, ionising radiation, for example alpha particles emanating from exposed HLW, can split the water molecules and thereby produce hydrogen and oxidising species (oxygen and hydrogen peroxide). The case of microbial recombination of radiolysis products was thoroughly discussed in SKB TR 95-10 (Pedersen and Karlsson 1995, section 2.4 and 5.6) and will not be discussed here.

3.5.1 Research task, conclusions and performance assessment relation

The research task:

Will bacterial recombination of radiolysis products significantly contribute to the removal of unwanted oxidised molecules such as oxygen?

Current conclusions:

- Microorganisms have the capability to enzymatically recombine radiolysis oxidants formed by radiation of water.

Current research:

There is not any research ongoing on this task at Göteborg University presently.

Performance assessment relation for recombination of radiolysis products:

Microbial recombination of radiolysis products will add to inorganic processes and, as it is a catalytic process, microbial recombination will be continuous.

3.6 MICROBIAL INFLUENCE ON RADIONUCLIDE MIGRATION

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 1990). 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. 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 microorganisms and microbial complexing agents constitute important factors in the evaluation of how radionuclides may travel to the terranean biosphere.

- *The content of microorganisms and microbial complexing agents constitute important factors in the evaluation of how radionuclides may travel to the terranean biosphere.*

The presence of microorganisms can influence the groundwater transport of radionuclides in different ways. Free-living microorganisms 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 microorganisms. On the other hand, if the majority of the microorganisms are growing in biofilms on fracture surfaces, transport of radionuclides may be reduced. Finally, microbial production of complexing agents and other metabolites can affect speciation and thus mobility of radionuclides independent of whether the microorganisms are attached or not.

- *Microbial production of complexing agents and other metabolites can affect speciation and thus mobility of radionuclides.*

See SKB TR 95-10 (Pedersen and Karlsson 1995, section 5.7) for a more detailed discussion on the potential for microbial migration of radionuclides.

3.6.1 Research task, conclusions and performance assessment relation

The research task:

To what extent, if any, can microbial dissolution of immobilised radionuclides and production of complexing agents increase radionuclide migration rates?

Current conclusions:

- The relevance of microbial effects on 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 has lead to fundamental questions about subterranean

microbiology and the interaction between microbes and radionuclides. Numbers, species and activities of subterranean microbial populations in Swedish granitic rock have been determined. It has earlier been concluded that the migration of radionuclides due to sorption on microorganisms can be neglected (Pedersen and Karlsson 1995, section 5.7.1).

- The influence from microbially produced complexing agents remains to be studied at realistic conditions in deep groundwater. This research task would require a designated site for microbiology at the Äspö HRL.

Current research:

There is not any ongoing research on this task at Göteborg University, presently.

Performance assessment relation for microbial influence on radionuclide migration:

Dissolution and transport with the groundwater is by far the most important migration mechanism for radionuclides, if released from an underground HLW repository. Microorganisms and microbial complexing agents may constitute important factors in the assessment of how radionuclides may travel to the terranean biosphere.

3.7 ALKALIPHILIC MICROBES AND CONCRETE

Many radioactive waste repository concepts envisage the use of large quantities of cement and cement-based materials which will create environments with initial pH values of up to 13.5. The natural springs of the Maqarin area in NW Jordan contain highly alkaline groundwater, with pH values as high as 12.9, occurring within an organic-rich marl formation (Khoury et al 1992). This environment is, therefore, regarded as a natural analogue for the study of processes that may take place in the hyperalkaline parts of low and intermediate radioactive waste repositories (Miller et al 1994). An earlier investigation has indicated the presence of microorganisms in the alkaline groundwater of Maqarin by the use of culturing techniques for the assessment of numbers and types of bacteria (Coombs et al 1994). A diversified microbial population was found with a pH tolerance in cultures up to the highest pH studied, pH 11. Paper 11 describes how molecular methods, microscopy, culturing techniques and chemical analysis were used in an attempt to study the culturability and diversity of microbial populations detected in the hyperalkaline ground water of Maqarin.

3.7.1 Research task, conclusions and performance assessment relation

The research task:

Do relevant microorganisms survive at pH equivalent to that of repository concrete and can they possibly influence repository performance by concrete degrading activities such as acid production?

Current conclusions:

- Microorganisms were found in all of the Maqarin groundwaters but it could not be conclusively demonstrated that they were *in situ* viable and growing, rather than just transported there from neutral groundwater.
- The diversity of the found microorganisms was similar to what has been detected with the 16S rRNA gene sequencing method earlier, but none of the sequences found were typical for known alkaliphilic organisms.
- A possible hypothesis based on the obtained results is that the investigated Maqarin springs may be a bit too extreme for active life even for the most adaptable microbe - but this remains to be demonstrated.

Current research:

There is not any ongoing research on this task at Göteborg University, presently.

Performance assessment relation for alkaliphilic microbes and concrete:

Some types of bacteria produce acids in their metabolism and may, therefore, be corrosive to concrete if they can survive the extreme pH.

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PAPER 1

Investigation of the potential for microbial contamination of deep granitic aquifers during drilling using 16S rRNA gene sequencing and culturing methods

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CONTENTS

1 ABSTRACT

Total number of bacteria, viable counts of aerobic and anaerobic heterotrophic bacteria and 16S rRNA gene diversity were investigated during drilling of three boreholes in the walls of the Äspö hard rock laboratory tunnel, at depths ranging from 380 to 446 m below sea level. Water samples were taken from the drill water source, the drilling equipment and from the drilled boreholes. The drill water was kept under nitrogen atmosphere and all equipment was steam cleaned before the start of a new drilling. Total and viable counts of bacteria in the drilled boreholes were several orders of magnitude lower than in the samples from the drilling equipment, except for sulphate reducing bacteria. A total of 158 16S rRNA genes that were cloned from the drill water source, the drilling equipment and the drilled boreholes were partially sequenced. The drilled boreholes generally had a 16S rRNA diversity that differed from what was found in samples from the drilling equipment. Several of the sequences obtained could be identified on genus level as one of the genera *Acinetobacter, Methylophilus, Pseudomonas* and *Shewanella.* In conclusion, the tubing used for drill water supply constituted a source of bacterial contamination to the rest of the drilling equipment and the boreholes. The results show, using molecular and culturing methods, that although large numbers of contaminating bacteria were introduced to the boreholes during drilling, they did not establish in the borehole groundwater at detectable levels.

Key words: 16S rRNA, granite, drilling, Äspö hard rock laboratory.

2 INTRODUCTION

Diverse and active populations of microorganisms have been found in granitic rock aquifers at depths ranging from 70 m down to 1240 m (Ekendahl and Pedersen, 1994; Pedersen and Ekendahl, 1990; Pedersen et al, 1996a; Pedersen, 1997). The drilling and excavation to access these microbial ecosystems are vigorous operations, and it can be argued that the observed life is an artifact of the access operations (Pedersen, 1993a). The risk for microbial contamination of the aquifers by the drill water used to cool the drill and transport the drill cuttings out of the borehole during drilling is obvious. The use of culturing methods alone for the control of such contamination is not enough due to the well documented plate versus total count anomaly of environmental samples (Amann et al, 1995). Non-culturing techniques, such as extraction and sequencing of the ribosomal 16S rRNA genes (Ekendahl et al, 1994; Pedersen, 1996; Amann et al, 1995; Pedersen et al, 1996a) can be applied to overcome this problem.

The Äspö hard rock laboratory (HRL) has been constructed as a part of the development of the Swedish concept for deep geological disposal of spent nuclear fuel and the work has been divided into three phases; the pre-investigation (1986-1990), the construction (1990-1995), and the operating (1995-) phases (Pedersen, 1996). The work presented here was performed during the end of the construction phase and the samples were collected from boreholes in the Äspö HRL tunnel during the geological, hydrological and hydrogeochemical characterization of designated experimental rock volumes (Winberg et al, 1996). This paper describes how 16S rRNA gene sequencing and culturing methods were used to investigate whether a lasting microbial contamination occurred in boreholes drilled in the granitic bedrock of the Äspö HRL tunnel. Samples were collected from the drill water source, the drilling equipment and from the drilled boreholes. Total numbers of bacteria, viable aerobic and anaerobic plate counts and most probable numbers (MPN) of sulphate reducing bacteria (SRB) were performed in parallel with the analysis of 16S rRNA gene diversity.

3 MATERIALS AND METHODS

3.1 DESCRIPTION OF THE SELECT SITE AND DRILLING PROCEDURES

The Äspö HRL is located at the Baltic coast under the Äspö island, in the vicinity of the Simpevarp nuclear power plant north of Oskarshamn, SE Sweden. The tunnel has a total length of 3,600 m, is approximately 5 x 5 m (height x width) and proceeds down with an inclination of about 14%. It starts at the coast line and continues about 1,700 m under the sea floor where it spirals down to 460 m below sea level under the Äspö island (Pedersen, 1997; Pedersen et al, 1996a). Microbiological data from boreholes in the tunnel down to tunnel length 1,420 m and in surrounding surface boreholes have been published earlier (Pedersen and Ekendahl, 1990 ; Pedersen et al, 1996a ; Pedersen and Ekendahl, 1992b).

Eight boreholes from tunnel length from 2,858 m to 3,385 m were drilled for a project denoted SELECT, aiming at the geological, hydrological and hydrogeochemical characterization of designated experimental rock volumes for redox and tracer retention experiments. Detailed information about the SELECT borehole set-up etc. can be found elsewhere (Winberg et al, 1996). Three of these boreholes were studied in detail; KA2858A, KA3005A and KA3105A (Table 1). A borehole denoted HD0025A was drilled from a side vault of the tunnel at tunnel length 3,200 m and was used as drill water supply for drilling of the SELECT boreholes (Table 1). The possibility to use surface water as drill water was considered during the planning process but was abandoned due to the obvious risk for introduction of oxidising water into the rock aquifers. Part of the SELECT site will be used for studies

on the reducing capacity of fracture minerals and microorganisms, and oxygenated water would have spoiled these experiments. The drill water was funneled from HD0025A to two 1.6 m^3 plastic containers where the water was continuously kept under nitrogen atmosphere to prevent introduction of oxygen. The two drill water containers were used interchangeably. The length of the tubing used was approximately 600 m. The drilling equipment can ideally be regarded as a homogenous and totally mixed water system, but in reality, sampling different sites of this equipment at one occasion may still give some variation in the results. For example, the water from the tubing is mixed with water already present in the containers and a deviation in water chemistry and bacterial numbers in the tubing will not immediately result in a similar deviation in the containers.

Table 1 *Drilling and 16S rDNA sampling schedule for the SELECT boreholes and the drilling equipment. The borehole HD0025A was used as the source of drill water during drilling of the other boreholes.*

Sampling date	Sampled site	Section sampled (meter)	16S rDNA extraction and sequencing	Drilled borehole
94-11-30	HD0025A	0-17.0	x	KA3005A
94-12-14	HD0025A	0-17.0	-	KA3105A
	Container	n.r.[a]	x	KA3105A
95-01-17	HD0025A	0-17.0	x	KA2858A
	Tubing	n.r.	x	KA2858A
	Container	n.r.	x	KA2858A
	Machine	n.r.	x	KA2858A
	KA3105A	0-70	x	n.r.
95-02-02	KA2858A	0-59.7	x	n.r.
96-06-27	KA2858A:2	39.8-40.8	x	n.r.
	KA3105A:3	17.0-19.5	x	n.r.
	KA3105A:4	22.5-24.5	x	n.r.

[a] Not relevant

A fluorescent dye, uranine, was added to the drill water in the containers to allow detection of the amount of drill water in the collected water samples. The fluorescence of the drill water was compared to the fluorescence of the sampled groundwater and the percentage of drill water left in the aquifer groundwater, the drill water contamination, could then be calculated (Table 2).

Table 2 *Borehole information with groundwater chemistry data and drill water contamination values.*

Borehole	Depth below ground (m)	Length of borehole (m)	Sampled section (m)	Sampling date (y-m-d)	pH	DOC mM	HCO_3^- mM	SO_4^{2-} mM	Fe^{2+} µM	Cl^- mM	Drill water contamination %
KA2858A	380	59.7	39.8-40.8	95-03-10	7.8	0.075	0.15	2.03	1.60	294	0.05
KA3005A	400	58.1	0-58.1	94-12-07	7.7	0.177	1.33	1.10	4.85	147	-[a]
KA3005A	400	58.1	36.9-37.9	95-03-10	7.6	0.117	0.93	1.16	4.00	154	0.09
KA3105A	414	69.0	0-70	95-12-16	7.6	0.233	1.67	0.88	3.45	113	-
KA3105A	414	69.0	22.5-24.5	95-03-10	7.6	0.258	2.05	0.82	2.50	101	0.08
HD0025A	420	17.0	0-17	95-01-09	7.6	-	2.88	-	-	96	0.86

[a] No data.

Great care was taken to reduce the possibility of introducing non-indigenous microorganisms into the target aquifers during drilling. This was achieved by steam cleaning of all temporary and permanent equipment, except the tubing, with a hot-water high pressure cleaner. It was practically impossible to steam clean the 600 m long tubing and it was, therefore, not included in the cleaning procedure. Each SELECT borehole was core drilled and the first 2-2.5 m were drilled with an 86 mm diameter drill bit after which a stainless steel casing, 3 m in length, was cemented in. Subsequently, core drilling using a 56 mm bit progressed to full borehole length. Prior to the onset of drilling, all down-hole parts were cleaned with the hot-water high-pressure steam cleaner. After drilling, flow logging and pressure build-up tests were performed using one set of packers in the outer end of the borehole. The first sampling occasion for microbiology coincided with this packer set-up. Then, the boreholes were packed-off by between two and six inflatable packers in smaller sections depending on the number of hydraulic aquifers found in each borehole during the above mentioned tests. One or two of the sections with the highest groundwater flow rates were made available for sampling from panels in the tunnel. The panels were mounted directly on top of the borehole and were equipped with valves. All sections were kept closed except during the sampling campaigns.

3.2 SAMPLING PROCEDURES

Boreholes and drilling equipment were sampled on site in the tunnel according to Table 1 and as follows. Samples were taken directly from HD0025A, from the tubing that funneled drill water to the containers from the HD0025A borehole and from the drill water container in operation. Samples were also taken from the outlet of drill water and drill debris on the drilling machine when drill water was flowing through the machine (not drilling at this sampling). Before sampling a drilled borehole, several borehole volumes were drained to ensure that groundwater from the aquifers intersected by the borehole was sampled. All tunnel boreholes were artesian

and were sampled from the packed-off section via 6 mm plastic tubing (outer diameter). The male Luer fittings of sterile syringes, 25-50 ml, were fitted directly into the orifice of the tubing. The samples obtained were immediately transferred to sterile, DNA free test tubes for DNA extraction, total counts and plate counts or to sterile and pregassed (nitrogen) anaerobic 100 ml serum bottles for determination of the most probable number of SRB.

3.3 GROUNDWATER CHEMISTRY

Groundwater for chemical analysis was sampled parallel with microbiological sampling. Detailed information about the sampling and analysis procedures have been published elsewhere (Nilsson, 1995).

3.4 DETERMINATION OF THE TOTAL NUMBER OF BACTERIA

Portions of 40 ml were collected from each sampling point as described above, preserved with formaldehyde (2% final concentration) and transported to the laboratory in Göteborg for counting. This sampling was repeated three times, directly after drilling and packing of the boreholes and on May 19, and October 24, 1995. The total number of bacteria was determined by the acridine orange direct count (AODC) method (Pedersen and Ekendahl, 1992b). The groundwater samples were diluted two-fold the volume with sterile filtered 0.1% oxalic acid and vigorously shaken to reduce clogging of the filters used. A portion of the sample was filtered onto a Sudan-black stained Nuclepore filter of 0.22 µm pore size and 13 mm in diameter at -20 kPa and stained for 6 min. with acridine orange. All solutions were filter sterilized 0.22 µm). Two filters were counted for each water sample. The number of bacteria was counted with an epifluorescence microscope (Olympus BH-2) using blue light (390-490 nm).

3.5 VIABLE COUNT AND MPN OF SULPHATE REDUCING BACTERIA

The viable count of bacteria was analyzed by plate count technique on a medium used previously (Pedersen and Ekendahl, 1990) containing 1.5 g l^{-1} of organic substrate. The medium was composed of peptone, 0.5 g; yeast extract, 0.5 g; starch, 0.25 g; sodium acetate, 0.25 g; $CaCl_2$ x$2H_2O$, 0.2 g; K_2HPO_4, 0.1 g; NaCl, 10 g; trace metal solution SL 10 (Widdel and Bak, 1991), 1ml; 1000 ml of double distilled water; 15 g agar, pH was adjusted to 7.5 after autoclaving. Medium for anaerobic plate counts was supplemented with 1 g l^{-1} KNO_3 and 1 g l^{-1} Na_2SO_4 and stored in a 100% nitrogen atmosphere. The samples were serially diluted and spread in triplicate on the agar plates. For dilution of the samples prior to the viable count, a phosphate buffer (pH 7.5) was used. Aerobic plates were incubated at room temperature for two weeks, and anaerobic plates were incubated in anaerobic jars with an atmosphere of 90 % N_2 and 10% CO_2 at room temperature for two weeks.

For the MPN of SRB, a pressure proof glass device and a defined multipurpose SRB medium, adjusted to a salinity of 1%, were used as previously described (Widdel and Bak, 1991). Samples of ten ml were taken with sterile syringes directly from the tubing from boreholes as described above and immediately transferred through the butyl rubber stoppers of 115 ml serum bottles containing 50 ml of the SRB-medium under nitrogen atmosphere. MPN counts of SRB were performed as serial dilutions in Hungate tubes (Bellco glass, Inc, type no. 2048, 27 ml) from the serum bottles. The tubes were incubated at room temperature for 40 days. They were registered as positive if growth (AODC) and hydrogen sulfide production occurred.

3.6 DNA EXTRACTION, PCR-AMPLIFICATION, CLONING AND SEQUENCING OF THE 16S-rRNA GENE

Samples from HD0025, KA2858A, KA3105, tubing, drill water containers and the drilling machine were collected using syringes (Table 1). Ten ml samples were filtered onto sterilized 0.2 μm pore-sized Nuclepore filters. The sampling was repeated twice with intervals of approximately 5-10 minutes concurrent with sampling for the different counts of bacteria. The filters were deep frozen and transported to the laboratory in Göteborg for DNA extraction. They were resuspended in 380 μl of 20 mM Tris-HCl, pH 8.0; 20 mM EDTA; 0.35 M sucrose and incubated with 2 mg ml^{-1} lysozyme (Sigma) at 37 °C for 1 h to destroy cell walls. Thereafter the cells were lysed by adding 20 μl 20% Sodium Dodecyl Sulphate (SDS) and proteins were digested with 200 μg ml^{-1} proteinase K (Sigma) during an additional incubation at 60 °C for 1 h. The mixture was extracted with an equal volume phenol:chloroform:isoamylalcohol (25:24:1), and thereafter 3 extractions with chloroform:isoamylalcohol (24:1, called chisam) so that no cell debris was visible. Obtained DNA was precipitated with 1/3 volume of 10 M NH_4Ac (final concentration 2.5 M) and 2.5 volumes of 99% ethanol. To ensure complete precipitation, 50 μg tRNA was added as a coprecipitant and the mixture was incubated at -70 °C over night. The precipitate was washed with 100 μl 70 % ethanol (v/v) and dried in vacuum for 30 s, dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) over night and stored at -20 °C.

One μl of the extracted DNA solution was added to a mixture of 10 μl of 10xPCR buffer (Stratagene), 0.2 mM of each nucleotide triphosphate, 0.25 μM of each primer and double distilled water to a final volume of 100 μl. The samples were treated with 10 mg ml^{-1} of RNase A (Sigma) for 15 min at 37°C and incubated at 95°C for 5 min, before addition of 1 μl Pfu DNA polymerase (Stratagene) and coating with 100 μl mineral oil (Sigma). A total of 30 cycles were performed at 95°C (30 s), 55°C (1 min), 72° C (2 min) followed by a final incubation at 72°C for 10 min. The 5' and 3' primers used matched the universally conserved positions 519-536 and 1392-1404, *E. coli* Brosius numbering (Brosius et al, 1978). These were chosen to ensure that bacterial, archaeal and eucaryotic species could be

amplified. All PCR amplification series were accompanied by negative controls prepared as described above but without extracted DNA. Any PCR series was discharged if the negative control showed any sign of amplified DNA.

The amplification products were purified with the QIAEX agarose extraction kit (Qiagen) following the specification of the manufacturer and were finally diluted in 20 µl TE-buffer and stored at -20°C. The purified PCR products were cloned with the pCR-Script SK(+) cloning kit (Stratagene) following the specification of the manufacturer. From each DNA extraction, a total of 10 white colonies containing the insert was randomly picked. The sequencing was accomplished by Autoread Sequencing kit (Pharmacia Biotech) following the manufacturers instructions. Approximately 350 bases of all clones were sequenced using the 3' primer 907-926r (Lane et al, 1985) labeled with fluorescein. Gel electrophoresis was performed on an ALF DNA Sequencer (Pharmacia Biotech). All clone sequences obtained (158) were pooled and compared directly on the monitor of the ALF manager OS/2 computer, using the ALF manager program, version 2.5. Clones that had an identical total sequence were put in the same group and given a name (see results).

3.7 SEQUENCE ANALYSIS

The obtained 16S rRNA gene sequences were compared to sequences available in the European Molecular Biology Laboratory (EMBL) database using the FastA procedure in the GCG program package (Genetic Computer Group, Wisconsin, USA). This procedure calculates identities between an unknown sequence (clone) and sequenced bacteria in the database. No structural adjustments of the automatic FastA alignments were made, so the percentage values in table 4 show the approximate identity of the compared sequences. The association of a clone sequence with a species in the database at identity values below approximately 95 % is rather meaningless because of the large phylogenetic difference at or below this level of identity. Therefore, closest species in the database is only given for clones with an identity at or larger than 95%.

4 RESULTS

4.1 GROUNDWATER CHEMISTRY

The sampled groundwater all had a pH between 7.6 and 7.8. The amount of drill water left in the sampled groundwater, the drill water contamination, was generally very low (Table 2). The borehole KA2858A had an atypical groundwater composition compared to the other three boreholes sampled and also compared to other boreholes in the Äspö tunnel (Pedersen et al, 1996a). It had more than double the salinity, reflected by the chlorine and sulphate concentrations, due to an unusually high proportion of old deep saline groundwater at this depth (Smellie et al, 1995).

4.2 TOTAL NUMBER OF BACTERIA

The total number of bacteria in the borehole groundwater was highest in the borehole HD0025A used as drill water supply, and lowest in KA3005A (Fig. 1). Differences in total number of bacteria were small between samples from the full borehole sections sampled at the first occasion compared to the shorter sections sampled up to at most a year after the initial sampling (Fig. 1). The total number of bacteria was between one and two orders of magnitude higher in samples from the drilling equipment than in samples from the boreholes drilled and from the drill water borehole (Fig. 2a).

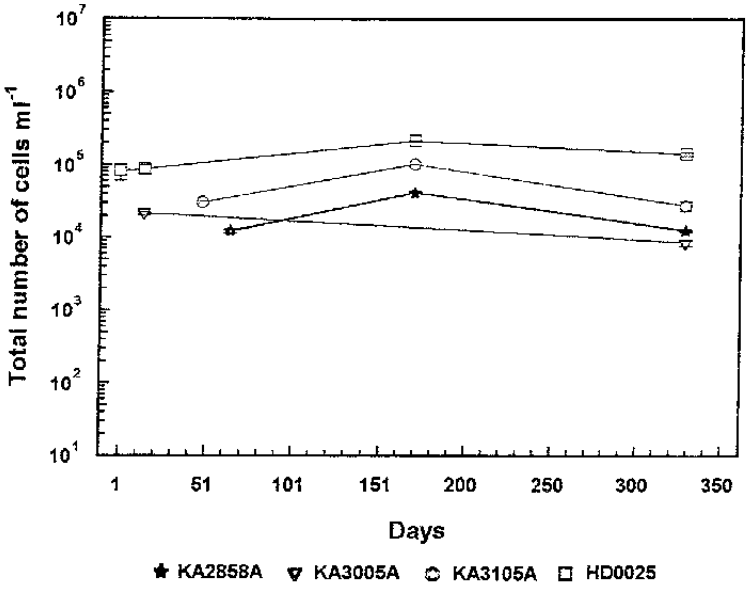
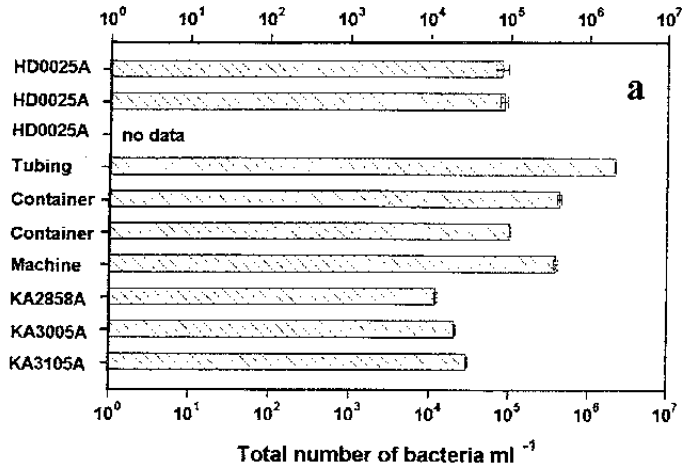
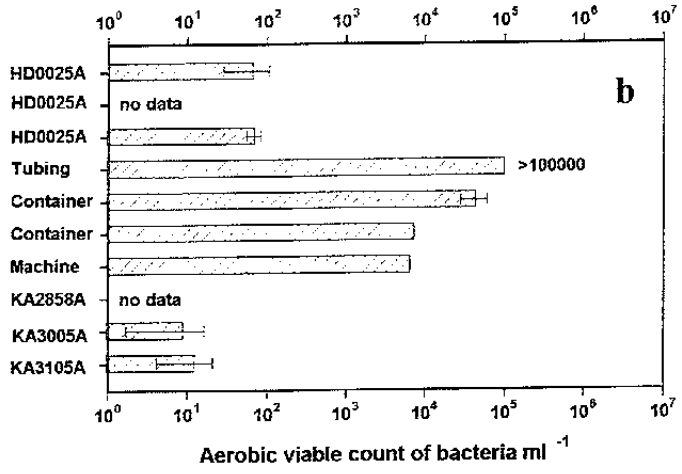
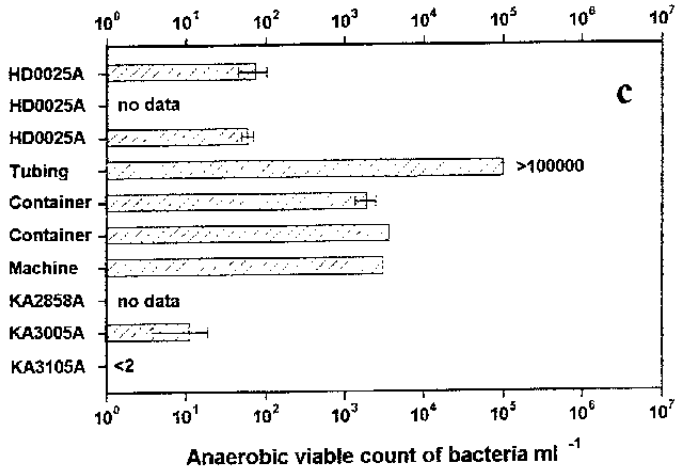


Figure 1 *The total count of unattached bacteria in the drilled boreholes and the borehole used for drill water supply (HD0025A). The x-axis shows the number of days that has passed after onset of the first drilling 30 November 1994 (Table 1). Error bars show standard deviation, the number of independent samples counted was 2.*







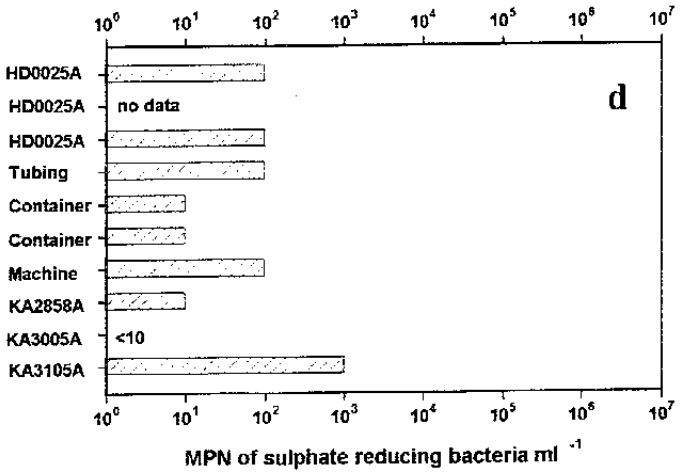


Figure 2 *Results from enumerations of different groups of bacteria in the drill water source HD0025A the drilling equipment and the drilled boreholes, KA2858A (0-59.7 m), KA3005A (0-58.1 m) and KA3105A (0-70 m). Sampling dates were as follows, from top of the y-axis: HD0025A 94-11-30, HD0025A 94-12-14, HD0025A 95-01-17, Tubing 95-01-17, Container 94-12-14, Container 95-01-17, Machine 95-01-17, KA2858A 95-02-02, KA3005A 94-12-14, KA3105A 95-01-17. Error bars in a-c show standard deviation, the number of independent samples counted was 2.*

4.3 VIABLE COUNTS AND MPN OF SRB

Aerobic and anaerobic viable counts were lower than 100 colony forming units (CFU) ml^{-1} in water from the boreholes (Fig. 2b, c). The highest CFU count was obtained from the tubing that funneled drill water from borehole HD0025A to the drill water container at the KA2858A site. The CFU counts were about one order of magnitude lower in the drill water container compared to the tubing, but still several orders of magnitude higher than in the borehole groundwater. The second highest CFU count was obtained from the container when sampled during drilling of KA3105A. The viable counts generally correlated positively with the total number of bacteria. Three isolates from the container samples with the most frequently occurring CFU colony characteristics were obtained from the plates inoculated with container water (95-01-17) and their 16S rRNA genes were partially sequenced. The sequences obtained were not identical to known and sequenced species (Table 3), nor identical to any of the sequences obtained through PCR of the extracted DNA (Table 4). The MPN for SRB indicated the presence of SRB in the range between 10 to 1000 SRB ml^{-1} (Fig. 2d)

Table 3 *Identification data for three of the most commonly occurring viable count CFU on the aerobic agar plates with samples from the tubing.*

Name of isolate	Culture collection identification number of the isolate	Phenotypic identification, closest species	EMBL accession number for 16S rRNA gene sequence of the isolate	Closest species in the EMBL DNA database	EMBL accession number for the closest species sequence	no. of basepairs compared	Identity %
SELECT-1	CCUG 36649	Unidentified	X99117	Not relevant	—	828	90.1
SELECT-2	CCUG 36648	*Pseudomonas alcaligenes*	X99118	*Pseudomonas aeruginosa*	M34133	669	95.4
SELECT-3	CCUG 36647	*Shewanella putrefaciens*	X99119	*Shewanella putrefaciens*	U39398	599	95.0

4.4 16S rRNA GENE DISTRIBUTION

A total of 129 16S rRNA gene clones were analysed in a first campaign; 20 clones were randomly selected and sequenced from each sample point and date, distributed over two independent DNA extractions. The PCR amplifications of DNA from both extractions from borehole KA2858A (95-02-02) and from one of the extractions from borehole KA3105A (95-01-17) were unsuccessful despite many repetitions and were, therefore, repeated with new samples collected 1.5 year later (96-06-27). This sampling comprised 29 sequenced clones from three extractions. A total of 158 clones was examined and clustered into 48 different, specific sequences, each of which was given a clone group name, ranging from S1 to S31 and T1-T17 and a clone number, 1-48, used below to identify the clones in Table 4. Between 2 and 10 different 16S rRNA genes were detected in each sample. One of the clone groups (no. 48) was represented by an 18S rRNA gene sequence, related to a yeast. Several clones were too distantly related to the database sequences for a meaningful closest species indication and the closest species in the database is only given for clones with identity values at or above 95% (Table 4).

Table 4 *Distribution of the clones between the different sampling sites during drilling of boreholes in the Äspö hard rock laboratory tunnel, November 1994 to February 1995 and June 1996. The clones are listed reflecting their phylogenetic relationship so that closely related clones are listed together. Clones that were found in samples from more than one site are written as bold face. The total number of clones in each clone group is followed, in parentheses, by the numbers achieved in extraction 1 and 2, respectively.* [a] *not relevant,* [b] *No data.*

				Number of identical clone sequences (Y-M-D)									
				Drill water	Drilling equipment				Drilled boreholes				
No	Clone group	Closest species in EMBL database	identity (%)	HD0025A 94-11-30	HD0025A 95-01-17	Container 94-12-14	Tubing 95-01-17	Container 95-01-17	Drilling machine 95-01-17	KA3105A 95-01-17	KA3105A:3 96-06-27	KA3105A:4 96-06-27	KA2858:2 96-06-27	Total
1	S31	n.r.[a]	77.8											
2	S23	n.r.	77.7						1(0+1)					1
3	S28	*Thiomicrospira denitrificans*	95.9		1(1+0)									1
4	S26	n.r.	85.3						3(1+2)					3
5	T10	Hydrothermal vent eubacterium	95.8		1(0+1)									1
6	T1	Aspo-1 SRB isolate	99.4								1	1		2
7	S2	Aspo-1 SRB isolate	99.1										4	4
8	T5	Aspo-1 SRB isolate	99.1							3(3+-b)				3
9	S5	*Desulfovibrio aespoeensis*	98.5										2	2
10	S21	*Desulfovibrio aespoeensis*	98.8							1(1+-)				1
11	S8	*Desulfovibrio* sp.	96.0		1(1+0)									1
12	S12	*Methylophilus methylotrophus*	98.3	1(1+0)	1(1+0)			5(3+2)	6(6+0)					13
13	S27	*Pseudomonas tolaasii*	98.6			9(3+9)		2(1+1)						11
14	S25	*Pseudomonas alcaligenes*	95.4						1(1+0)					1
15	S19	*Pseudomonas alcaligenes*	96.2						1(0+1)					1
16	S17	*Pseudomonas aeruginosa*	95.7			1(0+1)								1
17	S14	n.r.	89.7					1(0+1)						1
18	S30	n.r.	89.2			6(4+2)								6
19	S6	*Acinetobacter junii*	98.3					1(1+0)						1
20	S20	*Acinetobacter haemolyticus*	98.3						1(0+1)	1(1+-)				2
21	S15	*Acinetobacter haemolyticus*	98.6					3(0+3)						3
22	S11	*Escherichia coli*	99.1			1(1+0)								1
23	S9	*Shewanella putrefaciens*	98.8					1(1+0)						1
24	S10	*Shewanella putrefaciens*	98.3	19(9+10)	13(6+7)		1(0+1)	1(1+0)	1(0+1)					35
25	S29	*Shewanella alga*	98.0				18(9+9)	2(0+2)	2(0+2)					22
26	S3	n.r.	91.3				1(1+0)							1
27	S7	Aspo-4 (acetogenic isolate)	99.7							1(1+-)				1
28	T7	*Sphingomonas sp.*	96.8		2(0+2)			1(1+0)		1(1+-)				4
29	T6	*Flavobacterium capsulatum*	95.4									2		2
30	S24	n.r.	93.0								3	3		6
31	S13	*Propionobacter acnes*	98.3				1(0+1)							1
32	T9	n.r.	92.6					2(2+0)						2
33	T12	n.r.	84.6									1		1
34	S22	n.r.	86.7									1		1
35	S16	n.r.	84.5		1(1+0)									1
36	T4	n.r.	90.2			1(1+0)								1
37	S4	*Desulfobacula toluolica*	96.5										2	2
38	T2	*Desulfitobacterium hafniense*	95.0							1(1+-)				1
39	T14	n.r.	93.9										1	1
40	T17	n.r.	93.3								1			1
41	T11	n.r.	81.8								1			1
42	T16	n.r.	87.0									1		1
43	S18	n.r.	76.4								1			1
44	T15	n.r.	90.6			1(1+0)			3(2+1)					4
45	T3	n.r.	82.4								1			1
46	T8	n.r.	72.0										1	1
47	T13	n.r	71.3									1		1
48	S1	*Candida holmii*	99.7								1			1
						1(0+1)				2(2+-)				3
		Total number of clone groups		2	7	6	4	10	8	7	7	7	5	48
		Total number of sequenced clones		20	20	20	20	20	19	10	9	10	10	158

Some of the clone groups have several identical clone sequences, while others represent only a few or one clone sequence. The four most commonly occurring clone sequences were no. 11, 12, 23 and 24 as shown in Table 4. These clones correspond to 51 % of the sequenced clones (81 clones out of 158). Typically, each clone group predominated a separate sampling site with the exception of no. 11 that predominated in the drill water container and the drilling machine. Several of the clone groups were repeatedly detected in more than one DNA extraction (Table 4). Clone group no. 11 occurred in five, no. 23 in seven and no. 12 and no. 24 in four independent DNA extractions.

Several of the obtained clone sequences were very closely related to each other, but still different. Clone groups no. 23 and no. 24 differed only at two base positions, 649 and 760 (*Escherichia coli* Brosius numbering (Brosius et al, 1978)). This difference was significant throughout all 35 of the no. 23 clones and 22 of the no. 24 clones, distributed over seven and four independent extractions, respectively. The clone no. 27, observed in three samples, were 99.7 % identical with an acetogenic isolate (Aspo-4) and with a sequence observed in two other boreholes in the Äspö tunnel (see Pedersen et al. (1996)).

The clone groups no. 6 - 11 were all most closely related to SRB, and this observation correlates well with the MPN results of SRB that were found at all sites investigated for 16S rRNA gene diversity (Fig. 2d). Other common genera were *Acinetobacter* (no. 19 -21) and *Shewanella* (no. 23 - 25).

4.5 ACCESSION NUMBERS

The nucleotide sequence data reported in this paper, for DNA that was extracted from Äspö HRL groundwater, comprises 48 sequences that appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z69305-Z69335 corresponding to the clone groups S1 to S31 and under the accession numbers Z82958-Z82974 corresponding to the clone groups T1 to T17. The nucleotide sequence data reported for the isolated bacteria appear under the following accession numbers SELECT-1, X99117; SELECT-2, X99118; SELECT-3, X99119.

5 DISCUSSION

The measures taken to avoid microbial contamination of groundwater during drilling of the new boreholes included steam cleaning of all temporary and permanent equipment, except the tubing, with a hot-water high pressure cleaner and addition of a conservative fluorescent tracer, uranine, to the drill water as a control for drill water contamination of the sampled groundwater (Table 2). Microbiological methods were used to investigate the potential for microbial contamination after cleaning. They were applied to samples from the drill water source, the drilling equipment and groundwater from the

drilled boreholes and included determination of total number of bacteria, viable aerobic and anaerobic plate counts, MPN of SRB and analysis of the 16S rRNA gene diversity. The results obtained revealed the status of the studied microbial parameters along the drilling line and are evaluated below, first separately and then pooled to a common conclusion.

There were significantly higher counts of bacteria in the tubing passing water from the drill water source (HD0025A) to the drill water containers, as compared to the boreholes (Fig. 2a-c). This tubing was of a flexible, reinforced rubber type that could withstand the significant pressure of the groundwater in HD0025A; approximately 40 atmospheres over pressure. Rubber tubing material contains organic components such as softeners and stabilisers which slowly leak out to the water in the tubing. Therefore, such tubing is susceptible for biofilm formation of microorganisms that can grow on the released compounds (Pedersen, 1990; Schoenen and Schöler, 1985). This source of microbial contamination was not foreseen during the planning process of the drilling campaign, but it fortunately offered an excellent possibility to evaluate if high counts of microorganisms in the drill water caused similar counts in the new boreholes.

Fig. 2a shows that there were 10^5 cells ml^{-1} in the drill water source (HD0025A) and less than 10^6 cells per ml^{-1} in the container drill water introduced into the boreholes during drilling. As the drill water contamination of the drilled boreholes was below one part per thousand (<0.1%, Table 2), less than 10^3 cells ml^{-1} in the aquifers of the drilled boreholes can be expected to originate directly from the drilling operation. This number is more than one order of magnitude lower than what was actually found and can, therefore, not explain the origin of the observed total numbers in the new boreholes, unless growth occurred. However, all drilled boreholes had up to two orders of magnitude lower total counts than had the drill water from the container and up to one order of magnitude lower counts than the drill water source (Fig. 2a). The latter difference remained significant during the 250-300 days the investigation lasted (Fig. 1). A 0.1 % contamination of the sampled groundwater with drill water consequently cannot account for the total counts in the ground water from the drilled boreholes.

Viable counts reveal the number of culturable microorganisms, but usually, only a few percent or less of what is detected with total counts can be cultured (Amann et al, 1995). Here, less than 0.1 % of the total counts found in the drill water borehole and the drilled boreholes could be successfully cultured on agar plates (Fig. 2b-c). The situation was different when comparing total and viable counts in the drilling equipment. The viable aerobic and anaerobic counts were from two to three orders of magnitude higher in the drilling equipment than viable counts in the drilled boreholes and accounted for 1 to 10 % of the corresponding total counts. The highest viable counts were found in the tubing and correlate positively with the total counts, also highest in the tubing (Fig. 2). The observation of high viable counts in the tubing suggests that bacteria grew there. These bacteria did not establish at the same numbers in the drilled boreholes, doubtless due to the absence of appropriate growth conditions in the borehole groundwater.

The reproducibility of the 16S rRNA methods has been studied earlier with double repetitions of sampling, extraction, PCR and sequencing of groundwater from the Stripa research mine in Sweden (Ekendahl et al, 1994) and triple repetitions from the Oklo area in Africa (Pedersen et al, 1996b). It was found that the method is reproducible as it reports a specific sequence as predominating in all repeated extractions from different boreholes. Here, reproducible results were obtained as well. Several identical clones were obtained from two independent DNA extractions from one sample site (e.g. no. 12, 23 and 24) and in DNA extractions from different sample sites (no. 11, 12, 19, 23, 24, 27, 29, 42 and 48).

The 16S rRNA gene sequencing method has been applied previously to assess the diversity of microorganisms in different subterranean habitats without culturing and thorough discussions about possibilities and limitations of this method have been published elsewhere (Ekendahl et al, 1994; Pedersen et al, 1996a-b). Briefly, when PCR amplification is used for determination of species diversity, the result may be biased due to methodological problems, such as uneven extraction of DNA and biased PCR due to differences in genome size and variations in the growth rate dependent number of gene copies (Farrelly et al, 1995). The 16S rRNA protocol used here has been indicated to avoid some such biases in lab experiments examining different concentrations and mixtures of the Gram-negative groundwater bacteria *Gallionella ferruginea* and *Desulfomicrobium baculatum* (Ekendahl et al, 1994). One of the most important potential biases is that organisms belonging to the domain *Archaea* have been found to have only one or a few gene copies of the 16S rRNA gene while *Bacteria* can have from 1 up to several copies, 5-7 or more, which may bias towards *Bacteria* (Ward et al, 1992). In this investigation, the most important issue was to compare the 16S rRNA gene diversity in the drill water with that in the drilled boreholes. The sequencing method used here was reproducible as discussed above. Therefore, any of the possible bias effects described here, or any other bias caused by differences in the characters of the sequenced microorganisms will not have impaired this comparison. Instead, significant differences in the sequence diversity of two compared samples may possibly have been amplified by one or several bias effects. Consequently, species related bias, if present at all, will increase rather than decrease the sensitivity of the sequencing method with respect to its ability to detect differences in the microbial diversity of compared samples.

The deep groundwater at Äspö is reducing as inferred by the presence of ferrous iron (Table 1) and anaerobic (Pedersen, 1996). Enrichment and isolation of SRB from Äspö groundwater have been reported previously (Pedersen et al, 1996a) and 16S rRNA gene diversity analysis of attached and unattached groundwater bacteria in boreholes along the Äspö HRL tunnel has revealed sequences related to SRB in several boreholes (Pedersen et al, 1996a). Geological, hydrological, stable isotope and groundwater chemistry data, also obtained earlier, indicate that sulphate reduction has been ongoing in the deep aquifers of the Äspö granites long before the

tunnel was constructed (Pedersen, 1997; Laaksoharju et al, 1995). Altogether, previous results suggest the deep granitic aquifers of Äspö as being natural habitats for SRB communities. In this investigation, SRB were detected in all samples except for the groundwater from KA3005A, as judged from MPN counts (Fig. 2d). The 16S rRNA analysis showed a similar result with SRB related sequences in HD0025A, the container, the drill machine, KA2858A:2 and KA3105A (clones no. 6 - 11 in Table 4). SRB were probably present in the tubing too, as revealed by the MPN results (Fig. 2d) but their 16S rRNA genes were not detected. This is because the very high bacterial number in the tubing probably resulted in a high number of bacterial genes other than SRB, reducing the probability of getting a SRB sequence in the randomly selected and sequenced clones. Overall, the MPN and the 16S rRNA gene methods correlated well as they both revealed the presence of SRB in most samples. However, SRB cannot be used as indicators of contamination by drilling operations at Äspö, because they are obviously naturally occurring in the Äspö groundwater (Laaksoharju et al, 1995; Pedersen et al, 1996a) and can, as was observed, be expected along the drilling line, i.e. the drill water source, the drilling equipment and the drilled boreholes.

There is not an accepted value of % identity at which two 16S rRNA genes can be concluded to belong to the same genus or species. It can be quite different for different genera. It has been suggested based on a comparison of rRNA sequences and on DNA-DNA reassociation, that a relationship at species level does not exist at less than 97.5% identity in 16S rRNA. At higher identity values, species identity must be confirmed with DNA-DNA hybridisation (Fox et al, 1992). Accepting this level conservatively as identifying a sequence on the genus rather than the species level, some conclusions can be made about the sequences reported here. Several clones showed a high similarity with 16S rRNA genes from known and sequenced bacteria such as SRB (no. 6-10), discussed above, *Methylophilus* (no. 12), *Acinetobacter* (no. 19-21), *Shewanella* (no. 23-25), and an yeast, *Candida* (no. 48) (Table 4). The Äspö groundwater is anaerobic, while it is quite possible that the drill water in the containers may have taken up some oxygen from the tunnel air through the plastic container walls. The Äspö groundwater also contains methane (Pedersen, 1993b) and as most methanotrophs are aerobes or microaerophiles, the finding of a methanotroph (no. 12) in the container drill water, but not in the rock groundwater is plausible. *Acinetobacter* is a common groundwater genus and has been found among 16S rRNA sequences from several other subterranean sites (Pedersen, 1996). Yeast has been reported from subterranean environments previously (Pedersen et al, 1996a). The two predominant clone sequences no. 23 and 24 both represent the iron-reducing genus *Shewanella* which has been isolated from the HRL tunnel earlier (Pedersen et al, 1996a). The above mentioned bacteria seem as established in the Äspö aquifers as are the SRB and can, therefore, not be argued to derive from the drilling equipment during drilling of the new boreholes.

From 0.1 % up to 10% of the total count could be successfully cultured (Fig. 2) leaving more than 90% uncultured. We know form earlier experiments that the absolute majority of microorganisms found in deep ground water are viable (Ekendahl and Pedersen, 1994; Pedersen and Ekendahl, 1990, 1992a-b) and all viable microorganisms have a growth potential that must be considered when evaluating a possible contamination of new boreholes. The 16S rRNA gene sequences of the most frequently occurring CFUs from the container water (Table 3) were not identical to any of sequences obtained from the investigated drill water and borehole samples (Table 4). Although predominating among the CFUs, these bacteria still did not constitute more than a maximum of 1% of the total population in the container according to Fig. 2a-b, so the chance of picking up their DNA during random selection of 20 clones from a clone library is still very low. This result is typical and illustrates the rationale for applying the 16S rRNA method; to overcome the discrepancy between total and viable counts.

Comparing the container clones present during drilling of borehole KA3105A (94-12-14) with the borehole clones obtained later shows one single clone in common, no 48 (Table 4). The predominating container water clone, no 12, was not detected at all in any of the KA3105A borehole samples. Comparing KA2858A with the drilling equipment (95-01-17) shows that no sequence was in common and that the predominating clones no. 23 and no. 24 could not be detected in this borehole. An overall comparison of the 16S rRNA data (Table 4) obtained from the drill water source with the drilling equipment and results from the newly drilled boreholes obtained some weeks after drilling (Table 4) shows that three clones found in the drill water borehole HD0025A (no. 11, 23 and 27) were detected in the drilling equipment and that only one of the clones found in HD0025A could be detected in the drilled boreholes (no. 27). Comparing the clone data from the drilling equipment with the drilled boreholes demonstrates three clones in common (no. 19, 27 and 48). In contrast, five clones were detected in more than one sample within the drilling equipment (no. 11, 12, 23, 24 and 43). Many hundreds of litres of drill water are used per hour during drilling of boreholes in granitic rock. The exact amount differs depending on the permeability of the rock. Drill water is lost to the aquifers when intersecting permeable fracture zones, while most water returns out through the drilling machine when penetrating solid, non-fractured rock. After drilling, groundwater is allowed to flush out before packing off the borehole and this action significantly reduces the amount of drill water left in the aquifers. Usually, less than 0.1 % of drill water is left after such rinsing actions (Pedersen and Ekendahl, 1990; Pedersen et al, 1996a). A remaining contaminating population introduced by the drill water during drilling is consequently very dilute. Considering the total and viable count results in this investigation, the microbial contaminations introduced into the aquifers with the drill water from the drilling equipment must become established and multiply more than one order of magnitude in the aquifer environment to reach the total counts observed (Fig. 2a). If this was the case, more than 90% of the 16S rRNA sequences in new boreholes,

should be expected to be identical to what was found in the drilling equipment. However, only a few of the sequences detected in the borehole directly after drilling, and none of the 17 specific sequences obtained 1.5 years after drilling were similar to the sequences obtained from the drill water, indicating that the microbial populations that thrived in the drilled boreholes were very different from what was introduced during drilling, and therefore possibly intrinsic. Although the potential for a lasting contamination of the new boreholes from a high population of bacteria in the drilling line was obvious as revealed by total and viable counts, it could not be detected by the sensitive 16S rRNA method.

6 CONCLUSIONS

Proving that certain species of microorganisms found in the drilled boreholes are intrinsic and not introduced during drilling is extremely difficult. Instead, as was shown here, the opposite situation is easier to investigate, i.e. testing if a known contaminating microbial population establish or not in deep granitic aquifers during drilling. The tubing used for drill water supply constituted a source of bacterial contamination to the rest of the drilling equipment and the boreholes. Nevertheless, using molecular and culturing methods, it was shown that although large numbers of contaminating bacteria were introduced in the boreholes during drilling, they did not become established in the aquifers at detectable levels. Therefore, is seems reasonable to conclude that we find no evidences for lasting microbial contamination of boreholes drilled in granitic rock, and that the reason for this is the inability of foreign microbes to adapt to the prevailing oligotrophic, reducing, anaerobic and low temperature environmental conditions in deep granitic aquifers. Earlier documentation of in situ activity of microbial populations in deep granitic rock environments suggests that the microbes present are active at low but significant levels (Ekendahl and Pedersen, 1994; Pedersen and Ekendahl, 1990, 1992a-b). It can, therefore, be speculated that most of the microorganisms found in the new boreholes were present in the intersected aquifers before drilling. Recent findings of bacterial fossils in a granitic aquifer 207 m below ground at Äspö (Pedersen et al, 1997) support the hypothesis of a deep and intrinsic subterranean biosphere (Pedersen, 1993a).

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Evidence of ancient life at 207 m depth in a granitic aquifer

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CONTENTS

1 ABSTRACT

This paper reports electron-microscopy investigations of calcite precipitated in a water-conducting fracture in a ca. 1800 Ma granitic rock from 207 m below sea level at the island of Äspö on the southeastern (Baltic) coast of Sweden. The results are compared with measurements of carbon, oxygen and sulfur isotope composition of the calcite and embedded pyrite. Parts of the calcite had extremely low $\delta^{13}C$ values, indicative of biological activity, and contained bacteria-like microfossils occurring in colonies and as typical biofilms. X-ray microanalysis demonstrated these fossils to be enriched in carbon. Our results provide evidence for ancient life in deep granitic rock aquifers and suggest that the modern microbial life found there is intrinsic. Modeling historical and present geochemical processes in deep granitic aquifers should, therefore, preferably include biologically catalyzed reactions. The results also suggest that search for life on other planets, e.g. Mars, should include subsurface material.

2 INTRODUCTION

Diverse and active populations of microorganisms have been found in granitic rock aquifers at depths ranging from 70 m down to 1240 m (Ekendahl and Pedersen, 1994; Pedersen and Ekendahl, 1990; Pedersen et al., 1996; Pedersen, 1997). The drilling and excavation to access these microbial ecosystems are vigorous operations, and it can be argued that the observed life is an artifact of the access operations (Pedersen, 1993). Evidence of microbial life before penetration of the rock would confirm the existence of a deep subterranean biosphere in granitic aquifers (Pedersen, 1993).

This study was carried out on drill core samples from the site of the Äspö Hard Rock Laboratory, on the Baltic coast of Sweden, 400 km south of Stockholm. A vertical, hydraulically conductive fracture was transected at 207 m depth by a borehole denoted KAS02. Approximately 0.5 m of the entire fracture was exposed in the drill core. The fracture coating consisted of a thin rim of chlorite or clay minerals and fluorite overlain by calcite with embedded minute grains of precipitated pyrite. The host rock is a ca. 1800 Ma granodiorite belonging to the Fennoscandian Shield. Mineralogical and geochemical studies of the host rock close to the fracture show that no significant alteration of the minerals has taken place, and a low temperature (<150 °C) origin for the fracture has been suggested (Tullborg et al., 1996a; 1997). These findings indicate that the fracture is most probably younger than 700 Ma, as interpreted from fission-track analyses of apatite and

titanite from a neighboring borehole (Tullborg et al., 1996b). The carbon and oxygen isotope compositions of calcite precipitates and the sulfur isotope composition of embedded pyrite in this fracture were investigated, and thin sections of the fracture coating were studied with a scanning electron microscope and a transmission electron microscope equipped with an energy-dispersive X-ray system.

3 MATERIALS AND METHODS

3.1 STABLE ISOTOPE ANALYSIS

$\delta^{18}O$ and $\delta^{13}C$ analyses of calcite were performed at the Institutt for Energiteknikk (IFE), Kjeller, Norge. The samples were dried for 4 h at 400 °C, put into glass bottles together with 2 ml of 100% H_3PO_4, and evacuated to 5 x 10^{-3} mbar. The samples reacted with the acid for 2 h at 25 °C. The CO_2 produced was cleaned by freezing and analyzed in a VG Oprima gas mass spectrometer for measurements of the isotope ratio of stable carbon and oxygen isotopes.

The $\delta^{34}S$ analyses were performed at the Institute of Geology and Geochemistry at Stockholm University. The preparation of the sufide samples for sulfur isotope analyses was done according to the following technique: the pyrite was mixed with vanadium pentoxide, silica, and pure copper, placed in a quartz glass boat, and preheated at 450 °C for 30 min. Then the samples were roasted in an oven at 900 °C for another 30 min. The SO_2 gas generated was collected in a pentane trap using liquid nitrogen to remove the excess CO_2 gas. The SO_2 gas was then transferred to a sample vessel for analysis. The measurements of the sulfur isotopes were performed with a Finnegan Mat Delta E double-inlet mass spectrometer. The stable isotope results are related to a standard as follows:

$$\delta^{13}C_{sample} = [(({}^{13}C/{}^{12}C)_{sample}/({}^{13}C/{}^{12}C)_{standard}) - 1] \times 10^3$$

Similar equations are also valid for the $^{18}O/^{16}O$ and $^{34}S/^{32}S$ fractionation expressed as $\delta^{18}O$ and $\delta^{34}S$, respectively. The standard used for the carbon and oxygen analyses was related to the PDB (Peedee belemnite) standard and that for sulfur to the CDT (Cañon Diablo trolite) standard. The accuracy of the stable isotope analyses are ±0.1 ‰.

3.2 TRANSMISSION ELECTRON MICROSCOPY

Three drill core samples intersected by the investigated fracture were cut with a rock saw device into 0.5 x 1 cm rock sections, 0.5 cm thick, leaving the calcite and the granite immediately under it preserved. The calcite side of the rock sections was ground flat by using an aluminium oxide grinding tool and glued to plastic slides with a two-component epoxy glue. Subsequently, the rock sections were ground to 30 μm sections consisting of mainly calcite and some of the granite. Ten subsamples were collected with a razorblade from each such section, put into gelatine capsules, dehydrated with 99.5% ethanol for 30 min and embedded in epoxy plastic (LR White Resin, Hard Grade Acrylic Resin, London Resin Company Ltd, Reading, England). After 30 min, the plastic was replaced by a new batch to remove residual alcohol and left overnight at room temperature, before a final replacement of plastic and hardening at 60 °C for 24 h. Thin sections were made with a diamond knife on carbon-formvar-coated copper grids and were stained with uranyl acetate. A Jeol JEM 100S transmission electron microscope (at 60 kV) was used for viewing and photographing the samples. Analysis of the biofilm microfossils was done with a Philips CM200 TEM scanning microscope (120 kV), equipped with an EDAX energy-dispersive X-ray-system. For each X-ray analysis of the microfossils, one background analysis of the epoxy and grid film background was performed.

3.3 SCANNING ELECTRON MICROSCOPY

Pieces of rock were placed directly onto aluminium stubs with the untreated fracture in a side or upward position, attached with a two-component epoxy resin, and sputtered with gold-palladium for 2 min. The specimens were observed in a JSM 6400 scanning electron microscope.

4 RESULTS AND DISCUSSION

The present hydrogeologic situation at Äspö is characterized by a low hydraulic gradient, implying slow circulation (Smellie et al., 1995). The hydrochemistry is largely characterized by a near-surface fresh-water lens of varying depth (from a few meters down to 80 m) and then increasing salinity with depth. The prevailing ground water chemistry is described as a result of mixing between modern precipitation, glacial meltwater, Baltic Sea water, and old saline water of brine type; the glacial meltwater component is recognized down to considerable depth (Laaksoharju et al., 1996). The total numbers of microorganisms in ground water down to depths below 1000 m ranged between 10^4 and 10^6 cells per ml (Pedersen, 1996; Pedersen and Ekendahl, 1990, 1992), suggesting the presence of modern microbial life in the studied fracture.

On the basis of stable isotope data and trace element analyses, the processes responsible for the calcite formation in the fracture can be described as follows (Tullborg et al., 1996a; 1997): The fracture calcite precipitated from different types of water, ranging from marine-brackish to meteoric, possibly with a component of glacial meltwater. Of 18 samples analyzed from the fracture, 9 showed $\delta^{18}O$ values in the range of -9‰ to -11‰, i.e., close to equilibrium with the present ground water in this section of the drill hole (Smellie et al., 1995) (Fig 1).

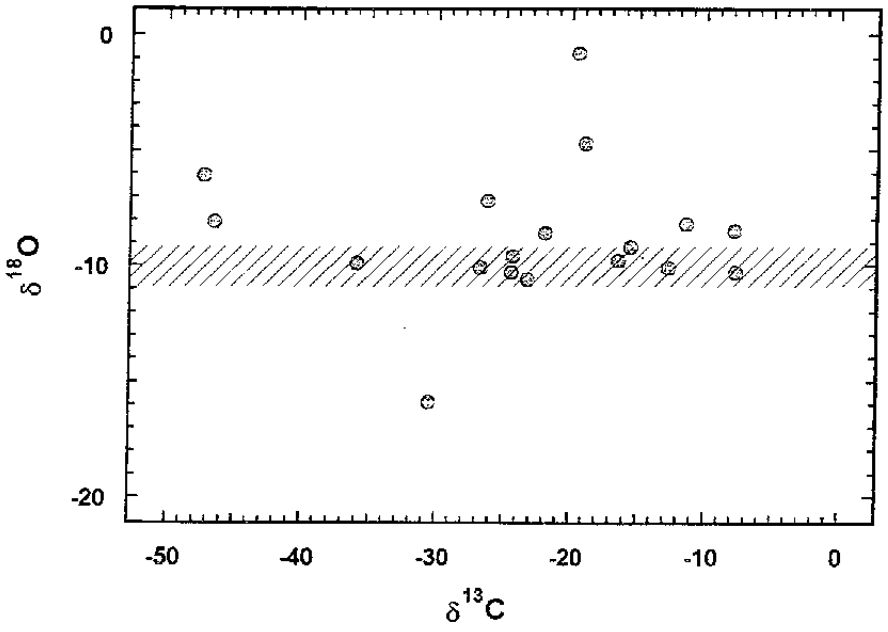

Figure 1 $\delta^{18}O$ *vs.* $\delta^{13}C$ *in calcite samples from one fracture at 207 m depth at Äspö, southeastern Sweden. The hatched area represents calcite precipitated in equilibrium with present ground water at ambient temperature (Tullborg et al., 1996a).*

The $\delta^{13}C$ values vary within a wide range, -7.0‰ to -46.5‰ (Fig 1), and the reason for the low $\delta^{13}C$ values is suggested to be biogenically produced bicarbonate. The low $\delta^{13}C$ values were not restricted to samples within any particular $\delta^{18}O$ interval. This lack of correspondence indicates that biogenic activity has taken place during different periods of time and in different hydrochemical environments. The $\delta^{13}C$ values vary also in samples with approximately the same $\delta^{18}O$ values, which suggests that the low $\delta^{13}C$ in the bicarbonate was produced locally, probably by degradation of organic material by bacteria (has caused local disequilibrium).

Three samples of pyrite embedded in the calcite were analyzed for $\delta^{34}S$, and the recorded values range from -2‰ to -16‰. Low values are typical for biogenic sulfide, but the higher values (close to 0‰) may have either a hydrothermal or a biogenic origin. However, no calcite with a hydrothermal isotopic signature has been identified. A likely interpretation of the stable isotope data from the fracture calcite and pyrite is that bacterial activity (sulfate-reducing bacteria) has been, and probably is, present in the fracture (Pedersen et al., 1996; Tullborg et al., 1996a).

One calcite sample shows a marine $\delta^{18}O$ signature (-0.8‰ relative to PDB), indicating that at least parts of the calcite coating are older than the Weichselian glaciation (older than 115 000 yr B.P.) because marine conditions have not prevailed since then in the region. As bacteria cannot be productive at temperatures much above 110 °C, it can be concluded that the formation of the low $\delta^{13}C$ in the bicarbonate must have taken place after the Devonian-Carboniferous interval (400-300 Ma) on the basis of the resetting of apatite ages in the area (Tullborg et al., 1996b).

The calcite surface appeared inhomogeneous with fissures and rims, as revealed by SEM (Fig. 2), offering many possibilities for microorganisms to attach and grow as biofilms if in contact with flowing ground water. The rims on the calcite grains suggest at least a 2nd generation of calcite precipitation. Three thin-sectioned subsamples from two of the three drill core samples investigated contained numerous bacteria-like microfossils, single or clustered in chains and colonies that are characteristic for growing bacteria (Fig. 3A). These microfossil structures were approximately 1 x 0.5 μm, i.e. typical bacteria size. The microfossil structures were found in the embedding epoxy plastic, indicating that they must have been attached to the calcite rather than buried in it. In one case, the microfossils appeared organized as a typical microbial biofilm (Characklis and Marshall, 1990), that had been attached to a surface (Fig. 3B). X-ray analysis of the biofilm microfossils showed a significant enrichment of carbon with an average net intensity of 165 ±57 in comparison to the background (average from 10 measurements), but none of the other elements analyzed (O, Na, Mg, Al, Si, P, S, Cl, K, Ca and U) differed significantly from the background. It can therefore be concluded that the structures in Figure 3 are not mineral grains or inclusions. In comparison, active bacteria that were used as a control for

possible contamination had significantly higher P values than the background and had visible structures typical for viable bacteria, such as cell wall, cell membrane, nucleotide and ribosomes. The lack of P and cellular structures of the microfossils confirms that they were not bacteria introduced during sample preparation. The drill core was stored dry in air for 6 yr before sampling, which excludes the possibility that the structures are contaminants introduced during drilling and sampling because desiccation of bacterial cells (they contain approximately 70% water) causes significant shrinkage that destroys their typical shape.

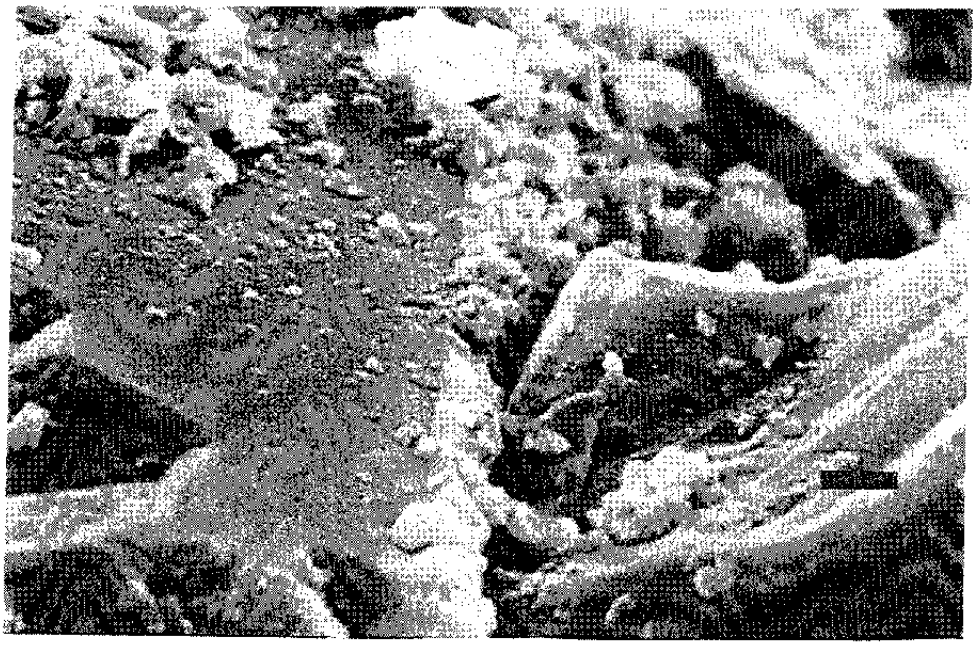

Figure 2 *Images from scanning electron microscopy of calcite precipitate that coated fracture in crystalline rock from 207 m below sea level in southeastern Sweden. Scale bar represents 1 μm.*

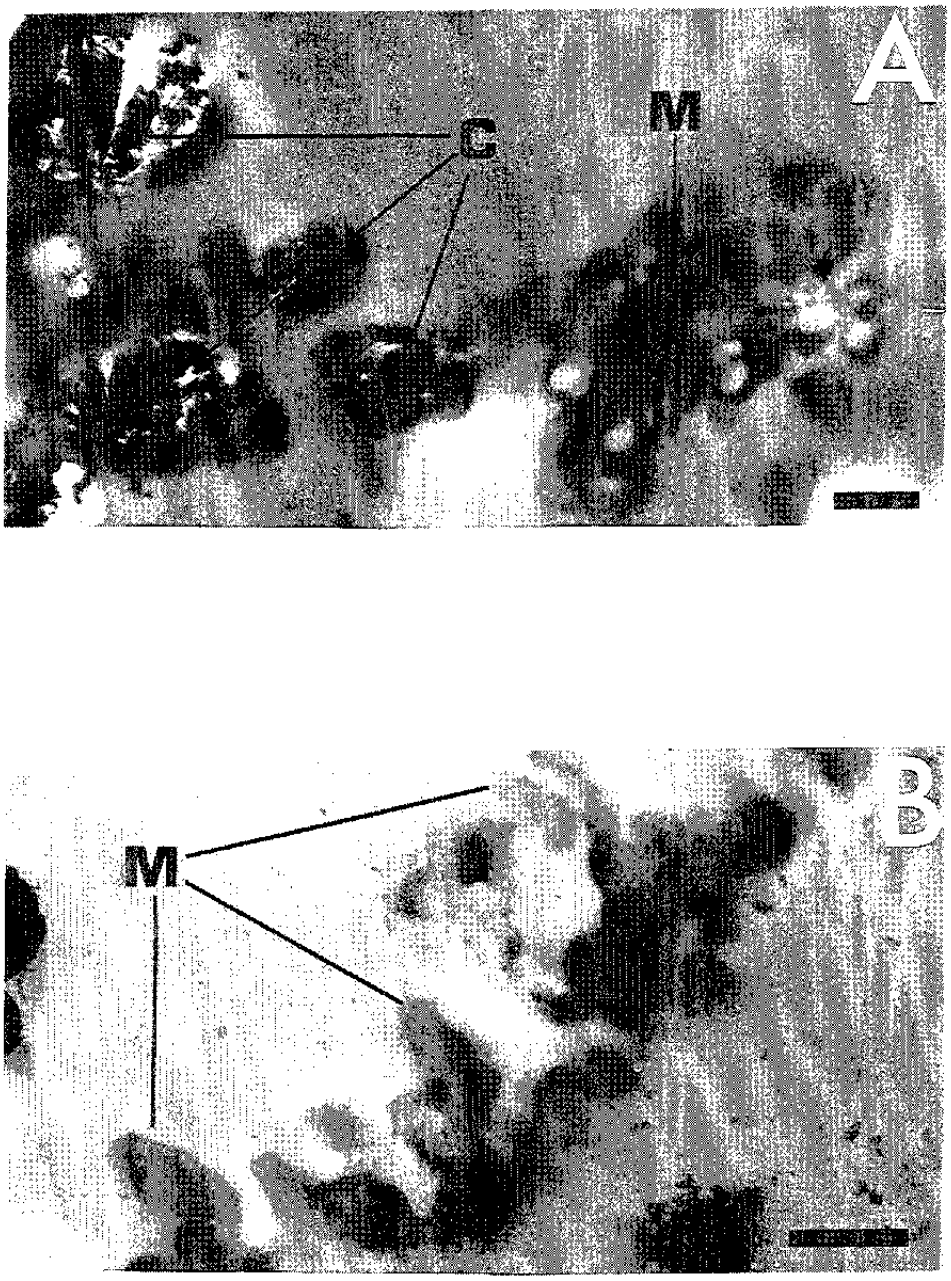

Figure 3 *Thin-section transmission electron microscopy of calcite precipitate that coated fracture in crystalline rock from 207 m below sea level in southeastern Sweden.* ***A:*** *Microcolony of fossil microorganisms (M) and calcite grains (C). Scale bar represents 1 μm.* ***B:*** *Fossil microorganisms (M) arranged in typical biofilm formation. X-ray microanalysis was performed on these biofilm microfossils. Scale bar represents 1 μm.*

The different generations of calcite have not been precipitated as uniform layers over the entire fracture surface. In contrast, a channellike pattern was observed. A possible process for the suggested preservation of ancient microorganisms as fossils in the studied aquifer may, therefore, be that some water-conducting channels (Moreno et al., 1985) with microbes growing attached to the calcite on the channel walls were closed by clogging or precipitation processes. Tiny volumes of ground water would then be captured in closed cavities together with the microbes, of which many probably can survive for years at low energy status (Kjelleberg et al., 1987). If there are no other microbes or bacteriophages that can degrade or lyse the attached microbes, they will eventually die and undergo fossilization processes. Fossilization of micro-organisms is not well understood and much experimental work still needs to be invested to study this process. Different bacterial species have been shown to be fossilized in different ways according to their cell wall structures (Westhall et al., 1995) and some studies suggest that inhibition of autolytic enzymes is crucial, perhaps through metallic ion binding to the cell wall polymers (Ferris et al., 1988). The deep ground water of Äspö contains iron, lithium, manganese and other dissolved metals (Smellie et al., 1995) which then may have counteracted autolysis of the microfossils reported here.

The electron-microscopy and isotopic results presented here strongly suggest that microbial activity had been ongoing in the deep granitic aquifers of Äspö before drilling of the sampled borehole. The presence of modern autotrophic and heterotrophic microbial life in aquifers in granite rock aquifers has been repeatedly demonstrated as well (Pedersen, 1997). These microbes consume and produce carbon dioxide, and they transform hydrogen and carbon dioxide to methane and acetate in deep granitic aquifers. They may therefore influence the distribution of carbon between dissolved and precipitated phases. Modeling historical and present geochemical processes in deep granitic aquifers should, therefore, include biologically catalyzed reactions to be correct, but it remains to be determined at what rates subterranean microorganisms can shuttle carbon between various such phases.

Considerable interest in subterranean microbial life has evolved out of the debate concerning the possibility of ancient and present life on Mars (McCoy, 1997). Fresh fracture surface of a martian meteorite was reported to contain polycyclic aromatic hydrocarbons, low temperature carbonate globules and small, bacteria like structures (McKay et al., 1996). A variety of chemical, mineralogical and morphological features of the carbonate globules were argued to be deposited by life on Mars. It was also suggested that such life must have existed under the surface of Mars. The data presented here implies that life on Earth indeed is adapted to a subterranean lifestyle and suggests that a planet with a lifeless surfaces may hide life deep under its surface provided there is liquid water and energy for life available. It has also been suggested that life is not only adapted to deep subterranean environments, it may rather have originated deep below the surface of Earth (and Mars?) (Bock and Goode, 1996).

5 ACKNOWLEDGEMENTS

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PAPER 3

16S rRNA gene diversity of attached and unattached bacteria in boreholes along the access tunnel to the Äspö hard rock laboratory, Sweden

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CONTENTS

1 ABSTRACT

A total of 155 16S rRNA genes that were cloned from unattached and attached bacteria in nine boreholes down to 626 m below ground were partially sequenced. Attached bacteria were examined with scanning electron microscopy (SEM). The distribution of the 16 rRNA genes found was related to the different types of groundwaters studied. Several of the sequences obtained could be identified on genus level as one of the genera *Acinetobacter, Bacillus, Desulfovibrio* or *Thiomicrospira.* The 16S rRNA genes from 20 selected isolates were closely related to the sulphate reducers *Desulfomicrobium baculatum* or *Desulfovibrio* sp., the iron reducer *Shewanella putrefaciens,* or distantly related to the Gram-positive genus *Eubacterium.* Viable counts confirmed the presence of sulphate-reducing bacteria.

Keywords: 16S rRNA; Äspö; gene diversity; DNA sequencing; Groundwater; Iron-reducing bacteria; Sulphate reducing bacteria.

2 INTRODUCTION

Radioactive waste with long-lived isotopes, produced by the nuclear energy industry and from other sources, must be safely stored for a hundred thousand years or more. Most countries are planning to build underground repositories for the disposal of such waste. Microbiology became a part of the Swedish scientific program for radioactive waste disposal in 1987 and the aims and progress of this sub-program were recently summarized (Pedersen 1993a; Pedersen and Karlsson 1995). The Äspö hard rock laboratory (HRL) has been constructed as a part of the development of the Swedish concept for deep geological disposal of spent nuclear fuel. The work has been divided into three phases: the pre-investigation (1986-1990), the construction (1990-1995), and the operating (1995-) phases. During the pre-investigation phase, diverse populations of active bacteria, from $1.5x10^4$ up to 17.5×10^5 cells ml^{-1}, were found in borehole groundwater down to 1078 m depths in the crystalline bed-rock of Äspö (Pedersen and Ekendahl 1990; Pedersen and Ekendahl 1992a; Pedersen 1993b). The work presented in this paper was performed during the construction phase and most samples were collected from boreholes in the access tunnel during the excavation operations.

Nucleic acid probes for specific RNA or DNA sequences can be used for the detection of bacteria. Most of this work has been performed using the gene for the 16S rRNA subunit of the bacterial ribosome (Amann et al. 1991; Braun-Howland et al. 1992; Giovannoni et al. 1990; Risatti et al. 1994; Weller and Ward 1989; Woese 1987). However, the use of nucleic acid probes requires 16S rRNA gene sequence information, or probes cannot be constructed that are specific for the species, genera or phylogenetic groups

of interest. Therefore, when probes are to be used in new environments with an unknown diversity of bacteria, the first step has to be an analysis of 16S rRNA gene diversity. This gene can be isolated from environmental samples (Johnson 1991), amplified with the polymerase chain reaction (PCR), sequenced (Ludwig 1991) and compared with DNA-sequences of known bacterial species.

This paper presents the 16S rRNA gene diversity of groundwater bacteria collected from boreholes along the access tunnel to the Äspö HRL. A total of 155 16S rRNA gene clones were collected from nine boreholes ranging from 10 m down to 626 m. Both attached and unattached bacteria were studied and the attached populations were examined with scanning electron microscopy (SEM). The 16S rRNA genes from enriched and isolated sulphate and iron-reducing bacteria (SRB and IRB) were also sequenced and compared with the groundwater sequences. The distribution and diversity of the bacteria detected were compared with groundwater chemistry data and with information about major groundwater bearing fractures and groundwater flow directions.

3 MATERIALS AND METHODS

3.1 DESCRIPTION OF STUDY SITE AND SAMPLING PROCEDURES

The Äspö HRL is located at the Baltic coast on the island Äspö, in the vicinity of the Simpevarp nuclear power plant north of Oskarshamn, SE Sweden. The access tunnel starts at the coast line and continues to about 1700 m tunnel length under the sea floor where it spirals down to 460 m below sea level under the Äspö island (see Fig. 1A). The tunnel is approximately 5 m x 5 m (height x width) and proceeds down with an inclination of about 14%. The borehole KAS03 was core-drilled during the pre-investigation phase. A major fracture zone (RZ, Fig. 1B) was extensively studied in another project that aimed at modeling shallow groundwater intrusion to the tunnel (Banwart et al. 1994). The boreholes HBH01, HBH02, (from the ground surface), and KR0012, KR0013 and KR0015 (from a side vault of the tunnel) were drilled for this purpose. Tracer tests were performed in these boreholes directly after sampling of DNA and bacteria. These tests revealed the residence time of groundwater flowing in the fracture zone (Gustafsson 1994). The boreholes SA813B, SA923A, SA1062A, HA1327B and SA1420A were all drilled 20-40 m horizontally into the tunnel walls. All surface boreholes were packed-off in sections and the tunnel boreholes were fitted with packers towards the tunnel. Unfortunately, the boreholes SA923A and SA1062A soon ran out of water and therefore only limited results were achieved from these boreholes. Depths, drill water contamination values and groundwater compositions of the boreholes can be found in Table 1.

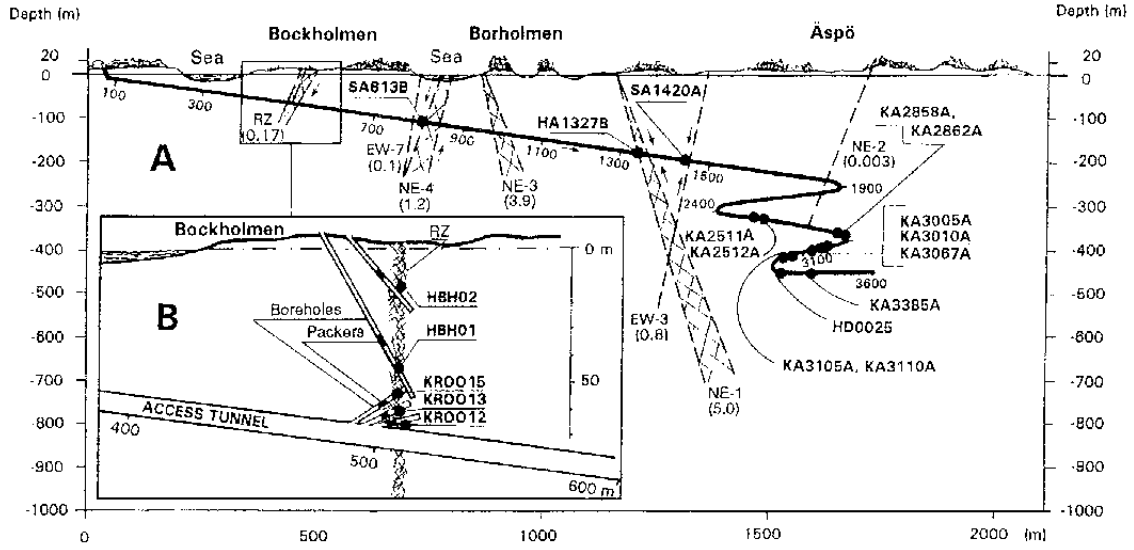

Figure 1 (**A**) *Site topology during the construction of the access tunnel to the Äspö hard rock laboratory in June 1995. The sample sites are depicted with their respective borehole names. These names show the type of drilling (HBH, HA = percussion drilled; KAS, SA = core drilled), the tunnel length where they were drilled and if they were drilled on the left (A) or the right (B) side of the tunnel when going down. Major fracture zones are marked with dashed lines and with their names given, generally indicating their geographic orientation. Possible flow directions of groundwater are indicated with arrows and the estimated inflow rates of groundwater via the fractures to the tunnel are shown in brackets as l* s^{-1}. (**B**) *A fracture zone (RZ) with boreholes that were drilled with the purpose to follow shallow groundwater intrusion through this major fracture zone into the tunnel. A side vault was constructed (not shown) from which the boreholes KR0012, -13 and -15 were drilled perpendicular through the zone. Note that these boreholes were all sampled at 68 m below sea level (not shown).*

Table 1 *Borehole information with groundwater chemistry data, the total number of attached and unattached bacteria (2 February 1993) and the number of cultivable SRB in groundwater from the Äspö hard rock laboratory tunnel.*

Borehole	Depth (m)	Clone letter[a]	Bacteria (ml^{-1} x 10^5)	Bacteria (cm^{-2} x 10^5)	SRB (ml^{-1})	pH	HCO_3^- (mM)	Cl^- (mM)	SO_4^{2-} (mM)	DOC (mg l^{-1})	Drill water %
HBH02	10	l	9.3	[b] -	<5[c]	6.4	1.04	0.260	0.15	21	-
HBH01	40	m	3.4	-	<5	7.5	4.70	18.4	1.34	15	-
KR0012	68	n	1.3	-	<5	7.7	4.98	23.7	1.42	15	<0.2[c]
KR0013	68	o/g	5.1	4.7	<5	7.5	4.90	50.5	1.45	15	<0.2
KR0015	68	p	1.5	-	<5	7.5	4.96	22.3	1.38	22	<0.2
SA813B	112	q/h	1.4	12.0	2420	7.0	6.64	94.7	2.36	11	<0.2
SA923A	134	-/i	-	-	<5	6.8	10.5	121	1.33	9.3	<0.2
SA1062A	143	r	-	-	-	7.3	6.46	123	1.97	9.1	<0.2
HA1327B	179	s/j	0.88	5.6	130	7.1	4.48	130	2.12	6.1	<0.2
SA1420A	192	t/k	0.44	0.15	<5	7.3	3.61	97.3	3.49	7.5	<0.2
KAS03	626	u	0.84	-	1390	7.1	0.78	99.5	1.83	1.0	0.3

a) Letter(s) given to the clone group names (Table 5) to show in which borehole they appeared, water/surface.
b) No data.
c) Detection limit.

Before sampling a borehole, several borehole volumes were drained to ensure that groundwater from the geological formation around the borehole was sampled. The boreholes HBH01, HBH02 and KAS03 were sampled from ground surface via stationary pumps placed in the respective borehole section. All tunnel boreholes were artesian and were sampled from the packed-off section via 6 mm plastic tubings (outer diameter). The male Luer fittings of sterile syringes, 25-50 ml, were fitted directly into the orifice of the tubings. The samples obtained were immediately transferred to sample tubes for DNA extraction or to anaerobic Hungate bottles for enrichment of anaerobic bacteria.

3.2 GROUNDWATER CHEMISTRY

Groundwater for chemical analysis was sampled parallel with sampling for microbiological analysis, on February 7, 1992. All boreholes were sampled at numerous other occasions as well, and the results have been reported together with detailed information about the sampling procedures (Nilsson 1995).

3.3 ATTACHMENT AND GROWTH OF BACTERIA

Laminar flow reactors (Pedersen 1982; Pedersen et al. 1986) were connected to the flowing groundwater from KR0013, SA813B, SA923A, HA1327B and SA1420A at the ambient flow rates of 31,14,8,12 and 0.7 mm s^{-1}, respectively. Glass surfaces, 60x24x1 mm, were heated in a muffle furnace at 475°C for 4h and used as hydrophilic substrata for attaching and growing bacteria. Slides from these reactors were sampled after 67 days (92-12-02 to 93-02-07), immersed in 0.22 µm filtered groundwater and transported on ice to the laboratory where the groundwater was decanted from the slides, subsequently stored at -80 °C until DNA extraction, or fixed with glutaraldehyde for SEM observation. Slides for total number of bacteria were preserved with formaldehyde to a final concentration of 2%.

3.4 SCANNING ELECTRON MICROSCOPY

Microscope slides with attached bacteria were sampled and immersed in groundwater as described above, fixed in 2% glutaraldehyde, cut into small pieces, dehydrated with alcohol-acetone, critical-point dried, sputtered with gold-palladium and observed in a Zeiss DSM 940 SEM.

3.5 DETERMINATION OF THE TOTAL NUMBER OF BACTERIA

Water samples of 40 ml were collected from the surface on Bockholmen (Fig.1) and from each borehole as described above, preserved with formaldehyde (2% final concentration) and transported to the laboratory in Göteborg for counting. This sampling was repeated three times: February 7, May 16, and August 16, 1993. The total number of bacteria in the groundwater and on surfaces was determined by the AODC method (Pedersen and Ekendahl 1992a). The groundwater samples were diluted

two-fold the volume with sterile filtered 0.1% oxalic acid and vigorously shaken to reduce clogging of the filters used. A portion of the sample was filtered onto a Sudan-black stained Nuclepore filter of 0.22 µm pore size and 13 mm in diameter at -20 kPa and stained for 6 min with acridine orange. All solutions were filter-sterilized (0.22 µm). Two filters were counted for each water sample. Surfaces from the laminar flow rectors were rinsed with sterile filtered groundwater, stained with acridine orange, washed, dried and counted. The number of bacteria was counted using blue light (390-490 nm) under an epifluorescence microscope (Olympus BH-2).

3.6 ENRICHMENT AND ISOLATION OF IRB AND SRB AND VIABLE COUNT OF SRB

For the enrichment of SRB, a pressure-proof glass device and a defined multipurpose SRB medium, adjusted to a salinity of 1%, were used as described previously (Widdel and Bak 1991). Samples of 10 ml were taken with sterile syringes directly from the tubings from boreholes as described above and transferred immediately to 100 ml serum bottles containing 50 ml of the SRB-medium under an atmosphere of nitrogen. Viable counts of SRB were performed as serial dilutions from the serum bottles in an anoxic agar medium in Hungate roller tubes, according to Widdel and Bak (Widdel and Bak 1991). The tubes were incubated at room temperature and counted after 40 days. A total of 19 different colonies appearing in the highest dilutions of the roller tubes were picked and subcultured. Different colony morphologies were deliberately chosen.

IRB were enriched with the same technique as used for enrichment and culturing of SRB, but using an iron citrate medium with appropriate salts. The salt medium consisted of 0.01 g peptone, 2.5 g $NaHCO_3$, 1.5 g NH_4Cl, 0.6 g $NaH_2PO_4 \cdot H_2O$, 0.1 g NaCl, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.005 g $MnCl_2 \cdot 4H_2O$, 0.001 g $Na_2MoO_4 \cdot 2H_2O$ and 1000 ml double distilled water. Iron citrate as electron acceptor was added to a concentration of 4 mM, and the energy source and electron donor used was lactate at a concentration of 57 mM. The medium was gassed with a mixture 80/20% of dinitrogen/carbon dioxide and adjusted to pH 7.

3.7 DNA EXTRACTION, PCR-AMPLIFICATION, CLONING AND SEQUENCING OF THE 16S rRNA GENE

Groundwater was sampled from the boreholes with syringes as described above and filtered onto sterilized Nuclepore filters of 0.22 µm pore size. Each filter was put in 380 µl of 20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.35 M sucrose and was transported to the laboratory on ice. Surfaces from the laminar flow reactors were sampled and treated for DNA extraction as described by Ekendahl et al. (1994). The DNA extraction protocol was based on procedures described by Marmur (1961) and Wallace (1987), but was modified. The filters were incubated with 2 mg ml^{-1} lysozyme (Sigma) at 37°C for 1 h, the cells lysed by adding 20 µl 20% SDS and the proteins digested with 200 µg ml^{-1} of proteinase K (Sigma) during an additional incubation at 60°C for 1 h. The DNA was extracted from the cell lysate

solution with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1), followed by extraction with chloroform:isoamylalcohol (24:1) until no cell debris was visible. The DNA was precipitated with 1/3 volume of 10 M ammonium acetate (final concentration 2.5 M) and 2.5 volumes of 99% ethanol. To ensure complete precipitation, 50 µg tRNA was added as a coprecipitant during incubation at -70°C overnight. The precipitate was washed with 100 µl 70% ethanol (v/v) and dried in vacuum for 30 s, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight at 4°C and stored at -20°C.

One µl of the extracted DNA solution was added to a mixture of 10 µl of 10 x PCR buffer (Stratagene), 0.2 mM of each nucleotide triphosphate, 0.25 µM of each primer and double distilled water to a final volume of 100 µl. The samples were treated with 10 mg ml^{-1} of RNase A (Sigma) for 15 min at 37°C and incubated at 95°C for 5 min, before addition of 1 µl Pfu DNA polymerase (Stratagene) and coating with 100 µl mineral oil (Sigma). A total of 30 cycles were performed at 95°C (30 s), 55°C (1 min), 72°C (2 min) followed by a final incubation at 72°C for 10 min. The 5' and 3' primers used matched the universally conserved positions 519-536 and 1392-1404, *E. coli* Brosius numbering (Brosius et al. 1978). These were chosen to enable bacterial, archaeal and eukaryal species to be amplified. The amplification products were purified with the QIAEX agarose extraction kit (Qiagen) following the manufacturer's specifications and were finally diluted in 20 µl TE-buffer and stored at -20°C.

The purified samples were cloned with the pCR-Script SK(+) cloning kit (Stratagene) following the manufacturer's specifications. From each DNA extraction, a total of 12 white colonies containing the insert was randomly picked. The colonies were inoculated into 3.5 ml Luria Broth (LB) and ampicillin overnight at 37°C. From each culture, 0.5 ml was suspended in 0.5 ml of concentrated glycerol and stored at -80°C until sequencing. The recombinant plasmids were extracted from the bacteria with the Magic miniprep kit (Promega).

The sequencing was performed using the double stranded sequencing method as described by Su et al. (1991) for denaturating the samples, followed by sequencing reactions using the DNA sequenase kit version 2.0 (USB). Standard procedures were followed. All clones were sequenced using the 3' primer 907-926 (Ekendahl et al. 1994), run on a 6% polyacrylamide gel and exposed to X-ray film over night.

3.8 SEQUENCING OF THE 16S rRNA GENES FROM CULTURES

The different 16S rRNA genes of 19 SRB culture isolates and of one IRB culture isolate were PCR amplified using the 5' and 3' primer positions 8-27 and 1522-1542, *E. coli* Brosius numbering (Brosius et al. 1978) and sequenced. Each specific 16S rRNA gene obtained was completely sequenced and the results were compared with available international databases for identification (September 1995), and with the sequences found in the groundwater samples from the HRL tunnel.

3.9 SEQUENCE ANALYSIS

The 16S rRNA gene clone sequences were compared to the sequences available in the European Molecular Biology Laboratory (EMBL) and GenBank databases using the FastA procedure in the GCG program package (Genetic Computer Group, Wisconsin, USA). This procedure shows identities between the unknown sequence (clone) and known and sequenced bacteria in the database. The phylogenetic analysis was performed by using the programs contained in the PHYLIP version 3.5c package compiled for PC. All nucleotide positions that could be unambiguously aligned for all clones were included in the analysis. The final data set comprised 342 nucleotide positions, position no.550 - 879 (*E.coli* Brosius numbering (Brosius et al. 1978), but not including 13 inserts in the analyzed data set). The distances were calculated using the DNADIST program and a tree was built running the KITCH program with contemporary tips. The KITCH program was run with a randomized input order of data with 10 jumbles and during the execution 172,443 trees were examined. The tree was drawn by using the drawing program DRAWTREE, also available in the PHYLIP package.

4 RESULTS

4.1 GROUNDWATER COMPOSITION AND NUMBER OF BACTERIA

Table 1 shows major parameters of the groundwater sampled in boreholes along the access tunnel to the Äspö HRL. At least four different types of groundwater were detected. The shallow borehole HBH02 had non-saline groundwater with the lowest pH, chlorine and sulphate concentrations and had among the highest values for DOC and total number of bacteria measured. The boreholes HBH01 and KR0012-13-15 were brackish, had higher carbonate, chlorine and sulphate concentrations than HBH02 and lower DOC concentration (except KR0015) and total number of bacteria. The boreholes HBH01-02 and KR0012-13-15 are all situated under the island of Bockholmen. The boreholes along the tunnel section below the Baltic Sea, SA813B down to SA1420A (see Fig. 1A), had a groundwater composition that was influenced by the Baltic Sea with high chlorine and sulphate concentrations. Finally, KAS03 had a composition typical for deep groundwater with low carbonate and DOC contents. The major fracture zones RZ, NE-4, NE-1 and EW-3 all transport groundwater to the tunnel as shown in Fig. 1. In addition, a flow component has been demonstrated along the tunnel from the fracture zone NE-3 to NE-1. Details of the composition, distribution and flow of groundwater in the Äspö HRL area can be found elsewhere (Nilsson 1995; Banwart 1994; Gustafsson et al. 1991). The drill water content was generally below the detection limit of 0.2%.

The total number of unattached bacteria decreased from approximately 10^6 down to below 10^5 bacteria ml^{-1} the first 100 m and was then fairly constant at just less than 10^5 bacteria ml^{-1} in the groundwater samples from 100 m downwards (Fig. 2). The total number at each sampling point was reproducibly stable at matching values over three seasons: winter, spring and summer 1993. The number of attached bacteria was highest on the surface exposed to SA813B groundwater and lowest on the SA1420A surfaces. Significant numbers of SRB could be found in SA813B, HA1327B and KAS03 (Table 1).

4.2 SEM OF ATTACHED BACTERIA

SEM showed that several morphologically distinct forms of bacteria were attached to the surfaces (Fig. 3). Those exposed to the KR0013 groundwater were colonized by three different morphological forms (Fig. 3a-b): one very long bacterium (approximately 5 μm), that predominated on the surfaces; a small, rod shaped bacterium that commonly was attached to the large bacterium; and cells with a polar flagellum were also observed. On the SA813B surfaces, 2 μm long cigar-shaped rods were observed partly buried in a dense precipitate (Fig. 3c-d) that gave the surface a white macroscopic appearance. The bacteria on the SA923A surface (Fig. 3e-f) had a shape and appearance that was similar to that of the bacteria on the SA813B surfaces. The HA1327B surfaces were colonized by small, 1 μm diameter, coccoid cells (Fig. 3g-h). It was not possible to see any bacteria on the SA1420A surfaces, possibly because the total number of bacteria was approximately 50 times lower on this surface compared to the other surfaces.

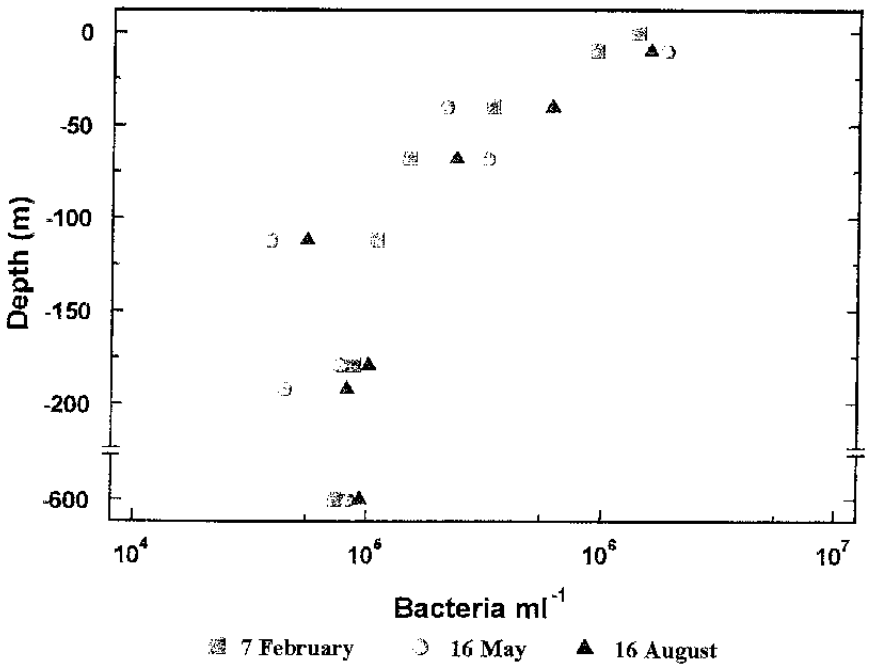

Figure 2 *The total number of unattached bacteria in the boreholes shown in Fig. 1, measured on three separate occasions during 1993.*

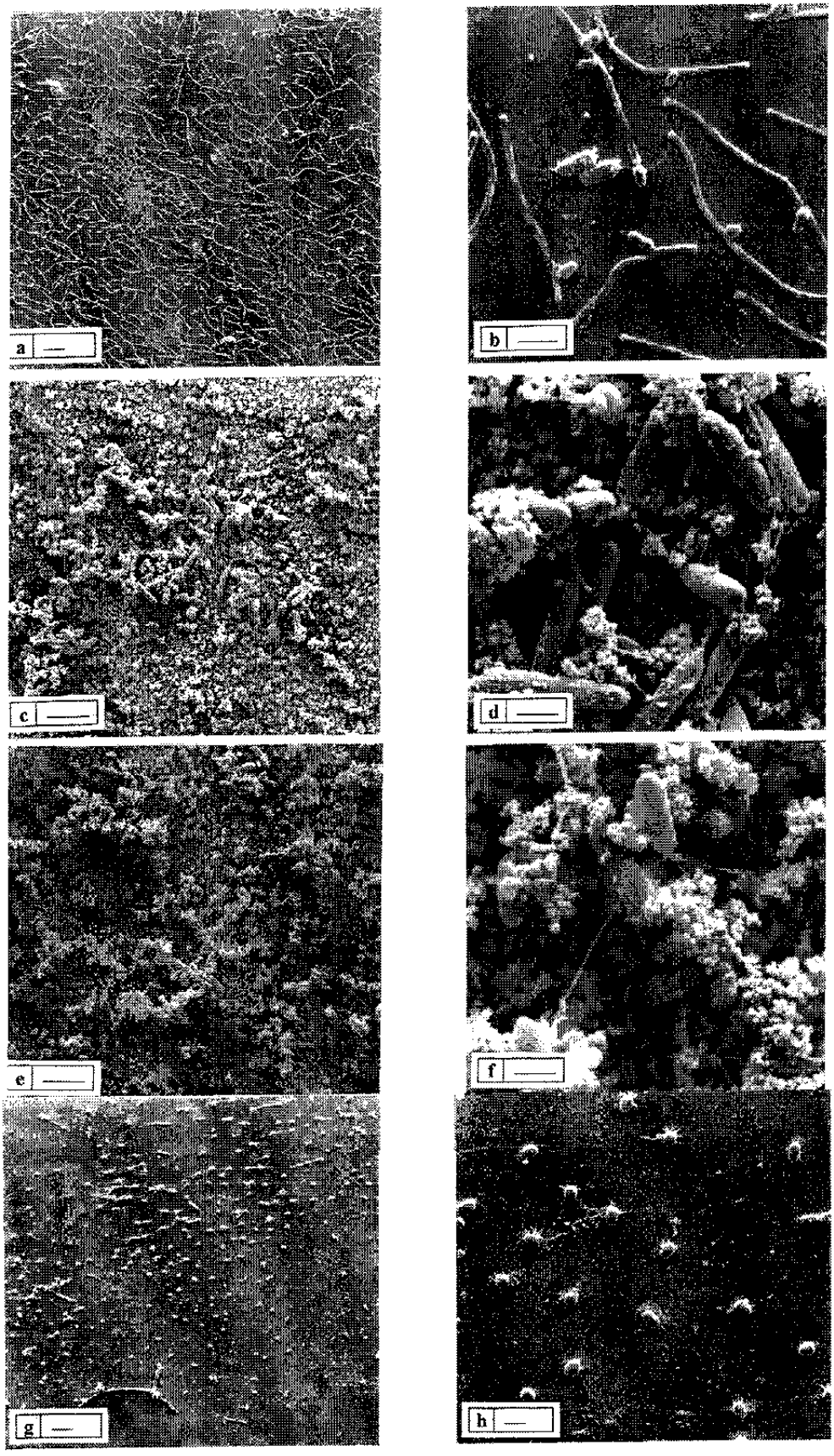

Figure 3 *SEM images of attached bacteria on glass slides exposed for 67 days to flowing groundwater from boreholes in the access tunnel to Äspö HRL. Boreholes, depths and flow rates were as follows:* ***a-b,*** *KR0013, 68m, 31 mm* s^{-1}; ***c-d,*** *SA813B, 112m, 14 mm* s^{-1}; ***e-f,*** *SA923B, 134m, 8 mm* s^{-1}; ***g-h,*** *HA1327B, 179m, 12 mm* s^{-1}; *Bars indicates 10 μm for a, c, e, g and 1 μm for b, d, f, h.*

4.3 16S rRNA GENE DIVERSITY OF ATTACHED AND UNATTACHED BACTERIA ALONG THE TUNNEL

A total of 155 16S rRNA gene clones were sequenced, 107 clones from unattached bacteria and 48 clones from attached bacteria; 12 clones were analysed from each sample (11 clones from KR0015). The boreholes SA923A and 1062A ran out of water and could, therefore, not be sampled in a proper way for DNA analysis. The 155 clones examined clustered into 61 different, specific sequences, each of which was given a clone group name, ranging from A1 to A62. The clone group names were given clone letters ranging from l to u. These letters show in which sample(s) the clone sequence was found (Table 1). Table 2 gives the number of clone groups obtained from unattached and attached bacteria for each of the boreholes investigated. Between 3 and 10 different 16S rRNA genes were detected in each sample.

Table 2 *The number of clone groups with specific sequences of unattached and attached bacteria detected in groundwater and on surfaces exposed to flowing groundwater, respectively.*

Borehole	Depth (m)	Number of clone groups	
		Unattached bacteria	Attached bacteria
HBH02	10	8	-[a]
HBH01	40	4	-
KR0012	68	10	-
KR0013	68	7	4
KR0015	68	9	-
SA813B	112	3	3
HA1327B	179	3	5
SA1420A	192	8	4
KAS03	626	7	-

a) Not determined.

Figure 4 shows a phylogenetic tree for 48 of these clone group sequences. Ten distinct groups of phylogenetically related bacteria were found (Woese, 1987), the alpha-, beta-, gamma-, delta- and epsilon-groups of the Proteobacteria, Grampositive bacteria and several sequences only very distantly related to known, named and sequenced bacteria reported to the databases. Table 3 and 4 give the total number of clones and the number of clone groups obtained from unattached and attached bacteria, respectively. Between 3 and 10 different 16S rRNA genes were detected in each sample. The clone group diversity was generally larger among the unattached than the attached bacteria. The unattached bacteria in the boreholes SA813B and HA1327B differ from this general pattern with only 3 different clone groups each. The Proteobacteria gamma-group was the largest phylogenetic group found among the unattached bacteria. To this group belonged 34 of the found clones (22%) and this was also the phylogenetic group with the largest diversity, having 11 different clone groups as shown in Table 3. The gamma-group could not be detected among the attached bacteria (Table 4). Instead, Grampositive bacteria, the Proteobacteria delta-group and the remaing clones comprised 85% of the sequences found (41 clones out of 48).

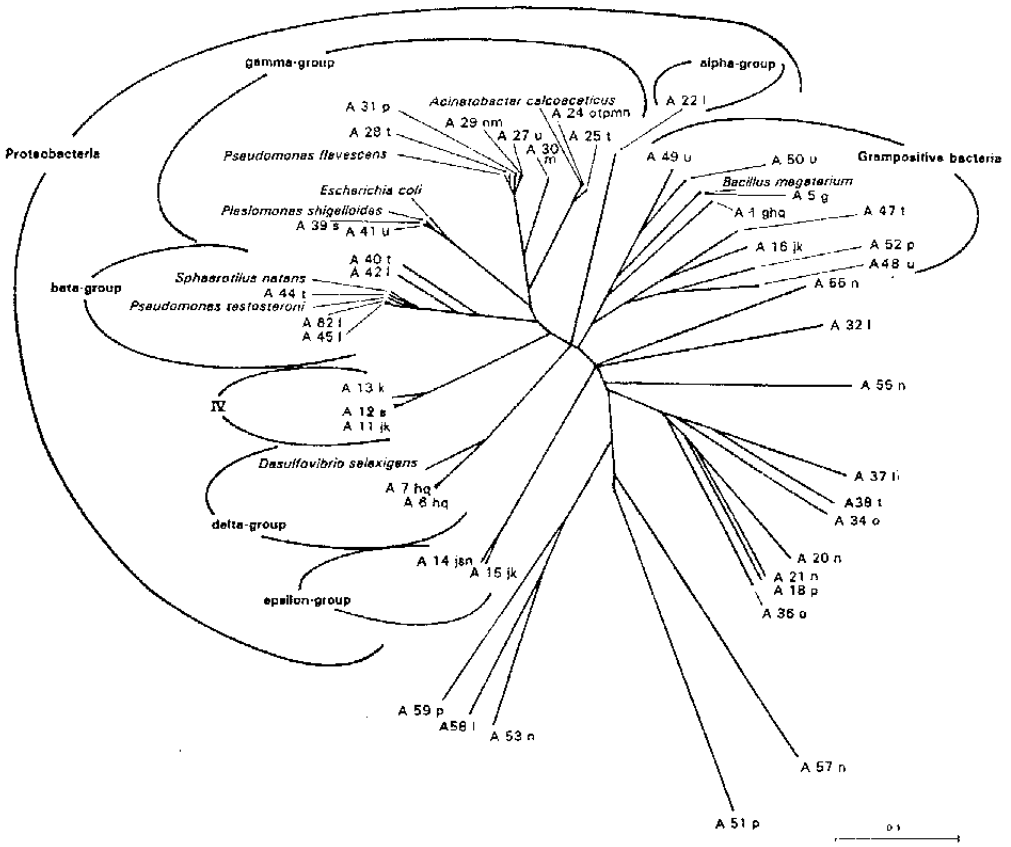

Figure 4 *Evolutionary distance tree based on the 16S rRNA gene sequences of clones from boreholes in the geological surroundings of the Äspö hard rock laboratory. Major phylogenetic groups of bacteria have been designated with their generally accepted names. As references, some 16S rRNA gene sequences of known bacteria from the EMBL database have been added to the tree and are indicated with their Latin names. The tree shows only 48 of the total of 62 different clone groups found, because there were 13 sequences that were too short for a proper alignment. Bar represents 10 nucleotide substitutions per 100 nucleotides.*

Some of the clone groups have several identical clone sequences, while others represent only a few or one clone sequence. The distribution of the 12 most commonly occurring clone sequences for unattached and attached bacteria, and the numbers of identical clone sequences in the clone groups, are shown in Table 5. These clones correspond to 63% of the sequenced clones (97 clones out of 155). Typically, each clone group predominated in a separate borehole with the exception for A11jk that predominated on two surfaces (SA1327B and SA1420A) and A24otpmn that occurred in five boreholes and comprised 10% of the sequences found (16 out of 155 clones). Several other clone groups were also detected in more than one borehole. The closest related species in the database for the predominating clones are shown in Table 6. High identities were obtained with the genus *Acinetobacter, Pseudomonas, Bacillus, Desulfovibrio, Saccharomyces* and *Thiomicrospira*. Identical clones appeared in two cases when groundwater and the corresponding surfaces samples were compared. The samples from SA813B groundwater and surfaces had 9 identical sequences, while HA1327B groundwater and surfaces had 1 identical sequence (Table 5).

4.4 16S rRNA GENE DIVERSITY OF ISOLATED SRB AND IRB

The specific 16S rRNA gene from a total of 20 isolates from the SRB cultures and one isolate from the IRB cultures were sequenced between bases 8 and 1542. Three different SRB sequences were found among the sequenced isolates. One of them, Aspo-1, could be identified as highly similar to the sequence of *Desulfomicrobium baculatum* (99.5%), while the other two, Aspo-2 and Aspo-3, were found to belong to the genus *Desulfovibrio* (Table 7); they had a vibrioid form when observed with light and SEM microscopy (not shown). Interestingly, Aspo-4 was not a SRB although isolated from an SRB enrichment. This was instead a Gram-positive bacterium with *Eubacterium limosum* as closest related species in the database and this isolate was also identical to the clone group A1ghq. Preliminary phenotypic characterization indicates this isolate to be an acetogenic species. The IRB isolate was found to be closely related to *Shewanella putrefaciens*, with a 99.1% identity value.

Table 3 *The numbers of clones that were affiliated major branches of the phylogenetic tree by PHYLIP as shown in Figure 4, distributed over the sampled boreholes. The total number of sequenced clones as well as the number of clone groups with a specific sequence are shown for each borehole. Note that some or several of the specific clone groups sequences reported for each borehole may occur in several boreholes, thereby resulting in a smaller sum of specific clone sequences than the sum of specific clone group sequences for each borehole. Cl. = Clones; Gr. = Clone groups.*

Phylogenetic branch in Figure 4	HBH02		HBH01		KR0012		KR0013		KR0015		SA813B		HA1327B		SA1420A		KAS03		Sum of clones and clone groups	
	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl	Gr.	Cl.	Gr.	Cl.	Gr.
Proteobacteria alfa-group	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
Proteobacteria beta-group	5	4	-	-	-	-	1	1	-	-	-	-	-	-	2	2	-	-	8	7
Proteobacteria gamma-group	1	1	10	3	2	2	7	2	2	2	-	-	1	1	7	3	4	3	34	11
Proteobactera delta-group	-	-	-	-	-	-	-	-	-	-	8	2	-	-	-	-	-	-	8	2
Proteobacteria epsilon-group	-	-	-	-	1	1	-	-	-	-	-	-	10	1	-	-	-	-	11	1
Grampositive bacteria	-	-	-	-	-	-	-	-	2	2	4	1	-	-	1	1	4	3	11	7
Remaning clones	5	2	-	-	9	7	4	4	5	4	-	-	1	1	2	2	-	-	26	20
Fungi	-	-	2	1	-	-	-	-	2	1	-	-	-	-	-	-	4	1	8	1
Sum of clones and groups	12	8	12	4	12	10	12	7	11	9	12	3	12	3	8	12	12	7	107	50

Table 4 *The numbers of clones that were affiliated in different major branches of the phylogenetic tree by PHYLIP as shown in Figure 4, distributed over the sampled surface. The total number of sequenced clones as well as the number of clone groups with a specific sequence are shown for each surface. Note that some or several of the specific clone group sequences reported for each surface may occur on several surface thereby resulting in a smaller sum of specific clone sequences, than the sum of specific clone group sequences for each surface. Cl. = Clones; Gr. = Clone groups.*

Phylogenicbranch in Figure 4	KR0013		SA813B		HA1327B		SA1420A		Sum of clones	Sum of clone groups
	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.	Cl.	Gr.
Proteobacteria alfa-group	-	-	-	-	-	-	-	-	-	-
Proteobacteria beta-group	2	2	-	-	-	-	-	-	2	2
Proteobacteria gamma-group	-	-	-	-	-	-	-		-	-
Proteobactera delta-group	-	-	11	2	-	-	-	-	11	2
Proteobacteria epsilon-group	-	-	-	-	3	2	2	1	5	2
Grampositive bacteria	10	2	1	1	2	1	1	1	14	3
Remaning clones	-	-	-	-	7	2	9	2	16	3
Fungi	-	-	-	-	-	-	-	-	-	-
Sum of clones and groups	12	4	12	3	12	5	12	4	48	12

Table 5 *Predominating clone groups from Äspö unattached and attached bacteria, and their distribution over sampled boreholes.*

Clone group[a]	HBH02	HBH01	KR0012	KR0013	KR0015	SA813B	HA1327B	SA1420A	KAS03	Sum of clones
unattached bacteria										
A1ghq	-	-	-	-	-	4	-	-	-	4
A6hq	-	-	-	-	-	6	-	-	-	6
A14jsn	-	-	1	-	-	-	10	-	-	11
A24otpmn	-	6	1	4	1	-	-	4	-	16
A26ou	-	-	-	3	-	-	-	-	1	4
A29mn	-	3	1	-	-	-	-	-	-	4
A58l	4	-	-	-	-	-	-	-	-	4
A61upm	-	2	-	-	2	-	-	-	4	8
attached bacteria										
A1ghq	-	-	-	1	-	1	-	-	-	2
A5g	-	-	-	9	-	-	-	-	-	9
A6hq	-	-	-	-	-	7	-	-	-	7
A7hq	-	-	-	-	-	4	-	-	-	4
A11jk	-	-	-		-	-	6	8	-	14
A15jk	-		-	-	-	-	2	2	-	4
Sum of clones and groups	4	11	3	17	3	22	18	14	5	97

[a]) Letters after the number show the borehole(s) in which the clone group was found, according to Table 1.

Table 6 *Comparison of identity between some of the sequenced and most predominating Äspö clones and 16S rRNA sequences in the EMBL database. The identity shows the percent identity between the obtained sequence and the most related organism in the database.*

No. of identical clone sequences	EMBL accession no. For clone group sequences	Most related species in EMBL	EMBL accession no for related species	Identity %
16	X91447	*Acinetobacter junii*	X81664	98.6
4	X91449	Unclassified	U12222	96.7
4	X91452	*Pseudomonas flavescens*	Z29622	95.6
13	X91429	*Desulfovibrio sp*	L40789	97.3
4	X91430	*Desulfovibrio sp*	L40789	97.7
11	X91437	*Thiomicrospira denitrificans*	L40808	97.4
4	X91438	*Thiomicrospira denitrificans*	L40808	96.0
6	X91424	*Eubacterium limosum*	M59120	91.7
9	X91428	*Bacillus megaterium*	D16273	98.6
4	X91481	*Beijerinkia indica*	M59060	73.3
14	X91434	Hydrothermal vent bacterium	U15116	94.7
8	X91484	*Saccharomyces cerevisiae*	U02969	97.6

Table 7 *Sequenced isolates of anaerobic bacteria obtained from enrichments for SRB and IRB in different boreholes at Äspö.*

Name of isolate	EMBL accession number for 16S rRNA gene sequence of the isolate	Borehole	No. of isolates sequenced	Closest species in the DNA database	EMBL accession number for the closest species sequence	Identity%
Aspo-1	X95570	HA1327B, KAS03	7	*Desulfomicrobium baculatum*	M37311	99.5[a]
Aspo-2	X95230	KR0013 HA1327B, KAS03	7	*Desulfovibrio sp.*	L42995	91.4
Aspo-3	X95331	SA1062A	2	*Desulfovibrio longreachii*	M24450	87.4
Aspo-4	X95232	SA813B	4	*Eubacterium limosum*	M59120	92.1
Aspo-5	X95233	KR0015	1	*Shewanella putrefaciens*	X81623	99.0

a) The percentage similarity is shown for this sequence as the database had too many unknown bases for a meaningful identity value.

4.5 THE CORRELATION BETWEEN FLOW AND 16S rRNA GENE DISTRIBUTION IN THE RZ FRACTURE ZONE

Fig. 5 compares the 16S rRNA sequences from the boreholes HBH01, HBH02, KR0012, KR0013 and KR0015 with the results from a tracer test using the same boreholes. The tracer test showed that water from HBH01 reached the vault boreholes KR0012, KR0013 and KR0015 after 2-9 days while it took 19 days for the HBH02 tracer to reach the HBH01 borehole and 26-39 days for the HBH02 tracer to reach the KR0012-15 boreholes. Obviously, the shallow (10 m) HBH02 borehole has a relatively poor hydraulic connection to the deeper part of the fracture zone, also indicated by its different groundwater composition (Table 1). A similar conclusion was obtained when the 16S rRNA sequences were compared. None of the clones obtained in HBH02 appeared in any of the other boreholes sampled. Furthermore, it was found that the borehole HBH01 shared 3, 2 and 4 clones with the vault boreholes KR0012-13-15, respectively. Finally, the vault boreholes shared one identical clone each (not shown).

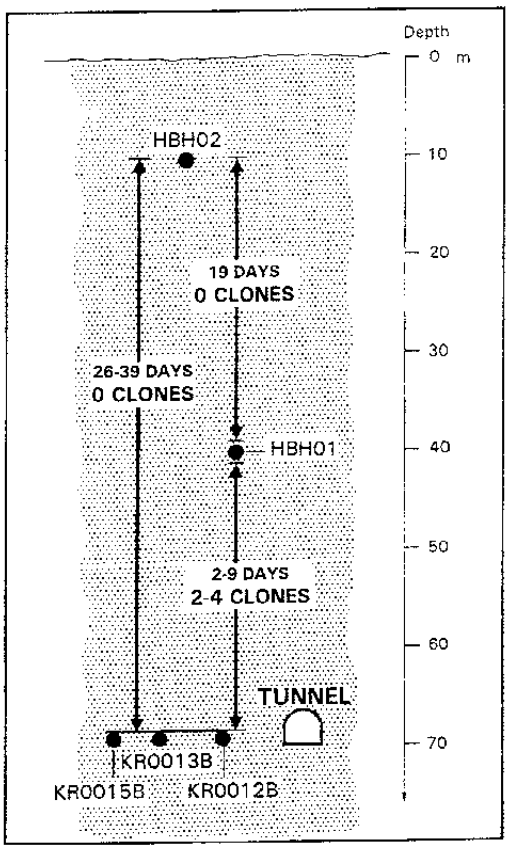

Figure 5 *The number of identical clone sequences shared between the boreholes drilled in the fracture zone RZ (Fig. 1B) that intersects the Äspö hard rock laboratory access tunnel at 68 m below level (Banwart et al. 1994) and the residence time of groundwater flowing down towards the tunnel as determined by injection of tracers (Gustafsson 1994).*

4.6 ACCESSION NUMBERS

The nucleotide sequence data reported in this paper for DNA that was extracted from Äspö HRL groundwater and surfaces comprises 61 sequences that appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X91424-X91482 and X91484-X91485 corresponding to the clone groups A1ghq to A59p and A61ump to A62l. The nucleotide sequence data reported for the isolated bacteria appear under the following accession numbers: Aspo-1, X95570; Aspo-2, X95230; Aspo-3, X95231; Aspo-4, X95232; Aspo-5, X95233.

5 DISCUSSION

5.1 GROUNDWATER COMPOSITION AND BACTERIA PRESENT

Geohydrochemical studies at Äspö HRL have helped to unravel the complex groundwater evolution that characterizes this area (Banwart 1994; Laaksoharju et al. 1995). Two main meteoric water bodies exist superimposed on deep regional and saline groundwater. These bodies consist of Baltic Sea-derived water and floating lenses of non-saline to

brackish water. There are at least three sources of meteoric waters that enter and mix within the upper 500 m: (a) recent non-saline to brackish near-surface waters, (b) modern Baltic Sea water and (c) deep saline waters. Subordinate amounts of ancient sea water and glacial melt water also contribute. The Bockholmen (RZ) groundwaters (Fig. 1B) were sampled via the boreholes HBH01, HBH02, KR0012, KR0013 and KR0015. The HBH02 groundwater was very diluted and non-saline, while the other RZ boreholes carried a brackish, meteoric water. The boreholes SA813B, SA923A, SA1062A and HA1327 had a modern Baltic Sea water profile, while SA1420A was influenced by a mix of brackish groundwater and modern Baltic Sea water. The KAS03 borehole sampled an old, deep lens of brackish groundwater floating on deeper very saline groundwater (see Table 1). The Äspö HRL groundwater situation is presently dynamic and changes continuously, because the drawdown of groundwater to the tunnel induces groundwater flows not present before the tunnel was made. This has resulted in an increasing inmixing of modern Baltic Sea water to the part of the access tunnel that runs below the sea (Laaksoharju et al. 1995).

Sampling the depths from 0 to 68 m was done from boreholes that represent non-saline to brackish water. These are influenced by the Bockholmen surface ecosystems as implied by the high concentrations of DOC (15 - 22 mg l^{-1}). The total number of bacteria found (Fig. 2, Table 1) decreased one order of magnitude from the surface of Bockholmen down to the tunnel at approximately 100 m depth and were thereafter fairly constant at greater depths. We have demonstrated earlier significant positive correlations between the total number of bacteria and the concentration of organic carbon (Pedersen and Ekendahl 1990; Pedersen et al. 1996) and a similar relationship exists here (not shown). Repeated sampling did not reveal any large variations in the total number of bacteria (Fig. 2). Thus the present microbial populations appear to be in a steady state with respect to their number. This situation is similar to what was found in the Stripa research mine, where the total numbers of bacteria did not change significantly during a sampling period of 5 years (Pedersen and Ekendahl 1992a; Ekendahl and Pedersen 1994).

A major conclusion from a large scale redox experiment using the Bockholmen boreholes (Fig. 1 B) was that IRB contribute to the redox control of the groundwater by producing ferrous iron (Banwart et al. 1994). This is in agreement with the isolation of an IRB from KR0015 that could be identified as closely related to *Shewanella putrefaciens* (Table 7); a well known iron reducing bacterium (Semple and Westlake 1987; Lovley et al. 1989; DiChristina and DeLong 1993). A succession from IRB to SRB along the flow path of groundwater has been suggested for uncontaminated subterranean areas (Lovley 1991). That model seems to fit well here as there were not any detectable SRB in the MPN samples from the shallow Bockholmen groundwater but IRB were detected there both by isolation and geochemical modeling. Instead, SRB were found at depths from approximately 100 m and deeper, as viable MPN counts (Table 1), as isolates from enrichments (Table 7) and as 16S rRNA sequences (Table 6,

clone groups A6hq and A7hq). They were found in the section of the tunnel going under the sea, and also at the depth of 626 m under Äspö (KAS03). Earlier, SRB have been reported from even greater depths of Äspö, 860 m (Pedersen and Ekendahl 1990). The reason for finding IRB and SRB at different sites of the tunnel is however not necessarily only depth related. A more likely explanation is that the types of groundwater sampled were different. Non-saline to brackish water under Bockholmen favors IRB and the saline, sulphate-rich Baltic Sea water reaching the tunnel under the sea, and the deep old groundwater under Äspö, favor SRB.

5.2 16S rRNA GENE DIVERSITY AND DISTRIBUTION OF UNATTACHED AND ATTACHED BACTERIA IN ÄSPÖ HRL

When PCR amplification is used for the determination of species diversity, the result may be biased due to methodological problems, such as uneven extraction of DNA and biased PCR due to differences in genome size (Farrely et al. 1995). One of the most important biases is that organisms belonging to the domain *Archaea* have only one or a few gene copies of the 16S rRNA gene while *Bacteria* can have several copies, 5-7 or more, and this will bias PCR amplification towards *Bacteria* (Ward et al. 1992; Amann et al. 1995). Therefore, using PCR primers that are specific for archaeal 16S rRNA gene sequences in parallel with universally conserved ones, will enhance the detection of microorganisms belonging to the domain *Archaea*. The results presented in this paper were obtained using the universal primers only and should therefore be expected to reveal mainly bacterial diversity and distribution.

By mixing bacteria in different ratios it has been shown that there exists a relationship between the ratio of clone groups obtained from a sample and the ratio of the corresponding species in the sample (Ekendahl et al. 1994). The clone groups with many identical sequences that are displayed in Table 6 then probably correspond to bacteria that occurred in relatively large number in the sampled boreholes. Another investigation has studied the effect on the diversity result from adding on 10 plus 10 plus 10 sequences, extracted and sampled in a series from the same borehole during 3-4 h (Pedersen et al. 1996). The four investigated boreholes were situated around the fossil nuclear reactor at Bangombé in Gabon, Africa. The result demonstrated a common pattern with one specific predominating sequence in each borehole, that did not predominate the other three boreholes. Generally, each set of ten sequences added new clone groups to the results, with from two up to six of the ten sequences common to different extractions from the same borehole. Similar observations were made earlier with this method with attached populations in two levels of the Stripa borehole V2 (Ekendahl et al. 1994).

The choice between sequencing many clones in a few samples or many samples with few clones is delicate. In the Äspö HRL investigation presented here, a total of 12 clones were sequenced from each clone library

(11 from KR0015) and this is certainly not enough to reveal the total 16S rRNA diversity of the samples, but it is probably enough to pick up the most predominating species, as inferred from the Bangombé and Stripa investigations. There probably exist many more species in the samples than was revealed here, but they were either too few to have a significant chance of being randomly picked from the clone libraries, or they were negatively biased for the reason discussed above and elsewhere Ekendahl et al. 1994). The interpretation of the data presented here has been restricted to discussion of the diversity and distribution of such predominating species. The conclusions will be used as a guideline for the choice and design of 16S rRNA in situ hybribization probes to be applied in the Äspö HRL environment.

The phylogenetic tree reveals that the Äspö HRL subterranean environment is inhabited by diverse microbial populations, mainly consisting of Proteobacteria and Grampositive bacteria (Figure 4). A closer inspection of the distribution of the clone groups from unattached bacteria shows a complex situation (Table 3). All samples had their own 16S rRNA gene diversity profile. The diversity, relative to the 12 clones sequenced, is high in all of the Bockholmen boreholes except HBH01 and also in SA1420A and KAS03, ranging from 7 to 10 clone groups. The two boreholes SA813B and HA1327A that were influenced by Baltic sea water had only 3 clone groups each. They also lack (with one exception) 16S rRNA sequences belonging to the Proteobacteria gamma-group, which is a clone group that occurs frequently in most of the other groundwater samples. This implies a different and a larger diversity of predominating bacteria in the recent non-saline to brackish near-surface waters than in groundwater influenced by Baltic Sea water.

There are clear general differences between the clone group profiles of unattached and attached bacteria in KR0013, SA813B, HA1327B and SA1420A. The major difference is that Proteobacteria gamma-group sequences could not be detected on the surfaces although they were found in the groundwater from all but the SA813B borehole (Table 3 and 4). Instead, Grampositive bacteria seem to prefer an attached situation as such sequences could be found on all surfaces investigated although they were absent in KR0013 and HA1327B groundwater. It was also found that one single clone group constituted 50% or more of the clones from each surface (6 or more clones) which was the case only for 3 of the 9 sampled groundwaters (Table 5). Comparing sequences in each borehole, from unattached bacteria with those from attached bacteria, shows a high similarity in clone group composition in one case, SA813B. The other three cases show different sequences in the groundwater and on the surfaces, with the exception of one clone sequence that appeared in both the groundwater and on the surface in the HA1327B borehole. The attached bacteria are much less susceptible to sampling variance than water populations which obviously is inherent with their attached lifestyle. The differences found may be due to that the surfaces select for bacteria other than those predominating in the groundwater populations, except for SA813B.

5.3 IDENTITY OF THE SEQUENCES FOUND

The finding of many new and unknown bacterial 16S rRNA sequences in natural environments is a commonly reported result (Giovannoni et al. 1990; Ward et al. 1992; Ward et al. 1990). This was also the case for most of the Äspö HRL clone group sequences when they were first compared with the EMBL database in September 1993. However, since then, the number of 16S rRNA gene sequences in the database had increased enough by September 1995 to assign identities to several clone groups. This is encouraging for the use of 16S rRNA as a tool for identifying bacteria. There is not any accepted value of the precentage identity at which two 16S rRNA genes can be concluded to belong to the same genus or species. It can be quite different for different genera (Fox et al. 1992) and is also dependent on whether total or partial 16S rRNA genes are compared. It has been suggested, based on a comparison of rRNA sequences and on DNA-DNA reassociation, that a relationship at the species level does not exist at less than 97.5% identity in the 16S rRNA sequence. At higher identity values, species identity must be confirmed by DNA-DNA hybridization (Stackebrandt and Goebel 1994). Accepting the level 97.5% conservatively, as identifying a sequence approximately on the genus level, some conclusions can be made about the sequences reported here. A comparison of the predominating 16S rRNA gene sequences (Table 6) with EMBL in September 1995, reveals three clone groups that can be identified with bacteria on the genus level. The *Bacillus* (A5g, 98.6%), *Desulfovibrio* (A6-7hq, 97.7%) and *Acinetobacter* (A24optmn, 98.6%) type sequences had an identity higher than 97.5% with 16S rRNA sequences in the database. These three genera and also some of the other genera indicated are discussed below in relation to the environment of their respective sampling site. All except one of the other seven predominating clone group sequences found had also high identities, between 93.6 and 97.4%. One of the clone groups could be identified as a member of the domain *Eukarya*, a yeast related to the genus *Saccharomyces* (A61upm, 97.6%).

The predominating sequence A5g was found on surfaces exposed to flowing groundwater from KR0013 and had a very high identity with the Gram-positive species *Bacillus megaterium* (98.6%, Table 6). This species is a large rod that can be 5 μm long or more, and Fig. 3a-b show that the surface from which A5g was collected was indeed colonized by a large rod. It is generally accepted that the primary habitat of *Bacillus* species is soil (Sneath 1984). The tracer test (Fig. 5) has demonstrated that shallow groundwater from the 40 m borehole HBH01 and a minor part from 10 m (HBH02) reach the borehole KR0013 - a link between the top soil of Bockholm and the KR0013 surface is established. Most *Bacillus*, including *B. megaterium*, degrade biopolymers and produce capsules that enhance attachment. An attached lifestyle may have been profitable as there were significant amounts of DOC in the groundwater flowing over the surface.

Acinetobacters are ubiquitous organisms that are present in soils, water and sewage and it has been estimated that *Acinetobacter* may constitute as much as 0.001% of the total aerobic population of soil and water (Tower 1992). It is a coherent genus and described and accepted species are phylogenetically well defined (Rainey et al. 1994). Finding *Acinetobacter* type sequences (A24optmn) in all 5 boreholes representing recent brackish near-surface waters (Table 1), is consequently in agreement with what is known about this group. It could not be found among the 72 clones sequenced from the deep KAS03 borehole, the non-saline HBH02 and the tunnel boreholes influenced by Baltic Sea water (SA813B and HA1327A). Sequences that relate closely to this genus have been obtained in four other subterranean environments as well (Pedersen 1996), further indicating this genus to be very common in groundwater.

Clone groups related to SRB were found only in groundwater and on surfaces from SA813B. Enrichments (Aspo-1, Aspo-2 and Aspo-3) and viable counts indicated SRB to be present in SA1062A, HA1327A and KAS03 as well but they were obviously not predominant enough to be detected by sequencing 12 clones. The only isolate whose sequence also was found in the clone libraries (clone A1ghq) was Aspo-4. The 16S rRNA of Aspo-4 showed 92.1% identity with the acetogenic bacterium *Eubacterium limosum* which is too low for identification. However, preliminary phenotypic characterization indicates this isolate to be an acetogenic species. It was isolated from SA813B (Table 7) and its 16S rRNA sequence was found in groundwater and on surfaces from this borehole and on surfaces from KR0013. The potential for classification of Aspo-2, Aspo-3 and Aspo-4 as new species is presently being investigated.

5.4 16S rRNA GENE SEQUENCES AS TRACERS FOR GROUNDWATER MIXING

Successful prediction and understanding of the dispersal of pollutants from various constructed as well as incidental sources requires information about groundwater flow and mixing. The pollutants can be radionuclides from future radioactive waste repositories, chlorinated hydrocarbons from industrial sites, metals from waste dumps etc. A commonly used technique is to add tracers, in solution or as particles, at defined sites and then register the time until breakthrough occurs at other sites. The degree of dilution of the tracer can be used to calculate mixing of different groundwater sources. The bacterial composition in a borehole is complex. In addition to acting as particles, specific species of bacteria grow and divide at optimal conditions but are reduced in number in other conditions. Therefore, DNA results presented here reveal the result of a combination of geochemical conditions that select for different bacteria and of groundwater mixing. Still, a considerable agreement was achieved between flow and the 16S rRNA gene signatures of the Bockholmen groundwater (Fig. 5). This, together with similar results from Bangombé in Africa (Pedersen et al. 1996), indicate that DNA analysis may add information that will help to unravel the flow and mixing of groundwater.

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RNA extraction from deep granitic groundwaters at Äspö and hybridization with group-specific 16S rRNA-directed oligonucleotide probes

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CONTENTS

1 ABSTRACT

The aim with this study was to evaluate the suitability of RNA extraction and hybridization with group-specific 16S rRNA-directed oligonucleotide probes as a method for investigation of abundance and activity of different microbial groups present in deep granitic groundwaters. RNA was extracted from groundwater sampled from boreholes in granitic rock at Äspö HRL, southeastern Sweden. Hybridization was primarily performed with a *Bacteria*-specific probe (EUB338), an *Acinetobacter*-specific probe (ACA652) and a negative control probe (NONEUB338). Other group-specific probes were evaluated as well. It was possible to extract RNA from one liter of pressure-filtered groundwater. Co-extracted organic material interfered, resulting in low RNA purity and inhibition of reliable quantification of RNA as well as proper probe binding in slot blot hybridization experiments. Several purification steps using Chelex-100, Sephadex G-75 and polyvinylpolypyrrolidone (PVPP) to remove organic material and trace metals were necessary and improved the hybridization results considerably. Hybridization signals were obtained from EUB338 and other probes. The developed RNA extraction method was suitable for pure cultures, for groundwater samples containing many bacteria, and for groundwater enrichment cultures for *Acinetobacter*. Hybridization of the ACA652 probe to RNA extracts from enrichments showed weak positive responses, indicating the presence of *Acinetobacter* in the subsurface. The positive responses supported indications from another study showing *Acinetobacters* is present in the deep granitic groundwaters analyzed. RNA extracts from larger volumes of groundwater than one liter or from samples containing more than 10^6 cells ml^{-1} are desirable in future RNA hybridization studies on deep groundwaters.

Key words: *Acinetobacter, Bacteria*, humic compounds, purification, 16S rRNA probes subsurface.

2 INTRODUCTION

It is often concluded important to identify groups or species of organisms that are present in a mixed environmental community. The presence of specific organisms can be of concern in polluted areas as well as at pristine sites where the community structures are unknown. Valuable information about the most dominant or most active populations can be obtained using molecular methods. The importance of specific populations for the activity within a community and their effect on the environment can be evaluated.

Studies on deep groundwater microbiology have increased the knowledge about subsurface microbial communities especially during the last two decades. In Sweden, microbial investigations have been performed on deep granitic groundwaters due to the plans for storing radioactive high level

nuclear waste 500 m underground in the granite. Microorganisms can affect a waste storage in negative or positive directions (Pedersen, 1996). To evaluate microbial effects on such storage knowledge about present organisms is important.

A molecular method for studying specific populations is characterized by RNA extraction followed by slot blot hybridization with group- or species-specific 16S rRNA-directed oligonucleotide probes (Stackebrandt and Goodfellow, 1991). In this paper, RNA extraction and hybridization was evaluated as a method for studying microorganisms in subsurface granitic groundwaters. The aim was to investigate if 16S rRNA could be extracted and if RNA yields would be sufficient for detecting positive hybridization signals. Focus was set primarily on groundwater from a granitic borehole called KA3105A due to the presence of sulfate reducing bacteria in this groundwater. Sulfate reducers are highly interesting bacteria because of their possible participation in anaerobic corrosion that might affect the future safe storage of nuclear waste in copper/steel canisters in the rock (Pedersen, 1996). The bacterial genus *Acinetobacter* is also interesting. Earlier studies using 16S rRNA gene sequencing methods have indicated that *Acinetobacters* is common in deep groundwaters (Pedersen et al. 1996). In addition to evaluation of RNA extraction and hybridization with granitic groundwater RNA, another aim with this study was therefore to detect *Acinetobacter* in the groundwater with an *Acinetobacter*-specific 16S rRNA oligonucleotide probe.

3 MATERIALS AND METHODS

3.1 STUDY SITE

Investigated deep groundwaters were sampled from boreholes in granitic rock at the Äspö Hard Rock Laboratory (HRL) on the Swedish southeast coast. The site and the boreholes have been described in detail elsewhere (Pedersen et al. 1996). Anoxic groundwater for RNA extraction was sampled from artesian boreholes in the Äspö HRL tunnel. The boreholes were: KR0012B, KR0013B, KR0015B (drilled at 68 m depth into the tunnel wall), SA1420A (at 192 m depth), and KA3105A (at 414 m depth). Groundwater for *Acinetobacter* enrichment cultures was also sampled from these boreholes.

3.2 SAMPLING OF GROUNDWATER MICROORGANISMS FOR RNA EXTRACTION

A sterile 25-mm high pressure stainless steel filter holder (Millipore) was connected directly to the tubings from the boreholes. Five hundred ml up to one liter of groundwater were pressure filtered from SA1420A and

KA3105A (using only the artesian pressure of the groundwater). The pressure was 15 or 35 bars prior to the filter holder and 10 or 30 bars after the filter unit for SA1420A and KA3105A, respectively. Groundwaters from KR0012B, KR0013B and KR0015B were vacuum filtered. Autoclaved, UV-irradiated hydrophilic Durapore membrane filters (25 mm diameter, 22 µm pore size, Millipore) were used in both filtration methods. After filtration, the filters were placed in 1 ml 0.1% diethyl pyrocarbonate-(DEPC) treated TE buffer in sterile, RNase-free 2 ml tubes (Sarstedt). They were put on ice, transported frozen to the laboratory the same day and stored at -20°C until extraction of RNA was performed.

3.3 RNA EXTRACTION

The filter samples were thawed to room temperature. Each of 0.4 ml Sephadex G-75 (gel filtration medium, DNA grade, Pharmacia) and Chelex-100 (chelating ion exchange resin for trace metal removal, analytical grade, BioRad), were added to each filter-containing tube. The Chelex and Sephadex solutions were washed three times in 0.1% DEPC-treated TE buffer and autoclaved before use. After gentle mixing with Chelex and Sephadex, the samples were centrifuged in an MSE MicroCentaur centrifuge (Sanyo) at 1000 rpm for 2 min. Supernatants and filters were subsequently transferred to new tubes containing 2.5 g of 2% HCl-washed, autoclaved and dried 0.1 mm zirconium/silica beads (Biospec Products). A mini-bead-beater (Biospec Products) was used to beat each filter for five minutes. Lysozyme (Boehringer Mannheim), 1 mg in 50 µl 0.1% DEPC-treated TE buffer, was added and mixed by vortexing for five seconds. The samples were left for 10 min at 30°C (due to the heat developed during beadbeating) and then transferred to sterile 13 ml polypropylene tubes (Sarstedt).

A modified protocol for isolating bacterial RNA with the RNeasy extraction kit (Qiagen) was used. Apart from added purification steps and beadbeating, the main change was the scaling up of volumes. Lysis buffer (which contained guanidinium isothiocyanate), 3.92 ml, and beta-mercaptoethanol, 40 µl, were used to rinse the 2 ml tubes and to transfer the remaining samples into the 13 ml tubes. The lysis mixture was then vortexed vigorously. One ml sterile PVPP (polyvinylpolypyrrolidone, washed in TE buffer) was added and vortexed briefly. After centrifugation at 1000 rpm for 2 min, the supernatants (leaving the PVPP, filters and beads) were transferred to new 13 ml tubes. Ethanol, 2.8 ml, 99.5%, was added and mixed gently.

The samples were applied to RNeasy spin columns and centrifuged, 700 µl at a time until all had passed the column. The columns were washed with buffers supplied in the RNeasy kit, first once with "RW1" wash buffer and four times (3x15 s and 1x2 min) with wash buffer "RPE" according to the RNeasy protocol. RNA was eluted from the columns with 2x30 µl 0.1% DEPC-treated double distilled water for 10 minutes each before spinning it down into a 1.5 ml tube.

RNA from pure cultures, representative of the domains Bacteria (*Acinetobacter calcoaceticus, Acinetobacter lwoffii, Thiobacillus ferrooxidans, Desulfovibrio aespoeensis, Streptomyces griseus, Desulfomicrobium baculatum), Archaea (Methanobacterium autotrophicum)* and *Eucarya* (*Saccharomyces cerevisiae*) were extracted according to the RNeasy extraction kit protocol (Qiagen) or with the modified method described above.

3.4 RNA QUANTIFICATION

Yields of RNA from both groundwater and pure cultures were measured with a Beckman DU-65 spectrophotometer using the Warburg nucleic acids program (Warburg and Chrisitian, 1942). The spectrophotometric values were compared with results from nucleic acid measurements using DNA dipsticks (Invitrogen Corporation). RNA from pure cultures was additionally visualized with standard 1% agarose gel electrophoresis.

3.5 RNA SLOT BLOTS

RNA was denatured with four volumes 2% glutaraldehyde for 10 min at room temperature, then diluted with 0.1% DEPC-treated water to 100 µl (if necessary) and filtered onto Hybond-N nylon membranes (Amersham) using a BioDot apparatus (BioRad). Membranes were washed in a 2xSSC, 0.1% SDS, 0.1% DEPC solution for 15 min, UV-crosslinked for one minute, baked in an oven at 80°C for two hours and stored dry at room temperature.

From groundwater extractions, all RNA from one extraction was put into one well in the slot blot apparatus. In dilution series with pure culture RNA, ten-fold dilutions from 100 ng per well and downwards (100, 10, 1, 0.1 and 0.01 ng) were added to each well. For probe specificity control, 100 ng of pure culture 16S rRNA was used.

3.6 OLIGONUCLEOTIDE PROBES AND SPECIFICITY CONTROL

The 16S rRNA oligonucleotide probes that were examined in RNA hybridizations are presented in Table 1. Two universal probes (UNI926 and UNI1406), a bacterial probe (EUB338), a eucaryal probe (EUK1209), a probe specific for *Archaea* (ARCH915), a probe specific for *Acinetobacter* (ACA652), two probes specific for sulfate reducing bacteria (SRB687 and SRB804) and a negative control probe (NONEUB338, complementary to EUB338) were used. To confirm that the extraction method mainly gave 16S and 23S rRNA and that probes were specific for 16SrRNA, standard Northern blots were run on gels in which RNA from pure cultures was compared with an RNA ladder (Life Technologies). The presence of DNA and smaller RNAs was lower using the RNeasy extraction as compared to phenol extraction methods.

Table 1 Oligonucleotide probes used for 16S rRNA hybridization with groundwater RNA.

Specificity	Probe	Reference
Universal	UNI926	(Hallbeck el al. 1993)
-"-	UNI1406	(Hallbeck el al. 1993)
Bacteria	EUB338	(Amann el al. 1990)
Neg. Control	NONEUB388	-
Acinetobacter	ACA652	(Wagner el al. 1994)
SRB	SRB687	(Devereux el al. 1991)
SRB	SRB804	(Devereux el al. 1991)
Archaea	ARCH915	(Stahl and Amann, 1991)
Eucarya	EUK1209	(Giovannoni el al. 1988)

3.7 SLOT BLOT HYBRIDIZATION WITH [GAMMA-^{32}P]-ATP-LABELLED PROBES

Hybridizations were performed largely according to Devereux et al. (1991) with the following modifications. Prehybridizations were made in rotating 50 ml polypropylene centrifuge tubes containing 3-5 ml hybridization solution (0.9 M NaCl, 50 mM $NaPO_4$ pH 7.0, 5 mM EDTA, 10xDenhardts solution, 0.5 mg ml^{-1} poly-A, (Devereux et al. 1991) and RNA membranes in a hybridization oven (Hybaid Ltd) at 40°C for 4 h.

The oligos were labelled in the following manner: 50 ng (1 µl) oligo, 1 µl 10xbuffer for T4 polynucleotide kinase and 4 µl 0.1% DEPC-treated double distilled water were mixed, heated at 90°C for two minutes and put on ice. Polynucleotide kinase (1.5 µl = 15 units, Boehringer Mannheim) and 2.5 µl of [gamma-^{32}P]-ATP, 5000 Ci/mmol (Amersham) were added and the mixture was incubated at 37°C for 30 min. After incubation, 300 µl 0.1% DEPC-treated double distilled water was added and the mixture spun through a column containing 1.5 ml TE-buffer-washed Sephadex G-25 (Pharmacia) for 4 min at 2000 rpm. All of the wash-through were added to the hybridization tube.

Hybridization was carried out over night for 16-20 h at 40°C, before washing 2x15 min in a 48°C-heated 1xSSC, 1% SDS, 0.1% DEPC solution at 45°C. The membranes were dried for one hour before being placed in a hybridization bag. They were exposed for between four hours and three days before visualization with a PhosphorImager (Molecular Dynamics) system.

3.8 ENRICHMENT CULTURES FOR ACINETOBACTER

Baumann's medium (Baumann, 1968) was used to enrich *Acinetobacter* species. It consisted of 2 g CH_3COONa x 3 H_2O, 2 g KNO_3, 0.2 g $MgSO_4$ x 7 H_2O, 1 ml trace element solution (SL-10, Pedersen and Ekendahl, 1990), 1 ml selenite-and-tungsten solution (Pedersen and Ekendahl, 1990), 1 ml vitamin B_{12} solution (50 mg l^{-1}) and 10 ml 10-vitamine solution in 1000 ml (final volume) of a 0.04 M KH_2PO_4-Na_2HPO_4 buffer with pH 6.0. The

vitamine solution contained 2 mg biotine, 2 mg folic acid, 0.1 mg pyridoscine (B_6), 5 mg thiamine-HCl (B_1), 5 mg riboflavine (B_2), 5 mg nicotinic acid, 5 mg panthothenic acid, 0.1 mg cyanocobalamine, 5 mg P-aminobenzoic acid and 5 mg lipoic acid.

Groundwater was inoculated aerobically in 10 ml samples into 100 ml serum bottles containing 40 ml sterile medium. The enrichments were shaken at room temperature aerobically, since *Acinetobacter* species are reported to be obligate aerobes (Tower, 1992). Four parallells were used.

RNA from these cultures was extracted with the modified RNeasy extraction kit method used for other groundwater samples (see above). 16S rRNA hybridizations with the EUB338 and ACA652 probes were done as described above.

4 RESULTS

4.1 RNA EXTRACTION AND PURIFICATION OF DEEP GROUNDWATER SAMPLES

It was possible to obtain rRNA from the deep groundwater samples. Beadbeating gave a ten-fold higher RNA yield than by only using the manufacturer's RNeasy protocol, but it also released organic compounds that inhibited proper RNA quantification and hybridization. The use of a Chelex-100 + Sephadex G-75 mixture before beadbeating and the use of PVPP after lysis buffer addition improved the sample purity considerably. Spectrophotometric values dropped ten times, indicating humic material and/or RNA to have disappeared. Results from DNA dipstick measurements on extracts generally agreed with spectrophotometric values after RNA purification.

However, although improved, the samples were still unpure according to spectrophotometric values. Different wavelengths used in spectrophotometry reveal different kinds of compounds in a sample. The absorbance at 230 nm (A_{230}) shows presence of proteins with peptide bonds and also phenolic compounds. A_{260} shows nucleic acid presence and A_{280} measures proteins (aromatic amino acids). The ratio A_{260}/A_{280} shows the level of protein contamination, while A_{260}/A_{230} reveals phenolic (humic) contamination (Steffan et al. 1988). The A_{260}/A_{280} values obtained in our groundwater RNA extracts stayed around 1.4-1.6 (Table 2). Pure samples have a quotient of 1.7 or higher (Steffan et al. 1988). The $A_{260}/_{230}$ ratio for pure samples is >2 (Steffan et al. 1988). Our values were between 0.1 and 0.7 depending on which purification steps that were used (Table 2). Steffan et. al (1988) report values of 1.2 for DNA samples with humic compounds treated with PVPP, and that further purification did not approve this

number. Using Sephadex and Chelex in a column, instead of just mixing with the sample, purified our groundwater samples from KA3105A effectively and A_{260}/A_{280} values reached 1.9 and A_{260}/A_{230} values reached 1.0, but simultaneously we lost 90% of the RNA, which was unacceptable. Extracted groundwater RNA could not be observed on minigels.

Hybridization signals obtained from a pure culture RNA dilution series and RNA extracted from the Äspö borehole KA3105A were compared using the *Bacteria*-specific EUB338 probe. Significantly lower hybridization signals of 16S rRNA from both unpurified and purified groundwater samples were observed than expected from spectrophotometric values (Table 3). A mixture of Chelex and Sephadex improved the hybridization signal. Roughly assuming that pixel values are linear, the difference between expected and measured pixel values was lowered from 652 times to 28 times in the groundwater samples. Results from a hybridization with RNA from 500-600 ml of KA3105A groundwater, extracted and purified with Chelex and Sephadex (Fig. 1), showed clearly that the best hybridization signal was obtained if Chelex and Sephadex were added before the beadbeating step.

Table 2 *Spectrophotometric values of RNA from the KA3105A deep granitic groundwater at Äspö, extracted and purified with Chelex/Sephadex and PVPP.*

Sample	A_{260}/A_{280}	A_{260}/A_{230}
Pure RNA	>1.7-2.0	>2
KA3105A (purified)	1.4-1.6	0.1-0.7
KA3105A (Chelex/Sephadex column + PVPP purified)	1.9	1.0

Table 3 *Ratios between expected and measured hybridization signals (in pixels) in slot blot hybridizations with RNA from groundwater and the EUB338* Bacteria*-specific probe. RNA yields were measured spectrophotometrically after purification. Expected pixel values are based on measured pixel values from the* A. calcoaceticus *pure culture pixel value 52 000 below.*

Sample	Purification (added to RNeasy)	16S rRNA amount (ng) added to well	Measured pixel value	Expected pixel value	Ratio expected / measured
Acinetobacter calcoaceticus	- [a]	100	52 000	-	-
A. calcoaceticus	-	10	5 400	-	-
A. calcoaceticus	-	1	250	-	-
KA3105A	-	376	300	195 520	652
KA3105A	Chelex-100	289	4 000	150 280	38
KA3105A	Chelex-100 + Sephadex G-75	53	1 000	27 560	28

[a] = not performed/not relevant.

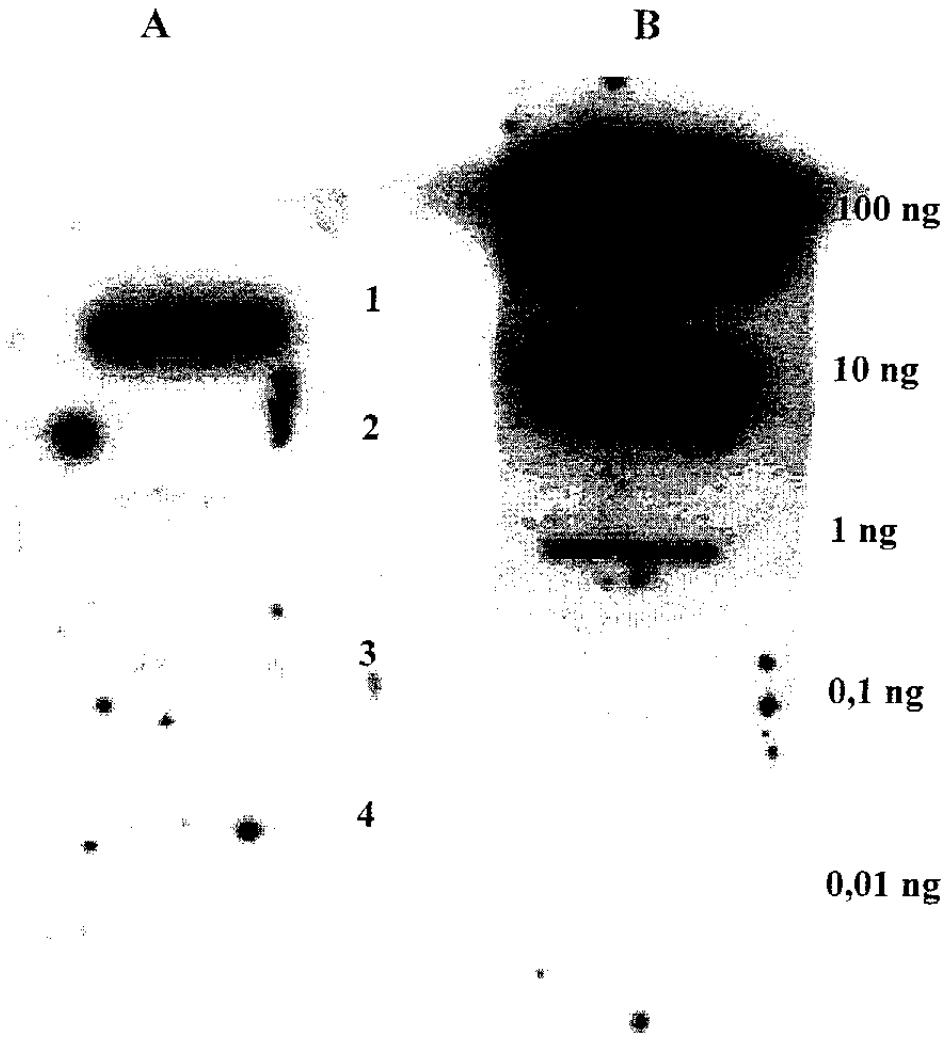

Figure 1 *Hybridization with the* Bacteria*-specific EUB338 16S rRNA oligonucleotide probe to;* ***A.*** *RNA extracted with a modified RNeasy extraction kit protocol (using Chelex-100 and Sephadex G-75 for purification) from the Äspö borehole KA3105A.;* ***1.*** *Chelex-100 and Sephadex G-75 mixed with sample before the beadbeating step in lysis. (RNA from 500 ml groundwater);* ***2.*** *The same compounds used in a column after beadbeating but before addition of lysis buffer (from 600 ml groundwater).;* ***3.*** *Chelex and Sephadex mixed wih samples after addition of lysis buffer but before ethanol addition (from 550 ml groundwater).;* ***4.*** *Same compounds mixed with samples after addition of both lysis buffer and ethanol (from 500 ml groundwater).;* ***B.*** *a dilution series of an* Acinetobacter calcoaceticus *pure culture (100, 10, 1, 0.1, 0.01 ng 16S rRNA). For extraction procedures, see main text.*

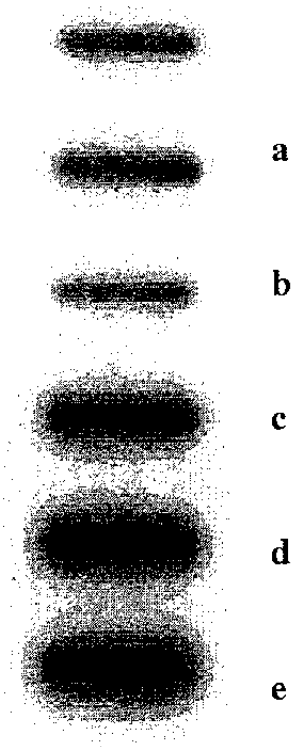

Figure 2 *Results from slot blot hybridizations with 16S rRNA, extracted from 1.5 ml of a KA3105A* Acinetobacter *enrichment culture, with different purification steps added to the RNeasy extraction protocol;* ***a.*** *Addition of 1 ml PVPP to samples after addition of lysis buffer but before ethanol addition;* ***b.*** *Addition of 0.4 ml PVPP and centrifuging (1000 rpm, 2 min) before beadbeating and lysis of the supernatant;* ***c.*** *Mixing of samples with 0.4 ml Chelex-100 and 0.4 ml Sephadex G-75 before centrifuging and beadbeating of supernatant, and then mixing of 1 ml PVPP after lysis buffer addition;* ***d.*** *Addition of 0.4 ml PVPP, mixing and centrifuging, followed by addition of 0.4 ml Chelex-100 and 0.4 ml Sephadex G-75 to supernatant before centrifuging and beadbeating of second supernatant;* ***e.*** *As in d, but mixing and centrifuging of PVPP followed after addition of Chelex and Sephadex instead of before (all before beadbeating).*

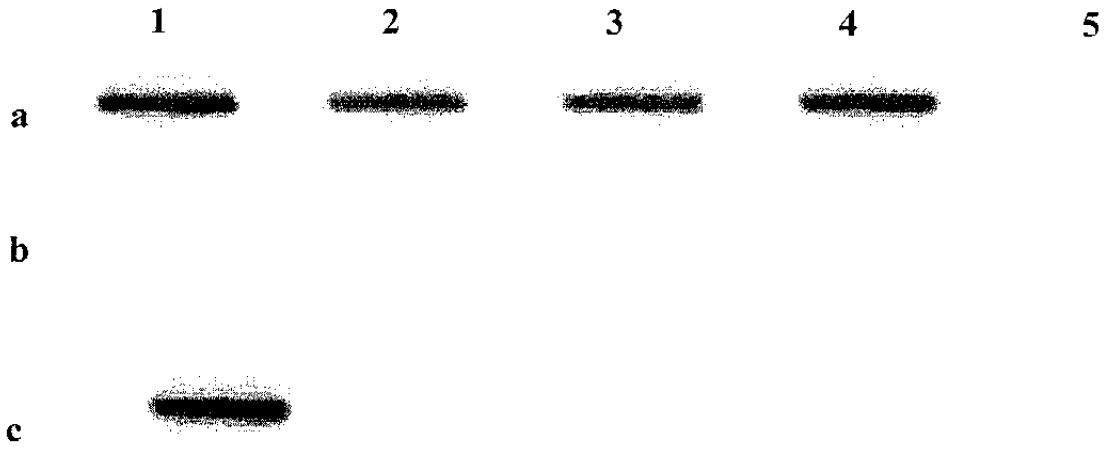

Figure 3 *Slot blot hybridizations to 100 ng of 16S rRNA from pure cultures with three [gamma-^{32}P]-ATP-labelled oligonucleotide probes: **A** EUB338, Bacteria-specific, **B**. NONEUB338, negative control probe and **C**. ACA652,* Acinetobacter-*specific. 1.* Acinetobacter calcoaceticus*; 2. Aspo-3 isolate,* Desulfovibrio-*related (Pedersen et al. 1996); 3.* Streptomyces griseus*; 4. Aspo-1 isolate,* Desulfomicrobium baculatum *(Pedesen et al. 1996); 5.* Saccharomyces cerevisiae.

Fig. 2 shows results from PVPP treatment (with or without Chelex and Sephadex purification) in extraction from *Acinetobacter* enrichment cultures containing 1/5 groundwater and 4/5 Baumann's medium. The best result was obtained by adding PVPP in the lysis buffer mixture before ethanol addition (Fig. 2C).

The hybridization signal from KA3105A groundwater RNA, extracted from one liter of water (containing $3x10^4$ cells ml^{-1}), was at best comparable to the signal from about 5-10 ng of pure culture RNA, using the EUB338 probe (compare Äspö RNA in Fig 1A1, where 100 ng 16S rRNA extracted from 500 ml of groundwater was comparable to 2-3 ng, with a pure culture in Fig. 1B). The ratio expected/observed number of pixels was about 40 times (no PVPP added).

4.2 PROBE DETECTION LIMITS

The hybridization detection limit for the EUB338 probe (Fig. 3) was 1 ng of pure 16S rRNA, as 0.1 ng was barely visible and considered as background (Fig 1). A probe for *Acinetobacter*, ACA652, had the same detection limit of (0.1-)1 ng as EUB338 (Fig. 4B). Specificity controls showed that the probe only bound to *Acinetobacter* species with none or one mismatch to the probe (Fig. 4C, see also 3C). It also bound to *Thiobacillus ferrooxidans* (one mismatch), but not to bacteria with two or more mismatches. ARCH915, EUK1209 and UNI926 needed (1-) 10 ng for detection, while the SRB probes and UNI1406 had a detection limit of (10-)100 ng.

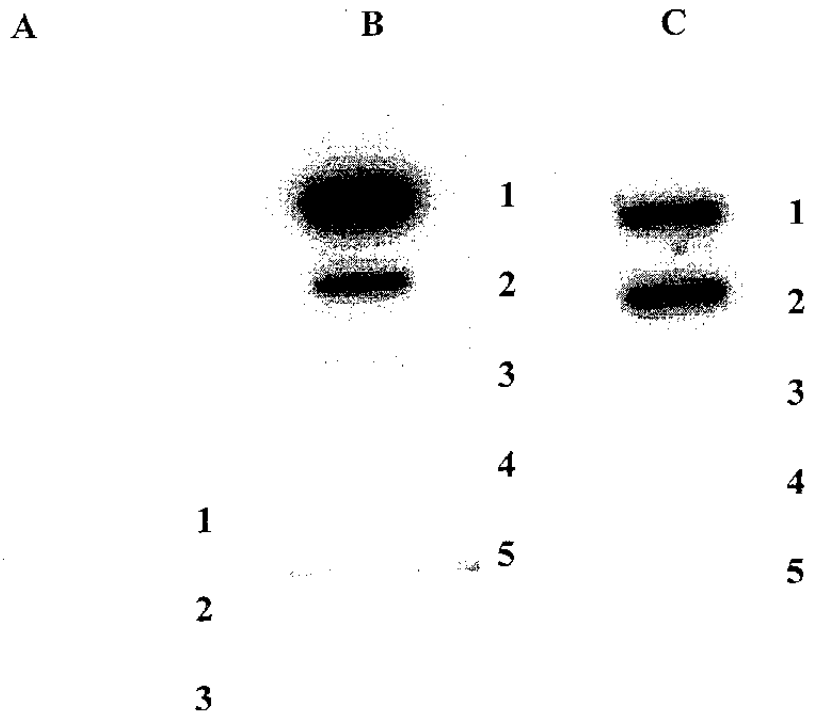

Figure 4 *Slot blot hybridizations with a [gamma-^{32}P]-ATP-labelled* Acinetobacter*-specific oligonucleotide probe ACA652 to 16S rRNA extracted with the RNeasy protocol from: A. mixed enrichment cultures for* Acinetobacter, *B. an* Acinetobacter lwoffii *pure culture dilution series, C. 100 ng 16S rRNA from different pure cultures for control of probe specificity;* ***A1.*** *From the Äspö tunnel borehole KR0012B;* ***A2.*** *From the Äspö tunnel borehole KR0013B;* ***A3.*** *From the Äspö tunnel borehole KR0015B;* ***B1-5.*** *RNA detection levels for the ACA652 probe in a dilution series with 100, 10, 1, 0.1, and 0.01 ng 16S rRNA from* Acinetobacter lwoffii, *strain ATCC 17986, DSM 2403, which has no mismatches to the probe;* ***C1.*** Acinetobacter calcoaceticus, *strain DSM 30006, 1 mismatch to the probe (compare B1);* ***C2.*** Thiobacillus ferrooxidans, *1 mismatch;* ***C3.*** Desulfovibrio-*asponium, Aspo-3 isolate (Pedersen el al. 1996), 2 mismatches;* ***C4.*** Streptomyces griseus, *4 mismatches;* ***C5.*** Desulfomicrobium baculatum, *Aspo-1 isolate, (Pedersen el al. 1996), 6 mismatches. Table 1. Oligonucleotide probes used for 16S rRNA hybridization with groundwater RNA.*

4.3 ACINETOBACTER ENRICHMENTS

Enrichments for *Acinetobacter* that were inoculated with groundwater from the three KR0012-13-15B boreholes became turbid after 4-6 days, from SA1420A after 7-9 days and from KA3105A after 13 days. The KR cultures contained mainly large or smaller rods. Some bacteria were more coccoid and single or two or three in a row, and some were swimming fast. A few bacteria were curved or spiral-shaped. The SA1420A enrichments gave mainly smaller coccoid-like cells in chains. One bottle from this borehole had a few filamentous "rectangular" cells. From KA3105A, two bottles

showed no growth while the remaining two showed dominating rod-shaped bacteria but also some of the "rectangular" cells and fungi. RNA extractions from the *Acinetobacter* enrichment cultures that were hybridized with the *Acinetobacter* genus-specific probe ACA652 showed weak positive responses for the three KR0012-13-15B boreholes (Fig. 4A).

5 DISCUSSION

5.1.1 RNA extraction and purification

Instead of phenol extraction, an rRNA extraction kit was used for groundwater samples. The RNeasy method gave less interfering DNA and small RNAs than phenol extraction both from pure cultures and groundwaters.

Many investigators have discussed the problems with detecting less active bacteria and with obtaining RNA enough for hybridization studies from environmental samples. This is due to the low number of cells (Ogram et al. 1995), the difficulty in getting clean samples (Moran et al. 1993) and the low activity in oligotrophic environments (Stevens et al. 1995).

Initially, there seemed to be much RNA in the extracts. Calculations from spectrophotometric values showed that, with the total counts we had, each cell had more rRNA than laboratory cultures of *Esherichia coli* in log phase (18 700 ribosomes per cell, (Neidhardt et al. 1990)! Although Äspö bacterial communities have been shown to be active (Pedersen and Ekendahl, 1990; Pedersen and Ekendahl 1992), this value seemed much too high. On minigels, 100 ng was the RNA detection limit for pure cultures, but when adding multiple amounts of groundwater RNA it could not be observed. This phenomenon has been reported previously by Abbaszadegan et. al (1993). Humic compounds can inhibit binding of ethidium bromide to nucleic acids (Akkermans et al. 1995). Many organic compounds, especially humic and fulvic acids, copurify with RNA (Ogram et al. 1995) during extraction. They absorb at the same UV wavelength as nucleic acids, which makes exact quantification of groundwater RNA difficult. Äspö groundwaters are rich in humic materials (Petterson et al. 1990) and purification during RNA extraction became necessary.

Beadbeating is reported not to disrupt RNA molecules, as it does with DNA (Stackebrandt and Goodfellow,1991). It improved our groundwater RNA yield but simultaneously released compounds that inhibited RNA quantification and hybridization. Abbaszadegan et. al (1993) showed that a mixture of Chelex-100 and Sephadex G-100 purified groundwater samples from inhibiting substances like humic acids significantly better than purification with Sephadex G-100 alone. Moran et. al (1993) used Sephadex G-75 for this purpose. Studies by Steffan et. al (1988), Steffan et. al (1991) and Weller and Ward (1989) showed that PVPP helped similarly. Keller and Manak (1993) list several protocols where PVPP and Chelex are used for extraction. What size fracture of Sephadex to choose for our groundwater

samples was difficult to decide due to the unknown size of humic particles, but G-75 was chosen and seemed to work. The developed extraction protocol was a compromise between RNA purity and RNA yield.

5.2 DETECTION LIMITS IN HYBRIDIZATION WITH DEEP GROUNDWATER 16S RRNA

It has been reported that as small amounts as 1-3 pg of RNA can be observed in ^{32}P-hybridizations (Hames and Higgins, 1985). In our case, 1 ng was the detection limit for pure cultures. If 1000 ribosomes per cell are assumed for stationary phase cells, calculations show that 1 ng of 16S rRNA can theoretically be obtained from $1.2x10^6$ cells (one 16S rRNA molecule weighs about $8.3x10^{-19}$ g). Even if all cells are inactive, one liter of groundwater containing $3x10^7$ cells (as in KA3105A) should then be about ten times more than needed for detection with hybridization probes. This assumes a probe detection limit of 1 ng and that all RNA can be extracted and observed in hybridization. Obviously, the present organic compounds in the groundwater not only inhibited detection on minigels and proper spectrophotometric calculations, but they also seemed to inhibit hybridization.

The highest EUB338 hybridization signal obtained from 500 ml of KA3105A water indicated about 2.5 ng of RNA (Fig. 2). Table 4 shows how many percent of a community that a specific genus has to comprise to be able to observe with RNA extractions from one liter of groundwater, providing the specific probe has a detection limit of 1 ng and that the hybridization signals from the other boreholes are comparable to signals from KA3105A. A population within a diverse community in deep groundwater (Pedersen el al. 1996) probably does not comprise more than ten percent of the community. To detect 1% of the community in KA3105A, with $3x10^4$ cells ml^{-1}, a 28 times lower hybridization signal than expected and a probe detection of 1 ng, at least 20 liters would be necessary (compare Table 4).

Table 4 *Total number of cells, potential 16S rRNA hybridization signal from one liter of groundwater (based on best signal from KA3105A), and percentages of specific populations within groundwater communities that are necessary for detection in 16S-rRNA hybridization, assuming a detection limit for the specific probe of 1 ng 16S rRNA.*

Borehole	Total number of cells ml^{-1}	Signal from 1 liter (ng)	Percentage of specific population
KR0012B	$1.3x10^5$	21.7	4.6
KR0013B	$5.1x10^5$	85	1.2
KR0015B	$1.5x10^5$	25	4.0
SA1420A	$4.4x10^4$	7.3	13.7
KA3105A	$3.0x10^4$	5	20

Positive hybridization results can be achieved by taking samples where cells are more abundant than in KA3105A or by getting larger samples. Giovannoni et. al (1990) used tangential flow filtration using the Pellicon system to concentrate cells from up to 8 000 l of sea water. The recovery of cells was about 37%. Tangential flow filtration does not seem realistic for the deep groundwaters. To pool 1-liter samples would be very time consuming. Wang and Wang (1995) developed a method that may reduce sampling time. They pooled all their ^{32}P-labelled probes and hybridized them together in a single hybridization. Then they eluted the hybridized probes, separated them on a denaturing polyacrylamide gel and read results from an autoradiograph. The probes must have different lengths and about the same melting temperature for this method.

5.3 THE USE OF RNA HYBRIDIZATION FOR STUDIES OF MIXED GROUNDWATER COMMUNITES

RNA extraction and hybridization is a method revealing which groups of organisms that are most active within a community, and to some extent also their abundance. Metabolic activity is reflected in the ribosome content of a cell. An organism comprising only a few per cent of a community may show the highest activity. Other more dominant groups may be less active. A second, and maybe more elegant method than RNA extraction and hybridization, is *in situ* whole cell hybridization, where RNA stays within the cell and individual cells of a certain group can be counted under the epifluorescence microscope. Quantitative numbers of the populations of interest may be obtained (Amann el al. 1995). The strength of the hybridization signal may show how active certain populations or individual cells are. For *in situ* studies, it is an advantage if it has been concluded earlier that the bacterial groups searched for are present (Pedersen el al. 1996) and active to give a good and detectable hybridization signal. By first using RNA extraction and hybridization, it can be evaluated if *in situ* whole cell hybridization would be worth the effort. To hybridize against the 16S rRNA gene itself would give too low a signal for detection. rRNA constitutes a greater part of the total 16S nucleic acid pool, gives a higher signal and is therefore usually chosen in such studies.

Ideally, probes for higher taxonomic groups should be hybridized first. One universal, one bacterial, one archaeal and one eucaryal probe can be used to screen the ecosystem. It is theoretically possible to quantitatively observe the percentage of 16S rRNA that different populations have (quantitative dot blot hybridization, (Amann el al. 1995), and then concentrate on the dominating populations first. There are problems with such quantitative calculations. Different probes do not bind with the same efficiency and this makes it difficult to calculate percentages. The EUB338 probe is one of the best-binding (and most used) probes available. Moran et al. (1993) calculated percentages of different microbial groups within one sample by using specific probes and a universal probe. They used standard RNA and environmental RNA dilution series to standardize signals from different probes. Ruff-Roberts et. al (1994) report that it is impossible to calculate

percentages within one sample by using many probes with different signals. Instead, they normalized the signal from a universal probe to 1, then showed that the signal from other probes can be lower or higher than the universal signal. Then it is possible to compare the relative signal from one probe, specific or not, among samples. It cannot be done within one sample only.

The RNA extraction method developed in this study was suitable for pure cultures and RNA samples from the Äspö tunnel walls and floor, where there are many sulfur oxidizing bacteria in dense communities (Pedersen, 1996). Evidently the method did not suit all kinds of samples, however. Due to the heterogeneity among boreholes and groundwaters, a special RNA extraction protocol for each of them would be ideal. This is not realistic. PCR can be used to amplify RNA but biases in the method would probably spoil the initial purpose to compare activities. A PCR amplification from purified KA3105 extracts showed that the SRB687 probe worked and that SRB were present in this borehole, while no amplification was seen with the ACA652 probe. Whether this depended on a lack of *Acinetobacter* or unpure samples is unknown.

5.4 ACINETOBACTER ENRICHMENTS AND HYBRIDIZATION

In samples from all boreholes investigated, several kinds of bacteria and even fungi grew in the enrichment medium for *Acinetobacter*. Not many of them had a morphology that resembled common *Acinetobacter*. Baumann (1968) reported that if such cultures are not heavily aerated, *Pseudomonas* species will take over due to their motile nature. This was probably what happened. *Pseudomonas*-like 16S rRNA gene sequences have been found in Äspö groundwaters (Pedersen el al. 1996). *Acinetobacter*-like 16S rRNA gene sequences have also been found in the KR0012-13-15B boreholes and in SA1420A (Pedersen el al. 1996). The hybridization results from the enrichments indicated that the sequence data was correct and that *Acinetobacter* bacteria were present in the deep granitic groundwaters at Äspö. The only bacteria in the EMBL database that have one mismatch were *Thiobacillus ferrooxidans, Rickettsia tsutsugamushi* and an unclassified gamma-proteo isolate. The first two are not likely found in these groundwaters. The relatively good binding of the ACA652 probe should make it useful for later *in situ* whole cell hybridizations.

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PAPER 5

Isolation and Characterization of a Mesophilic Sulfate-Reducing Bacterium, *Desulfovibrio aespoeensis* sp. nov. from Deep Ground Water at Äspö Hard Rock Laboratory, Sweden

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CONTENTS

1 ABSTRACT

A sulfate-reducing bacterium, strain Aspo-2, was isolated from granitic ground water sampled at a depth of 600 m. This and other strains of SRB frequently occur in the deep granitic rock aquifers studied. On the basis of its morphological, physiological and genotypical properties, and its unique habitat, we propose strain Aspo-2 as a new species of the genus *Desulfovibrio*, *Desulfovibrio aespoeensis* (DSM 10631).

2 INTRODUCTION

Over the last decade the attention to deep subsurface microbiology has increased markedly. Until the middle of this century, it was assumed that microorganisms only could live in shallow of aquifers. Bacteria have now been found in deep subterranean environments, deep-see thermal vents and at the bottom of oil wells (Madsen and Ghiorse, 1993; Pedersen, 1993). In recent years interest has increased considerably in the area of deep hard rock microbiology. One reason is that deep (500 m) fractured rock environments are suggested as possible repository sites for disposal of nuclear fuel waste material. In focus is to understand how subterranean microorganisms will interact with the performance of a future nuclear fuel waste repository (Pedersen, 1996, 1997b). The relevance of different microbial activities possibly influencing repository barriers can only be evaluated with knowledge about the properties and ecology of microorganisms that will inhabit a repository and its surroundings. In particular, an understanding of the ecology of sulfate-reducing bacteria (SRB) in subterranean environments is essential for evaluation of the adverse effects associated with their hydrogen sulfide formation which ultimately may corrode the metal canisters used for the disposal of nuclear fuel waste material.

SRB are an unique physiological group known for their strictly anaerobic nature and their ability to use elemental sulfur, sulfate or other oxidized sulfur compounds as electron acceptor during anaerobic respiration (Barton and Tomei, 1995; Biebl and Pfennig, 1977; Fauque et al, 1980; Widdel, 1988). SRB are harmful in industry because of their primary roll in the anaerobic corrosion of iron in pipelines, heating systems and other structures (Hamilton, 1985; Watkins Borenstein, 1994).

3 SOURCE OF ORGANISM

As part of an on-going study to investigate the microbial ecology of subterranean microorganisms (Pedersen, 1996; Pedersen and Ekendahl, 1990) SRB have been isolated from granitic ground water of southeastern

Sweden, where the Äspö hard rock laboratory (HRL) has been constructed to study the function of underground repositories for long-lived nuclear fuel waste. Eleven boreholes at Äspö HRL were sampled and screened for the presence of SRB (Laaksoharju et al, 1995) using repeated enrichments with water samples inoculated in a brackish medium containing lactate and sodium sulfate as the substrate and electron acceptor, respectively (Widdel and Bak, 1992) and incubated for 2 weeks at room temperature. The growth of SRB was confirmed by blackening of the medium and production of sulfide. Repeated application of the agar roll-tube (Widdel and Pfennig, 1984) resulted in 16 pure cultures of SRB (Laaksoharju et al, 1995). The purity of these isolates were routinely checked by microscopy and inoculation on complex sulfate-free media. The 16S rRNA gene of the isolates were partially sequenced and compared with the sequences from the EMBL database (Pedersen et al, 1996). Seven isolates were found to be identical to *Desulfomicrobium baculatum*, seven isolates were distantly related to *Desulfovibrio salexigens* and two isolates were distantly related to *Desulfovibrio longreachii*. The strain Aspo-2 was isolated from borehole KAS03 at depth of 600 m. Also, 6 other strains with 100% 16S rRNA identity with strain Aspo-2 were isolated from the depths of approximately 70 m (borehole KR0013, one isolate), 200 m (borehole HA 1327-B, two isolates) and 600 m (borehole KAS03, three isolates). Here, we describe isolate Aspo-2 in detail.

Strain Aspo-2 has been deposited in Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the collection number DSM 10631.

4 PHYLOGENY

The DNA of strain Aspo-2 was extracted, the 16S rRNA gene amplified and sequenced as described elsewhere (Pedersen et al, 1996). An almost complete sequence consisting of 1502 bases of the amplified and cloned 16S rRNA gene of strain Aspo-2 was obtained using 11 primers. The sequence is deposited in the EMBL data base under accession number X95230. It has 68 signatures which indicate that it is a member of the domain *Bacteria* and 28 signatures which show similarity with the delta subdivision of proteobacteria (Woese, 1987). Furthermore, the occurrence of distinctive features of *Desulfovibrio* signatures confirmed that strain Aspo-2 belongs to the genus *Desulfovibrio* (Devereux et al, 1990). The complete 16S ribosomal DNA sequence of strain Aspo-2 was compared with sequences of various members of the bacterial phylum obtained from the EMBL data base using the FASTA program in the GCG package (Genetic Computer Group, Wisconsin, USA). The isolate was found to be distantly related to an unnamed *Desulfovibrio* sp. accession number, K 42995 (Teske, A. P. et al; unpublished data) and a *Desulfovibrio* strain *Pib* accession number U 33316 (Krekeler, D. et al; unpublished data) isolated from a hypersaline

cyanobacterial mat, with an overall identity value of 91.2% with both strains. The third closest species was *Desulfovibrio salexigens* with an identity value of 90.3%. The nucleotide sequence was also compared with sequences representative of *Bacteria* and major groups of SRB using the pile-up program in the GCG package. The positions at which any sequences had unknown or ambiguous nucleotides were omitted and pair-wise evolutionary distances were calculated for the remaining 1320 bases using DNADIST program (Jukes-Cantor method (Jukes and Cantr, 1969)). A phylogenetic dendrogram (Fig. 1) was constructed from distance data using the Fitch-Margolish method (Felsenstein, 1988). The dendrogram was drawn using the DRAWTREE program. These programs are available in the PHYLIP package (Felsenstein, 1993).

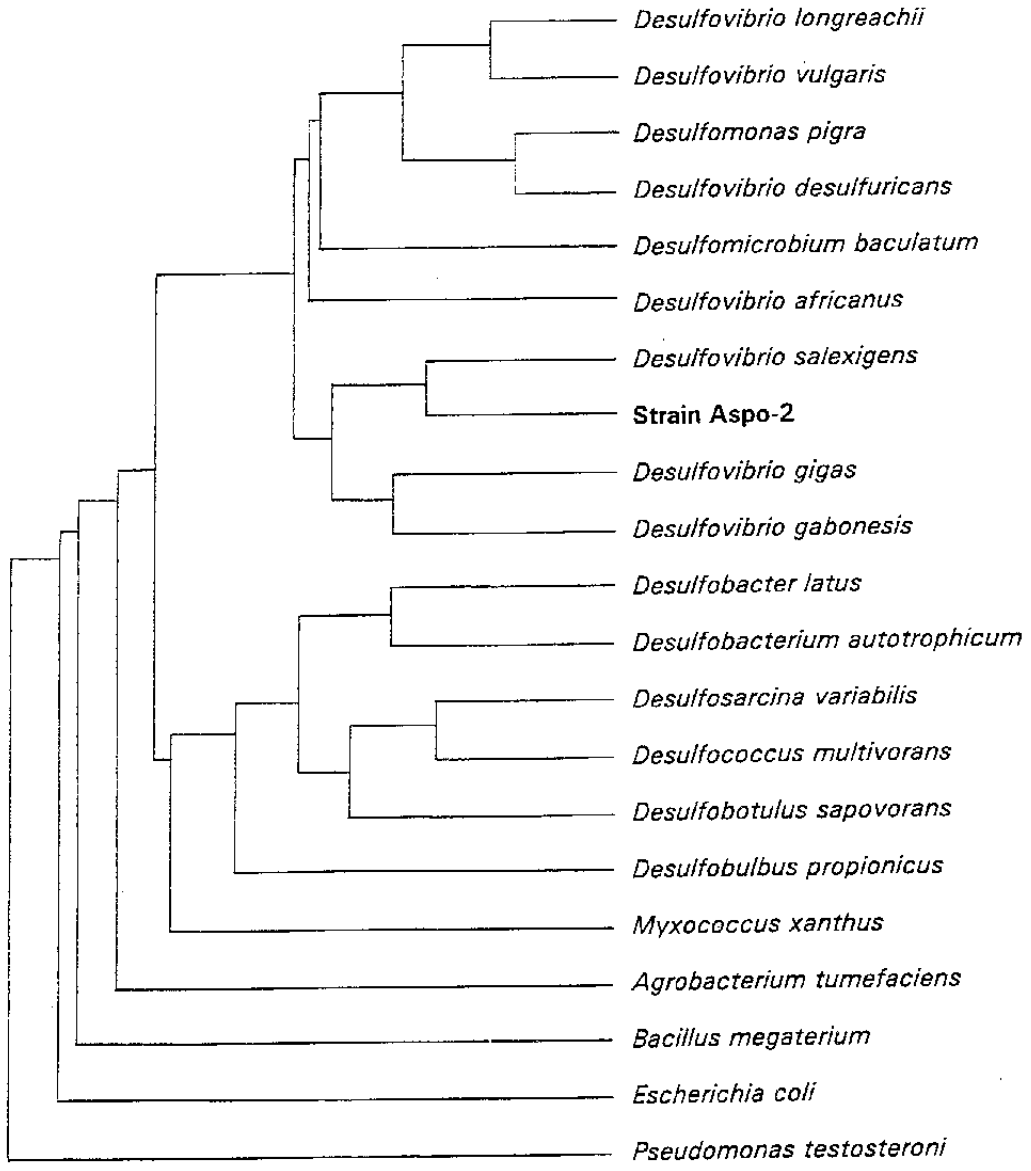

Figure 1 *Phylogenic dendrogram based on 16S rRNA sequence comparison, showing the positions of strain Aspo-2 within the radiation of members of sulfate-reducing bacteria and related taxa.*

5 DETERMINATION OF CHARACTERS

Determination of desulfoviridin and DNA base composition (using thermal melting point profile) was done as described previously (Johnson, 1985; Postgate, 1959). The isolate was desulfoviridin positive and the DNA guanine-plus-cytosine (G+C) content was 61± 0.5 mol %. Cells of the strain Aspo-2 were vibrioid and motile. The cells were approximately 0.5 µm in diameter and 1.7 to 2.5 µm long and occurred singly and in chains when lactate was used as the substrate (Fig. 2). The Gram reaction was negative, electron microscopy of sectioned cells revealed a typical Gram-negative cell wall and spores were never observed. Circular, entire to erose, yellowish, raised and rough colonies were produced on plates with 1.5% agar and lactate as the substrate. Strain Aspo-2 grew optimally between 25 and 30°C. No growth was observed at temperatures higher than 35°C or below 4°C. The optimum pH for growth was 7.5. It grew in medium containing up to 30 g of NaCl per liter. Optimum growth occurred at 7 g NaCl per liter.

Substrate utilization was determined in duplicate tests by using the basal growth medium (Widdel and Bak, 1992) supplied with 25 mM sodium sulfate as the electron acceptor and with different substrates at final concentrations between 5 and 57 mM depending on the substrate used. Production of acetate was determined using an acetic acid kit (Boehringer Mannheim). For cultivation with H_2 as the electron donor, duplicate basal medium was used supplied with 25 mM sodium sulfate with and without 4 mM acetate and a mixture of H_2/CO_2 (90/10, v/v) with an over-pressure of 0.5 atm. in the headspace corresponding to 2/3 of the culture volume. In order to determine the reduction of different electron acceptors by strain Aspo-2, the duplicate basal medium was added with 57 mM sterile sodium lactate and supplied with 10 to 70 mM of the electron acceptors considered (sterile, anaerobic from stock solutions). Growth was determined by Acridine Orange Direct Count (AODC ; Pedersen, 1991) and by following production of sulfide (Cord-Ruwisch, 1985). In the presence of sulfate, isolate Aspo-2 oxidized lactate (57 mM) incompletely to acetate. Pyruvate (11 mM) was fermented in a sulfate-free medium. No growth was observed on the following substrates with sulfate as the electron acceptor: formate (20 mM), methanol (25mM), fumarate (20 mM), malate (20 mM), 2-propanol (10mM), isobutanol (10 mM), alanine (10 mM), butyrate (10mM), phenol (1.25mM), benzoate (5 mM), palmitate (1 mM), acetate (20 mM) and ethanol (20 mM). Hydrogen served as energy source in the presence of sulfate with 4 mM acetate as a carbon source. Thiosulfate and sulfur were used as electron acceptors in the presence of lactate. Nitrate and iron(III)hydroxide could not serve as electron acceptors in a medium supplied with lactate. Fermentation of succinate and fumarate on sulfate-free medium was not observed.

According to the Bergey's Manual of Systematic Bacteriology (Widdel and Pfennig, 1984), considering the key to the genera of SRB, isolate Aspo-2 belongs to genus *Desulfovibrio*, because a positive desulfoviridin test and morphological characters such as motility and vibrioid shape distinguish most *Desulfovibrio* from *Desulfobacter, Desulfococcus, Desulfosarcina, Desulfomicrobium* and *Desulfobulbus*. Strain Aspo-2 and the closest described and accepted species in the 16S rRNA gene database, *D. salexigens,* differ phenotypically in several aspects. Strain Aspo-2 is a halotolerant microorganism that grows with up to 30 g/l NaCl and the optimum growth was observed in the presence of 7 g/l NaCl. In contrast, *D. salexigens* is a moderately halophilic microorganism that requires 20 g/l NaCl for growth (Widdel and Bak, 1992). In addition to the sequence differences, strain Aspo-2 differed from all other *Desulfovibrio* species (except *D. longus* (Magot et al, 1992)) by its inability to utilize ethanol. The G+C content of strain Aspo-2 (61% $\pm$ 0.5) falls within the genus *Desulfovibrio* (47-65% ; Widdel and Bak, 1992), but differs markedly from *D. salexigens* (49%). In contrast to *D. salexigens*, that can grow on pyruvate only in the presence of sulfate (Postgate, 1984), strain Aspo-2 grows on pyruvate without any additional electron acceptor. Growth of strain Aspo-2 on a medium containing elemental sulfur as electron acceptor and lactate as the carbon and energy source was observed and confirmed by AODC and production of sulfide.

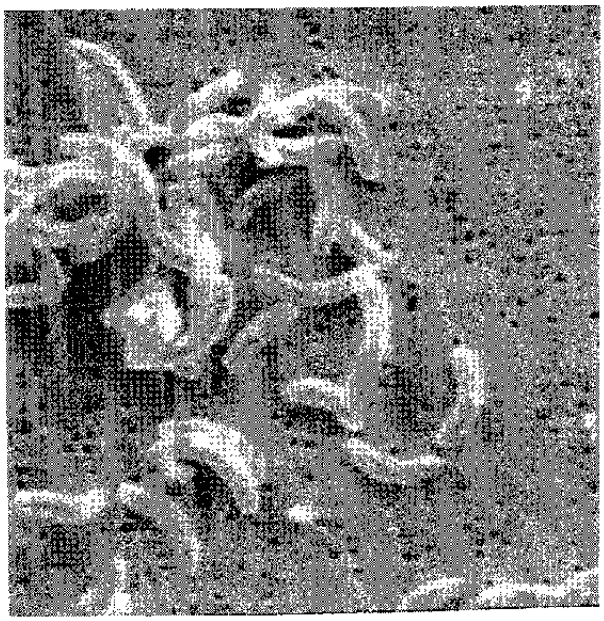

Figure 2 *Scanning electron micrograph after negative staining of strain Aspo-2, 1 cm = 1,5 µm.*

6 ECOLOGY

Aspo-2 was isolated from depth at Äspö HRL in southeastern Sweden together with two other SRB, a *Desulfomicrobium baculatum* and a *Desulfovibrio* sp. strain (Laaksoharju et al, 1995; Pedersen et al, 1996). The number of viable SRB in the boreholes KR0013 (70 m), HA1327-B (130 m) and KAS03 (600 m) where strain Aspo-2 was found, was >5, 130 and 1390 cell ml^{-1}, respectively. In these boreholes pH varied between 7.1 and 7.5, and the sulfate concentration varied between 1.45 and 2.12 mM. The amount of dissolved organic carbon (DOC) decreased with depth from 15 mg l^{-1} at 70 m to 1.0 mg l^{-1} at 600 m depth and salinity was approximately 1% in all three boreholes. The temperature increased with depth from 9°C at 70 m to approximately 20°C at 600 m. Overall, the deep ground water environment from which Aspo-2 was isolated shows potential for growth of SRB. The amounts of organic carbon and sulfate are sufficient to support the growth of SRB. Moreover, the pH and salinity observed is almost at the optimum values for growth of strain Aspo-2 and due to the ability of this bacterium to grow at a low temperature (≥4°C) temperature is not a limiting factor for *in situ* activity of strain Aspo-2. 16S rRNA gene diversity analysis of attached and unattached groundwater bacteria in boreholes at Äspö HRL (70-190 m depth) revealed sequences related to SRB in several boreholes as published previously (Pedersen et al, 1996). Recently, 16S rRNA sequences that are similar to Aspo-2 have been found in Äspö HRL at 400 m depth (EMBL accession numbers are Z69309 and Z69325, (Pedersen et al, 1997)). Geological, hydrological, stable isotope and groundwater chemistry data indicate that sulfate reduction was ongoing in the sites from which Aspo-2 was isolated before the tunnel was constructed (Pedersen, 1997a). Altogether, these results suggest that the deep granitic aquifers of Äspö are a natural habitat for SRB communities, of which Aspo-2 is a member. A potential for microbially induced corrosion of nuclear waste storage canisters is consequently possible and must be evaluated further.

In conclusion, on the basis of habitual, physiological and phylogenetic differences compared with other members of genus *Desulfovibrio*, strain Aspo-2 is not a member of any previously described *Desulfovibrio* species. We propose, therefore, that this organism belongs to a new species with deep granitic ground water as its major habitat, *Desulfovibrio aespoeensis*.

7 DESCRIPTION OF *Desulfovibrio aespoeensis SP. NOV*

Desulfovibrio aespoeensis (DSM 10631). Vibrioid-shaped cells are 0.5 μm in diameter and 1.7-2.5 μm long and occur singly. Cells are motile and contain desulfoviridin. Spores are never produced. In the presence of sulfate,

lactate is utilized as the sole electron donor and carbon source. Lactate is incompletely oxidized to acetate. It grows lithoheterotrophically on H_2 and acetate (as the carbon source). Pyruvate is fermented in the absence of sulfate. The following substrates are not used in the presence or absence of sulfate: formate, succinate, methanol, fumarate, malate, 2-propanol, isobutanol, alanine, butyrate, phenol, benzoate, palmitate, ethanol and acetate. The electron acceptors include sulfate, thiosulfate and sulfur. Nitrate and iron(III)hydroxide are not used as electron acceptors. Addition of NaCl is not necessary, but *Desulfovibrio aespoeensis* tolerates up to 30 g/l of NaCl in the medium, optimum growth occurs in the presence of 7 g/l of NaCl. The temperature range for growth is 4 to 35°C; the optimum temperature is between 25 and 30°C. The optimum pH for growth is 7.5. The G+C content of DNA is 61 ± 0.5 mol% (as determined by thermal melting point profile). *D.aespoeensis* was isolated from deep subterranean granitic ground water (600 m) of southeastern Sweden. The type strain is Aspo-2 (DSM 10631). The 16S rRNA sequence of strain Aspo-2 is deposited in the EMBL data base under accession number X95230.

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Methanobacterium subterraneum sp. nov., a new alkaliphilic, eurythermic and halotolerant methanogen isolated from deep granitic groundwater

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CONTENTS

1 ABSTRACT

Deep subterraneum granitic aquifers have not been explored regarding methanogens until now. Three autotrophic methane-producing *Archaea* were isolated from deep granitic groundwater at depths of 68, 409 and 420 meters. These organisms were non-motile, small, thin rods, 0.1-0.15 μm in diameter and they could use H_2+CO_2 or formate as substrates for growth and methanogenesis. One of the isolates, denoted A8p, was studied in detail. It grew with a doubling time of 2.5 h under optimal conditions (20- 40°C, pH 7.8-8.8 and 0.2 - 1.2 M NaCl). A8p is eurythermic as it can grow at a wide range of temperatures from 3.6 up to 45°C. It is resistant to bacitracin at concentrations up to 20 mg/l. The GC content is 54.5 mol %, as determined by thermal denaturation. Phylogenetic studies based upon 16S rRNA gene sequence comparisons placed the isolate A8p in the genus *Methanobacterium*. Phenotypic and phylogenetic characters indicate that the alkaliphilic, halotolerant strain A8p represents a new species. We propose the name *Methanobacterium subterraneum* for this species and strain A8p (=DSM 11074^T) is the type strain.

2 INTRODUCTION

Mesophilic, rod-shaped and hydrogen-consuming methanogenic *Archaea* are placed in the *Methanobacterium* and *Methanobrevibacter* genera. Typically, species belonging to the genus *Methanobrevibacter* are short rods with GC contents lower than 32 mole %, while *Methanobacterium* species are long rods with GC contents higher than 32 mole %. At present the genus *Methanobacterium* is composed of seven mesophilic and six thermophilic species. On the basis of phylogenetic comparative analysis of 16S rRNA, it was suggested to tranfer *Methanobacterium thermoautotrophicum, Methanobacterium thermophilum* and *Methanobacterium wolfeii* to the genus *Methanothermobacter* (Boone et al, 1993). The mesophilic species of the genus *Methanobacterium* were isolated from sewage sludge (Bryant et al, 1987), peat bog (Zellner et al, 1989), marshy soil (König, 1984), bleach kraft mill sludge (Patel et al, 1990) and an oil field (Belyev et al, 1986). *Methanobacterium alcaliphilum*, isolated from alkaline lake sediments, has a pH optimum between 8.1-9.1 (Boone et al, 1986). Four species, *Methanobacterium formicicum, Methanobacterium ivanovii, Methanobacterium palustre* and *Methanobacterium bryantii* are autotrophic, as they can grow in medium composed solely of inorganic substances. No members of *Methanobacterium* have been described before that can grow below 10°C.

In this paper we describe the phenotypic and phylogenetic characteristics of an autotrophic, halotolerant, eurythermic and alkaliphilic methanogen that is able to grow below 10° C. On the basis of these characteristics, we propose a new species, *Methanobacterium subterraneum.* The organism described here was isolated from deep granitic groundwater and is the first example of a methanogen isolated from the deep subterraneum biosphere (Pedersen, 1993a) . In this habitat, methanogens may represent chemoautolithotrophic organisms initiating food chains in the oligotrophic deep subsurface environment at the expense of geologically produced hydrogen.

3 MATERIALS AND METHODS

3.1 SOURCES OF ORGANISMS

The Äspö hard rock laboratory (HRL) tunnel is located on the Baltic coast under the island Äspö, in the vicinity of the Simpevarp nuclear power plant north of Oskarshamn, SE Sweden. The host rock is a ~1,800 Ma old granodiorite belonging to the Fennoscandian shield. The tunnel has a total length of 3,600 m, is approximately 5 x 5 m (height x width) and proceeds down with an inclination of about 14 degrees. It starts at the coast line and continues about 1,700 m under the sea floor where it spirals down to 460 m below sea level under the island Äspö (Pedersen et al, 1996, Pedersen, 1997). Microbiological data from boreholes in the tunnel to a depth of 192 m and a length of 1,420 m and in surrounding surface boreholes have been published earlier (Pedersen et al, 1990, 1992, Pedersen et al., 1996). Groundwater was sampled from core drilled surface and tunnel boreholes at depths from 10 to 440 m below ground. The methanogens described here were called A8p, 3067 and C2BIS and came from boreholes denoted KR0012A, KA3067A and HD0025A, respectively. These boreholes correspond to tunnel lengths 500, 3067 and 3200 m and depths below sea level of 68, 409 and 420 m, respectively. The samples were inoculated in the enrichment medium described below.

3.2 MEDIA AND CULTURING TECHNIQUES

The anaerobic technique described by Hungate (Hungate, 1969) was practiced. A total of 19 boreholes were screened during 1995-1996 for the presence of methanogens with various carbon and energy sources. Enrichment cultures were obtained in a medium prepared with filter sterilized groundwater that was collected from the boreholes KR0012A or KA3067A. The groundwater was supplemented with (per liter) 10 ml trace element solution (Wolin et al, 1963), 1.0 g yeast extract and 1 mg resazurine. The following carbon and energy sources were used (per liter): 3.4 g sodium acetete, 2.0 g formate, hydrogen + carbon dioxide (80:20%,

1,5 atm.), 2.0 g methanol or 1.0 g trimethylamine (TMA). Five ml portions of this medium were distributed under oxygen free nitrogen gas in Hungate type gas-tight, anaerobic culture tubes (Bellco glass, Inc, type no. 2047, 17 ml) and sterilized at 121°C for 20 min. After cooling, the following sterile, anoxic solutions were added (per liter): 5 ml of the vitamin solution SL-6 (Wolin et al., 1963), 1 mg coenzyme M, 2.0 g $NaHCO_3$, 0.25 g cysteine-HCl and 0.25 g Na_2S x $9H_2O$. The pH of the enrichment medium was adjusted twice, before sterilization and after the final additions, with 0.1 M NaOH or 0.1 M HCl to pH values corresponding to those of the groundwaters used for inoculation (7.25 -7.5). The enrichment tubes were inoculated with 0.5 ml groundwater from the boreholes within 2 h from sampling and incubated at room temperature for up to 5 months. The final head space in the enrichment tubes were approximately 11 ml. The enrichment cultures which actively produced methane were subcultured in serial dilutions in the presence of 0.5 g/l vancomycin. Pure cultures were obtained by mixing 1 ml of the last dilution of the culture which produced methane, with 5 ml of fresh enrichment medium plus 0.5 g/l vancomycin and solidified in a thin layer by rolling with 20 g/l agar (45°C) in butyl rubber stopped, aluminum crimp-sealed tubes (Bellco glass, Inc, type no. 2048, 22 ml). For the cultivation of pure cultures, including all physiological experiments, an artificial Äspö medium (ASPM) was used that mimicked the chemical composition of the groundwater from the KR0012B borehole. It contained (per liter): 0.4 g NH_4Cl, 0.03 g $MgCl_2$, 0.45 g NaCl, 0.5 g K_2HPO_4, 0.003 g $FeCl_3 \cdot 7H_2O$, 10 ml trace element solution (Wolin et al., 1963), 0.001 g rezasurin, 2.0 g $NaHCO_3$, 0.25 g cysteine-HCl and 0.25 g Na_2S x $9H_2O$. The following carbon and energy sources were used: 2.0 g/l formate, or hydrogen + carbon dioxide (80:20, 1.5 atm). The medium did not contain buffer before sterilization and the pH value of the medium, which became 7.0 after sterilization, was adjusted with anoxic bicarbonate (10% stock solution) and NaOH (0.1 M stock solution) to 7.8-8.0 before inoculation. All experiments were repeated at least once and all tests were done at least in duplicate. Averages of repeated tests are reported and used for the graphs presented.

3.3 MICROSCOPY

Phase-contrast and fluorescence microscopy were performed with an Olympus BH-2 phase-contrast microscope and a Carl Zeiss Axioscope equipped with UV lamps. Phase contrasted images were obtained with an Olympus C35AD camera and AD Exposure Control Unit (Japan). The autofluorescence of whole cells was observed with LP 420 excitation filter (Doddema et al, 1978). Acridine orange direct counts (AODC) were made as described earlier (Ekendahl et al, 1994). Ultrathin sections of the cells were obtained by glutaraldehyde fixation (2.5%, 2 h) followed by 1% osmium tetroxide fixation (2 h) in 0.1 M phosphate buffer (pH 7.6) at 4°C. The cells were Epon 812-embedded, thin-sectioned and stained with uranyl acetate and lead citrate. The thin sections were studied with an EAL 1200EX electron microscope.

3.4 GAS CHROMATOGRAPHY

Methane was determined with a Varian GC-3700 gas chromatograph equipped with a 2 m 1/8 inch steel column packed with Porapak Q, mesh 80/100 (Varian, Solna, Sweden) with nitrogen as carrier gas at flow rate 30 ml/min and a flame ionization detector. The response of the detector to methane was linear. The injector, column and detector temperatures were isothermal at 100, 100 and 200 °C, respectively. Calibration, registration and integration of methane peaks were done with a Star Chromatography Workstation, version 4.5 (Varian, Solna, Sweden).

3.5 GROWTH DETERMINATION

The studied isolate A8p grew in aggregates and it was, therefore, not possible to use measurements of optical density in the cultures for biomass determinations. Instead, the linear relation between methane production and biomass formation during the logarithmic growth phase (Powell, 1983) was used to calculate specific growth rates for the experiments described below. This relation was confirmed in experiments where methane production during growth of the isolates was found to be linearly correlated with the increase in cell number as determined by AODC. Growth at slow methane production rates ($< 0.05\ h^{-1}$) was confirmed with the AODC method. Inoculations which did not show a cell count increase and a corresponding methane production after 20 days of incubation were regarded as negative.

3.6 SUSCEPTIBILITY TESTS

The sensitivity of A8p to antibiotics was determined by adding 1-2000 mg/l of different antibiotics. Minimal concentration of the antibiotic causing 15% reduction in growth rate was considered inhibitory.

3.7 DETERMINATION OF GROWTH REQUIREMENTS

Cultures of A8p were transferred 3 times in a modified ASPM (pH 7.8), without vitamins and organic compounds, but with 40 mM formate. Subsequently, an array of possible growth factors were added to the modified ASPM and inoculated in duplicate with the vitamin depleted A8p culture. The control did not contain any additions. Growth of the cultures were monitored during incubation at 35°C for 120 h. The experiments were repeated twice. Growth curves were obtained for each repetition of the studied compounds and growth rates were calculated. The differences between the mean growth rate for an added compound compared to the control were evaluated with the Student's t-distribution test (Adler & Roessler, 1976) at a significance level of $p = 0.95$.

3.8 DETERMINATION OF GROWTH PARAMETERS

Methane production was monitored at from one to three days intervals in ASPM. The average specific growth rate for each incubation temperature was calculated from the exponential methane production phase. The effect of pH on growth of A8p was determined using different pH buffers in ASPM. They were: a mixture of 26 mM sodium acetate and 18 mM acetic acid (pH 4.8-5.5), 20 mM 2(N-morpholino)-ethanesulfonic acid (MES; pH 5.7-6.5), 20 mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES; pH 5.5-7.2), 20 mM piperazin-N,N-bis(2-ethanesulfonic acid; PIPES; pH 6.2-7.6), 20 mM sodium bicarbonate (pH 7.0-8.0), 30 mM N-tris(hydroxymethyl)methylglycine (TRICINE; pH 7.5-8.0) and 20 mM (hydroxymethyl)-aminomethane TRIS (pH 8.2-8.8). The culture was inoculated into 5 repetitions of each buffer. The pH was measured before and after inoculation, as well as during growth and never varied more than 0.15 units. The average specific growth rate for each incubation pH was calculated from the exponential methane production phase.

The salt tolerance of A8p was determined in ASPM at 35 °C and pH 8.5, supplemented with NaCl. The experiment was performed in duplicate and repeated twice. The specific growth rate at each NaCl concentration was calculated from the methane production between 20 and 177 hours of incubation.

3.9 ANTIGENIC FINGERPRINTING

Partial antigenic fingerprinting of the new isolates were performed using calibrated antibody probes as described previously (Macario et al, 1983, 1985).

3.10 DETERMINATION OF DNA COMPOSITION

Standard procedures (Marmur, 1961, Wallance, 1987) were used for DNA extraction from the culture with the following modifications. The cells were lysed by repeated freezing and thawing followed by 2 hours incubation in 50 mM ammonium bicarbonate, 50 mM EDTA buffer (pH 8.0) 10 mg/ml of SDS and 20 mg/ml of dithiothreitol at 60°C. Extracted DNA was dissolved in 0.1 SSC buffer and dialysed against the same buffer. The GC content was determined by a thermal denaturation method (Marmur et al, 1962) with a Cary Varian Thermal Spectrophotometer (USA), using the DNA of *Escherichia coli* strain L (51 mole % G+C) as a reference DNA. The GC base composition was calculated using the formula GC mole % = 2.08 x T_m - 106.4 (Owen et al, 1985). Relative standard deviation of the GC data was 0.74%.

3.11 16S rRNA GENE AMPLIFICATION

DNA was extracted from the isolates A8p, C2BIS and 3067 as described above, but in the last step DNA was dissolved in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0). New Archaea specific primers were designed matching the positions (7-26; *E.coli* Brosius numbering) GTC CGT TTG ATC CTG GCG GA and (1520-1538) GAG GTG ATC CAG CCG CAG A, to enable the 16S rRNA gene amplification of the studied methanogenic isolates. 1 µl of the extracted DNA solution was added into a mixture of 10 µl of 10 x PCR buffer (Stratagene), 0.2 mM of each nucleotide triphosphate, 0.25 µM of each primer and double distilled water in a final volume of 100 µl. The samples were treated with 50 µg/ml of RNA-ase A (Sigma) for 15 min at 37°C and incubated at 95°C for 5 min, before addition of 1 µl Pfu DNA polymerase (Stratagene) and 100 µl mineral oil (Sigma). A total of 34 cycles were performed at 95°C (30 s), 55°C (1 min), 72°C (2 min) followed by a final incubation at 72°C for 10 min.

3.12 16S rRNA GENE SEQUENCING

Repeated Primer Extension Sequencing (RPE-sequencing) was done as described in the thermo sequenase fluorescent labeled primer cycle sequencing kit manual (Amersham, Life Science; Amersham, 1995) with the following modifications. Sequencing was performed using fluorescent dye 5`-labeled primers: (7-26) GTC CGT TTG ATC CTG GCG GA, (352-368) CAG CAG GCG CGA AAM CT, (368-353) AGK TTT CGC GCC TGC T, (1225-1240) ACA CGC GGG CTA CAA T (designed in our laboratory) and (339-356) CTC CTA CGG GAG GCA GCA (Amann et al, 1990), (517-536) GCC AGC MGC CGC GGT AAT WC, (536-519) GWA TTA CCG CGG CKG CTG, (907-926) AAA CTY AAA KGA ATT GAC GG, (926-909) CCG CCA ATT CCT TTA AGT (Lane et al, 1985), (1099-1114) GCA ACG AGC GCA ACC C, (1114-1110) GGG TTG CGC TCG TTG (Lane, 1991). Base designations are standard International Union of Biochemistry designations for bases and ambiguity. Primer sequences are given from 5`to 3`. Nucleotide residues other than A, T,G and C are indicated by following ambiguity codes in mixtures of alternative primers: M, A or C; K, G or T; W, A or T; Y, T or C. Primer positions correspond to positions in the *E. coli* Brosius 16S rRNA gene sequence (Brosius et al, 1978). The master mixture contained 0.5 µl of the template (PCR-product), 10 pmol fluorescent 5'-labeled primer and water to a final volume of 25 µl. The mixture was distributed into four reaction tubes (6 µl in each tube) and to each tube 2 µl of one of the A, C, G or T reagent mixtures from the sequencing kit were added. One drop of mineral oil was added on the top of each tube to minimize evaporation. A total of 30 cycles were performed at 94°C (45 s), 55°C (45 s), 72°C (1 min). The sequencing product was separated from the residual oil by tip dropping. Four µl of loading buffer (formamide, EDTA and methyl violet) was added to each tube and about 7 µl was loaded into separate wells in a gel. Gel electrophoresis was performed on an A.L.F. DNA Sequencer (Pharmacia Biotech). Ready mix Long Ranger™ Gel

solution for DNA sequencing (FMS Bioproduct-Europe, Denmark) and urea (A.L.F. grade) were used in the gel solution. Sequenced fragments were obtained with 11 different primers and were aligned with the Gelassemble procedure (GCG-Genetic Computer Group, Wisconsin, USA) and verified manually.

3.13 SEQUENCE ANALYSIS

The 16S rRNA gene sequences of the isolates A8p, 3067 and C2BIS were compared to sequences available in the European Molecular Biology Laboratory (EMBL) database using the FastA and BestFit procedures in the GCG program package. The similarity percentages between the 16S rRNA gene sequences of the isolates and the most closely related organisms in the data base were calculated with BestFit not considering uncertain and unknown positions.

A phylogenetic analysis was performed on A8p and C2BIS strains using the programs contained in the PHYLIP version 3.5c package (Felsenstein, 1989) compiled for PC. Nucleotide positions that could be unambiguously aligned for all 16S rRNA genes compared were included in the analysis. The final data set comprised 1400 nucleotide positions, position no. 38-1438 (*E. coli* Brosius numbering (Brosius et al., 1978)) of 16 organisms. The sequences used for the tree construction were aligned using the Pileup procedure (GCG). The distances were calculated using the DNADIST program (Jukes et al, 1969) and a tree was built running the KITCH program with contemporary tips. The KITCH program was run with a randomized input order of data with 20 jumbles and during execution 11759 trees were examined. The organisms used for the tree construction are listed in the figure legend.

3.14 NUCLOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequence of the A8p, 3067 and C2BIS appear in EMBL and GeneBank Data Bases under the accession numbers: X99044, Y12592 and X99045, respectively.

4 RESULTS

4.1 ENRICHMENT AND ISOLATION

Groundwater from deep granitic rock aquifers (from 10 to 440 m below sea level) was used as the inoculant. These groundwaters were anoxic and oligotrophic (2.0-11.0 mg/l of organic carbon), with different salinities (537-13 300 mg Cl- per liter) and bicarbonate concentrations (53-326 mg H_2CO_3 per liter; Nilsson, 1995).

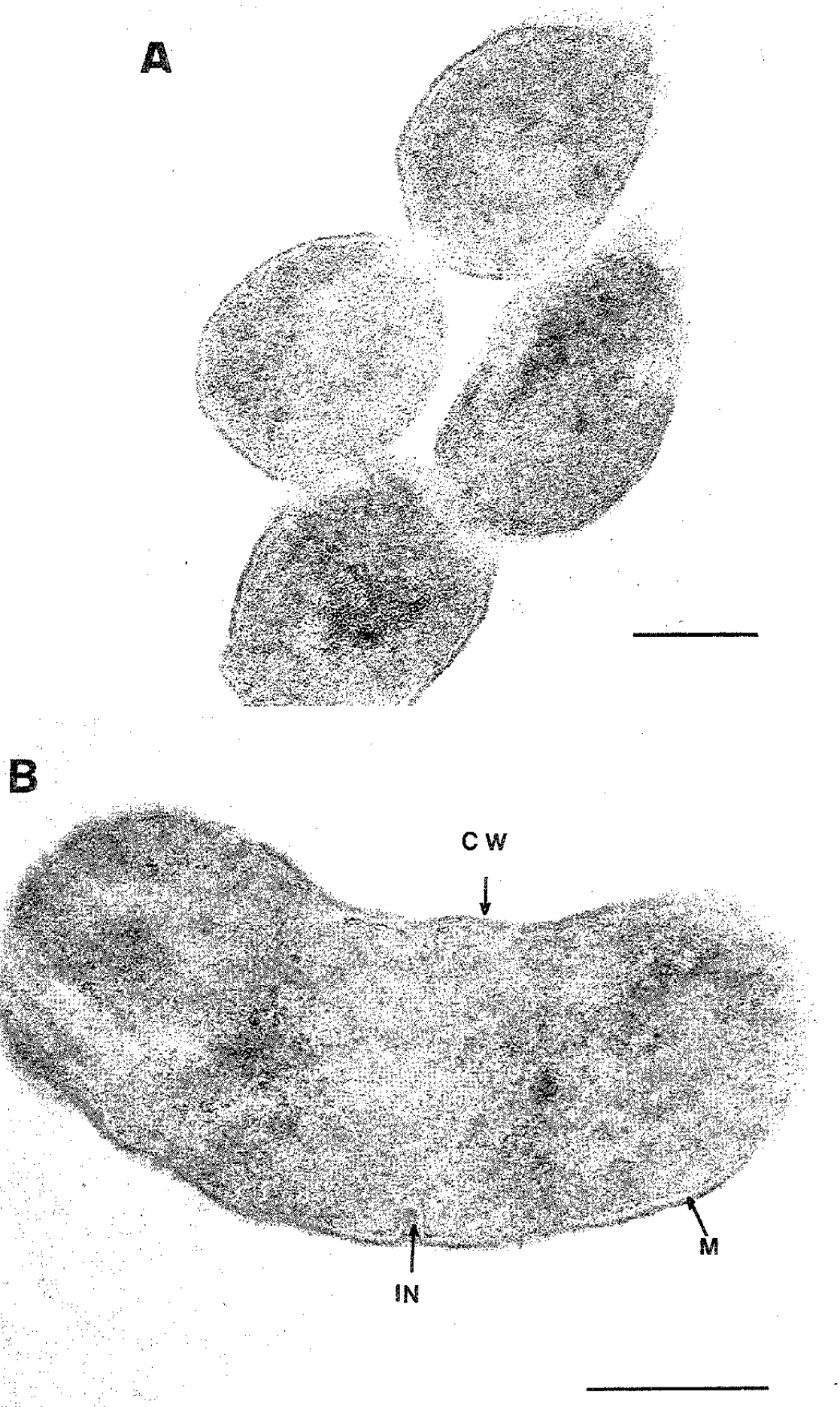
Active methanogenesis was observed in enrichment media containing 2.0 g/l formate and 1.0 g/l TMA inoculated with groundwater from the boreholes KR0012A and HD0025A, corresponding to sample dates 95-02-02, 95-11-25 and to 68 and 420 m below sea level, respectively. Abundant methane production was observed as well in an enrichment culture that was supplied with hydrogen and carbon dioxide (80:20, 1.5 atm.) inoculated 95-11-25 from borehole KA3067A, 409 m depth. The pH increased from 7.2-7.5 to 9.3-9.6 in these cultures concurrent with growth and methane production in the medium with formate. All the enrichment cultures could grow and produced copious amounts of methane when inoculated into the medium with formate or with hydrogen and carbon dioxide and they grew best at alkaline pH values (7.8-8.8). Small, non-motile and autofluorescing rod-shaped cells were observed using light microscopy in all these enrichments. The cultures were purified by serial dilution in the groundwater based media containing 2.0 g formate and 0.5 g vancomycin (per liter) and subsequently inoculation in roll-tubes. Colonies were observed after two weeks. Single colonies from tubes with methane production were selected and transferred into liquid ASPM medium with 2.0 g/l formate. The resulting cultures were morphologically homogeneous. Only autofluorescing cells were observed. Inoculation into ASPM medium without carbon and energy sources but instead containing 20 mM of sulfate and 20 mM of lactate or 2 g/l glucose and 1 g/l peptone, showed no growth. Thus, the cultures were axenic. The cells of the studied isolates had differing autofluorescence intensity and varied slightly in length. The isolates were designated as strains A8p (68 m depth), 3067 (409 m depth) and C2BIS (420 m depth).

4.2 COLONY AND CELL MORPHOLOGIES AND ULTRASTRUCTURE

Surface colonies of strain A8p were circular, granular, colorless and 0.5-1.5 mm in diameter. Cells of isolate A8p were non-motile, slightly curved, small and short rods, 0.6 -1.2 μm in length and 0.1-0.15 μm in diameter, often growing in aggregates (Fig. 1A, D). These aggregates floated with gas bubbles when cultivated in ASPM medium with formate. Ultrathin sections showed a single-layered electron dense cell wall of about 6.5 nm in thickness (Fig. 1B), typical of Gram-positive bacteria. The cell wall was tightly fitted to the cytoplasmic membrane. The cells divided after septum formation that was initiated peripherally (Fig. 1B, C).

Colonies of the C2BIS strain were circular, granular, yellow, 1.0-2.0 mm in diameter. The cells were straight rods with an ultrastructure of the cell wall typical of Gram-positive bacteria and with round ends, i.e., a morphology similar to A8p. Under 420 nm light, bright blue-green autofluorescence was observed. Colonies and cells of strain 3067 had a morphology similar to that of C2BIS. Because the 16S rRNA sequence of isolate 3067 was similar but not identical to those of the isolates A8p and C2BIS (that were identical, see

below), phenotypic studies were continued only with isolate A8p.from borehole KA3067A, 409 m depth. The pH increased from 7.2-7.5 to 9.3-9.6 in these cultures concurrent with growth and methane production in the medium with formate. All the enrichment cultures could grow and produced copious amounts of methane when inoculated into the medium with formate or with hydrogen and carbon dioxide and they grew best at alkaline pH values (7.8-8.8). Small, non-motile and autofluorescing rod-shaped cells were observed using light microscopy in all these enrichments. The cultures were purified by serial dilution in the groundwater based media containing 2.0 g formate and 0.5 g vancomycin (per liter) and subsequently inoculation in roll-tubes. Colonies were observed after two weeks. Single colonies from tubes with methane production were selected and transferred into liquid ASPM medium with 2.0 g/l formate. The resulting cultures were morphologically homogeneous. Only autofluorescing cells were observed. Inoculation into ASPM medium without carbon and energy sources but instead containing 20 mM of sulfate and 20 mM of lactate or 2 g/l glucose and 1 g/l peptone, showed no growth. Thus, the cultures were axenic. The cells of the studied isolates had differing autofluorescence intensity and varied slightly in length. The isolates were designated as strains A8p (68 m depth), 3067 (409 m depth) and C2BIS (420 m depth).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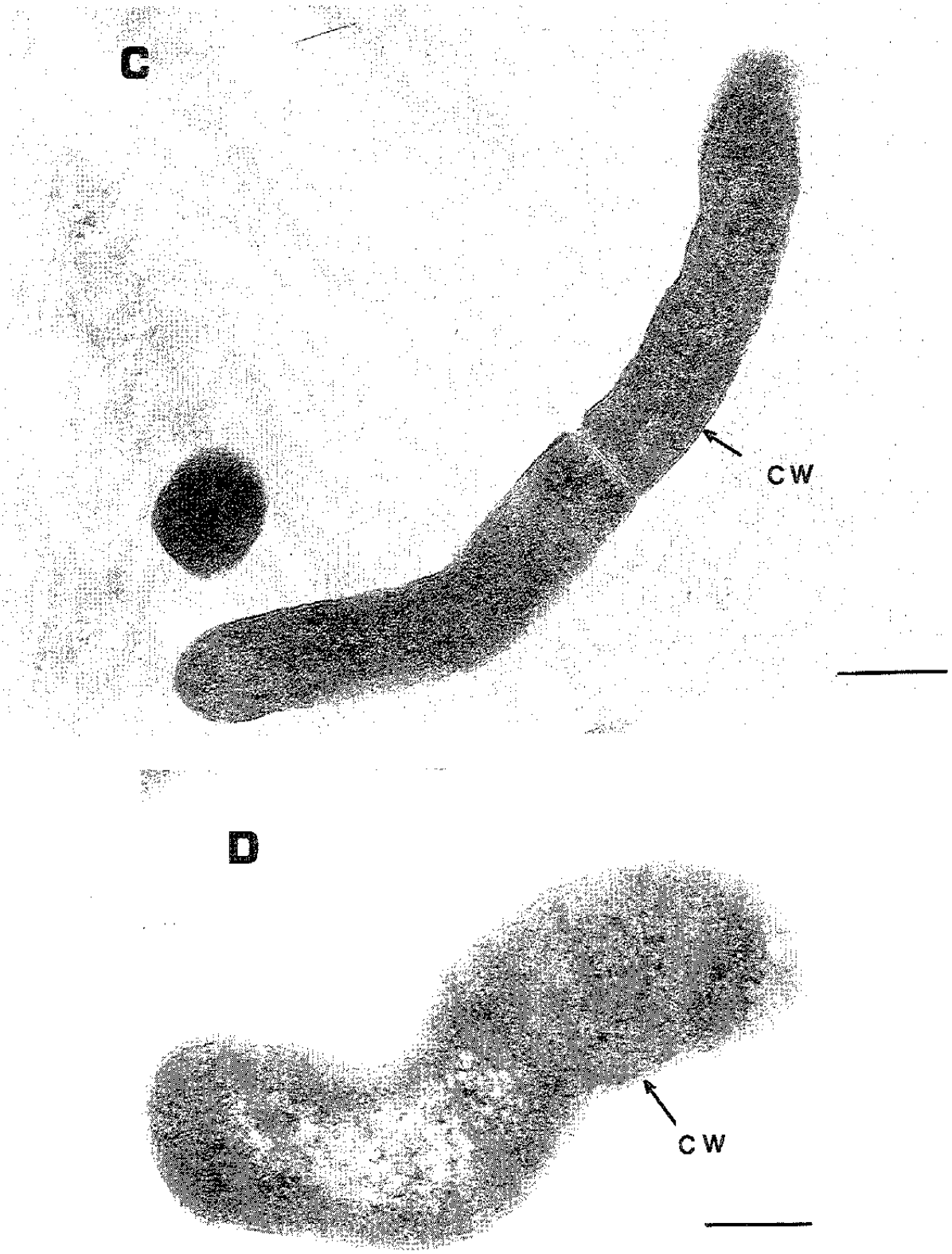

Figure 1 *Morphology of strain A8p.* **A**, **B** *and* **C** *- electron microphotographs of thin sections of cells.* **D** *- phase-contrast micrograph of cells and cell agreggate. The bar indicates 50 nm in A, B, 200 nm in C and 1 μm in D. Abbreviation means: M - cytoplasmic membrane, CW - cell wall, CP - cytoplasm, IN - invagination of cytoplasmic membrane before cell division, S - septum.*

4.3 ANTIBIOTIC SUSCEPTIBILITY

Growth of strain A8p was not inhibited by (per liter): 1 g vancomycin, 500 mg benzylpenicillin, 1 g ampicillin, 1g methycillin, 2 g streptomycin, 800 mg nalidixic acid, 14 mg neomycin or 20 mg bacitracin, while its growth rate was significantly reduced one order of magnitude by (per liter): 40-100 mg of chloramphenicol, 20 mg neomycin and 40 mg bacitracin. The minimal concentration of antibiotic which inhibited growth more than 15% was accepted as inhibiting concentration.

4.4 DETERMINATION OF GROWTH REQUIREMENTS

Strain A8p was transferred four times in ASPM medium with hydrogen as the energy source and bicarbonate as the electron acceptor and carbon source. The strain was autotrophic; it could use H_2/CO_2 (80:20;1.5 atm) with a growth rate of 0.2-0.3 h^{-1} and did not require any growth factors or vitamins for growth. Strain A8p grew in ASPM (specific growth rate, 0.2-0.4 h^{-1}) with formate (2 g/l) as the substrate. In the absence of exogenous bicarbonate or CO^2, methane formation from H^2 diminished. The addition of methanol, trimethylamine (TMA) or pyruvate did not stimulate methanogenesis from H_2. In the bicarbonate buffered medium without hydrogen, only small amounts of methane were formed (<0.5%). The presence of n-propanol (5 mM), isobutanol (5 mM), n-butanol (5 mM), or ethanol (5 mM) in the bicarbonate buffered medium did not increase methane production in comparison with the control. Thus, these alcohols were not hydrogen donors for methanogenesis. The additions of acetate (30 mM), methanol (20 mM), pyruvate (20 mM), TMA (20 mM), fructose (10 mM), glucose (10 mM), or dimethyl sulfide (4.5 mM) did not lead to an increase in methane production. Therefore, these compounds were also not substrates for methanogenesis by strain A8p.

Tryptone (1 g/l), sodium acetate (1 g/l), para-aminobenzoic acid (100 mg/l), vitamin B_{12}(4 mg/l), biotin (0.4 mg/l), coenzyme M (0.1 mg/l), $CaCl_2$ (0.5 g/l), $MgCl_2$ (0.5 g/l), Na_2MoO_4 (2-20 mg/l), Na_2WO_4 (2 mg/l), $FeCl_3$ (2 mg/l), $CuCl_2$ (2 mg/l) and $NiCl_2$ (0.2 mg/l) were tested as growth factors for strain A8p. Statistical evaluation showed that in the ASPM medium with hydrogen and carbon dioxide as energy and carbon sources supplemented with the different growth factors listed above, the strain A8p showed growth rates close to controls without additions. It means that these compounds did not influence methanogenesis by strain A8p. But growth was reduced by yeast extract, casamino acids, isobutyric acid, n-butyric acid, Na_2SeO_3, $ZnCl_2$, $CoCl_2$, $NiCl_2$ and $MnCl_2$ (Table 1). Growth in medium with vitamin solution SL-6 (5 ml/l; Wolin et al., 1963) was slower than in a vitamin-free medium. This observation was confirmed after 4 consecutive transfers into vitamin-free and vitamin-containing ASPM with H_2/CO_2 (80:20, 1.5 atm) by 5 vol % inoculations. Vitamins were not essential for growth. Relative standard deviation for the experiments with different growth factors were 2-12%.

Table 1 *Effect of addition of different growth factors on the growth of A8p.*

Growth factor added	Concentration (mg/l)	Growth rate (h^{-1})
ASPM (control)	-	0.178
$ZnCl_2$	2.0	0.082
$CoCl_2$	2.0	0.085
Na_2SeO_3	2.0	0.062
$NiCl_2$	2.0	0.095
$MnCl_2$	20.0	0.107
Yeast extract	2000	0.101
Casamino acids	1000	0.109
Isobutyric acid	5.0	0.121
n-butyric acid	5.0	0.113
Propionic acid	5.0	-[a]

[a] growth not observed

4.5 DETERMINATION OF GROWTH PARAMETERS

Methanogenesis of strain A8p was observed within a wide range of temperatures from 3.6 to 45°C (Fig. 2A). The optimum growth temperature was broad, 20-40°C. The length of the lag-phase differed greatly as a function of the incubation temperatures and varied from 5 to 144 h. Minimal lag-phases and maximal specific growth rates were observed at 20-40°C. The strain showed growth and methanogenesis at 3.6°C -13°C. Growth at 3.6, 13.6 and 20°C was confirmed by AODC method. At 3.6°C the number of cells had increased more than 2 orders of magnitude indicating active growth. In a separate experiment we verified that growth (increase in cell numbers) and methane production rates were linearly correlated during logarithmic growth at 6°C (Fig. 3). Growth and methane production were not detected at 2°C or 50°C. The organism is, therefore, mesophilic and eurythermic.

The optimum pH for growth and methane production at 35°C in ASPM medium was 7.8-8.8 (Fig. 2B). This optimum was confirmed by AODC at the end of incubation. Growth and methane production was observed at pH 9.2 and pH 6.5, but not above pH 9.2 or below pH 6.5. The length of the adaptation phase was the same at pH values ranging from 7.7 to 9.1 and increased at pH values below 7.7 and above 9.1. The reproducibility of cell growth at pH 9.0 was confirmed by subculturing strain A8p in ASPM at pH 9.0 twice. The level of growth and methane production observed during subculturing was reproducible. Thus, A8p strain is slightly alkaliphilic.

Although the growth rate of A8p was highest at a sodium chloride concentration of 0.2 M, this organism could grow up to a salinity of 1.25 M (Fig. 2C). In ASPM added with added sodium chloride (0.25-1.25 M) A8p cells formed huge aggregates (Fig. 1A,D). Strain A8p can grow and produce methane in wide range of salinity, consequently, it is halotolerant.

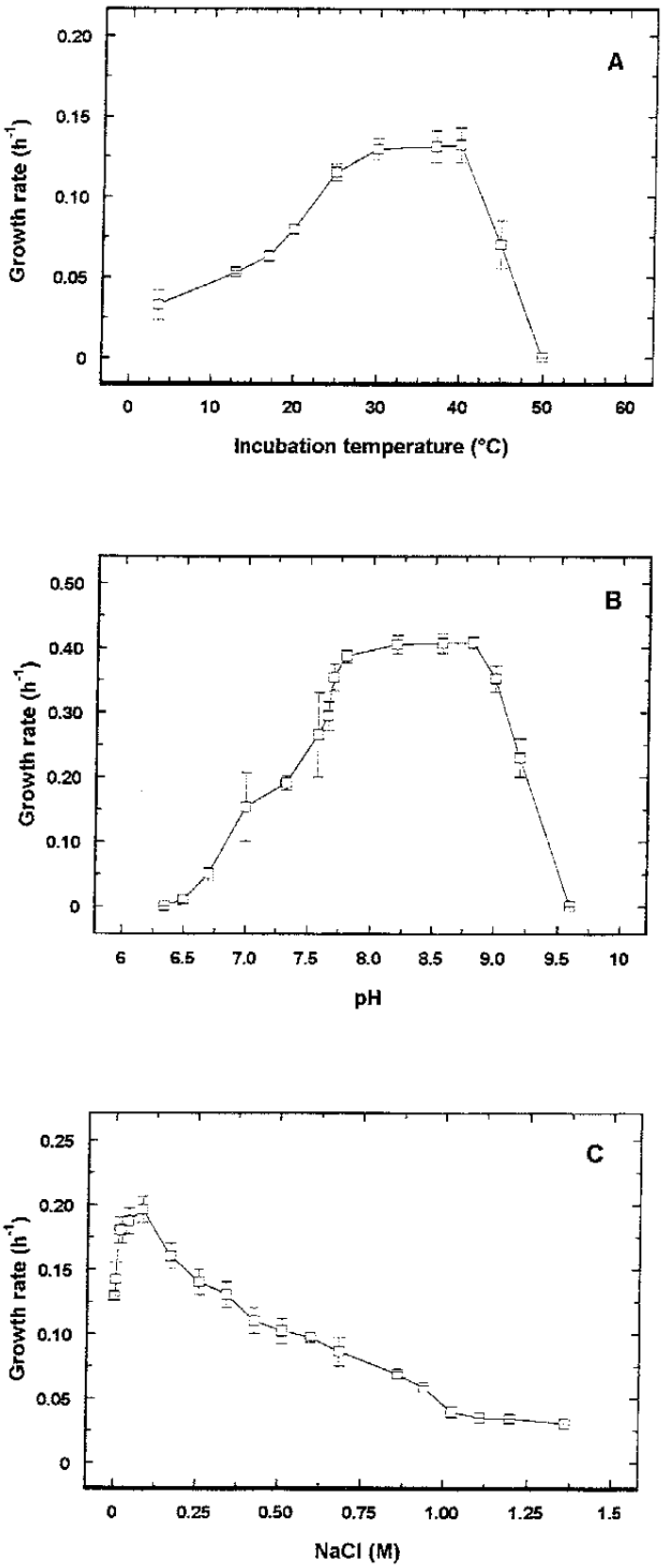

Figure 2 *Influence of temperature (A) at pH 8.5, pH (B) at 35 °C and NaCl concentration (C) at 35 °C and pH 8.5 on the growth of A8p, cultivated in ASPM. Specific growth rates were calculated from methane production values and are the means of duplicate cultures.*

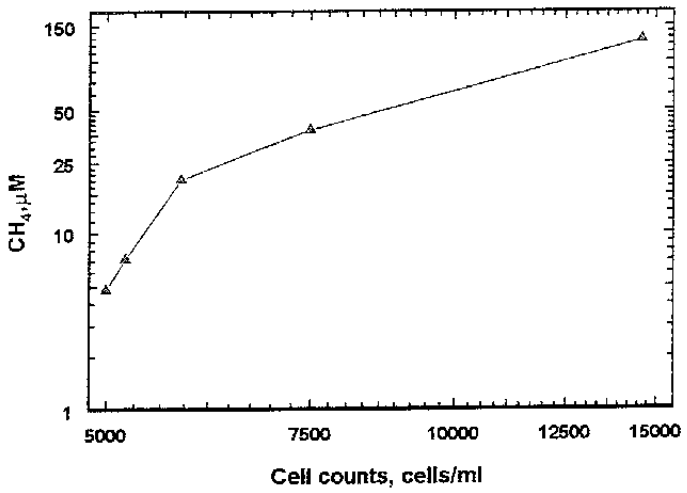

Figure 3 *AODC counted A8p cell numbers versus methane production. The cells were incubated at 6 °C for 250 hours.*

4.7 ANTIGENIC FINGERPRINTING

The partial antigenic fingerprints of the new isolates demonstrated that they were antigenically unrelated to reference methanogens from the genera *Methanobacterium* and *Methanobrevibacter*.

4.8 G+C CONTENT OF THE DNA

DNA base composition of strain A8p was 54.5 ± 0.5 mole %. The thermal melting point of DNA was 77.3 ± 0.2°C. These results are averages of 4 independent measurements.

4.9 PHYLOGENETIC ANALYSIS

The 16S rRNA genes of the isolates were amplified using specific archaeal primers and sequenced. The 16S rRNA genes of the isolates A8p and C2BIS were sequenced at positions 38 to 1534 and 22 to 1512, respectively (*E. coli* Brosius numbering). The 16S rRNA gene sequenses of isolates A8p and C2BIS were obtained without any unambiguous or unknown bases and were identified for 1474 positions. Isolate 3067 could not be sequenced completely (the sequenced fragment was between positions 570-1178) due to what seemed to be a tertiary structure blockage of the sequencing process at position 571 (*E. coli* Brosius numbering). The 16S rRNA gene of isolate 3067 differed 0.7% (13 bases) from A8p and C2BIS (610 bases compared). Only unambiguous bases were included in the comparison. The sequenced fragment of isolate 3067 included both variable and conservative regions of 16S rRNA gene and the differences were situated in the variable regions. The partial 16S rRNA gene sequence of the isolate 3067 was not included in the

The partial 16S rRNA gene sequence of the isolate 3067 was not included in the phylogenetic analysis. A phylogenetic tree was constructed based on the 16S rRNA gene sequences of A8p and C2BIS in comparison with organisms in the EMBL and GenBank databases (Fig. 3). The phylogenetic tree shows that the isolates A8p and C2BIS were most closely related to the mesophilic species of the genus *Methanobacterium*. The highest similarity, 97.2 %, was observed with *Methanobacterium formicicum*. The 16S rRNA genes of this organism differed in 38 positions per 1,450 bases from A8p and C2BIS. The percentages of sequence similarity of the A8p and C2BIS isolates 16S rRNA genes and the other organisms were as follows: *M. wolfeii*, 94.2%; *M. bryantii* 92.5%; *M. thermoautotrophicum CB-12*, 92.2%; *M. thermoautotrophicum TFT* and *M. thermoautotrophicum THF*, 92.1%; *M. thermoautotrophicum FTF*, 92.0%; *M. thermoautotrophicum Z-245* and *M. thermoautotrophicum delta H*, 91.8%.

5 DISCUSSION

The level of similarity among the 16S rRNA genes (99.3-100 %) of three new strains A8p, C2BIS and 3067, isolated from different granitic groundwater, implies that they can be regarded as closely related organisms unless evidence of phenotypic differences are revealed. The rod-like morphology and similar substrate specificity of the new isolates attest that they are phenotypically similar. Therefore, one organism, strain A8p, was chosen for detailed studies.

The resolution of 16S rRNA sequencing analysis between closely related organisms is generally low, but it is reliable for generic identification. The sequence similarities of the 16S rRNA gene of A8p showed that it was related to sequenced *Methanobacterium* species. The dissimilarity levels ranged between 2.8 and 8.2%. The levels of dissimilarity were within the range found between other species of this genus. Thus, the results of 16S rRNA gene comparison indicated that our isolate belongs to the genus *Methanobacterium*. Phylogenetically, A8p was most closely related to *M. formicicum* (97.2 % similarity). However, this evolutionary distance is sufficiently large that the two strains most probably belong in separate species (Boone et al., 1993, Devereux et al, 1990, Kadam et al, 1995, Stackebrandt et al, 1994). The phenotypic characteristics of A8p confirmed that the isolate belongs to the genus *Methanobacterium* and that it can be distinguished from phylogenetically related taxa as shown in Table 2.

Cells of strain A8p were small, thin rods. The cell diameter and the thickness of the cell wall were significantly smaller than those of previously studied methanogens in this genus (Table 2, Fig. 1). The length of the rod-shaped cells may vary, but the cell diameter and thickness of the cell wall are more stable cell characters. Thus, strain A8p differs morphologically from other members of the genus *Methanobacterium*.

Table 2 Comparison of phenotypic characteristics of mesophilic *Methanobacterium* species.

Property	*M. formicicum*	*M. bryantii*	*M. ivanovii*	*M. alcaliphilum*	*M. espanolae*	*M. palustre*	*M. uliginosum*	*M. subterraneum*
Morphology	rod 0.4-0.8x2-15 filaments, clumps	rod 0.5-1.0x1.5 filaments, clumps	rod 0.5-0.8x1.2 filaments	rod 0.5-0.6x2-5 filaments	rod 0.8x3-22 filaments	rod 0.5x3-5 filaments	rods 0.2-0.6x2-4 filaments	short rods 0.1-0.15x0.6-1.2 aggregates
Growth substrates	H_2-CO_2, formate, isopropanol +CO_2, isobutanol +CO_2	H_2-CO_2, isopropanol +CO_2, isobutanol +CO_2	H_2-CO_2	H_2-CO_2	H_2-CO_2	H_2-CO_2, formate, isopropanol +CO_2, isobutanol +CO_2	H_2-CO_2	H_2-CO_2, formate
Autotrophy	+	+	+	-	-	-	+	+
Stimulatory factors	Acetate, cysteine	yeast extract, acetate, cysteine B, cysteine, Fe,W,Ni	Acetate, cysteine	Trypticase pepton, yeast extract, pepton	vitamins	not reported	yeast extract	none
Growth factors	does not need	does not need	does not need	Trypticase pepton, yeast extract, pepton	vitamins	not reported	does not need	does not need
Temperature range,°C (optimum)	25-50 (37-45)	n.d. (37-39)	15-55 (45)	25-45 (37)	15-50 (35)	20-45 (33-37)	15-45 (40)	3.6-50 (20-40)
pH range (optimum)	n.d. (6.6-6.8)	n.d. (6.9-7.2)	6.5-8.5 (7.0-7.4)	7.0-9.9 (8.1-9.1)	4.6-7.0 (5.6-6.2)	7.0	6.0-8.5 (6.0-8.0)	7.0-9.2 (7.8-8.8)
Salinity, M (optimum)	0.25	0.26	0.19	0.012	n.d.	0-0.3 (0.2)	n.d.	0-1.4 (0.2-1.25)
GC mol%	38.0-48.0	31.0-38.0	36.6	57.0	34.0	34.0	29.0	54.5
Reference	(Bryant et al., 1987)	(Bryant et al, 1967;Boone, 1987)	(Belyaev et al..1986)	(Boone et al.,1986)	(Patel et al.,1990)	(Zellner et al., 1989)	(König, 1984)	This study

Strain A8p had a wider temperature range than other mesophilic *Methanobacterium* species. It was capable of growth at very low temperatures (3.6-20 °C) but also at mesophilic temperatures. Probably, this property allows A8p to survive at different depths in granitic rock aquifers, in which the temperature increases with depth from below 10°C at ground surface by 1-2 °C for every 100 meters. The increase in temperature over the sampled depth interval, 68 - 420 m, was from 8.5 to 16°C (Kotelnikova et al, 1997). The temperature in various parts of this environment is stable but the groundwater inhabited by microorganisms moves from one part to another through aquifers and may transport the cells between granitic layers at different depths. In addition, the isolates were distributed over at least three different depths, 68, 409 and 420 m below ground. The deep groundwater is an unique environment which is not limited by hydrogen and carbon dioxide availability (Kotelnikova et al., 1997), the main substrate for growth of our isolates. Thus, the varying temperature at different depths and absence of substrate limitation in the environment, where the strain A8p was isolated from, will favor development of eurythermic and hydrogenotrophic methanogens. None of the methanogens described in the comprehensive review by Boone et al. (Boone et al., 1993) could grow at a temperature

below 10°C. *Methanosarcina* and *Methanococcoides* species growing at psychrophilic conditions have been isolated earlier (Fransmann et al, 1992, Zhilina et al, 1991). Consequently, strain A8p is the first member of *Methanobacterium* that is able to grow at low temperatures. A8p is also capable of growth within a wide range of salinity values and at high pH values, which further suggests that it is well adapted to the deep granitic groundwater environment. The salinity and pH of the studied groundwater at Äspö HRL increase from 0 to 0.5 M and from 7.5 to 8.5, respectively, down to a depth of 950 m (Smellie et al, 1995). These figures coincide with the optimum salinity and pH values obtained for strain A8p. The temperature, salinity and pH ranges obtained for A8p are indicative of its habitual adaptation to an active life in deep granitic aquifers and support the suggestion of A8p as a new species.

The adaptations to the specific environment might determine the unique phenotypical properties of the isolated methanogen when compared to established methanogens. The levels of salinity and pH tolerated by strain A8p were higher than those tolerated by other *Methanobacterium* strains (Table 2). Although its pH optimum resembles that of *M. alcaliphilum* (Boone et al., 1986), strain A8p is salt tolerant and consumes formate. A8p is autotrophic like four other organisms of the genus *Methanobacterium*: *M. formicicum, M. bryantii, M. ivanovii* and *M. palustre* (Table 2), but all these organisms except *M. ivanovii* are able to use alcohols as electron donors, which strain A8p could not do. Strain A8p can grow on hydrogen + carbon dioxide or formate like *M. formicicum and M. palustre*, but strain A8p differed from these species by its temperature and salinity ranges and pH optimum. The difference in the mol % GC of strain A8p and *M. formicicum* was 16.5 GC % (Table 2). In contrast to *M. ivanovii* (Belyaev et al, 1986) and *M bryantii*, strain A8p uses formate, is alkaliphilic and halotolerant. Thus, strain A8p differs morphologically, physiologically, in sensitivity to antibiotics, in temperature and salinity ranges, in pH optimum, substrate spectrum and in 16S rRNA gene composition from known species of the *Methanobacterium* genus (Table 2, Fig. 4). These differences support placing A8p as a new species.

The antigenic fingerprinting data demonstrated that the three isolates described here are antigenically unrelated to reference methanogens and represent novel immunotypes within the genus Methanobacterium. Whether A8p, 3067 and C2BIS are of the same or different immunotypes, remains to be established. The antigenic fingerprinting indicated absence of cross reaction of the isolates with other members of order Methanobacteriaceae were negative. The immunotypes of the isolates could not be affiliated to any of the reference ones.

The requirement for growth factors is considered as a distinguishing phenotypic property for methanogens. The growth of strain A8p was inhibited by yeast extract, casamino acids, isobutyric acid and n-butyric acid. Low concentrations of Se^{+4}, Zn^{-2}, Co^{+2} and high concentrations of Ni^{+2} and Mn^{+2} ions inhibited methanogenesis (Table 1). Growth of strain A8p was not stimulated by any growth factors and did not require vitamins for growth and methanogenesis, unlike many previously studied mesophilic

species of the genus *Methanobacterium* (Table 2). The nutritional requirements of strain A8p are in accordance with the chemistry of the environment from which this organism was isolated. The deep groundwater contains trace amounts of metals and it is oligotrophic with organic matter consisting mostly of fulvic and humic acids at concentrations not exceeding 11 mg/l (Smellie et al., 1995). Anaerobic microbial decomposition of organic material at these low concentrations cannot produce inhibiting amounts of fatty acids. Thus, the nutritional requirements of A8p reflect its environmental adaptation.

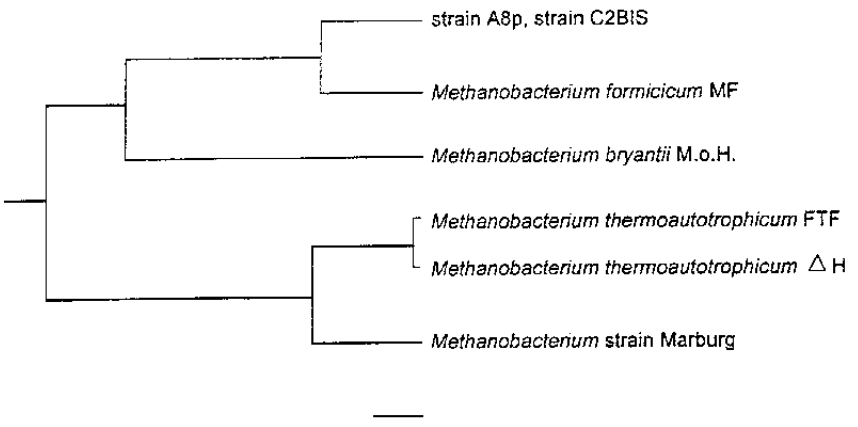

Figure 4 *Most probable phylogenetic tree constructed using Jukes-Cantor distance matrix calculated by comparing the 16S rRNA genes (1400 bases) with a random input order of sequences. Distances were calculated by the number of substitutions per 100 bases by the Fitch-Margoliash method with contemporary tips. The resulting tree was the most probable from 11759 trees. The branch length for the given topology is a least squares fit and is proportional to evolutionary distances. Bar = 1% difference in nucleotide sequences, as determined by measuring the length of the horizontal lines connecting two species. For the tree construction the 16S rRNA gene sequences from the following organisms with EMBL accession numbers were used:* Methanobacterium formicicum *MF, M36508;* Methanobacterium bryantii *M.o.H., M59124;* Methanobacterium thermoautotrophicum *FTF, X68711;* Methanobacterium thermoautotrophicum *Δ H, X68720;* Methanobacterium Marburg, *X15364,* Methanobacterium thermoformicicum *FTF, X68713;* Methanobacterium thermoformicicum *Z245, X68712;* Methanocorpusculum bavaricum, *X71838;* Methanothermus fervidus, *M59145;* Methanosphaera stadmanae *MCB-3, M59139,* Methanosaeta concilii, *X16932,* Methanosarcina barkeri *227, M59144;* Methanogenium cariaci *JR1, M59130;* Pyrococcus furiosus, *Z54172.*

The isolates described here are the first examples of methanogens from deep granitic groundwater, which can be 10,000 years old at depths of 400 to 500 m (Pedersen, 1997). They are representatives of life in the recently discovered deep biosphere (Pedersen et al., 1990) and may represent its autotrophic part of this deep biosphere, responsible for primary production

of organic matter (Pedersen, 1993b, Stevens et al, 1995). Such autotrophic microorganisms are suggested to live at the expense of geochemically produced hydrogen and the deep biosphere is then consequently postulated to be independent of the sun-driven ecosystems on the Earth's surface (Sherwood Lollar et al, 1993; Stevens et al., 1995; Kotelnikova et al., 1997, Martini et al, 1996, Pedersen, 1997). As our findings and other studies of the last years show, methanogenic *Archaea* probably play a significant role in subsurface biogeochemistry (Kotelnikova et al., 1997, Martini et al., 1996, Sherwood Lollar et al., 1993).

Because of the of the phylogenetic and phenotypic characteristics reported above, we propose that A8p is a new species. The proposed name is *Methanobacterium subterraneum,* with strain A8p as the type strain. The description follows.

5.1 METHANOBACTERIUM *SUBTERRANEUM* SP. NOV.

Methanobacterium subterraneum (sub.ter.ra'ne.um, L.adj. (neut) subterraneum - underground, below the earth/soil surface) Cells are nonmotile, small and thin rods, 0.6-1.2 μm in length and 0.1-0.15 μm in diameter, often in aggregates but not chains. The cells stain Gram-positive. The substrates used for growth and methane production include H_2-CO_2 and formate, but not methylamines, acetate, pyruvate, dimethyl sulfide, methanol or other alcohols plus CO_2. It grows autotrophically in mineral medium without any organic additions. Growth is inhibited by yeast extract (2 g/l), casamino acids (1 g/l), isobutyric acid (5 mg/l), n-butyric acid (5 mg/l), Na_2SeO_3 (2 mg/l), $ZnCl_2$ (2 mg/l), $CoCl_2$ (2 mg/l), $NiCl_2$ (20 mg/l) and $MnCl_2$ (20 mg/l) Vitamins are not essential for growth. Growth conditions: temperature 3.6 - 45°C, pH 7.0-9.2, salinity 0.2-1.2 M NaCl. The GC content of the DNA is 54.5 ± 0.5 mol% (as determined by thermal melting point).

The habitat from which it was isolated is granitic rock groundwater from the Äspö Hard Rock Laboratory tunnel, Southeastern Sweden. The type strain is $A8p^T$ = $DSM11074^T$ and the reference strains are 3067 and C2BIS = DSM11075, isolated from granitic groundwater at the depths 68, 409 and 420 m below sea level, respectively. The 16S rRNA gene sequences of A8p, 3067 and C2BIS appear in the EMBL and GenBank databases under the accession numbers: A8p, X99044, 3067, Y12592 and C2BIS, X99045.

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PAPER 7

Evidence for methanogenic *Archaea* and homoacetogenic *Bacteria* in deep granitic rock aquifers

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CONTENTS

1 ABSTRACT

The presence and diversity of methanogens and homoacetogens in deep granitic rock groundwater from Äspö Hard Rock Laboratory were studied. Concentrations of hydrogen and methane in Äspö groundwater were 45 nM-100 μM and 19 μM - 1000 μM, respectively. Groundwater-based media were used to count viable cells in the groundwaters from the subsurface. Different physiological groups of methanogens (autotrophic H_2/CO_2-consuming and heterotrophic acetate, methanol and trimethylamine consuming) were found at depths ranging from 68 to 446 m below sea level in numbers from 12 to 4.5 x 10^5 cells/ml. Viable numbers of different physiological groups of methanogens and autofluorescent cells were counted repeatedly during a period of 1 year in the groundwater. The counts were reproducible in the same boreholes at different sampling times. Methane production was observed in the groundwater with added methanogenic substrates, at anaerobic conditions, at 17°C, after 20-50 days of adaptation period. The most active methane production took place in those groundwaters which were sources of methanogenic enrichment and pure cultures. *Methanosarcina*-like organisms were observed in groundwater from depths of 45-68 m and active enrichment cultures. 16S rRNA gene sequencing of enrichment cultures indicated the presence of a psychrophilic *Methanohalophilus* related organism at a depth of 414 m. A new species of alkaliphilic *Methanobacterium* was isolated from depths of 68, 409 and 420 m and studied. Homoacetogenic *Bacteria* were found in the granitic groundwater as viable cells from 10 to 3.6 x 10^4 cells/ml and produced acetate autotrophically. It appears that deep granitic groundwater is inhabited by autotrophic methanogens and acetogens, which may produce methane and acetate at the expense of subterranean hydrogen and bicarbonate.

2 INTRODUCTION

In anoxic freshwater environments where bicarbonate is the dominant electron acceptor, hydrogen is mostly oxidized by methanogens and acetogenic bacteria (Drake, 1994). Indications of methanogenesis have been obtained from some subsurface environments including deep aquifers (Godsy, 1980), water flooded oil fields (Belyaev et al, 1986) and deep sediments (Fredrickson et al, 1989, Phelps et al, 1989). Reports of acetogenic bacteria from deep aquifers (Stevens et al, 1995) are extremely rare. Methanogenesis and acetogenesis in deep granitic rock have not been studied until now. The goal of this paper is to present some studies of the presence and diversity of methanogens and homoacetogens in the Äspö deep granitic subsurface.

3 SAMPLING SITES

For the research the Hard Rock Laboratory (HRL) at Äspö (Figure 1) was used (Pedersen et al, 1990, 1992, 1996). Nineteen boreholes at 3 sites in the tunnel were repeatedly sampled in this investigation during 11 expeditions for the period 1994-1996. One site comprised 4 core drilled boreholes down to 68 m below sea level around or near a shallow fracture zone, called the shallow site. Three boreholes were situated in an intermediate site (112-179 m below sea level). The deepest site comprised 14 boreholes. These boreholes penetrated the rock from 345 m below sea level down to 446 m.

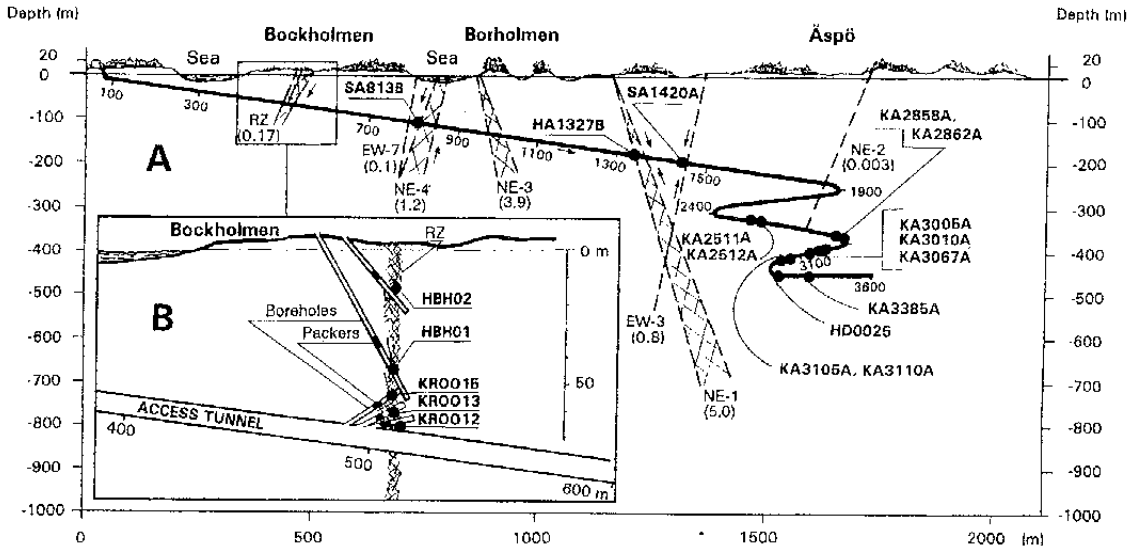


Figure 1 *Site topology along the tunnel in Äspö Hard Rock Laboratory, 1996. The sample sites are depicted with the respective borehole names. These names shows the type of drilling (HBH = percussion drilled, SA, KA = core drilled). Numbers in the names show place of drilling with length of tunnel. "A" and "B" letters indicate location of the borehole on left and right side of tunnel, respectively. Shallow Site (B) boreholes were drilled for the studying of the fate of shallow groundwater intrusion into the tunnel. Deep Site boreholes were drilled 200 below sea level down to 460 m.*

4 GASES DISSOLVED IN THE GRANITIC GROUNDWATERS

The gases in the gas phase of tightly stoppered bottles filled with groundwaters were analyzed with gas chromotography. Figure 2 shows methane content in the groundwater with depth. Methane content varied from 19 to 1000 μM and it constituted 0.1-5 % of the total gas. Results of 6 independent measurements deviated around a constant level of methane in the same groundwaters (Figure 2A), indicating production stability of the system. If living methanogens contribute to methane production, their input would be assumed on a constant level.

Free hydrogen was also found in the Äspö groundwaters. Figure 2B shows results of two independent measurements, which showed presence of hydrogen in the same aquifers. Concentrations of hydrogen in the groundwater varied from 45 nM to 100μM. Hydrogen has been detected in all boreholes tested except two boreholes in shallow site, where hydrogen was below the detection limit (45 nM). Concentrations of hydrogen were as a rule equal or higher in deep site boreholes in comparison with shallow site boreholes. Physico-chemical conditions in the groundwaters studied (Pedersen et al., 1990, 1992, 1996) the content of gases (hydrogen and methane) dissolved in the groundwaters suggested that the methane could be microbially produced.

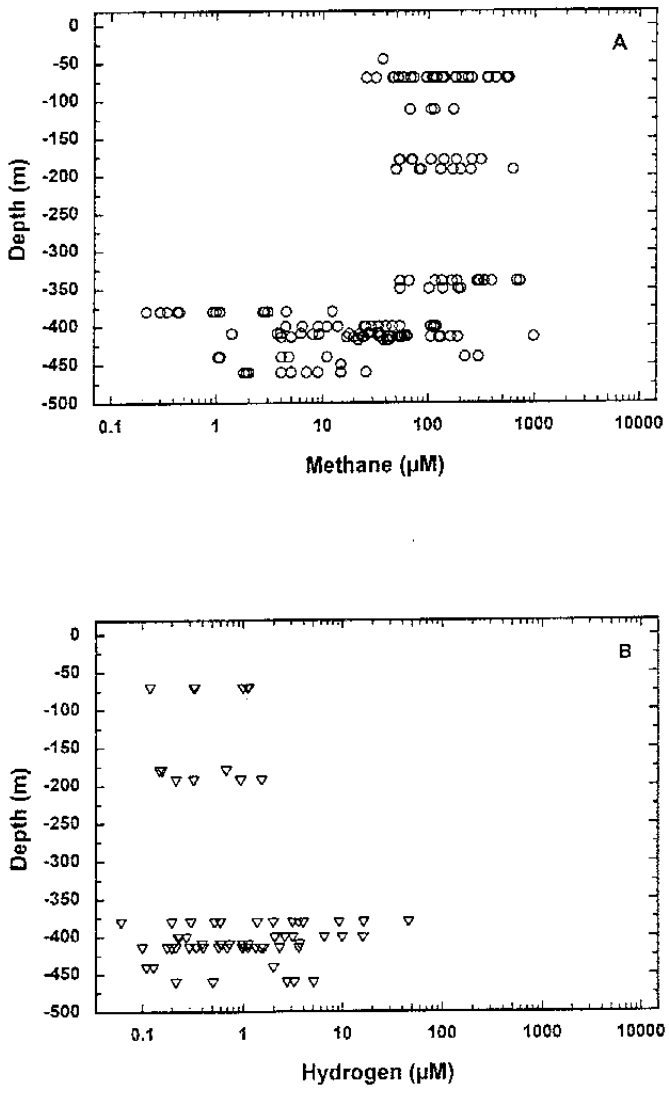


Figure 2 *(A) Concentrations of methane dissolved in the groundwater. Data of samples collected in October and November 1995, February, April, June and August 1996. (B) Concentrations of hydrogen dissolved in the groundwater. Data from samples collected in April and June 1996.*

5 METHANOGENIC *ARCHAEA*

5.1 COUNTS OF METHANOGENIC *ARCHAEA* IN THE DEEP GRANITIC SUBSURFACE

Methanogens in Äspö groundwaters were counted in two ways: under UV with an excitation at 420 nm and by a most probable number counting method (MPN). Autofluorescent cells were observed with an LP 420 excitation filter (Doddema et al, 1978). Methanogens contain high concentrations of the electron carrier F_{420} (Gorris et al, 1986). When cells illuminated with UV light had bright blue-green autofluorescence they were, therefore, considered to be methanogenic. Groundwater samples were cooled to 4° C overnight and autofluorescent cells were counted the day after sampling. Different volumes of the water were filtered in 3-4 repetitions on Nuclepore filters (0.22 μm pore size) and counted. The results were calculated as an average of the counts for each borehole.

The number of viable methanogens was estimated by the MPN method (Koch, 1994) in media containing groundwater taken from the HRL and autoclaved. For cultivation, anaerobic technique (Hungate, 1969) was used. Methane production was used as a positive indication of growth in the MPN tubes. To count different physiological groups of methanogens H_2/CO_2 (80:20), acetate (5 mM), methanol (10 mM) and trimethylamine (10 mM) were used as substrates added into the media. The MPN experiments were repeated seven times during 1994-1996.

Figure 3 shows viable numbers of different physiological groups of methanogens and autofluorescent cells counts in groundwater sampled repeatedly during period of 1 year from one borehole (KR0012B) at a depth of 68 m below sea level. The figure shows that viable methanogenic *Archaea* were present in the groundwater at numbers of 5 - 3.6 $x10^4$ cells/ml and autofluorescing cells varied from 4.5 $x10^4$ to 1.75 x 10^5 cells/ml. Counts of heterotrophic methanogens were higher than counts of autotrophic methanogens in this groundwater. Numbers of autofluoresent cells exceeded the MPN. This implies that a major part of the methanogens remained uncultivated. The number of cells were reproducible with the time implying that the population inhabiting the granite is rather stable and methanogens likely inhabit the environment indigenously. The viable methanogenic and fluorescing counts were compared with total number of bacteria with acridine orange staining for the borehole KR0012B (2.24 x 10^5 cells/ml). The comparison showed that cultivable methanogens constituted up to 16 % of the total microbial population inhabiting the groundwater. The autofluorescent cell count constituted between 20 and 78 %. Since we received surprising results indicating that methanogens are one of dominating groups inhabiting the deep granite environment, we used another set of methods which could show the diversity and activity of the methanogens.

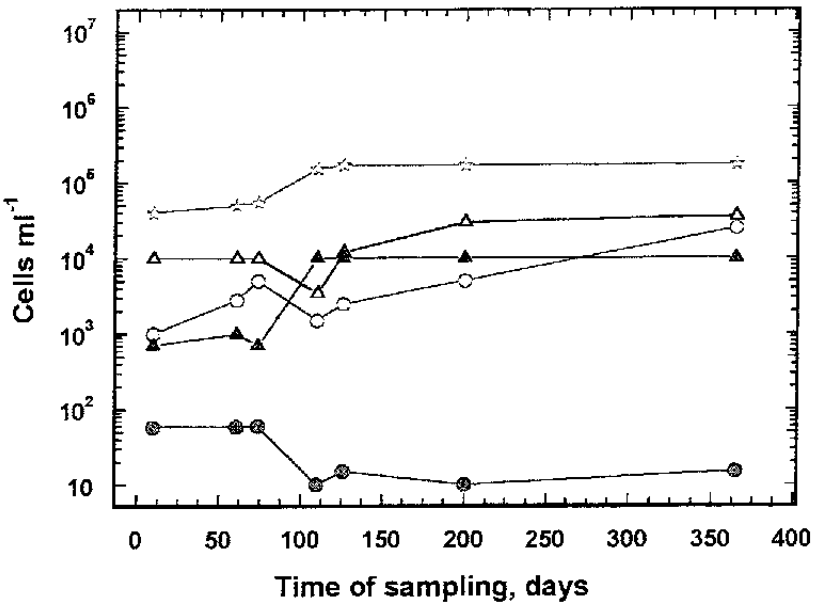


Figure 3 *Direct counts of autofluorescent (☆) and viable methanogens enumerated in the presence of H_2/CO_2 (●), acetate (○), methanol (▲) and trimethylamine (Δ), in the groundwaters from borehole KR0012B at 68 m depth, plotted with time of different sampling occasions. The MPN experiments were repeated on October 10, December 12, 1994 and January 17, February 2, May 16, October 10, November 15 and November 24, 1995. Three replicate tubes were inoculated with 0.5 ml from 6 to 10-fold dilution series of the samples. After every period of two to three weeks of incubation at 20 °C and 37 °C, methane was estimated. MPN values were calculated with an MPN determination program (Hurley et al, 1983). The standard deviation ranged between 10 and 30 %.*

5.2 DIVERSITY OF METHANOGIC *ARCHAEA* IN THE GRANITIC SUBSURFACE

One of the approaches which we used in our research was cultivation of methanogens with visual observation of morphologies of auto-fluorescent cells in enrichment cultures, actively producing methane. Morphologies of enriched methanogens were similar to morphologies observed directly in the groundwater. It is interesting that morphological types observed in different groundwater samples agreed with the distribution of different physiological groups of methanogens with the depth. Numerous fluorescent irregular nonmotile cocci about 2-3 μm in diameter dominated in the trimethylamine and methanol containing enrichment media. The cells multiplied and remained nondivided as diplo- or tricocci (Figure 4A). The 16S RNA gene sequence of the methanogenic culture enriched with trimethylamine showed the presence of *Methanohalophilus* related organisms at a depth of 414 m below the surface.

Similar fluorescing coccus shaped cells were observed in acetate containing medium and directly in groundwaters from a depth of 68 m. The coccus often formed *Methanosarcina*-like aggregates (Figure 4B). After acridine orange staining the grape-shaped aggregates of cocci became visible. Small rod-like fluorescing cells grew in H_2/CO_2 medium. Huge aggregates of small autofluorescent methane-producing cells were enriched on formate. The cells created clumps and films on the glass surface of cultivation tubes. Several autotrophic methanogens were isolated in pure cultures. One of them, *Methanobacterium subterranium,* was identified as a new species (Kotelnikova et al, 1997). The pure cultures of these *Archaea* were isolated from subsurface aquifers at 68, 409 and 420 meters depth. The isolated strains were named A8p, 3067 and C2BIS. They were enriched and isolated using formate as a carbon source and electron donor but later, it was found that they could also grow with hydrogen and carbon dioxide. The isolates were morphologically similar and could be characterized as non-motile, straight or slightly curved small rods (0.6-1.2 μm x 0.10-0.15 μm), often aggregate forming and with Gram-positive cell walls. The cells were autofluorescent when observed under UV using 420 nm light for excitation.

The physiology of strain A8p was studied in detail. Strain A8p is a hydrogenotrophic autotroph and also a formate consumer that grows in mineral medium without addition of vitamins. Growth was stimulated by addition of trypton, acetate, vitamin B_{12} and Mo^{+3}, Cu^{+2} and Ni^{+2} ions. Large growth ranges with active methanogenesis were observed with respect to temperature, 3.6 - 45 °C; pH, 7.0-9.5; and NaCl concentration, 0.1 - 1.3 M. The shortest doubling time, 1.7 h, was observed at 30-36 °C. The optimal pH for growth and methanogenesis was 8.5 - 8.8. Strain A8p was not affected by neomycin up to 20 mg/l or by bacitracin up to 20 mg/l, while growth of the previously studied strains of the *Methanobacterium* genus were inhibited at these concentrations. Morphologically and as evaluated by substrates utilized, strain A8p was found to be similar to the genus *Methanobacterium,* although several phenotypical differences from known *Methanobacterium* species were observed. Strain A8p differs in pH, temperature and salinity ranges and by its insensitivity to antibiotics.

The 16S rRNA genes of the isolated strains (A8p, 3067 and C2BIS) were amplified using specific *Archaea* primers and sequenced. The results of comparison of the sequences with EMBL-GenBank database indicated the isolates to be distantly related to *Methanobacterium formicicum,* which was the closest species in the database with 97 % identity. Because of the unique phylogenetic difference and phenotypical properties of strain A8p, we propose strain A8p as a new species of the genus *Methanobacterium, Methanobacterium subterranium* (Kotelnikova et al., 1997).

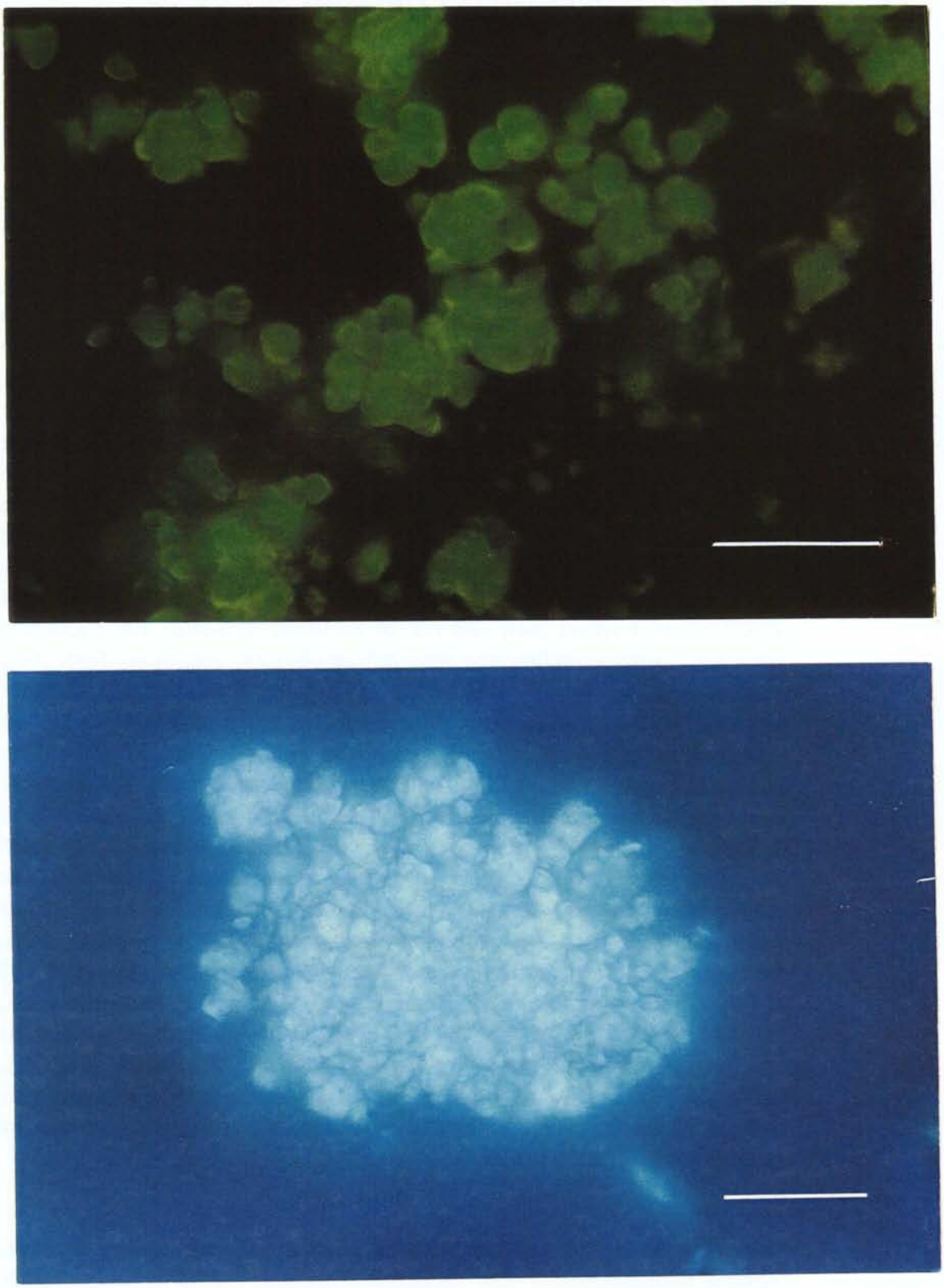


Figure 4 *Microscopy of subterranean methanogens. (A) Methylotrophic* Methanohalophilus*-like methanogen enriched from borehole KA3110A, depth 414 m at 17 °C. (B) Trimethylamine consuming* Methanosarcina*-like methanogens enriched from borehole KR0012B, depth 68 m, at 20 °C. Bar = 10μm*

5.3 METHANE PRODUCTION IN THE GROUNDWATERS

To elucidate relevant autotrophic and heterotrophic activities of methane production in different groundwaters, the rates of methane production were estimated by measuring methane produced in the sampled groundwaters with time. The bottles with groundwater were incubated at 17 °C in darkness for 60-160 days. Methane production was followed by gas chromatography every 10 days. The experiments were initiated on November 15, 1995 and April 16, 1996. Figure 5 shows methane production curves for groundwater sampled from KR0012B (A) and KA3110A (B) boreholes at 68 and 414m depths. The figure displays examples of the process observed in many other groundwaters at depths of 68, 192, 400, 409, 414 and 420 m. It was a convincing proof of the presence of methanogens in the groundwater because the progressive methane production was observed in all repetitions of the same boreholes during two independent experiments. The most active methane production took place in the groundwaters from which pure cultures were isolated. After adaptation to laboratory conditions (20-50 days) methane production was activated in the bottles with added substrates. Such a long lag-phase indicates that activation of dormant cells can not be excluded, however, the temporary oxidation during sampling and the shift of physical conditions from deep granites to the laboratory could inhibit an on-going physiological activity of the cells in the groundwaters. The extent of the adaptation phase was reproducible in repetitive experiments. Interestingly, the length of the adaptation period of shallow site methanogens in the presence of heterotrophic substrates were shorter (10 days) than in the presence of H_2/CO_2 (>100 days ; Figure 5A). The CH_4 production rates in different groundwaters and with different substrates are presented in Figure 6. The rates in the presence of substrates varied in different boreholes from 0.017 to 0.300 μM of methane per liter of groundwater per day. The most active methanogenesis on heterotrophic substrates was found at depths of 68 and 414 m. Active autotrophic methanogenesis was observed in boreholes at 68, 112, 345, 380, 400, 409, 414 and 420 m (Figure 6A). Methane production was not observed in three boreholes KA2858A, KA2862A (392 m), KA3385A (460 m; Figure 6 A, B). The results are in good agreement with enrichment results and distribution of different physiological groups of methanogens along the tunnel.

Interestingly, in the variants without addition of any energy sources, ongoing methanogenesis was observed. The rates varied between 0.008-0.159 μM of methane per liter of groundwater per day. The methanogenic activity in the flasks lacking substrate additions indicates the presence of methanogenic substrates in the tested groundwaters. Additions of bicarbonate stimulated the methanogenesis in these groundwaters. No increase of the methane concentrations was observed in bromethanesulfonic acid inhibited bottles.

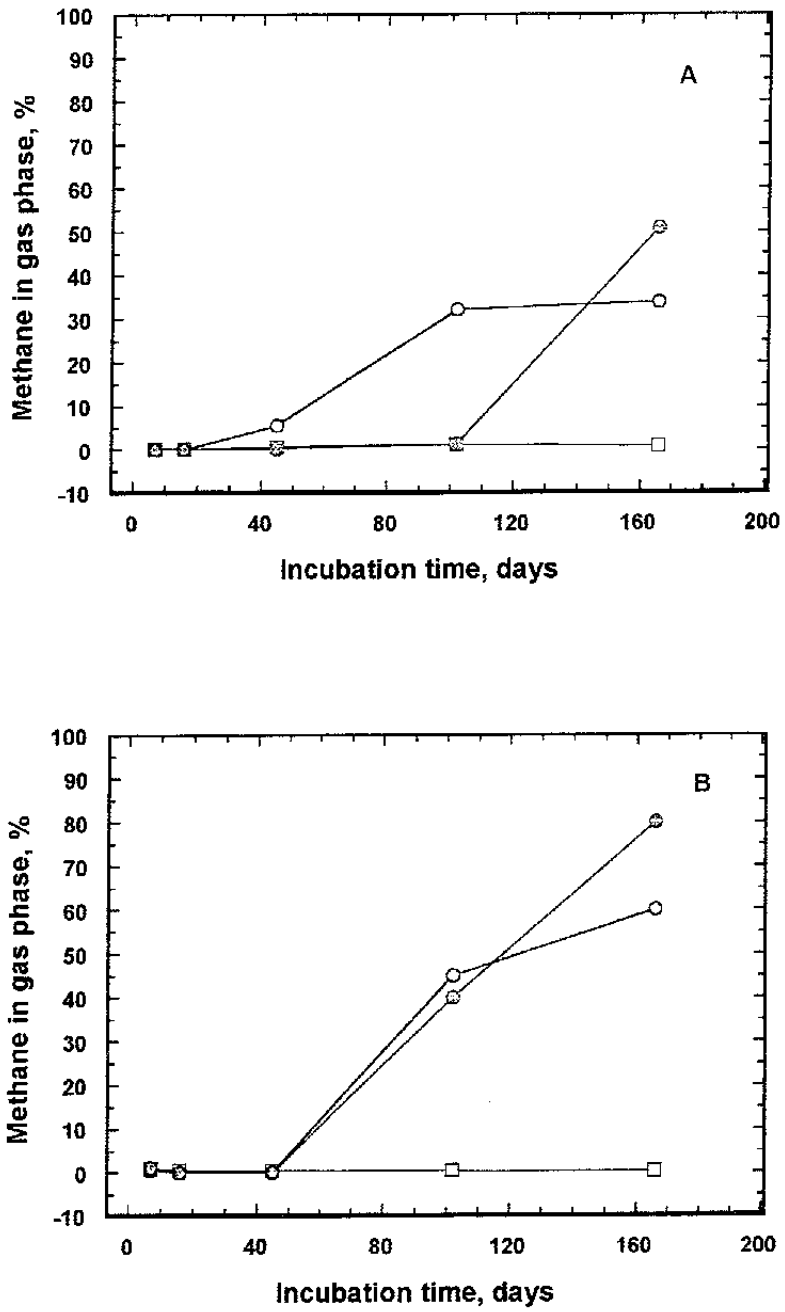
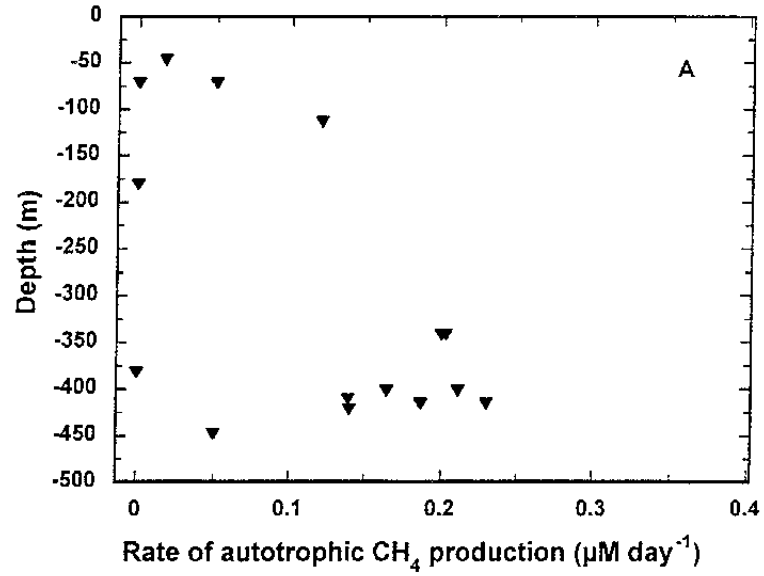


Figure 5 *Methane production in the groundwaters from borehole KR0012B (A) and KA3110A (B) in the presence of H_2/CO_2 (●), acetate, methanol and trimethylamine* (○) *and in controls without any substrates but with BES* (□) *plotted with the time of incubation. For the experiments sterile closed vacuum pumped bottles (115 ml volume) were filled with 100 ml of the freshly collected groundwater. Sterile and anaerobic resazurine solution was added (0.0002 %) as a redox potential indicator and new rubber stoppers were used. For each groundwater heterotrophic substrates (acetate, methanol, trimethylamine) at a concentration of 5 mM each were added to two bottles. In another pair of bottles H_2/CO_2 (80:20) was flushed at a pressure of 2 bars. Bromethanesulfonic acid (50 mM) was added to controls for abiotic methane production.*



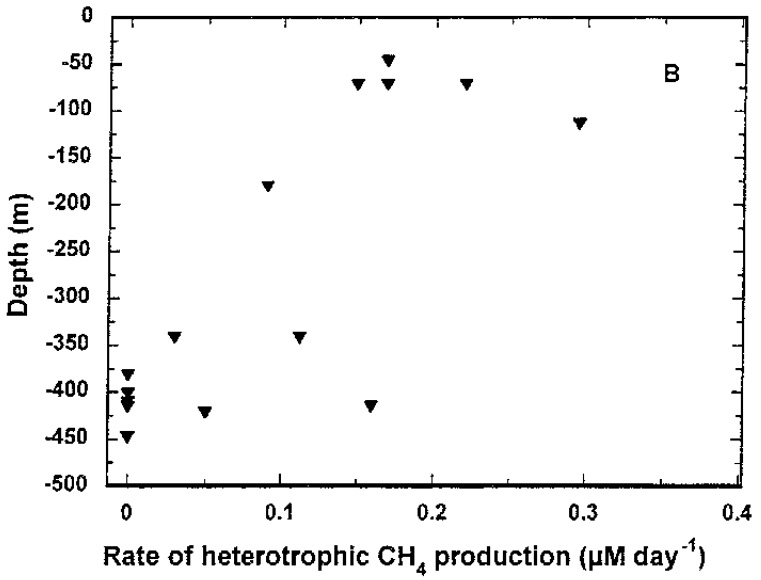


Figure 6 *Rates of autotrophic (A) and heterotrophic (B) methane production in the groundwaters sampled from different boreholes plotted with depth. Average of two repetitions of the experiment initiated in November 1995. The rates of methane production in the groundwater were calculated from the exponential phase of the processes.*

6 HOMOACETOGENIC *BACTERIA*

6.1 HOMOACETOGENIC *BACTERIA* IN THE ÄSPÖ GROUNDWATERS

Since we found acetoclastic methanogens in the deep subsurface at low organic carbon concentrations at numbers of 10^5-10^6 cells/ml, we decided to check the environment for the presence of homoacetogens. Acetogens play a significant role in low-temperature environments such as forest soils (Kusel et al, 1995), anoxic paddy soils and lake sediments (Peters et al, 1995), pond sediments, tundra soil (Nozhevnikova et al, 1994) and hypersaline waters (Zavarzine et al, 1994). Hydrogen-oxidizing acetogenic activities have been observed in eutrophic lake sediments, but only 2 % of the total acetate was derived from carbon dioxide (Lovely et al, 1983). Our MPN results indicated that acetogenic bacteria are present (up to 3.6 x 10^4 cells/ml) in subsurface granitic groundwater down to a depth of 440 m (Figure 7). Acetate (up to 300 mg/l) was produced in media inoculated with groundwater in the presence of H_2/CO_2 (80:20) in the gas phase. The formalin inhibited groundwater did not produce acetate from H_2/CO_2. Our cultivation data indicate that homoacetogens are present and may produce acetate autotrophically in Äspö granitic groundwater.

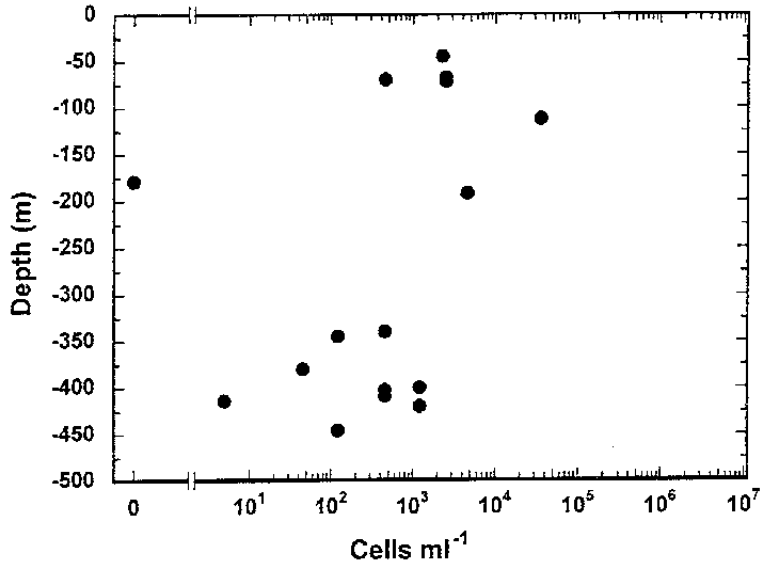


Figure 7 *Most probable numbers of homoacetogenic bacteria plotted with depth. Acetate production in the medium (Kotelnikova et al., 1997) with 4 g/l NaCl, H_2/CO_2 (80:20) and 20 mM bromethanesulfonic acid added was a positive indication of the acetogenic growth. MPN values were calculated with an MPN determination program, Yamanashi University, Ishikawajima-Harima Heavy Industries, LDT (Hurley et al., 1983). The standard deviation was 10 % . The enumerations were repeated on April 16 and June 27, 1996.*

One homoacetogenic bacterium has been isolated from the groundwater sampled along the Äspö HRL tunnel, strain Aspo-4 (Pedersen et al., 1996). Investigations of the 16S rRNA diversity of the groundwater samples from different boreholes along the tunnel revealed the presence of sequences identical to Aspo-4 in several boreholes. It was found in the groundwater from the depths of 68 m (KR0013B), 112 m (SA813B), 413 m (SA3105A) and 420 m (HD0025A). In all these boreholes the MPN of homoacetogens were high (Figure 7).

The distribution of methanogens and homoacetogens with different depths is governed by many environmental factors like dissolved organic carbon and molecular hydrogen content, salinity, pressure and temperature. Currently we are performing statistical analyses of variance, which will show the main factors responsible for the specific distribution of these anaerobes.

7 CONCLUSIONS

The presence of diverse populations of methanogenic *Archaea* and homoacetogenic *Bacteria* has been found in deep granitic groundwater in Äspö HRL. The results extend the known habitat of these organisms. Autotrophically produced acetate may be one of the organic energy sources in oligotrophic ecosystems such as a granitic subterranean aquifers. Molecular hydrogen observed in the groundwaters at comparatively high concentrations, is suggested to be one of the possible inorganic energy sources for the chemolithotrophs revealed in the investigated granitic rock aquifers.

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Distribution and activity of methanogens and homoacetogens in deep granitic aquifers at Äspö Hard Rock Laboratory, Sweden

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CONTENTS

1 ABSTRACT

This study reveals the existence of active chemolitoautotrophic microbial communities in deep granitic groundwater, containing hydrogen and methane. Homoacetogens and acetotrophic methanogens dominated in the groundwater down to a depth of 112 m, while autotrophic methanogens prevailed deeper, down to 446 m. The results from most probable number counts showed that viable methanogens and homoacetogens coexisted at all dephts investigated, from 45 m to 446 m below sea level. *In vitro* radiotracer experiments demonstrated these microorganisms to form methane and acetate from CO_2 with hydrogen and methane from [^{14}C]-acetate at close to *in situ* temperature (17°C). The distributions and *in vitro* activity of methanogens and homoacetogens correlated with depth, chlorinity, *in situ* temperature and the concentrations of dissolved organic carbon and bicarbonate. The data suggest that autotrophic methanogens and homoacetogens initiate a deep subterraneum food chain and that acetate depending methane formation is mediated by homoacetogenesis. The finding of an active, deep, hydrogen based autotrophic biosphere adds a significant but earlier overlooked reducing force to deep granitic rock.

2 INTRODUCTION

The Swedish research program on subterraneum microbiology (Pedersen et al, 1996a, Pedersen, 1997) has been performed at two sites in granitic rock aquifers at depths ranging from 70 m down to 1240 m; the Stripa research mine in the middle of Sweden (Ekendahl et al, 1994a, 1994b; Pedersen et al, 1992b) and the Äspö hard rock laboratory (HRL) situated on the Southeastern coast of Sweden (Pedersen et al, 1990, 1992c, 1996b, 1997a, 1997b; Pedersen, 1997). The Äspö HRL has been constructed as a part of the development of the Swedish concept for deep geological disposal of spent nuclear fuel and the work has been divided into three phases; the pre-investigation (1986-1990), the construction (1990-1995) and the operating (1995-) phases. The work presented here was performed during the transition from the construction to the operating phase.

Throughout the above mentioned work, results have indicated the presence of autotrophic microorganisms that utilise hydrogen as a source of energy in the studied deep granitic rock environments. Therefore, a hypothesis on a hydrogen driven biosphere in deep granite has been suggested (Pedersen et al, 1992a; Pedersen, 1993; Pedersen, 1997). The base for this biosphere is postulated to be composed of acetogenic bacteria that react hydrogen with carbon dioxide to acetate (homoacetogens) and methanogens that yield

methane from hydrogen and carbon dioxide (autotrophic methanogens) or from acetate produced by homoacetogens (acetotrophic methanogens). A similar hypothesis has recently been published for hydrogen consuming autotrophs found in deep basaltic rock aquifers (Stevens et al, 1995). The aim of this study was to collect evidence for a hydrogen driven biosphere in deep granitic aquifers and it was focused on acetogenic bacteria and methanogens as the autotrophic base for such a biosphere. Distribution, numbers and physiological diversity of homoacetogens and methanogens in deep granitic rock aquifers at the Äspö HRL were investigated using a variety of methods. Total numbers of auto-fluorescent methanogens and most probable numbers (MPN) of cultivable methanogens and homoacetogens were determined in the groundwater from 18 boreholes. *In vitro* activities of these microorganisms were measured using enzymatic and radio tracer techniques. The obtained results were compared with geochemical and physical data. Environmental variables governing the distribution of the studied microorganisms are suggested.

3 MATERIALS AND METHODS

3.1 SITE DESCRIPTION

The Äspö Hard Rock Laboratory (HRL) is situated on the island of Äspö, adjacent to the Baltic coast of Sweden approximately 400 km south of Stockholm. The access tunnel to the HRL proceeds with an inclination of 14 % from the Baltic shoreline under the sea-floor for a distance of approximately 1, 700 m, where it spirals down and terminates 460 m below the island of Äspö (Fig.1). The total length of the tunnel is 3600 m. Äspö itself, characteristic for the surrounding islands, comprises a slightly undulating topography (10 m above sea level) of well-exposed rock. The geology is characterised by a red to grey porphyritic granite-granodiotite known regionally as "Småland-type" belonging to the vast Trans-Scandinavian Granite-Porphyry Belt (Gaal et al, 1987) with intrusion ages (U-Pb) between 1760 and 1840 Ma (Johansson, 1988), i.e., late orogenic to postorogenic in relation to the Svecofennian origin (1800-1850 Ma). Major fractures and fracture zones control recharge, discharge and groundwater flow through the island (Smellie & Laaksoharju, 1992).

A total of 18 boreholes on 3 sites in the tunnel were repeatedly sampled in this investigation during 8 expeditions during the period 1994 to 1996. They were only opened at sampling, with exception for KR0013 that has been standing open and flowing since it was drilled in 1992. The sampling took place December 12 1994, January 17, February 22, October 10, November 24 1995, February 5, April 15-16 and June 27, 1996. The sites, borehole designations and depths of the boreholes can be found in Table 1. A shallow

site comprised five core drilled boreholes at tunnel length 520 m, intersecting a fracture zone (Fig. 1B) and four of these boreholes were included. This site has been studied thoroughly earlier for the understanding of the fate of shallow groundwater intrusion into the tunnel (Banwart et al, 1994, 1996). Two boreholes were drilled from ground, intersecting the fracture RZ at 15 and 45 m below sea level (HBH02 and HBH01 respectively, Fig 1). They were packed off around the sampled RZ feature as shown in Fig. 1B and equipped with stationary submersible pumps. HBH01 was sampled in this investigation. The three other sampled boreholes were drilled from a vault excavated 68 m below the sea level perpendicular to the main tunnel and parallel to the RZ fracture zone (KR0012, KR0013 and KR0015, Fig. 1).

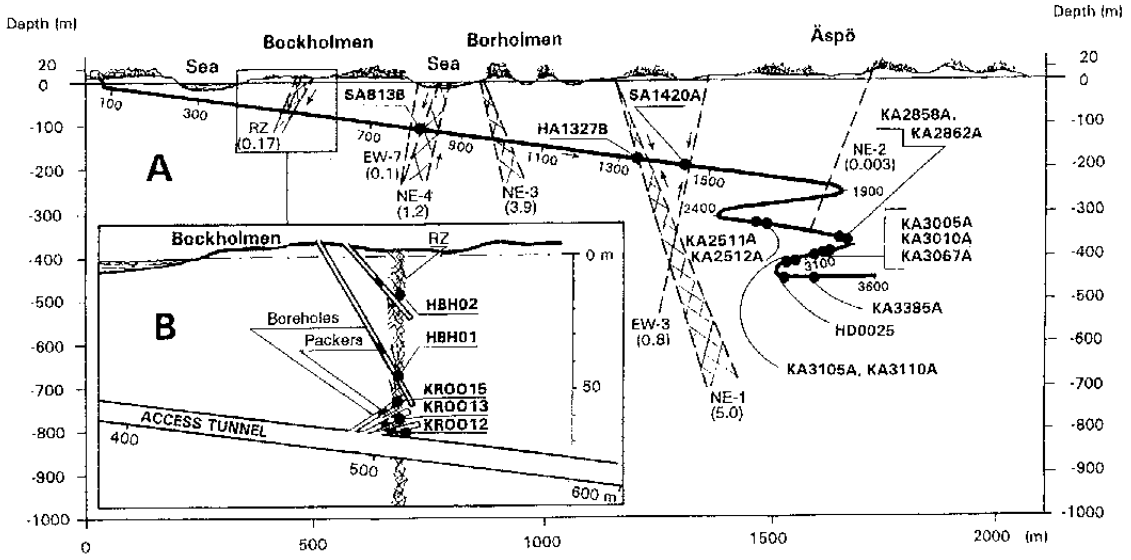


Figure 1 *(A). Site topology along the tunnel in Äspö Hard Rock Laboratory, in June 1996. The sampled sites are depicted with their respective borehole names. These names show the type of drilling (HBH, HA = percussion drilled, KAS, SA = core drilled), the tunnel length where they were drilled and if they were drilled on the left (A) or the right (B) side of the tunnel when going down. Major fracture zones are marked with dashed lines and with their given names, generally indicating their geographic orientation. Possible flow directions of ground water are indicated with arrows and the estimated inflow rates of ground water via the fractures to the tunnel are shown in brackets as L* s^{-1}. *(B). A fracture zone (RZ) with boreholes that were drilled with the purpose to follow shallow ground water intrusion through this major fracture zone into the tunnel. A side vault was constructed (not shown) from which the boreholes KR0012-13-15 were drilled perpendicular through the zone. Note that these three boreholes all sampled at 68 m below sea level (not shown).*

Table 1 *Borehole information with groundwater chemistry data.*

Borehole	Depth (m)	Analysis (D-M-Y)	pH	T °C	HCO_3^- mg L^{-1}	SO_4^{2-} mg L^{-1}	Cl^- mg L^{-1}	NH_4^+ mg L^{-1}	Fe mg L^{-1}	DOC mg L^{-1}
Shallow Site										
HBH01	45	05.09.94	7.2	n.a[a].	319	35	348	0.04	0.40	14.0
KR0012B	68	05.09.94	7.4	9.3	326	43	573	0.32	0.19	11.0
KR0013B	68	05.09.94	7.2	8.9	291	148	1737	0.24	0.37	11.0
KR0015B	68	05.09.94	7.2	8.9	422	39	535	0.27	0.30	18.0
Intermediate site										
SA813B	112	05.09.94	6.8	10.4	292	100	3112	n.a	5.00	7.1
SA1327B	179	15.10.92	7.4	11.0	295	225	3920	n.a.	2.30	6.5
SA1420A	192	06.09.94	7.1	11.4	199	102	2950	n.a.	1.60	2.6
Deep Site										
KA2511A	345	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
KA2512A	345	13.12.94	7.2	11.8	197	101	4750	n.a.	0.79	3.8
KA2858A	380	10.03.95	7.8	12.9	9	201	10280	n.a.	0.09	0.3
KA2862A	380	27.01.95	7.2	13.3	8	231	13300	n.a.	0.13	0.8
KA3005A	400	07.12.94	7.7	14.3	81	108	4870	n.a.	0.59	2.0
KA3010A	400	15.12.95	7.5	14.3	56	119	5770	n.a.	0.81	2.5
KA3067A	409	13.12.94	7.4	14.0	53	112	5650	n.a.	0.53	2.0
KA3105A	414	15.12.94	7.6	14.2	102	87	3960	n.a.	0.47	2.8
KA3110A	414	09.01.95	7.6	13.4	164	103	3820	n.a.	1.00	4.0
HD0025A	420	09.01.96	7.5	15.0	176	n.a.	3346	n.a.	n.a.	n.a.
KA3385A	446	11.01.95	7.7	14.5	10	154	6650	n.a.	0.14	0.9

[a] not analysed

An intermediate site comprised three boreholes between 813 and 1420 m tunnel length (SA813B, HA1327B and SA1420A, Fig. 1). These boreholes were core drilled and arthesian and were partly influenced by Baltic sea water (Laaksorju et al, 1995). They were packed of with a packer 2 m from the tunnel walls, fitted with tubings from the packed-off section to a valve in the tunnel and were kept closed.

Eleven deep site boreholes were drilled between tunnel length 2858 m and 3385 m (Fig. 1) for a project denoted SELECT, aiming at the geological, hydrological and hydrogeochemical characterisation of designated experimental rock volumes for redox and tracer retention experiments. Detailed information about the SELECT borehole set-up etc. can be found elsewhere (Winberg et al, 1996). A description of the drilling procedure, that included measures to avoid microbial contamination of the boreholes during drilling is also given by Pedersen et al. (1997b). Briefly, the drill water was taken from a borehole at 3, 200 m tunnel length and was kept under nitrogen atmosphere. All equipment was steam cleaned before the start of a new drilling. An investigation of the potential for microbial contamination during

this drilling was performed concomitant with the drilling (Pedersen et al., 1997b). Total number of bacteria, viable counts of aerobic and anaerobic heterotrophic bacteria and 16S rRNA gene diversity were investigated. Water samples were taken from the drill water source, the drilling equipment and from the drilled boreholes. The investigation showed that the tubing used for drill water supply constituted a source of bacterial contamination to the rest of the drilling equipment and the boreholes. The results also showed, using molecular and culturing methods, that although contaminating bacteria from the tubing were introduced to the boreholes during drilling, they did not establish in the borehole groundwater at detectable levels.

3.2 SAMPLING PROCEDURE

Before sampling a borehole, several borehole volumes were drained to ensure that groundwater from the geological formation around the borehole was sampled. All tunnel boreholes were artesian and were sampled from the packed-off section via 6 mm (outer diameter) plastic tubings draining specific sections of the boreholes. Sampling was done with syringes, flushed with 2.5 % sodium sulphide, from the tubings. The samples were injected in sterile, nitrogen flushed and oxygen-free anaerobic bottles equipped with butyl rubber stoppers. Inoculation of the different media was done not later than 1 hour after sampling in the field laboratory situated on the ground close to the tunnel entrance (Wikberg, 1987).

3.3 GROUNDWATER CHEMISTRY

Groundwater for chemical analysis was sampled parallel with sampling for microbiological analysis. Information about the sampling procedures have been published elsewhere (Nilsson, 1995).

3.4 SAMPLING FOR ANALYSIS OF DISSOLVED METHANE AND HYDROGEN

Bottles for anaerobic cultivation (115 ml volume) were equipped with new butyl rubber stoppers and aluminium crimp seals, autoclaved, evacuated (-1 bar) and subsequently filled with 100 ml of groundwater and chilled. The amounts of hydrogen and methane released from the water to the head space of the bottles were analysed within 24 h from the sampling occasion. The gas analysis were done on groundwater sampled in October 10 and November 24, 1995 and February 5, April 15-16 and June 27, August 22-23, 1996.

3.5 GAS ANALYSIS

Methane was determined with a Varian 3700 gas chromotograph (Varian, Solna, Sweden) equipped with a 2 m 1/8 inch steel column packed with Porapak Q, mesh 80/100 and a flame ionisation detector (FID). The flow rates were 300 ml min^{-1} for air and 30 ml min^{-1} for hydrogen to the FID with N_2 as carrier gas at 30 ml min^{-1}. The injector, column and detector temperatures were isothermal, 100, 100 and 200 °C, respectively. The response of the FID to methane was linear. Hydrogen was analysed on the same machine with a 5 m long 1/8 inch steel column packed with Molsieve 5A, mesh 60/80 and a thermal conductivity detector with N_2 as carrier gas at 30 ml min^{-1}. Filament, detector and column temperature were isothermal, 250, 125 and 30 °C, respectively. The helium peak could be separated from the hydrogen peak since the retention times for these chromatogram peaks were 1.2 and 1.6 min, respectively. The gases used for calibration and chromatography was 99.99 % pure (AGA, Gothenburg, Sweden). Relative standard deviation of samples analysed did not exceed 5 %. Calibration, integration of peaks and calculations of gas content were done with Star Chromatography Workstation, Varian, version 4.01 Software. The detection limits for methane and hydrogen dissolved in groundwater were 4.5 nM and 45 nM, respectively.

3.6 ANALYSIS OF ORGANIC ACIDS

Acetate was analysed using an enzymatic and photometric method (Boehringer Mannheim) at 340 nm. The groundwater or media to be analysed was filtered (DynaGard filters, 0.2 m pore diameter, Microgon, Inc.) and frozen in 10-20 ml and 0.2 ml portions, respectively. The analysed volumes were 1.0 ml, 0.5 ml and 0.1 ml. The effect from dissolved solids in the groundwater was assessed in control experiments using additions of acetate standards and distilled water.

3.7 METHANOGENIC AND TOTAL CELL MICROSCOPY COUNTS

Methanogens contain high concentrations of the electron carrier F_{420} (Gorris et al, 1986) that emits a bright blue-green auto-fluorescence when illuminated with light at 420 nm (Doddema et al, 1978). The number of cells with auto-fluorescence, when illuminated with 420 nm and acridine orange direct total counts (AODC) (Pedersen et al. 1990) were assayed the day after sampling of groundwater that had been stored at 4 °C. AODC samples were preserved with 2 % formaldehyde. Different volumes of groundwater were filtered in 3 or 4 repetitions on Nuclepore filters (0.22 µm pore size). Fluorescence microscopy was performed with epifluorescence microscopes (Olympus BH-2 or Carl Zeiss Axioscope) using blue light (390-490 nm).

Autofluorescent cells were counted using a LP 435 excitation filter. The results were calculated as an average of 3 to 5 repetitive counts for each borehole.

3.8 CULTURING CONDITIONS FOR VIABLE COUNTS AND ENRICHMENTS

A technique for cultivation of strict anaerobic microorganisms (Balch et al, 1979; Hungate, 1969) was applied for MPN assays and for the enrichment of methanogens. The medium used contained per liter of solution: yeast extract, 0.1 g; casamine acids, 0.1 g; tryptone, 0.1 g; coenzyme M (mercaptoethanesulfonic acid), 5 mg; propionic acid, 20 mg; N-butyric acid, 16 mg; isobutyric acid, 3 mg; N-valeric acid, 3 mg; DZ-2 methylbutyric acid, 3 mg; element solution SL-6, 10 ml (Wolin et al, 1963); vitamin solution, 5 ml (Wolin et al., 1963); resazurine, 2 mg; $NaHCO_3$ 2.0 g; L-cystein HCl, 0.5 g; Na_2S, 0.5 g; groundwater from the corresponding borehole analysed, 985 ml. pH of the medium was adjusted to the pH in each borehole according to Table 1. The following compounds were used separately as energy and carbon source for methanogenesis: H_2/CO_2 (80:20 % vol:vol) at 2 bars; sodium acetate, 20 mM; sodium formate, 30 mM; methanol, 30 mM; monomethylamine, 20 mM; trimethylamine (TMA), 20 mM. For the MPN of homoacetogens, the anaerobic technique and an artificial Äspö medium (ASPM) that imitates the groundwater composition as described elsewhere (Kotelnikova et al, 1997a) were used. ASPM was added with H_2/CO_2 as substrate for homoacetogens and with bromethansulfonic acid (BESA), 20 mM, to inhibit methanogenesis.

3.9 MPN OF METHANOGENS AND HOMOACETOGENS

The numbers of viable methanogens and homoacetogens were estimated by the MPN method (Koch, 1994). Methane or acetate formation were used as positive indications of growth in the MPN tubes (see above). The MPN experiments were conducted December 12, 1994, January 17, February 22, October 10, November 24, 1995, February, 1996 and April, 1996. Three replicate tubes were inoculated with 0.5 ml from 6 to 10-fold dilution series of the samples. Methane or acetate were measured after repeated periods of two to three weeks of incubation at 20° C and 37°C. BESA (20 mM) was added as a control for non-biogenic methane formation in the MPN tubes for methanogens. None of these control samples exhibited detectable methane formation. MPN numbers, 95 % confidence limits and statistical deviations were calculated using a MPN program from Yamanashi University, Ishikawajima-Harima Heavy Industries, LDT (Hurley et al, 1983). The standard deviation of the MPN determinations did not exceed 30 %.

3.10 *IN VITRO* AUTOTROPHIC METHANE PRODUCTION

The formation of $^{14}CH_4$ from $NaH^{14}CO_3$ was followed in a separate set of sterile and nitrogen flushed 55 ml serum bottles equipped with aluminium crimp sealed butyl rubber stoppers and added with 2 ml ASPM. $NaHCO_3$ was added to the ASPM after sterilisation from sterile anaerobic solution to a final concentration 250 mg L^{-1}. The pH of ASPM was adjusted with phosphate buffer to that of each groundwater sampled (Table 1) and reduced with 0.25 mg L^{-1} L-cystein HCl and 0.25 mg L^{-1} Na_2S. Four bottles per borehole were injected with 18 ml groundwater collected from 15 boreholes at February 5 and April 15-16, 1996. Two of them were added with formaldehyde to a final concentration of 2 % and served as killed controls. Finally, $NaH^{14}CO_3$ was added in various concentrations to final activities of between 0.15 to 13.68 μCi mL^{-1}, corresponding to 0.003 - 3.0 mg L^{-1} (Sodium bicarbonate, CFA3, Amersham, Sweden). The bottles were subsequently supplied with 0.5 bar hydrogen and incubated during 4-10 days at 17°C. The corresponding concentration of dissolved hydrogen in the medium became approximately 50 μM. The formation of $^{14}CH_4$ from $NaH^{14}CO_3$ was repeatedly measured after periods of 3-5 days using a toluene-based scintillation cocktail (Quickscint 501, Zinsser Analytic, Frankfurt, Germany, Scint Varuhuset AB) with 1M NaOH trapping of methane as described by Zehnder et al. (1979). A Beckman Scintillation Counter was used in automatic mode for the measurement of radioactivity with an average count time of 5 min and automatic subtraction of blank counts. For the blank count controls four scintillation vials were filled with the same volume of scintillation liquid but without addition of the trapped gas.

3.11 *IN VITRO* ACETOTROPHIC METHANE PRODUCTION

The potential for acetotrophic methanogenesis was studied in groundwater from 15 boreholes in 2 replicates with 2 controls as described above, but without addition of $NaHCO_3$, $NaH^{14}CO_3$ and hydrogen. Instead, 1, 2-^{14}C-acetate was supplied to a final activity of 0.113 μCi mL^{-1} (Acetic acid sodium salt, CFA229, Amersham, Sweden). Methane formation was measured as described above for *in vitro* methane formation.

3.12 *IN VITRO* AUTOTROPHIC ACETATE PRODUCTION

Autotrophic acetate formation was studied in groundwater using an enzymatic, photometric method (Boeringer Mannheim), the liquid scintillation technique and the procedures as describe above for autotrophic methane formation ($NaH^{14}CO_3$, 0.15 μCi ml^{-1}) with the following modifications. Groundwater from 15 boreholes was added with BESA (50

mM) in two replicates with two formaldehyde killed controls and incubated for four days at 17 °C. The samples were subsequently acidified over night and filtered (0.2 µm). The amount of radioactive acetate, produced from $NaH^{14}CO_3$ in groundwater, was determined in the filtrate as described elsewhere (Schnürer et al, 1994). Liquid scintillation was performed on 100 µl of filtered groundwater in 10 ml of Ready Safe scintillation cocktail (Beckman) using a Beckman Scintillation Counter as described above.

3.13 RATE CALCULATIONS

Methane and acetate formation rates were calculated from the observed increase in the concentration of respective labelled product over time. The amount (nM) of respective ^{14}C-product formed were calculated as:

$$\frac{(\text{disintegrations min.}^{-1}\ \text{sample}^{-1} - \text{disintegrations min.}^{-1}\ \text{control}^{-1})}{\text{specific activity of the } C^{14} \text{ substrate}}$$

The total amount of a product in the radiotracer experiments was calculated as follows. The substrate concentrations, which equalled the sum of the *in situ* pool of substrate and the amount of non-labelled substrate and ^{14}C-labelled substrate added, was divided by the ^{14}C-labelled substrate concentration, giving a coefficient denoted K. This coefficient shows the ratio of unlabelled to labelled substrate. The incorporation rates (µM product h^{-1}) of labelled carbon in methane from [1, $2\text{-}^{14}C$]-acetate or from $NaH^{14}CO_3$ or in acetate from $NaH^{14}CO_3$ were calculated as:

$$\frac{K \times (C_2 - C_1)}{time_2 - time_1}$$

where C_1, C_2 is the concentration of radio labelled product after time 1 and time 2. Standard deviations calculated for repetitions and results from different sampling dates did not exceed 28 %.

3.14 STATISTICAL ANALYSIS

Multiple stepwise linear regressions using SAS (1989) were made for the following parameters: AODC, numbers of autofluorescent cell, MPN of homoacetogens, autotrophic, methylotrophic and acetotrophic methanogens, rates of methanogenesis at expense of $H_2/^{14}CO_2$ or ^{14}C-acetate, acetogenesis

at expense of $H_2/^{14}CO_2$, methanogenesis from H_2/CO_2 or acetate, physical and chemical characteristics of the groundwater such as the concentrations of methane, hydrogen, acetate, bicarbonate and DOC and the depth, pH, temperature and chlorinity.

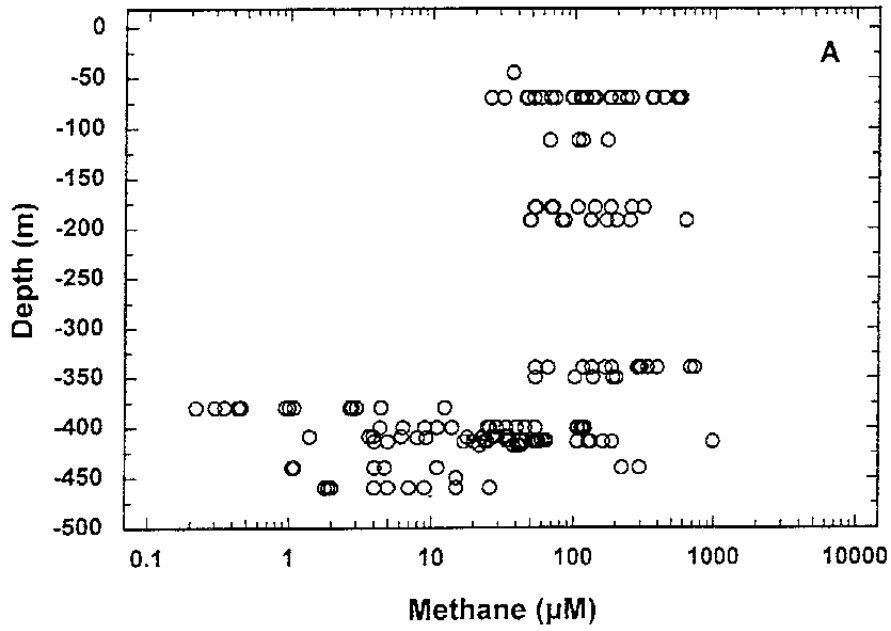
4 RESULTS

4.1 GROUNDWATER COMPOSITION

The chemical composition and the physical conditions in the investigated groundwater are presented in Table 1. The pH varied between 6.8 and 7.8 and the temperature increased with depth from 8.9°C up to 15°C. The chlorinity (mg Cl^- L^{-1} of the groundwater varied, generally increasing with the depth, from 348 up to 13 300 mg L^{-1}. The shallow site groundwater was slightly less alkalic than the deep site groundwater. The bicarbonate content were higher in the shallow site groundwater compared to deep site groundwater and ranged between 291 and 422 mg L^{-1} and between 8 and 197 mg L^{-1}, respectively. The content of dissolved organic carbon (DOC) ranged between 11 and 18.0 mg L^{-1} in shallow site groundwater and decreased with the depth to 0.3-4.0 mg L^{-1} in the deep site groundwater. The amount of sulphate was lower at the shallow site (35-148 mg L^{-1}) than in the intermediate and deep site groundwater (87-231 mg L^{-1}) and most of the borehole groundwaters had a higher bicarbonate content than sulphate. The intermediate site groundwater had transit chemical and physical features between the shallow and deep site compositions (Table 1). Thus, the studied groundwater varied significantly in its composition as a function of depth and site, but was generally, neutral to slightly alkalic, fresh to brackish and oligotrophic.

4.2 CONTENT OF METHANE AND HYDROGEN IN THE GROUNDWATER

Figure 2 A shows that the methane content varied over depth from 0.19 to 1000 μM. Results from up to six independent measurements deviated around a specific concentration of methane in respective borehole, indicating a stability of the system. Free hydrogen was also found in concentrations varying from 0.045 to 50 μM (Figure 2B). Hydrogen was detected in all boreholes investigated except two of the shallow site boreholes, where hydrogen was below the detection limit (45 nM). Concentrations of hydrogen were as a rule equal to, or higher in the deep site boreholes compared to the shallow and intermediate site boreholes.



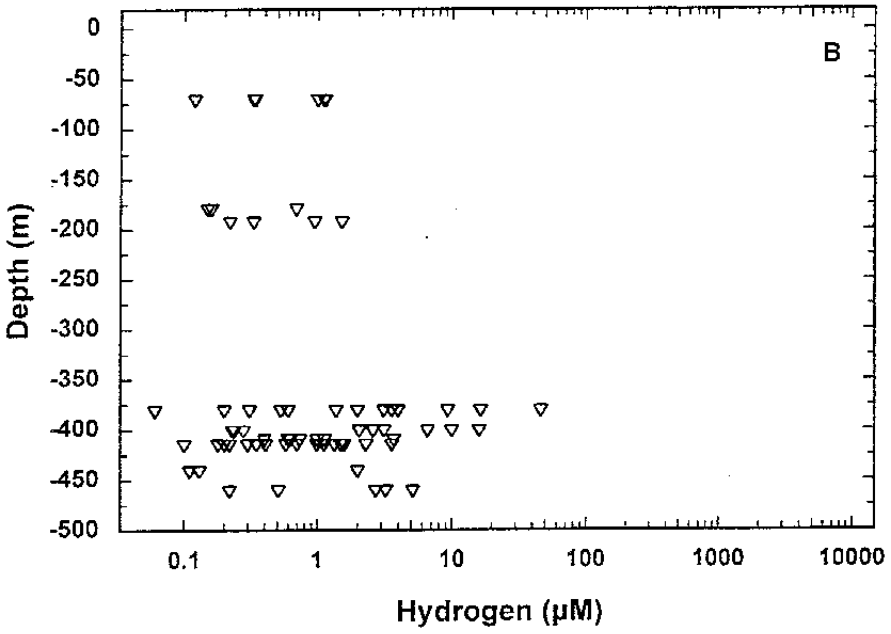


Figure 2 (A). *Concentrations of methane dissolved in the groundwater. Data for samples collected in October and November 1995, February, April, June and August 1996* **(B).** *Concentrations of hydrogen dissolved in the groundwater. Data for samples collected in April and June 1996.*

4.3 TOTAL AND METHANOGENIC MICROSCOPY CELL COUNTS

The distributions of fluorescent microscopy counts of auto-fluorescent cells and total number of cells (AODC) along the Äspö hard rock laboratory tunnel are shown in Figure 3. The microscopic counts of auto-fluorescent cells varied from 12 to 7.4 x 10^5 cells mL^{-1} while the total number of cells varied between 5.4 x 10^3 and 3.4 x 10^6 cells mL^{-1}. The total AODC numbers were always higher than the number of auto-fluorescent methanogens. A tendency for decreasing average cell numbers with depth was detected.

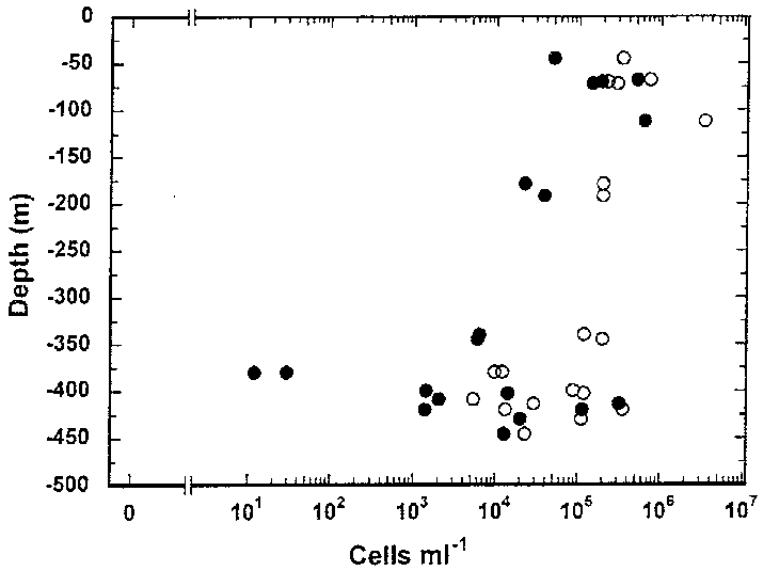


Figure 3 *Fluorescent microscopy counts of the total number of cells and the number of auto-fluorescent methanogens in groundwater sampled from 18 boreholes along the Äspö hard rock laboratory tunnel.* (○) *Total number of cells;* (●) *Auto-fluorescent (F_{420}) methanogens.*

4.4 VIABLE NUMBER OF METHANOGENS AND HOMOACETOGENS

Both autotrophic and heterotrophic methanogens were abundant in the Äspö groundwater. MPN of autotrophic and heterotrophic methanogens varied from 5 to 4.1 x 10^4 cells mL^{-1} and from 0 to 1.72 x 10^5 cells mL^{-1} groundwater, respectively (Fig. 4A-B). The numbers of autotrophic methanogens increased with depth while heterotropic methanogens decreased in numbers with depth (Figure 4). The MPN of autotrophic methanogens varied from 5 to 7.05 x 10^2 in the shallow site groundwater and from 5 x 10^2 to 4.12 x 10^4 cells mL^{-1} in the deep site groundwater. The heterotrophic methanogens observed were physiologically diverse. MPN varied from 470 to 2.5 x 10^4 and from 0 to 5.51 x 10^2 acetate utilising methanogens mL^{-1} groundwater in the shallow and deep sites, respectively. MPN of methanol and TMA consuming methanogens ranged from 70 to 1.72 x 10^5 and from 0 to 4.83 x 10^2 cells mL^{-1} groundwater at the shallow and deep sites, respectively.

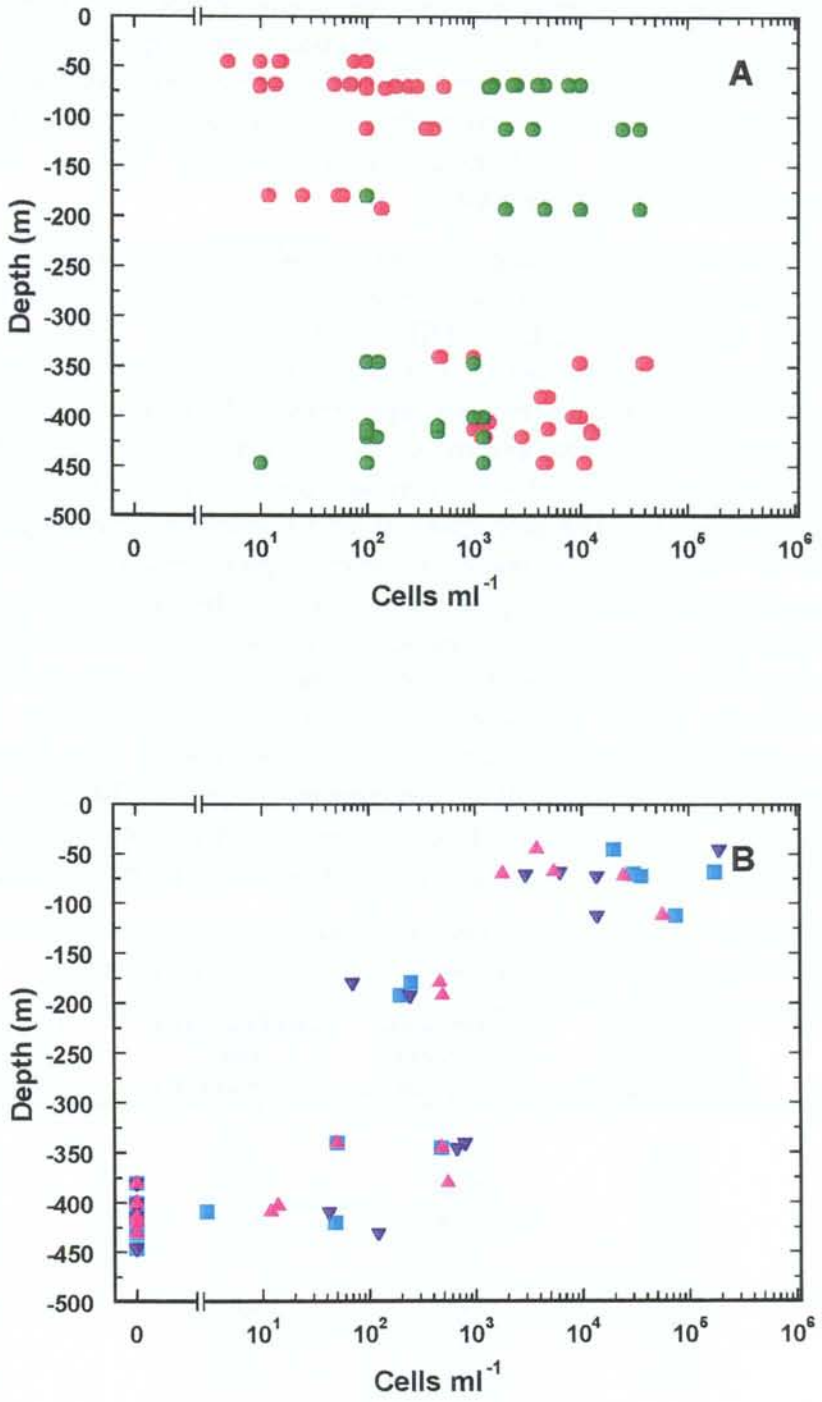


Figure 4 *Most probable numbers of autotrophic and heterotrophic methanogens and of homoacetogenic bacteria in groundwater sampled from 18 boreholes along the Äspö hard rock laboratory tunnel. The data represents 7 independent MPN counts conducted during the period 1994 to 1996. (A). The distribution of autotrophic methanogens and homoacetogenic bacteria over depth. (●) Autotrophic methanogens; (●) homoacetogenic bacteria; (B). The distribution of heterotrophic methanogens over depth. (▼) TMA-consumers; (▲) methanol consumers; (■) acetate consumers.*

MPN of homoacetogens is presented in Figure 4A. The observed acetate formation in the last growing MPN dilution ranged from 83 to 430 mg L^{-1}. This range significantly exceeded the acetate concentration in groundwater which generally was below the detection limit (approximately 1 mg L^{-1}). The homoacetogens predominated over autotrophic methanogens at the shallow and intermediate sites while the opposite was found at the deep site. The distribution of homoacetogens followed that of heterotrophic methanogens of (Figure 4A-B).

The percentages of average numbers of cultivable autotrophic, heterotrophic methanogens, homoacetogens and auto-fluorescent cells, as compared to the total number of cells (AODC) are shown in Table 2. Cultivable methanogens constituted from 0.03 to 59.7 % of the total number of cells and cultivable homoacetogens represented 0.2-13.84 %. The percentage of cultivable viable methanogens varied between 10 % and 18 % in 11 of the 18 investigated boreholes and homoacetogens constituted 0.2-4.0 % in 12 of these boreholes. The percentage of auto-fluorescent cells ranged from 10.3 to 83 %. The percentage of heterotrophic methanogens significantly exceeded that of autotrophic methanogens in the shallow site groundwater. Cultivable acetotrophic methanogens constituted 0.2-8.5 % of the total cell count in the shallow site boreholes while they were absent in the deep site boreholes except for boreholes KA2512A and KA3105A, where they constituted 0.2 to 0.4 % of the whole cell population. Note that autotrophic methanogens at the shallow site constituted only 0.03-0.3 % while at the deepsite, the numbers of autotrophic methanogens exceeded that of acetotrophic ones and ranged from 0.7 to 41.9 % of the AODC numbers.

Table 2 *Percentages of different methanogens and homoacetogens of the total number of cells (AODC) in groundwater from boreholes in the Äspö HRL tunnel.*

Borehole	Autotrophic methanogens (% of AODC)	Heterotrophic methanogens (% of AODC)	Autofluorescent cells (% of AODC)	Homoacetogenic bacteria (% of AODC)
Shallow site				
HB001B	0.03	11.3	13.2	n.a[a].
KR0012B	0.30	59.7	80.0	1.37
KR0013B	0.06	54.8	83.0	1.35
KR0015B	0.20	17.3	16.7	1.45
Intermediate site				
SA813B	12.6	25.5	44.1	3.90
SA1327B	0.05	0.4	11.2	0
SA1420B	11.78	0.3	14.7	3.90
Deep site				
KA2511A	n.a.	n.a.	n.a.	n.a.
KA2512A	0.73	2.4	16.8	1.50
KA2858A	0.83	0	n.a.	n.a.
KA2862A	14.9	0	n.a.	13.80
KA3005A	11.2	1.12	16.2	13.84
KA3010A	1.16	0	11.9	0.40
KA3067A	20.3	3.00	37.8	8.50
KA3105A	12.0	0.42	14.5	4.03
KA3110A	6.79	4.41	18.0	0.20
HD0025A	9.02	0	10.5	9.25
KA3385A	41.9	0	56.0	0.50

[a] not analysed

4.5 ENRICHMENT CULTURES

Enrichment cultures of methanogens were obtained in culture vessels with methanogenic growth conditions after a 3 months incubation period (Table 3). Methane formation (2.1-5.0 mM CH_4) was observed in enrichment cultures with acetate, methanol or TMA inoculated with groundwater from the shallow site and in some cultures inoculated with deep site groundwater. Enrichment cultures for autotrophic and formate consuming methanogens were obtained from many of the deep and some of the shallow site boreholes. Cultures with active methane formation were examined using phase contrast and fluorescent microscopy and the morphologies of auto-fluorescent cells were observed to be similar to the morphologies of auto-fluorescent cells observed directly in the groundwater (Kotelnikova et al, 1997b).

Table 3 *Positive enrichment cultures of methanogens in groundwater from boreholes the Äspö HRL tunnel. Enrichments that could be sub-cultured at laboratory conditions and that showed methane formation exceeding 2 mM CH_4 in the headspace of the culture vessel were regarded as positive (+).*

Borehole	Substrate for methanogenesis				
	H_2/CO_2 (80/20 %)	Formate 40 mM	Acetate 10 mM	Methanol 30 mM	TMA 25 mM
Shallow site					
HB001B	.	.	+	+	+
KR0012B	+	+	+	+	+
KR0013B	+	.	.	+	+
KR0015B	+	.	+	+	.
Intermediate site					
SA813B	.	.	+	+	.
SA1420A	.	.	.	.	.
SA1327B	.	.	.	.	.
Deep site					
KA2511A	.	.	.	.	.
KA2512A	.	.	.	+	+
KA2858A	.	.	.	.	.
KA2862A	.	.	.	.	.
KA3005A	+	+	+	.	.
KA3010A	+	+	.	.	.
KA3067A	+	+	.	.	+
KA3105A	+	+	+	.	.
KA3110A	+	+	.	+	+
HD0025A	+	+	.	.	.
KA3385A	.	.	.	.	.

4.6 *IN VITRO* METHANE FORMATION RATES

The methane formation rates from [1, 2-^{14}C]-acetate were from 0 to 12.46 μM CH_4 h^{-1} (Table 4). The fastest acetotrophic methane formation rate was registered in the shallow site borehole KR0012B. Acetotrophic activity was generally higher than autotrophic activity in boreholes with a high percentage of acetotrophic methanogens, i.e. at the shallow site (Table 2). The rates of autotrophic methane formation using $NaH^{14}CO_3$ with hydrogen as energy source ranged from 0.007 to 1.47 μM of CH_4 h^{-1} (Table 4). Methane formation could not be detected in the BESA-inhibited controls. The $^{14}CH_4$ formation was observed without any detectable adaptation phase indicating that the studied methanogens were in a state of active growth in the groundwater. The rates of autotrophic acetate formation, measured as $^{14}CO_2$ assimilation, are presented in Table 4 and ranged between 0.002 and 33.2 μM h^{-1}. The homoacetogenic activity predominated in the shallow and one of the intermediate site boreholes as compared to the deep site boreholes.

Table 4 *In vitro methane and acetate formation in Äspö groundwater. Acetate formation was measured with an enzymatic method in addition to the radiotracer technique.*

Boreholes	μM CH_4 h^{-1}		μM Acetate h^{-1}	
	Carbon source		Carbon source	
	[^{14}C]-Acetate	[^{14}C]-CO_2	[^{14}C]-CO_2	CO_2
Shallow site				
HBH01	n.a.[a]	n.a.	n.a.	n.a.
KR0012B	12.46 ±0.64[b]	1.17 ±0.31	9.63 ±1.32	3.16 ±0.24
KR0013B	3.10 ±0.025	0	4.62 ±1.30	3.12 ±0.15
KR0015B	12.37 ±2,50	1.47 ±0.20	15.3 ±2.14	2.03 ±0.11
Intermediate site				
SA813B	6.30 ±0.30	0	33.23 ±2.50	3.20 ±0.26
SA1327B	n.a.	n.a.	n.a.	n.a.
SA1420A	0.12 ±0.02	0	0.003 ±0.0005	0.06 ±0.01
Deep site				
KA2511A	0.30 ±0.05	0.53 ±0.03	2,22 ±1.64	2.10 ±0.12
KA2512A	1.29 ±0.26	0	0	0
KA2858A	n.a.	n.a.	n.a.	n.a.
KA2862A	0	0.007 ±0.001	0	0
KA3005A	0.03 ±0.005	0.150 ±0.02	0.03 ±0.003	0.05 ±0.01
KA3010A	0	0.059 ±0.004	0.002 ±0.0005	0.007 ±0.0005
KA3067A	0.11 ±0.02	0.98 ±0.07	0.002 ±0.0005	0.54 ±0.02
KA3105A	0.13 ±0.03	0.07 ±0.0004	0.007 ±0.001	0.007 ±0.001
KA3110A	0	0.51 ±0.12	0.065 ±0.005	0.49 ±0.02
HB0025A	0.13 ±0.003	0.25 ±0.04	0.09 ±0.002	1.55 ±0.35
KA3385A	0	0.02 ±0.001	0	0

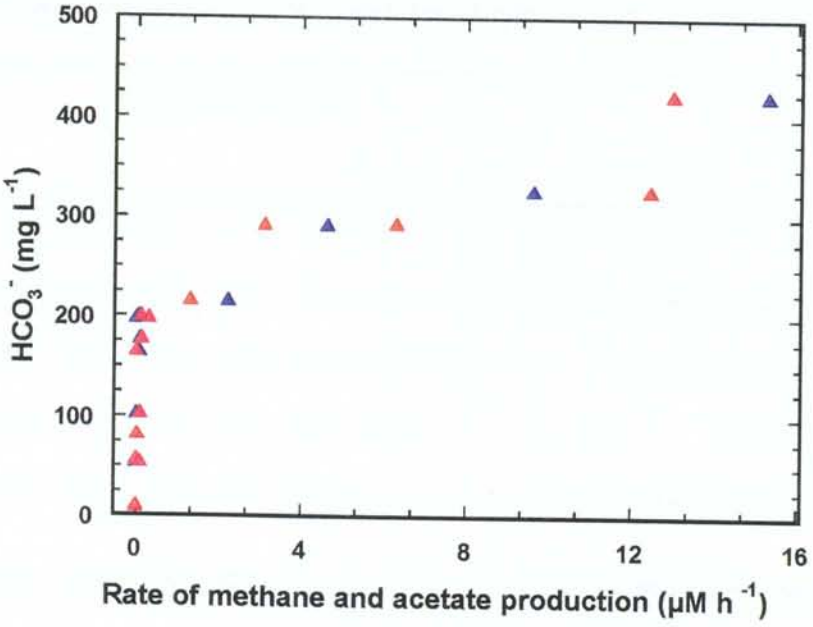
[a] *not analysed;* [b] *±SD (n=2-4)*

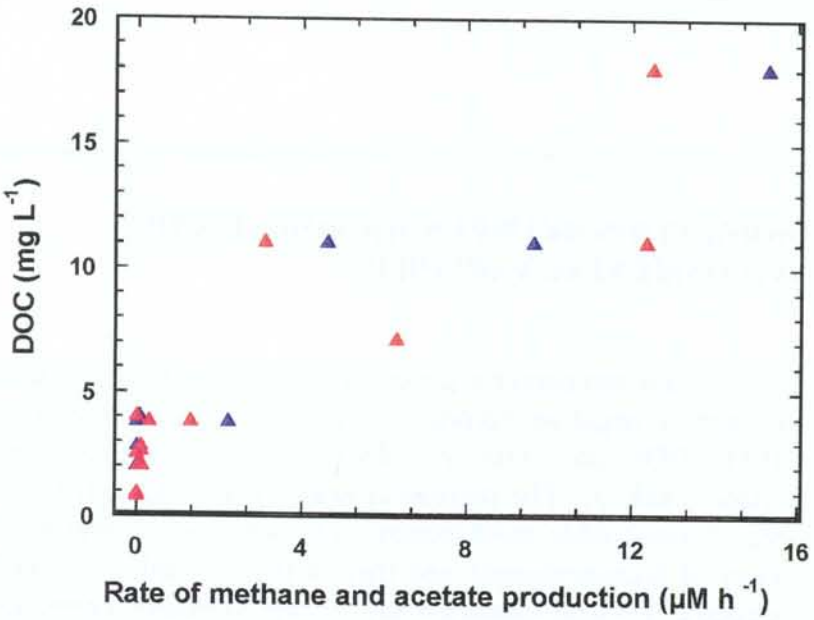
Table 5 *Selected correlation coefficients obtained from a multiple stepwise linear regression analysis of the relation between obtained numbers and in vitro activities of methanogens and homoacetogens, depth and Äspö HRL groundwater composition.*

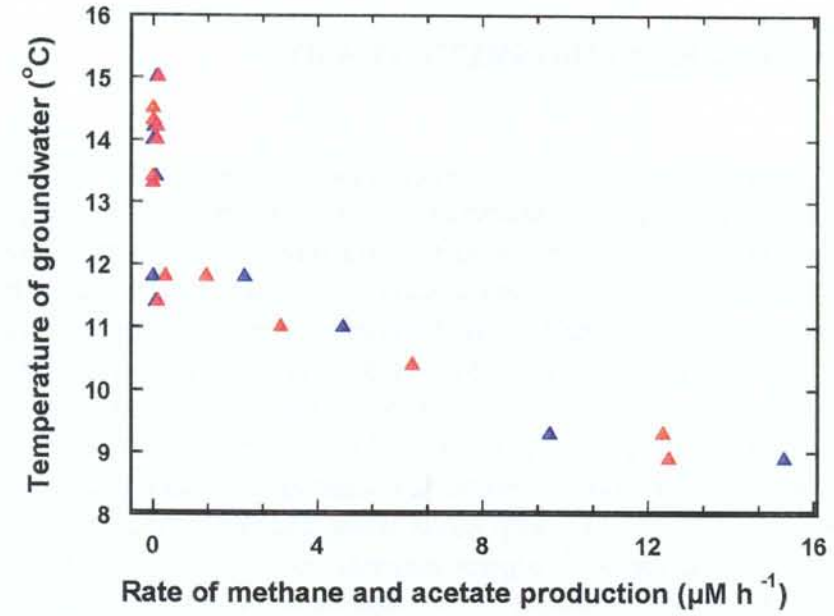
	Correlation coefficient (r^2)										
Main variable											
Main variable number	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
1.Autotrophic methanogens (cells mL^{-1})	-	0.47	-0.60	0.77	-0.2	-0.2	-0.03	-0.01	-0.05	-0.03	-0.06
2.Acetotrophic methanogens (cells mL^{-1})	-	-	0.84	0.03	0.70	0.81	-0.30	-0.30	-0.30	-0.30	-0.30
3.Homoacetogens (cells mL^{-1})	-	-	-	-0.10	0.94	0.86	-0.40	-0.30	-0.30	-0.40	-0.40
4.Autotrophic methane formation (μM CH_4 h^{-1})	-	-	-	-	-0.11	-0.14	0.12	0.04	-0.02	-0.15	0.02
5.Acetotrophic methane formation (μM CH_4 h^{-1})	-	-	-	-	-	0.78	-0.81	-0.60	0.83	0.86	-0.76
6. Acetate formation (μM acetate h^{-1})	-	-	-	-	-	-	-0.89	-0.65	0.81	0.86	-0.74
7. Depth (m)	-	-	-	-	-	-	-	0.62	-0.83	-0.88	0.95
8. Chlorinity (mg L^{-1})	-	-	-	-	-	-	-	-	-0.73	-0.80	0.54
9. Dissolved organic carbon (mg L^{-1})	-	-	-	-	-	-	-	-	-	0.89	-0.86
10. HCO_3^- (mg L^{-1})	-	-	-	-	-	-	-	-	-	-	-0.84
11. Temperature (°C)	-	-	-	-	-	-	-	-	-	-	-

4.7 CORRELATION BETWEEN MICROBIAL AND ENVIRONMENTAL VARIABLES

Chlorinity and temperature generally showed a positive correlation with depth and a negative correlation with the observed concentrations of $NaHCO_3$, DOC and with the observed viable numbers and microbial activities (Table 5). The regression analysis also demonstrated that viable counts of autotrophic methanogens was negatively correlated with viable numbers of homoacetogens and their activity (Table 5). Autotrophic and acetotrophic methane formation and acetate formation correlated positively with viable counts of autotrophic methanogens (r^2 = 0.77, p = 0.001), acetotrophic methanogens (r^2 = 0.70, p = 0.001) and homoacetogens (r^2 = 0.86, p = 0.001). Concentrations of $NaHCO_3$ and DOC correlated with increasing acetate and methane formation rates (r^2 = 0.86 and r^2 = 0.81-0.83, p = 0.001) (Fig. 5A-B). A low temperature (depth related) seemed to favour acetotrophic methane and acetate formations (r^2 = -0.74, p = 0.001) (Fig. 5C) while both acetotrophic methane and acetate formations were reduced with increasing chlorinity (Fig. 5D, Table 5).


500
400
300
200
100
0
HCO_3^- (mg L^{-1})
0
4
8
12
16
Rate of methane and acetate production (μM h^{-1})


20
15
10
5
0
DOC (mg L^{-1})
0
4
8
12
16
Rate of methane and acetate production (μM h^{-1})



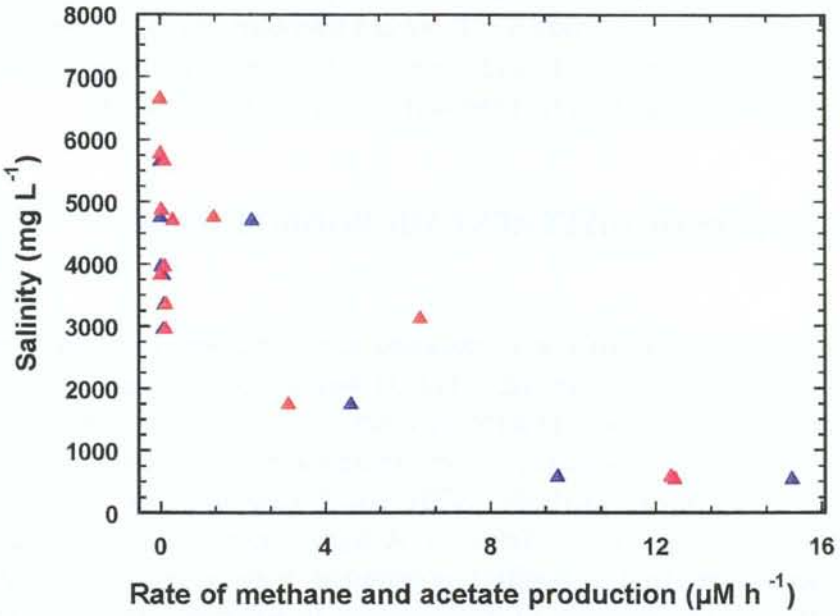


Figure 5 *Average values of $NaHCO_3$, DOC, temperature and chlorinity with homoacetogenesis (▲) and acetotrophic methanogenesis (▲), determined using the radiotracer technique (Table 4).*

5 DISCUSSION

5.1 THE ROCK ENVIRONMENT AT ÄSPÖ

The studied granitic rock environment is chemically and hydraulically heterogeneous and groundwater of very different origins are prevailing at the sites studied. The conductive fracture zone at tunnel length 520 m (Figure 1B) transport meteoric water that carries organic matter from surface ecosystems to the shallow site boreholes intersecting this feature (Wallin et al, 1995). Anaerobic respiration of this organic carbon has, therefore, been suggested to be a likely source of inorganic carbon to the shallow site groundwater (Banwart et al., 1996). The groundwater at the intermediate site is influenced by modern Baltic Sea water and the deep site is situated in the interface between old deep saline water and recharging groundwaters, with no or limited supply of organic material from surface ecosystems (Smellie et al, 1995). All transport of groundwater is restricted to hydraulically conductive fractures in the granite and the transmissivity of the rock and groundwater flow are generally extremely low at depths around 100m or deeper, although major faults locally transport more water. A significant part of the inflow to the tunnel comes from aquifers situated below the tunnel floor and most of it comes via fracture zones intersected by the tunnel (Fig. 1). The hydraulic situation and the prevailing environmental conditions at each of the sites studied will govern transport of cells, gases, organic and inorganic compounds to and from the sites and thus strongly influence cell numbers, their distribution and activity, as discussed below.

5.2 NUMBERS OF DIFFERENT MICROORGANISMS

The total number of microorganisms at the studied sites varied from 5.4 x 10^3 up to 3.4 x 10^6 cells mL^{-1} (Fig. 3) which is in the range of what has been found earlier in deep granitic groundwater (Pedersen 1996). A significant part of these microorganisms were represented by methanogens as shown by two independent methods, MPN numbering and the number of auto-fluorescent (F_{420}) cells (Table 2). A high number of auto-fluorescent cells generally showed a positive correlation with a high viable number of methanogens. Presence of methanogens in subterraneum environments has been reported in many other types of deep environments (Belyaev et al, 1986; Chapelle et al, 1987; Daumas et al, 1986; Fredrickson et al, 1989; Fry et al, 1997; Godsy, 1980; Ogram et al, 1995; Olson et al, 1981; Phelps et al, 1989; Stevens et al., 1995) and deep granitic rock aquifers can now be added to the list. Auto-fluorescent numbering does not show the types of different methanogens. That task is solved by the MPN numbering which showed that heterotrophic methanogens predominated at the shallow site and that autotrophic methanogens were more predominant at the deeper sites.

Homoacetogens generally constituted a smaller part of the total number than did methanogens, but still a significant part. Here, only MPN could be used for numbering, but the use of acetate formation from carbon dioxide and hydrogen as a positive indication of homoacetogenesis in MPN numbering is faultless and cannot be explained by inorganic reactions or by activity of other microorganisms than homoacetogens.

5.3 CULTIVABLE METHANOGENS AND HOMOACETOGENS

Subsurface microorganisms are potentially difficult to cultivate since they are highly adapted to deep subterraneum environment conditions such as high pressure with dissolved gases, unique compositions of microelements and a very large mineral surface area per volume of groundwater. Still, if auto-fluorescent cells are accepted as representing the total number of methanogens, Table 2 shows that the majority (> 50 %) of the present methanogens could be cultivated as MPN cultures in 14 out of 18 boreholes. The use of original groundwater probably contributed to this good recovery. In addition to the MPN determinations, positive enrichments of methanogens were obtained from most of the boreholes investigated and the type of methanogens enriched followed the same trend as was shown by the MPN results (Table 3). Heterotrophic methanogens could be more frequently enriched from the shallow site, while autotrophic methanogens prevailed among the deep site enrichments.

The 16S rRNA genes of methanogens isolated from different depths were closely related to the genera *Methanogenium, Methanobacterium and Methanohalophilus*. Selected isolates from the boreholes KR0012A, KA3067A and HD0025A have been further studied and the results are published elsewhere (Kotelnikova and Pedersen, in press). One of these isolates is described as a new species of *Methanobacterium, M. subterraneum* sp. nov. This species was characterised as being autotrophic, alkaliphilic, eurythermic and euryhalophilic and being able to form biofilms on surfaces. Drilling contamination could not be demonstrated for the deep site boreholes (Pedersen et al, 1997b) and recent findings of fossilised bacteria in calcite fracture fillings from 207 m below sea level (Pedersen et al 1997a) also suggest that granitic rock aquifers harbour intrinsic microbial populations. These results and the characters of *M. subterraneum*, which demonstrate the described methanogen as being adapted to a subterraneum life in granitic rock, suggest that it is a true dweller in deep rock aquifers and that it was not introduced during drilling.

The fraction cultivable homoacetogens of the total number in Table 3 is less precise because the absolute number is not known as was the case for autofluorescent methanogens. Earlier investigations have, however, indicated the presence of homoacetogens in Äspö groundwater (Pedersen et al., 1996a, Pedersen et al 1997b). Repeated retrieval of a 16S rRNA

sequence related to the homoacetogenic genus *Eubacterium* (92.1 % identity), from KR0013, SA813, KA3105A and HD0025A, was obtained. In addition was an acetogenic isolate obtained from SA813A (Aspo-4) with a 16S rRNA gene sequence that is identical to the 16S rRNA sequence obtained from these boreholes. The presence of cultivable homoacetogens is, consequently, repeatedly shown here and elsewhere for the deep granitic aquifers below Äspö.

5.4 *IN VITRO* METHANE AND ACETATE FORMATION

Significant formation rates of methane and acetate were obtained *in vitro* with Äspö groundwater at a temperature (17°C) close to the *in situ* temperature (Table 4). The general trends for heterotrophic methane formation and for acetate formation followed the trends observed with MPN and enrichments. Highest activities was found at the shallow site which also showed the highest numbers of heterotrophic methanogens and homoacetogens. Autotrophic methane formation did, however, not follow the culturability trend which may be due to the increasing difficulties to *in vitro* mimic *in situ* conditions such as pressure and dissolved gases with increasing depth. With this exception, the same trends were obtained with three independent methods, MPN, enrichments and radiotracer assays, which establish the presence of active heterotrophic methanogens and homoacetogens in the shallow site groundwater.

Stable isotope evidence for microbially generated methane has been obtained for Canadian and Fennoscandian shield rock (Sherwood Lollar et al, 1993) and methane was found in all of the studied boreholes at Äspö as well (Fig. 2). The *in vitro* methane formation activity (Table 4) attest that at least part of the methane in Äspö groundwater is of biological origin. The estimated conversion rates of hydrogen and carbon dioxide to methane are probably different *in vitro* as compared to the *in situ* conditions. The addition of $NaHCO_3$ with the ASPM medium increased the $NaHCO_3$ pool in some of the deeper site samples with low $NaHCO_3$ concentrations. The rates could also be altered by the absence of ambient pressures in the experimental bottles, which *in situ* ranged from 7 to 46 atmospheres. The concentration of dissolved hydrogen in these experiments was approximately 50 μM and this value did not exceed the maximum value measured in the groundwater (50 μM hydrogen), but were higher than measured in several discrete samples. The rates of methane formation obtained are similar to or higher than those observed in other aquatic systems such as a meromictic lake (Winfrey et al, 1979), fresh water sediments (Winfrey et al, 1977a, b) and flooded oil fields groundwater (Belyaev, 1996). The time needed for formation of the methane observed in Äspö groundwater was calculated as the quote of the methane concentrations and the methane formation rates. The resulting times varied from 3 to 74 days and would theoretically correspond to the time it would take methanogens to form the observed levels of methane in the Äspö

groundwaters. It is not possible to accurately assess if these time spans are correct, but most of them are at least in a realistic range, i.e. it will take weeks or months for the deep groundwater methanogens to produce the observed amount of methane (Fig. 2). Irrespective of uncertainties regarding the absolute *in situ* activity, it can be concluded that active methanogenesis could be demonstrated in most of the groundwater samples investigated.

5.5 DISTRIBUTION OF METHANOGENS AND HOMOACETOGENS

The high DOC and $NaHCO_3$ concentrations and low *in situ* temperature and chlorinity at the shallow site seemed to favour *in vitro* activity of acetotrophic methanogens and homoacetogens (Figure 5). The regression analysis also showed that viable counts and *in vitro* activity of homoacetogens and acetotrophic methanogens were strongly correlated (Table 5) and dominated at the shallow site over those of autotrophic methanogens. There are several possible explanations to these observations. The shallow site population of acetotrophic methanogens might have been favoured by the observed presence of many active homoacetogens. These homoacetogens probably competed successfully with autotrophic methanogens (Conrad and Wetter, 1990) at the shallow site due to their higher thermodynamic efficiency for hydrogen at low temperatures and due to their metabolic versatility that allows them to use organic electron donors in addition to hydrogen. Similar results have been obtained elsewhere for hydrogen. Recently, Wagner et al (1997) showed that formate and acetate consuming methanogens predominated in a low temperature (10 °C) marshland. In another investigation, Schultz et al. (1997) demonstrated that homoacetogenesis and acetotrophic methanogenesis were the only detectable metabolic activities at low *in situ* temperature (4 °C) in a profoundal lake sediment while autotrophic methaongensesis was observed only at temperatures above 20 °C. In the Äspö groundwater, numbers and activity of autotrophic methanogens dominated at the deep site (except borehole HD0025A) where the low organic carbon content may limit acetotrophic and versatile homoacetogenic activity and where a higher temperature (14-17 °C) may favour the hydrogen consuming efficiency.

5.6 THE ANAEROBIC METABIOSIS HYPOTHESIS

Radiometric dating of organic carbon from the deep site groundwater in Äspö yields ages greater than 10.000 years (Smellie et al., 1995), which implies that it does not originate from modern organic carbon sources. These data imply that carbonate carbon at the deep site has not been involved in biological reactions on the ground surface since that time. In addition, the ability of microorganisms to grow at expense of such old organic material may be effected not only by the low content, but also by its low degradability. Organic carbon in deep granitic aquifers generally

consists of a mixture of humic and fulvic acids (Wallin et al., 1995), which are derived from partial decomposition of plants and bacteria (Mann et al, 1995). The water solubility of these acids is low at circumneutral pH, but a larger fraction is soluble at alkalic conditions and consists of polymerised aromatic molecules, purines, pyrimidines and uronic acid polymers. Shallow carbon may be less degraded than such deep carbon, very resistant to microbial degradation. The high proportion of chemolitoautotrophes, the non-biodegradability of the organic dissolved in the deep site groundwater and its age suggest that microbes there, most likely, must use autotrophic ways for their anabolic supply of organic carbon (Fig. 6). Obviously, the high proportion of autotrophs and the simultaneous presence of methanogens and homoacetogens in the studied groundwater is possible because they have access to energy in the form of molecular hydrogen. This study also demonstrates that homoacetogens may constitute an energy link from hydrogen, via acetate to heterotrophic methanogens and possibly also to other acetate utilising organism such as iron and sulphate reducing bacteria, which have been found to be common in the studied groundwater (Pedersen et al. 1996a, 1997).

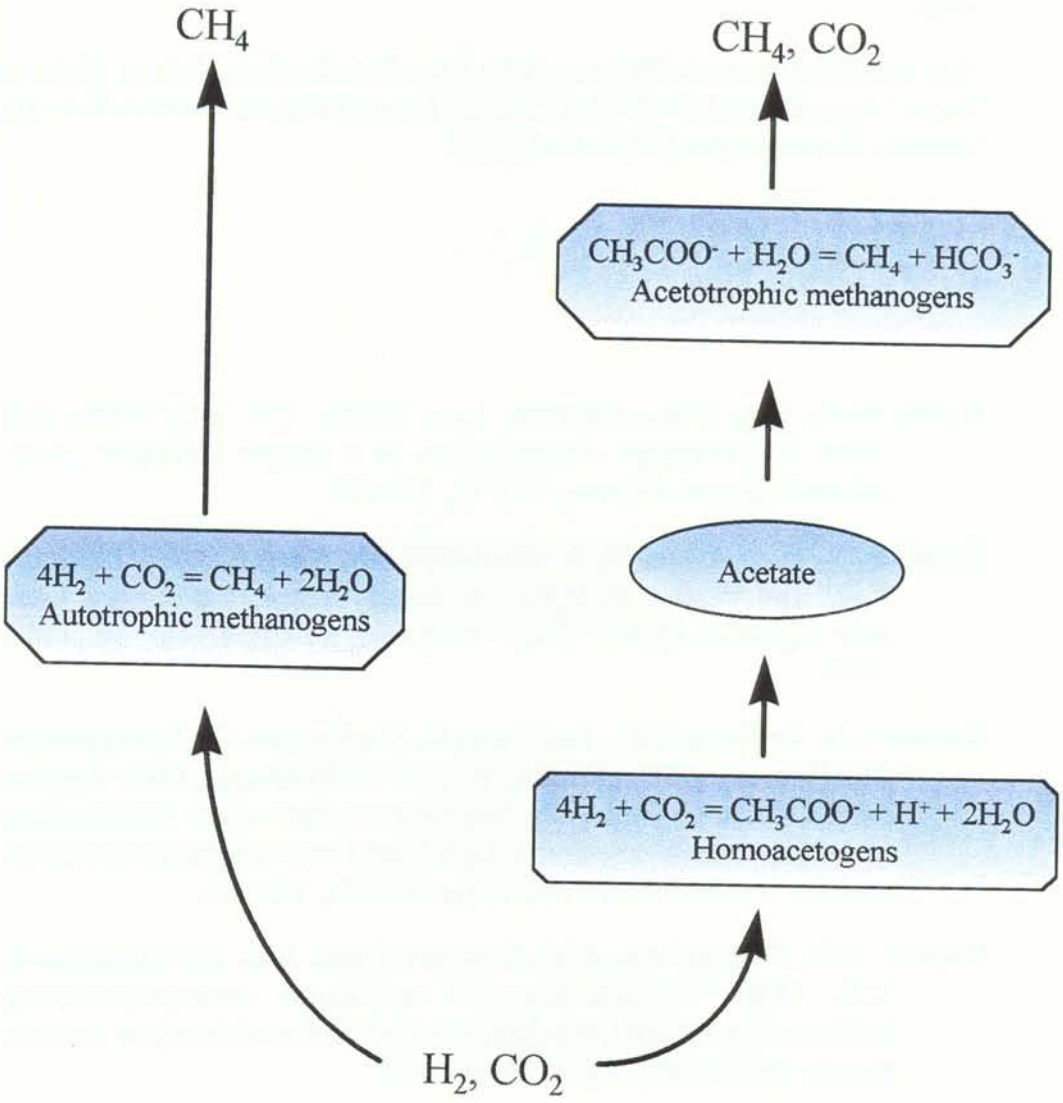


Figure 6 *Scheme of the anaerobic metabiosis hypothesis suggested to be important in the studied deep site granitic aquifers.*

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Survival of sulfate reducing bacteria at different water activities in compacted bentonite

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CONTENTS

1 ABSTRACT

Long-lived radioactive waste will be buried several hundred meters below ground in metal canisters surrounded by a buffer of compacted bentonite. Sulfate-reducing bacteria present in the bentonite may induce canister corrosion by production of hydrogen sulphide. Here we show that survival of sulfate-reducing bacteria in bentonite depends on the availability of water and that compacting a high quality bentonite to a water activity (a_w) of 0.96 was lethal for the species investigated.

2 INTRODUCTION

The concept of deep geological disposal of spent fuel is common to most national nuclear fuel waste programs. Long-lived radioactive waste will be encapsulated in canisters made of corrosion resistant materials (e.g. copper (Marsh et al, 1983; McCright, 1994)) and buried several hundred meters below ground in a geological formation (Pedersen and Karlsson, 1995). Different types of compacted bentonite clay, or mixtures with sand, will be placed as a buffer around the waste canisters (Atabek et al, 1985; Werme et al, 1992). A common demand to waste disposal concepts developed in countries concerned, is that canisters and buffers must remain intact for a very long time. Assessments of the performance of disposal systems are often done covering periods of up 10,000 years or more (Pedersen and Karlsson, 1995; Werme et al, 1992). Therefore, bentonite clay has been proposed as buffer material since it reduces the effects on the canister of a possible rock displacement and it minimizes water flow over the deposition holes (Puch, 1983). The transport through the buffer will thereby be reduced principally to diffusion both with respect to corrosive components in the ground water and to escaping radionuclides in case of a canister failure. Corrosion is an important process to consider in such an assessment for at least two reasons. The first is obvious: the canisters are an absolute barrier to radionuclide dispersal as long as they remain intact. A second reason is that a separate gas phase may form at a sufficiently high corrosion rate, which may exert a pressure on the system and add to the dispersion of radionuclides by gas bubble transport. Sulfate-reducing bacteria (SRB) may cause corrosion of canister materials due to their dissimilatory reduction of sulfate to hydrogen sulphide (Hamilton, 1985; Philip et al, 1991) (Pedersen, 1996). Consequently, it is very important to reveal if SRB can survive and produce hydrogen sulphide in bentonite buffers.

SRB are obligate anaerobic organisms and they can generally utilize a wide range of carbon substrates. Several species can survive using carbon dioxide and hydrogen as carbon and energy sources (Widdel, 1988). Bentonite clay contains organic material and hydrogen and carbon dioxide can also be found at repository depths (Pedersen, 1993; Stevens and McKinley, 1995). It has, therefore, been considered plausible that SRB may establish in a

repository bentonite environment, as it will be anaerobic, having reducing conditions together with nutrients and energy available for propagation. The temperature in bentonite surrounding the waste canisters will reach between 50°C and 80°C during the first 1000 years, but that does not pose any conceptual hindrance for growth of SRB since some are thermophiles that grow at an optimum temperature around 65°C, e.g. *Desulfotomaculum* and *Thermodesulfobacterium* (Widdel and Hansen, 1992). A corrosive effect on steel from *Desulfotomaculum nigrificans* in bentonite at 50°C has recently been demonstrated (Philip et al, 1991). Nor will the high pressure at repository depth constitute any limitation for bacteria as many can withstand among the highest hydrostatic pressures on the planet - those in the deepest parts of the ocean (Kato et al, 1994).

In contrast to nutrient, energy, pressure and temperature constraints, few bacteria can tolerate removal of water from the cell. The term water activity, a_w, is used to express quantitatively the amount of water available for microorganisms and is equivalent to the ratio of a solution's vapour pressure to that of pure water (Potts, 1994). Most bacteria grow well at an a_w around 0.98 (the approx. a_w for sea water) but relatively few species can grow at an a_w of 0.96 or lower. The halophilic bacteria are one exception; several can grow at an a_w as low as 0.75 (Kushner, 1978). Sulfate-reducing bacteria are found in natural waters with salinities from near zero to saturation. Certain SRB genera can withstand desiccation by spore formation, e.g. *Desulfotomaculum*, but spores are inactive and do not produce hydrogen sulphide. They are rarely found at above approximately 2 % NaCl, if present they are often not native (Postgate, 1984). Based on the discussion above, it was hypothesized that the only restriction for survival of different SRB in nuclear waste bentonite buffers is a water activity below certain values; this work was aimed at identifying such values.

3 MATERIAL AND METHODS

3.1 BACTERIAL STRAINS

Two strains of SRB, *Desulfomicrobium baculatum* and *Desulfovibrio sp.* isolated from deep crystalline bed-rock ground water of South-eastern Sweden at the Äspö hard rock laboratory were used (Pedersen and Ekendahl, 1990). Previous studies have shown that *D. baculatum* and *Desulfovibrio sp.* grow in up to 20 and 30 g l^{-1} NaCl respectively (paper 5).

3.2 CULTURE CONDITION

The bacteria were cultivated in an anoxic mineral medium (brackish medium containing 7 g l^{-1} NaCl (Widdel and Bak, 1992)) enriched with lactate as the substrate, sodium sulphate as the electron acceptor and incubated at 30°C.

3.3 BENTONITE COMPOSITION AND CHARACTERISTICS

The bentonite material is a natural mixture of smectite and several common minerals like quartz and feldspar. The composition varies considerably depending on the mining site but the smectite component, which normally is montmorillonite, dominates the material (Müller-Vonmoos and Kahr, 1983). In this study commercial MX-80 Wyomig bentonite clay from American Colloid Co. was used. The bentonite consisted approximately of: sodium montmorillonite clay, 75 %; quartz, 15 %; feldspars, 7 %; carbonates, 1.4 %; sulphides, 0.3 %; organic carbon, 0.4 % and other minerals, 2 % (Grim and Guven, 1978).

The smectite is characterized by a high water affinity which yields swelling when contacted with water. If swelling is restricted due to mechanical hindrance from the surrounding rock, the smectite will give rise to a swelling pressure and to a reduced water activity (a_w<1). Both effects are sensitive to the ratio between water and smectite. This ratio will be controlled in the deposition holes by compacting the bentonite to a relatively high density, approaching 2.0 g cm^{-3} (a_w=0.96), either by compaction in situ or by use of pre-compacted bentonite blocks. Bentonite with lower densities will likely be used in other constructions in the repository, e.g. in the tunnel backfill material. The bentonite was heat sterilized at 160°C for 2 h before start of the experiment.

3.4 PROCEDURE

Sodium bentonite was inoculated with two species of SRB and compacted to three different densities using the swelling pressure odometers. All the procedures (except compacting of the samples) were performed under nitrogen atmosphere in a glove box. The parts of the odometers that were in contact with the samples (sample holders and pistons) were heat sterilized at 160°C for 2h. Two fresh cell-suspensions of the strains harvested in late exponential growth phase were used. The initial number of *Desulfovibrio sp.* and *D. baculatum* in the clay was adjusted to 1.1 x 10^7 and 9.4 x10^7 cells g^{-1} respectively, as counted by acridin orange direct count (AODC (Pedersen and Ekendahl, 1990)).

The quantities of dry bentonite and SRB suspensions used in the mixtures were: 31.2 g bentonite and 27.8 g SRB suspension; 50.0 g bentonite and 20.8g SRB suspension and 62.4 g bentonite and 16.2 g cell suspension corresponding to water ratio of 89 %, 42 % and 26 % respectively. The SRB inoculated bentonite-water mixtures at ratio 89, 42 and 26 % were placed in the central cylindrical sample holder and compacted to densities of 1.5, 1.8 and 2 g cm^{-3} respectively by forcing the piston down (Fig. 1 A). These densities correspond to a_w of 1.0, 0.99 and 0.96 respectively (Kahr et al, 1986). The nitrogen gas in the sample was evacuated by the confining filters and pressurized water (2 MPa) was contacted to the sample by the upper three-way stop cock to simulate hydrostatic ground water pressure (Fig. 1 B). All samples were incubated at 30°C for 1 or 60 days.

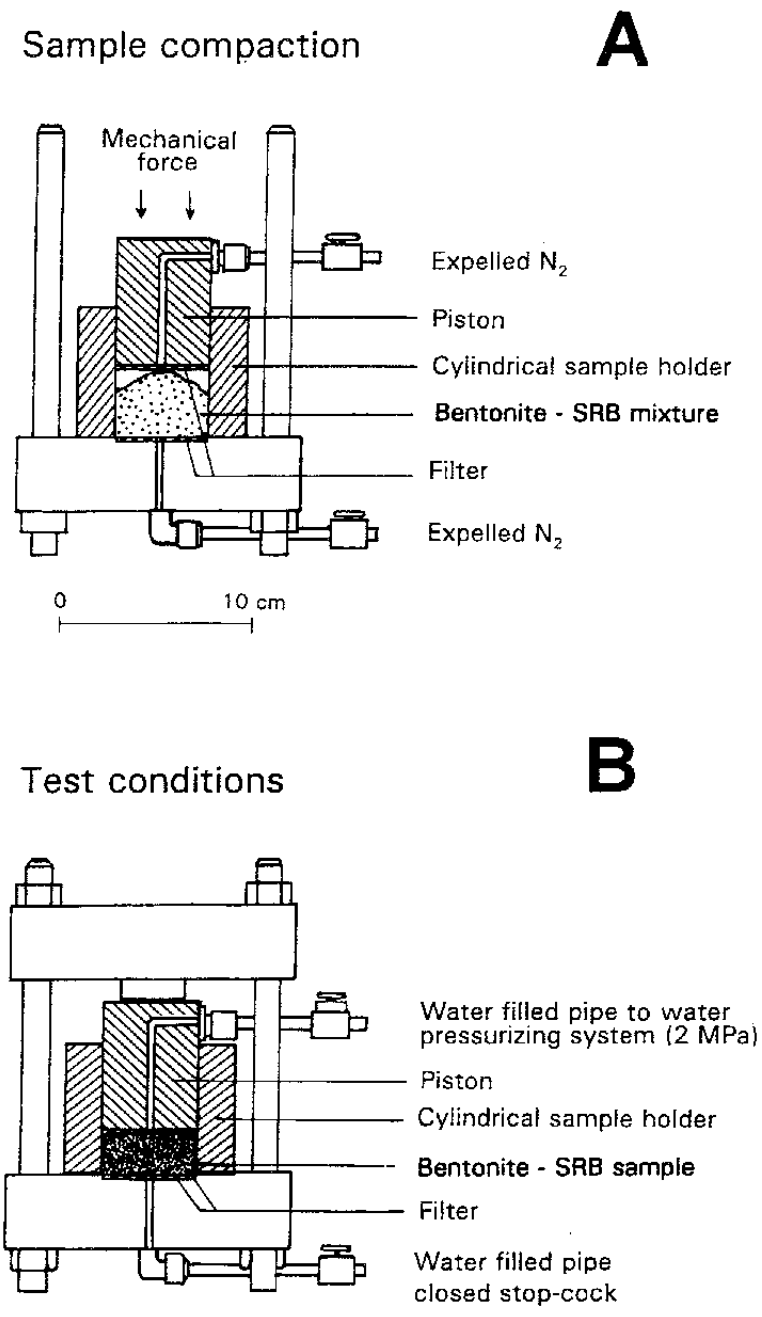

Figure 1 *Schematic drawing of the swelling pressure odometer.*

3.5 SAMPLING AND DETERMINATION OF THE NUMBER OF VIABLE CELLS

Sampling of the bentonite was performed after placement the odometers in an anaerobic box under a mixture of H_2, CO_2 and N_2. The number of viable cells was estimated by a MPN method (Koch, 1994). The tests were performed in 12 dilution levels with 5 repetitions in each level. The medium used for MPN was brackish medium (Widdel and Bak, 1992) containing 7 g l^{-1} NaCl enriched with lactate and sodium sulphate. The results were calculated according to a computer program.

4 RESULTS AND DISCUSSION

The amount of water available in the bentonite significantly influenced the survival of the studied SRB. Both strains were 100 % non-viable after 1 d at the lowest a_w studied, 0.96. The dry conditions at this density of 2 g cm^{-3} effectively killed more than 10^7 SRB per g bentonite in less than 24 h. The best survival was observed in the bentonite with an a_w of 1.0, but the survival differed markedly between the species. About 10 % of the initial population of *D. baculatum* survived for 60 days, but *Desulfovibrio sp* did not survive at all after this time (Table 1). Limitation in nutrients and energy sources, accumulation of hydrogen sulphide and interference of the redox potential may add constraints to a closed batch system like the one used here (Fig. 1). A better survival may be expected in an open system at non-limiting a_w values, i.e. at a_w close to 1.

Table 1 *The number of viable cells of* Desulfomicrobium baculatum *and* Desulfovibrio *sp. in bentonite samples with different densities and water activities (a_W) for 1 and 60 days incubation at 30 °C.*

Sample density g cm^{-3}	Water activity a_w	Hydrostatic pressure MPa	No. of viable bacteria per g clay		
			Incubation time (days)		
			0[a]	1[b]	60[b]
D.baculatum					
1.5	1.0	2.0	9.4×10^7	1.6×10^7	1.8×10^6
1.8	0.99	2.0	9.4×10^7	1.2×10^6	0.0
2.0	0.96	2.0	9.4×10^7	0.0	0.0
Desulfovibrio sp					
1.5	1.0	2.0	1.1×10^7	4.2×10^4	0.0
1.8	0.99	2.0	1.1×10^7	1.4×10^2	0.0
2.0	0.96	2.0	1.1×10^7	0.0	0.0

[a] Total number of bacteria as counted by AODC

[b] Determined by MPN, SD = 0.26

The pore size of highly compacted bentonite is in the nanometer range (Puch, 1983) which makes contamination of a compacted buffer with SRB, migrating into the buffer from groundwater, improbable. The only way with which bacteria can be seeded in nuclear waste buffers is during mixing of the bentonite and water (sometimes groundwater) before compaction (Fig. 1 A). A similar inoculation process has been suggested for viable bacteria that are found in subsurface confined clay layers. These bacteria were probably mixed into the clay when it was laid down during sedimentation and they have remained viable since then (Pedersen, 1993). Here, we used a compaction technique similar to what will be used for production of buffer at an industrial scale and we deliberately introduced very high levels of viable SRB to simulate a "worst case scenario" in such a production. The results show that survival of these SRB depended on the amount of water that was available (a_w). When a_w approached 0.96 in the bentonite, they were assumably killed by desiccation. In conclusion, the mechanism of microbiologically induced sulphide corrosion inside a nuclear waste bentonite buffer will probably be restrained if an a_w of 0.96, or lower, is maintained. Extreme halophilism in *Desulfovibrio* exists but has not been studied extensively (Postgate, 1984). Indeed, the types of sulfate-reducing bacteria which colonize highly saline soils and water need to be studied, preferably in full scale experiments at actual deposition depths.

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Occurrence and identification of microorganisms in compacted clay-based buffer material designed for use in a nuclear fuel waste disposal vault

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CONTENTS

1 ABSTRACT

A full scale nuclear fuel waste disposal container experiment was carried out 240 m below ground in an underground granitic rock research laboratory in Canada. An electric heater was surrounded by buffer material composed of sand and bentonite clay and provided heat equivalent to what is anticipated in a Canadian type nuclear fuel waste repository. During the experiment, the heat caused a mass transport of water and gradients of moisture content developed in the buffer ranging from 13 % closest to the heater to 24 % at the rock wall of the deposition hole. Upon decommissioning after 2.5 years, microorganisms could be cultured from all samples having a moisture content above 15 % but not in samples with a moisture content below 15 %. Heterotrophic aerobic and anaerobic bacteria were found in numbers ranging from 10^1 to 10^6 cells per gram dry weight buffer and approximately 10^2, or less, sulphate reducing bacteria and methanogens per gram dry weight buffer were also found. Identification of buffer population members was performed using Analytical Profile Index (API) strips for isolated bacteria and 16S rRNA gene sequencing for in situ samples. A total of 79 isolates from five buffer layers were identified with API strips as representing the beta, gamma and delta groups of Proteobacteria and Gram-positive bacteria while the 67 16S rRNA clones that were obtained from three buffer layers were classified in 21 clone groups representing alpha and gamma groups of Proteobacteria, Gram-positive bacteria and an yeast. Approximately 20 % of the population was indicated as being Gram-positive bacteria and members of the genera *Amycolatopsis, Bacillus* and *Nocardia* were suggested to predominate. Among Gram-negatives the genera *Acinetobacter* and *Pseudomonas* predominated. Analysis of lipid biomarker signatures and *in situ* leucine uptake demonstrated the found buffer population to be viable. The results suggest that a nuclear fuel waste buffer will be populated by active microorganisms only if the moisture content is above a value where free water is available for active life.

Keywords: 16S rRNA, bacteria, bentonite, nuclear fuel waste, PLFA, water activity

2 INTRODUCTION

The concept of deep geological disposal of spent nuclear fuel is common to most national nuclear energy programs. Long-lived radioactive waste will be encapsulated in metal canisters (e.g. copper, iron or titanium) and buried several hundred meters below ground in a geological formation. Different types of compacted bentonite clay, or clay/sand mixtures, will be placed as a buffer around the waste canisters (Kjartanson et al. 1995). Subsurface environments will not be sterile and the potential effects of microbial activity on the integrity of disposal systems for nuclear fuel waste are being

studied in a number of countries (Stroes-Gascoyne and West 1996). These studies cover topics such as the presence of microbes in geological media, tolerance to extreme conditions of radiation, heat and desiccation expected in nuclear fuel waste vaults, microbially influenced corrosion of waste containers, microbial effects on radionuclide migration in the surrounding rock, microbial gas production in relation to the potential for pressure build-up in a vault and mathematical modelling of microbial effects.

A full scale buffer-container experiment (BCE) was carried out at the Underground Research Laboratory near Pinawa, Manitoba, Canada, by Atomic Energy of Canada Limited to examine *in situ* performance of a compacted buffer material in a single emplacement borehole under field conditions. An electric heater, with the dimensions of a waste container, was used to provide heat equivalent to what is anticipated in a nuclear fuel waste repository. During the 2.5 years of the test, it became apparent that the BCE could provide data on the occurrence and survival of microorganisms naturally present in buffer materials. Upon decommissioning of the BCE, investigations were, therefore, initiated to assess the microbial population in the buffer environment.

The microbial investigation of the BCE involved aerobic and anaerobic viable counts of different bacteria at culture temperatures ranging from 20°C to 55°C, assays of *in situ* activity, phenotypic identification of enriched bacteria, genotypic identification of bacteria in the buffer, scanning electron microscopy and lipid analysis (Stroes-Gascoyne et al. 1996). This paper presents numbers and distribution of different microorganisms and *in situ* activity in the buffer material around the heater. In addition, it describes how phenotypic and genotypic information was obtained from enrichment cultures combined with Analytical Profile Index (API) strips and from 16S rRNA analysis of clay samples, respectively. Bacterial *in situ* activity was measured as uptake of tritiated leucine. Lipid analysis was used to study the community structure, its potential viability and nutritional status.

3 MATERIALS AND METHODS

3.1 THE BUFFER CONTAINER EXPERIMENT

The experiment was performed by Atomic Energy of Canada Limited in an excavation of granitic rock in the Canadian shield, 240 m below ground at the Underground Research Laboratory near Pinawa, Manitoba, Canada. A cylindrical electrical aluminum heater (2.25 x 0.64 m) was wrapped in a Teflon™ cloth and placed in a drilled hole (1.24 m diameter and 6 m deep). It was surrounded by an inner 5 cm pure sand layer and by an outer layer of compacted buffer material (Fig. 1) consisting of 50 % sand and 50 % Avonlea sodium bentonite, a Civil Engineering grade bentonite produced by Canadian Clay Products in Wilcox, Saskatchewan. The sand consisted of a

mix of six size portions of silica sand in equal dry weight fractions, from 12 up to 140 mesh (Dixon et al. 1992). The buffer was prepared on site by mixing sand, bentonite and groundwater to produce a buffer with a moisture content of 18 %. It was laid down stepwise during emplacement of the experiment in 13 layers, denoted G to R according to Fig. 1. In a separate investigation, this buffer mixture was found to contain approximately 10^6 culturable aerobic bacteria per gram dry weight (gdw) (Haveman et al. 1995). Thermocouples were distributed throughout the buffer to monitor temperature for the duration of the experiment. Eight weeks after the start, the temperature of the heater was stabilised at 85°C and did not change for the 2.5 years duration of the experiment (December 1991 to April 1994). There was a horizontal temperature drop of 20°C over the sand layer surrounding the heater and a further drop of another 20°C over the buffer layer. Sample temperature data were obtained by extrapolation of the temperature registered by the thermocouple nearest to the sample location. No *in situ* temperature readings were taken during decommissioning and sampling. Moisture content data were obtained from samples taken specifically for moisture content measurements from locations precisely above the microbiology samples and great care was taken to avoid moisture loss or gain in these samples (Chandler et al. 1995).

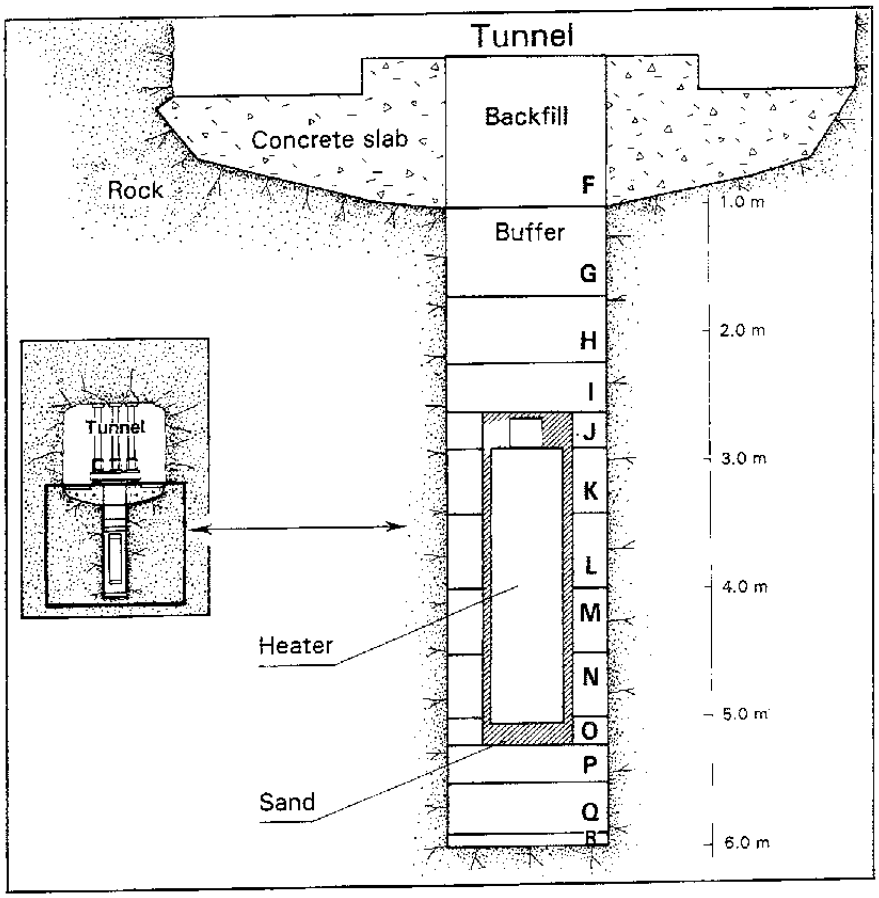

Figure 1 *Schematic drawing of the buffer-container experiment in an excavation at 240 m depth in Atomic Energy of Canada Limited's Underground Research Laboratory, near Pinawa, Manitoba, Canada.*

3.2 ENUMERATION OF CULTURABLE MICROORGANISMS

Four 15 cm long buffer cores were taken from each layer (Fig. 1) by pushing sterilised (300°C for 24 hours) metal tubes (2.5 cm diameter) into the compacted buffer using a pneumatic hammer. Metal tubes containing the buffer cores were pulled out of the buffer using an overhead crane (Chandler et al. 1995). For each layer, two samples were taken (90 or 120° apart) from the periphery of the buffer (about 5 cm from the granite borehole wall) and two samples were taken from the central region of the buffer near the heater surface (90° or 120° apart). Half of the samples were sealed with sterile plastic caps at both ends and taken immediately for analysis in an underground anaerobic glovebox located 20 m away from the experiment site. The other half were sealed immediately on both ends with hot sterile paraffin wax and sterile plastic caps, wrapped in double plastic bags containing iron filings to prevent exposure to oxygen and shipped to the Guigues Recherche Appliquée en Microbiologie (GRAM) laboratory in France. Samples for shipment were kept at 4°C until they were shipped in insulated wooden boxes on ice within 3 to 5 days of sample retrieval. Shipping time varied between 3 and 7 days.

Sample analysis in the underground facility and at the lab adjacent to the underground facility (Whiteshell Laboratories, WL), was initiated within 24 hours of sample retrieval (usually within 6 to 12 hours). The samples shipped to the GRAM laboratory were analysed within 2 weeks of sample retrieval. All analytical procedures at WL and GRAM were performed in anaerobic glove boxes. At WL, the buffer cores in the metal tubes were partially extruded with a specially designed tool. The first 2 to 3 cm were sliced off with a sterile knife and discarded. The adjacent 2 cm of core was aseptically sliced off, ground with a sterile mortar and pestle and diluted 1:20 in phosphate buffered saline (PBS; 0.01 M NaCl, 10 mM sodium phosphate buffer, pH 7.6). The resulting slurries were stirred for 30 minutes before inoculation into the various culture media. At GRAM, the first 5 cm were removed from the tube with a sterile spatula. Then ten gdw clay were removed and added to 90 mL of a sterile water solution of 9 g/l NaCl. The suspension was ultrasonicated for 10 min. and subsequently diluted and inoculated on the various media used.

Samples were enumerated for heterotrophic aerobic bacteria (HAB), heterotrophic anaerobic bacteria (HAnB), fermenting bacteria (FB), sulphate reducing bacteria (SRB), sulphur oxidising bacteria (SOB), methanogens and fungi. Table 1 gives a summary of the culture media and methods and the laboratories where the analyses were carried out. Selected isolates cultured from samples enumerated at GRAM were identified using the selective enrichment media in Table 1 combined with API strips.

Table 1 *Methods used for the enumeration of culturable microorganisms; for detailed protocols and media see Stroes-Gascoyne et al. (1996).*

Type of organisms[1]	Medium[2]	Culturing method	Detection limit (cells/gdw buffer)	Incubation temperature (°C)	Incubation time (days)	Laboratory[3]
HAB	R2A	pour plate count	200	17-25; 50	4	WL
HAB	PCA	spread plate count	50	25; 50	8	GRAM
HAnB	R2A	pour plate count	200	17-25; 50	28	WL
HAnB	mod. W+P	MPN[4]	5	25; 50	15	GRAM
FB	LOM	MPN	20	50	1	S
SRB	W+B	MPN	20	50	21	S
SRB	W+B	Hungate roll tube count	20	50	21	S
SRB	Labège	MPN	5	25; 50	15	GRAM
SOB	Starkey	MPN	5	25; 50	28	GRAM
SOB	THH	MPN	5	25; 50	28	GRAM
Methanogens	Whitman	MPN	20	50	21	S
Methanogens	Whitman	Hungate roll tube count	20	50	21	S
Methanogens	mod. W+P	MPN	5	25; 50	28	GRAM
Fungi	SC	spread plate count	50	25; 50	8	GRAM

[1] HAB = heterotrophic aerobic bacteria; HAnB = heterotrophic anaerobic bacteria; FB = fermenting bacteria; SRB = sulphate reducing bacteria; SOB = sulphur oxidising bacteria

[2] R2A = Reasoner and Geldreich (1985); PCA = plate count agar (Difco); mod. W+P = modified Widdel and Pfennig (1977); Widdel (1980); LOM = liquid organic medium without an inorganic electron acceptor; Stroes-Gascoyne et al. (1996); W+B = Widdel and Bak (1992); Labège = Labège test kits; Stroes-Gascoyne et al. (1996); Starkey = Starkey (1935); THH = Taylor; Hoare and Hoare (1971); Whitman = Whitman et al. (1992); SC = Sabouraud Chloramphenicol; Stroes-Gascoyne et al. (1996).

[3] WL = Whiteshell Laboratories; Canada; GRAM = Guigues Recherche Appliquée en Microbiologie; France; S = analysed by personnel from Sweden at WL

[4] MPN = most probable number

3.3 STATISTICAL EVALUATION OF THE VIABLE COUNTS

A total of 394 different viable count determinations were included in a statistical evaluation. Variables that may have had an effect on the number of culturable microorganisms (CMO) are expressed in the following analysis of variance model:

$$CMO_{fghijkl} = m + T2_f + W2_{fg}(T2_f) + lab_{fgh}\#T3_{fghi}\#ox_{fghij}\#medium_{fghijk} + E_{fghijkl} \quad (1)$$

$CMO_{fghijkl}$ is the lth measurement of the number of culturable microorganisms growing on the kth medium with the jth oxygen culturing conditions at the ith culturing temperature cultured by the hth laboratory sampled from a buffer mass with the gth percent moisture and the fth buffer mass temperature. m is the overall mean number of bacteria predicted. $T2_f$ is the effect from the *in situ* temperature of the buffer mass. This variable ranged from 19.8 to 60.9°C and was divided in four classes, 20-30 (25°C), 30-40 (35°C), 40-50 (45°C) and 50-61 (55°C). Degrees within parentheses are the class level names used. $W2_{fg}$ is the effect from the percent moisture of the sampled buffer mass. This variable ranged from 13.8 to 23.6 % moisture and was divided in four classes, 11-15, (13.5 %), 15-18 (16.5 %), 18-21 (19.5 %) and 21-24 (22.5 %). Percentages within parentheses are the class level names used. The lab_{fgh} is the effect from the different laboratories doing the analyses, including transportation effects. This variable had three classes, Canada (C), France (F) and Sweden (S). $T3_{fghi}$ is the effect from the culturing temperature used and was assigned two classes, 20°C (including the different culturing temperatures 17°C (C), 20°C (C) and 25°C (F)) and 50°C (C and S). The ox_{fghi} is the effect from the oxygen conditions used for culturing. This variable had two classes, aerobic (C, F) and anaerobic conditions (C, F and S). The $medium_{fghijk}$ is the effect from the different media used. This variable was given four classes: media for heterotrophic bacteria (C, F and S), methanogens (S), SRB (F and S) and sulphur oxidising bacteria (F). $E_{fghijkl}$ is the random sampling effect not explained by the other variables, i.e. the residual.

Each term in the model (1) is an independent effect that is a variable or a combination of variables specified with a notation using the variable names: $T2_f$, $W2_{fg}(T2_f)$ and $lab_{fgh}\#T3_{fghi}\#ox_{fghij}\#medium_{fghijk}$. It was assumed that the effect of moisture on CMO was nested within the buffer mass temperature effect because the obtained temperature gradient caused a redistribution of moisture in the buffer. Any effect from a change in moisture will then have been caused by a change in temperature, i.e. the effect from W2 on CMO was dependent on (nested within) T2. The effect of culturing conditions (the laboratories, culturing temperatures, oxygen conditions and the medium used, Table 1) was assumed to be crossed (#) because each set of such conditions were specific for the type of bacteria cultured. Any culturing procedure as described in Table 1 required a given set of culturing conditions that interacted to give the obtained results. It was also assumed that each effect was random; consideration of fixed effects was not relevant (Sheffe 1964). An analysis of variance and a pertaining estimate of the

variance components were executed running the General Linear Models procedure (GLM) for unbalanced data (SAS 1989) with the CMO data. Logarithms of the CMO data had to be considered as the data indicated multiplicative treatment effects.

3.4 LEUCINE UPTAKE ACTIVITY

The method used has been applied previously to groundwaters and surfaces from the Stripa and Äspö underground research areas (Pedersen and Ekendahl 1992*a*, 1992*b*) and involves assimilation of a nanomolar concentration of added tritiated leucine and registration with scintillation and autoradiography techniques. Two aliquots of 10 mL of the 10 g buffer in 200 mL PBS suspension prepared for culturing microbes were used for leucine uptake. An additional 10 mL aliquot was taken for acridine orange direct counting. To the leucine uptake bottles was added 1 mL of a 0.033 μM L-(4,5-^{3}H)-leucine, giving a final concentration of 3 nM and an activity of 4.56 x 10^{-7} Ci/mL. To the control bottles was immediately added 1 mL concentrated formaldehyde to stop all biological reactions (3.5 % final concentration). Both samples and controls were incubated on a shaker for 6 hours at 50°C in the dark. The reactions were stopped with formaldehyde as for the controls. Four portions of a predetermined volume from both controls and samples were filtered onto sterile Nuclepore filters (0.22 μm pore size). The filters used in the microautoradiography were rinsed with 2 x 1 mL of 1 % oxalic acid and the ones used in scintillation measurements were rinsed with 2 x 1 mL of PBS. The filters for scintillation counts were put in 10 mL Insta-Gel Plus (Packard) scintillation cocktail and the radioactivity was measured in a Packard scintillation counter.

3.5 16S rRNA GENE ANALYSIS

Additional 15 cm long and 2.5 cm diameter metal tube buffer cores were taken aseptically from layers H, M and P (depths of 1.9, 4.2 and 5.3 m, respectively). These samples were sealed and shipped to the Institute of Cell and Molecular Biology, Göteborg University, Göteborg, Sweden, as described for the GRAM samples. Maximum shipping time was 72 h. Two buffer mass subsamples, each with a weight of 0.30 to 0.33 g, were taken from each core. They were suspended in 760 μl of 20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.35 M sucrose and incubated with 2 mg mL^{-1} lysozyme (Sigma) at 37°C for 1 h to destroy cell walls. Thereafter the cells were lysed by adding 40 μl 20 % SDS and the proteins were digested with 250 μg mL^{-1} proteinase K (Sigma) during an additional incubation at 60 °C for 1 h, followed by a centrifugation at 8000 g for 5 min to separate the DNA from the sediment. The supernatant was carefully placed in a new tube. DNA was extracted with an equal volume phenol:chloroform:isoamylalcohol (25:24:1) followed by 3 extractions with chloroform:isoamylalcohol (24:1) so that no cell debris was visible. The DNA was precipitated with 1/3 volume of 10 M

NH_4Ac (final concentration 2.5 M) and 2.5 volumes of 99 % ethanol. To ensure complete precipitation, 50 µg tRNA was added as a coprecipitant and the mixture was incubated at -70°C. The precipitate was washed with 100 µM 70 % ethanol (v/v) and dried in vacuum for 30 s, dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight and stored at -20°C.

For PCR, 1 µL of the extracted DNA solution was added to a mixture of 10µL of 10x PCR buffer (Stratagene), 0.2 mM of each nucleotide triphosphate, 0.25µM of each primer and double distilled water to a final volume of 100 µL. The samples were treated with 10 mg mL^{-1} of RNase A (Sigma) for 15 min at 37°C and incubated at 95°C for 5 min, before addition of 1 µL Pfu DNA polymerase (Stratagene) and coating with 100µL mineral oil (Sigma). A total of 30 cycles were performed at 95°C (30 s), 55°C (1 min), 72°C (2 min) followed by a final incubation at 72°C for 10 min. The 5' and 3' primers used matched the universally conserved positions 519-536 and 1392-1404, *E. coli* Brosius numbering (Brosius et al. 1978). These were chosen to ensure that bacterial, archaeal and eucaryal species could be amplified. The amplification products were purified with the QIAEX agarose extraction kit (Qiagen) following the manufacturers specifications and were finally diluted in 20 µL TE-buffer and stored at -20°C.

The purified samples were cloned with the pCR-Script SK(+) cloning kit (Stratagene) following the manufacturers instructions. From each DNA extraction, a total of 11 or 12 white colonies containing the insert were randomly picked. Each colony was inoculated in 3.5 mL LB (Luria Broth)+Ampicillin and incubated overnight at 37°C. From each culture 0.5 mL was suspended in 0.5 mL of concentrated glycerol and stored at -80°C. The recombinant plasmids were extracted from the bacteria with the Magic miniprep kit (Promega). The sequencing was accomplished by an Autoread sequencing kit (Pharmacia Biotech) according to the manufacturers instructions. All clones were sequenced using the 3' primer 907-926 (Ekendahl et al. 1994) labelled with fluorescein. Gel electrophoresis was performed on an ALF DNA Sequencer (Pharmacia Biotech). Sequences obtained from the three separate samples were pooled and compared on the ALF manager OS/2 computer, using the ALF manager program, version 2.5. Clones that were identical were put in the same group.

3.6 SEQUENCE ANALYSIS

The 16S rRNA gene clones were compared to the sequences available in the European Molecular Biology Laboratory (EMBL) database using the FastA procedure in the GCG program package (Genetic Computer Group, Wisconsin, USA). This procedure shows identities between an unknown sequence (clone) and known and sequenced bacteria in the database. The phylogenetic analysis was performed by using the programs contained in the PHYLIP version 3.5c package (Felsenstein 1989) compiled for PC. All

nucleotide positions that could be unambiguously aligned were included in the analysis. The final data set comprised 360 nucleotide positions, position no. 542 - 880 (*E. coli* Brosius numbering, did not include 18 inserts in the analysed data set, Brosius et al. 1978). The distances were calculated using the DNADIST program and a tree was built running the KITCH program with contemporary tips. The KITCH program was run with a randomised input order of data with 10 jumbles and during the execution 36634 trees were examined (Felsenstein 1989). The tree was drawn using the drawing program DRAWTREE, also available in the PHYLIP package and was used to differentiate the main lines of descent of the found sequences, it will not unravel intragenic phylogeny (Stackebrandt and Rainey 1995).

3.7 LIPID ANALYSIS

Lipid analysis was performed on three 45 cm long x 5 cm diameter buffer core samples taken aseptically with a hollow stem auger from layers H, M and P (depth 1.9, 4.2 and 5.3 m respectively). Upon retrieval, samples were sealed immediately on both ends with hot sterile paraffin wax and sterile plastic caps, wrapped in double plastic bags containing iron filings to prevent exposure to oxygen and quickly frozen to -18°C until they were shipped on ice to Microbial Insights Inc., Knoxville, Tennessee, USA. The samples were analysed for: 1) total biomass, related to the quantity of the membrane component phospholipid fatty acid (PLFA) in the samples; 2) community structure as derived from the presence of specific signature lipids; 3) nutritional-physiological status as derived from the ratio of certain PLFA's and from the ratio of poly-β-hydroxyalkanoic acid (PHA) to PLFA. The lipid analysis method has been described in detail elsewhere (White et al. 1997). Briefly, the frozen buffer samples were extracted and fractionated into neutral, glyco- and polar lipids using column chromatography with silicic acid. The glycolipids were analysed for PHA. The polar lipids were then treated in a mild alkaline system containing methanol to transesterify the PLFA into methyl esters which subsequently were further separated and quantified by capillary gas chromatography/mass spectrometry.

4 RESULTS

4.1 TEMPERATURE AND MOISTURE CONTENT

The moisture content in the samples from near the granite-buffer interface (the peripheral samples) varied from about 19.5 to 23 % throughout all layers (Fig. 2b, 2d). The temperature in these samples varied from 20 to 45°C with the higher temperatures (40-45°C) occurring in the peripheral

samples taken from the layers around the heater (layers K, L, M and N; depth 2.95 to 5.0 m). For samples taken near the centre of the borehole (above and below the heater) and in layers surrounding the heater (the central samples), the moisture content dropped from about 19 % above the heater to about 13 % in the layers around the heater and then increased again to about 20 % in the layers below the heater (Fig. 2a, 2c). The temperature, which was responsible for this redistribution of the moisture, varied from about 20°C in the top layers to up to 60°C in the layers around the heater.

4.2 CULTURABLE MICROORGANISMS IN BUFFER SAMPLES

At WL, HAB at 25°C and 50°C could be cultured from the central and peripheral samples above and below the heater and from the peripheral samples around the heater, all with a moisture content above 15 % but HAB could not be cultured from the central samples of the buffer layers around the heater that had a moisture content below 15 % (Fig. 2a,2b). The culturing results for HAnB at 17-25°C and 50°C showed the same trend as the results for HAB, with negative results for samples with a moisture content below 15 %. The GRAM results for HAB and HAnB at 25°C were up to two orders of magnitude higher than the WL results, while the GRAM results at 50°C were all negative for HAB and HAnB (Fig. 2c,2d). The results from the GRAM analyses followed the same general trend along the layers, with low or negative results in samples with a moisture content below 15 %.

The numbers of culturable SRB detected did not exceed 3.9×10^2 colony forming units (CFU)/gdw (Fig. 2). No indications of active sulphate reduction, such as a H2S smell or black precipitates, were encountered during decommissioning of the BCE. Methanogens were detected sparsely at levels of up to 2.0×10^2 CFU/gdw in samples. They were distributed throughout most layers analysed, but generally not in those with moisture contents below 15 %. With one exception (layer P, depth 5.3 m, 7.2 CFU/gdw), no aerobic SOB were detected in any of the buffer samples using Starkey (1935) medium. In contrast, anaerobic SOB, enumerated with Taylor, Hoare and Hoare (1971) medium, were numerous in all buffer samples analysed (up to 3.0×10^5 CFU/gdw), except in samples taken from close to the heater (layers K, M and N; depth 3.2, 4.2 and 4.8 m, respectively). The maximum number of FB detected was 1.7×10^3 CFU/gdw. No growth was observed in samples that had a combination of high temperature and low moisture content. Analyses for fungi were carried out using Sabouraud Chloramphenicol standard solid medium (Stroes-Gascoyne et al. 1996) but culturable fungi were not detected in any of the buffer samples analysed.

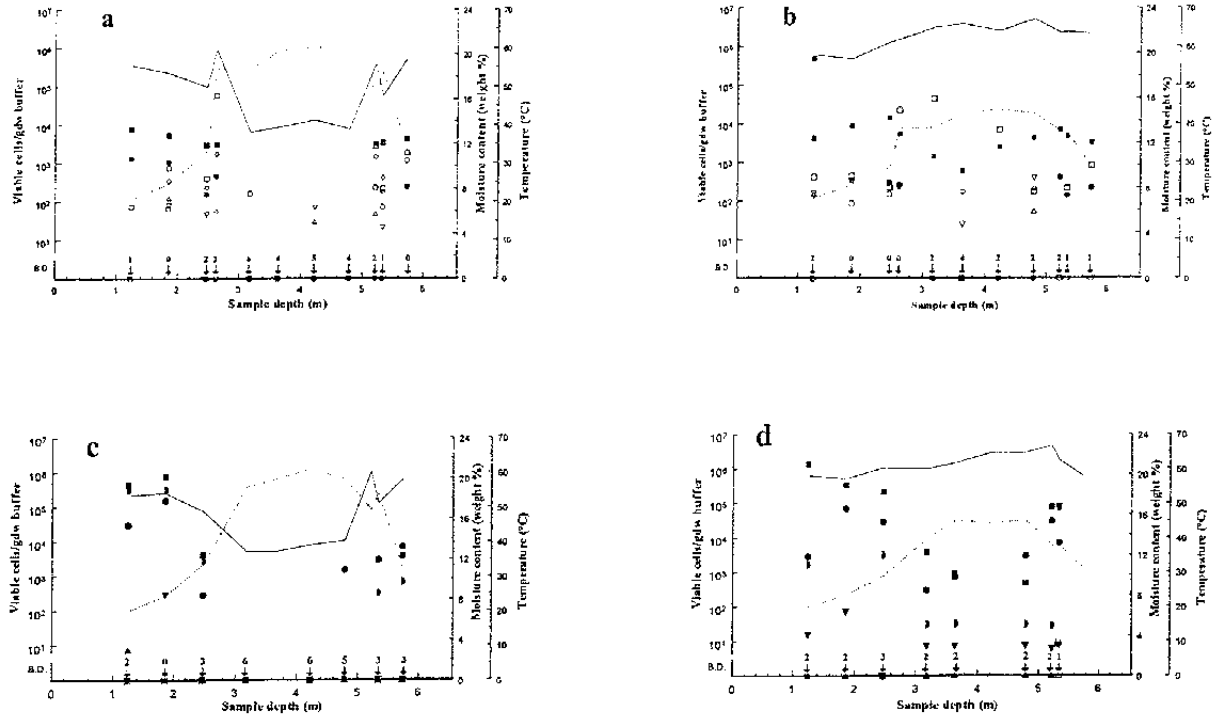

Figure 2. *Numbers of viable cells per gram dry weight buffer and moisture and temperature data distributed along a sand/bentonite nuclear fuel waste buffer material that surrounded a heater in a 6 m deep deposition hole drilled in granite for 2.5 years. The tunnel floor is the zero depth reference (confer Fig. 1). The heater position was between 2.65 m and 5.2 m from the tunnel floor.* ***a)*** *Central samples taken at a radial distance of about 5 cm from the heater surface and about 20 cm from the granite rock and analysed at WL.* ***b)*** *Peripheral samples taken at a radial distance of about 20 cm from the heater surface and about 5 cm from the granite rock and analysed at WL.* ***c)*** *Same as for a) but analysed at GRAM.* ***d)*** *Same as for b) but analysed at GRAM. Symbols: Filled symbols indicate culturing temperature between 17 and 25 °C, Open symbols indicate a culturing temperature of 50 °C.* ■ ❑, *HAB;* ● ○, *HAnB;* ◊, *FB;* ▽ ▼, *SRB;* ★, *Aerobic SOB;* ◗, *Anaerobic SOB;* ▲ Δ, *Methanogens. The dotted line shows temperature and the solid line shows moisture content. Numbers with arrows indicate the number of results below the detection limit (B.D.) for each sampled layer, see text for details.*

4.3 STATISTICAL EVALUATION OF THE VIABLE COUNTS

Figures 3a and 3b show the data used for the statistical analysis as a function of the *in situ* temperature and moisture content, respectively. Only 5 out of 75 samples with a moisture content lower than 15 % contained culturable microorganisms and the analysis of variance showed no significant effect of temperature on CMO (Table 2). The *in situ* temperature then did not have any significant effect on the results obtained. Contradictory, the F-test showed significance on the 0.001 significance level for the effect of the moisture variable, nested within the *in situ* temperature variable, as well as

for the crossed effect of the variables culture temperature, oxygen culturing conditions, the medium used and the laboratory doing the analysis, i.e. the culturing conditions (Table 1). The pertaining variance component analysis showed that the major variance components were those that were shown to be significant in the analysis of variance, the *in situ* moisture content of the buffer mass (32.4 %), the culturing conditions used to produce the CFU number (32.4 %) and the residual unexplained variance (30.8 %).

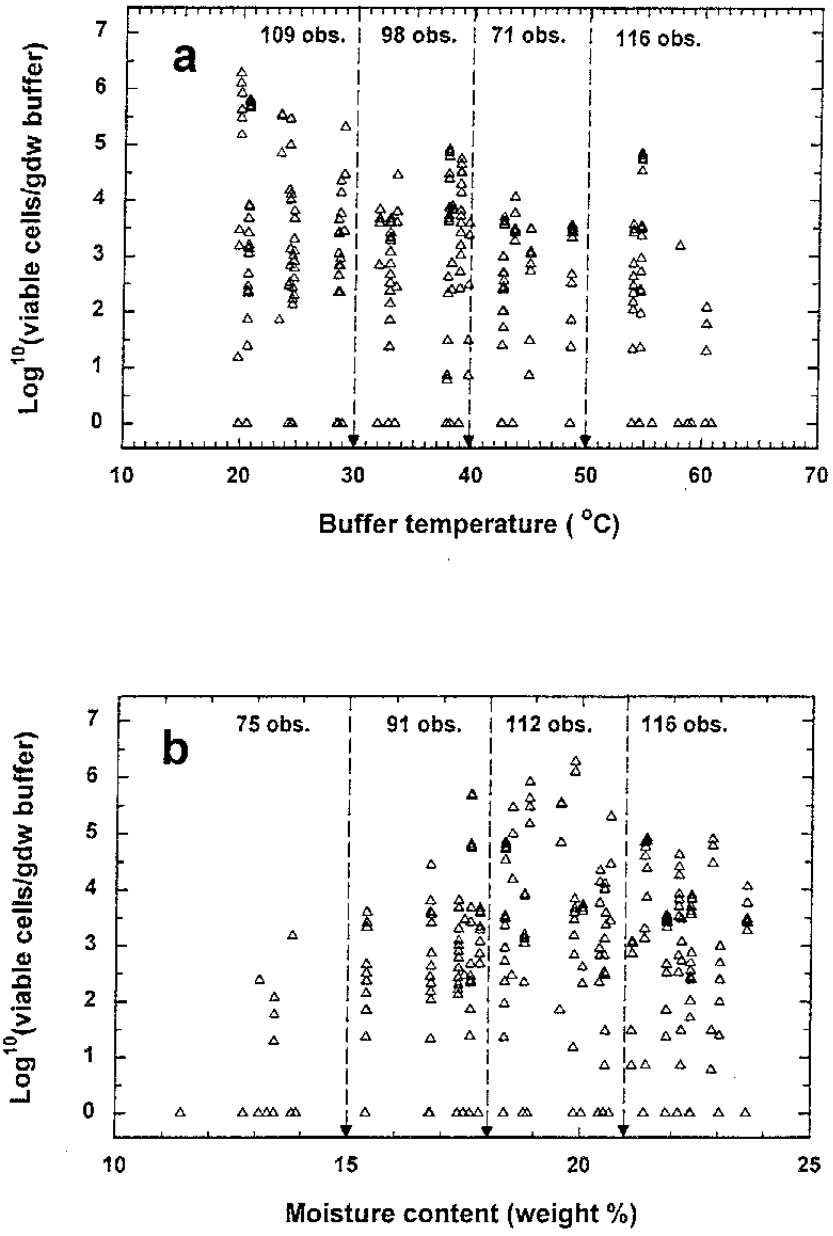

Figure 3. *The distribution of all colony forming unit (CFU) data obtained (394 observations) versus the in situ temperatures* ***(a)*** *and moisture contents* ***(b)*** *for the buffer samples investigated. The four temperature (a) and moisture content (b) classes that were used for the analysis of variance are indicated and the number of observations within each class level is presented. Note that many of the data points overlay each other.*

Table 2 *Results from analysis of variance with pertaining variance component estimation for effects hypothesized to influence the CFU of the buffer masses sampled. The effect from each variable on the CFU numbers is reflected by the variance component estimates. The total number of observations was 394, distributed over 4 temperature classes, 4 water content classes, 2 culturing temperature classes, aerobic or anaerobic culturing conditions, 4 different media and 3 laboratories. T2 is the effect from the in situ sample temperature, w2(t2) is the effect from the water content of the sample nested within the in situ sample temperature (dependent), t3#ox#medium#lab is the crossed (interacting) effect from culturing temperature, oxygen condition at culturing, the medium used and the laboratory doing the analysis. Var, variance; MS, mean square.*

Source	Degrees of freedom	Sum of squares (SS)	Mean square (MS)	F-test	F	Significance level of the F-test	Expected mean square model	Variance component	Estimate	Part of total variance component estimate (%)
T2	3	54.05	18.02	MS(W2(T2))MS(T2)	1.57	0.3770	Var(Residual) + 85.3 Var(T2) + Q(W2(T2))	Var (T2)	0.174	4.9
W2(T2)	5	141.51	28.30	MS(W2(T2) MS(Residual)	19.65	0.0001	Var(Residual) + Q(W2(T2))	Var (W2(T2))	1.160	32.4
T3#ox# medium#lab	10	383.77	38.38	MS(T3#ox#medium#lab) MS(Residual)	26.65	0.0001	Var(Residual)+ 33.7 Var(T3#ox#medium#T)	Var (T3#ox#medium#lab)	1.158	32.4
Residual	375	539.12	1.44						1.102	30.8

4.4 ACTIVITY OF BACTERIA

Assimilation experiments indicated a significant uptake of leucine in samples from the layers above the heater, but not in layers around the heater (Fig. 4). Layers N to Q were not investigated. The results from the microautoradiography investigation were all negative, indicating either methodological problems, or that the amount of leucine assimilated was too low to give a positive signal (less than 0.001 DPM per bacterium, Pedersen and Ekendahl 1992*a*). Total number of bacteria with the acridine orange direct count method could not be determined due to interference from clay particles.

4.5 PHENOTYPIC IDENTIFICATION OF OCCURRING BACTERIA

Selected isolates cultured from samples enumerated by GRAM were identified using the selective enrichment media described in Table 1 combined with API strips. Ten randomly chosen HAB isolates per sample were identified as well as some HAnB, SRB and SOB (Table 3). Only species present in large numbers (minimum 50 per sample) were included. More than 67 % of the aerobically grown isolates were identified as either *Pseudomonas stutzeri* or belonging to the genus *Bacillus*. The phenotypic identification of *P. stutzeri* was confirmed by comparing the 16S rRNA sequence of one of the isolates with the sequence of the type strain. *Thiobacillus denitrificans* was detected in every sample from which bacteria were isolated.

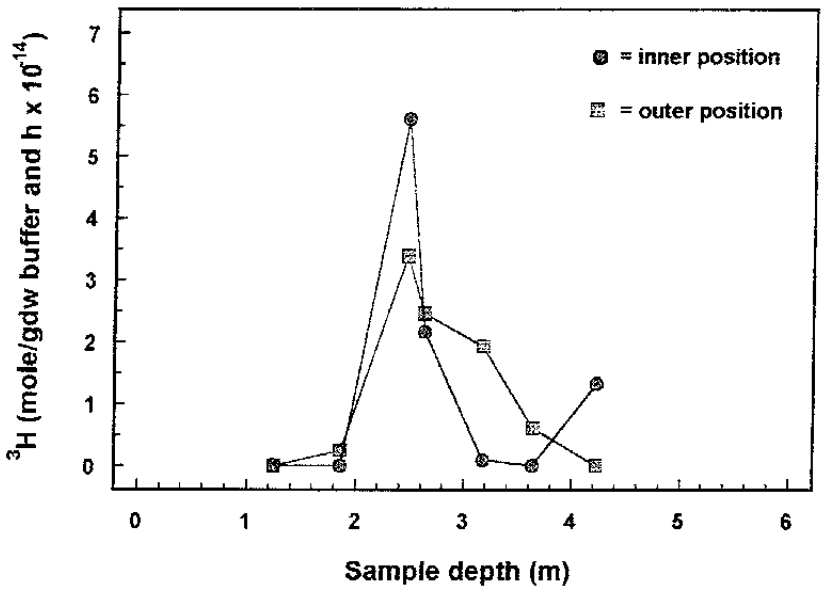

Figure 4 *The activity of microbes in the buffer mass samples measured as assimilation of the tritiated amino acid (^{3}H)-leucine at different depths of the buffer. The tunnel floor is the zero depth reference (confer Fig. 1). The heater position was between 2.65 m and 5.2 m from the tunnel floor.*

Table 3 *Bacteria identified in selected buffer samples at the GRAM laboratory using selective enrichment media (Table 1) and API strips.*

	Number of identified cultures for each layer, temperature and moisture content						
Genus or species identified	**H 23 °C 19.6 %**	**H 24 °C 18.5 %**	**L 45 °C 21.1 %**	**N 45 °C 22.2 %**	**O 38 °C 22.8 %**	**P 38 °C 21.5 %**	**P 54 °C 17.5 %**
Proteobacteria beta group							
Thiobacillus denitrificans	1	1	1	1	1	1	1
Thiobacillus thiooxydans	.[1]	.	.	.	.	1	.
Proteobacteria gamma group							
Acinetobacter	.	.	.	2	.	1	.
Pseudomonas stutzeri	6	5	.	.	9	9	.
Proteobacteria delta group							
Desulfovibrio	1	1	.	.	.	1	.
Gram-positive bacteria							
Bacillus	1	4	5	5	.	.	2
Bradyrhyzobium japonicum	.	1	.	.	.	.	.
Cellulomonas	.	.	.	.	.	.	2
Clostridium	1	.	1	1	.	.	.
Curtobacterium	2	.	.	1	.	.	.
Desulfotomaculum	1	.	1	1	.	1	.
Nocardia	.	.	5	.	1	.	.

[1] = not detected

4.6 16S rRNA GENE ANALYSIS

A total of 67 clones were sequenced; 23, 22 and 22 from layers H, M and P, respectively (depth 1.9, 4.2 and 5.3 m, respectively). The clones were segregated into 21 specific groups; those that were identical were placed in the same clone group and each such group, together with clone sequences appearing only once, was given a clone group name, ranging from K1 to K21. Fig. 5 depicts a phylogenetic tree for 20 of the clone group sequences. Three distinct groups of phylogenetically related bacteria were found (Woese 1987), the alpha and gamma groups of the Proteobacteria and Gram-positive bacteria. An eucaryotic yeast sequence was also found (not shown in Fig. 5). The Proteobacteria gamma-group was the largest phylogenetic group, to which 35 of the found clones belonged and had the highest diversity, with 13 different clone groups as shown in Fig. 5. Gram-positive bacteria was the second largest group with 16 clones and five different clone groups. All the found sequences were compared with the sequences available in the EMBL database by November 1996 and the closest species in the database for each clone is shown in Table 4. Note that 99.9 % similarity or more is regarded to be a potentially successful identification of a species by this method, unless DNA-DNA reassociation is also performed, because the 16S rRNA gene is a very conservative part of the bacterial genome. Accordingly, several clones were too distantly related to the database sequences for a meaningful closest species indication and the closest species in the database is only given for clones with identity values at or above 95 %. Three clones dominated, K1, K2 and K9 and they were related to the sequences of the actinomycete *Amycolatopsis orientalis* (97.7 %) *and* the Proteobacteria *Pseudomonas flavescens* (95.6 %) and *Acinetobacter junii* (99.1 %), respectively. Several identical clones appeared when different layers were compared. The three layers (H, M and P) examined shared 11 or 12 identical clone sequences and Table 4 also shows that 3 clone groups, K1, K2 and K9, were shared by all three layers investigated in the proportions 3, 3 and 2 clones respectively. As expected, the sampled buffer material seems to have been rather homogeneous with respect to the bacterial DNA present.

4.7 LIPID ANALYSIS

The sample size from buffer layers H, M and P varied from 64 up to 67 gdw buffer and contained a total amount of 405, 2211 and 1861 pmol PLFA, respectively. The proportions of different signature lipid biomarkers in the total PFLA sampled are shown in Table 5. The laboratory blank contained 35 pmol of PLFA, characteristic of human dandruff type contamination (eucaryotic) without classical bacterial lipid biomarkers and did, therefore, not compromise the PLFA analysis.

The amount of PFLA in the buffer samples from layers H, M and P was 6.3, 33 and 29.3 pmol PFLA/gdw buffer, respectively. The cell equivalent value for a specific amount of PLFA is calculated by using an established PLFA content from experiments performed with subsurface bacteria (Balkwill et

al. 1988). This cell equivalent is based on 1.0 x 10^8 pmol of PLFA/gdw cells and 2.5 x 10^{12} cells/gdw cells which gives 2.5 x 10^4 cells/pmol of PLFA. It is important to note that the number of cells/gdw cells may vary by up to one log unit depending on their environmental conditions. The total number of cells as indicated by the PLFA concentrations in the buffer samples from the layers H, M and P then becomes 1.58 x 10^6, 8.25 x 10^6 and 7.33 x 10^6 cells/gdw buffer, respectively.

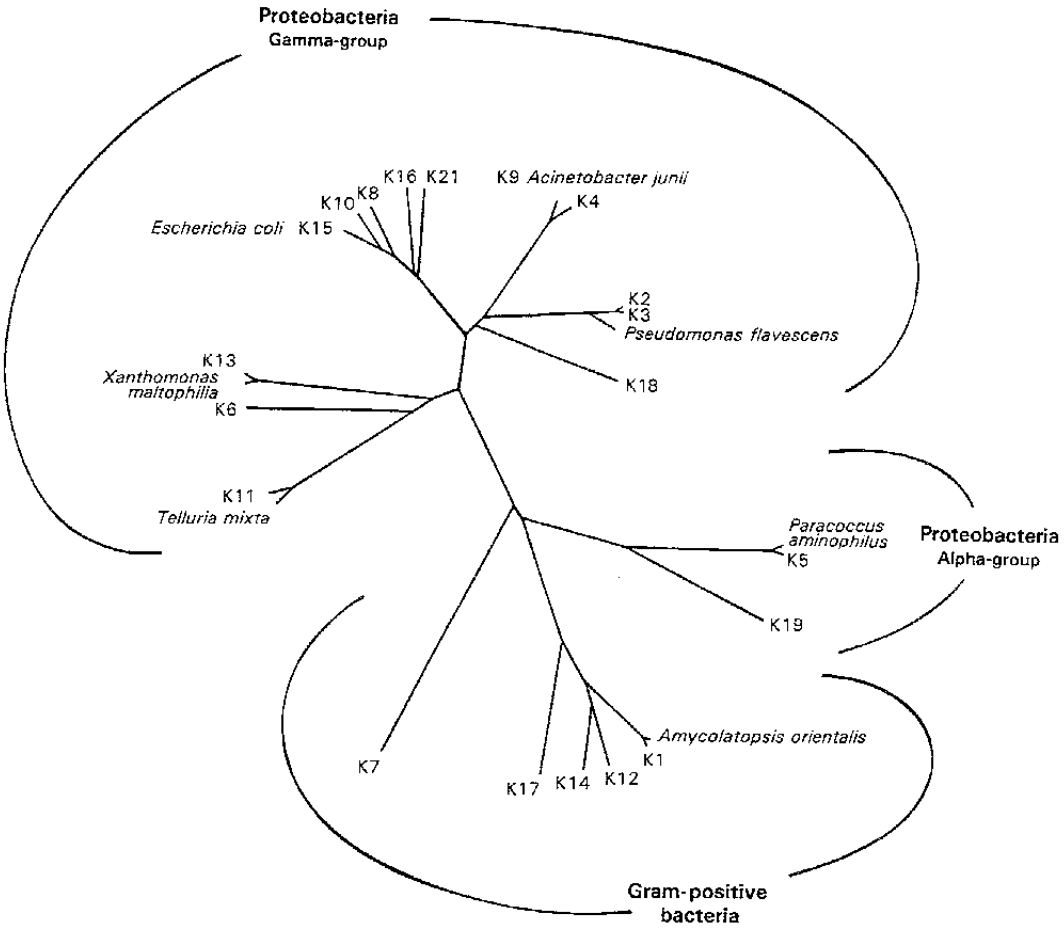

Figure 5. *Evolutionary distance tree based on the 16S rRNA gene sequences of clones from three BCE samples, layers H, M and P. Major phylogenetic groups of bacteria have been designated with their generally accepted names. As references, some 16S rRNA gene sequences of known bacteria from the EMBL database have been added to the tree and they are indicated with their Latin names. The tree was used to differentiate the main lines of descent of the found sequences, it will not unravel intragenic phylogeny (Stackebrandt and Rainey 1995). It shows 20 of the total of 21 specific clone groups found; 1 sequence was from an eucaryote and was not incorporated in this analysis as it would have significantly reduced the resolution of the tree*

Table 4. *Identity between the sequenced clone groups and 16S rRNA sequences in the EMBL database. The identity shows the percent identity between the obtained sequence and the most related microorganism in the database.*

Clone group	Closest species in the EMBL database	Identity %	Number of identical clone sequences for each layer, temperature and moisture content		
			H 25°C 18.4 %	M 44 °C 20.6 %	P 38°C 20.8 %
Proteobacteria alphagroup					
K5	*Paracoccus amonophilus*	97.4	.	.	1
K19	n.r.[1]	93.4	1	.	.
Proteobacteria gammagroup					
K9	*Acinetobacter junii*	99.1	6	4	2
K4	*Acinetobacter junii*	95.5	1	.	1
K8	*Aeromonas caviae*	97.4	.	.	1
K6	*Bordetella avium*	95.4	1	.	1
K15	*Escherichia coli*	98.5	.	1	.
K2	*Pseudomonas flavescens*	95.6	3	6	7
K3	*Pseudomonas flavescens*	95.4	1	.	2
K11	*Telluria mixta*	96.2	1	1	.
K13	*Xanthomonas maltophilia*	96.0	.	1	.
K10	n.r.	94.0	.	1	.
K16	n.r.	92.9	.	1	.
K18	n.r.	90.7	1	.	1
K21	n.r.	89.3	.	1	.
Gram-positive bacteria					
K1	*Amycolatopsis orientalis*	97.7	7	3	3
K17	*Arthrobacter globiformis*	95.9	1	.	.
K12	n.r.[1]	90.5	.	1	.
K14	n.r.	87.0	.	.	1
K7	n.r.	86.9	.[2]	.	1
Eucarya					
K20	*Torulaspora delbrueckii*	98.6	.	2	1

[1] = not relevant [2] = not detected

Monoenoic PLFA are found in most Gram-negative bacteria (White et al. 1997) and ranged from 11.6 to 38.7 % in the buffer samples (Table 5) while the amount of terminally branched saturated PLFA, common to Gram-positive bacteria (White et al. 1997), ranged from 7.2 to 12.6 %. Polyenoic PLFA represented between 9.7 and 20.9 % of the extracted PLFA and are generally thought to indicate the presence of microeucaryotes. The polyenoic PLFA 18:2w6, which is especially prominent in fungi (White et al. 1997), was found in samples M and P in small amounts. Mid-chain branched saturated PLFA, particularly 10Me16:0, are indicative of the SRB genus *Desulfotobacter* and were found in all three samples. The presence of the branched monoenoic PLFA i17:1w7c, which was found in layer P, often indicates the presence of another sulphate- (or iron-) reducer, *Desulfovibrio*, suggesting that SRB were present.

Table 5 *The proportion of different signature lipid biomarkers in the total PFLA sampled from three buffer mass samples from layers H, M and P. The in situ temperature and the moisture content of each analysed sample is also shown.*

PFLA component	Percent of total PLFA		
	H	**M**	**P**
	25 °C	**60 °C**	**55 °C**
	19.5 %	**13 %**	**18.5 %**
Terminally Branched Saturates			
i15:0	.[1]	1.0	1.2
a15:0	.	0.7	1.7
i16:0	3.8	2.4	3.1
i17:0	.	1.0	1.8
a17:0	5.0	2.2	4.9
Total Terminally Branched Saturates	**8.9**	**7.2**	**12.6**
Monoenoics			
16:1w7c	5.7	2.7	4.6
16:1w5c	.	1.6	2.1
cy17:0	5.1	1.2	5.9
18:1w7c	9.6	4.9	7.1
18:1w7t	.	.	1.5
cy19:0	10.5	1.3	17.5
Total Monoenoics	**30.9**	**11.6**	**38.7**
Normal Saturates			
14:00	.	.	.
15:00	.	1.0	0.6
16:00	21.5	30.7	16.0
17:00	.	.	.
18:00	13.0	22.1	8.0
22:00	5.6	3.2	2.2
24:00	.	1.1	1.9
Total Normal Saturates	**40.1**	**58.1**	**28.6**
Total Monoenoics + Normal Saturates	**71.0**	**69.7**	**67.3**
Polyenoics			
18:2w6	.	2.7	2.1
18:1w9c	15.4	18.2	7.6
Total Polyenoics	**15.4**	**20.9**	**9.7**
Mid-Chain Branched Saturates			
10me16:0	4.7	1.3	4.9
10me18:0	.	.	3.0
12me16:0	.	0.9	1.3
Total Mid-Chain Branched Saturates	**4.7**	**2.2**	**9.2**
Branched Monoenoics			
i17:1w7c	.	.	1.3

[1] = not detected

The state of growth for the studied microbes is indicated by the ratio of cyclopropyl fatty acids (cy#:0) to monoenoics (#1w7c). The monoenoics change to cyclopropyl fatty acids as microbes move from a log to a stationary phase of growth. The ratio is usually between 0.5 (log phase) and 2.5 (stationary phase) (White et al. 1997). The results for the buffer samples indicate that the microbes in sample M were in log phase growth, but those in samples H and P were in stationary phase (Table 6). Unbalanced growth, which often occurs in communities which are missing a necessary nutrient, is indicated by the ratio of the storage lipid PHA to the membrane lipid PLFA. Ratios greater than 0.2 usually indicate the beginnings of unbalanced

growth in at least part of the microbial community (White et al. 1997) and this was indicated for layer M (Table 6). Starvation or toxicity is indicated by the ratio of trans fatty acids (w7t) to cis fatty acids (w7c), because bacteria begin making trans fatty acids under environmental stress such as lack of nutrients or presence of a toxin. Healthy systems usually have w7t/w7c ratios between 0.05 and 0.1. Starved systems usually have ratios between 0.1 and 0.3 (White et al. 1997). The only trans fatty acids detected in the buffer samples were in sample P and a w7t/w7c ratio of 0.2 was calculated for this sample (Table 6), indicating starvation or toxicity for this layer.

Table 6 *Signature lipid biomarker ratios showing nutritional and physiological status of microbes present in the buffer samples from layers H, M and P. The in situ temperature and the moisture content of each analysed sample is also shown.*

Lipid biomarker indication	Ratios of lipid components		
	H 25 °C 19.5 %	M 60 °C 13 %	P 55 °C 18.5 %
Growth phase			
ratio of cy 17:0 to 16:1w7c	0.9	0.4	1.3
ratio of cy 19:0 to 18:1w7c	1.1	0.3	2.5
Unbalanced growth			
pmol PHA / gdw buffer	. [1]	8.94	.
ratio of PHA to PLFA	0	0.27	0
Starvation or toxicity			
ratio of 16:1w7t to 16:1w7c	0	0	0
ratio of 18:1w7t to 18:1w7c	0	0	0.2

[1] = not detected

4.8 NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequence data reported in this paper appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers X91516 - X91536 corresponding to K1-K21 respectively.

5 DISCUSSION

5.1 NUMBER OF MICROORGANISMS IN THE BUFFER

The trends for viable cells/gdw throughout the buffer appear reproducible for equivalent samples, despite slight differences in culture media, culturing temperatures and the culturing lab (Fig. 2). The overall numbers of HAB were up to two log unit higher in the samples analysed at GRAM compared to WL. The samples at WL were analysed within 24 hours of sampling whereas it took about 10 to 14 days before the samples reached GRAM. Fredrickson et al. (1995) subjected subsurface samples to various storage times and found that, generally, the number of culturable HAB increased by up to 6 orders of magnitude in some samples with sample storage times up to 77 d. Evidence pointed towards a complex interplay between different lag times for 'reactivation', growth and succession of various bacteria as well as

the heterogeneous distribution of microorganisms in subsurface samples. Storage effects were more pronounced in samples homogenised prior to storage than in samples left intact. The buffer samples shipped to GRAM were left intact, as they were retrieved and the distribution of microbes in these samples was homogeneous, because the buffer material underwent thorough mixing before emplacement in the BCE. The generally higher numbers of viable HAB in the samples analysed at GRAM might, therefore, have been caused by differences in culture techniques and the longer sample transportation time. The statistical analysis of variance demonstrated culturing conditions to be a significant effect, responsible for one third of the total variance (Table 2).

The buffer mass was mixed and laid down in layers aerobically. There is not much reducing capacity in the buffer mixture per se. Anaerobic and reduced conditions in a nuclear waste buffer will occur mostly as a result of exchange processes with surrounding reduced groundwater, a process expected to take many years (Motamedi et al. 1996). Therefore, it can be anticipated that oxidised conditions and aerobic or microaerophilic conditions prevailed in the studied buffer mass. Strict anaerobic microorganisms such as SRB and methanogens will not develop under these conditions and should consequently not be expected in high numbers. This appears to have been the case, as none of the laboratories could demonstrate significant numbers of such microorganisms using culturing methods. The numbers of anaerobes found were generally at or below some 100 viable cells/gdw or below the detection limits. Instead, relatively large numbers of HAB were detected. Similar results were obtained with the 16S rRNA gene sequencing and the PLFA methods which both revealed many genera representing HAB or facultative anaerobes, but no or only very few strict anaerobes.

5.2 FACTORS CONTROLLING CULTURABILITY

When the buffer masses were laid down at start of the experiment, the moisture content was homogeneous at 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 moisture content developed. Approximately 50 % of the buffer mass is sand and this part will not influence the amount of free water more than marginally by a matrix adsorption effect. However, the bentonite clay will sorb water until it is saturated and only water in excess of what is sorbed by the clay will be available for microorganisms in the buffer. The moisture content at which water becomes available in the 50:50 sand-bentonite mixture used in this experiment is about 15 %, which correlates well with the values below which bacteria could not be cultured (Fig. 3 b). This indicates that moisture content was an important factor controlling the number of culturable bacteria. The moisture content effect was confirmed as significant by the analysis of variance, corresponding to about one third of the total variance (Table 2). Many bacteria could be cultured from high temperature samples, provided the moisture content was above 15 % (Fig. 3a) and correspondingly, the effect from the *in situ* buffer mass temperature was

found to be statistically non-significant. The only limitation for the viability of microorganisms in the buffer masses then seems to have been water availability. Measurements at WL have shown that the total suction of this buffer material, containing 15 % moisture at 25°C, is 6.08 MPa (Wan 1996). This converts to a water activity (a_w) of approximately 0.96 (Brown 1990). An a_w value at or below 0.96, therefore, appears to form a boundary where microbial life in nuclear waste buffers is difficult due to the lack of free water. This conclusion was recently supported by studies of Motamedi et al. (1996) who showed that viable SRB, introduced at high concentrations (10^8 cells/g clay) in compacted 100 % bentonite, died within 24 h at an a_w of 0.96 but were still viable after 60 days at an a_w of 1.

5.3 IDENTIFICATION OF BACTERIA USING DIFFERENT METHODS

Three different methods were employed for identification of microbial species and groups present in the buffer material. They were based on lipid analysis, viable counts with API strips and 16S rRNA gene sequencing. Viable counts using plates and tubes under various general or specific culturing conditions reveal culturable microorganisms, but usually only a few percent or less of what is detected with total counts can be cultured (Amann et al. 1995). The plate versus total count anomaly of environmental samples has led to development of non-culturing techniques to overcome this problem, such as extraction and sequencing of the ribosomal 16S rRNA genes (Ekendahl et al. 1994, Pedersen 1996, Pedersen et al. 1996a) or extraction and analysis of lipids (White et al. 1997). The inability to reveal the large majority of microorganisms present with culturing techniques is of course a major drawback, but presently culturing is required for description of new species. It is also the only method which truly proves viability. The other methods are based on inferences and might be reporting preserved, dead or inactive, populations. This merits the continued use of culturing in microbial ecology.

Selected isolates from samples enumerated by culturing at GRAM were identified using API strips (Table 3). Approximately ten randomly chosen HAB isolates per sample were identified as well as some HAnB, SRB and SOB. From a total of 79 identified isolated strains, 65 % were identified as either *Pseudomonas stutzeri* or belonging to the genus *Bacillus*. Another bacterium frequently identified was the actinomycete *Nocardia*. The information obtained by this identification effort is only qualitative and reflects what can be cultured after a transportation time of approximately 14 days. Considering that culturing usually only reveals a few percent or less of the total population in environmental samples may explain why none of the cultured and identified isolates coincide on the species level with what was observed using 16S rRNA sequencing. The proportion of Gram-positives was greater in the API analysis than indicated by both the 16S rRNA gene sequencing and the PLFA results. The majority of the 12 species identified with the API system in layers H, M and P were Gram-negative (59 %) while the rest were Gram-positive (41 %). In comparison, five of the 16S rRNA gene clones detected out of 21 were Gram-positive genera (23 %) and the percentage of Gram-positive bacteria based on the proportion of terminally-

branched saturated PLFA varied between 7.2 and 12.6 % in the sampled layers (Table 5). Despite these differences, it is important to note that all three methods identified significant amounts and diversity of Gram-positive bacteria. Many of these may form spores or other survival forms that are resistant to desiccation which would be advantageous for survival in the dry buffer environment.

The value of the percent identity at which two 16S rRNA genes can be concluded to belong to the same genus or species can be quite different for different genera (Fox et al. 1992). It has been suggested, based on a comparison of rRNA sequences and on DNA-DNA reassociation, that a relationship at species level does not exist at less than 97.5 % identity in 16S rRNA (Stackebrandt and Goebel 1994). At higher identity values, species identity must be confirmed with DNA-DNA hybridisation. Accepting this level conservatively as identifying a sequence on the genus level, some conclusions can be made about the sequences reported here. Two of the three predominating species found using 16S rRNA gene sequencing had high identities with the typical soil and groundwater bacteria *Amycolatopsis orientalis* (97.7 %) and *Acinetobacter junii* (99.1 %). The third predominating species was a bacterium more distantly related to *Pseudomonas flavescens* (95.6 %). On the genus level, the detection of *Pseudomonas* and *Acinetobacter* coincides between the API system and 16S rRNA gene sequencing results and both genera are typical groundwater bacteria that may have originated from the groundwater used to mix the buffer.

The 16S rRNA gene sequencing method has been applied previously to assess the diversity of microorganisms in different subterranean habitats without culturing and thorough discussions about possibilities and limitations of this method have been published elsewhere (Ekendahl et al. 1994, Pedersen *et al.* 1996a, Pedersen *et al.* 1996b, Pedersen *et al.* 1997). Briefly, when PCR amplification is used for determination of species diversity, the result may be biased due to methodological problems, such as uneven extraction of DNA and biased PCR due to differences in genome size and variations in the growth rate dependent number of gene copies (Ekendahl et al. 1994, Farrelly et al. 1995). One of the most important potential biases is that organisms belonging to the domain *Archaea* have been found to have only one or a few gene copies of the 16S rRNA gene while *Bacteria* can have from 1 up to several copies, 5-7 or more, which may bias towards *Bacteria* (Ward et al 1992). In this investigation, an important issue was to analyse the microbial diversity and especially to search for groups of microorganisms that may be detrimental to a spent nuclear fuel repository, such as corrosive SRB or gas producing methanogens. SRB have been detected in other investigations using the 16S rRNA method (Pedersen et al. 1996a; Pedersen et al. 1997) and the absence of SRB clones here is, therefore, most probably due to a limited presence of this group of bacteria, as inferred by the viable counts (Fig. 2). The negative results with respect to methanogenic sequences is on the other hand not conclusive, due to the potential gene copy number bias described above.

A total of 21 clone groups could be identified in the buffer material examined. Three distinct phylogenetic groups of bacteria were found, proteobacteria alpha and gamma groups and Gram-positive bacteria (Fig. 5). Most clones belonged to the gamma group of the proteobacteria. Three identical and dominating bacterial sequences were shared between each of the three layers investigated (H, M and P, depth 1.9, 4.2 and 5.3 m, respectively), indicating a homogenous buffer mass along the heater (Table 4). Also, each layer shared about 50 % of its clone identities with each of the other two layers. Since all buffer mass layers were mixed from common sources of groundwater, clay and sand, this result is expected and demonstrates the reproducibility of the 16S rRNA diversity method. The temperature differences of the layers examined did not correlate with any drastic 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 eucaryotic DNA. The closest species in the database was the yeast *Torulaspora delbrueckii*. This sequence, clone K20, appeared only at the two lower layers analysed, M and P (depth 4.2 and 5.3 m, respectively). This observation correlates with the PLFA analysis indicating eucaryotic fungi in these layers as represented by the polyenoic 182w6 PLFA content (Table 5).

Two of the three applied identification methods did indicate significant amounts of actinomycetes in the buffer. Clone group K1 represents an actinomycete and was found in all three samples sequenced and the actinomycete genus *Nocardia* was identified among the viable counts as well (Table 3). Many actinomycetes are adapted to life in soils and their mycelia-like morphology make them well adapted to desiccation and a life in an environment such as the studied buffer. The actinomycetes contain a mixture of 10Me branched saturated PLFA and classically, they contain more of the PLFA component 10Me18:0 than 10Me16:0 (White et al. 1997), which is the opposite of what was detected in the buffer samples (Table 5). However, there are numerous very different types of actinomycetes and as all of their PLFA patterns have not yet been studied, the absence of actinomycets suggested by PLFA result is not a conclusive result.

5.4 VIABILITY OF THE BUFFER POPULATION

The measured assimilation of leucine in the layers around the top of the heater was significant, indicating the presence of viable bacteria. The activity was highest at the top of the container, at 2 to 3 m depth, where the temperature and moisture content coincided to among the highest values detected (Fig 2a-d). Most microbial activity is associated with temperature and moisture; a higher activity should be expected at higher temperature if moisture is present in enough quantities which was the case here. The leucine assimilation activity observed in the buffer was similar to what has been registered for the deep groundwater of the Stripa research mine (Pedersen and Ekendahl 1992*a*) and the Äspö hard rock laboratory environments (Pedersen and Ekendahl 1992*b*), which harbours between 10^4 and 10^5 cells per mL of groundwater. The percentage of bacteria active in

leucine uptake in Stripa and Äspö were from 9 to 99 % and it was possible to calculate the assimilation per bacterium for these results. However, as there were only negative results for the microautoradiography, such calculations cannot be done for the BCE.

The three samples analysed for PLFA (H, M and P, depth 1.9, 4.2 and 5.3 m, respectively) contained significant numbers of bacteria with intact membranes which were, therefore, potentially viable. The viable biomass indicated by the PLFA concentrations varied from 1.58 x 10^6 to 8.25 x 10^6 cells/gdw buffer. These numbers, at first inspection, do not fully corroborate the viable number results obtained in corresponding buffer samples (Fig. 2). The potentially viable biomass found in samples from layer H (depth 1.9 m), a cool sample with ample moisture present, was lower than in the other two samples, which came from hot and dry (M, depth 4.2 m) and warm and moist (P, depth 5.3 m) locations. Fig. 2 shows considerable growth in layer H in contrast with no growth in the samples from layer M (in the hot locations adjacent to the heater) and less growth in layer P. The PLFA analysis indicated a definite stationary growth phase for sample P (Table 6), while especially for sample M a log phase was indicated. All three samples had characteristics of starved bacteria and this was especially pronounced for sample P. Unbalanced growth, as indicated by the ratio of PHA/PLFA was indicated for sample M. These results suggest that shortly after the heater was started, the temperature in the layers around the heater (K, L, M and N, depth 2.95 to 5.0 m) increased rapidly, possibly stimulating growth more in these layers than anywhere else. The moisture content likely changed considerably more slowly than the temperature in these layers. When conditions became more harsh and dry, growth slowed and desiccation occurred, to the point that the bacteria were no longer culturable, but still detectable by PLFA analysis. The fact that less biomass was indicated for the coolest sample from layer H may reflect the absence of growth stimulation due to increased temperature.

5.5 CONCLUSIONS RELEVANT FOR NUCLEAR WASTE DISPOSAL

The results from this study suggest that, for some time after disposal of containers with used nuclear fuel waste in a subsurface vault, the area around these containers (i.e., the part of the buffer directly adjacent to the containers) would be virtually devoid of microbial activity because of the redistribution of the initial moisture as a result of the high temperatures of the waste containers. This 'sterilisation' effect likely would be further enhanced by the high initial radiation emanating from the containers (Stroes-Gascoyne et al. 1995; King and Stroes-Gascoyne 1995). It has been argued (Stroes-Gascoyne and West 1994) that microbial repopulation of an initially deplted zone around waste containers may be limited due to the extremely small pore size of the buffer material. This argument remains to be verified. Water activity in buffer materials is determined by the amount of bentonite in the mixture. Reducing the water activity by increasing the bentonite content of buffer material may, therefore, be a potential approach to limiting microbial activity in the vicinity of waste containers if it were found that repopulation is possible.

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Culturability and 16S rRNA gene diversity of microorganisms in the hyperalkaline groundwater of Maqarin, Jordan

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CONTENTS

1 ABSTRACT

The Maqarin site in northern Jordan is unique, situated in bituminous marls which have been thermally altered by natural *in situ* combustion. As a result of this, the ground waters discharging at Maqarin are hyperalkaline and geochemically similar to Portland cement pore water. The site is, therefore, considered to be an excellent natural analogue for the high pH environments that will dominate around and in low- and intermediate level waste repositories. Among the questions to be answered in this work was if microorganisms can survive and be active at the extreme pH values typical for the Maqarin groundwater. Molecular methods, microscopy, culturing techniques and chemical analysis were used in an attempt to study these questions. Microorganisms were found in all of the Maqarin groundwaters but it could not be conclusively demonstrated that they were viable and growing *in situ*, rather than just transported there from neutral groundwater. The diversity of the found microorganisms was similar to what has been detected with the 16S rRNA gene sequencing method earlier, but none of the sequences found were typical for known alkalophilic organisms. A possible hypothesis based on the obtained results is that the majority of the investigated Maqarin springs may be a bit to extreme for active life even for the most adaptable microbe - but this remains to be demonstrated.

2 INTRODUCTION

Many radioactive waste repository concepts envisage the use of large quantities of cement and cement-based materials which will create environments with initial pH values of up to 13.5. The natural springs of the Maqarin area in NW Jordan contain highly alkaline groundwater, with pH values as high as 12.9, occurring within an organic-rich marl formation (Khoury et al 1992). This environment is, therefore, regarded as a natural analogue for the study of processes that may take place in the hyperalkaline parts of low and intermediate radioactive waste repositories (Miller et al 1994).

An earlier investigation has indicated the presence of microorganisms in the alkaline groundwater of Maqarin by the use of culturing techniques for the assessment of numbers and types of bacteria (Coombs et al 1994). A diversified microbial population was found with a pH tolerance in cultures up to the highest pH studied, pH 11. A well documented problem when using culturing techniques to reveal microbial diversity is the "great plate count anomaly" of environmental samples as recently reviewed by Amann et al. (1995). Generally, only a few percent or less of the total microbial

population in environmental samples can be successfully cultured. Non-culturing techniques, such as extraction and sequencing of the ribosomal DNA can be applied to overcome that problem (Pedersen et al 1997). Still, if both culturing results and molecular data show that microbes can be found in the Maqarin alkaline spring water, such results have, however, not proven that the found microorganisms were actively growing in the highly alkaline ground water, rather than just transported there from neutral pH ground layers. Investigation of solid material that is exposed to high pH water for growth of microbes in biofilms or for other signs of attached growth may be a possible way to prove that microbes can be active at these high pH values. This paper describes how molecular methods, microscopy, culturing techniques and chemical analysis were used in an attempt to study the culturability and diversity of microbial populations detected in the hyperalkaline ground water of Maqarin.

3 MATERIALS AND METHODS

3.1 SAMPLING SITES AND SAMPLING SCHEDULE

The Maqarin area is situated 80 km North of Amman at co-ordinates E. 35°50′ and N. 32°40′, where the Yarmouk river constitutes the Jordanian-Syrian border. The rock formation of interest is a Cretaceous organic-rich marl, known locally as the "Bituminous Marl Formation". The geology of the site is described by Khoury et al. (1992). The marl is a biomicrite, composed essentially of calcite with accessory quartz, dolmite, apatite, pyrite and clay minerals, and has a high organic content, up to 20%. In places, the rock has undergone spontaneous combustion generating high temperature, low pressure metamorphism. Subsequent intrusion of groundwater led to the production of a suit of natural cementitious minerals. Further leaching of these retrograde phases has produced the current, hyperalkaline groundwaters with a maximum pH at 12.9.

The samples analysed here were taken during November 1994, April 1995 and November 1996 from three major sites referred to as the Eastern springs, Adit A-6 and Western springs (Fig. 1). Water was collected in sterile plastic tubes and treated according to the descriptions below. The November 1994 sampling followed heavy rainfall and a period of flooding of the Yarmouk river. In contrast, the April 1995 and November 1996 sampling occurred after dry periods. Table 1 summarizes sampling sites and gives relevant information about the sampling schedule. The Eastern springs M3 and M8 represents hyperalkaline discharge seeps through marls downstream of a metamorphic zone. These seeps have been opened by a former railway cut that toaday is a road cut. M18 represents a mixture of

hyperalkaline groundwater and rainwater collected from a borehole drilled in the road. This borehole was sampled in series during the 1994 expedition after that 1 liter (M18-1), 2 liter (M18-2) and 25 liter (M18-3) of groundwater had been removed from the borehole. It was also sampled once during the 1996 expedition, but the sample obtained was very turbid because malfunctioning pumps made it impossible to get a representative sample. The Adit A-6 is a long tunnel that cuts into the hillside at some 50 m above the river. There are active seeps on several sites in the tunnel but the most massive one, that is possible to reach safely, is situated 140 m from the tunnel entrance and is denoted M1. This seep represents a hyperalkaline active discharge from a metamorphic zone.

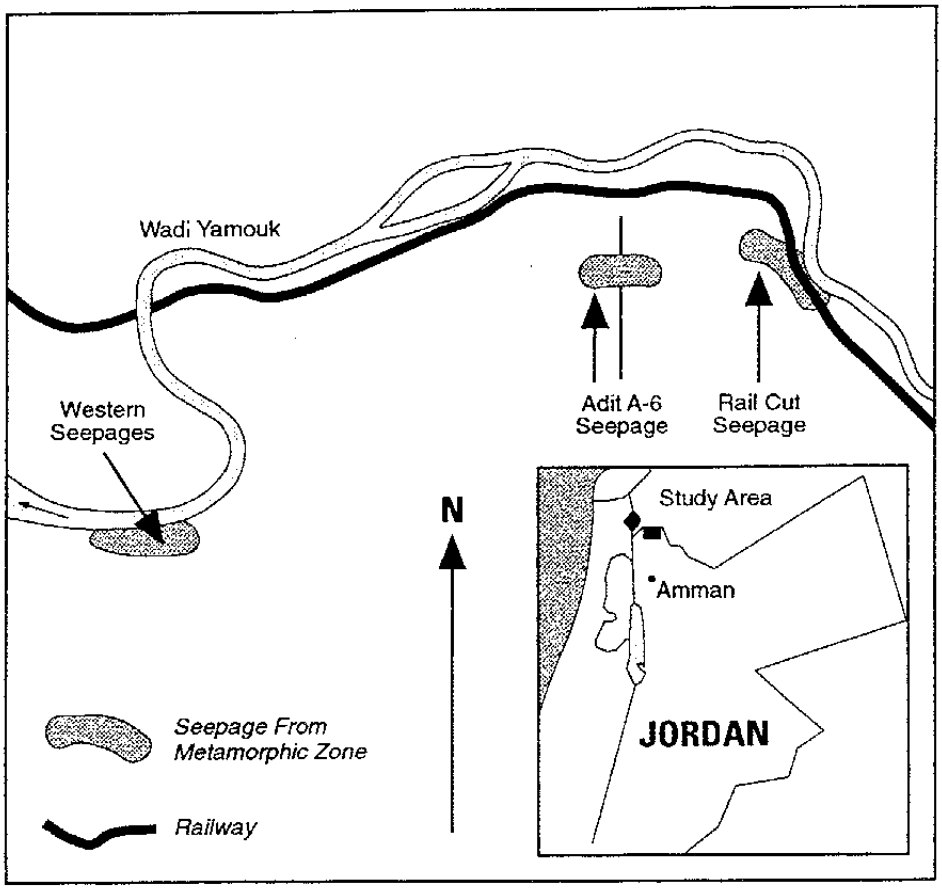

Figure 1 *Location of the Maqarin natural analogue study site in northern Jordan. The sites of the hyperalkaline springs are shown (From: Miller et al 1994).*

Table 1 *Site and sampling information for three expeditions to Maqarin in November 1994, April 1995 and November 1996.*

Sampling site	Site description	pH	Microbiology sampling year			Ground water chemistry
			1994	1995	1996	1996
Eastern springs						
M3	Road cut seep	alkaline		x	x	x
M8	Road cut seep	alkaline	x		x	x
M18	Borehole in the road	neutral	x		x	x
Adit A-6						
M1	Seeps 140 m inside a tunnel	alkaline		x	x	x
Western springs						
Wadi Sidjin (WS)	Seep in a valley	alkaline			x	x
M5	Seep at the rive side	alkaline	x	x	x	x
M6	Seep at the river side	alkaline			x	x
M17	Borehole in the road	neutral	x			-

The Western spring WS represents an alkaline seep in a small valley a couple of hundred m away from the river. The Western springs M5 and M6 are situated close to the river side of the Yarmouk River and represent hyperalkaline seeps through basalt-chert-limestone colluvium about 0.5-1 m above the river level. M17 represents a borehole containing neutral groundwater characterised by a smell of H_2S. It was sampled in series 1994 after that 0 liter (i.e. start of pumping, M17-1), 2 liter (M17-2), 3.5 liter (M17-3), 5 liter (M17-4) and 20 liter (M17-5) was removed from the borehole.

3.2 DETERMINATION OF THE TOTAL NUMBER OF BACTERIA

Samples for determination of total number of bacteria were taken in sterile plastic tubes and immediately preserved with formaldehyde (2% final concentration). All samples were transported to the laboratory in Göteborg for counting. The total number of bacteria in the groundwater was determined by the AODC method (Pedersen and Ekendahl 1992). Briefly, the groundwater samples were diluted to twice their volume with sterile filtered 0.1% oxalic acid and vigorously shaken to reduce clogging of the filters used. A portion of the sample was filtered onto a Sudan-black stained Nuclepore filter of 0.22 µm pore size and 13 mm in diameter at -20 kPa and stained for 6 min. with acridine orange. All solutions were filter sterilised (0.22 µm). One or up to three parallel samples were analysed and two filters were counted for each water sample. The number of bacteria was counted using blue light (390-490 nm) under an epifluorescence microscope (Olympus BH-2). Between 500 and 600 cells or a minimum of 15 fields

(0.0064 mm^2 each) were counted on each filter. This procedure will predict a sample mean with a precision of 5 % (Hallbeck and Pedersen 1990, Niemelä 1983). The results were calculated as mean values of all filters from each site.

3.3 *IN SITU* HYBRIDIZATION ON ROCK SAMPLES

Samples of stalactites and stalagmites from the M1 site in the Adit A-6 tunnel, and rock samples from the alkaline springs were collected and preserved with a sterile filtered cold 4 % paraformaldehyde. It was ensured that the samples had been in contact with flowing alkaline water at the respective site. After twenty-four hours in the 4% paraformaldehyde solution, the rock samples were removed from and washed with a phosphate buffer solution (PBS) and put into a tube with PBS and ethanol (50/50%). Autofluorescense of the samples were checked at the wavelength to be used. For Cy5 marked nucleic acid probes used, the wavelength is 647nm. If the minerals had a too high autofluorescense it was not possible to resolve the *in situ* hybridization signal from the background.

3.4 GROUND WATER COMPOSITION AND PH

A tube was connected to the WS, M5 and M6 springs and the water was allowed to flow for a couple of minutes before it was collected into a 1 litre bottle. At M3 and M8, the water was collected via plastic bags. At M18, ground water was pumped out of the borehole with a tube and submersible pumps. Unfortunately, the pumps did not work correctly, so it was not possible to pump the borehole free of standing water before sampling. Chemical analysis was subsequently made by KM Lab, Norrby Tvärgata 9, 504 37 Borås, Sweden (a laboratory accredited by Swedac).

The *in situ* pH was measured directly at each site with a pHep3-meter (Göteborgs termometer fabrik) calibrated with buffers 4.01 and 7.00. The pH was also measured 3 or 4 days and 27 days after sampling with a PHM 83 pH-meter (Radiometer Copenhagen) calibrated with buffer 4.01 and 7.00, and by KM lab.

3.5 SAMPLING AND ENRICHMENT OF ALKALOPHILIC MICROORGANISMS

During the 1996 expedition, a total of 100 ml spring water was collected directly from each alkaline spring in two separate 300 ml sterile Nalgene beakers (with a screw lid) supplied with different carbon sources. The beakers were put on a shaker in room temperature the third day after sampling, when back at the laboratory in Göteborg. Extra water was collected in sterile, empty beakers for use during sub-culturing and for counts of the total numbers of bacteria. Two different media were used and

inoculated with alkaline water from the springs: 1) 100 ml of water were collected to beakers with 0.1g peptone, 0.1g yeast extract, 0.5g glucose. 2) 100 ml of water were collected to beakers with 0.1g peptone, 0.1g yeast extract and 0.5 g lactate dissolved in 5 ml distilled H_2O. The enrichments were put on a shaker for 27 days in room temperature when they arrived to Göteborg three days after sampling. Subsequently, the total number of bacteria in the cultures were assayed using the AODC technique. Enrichments with growing cells were sub-cultured in fresh media.

One ml of the cultures that were growing with lactate were inoculated into the following three different media: 1) Site relevant medium: 0.1g peptone, 0.1g yeast extract, 0.5g lactate and 100 ml of sterile filtered water from the same source as the original culture was inoculated from. 2) Basal medium for alkalophilic mikroorganisms: Lactate 10g/l, polypeptone 5g/l, yeast extract 5g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2g/l, Na_2CO_3 10g/l, 100ml H_2O /beaker. 3) Basal medium for alkalophilic mikroorganisms. Lactate 10g/l, polypeptone 5g/l, yeast extract 5g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2g/l, Na_2CO_3 10g/l, 100ml H_2O /beaker. To beakers 2 and 3 we added 10 ml sterile filtered water from the same source as the original culture was inoculated from to ensure that necessary growth factors were included.

One ml of the cultures that were growing with glucose were sub-cultured in the following media: 1) 0.1g peptone, 0.1g yeast extract, 0.5g glucose and 100 ml of sterile filtered water from the same source as the original culture was inoculated from. 2) Basal medium for alkalophilic mikroorganisms: Glucose 10g/l, polypeptone 5g/l, yeast extract 5g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2g/l, Na_2CO_3 10g/l, 100ml H_2O /beaker. 3) Basal medium for alkalophilic mikroorganisms: Glucose 10g/l, polypeptone 5g/l, yeast extract 5g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2g/l, Na_2CO_3 10g/l, 100ml H_2O /beaker. To beakers 2 and 3 we added 10 ml sterile filtered water from the same source as the original culture was inocculated from. The sub-cultures were put on a shaker in room temperature for 19 days before they were observed in microscope using the AODC technique. The pH of the culture was measured simoultaneously with observation.

3.6 DNA EXTRACTION, PCR-AMPLIFICATION, CLONING AND SEQUENCING

Groundwater for DNA analysis was sampled from M5, M8, M17-1, M17-2, M17-5 and M18-3 in November 1994, and from the sites M1, M3 and M5 in April 1995. The groundwater was collected directly from the boreholes or seeps in sterile plastic tubes and was immediately deep frozen with CO_2 ice and transported rapidly to the laboratory in Göteborg, where 10 ml samples were filtered onto sterilised 0.2 µm pore-sized Nuclepore filters. DNA extraction, PCR amplification, cloning and sequencing followed a procedure published elsewhere (Pedersen et al 1996b). A Pfu DNA polymerase (Stratagene) was used, which has a proof reading ability that minimises the chance of chimer formation.

3.7 SEQUENCE ANALYSIS

The 16S rRNA gene clone sequences found were compared to sequences available in the European Molecular Biology Laboratory (EMBL) database using the FastA procedure in the GCG program package (Genetic Computer Group, Wisconsin, USA). This procedure calculates identities between an unknown sequence (clone) and sequenced bacteria in the database. The phylogenetic analysis was performed by using the programs contained in the PHYLIP version 3.5c package (Felsenstein 1989) compiled for PC. Nucleotide positions that could be unambiguously aligned for all clones were included in the analysis. The final data set comprised 301 nucleotide positions, position no. 537-833 (*E.coli* Brosius numbering, but not including 5 inserts in the analysed data set (Brosius et al 1978)). The distances were calculated using the DNADIST program and a tree was built running the KITCH program with contemporary tips. The KITCH program was run with a randomised input order of data with 10 jumbles and during execution, 60224 trees were examined. The tree was drawn by using a drawing program, DRAWTREE, also available in the PHYLIP package. The tree was used to differentiate the main lines of decent of the found sequences as described by Stackebrandt & Rainey (1995).

4 RESULTS

4.1 GROUND WATER CHEMISTRY

The Maqarin groundwater has been sampled for chemical analysis earlier and during the 1996 expedition. The results obtained before 1996 are presented elsewhere (Milodowski 1996) and are only briefly summarised below. The sampling sites M1, M3, M5 and M8 consist of alkaline water with a pH between 12.3 and 12.9 while the sites M17 and M18 were close to neutral with a pH of 7.2 and 8.2 respectively. The temperature was around 25 °C in all groundwater except for M18 which had a temperature of 18.5 °C. The total organic carbon contents (TOC) were 6-7 mg l^{-1} at M5, M8 and M18, with smaller amounts (1-3 mg l^{-1}) present in the other groundwater analysed. Nitrogen was present as nitrate in all waters (0.2 up to 35 mg l^{-1}), with the lowest concentration at M17 and the highest value in M5. There were high concentrations of calcium (87-1120 mg l^{-1}) and sulphate (280-1410 mg l^{-1}) in all waters analysed. Phosphorus was present in all sampled groundwaters, with highest concentrations in M5 (0.12 mg l^{-1}) and lowest at the sites M17 and M18 (less than 0.02 mg l^{-1}). The 1996 expedition data is shown in Table 2. It shows that Western springs, M5 and M6 are characterised by groundwater with higher pH, Ca, Na, K, NH_3, Cr, SO_4 and NO_3 concentrations than the Eastern Springs and M1.

Table 2 *Physical conditions and chemical composition of Maqarin spring and well water sampled during the 1996 expedition.*

Component	Method	Sort	M8	M18	M1	WS	M5	M6
Colour	SS028124-2		20	brown	5	15	yellow /green	yellow /green
Turbidity	SSEN 27027	FNU	0,44	37	0,68	1,3	0,12	3
Smell	SLV 900101		weak	strong	none	none	weak	weak
Smell, type	SLV 900101		-	wastewater	-	-	chalky	chalky
Precipitate	SLV 900101		small	very large	rather large	rather large	small	small
Precipitate, type	SLV 900101		brown particles	brown grains	white particles	sand particles	brown grains	brown grains
COD (Mn)	SS 028118	mg/l	4	19	<1	<1	<1	<1
Conductivity 25 °C	SSEN 27888	ms/m	483	203	579	764	935	927
pH	SS028122-2		12,1	7,4	12,2	12,3	12,3	12,3
Alkalinity, HCO_3	SS 028139	mg/l	1400	630	1800	2100	2300	2200
Hardness total	Calculated	odH	67,2	76,8	98	131,7	154	154
Hardness tot, Ca+Mg	Calculated	mg/l	480	549	700	941	1100	1100
Iron	FE-NI	mg/l	<0,05	0,8	<0,05	<0,05	<0,05	0,12
Manganese	MN-NI	mg/l	<0,01	0,03	<0,01	<0,01	<0,01	<0,01
Ammonium, NH_4-N	SS 028134	mg/l	0,11	0,024	<0,02	0,05	1,9	1,9
Nitrite, NO_2-N	SSEN 26777	mg/l	0,028	0,018	0,006	0,011	0,28	0,51
Nitrate, NO_3-N	SS028133-2	mg/l	2	<0,2	0,7	1,1	7,7	8,1
Phosphate, PO_4-P	SS028126-2	mg/l	<0,01	0,01	<0,01	<0,01	<0,01	<0,01
Fluoride	SS 028135	mg/l	0,39	1,7	0,34	0,45	1.0	1,1
Chloride	SS 028120	mg/l	53	163	55	87	47	48
Sulphate	SS 028182	mg/l	325	780	280	400	1300	1300
Sodium (ICP)	NA-A2I	mg/l	28	110	49	40	140	150
Potassium (ICP)	K(AIM)	mg/l	4	35	9	18	530	550
Zink (ICP)	ZN-AIM	mg/l	<0,01	0,06	0,09	0.12	0,02	0,02
Cadmium (ICP)	CD-AIM	mg/l	<0,01	<0,01	0,01	0,01	0,01	0,01
Nickel (ICP)	NI-AIM	mg/l	<0,02	0,03	<0,02	<0,02	<0,02	<0,02
Magnesium (ICP)	MG-NI	mg/l	<0,1	54	<0,1	0,4	<0,1	<0.1
Copper (ICP)	CU-AIM	mg/l	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Aluminium (ICP)	AL-AIM	mg/l	0,1	0,3	<0,1	0,1	0,1	0,3
Barium	BA-AIM	ug/l	30	70	30	30	20	20
Litium (ICP)	LI-NI	mg/l	0,02	0,08	0,02	0,06	0,48	0,48
Lead, Pb (ICP)	PB-AIM	mg/l	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1
Cobolt, Co (ICP)	CO	mg/l	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
Bor, B (ICP)	B-AIM	mg/l	<0,01	0,07	<0,01	<0,01	0,11	0,12
Chromium, tot, Cr (ICP)	CR-AIM	mg/l	0.88	<0.01	0,73	0,72	5.0	5.0
Calcium, Ca (ICP)	CR-A2IM	mg/l	480	460	700	940	1100	1100
Strontium, Sr (ICP)	SB-AIM	mg/l	3.7	11	6.4	15	14	14

4.2 TOTAL NUMBER OF BACTERIA

The five samples collected in series during 1994 from borehole M17 (1-5), and the three from borehole M18 (1-3) show an initial decrease in total number of bacteria ml^{-1} that was stabilised after that approximately 5 l of water had been flowing (Fig. 2). The total number of bacteria was highest in the neutral pH groundwaters, M17 and M18 (Fig. 2 and Table 3), and ranged between 1 up to 3 orders of magnitude higher than the total number of bacteria in water from the alkaline seeps M1, M3, WS, M5 and M8. There was a relatively large number of bacteria in M8 during the 1996 expedition.

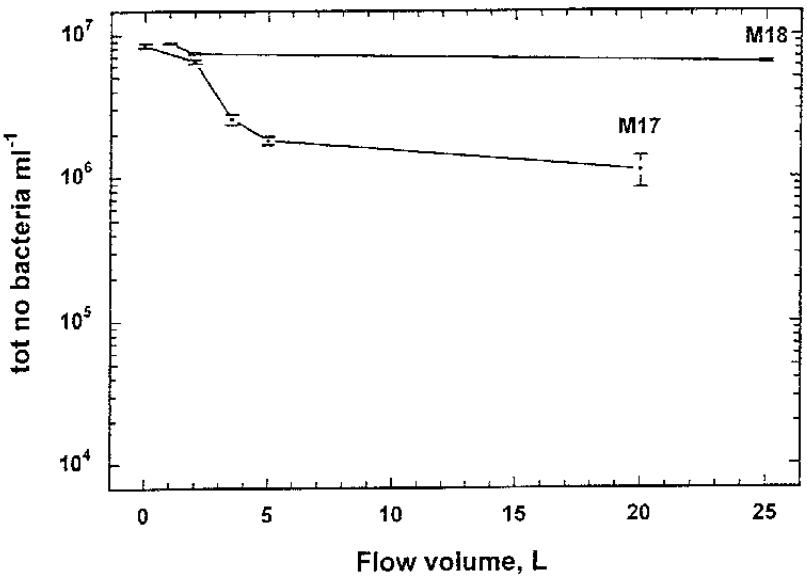

Figure 2 *The change in total number of bacteria in the boreholes M17 and M18 during pumping of groundwater in April 1994.*

Table 3 *Total number of bacteria in groundwater samples from the Maqarin area in Jordan as determined by AODC.*

Sampling site			Total number of bacteria ml^{-1}		
	letter	pH	Nov. 1994	Apr. 1995	Nov. 1996
Eastern springs					
M3	h	alkaline	-[a]	2.0×10^4	nd[b]
M8	b	alkaline	1.2×10^4	-	1.3×10^5
M18-3	f	neutral	6.4×10^6	-	-
Adit A-6					
M1	g	alkaline	-	2.5×10^3	nd
Western springs					
WS	-	alkaline	-	-	$9.8\ 10^3$
M5	a	alkaline	nd	3.0×10^3	$4.6\ 10^3$
M6	-	alkaline	-	-	$2.4\ 10^4$
M17-1	c	neutral	8.5×10^6	-	-
M17-2	d	neutral	6.5×10^6	-	-
M17-5	e	neutral	1.1×10^6	-	-

[a] No sampling
[b] Not determined due to high background caused by much precipitates

Table 4 *Enrichment cultures with glucose or lactate as carbon source and the subsequent action taken after assessing growth of enrichment cultures at day 27 after inoculation in Maqarin.*

Sampling site	Culture	Result	Action taken
Eastern springs			
M3	lactate	no growth	cancelled
M3	glucose	no growth	cancelled
M8	lactate	growth	sub-cultured, see Table 5
M8	glucose	growth	sub-cultured, see Table 5
M18	lactate	no growth	cancelled
M18	glucose	no growth	cancelled
Adit A-6			
M1	lactate	growth	sub-cultured, see Table 5
M1	glucose	no growth	cancelled
Western springs			
WS	lactate	no growth	cancelled
WS	glucose	no growth	cancelled
M5	lactate	no growth	cancelled
M5	glucose	no growth	cancelled
M6	lactate	no growth	cancelled
M6	glucose	no growth	cancelled

Table 5 *Results from the sub-culturing of the selected enrichment cultures after 19 days.*

Enrichment culture, from Table 4	Media used	pH	Result
M8-lactate	Lactate media 1	8.5	heavy growth
M8-lactate	Lactate media 2	10.1	growth
M8-lactate	Lactate media 3	10.1	weak growth
M8-glucose	Glucose media 1	8.6	no growth
M8-glucose	Glucose media 2	10.1	no growth
M8-glucose	Glucose media 3	10.0	no growth
M1-lactate	Lactate media 1	10.8	no growth
M1-lactate	Lactate media 2	10.1	no growth
M1-lactate	Lactate media 3	10.1	no growth

Table 6 *pH of the water sampled during the 1996 expedition and the pH values that developed during culturing experiments.*

Sampling site	Measured at the sampling site	Laboratory results	After 3 days in a glucose culture	After 3 days in a lactate culture
Eastern springs				
M3	12.1	n.a.[a]	11.6	12.7
M8	11.9	12.1	11.4	12.5
M18	7.6	7.4	n.a.	n.a.
Adit A-6				
M1	11.9	12.2	11.5	12.6
Western springs				
M5	12.0	12.3	11.8	12.9
M6	12.0	12.3	11.7	12.9
Wadi Sidjin	11.9	12.3	11.9	12.9

[a] not analysed

4.3 ENRICHMENT OF ALKALOPHILIC MICROORGANISMS

The cultures were filtered and stained for AODC and enrichments to be sub-cultured were chosen if growth could be detected. Only a few of the cultures had grown and were sub-cultured again in new medium (Table 4 and 5). The pH was followed during incubation of the enrichment cultures and decreased approximately 0.5 units in the glucose based medium and increased about 0.5 units whit lactate as carbon source (Table 6). pH was initially high when using the Maqarin ground water as water source, but the preparation of synthetic media with a pH that was stable at around 12.5 was unsuccessful.

4.4 SEQUENCING 16S rRNA OF ISOLATED BACTERIA

Two isolates were obtained from the M8 enrichments and their 16S rRNA gene could eventually be successfully sequenced but we encountered, for unknown reasons, several difficulties with our sequencing protocol. None of the found sequences coincided with earlier obtained sequences (Table 7).

4.5 *IN SITU* HYBRIDIZATION

A mineral sample that had been in contact with the M5 spring was analysed with epifluorescense microscope at wavelengths 390-490nm and 647nm. It had to much autofluorescense at both these wavelengths, so *in situ* hybridization could not be performed. The same procedure was done with a stalactite from the tunnel. It also had to much autofluorescense at the 647 nm wavelength. The mineral sample from M5 and the stalactite from the tunnel were additionally stained with acridine orange and studied under blue light but attached cells could not be detected.

Table 7 *Closest species in the EMBL database to 16S rDNA extracted from two of the enrichment cultures.*

Enrichment culture	Closest species in the EMBL database	identity %
M8-glucose	*Sphingomonas sp.*	92.8
M8-lactate	*Rhodobacter capsulatis*	97.9

4.6 DISTRIBUTION AND DIVERSITY OF BACTERIA AS ANALYSED BY 16S rRNA GENE ANALYSIS

A total of 87 clones were sequenced, 10 from each sampling site and sample occasion except for M8 with 9 clones and M17-2 with 8 clones (Table 8). The 87 clones examined clustered into 23 different, specific sequences, each of which was given a clone group name, ranging from JN1 to JN19 for samples obtained in November 1994 and JA21-JA24 for samples obtained in April 1995. The clone group names were given clone letters ranging from a to h. These letters show in which sample(s) the clone sequence was found (Tables 2 and 8). A clone number, 1-23, is used below to identify the clones in Table 8. Between 1 and 5 different 16S rRNA gene sequences were detected in each sample. Some of the clone groups have several identical clone sequences, while others represent only a few or one clone sequence. The 6 most commonly occurring clone sequences, No. 5, 6, 13, 16, 19, and 20 constituted 71 % of the sequenced clones (62 clones out of 87).

Typically, each of these clone group sequences predominated in a specific site with the exception for No. 16 that predominated in the three sites M1, M3 and M5 (Table 8) and comprised 29 % of all found sequences (25 clones out of 87). Three other clone groups were also detected on more than one site, No. 5, 19 and 20. The diversity was highest in the neutral pH groundwater from M17 with 5 different clone groups obtained for each of the three samples and a total of 14 different clone groups appeared here. The alkaline boreholes had a lower diversity with 1 or 4 clone groups per borehole. The lowest diversity was found in M1, with all 10 clone sequences belonging to the same clone group while 4 specific clone group sequences were found in M3, M5 and M8. Sampling site M18 also had low diversity with 2 clone groups.

The closest related species in the database for the clones are also shown in Table 8. The association of a clone sequence with a species in the database at identity values below approximately 95 % is rather meaningless because of the large phylogenetic difference at or below this level of identity. Therefore, closest species in the database is only given for clones with an identity at or larger than 95%. Most of the found sequences clustered with the Proteobacteria Alpha, Beta and Gamma groups, one, No 17, with Gram-positive bacteria and one, No 18, with the phylogenetic group *Thermus-Deinococcus* which is a rather ancient branch in the phylogenetic tree of *Bacteria* (Woese 1987) (Table 8 and Fig. 3). Four of the clones, No. 20, 21, 22 and 23 were only very distantly affiliated with known bacteria and may, therefore, represent new branches in the bacterial phylogenetic tree (Fig. 3). No. 20, occurring in M8 and M18-3, was, however, closely related to the clone sequence G15 found in groundwater from Bangombé in Gabon. The clone group sequence No. 13 was identical with the Bangombé sequence G21. High identities were also obtained with the genus *Acinetobacter* (No. 11, 12, 13, 14 and 15. Finally, one of the clone group sequence, No. 19, was closest related to an eukaryal species, a fungus, with 98.1 % identity.

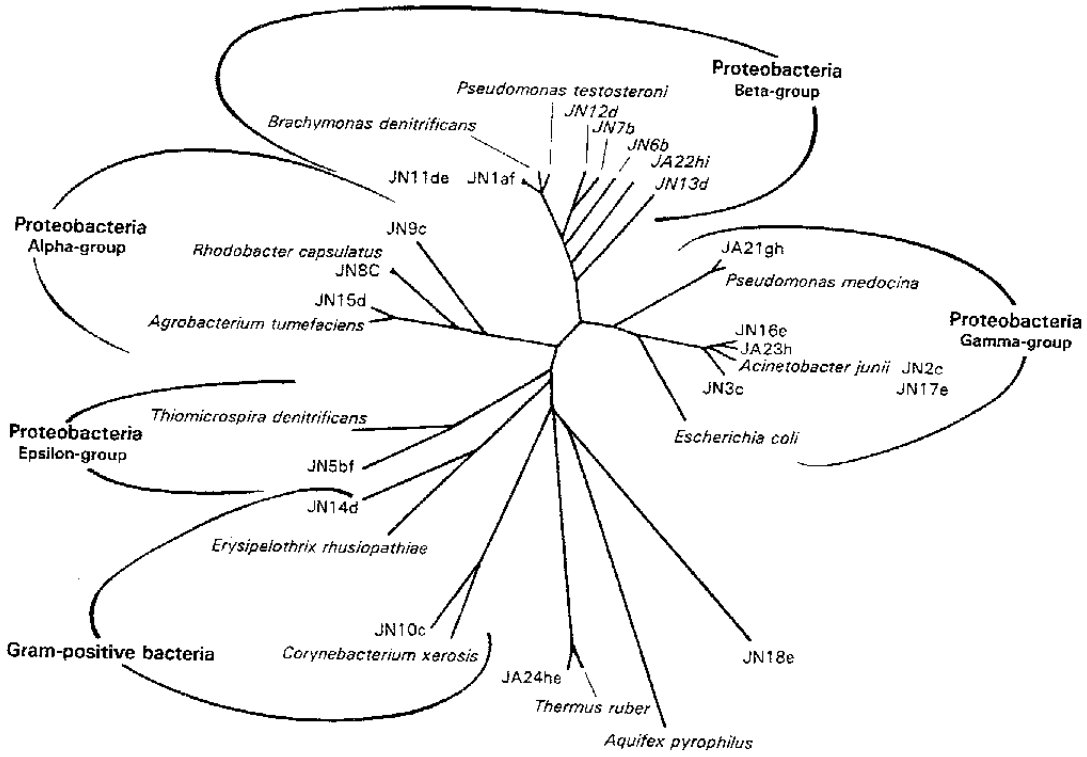

Figure 3 *Evolutionary distance tree based on the 16S rRNA gene sequences of clones from different localities in the Maqarin area. Four clone sequences were only very distantly related to known, sequenced and reported bacteria, and were, therefore, not affiliated in phylogenetic groups. Major phylogenetic groups of bacteria have been designated with their generally accepted names. As references, some 16S rRNA gene sequences of known bacteria from the EMBL database have been added to the tree and they are indicated with their Latin names. The tree was used to differentiate the main lines of descent of the found sequences, it will not unravel intragenic phylogeny.*

Table 8 *Distribution of the clones between the different Maqarin sample sites, sampled in November 1994 (clone groups JN1 to JN 19 from M5, M8, M17, M18) and April 1995 (clone groups JA21 to JA24 from M1, M3 and M5). Alkaline springs are shaded.*

No	Clone group	Closest sequence in the EMBL database	Identity (%)	Number of identical clone sequences: Eastern springs M3	M8	M18-3	Adit A-6 M1	M5	Western springs M17-1	M17-2	M17-5	Total
		Proteobacteria Alpha group										
1	JN15d	*Agrobacterium*										
		tumefaciens	95.4							1		1
2	JN8c	*Rhodobacter*										
		capsulatus	97.2						1			1
3	JN9c	n.ra.	93.5						1			1
		Proteobacteria Beta group										
4	JN7b	Bangombé, G16										
		(Rhodocyclus)	98.0		2							2
5	JN1af	Stripa clone group II										
		(*Pseudomonas*)	95.4			1		9				10
6	JN11de	Stripa clone group II										
		(*Pseudomonas*)	97.4							4	5	9
7	JN6b	n.r.	92.8		1							1
8	JN12d	n.r.	92.8							1		1
9	JA22ahi	n.r.	90.3	1				1				2
10	JN13d	n.r.	89.4							1		1
		Proteobacteria Gamma group										
11	JA23h	*Acinetobacter*										
		calcoaceticus	98.0	2								2
12	JN16e	*Acinetobacter*										
		lwoffii	97.4								1	1
13	JN2c	Bangombé, G21										
		(*Acinetobacter*)	100						6			6
14	JN3c	Bangombé, G21										
		(*Acinetobacter*)	95.0						1			1
15	JN17e	Äspö, S6										
		(*Acinetobacter*)	98.8								2	2
16	JA21agh	Äspö HRL, S25										
		(*Pseudomonas*)	98.8	6			10	9				25
		Gram-positive bacteria										
17	JN10c	n.r.	92.9						1			1
		***Deinococcus-Thermus* group**										
18	JA24he	n.r.	93.3	1								1
		Eukarya										
19	JN4ab	*Candida holmii*	98.1		3			1				4
		Remaing distant clones										
20	JN5bf	Bangombé, G15 (n.r.)	99.7		3	9						12
21	JN14d	n.r.	87.6							1		1
22	JN18e	n.r.	80.4								1	1
23	JN19e	n.r.	73.1								1	1
		Total no. of clone groups		4	4	2	1	2+2	5	5	5	23
		Total no. of clones		10	9	10	10	10+10	10	8	10	87

[a] To distant for a meaningful affiliation and, therefore, not relevant (n.r.).

4.7 ACCESSION NUMBERS

The nucleotide sequence data reported in this paper appears in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z69266 - Z69288 corresponding to JN1af - JN19e and JA21gh - JN24he, respectively.

5 DISCUSSION

So called "normal conditions" have been used to understand the foundations of bacterial cell functions and to describe the origin of physiological biochemistry. Normal conditions are usually defined as 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 conditions for life in a radioactive waste repository environments will in most respects be somewhat extreme, i.e. very different from what is understood as normal conditions. Modelling bacterial processes in radioactive 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 under normal conditions, when it comes to predicting bacterial processes in future waste repositories at hyperalkaline conditions, high temperature, radiation, etc. (Pedersen 1996, 1997b). 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 (Horikoshi and Akiba 1982). Although a high pH environment may be extreme for many bacteria, there appears to be no obvious limitation for adaptation of bacteria to a such environments.

Natural analogues and other sites with adverse conditions (e.g. radioactive waste sites) are, therefore, valuable sites to study for the task of modelling and assess the performance of future radioactive waste repositories. The Maqarin site in northern Jordan is unique, situated in bituminous marls which have been thermally altered by natural *in situ* combustion (Miller et al 1994). As a result of this, the ground waters discharging at Maqarin are hyperalkaline and geochemically similar to Portland cement pore water. The site is, therefore, considered to be an excellent natural analogue for the high pH environments that will dominate around and in low- and intermediate level waste repositories. Among the questions to be answered is if microorganisms can survive and be active at the extreme pH values typical for the Maqarin groundwater.

5.1 TOTAL NUMBER OF BACTERIA

A first approach to most environmental microbiology studies is to assay total numbers of bacteria which will reveal if microorganisms are present at all. The total number of bacteria found here, from 10^3 up to 10^7 bacteria ml^{-1} (Table 3), was within the range of what has been previously reported for other subterranean sites (Pedersen 1996). The decrease in total number

registered in the M17 and M18 boreholes during pumping (Fig. 2) was probably due to that stagnant water in the borehole contained more bacteria than the aquifer water subsequently pumped up after some time. Earlier investigations of the M1 and M3 sites by Coombs et al. (1994) in May 1992 indicated numbers in the same order of magnitude (M1 and M3 then had 1.7 x 10^4 [S.E. 2.6 x 10^3] and 6 x 10^4 [S.E. 2 10^4] bacteria ml^{-1} respectively). Coombs et al. (1994) also found attached bacteria on solid samples from M1 using scanning electron microscopy and the total number of attached bacteria on solids from the alkaline sites M1 and M3 were estimated by them to be 3.4 x 10^5 and 1.9 x 10^5 bacteria cm^{-2}, respectively. However, it should be stressed that the identification of bacteria using SEM only as reported, is not regarded conclusive unless structures typical for microorganisms are reported. The affected discussion concerning SEM images of what was suggested to be Martian microbes is a good example of the uncertainty that follows SEM identification alone (McKay et al 1996). We could not find any attached bacteria using *in situ* hybridisation and light microscopy investigation on samples collected during the 1996 expedition. This may be due to methodological problems or to that attached bacteria did not exist. Future experiments should include artificial surfaces introduced in flowing Maqarin alkaline water. Presence of microbial biofilms after a couple of months of exposure would be a conclusive evidence for that microbes can be active at the very high pH in these water, as indicated by the results by Coombs et al. (1994). Absence of microbial biofilms would indicate that microbial life is difficult in the studied ground waters.

5.2 ENRICHMENT AND ISOLATION OF ALKALIPHILIC MICROORGANISMS

The earlier investigation performed by Coombs et al. (1994) reported growth of bacteria at or below pH 11 but pH above 11 was not studied. Here, an attempt was made to grow bacteria at the high pH values prevailing in Maqarin with a partially successful result. It was possible to get cultures growing at the *in situ* pH using Maqarin groundwater as base, but it was not possible to make artificial media with pH above 12 as heavy precipitation occurred when the pH was raised. Growth occurred in *in situ* cultures with a pH above 12 (Table 6) but the pH did eventually decrease to below 10 after prolonged culturing (Table 5). It is, therefore, not possible to conclusively claim that the obtained growth occurred at high pH. Few of the enrichment cultures showed growth. It was mainly M8 that resulted in enrichment cultures which coincides with a total number of bacteria that was highest in M8 of the alkaline springs investigated. This particular spring seems to have been a bit less well buffered at high pH and to be a bit more diluted than the other alkaline springs (Table 2) and possibly thereby a bit more inhabitable. It was not possible to continue the enrichment experiments and isolate bacteria for identification, because they only grew with addition of Maqarin ground water as a growth factor additive and the availability of such groundwater was limited for transportation reasons.

5.3 16S rRNA GENE DIVERSITY AND DISTRIBUTION

A total of 23 different clone sequences was found which indicates the Maqarin area to be populated by a diverse microbial population. Most sequences were related to the Proteobacteria alpha, beta and gamma groups which agrees with what has been found on other subterranean sites (Ekendahl et al 1994, Pedersen 1997a, Pedersen et al 1996a, 1997). A few were only distantly related to sequences from known, cultured and sequenced bacteria contained in the database and may, therefore, represent new branches in the phylogenetic tree. One of these showed a high identity with other sequences from subterranean sites (Table 8), e.g. the clone group No. 20 (12 clones) that was closely related to the Bangombé sequence G15 frequently occurring there in two independent boreholes (13 clones). Also, several of the Maqarin site clone sequences had sequences from other subterranean sites as the closest related sequence in the database, (Table 8) implying that many of these sequences may represent a the subterranean biosphere (Pedersen 1993).

Each Maqarin sampling site generally had a clone sequence diversity that was specific and not related to the other sites, with the exception of the M1, M3 and M5 sites that shared 6 clone sequences of a *Pseudomonas* type bacterium (Table 8), and M8 and M18 that shared 3 sequences of the Bangombé G15 bacterium. This result is also consistent with what has been reported from three other subterranean areas investigated using a similar technique (Ekendahl et al 1994, Pedersen et al 1996a, 1997) and probably reflects a natural selection of species diversity due to differences in the geochemical situation between the investigated boreholes. The M17 borehole was sampled three times in series and the first sample, M17-1, did not share any similar sequences with the other two samples, M17-2 and M17-5, which shared 4 similar sequences, No. 6 (Table 8). The uniqueness of M17-1 seems due to that stagnant water in the borehole contained bacteria different from those in the aquifer water subsequently pumped up after some time. A repeated appearances of similar sequences in aquifer water from boreholes sampled in a series has been reported previously (Pedersen et al 1996b), and infer that the situation has stabilized in the pumped borehole with respect to species diversity and probably also with respect to the geochemical situation.

5.4 IDENTITY OF THE FOUND SEQUENCES

There is no accepted value of the percentage identity at which two 16S rRNA genes can be concluded to belong to the same genus or species. It can be quite different for different genera (Fox et al 1992) and is also due to whether total or partial 16S rRNA genes are compared. It has been suggested, based on a comparison of rRNA sequences and on DNA-DNA

reassociation, that a relation on the species level does not exists at less than 97.5% identity in the 16S rRNA sequence. At higher identity values, species identity must be confirmed by DNA-DNA hybridisation (Stackebrandt and Goebel 1994). Accepting the level 97.5% conservatively, as identifying a sequence approximately on the genus level, some conclusions can be made about the sequences reported here. A comparison of the 16S rRNA gene sequences with EMBL in March 1996, reveals 2 clone groups that can be identified with bacteria on the genus level and one with a fungus (Table 8). The *Acinetobacter* type 16S rRNA sequence No. 11 and the *Candida* type 18S rRNA sequence No. 19 had identities higher than 97.5 % with ribosomal rRNA sequences in the database. In addition, similarities higher than 97.4 % were obtained with several sequences of other subterranean bacteria detected previously, the clones No. 5-6 and 12-16. The sequence No. 13 from the neutral pH site M17 was 100% identical with the Bangombé G21 sequence representing a clone group that occurred in 3 of the 4 boreholes investigated in Bangombé, Gabon (Pedersen et al 1996b) (also a neutral pH groundwater).

In general, none of the closest species in the database is a typical alkaliphilic organism (Duckworth et al 1996) and it can, therefore, not be concluded from the 16S rRNA data that such organisms were present in Maqarin. Two possibilities remain: 1) the found sequences represent new and unknown alkaliphilic microorganisms that last to sequence. 2) The found sequences represent microorganisms that have been transported to the alkaline sites with groundwater from neutral pH environments.

6 CONCLUSIONS

- Microorganisms were found in all of the Maqarin groundwaters but it could not be conclusively demonstrated that they were *in situ* viable and growing, rather than just transported there from neutral groundwater.

- The diversity of the found microorganisms was similar to what has been detected with the 16S rRNA gene sequencing method earlier, but none of the sequences found were typical for known alkalophilic organisms.

- Finally, a possible hypothesis based on the obtained results is that the investigated Maqarin springs may be a bit to extreme for active life even for the most adaptable microbe (except M8) - but this remains to be demonstrated.

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APPENDIX 1

Sampling and analysis of gas content and composition in Äspö groundwaters

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CONTENT

1 BACKGROUND

The total amount of dissolved gas per volume of groundwater is important to study for several reasons. 1) A situation with oversaturation may cause gas bubble formation which can result in rapid two phase transport of possibly released radionuclides to the surface biosphere. 2) Experimental conditions close to the Äspö tunnel may include a decreased groundwater pressure, that in turn can generate a gasphase which will make interpretation of analysis data difficult unless the gas-content is known. 3) Methane and hydrogen are energy rich gases used by many microorganisms and their concentrations must be known for accurate modelling of microbial processes that may influence radioactive waste disposal. The reducing ability of microbes relates to the concentrations of these gases.

2 EXPERIMENTAL PROCEDURE

2.1 GAS SAMPLING

Sampling of groundwater for gas analysis can be done with two different methods. The so called BAT sampler has been used for deep boreholes earlier (Pedersen and Ekendahl 1992). Steel cylinders have more recently been used when sampling groundwater from boreholes in the Äspö tunnel.

2.1.1 BAT-sampler in boreholes

The BAT sampler is be used for sampling gas in deep boreholes. It consists of one or two evacuated steel cylinders that can be opened or closed at sampling depth by means of hypodermic needles. Problems frequently occurred with clogging of the needles by rubber material. There was also problem with uneven distribution of gas between sample containers if the two container configuration was used. The BAT sampler has, therefore, not been used for sampling in the Äspö tunnel. The analysis procedure described below will, however, allow analysis of BAT gas-samples if requested.

2.1.2 Sample cylinders in tunnel

A technically simple and very reliable sampling system has been developed for sampling of gas in Äspö tunnel groundwater. Stainless steel, teflon coated cylinders with a volume of 500 ml are fitted with valves and used as sample containers. The teflon coating will hinder anaerobic corrosion of the steel, which otherwise may generate hydrogen. All valves, tubing and pressure gauges are made of stainless steel (Swagelok). Sampling is done as follows (confer Fig. 1):

1. The lower end of the cylinder is connected to the borehole tubing and groundwater is allowed to flush through the cylinder until all air has been replaced by groundwater.

2. The groundwater flow is gradually reduced by means of a pressure valve that raises the sample cylinder pressure to a pressure just below where the flow stops. The exact pressure difference (approx 0.5 Bar) and flow depend on the maximum flow rate available in a borehole.

3. The cylinder is left with groundwater slowly passing for enough time to allow formation water that has not been de-pressurised to enter - preferably over night. This depends, however, on borehole section volume, flow rate and length of tubings.

4. Sampling site, section, date, formation pressure at start and end of sampling (valve 3 closed, valves 1 and 2 open), sampling pressure (valves 1-3 open, pressure valve just below formation pressure), flow rate during sampling and time with slow flow and possible parallel sampling are documented and reported with gas data.

5. The cylinders are closed and transported rapidly to the laboratory.

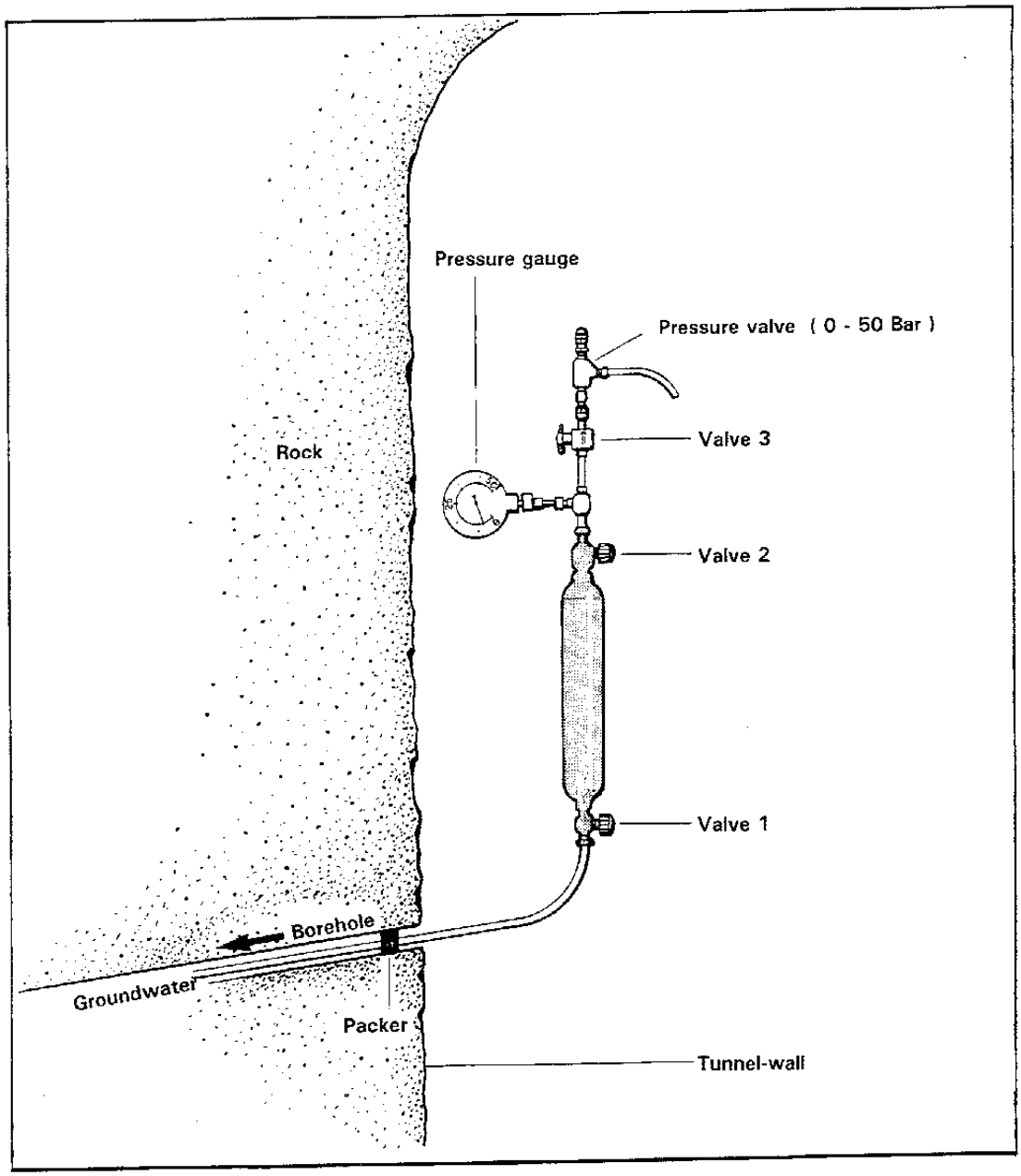

Figure 1 *Sampling of groundwater for gas analysis. See text, 2.1.2, for details.*

2.2 EXTRACTION AND MEASUREMENT OF GAS VOLUME

The dissolved gasses are extracted according to Fig 2.

1. The sample cylinder is connected to the extraction system by means of an NS14 fitting (stainless steel/glass).

2. The extraction system is evacuated to $< 10^{-3}$ mBar.

3. The lower sample cylinder valve is opened.

4. The extracted gas is collected in a calibrated burette (8) fitted with a septum (13) by stepwise pumping with mercury and compressed nitrogen (1.5 bar) (15).

5. The amount of dissolved gas is registered when the mercury levels in the burette and the manometer (9) coincides (at atmospheric pressure), and the volume is registered and adjusted to 760 mm Hg atmospheric pressure.

6. Gas samples are taken through the septum (13) and used for gas chromatography analysis of the gas composition as described below. Gastight 50 ml syringes are used to transfer the gas from the extraction system to the GC. High precision Hamilton syringes with small sample volumes (10-50 μL) are used for column injections. The injections volumes vary between 2 and 20 μL.

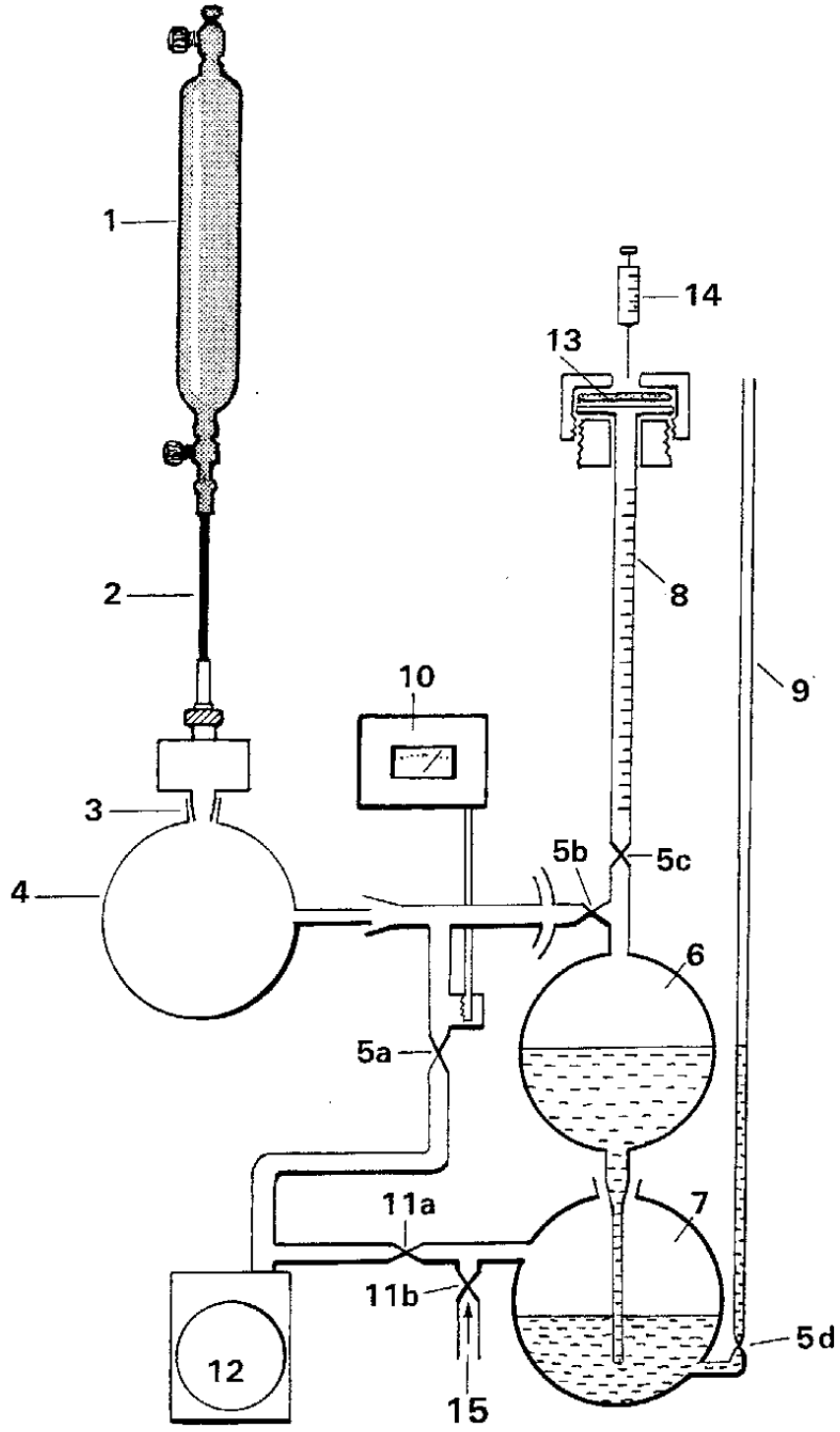

Figure 2 *Gas extraction equipment. See text, 2.2, for details. 1. sample cylinder; 2. flexible tubing; 3. brass/glass connection; 4. water sample container; 5a. valve 1; 5b. valve 2; 5c. valve 3; 5d. valve 4; 6 gas sample container; 7. mercury reservoir; 8. measuring burette; 9 manometer; 10. vacuum gauge; 11a valve 5; 11b. valve 6; 12. vacuum pump; 13. gas septum; 14. gas sample syringe; 15. compressed nitrogen, 1.5 Bar.*

2.3 MEASUREMENT OF GAS COMPOSITION

2.3.1 Gas chromatograpy conditions

Table 1 shows the detailed set-up of the two Varian GC machines in use and the detection limit for each gas analysed. Oxygen were analysed as a control for leakage of air during the sampling and analysis procedure. Undisturbed deep groundwater is generally anaerobic and should not contain oxygen.

Hydrogen, helium, nitrogen and oxygen were analysed on a Varian GC 3700, equipped with a 5 m long, 1/8 inch wide steel column packed with Molsieve 5A of a mesh size of 60/80, and with a thermal conductivity detector (TCD).

Carbon dioxide and carbon monoxide are analysed using a methaniser catalytic system on a Varian GC-3400 equipped a 2 m long, 1/8 inch wide steel column packed with Hayesep of a mesh size of 80/100 and with a flame ionisation detector (FID).

Methane, ethane and propane are determined with a Varian GC-3700 equipped with a 2 m long, 1/8 inch wide steel column packed with Porapak Q and with a flame ionisation detector (FID).

2.3.2 Calibration and integration of peaks

Calibration, integration of peaks and calculations of gas contents are done using a Star Chromatography Workstation, Varian, version 4.01 Software, running on an IBM 486 PC. Table 1 shows detailed information on methods used. Calibration for each gas is done with respective pure gas (analytical grade) and with a standard gas-mix of a composition typical for groundwater:

Reference gas composition, %:

Hydrogen	1.01
Helium	5.15
Nitrogen	83.8
Methane	4.02
Carbon dioxide	5.00
Carbon monoxide	1.02

Table 1 *Analysis conditions for gases dissolved in groundwater. FID=Flame Ionisation Detector; TCD=Thermal Conductivity Detector.*

Gas	Column material diameter and mesh size	Column length (m)	Injector Temp. (°C)	Column Temp. (°C)	Detector Temp. (°C)	Carrier gas flow (ml/min)	Retention time (min)	Sensitivity (nm)
Hydrogen	Mol Sieve 5A 1/8, 60/80	5	100	33	TCD +125 filament +250	Nitrogen 30-35	1.52	45
Helium	Mol Sieve 5A 1/8, 60/80	5	100	33	TCD +125 filament +250	Nitrogen 30-35	1.23	45
Oxygen	Mol Sieve 5A 1/8, 60/80	5	100	60	TCD +125 filament +250	Helium 30	2.85	0.4
Nitrogen	Mol Sieve 5A 1/8, 60/80	5	100	60	TCD +125 filament +250	Helium 30	5.94	90
Carbon monoxide	Hayesep Q 1/8, 80/100	2	100	40	FID +200	Nitrogen 30	0.64	5.0
Carbon dioxide	Hayesep Q 1/8, 80/100	2	100	40	FID +200	Nitrogen 30	1.56	2.0
Methane GC-3700	Porapack Q 1/8	2	100	100	FID +200	Nitrogen 30	0.6	4.5
Methane GC-3400	Hayesep Q 1/8, 80/100	2	100	40	FID +200	Nitrogen 30	0.81	5.0
Ethane	Porapack Q 1/8	2	100	100	FID +200	Nitrogen 30	1.5	4.5
Propane	Porapack Q 1/8	2	100	100	FID +200	Nitrogen 30	2.3	4.5

3 RESULTS

The boreholes KA3005A, KA3010A and KA3110A (see Fig. 1, paper 1) were repeatedly sampled during 1997 with the method described in Fig. 1. Each borehole was sampled once with two sample cylinders that subsequently were analysed after two different times. The difference in the extracted volume of gas was small between these two parallel samples and there was no correlation with the time span from sampling to analysis (Fig. 3).

The volume of dissolved gas (Fig. 3) and the gas composition (Fig. 4 and 5) obtained was in the range of what have been obtained earlier (see Table 1 in the summary). Occasionally, some oxygen appear in the results due to in-leakage of air during the extraction and analysis procedure. Initial problems with gas septa, sub-sampling and the extraction procedure probably caused those oxygen values.

Most of the extracted gas volume could be recovered with the gas chromatograph as different gas components, but there was occasionally gas "missing" (Fig. 6). Part of this "missing" gas may be Argon which was not analysed for.

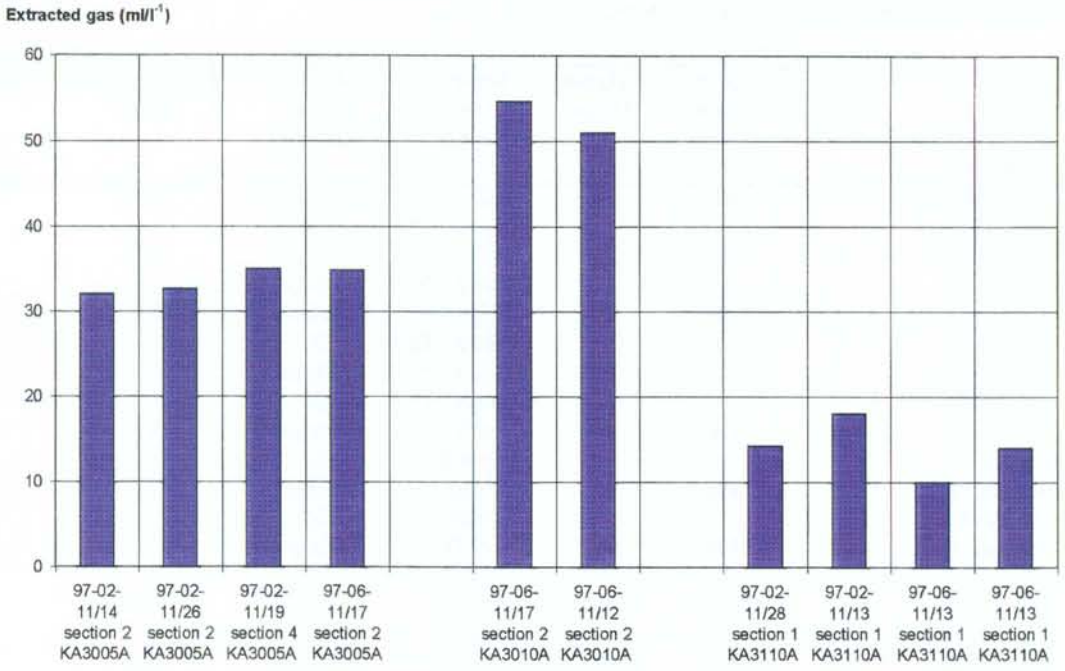

Figure 3 *The volume of extracted gas obtained with the device in Fig. 2. The diagram arrays the volume read on the measuring burette (8). The text under each bar denotes (from bottom) the sampled borehole, the borehole section sampled and sampling date/analysis date.*

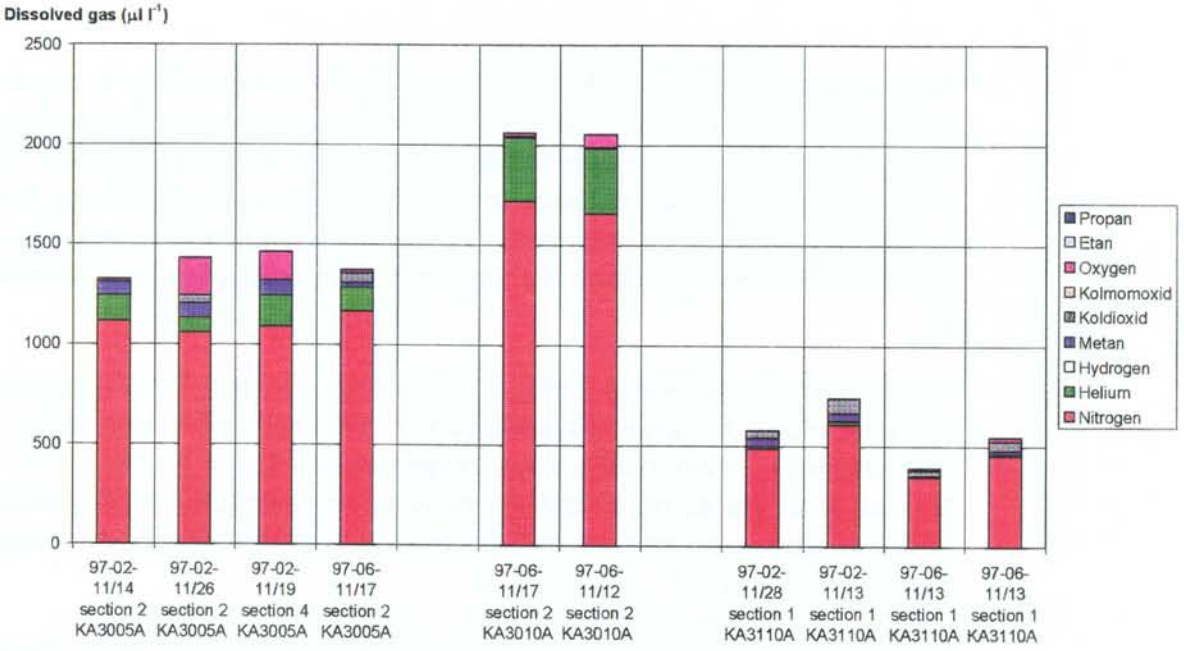

Figure 4 *The volumes of dissolved gas components as analysed with gas chromatography as described in section 2.3. The text under each bar denotes (from bottom) the sampled borehole, the borehole section sampled and sampling date/analysis date.*

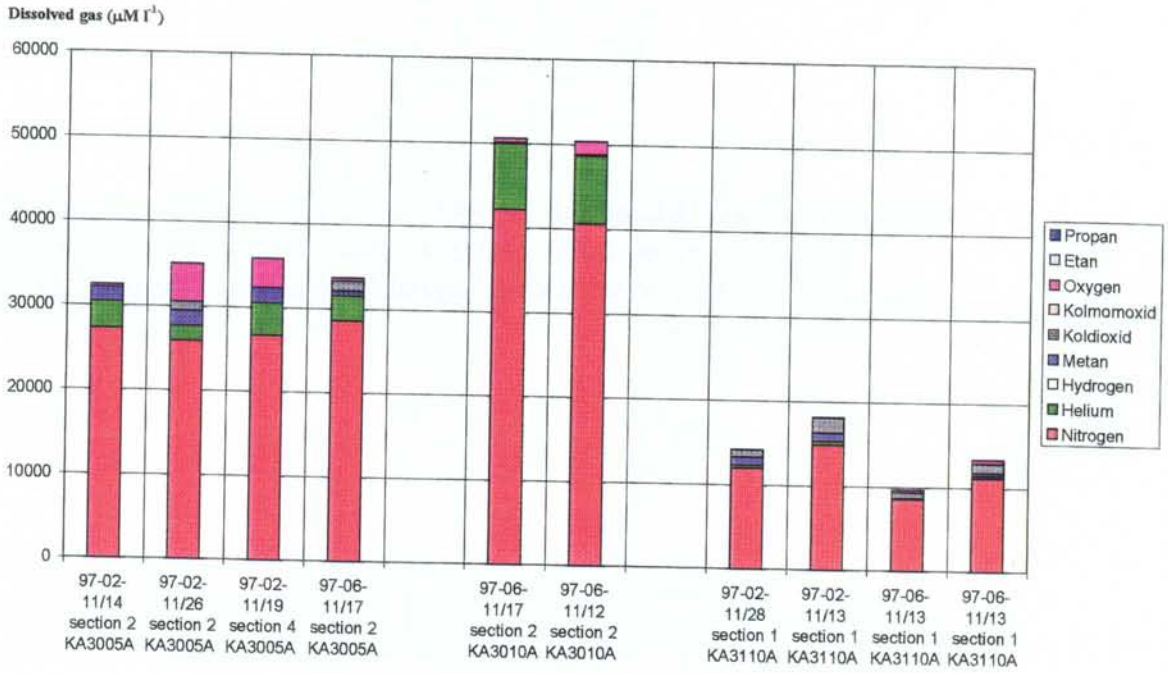

Figure 5 *The concentrations of dissolved gas components analysed with gas chromatography as described in section 2.3. The text under each bar denotes (from bottom) the sampled borehole, the borehole section sampled and sampling date/analysis date.*

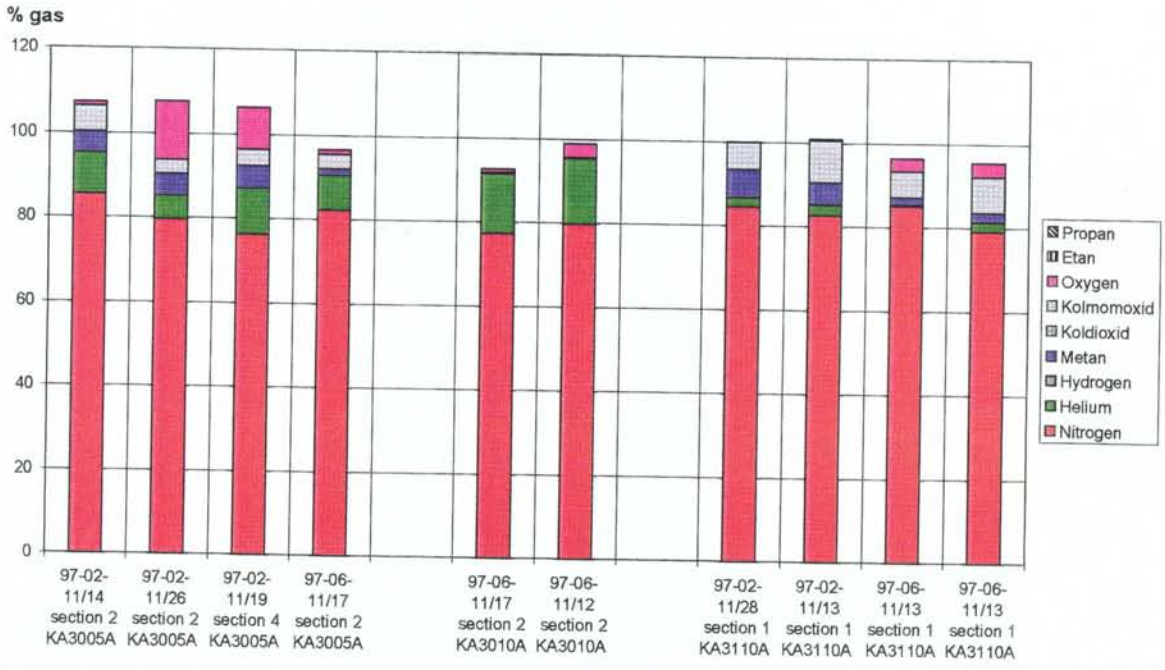

Figure 6 *The percent gas components recovered with gas chromatography in relation to the volume of gas extracted in Fig 3. The text under each bar denotes (from bottom) the sampled borehole, the borehole section sampled and sampling date/analysis date.*

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Pedersen, K. and Ekendahl, S. 1992. Assimilation of CO_2 and introduced organic compounds by bacterial communities in ground water from Southeastern Sweden deep crystalline bedrock. *Microbial Ecology,* Vol. 22, 1-14.

APPENDIX 2

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

2. **Pedersen, K. 1989.** Deep ground water microbiology in Swedish granitic rock and it's relevance for radionuclide migration from a Swedish high level nuclear waste repository. 13 p. *SKB Technical Report, 89-23.*

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APPENDIX 3

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Retention mechanisms and the flow wetted surface – implications for safety analysis

Mark Elert
Kemakta Konsult AB
February 1997

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Äspö HRL – Geoscientific evaluation 1997/1. Overview of site characterization 1986–1995

Roy Stanfors[1], Mikael Erlström[2], Ingemar Markström[3]
[1] RS Consulting, Lund
[2] SGU, Lund
[3] Sydkraft Konsult, Malmö
March 1997

TR 97-03

Äspö HRL – Geoscientific evaluation 1997/2. Results from pre-investigations and detailed site characterization. Summary report

Ingvar Rhén (ed.)[1], Göran Bäckblom (ed.)[2], Gunnar Gustafson[3], Roy Stanfors[4], Peter Wikberg[2]
[1] VBB Viak, Göteborg
[2] SKB, Stockholm
[3] VBB Viak/CTH, Göteborg
[4] RS Consulting, Lund
May 1997

TR 97-04

Äspö HRL – Geoscientific evaluation 1997/3. Results from pre-investigations and detailed site characterization. Comparison of predictions and observations. Geology and mechanical stability

Roy Stanfors[1], Pär Olsson[2], Håkan Stille[3]
[1] RS Consulting, Lund
[2] Skanska, Stockholm
[3] KTH, Stockholm
May 1997

TR 97-05

Äspö HRL – Geoscientific evaluation 1997/4. Results from pre-investigations and detailed site characterization. Comparison of predictions and observations. Hydrogeology, groundwater chemistry and transport of solutes

Ingvar Rhén[1], Gunnar Gustafson[2], Peter Wikberg[3]
[1] VBB Viak, Göteborg
[2] VBB Viak/CTH, Göteborg
[3] SKB, Stockholm
June 1997

TR 97-06

Äspö HRL – Geoscientific evaluation 1997/5. Models based on site characterization 1986–1995

Ingvar Rhén (ed.)[1], Gunnar Gustafson[2], Roy Stanfors[3], Peter Wikberg[4]
[1] VBB Viak, Göteborg
[2] VBB Viak/CTH, Göteborg
[3] RS Consulting, Lund
[4] SKB, Stockholm
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Paul La Pointe, Peter Wallmann, Andrew Thomas, Sven Follin
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Allan Hedin
Swedish Nuclear Fuel and Waste Management Co,
Stockholm, Sweden
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Anders Engqvist
A & I Engqvist Konsult HB, Vaxholm, Sweden
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Ignasi Casas[1], Isabel Pérez[1], Elena Torrero[1], Jordi Bruno[2], Esther Cera[2], Lara Duro[2]
[1] Dept. of Chemical Engineering, UPC
[2] QuantiSci SL
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Erik Larsson
Dept. of Geology, Chalmers University of Technology, Göteborg, Sweden
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Alex E Bond, Andrew R Hoch, Gareth D Jones, Aleks J Tomczyk, Richard M Wiggin, William J Worraker
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Yvonne Ohlsson, Ivars Neretnieks
Department of Chemical Engineering and Technology, Chemical Engineering, Royal Institute of Technology, Stockholm, Sweden
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Anders Boghammar[1], Bertil Grundfelt[1], Lee Hartley[2]
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