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Potential effects of bacteria on radionuclide transport from a Swedish high level nuclear waste repository

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ABSTRACT

Microorganisms can influence radionuclide migration if their concentration are high in comparison with other organic particles. Data on the numbers of microorganisms in undisturbed ground-water have been collected. The average number of cells in the samples from 17 levels in 5 boreholes was 3.0×10^5 cells ml^{-1}. A biofilm experiment indicated an active microbial rock surface population. Radiographic uptake experiments suggest inactive bulk water populations. The bulk water microbial cells in deep ground water might then be inactive cells detached from active biofilms. Enrichment cultures for anaerobic bacteria demonstrated the presence of anaerobic bacteria capable of growth on C-1 compounds with hydrogen and carbon dioxide, presumably methanogenic bacteria. Further, growth in enrichment cultures with sulphate as electron-acceptor and lactate as carbon source proved dissimilatory sulphate reducing bacteria to be present.

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SUMMARY

The disposal of high level radioactive waste in deep geologic formations has been suggested. The Swedish concept, presented in the KBS-3 study (1983), is to isolate the waste in copper canisters embedded in bentonite in excavated granitic rock repositories at 500 m depth. Dissolution and transport by the ground water is then the by far most important dispersion mechanisms for the radionuclides eventually released from the waste. Consequently, the chemistry and the flow of the ground-water plays an important role in the safety evaluation for how the biosphere on the ground, in lakes and wells will be affected by the repository. The ground-water biology might also play an important role, since the presence of microorganisms can affect the transport of radionuclides from a repository.

The physico-chemical environment in the ground water studied is rather extreme. It is anaerobic with a low E_h between -112 to -383 mV, a pH usually around 8 and a temperature of 10.2 - 20.5 °C, depending on the depth. The organic content is low, and ranges between <0.5 to 6.9 mg TOC l^{-1}. The salinity of the environment increases with the depth. Further, there is hydrogen-sulphide, methane, carbon-dioxid and hydrogen. The sulphate concentration increases with the depth. The nitrate, nitrite and phosphate concentrations are close to or below the detection limits, while there are detectable amounts of NH_4 in the range 4 - 330 $\mu g\ l^{-1}$.

Acridine orange stained direct count (AODC) was used to determine the total number of cells in the samples. The number of heterotrophs was determined with plate count technique on a medium with 1.5 g of organic substrate. An autoradiographic technique was used to determine the

substrate uptake activity of individual bacteria in the ground water collected. A biofilm reactor was connected to the flowing ground-water from a borehole for 10 days at a flow of 0.25 cm s^{-1}.

The average number of cells in the samples from 17 levels in 5 boreholes was 3.0×10^5 cells ml^{-1}. A biofilm experiment indicated an active microbial surface population. Radiographic uptake experiments suggested inactive bulk water populations. The bulk water microbial cells in deep ground water might then be inactive cells detached from active biofilms. Enrichment cultures for anaerobic bacteria demonstrated the presence of anaerobic bacteria capable of growth on C-1 compounds with hydrogen and carbon dioxide, presumably methanogenic bacteria. Further, growth in enrichment cultures with sulphate as electron-acceptor and lactate as carbon source proved dissimilatory sulphate reducing bacteria to be present.

FOREWORD

This report summarizes the work that have been done in microbiology during the investigations for a rock laboratory (Gustavsson et al., 1988, 1989). Two earlier reports have given "state of the research", (Pedersen 1987, 1989).

The concepts of geology, barriers and safety are based on the KBS-3 study (1983) for the final storage of spent nuclear fuel.

Karsten Pedersen

1. INTRODUCTION

1.1 THE PROBLEM

The disposal of high level radioactive waste in deep geologic formations has been suggested. The Swedish concept, presented in the KBS-3 study (1983), is to isolate the waste in copper canisters embedded in bentonite in excavated granitic rock repositories at 500 m depth. Dissolution and transport by the ground water is then the by far most important dispersion mechanisms for the radionuclides eventually released from the waste. Consequently, the chemistry and the flow of the groundwater plays an important role in the safety evaluation for how the biosphere on the ground, in lakes and wells will be affected by the repository. The groundwater biology might also play an important role, since the presence of microorganisms can affect the transport of radionuclides from a repository. Three principal mechanisms can be identified.

1. The microorganism constitutes a mobile particle which can have a high, selective and possibly irreversible radionuclide sorbing or uptake capacity. The radionuclide is sorbed on the outside of the cell or accumulated inside the cell (Beveridge & Fyfe, 1985; Strandberg et al., 1981).

2. The microorganism constitutes a part of a microbial biofilm on the rock surface in the groundwater aquifer. The microorganism is non-mobile and the sorbed radionuclides will be immobilized. Single cells or aggregates of cells might detach from the biofilm

(Characklis, 1984; McFeters, 1984; Pedersen, 1982c). The radionuclides will be mobilized again.

3. The microorganism may produce complexing agents that can affect speciation and thus mobility of radionuclides (Rudd et al., 1984).

The relevance of those different mechanisms for radionuclide transport can only be evaluated with knowledge about sorption and uptake properties and the ecology of the microorganisms that might inhabit a repository and its surroundings. This leads to fundamental questions about deep ground water microbiology and the interaction between microbes and radionuclides. What are the numbers, species and activities of deep ground water microbial populations in Swedish granitic rock? Which interactions will develop between a nuclear waste repository and indigenous and introduced microbial populations?

A more detailed analysis of the two questions put above reveals very complex and difficult research areas (West et al., 1985) obstacled by the classical problem of undisturbed sampling (Alföldi, 1988, Ghiorse & Wilson, 1988). The investigations for a Swedish hard rock laboratory (Gustafson et al., 1988, 1989; Wikberg et al., 1987) and the laboratory itself offer and will offer, respectively, close to ideal sampling possibilities which might solve the sampling problem and give answers on some of the questions put below.

1.2 FUNDAMENTAL QUESTIONS ON DEEP GROUND WATER MICROBIOLOGY

1.2.1 Numbers of microorganisms in the ground water

Microorganisms can influence radionuclide migration if its concentration is high in comparison with other organic particles. Data on the numbers of

microorganisms in undisturbed groundwater should be collected.

1.2.2 Microbial biofilms on the rock surfaces

This is an important question then it is known that microbial biofilms can interact with metals (e.g. Ferris et al., 1989; Polprasert & Charnpratheep, 1989).

Biofilms can be defined as microorganisms and their extracellular products associated with a substratum (McFeters, 1984). Initiation and subsequent development of a biofilm community follow the chemical conditioning of the surface and the attachment of microorganisms (Bitton and Marshall, 1980; Marshall, 1984). During the stages of biofilm development, exopolymers (Costerton *et.al.*, 1981) begin to accumulate and the biofilm takes on the macroscopic appearance of a gel (McFeters, 1984). This gel appears amorphous in the microscope as it acts as a trap for debris, corrosion products and other materials, usually including nutrients and dissolved metals, from the bulk phase. Growth of the attached cells to microcolonies, product formation and debris entrapment seems to be the dominant processes during the biofilm development. (Characklis 1984 and 1986; Characklis *et al.*, 1986; Pedersen 1982a and 1982b).

An approximation of if and how microorganisms will live on the aquifer rock surfaces preferably should be done after a period that allow the initially attaching, naturally occurring microorganisms to grow, reproduce and form a biofilm on surfaces exposed to flowing ground water. The biofilm reactor methodology described by Pedersen (1982a and 1986b) might be a valuable tool in such experiments.

1.2.3 Species of microorganisms in deep ground water

A detailed knowledge about the chemical and physical conditions in deep ground water promote predictions

over what species might be present. We do know those conditions (Table 3-5 in this report) from the investigations for a Swedish hard rock laboratory (Gustavsson et al., 1988, 1989; Wikberg et al., 1987). The physico-chemical environment in the ground water studied is rather extreme. It is anaerobic with a low E_h between -112 to -383 mV, a pH usually around 8 and a temperature of 10.2 - 20.5 °C, depending on the depth. The organic content is low, and ranges between <0.5 to 6.9 mg TOC l^{-1}. The salinity of the environment increases with the depth. Further, there are hydrogen-sulfide, methane, carbondioxide and hydrogen. The sulphate concentration increases with the depth. The nitrate, nitrite and phosphate concentrations are close to or below the detection limits, while there are detectable amounts of NH_4 in the range 4 - 330 $\mu g\ l^{-1}$. It seems reasonable to search for anaerobic or facultative anaerobic bacteria that can live under such circumstances. Denitrifying (Jeter and Ingraham, 1981,) sulphate-reducing (Pfennig et al., 1981), and methanogenic (Mah and Smith, 1981) bacteria would suit our present knowledge.

1.2.4 The activity of microorganisms in deep ground water

Though bacterial numbers yield valuable information on bacterial communities, they do not necessarily reflect the dynamics of these communities and their interdependence with a repository. It is essential to know the metabolic activity of each separate group of organism of interest.

One of the basic problems in the study of *in situ* bacterial activity is that bacteria respond very quickly to relatively slight changes in their environment. Because of the pronounced response of bacteria to changing environmental conditions, the prerequisite of a measurement should be that the natural situation is not significantly altered. Another requirement is that, in calculating actual activity or

biomass production, possible conversion factors are correct. As has been shown by Van-Es and Meyer-Reil (1982), these prerequisites are hard to fulfil.

Autoradiography has become a useful tool in ecological studies, since it enables investigators to relate activity to individual cells (eg. Meyer-Reil, 1978; Tabor and Neihof, 1982). This technique was used here.

1.2.5 The interactions between microbial populations and the repository

The building of a repository will drastically change the environment for the indigenous microbial flora. The explosives used to blast out the rock facilities will introduce an instantaneous increase in the nitrogen content of the surroundings. There will be smoke, oil and rubber from the machines used. Oxygen reaches the repository area and even if this stops when the repository is backfilled, radiolysis might produce oxygen and hydrogen in a far future. New species of bacteria will reach the repository. The backfill and canister materials are new for the indigenous bacteria. The cooling waste will raise the temperature to at most 80 °C. Even if the total amount of substances introduced and the physical changes made is moderate, they represent an extreme change in the prevailing environment for deep groundwater microorganisms.

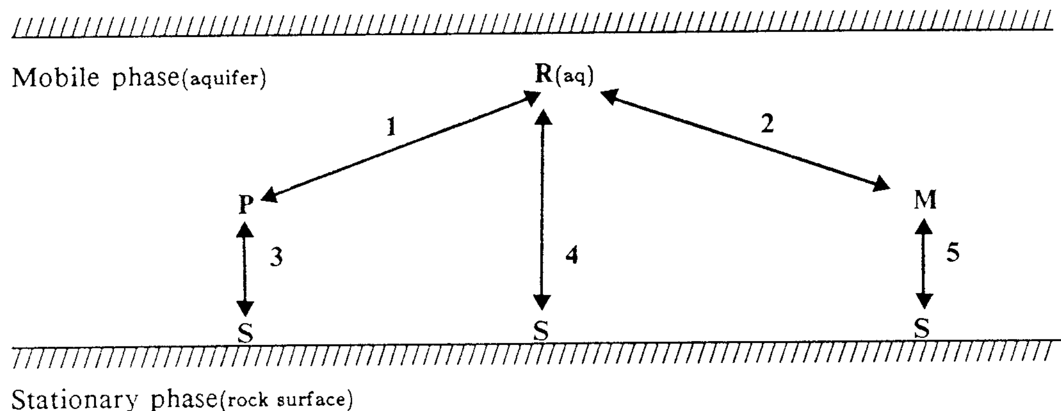
It should be judged if the activities around the building of the repository will have an effect, remaining long enough to give microbial populations relevant for radionuclide migration. It should also be evaluated if the activity from microbial populations can affect the safety barriers of the repository. (see West et al., 1982, 1985). A study of the deep groundwater microbiology before and after the building of the rock laboratory might give important clues of this question. This report is however limited to transport problems.

1.3 FUNDAMENTAL QUESTIONS ON THE RELEVANCE OF MICROBES FOR RADIONUCLIDE MIGRATION

1.3.1 Microbial uptake of radionuclides

Microorganisms are known to be able to take up radionuclides (e.g. Kepkay, 1986; Strandberg et al., 1981). The uptake can be either extracellular or

Figure 1-1 Distribution of a radionuclide between mobile and immobile phases (from Allard, 1989).



R **Radionuclide in solution**, organic or inorganic related to the hydrochemical conditions.

P **Solid mobile phase**, precipitate or coprecipitate that can be formed or dissolved when the chemical conditions are changed, possibly by *microorganisms*, in nearly saturated systems. E.g. $Fe(OH)_3 \cdot aq$, $Al(OH)_3 \cdot aq$, $CaCO_3$, macromolecular organics that *can be effected by microorganisms.*

M **Solid mobile phase**, natural colloids. E.g. *Microorganisms*, clay minerals, silica.

S **Solid stationary phase**, Rock surface with or without *microbial biofilms.*

1 Precipitation, coprecipitation - dissolution

2,5 Sorption, uptake - desorption, related to chemical speciation

3,4 Attachment, filtration, mineralisation, sedimentation - detachment, sloughing, resuspension, weathering.

intracellular. It has to be studied how microorganisms relevant to the repository can take up radionuclides. Fig. 1-1 gives an overview of potential ways of uptake and exchange of the radionuclides.

1.3.2 Microbial production of complexing agents

Microorganisms are able to produce extracellular materials that can work as chelating agents (Rudd et al., 1984). They can be amino acids, organic acids sugars, peptides and polysacharides (Corpe,1980; Costerton 1981). It has to be evaluated whether such production would be relevant for the safety analysis of a nuclear waste repository.

2. MATERIALS AND METHODS

2.1 SAMPLING EQUIPMENT

2.1.1 The field laboratory

The integrated mobile field laboratory described by Wikberg *et al* (1987) was used for water sampling and for the immediate counting and inoculating of the samples.

2.1.2 The gas sampler

A sampler adapted for the collection of undisturbed samples for gas analysis was used to collect samples for bacterial enumerations and enrichments. The sampler can be opened and closed from the surface at the actual sampling depth. The samples can subsequently be brought to the surface without contact with air and with the pressure at the actual sampling depth.

2.1.3 The bore holes

The bore holes examined are core drilled with a diameter of 76 mm. They were flushed with ground water from a 100 - 150 m deep percussion drilled hole during drilling. The drilling water was marked with uranine as a drilling water tracer. The examined level was closed of with packers in a section of 5 to 10 meters, or more when necessary for achieving a satisfactory water flow, approximately 100-150 ml min^{-1}.

2.2 TEST SITES

2.2.1 Ävrö, KAV

The bore hole KAV01 was investigated at 4 different levels, 420, 522, 558 and 635 meters, (Pedersen 1987).

2.2.2 Äspö, KAS

At an early stage of the investigations it was found that Äspö consist of two comparatively undisturbed blocks separated by a major tectonic zone. KAS02 and 03 were sited in the central parts of these blocks.
The bore hole KAS02 is a sub-vertical borehole to approximately 1000 m in the central of the SE block. It was investigated at 3 different levels, 202, 463 and 860 meters. The bore hole KAS03 is a sub-vertical borehole to approximately 1000 m in the central of the NW block. It was investigated at 2 levels, 129 and 860 meters. The bore hole KAS04 is an inclined borehole, approximately 60°, across a mylonite zone. It was investigated at 3 different levels, 195, 290 and 360 meters.

2.2.3 Laxemar, KLX

The bore hole KLX01 is situated in the central of a major block and is sub-vertical and 700 m deep. It was investigated at 272, 463 and 680 meters.

2.3 METHODS FOR ENUMERATION AND CULTURING OF BACTERIA

2.3.1 The total number of bacteria

Acridine orange stained direct count (AODC) (Hobbie *et al*. 1977) was used to determine the total number of cells in the samples form the different levels. Nuclepore filters (0.2 lm pore size, 13-mm diameter) were pre-stained with a Sudanblack solution which was prepared by dissolving 25 mg Sudanblack in 75 ml 99% ethanol and then diluted with 75 ml de-ionized water. The filters were thoroughly rinsed with de-ionized water before use. An acridine orange (AO) solution was prepared by dissolving 10 mg AO in 1 l of 6.6 mM sodium potassium phosphate, pH 6.7. The phosphate buffer was a mixture of 2.45 mM-KH_2PO_4 and 4.15 mM-Na_2HPO_4 in de-ionized water. The AO solution was stored as 10 ml

aliquots. All solutions and the water were filter sterilized (0.2 µm). A portion of the sample was filtered on a pre-stained nuclepore filter at -20 KPa and stained for 6 minutes with AO. The number of bacteria was counted using blue light (390-490 nm) in a epi-fluorescence microscope (filter 515 nm, Zeiss) at 1250 times enlargement. Between 500 and 600 cells or a minimum of fifteen microscopic fields (80 x 80 µm = 0.0064 mm^2) (n=15) were counted on each filter.

2.3.2 Viable count for heterotrophic bacteria

The number of heterotrophs was determined with plate count technique on a medium with 1.5 g of organic substrate. The medium used was composed of: peptone 0.5g, yeast extract 0.5g, 1.40 mM-glucose, 1.55 mM-starch, 1.36 mM-$CaCl_2 \cdot 2H_2O$, 0.57 mM-K_2HPO_4, NaCl corresponding to the salinity of the ground water sampled, trace metal solution, 1 ml agar 15 g, de-ionized water 1000 ml, pH was adjusted to 7.5 after sterilization in autoclave.

The trace metal solution consisted of 7.65 mM-$ZnSO_4 \cdot 7H_2O$, 33.5 mM-$CaCl_2 \cdot 6H_2O$, 12.6 mM-$MnCl_2 \cdot 4H_2O$, 2.10 mM-$CoCl_2 \cdot 6H_2O$, 0.40 mM-$(NH4)_6Mo_7O_{24} \cdot 4H_2O$, 18 mM-$FeSO_4 \cdot 7H_2O$, 0.80 mM-$CuSO_4 \cdot 5H_2O$, 134 mM-$Na_2EDTA \cdot 2H_2O$, NaOH for pH adjustment, Distiled water, pH 4.0.

For dilution of the samples prior to the plate count a dilution medium was used. It consisted of 1.36 mM-$CaCl_2 \cdot 2H_2O$, 1.62 mM-$MgSO_4 \cdot 7H_2O$, 0.57 mM-K_2HPO_4, NaCl according to the salinity of the ground water sampled, de-ionized water.

The samples were serially diluted to a concentration corresponding to the inverse of the total number of bacteria determined with AODC and spread in triplets on the agar plates. They were incubated at 20 °C for 7 days.

2.3.3 Isolation and identification of facultative anaerobic heterotrophic bacteria

A number of different bacteria from the highest dilutions (2.3.2) were reinoculated and isolated in an atmosphere of 90% N_2 and 10% CO_2. Biochemical identification for numerical classification were performed both by our lab and Culture Collection, University of Göteborg (CCUG).

2.3.4 Enrichment and isolation of obligate anaerobes

An anoxic mineral medium was used. It consisted of 1.5 mM-KH_2PO_4, 5 mM-NH_4Cl, 150 mM-NaCl, 8.5 mM-KCl, 2 mM-$MgCl_2 \cdot 6H_2O$, 2 mM-$CaCl_2 \cdot 6H_2O$, 60 mM-$NaHCO_3$, 1 mM-$Na_2S \cdot 9H_2O$, 10 mg sodiumdithionite, 1 ml trace elements, 1 ml selenite and tungsten solution, 0.5 ml 6-vitamin solution and 1 ml vitamin B_{12} solution, all dissolved in de-ionized water.

Trace element solution (SL 10, Widdel & Pfennig, 1981): 7.54 mM-$FeCl_2 \cdot 4H_2O$, 0.514 mM-$ZnCl_2$, 0.494 mM-$MnCl_2 \cdot 2H_2O$, 0.0970 mM-H_3BO_3, 0.798 mM-$CoCl_2 \cdot 6H_2O$, 0.0117 mM-$CuCl_2 \cdot 2H_2O$, 0.101 mM-$NiCl_2 \cdot 6H_2O$, 0.149mM-$Na_2MoO_4 \cdot 2H_2O$.

Selenite and tungsten solution: 12.5 mM-NaOH, 0.0114 mM-$Na_2SeO_3 \cdot 5H_2O$, 0.0121 mM-$Na_2WO_4 \cdot 2H_2O$.

Vitamin solution: 0.583 mM-P-aminobenzoic acid, 0,0819 mM-D(+)-biotin, 1.62 mM-Nicotinic acid, 0.210 mM-Ca-D(+)-pantothenic acid, 1.16 mM-Pyridoxamindihydrochloride, 0,593 mM-Thiaminiumdichloride.

Vitamin B_{12} solution: 50 mg l^{-1} Cyanocobalamin.

The medium was portioned in aliquotes of 50 ml in 100 ml serum bottles with aluminum crimp sealed butyl rubber stoppers under N_2/CO_2 (90/10%) atmosphere.

Series of bottles with medium were subsequently supplied with 10 mM-formate, 5 mM-methanol, 10 mM-sodiumacetate, 58 mM-lactate, 58 mM sodiumsulphate and 1 atmosphere overpressure of H_2/CO_2 in the following combinations.

KLX01, 272 m: formate, formate + H_2/CO_2, formate + H_2/CO_2 + yeast extract, methanol, methanol + yeast extract, acetate.

KLX01, 680 m, 891101: Formate, formate + H_2/CO_2, methanol, methanol + H_2/CO_2, acetate, acetate + H_2/CO_2, formate + SO_4, formate + H_2/CO_2 + SO_4, methanol + SO_4, methanol + H_2/CO_2 + SO_4, acetate + SO_4, acetate + H_2/CO_2 + SO_4, lactate + SO_4, lactate + H_2/CO_2 + SO_4.

KAS02, 860 m: Formate, formate + H_2/CO_2, formate + H_2/CO_2 + yeast extract, methanol, methanol + yeast extract, acetate.

KAS03, 860 m: Formate, formate + H_2/CO_2, methanol, methanol + H_2/CO_2, acetate, acetate + H_2/CO_2, formate + SO_4, formate + H_2/CO_2 + SO_4, methanol + SO_4, methanol + H_2/CO_2 + SO_4, acetate + SO_4, acetate + H_2/CO_2 + SO_4, lactate + SO_4, lactate + H_2/CO_2 + SO_4.

The bottles were inoculated with 5 ml of water from the field lab and the gas sampler and incubated for 3-4 weeks at room temperature. Bottles with H_2/CO_2 were incubated on a shake. Successful enrichment cultures were transferred to new bottles once for another 3-4 weeks of inoculation. Growth was confirmed with microscope. Agar shakes, from which isolation was made, were subsequently made on the enrichment cultures.

2.3.5 Most probable number of sulphate reducing bacteria and methane bacteria

The number of sulphate reducing bacteria was investigated with most probable number technique using the medium and culture vessels described under 2.3.4 complemented with 58 mM-lactate, 58 mM-sodiumsulphate and H_2/CO_2. Two levels were investigated in KAS04, 195 and 380 m. The presence of sulphate reducing bacteria was confirmed by analysis of the production of H_2S in the cultures. The number of sulphate reducing bacteria and the number of methane bacteria were investigated in KLX01, 680 m, 891101. A mixture of formate, methanol and H_2/CO_2 were used for the methane bacteria.

2.4 MICROAUTORADIOGRAPHIC STUDIES OF MICROBIAL ACTIVITY

The MARGE-E technique developed by Tabor and Neihof (1982) was used to determine the substrate uptake activity of individual bacteria in the ground water collected in the field lab. The following carbon sources were used at an activity of 0.36 µCi/ml of sample: Format, ^{14}C, 6360 nM, methanol, ^{3}H, 6000 nM, acetate, ^{3}H, 109 nM, lactate, ^{14}C, 14600 nM, glucose, ^{14}C, 1320 nM, and sodium-bi-carbonate, ^{14}C, 108000 nM (0.91 µCi/ml) or 6370 nM (0.36 µCi/ml). The following cell constituents were also tested for uptake: Leucine, ^{3}H, 3 nM, glutamine, ^{3}H, 9 nM, thymidine, ^{3}H, 2.9 nM and N-acetyl-glucose-amine, ^{3}H, 62 nM. KAS03, 860 m, KAS04, 380 m and KLX01, 680 m were investigated.

2.5 ATTACHMENT AND GROWTH OF BACTERIA IN FLOWING GROUNDWATER

A biofilm reactor was connected to the flowing groundwater from KLX01, 680 m, 891101 for 10 days at a flow of 0.25 cm s^{-1}. The reactor is described in detail by Pedersen et al. (1986). Glass slides, 60 x 24 x 0.17 mm, were used as a substratum for attaching bacteria. The number of bacteria on the surfaces were counted after staining with acridine orange for 6 minutes.

3. RESULTS

3.1 NUMBERS OF BACTERIA IN THE BOREHOLES ÄVRÖ 01, ÄSPÖ 02, 03, 04 AND LAXEMAR 01.

Table 3-1 shows the results from the enumerations of bacteria in the boreholes Ävrö 01 (KAV01), Äspö 02, 03 and 04 (KAS02, KAS03 and KAS04) and Laxemar 01 (KLX01).

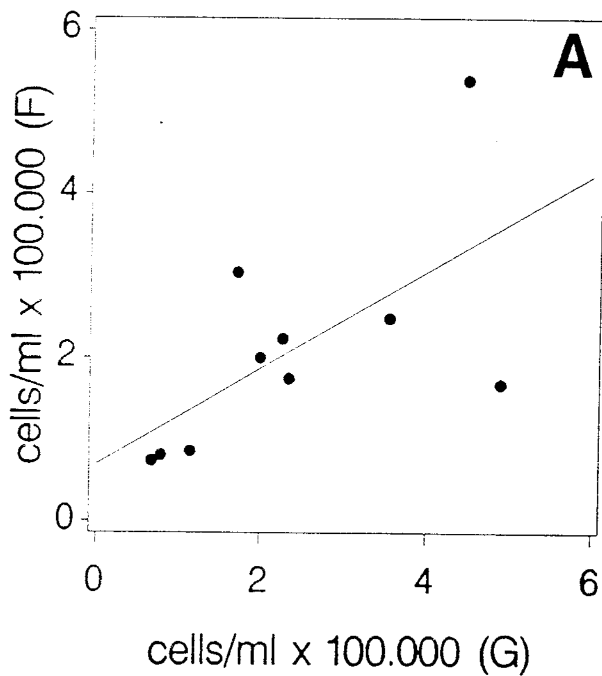
The numbers of bacteria were determined in samples from the gas sampler when this was possible and in the water that was pumped up to the field lab. The average total number of bacteria was 3 x 10^5 (N=17) bacteria ml^{-1} for the field lab and 2.4 x 10^5 (N=10) bacteria ml^{-1} for the gas sampler. The number of bacteria possible to recover with plate count arrays from 0.10 to 21.9 %.

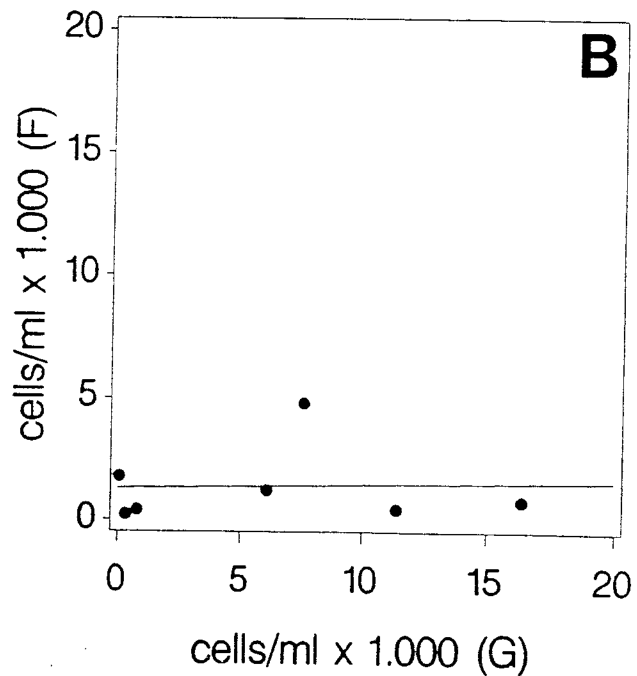
There was a correlation between the total numbers of bacteria sampled the field lab and in the gas sampler, (Fig 3-1A), R=0.628 at a probability of R=0 of 0.0517. There was no correlation between the viable counts from the field lab and from the gas sampler, (Fig 3-1B), R=0.060 at a probability of R=0 of 0.898.

Table 3-1 The table shows the total number of bacteria in the five studied boreholes, KAV01, KAS02, KAS03, KAS04 and KLX01. The total numbers of bacteria were determined with epi-fluorescence microscopy after staining with acridine orange. The viable counts were determined as plate counts. The percentage of the total number of bacteria that could be counted as a viable count (v.c) has been calculated. F=Field lab, G= Gas sampler.

Bore-hole-	Depth m	Date of samp-ling	Analyse number	Total cells ml^{-1} x 10^5 F/G	Viable count (v.c) cells ml^{-1} x 10^3 F/G	% v.c of total number F/G
KAV01	420	870923	KBS 1391	16.6/-	2.49/-	0.15/-
KAV01	522	870826	KBS 1383	3.31/-	2.19/-	0.66/-
KAV01	558	870604	KBS 1374	3.82/-	-	-
KAV01	635	870421	KBS 1354	1.30/-	-	-
KLX01	272	881209	KBS 1538	2.05/-	0.21/-	0.10/-
KLX01	466	881123	KBS 1528	1.10/-	15.3/-	13.9/-
KLX01	680	881102	KBS 1516	1.68/4.90	3.30/-	1.96/-
KLX01	680	891101	KBS 1633	1.75/2.35	0.45/11.4	0.26/4.85
KAS02	202	890111	KBS 1548	1.15/-	1.14/-	0.99/-
KAS02	314	880412	KBS 1419	0.81/0.79	-/4.93	-/6.24
KAS02	463	880425	KBS 1428	3.04/1.71	39.1/37.6	12.9/21.9
KAS02	860	890131	KBS 1560	0.86/1.15	0.41/0.81	0.48/0.70
KAS03	129	890222	KBS 1569	2.00/2.00	1.23/6.10	0.62/3.05
KAS03	860	890315	KBS 1582	2.23/2.27	0.23/0.35	0.10/0.15
KAS04	195	890417	KBS 1596	5.39/4.47	4.80/7.60	0.89/1.70
KAS04	290	890427	KBS 1603	2.48/3.55	0.81/16.4	0.33/4.62
KAS04	380	890403	KBS 1588	0.74/0.68	1.79/0.08	2.42/0.12

Figure 3-1 A. The total number of cells measured in the field lab (F) versus the total number of cells measured in the gas sampler (G) in 10 different borehole levels distributed over 4 different boreholes.
B. The viable count of cells measured in the field lab (F) versus the viable count of cells measured in the gas sampler (G) in 7 different borehole levels distributed over 4 different boreholes.





3.2 ISOLATION AND IDENTIFICATION OF COLLECTED BACTERIA.

3.2.1 Facultative anaerobic heterothrophic bacteria

Seven bacteria were isolated from the gas sampler, identified and characterized. Table 3-2 shows the data obtained. Y1-Y4 were isolated from KAS02, 463 m that had a large viable count (Table 3-1). Y5-Y6 were isolated from KAS03, 129 m and Y7 from KAS02, 860 m

Table 3-2 The species of bacteria identified in KAS02-03. CCUG=Culture Collection, University of Göteborg.

Viable no. of cells ml^{-1} in the ground water	sample code	species	CCUG-number
n.d.	Y1	*Shewanella putrefaciens*	CCUG-22946
2 x 10^3	Y2	*Pseudomonas vesicularis*-like	CCUG-22947
n.d.	Y3	*Shewanella putrefaciens* (atypical)	CCUG-22948
42 x 10^3	Y4	*Pseudomonas vesicularis*-like	CCUG-22949
2.5 x 10^3	Y5	*Pseudomonas fluorescens*	CCUG-25085
1 x 10^3	Y6	NaCl-dependant nonfermentative	CCUG-25086
0.5 10^3	Y7	*Pseudomonas fluorescens*	CCUG-25087

3.2.2 Obligate anaerobes

We have achieved growth in the enrichment cultures after 4-8 weeks of incubation as shown in table 3-3. Growth was obtained with formate, methanol and lactate as carbon sources. Occasionally, there was growth with acetate (KAS03, 860 m, gas sampler) and with CO_2 and H_2 (KLX01, 272 m, 680 m, KAS02, 860 m).

3.2.3 Most probable number of sulphate reducing bacteria and methane bacteria

The samples from KAS04, 380 meter was diluted to 10^6 and five parallels of serum bottles were inoculated

Table 3-3 The results from the enrichment cultures with different carbon sources and with sulphate and hydrogen as electron acceptor (e^--acc) and electron donor (e^--don) respectively. G.=Growth, N.G.= No growth.

Carbon sources	e^--acc	e^--don	KLX01 272 m	KLX01 680 m	KAS02 860 m	KAS03 860 m
Field lab						
CO_2		H_2	G.	G.	G.	G.
CO_2	SO_4	H_2		G.		N.G.
Formate			G.	N.G.	G.	N.G.
Formate +CO_2		H_2	G.	N.G.	G.	G.
Formate +CO_2 +ye		H_2	G.		G.	
Formate	SO_4			N.G.		G.
Formate +CO_2	SO_4	H_2		N.G.		G.
Methanol			G.	N.G.	G.	N.G.
Methanol +ye			G.		G.	
Methanol +CO_2		H_2		N.G.		G.
Methanol	SO_4			N.G.		G.
Methanol +CO_2	SO_4	H_2		N.G.		G.
Acetate			N.G.	N.G.	N.G.	N.G.
Acetate +CO_2		H_2		N.G.		N.G.
Acetate	SO_4			N.G.		N.G.
Acetate +CO_2	SO_4	H_2		G.		N.G.
Lactate	SO_4			G.		G.
Lactate +CO_2	SO_4	H_2		G.		G.
Gas sampler						
CO_2		H_2		G.	N.G.	N.G.
CO_2	SO_4	H_2		G.		N.G.
Formate				G.	N.G.	N.G.
Formate +CO_2		H_2		N.G.	N.G.	N.G.
Formate +CO_2 +ye		H_2			G.	
Formate	SO_4			N.G.		G.
Formate +CO_2	SO_4	H_2		N.G.		N.G.
Methanol				N.G.	G.	N.G.
Methanol + ye					G.	
Methanol +CO_2		H_2		N.G.		N.G.
Methanol	SO_4			N.G.		N.G.
Methanol +CO_2	SO_4	H_2		N.G.		N.G.
Acetate				N.G.	N.G.	N.G.
Acetate +CO_2		H_2		N.G.		G.
Acetate +CO_2	SO_4	H_2		G.		
Lactate	SO_4			G.		G.
Lactate +CO_2	SO_4	H_2		G.		G.

from 10^2 to 10^6 dilution. No growth was observed. The most probable number of sulphate reducing bacteria utilizing lactate was less than 100 ml^{-1} in this bore hole at 360 meter.

The samples from KAS04, 195 meters was diluted to 10^3. The most probable number of sulphate reducing bacteria was 900 cells ml^{-1} for the field lab and 1600 cells ml^{-1} for the gas sampler. Those numbers were not significantly different at the 0.05 significance level.

The sample from KLX01 680 m, 891101 was diluted to 10^5. The most probable number of sulphate reducing bacteria was 5.6×10^4 cells ml^{-1} for the field lab. There was no growth in the Formate-methanol medium.

3.3 MICROBIAL ACTIVITY

Table 3-4 shows the % of activity shown by the microbial populations in the investigated boreholes.

3.3.1 Uptake of cell constituents: glutamine, leucine, thymidine and N-acetyl-glucose-amine

The majority of the population incorporated the amino acids leucine and glutamine in KAS03, 860 m while there was a sparse uptake of leucine in KAS04, 380 m and a moderate uptake in KLX01, 680 m. There was a low uptake of glucose and no uptake of N-acetyl-glucose-amine in KAS03, 860 m and a lack of uptake of thymidine in KAS03, 860 m, KAS04, 380 m and KLX01, 680 m. A prolonged incubation time to 96 h allowed a growth on glucose and N-acetyl-glucose-amine in KAS04, 380 m.

Table 3-4 The uptake of different labelled substances with the microbial populations in 3 different boreholes.

I=Tot. no of cells at stop/tot no of cells at start.
R=Ratio of the sample showing uptake.
T=Time of incubation.

Carbon source	Isotop	Final activity µCi/ml	concentration nM	KAS03 860 m I	R	T	KAS04 380 m I	R	T	KLX01 680 m I	R	T
Field lab												
Carbon sources												
Format	^{14}C	0.36	6360	0.84	0 %	9.5 h	-			1	0	9.5 h
Methanol	^{3}H	0.36	6000	0.91	0 %	9.5 h	-			1	0	9.5 h
Acetate	^{3}H	0.36	109	0.91	0 %	9.5 h	14.5	56.7 %	96 h	1	0	9.5 h
Lactate	^{14}C	0.36	14600							1	0	9.5 h
Glucose	^{14}C	0.36	1320	0.86	1.4 %	9.5 h	5.2	97.8 %	96 h	1	0	9.5 h
Carbonate	^{14}C	0.91	108000				1	0 %	96 h			
Carbonate	^{14}C	0.36	6370							1	0	9.5 h
Cell constituents												
Leucine	^{3}H	0.36	2	0.96	81.2 %	9.5 h	0.94	6.2 %	4 h	0.77	17.6	4 h
							1.09	9.4 %	9.5 h	0.70	12.9	9.5 h
Glutamine	^{3}H	0.36	9	0.87	81.0 %	9.5 h	1	0 %	4 h	1	0	9.5 h
Thymidine	^{3}H	0.36	2.9	0.95	0	9.5 h	0.98	0 %	4 h	1	0	9.5 h
N-acetyl-glucose-amine	^{3}H	0.36	62	0.92	0	9.5 h	35	75 %	96 h	1	0	9.5 h
Gas sampler												
Carbon sources												
Format	^{14}C	0.36	6360				62.5	24.5%	96 h	1	0	9.5 h
Methanol	^{3}H	0.36	6000				-			1	0	9.5 h
Acetate	^{3}H	0.36	109				26.8	79.8 %	96 h	1	0	9.5 h
Lactate	^{14}C	0.36	14600							1	0	9.5 h
Glucose	^{14}C	0.36	955				22.2	96 %	96 h	1	0	9.5 h
Carbonate	^{14}C	0.91	108000				1	0	96 h			
Carbonate	^{14}C	0.36	6370							1	0	9.5 h
Cell constituents												
Leucine	^{3}H	0.36	2				1	0	4 h	1	0	9.5 h
							1	0	9.5 h	1	0	9.5 h
Glutamine	^{3}H	0.36	9				1	0	4 h	1	0	9.5 h
Thymidine	^{3}H	0.36	2.9				1	0	4 h	1	0	9.5 h
N-acetyl-glucose-amine	^{3}H	0.36	62				48.9	49.6	96 h	1	0	9.5 h

3.3.2 Uptake of carbon sources: formate, methanol, acetate, lactate, glucose and carbonate.

There was no uptake of the one and two-carbon sources introduced in the KAS03, 860 m and the KLX01, 680 m samples. Prolonged incubation time to 96 h allowed a growth on formate and acetate to up to 60 times more bacteria than initially assayed in KAS03. CO_2-fixation could not be detected.

3.4 ATTACHMENT AND GROWTH OF BACTERIA IN FLOWING GROUNDWATER

There were 3.45 x 10^5 bacteria cm^2 on the surfaces after 10 days of exposure to the flowing groundwater. The bacteria appeared in microcolonies with 2 to at most 20 cells in each colony. This indicate that the bacteria on the surfaces were active and in a state of growth.

3.5 THE COMPOSITION OF THE GROUND WATERS STUDIED

An important condition for the analysis of the ground water composition was that the continuously measured redox potentials were stabilized. Water had to be pumped from each bore hole section for 2 - 3 weeks with water flows between 61 - 200 ml min^{-1} (see bottom, table 3-5) to fulfil this requirement. This means that between 2000 and 4000 l of ground water were drawn before the final analysis, presented in table 3-5.

Table 3-5 shows that the physico-chemical environment in the ground water studied is rather extreme. It is anaerobic with a low E_h between -112 to -383 mV, a pH usually around 8 (except for KAV01) and a temperature of 10.2 - 20.5 °C, depending on the depth. The organic content is low, and ranges between <0.5 to 6.9 mg TOC l^{-1}. It decreases with the depth and thereby usually with increasing age of the water. The salinity of the environment increases with the depth. The chemical

composition and concentration at indicate that there is stagnant relict sea water at depths below about 500 m (Gustafson et al., 1988). Further, there are hydrogen-sulfide, methane, carbondioxide and hydrogen. The sulphate concentration increases with the depth. The nitrate, nitrite and phosphate concentrations are close to or below the detection limits, while there are detectable amounts of NH_4 in the range 4 - 330 $\mu g\ l^{-1}$.

There seemed to be a slight in leakage of oxygen to the water during its transport to the field lab (O_2-f).

Uranine was added as a trace substance to the drilling water and was subsequently analysed for in the water samples (See Table 3-5). The drilling water contamination was between 2.62-13.7% for KAV01 and KLX01 and between 0.06 to 0.51% for KAS02-04). The KAS boreholes were drilled with a technique different from the others resulting in significantly lower drilling water contamination.

Table 3-5 The composition of the groundwater of KAV01, KAS02, KAS03 KAS04 and KLX01. -f=field lab, -b=bore hole sonde, -=not determined.

Borehole		KAV01	KAV01	KAV01	KAV01	KLX01	KLX01
Level m		420	522	558	635	272	466
Date		870923	870825	870603	870421	881209	881123
Analyse number		1391	1383	1374	1354	1538	1528
Temp	°C	12.9	14.86	15.0	-	-	-
pH		6.9	7.0	7.2	6.5	7.93	8.2
E_h-f	mV	-213	-290	-230	-112	-240	-
E_h-b	mV	-215	-	-204	-	-	-
Cond.	mS/m	232	680	1310	2660	760	637
N_2	µl/l	-	-	-	-	25000	-
H_2	µl/l	-	-	-	-	88	-
He	µl/l	-	-	-	-	-	-
CH_4	µl/l	-	-	-	-	110	-
CO	µl/l	-	-	-	-	14	-
CO_2	µl/l	-	-	-	-	-	-
O_2-f	mg/l	0.18	0.16	0.10	-	0.35	-
TOC	mg/l	-	-	3.9	<0.5	1.5	1.4
SiO_2	mg/l	4.9	5.8	5.1	4.0	11.4	11.6
Na^+	mg/l	255	750	1500	3200	1011	854
K^+	mg/l	5.0	7.4	6	8	5.68	6.0
Li^+	mg/l	0.0051	0.21	0.55	1.2	-	0.15
Ca^{+2}	mg/l	162	440	1100	2800	244	225
Mg^{+2}	mg/l	29	42	60	31	26.2	17
Sr^{+2}	mg/l	5.9	6.0	20	-	-	4.0
Al^{+3}	mg/l	0.16	0.11	0.39	0.027	-	0.092
Mn^{+2}	mg/l	3.1	2.4	1.7	0.18	0.197	0.138
Fe^{2+}	mg/l	1.68	2.23	1.02	0.430	0.198	0.390
Fe_{tot}	mg/l	1.68	2.23	1.02	0.438	0.200	0.410
HCO_3	mg/l	1.87	81	42.3	9.9	80.6	77.0
Cl^-	mg/l	616	1970	4300	9700	2070	1698
F^-	mg/l	2.6	2.2	1.8	1.4	2.32	2.46
Br^-	mg/l	3.0	8.9	24	72	6.03	6.44
I^-	mg/l	0.06	0.10	0.32	0.72	0.13	0.12
S^{-2}	mg/l	0.59	1.20	0.81	<0.01	0.473	0.460
SO_4^{-2}	mg/l	47	118	220	400	48.5	105
PO_4^{-2}	µg/l	5	5	10	3	<1	4
NO_2^-	µg/l	<1	<1	<1	<1	<1	<1
NO_3^-	µg/l	<10	20	<10	<10	<10	<10
NH_4^+	µg/l	80	60	80	-	81	61
Drilling water	%	9.6	10.0	-	-	4.60	13.7
Water flow	ml/min.	178	165	200	120	130	138

Table 3-5 continuing

Borehole Level m Date Analyse number		KLX01 680 881102 1516	KLX01 680 891101 1633	KAS02 202 890111 1548	KAS02 314 880412 1419	KAS02 463 880425 1428	KAS02 860 890131 1560
Temp	°C	-	16.1	-	12.41	15.47	16.32
pH		8.05	7.9	7.43	8.52	8.34	8.35
E_h-f	mV	-280	-246	-258	-293	-218	-140
E_h-b	mV	-		-	-	-	-
Cond.	mS/m	1714	1540	1541	1560	1630	3130
N_2	µl/l	72000	33800	38600	-	-	48000
H_2	µl/l	-	-	610	3700		-
He	µl/l	-	-	-	3000		-
CH_4	µl/l	220	44	30	60		34
CO	µl/l	1.5	-	0.6	20		42
CO_2	µl/l	290	350	-	1400		490
O_2-f	mg/l	-		-	0.01	0.04	0.38
TOC	mg/l	1.2	3.3	6.0	2.4	3.0	<0.5
SiO_2	mg/l	13	5.78	13	5.23	3.6	8.38
Na^+	mg/l	1120	1619	1206	1560	1800	2845
K^+	mg/l	6.4	5.84	6.7	7.1	8.1	11.0
Li^+	mg/l	0.53	-	0.38	-	0.81	1.90
Ca^{+2}	mg/l	1400	-	998	1541	1580	3831
Mg^{+2}	mg/l	9.1	-	60.8	75	66	31.5
Sr^{+2}	mg/l	24	-	18	-	30	63
Al^{+3}	mg/l	0.085	-	-	-	0.046	0.062
Mn^{+2}	mg/l	0.191	0.620	1.0	0.81	0.73	0.28
Fe^{2+}	mg/l	0.029	0.139	0.483	0.792	-	0.485
Fe_{tot}	mg/l	0.031	0.139	0.500	0.793	0.964	0.500
HCO_3	mg/l	23.9	-	71	26.6	25	11.0
Cl^-	mg/l	4861	-	3822	5343	5440	11097
F^-	mg/l	1.63	1.73	1.36	1.33	1.4	1.62
Br^-	mg/l	38	28.5	13.4	22.6	28	74.1
I^-	mg/l	0.27	-	0.30	0.33	0.32	0.69
S^{-2}	mg/l	2.55	0.665	0.48	0.143	0.13	0.715
SO_4^{-2}	mg/l	351	392.5	106	271	290	518
PO_4^{-2}	µg/l	2	3	3	2	4	11
NO_2^-	µg/l	<1	<1	2	<1	<1	<1
NO_3^-	µg/l	<10	-	<10	<10	<10	<10
NH_4^+	µg/l	4	40	39	330	220	11
Drilling water	%	2.62	1.992	0.808	0.616	0.38	0.224
Water flow	ml/min.	138	96	61	180	160	135

Table 3-5 continuing

Borehole		KAS03	KAS03	KAS04	KAS04	KAS04
Level m		129	860	195	290	380
Date		890222	890315	890417	890427	8790403
Analyse number		1569	1582	1596	1603	1588
Temp	°C	10.2	20.5	10.83	-	-
pH		8.04	8.12	8.19	8.00	8.07
E_h-f	mV	-280	-270	-290		-285
E_h-b	mV	-260	-250	-		-
Cond.	mS/m	471	3532	264	1082	1926
N_2	µl/l	20000	40000	34000	50000	7000
H_2	µl/l	-	-	-	-	-
He	µl/l	-	-	720	2700	1800
CH_4	µl/l	16	37	85	28	3.6
CO	µl/l	11	35	0.7	-	1.3
CO_2	µl/l	1200	175	4500	960	330
O_2-f	mg/l	-	0.26	0.33	-	0.29
TOC	mg/l	2.0	0.5	6.9	5.3	1.3
SiO_2	mg/l	11.5	9.02	10.9	8.9	11.2
Na^+	mg/l	609	2998	384	1180	1883
K^+	mg/l	2.20	5.49	2.18	5.51	5.04
Li^+	mg/l	0.13	1.65	-	0.38	0.94
Ca^{+2}	mg/l	163	4376	91.5	769	1657
Mg^{+2}	mg/l	20.4	43.7	5.7	30.4	58.3
Sr^{+2}	mg/l	3.3	78	1.8	12.6	28.9
Al^{+3}	mg/l	0.039	-	-		-
Mn^{+2}	mg/l	0.105	0.25	0.075	0.29	0.45
Fe^{2+}	mg/l	0.123	0.076	0.040	0.324	0.255
Fe_{tot}	mg/l	0.124	0.077	0.041	0.325	0.260
HCO_3	mg/l	61.2	10.6	222.7	68.8	20.5
Cl^-	mg/l	1234	12297	500	3058	5845
F^-	mg/l	2.12	1.58	4.12	2.65	1.70
Br^-	mg/l	4.81	84.7	2.50	15.9	24.4
I^-	mg/l	0.10	-	0.07	0.16	0.44
S^{-2}	mg/l	0.586	1.28	1.09	0.42	0.61
SO_4^{-2}	mg/l	31.1	709	179	221	407
PO_4^{-2}	µg/l	5	<3	7	4	<3
NO_2^-	µg/l	<1	<1	<1	<1	<1
NO_3^-	µg/l	<10	<10	<10		<10
NH_4^+	µg/l	36	-	15	88	50
Drilling water	%	0.064	0.13	0.16	0.51	0.08
Water flow	ml/min.	122	118	100	108	93

3.6 CORRELATIONS BETWEEN THE NUMBERS OF BACTERIA, DEPTH, TOC AND URANINE

Table 3-6 shows the correlation between the numbers of bacteria, depth, TOC and uranine. It can be seen that significant correlation were obtained only between total number of cells and TOC (Figure 3-2) and between TOC and depth (Figure 3-3).

Table 3-6 The correlation between the numbers of bacteria, depth, TOC and uranine for some of the data in tables 3-1 and 3-5.

Variables	Significant on the 5 % level	N	Correlation coefficient (R)	Probability of R=0
Total number of cells - TOC	*	15	0.557	0.0311
Total number of cells - uranine		15	0.376	0.1850
Total number of cells - depth		17	-0.115	0.6617
Total number of cells - viable count		14	-0.014	0.9622
TOC - depth	*	15	-0.514	0.0500
TOC - uranine		12	-0.241	0.4514
TOC - viable count		12	0.019	0.9525
Viable count - depth		14	-0.026	0.9287
Viable count - uranine		13	0.068	0.8258
Depth - uranine		14	0.066	0.8218

Figure 3-2 The total number of cells versus the content of TOC in 15 different borehole levels distributed over 5 boreholes.

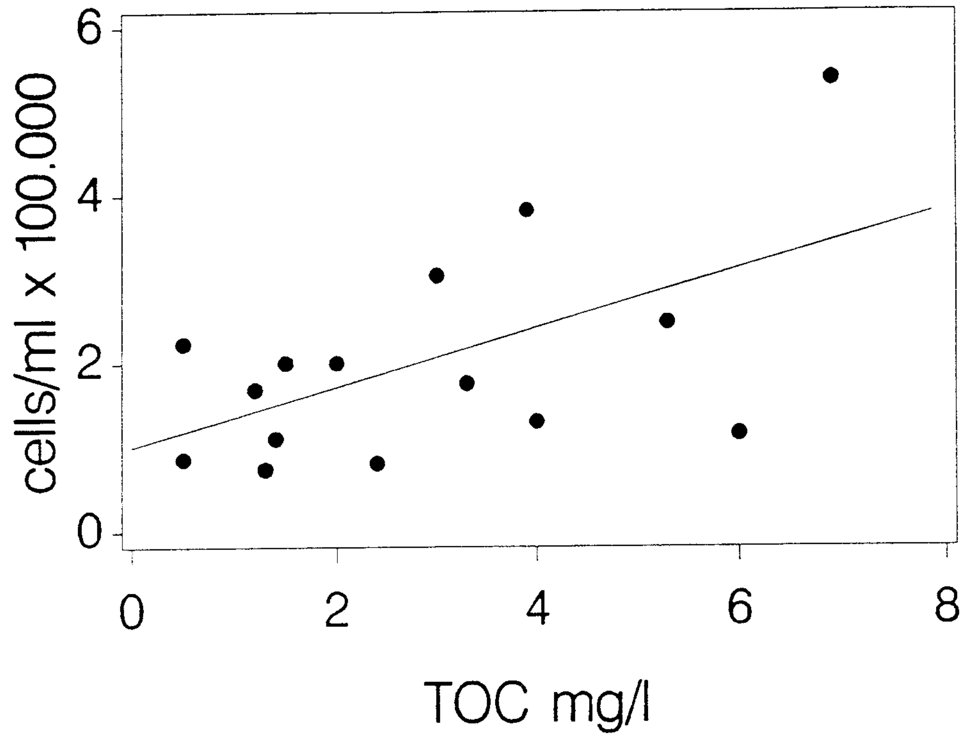
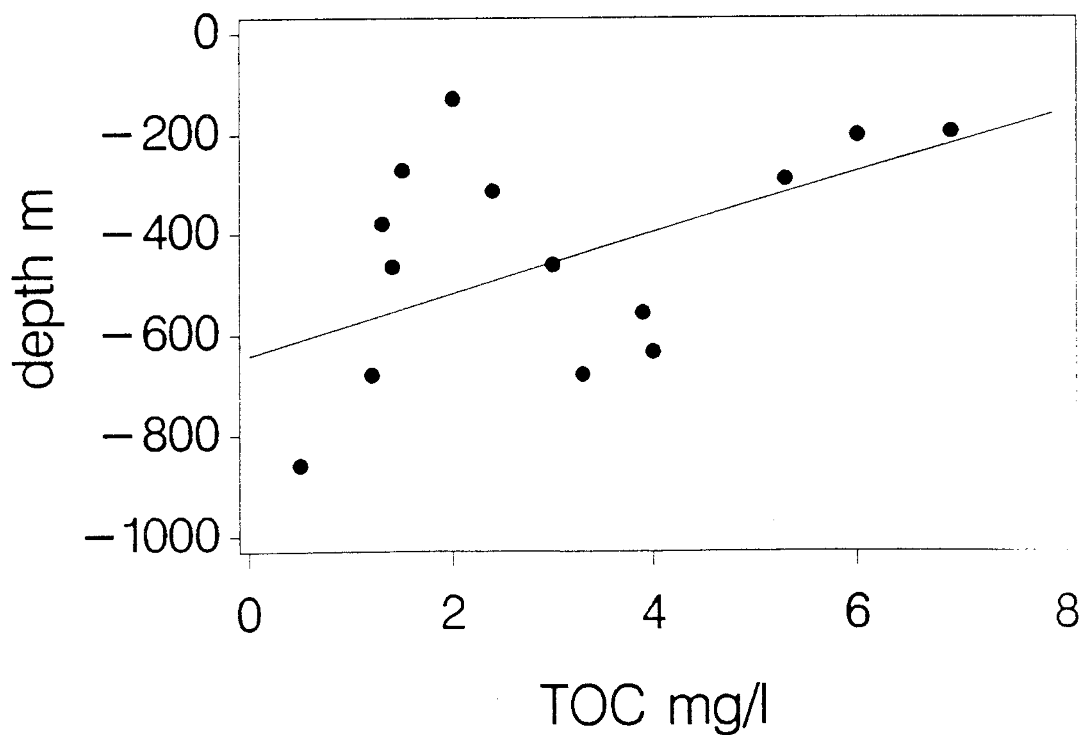


Figure 3-3 The content of TOC versus the depth in 15 different borehole levels distributed over 5 boreholes.



4. DISCUSSION

The KBS-3 report defines the biosphere as synonymous with the earths surface, including some meters of soil, lakes, rivers and wells. The research here and else (cf Alföldi, 1988, Ghiorse & Wilson 1988) have shown that the biosphere in it's true meaning extends to depths far below the planned repository. The geological, physical and chemical models over the ground have to be supplemented with biological data to be complete. At least should the impact of deep ground water biology with the safety analysis be evaluated.

4.1 NUMBERS OF MICROORGANISMS IN THE GROUND WATER

4.1.1 Sources of microorganisms in the groundwater

The average number of cells in the samples was 3.0 x 10^5 cells ml^{-1} (N=17) in the field lab and 2.4 x 10^5 cells ml^{-1} (N=10) in the gas sampler. The means were not significantly different. The source of those microorganisms must be discussed because there is always an uncertainty wether the microbes represent an natural or introduced flora.

4.1.2 Possible contamination from the drilling of the boreholes

There was no sterilisation of the drilling equipment or the drilling water used to drill the boreholes. A measure to check for drilling water contamination of the water analysis was taken. Uranine was added as a trace substance to the drilling water and was subsequently analysed for in the water samples (See Table 3-5). The drilling water contamination was between 2.62-13.7% for KAV01 and KLX01 and between 0.06

to 0.51% for KAS02-04). There was no correlation between the uranine content and the number of cells in the water samples (Table 3-6). This seems reasonable as many of the boreholes were made a year or more in advance of sampling. The possibility of inoculation of the ground water from the drilling water can however not be excluded at this point of the research. In such a case the groundwater is inoculated with microbial populations from approximately 100-150 m depth (*confer* 2.1.3). Artesian fissures in the rocklab will give water that is uncontaminated from drilling and possibly an answer on this question.

4.1.3 Sampling equipment effects

The samples for enumeration of the total number of microorganisms were taken both in the field lab and from the gas sampler. There was a correlation between the numbers achieved (Fig 3-1A). This result indicate that the pumping of the sample through a 1000 m long polyamid tube with a diameter of 6 mm (= 15 m^2 of wall surface) did not alter the total number of cells in most cases. This issue has been further discussed by Pedersen (1987). Whether the species and their viability are changed during pumping remains to be evaluated. The field lab gives water that has been slowly depressurized for some hours whilst being drawed. Further, some oxygen apparently dissolves in the water as it is drawn to the fieldlab. The gassampler is pressurized with the pressure at the sample level until opening when the water sample is instantaneously depressurized. This might affect the viability of the sampled microbes in different ways. Figure 3-1B showed a variability in the viable counts for samples from the gas sampler while there was almost no variability in viable counts in the field lab. The viability is obviously changed during pumping.

4.1.4 Total number of bacteria in relation to viable counts

The number of bacteria possible to recover with plate counts arrays from 0.10 to 21.9 % of the total numbers. The reasons for a lower viable count can be several. Firstly, far from all bacteria will grow on the medium used. Secondly, The fluorescence microscopy can not distinguish between viable and non-viable microorganisms. VanEs and Meyer-Reil (1982) gives 6 more general possibilities. In an investigation of a number of drinking water wells, Hirsch and Rades-Rohkohl (1988) found incubation time, sampling the aquifer versus the well, the diameter of the well, the age of the well, the medium composition and the sample storage time to influence the viable count and the microbial diversity. Different microbial populations and different status of the microbes harbouring different boreholes and levels will give a variability in the percentage of viable counts as was found here.

4.1.5 Total numbers of bacteria in other aquatic environments

Ghiorse and Wilson, (1988) have listed the numbers of bacteria in 11 pristine groundwaters. Their list arrays between 10^3 to 10^8 cells ml^{-1}. They report total numbers in the same order from subsurface materials from 13 contaminated sites. Fliermans and Balkwill (1989) report diverse, abundant communities of microbes to thrive 500 m underground in sedimentary formations. They claim terrestrial deep subsurface to be a habitat of great biological diversity and activity that does not decrease significantly with increasing depth (at least in their area of investigation; authors comment). VanEs and Meyer-Reil (1982) have compiled 44 total numbers of bacteria in aquatic marine environments over the world. Between 0.1 to 264 x 10^5 cells ml^{-1} are reported and these numbers can be compared with the between 0.68 to 16.6 x 10^5 cells ml^{-1} found here. It is rather fascinating that these deep groundwaters harbours bacteria in numbers within the range of what sea-water does. Their activity and origin remains to be evaluated.

4.1.6 Maintaining of deep ground water microbes

In most natural waters, freely suspended heterotrophic bacterial cells are assumed to exist under starving conditions. Growth is limited primarily by low concentrations of suitable carbon and energy sources. The cells are believed to be nongrowing at very low endogenous metabolic rates (Kjelleberg et al., 1987).

The organic content in deep groundwater is low in relation to surface waters, <0.5 - 6.9 mg TOC l^{-1} (Table 3-5). There was a correlation between the TOC content and the depth of sampling in this investigation (table 3-6). TOC decreased with the depth. (Fig. 3-3). It has been found in other deep ground waters in the KBS program that the major proportion of the TOC consists of carbon from humic material (approximately 50% of humic acids constitute carbon), at least when TOC is about 1 mg l^{-1} or less (B Allard, pers. com.) About 90% of the humic material is fulvic acids with a molecular weight of 1500-2000 u, the rest is humic acids. TOC concentration above 1 mg l^{-1} might indicate that there have been an inflow of water from the surface quite recently. However, 15.000 years old deep ground water with 10 mg humic acids have been found (B. Allard, pers. com.). Humic acids are comparatively resistant to bacteria and can not have contributed to microbial growth in waters with ages of a thousand year or more. Further, it must be assumed that this organic material has it's origin from biological activities on the ground surface or possibly from autolitothropic microbial activity in the groundwater (see section 4.3). The renewal is extremely slow for organic material as it has to move down with the groundwater and probably also very slow if production by autolitothropic activity exists. This means that any heterotrophic microbial activity must be very slow, probably more or less in a nongrowth state.

The correlation found between TOC and the total number of cells (Table 3-6, Fig 3-2) indicate the total number of cells to be partly dependant on the TOC-content. Assuming a TOC weight of a microbial cell to be 1.4 x 10^{-13} g (Ingraham et al. 1983), it turns out that the bacterial contribution to the TOC was approximately 3 % which is to little to explain the correlation. Heterotrophic utilization of parts of the TOC could explain the correlation obtained.

4.2 MICROBIAL BIOFILMS ON ROCK SURFACES

In the absolute majority of all microbial systems that exhibit microbes in the water phase, there also develop microbial biofilms (Bitton & Marshall, 1980, Marshall, 1984; Pedersen, 1982c). Even pure and disinfected drinking waters show this phenomenon (Pedersen, 1989b; Schoenen & Schöler, 1985).

This was also the case in KLX01, 680 m were we found 3.45 x 10^5 bacteria cm^2 on glass surfaces after 10 days of exposure to the flowing groundwater. The bacteria appeared in microcolonies with 2 to at most 20 cells in each colony. This indicate that the bacteria on the surfaces were active and in a state of growth.

It can the be assumed that the rock surfaces in the aquifers of this and probably also other boreholes are covered with microbial biofilms. It might be that the deep groundwater microbial activity should be looked for on the rock surfaces and not as was done here (for methodological reasons) in the ground water itself.

4.2.1 The impact of biofilms on the radionuclide sorption in the rock matrix.

Microbes and microbial biofilms are known to interact with metals like the radionuclides relevant for the

HLW-repository. (Ferris et al., 1989; Francis & Dodge, 1988; Kepkay, 1986; Strandberg et al., 1981).

If microbial biofilms grow in fissures with water that transport radionuclides from the repository, they might violate the radionuclide matrix sorption theory. If the affinity of the radionuclide is higher for the organic biofilm than for the rock it will result in organic complexes on the fissure surface. Detachment and sloughing processes can mobilize the radionuclide again and it will migrate with the flow instead of being retarded by the matrix. A second possibility might be that the biofilm-complexed metals are immobilized and mineralized permanently at the surface (cf Beveridge et al., 1983). The binding of metals by bacteria is discussed in section 4.5.

4.3 SPECIES OF MICROORGANISMS IN DEEP GROUND WATER

4.3.1 Deep ground water as a habitat for bacteria.

Table 3-5 shows that the physico-chemical environment in the ground water studied is rather extreme. It is anaerobic with a low E_h between -112 to -383 mV, a pH usually around 8 and a temperature of 10.2 - 20.5 °C, depending on the depth. The organic content is low, and ranges between <0.5 to 6.9 mg TOC l^{-1}. The salinity of the environment increases with the depth. Further, there are hydrogen-sulfide, methane, carbondioxide and hydrogen. The sulphate concentration increases with the depth. The nitrate, nitrite and phosphate concentrations are close to or below the detection limits, while there are detectable amounts of NH_4 in the range 4 - 330 $\mu g\ l^{-1}$.

Table 3-2 shows that some of the isolated bacteria were facultative, gram-negative, non-fermenting

heterotrophs. Their electron-acceptor is difficult to predict. Nitrate would have been good but there is a very low concentration of this element, eventually as a result of dissimilatory nitrate reduction to ammonium (Tiedje, 1988).

The presence of methane might indicate methanogens while the hydrogen sulfide could have been produced by dissimilatory sulphate reducing bacteria. Geological formation could also explain the presence of those gases. We know from other habitats that the formation of methane is insignificant in habitats where there is plenty of sulphate (Ormeland & Policin, 1982; Winfrey & Ward, 1983). It occurs only when the sulphate concentration falls to a very low level (Pfennig et al., 1981). This indicates that the sulphate reducing bacteria compete successfully with methanogenic bacteria for the available electron donors, e.g. hydrogen. Some work however report simultaneously methane production and sulphate reduction (Beeman & Sulflita, 1987; Ormeland & Policin, 1982).

The enrichment cultures for anaerobic bacteria has given positive results (table 3-3). They indicate the presence of anaerobic bacteria capable of growth of C-1 compounds with hydrogen and carbon dioxide, presumably methanogenic bacteria. Further, the enrichment with sulphate as electron-acceptor and lactate as carbon source, which would favour dissimilatory sulphate reducing bacteria, gave positive results.

4.3.2 Denitrifying bacteria

Reduction of nitrate is dominated by two dissimilatory processes: respiratory denitrification (RD) and dissimilatory nitrate reduction to ammonium (DNRA) (Tiedje, 1988). Those processes are inhibited by oxygen, they occur only in anaerobic environments. In anaerobic environments where the limitation of electron

acceptors often restricts metabolism, the accommodation of eight electrons per nitrogen in the nitrate to ammonium step makes this one of the most favourable electron acceptors available to anaerobic environment.

The fact that the ground waters studied lack measurable amounts of nitrate and exhibit ammonium in the range 4 - 330 $\mu g\, l^{-1}$ might indicate DNRA as the respiratory mechanism for heterotrophs in this environment.

4.3.3 Dissimilatory sulphate reducing bacteria

Dissimilatory sulphate reducing bacteria are probably one of the oldest forms of bacterial life on earth. Their activity have been traced back more than 3 billion years by sulphur isotope fractionation in minerals and rocks.

The sulphate reducing bacteria utilize sulphate mainly as the terminal electron acceptor in their anaerobic oxidation of organic substrates. As a consequence, they produce and accumulate large amounts of sulfide in their natural habitats and participate in the production and transformation of mineral deposits in nature.

All known sulphate reducing bacteria are strict anaerobes. Pure cultures of them require not only absence of oxygen for growth but also a low redox potential of about zero to -100 mV in the medium.

The known spectrum of organic carbon sources and electron donors used are rather limited. Lactate, pyruvate, fumarate, malate, ethanol and occasionally glucose and citrate are utilized with the formation of acetate and carbon dioxid as end products. Hydrogen or formate together with a carbon source , e.g., acetate plus bicarbonate , may serve as electron donor for growth. Some species of sulphate reducing bacteria are

capable of autotrophic growth with either hydrogen or formate as electron donor and carbon dioxid as sole carbon source.

The sulphate reducing bacteria are widely distributed in aquatic and terrestrial environments that are anaerobic due to the microbial decomposition of organic materials. Habitats in which sulphate reducing bacteria exhibit a high metabolic activity are readily apparent by the smell of hydrogen sulfide.

The ground waters sampled contained from 0.01 to 2.55 mg S^{-2} l^{-1}. KAS04, 195 m and KLX01 680 m could be shown to contain viable sulphate reducing bacteria (3.2.3). Those populations might have contributed to the sulfide in the waters.

4.3.4 The methanogenic bacteria

The methanogenic bacteria are unique among the prokaryotes because they produce a hydrocarbon, methane, as a major product of anaerobic metabolism.

Axenic cultures of the methanogenic bacteria establish that these organisms are morphologically diverse. Despite the diversity of morphological forms within the group, almost all members possess two unique cofactors, factor 420 and 2-mercaptoethanesulfonic acid. The factor 420 can be used for identification of methanogenic bacteria (Mink & Dugan 1977).

The methanogenes share the properties of strict anaerobiosis and the ability to reduce carbon dioxid with molecular hydrogen to produce methane. Some have the additional property of forming methane from simple substrates such as formate, methanol, methylamin, or acetate. Their physiological interaction with chemoheterotrophic bacteria via H_2-transfer reactions

establishes an important ecological function for the methanogenes in anaerobic habitats.

The methanogenic bacteria are widely distributed in nature. They occupy their terminal niche in the transfer of electrons by anaerobic decomposition of organic matter.

The enrichment cultures (Table 3-3) showed the presence of bacteria capable of anaerobic growth on C-1-compounds. Parts of the populations seem to have been methanogenic bacteria. Evidence for active presence of methanogenes in other geological formations as deep sediments have been reported (cf. Oremland, 1988).

4.4 THE ACTIVITY OF MICROORGANISMS IN DEEP GROUND WATER

4.4.1 Metabolic activity

Though bacterial numbers yield valuable information on bacterial communities, they do not necessarily reflect the dynamics of these communities and their interdependence with a repository. It is essential to know the metabolic activity of each separate group of organism of interest.

One of the basic problems in the study of *in situ* bacterial activity is that bacteria respond very quickly to relatively slight changes in their environment. Because of the pronounced response of bacteria to changing environmental conditions, the prerequisite of a measurement should be that the natural situation is not significantly altered. Another requirement is that, in calculating actual activity or biomass production, possible conversion factors are correct. As has been shown by Van-Es and Meyer-Reil (1982), these prerequisites are hard to fulfil. Further, many bacteria are sensitive to physical and chemical manipulations like sampling, incubation in a

container, disruption of dynamic equilibria (eg introduction of oxygen) and to be depressurized.

4.4.2 Heterotrophic uptake of glucose, glutamine, leucine, N-acetyl-glucose-amine and thymidine

In section 4.1.6 it was predicted that any heterotrophic microbial activity must be very slow, probably more or less in a nongrowth state. Assays of microbial heterotrophic activity should therefore indicate a low percentage of the population to be active. The three populations studied incorporated the amino acid leucine to different degrees and in KAS03, 860 m, also glutamine (Table 3-4). This results might be demonstrations of high affinity uptake by nongrowing cells as discussed by (Kjelleberg et al., 1987). Or, an indication of that growth was occurring. The very low uptake of glucose probably indicate the former of the two suggestions to be valid.

Thymidine is a nucleoside that can be used by many bacteria for the synthesis of RNA and DNA. It can therefore be used to estimate bacterial growth (Kirchman et al., 1982; Moriarty, 1986). The lack of uptake of thymidine might confirm that there was no growth occurring. A drawback of this method is that not all bacteria are able of taking up this substance. This has been shown by Pollard and Moriarty (1984) for some aquatic pseudomonads and is probably due to a deficiency in transport of thymidine. Bacteria with very limited nutrient requirements such as the chemolitotrophic bacteria may also lack such transport systems as suggested by Moriarty (1986) for sulphate-reducing bacteria. It has also been shown that several species of microorganisms lack the enzyme thymidine kinase, necessary for the conversion of the nucleoside to thymidine mono phosphate (TMP) (Moriarty, 1986).

Any uptake of N-acetyl-glucose-amine in KAS03, 860 m would have indicated synthesis of the peptidoglucan

structure of the bacterial cell-wall. No such activity was found.

The heterotrophs in KLX01, 680 m, KAS03, 860 m and KAS04, 380 m seem to have been in a state of non-growth (cf Kjelleberg et al., 1987).

A prolonged incubation time to 96 h allowed a growth on glucose and N-acetyl-glucose-amine in KAS04. It is known from marine environments that relatively small concentrations of glucose or amino acids have a rapid and considerable effect on aquatic bacterial communities (Vaccaro, 1969; Williams & Gray, 1970). This seems to be the case also for deep ground water populations and is an important conclusion when evaluating the influence from building a repository on microbes.

4.4.3 The anaerobic environment and uptake of formate methanol, acetate and carbonate.

Much of the work that have been done on microbial activity in nutrient poor environments were done aerobically. The groundwater studied is however anaerobic with a very low E_h. The sulphur and hydrogen oxidizers and the methanogenic bacteria that can inhabit this environment may show a very different response to radiographic assays. The enrichment cultures indicated the presence of bacteria capable of living on CO_2 and H_2, formate, methanol and lactate (table 3-3).

There was no uptake of the one and two-carbon sources introduced in the samples. This indicates that anaerobic populations were inactive or, that the physical and chemical manipulations introduced during sampling, incubation in a container, disruption of dynamic equilibria and depressurizing might have

disturbed their possible activity. This could also be the explanation to why CO_2-fixation was not detected.

A prolonged incubation time to 96 h allowed a growth on formate and acetate to up to 60 times more bacteria than initially assayed. Such long incubation times reflect the potential of a microbial population to metabolize carbon sources introduced in the repository.

4.4.4 Potential activity of microbial biofilms

The assays used can not give information on microbial processes in biofilms down in the aquifers. The biofilm experiment (3.4) indicated a very active microbial population in relation to the bulk water population. It might very well be that when activity measurements on the rock wall are possible to perform, a totally different picture of the deep ground water microbiology will show up. The work by Van der Wende et. al. (1989) demonstrated how microbial biofilm growth and detachment accounted for most, if not all, of the planktonic cells present in a drinking water. The bulk water microbial cell in deep ground water might then be inactive cells detached from an active biofilm.

4.5 MICROBIAL PRODUCTION OF COMPLEXING COMPOUNDS

In all, 78 elements comply with the definition of a metal. Several of those are heavy metals, essential for growth, reproduction and/or survival of many microorganisms as they may constitute central parts of cofactors and prosthetic groups of different enzymes. Manganese, Co, Cu and Zn are frequently essential for growth whereas Cr, Ni, Mo and Sn may be required for specific metabolic processes by particular organisms.

Of the various metal-complexing agents in aquatic systems, microorganisms and their constituent polymers are among the most efficient scavengers of metallic ions. For the intention of this report, two main types

of complexing agents can be identified (confer Fig 1-1). 1) Compounds, bound in one way or another to the cell, mainly the cell-wall. The metal will become a part of the cell. 2) Organic components, released to the environment by the microorganism, either to enhance the bio-availability of the metal or to reduce the concentration of a deleterious metal. The speciation of the metal will change.

4.5.1 Complexing compounds associated with microorganisms

Most bacteria has a cell-wall that, together with the cytoplasmatic membrane, separate the cell from the outer environment. The cell-wall contributes to the shape and form of the cell. It provides a rigid mechanical support, preventing turgor pressure from bursting the cell. It is also involved in the transport of molecules in and out of the cell.

The Gram-positive wall is essentially a multisheet peptidoglycan structure. Variation in peptidoglycan structure is particularly marked among Gram-positive species but it is the nature of teichoic acids present that really confers individuality on Gram-positive strains. Up to 50 % of the wall mass can be teichoic acids most of which is covalently linked to the 6-OH of occasional muramic acid residues of the peptidoglucan. It is known that teichoic acids bind Mg2+, and it has been suggested that they play a role in supplying this ion to the cell.

The Gram-negative cell characteristically has a very thin peptidoglycan layer, a periplasm and an outer membrane. The outer membrane has the familiar lipid bilayer common to all biological membranes. The outer leaflet contain a molecule unique to the outer membrane, the lipopolysaccharide. This molecule is a lipid with a core polysaccharide with polysaccaride side chains that projects outwards. Mg^{2+} ions stabilize the lipopolysaccaride molecules. The outer membrane

also has a number of different proteins for binding, transport, diffusion and chemotaxis.

Archaebacteria has a wall structure that seems to bee only quite distantly related to other bacteria. (Woese et al., 1978). Wall composition and ultrastructure is quite variable among archaebacteria (Kandler & Konig, 1978). Some contain a heteropolymer, termed pseudomurein, others have walls composed of protein (eg *Methanospirillum hungatii*) and still others have heteropolysaccharide walls.

Like most cell surfaces, the bacterial wall is anionic independent of if the cell is Gram-negative or positive. It is reasonable to assume that bacteria with their encompassing outer layers will interact strongly with metallic ions in dilute solution within natural bodies of water. Beveridge & Fyfe (1985) showed how Na, K, Mg, Ca, Mn, Fe^{3+}, Ni, Cu, and Au^{3+} bound to cell walls of *Bacillus subtilis, B. licheniformis* and *Escherichia coli.* The binding was in the order of μmoles per milligram dry-weight of cell walls. They propose a two step mechanism for the deposition process. The first step is a stoichiometric interaction between metal ion and active site within the cell wall. This interaction then acts as a nucleation site for the deposition of more metal from solution. The end result is a cell wall that contains copious amounts of metal, which is not easily replaced by water or the hydrated proton. If microbial biofilms exists in the rock around the repository, their interaction with the radionuclides must be determined. Ferris et. al., (1989) have shown that such interactions are significant in acidic and neutral aquatic environments.

Many bacteria can produce extra cellular polymers like capsules (eg. *Klebsiella aerogenes)* or a gelatinous matrix (eg. *Zoogloea ramnifera*). Such polymers have been shown to be involved in the formation of biofilms on surfaces

(Costerton et al., 1987) and might therefore be of interest here. Rudd et al.; (1984) studied the strength and nature of the binding of heavy metal ions to bacterial extracellular polymers. They found the conditional stability constants for complexes formed between K. aerogenes polymers and the metals Cu, Ni, Co and Cd to be comparable with those of naturally occurring organic polymers such as fulvic acids and humic acids determined under similar conditions. Mittelman and Geesey (1985) found copper-complexing exopolymers with an freshwater-sediment bacterium.

Intracellular uptake of uranium has been shown by Strandberg et al., (1981). The uptake mechanism is unclear. Perhaps metal-binding proteins, necessary for the supply of trace metals to the cell, are responsible. This is discussed under 4.5.2.

4.5.2 Complexing compounds released by microorganisms

The ability of microorganisms to release organic metabolites into the environment, capable of complexing metals is known. Mycobacteria produce mycobactins as iron-chelating growth factors (Snow, 1970). Many bacteria produce hydroxamic substances as sophisticated ligands for the uptake iron (Neilands 1967). Jardim and Pearson (1984) demonstrated copper-complexing compounds released by some species of cyanobacteria.

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