Deep ground water microbiology in Swedish granitic rock and it's relevance for radio-nuclide migration from a Swedish high level nuclear waste repository

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ABSTRACT

Data on numbers, species and activity of deep ground water microbial populations in Swedish granitic rock have been collected. Specific studies are performed on radio-nuclid uptake on bacteria judged to be probable inhabitants in Swedish nuclear waste repositories.

An integrated mobile field laboratory was used for water sampling and for the immediate counting and inoculation of the samples from bore holes at levels between 129 and 860 m. 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.

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

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SUMMARY

We know very little about the microbial inhabitants below our feets, mainly due to the obvious problem of how to collect undisturbed samples. However, the existence of microbes in ground water can be expected with respect to the enormous adaptability of different microbial populations. The report "Preliminary investigations of deep ground water microbiology in Swedish granitic rock" (Pedersen 1987) confirms the existence of microbial populations in deep ground water, at least in the areas investigated.

Data on numbers, species and activity of deep ground water microbial populations in Swedish granitic rock have been collected. Specific studies are performed on radio-nuclid uptake on bacteria judged to be probable inhabitants in Swedish nuclear waste repositories.

An integrated mobile field laboratory was used for water sampling and for the immediate counting and inoculation of the samples from bore holes at levels between 129 and 860 m. 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.

The number of bacteria were determined in samples from the gas sampler when this was possible. Else numbers are determined in the water that is pumped up to the field lab. The average total number of bacteria is 3×10^5 bacteria ml^{-1}. The number of bacteria possible to recover with plate count arrays from 0.10 to 21.9 %.

1. INTRODUCTION

We know very little about the microbial inhabitants below our feets, mainly due to the obvious problem of how to collect undisturbed samples. However, the existence of microbes in ground water can be expected with respect to the enormous adaptability of different microbial populations. The report "Preliminary investigations of deep ground water microbiology in Swedish granitic rock" (Pedersen 1987) confirms the existence of microbial populations in deep ground water, at least in the areas investigated.

Pedersen (1987) identified 3 main tasks that has to be solved before safe predictions on the influence from microbes on radio-nuclid migration can be made. They were:

1. Data on numbers, species and activity of deep ground water microbial populations in Swedish granitic rock have to be collected.
2. Models for the interactions between a nuclear waste repository and the developing microbial populations should be set up. Actual data collected in Swedish granitic rock should be used, merged with studies on the interactions between backfill materials and microbes.
3. Specific studies should be performed on the effect on radio-nuclid migration from bacteria judged by the models to be probable inhabitants in Swedish nuclear waste repositories.

The main research efforts this year have been concentrated on task 1 but have also aimed somewhat towards task 3.

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 inoculation 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. The examined level was closed of in a section of 5 to 10 meters, or more when necessary for achieving a satisfactory water flow.

2.2 TEST SITES

2.2.1 Ävrö

The bore hole AV01 was investigated at 4 different levels: 420, 522, 558 and 635 meters, (Pedersen 1987).

2.2.2 Äspö

The bore hole AS02 was investigated at 3 different levels: 202, 463 and 860 meters. The bore hole AS03 was investigated at 1 level, 129 meters.

2.2.3 Laxemar

The bore hole LX01 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 1000 ml of a 6.6 mM phosphate buffer, pH 6.7. The phosphate buffer was a mixture of 0.449 g KH_2PO_4 and 0.588 g Na_2HPO_4 in 1000 ml de-ionized water. The AO solution was stored as 10 ml aliquots. All solutions and the water were filter sterilized (0.2 lm). 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 lm = 0.0064 mm^2) (n=15) were counted on each filter.

2.3.2 Viable count for facultative anaerobic heterotrophs

The number of facultative anaerobic 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, glucose 0.25g, starch 0.25g, $CaCl_2 \cdot 2H_2O$ 0.2g, K_2HPO_4 0.1g, 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 agar was omitted and the medium divided into 100 ml portions in 300 ml Erlen-Meyer flasks in the growth experiments.

The trace metal solution consisted of $ZnSO_4 \cdot 7H_2O$ 2.2g, $CaCl_2 \cdot 6H_2O$ 7.34g, $MnCl_2 \cdot 4H_2O$ 2.5g, $CoCl_2 \cdot 6H_2O$ 0.5g, $(NH4)_6Mo_7O_{24} \cdot 4H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 5g, $CuSO_4 \cdot 5H_2O$ 0.2g, Na_2EDTA 50g, NaOH for pH adjustment, Distilled water 1000 ml, pH 4.0.

For dilution of the samples prior to the plate count a dilution medium was used. It consisted of $CaCl_2 \cdot 2H_2O$ 0.2g, $MgSO_4 \cdot 7H_2O$ 0.4g, K_2HPO_4 0.1g, NaCl according to the salinity of the ground water sampled, de-ionized water 1000 ml.

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 heterotrophes

A number of different bacteria from the highest dilutions (2.3.2) were reinoculated and isolated. Growth experiments in liquid media (2.3.2) were made. The growth rates were determined for the temperature range of the bacteria isolated. 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 KH_2PO_4 0.2g, NH_4Cl 0.27g, NaCl 10g, KCl 0.62g, $MgCl_2 \cdot 6H_2O$ 0.62g, $CaCl_2 \cdot 6H_2O$ 0.3g, $NaHCO_3$ 5g, $Na_2S \cdot 9H_2O$ 0.27g and vitamins and trace elements dissolved in 1000 ml de-ionized water. The medium was portioned in aliquotes of 50 ml in 100 ml serum bottles with butyl rubber stoppers under N_2/CO_2 (90/10%) atmosphere. Series of bottles with medium were subsequently supplied with the following combinations: Acetate, Formate, Formate + H_2/CO_2, Formate + H_2/CO_2 + yeast extract, H_2/CO_2, Metanol, Methanol + yeast extract. 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. Agar shakes were subsequently made on the enrichment cultures from which isolation to hungate tubes are made.

2.4 MICROAUTORADIOGRAPHIC STUDIES OF MICROBIAL ACTIVITY

The MARGE-E technique developed by Tabor and Neihof (1982) were used to determine the substrate uptake activity of individual bacteria in the ground water. So far we have used acetate (^3H), 0.2 $\mu Ci\ ml^{-1}$. Other carbon sources are being included.

2.5 BACTERIAL UPTAKE OF RADIO-NUCLIDS

Successfully isolated bacteria will be studied for their ability to take up radio nuclids. The bacterium is cultured over night, harvested by centrifugation and washed twice in 0.9% NaCl (centrifugated 20 min. at 17000g) at pH 7.

Two dilutions of the Gram-negative Y1 species and the Gram-positive *Bacillus megaterium*, 1×10^8 and 5×10^8 bacteria ml^{-1} Were mixed with 84 pmolar Pm at pH between 5 and 9. The samples were centrifugated at approximately 15000g and the remaining activity in the supernatant was measured.

This work is done in collaboration with Y. Albinsson at Dept. of Nuclear chemistry at Chalmers Technical Univ.

3. RESULTS

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

Table 3-1 shows the results from the enumerations of bacteria in the boreholes Ävrö 01 (KAV01), Äspö 02 and 03 (KAS02 and KAS03) and Laxemar 01 (KLX01). The number of bacteria were determined in samples from the gas sampler when this was possible. Else numbers are determined in the water that is pumped up to the field lab. The average total number of bacteria is 3 x 10^5 bacteria ml^{-1}. The number of bacteria possible to recover with plate count arrays from 0.10 to 21.9 %.

Table 3-1 The table shows the total number of bacteria in the three studied boreholes, KAV01, KAS02 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. G= Gas sampler, F=Field lab.

Date of sampling	borehole code	Analyse code	Depth m	Total no. cells ml^{-1} x 10^5	Viable count (v.c) cells ml^{-1} x 10^3	% v.c av total no.
870923	KAV01		420	16.6 (F)	2.49	0.15
870826	KAV01		522	3.31 (F)	2.19	0.66
870604	KAV01		558	3.82 (F)	-	-
870421	KAV01		635	1.30 (F)	-	-
881209	KLX01	KBS 1538	272	2.05 (F)	0.21	0.10
881123	KLX01	KBS 1528	466	1.10 (F)	15.3	13.9
881102	KLX01	KBS 1516	680	1.68 (F)	3.30	1.96
890111	KAS02	KBS 1548	202	1.15 (G)	1.14	0.99
880412	KAS02	KBS 1419	314	0.79 (G)	4.93	6.24
880426	KAS02	KBS 1428	463	1.71 (G)	37.6	21.9
890131	KAS02	KBS 1560	860	1.15 (G)	0.81	0.70
890222	KAS03	KBS 1569	129	2.00 (G)	6.1	3.05

3.2 ISOLATION AND IDENTIFICATION OF COLLECTED BACTERIA.

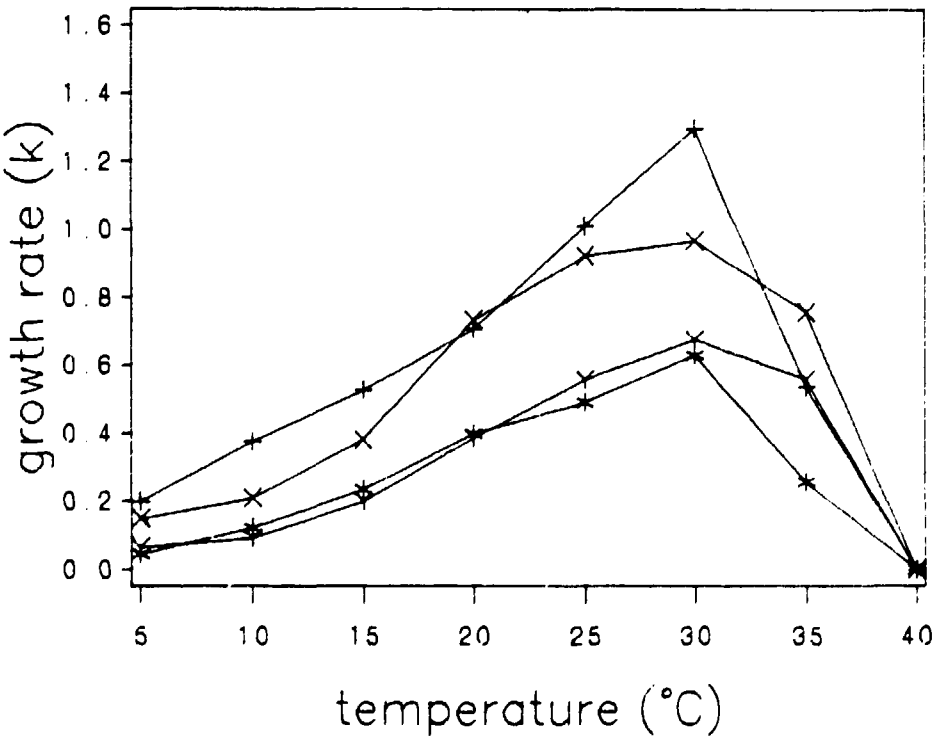
3.2.1 Facultative anaerobic heterothrophs

KBS 1428 showed the largest number of viable count (Table 3-1). Four bacteria were isolated from the gas sampler, identified and characterized. Table 3-2 shows the data obtained. Fig. 3-1 gives the growth rates versus culturing temperature.

Table 3-2 The species of bacteria identified in KBS 1428. CCUG=Culture Collection, University of Göteborg.

Viable no. of cells ml^{-1} in the round water	sample code	species	CCUG number
n.d.	Y1	*Shewanella putrefaciens*	CCUG-22946
2×10^3	Y2	*Pseudomonas vesicularis*-like	CCUG-22947
n.d.	Y3	*Shewanella putrefaciens* (atypical)	CCUG-22948
4.2×10^4	Y4	*Pseudomonas vesicularis*-like	CCUG-22949

Fig 3-1 Growth rates of the isolated bacteria (table 3-2) as a function of temperature. +=Y1, *=Y2, x=Y3, y=Y4.



3.2.2 *Obligate anaerobes*

We have achived growth in all enrichment cultures exept for cultures with acetate. Isolation and identification are undertaken during spring 1989.

3.3 MICROBIAL ACTIVITY

Samples from KBS 1538 were added with acetate, 0.2 μCi ml^{-1}. The total number of bacteria was 2.05×10^5 cells ml^{-1} wereof 15 % showed active acetate uptake.

Samples from KBS 1569 were added with acetate 0.4 μCi/ml. The total number of bacteria was $2.0.10^5$ cells ml^{-1} wereof 1.4 % showed active acetate uptake.

3.4 BACTERIAL UPTAKE OF RADIONUCLIDS

Those experiments are undertaken at the time for writing this report. We have only preliminary results mow. We have observed takup of the nuclid which seems to be dependant of pH, bacterial number and the species used.

3.5 THE COMPOSITION OF THE GROUND WATERS STUDIED

The ground water composition is shown in Table 3-3.

Table3-3 The composition of the groundwater of KAV01, KAS02, KAS03 and KLX01.

Level m		420	522	558	635	202	314
Borehole		KAV01	KAV01	KAV01	KAV01	KAS02	KAS02
KBS-Code		-	-	-	-	1548	1419
Temp	°C						12.41
pH		6.9	7.0	7.2	6.5	7.43	8.52
Redox mV						-258	-293
Cond.	ms/m	232	680	1310	2660	1541	1560
O_2	µl/l	-	-	-	-	- -	-
N_2	µl/l	-	-	-	-	-	-
H_2	µl/l	-	-	-	-	610	3700
He	µl/l	-	-	-	-	-	3000
CH_4	µl/l	-	-	-	-	30	60
CO	µl/l	-	-	-	-	0.6	20
CO_2	µl/l	-	-	-	-	25000	1400
TOC	mg/l	9.6	-	3.9	<0.5		
SiO_2	mg/l	4.9	5.8	5.1	4.0	13	5.23
Na^{+}	mg/l	255	750	1500	3200	1206	1560
K^{+}	mg/l	5.0	7.4	6	8	6.7	7.1
Li^{+}	mg/l	0.0051	0.21	0.55	1.2	-	-
Ca^{+2}	mg/l	162	440	1100	2800	998	1541
Mg^{+2}	mg/l	29	42	60	31	60.8	75
Sr^{+2}	mg/l	5.9	6.0	20	-	-	-
Al^{+3}	mg/l	0.16	0.11	0.39	0.027	-	-
Mn^{+2}	mg/l	3.1	2.4	1.7	0.18	1.0	0.81
Fe^{2+}	mg/l	1.68	2.23	1.02	0.430	0.483	0.792
Fe_{tot}	mg/l	1.68	2.23	1.02	0.438	0.500	0.793
HCO_3	mg/l	1.87	81	42.3	9.9	71	26.6
Cl^{-}	mg/l	616	1970	4300	9700	3822	5343
F^{-}	mg/l	2.6	2.2	1.8	1.4	1.36	1.33
Br^{-}	mg/l	3.0	8.9	24	72	13.4	22.6
I^{-}	mg/l	0.06	0.10	0.32	0.72	-	-
S^{-2}	mg/l	0.59	1.20	0.81	<0.01	0.48	0.143
PO_4^{-2}	mg/l	0.005	<0.003	0.010	<0.003	0.003	0.001
SO_4^{-2}	mg/l	47	118	220	400	106	271
NO_2^{-}	mg/l	<0.001	<0.001	<0.001	<0.001	0.002	0.001
NO_3^{-}	mg/l	<0.01	<0.01	<0.01	<0.01	-	-
NH_4^{+}	mg/l	0.08	0.06	0.08	-	0.39	0.33
Uranine	%	-	-	-	-	0.808	0.616

Table 3-3 continuing

Level m		463	860	272	466	701	129
Borehole KBS-Code		KAS02 1428	KAS02 1560	KLX01 1538	KLX01 1528	KLX01 1516	KAS03 1569
Temp	°C	15.47	16.32				
pH		8.34	8.35	7.93	8.2	8.05	8.04
Redox mV		-267	-140				
Cond.	ms/m	1630	3130	760	637	1714	471
O_2	µl/l		-	-	-	-	-
N_2	µl/l		-	-	-	-	-
H_2	µl/l		-	88	-	-	-
He	µl/l		-	-	-	-	-
CH_4	µl/l		34	110	-	220	16
CO	µl/l		42	14	-	1.5	11
CO_2	µl/l		490	25000	-	290	1200
O_2	mg/l	<0.01					
TOC	mg/l	3.0					
SiO_2	mg/l	3.6	8.38	11.4	11.6	13	11.5
Na^{+}	mg/l	1800	2845	1011	854	1120	609
K^{+}	mg/l	8.1	11.0	5.68	6.0	6.4	2.20
Li^{+}	mg/l	0.81	-	-	-	-	-
Ca^{+2}	mg/l	1580	3831	244	225	1400	163
Mg^{+2}	mg/l	66	31.5	26.2	17	9.1	20.4
Sr^{+2}	mg/l	30	-	-	-	-	-
Al^{+3}	mg/l	0.046	-	-	-	-	-
Mn^{+2}	mg/l	0.73	0.28	0.197	0.138	0.191	0.105
Fe^{2+}	mg/l	-	0.485	0.198	0.390	0.029	0.123
Fe_{tot}	mg/l	0.964	0.500	0.200	0.410	0.031	0.124
HCO_3	mg/l	25	11.0	80.6	77.0	23.9	61.2
Cl^{-}	mg/l	5440	11097	2070	1698	4861	1234
F^{-}	mg/l	1.4	1.62	2.32	2.46	1.63	2.12
Br^{-}	mg/l	28	74.1	6.03	6.44	38	4.81
I^{-}	mg/l	0.32	-	-	-	-	-
S^{-2}	mg/l	0.13	0.715	0.473	0.460	2.55	0.586
PO_4^{-2}	mg/l	0.004	0.011	-	0.004	0.017	0.005
SO_4^{-2}	mg/l	290	518	48.5	105	351	31.1
NO_2^{-}	mg/l	<0.001	<0.001	-	<0.001	<0.001	<0.001
NO_3^{-}	mg/l	<0.01	-	-	-	-	-
NH_4^{+}	mg/l	0.22	0.011	-	0.061	0.004	0.036
Uranine	%	0.38	0.224	-	13.7	2.62	0.064

4. DISCUSSION

4.1 DEEP GROUND WATER MICROBIOLOGY

4.1.1 Dissimilatory sulfate reducing bacteria

Dissimilatory sulfate 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 sulfur isotope fractionation in minerals and rocks.

The sulfate reducing bacteria utilize sulfate 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 sulfate 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 sulfate reducing bacteria are capable of autotrophic growth with either hydrogen or formate as electron donor and carbon dioxid as sole carbon source.

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

4.1.2 The methanogenic bacteria

The metanogenic 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 widley distributed in nature. They occupy their terminal niche in the transfer of electrons by anaerobic decomposition of organic matter.

4.1.3 Deep ground water as a habitat for bacteria.

Table 3-3 shows that the physico-chemical environment in the ground water studied is rather extreme. There is no oxygen, the redox is low and the salinity of the environment increases with the deepth. Further, there is hydrogen sulfide, methane and hydrogen together with sulfate and carbon mono- and dioxid. The organic content is low, a few mg TOC l^{-1}. The average number of bacteria is $3 \cdot 10^5$ cells ml^{-1}.

The presence of methane might indicate methanogens while the hydrogen sulfide could have been produced by dissimilatory sulfate reducing bacteria. Geological formation could also explain the presence of those gases. We know from marine habitats that the formation of methane is insignificant in marine and saline habitats and occurs only when the sulfate concentration falls to a very low level. This indicates that the sulfate reducing bacteria compete successfully with methanogenic bacteria for the available electron donors, e.g. hydrogen.

The enrichment cultures for anaerobic bacteria has given positive results with environments for anaerobic bacteria without sulfate. The results indicate the presence of anaerobic bacteria capable of growth of C-1 compounds with hydrogen and carbon dioxide, presumably methanogenic bacteria. The enriched organisms will be isolated and identified if possible. Further, we will include enrichment substrates with sulfate which would favour dissimilatory sulfate reducing bacteria.

The present hypothesis is that a large portion of the bacterial population in the investigated ground water

consists of methanogenes and/or sulfate reducing bacteria working at very slow growth rates.

4.1.4 Activity of deep ground water bacteria.

Two levels have been studied with acetate. The obtained activities correspond to 15 and 1.4% of the populations counted. This coincides with the enrichment cultures where low response was obtained when acetate was used as the sole carbon source. The carbon sources will be expanded to formate (^{3}H), methanol(^{14}C), leucine(^{3}H), glucose(^{3}H), and carbon dioxide (^{14}C). This might give a better understanding of what populations are present.

4.2 BACTERIAL UPTAKE OF RADIO-NUKLIDS

We have observed uptake of Pm as a function of pH, bacterial species and numbers. The results are preliminary.

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[1] Swedish Geological Company, Uppsala, Sweden
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