The Palmottu Natural Analogue Project

Technical Report 99-14

Analysis of diversity and distribution of micro-organisms in Palmottu groundwater and evaluation of their influence on redox potential and uranium (VI) reduction

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PREFACE

The Palmottu Natural Analogue Project is jointly funded by the Commission of the European Communities, the Geological Survey of Finland (GTK), Radiation and Nuclear Safety Authority, Finland (STUK), Empresa Nacional de Residuos Radioactivos S.A (ENRESA)/Centro de Investicationes Energéticas, Medioambientales y Tecnológicas (Ciemat), Svensk Kärnbränslehantering Ab (SKB) and Bureau de Recherches Géologiques et Minières (BRGM).

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Introduction

Extensive investigations of Finnish and Swedish deep groundwater have revealed that numerous populations of microorganisms dwell deep below the ground level (Haveman et al 1998, Pedersen 1997). These populations may influence the disposal of high level radioactive waste in a number of different ways (Pedersen 1999). Most microbial activities tend to lower the redox potential through a series of redox reactions including a set of electron donors and acceptors. The 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 Fig. 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. Several investigations have demonstrated that microorganisms can reduce U(VI) to U(IV) (Lovley and Phillips 1992a, 1992b, Lovley et al 1991).

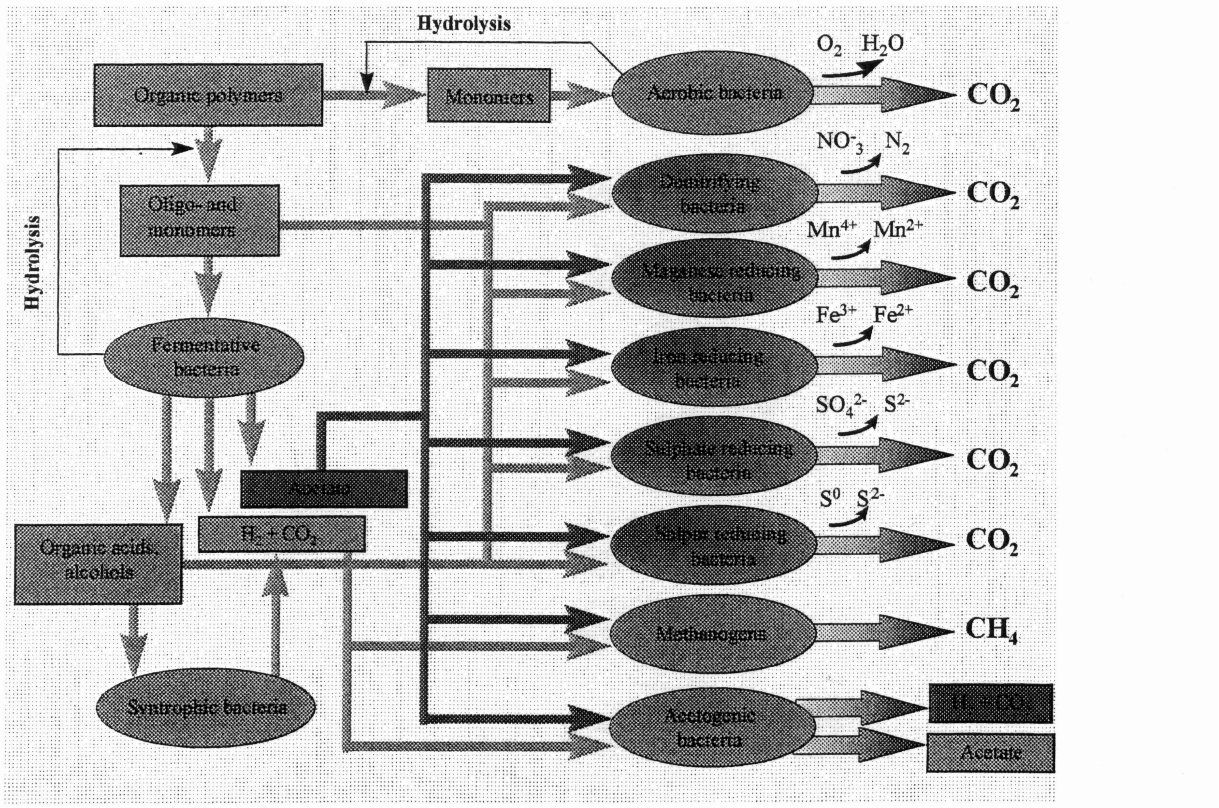

Figure 1 *The degradation of organic carbon can occur via a number of different metabolic pathways, characterised by the principal electron acceptor in the carbon oxidation reaction. A range of significant groundwater compounds are formed or consumed during this process. Of great importance for the groundwater redox potential is the production of ferrous iron and hydrogen sulphide and the turnover of gases such as carbon dioxide, hydrogen and methane.*

The possibility of microbially driven redox processes in the Palmottu motivated a search for most of the physiological groups of microorganisms indicated in figure 1. Iron and sulphate reducing bacteria (IRB and SRB, respectively), heterotrophic and autotrophic acetogens (HA and AA, respectively), and heterotrophic and autotrophic methanogens (HM and AM, respectively) were analysed in three Palmottu boreholes. The potential for uranium reduction of enrichment cultures of IRB and SRB was also studied.

Materials and Methods

Study Sites and samples

Groundwater was sampled from the Palmottu site in southwestern Finland. (Blomqvist et al., 1998). The boreholes and depths sampled are outlined in Table 1.

***Table 1**: Groundwater samples analysed*

Borehole	Depth (m)	Date Sampled	Sampling method
R337	80-100	980721	GTK
R302	80-132	980721	GTK
R387	119-127	981103	SKB
R387	304-309	980915	SKB
R387	32-36.8	990516	GTK

Groundwater Sampling

The SKB mobile lab wagon was used for sampling of the borehole R387. Borehole R302 and 337 were sampled with the GTK method. Each borehole section was pumped until readings stabilized. A groundwater sample was collected in a 5 liter polycarbonate bottle and shipped to the lab in Göteborg on ice within 12 hours of sample collection. This groundwater was used to make media as described below. Microbiologists traveled to Palmottu to collect the sample and inoculate the MPN tubes. Samples were collected in the mobile lab wagon, or at the borehole into sterile glass bottles under a stream of sterile N_2. Inoculation of media was started immediately after collection of the groundwater, and work with each sample was complete within 6 hours of sample collection. MPN tubes were transported back to the laboratory overnight and incubated at 17^{o}C on their sides. H_2 was added at 2 bar overpressure to autotrophic methanogen and autotrophic acetogen tubes upon return to the laboratory.

Geochemistry

Samples for geochemistry were collected during the same sampling period as the microbial samples. Analysis methods and results are reported elsewhere (Kaija, 1998; Pitkänen et al., in press). Table 2 shows data important for microbial processes.

Media

In previous sampling of groundwaters from Finland, viable microbes were cultured using media designed for each groundwater sample based on groundwater chemical data (Haveman et al. 1998). After inoculation, 10% 0.2μm filter sterilized groundwater (DynaGard filters, Microgon Inc., Laguna Hills, California) was added to provide any growth factors present in the groundwater but not in the media.

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

***Table 2**: Groundwater geochemistry data (Kaija, 1988f).*

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

The groundwater-based media were prepared anaerobically, according to the Hungate method (Hungate 1969). Media components were added from sterile, anaerobic stock solutions. Media contained (g/l): resazurin 0.0002; NH_4Cl, 0.4; KH_2PO_4, 0.01; Na_2SO_4, 0.002; Cysteine $HCl{\cdot}H_2O$, 0.25; $Na_2S{\cdot}9H_2O$, 0.25; Element solution (Haveman et al. 1998), 10 ml; and Vitamin solution (Wolin et al. 1963), 5 ml. Buffers were added from sterile, anaerobic stock solutions depending on borehole pH. Media for groundwater with pH 7.0-8.0 contained 1.72 g/l $NaHCO_3$. Media for groundwater with pH 8.0-9.0 contained 0.86 g/l $NaHCO_3$ and 1.21 g/l Tris HCl. The pH of the media was checked and adjusted to borehole pH, if necessary, with sterile, anaerobic HCl and NaOH solutions.

For the R387/304-309m sample, synthetic medium was prepared as a comparison to the groundwater-based media. This synthetic medium contained (g/l): resazurin 0.0002; NH_4Cl, 0.4; KH_2PO_4, 0.01; Na_2SO_4, 0.002; $CaCl_2{\cdot}2H_2O$, 0.1; $MgCl_2{\cdot}6H_2O$, 0.05; $FeCl_2{\cdot}4H_2O$, 0.001; $NaHCO_3$, 1.29; Tris HCl, 1.82; Cysteine $HCl{\cdot}H_2O$, 0.25; $Na_2S{\cdot}9H_2O$, 0.25; Element solution (Haveman et al. 1998), 10 ml; and Vitamin solution (Wolin et al. 1963), 5 ml. The pH of the media was checked and adjusted to pH 8.7 with sterile, anaerobic HCl and NaOH solutions.

Substrates were added to separate aliquots of media for the different physiological groups of microorganisms investigated. The medium for autotrophic methanogens (AM) contained no additions. The medium for heterotrophic methanogens (HM) contained 10 mM acetate, 10 mM trimethylamine (TMA), 50 mM methanol and 74 mM formate. The medium for autotrophic acetogens (AA) contained 50 mM 2-bromethanesulfonic acid (BESA) as an inhibitor of methanogenesis. The medium for heterotrophic acetogens (HA) contained 50 mM BESA, 10 mM TMA, 74 mM formate, and 2 g/l yeast extract. The medium for SRB contained 14 mM Na_2SO_4 and 6 mM lactate. The medium for IRB contained 7 g/l amorphous iron and 11 mM lactate. For the HH-KR6 sample, IRB were tested with a combination of acetate and lactate. This IRB+acetate medium contained 7 g/l amorphous iron, 11 mM lactate, and 10 mM acetate. The different types of media were dispensed anaerobically in 9 ml aliquots in sterile Hungate test tubes (Bellco) with N_2 in the gas phase, and stoppered with sterile blue rubber stoppers (Bellco).

Microbiology

Total cell numbers were determined according to the direct count method described previously (Pedersen & Ekendahl 1990), except that filters were rinsed twice with 1.0 ml of 0.2 μm filtered (DynaGard filters) double distilled water to dissolve salt crystals prior to staining for 10 minutes with 10 μg/ml acridine orange (AO) or 4',6-diamidino-2-phenylindole (DAPI). Cells were counted on an Olympus BH-2 microscope with blue filters for AO and UV filters for DAPI. Results were calculated as an average of 2 filters prepared for each stain, with sample standard deviation as the error.

The most probable number (MPN) of each physiological group of *Bacteria* or *Archaea* in each sample were determined (Koch 1994) using the media prepared for the various groups. For the groundwater-based media, negative controls were prepared with medium only and inoculated with 1 ml groundwater and immediately killed with 2% formaldehyde. In the case of synthetic media, a 1 ml aliquot of 0.2 μm filter sterilized groundwater (DynaGard filters) was added to each dilution to provide any growth factors present in the groundwater, but not in the media. Three types of negative controls were prepared for the synthetic media: with medium only, with addition of 1 ml 0.2 μm filter sterilized groundwater, and inoculated with 1 ml groundwater and immediately killed with 2% formaldehyde. The AM and AA tubes were gassed with 2 bar overpressure oxygen-free H_2. MPN tubes were incubated on their sides in the dark at 17°C for 6-8 weeks.

MPN tubes were analyzed for products of metabolism. The methanogenic tubes (AM and HM) were analysed for presence of CH_4 by gas chromatography as described previously (Kotelnikova et al. 1998). The acetogenic tubes (AA and HA) were also analysed for CH_4 as negative controls for methanogenesis. Acetate was analysed in the acetogenic tubes by an enzymatic and UV method (Boehringer Mannheim, Mannheim, Germany). SRB tubes were analysed for sulfide using the $CuSO_4$ method (Widdel & Bak 1992). IRB tubes were analysed for both total and ferrous iron using a spectrophotometric ferrozine method (Stookey 1970, Hallbeck 1993). Tubes were graded positive or negative in comparison with negative controls and MPN was calculated with a computer program from Yamanashi University, Ishikawajima-Harima Heavy Industries, Ltd. (Hurley & Roscoe 1983). The detection limit for MPN was 0.2 cells/ml.

Uranium Reduction

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

Results

Numbers of microorganisms

Results of total counting and MPN are presented in Table 3. These numbers represent the best results of synthetic or groundwater-based media if both were analysed. Comparisons between synthetic and groundwater-based media for R387/304-309 m are presented in Table 4.

Table 3*: Results of total counting and MPN analyses of Palmottu samples.*

Borehole	**R337**	**R302**	**R387**	**R387**	**R387**
Depth (m)	**80-100**	**80-132**	**32-36.8**	**119-127**	**304-309**
Total (±SD) x10^5	0.59	0.65	2.1	1.4	0.23
Autotrophic Methanogens (AM)	NT*	NT	NT	<0.2	<0.2
Heterotrophic Methanogens (HM)	NT	NT	<0.2	<0.2	<0.2
Autotrophic Acetogens (AA)	NT	NT	NT	<0.2	3.3
Heterotrophic Acetogens (HA)	NT	NT	<0.2	790	330
Sulfate Reducing Bacteria SRB)	<0.1	<0.1	0.8	24	170
Iron Reducing Bacteria (IRB)	<0.1	<0.1	54	13000	3300
% of total cells cultured	0	0	0.026	9.9	30.1

* NT, not tested

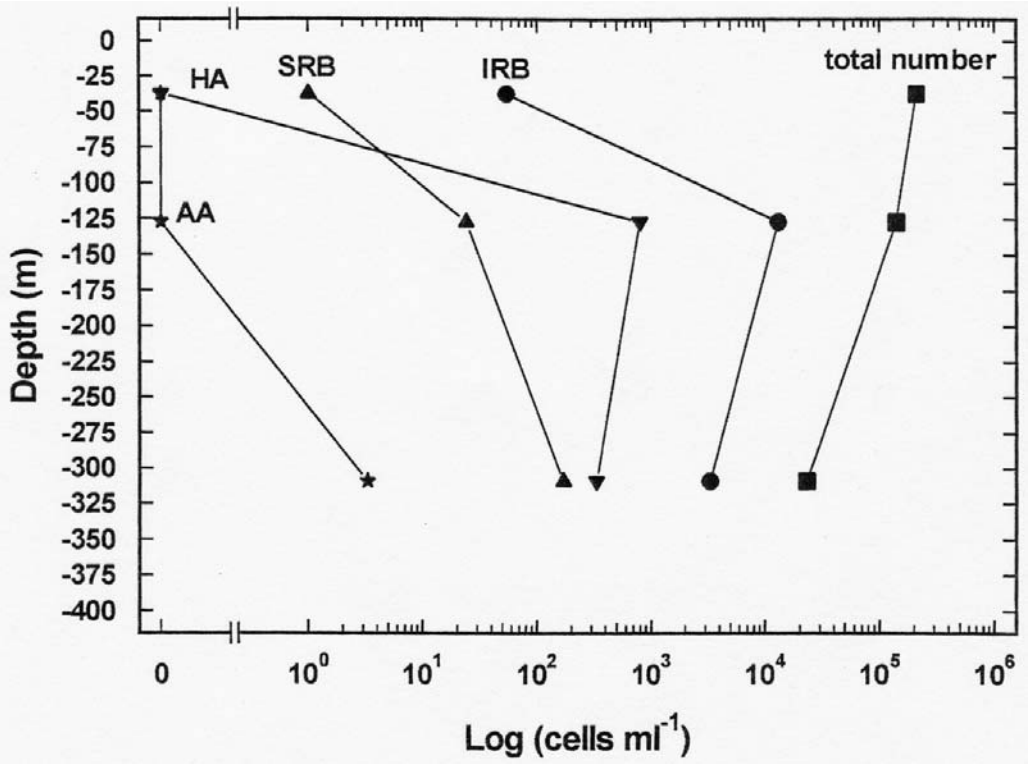

Figure 2 *The number of physiological groups of microorgenisms in R387. See table 3 for symbol descriptions*

Table 4*: Comparison of groundwater-based and synthetic growth media.*

Media type	**Borehole R387/304-309m**	
	Gw	**Syn**
Autotrophic Methanogens (AM)	<0.2	NT*
Heterotrophic Methanogens (HM)	<0.2	NT
Autotrophic Acetogens (AA)	3.3	NT
Heterotrophic Acetogens (HA)	330	NT
Sulfate Reducing Bacteria (SRB)	170	3300
Iron Reducing Bacteria (IRB)	3300	3300
% of total cells cultured	19.0	33.0

*NT, not tested

Bacterial reduction of uranium

The data from the uranium reduction test is shown in table 5. No difference was observed for uranium alone or uranium plus sulphate. With uranium plus iron, presence of bacteria resulted in greater precipitation of uranium compared to the sterile control.

Table 5. Summary of results from uranium reduction experiments with enrichment cultures.

Electron Acceptors	Average concentration dissolved U of triplicate samples (µg/l)	
	Palmottu Microbes	**Uninoculated control**
U	20400	21800
$U+SO_4^{2-}$	25700	23600
$U+Fe^{3+}$	644	1787

Discussion

Methodology

Sampling

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

Media

The test of synthetic and groundwater based media (Table 4) Showed good correlation between the obtained data. This result also demonstrates the reproducibility of the culturing technique applied. Two different media types inoculated with discrete samples from the same borehole level gave results that show similar trends for IRB and SRB.

Distribution of physiological groups of microorganisms

The total numbers of microorganisms decreased with depth (Table 3, Figure 2) and this relation has also been observed at Äspö hard rock laboratory (Pedersen et al 1996). The numbers of anaerobic reducing microorganisms increased with depth (Figure 2), which is in agreement with results from other Baltic Shield groundwater sites (Haveman et al 1999). There was a direct correlation between the amount of IRB and SRB detected with the

concentrations of total iron and sulphate (Table 2 and 3). Generally, a lower redox correlated with more IRB, SRB, HA and AA. These relations can be expected. It is not obvious from the data which of the correlation variables that depend on the others. Typically, microbial activity decrease the redox potential but it is premature to conclude if the redox of the sampled Palmottu groundwater are coupled to the reduction activities of the found microorganisms. Such information would require a much more extensive program in microbiology, including measurements of in situ activities with radiotracer techniques.

Possible microbial uranium reduction

The distribution of SRB and IRB showed an inverse correlation with dissolved uranium in R387. The attempt to mimic the groundwater situation in culture tubes inoculated with enrichment cultures of IRB and SRB were partly successful. Some uranium reduction was detected with cultures enriching IRB. This process has been demonstrated in laboratory environments earlier (Lovley et al 1991). It is, consequently, possible that microorganisms contribute to keeping the Palmottu groundwater system reduced and that they also may be directly involved in reducing uranium(VI) to U(IV).

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