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Geomicrobial investigations of groundwaters from Olkiluoto, Hästholmen, Kivetty and Romuvaara, Finland

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GEOMICROBIAL INVESTIGATIONS OF GROUNDWATERS FROM OLKILUOTO, HÄSTHOLMEN, KIVETTY AND ROMUVAARA, FINLAND

Tiivistelmä – Abstract

Groundwater from four deep hard rock sites being considered for nuclear waste disposal in Finland (Olkiluoto, Hästholmen, Kivetty and Romuvaara) were investigated for microbial populations. Bacteria will be present in a waste disposal vault, so it is important to understand the microbiology of any potential site.

Groundwater samples were collected from 200 to 950 m depth and included fresh, brackish and saline waters. Samples were collected with a pressurized groundwater sampler, PAVE, which is an excellent tool for microbiological sampling.

Total cell numbers were typical for deep groundwater, 10^5 to 10^6 cells/ml. Growth media designed using groundwater chemistry data were used for enumeration of methanogens, acetogens, sulfate reducing bacteria (SRB) and iron reducing bacteria (IRB). Microbial populations varied between sites. Iron sulfide fracture minerals are common in the brackish high sulfate groundwaters of Olkiluoto, where SRB predominated. Hästholmen groundwater has high dissolved iron, iron hydroxide fracture minerals and IRB were the main microbial population. Kivetty and Romuvaara had mixed populations.

It has been proposed that deep subsurface ecosystems are based on hydrogen and carbon dioxide which provide energy and carbon to support the food chain. Signs of such an ecosystem were seen in Olkiluoto. More study is needed to understand the basis for deep subsurface life.

From a microbiological point of view, all sites investigated are equally suitable for nuclear waste disposal.

Avainsanat - Keywords

acetogenesis, groundwater, hydrogen, iron reduction, methanogenesis, microbiology, redox, sulfate reduction

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OLKILUODON, HÄSTHOLMENIN, KIVETYN JA ROMUVAARAN TUTKIMUS-ALUEIDEN POHJAVESIEN MIKROBIOLOGISET TUTKIMUKSET

Tiivistelmä – Abstract

Posiva Oy tutkii Suomessa neljää kiteisen kallioperän aluetta (Olkiluoto, Hästholmen, Kivetty ja Romuvaara) ydinjätteiden loppusijoittamiseksi. Myös alueiden pohjavesien mikrobiologisia ominaisuuksia on tutkittu, koska loppusijoitustilassa tulee luonnollisesti olemaan bakteereja.

Makeita ja suolaisia pohjavesiä sekä murtovesiä kerättiin kairanrei'istä 200–950 m syvyydeltä paineellisten vesinäytteiden ottimella (PAVE), joka osoittautui soveltuvan erinomaisesti mikrobiologiseen näytteenottoon.

Solujen kokonaismäärät olivat tyypillisiä syville pohjavesille, 10^5–10^6 solua/ml. Metanogeenisten ja asetogeenisten sekä sulfaattia pelkistävien (SRB) ja rautaa pelkistävien (IRB) bakteerien kasvualustojen koostumus suunniteltiin vastaamaan pohjavesinäytteiden kemiallisia ominaisuuksia. Mikrobikoostumus vaihteli tutkimusalueittain. Sulfaattia pelkistävät mikrobit olivat dominoivia Olkiluodossa, jossa on todettu runsaasti rautasulfideja raontäytemineraaleina. Hästholmenin pohjavesissä on suuria määriä liuennutta rautaa sekä rautaoksideja ja -hydroksideja raontäytteinä. Vastaavasti rautaa pelkistävät bakteerit osoittautuivat Hästholmenin pohjavesien valtalajeiksi. Kivetyn ja Romuvaaran pohjavesissä on sekoittuneena erilaisia mikrobipopulaatioita.

Alan kansainvälisessä kirjallisuudessa on ehdotettu, että syvällä maan uumenissa sijaitsevien ekosysteemien elämä perustuu vetyyn ja hiilidioksidiin, joista bakteerit saavat energiaa ja hiiltä. Olkiluodon tutkimustulokset antavat viitteitä tällaisesta ekosysteemistä, mutta tarvitaan lisää tutkimuksia syvän, maanalaisen elämän ymmärtämiseksi.

Nyt tehtyjen mikrobiologisten tutkimusten perusteella kaikki neljä aluetta soveltuvat yhtä hyvin ydinjätteiden loppusijoittamiseen.

Avainsanat - Keywords

asetogeeni, metanogeeni, mikrobiologia, pohjavesi, raudan pelkistyminen, redox, sulfaatin pelkistyminen, vety

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PREFACE

We would like to thank the Posiva field personnel for collecting and shipping the samples, and Berit Ertman Ericsson, Johanna Arlinger, Agneta Welin and Farideh Taheri Nejad for laboratory assistance.

1 INTRODUCTION

1.1 Microbiology of deep hard rock environments

Microbes inhabit virtually all environments on earth where liquid water is available. They survive and grow in extremes of temperature, pH, salinity, pressure and redox. The basic requirements for their survival are water, energy and carbon sources. Therefore, it is not surprising that microbes are present and active in a wide range of subsurface environments (Bachofen 1997), including deep, hard rock aquifers. The microbial ecology of such environments has been studied in hard rock environments in Sweden and Canada for many years.

Stripa is a former iron mine located in central Sweden at which hydrology, geochemistry and microbiology of granitic rock were studied from 1976 to 1994. Stripa groundwaters were studied for number and activity of attached and planktonic microbes (Pedersen & Ekendahl 1992a, Ekendahl & Pedersen 1994). There were 10^3 to 10^5 cells/ml groundwater in Stripa, but it was shown that many more bacteria attached to surfaces in biofilms, on the order of 10^6 cells/cm^2. These microbes took up a wide range of substrates, indicating that both heterotrophic (using organic sources of carbon) and autotrophic (using inorganic sources of carbon, such as CO_2) microbes were present and active. In addition, isotope studies suggested that active populations of sulfate reducing bacteria (SRB) were present. Biodiversity in Stripa was investigated by determining the 16S rRNA sequences of attached microbes, which gives an idea of the different types of bacteria present (Ekendahl et al. 1994). All the sequenced bacteria represented species that had not been sequenced previously, indicating that a large number of uncharacterized bacteria were present in the subsurface environment of Stripa.

The Äspö Hard Rock Laboratory (HRL) is a tunnel located in southeastern Sweden which was constructed for research concerning the Swedish nuclear waste disposal program. At Äspö HRL, studies of number and activity of microbes and 16S rRNA sequencing were also carried out (Pedersen & Ekendahl 1990, 1992b, Pedersen et al. 1996). These studies showed once again that there were greater numbers and more active microbes attached to surfaces, as opposed to planktonic microbes. Both heterotrophic and autotrophic microbes were found, as well as SRB and iron reducing bacteria (IRB). Microbial investigations at Äspö and Stripa have been summarized by Pedersen and Karlsson (1995) and Pedersen (1997).

Atomic Energy of Canada Limited (AECL) has also built an underground research laboratory (URL) in central Canada for investigations related to nuclear fuel waste disposal. Microbes from depths of about 200 to 300 m in granitic rock have been characterized (Jain et al. 1997, Haveman et al. 1995, Brown & Hamon 1994). Total cell counts were on the order of 10^3 to 10^5 cells/ml. A wide range of active and viable bacteria were detected, including heterotrophs, IRB, and SRB.

The question has been raised whether subsurface microbes are natural communities or contaminants introduced during drilling and excavation. Recent studies indicate the former to be the case. A study at Äspö HRL indicated that contaminating microbes from the drilling process were not able to establish in the new boreholes (Pedersen et al. 1997a). In addition, fossil microorganisms have been found at a depth of 207 m in Äspö HRL, indicating that microbes were present prior to the excavation of the rock laboratory (Pedersen et al. 1997b).

1.2 Nuclear Waste Disposal Safety

Microbes will be present in any subsurface nuclear fuel waste repository and thus it is important to study them to predict any effects they may have on waste disposal. Microbes could have both positive and negative effects on subsurface disposal of nuclear waste. These effects are reviewed in Pedersen & Karlsson (1995).

1.2.1 Microbial Redox Effects

One of the major positive effects that subsurface microbes can have is on redox. A reducing environment in a waste disposal vault is important as it induces precipitation of metals, including uranium, as well as preventing corrosion of the metal container surrounding the waste. Microbes have the ability to reduce a subsurface environment much faster than by purely chemical reactions. Microbes consume oxygen and produce reducing compounds including ferrous iron, sulfide and methane. Microbial processes affecting redox are shown in Figure 1.

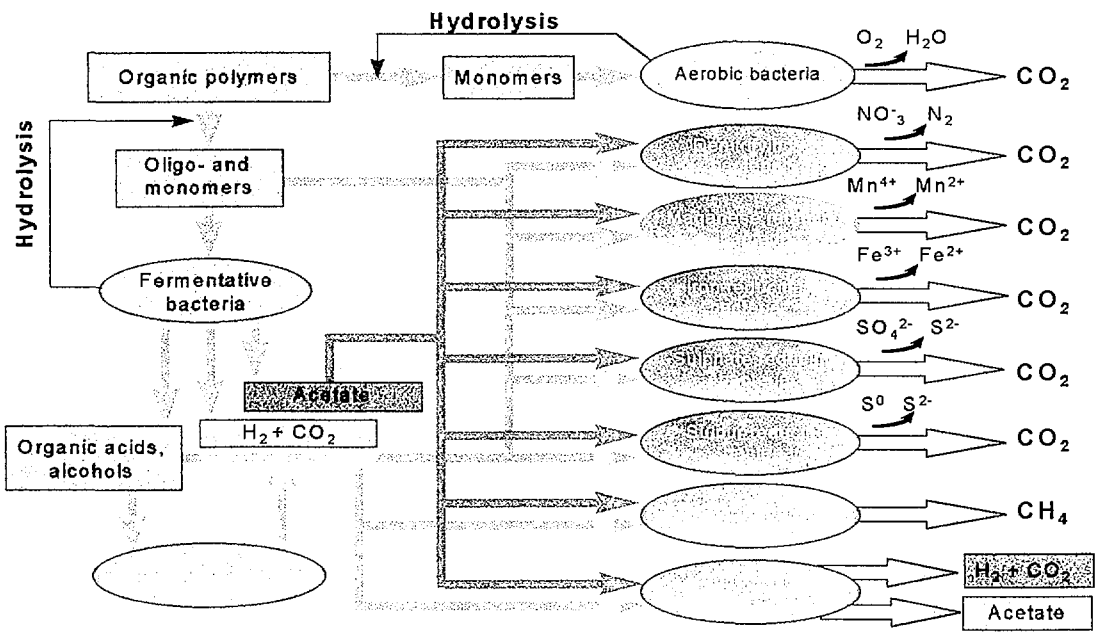

Figure 1: Microbial processes in groundwater (Pedersen 1997).

1.2.2 Microbially Induced Corrosion

A negative effect bacteria could have on waste disposal is production of sulfide. While sulfide is beneficial with respect to redox, as discussed above, it can also promote corrosion of metals, including copper, the material to be used in the container encapsulating the used fuel. In order for microbially produced sulfide to corrode the container, it must come into contact with the metal, and studies show that the bentonite buffer which will surround the canister is an environment which is not conducive to bacterial growth and survival, mainly due to the low water activity in the buffer (Motamedi et al. 1996, Stroes-Gascoyne et al. 1997). Studies are ongoing in this area.

1.3 The deep hydrogen based biosphere

Groundwater at depth in hard rock is quite old, and thus it is depleted in biodegradable organic material. The long residence times of groundwater mean that it is not continually replenished with organic material from surface ecosystems. It has been postulated that subsurface microbes live in an ecosystem which is isolated from surface organic material and thus totally independent of the sun's energy and photosynthesis. Such a deep biosphere would depend on hydrogen "geogas" produced at depth in the rock by radiolysis, mineral reactions or volcanic activity (Figure 2). The carbon source which would be the basis for such an ecosystem would be inorganic, in the form of carbon dioxide gas, dissolved bicarbonate, or carbonate minerals, which are present in most deep groundwater systems. This means that the ecosystem would be based on autotrophic microbes using H_2 and CO_2 to produce organic carbon for heterotrophic microbes. Thus, the subsurface hydrogen based biosphere is very similar to the surface photosynthetically based biosphere, with hydrogen replacing sunlight as the source of energy for the ecosystem.

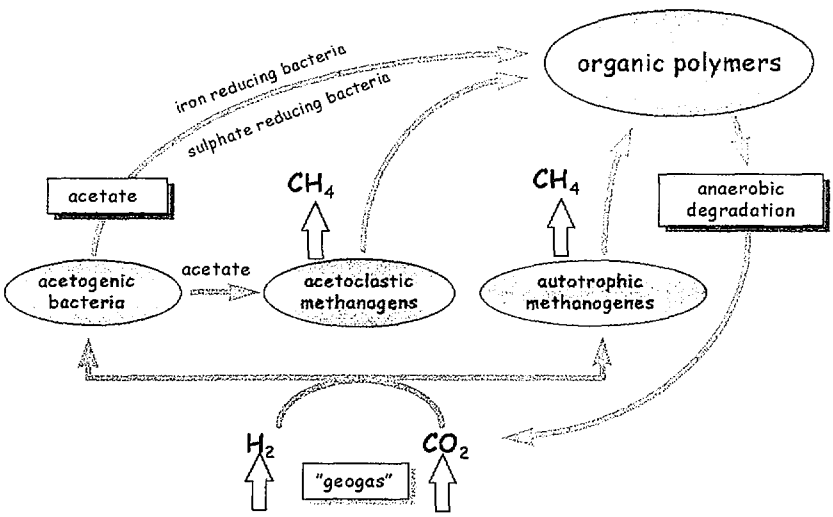

Figure 2: The deep hydrogen based biosphere (Pedersen 1997).

A hydrogen based biosphere would rely on organisms that can use H_2 and CO_2 to produce organic material, which can then support other heterotrophic bacteria. The acetogenic bacteria are the only known group of bacteria which can do this. The process is called autotrophic acetogenesis:

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \qquad \textbf{1-1}$$

The acetate thus produced can be used by a wide range of heterotrophs, including methanogens, SRB and IRB:

$$CH_3OOH \rightarrow CH_4 + CO_2 \qquad \textbf{1-2}$$
$$SO_4^{2-} + CH_3COOH + H^+ \rightarrow HS^- + 2\ CO_2 + 2\ H_2O \qquad \textbf{1-3}$$
$$8\ Fe^{3+} + CH_3COOH + 2\ H_2O \rightarrow 8\ Fe^{2+} + 2\ CO_2 + 8\ H^+ \qquad \textbf{1-4}$$

Autotrophic methanogens also use H_2 and CO_2 as the basis of their metabolism:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \qquad \textbf{1-5}$$

The main difference between autotrophic acetogenesis (1-1) and autotrophic methanogenesis (1-5) is that methanogens produce no organic carbon directly which can provide nutrients for heterotrophic bacteria. However, organic carbon is produced indirectly in the form of cell material in all autotrophic microbes. Once the cells die, they are degraded by other microbes, and the organic carbon enters the food chain.

The first indication of a hydrogen based biosphere in hard rock groundwater came with enrichment of microbes capable of growth on single carbon compounds and hydrogen from Äspö HRL (Pedersen & Ekendahl 1990). More in depth studies of autotrophs in Äspö HRL indicated that methanogens and homoacetogens were abundant in these groundwaters (Kotelnikova & Pedersen 1997, 1998). Studies in basalts in USA have also indicated a deep hydrogen based biosphere (Stevens & McKinley 1995). They found that autotrophic microorganisms outnumbered heterotrophic microorganisms, and that autotrophic acetogens and methanogens were ubiquitous. They also demonstrated abiotic hydrogen production from crushed basalt and anaerobic groundwater, which was hypothesized to support the autotrophically based ecosystem.

1.4 Microbiology of deep groundwaters in Finland

The aim of this study was to gain knowledge of redox related microbes, including methanogens, acetogens, SRB and IRB, at the four sites being investigated as possible sites for final disposal of spent fuel in the Finnish nuclear waste disposal program: Olkiluoto, Hästholmen, Kivetty and Romuvaara. The study of four geographically distinct sites with differences in rock type, groundwater chemistry and fracture minerals may help us to predict how differences in hydrogeochemistry can affect the microbial ecosystems present at each site. This study also expands knowledge of deep hard rock microbial ecosystems from three sites to seven. Since microbial sulfate reduction could have negative effects on waste disposal, it would be useful to be able to predict the occurrence of microbial sulfate reduction in groundwater, based on geochemical parameters.

2 MATERIALS AND METHODS

2.1 Site descriptions

Groundwater from four sites in Finland are being characterized as possible sites for nuclear waste disposal (Figure 3). The positions of the sites with respect to the Baltic sea and their levels throughout history directly relate to their level of salinity today. The ice sheet retreated from Finland about 10 000 years before present (BP). By 9500 years BP, both Romuvaara and Kivetty were above sea level, Romuvaara on the mainland, and Kivetty on a large island. These two sites are composed of freshwater today. At this time, however, Olkiluoto and Hästholmen were still below sea level. By 7500 years BP these two sites were located beneath the Litorina sea, which was more saline than the present Baltic sea. Hästholmen rose above sea level approximately 4000 years BP, while Olkiluoto rose above sea level approximately 2500 to 3000 years BP. Due to their longer residence times under the sea, Olkiluoto and Hästholmen are composed of quite saline groundwater, with brackish water as shallow as 100 m depth (Ruotsalainen & Snellman 1996).

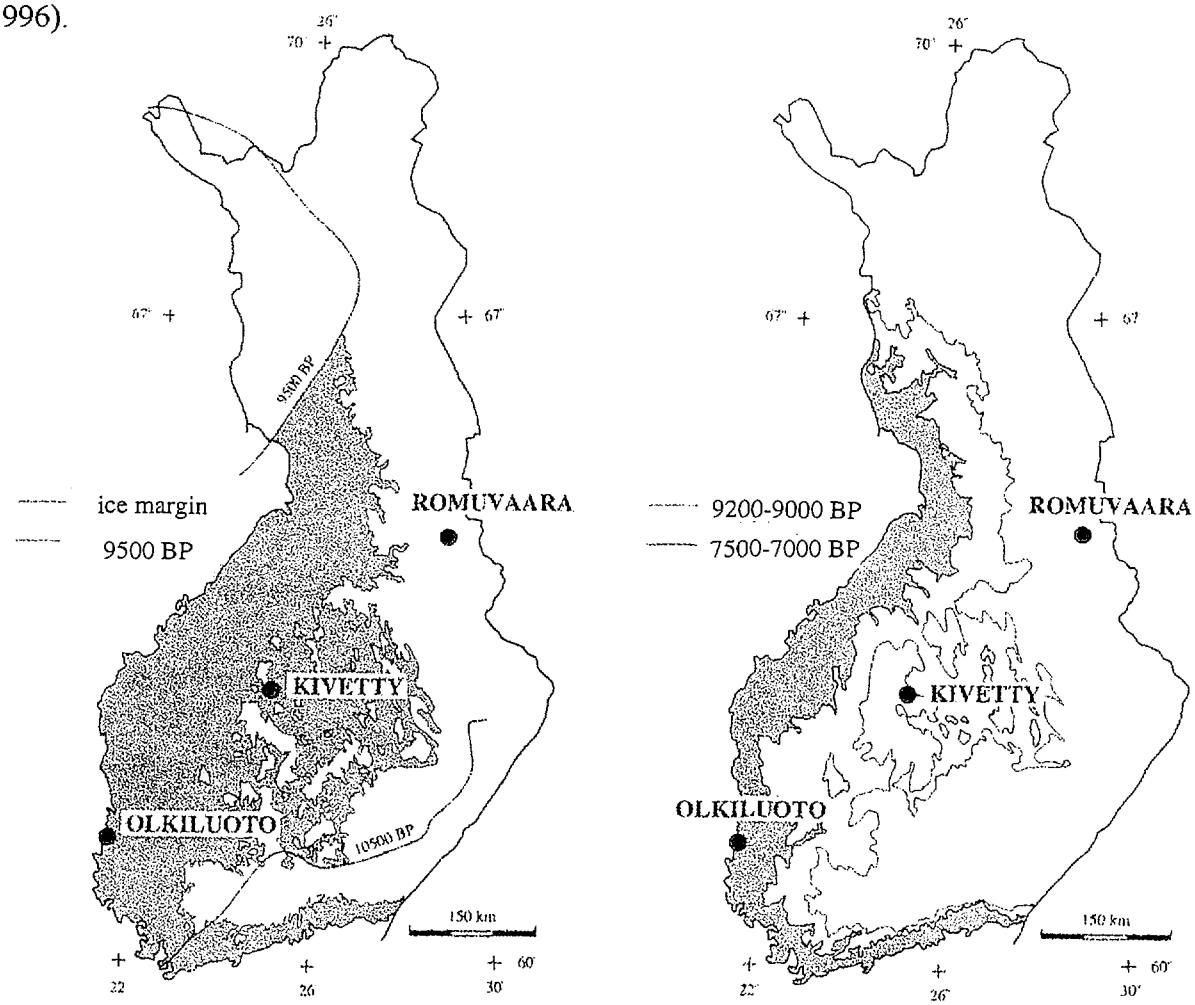

Figure 3: The Finnish shoreline (a), about 9500 years BP, and (b), about 7500 years BP. Based on Eronen et al. (1995).

2.1.1 Olkiluoto

The main rock types at the Olkiluoto site include gneisses, schists, granodiorites and granites (Simonen 1980, Anttila & Heikkinen 1996). The major fracture minerals include calcite ($CaCO_3$), chlorite (a clay mineral), iron sulfides and other clay minerals (Gehör et al. 1996). Pyrite (FeS_2, an iron sulfide) is the major sink for aqueous iron, which is hypothesized to precipitate along with microbially produced sulfide. It is noteworthy that there is no observation of iron oxides or iron hydroxides at Olkiluoto. Sulfur species have the most important role in controlling redox processes in Olkiluoto groundwaters. The groundwater ranges from fresh to brackish to saline, and is composed of several different end members as outlined in Table 1.

***Table 1**: Main water types at Olkiluoto (Pitkänen et al. 1996a).*

Depth (m)	Salinity	Water Type	Origin of dominant end members	Age estimate (BP)
0-150	Fresh to slightly brackish	Na-HCO_3	Precipitation and present Baltic seawater	modern-2500
100-300	Brackish	SO_4 rich, Na-Cl	Litorina seawater	2500-7500
100-500	Brackish	Na-Cl	Pre-Litorina seawater containing fresh glacial meltwater	7500-10000
> 500	Saline	Ca-Na-Cl	Preglacial meteoric water, possibly influenced by hydrothermal salts	» 10000

2.1.2 Hästholmen

The rock at the Hästholmen site consists of rapakivi type granite (Anttila 1986). The main fracture minerals include calcite, dolomite ($MgCa(CO_3)_2$), iron hydroxides, and clay minerals (Gehör et al. 1997a, 1997b). Iron sulfides occur only sporadically. Iron and iron hydroxides are very common in Hästholmen, and thus iron is more important than sulfur in controlling redox processes in Hästholmen groundwaters (Snellman et al. 1998). The groundwater ranges from fresh to brackish to saline, and is composed of several different end members as outlined in Table 2.

Table 2: Main Groundwater Types at Hästholmen (Snellman et al. 1998).

Depth (m)	Salinity	Water Type	Origin of Dominant End Members	Estimated Age BP (years)
< 50-100	Fresh-Brackish	HCO_3 rich	Rain water and present Baltic water	modern-4000
100-400	Brackish	SO_4 rich, Na-(Ca)-Cl	Litorina seawater	4000-7500
400-600	Brackish	Ca-Na-Cl, still SO_4 rich	Pre-Litorina water containing fresh meltwater of the ice sheet	7500-10000
>500-600	Saline	Ca-Na-Cl	Preglacial water, possibly influenced by brines	» 10000

2.1.3 Kivetty

The main rock types at the Kivetty site are granodiorite and granite (Front & Paananen 1996). Major fracture minerals include calcite, iron sulfides, iron oxyhydroxides and clay minerals. Iron hydroxides (goethite and limonite, FeOOH) and quartz are also present in some places (Gehör et al. 1995a). The iron oxyhydroxides are mostly found at shallow depths, down to about 130 to 170 m. Calcites and iron sulfides are found below 50 to 100 m depth. At Kivetty, some groundwater samples are characterized by colder infiltration conditions and glacial melt waters. All groundwater in Kivetty is freshwater, with a regional maximum TDS of less than 200 mg/l (Ruotsalainen & Snellman 1996). The age of groundwater in Kivetty ranges from modern to 15000 years BP (Pitkänen et al. 1998). Sulfur compounds are thought to control redox processes in Kivetty groundwaters, buffering the redox to below -300 mV in deep groundwater (Pitkänen et al. 1998).

2.1.4 Romuvaara

At the Romuvaara site, the main rock types include different types of gneisses, intersected by granodiorite and metadiabase dykes (Okko & Paulamaki 1996). The major fracture minerals are calcite, iron sulfides, iron oxyhydroxides (goethite and limonite, FeOOH) and clay minerals (Gehör et al. 1995b). The iron oxyhydroxides are found at shallow depths down to 150 m. Iron sulfides, including pyrite, are found at depths of greater than 100 m. All the Romuvaara groundwater is fresh, with maximum Cl concentrations of less than 300 mg/l at depth (Ruotsalainen & Snellman 1996). According to stable isotope data, Romuvaara groundwaters show no effects of glacial melt waters. Sulfur compounds are thought to control redox processes, buffering redox to below -200 mV (Pitkänen et al. 1996b).

2.2 PAVE sampler

As groundwater is pumped from a borehole, the pressure decrease causes degassing of the water. This may affect the results of analysis for gas content, some groundwater chemistry, colloids and microbiology. Posiva has developed pressurized groundwater sampling equipment (Ruotsalainen et al. 1996), called PAVE, which is short for "Paineellisten Vesinäytteiden otin" in Finnish. In the case of groundwater microbiology, the PAVE system is ideal since the sample can be kept at in situ pressure and temperature during transport to the lab, which hopefully minimizes changes in the microbial population due to sampling.

Prior to sampling, the PAVE (Figure 4) was disinfected with a chlorine dioxide solution (Freebact, Wecantech AB, Märsta, Sweden). Pure ELGA water was prepared in the IVO laboratory. 150 ml of Freebact was dissolved in 40 l of ELGA water to give a final concentration of 10 ppm chlorine dioxide. The PAVE was rinsed with this solution for 30 min, then flushed with ELGA water for about 10 min.

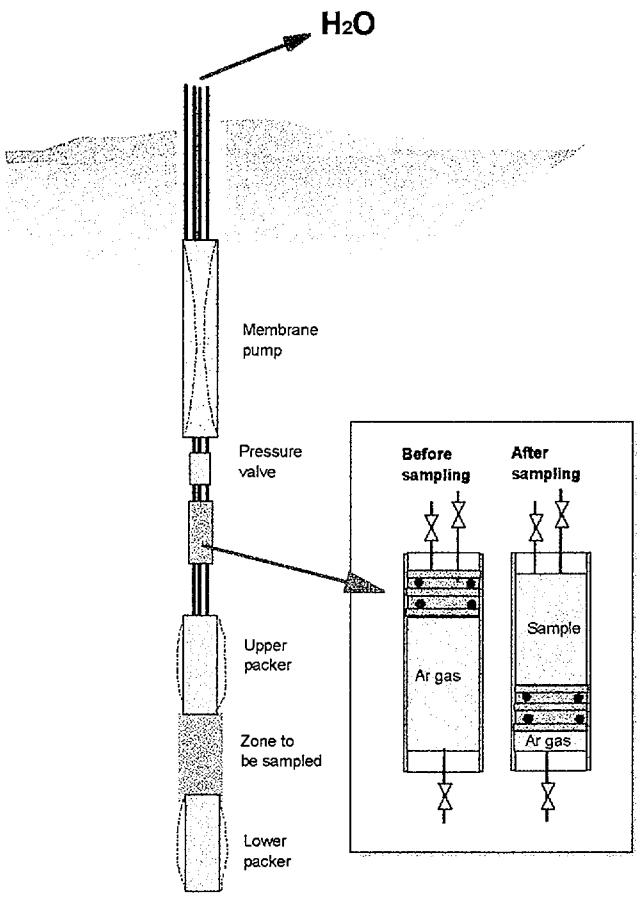

***Figure 4**: The PAVE groundwater sampler (Ruotsalainen et al. 1996).*

To ensure the efficiency of the sterilization, control samples were analyzed. These were a PAVE sampler containing ELGA water after the chlorine dioxide treatment, and a separate sample of ELGA water. The control samples were analysed for total cell counts as described below and inoculated into tubes of the freshwater growth medium described below for samples KI-KR5 and RO-KR10. The total cell counts were $(1.7 \pm 1.6) \times 10^3$ cells/ml for the ELGA water and $(6.3 \pm 1.4) \times 10^4$ cells/ml for the PAVE control. Since the control counts are less than the standard deviation of the samples (the range of standard deviations is 8×10^4 to 2×10^5 cells/ml, see below), they wouldn't significantly affect the total counts. No growth was detected in the MPN tubes for the control samples.

After sterilization, the pressure compartment of PAVE is filled with Ar gas and the movable piston moves to the top of the pressure vessel (Figure 4). Finally, the PAVE sampler is evacuated to eliminate atmospheric contamination. The section of the borehole to be sampled is packed off with inflatable rubber packers. The PAVE system, consisting of a rubber membrane pump and one or more sterile, evacuated and closed pressure vessels, is lowered into the borehole. Groundwater is pumped from the packed off zone past the closed pressure vessels and out of the borehole. Groundwater parameters (pH, Eh, EC, O_2, temperature) are monitored in N_2 shielded flow through cells in the field lab on the surface until they stabilize. At this point, samples for field and laboratory analysis for hydrogeochemical characterization are collected. After this phase, the pressure valve of the PAVE sampler is opened. Groundwater pressure pushes down a piston in the sampler to fill the sampler with groundwater. The PAVE is closed again and raised out of the borehole.

PAVE samplers were shipped on ice and arrived at the lab in Göteborg the same day or the morning after the sample was taken. At the lab, the groundwater was removed from the sampler into a sterile bottle under oxygen free N_2. Inoculation of the MPN tubes was started immediately after removal of the groundwater from the sampler, and work with the samples was complete within 4 to 6 hours of removal of groundwater from the sampler.

2.3 Gases and Geochemistry

The samples for gases and geochemistry were collected during the same sampling period as the microbial samples. Analysis methods (Ruotsalainen et al. 1994) and results (Helenius et al. 1998a, 1998b, 1998c) for the four sites are reported elsewhere. Tables 3 and 4 show data important for microbial processes.

***Table 3**: Gas results for PAVE sampling from Olkiluoto, Hästholmen, Kivetty and Romuvaara, 1997 (Helenius et al. 1998a, 1998b, 1998c).*

Borehole	Depth (m)	Volume gas (ml/l)	N_2 (mM)	O_2 (μM)	CO (μM)	CO_2 (mM)	H_2 (μM)	Ar (mM)	He (mM)	CH_4 (mM)	C_2H_2 (μM)	C_2H_4 (μM)	C_2H_6 (μM)	C_3H_6 (μM)	C_3H_8 (μM)
OL-KR3	243-253	82	1.87	17	<0.2	0.002	2.9	0.14	0.049	1.17	<0.02	0.002	4.1	<0.04	<0.04
OL-KR8	302-310	47	1.66	116	<0.08	0.006	<0.08	0.03	0.04	0.002	0.002	0.004	0.008	<0.02	<0.02
OL-KR10	324-332	145	2.24	2.9	<0.2	0.006	0.2	0.05	0.18	3.33	<0.02	<0.02	16	<0.05	0.3
OL-KR9	563-571	340	2.29	21	<0.6	0.006	15	0.04	0.48	10.5	<0.08	0.007	69.4	0.01	0.5
HH-KR1	937.5-947.5	158	4.8	510	<0.3	0.03	78	0.4	0.6	0.004	0.002	0.009	0.14	0.010	0.01
HH-KR2	907-912	228	6.5	643	<0.5	0.01	13	0.9	0.9	0.009	0.004	0.009	0.12	<0.009	0.02
HH-KR3	211-216	77	2.8	6.1	<0.2	0.02	0.6	0.08	0.009	0.002	<0.002	0.003	0.02	<0.003	<0.00
KI-KR5	717-725	147	4.9	571	<0.2	0.05	0.06	0.4	0.0007	0.006	<0.02	<0.02	0.2	<0.05	0.02
RO-KR10	561-566	33	1.1	2.9	<0.06	0.004	0.08	0.14	0.0002	0.003	<0.006	0.001	0.07	<0.01	0.01

***Table 4**: Chemistry results for PAVE sampling from Olkiluoto, Hästholmen, Kivetty and Romuvaara, 1997 (Helenius et al. 1998a, 1998b, 1998c).*

Borehole	Depth (m)	pH	Eh (mV)	Alk (meq/l)	HCO_3 (meq/l)	DOC (mM)	Fe_{tot} (mM)	Fe^{2+} (mM)	S^{2-}_{tot} (mM)	SO_4 (mM)	Na (mM)	Ca (mM)	Cl (mM)	TDS (mg/l)	$\delta C\text{-}13(CH_4)$ (‰ PDB)	$\delta S\text{-}34(SO_4)$ (‰ CDT)
OL-KR3	243-253	8.3	-180	0.27	0.38	0.14	0.0018	0.0011	0.011	0.01	62.9	7.0	77.8	4570	-58.4	
OL-KR8	302-310	7.8	-[1]	0.63	0.73	0.13	0.0003	0.0005	0.0006	4.89	88.3	25.5	134.5	8510	-22.4	26.69
OL-KR10	324-332	7.9	-[1]	0.25	0.36	0.12	0.0066	0.0061	0.0005	0.09	84.0	30.9	152.3	8730	-48.5	
OL-KR9	563-571	8.2	-[1]	0.07	0.22	0.13	0.0002	0.0002	<0.0003	0.01	182.7	81.1	323.8	19127	-42.9	
HH-KR1	937.5-947.5	7.0	-46	0.38	0.38	0.31	0.0286	0.025	<0.0003	1.44	213.1	92.32	433	24732	-36.7	27.87
HH-KR2	907-912	7.33	-214	0.45	0.45	0.30	0.0233	0.017	<0.0003	0.35	230.5	75.85	420	23910		
HH-KR3	211-216	7.8	-[1]	2.22	2.22	0.34	0.0215	0.012	0.0019	6.66	108.7	16.22	149	9537	-50.5	
KI-KR5	717-725	8.1	-[1]	1.44	1.44	0.18	0.0018	0.0016	0.0003	0.02	0.43	0.40	0.1	144	-46.3	
RO-KR10	561-566	8.4	-418	1.85	1.80	1.05	0.0009	0.0002	<0.0003	**0.03**[2]	1.09	0.26	0.1	172	-37	

[1] Technical problems
[2] **Bold** = an uncertain value

2.4 Direct Counting

Total numbers of cells in the groundwater samples were counted using two different stains that specifically stain DNA and RNA, allowing cells to be stained specifically with minimal background. Samples were stained according to the acridine orange (AO) direct count method described by Pedersen & Ekendahl (1990), except that filters were rinsed twice with 1.0 ml of 0.2 μm filtered double distilled water to dissolve salt crystals prior to staining for 10 minutes with AO. The same method was used with a second stain, 10 μg/ml 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.

2.5 Growth Media

Bacterial growth media were designed based on groundwater chemistry data measured in the field lab on groundwater collected by the PAVE bypass during pumping prior to sampling. This allowed media to be designed using current chemistry data for each borehole, as opposed to data from previous sampling occasions. The PAVE system thus allowed the media to come as close as possible to mimicking actual groundwater chemistry, giving the microbes a growth medium with minimal changes from their natural environment.

All media were prepared anaerobically, according to the Hungate method (Hungate 1969). Salt and buffer concentrations were varied according to borehole parameters as outlined in Table 5. In addition, all media contained (g/l): KH_2PO_4, 0.01; Na_2SO_4, 0.002; $FeCl_2 \cdot 4H_2O$, 0.001; Cysteine $HCl \cdot H_2O$, 0.25; $Na_2S \cdot 9H_2O$, 0.25; Element solution, 10 ml; Vitamin solution (Wolin et al. 1963), 5 ml; and resazurin, 0.2 mg. The element solution contained (g/l): Nitrilotriacetic acid, 12.8; $FeSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.17; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2$, 0.02; H_3BO_2, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01; NaCl, 1.0; and Na_2SeO_3, 0.01. The pH of the media was adjusted to borehole pH after autoclaving according to Table 5.

Table 5: Salt and buffer concentrations and pH of media for Olkiluoto, Hästholmen, Kivetty and Romuvaara groundwater samples.

Borehole	Component (g/l)							pH
	NaCl	$CaCl_2 \cdot 2H_2O$	$MgCl_2 \cdot 6H_2O$	NH_4Cl	KCl	$NaHCO_3$	Tris HCl	
OL-KR3	1.75	0.9	0.21	0.4	0.02	1.29	1.82	8.3
OL-KR8	2.5	2.2	0.47	0.4	0.02	1.29	1.82	7.8
OL-KR10	3.0	2.5	0.47	0.4	0.02	1.29	1.82	8.3
OL-KR9	10.0	8.0	0.47	0.4	0.02	1.29	1.82	8.4
HH-KR1	12.0	8.0	0.47	0.4	0.02	1.29	1.82	7.0
HH-KR2	11.0	13.0	0.47	0.4	0.02	2.58	0	7.3
HH-KR3	5.0	2.2	2.5	0.4	0.02	1.68	0	7.5
KI-KR5	0	0.05	0.02	0.01	0	0.84	1.21	8.1
RO-KR10	0	0.05	0.02	0.01	0	0.84	1.21	8.4

The medium for each groundwater sample was divided into 6 different anaerobic bottles, and substrates for different physiological groups of microorganisms were added to each anaerobically. 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 sulfate reducing bacteria (SRB) contained 14 mM Na_2SO_4 and 6 mM lactate. The medium for iron reducing bacteria (IRB) contained 7 g/l amorphous iron and 11 mM lactate. IRB were not tested for samples OL-KR8 and OL-KR9.

2.5.1 Most Probable Number

The different types of media were dispensed anaerobically in 9 ml aliquots into sterile test tubes with N_2 in the gas phase, which were used to determine the most probable number (MPN) of each physiological group of bacteria or archaea in each groundwater (Koch 1994). A 1 ml aliquot of 0.2 µm filter sterilized groundwater (DynaGard filters, Microgon Inc., Laguna Hills, California) 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: 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. Tubes were incubated on their sides at 17°C, except the HH-KR2 tubes which were incubated at 20°C due to a higher borehole temperature.

After a minimum of 2 months incubation, tubes were analyzed for products of metabolism. The gas phase of the methanogenic tubes (AM and HM) were analysed for presence of methane by gas chromatography (Kotelnikova et al. 1998). The acetogenic tubes (AA and HA) were also analysed as negative controls for methanogenesis. Acetate was analysed with a kit (Boehringer Mannheim, Mannheim Germany) which detected acetate by an enzymatic and UV method. SRB tubes were analysed for production of hydrogen sulfide by adding 0.1 ml of the culture to 2 ml of 5 mM $CuSO_4$ solution (Widdel & Bak 1992). If the solution turned brown, sulfide was produced in the culture. IRB tubes were analysed for both total and ferrous iron using a spectrophotometric ferrozine method (Stookey 1970, Hallbeck 1993).

2.6 Whole cell in situ hybridization

Groundwater was filtered onto 0.2 μm x 45 mm Nuclepore polycarbonate filters (Costar, Badhoevedorp, the Netherlands). The saline groundwaters from Olkiluoto and Hästholmen were rinsed twice with a volume of 0.2 μm filtered double distilled water equal to the groundwater volume to dissolve salt crystals. The cells were fixed overnight at 4°C with 4% paraformaldehyde, rinsed with 10 ml 0.2 μm filtered PBS (130 mM NaCl, 10 mM Na_xPO_4, pH 7.2), followed by a 50:50 mixture of 0.2 μm filtered PBS and ethanol. The filters were then dehydrated in 50, 80 and 98% ethanol, air dried, and frozen at -20°C.

Oligonucleotide probes specific for 16S rRNA sequences conserved within the domains *Bacteria* and *Archaea* were used. The probes were labelled with Cy5, a fluorochrome that has excitation and emission wavelengths of 650 and 660 nm, respectively, chosen because the wavelengths are well out of the range for background fluorescence from minerals or autofluorescent cells. The EUB probe, for members of the domain *Bacteria* (which includes acetogens, SRB and IRB) was specific to 16S rRNA positions 338 to 355 (*Escherichia coli* Brosius numbering, Brosius et al. 1978) and had the sequence 5'GCTGCCTCCCGTAGGAGT3' (Amann et al. 1990). The ARC probe, for members of the domain *Archaea* (including methanogens) was specific to 16S rRNA positions 915 to 934 (*E. coli* Brosius numbering) and had the sequence 5'GTGCTCCCCCGCCAATTCCT3' (Stahl & Amann 1991). In addition, a negative control probe, complementary to the EUB probe, and thus not complementary to any cellular RNA sequences, was used. This probe was called NONEUB, and had the sequence 5'CGACGGAGGGCATCCTCA3'. This probe is used to check for nonspecific hybridization. The percentage of cells with nonspecific hybridization, if any, was subtracted from the number of cells with hybridization with the EUB and ARC probes to give the percentage of total cells which corresponded to Bacteria and Archaea, respectively.

Each filter was cut up into 8 to 10 pieces. Each piece was covered with hybridization solution (0.9 M NaCl, 20 mM Tris, 0.01% SDS, pH 7.2) and prehybridized for 20 minutes at 45°C in a tempered water bath. Then 50 ng probe (in prewarmed hybridization solution) was added per filter piece and hybridization continued for 2 hours at 45°C in an

equilibrated chamber. Filters were washed for 20 minutes at 48°C in a 15 ml vial of prewarmed hybridization solution. Filters were then air dried and stained for 5 minutes with 10 μg/ml DAPI. Excess DAPI was poured off, the filters were air dried once again, and mounted on glass microscope slides with Citifluor AF87 (Citifluor Ltd., Canterbury, England).

Cells were viewed using a Molecular Dynamics 2010 confocal laser scanning microscope equipped with a Kr/Ar laser. Cells were observed with the software Image Space running on a Silicon Graphics UNIX based computer. Photographs taken with a CCD camera and viewed with ISee software were used to count hybridized cells. At least ten individual photographs were counted, for a total of at least 600 cells counted per photograph. Total cells were counted using the DAPI stained cells.

3 RESULTS

Total cell counts for the Finland samples were typical for deep hard rock groundwaters, in the order of 10^5 to 10^6 cells/ml groundwater (Table 6). Cells were various shapes of rods and cocci. Figure 5 shows some typical cell morphologies at the different sites. In the Kivetty groundwater sample, a large number of stalked cells were observed (Figure 5). These cells live in low nutrient conditions attached to surfaces or particles, and the stalk is a mechanism for the cell to increase its surface area and therefore to maximize its nutrient uptake (Madigan et al. 1997).

***Table 6**: Results of total counting and MPN analyses of PAVE samples.*

Borehole	Depth (m)	Total Cells $x10^5$	Autotrophic Methanogens	Heterotrophic Methanogens	Autotrophic Acetogens	Heterotrophic Acetogens	SRB	IRB	% of total cells cultured
OL-KR3	243-253	5.1±1.4	-[1]	-	$7.8x10^0$	$3.3x10^2$	$>1.6x10^4$	$1.5x10^3$	3.5
OL-KR8	302-310	2.8±1.0	-	-	-	-	$1.6x10^4$	NT[2]	5.7
OL-KR10	324-332	6.5±1.7	$4.5x10^{-1}$	$4.5x10^{-1}$	$2.2x10^1$	$9.2x10^3$	$>1.6x10^4$	$7.0x10^0$	3.9
OL-KR9	563-571	6.2±1.5	-	-	-	-	$1.7x10^0$	NT	0.00027
HH-KR1	937.5-947.5	4.1±1.2	-	-	-	-	-	$7.0x10^3$	1.7
HH-KR2	907-912	6.1±1.5	-	-	-	$1.3x10^2$	-	$1.4x10^1$	0.024
HH-KR3	211-216	3.5±0.8	-	-	-	$7.9x10^2$	$4.9x10^1$	$1.6x10^4$	4.8
KI-KR5	717-725	4.7±1.1	-	-	-	$3.3x10^1$	$2.2x10^3$	$2.2x10^2$	0.52
RO-KR10	561-566	11.8±2.3	-	-	-	$3.3x10^1$	$4.9x10^1$	$1.7x10^3$	0.15

[1] - not detected
[2] NT not tested

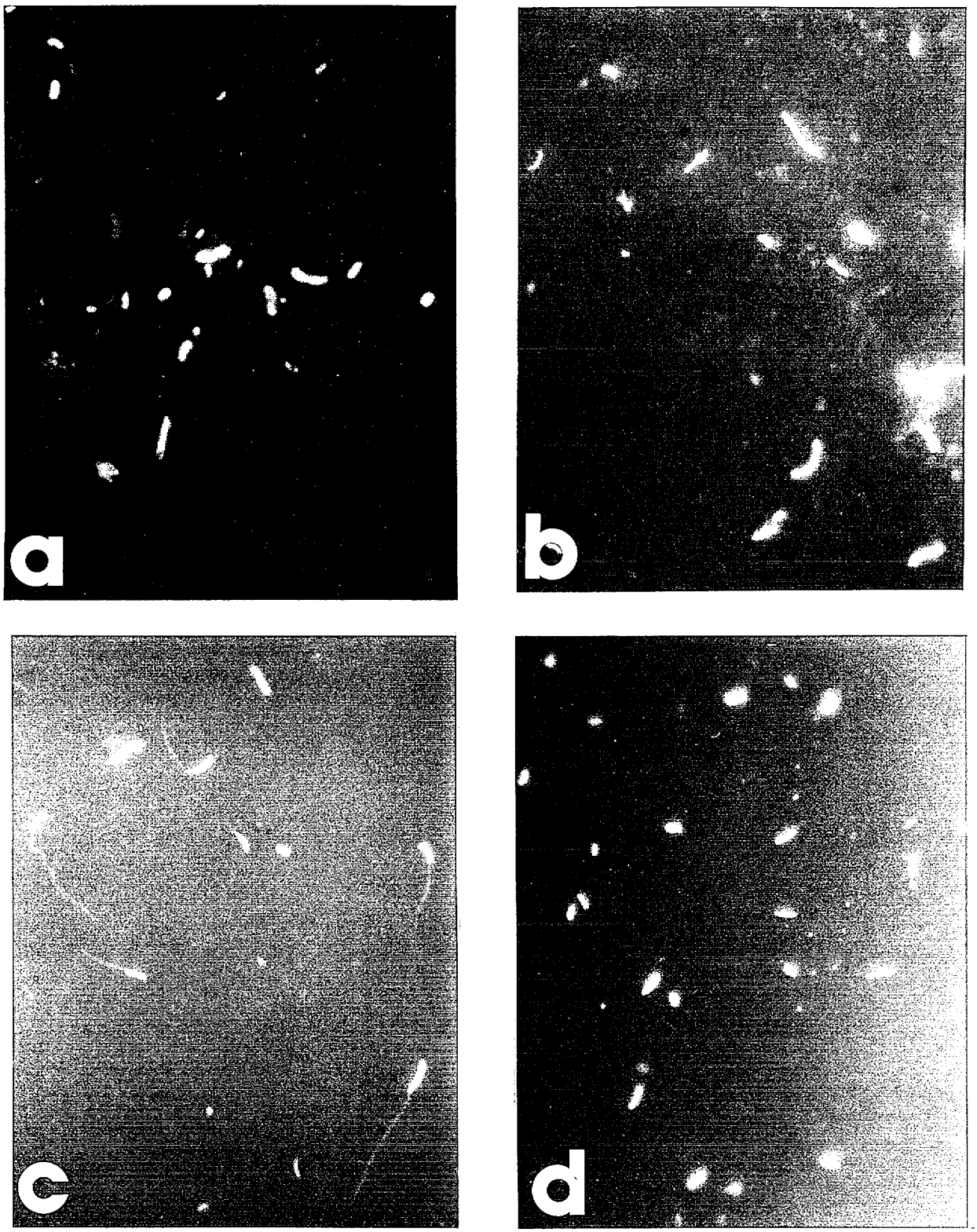

Figure 5: *Common cell morphologies from OL-KR10 (a), HH-KR1 (b), KIKR5 (c) and RO-KR10 (d) groundwaters. (a) and (b) stained with acridine orange, (c) and (d) stained with DAPI.*

MPN results are given in Table 6, and will be discussed in more detail below. The percent of total cells which were cultured by the MPN method are shown for each borehole in Table 6. Culturable cells ranged from 0.00027% in OL-KR9 to 5.9% in OL-KR8. The best culturing results were in the brackish water samples from Olkiluoto and Hästholmen, as the intermediate salt concentrations were most easily reproduced as growth media (Figure 6). For the very saline, deeper boreholes it was difficult to keep such high amounts of salt in solution, and most of the high salt media had precipitates. For the two freshwater samples, media contained much higher total dissolved solids (TDS) than the groundwaters, as the TDS of the buffers and reducers required to make anaerobic media exceeded that of the groundwater. However, the number of different groups of bacteria cultured from the freshwater samples was comparable with that for the brackish samples.

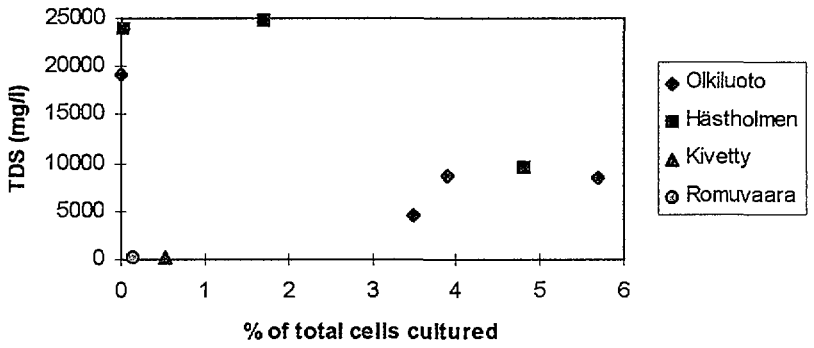

***Figure 6**: Percentage of cultured cells compared with TDS in groundwater samples.*

Probing results for the two freshwater samples are shown in Table 7. The results show that there are significant populations of both *Bacteria*, which include acetogens, sulfate reducers and iron reducers, and *Archaea*, which include methanogens, in both freshwater boreholes. Probing was attempted for the least saline of the remaining boreholes, OL-KR3, but there were no results, only nonspecific hybridization to salt crystals was observed. No cells could be seen. Method development for probing in brackish and saline groundwater is ongoing.

***Table** 7: Results of whole cell in situ hybridization.*

	% of total cells	
	RO-KR10	KI-KR5
Bacteria	57	44
Archaea	34	29
Bacteria + Archaea	91	73

4 DISCUSSION

4.1 Redox related bacterial processes

4.1.1 Olkiluoto

In Olkiluoto, three of the groundwater samples analyzed for microbes came from between 240 and 330 m depth in the brackish groundwaters (OL-KR3, OL-KR8 and OL-KR10), and only one these samples (OL-KR8) was sulfate rich (Table 4). The fourth sample came from the saline groundwater at a depth of greater than 500 m (OL-KR9).

The brackish groundwater between 100 and 300 m depth has the maximum concentrations of both sulfate and total iron in Olkiluoto, and iron sulfide fracture minerals are common at all depths. The iron concentrations in Olkiluoto are quite low in general, however, and this may limit iron reducing bacteria. Ferric iron containing minerals may provide a source of iron to these microbes, but the actual amount of solid ferric iron phases in the rock not known. Based on the chemical data, one would predict that the dominant microbial group in the brackish waters would be SRB and they could be accompanied by lower numbers of IRB. The results confirmed these hypotheses, as SRB were the most abundant group in all three brackish samples. The two boreholes with low levels of sulfate (OL-KR3 and OL-KR10) had higher numbers of SRB than the borehole with high sulfate (OL-KR8). IRB were also quite abundant in OL-KR3 but very low in OL-KR10. The third borehole, OL-KR8, was not tested for IRB. Sulfate reducing bacteria preferentially use S-32(SO_4), so the remaining SO_4 in groundwater would have a positive δS-34(SO_4) value. The only Olkiluoto sample which contained enough SO_4 to determine δS-34(SO_4) was OL-KR8, and the result was + 26.69 ‰CDT (Table 4), indicating that SRB have used the lighter S-32 isotope of sulfate, and confirming the presence of SRB in this borehole.

The deeper, more saline sample from OL-KR9 had very low levels of SRB (Table 6), which is expected since it is outside the depth which is characterized by high sulfate groundwater. IRB were not tested in OL-KR9. This sample had abundant methane and hydrogen indicating that the microbial population may depend more on autotrophy. The redox at these depths could therefore be buffered by methane rather than sulfur or iron compounds.

The IRB and SRB cultured from the brackish boreholes are heterotrophs, using lactate as their source of carbon and energy. The source of carbon in situ could be one of a number of things. The median concentration of DOC in groundwater is 0.06 mM (Thurman 1986), and similar average DOC concentrations (0.07 mM) were found in deep granitic groundwater in Canada (Vilks & Bachinski 1996). DOC in the boreholes tested in Olkiluoto has slightly higher but similar concentrations, ranging from 0.12 to 0.14 mM (Table 4). This organic carbon may or may not be available for use by microbes as

organic carbon in deep groundwater consists mainly of humic and fulvic acids which tend to be quite recalcitrant to anaerobic breakdown by microbes. However, Lovley et al. (1996) have shown that iron reducing bacteria can use these substances as electron shuttles to iron minerals, so that the microbes don't actually have to come in contact with the minerals to use them as electron acceptors. This process still requires an organic compound to be broken down as a source of energy. Although most SRB are heterotrophs, there are some autotrophic species, which use H_2 and CO_2 as energy and carbon sources (Widdel & Bak 1992), both of which are available in groundwater.

One final possibility is that the SRB use methane as their carbon and energy source, as has been previously suggested for Olkiluoto SRB (Pitkänen et al. 1996a). The high methane concentrations in Olkiluoto groundwaters are potentially an abundant source of carbon to microbes. However, thermodynamic calculations show that oxidation of methane to carbon dioxide doesn't provide a lot of energy, and it has yet to be demonstrated that this reaction can support cell growth. Net anaerobic methane oxidation has been observed by monitoring geochemical parameters in marine sediments (Hoehler et al. 1994, Blair & Aller 1995, Niewöhner et al. 1998) and in waters of an alkaline, saline lake (Iversen et al. 1987). Anaerobic methane oxidation is not well understood, but the most likely hypothesis is that this process is carried out by a consortium of SRB and methanogens (Hoehler et al. 1994, Niewöhner et al. 1998). The methanogens would oxidize methane in the reverse of their normal methanogenesis reaction:

$$CH_4 + 2\,H_2O \rightarrow CO_2 + 4\,H_2 \qquad \textbf{4-1}$$

High methane concentrations and/or low CO_2 and H_2 concentrations would be required to make this reaction thermodynamically feasible. The SRB would use the hydrogen produced by the methanogens as their energy source:

$$HSO_4^- + 4\,H_2 \rightarrow HS^- + 4\,H_2O \qquad \textbf{4-2}$$

The SRB would help with the thermodynamics of reaction 4-1 by keeping very low H_2 concentrations in the system. The net reaction would be:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \textbf{4-3}$$

Both the SRB and methanogens benefit from this relationship. The anaerobic methane oxidation reaction is made energetically favourable for the methanogens by the low H_2 concentrations, and H_2 is also a sink for electrons. The SRB gain a source of energy. The SRB would need an additional source of carbon, either organic or inorganic. Another possibility is that the process of anaerobic methane oxidation is carried out by a single unknown type of organism with methane as the sole carbon source and sulfate as the electron acceptor, but if such an organism exists, it is as yet unknown to microbiologists. This will be an interesting area of microbial ecology to investigate in the future.

The trends in Olkiluoto show methane concentrations decreasing at the depth of 100 to 300 m where there is high sulfate and abundant SRB, supporting the hypothesis of anaerobic methane oxidation (Figure 7). The methane results in this figure are for gas captured at the surface in ampoules, which do not give as large values as later gas samples analysed from the PAVE system. However, the limited data available from the PAVE (5 samples with gas results) does not allow such a trend graph to be constructed. The new results do seem to follow the same general trends, at higher concentrations, however.

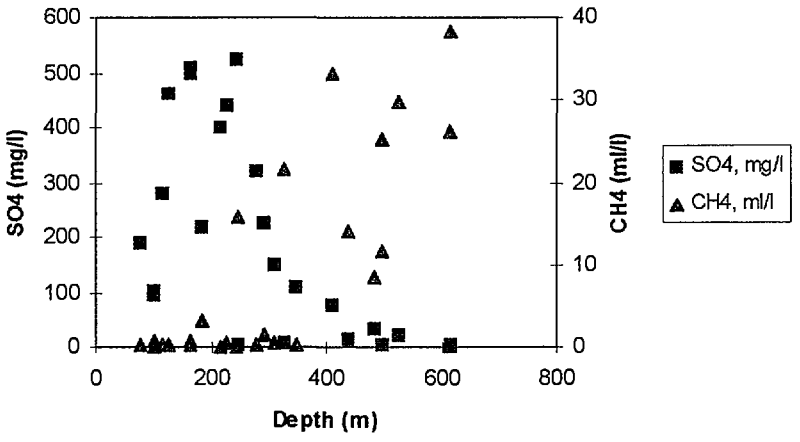

Figure 7: Relation between CH_4 and SO_4 with depth in Olkiluoto, from Ruotsalainen and Snellman (1996).

4.1.2 Hästholmen

Two of the Hästholmen samples (HH-KR1 and HH-KR2) come from saline groundwater, 900 to 950 m depth. The third (HH-KR3) is from brackish, sulfate rich water at just over 200 m depth. Hästholmen groundwaters are rich in iron, containing up to two orders of magnitude more total iron than is found in Olkiluoto. This is due to the higher levels of iron in the rapakivi granite in Hästholmen, which buffer the groundwater to a lower pH (< 8) as compared to the other sites. The lower pH also keeps more iron in solution, since at high pH iron precipitates from groundwater into pyrite or goethite minerals, according to the prevailing redox conditions, making the iron less easily available to microbes. In addition, these groundwaters are quite high in sulfate at all depths, but especially in the sulfate rich brackish groundwater between 100 and 400 m depth. Fracture minerals include iron hydroxides, but pyrites are only sporadically present. This indicates that the microbial population of these groundwaters would dominated by IRB. Microbial results confirm this, as IRB are present in all Hästholmen boreholes tested, at varying levels. SRB were not cultured at all from the deeper boreholes, although small numbers of SRB were detected in the sample from the sulfate rich groundwater.

The only Hästholmen sample for which we have a δS-34(SO_4) value is HH-KR1, with +27.87 ‰CDT (Table 4), indicating that SRB have used the lighter S-32 isotope of sulfate, which is in contrast with the lack of SRB cultured from this borehole. One possible explanation for this is that the sulfate was reduced at shallower levels of groundwater migration by SRB, since SRB were found in HH-KR3 at 200 m depth. As the groundwater passed through this depth, the sulfate was reduced to produce sulfide, but not all the sulfate was used due to low levels of SRB and abundant sulfate. As the groundwater migrated deeper and became more saline, SRB were inhibited by the increased salt or other factors, and died off, leaving unused sulfate with positive δS-34(SO_4) in groundwater without viable SRB.

It seems that the presence or absence of pyrite as a fracture mineral is a good indicator of the presence or absence of sulfate reducing bacteria in groundwater. The sulfate and sulfide concentrations are of less use in predicting the presence or absence of SRB. There is no correlation between sulfate concentration and SRB, the largest numbers of SRB in the Finland samples were found in boreholes with both very low and very high sulfate. Often δS-32 depleted sulfate is taken as an indicator of the presence of SRB, but this is not always the case as we have seen in Hästholmen. Sulfide is often found at low levels in groundwater, regardless of the presence or absence of SRB. The level is kept low because sulfide combines with ferrous iron to form pyrite, which has a low solubility, and is removed from the groundwater.

Carbon sources for the IRB in Hästholmen are unknown. The bacteria were cultured with lactate, and therefore heterotrophic. Possible carbon sources found in groundwater include DOC, which is at typical groundwater levels of 0.30 to 0.34 mM in Hästholmen (Table 4). Some IRB can grow autotrophically with H_2 and CO_2 (or HCO_3^-) as energy and carbon sources, respectively (Lovley et al. 1989). H_2 is present at quite high concentrations, especially in the two deeper Hästholmen boreholes, so it is possible that the IRB which were cultured heterotrophically could also grow autotrophically with H_2 as their energy source. Another possibility is that autotrophic microbes such as acetogens are producing organic carbon to support the heterotrophs, as will be discussed later.

4.1.3 Kivetty

In Kivetty, there are quite low levels of sulfate and total iron. Sulfate and sulfide increase with salinity, but there are no clear trends for iron apparently due to the very low contents (Ruotsalainen & Snellman 1996). Iron sulfides are quite common, as well as iron hydroxides to a lesser extent. This indicates that the microbial population will be a mixture of SRB and IRB, which is what was found. The presence of stalked cells in this borehole indicates that the groundwater is quite oligotrophic, and the dominant population, as seen in the total counts is stalked cells rather than SRB or IRB. These stalked cells are heterotrophic and require low levels of substrate, so they probably wouldn't have grown in any of the media used in this study.

4.1.4 Romuvaara

Both sulfate and total iron in Romuvaara are low (Ruotsalainen & Snellman 1996). Fracture minerals include iron sulfides and iron oxyhydroxides. This indicates a mixed population with both SRB and IRB. The largest population cultured from the Romuvaara sample was IRB (Table 6). This borehole has exceptionally high DOC, the highest for all boreholes investigated and higher than other boreholes at the Romuvaara site (Helenius et al. 1998b). This borehole also has the highest total cell count (Table 6). So, the IRB and SRB in this sample probably live heterotrophically in situ.

4.2 H_2 Based Biosphere

4.2.1 Olkiluoto

The deep biosphere is hypothesized to depend on H_2 and CO_2 as its driving forces. Both of these gases are present, and H_2 was at quite high concentrations in the Olkiluoto groundwaters tested, with the exception of OL-KR9 (Table 3). In addition, autotrophic acetogens, which convert H_2 and CO_2 to acetate were cultured in small numbers from the brackish groundwater from OL-KR3 and OL-KR10. The deepest borehole, OL-KR9 had the highest H_2, but no autotrophs were cultured from this borehole. This could have been due to problems with making a saline medium without precipitates.

Methanogens, another important component of the deep biosphere, could not be cultured from the Olkiluoto groundwaters, except in very small numbers from OL-KR10. However, methanogens tend to be difficult to culture. They are strict anaerobes which don't tolerate exposure to oxygen and they may not survive the stress of sampling and transport, so it is possible that methanogens are present but could not be cultured from the Olkiluoto samples.

Methanogens are not very competitive with other microbes at the low temperatures of the groundwater sampled. At low temperature, methanogenesis occurs faster via acetate rather than H_2/CO_2, so the methanogens would be dependent on close relationships with acetogens. In one laboratory study with lake sediments (Schulz et al. 1997), autotrophic methanogenesis did not occur at temperatures below 22°C even though the in situ temperature was constant at 4°C. Acetoclastic methanogenesis occurred from 2°C with rates increasing to the optimum temperature of 40°C (Schulz et al. 1997). Perhaps higher incubation temperatures would have shown more methane production, but the purpose of this study was to mimic what was happening in situ, so in situ temperatures were used. At low in situ temperatures, the autotrophic methanogens may be present but not active. However, heterotrophic methanogenesis should still have been observed at low rates at the in situ temperature. Another possibility is that the methanogens present may be carrying out anaerobic methane oxidation as discussed above, so their presence would not be detected by analysing for methane production. To avoid the problems of non culturable methanogens, in situ hybridization of the samples to detect *Archaea* (the Domain that includes methanogens) was attempted, but due to interference from the salt in the groundwater, there were no results.

Without culturing or probing results, carbon stable isotope data can give us information about sources of methane. Methane produced by microbes has a δC-13(CH_4) ratio more negative than about -50 ‰PDB (Oremland 1988). Microbial gas is also depleted in higher alkanes, such that they are less than 1% of the amount of methane (Oremland 1988). A plot of these two values for all four sites (Figure 8) shows biogenic and thermogenic sources of methane. The space in between presumably consists of a mixture of the two types. All of the Olkiluoto groundwaters plot either in the thermogenic or intermediate areas. This indicates that the methane in the Olkiluoto groundwaters is of thermogenic origin, with some mixing of biogenic methane, especially for samples from OL-KR3, and to a lesser extent OL-KR10 (from which we cultured small numbers of methanogens) and OL-KR4 (not tested for microbes). Presumably such a weak biogenic signal means that biogenic methane is produced elsewhere and a portion of it is transported to the fractures sampled by these boreholes or that methanogenesis takes place at low levels in these fractures. The main component of the methane seems to be thermogenic, however, diffusing up from deep in the rock. The values found for Olkiluoto groundwaters are similar for those found by Sherwood Lollar et al. (1993) for groundwaters from other shield sites in Finland and Canada. Based on a model, the explanation for the intermediate values in Sherwood Lollar et al. (1993) is mixing of different end members, with a bacterial end member consisting of anywhere between 20 and 94% of the groundwater.

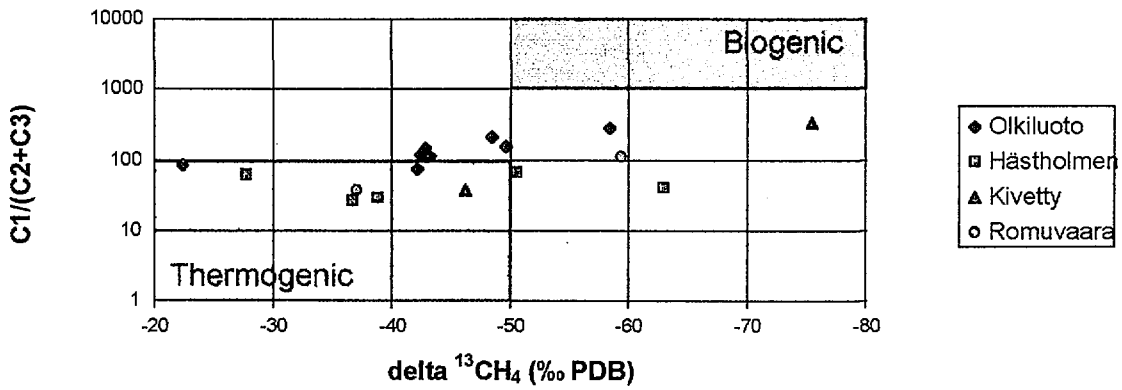

***Figure 8**: Origin of methane in Finland groundwater.*

It is well known that sulfate reducers outcompete methanogens in high sulfate environments, although small numbers of methanogens remain (Raskin et al. 1996). Since there are viable SRB in the brackish groundwaters at Olkiluoto, methanogens may be inhibited by the SRB. Methanogens could still be present at low numbers but not very active. In addition, the methanogens could be oxidizing methane rather than producing it, as discussed previously. If anaerobic methane oxidation was taking place, this would affect the carbon stable isotope ratios of methane, as they would preferentially use the lighter isotope of carbon, making the δC-13(CH_4) less negative. If this were the case, the methane present in these groundwaters could have more of a biogenic origin than is indicated by the δC-13(CH_4) values.

4.2.2 Hästholmen

Heterotrophic acetogens were cultured from two of the Hästholmen boreholes but no autotrophic acetogens were cultured (Table 6). No methanogens were cultured from Hästholmen, and probing was not attempted due to the high salinity of the groundwater. Looking at the isotope data, the methane from the two deep boreholes HH-KR1 and HH-KR2 seem to have a mainly thermogenic origin, with low δC-13(CH_4) and high relative levels of higher alkanes (Table 4, Figure 8). At more shallow depth in the borehole HH-KR3, there may be a mixture of biogenic and thermogenic methane, as the δC-13(CH_4) value is on the border between biogenic and thermogenic values. Since no methanogens were cultured from any of these boreholes, it is possible that methane is being produced biogenically elsewhere in the area and transported to these boreholes in smaller amounts mixed with the predominating thermogenic methane. The IRB in these boreholes could also outcompete methanogens by using any organic substrates more efficiently and keeping H_2 levels too low for methanogenesis (Lovley & Goodwin 1988). However, H_2 levels are quite high in the Hästholmen boreholes (Table 3), so this is probably not the case. No real support for a hydrogen based biosphere was found in Hästholmen.

4.2.3 Kivetty

Hydrogen gas is observed only in some boreholes in Kivetty, while methane is present, but at lower levels than in Olkiluoto and Romuvaara. The borehole investigated in this study had low levels of both hydrogen and methane. No methanogens or autotrophic acetogens were cultured from the Kivetty groundwater, however, a small number of heterotrophic acetogens were cultured (Table 6). Probing did indicate the presence of *Archaea*, about 30% of the total population. In the cold, low salt environment of these boreholes, these archaea could only be methanogens, of the known types of archaea. However, new uncultured types of archaea are being discovered all the time, so it is possible that the archaea detected with probes are not methanogens. Carbon stable isotope data for KI-KR5 indicates that the methane in this borehole is mainly of thermogenic origin. However, another borehole at Kivetty, KI-KR1, depth 816-831 m, is very depleted in C-13(CH_4), with a value of -75.5 ‰PDB (Snellman et al. 1995b). Values this low are indicative of biogenic methane. Kivetty has signs of the hydrogen based deep biosphere, but no autotrophs were cultured, possibly due to difficulties making an anaerobic medium with low TDS.

4.2.4 Romuvaara

Methane was not observed in all samples at Romuvaara, and when it was observed it was at low levels (Helenius et al. 1998b). Both methane and hydrogen are low in the borehole sampled for this study. Once again no methanogens or autotrophic acetogens were cultured, but low numbers of heterotrophic acetogens were found. In Romuvaara, the

data for RO-KR10 also indicates that the methane is not biogenic, with low δC-13(CH_4) value and high relative amounts of higher alkanes. Once again in Romuvaara, another borehole may consist at least partly of biogenic methane, with δC-13(CH_4) and C1/C2+C3 values similar to that found in OL-KR3 (Snellman et al. 1995a). But as with all other boreholes, the only real proof that methanogenesis is occurring is to culture methanogens, and problems with the high TDS required to make anaerobic media may have inhibited growth of freshwater microbes.

5 CONCLUSIONS

This study has demonstrated that microbes are present and active in subsurface groundwaters in Olkiluoto, Hästholmen, Kivetty and Romuvaara. Site variations of microbial groups agree with previous predictions based on groundwater chemistry. SRB predominate in Olkiluoto brackish groundwaters, and correlate with the presence of iron sulfide minerals. SRB contribute to redox control via sulfur species in Olkiluoto. Hästholmen has high dissolved iron, and iron hydroxide fracture minerals predominate while iron sulfides occur only sporadically. IRB are the dominant population in Hästholmen with no or very few SRB. Iron is more important than sulfur in controlling redox in Hästholmen and the IRB carry out redox buffering reactions. Kivetty and Romuvaara contained mixed microbial populations.

Signs of a deep hydrogen based biosphere were found especially in Olkiluoto. More study is needed to determine the basis for the subsurface ecosystem.

No signs of overly vigorous sulfate reduction, which is a concern for nuclear waste disposal, were seen in any of the sites. It seems that all sites being investigated are equally suitable for waste disposal from a microbiological point of view.

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