The ISME Journal (2008) 2, 760–775

www.nature.com/ismej

ORIGINAL ARTICLE

Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4–450 m in Olkiluoto, Finland

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Microbiology, chemistry and dissolved gas in groundwater from Olkiluoto, Finland, were analysed over 3 years; samples came from 16 shallow observation tubes and boreholes from depths of 3.9–16.2 m and 14 deep boreholes from depths of 35–742 m. The average total number of cells (TNC) was 3.9×10^5 cells per ml in the shallow groundwater and 5.7×10^4 cells per ml in the deep groundwater. There was a significant correlation between the amount of biomass, analysed as ATP concentration, and TNC. ATP concentration also correlated with the stacked output of anaerobic most probable number cultivations of nitrate-, iron-, manganese- and sulphate-reducing bacteria, and acetogenic bacteria and methanogens. The numbers and biomass varied at most by approximately three orders of magnitude between boreholes, and TNC and ATP were positively related to the concentration of dissolved organic carbon. Two depth zones were found where the numbers, biomass and diversity of the microbial populations peaked. Shallow groundwater down to a depth of 16.2 m on average contained more biomass and cultivable microorganisms than did deep groundwater, except in a zone at a depth of approximately 300 m where the average biomass and number of cultivable microorganisms approached those of shallow groundwater. Starting at a depth of approximately 300 m, there were steep gradients of decreasing sulphate and increasing methane concentrations with depth; together with the peaks in biomass and sulphide concentration at this depth, these suggest that anaerobic methane oxidation may be a significant process at depth in Olkiluoto.

The ISME Journal (2008) **2,** 760–775; doi:10.1038/ismej.2008.43; published online 24 April 2008
Subject Category: microbial population and community ecology
Keywords: ATP; most probable number; oxygen; methane oxidation; hard rock

Introduction

The subsurface biosphere of Earth appears to be far more extensive and metabolically and phylogenetically complex than previously thought (Amend and Teske, 2005; Lin *et al.*, 2006). Deep intraterrestrial microbial life is investigated to understand the diversity of life of Earth, the evolution and potential origin of life in the deep underground and the tolerances of intraterrestrial life towards extreme environmental conditions (Fredrickson and Balkwill, 2006). The discovery of a deep intraterrestrial biosphere (Pedersen, 1993) has several important implications for underground repositories for spent high-level radioactive wastes (HLRW, Pedersen, 2002). There are three main effects of microorganisms in the context of an HLRW repository situated approximately 500 m underground in bedrock of the Fennoscandian Shield (Anonymous, 1983). These are (1) oxygen reduction by microorganisms using organic carbon and methane as electron donors and the maintenance of anoxic and reduced conditions—oxygen is corrosive to the copper canisters that will be used to dispose of spent nuclear fuel, and radionuclides are generally more mobile under oxidizing than under reducing conditions (Kotelnikova, 2002; Pedersen, 2002); (2) sulphide production by sulphate-reducing bacteria (SRB) under anaerobic and reduced conditions—sulphide, in addition to

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Received 25 February 2008; revised 4 April 2008; accepted 4 April 2008; published online 24 April 2008

oxygen, is corrosive to the copper canisters (Pedersen, 2002) and (3) biomobilization and bioimmobilization of radionuclides, and the effects of microbial metabolism on radionuclide mobility (Pedersen, 2005).

Because of the potentially important effects of microorganisms, as listed above, microbiology research initiatives form part of the Finnish HLRW disposal programme (Pedersen, 2002). Microbes were found in all groundwater studied in the Finnish HLRW repository site selection investigations performed on four sites from 1996 to 2000 at depths of 65–948 m (Haveman *et al*., 1999; Haveman and Pedersen, 2002a). Relevant microbiology research in Finland can now be site related, because a disposal site for Finland's HLRW, Olkiluoto, has been selected.

The site selection investigation of microbiology in Olkiluoto started at a depth of 243 m and went to a depth of 866 m, leaving the shallow and intermediate deep groundwater unexplored. To fill this gap in our knowledge of the shallow and intermediate/deep Olkiluoto groundwater environment, a series of investigations of shallow boreholes was performed concurrently with new deep Olkiluoto groundwater investigations. Microbiology, chemistry and dissolved gas data from four sampling periods were assembled from measurements from 16 shallow observation tubes and boreholes ranging in depth from 3.9 to 16.2 m, resulting in 39 analytical data sets. In addition, 21 data sets on microbiology and geochemistry covering 13 deep boreholes ranging in depth from 34.6 to 449.6 m were produced. Finally, 33 gas analyses covering 14 deep boreholes ranging in depth from 40 to 742 m were carried out. Sampling and analysis protocols were adapted and tested for quality and reproducibility; contamination controls were also performed. Biomass determinations included total numbers of microorganisms and concentrations of ATP. Aerobic cultivation methods consisted of aerobic plate counts and most probable number determinations (MPN) of aerobic methane-oxidizing bacteria (MOB). Anaerobic MPN determinations included nitrate-reducing bacteria (NRB), iron-reducing bacteria (IRB), manganese-reducing bacteria (MRB) and SRB, and acetogenic bacteria and methanogens. The concentrations of dissolved nitrogen, oxygen, methane, helium, carbon dioxide, hydrogen, carbon monoxide and ethane were analysed. Geochemical and physical parameters analysed included pH, E_h, temperature, conductivity, sulphate, sulphide, ferrous iron and dissolved organic carbon (DOC). The results of these analyses have been merged and interpreted, and the outcome is reported here.

Materials and methods

Sampling groundwater from shallow observation tubes and boreholes

Samples were collected on four different occasions from 16 shallow boreholes ranging in depth from 3.9 to 16.2 m. The sampling periods were 3–6 May 2004, 10–14 October 2005, 24–28 April 2006 and 9–13 October 2006. All sampling sites were pumped out using immersed borehole pumps (Whale self-venting 12 V DC, pump model 9216; Whale Water Systems, Bangor, Northern Ireland) for at least 1.5 h before any field measurements or sample retrieval (Supplementary Table 1). The pumps and tubing assemblies were sterilized for approximately 2 h in an 11 p.p.m. chlorine dioxide solution (FreeBact 20; XINIX, Märsta, Sweden) in a 100-litre plastic barrel. The pumps were soaked in the chlorine dioxide solution and the solution was also pumped through the tubing.

The boreholes sampled at Olkiluoto (PVP1, PVP3A, PVP3B, PVP4A, PVP4B, PVP13, PVP14, PVP20, PR1, PP2, PP3, PP7, PP8, PP9, PP36 and PP39) penetrated groundwater, which was present in either the overburden (PVP) or water-conducting fractures in the bedrock (PR, PP; Supplementary Figure 1). Several of the boreholes and tubes selected for the 2004 sampling period turned out to be problematic due to bad casing, collapsing rock, identical water chemistry or insufficient water flow (PVP3A, PVP3B, PVP4B, PP3, PP7 and PP8). These were abandoned and new sampling points were selected for the three subsequent sampling periods. Overburden extended at most to a depth of approximately 13 m and consisted of sand and silt with an organic soil layer approximately 0.8 m thick. Bedrock groundwater samples extended to a depth of 16.2 m. The local bedrock at Olkiluoto is Precambrian and consists of metamorphic rocks (predominantly migmatitic mica gneisses) intruded by igneous rocks (granodiorites, coarse-grained granites and granitic pegmatites). Local land use above the aquifers ranges from undisturbed forest to open areas cleared for repository construction. Further details can be found in the Posiva 2006 site description (Andersson *et al*., 2007a).

Groundwater for microbiological analyses was collected in the spring 2004 and fall 2005 sampling periods using a Solinst Model 425 Discrete Interval Sampler (Solinst Ltd, Georgetown, ON, Canada) immediately after the pumping period was finished and the borehole pump was hoisted out of the borehole. The sampling depth coincided with the depth of the borehole pump, positioned between depths of 2 and 10 m, depending on borehole length (Supplementary Table 1). Samplers of two different diameters (26 and 51 mm) were used, depending on the diameter of the observation tube or borehole used. Before sampling, all exterior and interior fittings of the Solinst sampler were sterilized with a 20 p.p.m. chlorine dioxide solution (FreeBact 20; XINIX) and then rinsed with sterile, autoclaved analytical grade water (AGW, Millipore Elix 3; Millipore, Solna, Sweden) to prevent microbial contamination of the groundwater. To collect *in-situ* groundwater from the required depth interval, the sampler was kept pressurized to 0.32 MPa with N_2

gas until it was at depth; then it was de-gassed (that is, vented to the surface) allowing the ambient water in, and finally re-pressurized once the sampler was full, before surface retrieval. Pressurizing the sampler to a pressure at least double that of the highest water pressure experienced by the sampler (0.16 MPa) ensured that it remained closed until reaching the sampling depth. Water from the sampler was then dispensed to the various containers for the analyses described below.

Quality control tests of the sampling procedures for shallow groundwater

In the spring 2004 sampling period, oxygen was detected in all boreholes. It was not possible to determine whether the sampling procedure had introduced this oxygen, or it was actually present in the groundwater that entered the boreholes. Therefore, a packer that allowed passage of sampling tubes and the wire for the borehole pump was constructed and tested in the fall 2005 sampling period. Nitrogen gas was flushed into the borehole at a rate of approximately 1 litre per min, starting before pumping, through five of the ten boreholes that were being sampled. In that way, the air above the water level was replaced with nitrogen while the groundwater level was being lowered by the pumping. This eliminated the possibility of oxygen entering the sampled groundwater from the atmosphere in the borehole. Oxygen was below the detection limit in all boreholes without packers except for PP9, which exhibited oxygen values above the detection limit (0.2) of 0.4 mg per litre. Irrespective of whether or not a packer was used, oxygen was not detected except in PP9, and did not appear to mix significantly with the groundwater; consequently, the packers were not needed.

The procedure for sterilizing the borehole pumps and tubing used for sampling shallow groundwater was tested by pumping sterile water through the equipment after a completed sterilization. The borehole pumps were first soaked in a chlorine dioxide solution for 2 h in a 100-litre barrel, as described above. The barrel was then washed with 500 ml of AGW. An adaptor used for microbiology sampling was installed on the orifice of the pump tube. AGW (2 litres) was then pumped through the system. Thereafter, 10 litre of AGW was added to the barrel and pumped (1 litre per min) out through the sampling adaptor. The adaptor was removed after 2 min and the remaining 8 litres of water was pumped out (8 litres per min). This procedure simulated the pumping out of a borehole before starting sampling. The adaptor was flushed with 1 litre of sterile AGW and then remounted. Finally, 5 litre of sterile AGW was pumped through the tubing, after which a full sampling for microbiology was performed on the AGW according to the procedures used in the field.

While taking samples from the overburden holes (PVP) in the fall 2005 sampling period, it was noted that the water sampled using the Solinst sampler was sometimes slightly more turbid than that sampled using the borehole pump; this effect was not observed in the bedrock holes (PR, PP). It was assumed that hoisting the pump and lowering the Solinst sampler may have caused some hydrodynamic disturbance that increased the concentration of suspended material in the borehole. Microorganisms attach to such particles, which could create some uncontrolled variability in the data. In fall 2005, a comparison was made in the PVP20 borehole in which water was sampled twice for microbiology, first using the borehole pump and then the Solinst sampler. The assumed effect was confirmed. Therefore, groundwater was taken directly from the pump to sample tubes and bottles in the 2006 spring and fall sampling periods.

Pumping groundwater out of a borehole results in the transport of water from the surrounding aquifers into the borehole. It was deemed important to test for sensitivity in the data obtained from prolonged pumping. Borehole PVP4A had an inflow of approximately 4 litres per min, and this borehole was selected for a reproducibility test in April 2006; it was sampled twice within a 6-h interval, which allowed 1440 litres of groundwater to be pumped out of the borehole between the sampling occasions.

Sampling groundwater from deep boreholes

A total of 21 samples for microbiological analysis were taken between October 2004 and November 2006 from 13 boreholes ranging in depth from 34.6 to 449.6 m (Supplementary Table 2). The groundwater was sampled using the PAVE system (Haveman *et al.*, 1999). The procedures for sampling and for microbiological analysis using pressure vessels, such as PAVE vessels, have been evaluated with the appropriate quality controls, including contamination tests as described elsewhere (Hallbeck and Pedersen, 2008).

Field measurements of physical parameters of shallow boreholes and groundwater observation tubes

Field measurements were made in a 1-litre container at the surface while groundwater was being pumped to the surface; the measurements and sampling for chemistry were done at the end of the pumping period. The temperature of the groundwater was measured using a pIONeer 10 portable pH meter equipped with a pHC5977 cartrode combined pH electrode (pH range 0–14, ±0.5 at 0; temperature range −10 to 110 °C, ±0.3 °C; Radiometer, Labora, Stockholm, Sweden). Redox was measured using the same pH meter, but equipped with a MC3187Pt combined platinum electrode with an Ag/AgCl reference system (range −2000 to 2000 mV, ±0.01% of reading; Radiometer). The dissolved

oxygen concentration was measured using two different meters and electrodes: (1) a pIONeer 20 portable oxygen meter equipped with a DOX20T-T oxygen probe with a concentration range of 1–20 mg per litre (0–200 ± 1%) (Radiometer) was used during the spring 2004 sampling period and (2) an HQ10 Hach Portable LDO dissolved oxygen meter, no. 51815-00 (Hach, Stockholm, Sweden) was used during the 2005–2006 sampling periods. The probes were calibrated *in situ* per the manufacturer's instructions. In addition, the dissolved oxygen was measured with the LDO electrode in a series of five measurements made over 1 year to analyse for seasonal variations; the sampling months were October 2005 and April, May, July and October 2006.

Oxygen was also analysed in the laboratory using a modified Winkler method as described in detail in Carritt and Carpenter (1966). Briefly stated, three approximately 115-ml, glass-stoppered Winkler bottles were flushed with at least three volumes of groundwater from the pump to remove all oxygen from atmospheric sources. Then added manganese ions were precipitated with dissolved oxygen directly in the field in an alkaline medium, forming manganous hydroxide that subsequently was dissolved in the laboratory with acid and reduced by iodine. Finally, the iodine produced was determined by titration, with thiosulphate ions and soluble starch used as the titration indicator.

Chemical analyses of shallow and deep groundwater

Water samples were transferred from the investigation site to the Teollisuuden Voima Oy (TVO) laboratory directly after sampling. The chemical analyses were performed by TVO according to their protocols, or were subcontracted to external laboratories (Supplementary Table 3). Groundwater samples for laboratory analysis were collected during pumping in a 5-litre plastic canister (for testing for Br^-, Cl^-, F^-, SO_4^{2-}, S_{tot}, pH and conductivity), 1-litre glass bottles (for testing for alkalinity, acidity and DIC/DOC) and 1-litre nitric acid-washed glass bottles (for testing for metals). Groundwater samples for sulphide analysis were collected in three 100-ml Winkler bottles. Some of the water chemistry samples were filtered with a 0.45-μm membrane filter and then bottled; preserving chemicals were added to some of the samples (Supplementary Table 3).

Sampling and analysis of dissolved gas

Water samples from all shallow boreholes and observation tubes sampled in spring and fall 2006 were analysed for dissolved gas. Shallow groundwater was sampled in triplicate in nitrogen-flushed 120-ml glass bottles equipped with butyl rubber stoppers (no. 2048-117800; Bellco Glass, Vineland, NJ, USA) and sealed with aluminium crimp seals (no. 2048-11020; Bellco Glass). The pressure in the bottles was set to 1 Pa 2–4 h before sampling. Water from the immersed borehole pump was led via polyetheretherketon tubing through a syringe into the bottles, which were filled with approximately 100 ml of groundwater. In the laboratory, the sample was transferred to a vacuum container and any gas in the water was boiled off under vacuum (that is, water vapour pressure) at room temperature; the transfer time was approximately 20–30 min. Deep groundwater (Supplementary Table 4) was sampled using the PAVE sample vessel, which was attached to the extraction unit as was done for the glass bottles; groundwater transfer typically took 5 min. After extraction, the gas was compressed and transferred to a 10-ml syringe (SGE Analytical Science, Victoria, Australia) and the volumes of extracted gas and water were measured. The captured gas was subsequently transferred to a 6.6-ml glass vial stoppered with a butyl rubber stopper and sealed with an aluminium crimp seal. The vial had previously been evacuated and flushed twice with nitrogen, in two cycles, and left at high vacuum (1 Pa). Copper sulphate (dehydrant) was added to adsorb any traces of water remaining in the gas (water causes troublesome baseline drifts in the gas chromatographs). Thereafter analysis was performed using gas chromatography.

Two different chromatographs were used and equipped as follows. Hydrogen and carbon monoxide were analysed on a KAPPA-5/E-002 analytical gas chromatograph (AMETEK/Trace Analytical, formerly Trace Analytical, Menlo Park, CA, USA) equipped with a 156 × 1/16-inch stainless steel HayeSep column in line with a 31 × 1/8-inch stainless steel molecular sieve 5A column, which was subsequently attached to a reduction gas detector. Helium, argon and nitrogen were analysed on a Varian Star 3400CX gas chromatograph (Varian Analytical Instruments, Varian AB, Bromma, Sweden) using a thermal conductivity detector with an oven temperature of 65 °C, a detector temperature of 120 °C and a filament temperature of 250 °C. The gases were separated using a Porapak-Q column (2 m × 1/8 inch diameter) followed by a molecular sieve 5A column (6 m × 1/8 inch) with argon (for helium and nitrogen) and nitrogen (for argon) as the respective carrier gases. Methane and ethane were analysed on a Varian Star 3400CX gas chromatograph using a flame ionization detector (FID) with an oven temperature of 65 °C and a detector temperature of 200 °C. The gases were separated using a Porapak-Q column (2 m × 1/8 inch diameter) and analysed on the FID with nitrogen as the carrier gas. Carbon dioxide was transformed to methane using a 10% Ni_2NO_3 'methanizer' fed with hydrogen gas (9.375 × 1/8 inch diameter, temperature 370 °C) and analysed as methane on the FID with nitrogen as the carrier gas.

Determining total number of cells

The total number of cells (TNC) was determined using the acridine orange direct count method as

devised by Hobbie *et al*. (1977) and modified by Pedersen and Ekendahl (1990).

ATP analysis
The ATP Biomass Kit HS for determining total ATP in living cells was used (no. 266-311; BioThema, Handen, Sweden). This analysis kit was developed based on the results of Lundin *et al*. (1986) and Lundin (2000). The ATP biomass method used in this work has been described, tested in detail and evaluated for use with Fennoscandian groundwater, including Olkiluoto groundwater (Eydal and Pedersen, 2007).

Determining cultivable aerobic bacteria
Petri dishes containing agar with nutrients were prepared as described elsewhere (Pedersen and Ekendahl, 1990) for determining the numbers of cultivable heterotrophic aerobic bacteria (CHAB) in groundwater samples. Ten-times dilution series of culture samples were made in sterile AGW with 1.0 g per litre of NaCl and 0.1 g per litre K_2HPO_4; 0.1-ml portions of each dilution were spread with a sterile glass rod on the plates in triplicate. The plates were incubated for between 7 and 9 days at 20 °C, after which the number of colony-forming units was counted; plates with between 10 and 200 colonies were counted.

Preparation of media, inoculations and analysis for most probable numbers of cultivable anaerobic microorganisms
The procedures described by Widdel and Bak (1992) for the preparation of anaerobic media for determining the MPN of microorganisms were modifed and applied as described elsewhere (Hallbeck and Pedersen, 2008). Media were prepared for the NRB, IRB, MRB, SRB, autotrophic acetogens (AA), heterotrophic acetogens (HA), autotrophic methanogens (AM) and heterotrophic methanogens (HM). The specific media compositions (Supplementary Table 5) were formulated based on previously measured chemical data from Olkiluoto. This allowed the formulation of artificial media that most closely mimicked in-situ groundwater chemistry for optimal microbial cultivation (Haveman and Pedersen, 2002a).

Inoculations and analysis for aerobic methane-oxidizing bacteria
Sets of MPN tubes for samples requiring a nitrate mineral salts medium (Whittenbury *et al*., 1970) were prepared as follows: 1.0 g per litre of KNO_3, 1.0 g per litre of $MgSO_4 \times 7\ H_2O$, 0.2 g per litre of $CaCl_2\ \times 2\ H_2O$, 1 mg per litre of $CuCl_2\ \times 2H_2O$, 7 g per litre of NaCl, 1 ml per litre of an iron solution made of 0.5 g of ferric (III) chloride in 1000 ml of AGW, 1 ml per litre of a trace element solution (Widdel and Bak, 1992) and 2 ml per litre of a phosphate buffer solution made of 3.6 g Na_2HPO_4 and 1.4 g NaH_2PO_4 in 100 ml of AGW. The pH was adjusted to 6.8–7.0. Cultural conditions were optimized to support the growth of both types I and II MOB by adding 1 mg per litre of copper chloride dehydrate. The gas atmosphere in the tubes was air.

MPN inoculations were completed within 2 h of sample collection for all shallow borehole samples. Five parallel dilution tubes were prepared for each dilution. All transfers were performed aseptically using new sterile syringes and needles. After each transfer, the tubes were vortexed to achieve homogeneity. Control tubes contained nitrate mineral salt medium and 1 ml of groundwater sample filtered through a 0.2-μm filter. After inoculation, filter-sterilized (using 0.2-μm Millipore filters) methane was injected into the headspace of each tube to 0.1 MPa overpressure. The tubes were then incubated horizontally in the dark at 20 °C. Growth of cells was detected after 2–4 weeks, as judged by turbidity compared with that of negative controls and the concomitant production of carbon dioxide via methane oxidation in turbid tubes. Carbon dioxide was analysed as described above for the analysis of groundwater gas. MPN calculations were made using a combination of positive tubes in a three-tube dilution series (that is, 15 tubes) according to Greenberg *et al*. (1992). The detection limit was 0.2 cells per ml.

Results

Quality control and contamination tests of the sampling procedures
Consecutive tests were performed in the 2004, 2005 and 2006 sampling periods to develop and test the quality of the sampling procedures. The procedures for sterilizing the borehole pump and samplers were analysed and the data obtained using the Solinst tube sampler were compared to those obtained using the borehole pump. The influence of pumping time on the results was also studied.

AGW water sterilized in an autoclave yielded ATP, TNC and CHAB readings that were not significantly different from 0 (not shown). The sterilized pump came directly from the field and had been in use for several years. Even so, the sterilization testing produced very good results, with values at the detection limits for TNC, ATP and CHAB (10^3 cells per ml, 10^3 amol per ml and 10 cells per ml, respectively). All MPN analyses were below the detection limit (<0.2 cell per ml), except in the case of NRB, for which the MPN was 0.4 cell per ml. It can thus be concluded that the sterilization procedures worked properly and that the sampling pump systems did not cross-contaminate the sampled boreholes or samples.

On 13 October 2005, sampling with the Solinst sampler was compared to sampling directly from the

borehole pump in the overburden borehole PVP20. The microbiology results obtained from samples taken with the borehole pump were generally equal to or lower than those obtained from samples taken with the Solinst sampler, except in the case of MOB (Figure 5a). The largest differences were found in the cases of NRB and AA, likely due to hydrodynamic disturbance caused by raising and lowering the pump and sampler in the boreholes. Sediment and colloids that became suspended in groundwater due to disturbance during sampling would certainly harbour attached microorganisms, which would subsequently increase the biomass estimates in turbid as compared with non-turbid groundwater. In choosing whether to use the Solinst or pump method for sampling, it can be argued that the Solinst method gives higher microorganism numbers related to particles in the groundwater. These are of course true results, in that these organisms were indeed present and possibly active in the sampled borehole. At the same time, the Solinst method introduced uncertainty into the results, as it is impossible to reproducibly cause turbidity in the boreholes. For comparative purposes, it was thus deemed better to sample from the borehole pump in the field activities in 2006. Otherwise, results from overburden borehole samples may overestimate the planktonic cell numbers compared with results from bedrock borehole samples that were free of turbidity caused by sampling activities in the borehole.

In testing the effect of pumping time on the results, the largest effects of pumping PVP4A (27 April 2006) for 6 h were found for the CHAB value, which decreased approximately 10-fold, and for the ATP value, which decreased approximately 50%. All remaining values, except for AM, were not significantly different, as the standard deviations of all MPN values overlapped (Figure 5a). The groundwater chemistry conditions also appeared to be very stable in this borehole. The only chemical parameter that changed significantly was the ferrous iron content, which decreased from 3.05 to 1.64 mg per litre.

The reproducibility of the pressure vessel method (PAVE) has been analysed and reported elsewhere (Hallbeck and Pedersen, 2008). The results indicated that MPN analyses of groundwater samples taken simultaneously using different pressure vessels reproduced very well within the 95% confidence intervals of the MPN analysis. The TNC determinations and ATP analysis also displayed good reproducibility; this included the sampling procedure, transportation logistics, and MPN inoculation, cultivation and analysis for each physiological group of microorganisms. A second test explored the reproducibility of two different analytical rounds on groundwater from two different borehole sections and of repeated sampling over time. This test also included the effects of different personnel involved and of different batches of chemicals and media. In general, groundwater from the two borehole sections had very different result profiles that reproduced well over time.

Physical parameters of shallow groundwater

The pH ranged from 4.8 to 8.2. All values below pH 6 were found in groundwater from a depth of less than 10 m (Figure 1a), except for samples from borehole PP36 (12.1 m deep), which had a stable pH of 5.8 over all sampling periods. Most of the shallow groundwater samples had pH values of 6.5–7.5, whereas the deep groundwater had pH values of 7–8.2. The conductivity ranged from 10 to 10 000 mS per m (Figure 1b), except in groundwater from boreholes PR1 (sampled 11 October 2006) and PP39 (sampled 24 April 2006), which were diluted to below and at the detection limit, respectively. The conductivity increased exponentially with depth within a range of approximately plus-minus five times the observed average value for each depth.

Oxygen was found in several shallow groundwater samples (Figure 1c) but was absent from deep groundwater (not shown). Two different methods were used to analyse oxygen in shallow groundwater, one electrochemical and one wet chemistry method. The HQ10 Hach Portable LDO dissolved oxygen meter was used in the field starting in fall 2005. Titrating oxygen using the Winkler method (Carritt and Carpenter, 1966) was introduced in spring 2006. The Winkler analysis is reliable over a wide range, extending from the detection limit of 0.05 mg of O_2 per ml to oversaturated samples. The LDO electrode results were very well correlated with the Winkler results at high oxygen concentrations (not shown). The LDO electrode was less precise at values below 0.5 mg O_2 per ml. Both the electrode and the Winkler analyses revealed rapidly decreasing oxygen values with increasing depth. Small amounts of oxygen remained in the groundwater from depths greater than 10 m (Figure 1c), except for one PP9 sample (14.7 m) that displayed a high oxygen value due to the drawdown of oxygenic surface groundwater through a corrosion-damaged steel casing tube. The four sampling periods were two in April and two in October. The concentration of dissolved oxygen was expected to vary seasonally, and when dissolved oxygen was repeatedly analysed over a year this was confirmed (Figure 1d). Oxygen concentration decreased in summer and increased in fall and spring. The E_h values over depth in shallow groundwater were very scattered, displaying only a very weak decreasing trend with increasing depth, and the measured E_h was lower at low oxygen concentrations (not shown). The average shallow groundwater temperature was 7.1 °C in spring and 9.1 °C in fall. The difference in temperature between seasons was most pronounced at depths of less than 10 m, where the water temperatures in shallow boreholes such as PVP1 and PR1 differed by up to 6 °C.

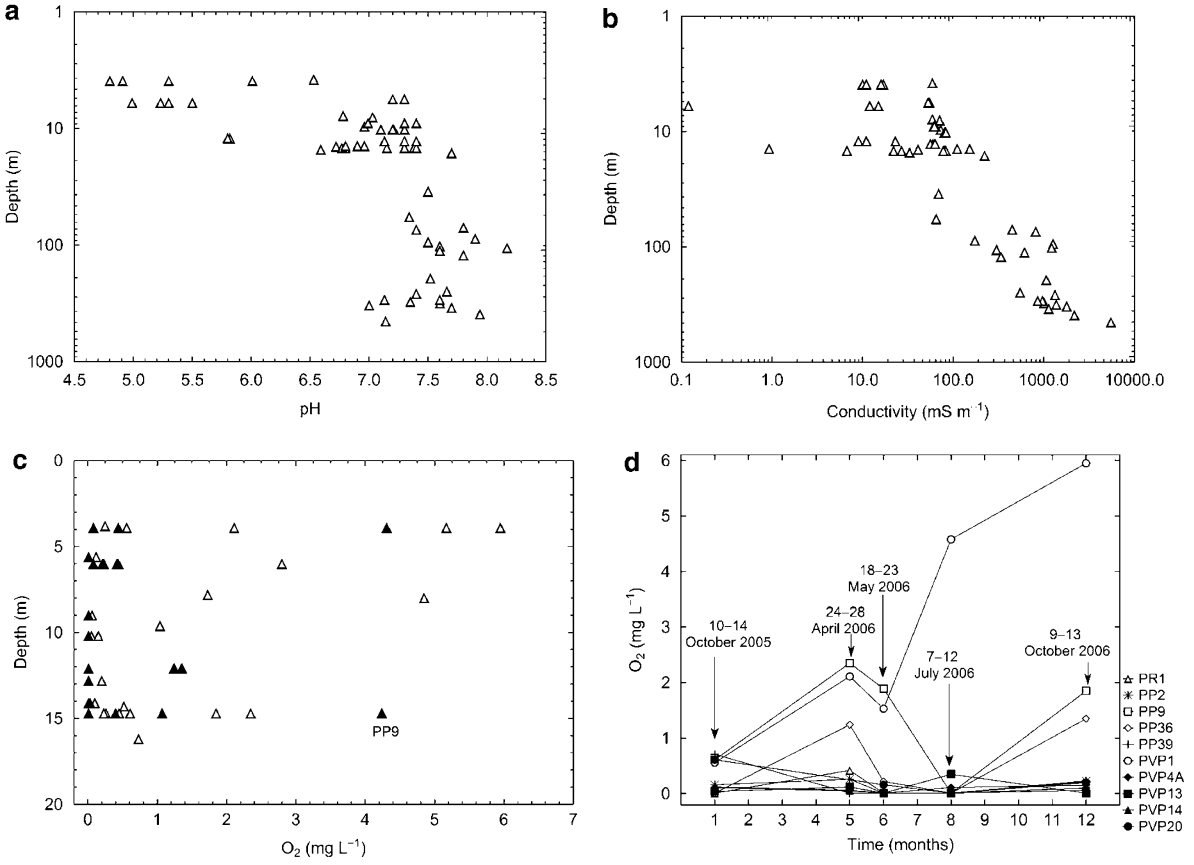

Figure 1 (**a**) The pH in groundwater samples from Olkiluoto against depth. (**b**) The electrical conductivity in groundwater samples from Olkiluoto against depth. (**c**) The concentration of dissolved oxygen in shallow groundwater analysed using the HQ10 Hach Portable LDO dissolved oxygen meter (open symbols) and by means of Winkler titration in the laboratory (filled symbols; the average value of three titrations is shown). (**d**) The seasonal variation of dissolved oxygen in shallow Olkiluoto groundwater analysed using the HQ10 Hach Portable LDO dissolved oxygen meter.

Chemical analyses of groundwater

The general trend was for dissolved solids to increase with depth, as reflected by the conductivity measurements (Figure 1b). The concentration of DOC is of special interest for microbiological interpretations, as DOC can be expected to relate positively to heterotrophic microbial activity. When analysed, no correlation was found between DOC and depth (Figure 2a). Instead, the DOC values were scattered from below the detection limit of 1.8 mg DOC per ml up to 39 mg DOC per ml. One sample displayed an exceptionally high DOC value of 196 mg DOC per ml. This was from the shallow PVP1 observation tube that was completely flooded by snow meltwater until the day before sampling (27 April 2006). This was not persistent contamination, as the DOC value was less than a tenth of that found 6 months later (12 October 2006). The concentrations of ferrous iron and sulphide displayed inversely related trends, with decreasing ferrous iron and increasing sulphide values with depth (Figure 2b). The ferrous iron concentration was up to 100 times higher in shallow than in deep groundwater. The dissolved sulphide concentration was at or below the detection limit down to a depth of 70 m and peaked at a depth of approximately 300 m.

Sulphate was scattered over a large concentration range at depths down to 400 m, after which the sulphate concentration approached zero (Figure 2c). Some of the shallowest groundwater samples were very dilute and had low sulphate concentrations as well. This profile implies that microbial sulphate reduction was possible to a maximum depth of 400 m at Olkiluoto. A peak in sulphide concentration at a depth of approximately 300 m is very obvious in the scatter plot of all available Olkiluoto data (Figure 2d); the peak values were approximately 100 times the sulphide concentrations at all other analysed depths.

Distribution of gases in Olkiluoto groundwater

The average total amount of dissolved gas per volume of groundwater increased exponentially with depth (Figure 3a). In the shallow groundwater, volumes of 25–70 ml of gas per litre groundwater were found. The amounts then increased up to a maximum of 1380 ml of gas per litre groundwater in the deepest groundwater sample from borehole

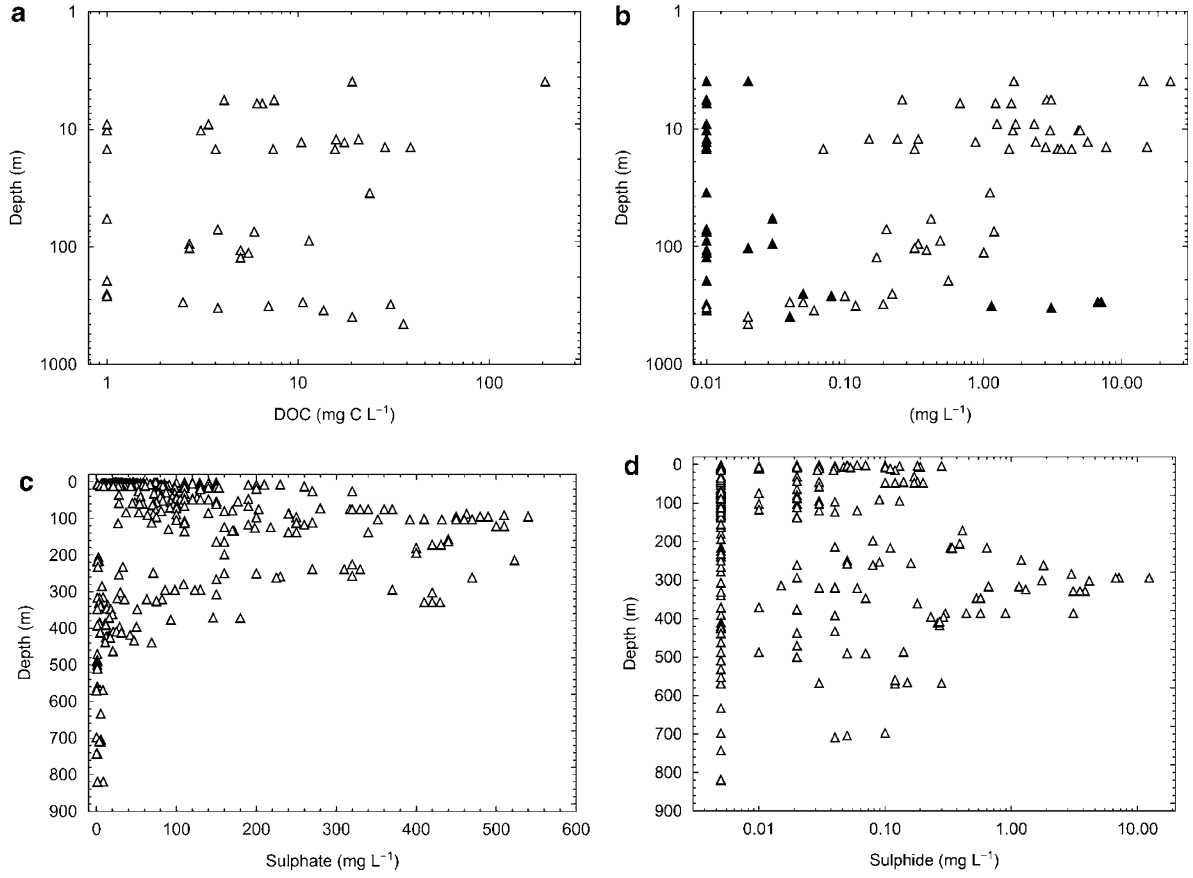

Figure 2 (**a**) The concentration of dissolved organic carbon (DOC) in groundwater samples from Olkiluoto against depth. (**b**) The concentration of dissolved ferrous iron (open symbols) and sulphide (filled symbols) in groundwater samples from Olkiluoto against depth. (**c**) The distribution of sulphate in Olkiluoto groundwater against depth. All available Olkiluoto data between 1992 and 2006 have been included in the scatter plot. (**d**) The distribution of sulphide in Olkiluoto groundwater against depth. All available Olkiluoto data between 1992 and 2006 have been included in the scatter plot. The value of observations of sulphide below the detection limit was set to 0.005 in the scatter plot.

OL-KR29, at a depth of 742 m. The concentration of dissolved nitrogen per litre groundwater increased with depth, but its concentration range was narrow compared with those of other gases. Nitrogen concentration increased approximately 20-fold with depth, rising from 15 to 250 ml of nitrogen per litre groundwater. In comparison, helium concentration increased approximately 1000-fold over the depth range analysed, rising from 30 to 20 000 µl of gas per litre groundwater. The trend was for average helium concentrations to increase exponentially over most of the depth range analysed, except in the deepest sample. Methane displayed a two-layer profile with values between 1 and 1000 µl of gas per litre groundwater down to a depth of 300 m (Figure 3b). At this depth, there was a distinct 100-fold increase in the methane concentration to 100 000 µl of gas per litre groundwater; the methane concentration then increased 10-fold by a depth of 742 m, the depth of the deepest sample analysed. Overall, the concentrations of methane were distributed over a million-times range. The concentration of the last of the major gases analysed, carbon dioxide, decreased approximately 10-fold from the shallow groundwater samples to a depth of approximately 17 m (Figure 3c). Thereafter, the average concentration decreased slightly, except in the deepest sample, which had a high concentration relative to the other deep (300–560 m) groundwater samples. The average concentration of dissolved hydrogen per litre groundwater displayed a weak increasing trend with depth, but the data points were very scattered (Figure 3d). Carbon monoxide concentrations were, as with hydrogen, scattered and ranged from 0.5 to 5 µl of gas per litre groundwater but did not change with depth. Average ethane concentrations increased exponentially with depth (3.9–742 m) from 0.1 up to 5000 µl of gas per litre groundwater.

Total number of cells and ATP

The TNC ranged from 8×10^3 to 2.5×10^6 cells per ml in the shallow groundwater (Figure 4a) and the overall average was 3.9×10^5 cells per ml. There were fewer cells in the deep groundwater, which had a maximum of 1.5×10^5 cells per ml at a depth of 450 m in groundwater from borehole OL-KR19.

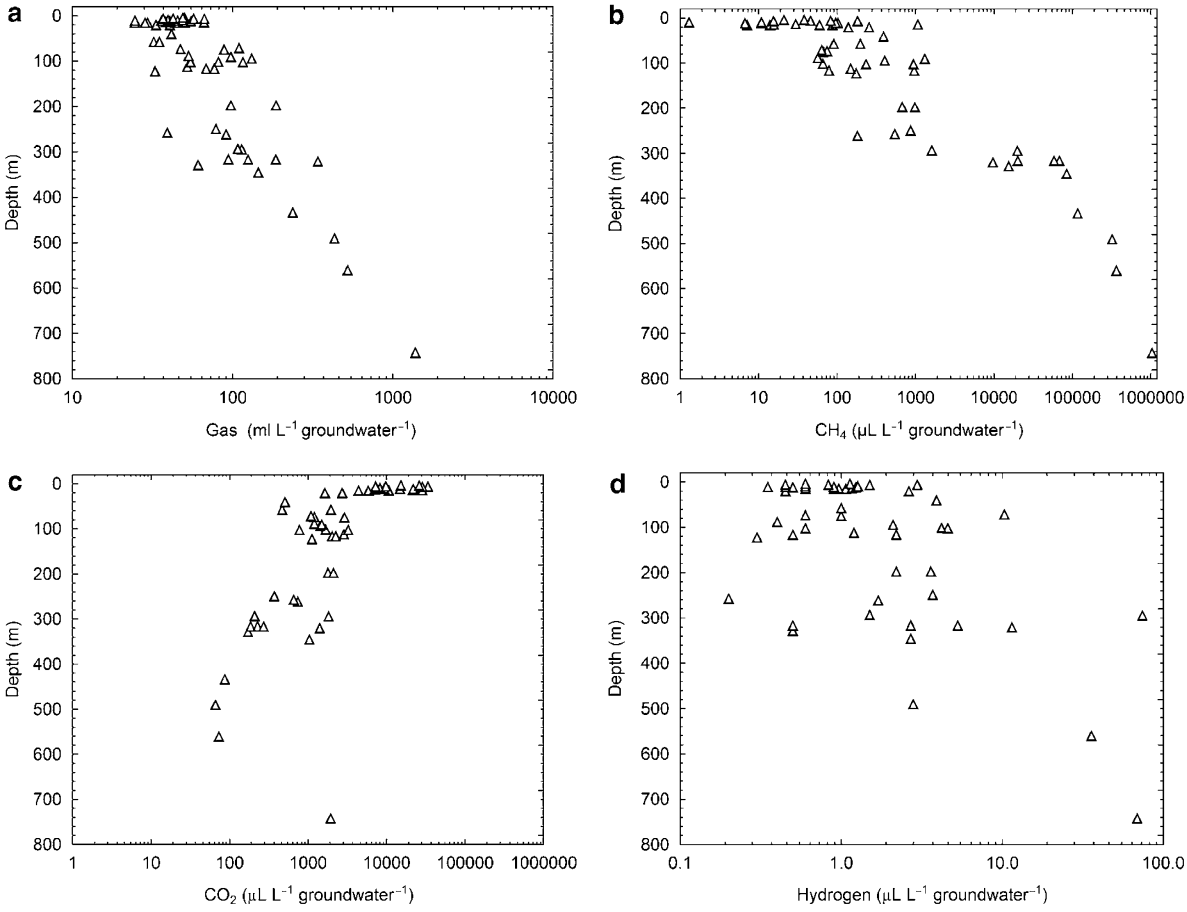

Figure 3 The total amount of extractable dissolved (**a**) gas, (**b**) methane, (**c**) carbon dioxide and (**d**) hydrogen in Olkiluoto groundwater against depth.

The overall average TNC over depth in deep groundwater was 5.7×10^4 cells per ml, which was almost 10 times lower than the average TNC over depth in shallow groundwater. The average TNC over depth in deep groundwater did not display any trend with depth.

The concentration of ATP in the sampled groundwater ranged over approximately four orders of magnitude if the highest ATP value of 10^7 amol per ml, obtained from borehole PVP1, was included (Figure 4b). This was, however, an extreme value from spring 2006, when the borehole had been completely flooded by meltwater until the day before sampling. The remaining data ranged over three orders of magnitude. There were two peaks in the ATP values over depth: the first was found in the shallow groundwater and the second appeared between depths of 300 and 400 m. The range of the ATP values in shallow versus deep groundwater did not differ markedly.

Cultivable heterotrophic aerobic bacteria

The numbers of CHAB in shallow groundwater were scattered, displaying no recognizable trend over depth (not shown). The overall average number of CHAB in shallow groundwater was 3.2×10^3 cells per ml. Deep groundwater had a narrower range of CHAB values than did shallow groundwater (not shown). The overall average number of CHAB in deep groundwater, that is, 3.1×10^3 cells per ml, was similar to the number in shallow groundwater. CHAB analysis was not done from the start of the sampling programme, only having been introduced in 2005.

Most probable number of metabolic groups of microorganisms

Base-10 logarithms were calculated for the MPN values for each metabolic group and stacked in bar graphs (Figure 5). The obtained stacked number value then represents both the diversity, that is, how many metabolic groups could be cultivated, and the numbers of cultivable organisms within each metabolic group in a borehole. A large stacked value will be obtained if both the diversity and the numbers of cultivated metabolic groups were large. The numbers cultivated of each group can be appreciated from the bar length for the respective metabolic

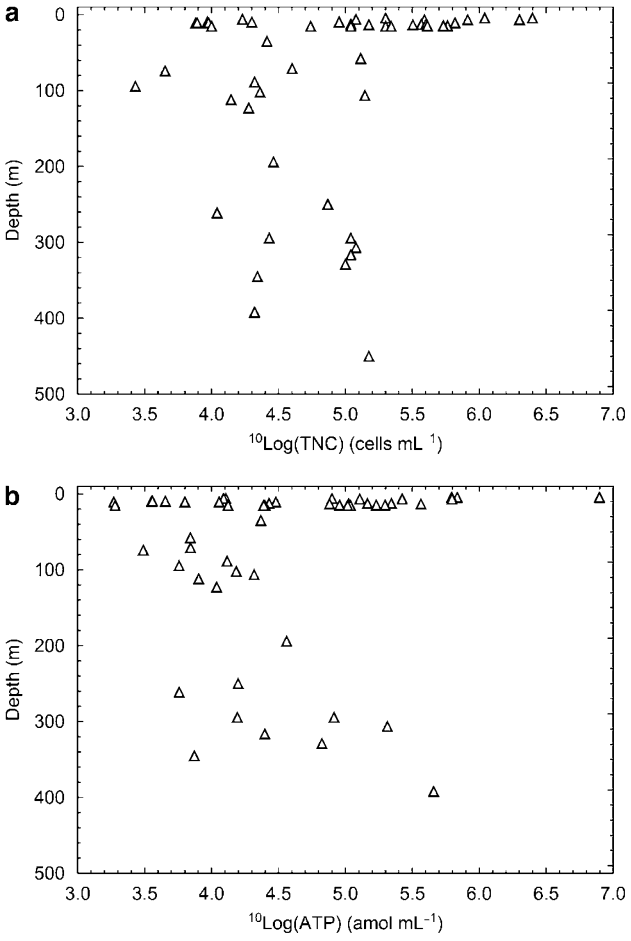

Figure 4 (**a**) The distribution of total number of cells (TNC) against depth in Olkiluoto groundwater. (**b**) The concentration of ATP distributed against depth in Olkiluoto groundwater.

group. The stacked MPN values in shallow groundwater remained similar from season to season and were borehole specific in the case of groundwater from several boreholes (Figure 5a). The spring 2004 values are excluded from the stacked MPN figures, as most of these values refer to boreholes that were not analysed again. Groundwater from boreholes PP2 and PP9 had low MPN values and the least diverse communities in all three seasons, whereas samples from boreholes PR1 and PP39 had the highest stacked MPN values and diversities of all shallow groundwater samples. The spring 2006 value for borehole PVP1 groundwater was much higher than in the other two seasons, due to the above-mentioned flooding event. There was no clear difference in the stacked MPN values between overburden (PVP) and shallow rock (PR, PP) boreholes.

The stacked MPN values in deep groundwater remained homogenous over depth for the first 120 m (Figure 5b). Thereafter, the MPNs decreased until a depth of 294 m, where the values increased, the highest value being found at a depth of 328 m. The NRB analysis was introduced into the sampling programme in 2005, so values are missing from six

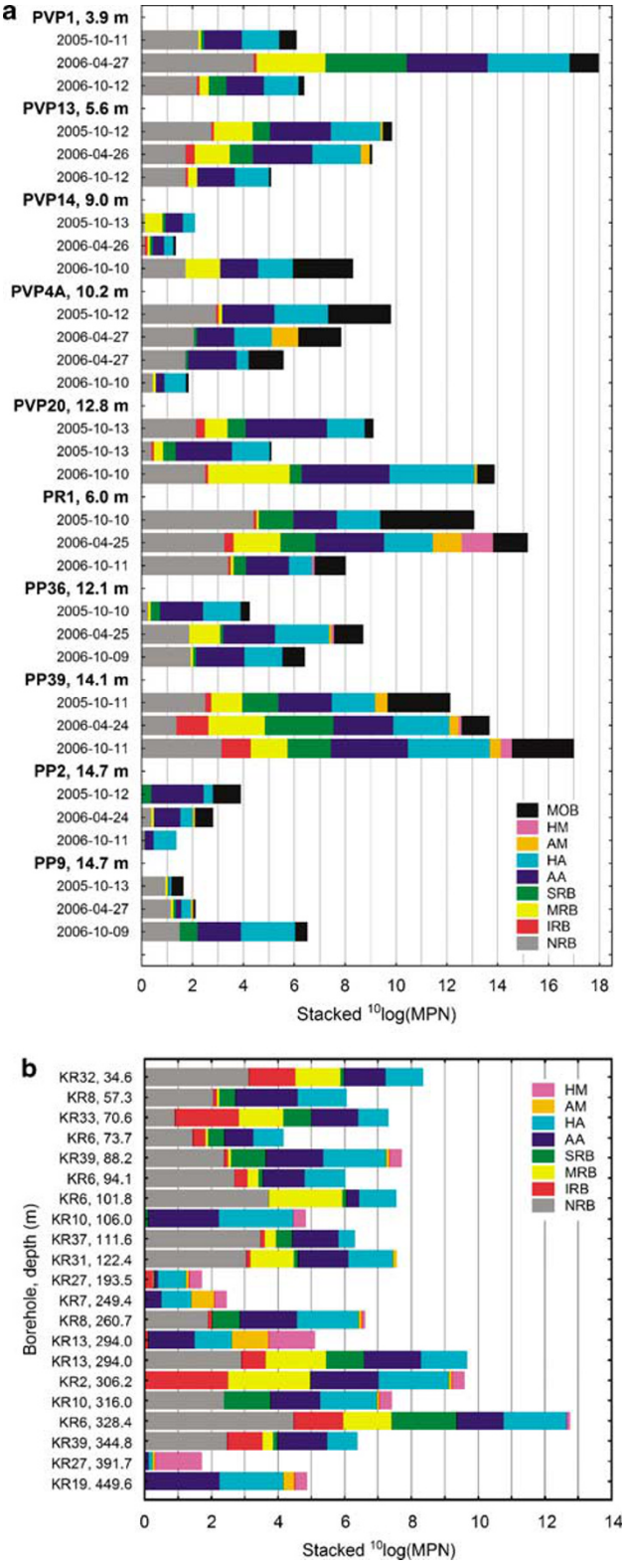

Figure 5 (**a**) Stacked values of MPN of various physiological groups of microorganisms in shallow Olkiluoto groundwater. (**b**) Stacked values of MPN of various physiological groups of microorganisms in deep Olkiluoto groundwater. NRB, nitrate-reducing bacteria; IRB, iron-reducing bacteria; MRB, manganese-reducing bacteria; SRB, sulphate-reducing bacteria; AA, autotrophic acetogens; HA, heterotrophic acetogens; AM, autotrophic methanogens; HM, heterotrophic methanogens; IRB; iron-reducing bacteria; MOB, methane-oxidizing bacteria.

of the stacked MPN bars for deep groundwater sampled earlier. This may partly explain why the stacked bars for boreholes OL-KR2, OL-KR7, OL-KR10, OL-KR19 and OL-KR27 are shorter than the remaining bars, which incorporate NRB data.

The MPN of NRB over depth displayed a range over four orders of magnitude in the groundwater samples. The highest NRB value was found at a depth of 328 m in borehole OL-KR6. The MPN of IRB was low in most samples, with a few values above 10 cells per ml in the shallow groundwater. The deep groundwater samples displayed a peak relative to the other MPN values of IRB, with two IRB values significantly above the detection limit at a depth of approximately 300 m. In the case of MRB, the situation was similar to that of IRB, but with several more values above 10 and 100 cells per ml in shallow- and intermediate-depth groundwater, respectively. As for IRB, three of the MRB values peaked at a depth of approximately 300 m. The MPN of SRB followed the trends of IRB and MRB, with scattered values of up to approximately 1000 cells per ml in shallow groundwater and four values above the detection limit at a depth of approximately 300 m. The MPN results for AA and HA displayed similar patterns. The data were scattered over a range of four orders of magnitude (10^0 up to 10^3 cells per ml) in the shallow groundwater. At a depth of approximately 300 m, there was a peak in the MPN values as was also observed for NRB, IRB, MRB and SRB. There were some detectable AM and HM in shallow groundwater and there were very few detectable methanogens at depth. The MPN analysis of MOB was only performed on shallow groundwater (Figure 5a); this metabolic group of microorganisms was present in all shallow groundwater samples analysed.

The MPN cultivability of the TNC values varied within wide ranges. Shallow groundwater samples displayed a range from 0.007 to 3.85% cultivability of the TNC values. The average cultivability in shallow groundwater was 0.72% ($n = 29$). For deep groundwater, the range was 0.16–30.25% with an average of 8.34% ($n = 14$); this range and average for deep groundwater excludes the six samples without analysis of NRB (this analysis was introduced in 2005).

Data

The complete data set for all analyses, except for the data in Figures 2c and d and for some of the control tests, can be found in the Supplementary Data.

Discussion

Microorganisms are generally more active at the boundaries between systems, where gradients of electron donors, carbon sources and electron acceptors meet, than in homogenous, mixed systems. Such gradients can be found, for example, between shallow organic-rich sediments and the overlying water, or near subseafloor hot springs. The observed underground geological and geochemical conditions at Olkiluoto suggest the presence of two zones that would fulfil the requirements for the occurrence of gradients of electron donors, carbon sources and electron acceptors for microbial activity. The first zone was the investigated shallow depth profile from depths of 3.9–16.2 m in Olkiluoto, which differs significantly from deeper layers in several respects. This zone includes porous geological layers of overburden materials such as organic soil, sand and silt, whereas deeper layers consist of various Precambrian, metamorphic fractured rocks that are intruded by igneous rocks (Andersson *et al.*, 2007a). The shallow zone delineates the oxygenic, photosynthetic surface biosphere from the anaerobic, reduced deep biosphere. Rainwater transports oxygen and organic and inorganic material from the surface biosphere downwards. Reduced gases such as methane and hydrogen from deep geological and biological processes ventilate to the atmosphere, upwards through the shallow zone (Figures 3b and d). Microbial populations can thus be hypothesized to be more numerous, diverse and active in shallow groundwater where oxygen, organic carbon and methane mix than in the underlying, deeper, anaerobic groundwater. The reduction of oxygen during the degradation of organic material and methane is hypothesized to be continuous in shallow groundwater, which would preserve the anaerobic and reduced environment of the future HLRW repository. The results, as presented and discussed here, support this hypothesis.

The second typical boundary zone in Olkiluoto was found at a depth of approximately 300 m, where the concentration of methane with depth increased abruptly up to 100 times and the content of sulphate decreased significantly, approaching nil below 400 m (Figures 2c and 3b). Similar methane and sulphate profiles in anaerobic aquatic sediments, albeit at a much narrower depth scale, have been interpreted as evidence of active anaerobic methane oxidation (ANME), (Zehnder and Brock, 1980; Thomsen *et al.*, 2001). However, the sulphate-rich groundwater is mobile relative to the underlying methane-rich, saline groundwater (Andersson *et al.*, 2007a). An alternative explanation of the observed methane gradient could then be the mixing and dilution of the deep saline groundwater with the overlying groundwater. These alternatives are discussed in detail in relation to the obtained data.

Amount and distribution of biomass

The overall average TNC in shallow Olkiluoto groundwater was almost 10 times the average in deep Olkiluoto groundwater. The TNC range in shallow groundwater was from 8.0×10^3 to 2.5×10^6 cells per ml, whereas in deep Olkiluoto groundwater

the TNC ranged from 2.7×10^3 to 0.15×10^6 cells per ml at a depth of 450 m in groundwater from borehole OL-KR19. This deep-groundwater TNC range compares with a TNC range of 10^3–10^6 cells per ml in deep groundwater from the Fennoscandian Shield, as analysed for almost two decades (Pedersen, 2001). The range of TNC in groundwater has been suggested to be under control of bacteriophages (Kyle *et al.*, 2008). The 10-fold change in TNC averages and the downshift in the TNC range had already occurred in the 16.2–35 m depth interval, suggesting that the influence of the surface biosphere rapidly fades with depth and is becoming absent in this depth interval in Olkiluoto. Accordingly, a deep biosphere signature with respect to TNC is already found in hard rock fractures at a depth of 16 m, just some 5–10 m below the porous overburden of hard rock. However, TNC values do not reveal the activity of the counted cells, so a method for estimating the total amount of viable biomass in groundwater was required. Analysis of the ATP concentrations supplied additional information about the metabolic state and biomass of the bacteria present. The analysed ATP concentrations in Olkiluoto groundwater (Figure 4b) were found to correlate with the TNC counts ($^{10}\log(\mathrm{ATP}) = 0.83 \times {}^{10}\log(\mathrm{TNC}) + 0.45$; $r = 0.78$, $P = 0.0001$, $n = 49$), as previously found for analysed Fennoscandian groundwater samples (Eydal and Pedersen, 2007). The TNC and ATP results thus suggested that bacterial populations deep underground at Olkiluoto were most active in the shallow groundwater and at the 300-m depth (Figure 4).

The distribution of DOC was scattered over the analysed depth range (Figure 2a), with no clear trends over depth. However, when compared with the ATP concentration, a weak, but non-significant, trend became evident, high concentrations of ATP being correlated with high concentrations of DOC in several samples. This suggests a relationship between microbial activity and DOC concentration in Olkiluoto groundwater, which is in line with our understanding of microbial processes. Heterotrophic microorganisms consume DOC and autotrophic ones produce DOC, and they all contain ATP. It is, however, impossible to conclude from concentrations only which of these two processes dominates. Methods such as cultivation (used here), tracer uptake experiments (Pedersen and Ekendahl, 1990, 1992a, b; Ekendahl and Pedersen, 1994) or molecular DNA analysis using real-time polymerase chain reaction (RT-PCR), are needed to identify the dominant microorganisms in the studied populations, and their activity with respect to hetero- versus autotrophic metabolism.

Distribution of cultivable heterotrophic aerobic bacteria and metabolic groups of microorganisms

The expression 'the great plate count anomaly' was coined by Staley and Konopka (1985) to describe the difference in orders of magnitude between the numbers of cells from natural environments that form colonies on agar media (CHAB) and the numbers countable by means of microscopic examination (TNC). In general, only 0.01–0.1% of bacterial cells sampled from various environmental aquatic systems produce colonies when using standard plating techniques. The anaerobic MPN cultivation methods applied here represent the culmination of more than 10 years of development, testing and adaptation for deep groundwater (Kotelnikova and Pedersen, 1998; Haveman *et al.*, 1999; Haveman and Pedersen, 2002a, b; Hallbeck and Pedersen, 2008). The success and usefulness of these methods are reflected in the maximum MPN cultivability of 30.25% of the TNC in the sample from borehole OL-KR6 at a depth of 328 m and the average cultivability of 8.34% of the TNC in deep groundwater. The TNC cultivability in shallow groundwater was about 10 times lower, but still about 10 times above the plate count anomaly range (0.01–0.1%). The use of multiple, liquid anaerobic media has obviously overcome some of the discrepancy found between TNC and cultivations that use agar media only for environmental groundwater samples.

Microorganisms in groundwater must be adapted to anoxic conditions but, if oxygen should appear, it is advantageous for a microbe to be able to switch to oxygen respiration. Indigenous groundwater microorganisms should consequently be detectable as facultative anaerobes, whereas contaminants from the surface should have a smaller tendency to be detectable in this way. There were no correlations between TNC and CHAB data for Olkiluoto groundwater. However, comparing the CHAB data to the NRB data indicated a good correlation ($^{10}\log(\mathrm{NRB}) = 0.81 \times {}^{10}\log(\mathrm{CHAB}) - 0.2$; $r = 0.67$, $P = 0.0001$, $n = 45$), suggesting that the microorganisms analysed as CHAB were generally facultative anaerobes and thus indigenous. Some of the metabolic groups analysed using MPN may overlap in numbers. At the outset of this investigation it was unclear whether AA and HA would differ in numbers. The acetogens are known to be a diverse group of organisms that may switch between different metabolic states (Drake *et al.*, 2002). Comparing the MPN numbers of AA and HA indicates that they correlated well, although there was a clear tendency for AA to outnumber HA in several samples ($^{10}\log(\mathrm{HA}) = 0.71 \times {}^{10}\log(\mathrm{AA}) + 0.29$; $r = 0.78$, $P = 0.0001$, $n = 52$). Similarly, it is known that a single organism can have the abilities to reduce both iron and manganese (Nealson *et al.*, 1988; DiChristina and DeLong, 1993). Comparing the IRB with the MRB numbers indicated that MRB tended to outnumber IRB in several samples. More research will be needed before we have a full understanding of the potential differences between AA and HA, and between IRB and MRB numbers in Olkiluoto groundwater; however, some overlap

between the MPN numbers of these groups can be anticipated.

Three different methods were used to analyse the groundwater samples: TNC returns numbers, ATP returns a measure of biomass and cultivation returns a measure of diversity and numbers. It has been demonstrated here and elsewhere that TNC and ATP values correlate in Fennoscandian Shield groundwater. It was demonstrated by Eydal and Pedersen (2007) that the amount of ATP in groundwater samples reflects the number of cells, biomass and activity. When the amount of ATP in the samples was scatter plotted versus the stacked MPN values for shallow and deep groundwater (Figure 5) samples, respectively, significant correlations were found (Figure 6). The scatter plot also shows that there was about 10 times more ATP per cultivated microorganism in shallow groundwater than in deep groundwater. This is because the percentage of TNC that could be cultivated was 10 times larger in deep than in shallow groundwater. The shallowest borehole examined in this investigation, the PVP1 (3.9 m) borehole, had significantly more ATP per cultivated cell than any other sample (Figure 6). That borehole was so shallow that insects, protozoa and other small animals were likely to appear in the samples, which of course, due to their large volumes, would raise the ATP values significantly. In conclusion, the outputs of the three independent methods were found to correlate with each other: ATP and TNC results have previously been demonstrated to correlate, but the correlation between ATP and MPN cultivations is demonstrated here for the first time. Adding RT-PCR analysis to groundwater investigations, combined with isolating and characterizing cultivable microorganisms from the highest dilutions of the MPN tubes, will reveal specific details regarding the diversity and activity of the studied populations.

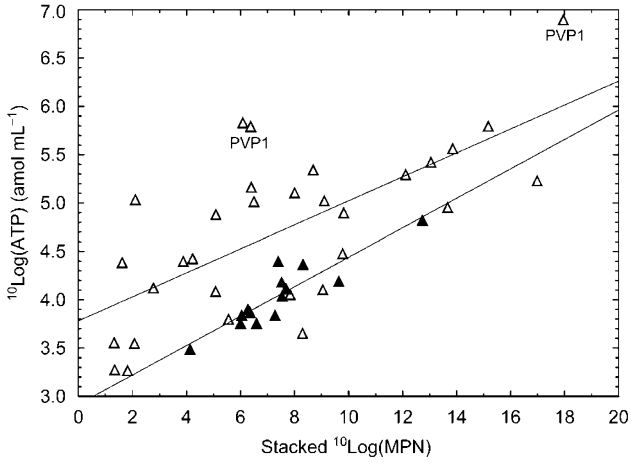

Figure 6 The relationship between stacked values of most probable numbers (MPN) of various physiological groups of microorganisms (Figure 5) in shallow (open symbols) and deep (filled symbols) Olkiluoto groundwater and ATP concentration. The deep groundwater correlation only includes data sets that include analysis of nitrate-reducing bacteria. The least square regression lines for ATP versus stacked MPN are shown for shallow ($^{10}\log(\mathrm{ATP}) = 0.12 \times {}^{10}\log(\mathrm{MPN}) + 3.78$; $r = 0.69$, $P = 0.00002$, $n = 31$) and deep ($^{10}\log(\mathrm{ATP}) = 0.15 \times {}^{10}\log(\mathrm{MPN}) + 2.92$; $r = 0.90$, $P = 0.00001$, $n = 14$) groundwater samples. PVP1 denotes data for the shallowest borehole (3.9 m).

The borehole-to-borehole variability of TNC, ATP and MPN data for shallow groundwater was great (Figure 5a). Still, several of the boreholes displayed good reproducibility between sampling occasions distributed over 1 year; PP2, PP2, PP39 and PVP13 are good examples of such boreholes (Figure 5a). Consequently, there were borehole-specific conditions that appear to have controlled the abundance and diversity of the microbial populations. It is not clear from the data what these controlling conditions were, except for a notable trend for highly stacked MPN values to correlate with high DOC concentrations. This is in line with previously reported observations of a correlation between the content of total organic carbon and TNC (Pedersen and Ekendahl, 1990). The variability in TNC, ATP and MPN data between deep boreholes was less pronounced. The stacked MPN values remained fairly constant at approximately 6 over the first 250 m of depth. At a depth of approximately 300 m, there was an increase in these values (Figure 5b) that coincided with an increase in the ATP concentrations (Figure 4b). The comparison between analyses presented in Figure 5b is somewhat impaired by the lack of NRB analyses for six of the deep boreholes. Taking both shallow and deep groundwater samples into consideration, it becomes clear that there were two zones with elevated TNC, ATP and MPN values; the first such zone was found in the 4–16 m depth interval, the second at a depth of approximately 300 m. The peaks in numbers, biomass and diversity may have very different explanations for each of these two zones, as discussed next.

Microbial oxygen-reduction processes in shallow groundwater

The average shallow groundwater temperature was 7.1 °C in spring and 9.1 °C in fall. The difference in temperature between seasons was most pronounced at depths of less than 10 m, where the water temperatures in shallow boreholes such as PVP1 and PR1 differed by up to 6 °C. Seasonal temperature effects on the microbial process rate could thus be significant in the shallow groundwater, but were absent at depth. The pH range was 4.7–7.7 in the shallow groundwater, and stabilized above 7 at depth (Figure 1a). The effect of the different pH values on microbial processes will be indirect, as pH influences many geochemical parameters, such as mineral dissolution and precipitation, carbon dioxide solubility and various solid–aqueous phase equilibria. Microbial processes produce carbon dioxide from the respiration of DOC, which tends to boost the acidity and lower the pH. Less carbon dioxide will precipitate as solids (for example,

calcite) at low pH in dilute shallow groundwater than in deep groundwater where the pH is buffered at 7 or higher by rock minerals and the dissolved solids concentrations are higher. This is reflected in the carbon dioxide concentrations, which were much higher in shallow than in deep groundwater (Figure 3c). The input of biodegradable organic carbon from surface plant and animal ecosystems can be safely assumed to be higher in shallow than in deep groundwater; thus, the production rate of carbon dioxide by microorganisms will be higher in shallow than in deep groundwater. This was also obvious from gas results, where carbon dioxide represented 20–50% of the total shallow gas content (not shown).

The shallow groundwater of Olkiluoto is clearly in close contact with plant and animal life on the surface. There is an input of rainwater to the ground that will transport dissolved organic material from degradation processes in the surface soils into shallow groundwater. Oxygen from the air will dissolve in the recharging rainwater and follow it into the ground. Life processes in the topsoil and deeper in the overburden will actively degrade particulate and dissolved organic material, which will reduce the oxygen. This is a continuous biological process with a clear seasonal variation: freezing conditions in winter will slow down the processes significantly, and the recharging will stop when the ground freezes. The shallow groundwater environment can consequently alternate between aerobic and anaerobic conditions, which most microorganisms are able to handle. Analysis of dissolved oxygen over a year demonstrated this to be the case for all but the shallowest borehole, PVP1 (3.9 m), which remained aerobic over the summer (Figure 1d).

The shallow groundwater investigations have documented the presence of MOB that oxidize methane with oxygen (Figure 5a). Active MOB populations are expected to reduce the oxygen concentration. Inspecting the relationship between the MPN of MOB and the concentration of dissolved oxygen in shallow groundwater revealed a weak relationship. The MOB population was higher in groundwater with low concentrations of oxygen than in groundwater with high oxygen concentrations. Six samples contained no detectable oxygen and had a range of different MOB numbers (that is, 1–250 cells per ml). Comparing MOB with the amount of dissolved methane revealed a scattered pattern with no clear trend (not shown). The interpretation of these observations is complex, because they represent snapshots of ongoing processes. It is clear, however, that low oxygen concentrations coincided with high numbers of MOB in several samples. This suggests that aerobic methane oxidation is a significant process in removing oxygen from intruding oxygenated recharge water, in addition to the oxidation of organic carbon. The safety calculations for a future HLRW repository in Olkiluoto may, consequently, take advantage of two different microbial oxygen-reducing processes in the shallow groundwater zone: organic carbon and methane oxidation. These processes will be continuous, at least for periods between glaciations, with the highest activity during summer.

Microbial sulphate-reducing processes in deep groundwater

The production of sulphide by SRB in Olkiluoto groundwater is important, because sulphide has the potential to corrode the copper canisters used to store spent nuclear fuel in an HLRW repository. The production of acetate by AA may also be important, because acetate can be utilized by many species of SRB, thereby contributing to the amount of produced sulphide. The safety analysis of a future repository requires detailed information regarding how much sulphide can be formed under various conditions in the deep aquifers surrounding a repository and in the near field of such a repository, as outlined by Hallbeck and Pedersen (2008).

Inspecting the concentration profiles of sulphide (Figures 2b and d) reveals a clear peak in sulphide concentration at several sampling points at a depth of approximately 300 m. These aquifers in Olkiluoto can be hypothesized to harbour very active sulphate-reducing microbial populations. At the same time, the concentration of dissolved methane increased markedly by approximately 100 times in analysed groundwater from depths of approximately 300 m compared to depths above 250 m. There are two possible hypotheses explaining this sharp shift. Hypothesis 1 (H1): if there were a flow of groundwater containing low concentrations of methane in the hydrogeological zone HZ20, which has been identified and defined at a depth of approximately 300 m (Andersson *et al.*, 2007b), it would replace high-methane-concentration groundwater and a rapid drop in the concentrations would result, just as was observed. Hypothesis 2 (H2): a methane-consuming process occurs at a depth of approximately 300 m. Stable isotope analysis suggests that most methane in the area is of deep thermocatalytic origin (Sherwood Lollar *et al.*, 1993; Haveman *et al.*, 1999). Such methane is accompanied by other gases on its transit towards the surface from their respective origins at great depths in the crust. Helium is an inert gas and thus cannot be consumed or precipitated in any way. A dilution effect arising from flowing groundwater, according to H1, should result in the equal dilution of both helium and methane, the ratio of which should not change. If H1 is valid, then the methane/helium ratio should be approximately the same over most of the analysed depth range. Helium diffuses to the atmosphere somewhat faster than methane does, so a slight increase in the ratio can be assumed under H1. Inspecting this ratio over depth reveals that the ratio decreases distinctly

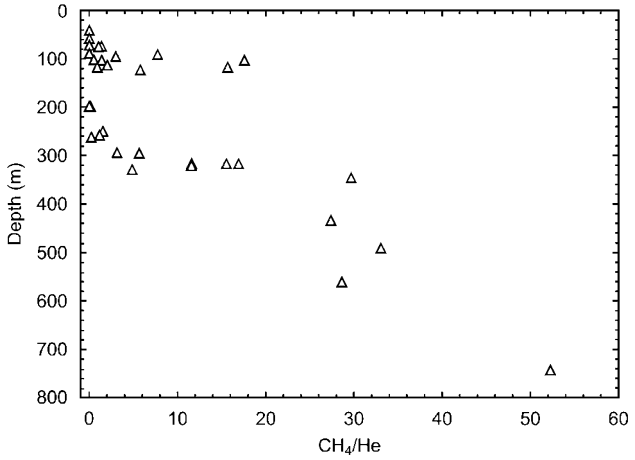

Figure 7 The methane/helium ratios for Olkiluoto groundwater gas samples.

by approximately 10-fold from a depth of 350 up to 200 m (Figure 7). It seems clear that H2 is valid for methane. However, the methane/helium ratio can also drop if the concentration of helium should increase for some unexpected reason. Plotting nitrogen (like helium, an inert gas) against helium results in a ratio that increases significantly at depths of less than 300 m. This indicates that helium is decreasing in concentration relative to nitrogen; it could also indicate that the nitrogen concentration is increasing, except that such an increase was not observed at depths of less than 300 m. If helium is decreasing in concentration due to its more rapid diffusion into the atmosphere, then the methane/helium ratio actually underestimates the methane consumption.

Scientists have long observed profiles of methane, sulphate, sulphide and carbon dioxide in anaerobic aquatic sediments that strongly suggested the presence of active ANME (Zehnder and Brock, 1980; Thomsen *et al.*, 2001). It was not until recently, however, that the ANME microorganisms underlying these profiles were identified (Boetius *et al.*, 2000). It is obvious that strong methane and sulphate gradients meet in several locations at a depth of 300 m in Olkiluoto (Figures 2c and 3b). Furthermore, it is obvious from determinations of ATP levels (Figure 4b) and from the MPNs of various physiological groups of bacteria (Figure 5b) that both microbial abundance and activity peak at these sample locations. Finally, sulphide concentrations are also very high at the same locations (Figure 2d). Of the sites evaluated and discussed here, OL-KR6 (328 m), OL-KR10 (316 m), and OL-KR13 (294 m) have the greatest potential for pronounced anaerobic methane oxidation; these three locations had high concentrations of ATP (Figure 4b) and DOC (Figure 2a) and high MPNs of NRB, SRB, AA and HA (Figure 5b) relative to those of other deep groundwater samples. The last piece of evidence needed for the conclusion that an ANME process is active in Olkiluoto is proof of the presence of ANME microorganisms in groundwater at these locations. Ongoing investigations are focusing on this task, using enrichment and cultivation approaches and RT-PCR methods using available genetic information (for example, Lösekann *et al.*, 2007). As the HLRW repository in Olkiluoto is planned to be situated at a depth of 500 m in Olkiluoto, it is likely to be safe from pronounced sulphide production via ANME processes. Future research, however, is needed to understand the depth stability of conditions that make the ANME process possible. One obvious scenario to be investigated is the influence of a glaciation period on the stability of the methane and sulphate gradients with respect to depth.

Acknowledgements

The first expedition to Olkiluoto, Finland, in April 2004 was a pioneering adventure involving a large group of PhD students, post-doctoral fellows and laboratory personnel. We learnt a lot about how investigations of shallow groundwater can be successfully performed. We are very happy to have done field work in Olkiluoto in April 2004 with the following people: Ernest Chi Fru, Hallgerd Eydal, Annika Kalmus and Sara Wikstrand from Göteborg University and Chris Kennedy and Rachel James from the University of Toronto, Canada. Marcus Olofsson contributed to the analytical work when we brought samples back to the laboratory in Göteborg. The work of these people ensured that the ensuing three expeditions went smoothly and produced an extensive amount of high-quality microbiology data. During our field work sampling periods in Olkiluoto, we were treated very well, receiving experienced, professional and efficient support from the following people: Anne Lehtinen, Tero Jussila, Kari Kovanen and Janne Laihonen. Mia Ylä-Mella played an important part in initiating and planning this work. Finally, behind-the-scenes personnel at the Teollisuuden Voima Oy (TVO) and other analytical laboratories capably supported us by performing high-quality chemical analyses. Proper English AB is acknowledged for excellent language and editing control. This work was supported by Posiva OY and the Swedish Research Council.

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