Subterranean microbial populations metabolize hydrogen and acetate under in situ conditions in granitic groundwater at 450 m depth in the Åspö Hard Rock Laboratory, Sweden

Karsten Pedersen

Microbial Analytics Sweden AB, Mölnlycke, Sweden

Abstract

Pressure-resistant circulating systems were constructed to enable the investigation of attached and unattached microbial populations under in situ pressure (2.5 MPa), diversity, dissolved gas and chemistry conditions. Three parallel flow cell cabinets were configured to allow observation of the effect on microbial metabolic activity of adding 3 mM hydrogen or 2.4 mM acetate, compared with an untreated control. Hydrogen addition reduced the generation time fourfold to 2 weeks, doubled the sulphide production rate and increased acetate production by approximately 50%. The acetate addition induced acetate consumption. The studied subterranean microbial processes appeared to proceed very slowly in terms of volume and time, although the results suggest that individual cells could be very active. Lytic bacteriophages are hypothesized to have caused this contradictive observation. Phages may consequently significantly reduce the rates of subterranean microbial processes. Furthermore, the results suggest that hydrogen from corroding underground constructions could induce significant local microbial activity and that the low concentrations of hydrogen often observed in pristine subterranean environments may support slow but sustainable microbial activity in deep groundwater.

Introduction

Plans to dispose of spent nuclear fuel (SNF) wastes in deep underground hard-rock repositories in the Fennoscandian Shield (SKB, 2010) have prompted research into the diversity and activity of subterranean microorganisms (Pedersen, 2001; Amend & Teske, 2005) and their influence on radionuclides (Anderson et al., 2011). Investigations of potential sites for these repositories in Sweden (Hallbeck & Pedersen, 2012) and Finland (Pedersen et al., 2008) have revealed diverse culturable populations in all analysed samples from depths of a few metres down to approximately 1000 m. Oxygen-, nitrate-, iron-, manganese- and sulphate-reducing bacteria (SRB) as well as acetogens and methanogens could be cultured in numbers ranging from a few cells mL<sup>-1</sup> to more than 1 × 10<sup>7</sup> cells mL<sup>-1</sup>. Evaluation of geochemical data pertaining, for example, to E<sub>H</sub>, Mn<sup>2+</sup>, Fe<sup>2+</sup>, S<sup>2-</sup> and δ<sup>34</sup>S, from site investigations has suggested that the cultured microorganisms were actively metabolizing in their deep aquifers of origin (Pedersen et al., 2008; Hallbeck & Pedersen, 2012). This is in line with earlier findings that the incorporation of 14C-labelled CO<sub>2</sub>, formate, acetate, lactate and glucose, and 3H-labelled leucine indicated active autotrophic and heterotrophic metabolisms in the studied populations (Pedersen & Ekedahl, 1992a, b; Ekedahl & Pedersen, 1994).

Prior cultivation and radiotracer results have generally been obtained using depressurized samples withdrawn to the surface with pumps through very long tubing or using sample vessels with limited sample volumes. These sampling procedures changed pressure-related parameters, such as gas solubility, which in turn may have influenced pH and E<sub>H</sub> relative to the in situ conditions in the aquifers. Moreover, data obtained on microbial activity over short times, that is, hours, were difficult to extrapolate to the long-term in situ conditions of the microorganisms. The obvious way to better understand in situ microbial activity was to install experiments underground under in situ pressure conditions when the Åspö Hard Rock Laboratory (HRL) became available for research (Pedersen,
Consequently, pressure-resistant circulating systems were constructed that enabled the investigation of attached and unattached microbial populations under in situ pressure. Diversity, dissolved gas and chemistry conditions in the Åspö HRL tunnel (Hallbeck & Pedersen, 2008). These systems were set up in an underground laboratory container, combining controlled laboratory conditions with the in situ conditions prevailing in an intersected aquifer at a depth of 450 m. The systems were first used to investigate sorption of radionuclides onto biofilms on glass and rock surfaces (Anderson et al., 2006, 2007), after which nitrate reduction activity, using lactate as a carbon source, was studied (Nielsen et al., 2006). The obtained results were of high quality, and the laboratory with its circulation systems operated as planned. The experience gained from these studies was used in developing the research presented here.

Investigation of Åspö HRL aquifers has demonstrated the presence of active methanogenic and acetogenic (Kotelnikova & Pedersen, 1998), and a novel methanogenic species, Methanobacterium subterraneum, was isolated and described (Kotelnikova et al., 1998). Similarly, diverse and abundant populations of SRB have been found (Eydal et al., 2009), and a novel species, Desulfovibrio aespoeensis, was isolated and described (Motamedi & Pedersen, 1998). The hypothesis of a deep hydrogen-driven biosphere has gained support from several independent investigations around the world (Stevens & McKinley, 2005; Eydal et al., 2008). A lytic phage specific to D. aespoeensis was subsequently isolated and described (Eydal et al., 2009). The action of lytic phages on microorganisms releases cell components, including short organic acids such as acetate, in a process commonly known as the viral shunt (Suttle, 2007). Given the large number of phages in Åspö HRL groundwater, the viral shunt is likely active there, and acetate released by this shunt could constitute an important carbon source for the microbial populations in Åspö HRL groundwater. One possible source of sustainable energy driving this shunt could be hydrogen from the underground environment (Apps & van de Kamp, 1993).

In the present work, three parallel flow cell cabinets (FCCs) with attached and unattached microorganisms were configured to permit observation of the effect on microbial metabolic activity of adding hydrogen or acetate to one FCC each, compared with an untreated control FCC. The FCCs were inoculated under in situ pressure with circulating groundwater from the selected aquifer for 116 days, after which they were isolated as three independent units with their respective additions. The concentrations of hydrogen, methane, sulphide and acetate were subsequently recorded under batch conditions for 91 days. Total numbers of microorganisms and the numbers of culturable SRB, acetogens and methanogens were also monitored over this period. The 16S rRNA gene diversity of the attached microorganisms in the FCCs has been presented elsewhere (Jägevall et al., 2011) and that information was compared with the observed microbial activities as a function of treatment. The results for the two treatment FCCs and the control FCC were compared and metabolic rates were calculated.

### Materials and methods

#### Site description and characterization

The Åspö HRL is located approximately 20 km north of Oskarshamn on the east coast of southern Sweden (N. 57° 26′ 4″, E. 16° 39′ 36″). An underground, air-conditioned laboratory container (7.5 × 2.5 × 2.5 m) was installed at a tunnel site denoted MICROBE, located 450 m below sea level in a niche denoted ‘J’. The MICROBE site has three boreholes, each intersecting a hydraulically conductive aquifer behind the tunnel rock face; these boreholes were denoted KJ0050F01, KJ0052F01 and KJ0052F03. The drilling procedure, instrumentation and groundwater chemistry at the time these boreholes were drilled have previously been outlined by Pedersen (2000b). In this study, a metal-free packer system isolated an aquifer in borehole KJ0052F01 located 43.7–43.9 m from the tunnel rock face; the groundwater was directed by this system to the FCCs and then back to the aquifer via 1/8 inch polyetheretherketone (PEEK) thermoplastic tubing of high-pressure liquid chromatography quality (IDEX Health and Science, Oak Harbor, WA).

#### FCCs

Three FCCs were installed in the circulating system running to and from the aquifer in KJ0052F01. Each cabinet
was temperature controlled (range: 16–18 °C) and had four flow cells installed in series through which groundwater from the intersected aquifer was pumped before returning to the aquifer. This ensured that the groundwater chemistry remained unaltered and that the in situ temperature of 16 °C and pressure of 2.5 MPa were maintained. The flow cells had a stainless steel shell (length 300 mm, diameter 65 mm) and were lined with polyvinylidifluoride (PVDF) plastic. A 120-mm-long PVDF insert supported 20 glass slides (exposed dimensions, 60 × 22 × 0.8 mm) in 10 layers separated by 2-mm flow spaces offering a total of 2112 cm² per FCC for microbial adhesion and biofilm development. All glass slides used in the flow cells were heated for 4 h at 475 °C in a muffle furnace to ensure that the glass surfaces were hydrophilic and free of organic material before insertion into the flow cells. Three flow stabilizers at each end of the insert ensured even distribution and the slow laminar flow of water through each flow cell (Pedersen, 1982). The circulation systems, that is, the packers isolating the aquifer in the borehole, PEEK tubing, valves and flow cells, were all made of metal-free materials to exclude the possibility of hydrogen production from anaerobic corrosion of metals during the experiments. The flow rate to and from the aquifer was kept at 32 mL min⁻¹, corresponding to a flow of approximately 1 mm s⁻¹ over the surfaces. Groundwater flowed at a rate of 1.26 mL min⁻¹ through the intersected aquifer in the KJ0052F01 borehole, as determined by means of saturation measurements (Pedersen, 2005). Consequently, there was a continuous supply of groundwater constituents, including microorganisms, phages and dissolved gases, to the flow cells. The basic requirements for attachment and growth of microorganisms in the flow cells were thus fulfilled. A detailed description of the FCC functions can be found elsewhere (Pedersen, 2005; Hallbeck & Pedersen, 2008).

Gas sampling and analysis

Water samples were collected in evacuated, nitrogen-flushed 120-mL glass bottles equipped with butyl rubber stoppers (no. 2048-117800; Belco Glass, Vineland, NJ) and sealed with aluminium crimp seals (no. 2048-11020; Belco Glass). The pressure in the bottles was set to 1 Pa before sampling. Water from the circulations was led via PEEK 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 (i.e. water vapour pressure) at room temperature (RT); the transfer time was approximately 20–30 min. After extraction, the gas was compressed and transferred to a 10-mL syringe (SGE Analytical Science, Melbourne, Vic., 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 under high vacuum (1 Pa). A dehydrant (cobalt chloride) was added to adsorb any traces of water remaining in the gas. Analysis was thereafter performed using gas chromatography.

Two different chromatographs were used and equipped as follows. Hydrogen (< 20 µL L⁻¹) was analysed on a KAPPA-5/E-002 analytical gas chromatograph (AMETEK/Trace Analytical, formerly Trace Analytical, Menlo Park, CA) 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 reductive gas detector, and nitrogen was used as a carrier gas. Helium and hydrogen (> 20 µL L⁻¹) were analysed on a Varian Star 3400CX gas chromatograph (Varian Analytical Instruments, Varian AB, Bromma, Sweden) using a thermal conductivity detector with oven, detector and filament temperatures of 65, 120 and 250 °C, respectively. 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 as the carrier gas. Methane was 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 gas was separated using a Porapak-Q column (2 m × 1/8 inch diameter) and analysed on the FID with nitrogen as the carrier gas.

**Total number of cells (TNC)**

The TNC mL⁻¹ was determined in 10-mL samples using the acridine orange direct count (AODC) method as devised by Hobbie et al. (1977) and modified by Pedersen & Ekendahl (1990). Glass slides from the flow cells were stained according to the AODC method, modified as described by Anderson et al. (2006). A minimum of 20 positions were counted on each side of a sampled glass.

**ATP analysis**

The ATP Biomass Kit HS (no. 266-311; BioThema, Händen, Stockholm, Sweden) was used to determine total ATP in living cells. The ATP biomass method used here has been described, tested in detail and evaluated for use with Fennoscandian Shield groundwater (Eydal & Pedersen, 2007).
Acetate and sulphide analysis

Acetate concentrations were determined using an enzymatic UV method (Kit # 10148261035; Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany), utilizing a Genesys 10UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA) for detection. Samples were diluted 1–100 times before analyses to obtain the optimal analytical concentration range. Sulphide was analysed using a colorimetric methylene blue method (Swedish standard method SIS 028115; the uncertainty of the analysis was ±17%).

Preparation of media, inoculations and analysis for most probable number (MPN) of culturable anaerobic microorganisms

The procedures described by Widdel & Bak (1992) for preparing anoxic media were modified and used to determine the MPN of microorganisms, as described elsewhere (Hallbeck & Pedersen, 2008). Five tubes were used for each 10 times dilution, resulting in an approximate 95% confidence interval lower limit of one-third of the obtained value and an upper limit of three times the value (Greenberg et al., 1992). Media were prepared for SRB, autotrophic acetogens (AA), autotrophic methanogens (AM) and heterotrophic methanogens (HM). The cultivation time was about 8 weeks to ensure that slow-growing microorganisms were included in the results. The specific media compositions were based on previously measured chemical data from KJ0052F01. The sodium chloride concentration was adjusted to obtain a medium salinity corresponding to the 15%o salinity of the sampled borehole water. This allowed the formulation of artificial media that closely mimicked in situ groundwater chemistry for optimal microbial culturing, as was previously found to be successful (Haveman et al., 1999; Hallbeck & Pedersen, 2012).

Configuration of growth experiments

Three FCCs, each equipped with four flow cells, were connected to the KJ0052F01 aquifer, and groundwater was pumped at a flow rate of 32 mL min⁻¹ through the FCC and back to the aquifer for a total of 116 days, from 26 October 2005 to 23 March 2006. The total volumes of groundwater circulated over 116 days were 6530, 6314 and 6485 dm³ in the control, acetate-treated and hydrogen-treated FCCs, respectively, as registered by flow meters. These volumes corresponded perfectly to the flow of 30 mL min⁻¹ set at the start of the CM experiments.

Sampling procedures

Sampling was performed seven times at about 2-week intervals, except for the last sampling interval of 34 days. Each sampling occasion required 2 days. On day 1, 20 mL of circulating water was drained and disposed of; thereafter, 100 mL of water was sampled for gas analysis as described previously. Then 25 mL of water was collected in a sterile 50-mL polypropylene tube (Sarstedt, Landskrona, Sweden) and immediately frozen at −20 °C until analysis of acetate. Thereafter, one glass slide was removed from each flow cabinet and stored in 40 mL of sterile filtered (0.2 µm) circulation water, preserved with a final concentration of 2.5% neutralized formaldehyde, for determination of TNC cm⁻². Three glasses were sampled on the first sampling occasion. The removed glasses were replaced with new sterile glasses. Finally, on day 1, a pressure vessel (PVB) was installed in line with the circulation flow, filled with 170 mL of groundwater and left overnight. On day 2, the PVB was detached and transported, at RT and with the in situ pressure maintained, to the laboratory where it was subsampled on the same day for analysis of TNC, ATP and MPN of SRB, AA, AM on 22 March 2006, resulting in a total volume of 5000 mL per FCC. Thereafter, each FCC circulation was disconnected from the borehole, and internal circulation was started under in situ pressure of approximately 2.5 MPa. This configuration was denoted the ‘closed batch mode’ (CM). Immediately after the switch from OM to CM, acetate and hydrogen were added as follows. A 1.21 M solution of sodium acetate in analytical-grade water was prepared and 10 mL was subsequently added to one FCC, resulting in a final concentration of 2.4 mM acetate; this experiment was denoted ‘acetate’. A 500-mL Teflon-lined, stainless steel cylinder was filled at RT (20 °C) with a gas mixture of 80% hydrogen and 20% carbon dioxide to a pressure of 100 KPa and connected in line with the FCC denoted ‘hydrogen’. The cylinder was filled with groundwater under in situ pressure (2.5 MPa) for a total circulating volume of 5500 mL for the hydrogen-treated FCC. This gas addition then corresponded to the final theoretical concentrations of 3 mM hydrogen and 0.75 mM carbon dioxide. The third FCC was left untreated and was denoted ‘control’. The start date for the CM configuration was 22 March 2006 (day 0) and the end date was 21 June 2006, resulting in a total CM experiment time of 91 days. The total volumes of water circulated over 91 days were 3819, 3906 and 3861 dm³ in the control, acetate-treated and hydrogen-treated FCCs, respectively, as registered by flow meters. These volumes corresponded perfectly to the flow of 30 mL min⁻¹ set at the start of the CM experiments.
Effect of hydrogen and acetate on subterranean microorganisms

and HM. The sampler and the detailed sampling and analysis procedures were described by Hallbeck & Pedersen (2008). The last sample taken on day 2 was for sulphide analysis. First, 20 mL of circulating water was drained and disposed of, after which a 100-mL glass bottle with a glass stopper was filled under an atmosphere of nitrogen. The sample was immediately preserved with sodium hydroxide and zinc acetate and sent for analysis (Swedish standard method SIS 028115). The volume of water circulating in the FCCs was reduced by the total sample volume of 475 mL. The final circulating volume before the last sampling occasion was 2400 mL for the control and acetate-treated FCCs and 2900 mL for the hydrogen-treated FCC. The in situ pressure was maintained by an expansion vessel that was in water contact with the aquifer pressure (Hallbeck & Pedersen, 2008).

16S rDNA gene diversity of the biofilm populations

The 16S rDNA diversity of the biofilms on the glass slides has previously been analysed and described (see table 4 in Jägevall et al., 2011). The total number of clones analysed was only 76, so the results should be treated as indicative only. SRB constituted 41% of a total of 76 sequenced clones, and the observed genera were distributed differently among the two treatments and control. The most common clone was Desulfovibrio aespoeenis (Motamedi & Pedersen, 1998) like (99%) and constituted 17% of the sequenced 76 clones. The D. aestoeenis-like clones were found in the biofilms of all three FCCs, and Desulfoferrophilus-like (97%) clones were detected in the acetate- and hydrogen-treated FCCs, but not in the control FCC. Desulfobacterium cattellicum-like and Desulfuribaculum sp-like (96–98%) clones were found in the control and acetate-treated FCCs, but not in the hydrogen-treated FCC. Overall, 24 unique clones were detected. Brevundimonas mediterranea-like clones were the most abundant (12%) of the clones not similar to SRB and were observed only in the hydrogen-treated FCC.

Statistical analyses

Data graphics design and statistical analyses were performed in STATISTICA 10 (Statsoft Inc., Tulsa, OK).

Results

Chemistry

Detailed chemical characterization of the studied groundwater has been published elsewhere (Pedersen, 2000b, 2005). At the start of the CM experiments, the groundwater had a pH of 7.31, a temperature of 16 °C and conductivity of 2470 mS m⁻¹. The major dissolved solids were 0.27 M Cl⁻, 0.11 M Na⁺, 0.08 M Ca²⁺ and 6 mM SO₄²⁻. The following were also analysed: PO₄³⁻ and NO₃ at < 0.01 µM, 2.7 µM NH₄⁺, 7 µM HSO₄⁻, 164 µM HCO₃⁻, 6.9 µM CO₂, 14.8 µM CH₄ and 1.67 µM H₂. The pH was analysed at the end of the experiments and was found to be 6.28, 6.61 and 5.45 in the control, acetate-treated and hydrogen-treated FCCs, respectively.

TNC and ATP

At the start, the TNC and ATP were 1.7 × 10⁵ cells mL⁻¹ and 4.28 × 10⁴ amol mL⁻¹, respectively, in the circulating water (Fig. 1a and b). The TNC increased to at most 6.3 × 10⁵ cells mL⁻¹ in the hydrogen-treated FCC after 30 days, after which it decreased to 3 × 10⁴ cells mL⁻¹. Similarly, the TNC increased in the acetate-treated FCC over the first 30 days, after which it decreased. The slowest increase in TNC was observed in the control FCC, but the TNC reached the same level, approximately 3 × 10⁵ cells mL⁻¹, in all three FCCs after 91 days, at the end of the experiment. The ATP data mimicked the TNC data; most ATP being found in the hydrogen-treated FCC followed by the acetate-treated and control FCCs. The glass surfaces were colonized by approximately 4 × 10⁵ cells cm⁻² at the start of the CM experiments after 116 days of OM circulation (Fig. 1c). The TNC on the glass surfaces increased linearly, approximately doubling over the 91 days of observation. The acetate-treated FCC surfaces harboured a somewhat lower TNC than did the other two FCC surfaces in the last 40 days.

Sulphide and sulphate-reducing bacteria

The concentration of sulphide was 7 µM at the start of the experiments. The sulphide concentration increased faster in the hydrogen-treated FCC than in the acetate-treated and control FCCs, which displayed similar increases (Fig. 2a). However, sulphide concentration reached approximately 0.22 mM in all three FCCs by the end of the experiment. Likewise, the MPN of SRB increased mostly in the hydrogen-treated FCC, reaching numbers above the detection limit, that is, > 1.6 × 10⁵ SRB mL⁻¹, on days 8 and 15 (Fig. 2b). Because of these over-range results, the number of dilutions was increased starting on day 29. After day 15, the MPN of SRB in the hydrogen-treated FCC started to decrease, and the final MPN value was at least 10 times lower than the maximum values observed on days 8 and 15. The starting MPN of SRB, that is, 0.3 × 10⁵ SRB mL⁻¹, decreased after 8 days in the acetate-treated and control FCCs, after

FEMS Microbiol Ecol 81 (2012) 217–229

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsor/article-abstract/81/1/217/567267 by guest on 26 July 2018
which the MPN of SRB in the acetate-treated FCC increased to $1.3 \times 10^5$ SRB mL$^{-1}$ by day 29. The MPN of SRB in the control FCC also increased after day 8, reaching $2.2 \times 10^5$ SRB mL$^{-1}$ on day 44. The MPN of SRB reached approximately $0.3 \times 10^5$ SRB mL$^{-1}$ in all three FCCs by the end of the experiments.

**Acetate and acetogens**

The groundwater acetate level at the start of the CM experiments was approximately 0.05 mM. The acetate concentration in the hydrogen-treated FCC increased linearly to 0.66 mM by the end of the experiment (Fig. 3a). The acetate-treated FCC started with approximately 2.3 mM acetate, and the concentration increased to 2.45 mM by day 29, after which it decreased back to approximately 2.3 mM by the end of the experiment. The acetate concentration in the control FCC increased to 0.25 mM by day 29, after which it levelled out and remained stable until the end of the experiment. The MPN of AA increased significantly in the hydrogen-treated FCC, exponentially after day 8, from a starting value of $1.7 \times 10^3$ AA mL$^{-1}$ to $3.5 \times 10^4$ AA (Fig. 3b) by day 90. The acetate-treated and control FCCs had MPNs of approximately $6 \times 10^3$ AA mL$^{-1}$ without any clear trend.
over time, with a variation of approximately 50% (i.e. within the 95% confidence interval limits of the applied MPN method). Both the acetate-treated and control FCCs had MPNs of AA at experiment end that were near the starting value of $1.7 \times 10^3$ AA mL$^{-1}$.

**Methanogens**

The MPNs of both AM and HM were stabilized at approximately $1 \times 10^2$ AM/HM mL$^{-1}$ after day 29 in the acetate-treated and control FCCs (Fig. 4a and b). In contrast, the MPN of AM and HM decreased to just a few AM/HM mL$^{-1}$ after day 8 in the hydrogen-treated FCC.

**Dissolved gases**

The analysis of gas went well on all but the last sampling occasion when the gas samples were unfortunately lost because of technical problems. The hydrogen concentration in the hydrogen-treated FCC increased slightly over the first 8 days, possibly owing to the slow dissolution rate of the added hydrogen (Fig. 5a). Thereafter, the hydrogen concentration decreased exponentially, probably due to the diffusion of hydrogen through the PEEK tube walls of the FCC and, to some extent, to microbial consumption. Extrapolation of this line suggests that the hydrogen concentration in the hydrogen-treated FCC would have decreased to $1–10$ $\mu$M by the end of the experiment. The hydrogen concentration increased in the acetate-treated FCC for the first 15 days; thereafter, the amount of hydrogen in both the acetate-treated and control FCCs decreased to $<1$ $\mu$M. The concentration of methane was approximately 10 $\mu$M at the start of the CM experiments (Fig. 5b); this concentration approximately doubled by day 8, after which there was no significant change up to the last successful analysis (day 57). The helium concentration decreased exponentially, just as hydrogen did, but more quickly (Fig. 5c), leaving no detectable helium in the groundwater after approximately 30 days.
Cell numbers, growth and metabolic rates

When the FCCs were switched to CM after 116 days in OM, microorganisms had attached themselves to the glass slides in the flow cells, and there were microorganisms in the 5000 mL of groundwater in the control and acetate-treated FCCs and in the 5500 mL in the hydrogen-treated FCC. The numbers of attached and unattached microorganisms were similar, and together, their numbers totalled $1.52 \times 10^9$ to $1.87 \times 10^9$ (Table 1). Over the first 29 days, most analysed parameters increased steadily. Several parameters then levelled out or started to decrease (Figs 1–5). Day 29 was therefore used to calculate \textit{in situ} rates and amounts assumed to reflect the OM activity and growth situation. The population size doubled in the hydrogen-treated FCC after 29 days and increased in the acetate-treated FCC, but did not change in the control FCC (Table 1). Because 475 mL of water was removed on every sampling occasion, the number of unattached microorganisms decreased correspondingly; this decrease is considered when calculating the population numbers.

The generation times of the unattached populations were calculated using two independent analyses, TNC and ATP. Both methods indicated a very slow observed growth; the doubling time (g) was almost 2 months in the control FCC, but only about 3 weeks in the acetate-treated FCC and 2 weeks in the hydrogen-treated FCC. Consequently, the two different treatments did influence microbial activity expressed as generation time. The biofilm populations displayed very slow growth with doubling times of 2–3 months. The amount of ATP per unattached cell was approximately 0.3 amol, near the average of 0.43 amol per cell found in Fennoscandian groundwater (Eydal & Pedersen, 2007). Finally, the production of acetate and sulphide h$^{-1}$ was calculated (Table 1).

Discussion

Experimental conditions

The FCCs were constructed and installed under the assumption that they would reproduce the \textit{in situ} microbial diversity, pressure, dissolved gas and chemistry conditions present in natural aquifers. It was further assumed that microbial processes similar to those in the aquifer’s groundwater and on its rock surfaces would become established in the FCCs. In the OM, a steady-state situation similar to that of a continuous culture was hypothesized to prevail in the FCCs after some weeks of circulation. The dilution rate was controlled by the flow through the intersected aquifer and was previously determined to be 0.064 h$^{-1}$, corresponding to a groundwater half-life in each FCC of approximately 11 h (Pedersen, 2005). However, unlike a classical chemostat in which the feeding nutrient solution is sterile, the feeding groundwater carried microorganisms to the FCCs. When the CM experiments started, metabolites from microbial processes and cell numbers initially started to build up at rates similar to those in the OM, and substrates were likewise
Table 1. Total populations of attached and unattached microorganisms in the three FCCs as analysed on days 0 and 29

| Day   | Treatment | Population size, TNC per FCC | Unattached TNC generation time, g (days) | Attached TNC generation time, g (days) | Unattached ATP generation time, g (days) | ATP, amol per cell | Acetate, µM h⁻¹ | Sulphide, µM h⁻¹ |
|-------|-----------|-----------------------------|-----------------------------------------|----------------------------------------|------------------------------------------|-------------------|----------------|----------------|
| 0     | Control   | 1.87 × 10⁹                  | b.d.                                     | 84.5                                   | 53.7                                     | 0.37              | 0.236          | 0.090          |
|       | Acetate   | 1.52 × 10⁹                  | 2.39 × 10⁹                              | 62.8                                   | 23.7                                     | 0.25              | 0.217          | 0.068          |
|       | Hydrogen  | 1.70 × 10⁹                  | 3.52 × 10⁹                              | 87.9                                   | 13.6                                     | 0.30              | 0.351          | 0.208          |
| 29    |           | 10⁹ b.d.                    | 84.5                                     | 53.7                                   | 0.37                                     | 0.236             | 0.090          |                |

*The growth rates of attached and unattached microorganisms and the acetate and sulphide production were calculated for the first 29 days.

b.d., below detection because of identical TNC values on days 0 and 29.*

consumed. A batch culture, such as the CM configuration, typically displays an exponential growth phase followed by stationary and declination phases. Such a batch growth pattern was indeed observed in the experiments presented here, in which the exponential growth phase lasted approximately 30 days in all three FCCs, as reflected by the TNC and ATP data (Fig. 1a and b). The observed growth rate, presented as generation time in Table 1, was 53 days for the control without additions; this was very slow compared with typical laboratory cultures, which usually exhibit generation times of just a few hours. The studied populations all appeared to be very slow growing, which was not expected in the two FCCs to which ample hydrogen or acetate was added.

The construction of an underground laboratory offered the advantage of access to *in situ* conditions in an aquifer. However, construction of the Åspö HRL may have disturbed the hydrogeochemical conditions, because groundwater leakage into the tunnels could in turn trigger new flow conditions in the aquifers compared with before the excavation. The observed flow of 1.26 mL min⁻¹ in the KJ0052F01 aquifer could have been caused by such a disturbance. The previously observed slowly increasing salinity in the KJ0052F01 groundwater indicated the presence of a particular hydrological process, the ‘up-coning’ of saline groundwater from deeper aquifers into the studied aquifer (Pedersen, 2005). Consequently, there was inflow of groundwater from deeper rather than shallower aquifers into the FCCs during the OM operation. The subterranean character of the microbial population in the KJ0052F01 aquifer must have been substantial, judging from the observed up-coning flow situation.

PEEK tubing and PVDF flow cells were used to prevent hydrogen production because of anaerobic corrosion. It became clear from the helium data that gas did diffuse through the PEEK tubing (Fig. 5c), because that was the only process by which the concentration of this noble and inert gas could have decreased. A similar process was noted for the hydrogen in the hydrogen-treated FCC (Fig. 5b), but because the starting concentration of hydrogen was high, a significant hydrogen concentration was maintained throughout most of the CM experimental time. The methane concentrations did not decrease (Fig. 5c), probably due to a smaller diffusion coefficient for methane through PEEK than for helium or hydrogen. Methanogenesis was unlikely to have compensated for the diffusion loss of methane because the MPN of methanogens was very low in the hydrogen-treated FCC compared with the control and acetate-treated FCCs, although the concentrations of methane were similar in all three FCCs. Otherwise, the FCCs performed very well, and the obtained data display trends in which the treatment data differ significantly from the control data. In other words, the unavoidable influence of the applied experimental conditions, such as the choice of FCC material, must have been similar in all three FCCs, and the observed and varied responses in microbial activity from the hydrogen and acetate treatments compared with the control were significant and valid.

**Growth-controlling variables**

The values for the TNC, ATP and MPN of SRB all increased over the first 15–30 days, after which they levelled out or decreased, irrespective of whether large amounts of energy were available in the form of hydrogen or acetate compared with the control FCC. The onset of decreasing values was not likely due to energy or carbon limitations, at least not in the hydrogen- and acetate-amended FCCs. The growth curves of SRB as analysed in terms of MPN were very similar to the curves obtained in phage-infected pure cultures of *D. aestuenden* (Eyda et al., 2009). Phage infections become more virulent at large cell numbers, and all three FCCs experienced a similar assumed phage lysis effect when the MPN of SRB reached approximately 1.5 × 10⁹ SRB mL⁻¹. First, the hydrogen-treated FCC, then the acetate-treated FCC and finally the control FCC experienced a reduced MPN of SRB.
cannot be excluded that these FCC systems were under phage control that kept the cell numbers at or below values at which the phage infection became very virulent. TNC and ATP data indicated similar growth and declination patterns, with the highest TNC value of $6.3 \times 10^5$ cells mL$^{-1}$ occurring in the hydrogen-treated FCC after 29 days. The TNC decreased in the hydrogen- ($\sim 3.5 \times 10^5$ cells mL$^{-1}$) and acetate-treated ($\sim 1 \times 10^5$ cells mL$^{-1}$) FCCs between 29 and 91 days, which only can be explained by attachment or sedimentation out of the groundwater or by cell lysis. The MPN of SRB displayed a much larger decrease compared with the highest observed numbers (Fig. 2b). The biofilm TNC data could approximately account for a corresponding increase because of the attachment or sedimentation of the TNC lost from the circulating groundwater, assuming that the biofilm TNC did not increase at all because of growth.

Alternatively, attacks of lytic phages could explain the TNC decrease in the hydrogen- and acetate-treated FCCs. The TNC from deep boreholes in Sweden never exceeded $4.7 \times 10^5$ cells mL$^{-1}$ ($n = 30$) (Hallbeck & Pedersen, 2012), and the TNC in deep groundwater in Finland likewise never exceeded $1.5 \times 10^5$ cells mL$^{-1}$ ($n = 48$) (Pedersen et al., 2008). Although their presence and lytic activity in the FCCs remains to be proven, it is at least a reasonably plausible hypothesis that the numbers of microorganisms in the FCCs were under the control of lytic phages.

Biofilm processes

The attached populations constituted about half of the total population, the proportion increasing somewhat with the collection of water for analyses. The total number of attached cells was similar to what has been observed previously on glass surfaces at another subterranean site (Pedersen & Ekendahl, 1992a; Ekendahl & Pedersen, 1994) and in the FCCs at the Åspö HRL site (Anderson et al., 2006; Nielsen et al., 2006). Replacing glass surfaces with granitic rock surfaces did not change the numbers of attached cells (Anderson et al., 2006). Typically, the attached microorganisms increasingly appeared in colonies over time (Ekendahl et al., 1994; Anderson et al., 2006), suggesting that growth, rather than attachment, was responsible for the increase in attached TNC. The slow growth of TNC observed on the glass surfaces in this experiment (Table 1) was therefore in line with previous observations. It is unclear whether the slow growth was owing to phage control or some other factor. It could simply be that the studied populations were adapted to slow growth, which would be energy effective in the oligotrophic environment in which they exist. However, the biofilm populations consisted largely of SRB, dominated by *D. aespoeenis* (Jägevall et al., 2011). Phages lytic to this species have been found in Åspö HRL groundwater (Eydal et al., 2009), so the possibility of a phage-governed mechanism controlling the numbers of attached cells cannot be ruled out.

Available sources of energy for microbial processes

Rough and simple stoichiometric calculations were helpful in revealing the reducing power needed to produce the observed amounts of sulphide and acetate. All three FCCs had produced approximately 225 $\mu$M sulphide after 91 days. Reduction of sulphate to sulphide requires eight electrons, which would theoretically be available from the complete oxidation of 450 $\mu$M organic carbon to carbon dioxide. If hydrogen instead constituted the electron donor, 900 $\mu$M would be needed to produce 225 $\mu$M sulphide. The control FCC produced 250 $\mu$M acetate, which, using a stoichiometric calculation similar to that used for hydrogen, would require 500 $\mu$M organic carbon or 1 mM hydrogen plus 500 $\mu$M carbon dioxide. These numbers are of course ideal and do not include the electrons needed for biomass increase and other losses related to reaction efficiencies under 100%. Still, they demonstrate a need for at least 1 mM organic carbon or 2 mM hydrogen to form the observed amounts of sulphide and acetate. Organic carbon was not analysed in these experiments, but the requirement of 1 mM organic carbon corresponds to 12 mg organic carbon L$^{-1}$, which is in the range found in deep groundwater (Hallbeck & Pedersen, 2008; Pedersen et al., 2008). The hydrogen-treated FCC was supplemented with 3 mM hydrogen, which supplied more energy than needed to produce the observed acetate and sulphide.

Effect of acetate addition

The addition of acetate increased the growth rate compared with that of the control for the first 29 days (Fig. 1, Table 1). In addition, acetate was produced during the first 29 days, after which the acetate concentration decreased, possibly owing to the consumption of acetate. The presence of attached SRB (Jägevall et al., 2011) belonging to the acetate oxidizers (Detmers et al., 2001) in the acetate-treated FCC indicated that SRB capable of acetate oxidation were present. Sulphate was the main available electron donor in the system, because oxygen, nitrate and ferric iron were all below detection, leaving SRB as the most plausible group of organic-carbon-consuming microorganisms in the acetate-treated and other two FCCs.
Effect of hydrogen addition

All analysed variables indicated the highest rates of formation and growth in the hydrogen-treated FCC except for the MPN of AM and HM, which decreased. The addition of 3 mM hydrogen was combined with the addition of 0.75 mM carbon dioxide to ensure that AA would have access to both energy and inorganic carbon for acetate production. This was obviously successful, in that the hydrogen-treated FCC produced more acetate than did the control FCC, and the MPN of AA became significantly greater there (Fig. 3a). A drawback of adding carbon dioxide was a lower pH of 5.45 in the hydrogen-treated FCC by the end of the experiment, vs. a pH of 6.28 in the control FCC. The pH decrease in the hydrogen-treated FCC probably explains the decrease in the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4). Many methanogens prefer a pH above 7; for example, the MPN of AM and HM (Fig. 4).

Adding hydrogen increased the acetate production rate by approximately 50% compared with those observed in the acetate-treated and control FCCs (Table 1). Obviously, significant amounts of acetate were produced in the FCCs containing only minor amounts of hydrogen. Many acetogens are facultative autotrophs that produce acetate from organic carbon (Drake et al., 2002). The MPNs of AA and heterotrophic acetogens found in deep groundwater from two other sites correlated very well ($r = 0.91$, $n = 28$) (Hallbeck & Pedersen, 2012). The difference (0.42 mM) between the amounts of acetate produced in the hydrogen-treated and control FCCs after 91 days should then represent acetate produced by AA, which indeed increased significantly in MPN in the hydrogen-treated FCC.

Adding hydrogen doubled the rate of sulphide production in the hydrogen-treated FCC compared with that found in the acetate-treated and control FCCs (Table 1). Several genera of SRB can grow chemolithotrophically on hydrogen and carbon dioxide (Bryschat et al., 1987). The observed hydrogen-induced increase in sulphide production could then be related to sulphate reduction using hydrogen as the electron donor (Nedwell & Banat, 1981). The MPN of SRB constituted up to 25% of the TNC in the hydrogen-treated FCC on day 29. Assuming that the MPN represented most of the SRB, approximately 25% of the attached and unattached population was sulphate reducing. The sulphide production rates of SRB then range from $0.48 \times 10^{-15}$ mol h$^{-1}$ per cell in the acetate-treated FCC to $1.09 \times 10^{-15}$ mol h$^{-1}$ per cell in the hydrogen-treated FCC. This is within the range found in pure cultures under good growth conditions (i.e. 0.01–2 $\times 10^{-15}$ mol h$^{-1}$ per cell) (Jørgensen, 1978). It is obvious that the SRB must have had good growth conditions in the FCC. Still, the amount of sulphide produced after 91 days, that is, 0.22 mM, was very modest compared with what is often obtained in pure cultures, in which 15 mM can be produced in one to several days (Reis et al., 1992). This could be explained by phage control of the studied populations. If phages grazed the SRB, they would have kept the numbers of SRB below approximately $2 \times 10^5$ SRB mL$^{-1}$. This would result in a sustainable situation with respect to available electron donors and acceptors in which the SRB that survived phage attacks could be metabolically very active. The observed long generation times (Table 1) could then represent the sum of cell growth and phage-induced cell lysis. Individual SRB cells may consequently have been growing and metabolizing at much higher rates than those observed for the total population, something that was clearly indicated by the high sulphide production rate per cell.

Conclusions

Underground constructions where metals corrode anaerobically constitute local sources of hydrogen that can induce significant reduction of sulphate to sulphide when sulphate is present, while acetate can be produced from hydrogen and carbon dioxide. Low concentrations of hydrogen are often found in pristine subterranean environments (Pedersen et al., 2008); however, the production rate of this hydrogen, if present, and its transport rate in the deep subsurface are poorly understood, although they have been modelled (Sherwood Lollar et al., 1993b; Lin et al., 2005). Hydrogen-sustained microbial activity may consequently be present in deep groundwater. More research is needed to deepen our understanding of hydrogen production and transport in deep hard-rock aquifers and in the rock matrix. In a wider perspective, using steel as a casing material and in other constructions used for subsurface research may introduce a growth stimulation artefact because of the production of hydrogen.

The studied subterranean microbial processes appeared to be very slow on a quantitative basis in terms of volume and time, although the results also suggested that individual SRB cells could be very active. The data obtained here present a contradiction: the studied populations seemed slow growing with long generation times, but individual cells could at the same time have been growing fairly quickly, assuming nearly equivalent death and lysis rates.
The influence of phages on subterranean microbial processes is poorly understood. The work presented here suggests that phages may strongly reduce the total amount of sulphate reduced to sulphide in an SRB population. Phages may also exert a strong selective pressure on the diversity of subterranean microbial populations. More research is needed analysing phage numbers, diversity and lytic activity.

Acknowledgements

The author is grateful to Johanna Arlinger, Anna Hallbeck, Lotta Hallbeck, Jessica Johansson, Sara Lydmark and Lisa Rabe at Microbial Analytics Sweden for their excellent laboratory work. The research leading to these results has received funding from the Swedish Nuclear Fuel and Waste Management Co. and the Swedish Research Council.

References

Amend JP & Teske A (2005) Expanding frontiers in deep subsurface microbiology. *Palaeoecogr Palaeoclimatol Palaeoecol* 219: 131–155.
Anderson C, Pedersen K & Jakobsson A-M (2006) Autoradiographic comparisons of radionuclide adsorption between subsurface anaerobic biofilms and granitic host rocks. *Geomicrobiol J* 23: 15–29.
Anderson C, Jakobsson A-M & Pedersen K (2007) Influence of *in situ* biofilm coverage on the radionuclide adsorption capacity of subsurface granite. *Environ Sci Technol* 41: 830–836.
Anderson C, Johnsson A, Moll H & Pedersen K (2011) Radionuclide geomicrobiology of the deep biosphere. *Geomicrobiol J* 28: 540–561.
Apps JA & van de Kamp PC (1993) Energy gases of abiotic origin in the Earth’s crust. *The Future of Energy Gases, U.S. Geological Survey Professional Papers, Vol. 1570* (Howell G, ed), pp. 81–132. United States Government Printing Office, Washington, DC.
Brysch K, Schneider C, Fuchs G & Widdel F (1987) Lithoautotrophic growth of sulphate-reducing bacteria, and description of *Desulfobacterium autotrophicum* gen. nov., sp. nov. *Arch Microbiol* 148: 264–274.
Detmers J, Brüchert V, Habicht K & Kuever J (2001) Diversity of sulphur isotope fractionations by sulphate-reducing prokaryotes. *Appl Environ Microbiol* 67: 888–894.
Drake HL, Küsel K & Matthies C (2002) Ecological consequences of the phylogenetic and physiological diversities of acetogens. *Antonie Van Leeuwenhoek* 81: 203–213.
Ekendahl S & Pedersen K (1994) Carbon transformations by attached bacterial populations in granitic ground water from deep crystalline bed-rock of the Stripa research mine. *Microbiology* 140: 1565–1573.
Ekendahl S, Arlinger J, Ståhl F & Pedersen K (1994) Characterization of attached bacterial populations in deep granitic groundwater from the Stripa research mine with 16S-rRNA gene sequencing technique and scanning electron microscopy. *Microbiology* 140: 1575–1583.
Eydal HSC & Pedersen K (2007) Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1000 m. *J Microbiol Methods* 70: 363–373.
Eydal HSC, Jägellvall S, Hermansson M & Pedersen K (2009) Bacteriophage lytic to *Desulfovibrio aespoeensis* isolated from deep groundwater. *ISME J* 3: 1139–1147.
Greenberg AE, Clesceri LS & Eaton AD (1992) Estimation of bacterial density. *Standard Methods for the Examination of Water and Wastewater, 18th edn.* American Public Health Association, Washington, DC, pp. 9–49.
Hallbeck L & Pedersen K (2008) Characterization of microbial processes in deep aquifers of the Fennoscandian Shield. *Appl Geochem* 23: 1796–1819.
Hallbeck L & Pedersen K (2012) Culture-dependent comparison of microbial diversity in deep granitic groundwater from two sites considered for a Swedish final repository of spent nuclear fuel. *FEMS Microbiol Ecol*, DOI: 10.1111/j.1574-6941.2011.01281.x.
Haveman SH, Pedersen K & Routsalainen P (1999) Distribution and metabolic diversity of microorganisms in deep igneous rock aquifers of Finland. *Geomicrobiol J* 16: 277–294.
Hobbie JE, Daley RJ & Jasper S (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* 33: 1225–1228.
Jägellvall S, Rabe L & Pedersen K (2011) Abundance and diversity of biofilms in natural and artificial aquifers of the Åspö Hard Rock Laboratory, Sweden. *Microbial Ecol* 61: 410–422.
Jörgensen BB (1978) A comparison of methods for the quantification of bacterial sulphate reduction in coastal marine sediments. *Geomicrobiol J* 1: 46–64.
Kotelnikova S & Pedersen K (1998) Distribution and activity of methanogens and homoacetogens in deep granitic aquifers at Åspö Hard Rock Laboratory, Sweden. *FEMS Microb Ecol* 26: 121–134.
Kotelnikova S, Macario AJL & Pedersen K (1998) *Methanobacterium subterraneum*, a new species of Archaea isolated from deep groundwater at the Åspö Hard Rock Laboratory, Sweden. *Int J Syst Bacteriol* 48: 357–367.
Kristjansson JK, Schönheit P & Thauer RK (1982) Different Ks values for hydrogen of methanogenic bacteria and sulphate reducing bacteria: an explanation for the apparent inhibition of Methanogenesis by Sulfate. *Arch Microbiol* 131: 278–282.
Kyle JE, Eydal HSC, Ferris FG & Pedersen K (2008) Viruses in granitic groundwater from 69 to 450 m depth of the Åspö Hard Rock Laboratory, Sweden. *ISME J* 2: 571–574.
Lin L-H, Slater GF, Sherwood Lollar B, Lacrampe-Couloumbe G & Onstott TC (2005) The yield and isotopic composition
of radiolytic H₂, a potential energy source for the deep subsurface biosphere. *Geochim Cosmochim Acta* **69**: 893–903.

Motamedi M & Pedersen K (1998) *Desulfovibrio aespoeensis* sp. nov. a mesophilic sulfate-reducing bacterium from deep groundwater at Åsö Hard Rock Laboratory, Sweden. *Int J Syst Bacteriol* **48**: 311–315.

Nedwell DB & Banat IM (1981) Hydrogen as an electron donor for sulfate-reducing bacteria in slurries of salt marsh sediment. *Microbiology* **47**: 305–313.

Nielsen ME, Pedersen K, Fisk M & Istok J (2006) Microbial nitrate respiration of lactate at in situ conditions in groundwater from a granitic aquifer situated 450 m underground. *Geobiology* **4**: 43–52.

Pedersen K (1982) Method for studying microbial biofilms in flowing-water systems. *Appl Environ Microbiol* **43**: 6–13.

Pedersen K (1997) Microbial life in granitic rock. *FEMS Microbiol Rev* **20**: 399–414.

Pedersen K (2000a) Exploration of deep intraterrestrial life: current perspectives. *FEMS Microbiol Lett* **185**: 9–16.

Pedersen K (2000b) *The Microbe Site: Drilling, Instrumentation and Characterisation*. International Progress Report IPR-00-36 (available from www.skb.se), Swedish Nuclear Fuel and Waste Management Co., Stockholm.

Pedersen K (2001) Diversity and activity of microorganisms in deep igneous rock aquifers of the Fennoscandian Shield. *Subsurface Microbiology and Biogeochemistry* (Fredrickson JK & Fletcher M, eds), pp. 97–139. Wiley-Liss, New York.

Pedersen K (2005) *The MICROBE Framework: Site Descriptions, Instrumentation, and Characterization, Åsö Hard Rock Laboratory*. International Progress Report IPR-05-05 (available from www.skb.se), Swedish Nuclear Fuel and Waste Management Co., Stockholm.

Pedersen K & Ekendahl S (1999) Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. *Microbial Ecol* **20**: 37–52.

Pedersen K & Ekendahl S (1992a) Incorporation of CO₂ and introduced organic compounds by bacterial populations in groundwater from the deep crystalline bedrock of the Stripa mine. *J Gen Microbiol* **138**: 369–376.

Pedersen K & Ekendahl S (1992b) Assimilation of CO₂ and introduced organic compounds by bacterial communities in groundwater from southeastern Sweden deep crystalline bedrock. *Microbial Ecol* **23**: 1–14.

Pedersen K, Arlinger J, Hallbeck A, Hallbeck L, Eriksson S & Johansson J (2008) Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4 to 450 m in Olkiluoto, Finland. *ISME J* **2**: 760–775.

Reis MAM, Almeida JS, Lemos PC & Carrondo MJT (1992) Effect of hydrogen sulfi de on growth of sulfate reducing bacteria. *Biotechnol Bioeng* **40**: 593–600.

Sherwood Lollar B, Frape SK, Fritz P, Macko SA, Welhan JA, Blomqvist R & Lahermo PW (1993a) Evidence for bacterially generated hydrocarbon gas in Canadian Shield and Fennoscandian Shield rocks. *Geochim Cosmochim Acta* **57**: 5073–5085.

Sherwood Lollar B, Frape SK, Weise SM, Fritz P, Macko SA & Welhan JA (1993b) Abiogenic methanogenesis in crystalline rocks. *Geochim Cosmochim Acta* **57**: 5087–5097.

SKB (2010) *Design and Production of the KBS-3 Repository*. SKB Technical Report TR-10-12 (available from www.skb.se), Swedish Nuclear Fuel and Waste Management Co., Stockholm.

Stevens TO & McKinley JP (1995) Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270**: 450–453.

Suttle CA (2007) Marine viruses: major players in the global ecosystem. *Nat Rev Microbiol* **5**: 801–812.

Widdel F & Bak F (1992) Gram-negative, mesophilic sulphate-reducing bacteria. *The Prokaryotes*, Vol. 4 (Balows A, Truper HG, Dworkin M, Harder W & Schleifer K-Z, eds), pp. 3352–3378. Springer-Verlag, New York.