Incorporation of CO₂ and introduced organic compounds by bacterial populations in groundwater from the deep crystalline bedrock of the Stripa mine

KARSTEN PEDERSEN* and SUSANNE EKENDAHL

Department of General and Marine Microbiology, University of Göteborg, Carl Skottsbergs gata 22, S-413 19 Göteborg, Sweden

(Received 20 May 1991; revised 19 August 1991; accepted 8 November 1991)

The incorporation of CO₂ and assimilation of introduced organic compounds by bacterial populations in deep groundwater from fractured crystalline bedrock has been studied. Three depth horizons of the subvertical borehole V2 in the Stripa mine, Sweden, 799–807 m, 812–820 m and 970–1240 m, were sampled. The groundwaters, obtained from fracture systems without close hydraulic connections, were anoxic and had the following physicochemical characteristics: pH values of 9.5, 9.4 and 10.2; E_h values of +205, +199 and –3 mV; sulphide, 0, 106 and 233 µM; CO₃²⁻, 158, 50 and 57 µM; CH₄, 245, 170 and 290 µl l⁻¹; and N₂, 25, 31 and 25 ml l⁻¹. Biofilm reactors, each containing a series of parallel glass surfaces, were connected to the groundwaters issuing from these depth horizons at flows of approximately 1×10^{-3} m s⁻¹ during two periods of two and four months. There were from 1.8×10^3 to 1.2×10^5 bacteria per ml groundwater and from 1.2×10^6 to 7.1×10^6 bacteria per cm² of colonized test surface. These results imply that the populations of attached bacteria are several orders of magnitude greater than those of unattached bacteria in bedrock fractures with flowing groundwater. The incorporation of ¹⁴CO₂, [¹⁴C]formate, [U-¹⁴C]lactate, [U-¹⁴C]glucose and L-[4,5-³H]leucine by the bacterial populations was demonstrated using microautoradiographic and liquid scintillation counting techniques. The measured CO₂ incorporation reflected the *in situ* production of organic carbon from CO₂. Incorporation of formate followed that of CO₂ and indicated the presence of bacteria able to substitute formate for CO₂, e.g. methanogenic bacteria. The presence of sulphate-reducing bacteria is suggested by the observed incorporation of lactate by up to 74% of the bacterial populations. The recorded uptake of glucose indicates the presence of heterotrophic bacteria other than sulphate-reducing bacteria. Up to 99% of the populations incorporated leucine, showing that major fractions of the populations were viable.

Introduction

In recent years there has been an increasing interest in the microbiology of fractured rock. One reason is that many countries are seriously considering using fractured rock as the final repository for nuclear waste, at depths ranging from a few tens of metres for low and intermediate waste, up to more than a kilometre for high-level waste. There is considerably less information and experience on depths below a few hundred metres than at shallow depths (Ghiorse & Wilson, 1988; West *et al.*,

1982, 1985). A more detailed analysis of microbial interactions with nuclear waste reveals complex and difficult research areas (West *et al.*, 1985), made more difficult by the classical problem of sample disturbance and contamination during the drilling of boreholes. Further, the investigations have to be aimed at assessing the long-term safety of nuclear waste disposal, where time scales range from hundreds to several millions of years due to the very long half-lives of many radionuclides.

Dissolution and transport by the groundwater are the most important dispersion mechanisms for radionuclides eventually released from the waste. There are currently large national research programmes in Canada, Switzerland and Sweden for the study of radionuclide transport in crystalline rock. In addition an international field

* Author for correspondence. Tel. 031 418700; fax 031 826790.

Abbreviations: AODC, acridine orange direct count; DOC, dissolved organic carbon; LSC, liquid scintillation counting; MARG, microautoradiography; SRB, sulphate-reducing bacteria.

research programme organized by OECD/NEA in the Stripa research mine, Sweden, has entered its final phase.

The presence of bacteria can influence the transport of radionuclides in different ways. Free-living bacteria constitute mobile suspended particles which may have a radionuclide-sorbing capacity higher than that of the surrounding rock (Strandberg *et al.*, 1981; Beveridge & Fyfe, 1985; Pedersen & Albinsson, 1991). Radionuclide transport will then proceed faster with, than without bacteria. If, on the other hand, the majority of the bacteria are growing in biofilms on fracture surfaces, transport of radionuclides may be reduced. Finally, bacterial production of complexing agents and other metabolites may affect speciation and thus mobility of radionuclides independently of whether the bacteria are attached or not.

The relevance of different mechanisms for radionuclide transport by bacteria can only be evaluated with a comprehensive knowledge of the ecology and physiology of the bacterial populations that may inhabit a nuclear waste repository and its surroundings. Identification of their nutritional strategies is an important step in this understanding. Autotrophic bacteria may provide organic matter for heterotrophic organisms if H_2 and CO_2 are available for the autotrophs, e.g. for acetogenic bacteria (Fuchs, 1986; Wood & Ljungdahl, 1991), methanogenic bacteria (Belyaev *et al.*, 1983; Belyaev & Ivanov, 1983; Godsy, 1980) and several species of sulphate-reducing bacteria (SRB) (Fauque *et al.*, 1991). Fermentative or respiratory utilization of geological deposits of organic material are other possible explanations as to why heterotrophic bacteria have been found in deep geological formations (Chapelle *et al.*, 1987, 1988; Hicks & Fredrickson, 1989; Pedersen & Ekendahl, 1990).

In this study, we have assayed the total numbers of bacteria in the groundwater and on surfaces exposed to slowly flowing groundwater from a borehole called V2, which has been well characterized during the geochemical investigations of the Stripa groundwaters (Nordstrom *et al.*, 1985). The nutritional responses of the unattached and attached bacterial populations were studied by measurement of their incorporation of CO_2 and different radiolabelled organic compounds using microautoradiographic (MARG) and liquid scintillation counting (LSC) techniques. Incorporation of CO_2 was used to assay the *in situ* production of organic carbon from CO_2 .

Methods

Description of study site. The Stripa mine (central Sweden; $15^\circ 5'E$, $59^\circ 40'N$) has been a deep underground research facility since 1976 when the iron ore was mined out. The ore consisted of a quartz-banded

haematite and occurred in a lepatite formation. Adjacent to the lepatite is a large body of medium-grained granite through which borehole V2 runs. It is a subvertical shaft with a diameter of 76 mm and runs from one of the deepest drifts of the mine, 410 m, down to a depth of 1240 m. It was drilled to 860 m in 1977 and continued to 1240 m in 1981. A number of sampling depths of this artesian borehole were closed off with packers made of inflatable 76 mm rubber tubes and connected to the drift with 6 mm Teflon tubing. The sampling depths used in this study were 799–807 m, 812–820 m and 970–1240 m below ground. There were approximately 2 fractures per m of the borehole, sealed or coated mainly with chlorite, epidote and calcite (Nordstrom *et al.*, 1985). Because of silicate weathering, the pH of the groundwater approaches 10.

Water and gas analysis. The pH and the E_h were measured *in situ* in the mine with a PHM Autocal pH meter (Radiometer), a GK2421C combined pH electrode and redox electrode PK1401. Sulphate was measured turbidimetrically with $BaCl_2$ (Franson, 1985), sulphide with an iodometric method (Franson, 1985) and the CO_3^{2-} content with a coulometer (model 5011 CO_2 Coulometer) (Huffman, 1977). These procedures were repeated after a 30 d interval.

Gas pipettes (100 ml) were connected to the flowing groundwaters, left for 17 h and closed. The dissolved gases of the waters were extracted by degassing the samples at <40 Pa, after which the total gas was collected in a burette fitted with a septum by step-wise pumping with mercury and compressed air. The gas composition was analysed with a Perkin-Elmer gas chromatograph supplied with two columns [Porapak N 80–100 mesh $4\text{ m} \times 1/8"$ (0.32 cm) and Molecular Sieve 5A 60–80 mesh $2' (61\text{ cm}) \times 1/8"$ (0.32 cm)], a thermal conductivity detector and a flame ionization detector. Carbon monoxide and carbon dioxide were converted to methane before detection. The carrier gas used was argon except for the hydrogen analysis, when helium was used. This procedure was repeated after a 30 d interval.

Attachment and growth of bacteria. Biofilm reactors (Pedersen, 1982; Pedersen *et al.*, 1986) were connected to the flowing groundwaters from V2 at flows of approximately $1 \times 10^{-3}\text{ m s}^{-1}$. Microscope slides, $60 \times 24 \times 1\text{ mm}$, were heated in a muffle furnace at 475°C for 4 h and used as hydrophilic substrata for the attachment and growth of bacteria.

Total number of bacteria. Acridine-orange-stained direct counts (AODC) were used to determine the total number of unattached bacteria as described by Pedersen & Ekendahl (1990). Surfaces from the biofilm reactors were rinsed with a 1% (w/v) NaCl solution in a surface rinse as described by Pedersen *et al.* (1986) to remove unattached bacteria, stained with acridine orange, rinsed with deionized water, dried and the stained bacteria counted.

Precision of the AODC method. The frequency distribution of the number of bacteria counted per microscope field follows a Poisson distribution (Hallbeck & Pedersen, 1990). This means that the precision of the mean of the counted bacteria is only dependent on the number of bacteria counted. One filter then predicts the sample mean with a precision of 5% if 400 or more bacteria are counted (Niemelä, 1983).

Variability of the total number of bacteria determined on different sampling occasions and in different samples. The total numbers of unattached bacteria per ml flowing groundwater were counted on four different occasions, 17 September 1987, and 17 April, 1 June and 1 October 1990. The total number of attached bacteria was counted after two different experimental periods: after 56 d attachment and growth between 21 February and 17 April 1990, and after 117 d between 8 June and 1 October 1990. Each serum bottle with a sample as described below constituted an independent sample of the flowing groundwaters from the different sampling depths and the results from all sampled

bottles were used to assay the random variability between samples in this investigation.

Incorporation of CO_2 and organic compounds by unattached bacteria. Ten-millilitre volumes of groundwater, sampled on 1 October 1990, were added to 100 ml sterile serum bottles with aluminium crimp-sealed butyl rubber stoppers under a 100% nitrogen atmosphere via hypodermic syringes mounted directly on the tubings from the different sampling depths. A number of different ^{14}C - or ^3H -labelled organic compounds and $\text{Na}_2^{14}\text{CO}_3$ (Amersham Sweden) were subsequently added in 1 ml oxygen-free portions to assay the nutritional responses of the populations studied. The radioactive concentrations of the ^{14}C -labelled organic compounds were adjusted to 14 MBq per ml sample. The sodium carbonate was adjusted to 73 MBq per ml to compensate for the isotope dilution caused by the CO_3^{2-} and CO_2 present in the groundwaters. The following final concentrations and specific activities were used: $38\text{ }\mu\text{M}\text{-Na}_2^{14}\text{CO}_3$ (1.92 GBq mmol^{-1}); $7\text{ }\mu\text{M}\text{-}[^{14}\text{C}]\text{formate}$ (2.05 GBq mmol^{-1}); $2.6\text{ }\mu\text{M}\text{-}[\text{U-}^{14}\text{C}]\text{-lactate}$ (5.7 GBq mmol^{-1}); $1.6\text{ }\mu\text{M}\text{-}[\text{U-}^{14}\text{C}]\text{-glucose}$ (9.1 GBq mmol^{-1}) and $6\text{ nM}\text{-L-[4,5-}^3\text{H]leucine}$ (5.63 TBq mmol^{-1}). The samples were incubated at 10°C for 6 h, after which formalin was added to a final concentration of 2% (v/v). Controls for abiotic adsorption of the labelled compounds were achieved by addition of formalin (2%, v/v) together with the labelled compounds at sampling, and were processed like the other samples. Control counts were subtracted from sample counts. Subsamples of $2 \times 2.5\text{ ml}$ (970–1240 m samples) or 5 ml (799–807 and 812–820 m samples) were filtered through Nuclepore filters (pore size $0.2\text{ }\mu\text{m}$, 13 mm), rinsed three times with 1 ml portions of Milli-Q filtered water (pore size $0.2\text{ }\mu\text{m}$) and subsequently placed in 10 ml scintillation cocktail (Ready-Safe, Beckman) and their radioactivity measured in a Beckman LS 3801 scintillation counter.

Incorporation of CO_2 and organic compounds by attached bacteria. Groundwater, sampled on 1 October 1990, was filtered in 20 ml volumes under a 100% nitrogen atmosphere through Dynagard hollow-fibre syringe filters (pore size $0.2\text{ }\mu\text{m}$), into sterile 50 ml polypropylene centrifuge tubes with lids (Nunc). Labelled compounds were added as above. Microscope slides from the biofilm reactors (experimental period 8 June–1 October 1990, 117 d) were transferred under a 100% nitrogen atmosphere to tubes with corresponding filtered groundwater, one slide per tube. The $\text{Na}_2^{14}\text{CO}_3$ was added after this step. The microscope slides were incubated with the equivalent controls as described above, rinsed with the surface rinse (Pedersen *et al.*, 1986), cut into four pieces with a diamond knife, placed in 20 ml scintillation cocktail (Ready-Safe, Beckman) and their radioactivity measured. There was no difference in the counting efficiency between pieces placed individually in scintillation vials or when all the pieces were counted in a single vial.

MARG studies of unattached bacteria. The MARG procedure followed was the MARG-E method described by Tabor & Neihof (1982). Briefly, residual $2 \times 2.5\text{ ml}$ (970–1240 m) or 5 ml (799–807 and 812–820 m) volumes from the sample bottles used for the incorporation studies, including the control samples, were filtered through Nuclepore filters (pore size $0.2\text{ }\mu\text{m}$) and rinsed three times with 1 ml portions of filtered 1% (w/v) NaCl in Milli-Q water. The filters were transferred to clean microscope slides previously dipped in Kodak NTB-2 autoradiographic emulsion, placed in a water-chilled PVC container (10°C), moved to a desiccator after solidifying and left for exposure under vacuum over silica gel for 3 d at 4°C . A bacterium was scored to be active, incorporating the labelled compound, if at least three silver grains were located at a maximal distance of $1\text{ }\mu\text{m}$ away from the bacteria labelled with ^3H and $3\text{ }\mu\text{m}$ away from the bacteria labelled with ^{14}C .

The light-sensitive part of the autoradiographic work was performed in a stainless steel dark box ($0.6 \times 0.45 \times 0.35\text{ m}$), with a top lid and two

neoprene seals through which material inside the box could be manipulated in complete darkness. A water bath inside the box, connected to a heater-circulator bath on the outside, was used to maintain the NTB-2 autoradiographic emulsion at 46°C . The PVC container was chilled inside the box with a heat exchanger, flushed with tap water.

MARG studies of attached bacteria. The MARG procedure followed the procedure described for unattached bacteria, with the following modifications. The microscope slides with the attached bacteria were rinsed and dipped directly into NTB-2 emulsion, allowed to gel, and then exposed as above.

Estimate of the lower limit of detection of the MARG method. The facultative anaerobe *Pseudomonas fluorescens* (CCUG-25085) and a SRB, isolated from the 860 m level in a borehole called KAS02 and the 680 m level in KLX01, respectively (Pedersen & Ekendahl, 1990), were used to estimate the lower limit of detection of the MARG method. The bacteria were cultured as described by Pedersen & Ekendahl (1990) with the addition of 0.2 to $20\text{ nM}\text{-L-[4,5-}^3\text{H]leucine}$, $2.3\text{ }\mu\text{M}\text{-}[\text{U-}^{14}\text{C}]\text{-lactate}$ or $1.4\text{ }\mu\text{M}\text{-}[\text{U-}^{14}\text{C}]\text{-glucose}$, incubated for 30 min to 24 h before sampling and processed as for the LCS and MARG studies with unattached bacteria.

Results

The composition of the groundwaters

Tables 1 and 2 show the major parameters and the gas content of the groundwaters in the Stripa borehole V2. The sulphate, sulphide, carbonate and conductivity data differed between the sampling depths, indicating that the groundwaters were obtained from fracture systems with no close hydraulic connections. The temperatures were measured earlier with a borehole sond during the geological well logging programme (Nordstrom *et al.*, 1985). The waters were chilled to 10°C when flowing from the sampling depths up to the drift and this was the temperature at which all incubations were done. The flow was measured as ml min^{-1} and converted to cm s^{-1} over the surfaces in the biofilm reactors.

Numbers of bacteria

The numbers of bacteria counted on different occasions in groundwater and on surfaces exposed to flowing groundwater from the different sampling depths are shown in Table 3. There were 10- to 100-fold more bacteria in the groundwater from 970–1240 m depth than from the other two depths, but this difference was not reproduced on the surfaces exposed to the flowing groundwaters. The random variability of the total number of unattached and attached bacteria in samples from the same depth examined, on one occasion, ranged between 6 and 45% of the mean. The bacteria on the surfaces were distributed in uneven patterns in clusters, indicating that they had grown on the surfaces rather than just attached randomly from the passing waters.

Table 1. *The major parameters of the groundwaters of the Stripa borehole V2*

A detailed description of the Stripa groundwaters is given by Nordstrom *et al.* (1985). Values in parentheses are standard deviations (%).

Sampling depth (m)	pH	E_h (mV)	Temp.* (°C)	SO_4^{2-} (μM)	S^{2-} (μM)	CO_3^{2-} (μM)	Conductivity ($\mu S\ cm^{-1}$)	Flow ($m\ s^{-1}$)
799–807	9.5	+205	18	52 (10)	<0.01 (–)	158 (–)	425 (–)	1.4×10^{-3}
812–820	9.4	+199	18	1433 (6)	106 (–)	50 (12)	1640 (–)	2.8×10^{-3}
970–1240	10.2	–3	26	520 (0)	233 (8)	57 (5)	1180 (–)	0.5×10^{-3}

* The water had been chilled to 10 °C when it reached the drift (410 m).

Table 2. *The content of nitrogen, hydrogen and carbon-containing gases and the total volumes of gas extracted from the samples of the groundwaters of the Stripa borehole V2*

Values in parentheses are standard deviations (%).

Sampling depth (m)	N_2 ($\mu l\ l^{-1}$)	H_2 ($\mu l\ l^{-1}$)	CO ($\mu l\ l^{-1}$)	CO_2 ($\mu l\ l^{-1}$)	CH_4 ($\mu l\ l^{-1}$)	C_2H_6 ($\mu l\ l^{-1}$)	C_2H_2-4 ($\mu l\ l^{-1}$)	Volume of extracted gas (%)
799–807	25000 (27)	<10	<1	32 (44)	245 (14)	0.3 (47)	<0.1	2.4 (25)
812–820	31000 (39)	<10	<1	11 (6)	170 (25)	0.6 (12)	<0.1	3.4 (44)
970–1240	24500 (3)	<10	<1	10 (0)	290 (5)	2.9 (2)	<0.1	2.7 (1)

Table 3. *The total number of unattached bacteria (ml^{-1}) in groundwater, and attached bacteria on surfaces (cm^{-2}) exposed to flowing groundwater from three sampling depths of the Stripa borehole V2, 799–807 m, 812–820 m and 970–1240 m, measured on different occasions*

	N^*	799–807 m		812–820 m		970–1240 m	
		$10^{-5} \times$ No. of bacteria†	SD (%)	$10^{-5} \times$ No. of bacteria†	SD (%)	$10^{-5} \times$ No. of bacteria†	SD (%)
Groundwater sampling							
1 October 1990	6	0.054	26	0.018	45	1.2	12
8 June 1990	1	0.240	–	0.047	–	2.3	–
18 April 1990	1	0.036	–	0.016	–	1.6	–
17 September 1987	1	0.097	–	0.061	–	2.3	–
Surfaces exposed to flowing groundwater							
1 October 1990, 117 d	6	12.0	30	71.0	38	59.0	31
17 April 1990, 56 d	3	10.0	6	72.0	20	22.0	42

* N is the number of independent samples.

† Populations are per ml or per cm^2 .

Estimate of the lower limit of detection of the MARG method

Fig. 1 shows the relation between the percentage of bacteria scored to be active using the MARG method and the mean number of disintegrations per minute (d.p.m.) per bacterium of the samples. The lowest radioactivity that resulted in active bacteria was 10^{-3} d.p.m. per bacterium and 80% of the bacteria were

scored active at 10^{-2} d.p.m. per bacterium. This corresponds to $0.1\text{--}1 \times 10^{-16}$ mol ^{14}C per bacterium and $0.21\text{--}2.1 \times 10^{-19}$ mol 3H per bacterium and these are the minimum amounts of the isotopes per bacterium that can be detected with the MARG method. A comparison of the data in Table 4 shows that several samples demonstrated significant incorporation of the ^{14}C -labelled Na_2CO_3 , formate and glucose using LSC, but bacteria to be scored active in the MARG procedure

Table 4. Incorporation of ^{14}C and ^3H from CO_2 and labelled organic compounds by unattached bacteria in groundwater and attached bacteria on surfaces exposed to flowing groundwater from three sampling depths of the Stripa borehole V2 and the percentage of the population scored to be active, using the MARG method, in incorporating the labelled compounds

Labelled compound	Depth (m)	Unattached bacteria		Attached bacteria	
		$10^{14} \times \text{Mol isotope ml}^{-1}$	Percentage active bacteria	$10^{14} \times \text{Mol isotope cm}^{-2}$	Percentage active bacteria
CO_2^* (^{14}C)	799–807	312	5	–	–
	812–820	66	5	1200	–
	970–1240	88	–	4380	–
Formate (^{14}C)	799–807	11	4	–	–
	812–820	3	6	29	8
	970–1240	–	–	81	–
Lactate (^{14}C)	799–807	26	16	5100	11
	812–820	10	34	13700	24
	970–1240	68	6	92400	74
Glucose (^{14}C)	799–807	19	5	123	–
	812–820	19	8	4800	5
	970–1240	66	–	2600	–
Leucine (^3H)	799–807	1	55	160	77
	812–820	5	23	290	99
	970–1240	5	9	280	38

–, Not detected.

* The data have been corrected for isotope dilution caused by the measured CO_3^{2-} and CO_2 contents of the groundwaters (Tables 1 and 2).

Fig. 1. Relation between the percentage of bacteria scored to incorporate the labelled compounds using the MARG method and the mean number of d.p.m. per bacterium in cultures with *Pseudomonas fluorescens* (CCUG-25085) (Δ) and a SRB ($\blacksquare$), amended with 0.2–20 nM-L-[4,5- ^3H]leucine, 2.3 μM -[U- ^{14}C]lactate or 1.4 μM -[U- ^{14}C]glucose between 30 min and 24 h before sampling.

could not be detected. This is explained by the higher sensitivity of the LSC technique compared to the MARG technique on the assumption that the bacteria incorporated too little ^{14}C ($< 0.1 \times 10^{-16}$ mol per cell) to be scored active using the MARG technique, but

sufficient ^{14}C to give a measurable contribution using LSC.

Incorporation of CO_2 and organic compounds by attached bacteria

The incorporation of ^{14}C and ^3H from labelled compounds by unattached (ml^{-1}) and attached (cm^{-2}) bacterial populations after 6 h (from 1 October 1990), measured with the LSC ($\text{mol } ^{14}\text{C}$ and $^3\text{H ml}^{-1}$ or cm^{-2}) and MARG (percentage of bacteria scored to be active) techniques is shown in Table 4. The LSC results are presented as mol isotope atoms incorporated because the metabolic pathways of the organic compounds used are unknown for the populations studied and stoichiometric calculations back to mol organic compounds might therefore be misleading. For instance, many SRB split lactate into acetate, which is incorporated via the TCA pathway, and to CO_2 , which is expelled from the cell. The content of dissolved organic carbon (DOC) in the borehole V2 groundwater has previously been determined to be 1.1 mg l^{-1} (0.4–4 mg l^{-1} , eight measurements) (Nordstrom *et al.*, 1985), which is in agreement

with the range of what has been found in 22 other deep groundwaters from crystalline bedrock (average 2 mg l^{-1}) (Lakksoharju, 1990). A significant part of this DOC consists of fulvic acids (Pettersson *et al.*, 1990). The amounts of ^{14}C -labelled organic compounds used were between 0.23 (lactate) and 0.32 (formate) mg l^{-1} . The presence of sufficient *in situ* concentrations of unlabelled molecules of the corresponding organic compounds to give significant misleading data due to isotope dilution is thus unlikely.

There was significant incorporation of CO_2 in all samples, except for the attached bacteria at 799–807 m depth (Table 4), indicating the *in situ* production of organic carbon from CO_2 . Incorporation of formate followed that of CO_2 except for the unattached bacteria at 970–1240 m depth and indicated the presence of bacteria able to substitute formate for CO_2 . The lactate and glucose incorporation demonstrated the presence of heterotrophic bacteria. The incorporation of lactate by the attached bacteria dominated over glucose at all depths and gave MARG responses up to 74%. Leucine was incorporated by up to 99% of the populations, which showed the major fractions of the populations studied were viable.

Discussion

The study by Nordstrom *et al.* (1985) showed that the salinity profile of the borehole V2 is heterogeneous, which is typical for groundwaters in crystalline bedrock that have not been intruded by saline waters such as that from the sea. The different conductivities, carbonate, sulphate and sulphide concentrations of the sampling depths (Table 1) reflect this heterogeneity and indicate that the groundwaters came from fracture systems without close hydraulic connections. The two lower sampling depths had a considerably higher salinity than the upper depth, indicating that these waters are old (in excess of 20000 years using conventional ^{14}C measurements; cf. Nordstrom *et al.*, 1985). The 799–807 m groundwater is probably mixed with surface water via the mine, which may have diluted the salinity above 810 m (Nordstrom *et al.*, 1985). The sulphide content of the two lowest depths indicates that these habitats were anaerobic, and that facultative or obligate anaerobic bacteria should be expected.

The physical and chemical parameters and the flows measured have not differed significantly since the borehole V2 was drilled in 1978 (Nordstrom *et al.*, 1985). In addition, each sampling depth exhibited an amount of bacteria per volume of water that differed little between sampling occasions from 1987 to 1990 (Table 3). This implies that the bacterial populations studied were in

steady states with their environments and that the fractures of the Stripa bedrock constitute stable habitats for bacteria. The possibility of contamination of the groundwater during drilling cannot be excluded at this point, but it can also be argued that bacteria probably inhabited this environment long before the mine was constructed. A population of bacteria, slowly migrating vertically at a rate of 0.1 to 1 m a year in the present chemical and physical gradients and with the groundwater movements, would need 1000 to 10000 years to reach a depth of 1000 m. This is rapid in relation to the geological age of many million years of the Stripa bedrock.

Bacteria, irrespective of whether they are migrating, contaminant or indigenous populations, need carbon and energy sources to survive. The incorporation of CO_2 (Table 4) reflects an *in situ* production of organic carbon from the CO_2 which might be sufficient to sustain autotrophic and also heterotrophic populations with organic matter. The energy source for autotrophy could be hydrogen migrating from the earth's crust. Hydrogen has been detected elsewhere in the Stripa ground waters (borehole V1; Carlsson *et al.*, 1983) but not in borehole V2 (Table 2), probably as a consequence of its utilization by resident bacterial populations. Heterotrophic incorporation of CO_2 during fermentative or respiratory utilization of geological deposits of organic material is another possible explanation for the recorded uptake of CO_2 .

There was a significant content of methane in the groundwaters (Table 2), which may have two different origins. Methanogens can use CO_2 and formate as carbon sources, and use CO_2 as terminal electron acceptor in their energy metabolism, producing methane (Fuchs, 1990; Ormeland, 1988). The small but significant incorporation of formate supports the presence of methanogenic bacteria. Another methane source might be of a geological origin, of the type proposed for the Siljan deep gas project area, approximately 100 km north of Stripa. Large gas and oil deposits, formed as a result of an ancient collision of a meteorite with the earth, are postulated to lie several kilometres below ground.

Incorporation of lactate by the attached bacteria was substantially greater than that of glucose at all depths and gave MARG responses in all samples (Table 4). The deep groundwaters of the Stripa mine site are anoxic and depleted of nitrate and nitrite. The only available electron acceptor for respiration was sulphate (Nordstrom *et al.*, 1985) and such an environment will be selective for fermenting bacteria and SRB. Propionate-producing bacteria are among the few bacteria known to ferment lactate without involving a respiratory chain; however, they thrive in nutrient-rich habitats like cheese, on skin and in mud but not in oligotrophic

environments like the Stripa deep groundwater. Consequently, it is likely that the bacteria that utilized lactate in the anaerobic incubations were the SRB. This suggests that SRB constitute a substantial part of the bacterial populations in the fractures of the Stripa crystalline bedrock, as has been reported for other deep geological formations (Olson *et al.*, 1981; Pedersen & Ekendahl, 1990). Sulphate reduction by SRB will increase the $\delta^{34}\text{S}$ isotopic content of a groundwater due to the preference for ^{32}S by sulphate reducers (Widdel, 1988) and also result in increasing amounts of sulphide. Fontes *et al.* (1989) found high $\delta^{34}\text{S}$ values in borehole V2 and they postulated the presence of viable populations of SRB. The 970–1240 m depth revealed the highest $\delta^{34}\text{S}$ value during their investigations; this depth had the highest lactate incorporation ($9.2 \times 10^{-10} \text{ mol cm}^{-2}$), with 74% of the bacteria actively incorporating lactate and the highest sulphide content in our study (233 μM) (Tables 1 and 4). Our data confirm the hypothesis proposed by Fontes *et al.* (1989).

Glucose utilization is a constitutive metabolic pathway, common among mixotrophic bacteria (Kuenen & Bos, 1989) and most fermenting, as well as respiring, heterotrophic bacteria. There was incorporation of glucose, which was below the detection limit for active bacteria ($<0.1 \times 10^{-16} \text{ mol per cell}$) in several samples analysed with the MARG method. Since only a few SRB are known to utilize glucose, the observed uptake probably indicates the presence of heterotrophic bacteria.

Leucine incorporation is virtually specific for bacteria provided low (nanomolar) concentrations are used (Kirchman *et al.*, 1985) and is used by many bacteria during growth for protein synthesis (Kirchman *et al.*, 1985). Leucine can also be used as a carbon and energy source and can be fermented by proteolytic clostridia via the Stickland reaction. High percentages of the populations, up to 99% of the attached bacteria at 812–820 m depth, incorporated leucine. The leucine incorporation showed that major parts of the studied populations were viable.

A fracture in crystalline bedrock is made of two surfaces which are wavy and rough. They are in contact with each other at some points but are at a distance from each other at others. The openings in the fractures are potential channels for groundwater. Recently several model studies have been made on flow and transport in fractures with variable apertures (Moreno *et al.*, 1988; Tsang *et al.*, 1988). The results indicated that considerable channelling is to be expected in such fractures and that there is a tendency for some pathways to carry much more water than others. In a limited mass of rock one or a few channels will dominate flow, radionuclide transport and transport of nutrients for bacteria. Assuming a mean

channel width of 0.1 mm (Moreno *et al.*, 1985), our results imply that there would be from 4×10^3 to 8×10^5 more attached than unattached bacteria in a channel after 4 months of contact with Stripa borehole V2 groundwater flowing at $0.5\text{--}2.8 \times 10^{-3} \text{ m s}^{-1}$ (Table 3). The average hydraulic conductivity, K , has been determined to be 10^{-6} m s^{-1} or less in fractured rock of Stripa (Carlsson *et al.*, 1983) but it will be considerably higher in individual channels (Neretnieks, 1990). K is a function of the injection flow rate, the injection excess head, the length of the injection interval and the radius of the borehole (Andersson *et al.*, 1989). The flows used here were probably even higher than in a channel with a high conductivity; instead the experiment time was very short in relation to the time a channel will be open for flowing groundwater and bacteria.

The availability of energy and nutrients over time for a biofilm is flow dependent and will determine whether a biofilm will develop *in situ* and how many bacteria can be maintained. The slower the flow, the slower the development rate of a biofilm down to a limit where the bacteria can no longer grow or maintain a non-growth metabolism. This limit is probably very low for bacteria in an oligotrophic environment like deep groundwater, and will select for bacteria with advanced morphological and physiological mechanisms to survive a very limited availability of nutrients (Kjelleberg *et al.*, 1987). Assessing the influence of groundwater microbiology on the long-term safety of nuclear waste disposal involves time scales ranging from hundreds to many millions of years, thus there is practically no time limit for even the slowest developing biofilm to reach a steady state. The presence of attached bacteria might retard transport of radionuclides from a nuclear waste repository unless they produce complexing agents and other metabolites that affect speciation and thus mobility of radionuclides in a contrary way. The possibility of such *in situ* production by bacteria in fractured bedrock will be an important task for future research, aimed to assess the influence of groundwater microbiology on the long-term safety of nuclear waste disposal.

This research was supported by the Swedish Nuclear Fuel and Waste Management Co. The collaboration of our mine guide, Birger Ekstrand, and the director of the mine, Gunnar Ramqvist, at the Stripa mine has been a pleasant experience. We would like to thank Dr Amanda Goodman and Dr Malte Hermansson for valuable comments on the content of the manuscript.

References

- ANDERSSON, P., ANDERSSON, P., GUSTAVSSON, E. & OLSSON, O. (1989). Investigation of flow distribution in a fracture zone at the Stripa mine, using the radar method, results and interpretation. *SKB Technical Report* 89-33. Swedish Nuclear Fuel and Waste Management Co., Box 5864, S-10248 Stockholm.

- BELYAEV, S. S. & IVANOV, M. V. (1983). Bacterial methanogenesis in underground waters. In *Environmental Biogeochemistry (Proceedings of the 5th International Symposium of Environmental Biogeochemistry)*, pp. 273–280. Edited by R. Hallberg. Stockholm: Liber Tryck.
- BELYAEV, S. S., WOLKIN, R., KENEALY, W. R., DENIRO, M. J., EPSTEIN, S. W. & ZEIKUS, J. G. (1983). Methanogenic bacteria from the Bondyuzhshoe oil field: general characterization and analysis of stable-carbon isotopic fractionation. *Applied and Environmental Microbiology* **45**, 691–697.
- BEVERIDGE, T. J. & FYFE, W. S. (1985). Metal fixation by bacterial cell walls. *Canadian Journal of Earth Science* **22**, 1893–1898.
- CARLSSON, L., OLSSON, T., ANDREWS, J., FONTES, J.-C., MICHELOT, J. L. & NORDSTROM, K. (1983). Geochemical and isotope characterization of the Stripa ground waters – progress report. *Stripa Project Technical Report* 83-01. Swedish Nuclear Fuel and Waste Management Co., Box 5864, S-10248, Stockholm.
- CHAPELLE, F. H., ZELIBOR, J. L. J., GRIMES, D. J. & KNOBEL, L. L. (1988). Bacteria in deep coastal plain sediments of Maryland: a possible source of CO₂ to ground water. *Water Resources Research* **23**, 1625–1632.
- CHAPELLE, F. H., MORRIS, P. B., MCMAHON, P. B. & ZELIBOR, J. L. (1988). Bacterial metabolism and the $\delta^{13}\text{C}$ composition of ground water, Floridan aquifer system, South Carolina. *Geology* **16**, 117–121.
- FAUQUE, G., LEGALL, J. & BARTON, L. L. (1991). Sulfate-reducing and sulfur-reducing bacteria. In *Variations in Autotrophic Life*, pp. 271–337. Edited by J. M. Shively & L. L. Barton. London: Academic Press.
- FONTES, J. C., FRITZ, P., LOUVAT, D. & MICHELOT, J. L. (1989). Aqueous sulfates from the Stripa ground water system. *Geochimica et Cosmochimica Acta* **53**, 1783–1790.
- FRANSON, M. A. H. (1985). *Standard Methods for the Examination of Water and Waste Water*, 16th edn. Washington, DC: AWWA-APHA-WPCF.
- FUCHS, G. (1986). CO₂ fixation in acetogenic bacteria: variations on a theme. *FEMS Microbiology Reviews* **39**, 181–213.
- FUCHS, G. (1990). Alternative pathways of autotrophic CO₂ fixation. In *Autotrophic Bacteria*, pp. 365–382. Edited by H. G. Schlegel & B. Bowien. Berlin: Springer-Verlag.
- GHORSE, W. C. & WILSON, J. T. (1988). Microbial ecology of the terrestrial subsurface. *Advances in Applied Microbiology* **33**, 107–172.
- GODSY, E. M. (1980). Isolation of *Methanobacterium bryantii* from a deep aquifer by using a novel broth-antibiotic disk method. *Applied and Environmental Microbiology* **39**, 1074–1075.
- HALLBECK, L. & PEDERSEN, K. (1990). Culture parameters regulating stalk formation and growth rate of *Gallionella ferruginea*. *Journal of General Microbiology* **136**, 1675–1680.
- HICKS, R. J. & FREDRICKSON, J. K. (1989). Aerobic metabolic potential of microbial populations indigenous to deep subsurface environments. *Geomicrobiology Journal* **7**, 67–78.
- HUFFMAN, E. D. W. (1977). Performance of a new automatic carbon dioxide coulometer. *Microchemical Journal* **22**, 567–573.
- KIRCHMAN, D., K'NEES, E. & HODSON, R. (1985). Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. *Applied and Environmental Microbiology* **49**, 599–607.
- KJELLEBERG, S., HERMANSSON, M., MÅRDEN, P. & JONES, G. W. (1987). The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. *Annual Review of Microbiology* **41**, 25–49.
- KUENEN, J. G. & BOS, P. (1989). Habitat and ecological niches of chemolitho(auto)trophic bacteria. In *Autotrophic Bacteria*, pp. 53–96. Edited by H. G. Schlegel & B. Bowien. Berlin: Springer-Verlag.
- LAKKSOHARJU, M. (1990). Colloidal particles in deep Swedish granitic ground water. *SKB Progress Report* 90-37. Swedish Nuclear Fuel and Waste Management Co., Box 5864, S-10248 Stockholm.
- MORENO, L., NERETNIEKS, I. & KLOCKARS, C.-E. (1985). Analysis of some laboratory tracer runs in natural fissures. *Water Resources Research* **21**, 951–958.
- MORENO, L., TSANG, Y. W., TSANG, C. F., HALE, F. V. & NERETNIEKS, I. (1988). Flow and tracer transport in a single fracture. A stochastic model and its relation to some field observations. *Water Resources Research* **24**, 2033–2048.
- NERETNIEKS, I. (1990). Soluble transport in fractured rock – applications to radionuclide waste repositories. *SKB Technical Report* 90-38. Swedish Nuclear Fuel and Waste Management Co., Box 5864, S-10248 Stockholm.
- NIEMLÄ, S. (1983). Statistical evaluation of results from quantitative microbiological examinations. *NMKL Report* no. 1, 2nd edn. Uppsala: Nordic Committee on Food Analysis.
- NORDSTROM, D. K., ANDREWS, J. N., CARLSSON, L., FONTES, J.-C., FRITZ, P., MOSER, H. & OLSSON, T. (1985). Hydrogeological and hydrogeochemical investigations in boreholes – final report of the phase 1 geochemical investigations of the Stripa ground waters. *Stripa Project Technical Report* 85-06. Swedish Nuclear Fuel and Waste Management Co., Box 5864, S-10248 Stockholm.
- OLSON, G. J., DOCKINS, W. S. & MCFETHERS, G. A. (1981). Sulfate-reducing and methanogenic bacteria from deep aquifers in Montana. *Geomicrobiology Journal* **2**, 327–340.
- ORMELAND, R. S. (1988). Biogeochemistry of methanogenic bacteria. In *Biology of Anaerobic Microorganisms*, pp. 641–705. Edited by A. J. B. Zehnder. New York: John Wiley.
- PEDERSEN, K. (1982). Method for studying microbial biofilms in flowing-water systems. *Applied and Environmental Microbiology* **43**, 6–13.
- PEDERSEN, K. & ALBINSSON, Y. (1991). Effect of cell number, pH and lanthanide concentration on the sorption of promethium by *Shewanella putrefaciens*. *Radiochimica Acta* **54**, 91–95.
- PEDERSEN, K. & EKENDAHL, S. (1990). Distribution and activity of bacteria in deep granitic ground waters of southeastern Sweden. *Microbial Ecology* **20**, 37–52.
- PEDERSEN, K., HOLMSTRÖM, C., OLSSON, A.-K. & PEDERSEN, A. (1986). The influence of species variation, culture conditions, surface wettability and fluid shear on attachment and biofilm development of marine bacteria. *Archives of Microbiology* **145**, 1–8.
- PETTERSSON, C., EPHRAIM, J., ALLARD, B. & BÖREN, H. (1990). Characterization of humic substances from deep ground waters in granitic bedrock in Sweden. *SKB Technical Report* 90-29. Swedish Nuclear Fuel and Waste Management Co., Box 5864, S-10248 Stockholm.
- STRANDBERG, G. W., STARLING, E., SHUMATE, II. & PARROTT, J. R. (1981). Microbial cells as biosorbents for heavy metals: accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **41**, 237–245.
- TABOR, P. S. & NEIHOF, R. A. (1982). Improved microautoradiographic method to determine individual microorganisms active in substrate incorporation in natural waters. *Applied and Environmental Microbiology* **44**, 945–953.
- TSANG, Y. W., TSANG, C. F., NERETNIEKS, I. & MORENO, L. (1988). Flow and tracer transport in fractured media. A variable aperture channel model and its properties. *Water Resources Research* **24**, 2049–2060.
- WEST, J. M., CHRISTOFI, N. & MCKINLEY, I. G. (1982). Microbes in deep geological systems and their possible influence on radioactive waste disposal. *Radioactive Waste Management Nuclear Fuel Cycle* **3**, 1–15.
- WEST, J. M., CHRISTOFI, N. & MCKINLEY, I. G. (1985). An overview of recent microbiological research relevant to the geological disposal of nuclear waste. *Radioactive Waste Management Nuclear Fuel Cycle* **6**, 79–95.
- WIDDEL, F. (1988). Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In *Biology of Anaerobic Microorganisms*, pp. 469–585. Edited by A. J. B. Zehnder. New York: John Wiley.
- WOOD, H. G. & LJUNGAHL, L. G. (1991). Autotrophic character of the acetogenic bacteria. In *Variations in Autotrophic Life*, pp. 201–250. Edited by J. M. Shively & L. L. Barton. London: Academic Press.