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Distribution, diversity and activity of microorganisms in the hyper-alkaline spring waters of Maqarin in Jordan

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Abstract The hyper-alkaline, high-Ca^{2+} springs of Maqarin, Jordan, were investigated as an analogue for various microbial processes at the extremely high pH generated by cement and concrete in some underground radioactive waste repositories. Leaching of metamorphic, cementitious phases in Maqarin has produced current, hyper-alkaline groundwater with a maximum pH of 12.9. Six consecutive expeditions were undertaken to the area during 1994–2000. The total number of microorganisms in the alkaline waters was 10^3–10^5 cells/ml. Analysis of the 16S-ribosomal ribonucleic acid (rRNA) diversity revealed microorganisms mainly belonging to the Proteobacteria. Obvious similarities between the obtained sequences and sequences from other alkaline sites could not be found. Numerous combinations of culture media compositions were inoculated with spring, seepage and groundwaters and incubated under aerobic and anaerobic conditions with various carbon sources. Assimilation studies were performed using identical radio-labeled carbon sources. Glucose seemed to be the preferred carbon source for assimilation, followed by acetate, lactate, and leucine. The results demonstrate that microorganisms from the hyper-alkaline springs of Maqarin could grow and be metabolically active under aerobic and anaerobic hyper-alkaline conditions. However, the growth and activity found were not vigorous; instead, slow growth, low numbers, and a generally low metabolic activity were found. This suggests that microbial activity will be low during the hyper-alkaline phase of cementitious repositories.

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Introduction

Present and future underground repositories for radioactive waste generate different combinations of extreme conditions for possible microbial communities to establish themselves in. Two main combinations can be identified. Repositories for high-level radioactive waste (HLW), such as spent nuclear fuel, will initially have a high level of radiation combined with a high temperature and a low concentration of organic carbon. Repositories for low- and intermediate-level radioactive waste (LILW) commonly contain large amounts of concrete, cement and waste materials rich in organic carbon. The pore water of LILW repositories may initially reach hyper-alkaline levels at pH 12–13 and will have a high Ca^{2+} concentration. Microorganisms are an inherent part of groundwater and many sedimentary geological formations, down to depths that greatly exceed the depths of existing and future radioactive waste repositories (Fredrickson and Fletcher 2001). Microorganisms suspended in the groundwater will therefore enter the repositories. In addition, there will be microorganisms in the repositories that were introduced during the construction of the repositories or that were associated with the disposed wastes.

The possible interactions between microorganisms and repositories and radionuclides have been well documented (Keith-Roach and Livens 2002). Microbial production of gas, acids and organic ligands in LILW repositories is of major concern since these processes may enhance radionuclide migration to the biosphere and eventually to the ground surface. The use of natural analogues for understanding processes related to radioactive waste disposal is well established (Miller et al. 1994). However, naturally occurring environments with

hyper-alkaline, high-Ca^{2+} groundwater are extremely rare. The hyper-alkaline springs of Maqarin (Khoury et al. 1992) are probably the best analogue out of very few known sites in the world for various processes, including microbial processes, at extremely high pH in LILW repositories.

The Maqarin area is situated 80 km north of Amman, Jordan, where the Yarmouk River constitutes the border between Jordan and Syria (Khoury et al. 1992). The rock formations of interest comprise a Cretaceous organic-rich marl, known locally as "bituminous marl formation". The marl is a biomicrite composed essentially of calcite with accessory quartz, dolomite, apatite, pyrite and clay minerals and with an organic content of up to 20% by weight. In some places the rock has undergone spontaneous combustion, generating high-temperature, low-pressure metamorphism. Subsequent intrusion of groundwater has led to production of a suite of natural cementitious minerals. Further leaching of retrograde phases has produced the current, hyper-alkaline groundwaters, with a maximum pH of 12.9.

Multi-national research teams have explored microbial life in the spring, seepage and groundwaters of Maqarin during four defined phases stretching over about a decade. Phase I of the Maqarin Natural Analogue Project, initiated in 1989, included a microbiological study to establish the likely impact of microbes in hyper-alkaline LILW repositories (West et al. 1992). One of the conclusions of that work was that sulfate-reducing bacteria (SRB) were present. This result agreed well with the laboratory studies by the same authors, which had shown microbes, especially SRB, capable of growing in hyper-alkaline environments containing repository structural materials (West et al. 1992). The presence of SRB suggested that further investigations were required, since sulfide poses a potential corrosion problem when in contact with metal waste canisters. During Phase II of the Maqarin Natural Analogue Project, which was initiated in 1991, extensive work was carried out on the microbiology of the springs (Coombs et al. 1994). The investigators concluded that diverse populations of microbes were present at all sites sampled. The unaltered, organic-rich biomicrites seemed to have the greatest potential for growth. However, all populations were only capable of exploiting nutrient-rich environments, which would indicate a need for a steady supply of nutrients and energy. Microbial tolerance for a pH of up to 11 was confirmed, and appeared to be associated with SRB, which were found at all sampling sites.

This paper reports on results achieved during Phase III, initiated in 1993, and Phase IV, which started in 1999, of the Maqarin Natural Analogue Project. The total numbers of microorganisms in seven alkaline water locations were analyzed and the diversity of the microbes was explored using 16S ribosomal deoxyribonucleic acid (rDNA) gene sequencing and comparison. Numerous combinations of culture media compositions were inoculated with spring, seepage and groundwater and incubated. The in situ activities of unattached and attached microorganisms were studied using various radio-labeled carbon sources. For comparison, two neutral groundwater wells in the area were also included in the investigations.

Materials and methods

Site description

The Maqarin area comprises Cretaceous-Tertiary carbonate rocks overlain by Quaternary basalt soils and alluvium. The east–west oriented Yarmouk river valley is a relatively recent geomorphological feature, having eroded through some 400 m of the Irbid Plateau, exposing the bituminous marl formation, in turn capped by basaltic lava flows. The river valley slopes are very steep, approximately 45°. The regional groundwater flow is generally northwards from the central Jordan Plateau towards Yarmouk valley. Within the Maqarin area a portion of this older groundwater, supplemented by the direct recharge of young waters, passes through and reacts with the metamorphosed "cement zones", eventually discharging as hyper-alkaline seeps and springs along the Yarmouk valley sides. A gallery denoted "Adit A-6" cuts into the hillside at 50 m above the river. Here, hyper-alkaline groundwater seeps out at several sites along the tunnel, the most active seep (M1) is situated at about 125 m from the gallery entrance. More detailed descriptions of the Maqarin site geology and geochemistry have been published by Khoury et al. (1992) and Alexander et al. (1992). Samplings were performed at three major sites referred to as the "Eastern springs", "Adit A-6" and the "Western springs". Table 1 gives further details about the sampled sites.

Groundwater chemistry

Groundwater from seeps and boreholes (Table 1) was collected in 1-liter polyethylene bottles and analyzed chemically. Swedish standard analytical protocols were used, as indicated in the Results section (Table 2). The groundwater was sampled simultaneously with groundwater used for microbiology analysis and experiments.

pH measurements

Field measurements of pH (± 0.01) were made using a portable pH meter (Orion Research, Model SA250) with a Ross combination glass electrode. The flow from the hyper-alkaline seeps was commonly too low to enable sealed flow-through cell measurements. Therefore, pH was measured by immersing the electrode directly in the discharging water or in standing pools of water, from which samples were taken. In addition, water samples for groundwater chemistry were analyzed for pH in the laboratory using a Swedish standard method (SS028122–2 mod/PH-25). The field and laboratory pH values differed, at most, a few tenths of a pH unit. Measurement of pH in cultivation vessels was made using color-fixed indicator sticks (Macherey Nagel, Germany). Subsamples were withdrawn to a small container and analyzed. pH was registered in 0.5 increments, as indicated by the pH interpreter on the stick box.

Sampling for total numbers of unattached and attached cells

All samples of unattached cells were obtained concomitantly with sampling of groundwater used for inoculation of cultures and the assimilation studies (see below). The samples were collected in 50-ml, sterile, screw-capped polypropylene tubes (Sarstedt, Landskrona, Sweden). Immediately after sampling, 2.6 ml of acid-free 37% (v/v) formaldehyde was added to each tube to kill the microorganisms and preserve the samples until the microscopic counts were carried out. The final concentration of formaldehyde was 1.8% v/v.

Flow-through systems with solid surfaces for attachment of microorganisms were prepared. Flow-through boxes (FTBs) (Pedersen et al. 1986) were used during the expedition in November

Table 1 Site and sampling information for six expeditions to Maqarin. Two expeditions were undertaken during 2000. For further details, see the report by Milodowski (1996)

Sampling site	pH condition	Site description	Sampling performed				
			1994	1995	1996	1999	2000
Eastern springs							
M3	Hyper-alkaline	Seeps from unmetamorphosed clay biomicrites exposed by a road cut. The rock face displays numerous fractures from which the water flows and drips As a result of the water flow, tufa precipitates have developed on the rock face		x	x	x	x
M8	Hyper-alkaline	Similar to M3	x		x		
M18	Neutral	A borehole drilled in December 1993 in the road next to the M3 and M8 sites. It represents a mixture of mostly rainwater and some hyper-alkaline groundwater	x		x		
Adit A-6 (tunnel)							
M1	Hyper-alkaline	A seep site in the metamorphic/ cement zone situated approximately 125 m from the adit entrance		x	x	x	x
D3	Hyper-alkaline	A borehole drilled in 1999 in the unaltered clay biomicrites downstream of the metamorphic zone. The borehole is situated approximately 51 m from the Adit				x	x
D6	Hyper-alkaline	Similar to D3, but 55 m from the Adit entrance				x	x
Western springs							
M5	Hyper-alkaline	A seep through basalt-chert-limestone situated about 0.5–1 m above the Yarmouk river level. The water is greenish because of the high content of chromium	x	x	x	x	x
M6	Hyper-alkaline	Similar to M5			x		
M17	Neutral	A borehole drilled during December 1993 representing neutral groundwater and characterized by a smell of hydrogen sulfide	x				

1999 (Fig. 1). With the exception of the aluminum lid, all components were made of polycarbonate. The test pile contained 12 layers of two adjacent glass slides (60×24×1 mm), with a 1×20 mm flow channel between each slide layer. Hydrophilic surfaces were obtained by heating the glass slides for 4 h at 450°C in a muffle furnace (VRB4–1200–017; Phoenix Furnaces, Sheffield, UK) before installation. The FTB was connected with the glass surfaces standing in a vertical position, in order to avoid sedimentation on the surfaces. Connection to the sampled boreholes was made via plastic tubing. Unfortunately, the high pH made the polycarbonate brittle and all the FTBs cracked soon after installation. Only one FTB could finally be sampled. After these problems with the FTBs, flow-through cylinders (FTCs) were developed and used. The polycarbonate flow-through box (Fig. 1) was replaced with a flow-through cylinder made of polyethylene. It was connected to the sampled boreholes in the same manner as was previously done with the FTB.

The sampled FTB was connected to seepage locality M1, in the Adit A-6 tunnel, on 29 November 1999 and sampled on 7 December 1999. Two FTCs were connected to M1 and borehole D6 on 6 March 2000. The FTC connected to D6 was removed and initially sampled on 29 April 2000, while the one connected to M1 was sampled for the first time on 1 May. The M1 test pile was thereafter replaced with a new set and the FTC was reconnected. This flow cell was sampled a second time on 6 May and was then finally removed. The glass slides were removed from the FTB/FTC with a pair of heat-sterilized tweezers and rinsed with 5 ml of 0.2-μm-filtered groundwater. The slides were stored in sterile 50-ml polypropylene, screw-capped tubes containing a sterile preservation mixture consisting of 18 ml 0.2-μm-filtered groundwater from the respective sites and 2 ml acid-free formaldehyde (37% v/v).

Enumeration of unattached and attached cells

The total numbers of cells in the groundwater samples and the total number of cells attached to the glass slides were counted using blue-stain 4′6-diamino-2-phenylindole (DAPI). Acridine orange (AO) could not be used because of its high interference with calcite crystals, which created too high a background fluorescence. A standard protocol for fluorescent staining was used (Pedersen and Ekendahl 1990). Salt crystals from the groundwater on the filters

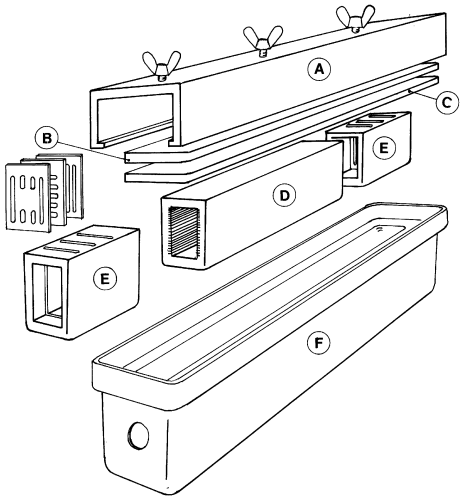


Fig. 1 The flow-through system. *A* Aluminum sliding lid, *B* aluminum lid, *C* lid, *D* test pile, *E* diffusors, *F* flow-through box

(diameter 13 mm) were dissolved by rinsing twice with 5 ml of 0.2-μm-filtered, double-distilled water heated to 40°C prior to staining. The filters were stained with 0.15 ml DAPI (10 μg ml^{-1}) for 10 min and counted under an Olympus BH-2 microscope (Olympus, Göteborg, Sweden) with ultraviolet (UV) filters for DAPI. For each filter 30 microscopic fields were counted, with each field measuring 80×80 μm = 0.0064 mm^2. The results were calculated as an average of two filters, with standard deviation (SD) as the error. The glass slides with attached microbes were rinsed with 5 ml of 0.2-μm-filtered, double-distilled water to remove possible unattached cells. The slides were then air-dried, stained with DAPI and counted as described above.

16S-ribosomal deoxyribonucleic acid diversity

Groundwater for DNA analysis was sampled from locations M5, M8, M17 and M18 in November 1994, and from locations M1, M3 and M5 in April 1995. The groundwater was collected directly from the boreholes or seeps in sterile 50-ml polypropylene, screw-capped tubes and was immediately deep-frozen with CO_2 ice and transported frozen to the laboratory in Göteborg, where 10 ml samples were filtered onto sterilized 0.2-μm pore-sized Nuclepore filters. Thereafter, DNA extraction, polymerase chain reaction (PCR) amplification, cloning and sequencing followed, using a procedure published elsewhere (Pedersen et al. 1996). A Pfu DNA polymerase (Stratagene) was used, which has a proof-reading ability that minimizes the chance of chimera formation.

PCR amplification of 16S rDNA was performed using a 27f/1492r primer set encompassing all bacteria. Partial sequences were imported and taxa selected for analysis with the ARB software environment for sequence data (Strunk et al. 1998). After masking of unambiguously alignable segments, phylogenetic trees were constructed including 258 nucleotides for analysis with PHYLIP (Felsenstein 1989). The reliability of branching points was determined by performing 1,000 bootstrap replications of the data, using both evolutionary distance with the Kimura-2-Parameter (Kimura 1980) correction, and maximum parsimony methods (Felsenstein 1985, 1989).

Cultivation

Media for cultivation were prepared with groundwater from the sampled sites. Growth factors such as carbon sources, electron acceptors, vitamins, trace elements and, in the case of anaerobic cultures, reducing agents, were added to the groundwater. This provided appropriate hyper-alkaline pH media for growth of different physiological groups of microbes. Since groundwater from Maqarin could not be obtained in advance, the addition of growth factors to empty bottles was initially done in the laboratory. The different types of Maqarin groundwater were then added at the sampling sites, serving both as in situ pH buffers and as inoculum. Bottles used for the cultures were 300-ml, hard plastic, screw-capped Erlenmeyer flasks (Nalgene) (November 1996) and 100-ml (November 1999) and 50-ml (May 2000) serum-type glass bottles (Bellco Biotechnology, Bie and Berntsen, Malmö, Sweden). For subcultures prepared from the enrichment cultures, 300-ml Erlenmeyer flasks (Nalgene) were used in 1996 and 50-ml serum bottles were used in 1999 and 2000. All culture vessels were acid-washed with 10% v/v HCl and sterilized by autoclaving prior to use.

During the 1996 expedition, a total of 100 ml of spring and seepage water was collected directly from each alkaline locality in two separate 300-ml sterile Erlenmeyer flasks (with screw lids) supplied with different carbon sources, and stored at room temperature. Extra water was collected in sterile, empty flasks for use during subculturing. Two different media were prepared and inoculated with alkaline water from the sampled localities: medium 1 contained 1 g/l peptone (Difco), 1 g/l yeast extract (Difco), and 5 g/l glucose. Medium 2 contained 1 g/l peptone (Difco), 1 g/l yeast extract (Difco), and 5 g/l lactate (dissolved in 5 ml distilled, sterile H_2O). When they arrived in Göteborg 3 days after sampling, the enrichments were put in a shaker for 27 days at room temperature. Subsequently, the total number of cells in the cultures was assayed using an AO direct-count technique (Pedersen and Ekendahl 1990). Enrichments with growing cells were subcultured in fresh media.

During the 1999 and 2000 expeditions, all water was collected in one container and distributed to the separate experiments. Briefly, the groundwater was sampled in sterile 3-liter hospital intravenous (IV) bags (Pharmacia and Upjohn; Letterkenny, Donegal, Ireland). The IV bag was connected through one of its three inlets to the sampled seepage (M1) or groundwater (D3 and D6) source via a plastic tube. After sampling, the used inlet was closed and permanently sealed. This procedure enabled sampling without exposure to air during the prolonged sampling times required owing to the low flow rates of the boreholes, springs and seepages. It was not possible to sample M3 with an IV bag. Instead, dripping groundwater at this location was collected in sterile plastic bottles. To the cultivation bottles were allocated 60 and 20 ml of sampled groundwater, in 1999 and 2000, respectively, from the IV bags using 60- and 20-ml sterile plastic syringes.

Aerobic media were prepared in acid-washed, sterile bottles sealed with blue silicon septa aluminum clamps (Chromacol, Trumbull, Conn., USA) that allowed diffusion of gases, including oxygen. All media components were added from sterile, aerobic stock solutions. With the exception of R2A, 1 ml trace element solution (Haveman et al. 1999) and 1 ml vitamin solution (Wolin et al. 1963) were added to all culture vessels. The final concentrations of added growth factors were as follows: standard aerobic media contained (mg/l) glucose, lactate, acetate or formate, 8 (1999) or 50 (2000). The CH_4 oxidation medium contained (per culture vessel) 5 ml methane (1 atm), which was added after inoculation, while the R2A medium (Reasoner and Geldreich 1985) contained (mg/l) peptone (Difco) 30/90, casamino acid (Difco) 30/90, yeast extract (Difco) 30/90, glucose 30/90, soluble starch 30/90, and sodium pyruvate 20/60 (1999/2000). Enrichment cultures were subcultured in the respective fresh media, with 20 ml of 0.2-μm sterile-filtered groundwater from the respective sampled site added.

Anaerobic media were prepared in acid-washed bottles placed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich., USA) with an atmosphere of approximately 4% H_2, 5% CO_2 and balance N_2 v/v. The bottles were left for at least 12 h in the anaerobic atmosphere before they were sealed with blue butyl rubber stoppers and thereafter sealed with aluminum clamps (Bellco Biotechnology, Bie and Berntsen, Malmö, Sweden). The bottles were then sterilized in an autoclave. All media components were added from sterile, anaerobic stock solutions and all contained 0.2 mg/l resazurin. Each bottle was also amended with 1 ml of a reducing solution consisting of 0.25 g/l cysteine-$HCl{\cdot}H_2O$ and 0.25 g/l $Na_2S{\cdot}9H_2O$. To all media except R2A were also added 1 ml trace

element solution and 1 ml vitamin solution, as described for aerobic cultures, and 8 mg (1999) or 50 mg (2000) glucose, lactate, acetate, pyruvate, formate, or trimethylamine (TMA) (1999 only) . The Fe(III)-reducer medium also contained 125–160 mg/l amorphous FeOOH, (Kostka and Nealson 1998). The manganese(IV)-reducer medium also contained MnO_2 [25–40/150–250 mg/l (1999/2000); Kostka and Nealson (1998)]. The sulfate reducer medium contained 17/50 mg/l Na_2SO_4 (1999/2000), and the nitrate reducer medium had 17 mg/l KNO_3 (1999). The fermentation medium was not amended with an external electron acceptor. The yeast/peptone media also contained 17 mg/l yeast extract (Difco; 1999); and 17 mg/l peptone (Difco). The R2A medium was prepared as described for aerobic media. Enrichment culture cells were subcultured in the respective fresh media, with 20 ml of 0.2-μm sterile-filtered groundwater from the respective sampled site added.

Assimilation of organic carbon sources by unattached cells

Acid-washed, sterile 50-ml serum bottles were prepared as described above for aerobic and anaerobic media. The 50-ml bottles were amended with the following radio-labeled carbon sources (all from Amersham Pharmacia Biotech, UK): D-[U-^{14}C]-glucose, 113 nM, specific activity 323 mCi/mmol; L-[U-^{14}C]-lactate, 239 nM, specific activity 152 mCi/mmol; [1,2-^{14}C]-acetate, 649 nM, specific activity 56 mCi/mmol; [^{3}H]-acetate, 1.1 nM, specific activity 970 mCi/mmol; L-[4,5-^{3}H]-leucine, 0.3 nM, specific activity 141 mCi/mmol. In addition to these bottles, during the 2000 expedition 500-ml glass bottles with butyl rubber stoppers and aluminum screw caps were prepared for aerobic and anaerobic batch assimilation tests. To the 500-ml bottles were added 16 ml of a D-[U-^{14}C]-glucose solution, 1,130 nM, specific activity 323 mCi/mmol. For each 500-ml bottle, 16 aerobic or anaerobic 50-ml bottles were prepared for subsequent uptake tests as described below.

To sets of eight 50-ml bottles amended with each of the radio-labeled carbon sources 10 ml groundwater was added from the respective IV bag. The groundwater was transferred using a sterile 10-ml syringe and needle. Groundwater from all sites was combined with carbon sources in discrete assimilation tests. The contents of two bottles from each combination were killed simultaneously with formaldehyde (3.4% final concentration v/v) at the start of each assimilation time series, as shown in the Results section. To the 500-ml bottles 160 ml of groundwater was added from the same IV bag that was used for cultivations. Two 11 ml aliquots of the isotope/groundwater mixture were removed from the batch bottles with sterile 20-ml syringes and added to 50-ml serum bottles containing 1 ml formaldehyde (3.4% final concentration v/v). This procedure was repeated in a time series, as shown in the Results section. The final concentration of radio-labeled glucose in this batch of 50-ml bottles was 113 nM, and the specific activity was 323 mCi/mmol.

The samples were first transported from the field sites and stored at room temperature at the University of Jordan, Amman. They were then shipped to the analysis laboratory by air cargo and arrived at Göteborg University on 28 December 1999 and 22 May 2000, respectively. All cultures were stored at room temperature.

Assimilation of organic carbon sources by attached cells

Assimilation studies of various organic carbon sources by attached microbes were performed as for unattached microbes, with the following modifications: to sterile 50-ml screw-capped polypropylene tubes isotope solutions were added in the laboratory. The final concentrations of the radio-labeled carbon sources were similar to those in the assimilation studies of unattached cells. To each such tube was added 18 ml of 0.2-μm-filtered groundwater from the respective sampling site. The glass slides were removed from the FTB or FTC with a pair of tweezers and rinsed with 5 ml of 0.2-μm-filtered groundwater and thereafter placed in the 50-ml tubes.

Scintillation procedure

The aluminum clamp seal was removed from each bottle for unattached assimilations. The bottle was vortexed and two 5.0 ml aliquots of the assimilation mixture were removed with a pipette. Each aliquot was filtered (0.2 μm), and the filters were rinsed with 2×2.5 ml of 0.2-μm-filtered groundwater from the respective sampling site. The filters were subsequently placed in scintillation vials (Poly-Q Vial; Beckman Instruments, Fullerton, Calif., USA) and left to dry. After drying, 15 ml scintillation cocktail for organic samples (NOCS104; Amersham Pharmacia Biotech) was added and the vial was sealed and counted using a Beckman LS 6000LL scintillation instrument (Beckman Instruments). Each glass slide from the attached assimilation tests was split into two or more pieces and added to a scintillation vial and allowed to air-dry. After drying, 15 ml scintillation cocktail for organic samples was added and the vial was sealed and counted as for the unattached cells. The mean assimilation per time and radio-labeled carbon source was calculated and subtracted from the control counts obtained from the killed controls at time 0. For all radioactive isotopes, 1 dpm = $1/2.22 \times 10^6$ μCi. Using this expression, the counts obtained were converted from dpm to μCi. As the specific activities of the isotopes used were known, the assimilation was calculated in moles. Finally, the mean assimilation per cell and hour was calculated from obtained data on the total number of cells in each bottle.

Results

Groundwater chemistry

All sampled sites except M17 and M18 were hyperalkaline water with a pH of between 12.1 and 12.7 (Table 2). The pH at M17 and M18 was close to neutral, 7.2 and 7.4, respectively, and there was lower conductivity. Compared to M17, M18 is believed to be mixed with some alkaline water (Milodowski 1996), as indicated by its higher calcium and alkalinity values than M17. The temperature varied from 16° to 27°C in all groundwaters, depending on the site and season. In general, November temperatures were lower than May temperatures, and Adit A-6 boreholes had lower temperatures than the ground surface springs and seepages. Nitrogen was present as nitrate in the surface spring (M5 and M6) and seepage (M3 and M8) waters but below detectable levels at the Adit A-6 sites and M18. Nitrogen was, however, present as ammonium and nitrite in most waters. There were high concentrations of calcium in all alkaline groundwater (480–1100 mg/l) and sulfate was generally high in those waters analyzed for sulfate (270–1500 mg/l). Phosphate was not detected, except at M18. The Western springs (M5 and M6) were characterized by higher alkalinity and concentrations of calcium, sulfate and chromium than seen at the Eastern spring seepage localities (M3 and M8) and the Adit A-6 boreholes (D3 and D6) and the Adit-6 seepage locality M1.

Total number of microbes

The total number of unattached cells for all expeditions is given in Table 3. The numbers obtained during the November expeditions were generally higher than the

Table 2 Chemistry results sampled and analyzed in November 1995 for M17, in 1996 for M6, M8, and M18, and in 1999 for M1, M3, M5, D3, and D6

Component	Units	Method	M1	M3	M5	M6	M8	D3	D6	M17	M18
Field pH			12.7	12.6	12.7	12.3	12.1	12.7	12.6	7.2	7.4
Conductivity at 25°C	mS/cm	SSEN 27888	586	669	948	927	483	573	573	–*	203
Chloride, Cl	mg/l	SS 028120	54	71	35	48	53	63	67	46	163
Alkalinity, HCO_3	mg/l	SS 028139	1 700	1 900	2 300	2 200	1 400	1 600	1 600	79	630
Calcium, Ca	mg/l	CR-A2IM	666	742	993	1 100	480	648	646	87	460
Chromium total, Cr	mg/l	CR-AIM	0.66	0.61	4.9	5.0	0.88	0.49	0.36	<0.01	<0.01
Iron, Fe	mg/l	FE-NI	0.20	<0.02	<0.02	0.12	<0.05	<0.02	<0.02	<0.01	0.8
Ammonium, NH_4	mg/l	SS 028134	<0.02	0.11	2.2	1.9	0.11	0.13	0.07	–	0.024
Nitrate, NO_3	mg/l	SS 028133–2	<1.0	9	6.6	8.1	2	<1.0	<1.0	–	<0.2
Nitrite, NO_2	mg/l	SSEN 26777	0.007	0.037	0.24	0.51	0.028	0.13	0.008	<0.04	0.018
Phosphate, PO_4	mg/l	SS 028126–2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.02	0.01
Sulfate, SO_4	mg/l	SS 028182	300	340	1 500	1 300	325	270	300	–	780

* – Not analyzed

Table 3 Total number of unattached cells for the sampled sites

Sampled site	Total number of cells (cells/ml)				
	November 1994	April 1995	November 1996	November 1999	May 2000
M1	–*	2.5×10^3	–	3.3×10^5	1.7×10^4
M3	–	2.0×10^4	–	7.8×10^3	7.0×10^3
M5	–	3.0×10^3	4.6×10^3	2.3×10^4	2.2×10^4
M6	–	–	2.4×10^4	–	–
M8	1.2×10^4	–	1.3×10^5	–	–
D3	–	–	–	6.9×10^4	n.s.
D6	–	–	–	4.3×10^5	1.6×10^4
M17	1.1×10^6	–	–	–	–
M18	6.4×10^6	–	–	–	–

* – Not sampled; n.s. = not significant

Table 4 Total number of attached cells, with standard deviation (SD) as error

Sampling site	Expedition	No. of sampling days	Total number of cells (cells/cm^2)
M1	November 1999	7	1.8×10^5 ($\pm1.0\times10^5$)
D6	May 2000	54	1.2×10^5 ($\pm0.7\times10^5$)
M1	May 2000	56	1.1×10^5 ($\pm0.4\times10^5$)
M1	May 2000	5	8.4×10^4 ($\pm6.4\times10^4$)

numbers obtained during April/May, possibly because there was more precipitation preceding the autumn sampling expeditions compared with the spring expeditions. Rainfall may have induced more groundwater flow, which transports more microbes into the aquifer system. The total numbers in the neutral groundwater from the boreholes M17 and M18 exceeded the alkaline waters ten- to a hundredfold. The Adit A-6 sites tended to show higher numbers than the ground surface springs and seepages. The SDs for the total number of unattached cells were in the range of 25–50%, with the highest SDs seen for the lowest numbers. These relatively high SDs were due to the fact that some of the numbers counted were close to the detection limit for the method (1×10^3 cells/ml), which increased the uncertainty of the counts. The total number counts of attached cells are shown in Table 4. The numbers of attached cells did not differ significantly between the sampling periods, or between the number of days' exposure to groundwater.

16S-ribosomal deoxyribonucleic acid diversity

A total of 82 clones were sequenced, distributed over six sample localities (Fig. 2). The diversity of clones found in neutral waters was double that found in hyper-alkaline groundwater. Of 37 sequenced clones, 15 unique sequences were found in the neutral water, but only 8 unique sequences out of 35 sequenced clones were found in the alkaline water. The majority of the sequences belonged to Proteobacteria. There was no overlap between clone libraries obtained from the alkaline water compared with M17. Two alkaline clones coincided with M18, which was believed to have a component of alkaline water similar to M3 and M8. The 25 clones representing clone group JA21agh were closely related to a *Pseudomonas* sp. ML-124 (AF139998) isolated from Mono Lake (California, USA), a well-studied saline and alkaline (pH 10) environment. The relationship of JA21agh to ML-124 was supported 100% by both evolutionary distance and parsimony bootstrap analysis, and these sequences shared 97.5% identical sequence positions.

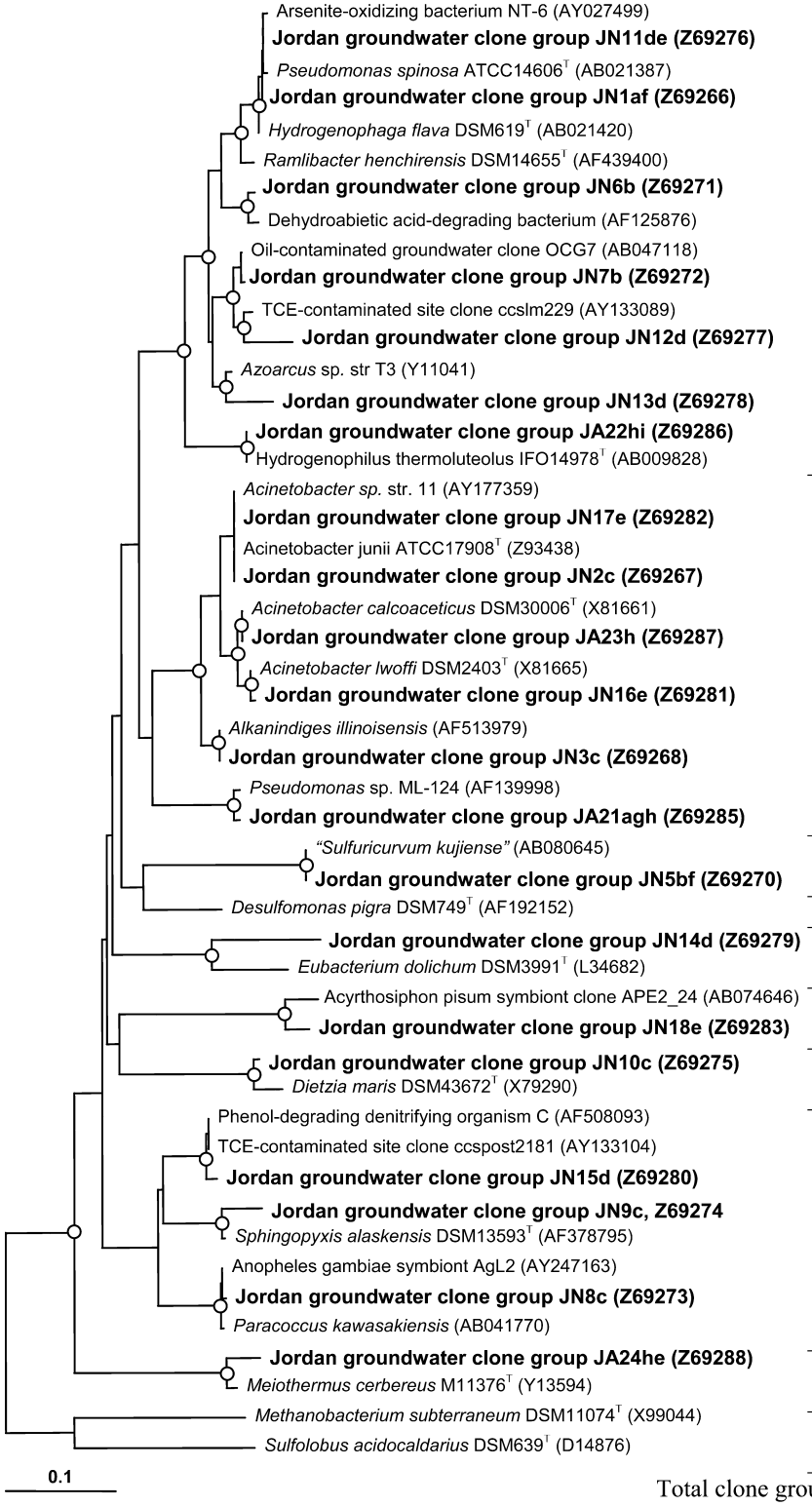
Cultivation

The cultures from the 1996 expedition were filtered and stained in the AO direct-counting (AODC) method.

►

Fig. 2 Neighbor-joining tree showing the 16S-rDNA relationships of the Maqarin clones to data available in the GenBank database. Bootstrap support of the branching topology of more than 75% from both evolutionary distance and maximum parsimony methods is indicated at the branching points of the tree by an *open circle*. The *scale bar* represents the number of nucleotide substitutions per position. *Gr* phylogenetic groupings, *EP Epsilonproteobacteria*, *DP Deltaproteobacteria*, *FM Firmicutes*, T7 candidate division TM7, *AC Actinobacteria*, *DT Deinococcus–Thermus*, *AR* domain Archaea

Enrichments to be subcultured were chosen where growth could be detected. Only a few of the cultures had grown and were subcultured in new medium (data not shown). They included glucose and lactate media inoculated with M8 water and the lactate medium inoculated with M1 water. Subcultures from M8 showed growth,



Gr		Alkaline			Neutral		Total
	M1	M3	M5	M8	M17	M18	
Betaproteobacteria							
Jordan groundwater clone group JN11de (Z69276)					9		9
Jordan groundwater clone group JN1af (Z69266)			9			1	10
Jordan groundwater clone group JN6b (Z69271)				1			1
Jordan groundwater clone group JN7b (Z69272)				2			2
Jordan groundwater clone group JN12d (Z69277)					1		1
Jordan groundwater clone group JN13d (Z69278)					1		1
Jordan groundwater clone group JA22hi (Z69286)		1	1				2
Gammaproteobacteria							
Jordan groundwater clone group JN17e (Z69282)					2		2
Jordan groundwater clone group JN2c (Z69267)					6		6
Jordan groundwater clone group JA23h (Z69287)		2					2
Jordan groundwater clone group JN16e (Z69281)					1		1
Jordan groundwater clone group JN3c (Z69268)					1		1
Jordan groundwater clone group JA21agh (Z69285)	10	6	9				25
EP							
Jordan groundwater clone group JN5bf (Z69270)				3		9	12
D							
FM							
Jordan groundwater clone group JN14d (Z69279)					1		1
T7							
Jordan groundwater clone group JN18e (Z69283)					1		1
AC							
Jordan groundwater clone group JN10c (Z69275)					1		1
Alphaproteobacteria							
Jordan groundwater clone group JN15d (Z69280)					1		1
Jordan groundwater clone group JN9c, Z69274					1		1
Jordan groundwater clone group JN8c (Z69273)					1		1
DT							
Jordan groundwater clone group JA24he (Z69288)		1					1
AR							
Total clone groups	1	4	3	3	13	2	26
Total clones	10	10	19	6	27	10	82

but not M1 subcultures did not. The pH of the cultures was monitored during incubation of the enrichment cultures and showed a decrease of approximately 0.5 units in the glucose-based medium and an increase of about 0.5 units in the lactate-based medium, compared with the sample water pH. The pH was initially high in the subcultures when using the Maqarin groundwater as water source, but subsequent preparations of synthetic media with a pH stable at around 12.5 were unsuccessful.

The total number of cells was used to calculate the number of generations produced (n) in the aerobic and anaerobic cultures obtained in 1999 and 2000. The numbers of generations (n) for the aerobic cultures, together with the pH values registered after the cultivation, are shown in Table 5. None of the cultures with CH_4 medium or H_2 medium inoculated in November 1999 showed visible growth. These media types were therefore not used in May 2000. Visible growth was obtained in several of the R2A cultures inoculated in November 1999. The R2A medium was not used in May 2000.

The pH values dropped by about 2 units for most of the May 2000 cultures, while staying closer to initial values in the November 1999 cultures (Table 5). There were 2–4 times more cultured cells in samples cultured from the November 1999 expedition than there were in those from the May 2000 expedition. The number of obtained generations (n) was higher in the November 1999 than in the May 2000 cultures for M5, and similar for M3 and D3 versus D6 between the expeditions, while it was lowest in M1 compared with all other cultures. The n depended strongly upon the number of cells in the groundwater used for inoculation. This suggests that there is an upper limit for the number of cells, which cannot easily be exceeded by growth, at or just above 1,000,000 cells per milliliter of the cultures. Subcultures from selected original cultures showed moderate growth with between three and five generations after about 190 days (data not shown).

The numbers of cultured cells in the anaerobic cultures were lower in M1 and D3 (Tables 6, 7) than those obtained for aerobic cultures. November 1999 cultures from M1 and D3 did not grow at all. The highest growth numbers and number of generations in anaerobic cultures were obtained from M5 cultures inoculated in November 1999. Otherwise, the number of generations was approximately the same as obtained for aerobic cultures. The pH dropped by between 1 and 2 units for all cultures. The smallest drop was observed in the M5 November 1999 cultures, which also showed the best growth. The anaerobic subcultures multiplied for approximately 4–7 generations from the start values (data not shown). The pH in these cultures dropped to values of about 2–4 units lower that the values observed in the respective sampled waters.

Assimilation of organic carbon sources by unattached microbes

The assimilation data appeared very scattered, which obscured trends. To overcome this problem, two criteria were set that had to be met in order for assimilation of the respective carbon source to be considered significant. Firstly, all repetitions (N) of the uptake series and batches were plotted against time and the slope of the linear regression line through the data points was calculated.

Table 5 Number of generations (n) for aerobic cultures inoculated with alkaline groundwater and total number of cells at the end of the incubations. Cultures were inoculated in November 1999 and May 2000 with starting values for cell numbers and pH shown next to the name of the sampled localities, and cultured for about 300 and 150 days, respectively. The pH values in the culture vessels after inoculation are shown for each culture

Medium	November 1999			May 2000		
	cells/ml	n	pH	cells/ml	n	pH
M1	2.9×10^5		12.7	1.3×10^4		12.5
R2A	1.2×10^6	2	11.5	–*	–	–
Glucose	1.2×10^6	2	11.0	2.7×10^5	4.3	8.0
Lactate	1.2×10^6	2	12.5	3.3×10^5	4.6	10.0
Acetate	8.8×10^5	1.6	12.5	4.3×10^5	5.0	11.0
Formate	8.2×10^5	1.5	12.0	3.8×10^5	4.8	11.0
M3	7.4×10^3		12.6	5.5×10^3		12.5
R2A	1.9×10^6	8.1	11.5	–	–	–
Glucose	2.0×10^6	8.2	10.5	8.7×10^5	7.3	10.0
Lactate	1.2×10^6	7.4	12.0	6.9×10^5	6.9	10.0
Acetate	7.6×10^5	6.8	12.0	5.8×10^5	6.7	9.0
Formate	1.2×10^6	7.3	12.5	3.1×10^5	5.8	10.0
M5	2.1×10^3		12.6	1.7×10^4		12.6
2A	1.7×10^6	9.7	12.5	–	–	–
Glucose	1.3×10^6	9.3	10.5	2.3×10^5	3.7	11.0
Lactate	1.8×10^6	9.8	12.0	6.6×10^5	5.3	12.0
Acetate	1.2×10^6	9.2	12.0	4.2×10^5	4.6	12.0
Formate	1.1×10^6	9.1	12.0	6.6×10^5	5.3	12.0
D3/D6	3.8×10^5		12.7	1.3×10^4		12.6
R2A	1.2×10^6	4.3	10.0	–	–	–
Glucose	9.2×10^5	3.9	10.0	3.8×10^5	4.9	10.0
Lactate	1.0×10^6	4.0	12.0	2.1×10^5	4.1	10.5
Acetate	7.6×10^5	3.6	12.0	5.2×10^5	5.4	11.0
Formate	7.4×10^5	3.6	12.0	6.7×10^5	5.7	10.5

* Not sampled

Table 6 Number of generations (*n*) for anaerobic cultures from collection sites M1 and M5 inoculated with alkaline groundwater and total number of cells at the end of the incubations. Cultures were inoculated in November 1999 and May 2000 with start values for cell numbers and pH as shown, and cultured for about 300 and 150 days, respectively. The pH values in the culture vessels after inoculation are shown for each culture

Medium	Electron acceptor	M1						M5					
		November 1999			May 2000			November 1999			May 2000		
		cells/ml	*n*	pH	cells/ml	*n*	pH	cells/ml	*n*	pH	cells/ml	*n*	pH
Start values		2.9×10^5		12.7	1.4×10^4		12.5	2.1×10^4		12.7	1.7×10^4		12.7
R2A	–	3.2×10^5	0.1	10.0	–*	–	–	2.8×10^6	10.4	12.0	–	–	–
Glucose	Mn	2.8×10^5	–0.1	11.0	4.6×10^5	5.1	10.0	2.9×10^6	10.5	12.0	1.2×10^5	2.9	11.0
Lactate	Mn	2.1×10^5	–0.5	11.0	2.1×10^5	3.9	11.0	2.4×10^5	6.9	12.0	1.8×10^5	3.4	11.0
Acetate	Mn	1.2×10^5	–1.2	11.0	1.1×10^5	2.9	11.0	3.3×10^5	7.3	12.0	1.5×10^5	3.2	11.5
Formate	Mn	1.2×10^5	–1.2	11.0	2.2×10^5	4.0	11.5	1.3×10^5	6.0	12.0	1.7×10^5	3.3	11.5
Pyruvate	Mn	2.6×10^5	–0.2	11.0	5.3×10^5	5.2	10.5	1.2×10^6	9.2	12.0	1.7×10^5	3.3	12.0
Glucose	Fe	–	–	–	–	–	–	–	–	–	–	–	–
Lactate	Fe	–	–	–	5.0×10^5	5.2	11.5	–	–	–	–	–	–
Pyruvate	Fe	2.3×10^5	–0.4	11.0	2.2×10^5	4.0	11.0	8.0×10^5	8.6	11.0	2.7×10^5	4.0	12.0
Glucose	SO_4^{2-}	1.2×10^5	–1.4	11.0	1.6×10^5	3.6	10.5	2.6×10^5	7.0	12.0	1.7×10^5	3.3	11.0
Lactate	SO_4^{2-}	–	–	–	–	–	–	4.0×10^5	7.6	12.0	2.2×10^5	3.7	12.0
Pyruvate	SO_4^{2-}	1.9×10^5	–0.6	11.0.	1.5×10^5	3.5	12.0	–	–	–	–	–	–
TMA and methanol	SO_4^{2-}	1.2×10^5	–1.4	11.0	–	–	–	–	–	–	–	–	–

* – Not sampled;
TMA trimethylamine

Table 7 Number of generations (*n*) for anaerobic cultures from localities D3 and D6 inoculated with alkaline groundwater and total number of cells at the end of the incubations. Cultures were inoculated in November 1999 and May 2000 with start values for cell numbers and pH as shown, and cultured for about 300 and 150 days, respectively. The pH values in the culture vessels after inoculation are shown for each culture

Medium	Electron acceptor	D3			D6		
		November 1999			May 2000		
		cells/ml	*n*	pH	cells/ml	*n*	pH
Start values		3.8×10^5		12.7	1.3×10^4		12.6
R2A	–	3.4×10^5	–0.2	11.0	–*	–	–
Glucose	Mn	6.5×10^5	0.8	11.0	5.8×10^5	5.5	10.5
Lactate	Mn	2.5×10^5	–0.6	11.0	2.3×10^5	4.2	11.5
Acetate	Mn	2.0×10^5	–0.9	11.0	1.2×10^5	3.2	11.0
Formate	Mn	3.2×10^5	0.3	11.0	4.6×10^5	5.2	11.0
Pyruvate	Mn	5.7×10^5	0.6	11.0	3.8×10^5	4.9	11.5
Glucose	Fe	–	–	–	3.1×10^5	4.6.	11.0
Lactate	Fe	–	–	–	–	–	–
Pyruvate	Fe	2.0×10^5	–0.9	11.0	1.6×10^5	3.7	11.0
Glucose	SO_4^{2-}	1.8×10^5	–1.1	11.0	1.6×10^5	3.6	10.5
Lactate	SO_4^{2-}	–	–	–	–	–	–
Pyruvate	SO_4^{2-}	2.1×10^5	–0.9	11.0	1.7×10^5	3.8	11.5
TMA and methanol	SO_4^{2-}	1.7×10^5	–1.2	11.0	–	–	–

* – Not sampled;
TMA trimethylamine

Secondly, the average assimilation per cell and hour was calculated for all samples (*N*) and subtracted from the start value for the killed controls. Assimilation was regarded as significant only for series and batches that had a positive slope and a positive average uptake per cell and hour.

The samples from Adit A-6 (M1, D3, and D6) all showed significant assimilations (Table 8). The anaerobic series assimilated more diversely than did the aerobic ones. All tested carbon sources scored significantly in the anaerobic tests, while in the aerobic series glucose and lactate were significant only with D6 groundwater. Glucose assimilation was also significant in the M3 series (data not shown). All M5 series were negative in one or both of the defined test criteria (data not shown). Glucose seemed to be the preferred carbon source, since assimilation was found to be significant in seven out of the 11 series investigated, followed by six acetate, three lactate and one leucine series assimilation (Table 8). The same trend was obtained using a balanced comparison excluding one of the glucose and the acetate series, tested pair-wise with different isotope configurations. This approach gave five glucose, five acetate, four lactate and one leucine assimilation series.

Assimilation of organic carbon sources by attached microbes

The assimilation data from attached microbes appeared less scattered than were those from the unattached series. However, for consistency's sake, the same approach as for unattached microbes was applied here (Table 9). Three acetate, two lactate, one glucose and one leucine assimilation series were regarded to be significant (Table 9).

16S-ribosomal deoxyribonucleic acid accession numbers

The nucleotide sequence data reported in this paper appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers

Table 8 In situ assimilation of added carbon sources by unattached microorganisms in M1, D3 and D6 groundwater. The experiments were started on 5 December 1999 with 3.4×10^5 cells ml^{-1} (M1) and on 4 May 2000 with 1.7×10^4 cells ml^{-1} (M1), and run for 11 h and 6 h, respectively, as well as on 26 November 1999 with 3.4×10^5 cells ml^{-1} (D3) and on 4 May 2000 with 1.7×10^4 cells ml^{-1} (D6), and run for 7 h. The average slope of all results is shown as negative or positive and the assimilation is calculated per cell. N shows the number of bottles used for each experiment, excluding the four bottles used for each background determination. The pH of the groundwater used for the assimilations was 12.7 in November 1999 (M1), 12.5 in May 2000 (M1), 12.7 in November 1999 (D3), and 12.6 in May 2000 (D6)

Carbon source	November 1999 (M1)			May 2000 (M1)			November 1999 (D3)			May 2000 (D6)		
	N	Slope*	amol/(cell h)	*N*	Slope*	amol/(cell h)	*N*	Slope*	amol/(cell h)	*N*	Slope*	amol/(cell h)
Aerobic series												
[^{14}C]-acetate	12	–	≤0	12	–	≤0	12	–	≤0	12	–	≤0
[^{3}H]-acetate	12	–	≤0	12	–	≤0	12	–	≤0	12	–	≤0
[^{14}C]-glucose	12	–	≤0	28	–	≤0	12	–	≤0	28	+	≤0
[^{14}C]-glucose (batch)	12	**	**	28	+	≤0	**	**	**	28	+	0.020
[^{14}C]-lactate	12	–	0.021	12	–	≤0	12	–	0.266	12	+	0.001
[^{3}H]-leucine	12	–	≤0	12	–	≤0	12	–	≤0	12	–	≤0
Anaerobic series												
[^{14}C]-acetate	12	–	2.840	12	+	0.066	12	+	6.758	12	+	0.0289
[^{3}H]-acetate	12	+	0.004	12	–	≤0	12	+	0.001	12	+	≤0
[^{14}C]-glucose	12	+	2.348	28	–	0.003	12	+	18.59	28	+	0.330
[^{14}C]-glucose (batch)	12	**	**	28	+	≤0	**	**	**	28	+	0.600
[^{14}C]-lactate	12	+	0.505	12	+	0.141	12	–	4.635	12	+	0.094
[^{3}H]-leucine	12	+	≤0	12	–	≤0	12	+	0.001	12	+	≤0

* Negative (–) or positive (+) value
** Not sampled

Table 9 In situ assimilation of added carbon sources by attached microorganisms in M1 groundwater. The experiments were started on 7 December 1999 with 1.8×10^5 cells cm^{-2} and run for 7 h and on 1 May 2000 with 1.1×10^5 cells cm^{-2}, as well as on 6 May 2000 with 8.4×10^4 cells cm^{-2} and run for 6 h. The average slope of all results is shown as negative or positive and the assimilation is calculated per cell. *N* shows the number of bottles used for each experiment, excluding the four bottles used for each background determination. The pH of the M1 groundwater used for the assimilations was 12.7 in November 1999 and 12.5 in May 2000

Carbon source	November 1999			1 May 2000			6 May 2000		
	N	Slope*	amol/(cell h)	*N*	Slope*	amol/(cell h)	*N*	Slope*	amol/(cell h)
Aerobic series									
[^{14}C]-acetate	6	+	0.531	6	+	0.810	6	+	2.403
[^{3}H]-acetate	6	+	≤0	6	+	≤ 0	6	–	≤0
[^{14}C]-glucose	6	+	9.544	6	–	≤ 0	6	+	≤0
[^{14}C]-lactate	6	–	≤0	6	+	0.164	6	+	1.256
[^{3}H]-leucine	6	–	≤0	6	+	≤ 0	6	+	0.001

* Negative (–) or positive (+) value

Z69266–Z69288, corresponding to JN1af–JN19e and JA21gh–JN24he, respectively.

Discussion

Alkaline environments

A combination of geological and climatic features give rise to high surface water alkalinity. Low-calcium-concentration environments are characterized by high concentrations of sodium carbonate. Ions, particularly sodium and chloride, become concentrated owing to evaporation and this eventually results in a saline, high-pH environment. When the concentration of carbonate is greater than that of Ca^{2+} and Mg^{2+}, alkalinity rises, usually with Na^+ as the dominant cation (Grant et al. 1990). Soda and desert lakes constitute the most stable, naturally occurring alkaline environments, with pH values exceeding 11.5. The most extensively studied soda lakes are those of the East African Rift valley (Grant et al. 1990; Jones et al. 1998), but soda lakes are also found in central Asia (Balashova and Zavarsin 1980). Soda lakes are among the most productive naturally occurring aquatic environments in the world because of their almost unlimited supply of CO_2, high temperature, and high daily light intensity. The predominance of microorganisms, particularly phototrophs (*Cyanobacteria*), is striking (Grant et al. 1990). Jones et al. (1998) recently reviewed the diversity of soda lakes and a phylogenetic study on soda lakes has been published by Duckworth et al. (1996).

Alkaline soda lakes with low calcium and high sodium concentrations have little in common with the environment that will develop in underground cementitious repositories. The water–cement interaction in the repository will cause leaching of calcium and thereby create a high-calcium alkaline environment. Naturally occurring high-calcium alkaline environments have only been found underground, in groundwater (Grant et al. 1990). High-calcium-concentration groundwater with a pH exceeding 11 has been identified in some geological locations in the former Yugoslavia and Oman (Barnes et al. 1978; Bath et al. 1987) as well as Jordan (Barnes et al. 1982). Only two of these sites, Oman and Jordan (West et al. 1992; Coombs et al. 1994; Pedersen et al. 1997), have thus far been the subject of microbial studies. Most of the reported studies have been performed as part of the Maqarin Natural Analogue Project.

In a high-calcium environment the amounts of Ca^{2+} and/or Mg^{2+} present exceed that of carbonate. Normally, water rapidly becomes saturated with respect to Ca^{2+}, resulting in precipitation of calcite ($CaCO_3$) and inhibition of the genesis of alkaline brine due to the lack of carbonate. However, if the host rock contains either of the two primary minerals olivine or pyroxene, high-calcium alkaline groundwater can be produced. In near-surface zones, CO_2-containing water decomposes these minerals and Ca^{2+} and OH^- are released. Initially this will lead to calcite precipitation, but the carbonate soon becomes depleted, after which $Ca(OH)_2$-dominated brine is produced. The alkalinity will then be caused by OH^- which will be in equilibrium with solid $Ca(OH)_2$ in the absence of carbonate. In addition, these chemical conditions are profoundly reducing in most cases (Bath et al. 1987; Grant et al. 1990).

In Jordan, at least three different sites with hyper-alkaline groundwater have been identified. In the analogy they appear to represent three different stages in the theoretical evolution of a cementitious repository. The three stages are: (1) early, active, high-pH Na/KOH leachates (M5 and M6, Western springs, Maqarin) (see Table 2); (2) intermediate, active, lower-pH $Ca(OH)_2$-buffered leachates (M1, D3 and D6, Adit A-6; M3, M8, Eastern spring seepages, Maqarin) (see Table 2); and (3) late, inactive, lower-pH silica-dominated leachates (Daba, Central Jordan). The latter site, because of an absence of water, could not be used for studies of active microorganisms. The pH in Maqarin is believed to be the highest ever reported from a natural environment. Maqarin also appears to be unique in the sense that the hyper-alkaline groundwater in the area is a product of leaching of an assemblage of natural cement minerals produced as a result of high-temperature/low-pressure metamorphism of marls and limestone.

16S-ribosomal ribonucleic acid diversity

The types of microbes in the Maqarin waters identified by 16S-rRNA sequencing (Fig. 2) could not be linked to species that have been reported from surface alkaline environments by Duckworth et al. (1996). These sequences are, however, from surface water environments with intensive solar radiation, which are high in carbonate and calcium concentrations, and which are consequently very different from the Maqarin localities. In Maqarin, it is plausible that microorganisms in the surrounding groundwater have adapted over a long time to the alkaline conditions under the cement zone. The absence of deeply branching 16S rRNA species supports the assumption that the populations in Maqarin have adapted to the Maqarin environment in relatively modern evolutionary times. If this is true, it cannot be excluded that similar adaptations may occur in high-pH areas of future radioactive waste repositories.

Total number of cells

The objective of counting cell numbers was to obtain the total numbers of unattached and attached, viable and non-viable microbes in groundwater from Adit A-6 boreholes, the Western and Eastern springs and the boreholes M17 and M18. All data ranged within about three orders of magnitude from 2.5×10^5 to 6.4×10^6 cells/ml of groundwater (Table 3). The numbers were generally highest in November 1999. The database of total numbers of microorganisms in Fennoscandian Shield groundwater is large and comprises analytical data from more than 100 sites at depths down to 1,500 m in deep crystalline bedrock (Pedersen 2001). The numbers range from about 1×10^3 to 5×10^6 cells/ml of groundwater. The highest numbers tend to come from shallow sites. The data from Maqarin alkaline waters (Table 3) cluster around the lower part of the Fennoscandian Shield numbers, suggesting that microbial abundance and activity are lower in Maqarin compared with circum-neutral, hard-rock groundwater.

The total numbers of attached cells did not exceed 1.8×10^5 cells/cm^2 (Table 4), irrespective of whether the exposure to the alkaline water was 1 week or 8 weeks. There can be two explanations for this. One is that the attaching microbes were inactive and attached only as a result of surface interaction forces (Marshall 1984). Attached microbes depend on transport of nutrients to the surface to be able to proliferate. The flow in the FTB/FTC may have been too low to allow growth of attaching microbes. Surfaces introduced in slowly flowing Fennoscandian rock groundwater commonly collect about 10^5 attaching cells/cm^2 within the first week. Thereafter, there is a slow growth and the numbers may increase to about 10^7 cells/cm^2 after 8 weeks or more (Pedersen 2001). This growth phase could not be observed in the M1 and D6 FTB/FTCs (Table 4).

Overall, the low total numbers of unattached cells relative to Fennoscandian Shield groundwater and the absence of attached cell growth suggest that the microorganisms found in the investigated alkaline waters are inactive, though not necessarily dead. Cultivation

experiments were performed to assess the live/dead status of the microorganisms found in the hyper-alkaline groundwaters of Maqarin (see below).

Cultivability

The objective of these cultivation experiments was to cultivate, at in situ pH, the main physiological groups of microbes expected to be present at Adit A-6 and the Western and Eastern springs using different carbon and energy sources. The first attempt, in 1996, to cultivate microorganisms from Maqarin was not very successful; only two out of 14 cultures grew. It was deduced that the concentrations of nutrients that had been applied were too high and at the next attempt, in November 1999, the concentration of organic carbon was lowered from one or a few grams per liter to 10–50 mg/l. This was an adaptation to the actual concentrations of organic carbon in the Maqarin alkaline waters, reported to be in the order of some tens of milligrams per liter (Milodowski 1996).

All aerobic cultures from the 1999 and 2000 expeditions increased in numbers and multiplied from two to, at the most, ten times (Table 5). These results show that at least some of the total numbers of microorganisms in the alkaline waters were viable and able to grow. The pH remained high and close to the ambient values in the November 1999 cultures and in many of the May 2000 cultures, suggesting that the observed growth occurred at hyper-alkaline pH. Most November 1999 cultures reached around 10^6 cells ml^{-1}, while most May 2000 cultures stopped at about 5×10^5 cells/ml, irrespective of the number of cells when culturing commenced, which varied from 2.1×10^3 to 2.9×10^5 cells/ml. The culture media used would most probably have produced numbers at least 100 times higher if inoculated with neutral pH lake or stream water. It appears as if the high pH did put a limitation on the highest number that could develop in the cultures. A low highest obtainable cultivable number is not unique; for instance, the iron oxidizing-bacterium *Gallionella ferruginea* never grows to numbers greater than 2×10^6 cells/ml when cultured in the laboratory (Hallbeck and Pedersen 1990). It is not clear why this is so, but it can be assumed that the cells expel signal substances that terminate growth at a specific concentration, which is defined by evolution. More cells than this number would result in ''overpopulation'' of the environment, which would pose a threat to the survival of the whole population. Therefore, the cultivation results suggest that there is a maximum number range of cells that could be reached by growth in the hyper-alkaline cultures, at between 5×10^5 and 1×10^6 cells/ml. Subculturing was done with the aim of enriching the microorganisms that adapted to the culture conditions. However, subculturing did not result in any numbers higher than this assumed maximum number range. Instead, the numbers obtained were close to those obtained in the first cultures. Limited growth was therefore observed again.

Anaerobic growth was not obtained in the M1 and D3 cultures of November 1999 (Table 6). This was possibly because the number of cells in the hyper-alkaline waters used for inoculation was equal to, or almost equal to, the supposed maximum number of cells that can be obtained via growth in the hyper-alkaline cultures employed. Growth would then have been restricted directly at inoculation. The best anaerobic growth was obtained with M5 water in November 1999. R2A, glucose and also pyruvate showed good growth relative to the other investigated carbon sources in all tested waters. As with the aerobic cultures (Table 5), the overall numbers obtained in the anaerobic subcultures (Table 6) were, however, close to those obtained in the first cultures.

In general the culturing task proved that microorganisms can be cultured under hyper-alkaline conditions. Both aerobic and anaerobic conditions were found to support growth. However, although the obtained growth was significant, it was not vigorous.

Assimilation activity

The objective of the assimilation task was to investigate whether microorganisms flowing with alkaline groundwater at Adit A-6 and the Western and Eastern springs are active in situ, i.e., whether they could assimilate various organic carbon compounds under in situ conditions in the flowing seepage and groundwaters.

Cultivation of microbes from environmental samples is frequently carried out and the results obtained are useful. However, it is also commonly recognized that many microorganisms in the environment do not grow in cultures. In other words, a positive result from cultivation demonstrates that living organisms were present but a negative cultivation result is not conclusive: there may have been organisms in the sample that simply did not grow under the conditions offered. To circumvent this problem with the Maqarin samples, activity assays were employed. This methodology assumes that if microorganisms in a sample are living and active, they will assimilate introduced carbon sources, and this assimilation is not growth-dependent, as are cultures. Assimilation studies have previously been successfully performed with samples from deep groundwater (Pedersen and Ekendahl 1992a, 1992b; Ekendahl and Pedersen 1994).

Assimilation was more frequent under anaerobic than aerobic conditions (Table 8). This suggests that the microorganisms in the hyper-alkaline waters of Maqarin are adapted to conditions with little or no dissolved oxygen. Some hydrochemical results (data not shown) have indicated large concentrations of dissolved oxygen, but the accuracy of the oxygen analysis was possibly disturbed by the slow flow of water. It is very difficult to sample for oxygen in very slowly flowing water because oxygen from the atmosphere easily mixes with the sample unless a fast flow flushes the sample container. In

Maqarin, most of the water sampled was dripping rather than flowing fast, which adds a large amount of uncertainty to the obtained oxygen readings. The presence of nitrite in the samples (Table 2) may indicate that anaerobic microbial nitrate respiration has been ongoing, either before the water reached the cement zone, or all along the flow path. Nitrate respiration is an anaerobic metabolic process that operates in the absence of oxygen, and this suggests that the Maqarin aquifers are anaerobic.

Both [^{3}H]-acetate and [^{14}C]-acetate were used in the assimilation tests. In several cases where [^{3}H]-acetate assimilation was negative [^{14}C]-acetate resulted in positive assimilations. This is probably explained by the much higher concentration of [^{14}C]-acetate, namely 6 490 nM, compared with that of [^{3}H]-acetate, namely 11 nM. The scintillation is about 500 times more sensitive to ^{3}H than it is to ^{14}C due to the much shorter half-life of ^{3}H; however, this sensitivity can be masked by isotope dilution with naturally present acetate in the water studied. The discrepancy between the two differently labeled acetates suggests that there may have been some acetate in the spring water.

The total number of cells per sample may also have had a significant influence on the assimilation results, assuming a constant percentage of viable cells in the different samples. The more viable the cells, the greater the total uptake that will generally occur. In our study, M1 November 1999 and M3 November 1999 cultures had higher total numbers of cells and also, more assimilation per cell of different sources. The same situation was observed for D3 compared with D6.

As a whole, the assimilation investigations strongly suggest that some or all of the microbes in the studied Maqarin waters were viable and responded to introduced carbon sources by assimilation. The assimilations all occurred under hyper-alkaline conditions, suggesting that microbial activity is possible under these extreme conditions, such as in the Maqarin alkaline waters.

An additional task of the assimilation studies was to investigate whether microorganisms at Adit A-6 would attach to surfaces and show metabolic activity, i.e., whether they assimilate organic carbon sources and metabolize them in situ at ambient pH (i.e., pH 12–13). As mentioned above, microorganisms did attach to the surfaces in the FTB/FTC systems, but they did not proliferate over time. The assimilation results were positive for all carbon sources tested with M1 water (Table 9). A comparison of these results with the results for unattached microorganisms (Table 8) revealed that the attached cells seemed to have been more active that the unattached ones. This result is consistent with what has previously been found in circum-neutral pH hard-rock groundwater (Pedersen and Ekendahl 1992a, 1992b; Ekendahl and Pedersen 1994). Attachment to a surface is commonly looked upon as a more favorable state of living compared with cells in a planktonic state, at least in systems with flowing water. This is because microorganisms will be exposed to more nutrients over time when attached in a flowing system, than when flowing with the water.

Microbial activity in low- and intermediate-level radioactive waste repositories

This study demonstrates that microorganisms from the hyper-alkaline waters of Maqarin could be cultured at low but significant numbers and that some were metabolically active under hyper-alkaline conditions. However, the growth and activity found were not vigorous. Rather, slow growth was related to low numbers and a generally low metabolic in situ activity. This implies that microbial activity will be low during the hyper-alkaline phase of a LILW repository. However, such repositories will not be sterile, so that when the pH drops over time, microorganisms that are present may increase their activity.

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