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Bacterial Diversity in the Hyperalkaline Allas Springs (Cyprus), a Natural Analogue for Cementitious Radioactive Waste Repository

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The biogeochemical gradients that will develop across the interface between a highly alkaline cementitious geological disposal facility for intermediate level radioactive waste and the geosphere are poorly understood. In addition, there is a paucity of information about the microorganisms that may populate these environments and their role in biomineralization, gas consumption and generation, metal cycling, and on radionuclide speciation and solubility. In this study, we investigated the phylogenetic diversity of indigenous microbial communities and their potential for alkaline metal reduction in samples collected from a natural analogue for cementitious radioactive waste repositories, the hyperalkaline Allas Springs (pH up to 11.9), Troodos Mountains, Cyprus. The site is situated within an ophiolitic complex of ultrabasic rocks that are undergoing active low-temperature serpentinization, which results in hyperalkaline conditions. 16S rRNA cloning and sequencing showed that phylogenetically diverse microbial communities exist in this natural high pH environment, including *Hydrogenophaga* species. This indicates that alkali-tolerant hydrogen-oxidizing microorganisms could potentially colonize an alkaline geological repository, which is predicted to be rich in molecular H_2, as a result of processes including steel corrosion and cellulose biodegradation within the wastes. Moreover, microbial metal reduction was confirmed at alkaline pH in this study by enrichment microcosms and by pure cultures of bacterial isolates affiliated to the *Paenibacillus* and *Alkaliphilus* genera. Overall, these data show that a diverse range of microbiological processes can occur in high pH environments, consistent with those expected during the geodisposal of intermediate level waste. Many of these, including gas metabolism and metal reduction, have clear implications for the long-term geological disposal of radioactive waste.

Keywords: hyperalkaline conditions, intermediate level radioactive waste disposal, metal reduction, serpentinization

Introduction

The United Kingdom has an extensive legacy of radioactive waste from more than 60 years of civil and military nuclear technology, and this inventory will only increase with the decommissioning of old facilities and the development of new nuclear power options. In 2008, deep geological disposal of intermediate- and high-level radioactive wastes, which are the most hazardous components of the UK waste legacy, was adopted as UK Government policy (Department for Environment, Food & Rural Affairs (DEFRA) 2008) and this situation is echoed throughout Europe and globally. Currently, in the United Kingdom, the proposed concept for intermediate level radioactive waste (ILW) disposal is based on a multibarrier system. The current generic model for disposal is that ILW will be grouted in steel containers emplaced in the geological disposal facility and eventually the waste will be sealed in the deep subsurface with a cementitious backfill (DEFRA 2008; NDA 2010a; Nirex 2003). When the host environment becomes saturated with groundwater, the highly alkaline conditions that will develop are intended to minimize radionuclide solubility and thus the risk of radionuclide transport to the biosphere (NDA 2010a; Nirex 2003). However, the biogeochemical gradients that will develop across the interface between the highly alkaline deep cementitious geological disposal facility and the geosphere are poorly understood in terms of their impact on the long-term

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performance of the geological disposal facility. It is now recognized that the wastes, which can include nitrate, iron, metal oxides, radionuclides, H_2 gas (produced by the corrosion of steel waste) and organic carbon (e.g., cellulose-derived compounds) are likely to create conditions favorable for microbial growth. Microbial transformations of radionuclides under reducing conditions are well reported at circumneutral pH (e.g., Anderson et al. 2003; Bernier-Latmani et al. 2010; Boyanov et al. 2011; Newsome et al. 2014). However, very little is known about microorganisms that can potentially reduce metals and radionuclides under alkaline conditions, although they may potentially control the speciation and solubility of several key radionuclides via complexation with ligands produced by microbial metabolism, by reduction or by mineralization processes. Therefore, understanding their activities will be critical in underpinning any safety case for a cementitious geodisposal facility.

To extrapolate results from laboratory and field experiments to the repository, the study of natural analogues of cementitious-based geological repositories is of high importance (Alexander and Milodowski 2011). Several sites with active hyperalkaline (pH $>$ 10) groundwater systems have been studied as natural analogues for cementitious geological repositories. These include the Maqarin site in northern Jordan (Nagra 1992), the Semail ophiolitic complex in Oman (Bath et al. 1987), the Troodos ophiolite in Cyprus (Alexander and Milodowski 2011), and the Zambales ophiolite in the Philippines (Alexander et al. 2008). Ophiolites are sequences of mafic and ultramafic rocks representing ancient oceanic crust and upper mantle rocks that have been tectonically emplaced onto a passive continental margin (e.g., Troodos in Cyprus and Semail in Oman) or have uplifted within subduction zone accretionary complexes or subduction complexes, such as the Josephine and Coast Range ophiolites of California (Harper et al. 1994; Shervais et al. 2004).

Highly alkaline groundwaters are often encountered within these systems and are associated with the reaction of percolating water with olivine and pyroxene within the mafic and ultramafic rocks to form serpentine minerals (serpentinization). Serpentinization occurs along several pathways (Moody 1976), although it is the low-temperature serpentinization (e.g., Barnes and O'Neil 1969; Barnes et al. 1972) that is particularly relevant to the active serpentinization sites in Cyprus, Oman and the Philippines. In this case, $Mg(HCO_3)_2$-type meteoric groundwaters react with the ultramafic rocks of the ophiolite in an essentially open system and produce highly alkaline Ca-rich (spring) waters (generally between pH 10 and 11) rich in K^+, Na^+, Ca^{2+}, Mg^{2+}, and results in the production of serpentine minerals, magnetite, hydroxide, hydrogen (H_2) and methane (CH_4) gas, depending on the mineralogy of the site (Blank et al. 2009; Schulte et al. 2006).

Active serpentinization systems in ophiolites are sometimes studied as analogues for potential early ecosystems on Earth and Mars (Russell et al. 2010; Schulte et al. 2006; Sleep et al. 2011). However, most studies on serpentinization and the affiliated microbial communities have focused on deep-sea hydrothermal fields (Brazelton et al. 2006; Brazelton et al. 2012; Kelley et al. 2005), where it has been shown that serpentinization can generate large volumes of hydrogen gas (H_2), variable quantities of methane (CH_4) and low molecular weight compounds (McCollom and Seewald 2007; Proskurowski et al. 2008), and that microbial communities are dominated by methane- and sulfur-metabolizing *Bacteria* and *Archaea* (Brazelton et al. 2006).

Microbial studies in serpentinization-driven (terrestrial) ophiolitic environments have been carried out only recently, at the Cabeço de Vide aquifer in Portugal (Tiago et al. 2004; Tiago and Veríssimo 2013), the Del Puerto ophiolite in California (Blank et al. 2009), the Tablelands ophiolite in Canada (Brazelton et al. 2012; Brazelton et al. 2013), and the Leka ophiolite in Norway (Daae et al. 2013). Nevertheless, no comprehensive microbiological studies have been carried out yet on the ophiolitic sites that have been studied as natural analogues to geological repositories for radioactive wastes, despite the potential significance of these sites in informing radioactive waste disposal options. Furthermore, the potential for microbial metal reduction in terrestrial alkaline serpentinization-associated systems has not been explored to date and our knowledge on alkaline microbial metal reduction is restricted to only a few isolated microorganisms, such as *Alkaliphilus metalliredigens* QYMF (Roh et al. 2007), *Bacillus* sp. strain SFB (Pollock et al. 2007), and two *Natronincola* strains (Zhilina et al. 2009b).

The aim of this study was to investigate, for the first time, the microbial ecology of samples from the hyperalkaline (pH up to 11.9) Allas Springs (Troodos Mountains, Cyprus), a site of active low-temperature serpentinization within the Troodos ophiolite that has been studied as a natural analogue for cementitious radioactive waste repositories (Alexander and Milodowski 2011). The objectives were: i) to describe the phylogenetic diversity of natural microbial communities from an analogue of cementitious geological radwaste disposal site using molecular microbiology techniques; ii) to investigate the potential of indigenous microbial communities to catalyze metal reduction at alkaline pH; and iii) to obtain pure cultures capable for Fe(III) reduction at alkaline pH, for future studies on the microbial transformation of metals and radionuclides. Our findings are discussed in the context of the potential for microorganisms to colonize and influence the evolution of (alkaline) cementitious-based geological repositories for radioactive wastes.

Methods

Samples Collected from the Hyperalkaline Allas Springs

A suite of samples were collected from the Allas Springs site in the Argaki tou Karvouna valley, near Platania in the Troodos Mountains in October 2010. The sampling site corresponds to location A1-1, as described by Alexander and Milodowski (2011), where Ca-rich hyperalkaline groundwater (up to pH 11.9) discharges under artesian flow through a steeply inclined fracture in a large outcrop of harzbergite up to 4 m high (supplemental materials, Figure S1). As the water discharges from the fracture and flows over the outcrop it reacts with atmospheric CO_2, resulting in the precipitation of calcite, aragonite and dolomite to form travertine (tufa) deposits on the bedrock surface. At the foot of the outcrop, the springwater also pecolates through a dense covering of forest litter and the underlying highly fractured and altered bedrock.

Samples Cyp1, Cyp2, Cyp3, Cyp5, CypR were collected from a broken stalactite/flowstone "rib" on the underside of the travertine-coated harzbergite outcrop (Figures S1 and S2). They consisted of: dripping hyperalkaline groundwater (Cyp1); a suspension of brown flowstone in groundwater (Cyp2); fragments of brown-stained flowstone in groundwater (Cyp3); a suspension of green microbial mats in groundwater (Cyp5); and a brown/green-stained flowstone-coated rock sample (CypR). A further sample (Cyp4) was taken from beneath the surface of the forest litter at the base of the harzbergite outcrop where the other samples in this study were collected.

This corresponds to Site A1-3 (Alexander and Milodowski 2011), and the sample taken consisted of a pink gelatinous layer, that was observed to occur at a depth of about 10 cm immediately beneath the buff/brown unconsolidated tufa (Figure S3) that impregnates the base of the forest litter. The water seeping through this material was still hyperalkaline (pH 10) at the point of sampling. The water chemistry of the collected samples is shown in Table 1. More details about the location of the site and the collected samples are included in the supplemental materials. The tufa-coated rock sample CypR was carefully chiselled from the flowstone surface and wrapped in a plastic Ziplock bag that was rinsed (5 times) with the same hyperalkaline groundwater discharging over the rock surface at this point. All other samples were collected into clean, sterile 30-ml plastic bottles, which had been thoroughly rinsed (5 times) with the hyperalkaline groundwater associated with each respective sampling point prior to collecting the samples, and topped up so that no head space was left in the bottle. All samples were stored at 4°C prior to further analysis.

Water Chemistry Measurements

In the groundwater-containing samples (Cyp1-5), pH and Eh measurements were taken using a Cole-Parmer 5990-45 electrode (Cole-Parmer Instrument Co. Ltd., London, UK) and a Mettler Toledo InLab Redox Micro electrode (Mettler-Toledo, Inc., OH, USA), respectively. Moreover, in filtered subsamples (<0.2 μm), the concentrations of cations were measured using a Perkin-Elmer Optima 5300 inductively coupled plasma atomic emission spectroscopy (ICP-AES) system (Perkin-Elmer Inc., Waltham, MA, USA), while the concentrations of anions were determined using a Metrohm 761 compact ion exchange chromatograph (Metrohm UK Ltd, Runcorn, UK).

Microbial Community Analyses

DNA was isolated from 0.25 g of the rock sample and 0.5 ml of the Cyp1a, Cyp2, Cyp3, Cyp4, and Cyp5 water suspensions using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's instructions. For the profiling of the bacterial communities present, PCR amplification was performed using universal bacterial 16S rRNA gene primers 8F (Edwards et al. 1989) and 1492R (Lane 1991). PCR products were purified using a Qiagen PCR purification kit (Qiagen, Inc., Valencia, CA, USA) and then ligated into the pGEM-T Easy Vector system (Promega, Madison, WI, USA) and transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Inc., Carlsbad, CA, USA).

Positive clones were screened by PCR using primers SP6 and T7, and sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, USA) and forward primer 8F (Edwards et al. 1989). The obtained 16S rRNA gene sequences were checked for chimera formation using Mallard (Ashelford et al. 2006). All nonchimeric bacterial 16S rRNA gene sequences from this study were clustered into OTUs (Operational Taxonomic Units) at a level of similarity of 97% using Mothur v.1.24.1 (Schloss et al. 2009).

Mothur was also used to calculate and compute alpha diversity indices, rarefaction curves, and unweighted pair group method with arithmetic mean (UPGMA) clustering based on Bray–Curtis dissimilarity values. The phylogenetic classification for all obtained nonchimeric 16S rRNA gene sequences of this study was performed using the RDP classifier (at 80% confidence threshold) of the Ribosomal Database Project (Release 10, update 31; (Cole et al. 2009). In addition, the closest phylogenetic relatives (environmental sequence, cultured organism or bacterial type strain) for the OTUs with the highest number of reads were identified by nucleotide

Table 1. pH, Eh measurements and concentration of major anions and cations in samples taken from the hyperalkaline Allas Springs in Cyprus

Sample	pH	Eh mV	Na^+ mg L^{-1}	Cl^- mg L^{-1}	Ca^{2+} mg L^{-1}	Mg^{2+} mg L^{-1}	K^+ mg L^{-1}	HCO_3^- mg L^{-1}	SO_4^{2-} mg L^{-1}	NO_3^- mg L^{-1}	S mg L^{-1}
Cyp1	11.68	158	1371	2230	38.8	0.1	69.0	120	130	<0.1	56.8
Cyp2	11.71	73	1379	2010	14.3	0.1	69.0	60	130	ND	56.8
Cyp3	11.52	68	1387	2030	4.4	0.0	70.9	160	130	ND	56.4
Cyp4	9.25	93	1636	1880	3.8	0.5	82.1	480	72	ND	50.6
Cyp5	9.40	−158	1348	1910	2.0	0.2	67.9	ND	54	<0.1	43.2

A complete list of the water chemistry analysis is shown in Table S1. ND stands for not detected.

Blastn search. All partial bacterial 16S rRNA gene sequences for this study were deposited to GenBank, under accession numbers JQ766531- JQ766937.

In addition to the profiling of the bacterial communities, 16S rRNA gene PCR amplifications were carried out to investigate the presence of Archaea and methanogens in our samples, using Archaeal-specific primers Arch21F and Arch958R (DeLong 1992), and methanogen-specific primers 1AF and 1100AR (Hales et al. 1996), respectively.

Setup and Sampling of Anaerobic Enrichment Cultures

Enrichment cultures were set up anaerobically by supplementing each of samples Cyp1, Cyp2, Cyp3, Cyp4, Cyp5 with an equal volume of a ferric-citrate containing medium that was largely based on a medium that has been used previously to isolate metal-reducing alkaliphilic bacteria (Ye et al. 2004). The medium used in this study contained 9.4 mM NH_4Cl, 4.3 mM K_2HPO_4, 4 mM $NaHCO_3$, 6.1 μM Na_2SeO_4, 17.1 mM NaCl, 10 ml L^{-1} mineral stock solution (Lovley et al. 1984), 7 mM sodium lactate, 7 mM sodium acetate, 0.025 g L^{-1} yeast extract, and 15 mM Fe(III)-citrate. The pH of the medium was adjusted to 10 with the addition of NaOH. Following sparging with N_2 gas and sterilization by autoclaving, 20 ml of the medium was mixed with approximately 20 ml of either of the Cyp1-Cyp5 samples in sterile 100 ml serum bottles, under a N_2:H_2 (98% : 2%) atmosphere in an anaerobic glove box. The serum bottles were sealed with butyl rubber stoppers and aluminium crimps, incubated at 20°C and sampled aseptically after 7 and 18 days, after which pH and Eh measurements were taken as described above, and the concentration of reduced Fe(II) was determined with the ferrozine assay (Lovley and Phillips 1987).

Isolation of Pure Cultures and Identification by 16S rRNA Gene Sequencing

For the isolation of pure cultures, the enrichment medium was solidified with 1.8% gellan gum, plated under a N_2:H_2 (98% : 2%) atmosphere in an anaerobic glove box, and inoculated with 30 μl of the Cyp4 and Cyp5 enrichment cultures. In addition, 30 μl of the Cyp4 and Cyp5 enrichment cultures were plated onto solidified medium that contained 15 mM Na_2SO_4 instead of Fe(III)-citrate as the electron acceptor to assess the potential for sulfate reduction in the experiments. After incubation at 20°C for 7 days under anaerobic conditions, single colonies were picked randomly and used to streak new gellan gum plates that contained either ferric citrate or sodium sulfate. One week later, 135 single colonies (65 from the ferric-citrate and 70 from the sodium sulfate-containing plates respectively) were used to inoculate 10 ml liquid enrichment medium containing either Fe(III) (as citrate) or sulfate (as the $NaSO_4$ salt) as the electron acceptor.

After incubation at 20°C for 7 days, a 250-μl subsample was used for DNA extraction with the MoBio PowerSoil DNA isolation kit. A total of 84 of the isolated bacteria were identified by 16S rRNA gene sequencing, after PCR amplification of the 16S rRNA gene using primers 8F and 1492R, PCR purification using a Qiagen PCR purification kit, and 16S rRNA gene sequencing using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit and primer 8F (as described here for the clone libraries). The closest phylogenetic relatives of the isolated bacteria were found by nucleotide Blastn search. Then, phylogenetic tree showing their phylogenetic associations was constructed with MEGA version 5.10 (Tamura et al. 2011), after alignment with ClustalW v.1.4 (Thompson et al. 1994). Evolutionary relationships were inferred by the neighbor-joining method (Saitou and Nei 1987) and the evolutionary distances were computed using the Jukes–Cantor model (Jukes and Cantor 1969). All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option) and bootstrapping was performed for 1,000 replicates.

Fe(III)-Reduction by Isolated Bacteria

The potential of the isolated microorganisms of this study to reduce Fe(III) was tested in 10- ml liquid cultures, containing the same medium that was used for the enrichment cultures, i.e., with 15 mM ferric citrate as the electron acceptor and 7 mM lactate and 7 mM acetate as the electron donor, but with the pH adjusted to 9. The pure cultures were incubated at 20°C for 7 days, before pH, Eh and Fe(II) measurements were taken, as described above.

Results

Water Chemistry of Collected Samples

Six samples were collected from the hyperalkaline Allas Springs, including groundwater only (Cyp1), brown-stained flowstone fragments in groundwater (Cyp2, Cyp3), a green-stained microbial mat in groundwater (Cyp5), a sample from the base underneath the artesian flowstone (Cyp4), and a rock sample (CypR). The chemical compositions of the collected samples are presented in Table 1 and are similar to compositions previously reported for groundwaters from this site (Alexander and Milodowski 2011).

The pH measurements indicated that samples Cyp1-3 were highly alkaline (pH between 11.52 and 11.71; Table 1), reflecting the alkalinity of the water that discharges from the harzbergite. The pH in samples Cyp4 and Cyp5 was lower (9.24 and 9.40, respectively), presumably as a result of atmospheric CO_2 reaction with sample Cyp5 and by reaction with the forest litter in the case of Cyp4. The redox potential was reducing in Cyp5 (−158 mV), while the Eh in the other samples was mildly oxic, between +68 and +158 mV. All samples were characterized by very high concentrations of Na^+ (1348–1635 mg L^{-1}), Cl^- (1880 to 2230 mg L^{-1}), and elevated concentrations of K^+ (69–82 mg L^{-1}) and sulfate (54–130 mg L^{-1}).

The concentration of Ca^{2+} was elevated in samples Cyp1 and Cyp2 (38 and 14 mg L^{-1} respectively), while in the remaining samples Ca^{2+} ranged from 2.2 to 4 mg L^{-1}. This variation in Ca^{2+} concentrations may also reflect the interaction of the hyperalkaline water with CO_2, which results in the removal of Ca^{2+} from solution through the precipitation of the extensive calcite and aragonite tufa on site. The concentration of HCO_3^- also varied, from below detection limits in Cyp5, 60–130 mg L^{-1} in Cyp1-3, and 480 mg L^{-1} in Cyp4. The water chemistry is not untypical for ophiolites, but the Na^+ and Cl^- levels are somewhat high compared to most ophiolite systems, possibly reflecting the presence of relict seawater in the host rock (see Alexander and Milodowski 2011). All other measured concentrations were either below 1 mg L^{-1} (Mg^{2+}, Al, Si, Ba) or negligible/nondetectable (nitrate, nitrite, total Fe, Mn, Sr). The complete set of measurements is shown in Table S1.

Phylogenetic Diversity of Bacterial Communities

The phylogenetic analysis of the 16S rRNA gene clone libraries from six samples collected from the hyperalkaline Allas Springs indicated that at the phylum level, most were dominated by *Proteobacteria*, while *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria* and *Firmicutes* phyla were also present in some of the samples (Figure 1). Based on RDP classification at the genus level (Figure 2), the sequences in the Cyp1 clone library were affiliated to the *Pseudomonas* (60%), *Propionibacterium* (7.5%), *Paracoccus* (7.5%) and *Hydrogenophaga* (2.5%) genera, while the Cyp3 clone library contained sequences affiliated to the *Hydrogenophaga* (53.8%), *Silanimonas* (4.6%) and *Acidovorax* (1.5%) genera and group IV *Cyanobacteria* (9.2%). The Cyp2 clone library was a mixture between Cyp1 and Cyp3, with sequences belonging to the *Silanimonas* (15.9%), *Acinetobacter* (14.3%), *Acidovorax* (6.3%), *Pseudomonas*, *Hydrogenophaga*, *Paracoccus* (4.8% each) genera and GpIV *Cyanobacteria* (3.2%).

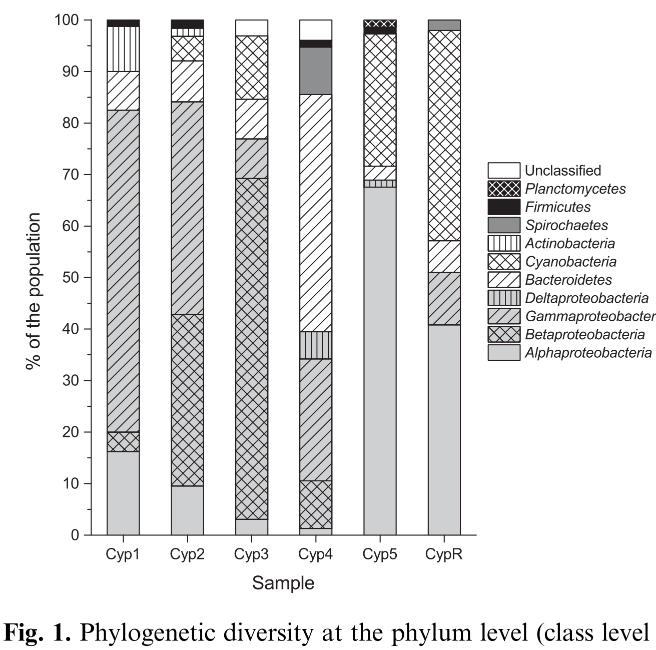

Fig. 1. Phylogenetic diversity at the phylum level (class level for the Proteobacteria) in six samples from the hyperalkaline Allas Springs, Cyprus.

The Cyp5 clone library was dominated by unclassified *α-Proteobacteria* (63.5%) and to a lesser degree by GpIV *Cyanobacteria* (17.6%), while the rock sample CypR contained group GpIV *Cyanobacteria* (40.8%) and *Rhodobacter* (16.3%), *Pseudomonas* (2%), *Silanimonas* (2%) genera. The microbial community in the sample that was taken from beneath forest litter at the base of the harzbergite outcrop (Cyp4) was significantly different to the microbial communities of the other samples as indicated by the UPGMA cluster dendrogram (Figure S5), with most of the sequences grouping within unclassified *α-Proteobacteria* (46%) and *γ-Proteobacteria* (22.4%), as well as in the *Spirochaeta* (7.9%) genus. The number of OTUs (at 97% similarity level) identified in each sample varied between 14 and 36 (total number of identified OTUs = 104), indicating moderately diverse microbial communities, in agreement with the calculated diversity indices (Table S2) and rarefaction curves (Figure S4). The OTUs that contained the highest number of sequences (more than 1.9% of the total number of sequences of this study) and their closest phylogenetic affiliations are shown in Table 2.

In addition to the bacterial diversity detected, PCR amplification using Archaeal- or methanogen-specific primers indicated that these microorganisms were present in samples Cyp4, Cyp5 and CypR, but probably in low abundances, as indicated by the low PCR yields (faint bands in the agarose gels; Figure S6). Further description of the phylogenetic diversity in these communities was beyond the scope of this study and it remains to be investigated by future sequencing efforts.

Isolated Bacterial Strains from the Hyperalkaline Allas Springs

Eighty-four of the bacterial isolates were identified by 16S rRNA gene sequencing. The phylogenetic analysis indicated that the isolates were grouped within 5 OTUs (at OTU similarity level of 99%). Most of the isolates (82 isolates, OTUs/strains P1, P2, P3) belonged to the *Paenibacillus* genus (Figure 3), and they shared 98% ID similarity to various facultative anaerobic *Paenibacillus* type strains, such as *P. odorifer* TOD45 (NR_028887), *P. borealis* KK19 (NR_025299), and *P. wynnii* LMG 22176 (NR_042244). The remaining two isolates (OTU/strains A1 and A2) were most closely related (99% ID similarity) to the uncultured bacterial clone Alchichica_AQ2_2_1B_147 (JN825558) from the alkaline lake Alchichica in Mexico (Couradeau et al. 2011) and the uncultured bacterial clone TX2_2O07 (JN178047) from an extreme saline-alkaline soil of the former lake Texcoco in Mexico (Figure 3).

They were also distantly affiliated (92 – 94% ID similarity) to various *Alkaliphilus* and *Natronincola* species (Figure 3), including known metal-reducing strains such as *A. metalliredigens* QYMF (NR_074633), *A. peptidofermentans* Z-7036 (EF382660), *N. peptidovorans* Z-7031 (EF382661) and *N. ferrireducens* Z-0511 (EU878275). Both *Alkaliphilus*-related isolates, strain A1 (KF954220) and strain A2 (KF954221) were obtained from sample Cyp4 and were isolated on sodium

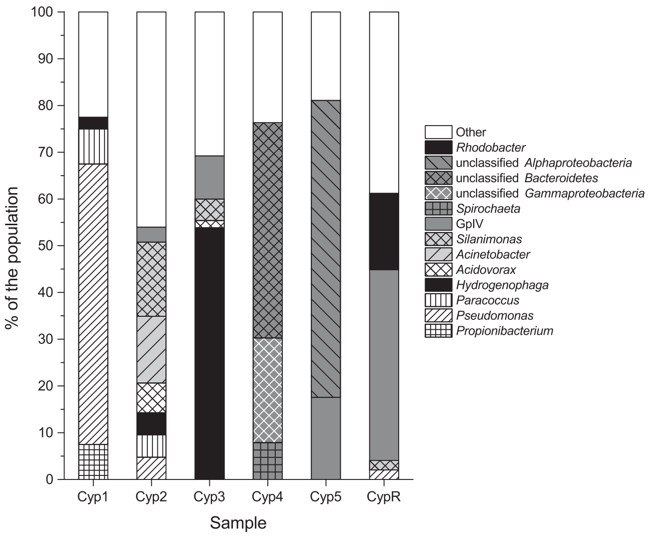

Fig. 2. Phylogenetic diversity at the genus level. Only the genera that contained more than 1.2% of the total number of sequences are shown.

sulfate medium. The *Paenibacillus* related isolates were obtained from both Cyp4 and Cyp5 samples. Strains P1 (KF954217) and P2 (KF954218) originated from sample Cyp5 and they were isolated on Fe(III)-citrate containing medium, while strain P3 (KF954219) originated from sample Cyp4 and it was isolated on sodium sulfate medium.

Alkaline Fe(III)Reduction in Enrichment Cultures and by Isolated Bacteria

The potential for Fe(III) reduction was tested by supplementing the Cyp1-Cyp5 samples from the hyperalkaline Allas Springs with an equal volume of an enrichment medium containing 15 mM Fe(III)-citrate as the electron acceptor, and 7 mM lactate and 7 mM acetate as the electron donors. Thus, the initial concentration of soluble Fe(III) in the enrichment cultures was approximately 7.5 mM. During the 18-day incubation, no Fe(III) reduction was observed in the Cyp1-3 enrichment cultures (Figure 4), while the pH decreased slightly to approximately 9.40 and the redox potential decreased from 78-168 mV to 56-112 mV. In contrast, in the Cyp4 and Cyp5 enrichment cultures, up to 40% and 32% of the Fe(III) was reduced, respectively, during the same incubation period (Figure 4). The reduction of Fe(III) was accompanied by a notable decrease in the redox potential to around -240 mV, and a drop in pH to 8.76 and 8.34 for the Cyp4 and Cyp5 enrichment cultures, respectively (Figure 4).

Furthermore, preliminary experiments with five isolated microorganisms (*Paenibacillus* affiliated strains P1, P2, P3, and *Alkaliphilus* related strains A1 and A2) showed that they can all reduce Fe(III)-citrate at alkaline pH 9 but to a different extent. After 7 days of incubation, the concentration of Fe(II) in solution ranged from 1.7 mM (A1 strain) to 7.6 mM (A2 strain), and it was between 2.6 and 3.7 mM in the cultures of the *Paenibacillus* strains (Figure 5). During incubation, the pH dropped significantly from pH 9 to 6.54 in the medium inoculated with strain P1, while in the remaining cultures the pH remained alkaline, between 8.3 and 8.8 (Figure 5).

Discussion

Microbial Diversity in Samples from the Hyperalkaline Allas Springs, Cyprus

Despite the potential role of microbial metabolism on the long-term performance of geological repositories for radioactive wastes, comparatively few studies have examined the microbial ecology of high pH natural environments, analogues to these systems. In this study the bacterial diversity in samples from a natural analogue for a cementitious based geological repository, the hyperalkaline Allas Springs (Troodos Mountains ophiolite) in Cyprus, was investigated by 16S rRNA gene cloning and sequencing. The results showed diverse bacterial communities present, but many of the sequences were not closely related (less than 95% sequence similarity) to isolated microorganisms (Table 2), an indication that these systems have not been studied extensively yet and/or that isolation of these highly alkaliphilic microorganisms is challenging.

Of the sequences that were closely affiliated to cultured genera, only a few were related to known alkaliphilic genera (for example *Silanimonas* genus; Table 2), and the majority were related to genera with type strains that are not known as alkaliphilic (*Hydrogenophaga*, *Pseudomonas*, *Acidovorax*, *Acinetobacter*, *Paracoccus*, *Propionibacterium*; Figure 2). Some of these genera have also been detected in samples from other highly alkaline sites, such as the Maqarin site (Pedersen et al. 2004) and the Cabeço de Vide aquifer (Tiago and Veríssimo 2013), which could indicate that alkali-tolerant microorganisms, adapted to environmental niches, may persist in such highly alkaline environments. Interestingly, the presence of a large number of sequences related to the *Hydrogenophaga* genus is observed among most of the studies on terrestrial ophiolitic environments.

In fact, *Hydrogenophaga* related sequences were present in three of the clone libraries of this study (Cyp3, and to a lesser degree in Cyp1 and Cyp2; Figure 2), made up more than 35% of a 16S rRNA gene clone library and 20% of the pyrosequencing 16S rRNA reads in samples from the alkaline Cabeço de Vide aquifer (Tiago and Veríssimo 2013), and were the most dominant genus in samples from the Leka ophiolite (Daae et al. 2013). *Hydrogenophaga* related sequences also dominated the bacterial communities in two spring water samples from the Tablelands ophiolite (Brazelton et al. 2013), and were detected among the metagenomic reads and contigs of one of these samples (Brazelton et al. 2012).

Although *Hydrogenophaga* spp. are not known alkaliphilic microorganisms, one *Hydrogenophaga* sp. isolate has been shown previously to grow on benzene at pH up to 8.5, with optimum growth at pH 8 (Fahy et al. 2008). In general, members of the *Hydrogenophaga* genus are facultative anaerobic, chemoorganotrophic or chemolithoautotrophic, such as *H.*

Table 2. Phylogenetic affiliations of the OTUs that contained more than 1.9% of the environmental sequences of this study (8 sequences or more)

OTU ID/ representative sequence (Accession number)	% of total population						Closest phylogenetic relative	Accession number	% ID similarity	Environment/characteristic (Reference)
	Cyp1	Cyp2	Cyp3	Cyp4	Cyp5	CypR				
Cyp1_46 (JQ766563)	57.5						*Pseudomonas peli* type strain R-20805	NR_042451	99	nitrifying inoculums (Vanparys et al. 2006)
Cyp1_06 (JQ766532)	7.5	4.8					*Paracoccus denitrificans* type strain 381	NR_026456	98	- (Rainey et al. 1999)
Cyp3_45 (JQ766700)	2.5	7.9	60.0				*Hydrogenophaga defluvii* type strain BSB 9.5	NR_029024	97	activated sludge (Kämpfer et al. 2005)
Cyp2_07 (JQ766616)		15.9	4.6			2.0	*Silanimonas lenta* type strain 25-4	NR_025815	97	hot spring, thermophilic and alkaliphilic (Lee et al. 2005)
Cyp3_62 (JQ766712)		4.8	10.8				uncultured bacterium clone PMB-63	AB757744	99	Padang Cermin hot spring water
Cyp5_45 (JQ766849)					62.2		uncultured *a-Proteobacterium* Alchichica_ AQ1_2_1B_102	JN825362	95	alkaline lake Alchichica, Mexico (Couradeau et al. 2011)
CypR_52 (JQ766918)					16.2	38.8	*Leptolyngbya antarctica* ANT.LAC.1	AY493588	99	benthic microbial mats, Antarctic lake (Taton et al. 2006)
CypR_21 (JQ766901)					2.7	16.3	*Rhodobacter blasticus* type strain ATCC 33485	NR_043735	97	small freshwater pond, England (Helsel et al. 2007)
Cyp4_25 (JQ766761)				26.3			uncultured bacterium clone Asc-w-9	EF632712	94	high altitude Andean Altiplano, Chile
Cyp4_55 (JQ766787)				22.3			uncultured *γ-Proteobacterium* clone P2U_16	FN687068	100	siliceous stromatolites, Lake Specchio di Venere, Italy
Cyp4_59 (JQ766791)				10.5			uncultured *Bacteroidetes* clone EK_Ca765	JN038302	95	petroleum contaminated soil
No. of sequences	80	63	65	76	74	49				

The closest type strain or cultured relative is given instead of the first GenBank match, in case sequence similarity is 95% or higher.

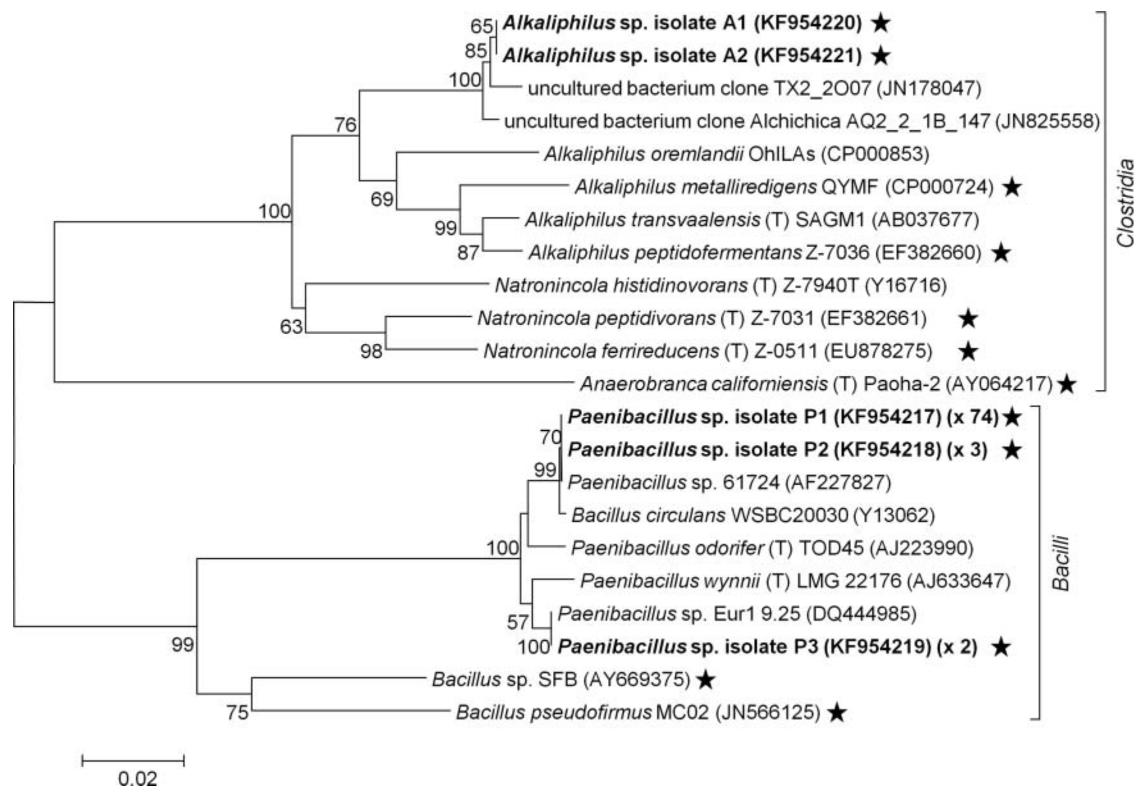

Fig. 3. Phylogenetic diversity of the isolated bacterial strains of this study (indicated in bold), in relation to other uncultured or cultured organisms or type strains (T). Metal-reducing organisms are indicated with a star.

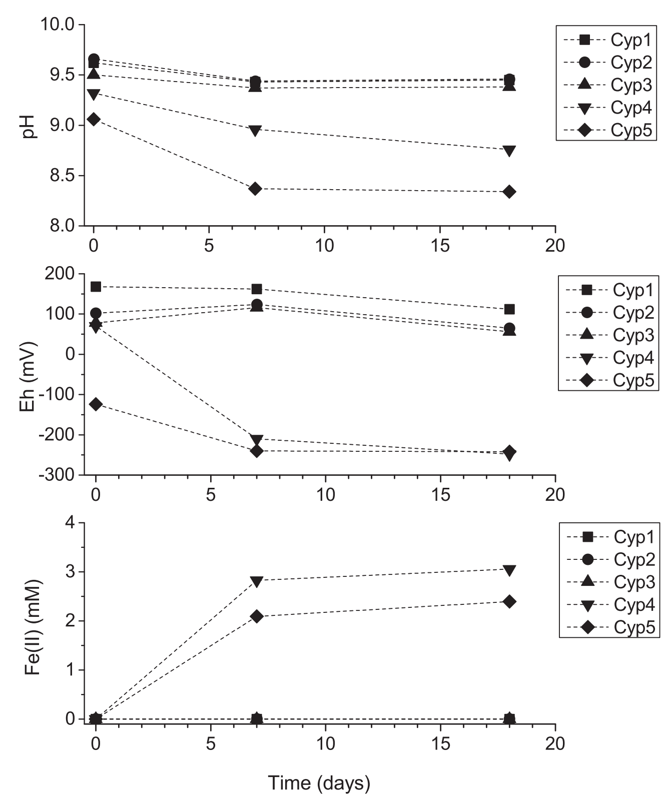

Fig. 4. pH, Eh and Fe(II) measurements in the anaerobic enrichment microcosms (containing approximately 7.5 mM ferric-citrate) set up with the samples from the hyperalkaline Allas Springs.

defluvii, which can use the oxidation of H_2 as an energy source and CO_2 as a carbon source, and their growth is not inhibited by high levels of O_2 in the atmosphere (Kämpfer et al. 2005). Thus, it appears that abiotic liberation of H_2 gas during the serpentinization of ophiolites, potentially combined with the biotic generation of H_2 by fermentative microorganisms, may have led to the enrichment of *Hydrogenophaga* species in these systems, despite the presumptively adverse alkaline pH.

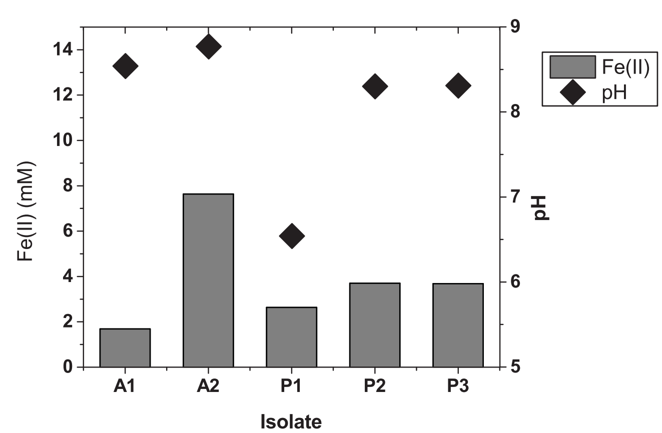

Fig. 5. Fe(II) concentration and pH measurements in anaerobic liquid cultures supplemented with 15 mM Fe(III)-citrate and inoculated with five isolated bacteria from the hyperalkaline Allas Springs (*Alkaliphilus* affiliated A1 and A2; *Paenibacillus* affiliated P1, P2, P3), after 7 days of incubation.

This is significant in the context of a geological repository for radioactive wastes, because alkali-tolerant hydrogen oxidizing bacteria (such as *Hydrogenophaga* species) in this setting could also potentially utilize excess H_2 that is expected to be produced during the abiotic corrosion of steel (NDA 2010b) and the biotic release of H_2 by fermentative microorganisms (particularly those degrading cellulose in low and intermediate-level wasteforms). This has significant implications as consumption of hydrogen by this previously poorly recognized biotic pathway has the potential to mitigate any over pressurization and transport effects in the geological disposal facility and/or host rock associated with hydrogen production from corrosion of steel (Libert et al. 2011). Oxidation of hydrogen could also be potentially linked to the reduction of a range of electron acceptors, including radionuclides (Lloyd 2003).

Another finding of this study was that samples Cyp5 and CypR, which contained visible green-colored microbial mats, yielded a relatively high number of sequences related to *Cyanobacteria* (17.6% and 40.8% respectively; Figure 1). Interestingly, these Cyanobacterial sequences were closely related to *Leptolyngbya* isolates from Antarctica (Taton et al. 2006), and a separate study has shown that *Leptolyngbya* species dominate the microbial communities on stromatolite benthic samples from the alkaline (pH 10.4) lake Untersee in Antarctica (Andersen et al. 2011). In addition, Cyanobacterial stromatolite-related sequences were also detected in the microbial mat sample (40% of the clones) from the Del Puerto Ophiolite, and have been linked to biological precipitation of carbonates (Blank et al. 2009).

However, microbial precipitation or dissolution of calcium carbonates and other minerals is not limited to photosynthetic *Cyanobacteria*, as it may also be promoted by other microbial processes linked to sulfate-reducing, nitrate-reducing or fermenting bacteria, under alkaline anaerobic conditions (Dupraz and Visscher 2005), and within the microbial community of the Cyp4 sample of this study, siliceous stromatolite-related sequences affiliated to *γ-Proteobacteria* were detected (Table 2). Thus, the potential significance of the microbial precipitation and dissolution of calcium carbonate (or other minerals, such as silicates) within the calcium-rich cement leachate that will be formed within the radwaste geological repository should not be overlooked, as it may influence the alkalinity and the evolution of the leachate plume during long-term radwaste disposal.

In regard to other microbial groups, although sulfate-reducing bacteria (SRB) or dissimilatory sulfite reductase gene fragments have been detected in other terrestrial highly alkaline or ophiolitic environments (Blank et al. 2009; Pedersen et al. 2004), and high concentrations of sulfate were present in most of the Cyprus samples, no sequences closely related to known SRB were detected in this study. Furthermore, methanogenic and anaerobic methane-oxidizing Archaea have been found to dominate deep hydrothermal vents (Brazelton et al. 2006) and to be present in terrestrial environments too (Blank et al. 2009), and their presence was confirmed by PCR amplification in some samples from the hyperalkaline Allas Springs (Figure S6). However their phylogenetic diversity remains to be investigated.

Potential for Metal Reduction at Alkaline pH

Microbial metal reduction at alkaline pH is likely to be a significant factor in controlling the solubility and mobility of key radionuclides such as uranium and technetium, during disposal in cementitious-based geological repositories. Recently it was shown that a clear succession of electron acceptor utilization existed in alkaline microcosm cultures up to pH 11, as rapid nitrate reduction was followed by slower soluble Fe(III)-citrate, insoluble Fe(III)-oxyhydroxide, and sulfate reduction (Rizoulis et al. 2012). Further sediment microcosm studies from our group have also shown that alkaline bioreduction of Fe(III)-oxyhydroxide can lead to the formation of magnetite (Williamson et al. 2013) and that microbially mediated reduction of U(VI) can occur at pH 10 (Williamson et al. 2014).

In this study, we have found that two out of the five microbial communities sampled from a natural analogue of cementitious-based geological repositories (the hyperalkaline Allas Springs) also exhibited the ability to reduce soluble Fe(III) (samples Cyp4 and Cyp5, Figure 4). Considering that the clone libraries of the Cyp4 and Cyp5 samples did not contain any sequences related to known Fe(III)-reducing bacteria, it is evident that complementary to molecular microbiology approaches, enrichment and isolation studies should be carried out when examining the potential for metal/radionuclide reduction by diverse natural microbial communities.

Furthermore, five bacterial strains isolated during this study, three of them belonging to the *Paenibacillus* genus and two of them associated with the *Alkaliphilus* genus, were also capable of Fe(III) reduction at alkaline pH. These metal-reducing strains were not detected among the environmental sequences of the corresponding microbial communities prior to the establishment of the enrichment cultures (i.e., the Cyp4 and Cyp5 clone libraries), indicating either culturing selectivity or that microbial species can be enriched and drive biogeochemical processes when new electron acceptors become available, under specific geochemical conditions.

To date, alkaline metal reduction by pure cultures has been reported (at least for Fe(III) reduction) for *Anaerobranca californiensis* (Gorlenko et al. 2004), *Alkaliphilus metalliredigens* (Ye et al. 2004), *Alkaliphilus metalliredigens* QYMF (Roh et al. 2007), *Alkaliphilus peptidofermentans* Z-7036 (Zhilina et al. 2009a), *Natronincola ferrireducens* and *Natronincola peptidovorans* (Zhilina et al. 2009b), *Bacillus* sp. strain SFB (Pollock et al. 2007), *Bacillus pseudofirmus* MC02 (Ma et al. 2012), and a *Serratia* sp. strain (Thorpe et al. 2012).

Noticeably, with the exception of the *Serratia* strain, all the other microorganisms that have been shown to reduce Fe (III) at alkaline pH (from this or other studies) are Gram-positive Bacilli (*Bacillus* or *Paenibacillus* genera) or Clostridial (*Alkaliphilus* and *Natronincola* genera) strains of the Firmicutes phylum (Figure 3). It is not clear whether this is due to culturing bias (Firmicutes are often obtained by culturing on solidified rich media) or because alkaline metal reduction is carried out preferentially by Gram-positive bacteria. To date, the mechanisms of microbial Fe(III)/metal reduction have been studied mostly at circumneutral pH using model Gram-negative bacteria, such as *Shewanella oneidensis* MR-1 and various *Geobacter* species. Extracellular electron transfer by *Geobacter and Shewanella*, implicated in the reduction of insoluble Fe(III) minerals, is mediated via *c*-type membrane cytochromes and/or conductive pili, as reviewed by Shi et al. (2009).

Shewanella species are also able to mediate extracellular reduction via the secretion of redox-active flavins, which act as electron shuttles (von Canstein et al. 2008), and may also play a role in Fe(III) reduction in high pH systems (Fuller et al. 2014). For Gram-positive bacteria, the mechanisms of extracellular electron transfer are largely unexplored and only recently has it been suggested that (at circumneutral pH) *c*-type cytochromes may be involved in the respiratory Fe (III) reduction by *Thermincola potens* strain JR (Carlson et al. 2012; Wrighton et al. 2011). Thus, more targeted studies are needed in order to determine the potential for and the fundamental mechanisms of microbial metal and radionuclide reduction at alkaline pH as these questions are highly pertinent to the management and disposal of the global legacy of radioactive waste materials.

Conclusions

The findings of this study indicate that phylogenetically diverse microbial communities can exist in a natural serpentinization-driven environment, analogous to a cementitious-based geological repository for radioactive waste. The presence of sequences affiliated to non-alkaliphilic genera may indicate that alkali-tolerant bacteria can persist at high pH. The phylogenetic results also indicate that microbial metabolism may have a significant role on important biogeochemical processes that have been largely overlooked to date in the context of geological repositories, such as (but not limited to), microbial oxidation of H_2 gas, reduction of metals and radionuclides, and precipitation or dissolution of calcium carbonates and other minerals. Therefore, this study highlights the potentially significant impact of microbial activity on long-term geological disposal of radioactive waste.

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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