

The Microbial Ecology of a Hyper-Alkaline Spring, and Impacts of an Alkali-Tolerant Community During Sandstone Batch and Column Experiments Representative of a Geological Disposal Facility for Intermediate-Level Radioactive Waste

Sarah L. Smith^{a,b}, Athanasios Rizoulis^a, Julia M. West^b, and Jonathan R. Lloyd^a

^aResearch Centre for Radwaste and Decommissioning & Williamson Research Centre for Molecular Environmental Science, School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Manchester, United Kingdom; ^bBritish Geological Survey, Environmental Science Centre, Nicker Hill, Keyworth, Nottingham, United Kingdom

ABSTRACT

Naturally occurring hyper-alkaline springs and associated hyper-alkaline environments may have components that are analogous to a cement-based deep geological disposal facility (GDF) for intermediate level radioactive waste (ILW). Such high pH environments could give insights into the biogeochemical processes that could occur in the region of a GDF environment after the ingress of GDF-derived groundwater leads to the formation of a hyper-alkaline plume in the surrounding rock mass. This study focuses on the microbial community composition found at a highly alkaline spring near Buxton, Derbyshire, England, and the variation in community structure across spatially separated sample points of contrasting pH values (ranging from pH 7.5–13). Communities containing alkaliphilic and alkalitolerant bacteria were observed across the site by PCR amplification and 16S rRNA gene pyrosequencing and included members of the families *Comamonadaceae* and *Xanthomonadaceae*. At pH 13, the sequence library was dominated by *Gammaproteobacteria* of the families *Pseudomonadaceae* and *Enterobacteriaceae*. Bacterial communities from the site demonstrated the ability to reduce Fe(III) in microcosm experiments up to pH 11.5, suggesting the potential to reduce other metals and radionuclides of relevance to cement-encapsulated intermediate level radioactive waste (ILW) disposal. In laboratory column flow-through experiments, microbial communities present at the field site were also able to colonize crushed sandstone. Bacterial community composition varied between columns that had been supplied with alkali surface waters from the site amended with carbon (lactate and acetate, as proxies for products of cellulose degradation from ILW), and control columns that were not supplied with added carbon. Members of the family *Clostridiaceae* dominated the sequence library obtained from the carbon amended column inlet (45.8% of library), but became less dominant at the outlet (20.8%). Members of the family *Sphingomonadaceae* comprised 11.8% of the sequence library obtained from the control column inlet, but were not present in sediments collected from the column outlet, whereas the relative abundance of members of the family *Comamonadaceae* increased from the column inlet (35.2%) to the column outlet (57.2%). The spatial variation in community composition within the columns is indicative of discrete biogeochemical zonation in these flow-through systems.

ARTICLE HISTORY

Received November 2014
Accepted May 2015

KEYWORDS


Intermediate level radioactive waste disposal; alkaliphile; microbial iron reduction

Introduction

The potential role of microorganisms on the containment of radioactive waste has long been recognized (e.g., Pedersen 1996; Stroes-Gascoyne and West, 1996; West and McKinley 2002). Microbes will be present in the host rock of a geological disposal facility (GDF), repository structural materials and in the waste forms themselves, and contamination from the surface will occur during the operational period.

The transport properties of a GDF host rock may be altered by microbial processes, for example by the formation of biofilm across pore throats, leading to porosity and permeability alteration (Coombs et al. 2010). The temporal and spatial extent to which these processes will occur in the host rock of a GDF are uncertain,

and is dependent on numerous factors including flow velocity and grain size (Surasani et al. 2013), angularity and surface roughness (Crawford et al. 2012), the hydraulic pressure gradient (Ginn et al. 2005), and biological factors including the metabolic state of cells, the isoelectric point of cell surface polymers, and processes such as filtration and dispersion (Ginn et al. 2005). Microbial growth in the geosphere may alter the size and shape of pore spaces, the roughness of grain surfaces, and may even cause aggregation of sediments (Atekwana et al. 2006). Microorganisms may also directly influence radionuclide migration through the geosphere, for example by redox state alteration (Lloyd 2003), or interactions between extracellular biofilm components (e.g., polysaccharides) and radionuclides (Kazy et al. 2008).

CONTACT Jonathan R. Lloyd  Jon.Lloyd@manchester.ac.uk  Research Centre for Radwaste and Decommissioning & Williamson Research Centre for Molecular Environmental Science, School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Williamson Building, Oxford Road, Manchester M13 9PL, United Kingdom.

Color versions for one or more of the figures in the article can be found online at www.tandfonline.com/ugmb.

© 2016 Sarah L. Smith, Athanasios Rizoulis, Julia M. West, and Jonathan R. Lloyd. Published with license by Taylor & Francis.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

The UK's intermediate level radioactive wastes will be disposed of in a deep (up to 1000 m) GDF, which utilizes a multi-barrier concept that could involve significant quantities of cementitious materials (Chapman and Hooper 2012). In a cementitious GDF, groundwater flow through the repository will generate a hyperalkaline plume which will interact with the host rock (Savage 2011), forming an alkali disturbed zone (ADZ), potentially impacting on radionuclide transport processes. Alteration of host rock porosity as a result of the increased solubility of silicates and aluminosilicates under high pH conditions may occur in the early stages of hyper-alkaline plume development (Savage et al. 1992), although this process will be counteracted by the precipitation of calcium-silicate-hydrate minerals (Hodgkinson and Hughes 1999; Savage 2011). These processes are thought to occur to the greatest extent in the near-field of a GDF (Savage 2011).

During the initial stages of repository evolution, pore fluid pH (pH 12.5–13; Bateman et al. 1999) may be too high to facilitate microbial activity (Rizoulis et al. 2012); decreases in pH over time as a result of $\text{Ca}(\text{OH})_2$ removal will however, provide conditions more favorable for microbial growth. Natural analogue sites are potentially very useful to help understand the biogeochemical processes that may develop within the ADZ. Naturally occurring highly alkaline environments, such as soda lakes, have been reported to be highly productive and contain a wide diversity of microorganisms (Dimitriu et al. 2008). For example, VanEngelen et al. (2008) isolated alkaliphilic microorganisms that were capable of Cr(VI) reduction at pH 9. Pollock et al. (2007) demonstrated the ability of soda lake microorganisms to reduce Fe(III) up to a pH of 10.4.

This latter process has been found to be significant in soda lake environments, as it may influence sediment geochemistry, for example by acting as an electron acceptor during the oxidation of organic contaminants, and by facilitating the release of toxic trace metals into surrounding waters (Lovley 1991). Studies of other naturally occurring alkaline environments, including saline-alkaline soils, have also identified several dominant phyla such as *Actinobacteria* and *Proteobacteria*, similar to those found in a range of other extreme environments (Keshri et al. 2013). Identification of several functional genes from these types of environment suggest that the microbes present could potentially utilize sulphur compounds for respiration, and catalyze a range of other biogeochemical reactions (Keshri et al. 2013), although the range of coupled processes that can proceed may be constrained by high pH (Burke et al. 2012).

Other naturally occurring highly alkaline environments include highly alkaline springs such as those that have formed at Maqarin, Jordan, as a result of groundwater interaction with naturally occurring cement materials (Khoury et al. 1992). The bacterial community in the surface waters at Maqarin is thought to have adapted to the highly alkaline conditions relatively quickly, as indicated by the lack of deeply branching 16S rRNA species (Pedersen et al. 2004). Naturally occurring alkaline springs are also present in northern Oman, where serpentinization causes the formation of highly alkaline waters populated with microorganisms including *Clostridia* and sulfate-reducing bacteria (Bath et al. 1987). In other serpentinising systems present in ophiolites, members of the genus *Hydrogenophaga* have been identified (Brazelton et al. 2013; Rizoulis

et al. 2014), under highly alkaline conditions; members of this genus are capable of oxidising hydrogen, and their presence in these systems may be attributed to the generation of H_2 gas during the serpentinization process.

Several studies have demonstrated tufa deposits (occurring where aqueous environments are supersaturated with calcium carbonate (Perri et al. 2012)) and their associated bodies of water contain diverse microbial communities, including members of the phylum *Cyanobacteria* (Ng et al. 2006), alkaliphilic members of the genus *Bacillus* and bacterial sequences exhibiting similarities to nonalkaliphilic bacteria, including *Planctomycetes* and *Pirellula* species (Stougaard et al. 2002). The formation of other types of highly alkaline environments may occur as a result of the influence of anthropogenic activities. For example at Brook Bottom spring, at Harpur Hill near Buxton, UK, the extensive deposition of lime burning waste has led to the formation of a hyper-alkaline spring; soils affected by the spring have been shown to contain microbial communities that have evolved relatively quickly to the high pH conditions, and are carrying out processes commonly seen in circum-neutral soils (Burke et al. 2012). Rizoulis et al. (2012) demonstrated that microorganisms from this field site were capable of nitrate and Fe(III) reduction in microcosm experiments up to pH 11.

This study aimed to investigate microbial community composition of shallow surface sediments taken from a hyperalkaline spring at Harpur Hill near Buxton, to give insights into bacterial community changes under varying pH conditions, and to help understand the range of biogeochemical processes that are potentially carried out at high pH in such an environment. This study also investigates the impact of alkaliphilic and alkalitolerant microorganisms on transport in crushed sandstone, in a laboratory analogue intended to mimic the alkali disturbed zone that could surround a cement-based GDF for ILW. Extensive studies have been carried out investigating microbial impacts on the transmissive properties of rock (e.g. Coombs et al. 2010), although little is known about these processes under hyper-alkaline conditions, even though they could potentially play a role in controlling radionuclide transport in the geosphere surrounding a GDF.

Methods

Site description

Samples for microbial community analysis and experimental investigations were collected from Brook Bottom Springs near Harpur Hill, 2 km south of Buxton (Ford and Pedley 1996). A Hoffman lime kiln was formally in operation at the site, producing extensive quantities of lime from the lime roasting process. The waste was deposited in a valley at the site, resulting in saturation of the groundwater with calcium hydroxide, which discharges from the waste pile causing significant increases in pH. Interactions between this calcium rich fluid and atmospheric CO_2 leads to the precipitation of tufa. In some areas of the site, particularly at the base of the waste pile, where a hyper-alkaline spring has formed, the pH of the water is above 13, although the pH of the fluids at the site vary drastically, and is influenced



Figure 1. Aerial photograph of Brook Bottom Spring at Harpur Hill in Buxton. Sampling points, and corresponding pH values at the time of sampling are also shown (Ordnance Survey data 2013; Grid reference at centre: SK 056 717).

by factors such as rainfall. After periods of heavy rainfall, streamwater flowing through a culvert (HH 7.5, Figure 1) is of circumneutral pH, creating mixing zones across the site facilitating calcium carbonate precipitation.

Samples were collected from five areas around the site varying in pH from 7.5–13, in April 2012. At each sampling point, pH and Eh values were measured in the field using a HANNA HI 9828 multiprobe calibrated to pH 7 and pH 10 prior to use. Surface sediment and fluid were collected in sterile containers. Samples were stored in airtight bottles in the dark at 4°C until needed.

Microcosm experimental design

Microcosm experiments were assembled with sediment and fluid collected from the variable pH sampling points to investigate microbial activity across the pH ranges found at the Harpur Hill site. Sandstone from the Hollington Sandstone Quarry (Sherwood Sandstone group) was used in these experiments as it contains a range of minerals, some of which may be found in a host rock for a GDF including quartz, feldspar, and micas. The friable nature of the sandstone also means that when the material is crushed, the creation of fresh (highly reactive) mineral surfaces is kept to a minimum, so reactions taking place within experiments occur with aged surfaces (AE Milodowski, personal communication, July 2014). Serum bottles (100 mL) contained 30 g

crushed sandstone (Particle size < 500 μm), 5 g of Harpur Hill sediment, and 60 mL of fluid from the corresponding sampling point.

Acetate (5 mM) and lactate (5 mM) were added to each microcosm bottle as electron donors, along with low amounts of yeast extract (50 mgL^{-1} ; DIFCO, UK). Electron acceptors included Fe(III) in the sandstone, along with nitrate (concentrations ranging from around 0–4 mgL^{-1} in Harpur Hill fluids) and sulfate (concentrations varying from 3–15 mgL^{-1} in Harpur Hill fluids) in the Harpur Hill groundwater. Microcosms were assembled under anaerobic conditions under a headspace of 95% N_2 /5% H_2 and sealed with a grey butyl rubber septa, held in place with an aluminium crimp cap. A range of control experiments were assembled ((i) autoclaved microcosm, (ii) surface water with no sediment, (iii) no electron donor control, and (iv) a sandstone free control) with fluid from each of the Harpur Hill sample sites. Microcosms were sampled aseptically under anaerobic conditions on a weekly basis for 4 weeks.

Column experimental design

Fluid samples were collected from the pH 11.4 sampling point in June 2012, and stored at 4°C for 2 months until needed. The experimental design, as shown in Figure 2 was as follows: PEEK columns (15 cm long, 0.75 cm internal diameter; Applied Research Europe, Berlin, Germany) were packed with crushed sandstone (Grain size < 500 μm ; approximately 10 g). Columns were assembled in duplicate, with two receiving fluid with the addition of 50 mgL^{-1} yeast extract, along with 5 mM of both sodium acetate and sodium lactate as electron donors. Two columns received unamended fluid (controls). Under anaerobic conditions (95% N_2 / 5% H_2), fluid was pumped through the columns by a peristaltic pump at an initial rate of approximately 1 mLh^{-1} , for 46 days. The outlet fluid was sampled on a weekly basis; total cell counts were made and the fluid chemistry was analyzed; the flow rate for each of the columns was calculated.

At the end of the experiments, the columns were cut in to 5 × 3-cm sections, the exposed sample at the ends of the sections was removed aseptically, and sediment samples were

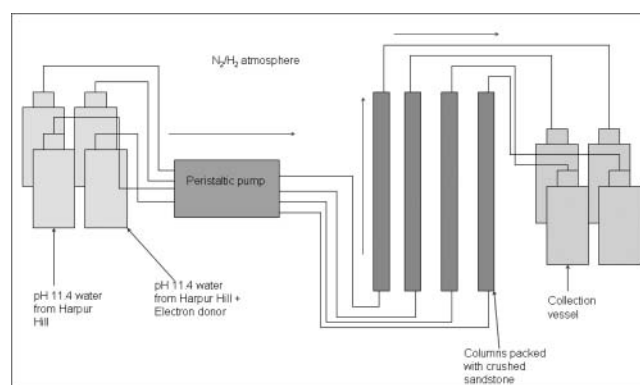


Figure 2. Schematic diagram showing the experimental setup of the column experiments. Surface water collected from the Harpur Hill site was pumped through the columns via a peristaltic pump, with collection vessels collecting the outlet fluids, under anaerobic conditions (95% N_2 /5% H_2).

taken from each section and preserved for analyzes as described next.

DNA extraction, PCR amplification and sequencing

DNA was extracted from the five slurry samples collected from the pH variable sites at Harpur Hill, using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc.) following the manufacturer's protocol, and also from each of the sediment samples taken from along the columns after operation. DNA was stored at -20°C until needed. Ribosomal Intergenic Spacer Analysis (RISA; Cardinale et al. 2004) was performed on the DNA extracts to give an indication of microbial diversity in the samples, and along the columns. Primer set ITSF/ITSFRaub consisted of 5'-GTCGTAACAAGGTAGCCGTA-3' (forward primer) and 5'-GCCAAGGCATCCACC-3' (reverse primer). PCR conditions were described previously by Cardinale et al. (2004). PCR products were visualized on a 3% agarose gel.

A pyrosequencing methodology was then applied to investigate the microbial community composition within the samples. DNA extracts were subjected to a 16S rRNA PCR targeting a 311bp region of the gene. Primers used were 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') (Lane 1991) and 338 (5'-GCTGCCTCCCGTAGGAGT-3') (Daims et al. 1999). 454 Life Sciences adaptor primers were included in the forward primer, along with a 10-bp sample-specific multiplex identifier (MID). PCR products were verified on an agarose gel, and then purified with a QIAquick gel extraction kit (Qiagen, Crawley, UK).

Positive and negative controls were also subjected to PCR to ensure amplification of contaminants was minimal prior to pyrosequencing; the negative control used was sterile purified water, and no amplification was noted from this sample. The positive control was DNA extracted from *Geobacter sulfurreducens*. Pyrosequencing on a GS Junior platform (454 Life Sciences, Roche Diagnostics, UK) was used to sequence amplicons according to the manufacturer's instructions. Pyrosequencing data were processed using the QIIME software pipeline (Caporaso et al. 2010) for quality control and primer removal. Sequences less than 300bp and more than 400bp were then removed.

Phylogenetic analysis

The QIIME software pipeline (Caporaso et al. 2010) was used to pick and assign OTUs (operational taxonomic units) using usearch (Edgar 2010). Phylogeny was assigned using the Ribosomal Database Project (Cole et al. 2009), with a minimum confidence of 80%. Blastn nucleotide search was then used to identify representative sequences for each OTU.

Geochemical and mineralogical analyses

pH measurements were carried out using a Mettler Toledo pH probe, calibrated with pH 7 and pH 10 buffer solutions. Redox potential was measured with a HANNA ORP probe. Alkalinity measurements were carried out with acid titration (Burden and Cave 1999). Trace metal analysis was carried out using an ICP-MS (Agilent 7500cx), anions and cations were quantified with IC (Dionex DX-600). Acetate, lactate and propionate were quantified using capillary ion exchange chromatography on a

Dionex BioLC with a Dionex ICE AS1 column, as previously described by Bassil et al. (2015). Standards were prepared using Spex Certiprep 1000 mgL^{-1} Acetate solution, Accuspec 1000 ppm Lactate solution and Sigma Gold Label Propionic Acid. Quantitative X-ray diffraction analysis was carried out on the crushed sandstone before and after the experiments. Samples were finely ground with the addition of amyl acetate, and slides prepared. Slides were scanned with Bruker D8Advance X-Ray Diffractometer. Pore water and 0.5N HCl extractable Fe (II) was quantified using 2,2'-Bipyridyl.

Microbial imaging and cell counts

For bacterial cell counts, samples were fixed in 1% glutaraldehyde, and stored at 4°C until analyzed. Direct cell counts were carried out using epifluorescence microscopy. Cells were stained with acridine orange, and slides were viewed with a Zeiss universal microscope with a Zeiss III RS epi-fluorescence head, filter set 09 (40–490 nm). For viewing with a confocal laser scanning microscope (CLSM), approximately 0.1 g of sediment was transferred to a petri dish. Stains from a LIVE/DEAD *bacLight*TM Bacterial Viability Kit (Life Technologies) were diluted 1:200 with deionized water; 10 μl of each of the dilute stains were applied directly to the sediment. The sample was then immersed in deionized water. Images were collected on a Leica TCS SP5 AOBs upright confocal microscope using a 63 \times HCX Apo L Objective and 4 \times zoom. Images were collected using the following settings: FITC 494–530 nm and Texas Red 602–665 nm, using the 488 nm and 594 nm laser lines, respectively. Images collected using the confocal microscope were analyzed using ImageJ (Abramoff et al. 2004).

Results

Harpur hill microbial community analysis

To investigate the molecular ecology of the hyper-alkaline spring at Harpur Hill, samples were taken from across the site, at a range of pH values from 7.5–13. Total cell numbers decreased with increasing pH from approximately 2.5×10^6 cells mL^{-1} at pH 7.5 to approximately 3.0×10^5 cells mL^{-1} at pH 13 (Figure 3).

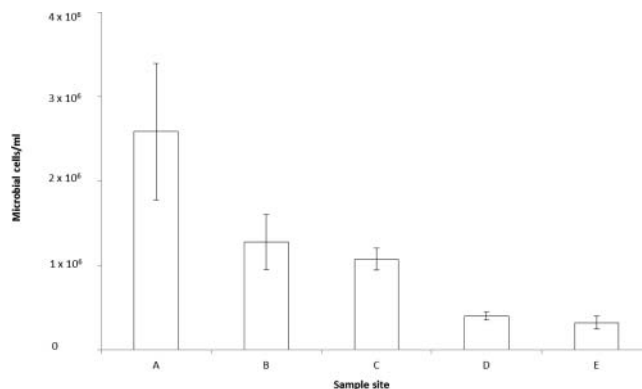


Figure 3 Total cell counts found under varying pH sample sites at Harpur Hill. A pH 7.5; B pH 8.9; C pH 11.4; D pH 12; E pH 13. Error bars show standard error calculated from ten replicate values.

A pyrosequencing approach was utilized to characterize the composition of microbial populations found at the site. A total of 45,013 partial 16S rRNA gene sequences were obtained from the five Harpur Hill sampling sites. OTUs were assigned using usearch to cluster sequences to the genus level. The largest number of OTUs were observed in the pH 8.9 sample (2090); the pH 13 sample site contained the smallest number of OTUs, with 390 observed. Rarefaction curves (not shown) confirmed that the pH 13 site contained a much lower diversity of bacterial species than the pH 7.5–12 sites.

Table 1 shows the relative abundance of bacterial genera from the five sampling sites. The sequence libraries from the site were dominated by members of the bacterial classes alphaproteobacteria, betaproteobacteria and gammaproteobacteria.

The sequence library obtained from the pH 7.5 site was dominated by organisms affiliated with the families *Xanthomonadaceae* and *Comamonadaceae* (21.1%). Other families made up smaller percentages of the library, for example organisms affiliated with *Acidobacteria* (4.3%), and *Koribacter* (4.2%). The sequence libraries obtained from the pH 8.9 and pH 12 sites contained complex bacterial communities, with no single family dominant. Significant bacterial families in the pH 8.9 sequence library included *Acidobacteria* (3.6%), *Chitinophagaceae* (2.9%), *Bradyrhizobiaceae* (2.5%), *Sphingomonadaceae* (3%) and *Sinobacteriaceae* (2.9%). A total of 1276 OTUs were observed, although most families made up a very small percentage of the library. At pH 12 organisms affiliated with *Paenibacillaceae* (9.4%) and *Cyclobacteriaceae* (7.5%) were dominant;

Table 1. Relative abundance of bacterial genera in samples collected from Harpur Hill.

Phylum	Family	Genus	HH 7.5	HH 8.9	HH 11.4	HH 12	HH 13
AD3	Unknown	Unknown	4.70%	3.70%	2.50%	4.60%	
	Unknown	Unknown	1.50%	1.90%		1.60%	
Acidobacteria	Unknown	Unknown	4.30%	3.60%	3.10%	4.70%	
	Koribacteraceae	Unknown	4.20%	3.30%	3.00%	3.80%	
	Koribacteraceae	<i>Candidatus Koribacter</i>		1.30%			
	Unknown	Unknown		1.20%			
	Other	Other				1.00%	
Actinobacteria	Solibacteraceae	<i>Candidatus Solibacter</i>	2.50%	3.90%	1.30%	2.00%	
	Other	Other		2.20%			
Bacteriodetes	Propionibacteriaceae	<i>Microlunatus</i>					3.30%
	Flavobacteriaceae	<i>Flavobacterium</i>					3.60%
Firmicutes	Other	Other		1.10%			
	Unknown	Unknown		2.90%			
	Chitinophagaceae	Unknown		2.90%		5.60%	
	Cyclobacteriaceae	Unknown			4.30%	7.50%	
	Flammeovirgaceae	<i>A4</i>		1.10%			
	Flexibacteraceae	<i>Arcicella</i>		1.10%			
	Alicyclobacillaceae	<i>Alicyclobacillus</i>					1.20%
	Paenibacillaceae	<i>Cohnella</i>			12.10%		
	Paenibacillaceae	<i>Paenibacillus</i>			10.20%	8.90%	
	Gal 15	Unknown	Unknown	1.10%			1.10%
Other	Other	Other		1.60%			
Proteobacteria	Beijerinckiaceae	Unknown					1.20%
	Bradyrhizobiaceae	<i>Bradyrhizobium</i>					1.70%
	Bradyrhizobiaceae	Other	1.00%	2.50%	1.10%	1.60%	1.80%
	Hyphomicrobiaceae	<i>Rhodoplanes</i>	1.10%	2.10%	1.00%	1.20%	
	Methylobacteriaceae	<i>Methylobacterium</i>					1.50%
	Other	Other		1.10%		1.60%	
	Acetobacteraceae	<i>Roseococcus</i>	1.00%		1.20%		
	Rhodobacteraceae	<i>Rhodobacter</i>				2.10%	
	Rhodospirillaceae	Unknown		1.00%			
	Erthobacteraceae	Other				1.00%	
	Sphingomonadaceae	Unknown		3.00%			
	Sphingomonadaceae	Other				1.20%	
	Sphingomonadaceae	<i>Sphingomonas</i>					4.90%
	EB1003	Unknown		1.20%			
	Comamonadaceae	<i>Hydrogenophaga</i>				2.70%	
	Comamonadaceae	Other	21.10%	2.10%	24.40%	3.80%	7.70%
	Comamonadaceae	<i>Rubrivivax</i>				1.60%	
	Comamonadaceae	<i>Variovorax</i>					1.60%
	Oxalobacteraceae	Other	2.10%	2.60%	1.10%	3.10%	
	Other	Other	1.10%	1.10%	1.10%		
	Unknown	Unknown			1.00%		1.40%
	Enterobacteriaceae	<i>Escherichia</i>					1.80%
	Enterobacteriaceae	Other					7.30%
211ds20	Unknown			1.00%			
Pseudomonadaceae	<i>Pseudomonas</i>	1.90%	2.60%	1.40%	2.10%	25.90%	
Sinobacteraceae	Unknown	1.40%	2.90%	1.00%	1.40%	1.20%	
Sinobacteraceae	Other			1.20%			
Xanthomonadaceae	Other	26.00%	1.50%	12.70%	5.40%		
Other	Other		1.10%				

HH 7.5: pH 7.5, HH 8.9: pH 8.9, HH 11.4: pH 11.4, HH 12: pH 12, HH 13: pH 13. Only genera comprising more than 1% of the population are shown.

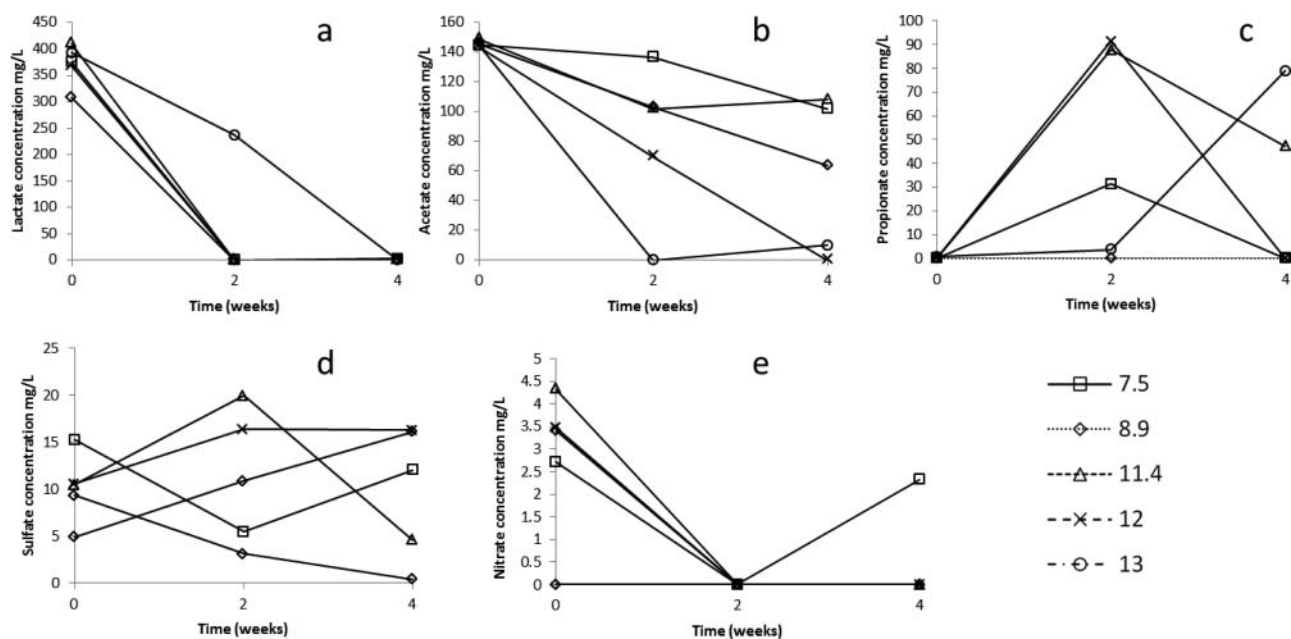


Figure 4. Lactate (a), acetate (b), propionate (c), sulfate (d) and nitrate (e) concentrations in fluids collected from carbon amended microcosms (starting pH values of microcosms are shown in the legend).

other significant families include *Chitinophagaceae* (5.6%), *Xanthomonadaceae* (5.4%), *Acidobacteria* (4.7%), *Oxalobacteriaceae* (3.1%) and *Hydrogenophaga* (2.7%).

The sequence library obtained from the sample collected at pH 11.4 was dominated by organisms affiliated with the families *Comamonadaceae* (24.4%), *Xanthomonadaceae* (12.7%), *Paenbacillaceae* (22.3%), *Sphingobacteriales* (4.3%), and *Acidobacteria* (3.1%). *Pseudomonadaceae* dominated the pH 13 community (25.9%), with other families highly abundant, including *Enterobacteriaceae* (10%), *Comamonadaceae* (7.7%), *Sphingomonadaceae* (4.9%), *Flavobacteriaceae* (3.6%), *Propionibacteriaceae* (3.4%), and *Methylobacteriaceae* (1.5%).

Microcosm experiments

Microcosm experiments were assembled to investigate activity of the microbial communities found under varying pH conditions, especially metal-reducing bacteria that have been identified at this site (Rizoulis et al. 2012) and have the potential to reduce and precipitate a range of redox active priority radionuclides. Increases in Fe(II) concentrations were observed only in non-autoclaved microcosms at pH 7.5, 8.9 and 11.4 (Figure 5), and attributed to the microbial reduction of Fe(III) in the sandstone, the Harpur Hill sediment or the Harpur Hill fluid (containing approx. 5 ppb Fe by ICP-MS analyses). In these microcosms there were concomitant increases in total cell counts consistent with respiration of Fe(III) or other electron acceptors in the microcosms that were used under anoxic conditions. Increases in Fe(II) concentrations were not observed throughout the experiment in the pH 12 and pH 13 microcosms, suggesting that Fe(III) was not respired under these conditions. Increases in Fe(II) were, however, observed in the pH 7.5, 8.9 and 11.4 microcosms over the course of the experiment.

Microbial processes are most likely responsible for Fe(III) reduction in these microcosms, and at these pH values,

increases in total cell counts were observed, consistent with energy conservation coupled to Fe(III) respiration during the experiment. The highest Fe(II) concentrations were observed in the pH 8.9 microcosms, reaching over 4 mM Fe(II) after 3 weeks (Figure 5). In autoclaved microcosms, no increases in Fe(II) were observed. The pH values of the microcosms, initially poised at pH 7.5 and pH 8.9, remained relatively constant throughout the experiment; in the microcosms with starting fluids of pH 11.4, 12 and 13, rapid pH buffering occurred.

The pH 11.4 and 12 microcosms buffered to around pH 8.5 after approximately 1 week, whereas the pH 13 microcosms remained at around pH 10 (data not shown). Lactate was utilized in biotic microcosms containing crushed sandstone (Figure 4a), although it was utilized more slowly in the microcosms containing fluid from the pH 13 sample site [and propionate production occurred at a slower rate (Figure 4c)] compared to the microcosms assembled with fluids of a lower pH. Decreases in sulfate concentration were observed in the microcosms poised initially at pH 7.5, 8.9 and 11.4 and containing crushed sandstone, and decreases in nitrate concentration were observed in microcosms containing crushed sandstone at all pH values (Figures 4d and 4e).

Column experiments

Both Fe(III) reduction and sulfate reduction was observed in microcosms containing fluid from the pH 11.4 sample site, so fluid from this sample site was subsequently used in column experiments. These experiments investigated the ability of alkaliphilic and alkalitolerant microorganisms from the site to colonize sandstone, and helped gain an understanding of the processes that they are capable of carrying out under high pH conditions, including biofilm formation and organic acid utilization. Column experiments ran for a total of six weeks. Initially, a rapid drop in the pH of the outlet fluid (from 11.5 to 8)

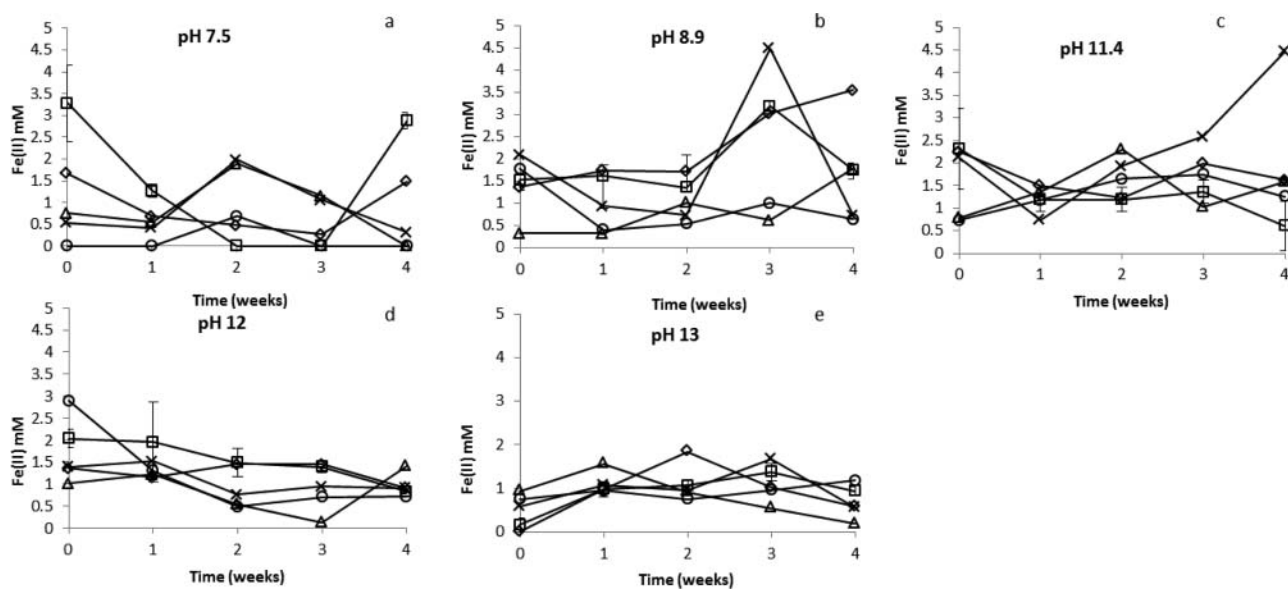


Figure 5. (a-e) Fe(II) measurements in anaerobic microcosms assembled with crushed sandstone, sediment and fluid from sampling sites at Harpur Hill poised at a range of pH values, with additions of acetate and lactate as carbon sources. ○: sterile control (autoclaved microcosm); △: sterile medium (Harpur Hill fluid); x: Harpur Hill microcosm containing sediment and fluid from the site, with no sandstone; ◇: Unamended control; □: Carbon amended microcosms. Error bars show standard error calculated from three replicate values.

was observed in both carbon (lactate/acetate) amended and control (no added carbon) experiments (Figure 6).

The pH of the control column outlet fluid (Figure 6) gradually increased throughout the experiment to 11.5, whereas the pH of the carbon amended column outlet fluid remained at around pH 8.5. A rapid increase in Eh (Figure 6) was observed initially in the outlet fluids compared to the Eh of the starting fluid in both carbon amended and control experiments; Eh then gradually decreased to approximately -200 mV. Total cell counts in the outlet fluid remained at around 1×10^5 cells mL^{-1} in the control experiments, whereas in the carbon-amended columns total cell counts increased after 4 weeks to almost 1×10^7 cell mL^{-1} (Figure 7).

Acetate concentration in the outlet fluids from the biotic columns remained relatively constant at around 350 mgL^{-1} .

Lactate concentration decreased rapidly, reaching 13 mgL^{-1} after 4 weeks. Propionate was observed in the outlet fluid after 2 weeks, eventually reaching 192 mgL^{-1} (Figure 8).

RISA of sediment samples collected from intervals along the length of carbon amended and control columns (Figure 9) demonstrated differences in microbial community composition between carbon amended and control columns, with microbial biomass decreasing (indicated by decreasing band intensity) moving from the inlet towards the outlet ends of columns. Figure 9 shows the presence of distinct microbial communities within the columns that differ from the community present in the Harpur Hill fluid supplied to the columns throughout the experiment. Colonization of the carbon amended columns occurred to a greater extent than the control columns, with a higher intensity banding pattern (indicating increased biomass)

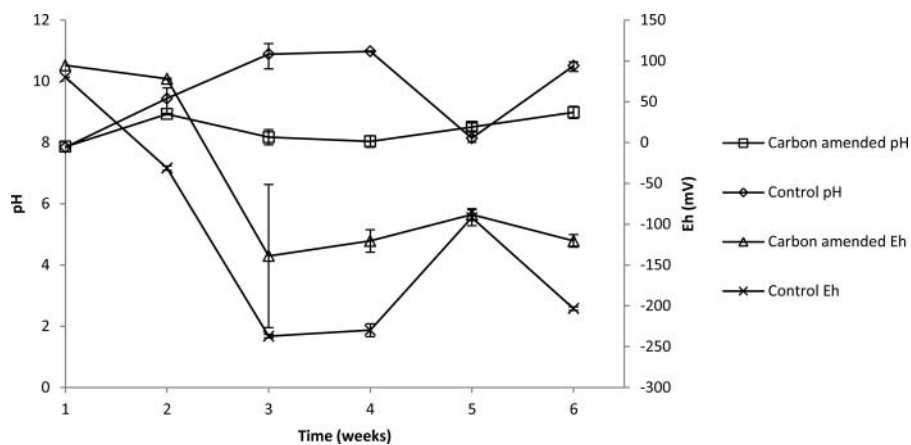


Figure 6. pH and Eh of outlet fluids of carbon amended and control columns. Error bars show standard error calculated from duplicate values. The starting fluid pH was around 11.6 and the Eh was around -260 mV.

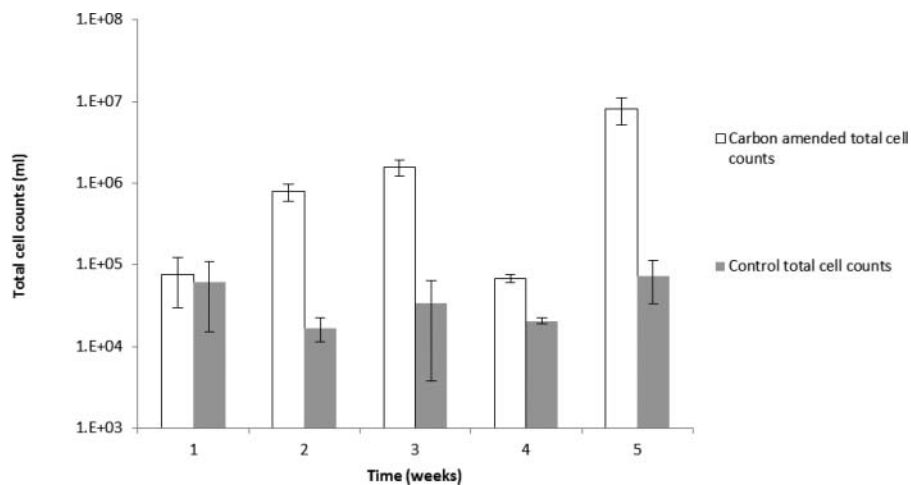


Figure 7. Total cell counts over the course of the column experiments. Error bars show standard error calculated from ten fields of view.

along the whole length of the column. The bacterial community composition of samples taken from the inlet and outlet ends of both carbon amended and control columns were investigated, to gain an insight into community differences both spatially, and under carbon amended and control conditions.

Genomic DNA extraction was attempted on the starting material (crushed sandstone) although when this extract was run on a 1% agarose gel, no product was observed, suggesting that biomass associated with the sandstone was low. Table 2 demonstrates the differences between the carbon amended and control columns. Members of the family *Clostridiaceae* dominated the inlet end of the carbon amended columns (45.8% of the sequence library), along with organisms affiliated with the family *Comamonadaceae* (35.2%). The relative abundance of *Clostridiaceae* decreased along the column, making up 20.8% of the sequence library at the outlet. There was a slight increase in the relative abundance of organisms affiliated with the family *Comamonadaceae* towards the end of the column to 38.3%.

Towards the outlet end of the control column, *Comamonadaceae* made up 52.2% of the sequence library, while the proportion of organisms that were relatively abundant at the inlet end decreased significantly. For example, members of the family *Spingomonadaceae* decreased from 11.8% to 0.3%; *Oxalobacteraceae* decreased from 8.2% to 0.3%, and *Pseudomonadaceae*

decreased from 10.9% to 4.9%. Microbial cells were clearly capable of colonising the surface of the sandstone under carbon amended conditions as demonstrated by CLSM (Figure 10).

Discussion

This study investigated the bacterial communities present in and around a hyper-alkaline spring at Harpur Hill near Buxton, Derbyshire, and assessed colonization and the potential impacts of microbial growth on the fluid transport characteristics of crushed sandstone. The study provides insights into the types of communities that may be relevant in relation to a cement-based GDF for ILW after the formation of a hyper-alkaline plume, and the processes that they are able to carry out under such conditions.

Harpur hill molecular ecology

Total cell counts carried out on samples collected from points with pH varying from 7.5–13 indicated that total cell numbers decreased as pH increased. Previous studies investigating microbial communities found in high pH environments have reported comparable levels of planktonic biomass, for example 10^5 cells mL^{-1} were observed in fluid from a hyper-alkaline spring in Maqarin at pH 12.9 (Pedersen et al. 2004). This is

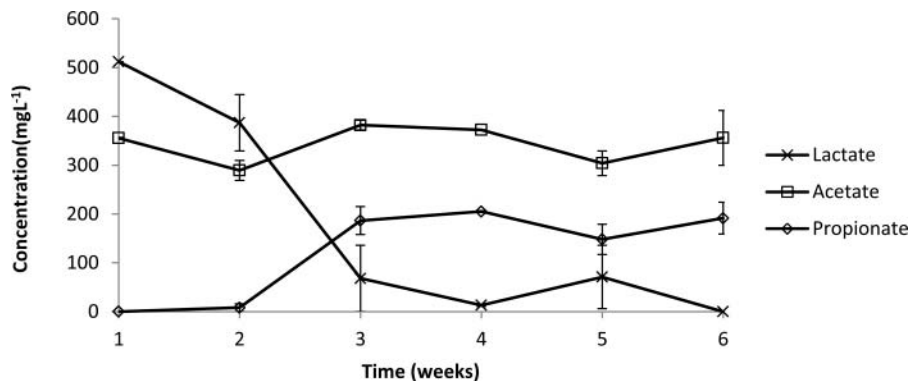


Figure 8. Lactate, acetate and propionate concentration in carbon amended columns outlet fluid. Error bars show standard error calculated from duplicate values.

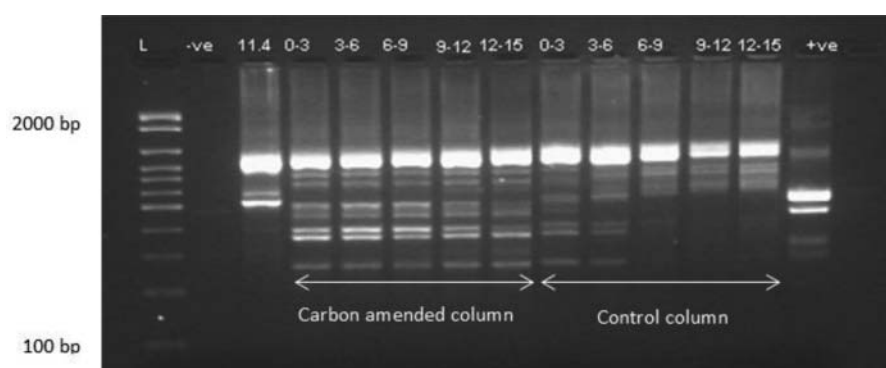


Figure 9 Ribosomal Intergenic Spacer Analysis (RISA) of DNA extracts from the column starting fluid and from sediment samples collected from along a carbon amended and control column. DNA was extracted from 3-cm intervals along the columns and subject to PCR-based analysis by RISA. Lane 11.4: Microbial community in the starting fluid before being pumped through the columns. Distance from the inlet (in cm) is shown at the top of the lanes. Positive (*E.coli* genomic DNA extract) and negative (sterile deionised water) controls are labelled +ve and -ve, respectively.

significantly lower than predictions of total numbers of cells (1.3×10^8 cells mL^{-1}) thought to be able to be supported in the hyper-alkaline waters based on availability of nutrients and energy sources present at the site (West et al. 1995).

In this study, cells were observed at pH 13 (as shown by acridine orange staining), although the potential for metabolism and growth under these conditions is unlikely; the upper pH limit for bacterial growth is not thought to extend above 12 (Rizoulis et al. 2012; Sorokin 2005). Neutrophilic bacteria are able to grow up to an external pH of 9 because of their ability to maintain cytoplasmic pH at around 7.5 via a range of mechanisms including up-regulation of “key cation-proton antiporters” (Krulwich et al. 2011), while obligately alkaliphilic species are thought to better tolerate increased cytoplasmic pH (Padan et al. 2005). The composition of the bacterial communities found at the varying pH sampling sites at Harpur Hill was investigated to determine which organisms may be carrying out geochemically important processes, and to determine the key differences in the communities living under different pH conditions.

Bacterial community composition from each of the sampling sites appeared to be distinct (Table 1). PCR-based pyrosequencing analyses indicated that although the communities

differed between sampling sites, some families were common to several sites. For example, *Acidobacteria* spp were present in both neutral and hyper-alkaline samples. Previous studies have also observed *Acidobacteria* in alkaline environments (e.g., Castro-Silva et al. 2013; Keshri et al. 2013; Tiago and Veríssimo 2013). Several genera in the family *Comamonadaceae* were also identified in samples taken from across the site up to pH 13.

Members of this family have been found to include denitrifying bacteria (Khan et al. 2002) and have been found in other studies of hyper-alkaline environments (Burke et al. 2012). Genera in the family *Comamonadaceae* found at the site include *Hydrogenophaga*, which are capable of oxidising hydrogen (Willems et al. 1989). *Hydrogenophaga* spp have been found previously in hyper-alkaline environments, for example Tiago and Veríssimo (2013) found that in samples collected from Cabeço de Vide of pH 11.4, 35.1% of their clone library was closely affiliated with *Hydrogenophaga flava*. *Hydrogenophaga* spp were present in travertine springs in Winter House Canyon at pH 12 (Brazelton et al. 2012), and were dominant in alkaline groundwater present in the Leka ophiolite (Daae et al. 2013).

Harpur Hill represents a highly alkaline environment where freshwater inputs and rainfall significantly alter the range of pH values found across the site over time. Indeed the presence

Table 2. Relative abundance of bacterial genera at inlet and outlet ends of a carbon amended and control column.

Phylum	Family	Genus	C. amended inlet	C. amended outlet	Control inlet	Control outlet
Acidobacteria	Koribacteriaceae	Unknown				1.00%
	Solibacteriaceae	<i>Candidatus Solibacter</i>				1.00%
Bacterioidetes	Other	Other		9.70%		1.00%
Firmicutes	Unknown	Unknown	7.80%	19.80%		3.10%
	Clostridiaceae	Other	45.80%	20.80%		
	Clostridiaceae	Unknown	1.50%			
	Peptostreptococcaceae	<i>Clostridium</i>	1.00%			
	Erysipelotrichaceae	<i>PSB-M-3</i>	7.50%			
Proteobacteria	Acetobacteraceae	<i>Roseococcus</i>		9.50%		1.00%
	Sphingomonadaceae	Other			11.80%	2.80%
	Comamonadaceae	Other	31.50%	38.30%	35.20%	57.20%
	Oxalobacteraceae	Other			3.40%	
	Oxalobacteraceae	Unknown			8.20%	
	Enterobacteriaceae	Other			5.90%	2.10%
	Pseudomonadaceae	<i>Pseudomonas</i>	1.00%		10.90%	4.90%
	Xanthomonadaceae	Other			2.80%	5.20%

Only genera comprising more than 1% of the population are shown.

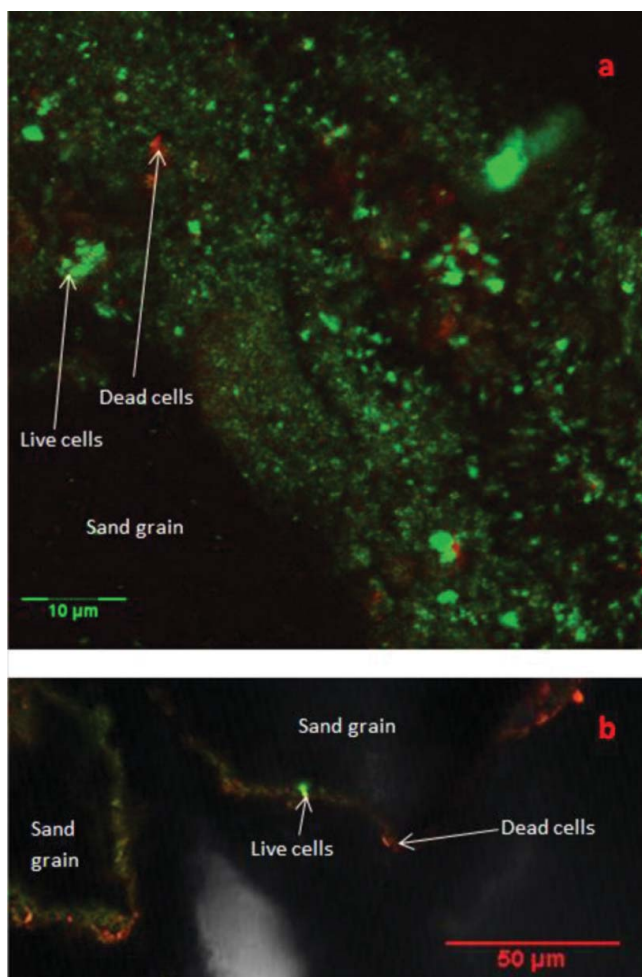


Figure 10. CLSM micrograph showing bacterial cells coating sand grains collected from the inlet end of a carbon amended column. Cells were stained with a LIVE/DEAD *backlight* Bacterial Viability Kit. Green: live cells, Red: dead cells. a: An image showing one view of a 3D projection of a stack comprised of 62 images of a sand grain collected over a depth of 18.08 μm . b: A single image of depth 0.3 μm showing live and dead cells attached to the surfaces of two sand grains.

of a diverse bacterial community at pH 13 may have been linked to heavy rainfall at the time of sampling, causing runoff from the surrounding grassland, as the dominant members of the sequence library obtained from this site were not affiliated with known alkaliphiles, and may not be adapted to life at high pH. Surface waters of pH > 13 have been observed at the site, particularly at the base of the limestone waste, but hyper-alkaline values have been observed across the whole site (pH 12–13) at times of low rainfall.

At the time of sampling (April 2012), heavy rainfall had caused an influx of freshwater from the culvert (HH 7.5, Figure 1) at a pH of 7.5, and flow rates of fluid out of the culvert of up to 1.1 ms^{-1} were recorded here. The input of water of a circum-neutral pH value caused significant changes in the pH values of fluids found across the site, ranging from pH 7.5–13. Bacterial cell counts collected at this time reflect these changes in pH, as total cell numbers decrease with increasing pH (Figure 3). The predicted evolution of the ADZ will also deliver pH variations in and around a GDF over time, in this case with increasing distance from the

waste, suggesting there may be distinct differences in microbial activity in this scenario also.

Lower pH niche environments may also occur within the ADZ, perhaps resulting from factors such as variations in groundwater flow velocity, or heterogeneity related to organic inputs providing more favorable conditions for microbial activity to occur. The results from this investigation suggest that a number of bacterial groups are able to tolerate highly-alkaline conditions, although they may only be active when the pH decreases to more favorable levels in and around the GDF. The occurrence of microbial activity in column experiments carried out under highly-alkaline (pH 11.5) conditions may suggest that microbial activity could be significant in a GDF where the pH conditions are slightly more favorable.

Microcosm experiments

Microcosm experiments were conducted to further explore the functional diversity within communities in the Harpur Hill samples, focusing especially on anaerobes with the potential to impact on radionuclide speciation via enzymatic or sulfide/Fe(II)-mediated reduction mechanisms. Results from microcosm experiments containing crushed sandstone and microbial inocula from Harpur Hill indicated that the microorganisms from the pH 11.4 site were capable of reducing Fe(III) and sulfate, whereas no increases in Fe(II) were seen in pH 12 and pH 13 microcosms. These findings are consistent with results from previous experiments carried out at hyper-alkaline pH, where anaerobic metabolism (including Fe(III) reduction) was limited at pH 12 (Rizoulis et al. 2012). It should be noted that pH buffering of the alkaline microcosms occurred during incubation; pH 11.5 microcosms eventually buffered to around pH 8.5, potentially creating a more favorable environment for biological activity.

Metal reduction by bacteria under alkaline conditions has been observed in several other studies, for example Pollock et al. (2007) isolated a strain similar to *Bacillus agaradhaerens* capable of Fe(III) reduction up to pH 11. Williamson et al. (2013) observed microbial Fe(III) reduction in microcosm experiments at pH 10; with enhanced Fe(III)-reducing conditions observed in the presence of electron shuttles including humics. Fe(III)-reducing bacteria have been found previously to have a key role in controlling radionuclide mobility, as Fe(III)-reducing bacteria have the ability to reduce a broad range of redox active radionuclides (e.g., Tc(VII), U(VI), Np(V)) via enzymatic and Fe(II)-mediated mechanisms (Lloyd et al. 2002). Organic acid utilization and nitrate reduction was also observed in biotic (nonautoclaved) microcosms containing crushed sandstone at all pH values; the pH 13 microcosms were buffered to around pH 10 during the experiment, allowing more favorable conditions for microbial activity to occur.

Column experiments

Sequence libraries from the inlet and outlet ends of the carbon amended and control (non-carbon amended) columns were compared to determine which organisms from the original community were able to colonize the sandstone in flow-through experiments, and how the communities differ when

they are stimulated with the addition of carbon sources. Lactate and acetate were added as a surrogate for the cellulose degradation products that may be present in an ILW GDF, and the naturally occurring organic matter that may be present in the pore water of the host rock environment. RISA banding patterns on DNA extracts (Figure 9) from samples taken from 3cm intervals along the columns demonstrated gradual changes in community composition along the columns, and also indicated that more DNA was extracted from the inlet ends of the columns (suggesting more biomass was present here); other studies have witnessed similar decreases in microbial attachment with distance travelled, even in field studies (Ginn et al. 2005). This behavior may be a result of cell surface properties ensuring microorganisms that are more capable of attaching to surfaces are deposited close to the column inlet (Tong et al. 2005).

The presence of more biomass at the carbon amended column inlet is also potentially because of greater utilization of lactate and acetate at the column inlet; the sequence library obtained from sediment from the carbon amended column inlet is dominated by members of the family *Clostridiaceae*, which became less dominant towards the column outlet. Several studies have demonstrated the changes to microbial community composition that take place as a result of variations in organic carbon availability, which could account for the variation observed along the columns. As an example, soil microbial populations are known to vary vertically along a soil profile as a result of declines in soil carbon availability with increasing depth (Fierer et al. 2003). Carbon availability is also known to impact the mechanisms of bioclogging; as an example Hand et al. (2008) found that grain size impacted upon bioclogging, until carbon concentration decreased below a certain threshold, at which point grain size no longer had significant impacts on biomass build up.

Other environmental characteristics are thought to lead to spatial variations in microbial community composition, including flow heterogeneity through porous media (Besemer et al. 2009), and variations in dispersal mechanisms of microorganisms and nutrients (Lin et al. 2012). It is likely that during these column experiments, a combination of these factors led to the spatial variation in community composition, perhaps with lower pH niches at the column inlet occurring as a result CO_2 generation from organic acid utilization. The data shown in Figure 7 could suggest that biomass builds up over time in carbon amended columns, and then processes such as clogging and dispersal occur. The spatial differences in microbial community composition may also be a result of the short timescale over which the experiment was carried out, and perhaps may have become more uniform over the length of the column over a greater timescale.

The sequence libraries obtained from the control column were dominated by *Comamonadaceae*, but far more phylogenetic groups were present in low abundance compared to the carbon amended columns. Previous studies have shown members of the family *Comamonadaceae* to be present in the mature stage of mixed biofilm development (Fernandez et al. 2008). Members of the family *Clostridiaceae* only made up 0.1% of the community in the control column, whereas they dominated the carbon amended column, suggesting the

populations of these known fermentative bacteria were stimulated by the addition of a carbon source to the system.

The bacterial populations present in the control columns may be representative of the starting population in the Harpur Hill fluid collected from the pH 11.4 sample site. Although the starting fluids were collected on different sampling days, sequence libraries obtained from both the pH 11.4 sample site, and the control column are dominated by members of the family *Comamonadaceae*.

Microbial communities in the biotic columns were capable of fermenting lactate to propionate; members of the bacterial family *Clostridiaceae* were dominant in the carbon amended column sequence libraries, and have previously been shown to carry out this fermentation process (e.g., Kuchta and Abeles 1985). As the acetate concentration remained relatively constant throughout the experiment, this may suggest that acetate was not utilized, or more likely that it was both further oxidized and produced (e.g., via lactate metabolism) at similar rates. Fermentation of lactate to acetate and propionate can occur via several different pathways including the methylmalonyl-CoA pathway and the acrylyl-CoA pathway (Seeliger et al. 2002). Other alkaliphilic/ alkalitolerant bacteria have been known to produce acetate and propionate as fermentation products under high pH conditions (Zhilina et al. 2004). This process may have implications for a GDF, as production of carbon dioxide gas can occur during fermentation (Seeliger et al. 2002).

Microbial CO_2 generation in a cementitious GDF may lead to changes in the host rock transport properties as CaCO_3 precipitation will occur if the Ca-rich cement pore waters come into contact with CO_2 (Ranaivomanana et al. 2013), potentially blocking pore throats. Indeed, the decreased calcium concentration (data not shown) in the outlet fluids of the carbon amended columns throughout the course of the experiment may be a result of organic acid fermentation (Figure 8) leading to the production of carbon dioxide gas, facilitating CaCO_3 precipitation. Another potential mechanism for the removal of calcium from solution is the microbially mediated precipitation of CaCO_3 ; it is thought that under appropriate conditions, most bacteria are capable of precipitating calcite crystals (Boquet et al. 1973).

In summary, diverse bacterial communities were identified at a highly alkaline spring, demonstrating the ability to tolerate highly alkaline conditions that could be representative of some of the conditions likely to be found in the ADZ around a cement-based GDF for ILW. The bacterial genera dominating the site have been observed previously in other hyper-alkaline environments. The microbial community present at pH 11.5 at the field site were able to reduce Fe(III) in microcosm experiments, and were capable of colonising sandstone in column experiments under alkaline conditions. The microbial communities within the columns also demonstrated the ability to utilize lactate, a surrogate for cellulose degradation products that will be present in a GDF, suggesting that these processes may be able to occur in a host rock environment.

Fermentation resulting in the production of CO_2 could potentially cause the precipitation of calcium carbonate in a cement-based GDF environment, potentially altering porosity and inhibiting radionuclide transport through the geosphere,

and this could be accentuated by the bioreduction of radionuclides under anoxic conditions. Although these experiments demonstrate that microbial processes impact on the transport properties of sandstone under high pH conditions over relatively short spatial and temporal scales, the importance of these processes over the lifetime of a GDF remain uncertain. Because of the nature of the hyper-alkaline plume development, it is likely that during ILW repository evolution, microbial activity will be limited to localities where a pH decrease in the plume has occurred.

The presence of a carbon source (in the form of cellulose degradation products) may stimulate microbial activity in a GDF host rock environment, although the extent to which alkaline degradation reactions generating these products will occur, before reaching conditions that will favor competing microbial degradation processes (< pH 12; Rizoulis et al. 2012) is uncertain, and will depend on parameters such as the rate of evolution of the hyper-alkaline plume. There also remain uncertainties regarding the evolution of microbial populations over the lifetime of a GDF, because the rapid changes that occurred during the short-term experiments carried out in this study are not representative of the timescales relevant to a GDF. Clearly further research is necessary to ensure that the microbial impacts over the lifetime of a GDF for ILW are better understood. Longer term flow-through experiments under conditions more representative of a potential GDF (with regards to environmental factors including temperature, groundwater velocity, pH, organic carbon availability and changes in the porosity and permeability of the media over time) may provide further insights into the possible impacts of microbial activity on GDF evolution and radionuclide transport.

Acknowledgments

The authors would like to thank Alastair Bewsher and John Waters (University of Manchester) for their help with data acquisition, and Keith Bateman, Simon Gregory and Kay Green (BGS) for their contributions to this work. Special thanks go to Steven Marsden for help with the microscopy results presented. This article is also published with the permission of the Executive Director, BGS (NERC).

Funding

This work was supported by a Natural Environment Research Council (NERC) CASE studentship with the Nuclear Decommissioning Authority Radioactive Waste Management Directorate (NDA RWMD). JRL and AR acknowledge the financial support of the NERC BIGRAD consortium. JMW acknowledges BGS support of the BIOTRAN project. The Bioimaging Facility microscopes used in this study were purchased with grants from the Biotechnology and Biological Sciences Research Council (BBSRC), Wellcome and the University of Manchester Strategic Fund.

References

- Abramoff MD, Magalhaes PJ, Ram SJ. 2004. Image processing with ImageJ. *Biophoton Inter* 11:36–42.
- Atekwana EA, Werkema DD, Atekwana EA. 2006. Biogeophysics: The effects of microbial processes on geophysical properties of the shallow subsurface, *Applied Hydrogeophysics*, Chapter 6. In: Vereecken H, Binley A, Cassiani G, Revil A, Titov K, editors. *Applied Hydrogeophysics*. The Netherlands: Springer, p161–193.
- Bassil NM, Bryan N, Lloyd JR. 2015. Microbial degradation of isosaccharinic acid at high pH. *ISME J* 9:310–320.
- Bateman K, Coombs P, Noy DJ, Pearce JM, Wetton P, Haworth A, Linklater C. 1999. Experimental simulation of the alkaline disturbed zone around a cementitious radioactive waste repository: numerical modelling and column experiments. In: *Chemical Containment of Waste in the Geosphere*, Vol. 157. Geological Society of London, p183–194.
- Bath, AH, Christofi, N, Neal, C, Philp JC, Cave MR, McKinley, I G, Berner, U. 1987. Trace element and microbiological studies of alkaline groundwaters in Oman, Arabian Gulf: A natural analogue for cement porewaters. Keyworth, Nottingham, UK: NERC British Geological Survey Report FLP0 87–2.
- Besemer K, Singer G, Hödl I, Battin TJ. 2009. Bacterial community composition of stream biofilms in spatially variable-flow environments. *Appl Environ Microbiol* 75:7189–7195.
- Boquet E, Boronat A, Ramos-Cormenzana A. 1973. Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* 246:527–529.
- Brazelton WJ, Nelson B, Schrenk MO. 2012. Metagenomic evidence for H₂ oxidation and H₂ production by serpentinite-hosted subsurface microbial communities. *Front Microbiol* 2:268.
- Brazelton WJ, Morrill PL, Szponar N, Schrenk MO. 2013. Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. *Applied and Environ Microbiol* 79:3906–3916.
- Burden SR, Cave MR. 1999. Validation of the method to determine pH and alkalinity by potentiometric titration. Keyworth, Nottingham, UK: British Geological Survey. BGS Technical Report No WI/99/009C.
- Burke IT, Mortimer RJG, Palaniyandi S, Whittleston RA, Lockwood CL, Ashley DJ, Stewart DI. 2012. Biogeochemical reduction processes in a hyper-alkaline leachate affected soil profile. *Geomicrobiol J* 29:769–779.
- Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia Am, Rizzi A, Zanardini E, Sorini C, Orselli C, Daffonchio D. 2004. Comparison of different primer sets for use in Automated Ribosomal Intergenic Spacer Analysis of complex bacteria communities. *Appl Environ Microbiol* 70:6147–6156.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello E, Fierer N, Peña AG, Goodrich JK, Gordon JI et al. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Meth* 7:335–336.
- Castro-Silva C, Ruiz-Valdivieso VM, Valenzuela-Encinas C, Alcántara-Hernández EJ, Navarro-Noya YE, Vázquez-Núñez E, Luna-Guido M, Marsch R, Densooven L. 2013. The bacterial community structure in an alkaline saline soil spiked with anthracene. *Electron J Biotechnol* 16:1–20.
- Chapman N, Hooper A. 2012. The disposal of radioactive wastes underground. *Proc Geol Asso* 123:46–63.
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. *Nucl Acids Res* 37:D141–D145.
- Coombs P, Wagner D, Bateman K, Harrison H, Milodowski AE, Noy D, West JM. 2010. The role of biofilms in subsurface transport processes. *Quart J Eng Geol Hydrogeol* 43:131–139.
- Crawford RJ, Webb HK, Truong VK, Hasan J, Ivanova EP. 2012. Surface topographical factors influencing bacterial attachment. *Advan Coll Interf Sci* 179–182:142–149.
- Daae FL, Økland I, Dahle H, Jørgensen SL, Thorseth IH, Pedersen RB. 2013. Microbial life associated with low-temperature alteration of ultramafic rocks in the Leka ophiolite complex. *Geobiology* 11:318–339.
- Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. 1999. The Domain-specific Probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *System Appl Microbiol* 22:434–444.
- Dimitriu PA, Pinkart HC, Peyton BM, Mormile MR. 2008. Spatial and temporal patterns in the microbial diversity of a meromictic soda lake in Washington State. *Applied and Environmental Microbiology*. 74:4877–4888.
- Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461.

- Fernandez N, Diaz EE, Amils R, Sanz JL. Analysis of microbial community during biofilm development in an anaerobic wastewater treatment reactor. *Microbial Ecology*. 56:121–132.
- Fierer N, Allen AS, Schimel JP, Holden PA. 2003. Controls on microbial CO₂ production: a comparison of surface and subsurface soil horizons. *Global Change Biology* 9:1322–1332.
- Ford TD, Pedley HM. 1996. A review of tufa and travertine deposits of the world. *Earth-Sci Rev* 41:117–175.
- Ginn TR, Camesano T, Scheibe TD, Nelson KE, Clement TP, Wood BD. 2005. Microbial transport in the subsurface. In: Anderson MG. (Ed.), *Encyclopedia of Hydrological Sciences*. 2005. pp: 1603–1626.
- Hand VL, Lloyd JR, Vaughan DJ, Wilkins MJ, Boulton S. 2008. Experimental studies of the influence of grain size, oxygen availability and organic carbon availability on bioclogging in porous media. *Environ Sci Technol* 42:1485–1491.
- Hodgkinson ES, Hughes CR. 1999. The mineralogy and geochemistry of cement/rock reactions: high-resolution studies of experimental and analogue materials. In: Metcalfe R, Rochelle CA, editors. *Chemical Containment of Waste in the Geosphere*, Vol. 157. Geological Society of London Special Publications 157, pp: 195–211.
- Kazy SF, Sar P, D'Souza SF. 2008. Studies on uranium removal by the extracellular polysaccharide of a *Pseudomonas aeruginosa* strain. *Bioremed J* 12:47–57.
- Keshri J, Mishra A, Jha B. 2013. Microbial population index and community structure in saline-alkaline soil using gene targeted metagenomics. *Microbiol Res* 168:165–173.
- Khan ST, Hiraishi A. 2002. *Diaphorobacter nitroreducens* gen. nov., sp. nov., a poly(3-hydroxybutyrate)-degrading denitrifying bacterium isolated from activated sludge. *The Journal of General and Applied Microbiology* 48:299–308.
- Khoury HN, Salameh E, Clark ID, Fritz P, Bajjali W, Milodowski AE, Cave MR, Alexander WR. 1992. A natural analogue of high pH cement pore waters from the Maqarin area of northern Jordan. I: introduction to the site. *Journal of Geochemical Exploration* 46:117–132.
- Krulwich TA, Sachs G, Padan E. 2011. Molecular aspects of bacterial pH sensing and homeostasis. *Nat Rev Microbiol* 9:330–343.
- Kuchta RD, Abeles RH. 1985. Lactate reduction in *Clostridium propionicum*. Purification and properties of lactyl-CoA dehydratase. *J Biol Chem* 260:13181–13189.
- Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. *Nucleic Acid Techniques in Bacterial Systematics*. London, England: John Wiley & Sons Ltd., p115–175.
- Lin X, McKinley J, Resch CT, Kaluzny R, Lauber CL, Fredrickson J, Knight R, Konopka A. 2012. Spatial and temporal dynamics of the microbial community in the Hanford unconfined aquifer. *ISME J* 6:1665–1676.
- Lloyd JR. 2003. Microbial reduction of metals and radionuclides. *FEMS Microbiol Rev* 27:411–425.
- Lloyd JR, Chesnes J, Glasauer S, Bunker DJ, Livens FR, Lovley DR. 2002. Reduction of actinides and fission products by Fe(III) reducing bacteria. *Geomicrobiol J* 19:103–120.
- Lovley DR. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiol Rev* 55:259–287.
- Ng CC, Huang WC, Chang CC, Tzeng WS, Chen TW, Liu YS, Shyu YT. 2006. Tufa microbial diversity revealed by 16S rRNA cloning in Taroko National Park, Taiwan. *Soil Biol Biochem* 38:342–348.
- Ordnance Survey data © Crown Copyright and database rights 2013, Harpur Hill, Derbyshire. Accessed January 14, 2014. Available at <http://www.getamap.ordnancesurveyleisure.co.uk/>.
- Padan E, Bibi E, Ito M, Krulwich TA. 2005. Alkaline pH homeostasis in bacteria: New insights. *Biochim Biophys Acta* 1717:67–88.
- Pedersen K. 1996. Investigations of subterranean bacteria in deep crystalline bedrock and their importance in the disposal of nuclear waste. *Can J Microbiol* 42:383–391.
- Pedersen K, Nillson E, Arlinger J, Hallbeck L, O'Neill A. 2004. Distribution, diversity and activity of microorganisms in the hyper-alkaline spring waters of Maqarin in Jordan. *Extremophiles* 8:151–164.
- Perri E, Manzo E, Tucker ME. 2012. Multi-scale study of the role of the biofilm in the formation of minerals and fabrics in calcareous tufa. *Sediment Geol* 263–264:16–29.
- Pollock J, Weber KA, Lack J, Achenbach LA, Mormille MR, Coates JD. 2007. Alkaline iron (III) reduction by a novel alkaliphilic, halotolerant, *Bacillus* sp. isolated from salt flat sediments of Soap Lake. *Appl Microbiol Cell Physiol* 77:927–934.
- Ranaivomanana H, Verdier J, Sellier A, Bourbon X. 2013. Sealing process induced by carbonation of localized cracks in cementitious materials. *Cement Concrete Compos* 37:37–46.
- Rizoulis A, Steele HM, Morris K, Lloyd JR. 2012. The potential impact of anaerobic microbial metabolism during the geological disposal of intermediate-level waste. *Mineral Mag* 76:3261–3270.
- Rizoulis A, Milodowski AE, Morris K, Lloyd JR. 2014. Bacterial diversity in the hyperalkaline Allas Springs (Cyprus), a natural analogue for cementitious radioactive waste repository. *Geomicrobiology Journal*. doi:10.1080/01490451.2014.961107.
- Savage D. 2011. A review of analogues of alkaline alteration with regard to long-term barrier performance. *Mineral Mag* 75:2401–2418.
- Savage D, Bateman K, Hill P, Hughes C, Milodowski A, Pearce J, Rae E, Rochelle C. 1992. Rate and mechanism of the reaction of silicates with cement pore fluid. *Appl Clay Sci* 7:33–45.
- Seeliger S, Janssen PH, Schink B. 2002. Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. *FEMS Microbiol Lett* 211:65–70.
- Sorokin DY. 2005. Is there a limit for high-pH life? *Inter J System Evol Microbiol* 55:1405–1406.
- Stougaard P, Jørgensen F, Johnsen MG, Hansen OC. 2002. Microbial diversity in ikaite tufa columns: an alkaline, cold ecological niche in Greenland. *Environ Microbiol* 4:487–493.
- Stroes-Gascoyne S, West JM. 1996. An overview of microbial research related to high-level nuclear waste disposal with emphasis on the Canadian concept for the disposal of nuclear fuel waste. *Can J Microbiol* 42:349–366.
- Surasani VK, Li L, Ajo-Franklin JB, Hubbard C, Hubbard SS, Wu Y. 2013. Bioclogging and permeability alteration by *L. mesenteroides* in a sandstone reservoir: a reactive transport modelling study. *Energy Fuels* 27:6538–6551.
- Tiago I, Verissimo A. 2013. Microbial and functional diversity of a subterranean high pH groundwater associated to serpentinization. *Environ Microbiol* 15:1687–1706.
- Tong M, Camesano TA, Johnson WP. 2005. Spatial variation in deposition rate coefficients of an adhesion-deficient bacteria strain in quartz sand. *Environ Sci Technol* 39:3679–3687.
- VanEngelen MR, Peyton BM, Mormille MR, Pinkart HC. 2008. Fe(III), Cr(VI), and Fe(III) mediated Cr(VI) reduction in alkaline media using a *Halomonas* isolate from Soap Lake, Washington. *Biodegradation* 19:841–850.
- West JM, Coombs P, Gardner SJ, Rochelle CA. 1995. The microbiology of the Maqarin site, Jordan - a natural analogue for cementitious radioactive waste repositories. *Materials Research Society Symposium on 'The Scientific Basis for Nuclear Waste Management'*, Kyoto, Japan. *Mater Res Soc Sympos Proceed* 353:181–188.
- West JM, McKinley IG. 2002. The geomicrobiology of radioactive waste disposal. In: Bitton G, editor. *The Encyclopedia of Environmental Microbiology*. New York, USA: John Wiley. p2661–2674.
- Willems A, Busse J, Goor M, Pot B, Falsen E, Jantzen E, Hoste B, Gillis M, Kersters K, Auling G, De Ley J. 1989. *Hydrogenophaga*, a new genus of hydrogen-oxidising bacteria that includes *Hydrogenophaga flava* comb. nov. (Formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (Formerly *Pseudomonas palleronii*), *Hydrogenophaga psudoflava* (Formerly *Pseudomonas psudoflava* and '*Pseudomonas carboxydoflava*'), and *Hydrogenophaga taeniospiralis* (Formerly *Pseudomonas taeniospiralis*). *Inter J System Evolution Microbiol* 39: 319–333.
- Williamson AJ, Morris K, Shaw S, Byrne JM, Boothman C, Lloyd JR. 2013. Microbial reduction of Fe(III) under alkaline conditions relevant to geological disposal. *Appl Environ Microbiol* 79: 3320–3326.
- Zhilina TN, Appel R, Probain C, Brossa EL, Harder J, Widdel F, Zavarzin GA. 2004. *Alkaliflexus imshenetskii* gen. nov. sp. nov., a new alkaliphilic gliding carbohydrate-fermenting bacterium with propionate formation from a soda lake. *Arch Microbiol* 182: 244–253.