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Biogeochemical Reduction Processes in a Hyper-Alkaline Leachate Affected Soil Profile

Ian T. Burke,[1] Robert J. G. Mortimer,[1] Shanmugam Palaniyandi,[2] Robert A. Whittleston,[1] Cindy L. Lockwood,[1] David J. Ashley,[1] and Douglas I. Stewart[2]

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Hyperalkaline surface environments can occur naturally or because of contamination by hydroxide-rich wastes. The high pH produced in these areas has the potential to lead to highly specialized microbial communities and unusual biogeochemical processes. This article reports an investigation into the geochemical processes that are occurring in a buried, saturated, organic-rich soil layer at pH 12.3. The soil has been trapped beneath calcite precipitate (tufa) that is accumulating where highly alkaline leachate from a lime kiln waste tip is emerging to atmosphere. A population of anaerobic alkaliphilic bacteria dominated by a single, unidentified species within the *Comamonadaceae* family of *β-proteobacteria* has established itself near the top of the soil layer. This bacterial population appears to be capable of nitrate reduction using electron donors derived from the soil organic matter. Below the zone of nitrate reduction a significant proportion of the 0.5N HCl extractable iron (a proxy for microbial available iron) is in the Fe(II) oxidation state, indicating there is increasing anoxia with depth and suggesting that microbial iron reduction is occurring. Supplemental materials are available for this article. Go to the publisher's online edition of *Geomicrobiology Journal* to view the free supplemental files.

Keywords anaerobe, alkaliphile, bacteria, contaminated land, iron-reduction, nitrate-reduction, microbial-reduction

INTRODUCTION

There are many different environments that occur both on and in the Earth that are characterized by high pH. Some are entirely natural, e.g., soda lakes, hot springs, oceanographic cold seeps, deep mine waters (Brazelton et al. 2010; McMillan et al. 2009; Pollock et al. 2007; Takai et al. 2001; 2005), but many are also due to human activities. These anthropogenic sites occur as a result of the presence of residues from a range of industrial processes, e.g., lime production waste, steelworks slags, coal combustion residues, Solvay process waste, chromite ore processing residues, bauxite processing wastes, borax wastes and cementitious construction wastes (Carlson and Adriano 1993; Deakin et al. 2001; Effler et al. 1991; Hartland et al. 2009; Mayes et al. 2006; 2008; 2011; Townsend et al. 1999; Ye et al. 2004). Weathering of these wastes typically produces highly alkaline leachate (pH 10–13) due to the ubiquitous presence of Ca, Na and K oxides (primarily CaO) that hydrolyze in natural waters to produce soluble metal hydroxides. As these wastes are often a legacy from times when disposal was poorly controlled, alkaline leachate emanating from such wastes can contaminate any groundwater resources beneath disposal sites (Hartland et al. 2009; Stewart et al. 2010).

The trace metal composition of these leachates varies greatly with the composition of individual wastes but elevated concentrations of contaminant trace metals and metalloids such as As, Pb, V and Cr are commonly reported (Chaurand et al. 2007; Mayes et al. 2009; Stewart et al. 2007). In addition, due to the leaching of sulphur bearing minerals, elevated sulphate concentrations are often reported to affect overall water quality (Mayes et al. 2008; Schwab et al. 2006; Whittleston et al. 2010). When Ca concentrations are not limiting, the alkaline leachate reacts rapidly with atmospheric CO_2 where it emerges into sub-aerial environments, sometimes producing very high rates of calcite precipitation (Deakin et al. 2001; Hartland et al. 2009). This has a detrimental impact on surface environments due to the build up of tufa deposits that smother natural vegetation and benthic organisms (Effler et al. 1991). As disposal sites rarely have impermeable barriers underneath the waste, the fate of the alkaline leachate, and particularly any contaminants within that leachate, depends solely on biogeochemical interactions with soils and sediments present beneath or adjacent to waste disposal sites.

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TABLE 1
Microbially significant half-reaction reduction potentials: Standard reduction potential, E^0, and redox potential, Eh, at pH 12 (at 25°C and atmospheric pressure)

Transformation	Reaction	E_0 (V)	Eh @ pH 7 (V)	Eh @ pH 12 (V)	Assumptions
O_2 depletion$^+$	$O_2 + 4H^+ + 4e^- = 2H_20$	1.230	0.816	0.510	$P_{O2} = 0.2$ bar
Nitrate reduction to nitrite$^+$	$NO_3^- + 2H^+ + 2e^- = NO_2^- + H_2O$	0.845	0.431	0.135	$[NO_3^-] = [NO_2^-]$
Nitrite reduction to nitric oxide$^\$$	$NO_2^- + 2H^+ + e^- = NO + H_2O$	1.192	0.484	−0.106	$[NO_2^-] = 100\ \mu$mol L^{-1} $P_{NO} = 10^{-6}$ bar
Nitric oxide reduction to nitrous oxide$^\$$	$NO + H^+ + e^- = \frac{1}{2}N_2O + \frac{1}{2}H_2O$	1.588	0.996	0.700	$P_{NO} = P_{N2O} = 10^{-6}$ bar
Nitrous oxide reduction to nitrogen$^\$$	$\frac{1}{2}N_2O + H^+ + e^- = \frac{1}{2}N_2 + \frac{1}{2}H_2O$	1.769	1.180	0.884	$P_{N2O} = 10^{-6}$ bar $P_{N2} =$ 0.8 bar
Mn reduction* Mn(III) to Mn(II)	$Mn_3O_4 + 2H^+ + 2H_2O + 2e^- = 3Mn(OH)_2$	0.480	0.066	−0.230	—
Fe reduction$^+$ Fe(III) to Fe(II)	$Fe(OH)_3 + 3H^+ + e^- = Fe^{2+} + 3H_2O$	0.975	0.014	−0.453	[Fe2+] = 18 μmol L^{-1}
Fe reduction* Fe(III) to Fe(II)	$Fe(OH)_4{}^- + H^+ + e^- = Fe(OH)_3^- + H_2O$	0.308	−0.106	−0.402	$[Fe(OH)_4{}^-] = [Fe(OH)_3^-]$
Sulfate reduction$^+$ S(VI) to S(-II)	$SO_4{}^{2-} + 10H^+ + 8e^- = H_2S + 4H_2O$	0.301	−0.217	−0.587	$[SO_4^{2-}] = [H_2S]$
Carbonate reduction$^\times$ C(VI) to C(0)	$2CO_3{}^{2-} + 11H^+ + 8e^- = CH_3COO^- + 4H_2O$	0.340	−0.292	−0.648	$[CO_3^-] = [CH_3COO^-] =$ 20 mmol L^{-1}

$^+$after Langmuir (1997).
*calculated using thermodynamic data from Stumm and Morgan (1996)
$^\times$calculated using thermodynamic data from Thauer (1977).
$^\$$calculated using thermodynamic data from Latimer (1952).

The ability of indigenous soil microorganisms to couple organic matter oxidation to the reduction of soluble oxyanions such as nitrate and sulphate, and transition metals such as iron and manganese, is well documented (Lovley 1993; 1997; Lovley et al. 2004; Tebo and Obraztsova 1998). Where sufficient organic matter is available for oxidation, progressively more anoxic conditions develop and a cascade of terminal-electron-accepting processes occur in sequence (but dependent on the availability of electron acceptors), with either increasing incubation time (Burke et al. 2005), or depth within sediments (Froelich et al. 1979). Microbial processes releasing most energy are favoured, so the sequence in which electron acceptors are used typically follows the decreasing order of redox potentials shown in Table 1 (calculated from standard thermodynamic data using the Nernst equation).

A broad range of microorganisms have been isolated and identified that can grow optimally and robustly in high pH environments. These microbes, called alkaliphiles, have adapted to this challenging environment with mechanisms for regulating cytoplasmic pH and by producing surface layer enzymes that function at high pH. For example many alkaliphiles use a Na^+ electrochemical gradient to maintain pH homeostasis and to energize solute uptake and motility (Detkova and Pusheva 2006; Krulwich et al. 2001).

Alkaliphiles are commonly divided into two broad groups; those that grow from circum-neutral to alkaline conditions are classified as facultative alkaliphiles (Sturr et al. 1994), however, those that only growth at around pH 9 or above are classified as obligate alkaliphiles (McMillan et al. 2009). Although for all alkaliphiles optimum growth commonly occurs at around pH 9–10, some species are reported to continue to grow up to around pH 12.5 (Pollock et al. 2007; Takai et al. 2001; Ye et al. 2004).

Another important consideration in alkaline environments is the nature of the organic matter (electron donors) present in affected soils. Indeed, some alkaliphiles show facultative or obligate behavior dependant on whether fermentable or non-fermentable electron donors are present respectively (McMillan et al. 2009). This has importance to alkaline affected soils, where at high pH, humic substances can be solubilized and leached from soils and sediments (Macleod and Semple 2000). Removal of such humic substances will leave behind less labile organic carbon that is more difficult for microbes to breakdown and metabolise and thus the soil microbial communities may differ significantly from those found at less alkaline pH.

The purpose of this study is to investigate the potential for biogeochemical redox cycling in a soil environment affected by hyperalkaline pH 13 leachate from a former lime burning waste disposal site near Buxton, UK. We have produced detailed vertical porewater profiles of redox active chemical species in order to characterize the range of biogeochemical processes that are supported within these soils; and used molecular ecology techniques to determine the genetic diversity of anaerobes present in the soil.

METHODS AND MATERIALS

Site Description

The site is near Harpur Hill, in Derbyshire, UK, in an area of upland farming. The Hoffman lime kiln operated by the Buxton Lime Company was one of the biggest of its type and was in operation continuously from 1872 until it was closed in 1944; the kiln was demolished and removed in 1980 (Anon 2008). The lime works has been restored for commercial/light industrial use. Limestone-roasting for the alkali-carbonate industry on this site produced huge volumes of alkali-generating waste. This was deposited into an adjacent valley to the north west of the kiln. Highly alkaline groundwater springs at the base of the waste (Figure 1; 53°14′07N, 001°55′02W) and flows northwards along the valley (known locally as Brook Bottom). A carbonate tufa is actively forming where this highly alkaline leachate emerges to atmosphere that is infilling the valley and completely smothering the natural valley floor to a depth in excess of 2 m for a distance of over 250 m.

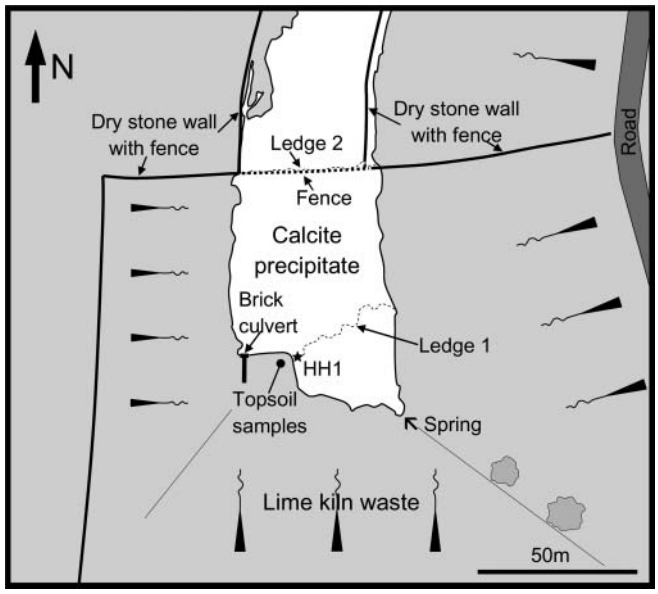

FIG. 1. Sketch map of Brook Bottom area affected by alkaline waters, near Harpur Hill, Derbyshire, UK, showing core locations and location of the alkaline spring. Area of carbonate precipitate shown in white. Highly alkaline water (up to pH 13.1) pools behind the ledge 1 and is diluted by mixing below that ledge with natural runoff water (pH 7–8) that issues from the brick culvert. At times of low water flow, however, no water issues from the culvert and alkaline water can be found flowing across the whole site. (Sketch redrawn from a Google Earth image of the site.)

Field Sampling

In July 2009 a borehole (HH1) was advanced through the carbonate precipitate into soil layers below using a 6 cm diameter auger, and a 39 cm core sample was recovered. Minor disturbance of the core material was observed during sampling, and groundwater was found within 5 cm of the surface. The core was sliced into approximately 4 cm sections using a clean stainless steel palate knife and stored in polypropylene containers (see Figure 2). Each core section was centrifuged at 6000 *g* within 24 hours to recover porewater samples for analysis. The pH of the spring water was measured on site using a Hanna HI 98129 Combo meter that had been calibrated using pH 7.01 and 10.01 buffer solutions. In May 2010 two samples of topsoil were recovered from a location about 5 m away from the carbonate deposit at depths of about 12.5 and 17.5 cm (see Figure 1).

Soil Sample Characterization

Soil samples (dried and ground to $< 75\ \mu$m) were characterized with a Bruker D8 powder X-ray diffractometer fitted with a GE (111) monochromator (XRD, CuKa = 1.54Å; 2theta = 5–70° range; patterns recorded at 0.001° steps at 0.5 sec/step) to identify their mineralogical composition. X-ray fluorescence (XRF) analysis was undertaken using a fused sample on a PANalytical Axios Advanced spectrometer (data were corrected for

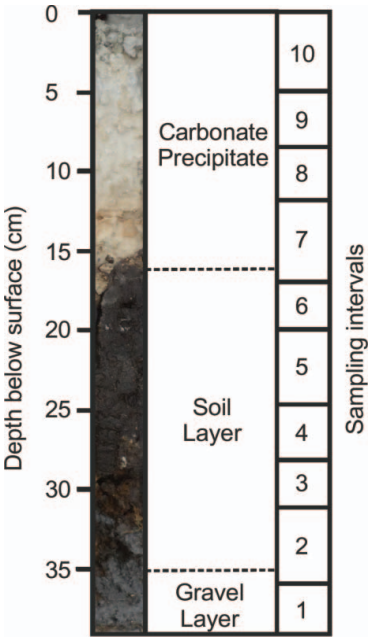

FIG. 2. Digital photograph of core HH1 and log of sediment types found (color figure available online).

loss on ignition). The total organic carbon content was determined using approximately 10 g of homogenized soil that was oven dried at 105°C, disaggregated with a mortar and pestle, and pre-treated with 10% HCl to remove any carbonates present. The carbon content was then measured using a Eurovector EA 3000 series combustion analyser (Pella 1990).

Geochemical Methods

Eh and pH were measured on extracted porewater using an Hanna bench top meter and calibrated electrodes (the pH electrode was calibrated between 4 and 10 using standard buffer solutions). Soil pH was measured using a 1:1 suspension in deionized water (ASTM 2006). Sulphate and nitrate concentrations were determined by ion chromatography on a Dionex DX-600 with AS50 autosampler using a 2 mm AS16 analytical column, with suppressed conductivity detection and gradient elution to 15 mM potassium hydroxide over 10 minutes. Samples were loaded in a random order to avoid systematic errors.

A standard UV/Vis spectroscopy method based on the reactions with Ferrozine was used to determine aqueous Fe concentrations using a Cecil CE3021 UV/VIS Spectrophotometer (Goto et al. 1977; Viollier et al. 2000). Nitrite was determined colourimetrically after reaction with N-(1-napthyl)-ethylenediamine (Shinn 1941). Fe(II) in solids was determined after extraction by 0.5 N HCl and reaction with ferrozine (Lovley and Phillips 1986). Standards for each analyte were used regularly. Calibration graphs exhibited good linearity (typically $r^2 > 0.99$).

DNA Extraction

Microbial DNA was extracted from ~0.5 g of sample HH1-5 (soil from a depth of 21–25 cm: Figure 2) using a FastDNA spin kit for soil (Qbiogene, Inc.) and FastPREP instrument (unless explicitly stated, the manufacturer's protocols supplied with all kits employed were followed precisely). DNA fragments in the size range 3 kb ~20 kb were isolated on a 1% "1x" Tris-borate-EDTA (TBE) gel stained with ethidium bromide to enable viewing under UV light (10x TBE solution from Invitrogen Ltd., UK). The DNA was extracted from the gel using a QIAquick gel extraction kit (QIAGEN Ltd., UK.). This purified DNA was used for subsequent analysis.

16S rRNA Gene Sequencing

A fragment of the 16S rRNA gene of approximately ~500 bp was PCR amplified using broad-specificity bacterial primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Eden et al. 1991) and 519r (5′-GWATTACCGCGGCKGCTG-3′) (Lane et al. 1985) where K = G or T, W = A or T. The PCR reaction mixture contained 25 μl of purified DNA solution, GoTaq DNA polymerase (5 units), 1× PCR reaction buffer (containing 1.5mM $MgCl_2$), PCR nucleotide mix (0.2 mM) and primers (0.6 μM each) in a final volume of 50 μl. The reaction mixture was incubated at 95°C for 2 min, and then cycled 30 times through three steps: denaturing (95°C, 30s), annealing (50°C, 30s), primer extension (72°C, 45s). This was followed by a final extension step at 72°C for 7min. The PCR product was purified using a QIAquick PCR Purification Kit. Amplification product size was verified by electrophoresis of 10 μl samples in a 1.0% agarose TBE gel with ethidium bromide straining.

The PCR product was ligated into the standard cloning vector pGEM-T Easy (Promega Corp., USA), and transformed into E. coli XL1-Blue supercompetent (Stratagene). Transformed cells were grown on LB-agar plates containing ampicillin (100 μg.ml^{-1}) at 37°C for 17 hours. The plates were surface dressed with IPTG and X-gal (as per Stratagene protocol) for blue-white colour screening. Colonies containing an insert were restreaked on LB-ampicillin agar plates and incubated at 37°C. Single colonies from these plates were incubated overnight in liquid LB-ampicillin. Plasmid DNA was extracted using the PureYield Plasmid Miniprep System (Promega, UK) and sent for automated DNA sequencing on an ABI 3100*xl* Capillary Sequencer using the T7P primer.

The quality of each 16s rRNA gene sequence was evaluated with Mallard 1.02 (Ashelford et al. 2006) and putative chimeras were excluded from subsequent analyses. Each non chimeric sequence was then classified using the Ribosomal Database Project (RDP) naïve Bayesian Classifier version 2.2 (Wang et al. 2007) in August 2010. Sequences were submitted to the GenBank database (accession numbers JF827038 - JF827074).

Multidimensional Scaling Analysis

Multidimensional scaling (MDS) was employed to represent the phylogenetic relationships among 16s rRNA gene sequences. Multidimensional scaling is a popular visualisation tool that can create a representation of a data set (usually in two- or three-dimensions) from information about the pairwise dissimilarity of the objects (Coxon 1982; Kruskal and Wish 1978). It transforms dissimilarity values into Euclidean distance in multidimensional space using a "stress" function that is optimized to minimise the error in the final representation. The 16s rRNA gene sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the distance matrix (a matrix of pair-wise dissimilarity scores) was downloaded into NewMDSX (Roskam et al. 2005). Basic non-metric MDS was undertaken using the Minissa-N algorithm within NewMDSX.

Phylogenetic Tree Building

The 16S rRNA gene sequences were grouped into operational taxonomic units (OTUs) by using >98% furthest neighbour similarity as a cut-off value in the MOTHUR software (Schloss et al. 2009). Representative sequences from selected OTUs were then aligned with known bacterial 16S rRNA gene sequences from the GeneBank database using the ClustalX software package (version 2.0.11), and phylogenetic trees were constructed from the distance matrix by neighbour joining. Bootstrap analysis was performed with 1000 replicates, and resulting phylograms drawn using the TreeView (version 1.6.6) software package.

TABLE 2
Composition of soil samples from HH1

Sample	Depth (cm)	Description	Predominant minerals	TOC (%)
HH1-9	6.7	tufa deposit	calcite	—
HH1-8	10.5	tufa deposit	calcite	—
HH1-6	19.3	brown soil	—	6.0
HH1-5	23.3	brown soil	calcite, quartz	6.4
HH1-4	27.5	brown soil	—	3.4
HH1-3	31.0	brown soil	calcite, quartz	4.6
HH1-2	34.8	brown soil	—	3.8
HH1-1	38.8	grey gravel	calcite, quartz	0.9

RESULTS

Soil Profile

Borehole HH1 (Figure 2) penetrated through the surface precipitate and encountered a dark brown clayey silt layer containing occasional plant matter at 17.5 cm, and a grey gravelly layer at about 35 cm, and it terminated at a depth of 39 cm. Ten samples were recovered: sample 1 was from the grey gravelly layer, samples 2–6 were from the brown soil layer, and samples 7 to 10 were from the surface precipitate. The pH of the surface water immediately adjacent to the spring was 13.1 in July 2009. A small volume of water with a pH value of 7.9 enters the stream from the brick culvert; however the pH of the surface water less than 10 m downstream of the culvert was 12.8.

Soil Characterization

XRD analysis of the surface precipitate (samples HH1-10 and HH1-8) identified it as calcite. XRD and XRF analyses of the brown soil (samples HH1-5 and HH1-3) identified that the main minerals as calcite and quartz (see Tables 2 and 3). The average TOC content was about 5%. Similarly, XRD and XRF analyses of the grey gravelly material identified that the main minerals were also calcite and quartz, although the TOC content was only 0.9%.

Geochemical Profiles

The pore water collected from core HH1 was consistently highly alkaline and slightly reducing with an average pH of 12.3 ± 0.1 and Eh of -77 ± 12 mV respectively (Figure 3). Nitrate concentrations in the top 15 cm were consistently above 100 μmols L^{-1} but dropped to below detection limits at 30 cm. Conversely nitrite concentrations were less than 15 μmols L^{-1} in the top 7 cm but increased to a peak of over 200 μmols L^{-1} at 27 cm, below 27 cm nitrite concentration dropped rapidly to less than 5 μmols L^{-1} by 35 cm. Solid phase 0.5 N HCl extractable Fe(II) was not detected in samples from the top 10 cm, but rose in the soil horizon to above 30% Fe(II), peaking at 69% Fe(II) at 30 cm. Aqueous Fe was below 3 μmols L^{-1} in the top 23 cm, but peaked at 21 μmols L^{-1} at 27cm depth and reduced to 8 μmols L^{-1} by 34 cm. Sulphate concentration was between 50–60 μmols L^{-1} throughout most the core with the exception of two peaks of around 90–100 μmols L^{-1} at 7–10 cm and 27 cm.

Microbial Community Analysis

A total of 37 rRNA gene sequences were obtained from HH1-5. These were assigned to 6 different bacterial phyla by the RDP classifier (confidence threshold >98%), with 16% of sequences remaining unassigned (see Figure 4). Sequences were allocated to an OTU that corresponded very approximately to a genus level assignment using MOTHUR (detailed listings of these assignments is given in Supplementary Table A). The rarefaction curve (Supplementary Figure A) and the Shannon indices ($H' = 1.72 \pm 0.39$) indicate that species richness is low (just eleven different OTUs were represented in the clone library).

The two dimensional MDS representation of the sequence dissimilarity scores (Figure 5) confirms the low species diversity described above. In this figure sequences from the same phylum group together, with sequences in the same OTU forming

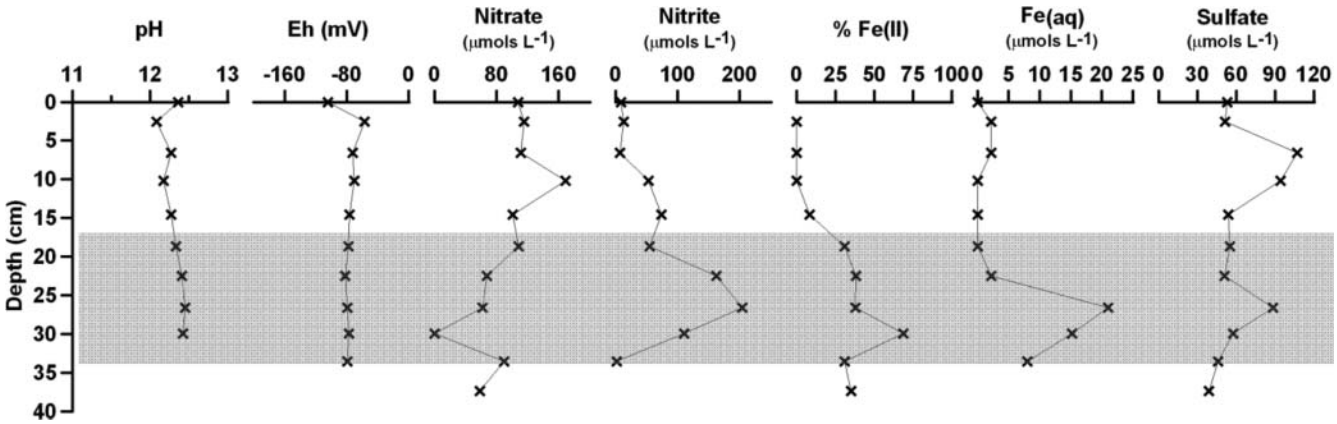

FIG. 3. Vertical geochemical profile from porewater recovered from core HH1. Position of the brown soil layer in the profile is shown by grey shading.

TABLE 3
Major elements in fused HH1 and nearby soil samples (A and B) measured by XRF (corrected for loss on ignition at 1000°C)

Sample	Depth cm	SiO_2%	Al_2O_3%	CaO%	MgO%	Fe_2O_3%	TiO_2%	MnO%	Na_2O%	K_2O%	P_2O_5%	SO_3%	LOI%
Topsoil A	12.5	13.63	7.14	32.07	0.69	2.65	0.265	0.062	0.05	0.421	0.121	n.d.	44.10
Topsoil B	17.5	17.59	9.49	31.75	0.77	3.43	0.345	0.055	0.04	0.533	0.092	0.029	37.45
HH1-9	6.7	0.14	n.d.	55.32	0.22	0.01	0.002	n.d.	n.d.	0.005	n.d.	n.d.	44.45
HH1-6	19.3	11.09	5.17	35.19	0.42	2.94	0.217	0.034	0.02	0.439	0.155	0.008	44.36
HH1-5	23.3	12.80	6.07	36.31	0.51	2.74	0.247	0.040	0.04	0.499	0.146	n.d.	41.20
HH1-1	38.8	9.14	4.90	44.11	0.51	1.46	0.180	0.029	n.d.	0.227	0.083	n.d.	40.16

n.d.—not detected.

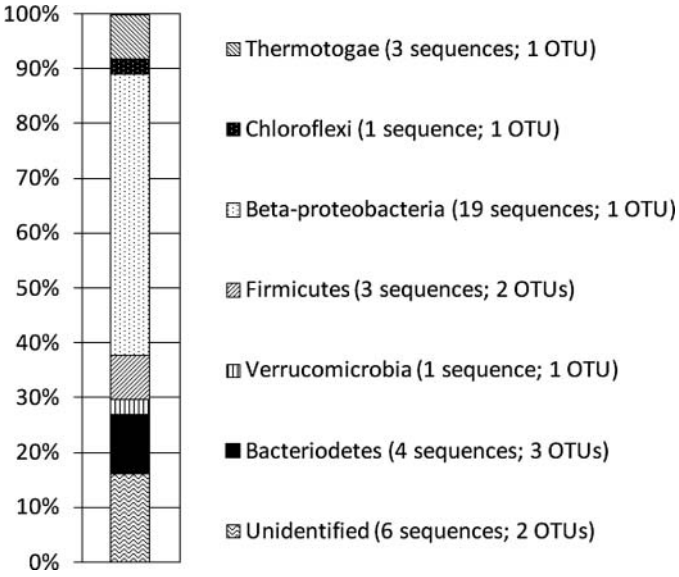

FIG. 4. Phylogenetic diversity of 16S rRNA gene sequences extracted from sample HH1-5 (20–25 cm below surface). Key shows the number of operationally defined taxonomic units within each phylum.

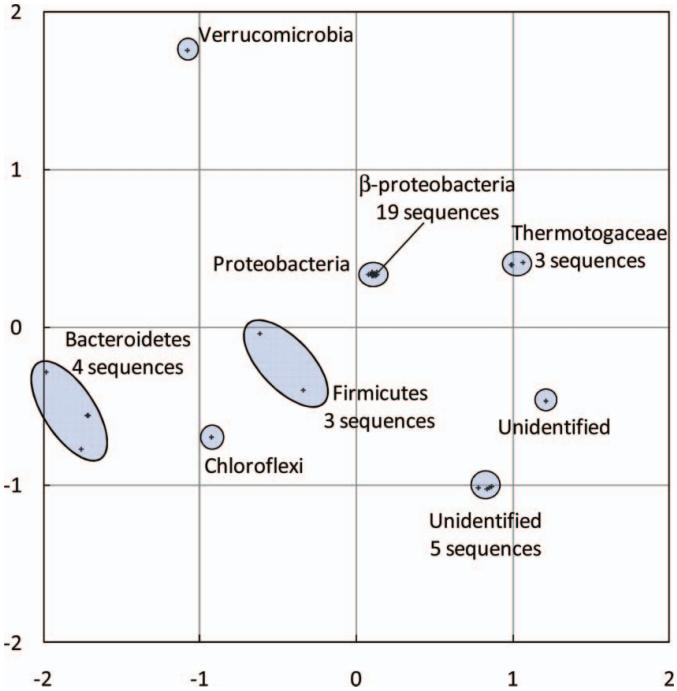

FIG. 5. Two-dimensional configuration from the MDS analysis of the pairwise sequence dissimilarity scores (distance scale within this Euclidean space is an arbitrary function of dissimilarity). The stress (lack of fit) associated with this two-dimensional representation decreased marginally from 0.31 to 0.28 when the number of dimensions was increased to three, which suggests that two dimensions adequately represent the dissimilarities in the data (color figure available online).

closely grouped clusters. The most significant cluster contains 19 sequences that were assigned to the β-class of *proteobacteria*. These sequences form a single OTU (labelled A6 in Supplementary Table A) and represent 57% of the population. A phylogenetic tree was constructed for the characteristic representative of this OTU (i.e., the sequence that is the minimum distance from the other members of this OTU; Schloss et al. 2009). This tree (Figure 6) suggests that sequences from this OTU are probably from members of a single genus within the *Comamonadaceae* family of β-proteobacteria, and appear to be most closely related to the genera *Rhodoferax*, *Pseudorhodoferax, Hydrogenophaga* and *Malikia*.

The second-largest OTU contained five of the six unidentified sequences. The third largest OTU (A4) contained only three sequences (8% of the population) that are closely related to the genus Petrotoga in the *Thermotogaceae* family of the phylum *Thermotogea* (a phylogenetic tree showing the characteristic representative of this OTU is shown in Supplementary Figure B). The remaining 10 sequences (27% of the population) fell in 8 different OTUs from across the phyla *Bacteroidetes*, *Firmicutes, Chloroflexi* and *Verrucomicrobia,* and one further unidentified sequence.

DISCUSSION

Lime kiln waste is rich in CaO, so groundwater that percolates through it becomes saturated with $Ca(OH)_2$. When this highly alkaline, calcium rich water emerges to atmosphere it absorbs $CO_2(g)$, precipitates calcite, and the pH buffers downwards towards the value for calcite equilibrium. The reaction scheme is (Clark et al. 1992).

$$CO_2(g) \rightarrow CO_2(aq) \quad [1]$$

$$CO_2(aq) + OH^- \rightarrow HCO_3^-(aq) \quad [2]$$

$$HCO_3^-(aq) \rightarrow CO_3^{2-}(aq) + H^+ \quad [3]$$

$$Ca^{2+}(aq) + CO_3^{2-}(aq) \rightarrow CaCO_3(precip) \quad [4]$$

Reaction (2), which is rate limiting, occurs slowly in neutral conditions but proceeds rapidly at high pH. Microbial activity can influence the rate of calcite precipitation by increasing the CO_2 flux and by providing potential nucleation (Mayes et al. 2006), however the reaction scheme above accounts for the formation of a large tufa deposit immediately where the spring water emerges and the gradual reduction in the stream's pH value down the site (in July 2009 the pH of stream just below the area of continuous surface precipitate was 8.6).

The brown soil layer seen in borehole HH1 has a total organic carbon content of ~5% and contains occasional plant matter. It has a similar composition to topsoil samples recovered from nearby. Thus, the brown soil was probably the original surface deposit which has become buried as the precipitate level has risen. This soil layer is now saturated with hyperalkaline groundwater (currently the water table is just below the top of the precipitate).

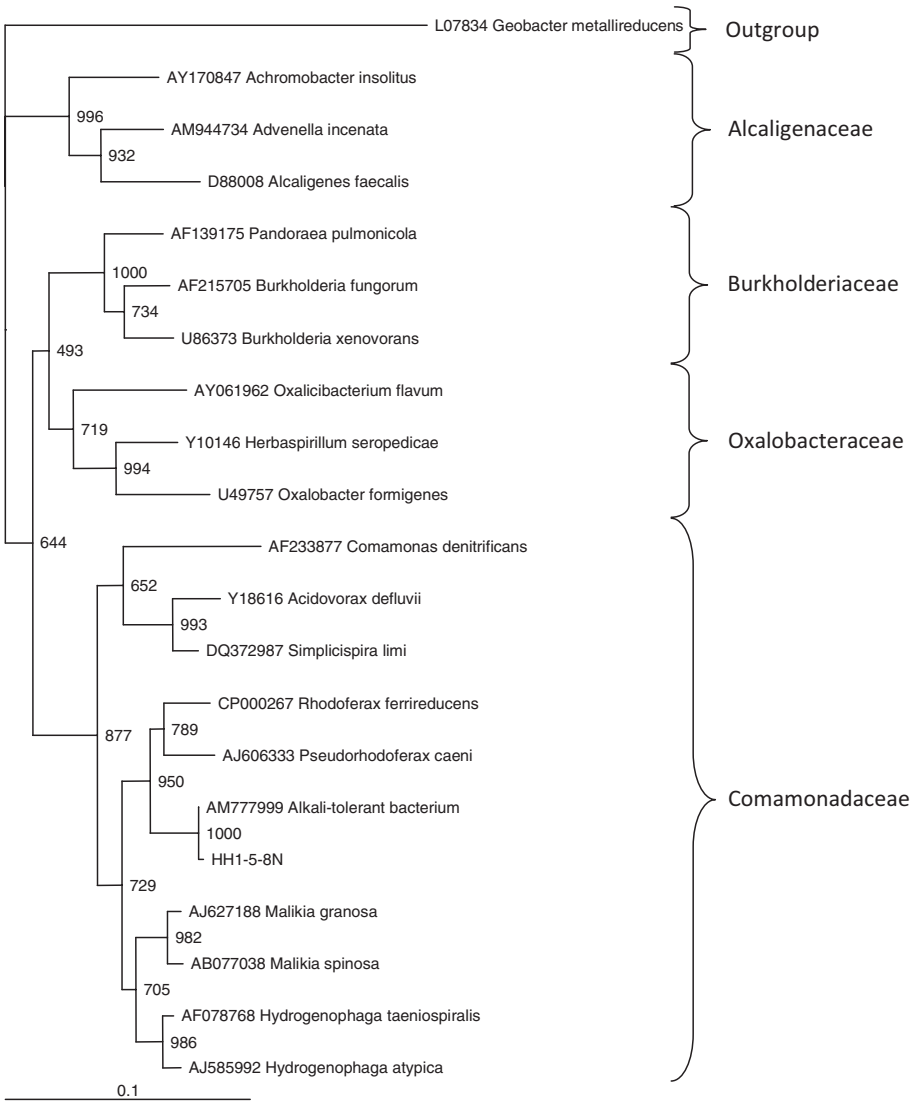

FIG. 6. Phylogenetic tree showing the relationship between a representative sequence from OTU A6 (HH1-5-8N) and other members of the order Burkholderiales of β-proteobacteria. Geobacter metallireducens (δ-proteobacteria) is included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.

The geochemical profile indicates that the pore fluid nitrate concentration in the brown soil is lower than in the precipitate layer above, whereas the pore fluid nitrite concentration is significantly higher in the top half of the soil layer. As the pore water originates from a single source (the lime kiln waste), and diffusion acts to eliminate spatial differences in concentration, these profiles indicate that nitrate reduction to nitrite must be occurring in-situ in the brown soil around the time of sampling. The nitrite concentration peaks at a depth of 27cm, and then decreases towards zero at the base of the brown soil layer. In this zone there is a peak in the aqueous iron concentration and, more importantly, a peak in the proportion of 0.5 HCl extractable iron in the Fe(II) oxidation state, which suggests that iron reduction may also have been occurring within the lower part of the brown soil layer (it is unlikely that a high proportion of the acid extractable iron in a former surface layer would be Fe(II) prior to inundation and burial).

The presence of organic matter and a bacterial population in the brown soil layer suggest that the mechanism responsible for nitrate reduction is microbial. Initially this may seem surprising as the pH value is above 12, however microbial mediated nitrate reduction has been widely reported in high pH systems where the microbial community has adapted to the ambient pH (Dhamole et al. 2008; Glass and Silverstein 1998; Whittleston et al. 2010). It is also reported that nitrite reduction to N_2 in these systems tends to lag behind nitrate reduction to nitrite (Glass and Silverstein 1998). As the nitrate deficit in the top half of the brown soil equates quite well with the nitrite

concentration it appears that nitrite reduction is lagging in this system.

The depletion of terminal electron acceptors with depth in the order associated with decreasing energy yield suggests the mechanism responsible for iron reduction may also be microbial. Microbial growth supported by iron reduction has been observed at pH values up to 11 (Pollock et al. 2007; Wu et al. 2011; Ye et al. 2004) suggesting that the brown soil with a pH value >12 is a rather marginal environment for iron reducing bacteria. However an average pH value does not fully characterize the geochemical environment of a soil, where there may be micro-environments.

The bacterial population in sample HH1-5, which was recovered from the zone of nitrate reduction is dominated by members of a single genus of bacteria in the *Comamonadaceae* family (OTU A6). Genera in the Comamonadaceae family and neighbouring phylogenetic groups are phenotypically highly diverse, even if they are phylogenetically closely related (Spring et al. 2005), so the similarity is not evidence of shared metabolic pathways. Nonetheless, it is interesting to note that several species in the closely related genera of *Rhodoferax* and *Hydrogenophaga* are facultive anaerobes that can couple oxidation of simple organic molecules such as glucose, lactate and acetate to the reduction of nitrate (Finneran et al. 2003; Kampfer et al. 2005). Thus the dominance of OTU A6 within the bacterial population suggests that it is capable of nitrate reduction. It is also interesting that this OTU shares $\geq$99% identity with 16s rRNA gene sequences isolated in four separate studies of alkaline soil and groundwater (GenBank accession numbers AM777999 shown in Figure 6, and AM884725, DQ266899 and AM980998).

The electron donors that support nitrate reduction are most likely derived from the soil organic matter. However, the soil has been buried below the calcite precipitate for a number of years and, without replenishment, any labile organic carbon would have been consumed by the bacterial population or leached from the soil over that period (humic substances, for example, are soluble at high pH; Macleod and Semple 2000).

Therefore, dissimilative nitrate reduction, which requires labile organic carbon that can be taken-up by bacterial cells (Gottschalk 1986; Kim and Gadd 2008), must be indicative of the continued breakdown of less labile organic substrates. In anaerobic systems the complete oxidation of organic matter requires the cooperative activity of a community of microorganisms collectively exhibiting several different metabolic pathways (e.g., hydrolysis of complex organic matter, fermentation of sugars, and oxidation of fatty acids, lactate, acetate and H2; Leschine 1995; Lovley 1993). The second largest identifiable OTU (A4) may be an important part of this symbiotic population. It was classified as a *Petrotoga* specie within the phylum *Thermotogae*.

Typically, *Petrotoga* species are strictly anaerobic fermentative bacteria that can oxidise sugars and some polymers during energy metabolism to produce lactate, acetate, CO_2 and H_2 as metabolites (L'Haridon et al. 2002; Lien et al. 1998; Miranda-Tello et al. 2004; 2007). Most members of this phylum that have been isolated to date are tough thermophiles recovered from deep oil reservoirs, and have adapted to this harsh environment with a sheathlike outer structure that helps them resist environmental stress (Miranda-Tello et al. 2004).

Recently, similar gene sequences have been detected in mesophilic anaerobic environments under stress from toxic chemicals (Nesbo et al. 2006), suggesting that adaptations to survive at high temperatures may also provide resistance to chemical stress. The characteristic member of OTU A4 (HH1-5-39) shares $\geq$99% identity with 16s rRNA gene sequences from thermophilic species isolated from an anaerobic digester and an anaerobic reactor (GenBank accession numbers EF559065, FN436142).

In summary, the primary finding of this study is that the bacterial population of a soil that has been in contact with highly alkaline groundwater for an extended time period has evolved to tolerate the high pH and is now undertaking geo-microbiological processes similar to those commonly observed in anoxic soils at near neutral pH values. The exact sequence of the cascade of terminal electron accepting processes varies slightly from circum-neutral pH (e.g., nitrite reduction lags behind nitrate reduction) because the relative thermodynamic favorability of these reactions varies with pH and possibly because some enzymatically catalysed reactions may be inhibited.

However, it appears that the process of adaptation to pH is not unique to the study site, as bacterial species similar to the dominant members of the observed population have been found in several other near-surface hyperalkaline environments. This suggests that, given enough time, soil bacterial populations can readily adapt to high pH.

CONCLUSIONS

A population of anaerobic alkaliphilic bacteria has established itself in the organic rich soil layer that is buried beneath calcite precipitate and receiving groundwater from a lime kiln waste tip despite the pH value >12. This bacteria population, which is dominated by a single bacterial species within the Comamonadaceae family of β-proteobacteria, appears to be capable of nitrate reduction while respiring on an electron donor(s) that is probably derived from the soil organic matter. Deeper in the same soil layer there is evidence that anoxia has developed further to the point where microbial iron reduction is occurring.

SUPPLEMENTARY INFORMATION

Supplementary information with this article, available online, gives full details of the 16S rRNA gene sequence assignments (Table A), a rarefaction curve for sequences from HH1-5 (Figure A), and phylogenetic tree for sequence HH1-5-39 and other Thermotogaceae species (Figure B).

REFERENCES

Anon. 2008. Flame which burned in Harpur for decades. Buxton Advertiser. Buxton: Johnston Press Digital Publishing.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ. 2006. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. Appl Environ Microbiol 72(9):5734–5741.

ASTM. 2006. D4972-01: Standard test method for pH of soils. Annual book of ASTM standards. Amer Soc Test Mater 4:963–965.

Brazelton WJ, Ludwig KA, Sogin ML, Andreishcheva EA, Kelley DS, Shen CC, Edwards RL, Baross JA. 2010. Archaea and bacteria with surprising microdiversity show shifts in dominance over 1,000-year time scales in hydrothermal chimneys. Proc Natl Acad Sci USA 107(4):1612–1617.

Burke IT, Boothman C, Lloyd JR, Mortimer RJG, Livens FR, Morris K. 2005. Effects of progressive anoxia on the solubility of technetium in sediments. Environ Sci Technol 39(11):4109–4116.

Carlson CL, Adriano DC. 1993. Environmental impacts of coal combustion residues. J Environ Qual 22(2):227–247.

Chaurand P, Rose J, Briois V, Olivi L, Hazemann JL, Proux O, Domas J, Bottero JY. 2007. Environmental impacts of steel slag reused in road construction: A crystallographic and molecular (XANES) approach. J Hazard Mater 139(3):537–542.

Clark ID, Fontes J-C, Fritz P. 1992. Stable isotope disequilibria in travertine from high pH waters: Laboratory investigations and field observations from Oman. Geochim Cosmochim Acta 56(5):2041.

Coxon APM. 1982. The user's guide to multidimensional scaling. London: Heinemann Educational Books.

Deakin D, West LJ, Stewart DI, Yardley BWD. 2001. Leaching behaviour of a chromium smelter waste heap. Waste Mgmt 21(3):265–270.

Detkova E, Pusheva M. 2006. Energy metabolism in halophilic and alkaliphilic acetogenic bacteria. Microbiology 75(1):1.

Dhamole PB, Nair RR, D'Souza SF, Lele SS. 2008. Denitrification of highly alkaline nitrate waste using adapted sludge. Appl Biochem Biotechnol 151:433–440.

Eden PA, Schmidt TM, Blakemore RP, Pace NR. 1991. Phylogenetic analysis of *Aquaspirillum-magnetotacticum* using polymerase chain reaction-amplified 16s ribosomal-RNA-Specific DNA. Inter J System Bacteriol 41(2):324–325.

Effler SW, Brooks CM, Addess JM, Doerr SM, Storey ML, Wagner BA. 1991. Pollutant loadings from solvay waste beds to lower Ninemile Creek, New-York. Water Air Soil Poll 55(3–4):427–444.

Finneran KT, Johnsen CV, Lovley DR. 2003. *Rhodoferax ferrireducens* sp nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). Intern J System Evolution Microbiol 53: 669–673.

Froelich PN, Klinkhammer GP, Bender ML, Luedtke NA, Heath GR, Cullen D, Dauphin P, Hammond D, Hartman B, Maynard V. 1979. Early oxidation of organic-matter in pelagic sediments of the Eastern equatorial Atlantic - suboxic diagenesis. Geochim Cosmochim Acta 43(7):1075–1090.

Glass C, Silverstein J. 1998. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. Water Res 32(3):831–839.

Goto K, Taguchi S, Fukue Y, Ohta K, Watanabe H. 1977. Spectrophotometric determination of manganese with 1-(2-pyridylazo)-2-naphthol and a nonionic surfactant. Talanta 24(12):752–753.

Gottschalk G. 1986. Bacterial Metabolism. New York: Springer-Verlag.

Hartland A, Fairchild IJ, Lead JR, Dominguez-Villar D, Baker A, Gunn J, Baalousha M, Ju-Nam Y. 2009. The dripwaters and speleothems of Poole's Cavern: a reveiw of recent and onging research. Cave Karst Sci 36(2):37–46.

Kampfer P, Schulze R, Jackel U, Malik KA, Amann R, Spring S. 2005. Hydrogenophaga defluvii sp. nov. and *Hydrogenophaga atypica* sp. nov., isolated from activated sludge. Int J Syst Evol Microbiol 55(1):341–344.

Kim BH, Gadd GM. 2008. Bacterial physiology and metabolism. Cambridge: Cambridge University Press.

Krulwich TA, Ito M, Guffanti AA. 2001. The Na+-dependence of alkaliphily in Bacillus. Biochim Biophys Acta-Bioenerg 1505(1):158–168.

Kruskal JB, Wish M. 1978. Multidimensional scaling. London: Sage Publications.

L'Haridon S, Miroshnichenko ML, Hippe H, Fardeau ML, Bonch-Osmolovskaya EA, Stackebrandt E, Jeanthon C. 2002. *Petrotoga olearia* sp nov and *Petrotoga sibirica* sp nov., two thermophilic bacteria isolated from a continental petroleum reservoir in Western Siberia. Inter J System Evolution Microbiol 52:1715–1722.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NL. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc Natl Acad Sci USA 82(20):6955–6959.

Langmuir D. 1997. Aqueous environmental geochemistry. Englewood Cliffs, New Jersey: Prentice Hall.

Latimer WM. 1952. The oxidation states of the elements and their potentials in aqueous solutions. Englewood Cliffs, NJ: Prentice-Hall.

Leschine SB. 1995. Cellulose degradation in anaerobic environments. Ann Rev Microbiol 49:399–426.

Lien T, Madsen M, Rainey FA, Birkeland NK. 1998. *Petrotoga mobilis* sp. nov., from a North Sea oil-production well. Inter J System Bacteriol 48:1007–1013.

Lovley DR. 1993. Dissimilatory metal reduction. Ann Rev Microbiol 47:263–290.

Lovley DR. 1997. Microbial Fe(III) reduction in subsurface environments. FEMS Microbiol Rev 20(3–4):305–313.

Lovley DR, Holmes DE, Nevin KP. 2004. Dissimilatory Fe(III) and Mn(IV) reduction. Adv Microb Physiol, 49: 219–286. London: Academic Press Ltd.

Lovley DR, Phillips EJP. 1986. Availability of ferric iron for microbial reduction in bottom sediments of the fresh-water Tidal Potomac River. Appl Environ Microbiol 52(4):751–757.

Macleod CJA, Semple KT. 2000. Influence of contact time on extractability and degradation of pyrene in soils. Environ Sci Technol 34(23):4952–4957.

Mayes WM, Aumonier J, Jarvis AP. 2009. Preliminary evaluation of a constructed wetland for treating extremely alkaline (pH 12) steel slag drainage. Water Science Technol 59(11):2253–2263.

Mayes WM, Jarvis AP, Burke IT, Walton M, Feigl VR, Klebercz O, Gruiz K. 2011. Dispersal and attenuation of trace contaminants downstream of the ajka bauxite residue (red mud) depository failure, Hungary. Environ Sci Technol 45(12):5147–5155.

Mayes WM, Younger PL, Aumonier J. 2006. Buffering of alkaline steel slag leachate across a natural wetland. Environ Sci Technol 40(4):1237–1243.

Mayes WM, Younger PL, Aumonier J. 2008. Hydrogeochemistry of alkaline steel slag leachates in the UK. Water Air Soil Poll 195(1–4):35–50.

McMillan DGG, Keis S, Berney M, Cook GM. 2009. Nonfermentative thermoalkaliphilic growth is restricted to alkaline environments. Appl Environ Microbiol 75(24):7649–7654.

Miranda-Tello E, Fardeai M-L, Joullan C, Magot M, Thomas P, Tholozan J-L, Ollivier B. 2007. *Petrotoga halophila* sp nov., a thermophilic, moderately halophilic, fermentative bacterium isolated from an offshore oil well in Congo. Inter J System Evolution Microbiol 57:40–44.

Miranda-Tello E, Fardeau MI, Thomas P, Ramirez F, Casalot L, Cayol J-L, Garcia J-L, Ollivier B. 2004. *Petrotoga mexicana* sp. nov., a novel thermophilic, anaerobic and xylanolytic bacterium isolated from an oil-producing well in the Gulf of Mexico. Int J Syst Evol Microbiol 54(1):169–174.

Nesbo, CL, Dlutek M, Zhaxybayeva O, Doolittle WF. 2006. Evidence for existence of "Mesotogas," members of the order Thermotogales adapted to low-temperature environments. Appl Environ Microbiol 72(7): 5061–5068.

Pella E. 1990. Elemental organic analysis. Part 2: State of the art. Amer Labor 22(12):28–32.

Pollock J, Weber KA, Lack J, Achenbach LA, Mormile 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 Biotechnol 77(4):927–934.

Roskam EE, Coxon APM, Brier AP, Hawkins PK. 2005. The NewMDSX Program Series, Version 5. Edinburgh: NewMDSX Project.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn J, Weber CF. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75(23):7537–7541.

Schwab AP, Hickey J, Hunter J, Banks MK. 2006. Characteristics of blast furnace slag leachate produced under reduced and oxidized conditions. J Environ Sci Health Pt A-Toxic/Hazard Subst Environ Eng 41(3): 381–395.

Shinn MB. 1941. Colorimetric method for determination of nitrite. Indust Eng Chem-Anal Ed 13:0033–0035.

Spring S, Wagner M, Schumann P, Kampfer P. 2005. *Malikia granosa* gen. nov., sp nov., a novel polyhydroxyalkanoate—and polyphosphate—accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. Inter J System Evol Microbiol 55:621–629.

Stewart DI, Burke IT, Hughes-Berry DV, Whittleston RA. 2009. Microbially mediated chromate reduction in soil contaminated by highly alkaline leachate from chromium containing waste. Ecol Eng 36:211–221.

Stewart DI, Burke IT, Mortimer RJG. 2007. Stimulation of microbially mediated chromate reduction in alkaline soil-water systems. Geomicrobiol J 24(7–8):655–669.

Stumm W, Morgan JJ. 1996. Aquatic geochemistry. New York: John Wiley and Sons.

Sturr MG, Guffanti AA, Krulwich TA. 1994. Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at High pH. J Bacteriol 176(11):3111–3116.

Takai K, Moser DP, Onstott TC, Spoelstra N, Pfiffner SM, Dohnalkova A, Fredrickson JK. 2001. *Alkaliphilus transvaalensis* gen. nov., sp nov., an extremely alkaliphilic bacterium isolated from a deep South African gold mine. Intern J Syst Evol Microbiol 51:1245–1256.

Takai K, Moyer CL, Miyazaki M, Nogi Y, Hirayama H, Nealson KH, Horikoshi K. 2005. *Marinobacter alkaliphilus* sp nov., a novel alkaliphilic bacterium isolated from subseafloor alkaline serpentine mud from Ocean Drilling Program Site 1200 at South Chamorro Seamount, Mariana Forearc. Extremophiles 9(1):17–27.

Tebo BM, Obraztsova AY. 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. FEMS Microbiol Lett 162(1):193–198.

Thauer RK, Jungermann K, Decker K. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41(1):100–180.

Townsend TG, Jang Y, Thurn LG. 1999. Simulation of construction and demolition waste leachate. J Environ Eng-ASCE 125(11):1071–1081.

Viollier E, Inglett PW, Hunter KW, Roychoudhury AN, Van Cappellen P. 2000. The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. Appl Geochem 15(6):785–790.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73(16):5261–5267.

Whittleston RA, Stewart DI, Mortimer RJG, Ashley DJ, Burke IT. 2010. Effects of microbially induced anoxia on Cr(VI) mobility at a hyperalkaline, chromite ore processing residue contaminated site. Geomicrobiol J 28:68–82.

Wu CY, Zhuang L, Zhou SG, Li FB, He J. 2011. Corynebacterium humireducens sp. nov., an alkaliphilic, humic acid-reducing bacterium isolated from a microbial fuel cell. Inter J System Evolution Microbiol 61:882–887.

Ye Q, Roh Y, Carroll SL, Blair B, Zhou JZ, Zhang CL, Fields MW. 2004. Alkaline anaerobic respiration: Isolation and characterization of a novel alkaliphilic and metal-reducing bacterium. Appl Environ Microbiol 70(9):5595–5602.