Biogeochemical reduction processes in a hyper-alkaline affected leachate soil profile

Journal:	*Geomicrobiology Journal*
Manuscript ID:	UGMB-2011-0080.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Burke, Ian; University of Leeds, School of Earth and Environment Whittleston, Rob; University of Leeds, School of Earth and Environment Palani, Shanmugam; Central Leather Research Institute Mortimer, Rob; University of Leeds, School of Earth and Environment Lockwood, Cindy; University of Leeds, School of Earth and Environment Ashley, David; University of Leeds, School of Earth and Environment Stewart, Doug; University of Leeds, School of Civil Engineering
Keywords:	alkaliphile , bacteria, nitrate-reduction, iron-reduction, contaminated land

Biogeochemical reduction processes in a hyper-alkaline affected leachate soil profile

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

[1] Earth Surface Science Institute, School of Earth and Environment, University of Leeds, Leeds LS2 9JT, UK

[2] School of Civil Engineering, University of Leeds, Leeds LS2 9JT, UK

[*]Corresponding Author e-mail: i.burke@see.leeds.ac.uk / d.i.stewart@leeds.ac.ukmailto:i.burke@see.leeds.ac.uk

[#] Present address: Central Leather Research Institute, Chennai, India

Abstract

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 specialised microbial communities and unusual biogeochemical processes. This paper 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.

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 characterised by high pH. Some are entirely natural, e.g., soda lakes, hot springs, oceanographic cold seeps, deep mine waters (Takai et al. 2001; Takai et al. 2005; Pollock et al. 2007; McMillan et al. 2009; Brazelton et al. 2010), 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 (Effler et al. 1991; Carlson and Adriano 1993; Townsend et al. 1999; Deakin et al. 2001; Ye et al. 2004; Mayes et al. 2006; Mayes et al. 2008; Hartland et al. 2009; Mayes et al. 2011). 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. 2009).

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; Stewart et al. 2007; Mayes et al. 2009). In addition, due to the leaching of sulphur bearing minerals, elevated sulphate concentrations are often reported to affect overall water quality (Schwab et al. 2006; Mayes et al. 2008; 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.

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; Lovley 1997; Tebo and Obraztsova 1998; Lovley et al. 2004). 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 (Krulwich et al. 2001; Detkova and Pusheva 2006). 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 (Takai et al. 2001; Ye et al. 2004; Pollock et al. 2007). 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 behaviour 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 solubilised 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 characterise 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.

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 5m away from the carbonate deposit at depths of about 12.5 and 17.5 cm (see Figure 1).

Soil sample characterisation

Soil samples (dried and ground to < 75 µm) 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 loss on ignition). The total organic carbon content was determined using approximately 10 g of homogenised 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 deionised water (ASTM 2006). Sulphate and nitrate concentrations were determined by ion chromatography on a Dionex DX-600 with AS50 autosampler using a 2mm 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 95ºC for 2 min, and then cycled 30 times through three steps: denaturing (95ºC, 30s), annealing (50ºC, 30s), primer extension (72ºC, 45s). This was followed by a final extension step at 72ºC 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 $\mu g.ml^{-1}$) at 37ºC 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 37ºC. 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 dataset (usually in two- or three-dimensions) from information about the pair-wise dissimilarity of the objects (Kruskal and Wish 1978; Coxon 1982). It transforms dissimilarity values into Euclidean distance in multidimensional space using a "stress" function that is optimised 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.

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 10m downstream of the culvert was 12.8.

Soil characterisation

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 27cm.

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 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).

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 (Glass and Silverstein 1998; Dhamole et al. 2008; 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 (Ye et al. 2004; Pollock et al. 2007; Wu et al. 2011) 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 characterise 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 ≥99% identity with 16s rRNA gene sequences isolated in four separate studies of alkaline soil and groundwater (Genebank accession numbers AM777999 shown in Figure 6, and AM884725, DQ266899 & 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; Lovley 1993; Leschine 1995). 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 (Lien et al. 1998; L'Haridon et al. 2002; Miranda-Tello et al. 2004; Miranda-Tello et al. 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 sheath-like 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 ≥99% identity with 16s rRNA gene sequences from thermophilic species isolated from an anaerobic digester and an anaerobic reactor (GeneBank 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 favourability 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 associated with this paper 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).

Acknowledgements

The authors would like to thank Martin Pedley and Mike Rogerson for introducing us to the site and Juan Diego Rodriguez-Blanco for organising XRD and XRF analysis.

References

Anon. (2008). Flame which burned in Harpur for decades. Buxton Advertiser Buxton, Johnston Press Digital Publishing **24 October 2008**.

Ashelford, K. E., N. A. Chuzhanova, J. C. Fry, A. J. Jones and A. J. Weightman (2006). "New Screening Software Shows that Most Recent Large 16S rRNA Gene Clone Libraries Contain Chimeras." Applied and Environmental Microbiology **72**(9): 5734-5741.

ASTM (2006). "D4972-01: standard test method for pH of soils. Annual book of ASTM standards." American Society for Testing and Materials **4**: 963-965.

Brazelton, W. J., K. A. Ludwig, M. L. Sogin, E. N. Andreishcheva, D. S. Kelley, C. C. Shen, R. L. Edwards and J. A. Baross (2010). "Archaea and bacteria with surprising microdiversity show shifts in dominance over 1,000-year time scales in hydrothermal chimneys." Proceedings of the National Academy of Sciences of the United States of America **107**(4): 1612-1617.

Burke, I. T., C. Boothman, J. R. Lloyd, R. J. G. Mortimer, F. R. Livens and K. Morris (2005). "Effects of progressive anoxia on the solubility of technetium in sediments." Environmental Science & Technology **39**(11): 4109-4116.

Carlson, C. L. and D. C. Adriano (1993). "Environmental Impacts of Coal Combustion Residues." Journal of Environmental Quality **22**(2): 227-247.

Chaurand, P., J. Rose, V. Briois, L. Olivi, J. L. Hazemann, O. Proux, J. Domas and J. Y. Bottero (2007). "Environmental impacts of steel slag reused in road construction: A crystallographic and molecular (XANES) approach." Journal of Hazardous Materials **139**(3): 537-542.

Clark, I. D., J.-C. Fontes and P. Fritz (1992). "Stable isotope disequilibria in travertine from high pH waters: Laboratory investigations and field observations from Oman." Geochimica et Cosmochimica Acta **56**(5): 2041.

Coxon, A. P. M. (1982). The User's Guide to Multidimensional Scaling. London, Heinemann Educational Books.

Deakin, D., L. J. West, D. I. Stewart and B. W. D. Yardley (2001). "Leaching behaviour of a chromium smelter waste heap." Waste Management **21**(3): 265-270.

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

Dhamole, P. B., R. R. Nair, S. F. D'Souza and S. S. Lele (2008). "Denitrification of Highly Alkaline Nitrate Waste Using Adapted Sludge." Applied Biochemistry Biotechnology **151**: 433-440.

Eden, P. A., T. M. Schmidt, R. P. Blakemore and N. R. Pace (1991). "Phylogenetic Analysis of Aquaspirillum-Magnetotacticum Using Polymerase Chain Reaction-Amplified 16s Ribosomal-Rna-Specific DNA." International Journal of Systematic Bacteriology **41**(2): 324-325.

Effler, S. W., C. M. Brooks, J. M. Addess, S. M. Doerr, M. L. Storey and B. A. Wagner (1991). "Pollutant Loadings from Solvay Waste Beds to Lower Ninemile Creek, New-York." Water Air and Soil Pollution **55**(3-4): 427-444.

Finneran, K. T., C. V. Johnsen and D. R. Lovley (2003). "Rhodoferax ferrireducens sp nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III)." International Journal of Systematic and Evolutionary Microbiology **53**: 669-673.

Froelich, P. N., G. P. Klinkhammer, M. L. Bender, N. A. Luedtke, G. R. Heath, D. Cullen, P. Dauphin, D. Hammond, B. Hartman and V. Maynard (1979). "Early Oxidation Of Organic-Matter In Pelagic Sediments Of The Eastern Equatorial Atlantic - Suboxic Diagenesis." Geochimica Et Cosmochimica Acta **43**(7): 1075-1090.

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

Goto, K., S. Taguchi, Y. Fukue, K. Ohta and H. Watanabe (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., F. I. J., J. R. Lead, D. Dominguez-Villar, A. Baker, J. Gunn, M. Baalousha and Y. Ju-Nam (2009). "The dripwaters and speleothems of Poole's Cavern: a reveiw of recent and onging research. ." Cave and Karst Science **36**(2): 37-46.

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

Kim, B. H. and G. M. Gadd (2008). Bacterial physiology and metabolism. Cambridge, Cambridge University Press.

Krulwich, T. A., M. Ito and A. A. Guffanti (2001). "The Na+-dependence of alkaliphily in Bacillus." Biochimica Et Biophysica Acta-Bioenergetics **1505**(1): 158-168.

Kruskal, J. B. and M. Wish (1978). Multidimensional Scaling. Beverley Hills and London, Sage Publications.

L'Haridon, S., M. L. Miroshnichenko, H. Hippe, M. L. Fardeau, E. A. Bonch-Osmolovskaya, E. Stackebrandt and C. Jeanthon (2002). "Petrotoga olearia sp nov and Petrotoga sibirica sp nov., two thermophilic bacteria isolated from a continental petroleum reservoir in Western Siberia." International Journal Of Systematic And Evolutionary Microbiology **52**: 1715-1722.

Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin and N. R. Pace (1985). "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis." Proceedings of the National Academy of Sciences of the United States of America **82**(20): 6955-6959.

Langmuir, D. (1997). Aqueous Environmental Geochemistry. New Jersey, Prentice Hall.

Latimer, W. M. (1952). The oxidation states of the elements and their potentials in aqueous solutions. New York, Prentice-Hall.

Leschine, S. B. (1995). "Cellulose Degradation in Anaerobic Environments." Annual Review of Microbiology **49**: 399-426.

Lien, T., M. Madsen, F. A. Rainey and N. K. Birkeland (1998). "Petrotoga mobilis sp. nov., from a North Sea oil-production well." International Journal of Systematic Bacteriology **48**: 1007-1013.

Lovley, D. R. (1993). "Dissimilatory Metal Reduction." Annual Review of Microbiology **47**: 263-290.

Lovley, D. R. (1997). "Microbial Fe(III) reduction in subsurface environments." Fems Microbiology Reviews **20**(3-4): 305-313.

Lovley, D. R., D. E. Holmes and K. P. Nevin (2004). Dissimilatory Fe(III) and Mn(IV) reduction. Advances in Microbial Physiology, Vol. 49. London, Academic Press Ltd. **49:** 219-286.

Lovley, D. R. and E. J. P. Phillips (1986). "Availability of Ferric Iron for Microbial Reduction in Bottom Sediments of the Fresh-Water Tidal Potomac River." Applied and Environmental Microbiology **52**(4): 751-757.

Macleod, C. J. A. and K. T. Semple (2000). "Influence of contact time on extractability and degradation of pyrene in soils." Environmental Science & Technology **34**(23): 4952-4957.

Mayes, W. M., J. Aumonier and A. P. Jarvis (2009). "Preliminary evaluation of a constructed wetland for treating extremely alkaline (pH 12) steel slag drainage." Water Science and Technology **59**(11): 2253-2263.

Mayes, W. M., A. P. Jarvis, I. T. Burke, M. Walton, V. r. Feigl, O. Klebercz and K. Gruiz (2011). "Dispersal and Attenuation of Trace Contaminants Downstream of the Ajka Bauxite Residue (Red Mud) Depository Failure, Hungary." Environmental Science & Technology **45**(12): 5147-5155.

Mayes, W. M., P. L. Younger and J. Aumonier (2006). "Buffering of alkaline steel slag leachate across a natural wetland." Environmental Science & Technology **40**(4): 1237-1243.

Mayes, W. M., P. L. Younger and J. Aumonier (2008). "Hydrogeochemistry of alkaline steel slag leachates in the UK." Water Air and Soil Pollution **195**(1-4): 35-50.

McMillan, D. G. G., S. Keis, M. Berney and G. M. Cook (2009). "Nonfermentative Thermoalkaliphilic Growth Is Restricted to Alkaline Environments." Applied and Environmental Microbiology **75**(24): 7649-7654.

Miranda-Tello, E., M.-L. Fardeai, C. Joullan, M. Magot, P. Thomas, J.-L. Tholozan and B. Ollivier (2007). "Petrotoga halophila sp nov., a thermophilic, moderately halophilic, fermentative bacterium isolated from an offshore oil well in Congo." International Journal Of Systematic And Evolutionary Microbiology **57**: 40-44.

Miranda-Tello, E., M.-L. Fardeau, P. Thomas, F. Ramirez, L. Casalot, J.-L. Cayol, J.-L. Garcia and B. Ollivier (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, C. L., M. Dlutek, O. Zhaxybayeva and W. F. Doolittle (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." American Laboratory **22**(12): 28-32.

Pollock, J., K. A. Weber, J. Lack, L. A. Achenbach, M. R. Mormile and J. D. Coates (2007). "Alkaline iron(III) reduction by a novel alkaliphilic, halotolerant, Bacillus sp isolated from salt flat sediments of Soap Lake." Applied Microbiology and Biotechnology **77**(4): 927-934.

Roskam, E. E., A. P. M. Coxon, A. P. Brier and P. K. Hawkins (2005). The NewMDSX Program Series, Version 5. Edinburgh, NewMDSX Project.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, J. Van Horn and C. F. Weber (2009). "Applied and Environmental Microbiology." Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. **75**(23): 7537-41.

Schwab, A. P., J. Hickey, J. Hunter and M. K. Banks (2006). "Characteristics of blast furnace slag leachate produced under reduced and oxidized conditions." Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering **41**(3): 381-395.

Shinn, M. B. (1941). "Colorimetric method for determination of nitrite." Industrial and Engineering Chemistry-Analytical Edition **13**: 0033-0035.

Spring, S., M. Wagner, P. Schumann and P. Kampfer (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." International Journal of Systematic and Evolutionary Microbiology **55**: 621-629.

Stewart, D. I., I. T. Burke, D. V. Hughes-Berry and R. A. Whittleston (2009). "Microbially mediated chromate reduction in soil contaminated by highly alkaline leachate from chromium containing waste." Ecological Engineering, doi:10.1016/j.ecoleng/2008.12.028.

Stewart, D. I., I. T. Burke and R. J. G. Mortimer (2007). "Stimulation of microbially mediated chromate reduction in alkaline soil-water systems." Geomicrobiology Journal **24**(7-8): 655-669.

Stumm, W. and J. J. Morgan (1996). Aquatic Geochemistry New York, John Wiley and Sons.

Sturr, M. G., A. A. Guffanti and T. A. Krulwich (1994). "Growth and Bioenergetics of Alkaliphilic *Bacillus Firmus* OF4 in Continuous Culture at High pH." Journal Of Bacteriology **176**(11): 3111-3116.

Takai, K., D. P. Moser, T. C. Onstott, N. Spoelstra, S. M. Pfiffner, A. Dohnalkova and J. K. Fredrickson (2001). "Alkaliphilus transvaalensis gen. nov., sp nov., an extremely alkaliphilic bacterium isolated from a deep South African gold mine." International Journal of Systematic and Evolutionary Microbiology **51**: 1245-1256.

Takai, K., C. L. Moyer, M. Miyazaki, Y. Nogi, H. Hirayama, K. H. Nealson and K. Horikoshi (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, B. M. and A. Y. Obraztsova (1998). "Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors." Fems Microbiology Letters **162**(1): 193-198.

Thauer, R. K., K. Jungermann and K. Decker (1977). "Energy conservation in chemotrophic anaerobic bacteria." Bacteriological Reviews **41**(1): 100-180.

Townsend, T. G., Y. Jang and L. G. Thurn (1999). "Simulation of construction and demolition waste leachate." Journal of Environmental Engineering-Asce **125**(11): 1071-1081.

Viollier, E., P. W. Inglett, K. Hunter, A. N. Roychoudhury and P. Van Cappellen (2000). "The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters." Applied Geochemistry **15**(6): 785-790.

Wang, Q., G. M. Garrity, J. M. Tiedje and J. R. Cole (2007). "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. ." Applied and Environmental Microbiology **73**(16): 5261-5267.

Whittleston, R. A., D. I. Stewart, R. J. G. Mortimer, D. J. Ashley and I. T. Burke (2010). "Effects of microbially induced anoxia on Cr(VI) mobility at a hyperalkaline, chromite ore processing residue contaminated site." Geomicrobiology Journal.

Wu, C. Y., L. Zhuang, S. G. Zhou, F. B. Li and J. He (2011). "Corynebacterium humireducens sp. nov., an alkaliphilic, humic acid-reducing bacterium isolated from a microbial fuel cell." International Journal Of Systematic And Evolutionary Microbiology **61**: 882-887.

Ye, Q., Y. Roh, S. L. Carroll, B. Blair, J. Z. Zhou, C. L. Zhang and M. W. Fields (2004). "Alkaline anaerobic respiration: Isolation and characterization of a novel alkaliphilic and metal-reducing bacterium." Applied and Environmental Microbiology **70**(9): 5595-5602.

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 μmol L^{-1} $P_{NO} = 10^{-6}$ bar
Nitric oxide reduction to nitrous oxide$^\$$	$NO + H^+ + e^- = ½N_2O + ½H_2O$	1.588	0.996	0.700	$P_{NO} = P_{N2O} = 10^{-6}$ bar
Nitrous oxide reduction to nitrogen$^\$$	$½N_2O + H^+ + e^- = ½N_2 + ½H_2O$	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	$[Fe^{2+}]$ = 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)

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

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	SiO_2	Al_2O_3	CaO	MgO	Fe_2O_3	TiO_2	MnO	Na_2O	K_2O	P_2O_5	SO_3	LOI
	cm	%	%	%	%	%	%	%	%	%	%	%	%
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

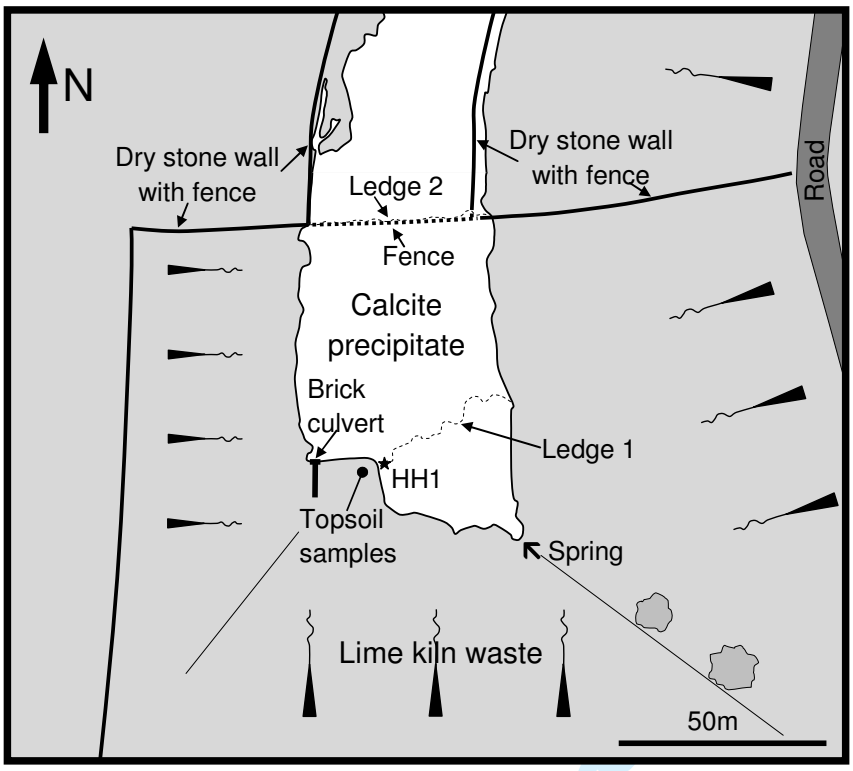


Figure 1: Sketch map of Brook Bottom area affected by alkaline waters, near Harpur Hill, Derbyshire, UK, showing core locations and location of the alkaline spring. Area of carbonate precipitate shown in white. Highly alkaline water (up to pH 13.1) pools behind the ledge 1 and is diluted by mixing below 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).

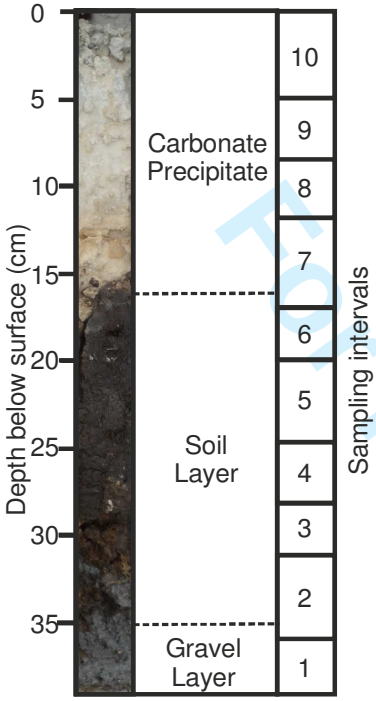


Figure 2: Digital photograph of core HH1 and log of sediment types found.

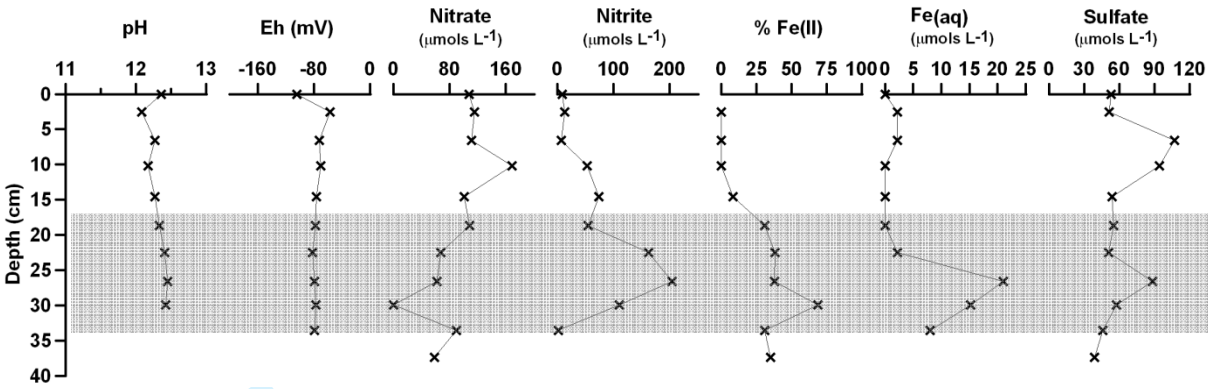


Figure 3: Vertical geochemical profile from porewater recovered from core HH1. Position of the brown soil layer in the profile is shown by grey shading

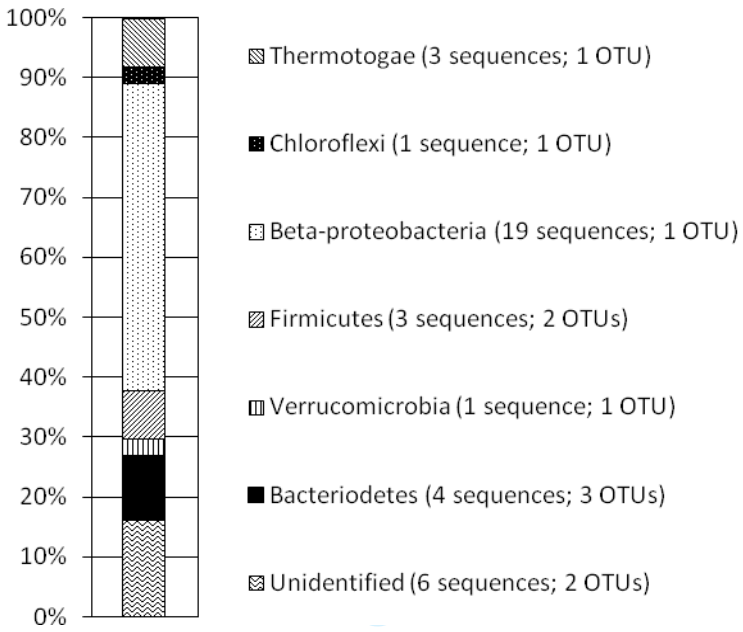


Figure 4. Phylogenetic diversity of 16S rRNA gene sequences extracted from sample HH1-5 (20-25 cm below surface). Key shows the number of operationally defined taxonomic units within each phylum.

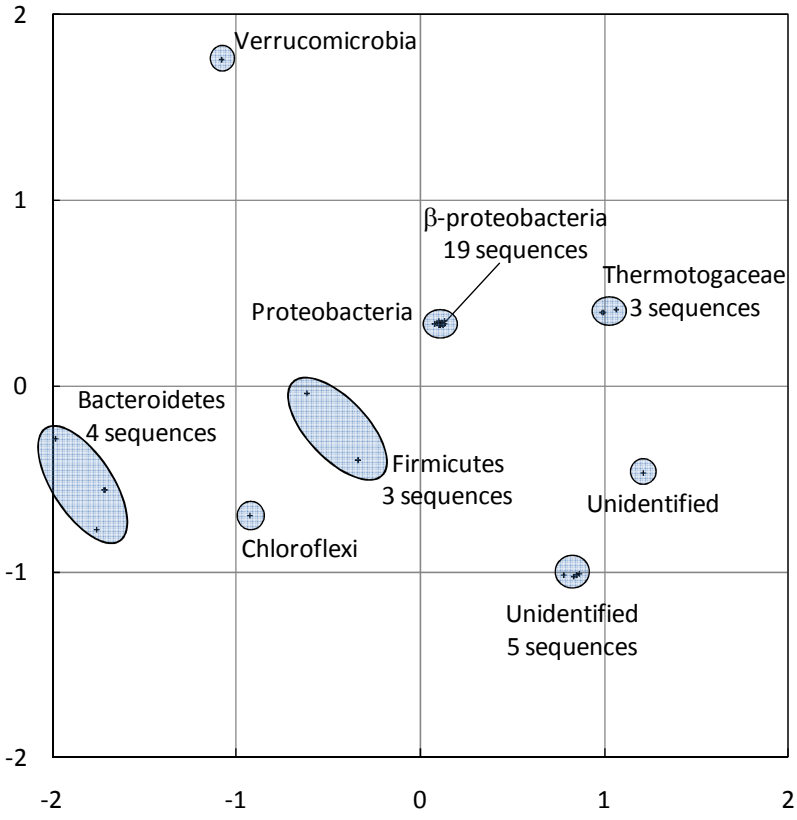


Figure 5. Two-dimensional configuration from the MDS analysis of the pair-wise sequence dissimilarity scores (distance scale within this Euclidean space is an arbitrary function of dissimilarity). The stress (lack of fit) associated with this two dimensional representation decreased marginally from 0.31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

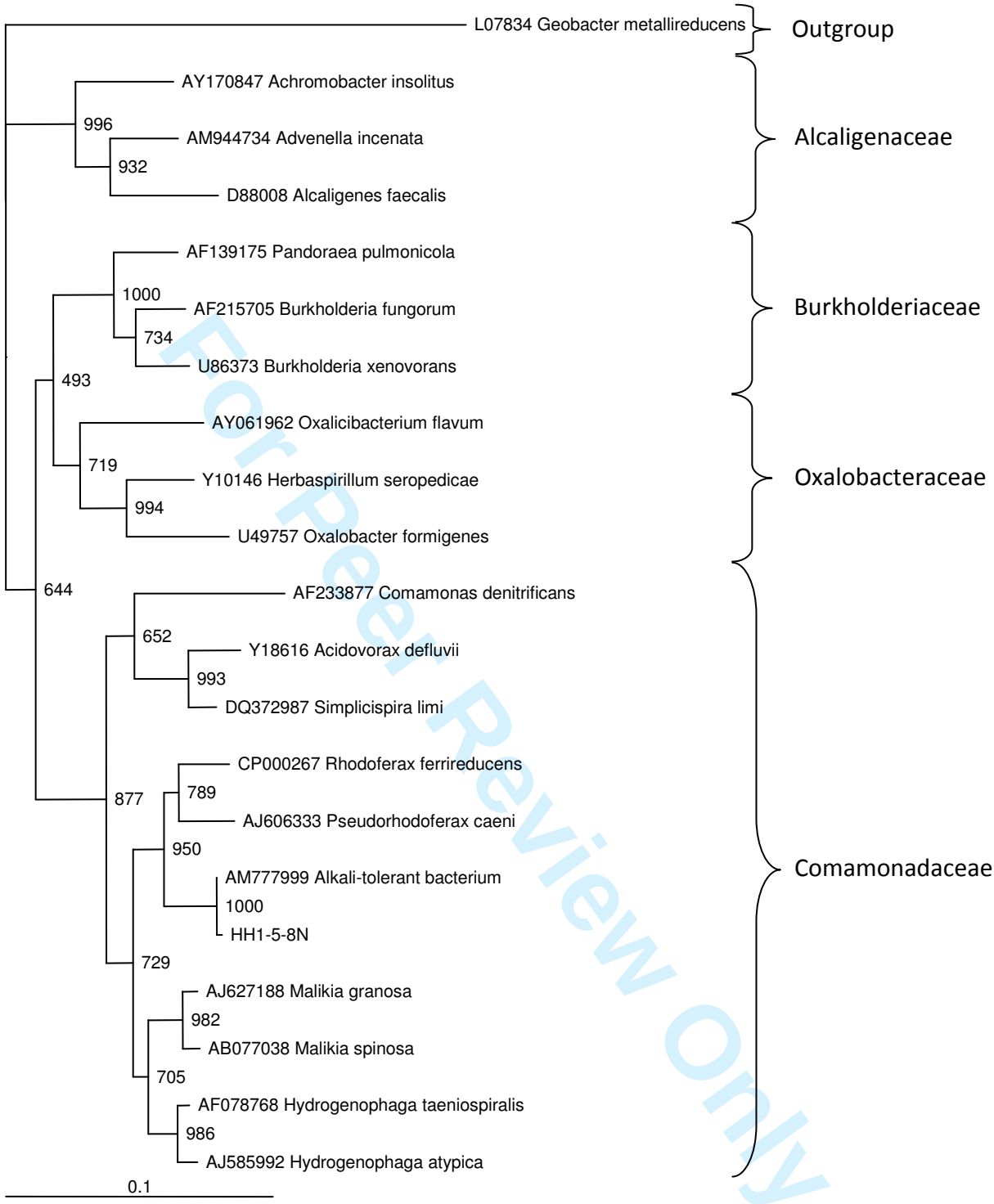


Figure 6: Phylogenetic tree showing the relationship between a representative sequence from OTU A6 (HH1-5-8N) and other members of the order Burkholderiales of β-proteobacteria. Geobacter metallireducens (δ-proteobacteria) is included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions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

Supplementary Information for:

Biogeochemical reduction processes in a hyper-alkaline affected leachate soil profile

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

[1] Earth Surface Science Institute, School of Earth and Environment, University of Leeds, Leeds LS2 9JT, UK

[2] School of Civil Engineering, University of Leeds, Leeds LS2 9JT, UK

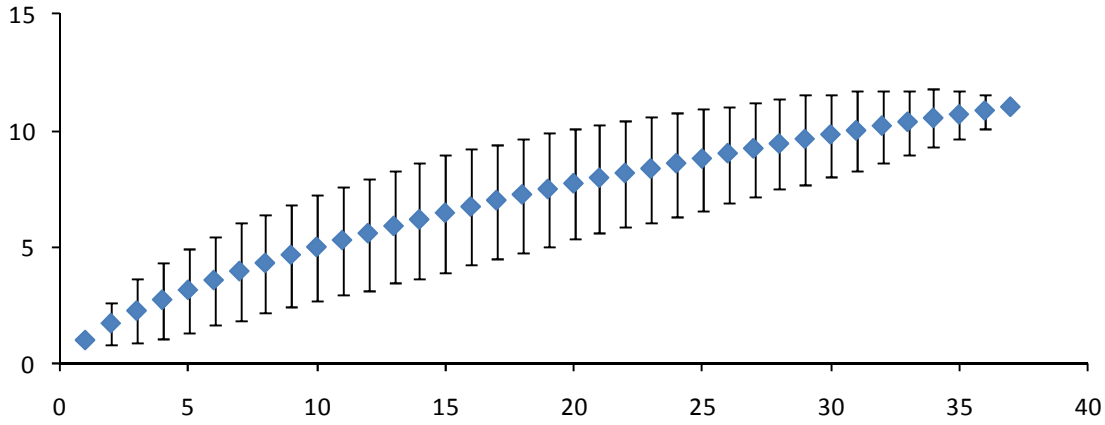
[*]Corresponding Author e-mail: i.burke@see.leeds.ac.uk / d.i.stewart@leeds.ac.ukmailto:i.burke@see.leeds.ac.uk

[#] Present address: Central Leather Research Institute, Chennai, India

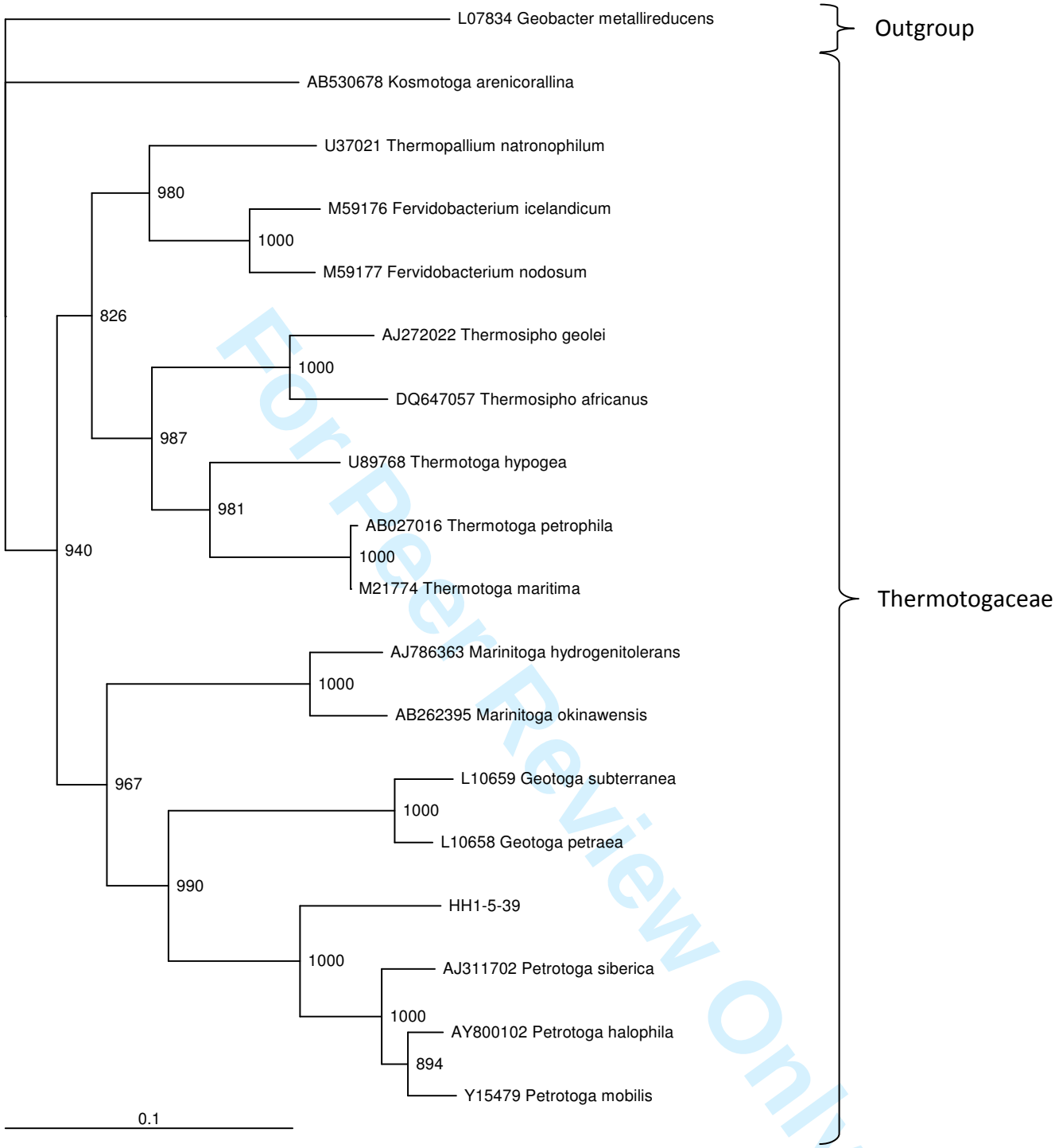
Supplementary Table A: Assignment of the 16S rRNA gene sequences obtained from sample HH1-5 (determined using the RDP Classifier[1] in August 2010).

ID	Accession number	Sequence length	OTU	Classification using the RDP classifier (95% Confidence threshold)
HH1-5-1N	JF827038	527	A3	-
HH1-5-3N	JF827039	546	A1	Verrucomicrobia
HH1-5-4N	JF827040	527	A3	-
HH1-5-5N	JF827041	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-6N	JF827042	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-7N	JF827043	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-8N	JF827044	515	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-9N	JF827045	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-10N	JF827046	527	A9	Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae, Petrimonas
HH1-5-11	JF827047	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-12N	JF827048	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-13N	JF827049	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-14	JF827050	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-15N	JF827051	505	A2	Firmicutes, Clostridia, Clostridiales, Ruminococcaceae
HH1-5-17	JF827052	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-18	JF827053	527	A3	-
HH1-5-20	JF827054	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-21	JF827055	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-22	JF827056	494	A5	-
HH1-5-23	JF827057	519	A7	Firmicutes
HH1-5-25	JF827058	527	A8	Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae, Parabacteroides
HH1-5-26	JF827059	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-28	JF827060	525	A10	Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae
HH1-5-29	JF827061	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-30	JF827062	494	A11	Chloroflexi, Anaerolineae, Anaerolineales, Anaerolineaceae
HH1-5-32	JF827063	527	A3	-
HH1-5-34	JF827064	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-35	JF827065	527	A3	-
HH1-5-36	JF827066	518	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-37	JF827067	504	A4	Thermotogae, Thermotogae, Thermotogales, Thermotogaceae, Petrotoga
HH1-5-38	JF827068	527	A8	Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae, Parabacteroides
HH1-5-39	JF827069	503	A4	Thermotogae, Thermotogae, Thermotogales, Thermotogaceae, Petrotoga
HH1-5-40	JF827070	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-43	JF827071	503	A4	Thermotogae, Thermotogae, Thermotogales, Thermotogaceae, Petrotoga
HH1-5-45	JF827072	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-46	JF827073	517	A6	Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae
HH1-5-48	JF827074	519	A7	Firmicutes

1. Wang, Q., et al., *Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. .* Applied and Environmental Microbiology 2007. **73**(16): p. 5261-5267.



Supplementary Figure A: Rarefaction curve for sequences from HH1-5. The curve is derived from a MOTHUR analysis using furthest neighbour assignment to OTUs for a distance level of 0.02. Error bars represent the 95% confidence interval.



Supplementary Figure B: Phylogenetic tree showing the relationship between a representative sequence from OTU A4 (HH1-5-39) and other members of the family Thermotogaceae family of phylum Thermotogae. Geobacter metallireducens (β-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.