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**Enhancing microbial iron reduction in hyperalkaline, chromium contaminated sediments
by pH amendment.**

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Abstract

Soil collected from beneath a chromite ore processing residue (COPR) disposal site contained a diverse population of anaerobic alkaliphiles, despite receiving a continuous influx of a Cr(VI) contaminated, hyperalkaline leachate (pH 12.2). Chromium was found to have accumulated in this soil as a result of an abiotic reaction of Cr(VI) with Fe(II) present in the soil. This sediment associated Fe(II) was therefore acting as a natural reactive zone beneath the COPR and thereby preventing the spread of Cr(VI). In anaerobic microcosm experiments soil microorganisms were able to reduce nitrate at pH 11.2 coupled to the oxidation of electron donors derived from the original soil organic matter, but progressive anoxia did not develop to the point of iron reduction over a period of 9 months. It is not clear, therefore, if Fe(II) can be actively replenished by microbial processes occurring within the soil at in situ conditions. Sodium bicarbonate was added to this soil to investigate whether bioreduction of iron in hyperalkaline chromium contaminated soils could be enhanced by reducing the pH to a value optimal for many alkaliphilic bacteria. The addition of sodium bicarbonate produced a well buffered system with a pH of ~9.3 and iron reducing conditions developed within 1 month once complete denitrification had occurred. Iron(III) reduction was associated with an increase in the proportion of genetic clone libraries that were from the phylum *Firmicutes*, suggesting that these species are responsible for the Fe(III) reduction observed. Amendment of the pH using bicarbonate may provide a suitable strategy for stimulating the bioreduction of Fe(III) in COPR leachate contaminated soils or other environments where microbial reduction is inhibited by elevated pH.

Keywords: Microbial iron-reduction, alkaliphile, biostimulation, Chromium, COPR, Cr(VI) contaminated groundwater, pH amendment.

Introduction

Disposal of chromium ore processing residue (COPR) is a globally widespread concern due to the risks associated with potentially harmful Cr(VI) contaminated hyperalkaline liquors leaching into the wider environment (Deakin *et al.*, 2001, Stewart *et al.*, 2010, Weng *et al.*, 2000). Chromium is extracted from its ore, chromite, by roasting it in the presence of air and an alkali carbonate to oxidise Cr(III) to Cr(VI) which can then be extracted with water due to its increased solubility. Originally lime (CaO), and then limestone, was added to the kiln to increase air penetration and thus provide sufficient O₂ for chromite oxidation in a practice known as the “high-lime” process (Farmer *et al.* 1999). Although technologically superseded by lime-free processing in the 1960s, the high-lime process, until recently, still accounts for 40% of chromium production worldwide (Darrie 2001). The high-lime process is notoriously inefficient and produces large volumes of hyperalkaline wastes with pH > 12 (Higgins *et al.*, 1998). Residual Cr concentrations within COPR typically range from 2000–40,000 mg.kg⁻¹, with up to 35% present in the oxidised Cr(VI) form (Geelhoed *et al.*, 2002, Tinjum *et al.*, 2008). Chromium can easily become mobile in water as the toxic and carcinogenic chromate anion (CrO₄²⁻) (Farmer *et al.*, 2006, James, 1994, Tinjum *et al.*, 2008). Remediation of legacy sites contaminated with COPR is challenging, particularly because these sites are often in urban areas and date from times when COPR disposal was quite poorly managed (Breeze, 1973, Geelhoed *et al.*, 2002, Higgins *et al.*, 1998, Jeyasingh and Philip, 2005, Stewart *et al.*, 2007). Traditional “dig and dump” remediation strategies are not only costly due to the large volumes involved, but also inadvisable due to the risk of forming carcinogenic Cr(VI) bearing dusts (USEPA, 1998). In contrast to the harmful properties of Cr(VI), the reduced form Cr(III) is an essential trace nutrient in plants and animals (Richard and Bourg, 1991), readily sorbs to soil minerals, and (co)-precipitates as insoluble Cr(III) hydroxides in neutral and alkaline environments (Rai *et al.*, 1987, Richard and Bourg, 1991). Amorphous Fe(II) is often present in reduced soils and aquifers and can readily react abiotically with Cr(VI) to produce Cr(III) and Fe(III) (Lin, 2002). Where this occurs it significantly reduces the hazard posed by Cr contaminated groundwater. Thus, the promotion of iron(III) reduction in soils beneath (or adjacent to) COPR may be a viable method to control Cr(VI) leaching from the waste.

Soil microorganisms can couple the oxidation of soil organic matter to the reduction of transition metals, such as iron and manganese, in a process known as dissimilatory metabolism (Lovley, 1993b). Iron is the most abundant redox-active metal in soils, and hence microbial Fe(III) reducing conditions are very important for controlling trace metal behaviour during the development of anaerobic soils (Lovley, 1991, Lovley, 1997, Pollock *et al.*, 2007, Zavarzina *et al.*, 2006). Numerous iron reducing microorganisms from a range of microbial taxa have been isolated

from a broad range of environments, including some alkaliphilic bacteria such as *Bacillus* sp and *Geoalkalibacter ferrihydriticus* (Pollock *et al.*, 2007, Zavarzina *et al.*, 2006). During dissimilative reduction, bacteria transfer electrons from organic carbon to Fe(III) and use the energy released from these coupled reactions to translocate protons from the cytoplasm to the periplasm. This produces an electrochemical gradient (or proton motive force) across the cytoplasmic membrane that drives adenosine triphosphate (ATP) synthesis via oxidative phosphorylation of adenosine diphosphate (ATP is the unit used for intracellular energy transfer). In contrast, fermentative bacteria synthesise ATP from the action of internal cytoplasmic enzymes which catalyse the transfer of a phosphate group from the substrate to adenosine diphosphate in a process known as substrate-level phosphorylation (Nelson and Cox, 2005). High pH is a challenging environment for bacteria as it is difficult to maintain a proton motive force when the external pH exceeds that of the cytoplasm, thus highly alkaline conditions may favour fermentative metabolism over respiration. Some fermentative alkaliphiles in the order Clostridiales have been demonstrated to indirectly reduce iron in soils and sediments through the external dumping of electrons to Fe(III) as a method of maintaining internal redox balance within cells (e.g. *Tindallia magadii* (Kevbrin *et al.*, 1998); *Clostridium beirjerinckii* (Dobbin *et al.*, 1999); *Anoxynatronum sibiricum* (Garnova *et al.*, 2003); *Anaerobranca californiensis* (Gorlenko *et al.*, 2004). Soil microorganisms can also reduce contaminant metals, such as chromium, during dissimilatory metabolism. Microbial reduction of Cr(VI) has been reported in a number of Gram negative genera including *Desulfovibrio* and *Shewanella*, and members of the Gram positive *Bacillus* and *Cellulomonas* genera (Francis *et al.*, 2000, Lovley, 1993a, Romanenko and Koren'kov, 1977, Sani *et al.*, 2002, Sau *et al.*, 2008), however only a few studies have demonstrated direct microbial Cr(VI) reduction at high pH (Chai *et al.*, 2009, VanEngelen *et al.*, 2008, Zhu *et al.*, 2008). Thus it has been suggested that microbially mediated Cr(VI) reduction in alkaline, chromium contaminated environments usually occurs by an indirect pathway involving extracellular reaction with reduced species (Higgins *et al.*, 1998), for example Fe(II) produced during dissimilative iron reduction coupled to oxidation of soil organic matter (Lovley and Phillips, 1986b).

The stimulation of iron bioreduction to achieve a remediation goal is usually induced by addition of an electron donor to environments where the growth of Fe(III)-reducing bacteria is limited by lack of organic matter. However, at high pH and in the presence of Cr(VI), it may be these harsh conditions that limit growth. This study investigates a soil recovered from beneath a COPR tip which contains organic matter and acid extractable Fe(II). A previous study found that an abiotic reaction between soil associated Fe(II) and aqueous Cr(VI) effectively reduced the Cr(VI) concentration to below detection limits and produced a mixed Cr(III)-Fe(III) oxyhydroxide precipitate

(Whittleston *et al.*, 2011b). This soil was also found to contain bacteria capable of Fe(III) reduction when incubated in suitable growth media at pH 9.2 (Whittleston *et al.*, 2011b). At elevated pH (i.e. above pH ~10.5) the rates of microbial Fe(III)-reduction supported by many alkaliphilic bacteria are known to reduce significantly (Pollock *et al.*, 2007) and in previous sediment microcosm experiments neither Fe(III) or Cr(VI) reduction were observed at pH ~11 (Whittleston *et al.*, 2011a). At the site being investigated in this study, the continual production of Fe(II) is vital to maintain the adventitious natural reactive zone currently present in soils beneath the COPR. The objective of this paper, therefore, is to investigate the feasibility of enhancing microbial iron reduction within these highly alkaline soils by buffering the soil pH down to about 9.5 (close to the optimum pH value for many alkaliphilic micro-organisms). It reports differences in the microbial community and the rate of development of Fe(III) reducing conditions that occur with and without pH buffering, and discusses the potential this may have as a remediation strategy in alkaline contaminated soil water systems.

Materials and methods

Field sampling and sample handling. Samples were obtained from a 19th century COPR tip in the north of England (see Whittleston *et al.*, 2011a). A soil sample (B2-310) was collected from a grey-clay horizon immediately beneath the COPR waste using a hand auger and 1m core sampler in March 2009 (sample locations are shown on Figure 1). Water from within the COPR waste was collected at the same time from a near-by monitoring well (BH5) using disposable PVC bailers (there is a perched water table within the waste, and this water is percolating downwards into the underlying soil horizons (Atkins, 2009)). Samples were stored in sealed polythene containers with as little headspace as possible, and kept in the dark at 4°C.

Sample characterisation. Soil pH was measured following the ASTM standard method (ASTM, 2006). The total organic carbon content of oven dried and HCl treated subsamples was measured using Carlo-Erba 1106 elemental analyser (Schumacher, 2002). The acid neutralisation capacity was determined with both 1 M HCl and 1 M NaHCO₃. Freeze dried soil (10g) was suspended in deionised water (100 ml) in a sealed flask stirred by magnetic flea (to limit CO₂ in-gassing). The titrating solution was added in increments (1 ml) and allowed to equilibrate (4 hrs).

Reduction microcosm Experiments. A *pH amended* microcosm series was established in triplicate with corresponding sterile controls. Each microcosm consisted of 10g of homogenised B2-310 soil and 100ml of COPR water in a 120ml glass serum bottle . After sealing these were buffered to pH 9 using 1 M sodium bicarbonate solution, and the headspace purged with nitrogen. Sterile controls

were prepared by autoclaving soil with a nitrogen purged headspace (120°C, 20min) and adding filter sterilised (0.2 µm) COPR leachate upon cooling (only one control microcosm was routinely sampled to ensure the sterility of the other replicates). An active *unamended* control microcosm series was prepared in triplicate using only the soil and COPR water. Bottles were incubated in the dark at 21°C and periodically sampled aseptically for geochemical analysis. During sampling microcosms were shaken and 3ml soil slurry extracted. Samples were centrifuged (3min, 16,000g) and the water analysed for pH and Cr(VI), and soil for acid extractable Fe(II). The soil pellets were pooled for each microcosm series and retained for microbiological community analysis.

Geochemical methods. Eh and pH were measured using an Orion meter (pH calibrated at 7 and 10). 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, and the column was flushed between samples with deionised water for 1.5 minutes. UV/VIS spectroscopy methods were used to determine aqueous Cr(VI) concentrations based on reaction with diphenylcarbazide (USEPA, 1992) and aqueous nitrite concentrations following reaction with sulphanilamide (SAN) and naphthylethylenediamine dihydrochloride (Harris and Mortimer, 2002). Iron was extracted from solids using 0.5N HCl (a proxy for microbial available iron (Burke *et al.*, 2006, Weber *et al.*, 2001), and the percentage Fe(II) was determined by reaction with ferrozine (Lovley and Phillips, 1986a). UV/VIS spectroscopy methods were performed on a Cecil CE3021 UV/VIS Spectrophotometer; calibration standards were used regularly.

X-ray Absorption Spectroscopy (XAS). XANES spectra were collected from soil recovered from the *pH 9 amended* microcosms on day 153 on station I18 at the Diamond Light Source, UK. Spectra were also collected from potassium chromate and amorphous Cr-hydroxide (precipitated by drop-wise neutralisation of CrCl₃ solution using 10M NaOH (Saraswat and Vajpei, 1984). XANES spectra were summed and normalised using Athena v0.8.056 (see S.I. for details).

Microbial Community Analysis. Microbial DNA was extracted from a sample of B2-310 soil, and the *pH amended* microcosms on day 153 and *unamended* control microcosms on day 270. A 16S rRNA gene fragment (~500 bp) was amplified from each sample by polymerase chain reaction (PCR) using broad specificity primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') (Eden *et al.*, 1991) and 519r (5'-GWATTACCGCGGCKGCTG-3') where K = G or T, W = A or T (Lane *et al.*, 1985). The PCR product was ligated into a standing cloning vector (pGEM-T Easy; Promega Corp., USA), and transformed into *E. coli* competent cells (XL1-Blue; Agilent Technologies UK Ltd) to isolate plasmids containing the insert,

which were sent for sequencing. The quality of each gene sequence was evaluated, and non-chimeric sequences were classified using the Ribosomal Database Project (RDP) naïve Bayesian Classifier (Wang *et al.*, 2007) in August 2010 (see S.I. for details). Sequences were grouped into operational taxonomic units (OTUs) using the MOTHUR software (Schloss *et al.*, 2009) (>98% nearest neighbour sequence similarity cut-off). Rarefaction curves were constructed to characterize the diversity of each clone library using the Shannon Index (H') (Krebs, 1999). Phylogenetic trees were constructed using representative sequences from selected OTUs, aligned with type species from the EMBL database using ClustalX (version 2.0.11), and drawn with TreeView (version 1.6.6). Trees were constructed from the distance matrix by neighbour joining, with bootstrap analysis performed with 1000 replicates. Sequences were submitted to the EMBL Nucleotide Sequence Database (accession numbers: FR695903-FR696047 and HE573872-HE573888).

Multidimensional Scaling Analysis. Multidimensional Scaling Analysis (MDS), which is also known as principle coordinate analysis, configures the position of objects in Euclidean space based on their pair-wise dissimilarity, and is used in a number of scientific fields to visualize datasets (Son *et al.*, 2011). Here it is used to investigate changes in initial B2-310 microbial community after incubation in the microcosm experiments (*unamended* and *pH amended*). 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 (Coxon, 2004, Roskam *et al.*, 2005). Basic non-metric MDS was undertaken using the Minissa-N algorithm within NewMDSX.

Results

Sample recovery

Borehole B2 (Figure 1) was located near the south-western edge of the COPR tip. It encountered a topsoil layer then entered COPR waste at 190cm, grey sandy clay at 310cm, and terminated in a gravel layer at 365cm. Soil sample B2-310 was collected from the grey clay just below the soil-waste interface (sample depth 310 – 320 cm). This layer was probably the original surface deposit before the waste was tipped approximately 100 years ago (Stewart *et al.*, 2010).

Sample geochemistry

Soil sample B2-310 has been described in detail by Whittleston *et al* (2011b). Briefly, it had a pH value of 12.2, contained 1.0% TOC and 53% of 0.5 N HCl extractable iron was present as Fe(II). The major minerals present were quartz, kaolinite and albite. The chromium concentration was

approximately 3400 mg.kg⁻¹, which chromium K-edge XAS analysis showed was predominately present in Cr(III) oxidation state within a mixed Cr(III)-Fe(III) oxy-hydroxide phase. Tilt (2009) studied the heterogeneity of this soil layer at cm resolution and found that the proportion of 0.5N HCl acid extractable iron in the Fe(II) oxidation state can vary at this scale from 5% to 90%.

The water from within the COPR collected from BH5 (Figure 1) had a pH of 12.2 and Eh of +90 mV (Eh was measured upon sampling) and a Cr(VI) concentration of 990 µM (51.5 mg.L⁻¹). The acid neutralisation capacity of B2-310 soil determined using HCl was approximately 1 mole H⁺ per kg of soil (Figure 2). The addition of NaHCO₃⁻ produced a slower decline in pH, with 0.6 moles HCO₃⁻ per kg of soil required to reach pH 9.5. However, this then remained stable about this value for the remainder of the experiment, up to 1.2 moles HCO₃⁻ per kg of soil.

Reduction Microcosms

The initial pH of the *pH amended* microcosm series was 8.9, while the corresponding sterile control had a slightly lower initial value of 8.6 (see Figure 3). In contrast the initial pH of the *unamended* control microcosm series was 12.2. The pH value of *pH amended* microcosms increased slightly over the first 5 days before levelling off at 9.3, and remained steady about this value until the tests were sampled for microbial community analysis on day 153. The pH value of the sterile control increased from 8.6 → 9.2 over the first 15 days, and remained steady about this value for the duration of the experiment. The pH value of the *unamended* control decreased from 12.2 → 11.7 over the first 20 days before remaining steady about this value until the tests were terminated for microbial community analysis on day 270.

At the first sample point (~1 hr) the aqueous Cr(VI) concentration in the *pH amended* microcosms had dropped from the initial leachate value of 990 → 656 ± 4 µmol L⁻¹, with complete removal observed by 20 days. Similar removal rates were observed in the corresponding sterile and *unamended* controls. Aqueous nitrate concentrations decreased rapidly in the *pH amended* active experiments from an initial value of 144 ± 13 µmol L⁻¹ → 0 by day 5, with concentrations decreasing at a similar rate in the *unamended* control. Nitrate was also removed from the sterile control, but this occurred very slowly over 137 days (nitrate was not detected in either backup control when they were sampled on day 137). Aqueous nitrite concentration in *pH amended* microcosms increased slightly over the first 5 days, before decreasing over the next 15 days. In contrast, nitrite concentration in the corresponding sterile control increased slightly over the duration of testing (confirmed by both backup sterile experiments). Nitrite concentrations also behaved differently in

the *unamended* control, increasing from $66 \pm 2 \rightarrow 296 \pm 26 \mu\text{mol L}^{-1}$ by day 41 before decreasing steadily over the remainder of the test.

The percentage of 0.5 N HCl extractable iron present as Fe(II) in the *pH amended* microcosms decreased slightly before recovering to its initial value by day 20, although this change is less than the measurement error. Beyond day 20, the percentage acid extractable iron present as Fe(II) increased steadily from $43 \rightarrow 96\%$ on day 137. No significant increase in Fe(II) percentage was observed in the corresponding sterile and *unamended* controls over the duration of the experiment.

X-ray Absorption Spectroscopy

The chromium XANES spectra from soil recovered from the pH 9 amended microcosms on day 153 (Figure 4) indicated that all the Cr associated with the solid phase was in the Cr(III) oxidation state (i.e. all the Cr(VI) initially present in the COPR water was removed from solution and had been reduced to Cr(III)).

Microbial community analysis

A total of 62 rRNA gene sequences were obtained from B2-310. These were assigned to 9 different bacterial phyla (confidence threshold >98%), with approximately 10% of sequences remaining unassigned (see Table 1). Three phyla were dominant, with 31%, 19%, and 16% of sequences assigned to *Proteobacteria*, *Firmicutes* and *Bacteriodetes*, respectively. The 57 sequences from the *pH amended* microcosms on day 153 were assigned to just two bacterial phyla, *Firmicutes* and *Proteobacteria* (68% and 9% of sequences, respectively), with the remaining 23% of sequences unassigned. In contrast the 43 sequences obtained from the *unamended* control microcosms on day 270 were also assigned to just two bacterial phyla, *Deinococcus-Thermus* and *Firmicutes* (79% and 12% of sequences, respectively), with 9% of sequences unassigned (see S.I. Table S1-3 for full assignments).

Rarefaction analysis of the three populations (S.I. Figure S1) indicates that species richness is highest in the B2-310 sample prior to incubation in microcosm systems (Shannon Index, $H' = 3.64 \pm 0.24$). Species richness was slightly lower after 153 days incubation in the *pH amended* microcosm ($H' = 3.07 \pm 0.25$), and lowest in the *unamended* control microcosm on day 270 ($H' = 0.62 \pm 0.36$). The two dimensional MDS representation of the sequence dissimilarity scores (Figure 5) largely

reflects this species diversity. The sequences recovered from the B2-310 population are scattered widely across the whole area of the MDS plot, demonstrating relatively wide diversity. In contrast the sequences recovered from the *pH amended* microcosm population are grouped more closely together on the plot, with only a small degree of scattering, reflecting that the species present were from significantly fewer phyla (Table 1). The sequences from the *unamended* control population show the least degree of spread across the plot, with most confined to a single area, demonstrating the very limited diversity recovered from this population.

Firmicutes species represented 21% of the initial (B2-310) population, 68% of the *pH-amended* microcosm population on day 153, and just 12% of the *unamended* control microcosm population on day 270. Two genera were common to all three populations. About 6% of the B2-310 population were *Dethiobacter*-like species (4 sequences), as were 5% of the *unamended* control population (2 sequences) and 42% of the *pH amended* population (24 sequences). Similarly, about 3% of the B2-310 population were *Anaerobranca*-like species (2 sequences), as were 2% of the *unamended* control microcosm population on day 270 (1 sequence) and 4% of the *pH9-amended* microcosm population on day 153 (2 sequences). A phylogenetic tree showing characteristic members of the *Dethiobacter*-like and *Anaerobranca*-like OTUs is shown in Figure 6 (the characteristic sequence is the sequence that is the minimum distance from the other members of the OTU (Schloss *et al.*, 2009)). In contrast to the other two populations, the bacterial population of the *unamended* control microcosms on day 270 was dominated by a single OTU (37 of the 43 sequences). This was a *Truepera*-like species within the phylum Deinococcus-Thermus.

Discussion

The soil immediately beneath the COPR tip is thought to have been the surface layer prior to waste tipping at the end of the 19th century. Cr(VI) bearing leachate from the COPR waste has probably been entering this soil for over 100 years (Stewart *et al.*, 2010). Despite this flux the soil contains a population of bacteria which is relatively diverse (46 different OTUs from 9 different phyla were identified within a clone library of 62 gene sequences). The soil has accumulated ~3400 mg.kg⁻¹ Cr, predominately present as Cr(III). As the Cr(III) is within a stable Fe(III) oxide host phase, it has previously been suggested that it has been reduced by microbially produced Fe(II) (Whittleston *et al.*, 2011b).

When this soil is incubated in microcosm experiments with water from within the waste pile under pH amended conditions, the microbial diversity decreases slightly. In contrast, microbial

diversity decreases significantly when there is no pH buffering in the *unamended* control. Initially it might seem surprising that there is loss of diversity in the *unamended* control microcosm experiments, which appear to replicate the conditions *in-situ*. However geochemical conditions vary rapidly in the former topsoil layer as the relatively oxic (Eh +90 mV) COPR water seeps into a reducing environment. Undisturbed the soil would have had fabric that affects seepage at a local scale, and it contains a variety of minerals that would buffer the pH of the COPR water. Together these will have produced a range of geochemical micro-environments within individual soil pores. Different micro-environments within the soil pores would have favoured different bacteria, which would produce a diverse clone library when the sample size is very much larger than the particle size. In contrast the microcosm experiments were prepared from a homogenised soil sample, and were shaken to produce a soil suspension as part of microcosm sampling. This would have produced more uniform geochemical conditions, which would have put a selective pressure on the microbial population, favouring a sub-set of the initial population to produce a less diverse population under essentially the same bulk average geochemical conditions as those observed *in-situ*.

Aqueous Cr(VI) was completely removed from solution at similar rates in all microcosm experiments, including the sterile *pH amended* control. Cr(VI) readily reacts abiotically with Fe(II) to produce Cr(III) and Fe(III) (Lin, 2002). Therefore the observed Cr(VI) removal in these experiments was probably the result of an abiotic reaction with the amorphous Fe(II) already present within the soil (53% of 0.5 N HCl extractable iron was deemed present as Fe(II)). XANES spectra collected from soil from a pH-amended microcosm after Cr(VI) removal confirmed that soil-associated chromium was predominately present in the Cr(III) oxidation state. A small decrease in the initial percentage acid extractable iron in the Fe(II) oxidation state concurrent with Cr(VI) removal was also observed in all experiments over the early sampling points, but this was less than the measurement error and therefore not significant. However, this minor reduction in Fe(II) during Cr(VI) removal is consistent with the calculated Fe(II) : Cr(VI) ratio of 20-30 : 1 present in these experiments.

In the *pH amended* microcosms aqueous nitrate was removed from the active experiments at least an order of magnitude more quickly than in the corresponding sterile control. In the microbially active experiments, aqueous nitrite concentration increased slightly during nitrate removal but decreased again once the nitrate was gone. The removal of nitrite was then rapidly followed by an increase in the proportion of acid extractable iron present as Fe(II). The rate of nitrate removal in the active microcosms (in comparison with the control) and subsequent depletion of nitrite and Fe(III) suggests that the reactions are microbially mediated and part of a cascade of terminal electron-accepting processes. The gradual removal of nitrate in the *pH-amended* sterile

control was most likely an abiotic process as nitrate was also removed from both backup controls (i.e. if nitrate removal was due to loss of sterility in the sampled control then it is unlikely to have occurred in both back-up controls as these were not sampled until day 137). The reduction of nitrate by Fe^{2+} (aq) is thermodynamically very favourable (even at high pH), but a direct reaction between these ions in solution is kinetically inhibited (Choe *et al.*, 2004, Hansen *et al.*, 1996, Ottley *et al.*, 1997). However, the reaction can be surface catalysed by transition metals and freshly formed Fe(III) precipitates (Fanning, 2000, Ottley *et al.*, 1997, Postma, 1990). Fe(II) oxyhydroxides are still sparingly soluble around pH9, where Fe^{2+} is the main aqueous species (Langmuir, 1997). It therefore seems reasonable that nitrate reduction by Fe^{2+} (aq) in a surface catalysed reaction was the mechanism by which nitrate was removed from the *pH amended* sterile microcosms.

The biogeochemical behaviour of the *unamended* control microcosms differed from the pH amended microcosms. Nitrate removal was observed over the first 2-3 weeks concurrent with a significant increase in nitrite concentration. Subsequently the nitrite concentration decreased steadily over the remainder of the experiment, suggesting that nitrite reduction was following-on from nitrate reduction. 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.*, 2011a), and in such systems it has been observed that nitrite reduction to N_2 tends to lag behind nitrate reduction to nitrite (Glass and Silverstein, 1998). Furthermore, the abiotic reduction of nitrate by Fe^{2+} (aq) is limited by its solubility minima around pH 11 (Smith, 2007), above which Fe(OH)_3^- is the dominant aqueous species (Langmuir, 1997). Thus it seems that nitrate and nitrite reduction in the *unamended* (pH ~11.7) control microcosms were microbially mediated processes, albeit occurring more slowly than with pH amendment. However, the absence of a significant increase in the proportion of acid extractable iron present as Fe(II) over the duration of the *unamended* control experiments suggests that microbial metabolism linked to Fe(III) reduction was inhibited at the unamended pH value.

The microbial community of the *pH-amended* microcosms was sampled when iron reduction was the predominant biogeochemical process. Amendment of the pH to ~9 and the subsequent development of iron reducing conditions favoured bacteria from the phylum *Firmicutes*. Two species, *Dethiobacter* and *Anaerobranca*, were also found in B2-310 soil and *unamended* microcosms. The *Dethiobacter*-like sequences are closely related to a clone, AJ431345, recovered from an alkaline tufa environment (Stougaard *et al.*, 2002) and also to the type species for this genus, *Dethiobacter alkaliphilus* (EF422412), an obligate anaerobic alkaliphile isolated from a soda lake environment (Sorokin *et al.*, 2008). *D. alkaliphilus* is reported to be involved in the reductive

sulphur cycle, and is capable of reducing elemental sulphur (Sorokin *et al.*, 2008), but to date no capacity for iron reduction has been reported. *Dethiobacter*-like species were a minor constituent of the original population but clearly thrived when the pH was buffered to 9.3, however it must be noted that there is no direct evidence that they were responsible for the Fe(III) reduction observed.

The *Anaerobranca*-like species from B2-310 soil and the two actives microcosm series form a distinct clade within the Clostridiales Incertae Sedis XIV, but are closely related to several species of *Anaerobranca*, a genus containing fermentative anaerobic extremophiles (see Figure 6). Whilst similarity of the 16S rRNA gene is not evidence that organisms share other genes (e.g. those associated with adaptation to a particular environment) it is nevertheless interesting that *A. californiensis*, *A. gottschalkii*, and *A. zavarzinii* are all alkaliphilic thermophilic anaerobes (Gorlenko *et al.*, 2004, Kevbrin *et al.*, 2008, Prowe and Antranikian, 2001). Similarly *A. horikoshii* is an alkali-tolerant thermophilic anaerobe (Engle *et al.*, 1995). *A. californiensis*, *A. gottschalkii* and *A. horikoshii* are able to reduce iron and sulphur in the presence of organic matter (Gorlenko *et al.*, 2004). There is also evidence that *A. zavarzinii* can reduce Fe(III) to Fe(II) but this is less conclusive (Kevbrin *et al.*, 2008).

The microbial community of the *unamended* microcosms was sampled when nitrite reduction was the predominant biogeochemical process. This population was dominated by a single OTU (37 of the 43 sequences), which is assigned to the order *Deinococcales* and were most probably a *Truepera* species. Phylogenetic tree construction (S.I Figure S2) confirms this classification. The only *Truepera* specie that has been studied in detail is *Truepera Radiovictrix*, the type genus of the family Trueperaceae, which is a radiation resistant, alkaliphilic, slightly halophilic aerobe (Albuquerque *et al.*, 2005).

No exogenic carbon source was added to the microcosm experiments, so microbial metabolism is supported by electron donors derived from the soil organic matter (the soil contains 1% total organic carbon). 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, 1993a). As processes such as dissimilative nitrate and iron reduction require labile organic carbon (Gottschalk, 1986, Kim and Gadd, 2008) the soil must contain consortium of bacteria capable of the continued breakdown of less labile organic substrates (without replenishment, labile substrates would have been consumed years ago). The development of a cascade of terminal electron accepting processes in the *pH-amended* system suggests that it retained this capability despite the slight decrease in microbial

diversity. However, the failure of the *unamended* control microcosms to progress beyond nitrate reduction after 270 days may be due in part to loss of microbial diversity impacting the community's ability to breakdown soil organic matter.

Environmental Implications

Soil recovered from beneath the COPR at this study site contained iron in the Fe(II) oxidation state that was available for Cr(VI) reduction. The Fe(II) presented in the soil was not significantly depleted by addition of a single aliquot of Cr(VI) contaminated groundwater in microcosms. However prolonged exposure to Cr(VI)-containing groundwater would eventually consume all the Fe(II) present unless it is replenished by Fe(III) reduction. Therefore, promoting *in-situ* bioreduction of iron may provide a promising way of enhancing and maintaining the amount of Fe(II) in the soil that is available for Cr(VI) reduction, ultimately preventing groundwater contamination or damage to aquatic ecosystems. However the very high pH of COPR leachate produces an extremely challenging environment to microorganisms capable of dissimilative metal reduction, and thus metabolic rates are slow even where the microbial community has adapted to high pH. This study shows that reducing the pH to closer to the optimum for many alkaliphiles (~pH 9.5) using sodium bicarbonate promoted microbial nitrate and iron reduction coupled to oxidation of electron donors derived from the soil organic matter. The advantage of pH amendment is that supplementing natural groundwater ions such as Na^+ and HCO_3^- may be more acceptable to regulating authorities responsible for groundwater quality than the addition of either nutrients (bio-stimulation) or non-native microorganisms (bio-augmentation). It required about five times more HCO_3^- than acid to buffer the soil pH to pH 9.5 (Figure 2). This is because equilibrium is established between CO_3^{2-} and HCO_3^- as this pH value is approached, reducing the effect of further HCO_3^- addition. However, despite the higher addition rate, HCO_3^- is probably the better pH buffer for promoting bioremediation as the pH stability of the $\text{CO}_3^{2-}/\text{HCO}_3^-$ buffered system will be better for bacterial growth.

Conclusions

A diverse population of novel anaerobic alkaliphiles exists in the former topsoil layer beneath a COPR tip, despite long-term exposure to oxidising Cr(VI) contaminated hyperalkaline leachate. This population contains taxa capable of coupling the oxidation of soil organic matter to the biogeochemical cycling of nitrate and iron during the progression of microbial anoxia. Addition of sodium bicarbonate to this soil produces a system with a high buffering capacity at pH values close

to the growth optima of many alkaliphiles (Horikoshi, 2004). This promotes bioreduction of Fe(III), with phylogenetic community analysis indicating that bacteria in the phylum *Firmicutes* are most likely responsible. Bicarbonate amendment of pH is a promising method of stimulating microbial iron reduction where the microbial community have adapted to alkaline conditions and subsequently may provide a strategy [for protecting environments](#) affected by highly alkaline Cr(VI) contaminated groundwater.

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Table 1. Summary of phylogenetic and OTU assignment from RDP and MOTHUR analysis using 95 and 98% confidence threshold and similarity cut off respectively.

	B2-310		pH9 amended		Unamended control	
	Sequences	OTUs	Sequences	OTUs	Sequences	OTUs
Deinococcus-Thermus	-		-		34	1
Acidobacteria	1	1	-		-	
Verrucomicrobia	2	2	-		-	
Planctomycetes	2	2	-		-	
Nitrospirae	3	2	-		-	
Actinobacteria	6	6	-		-	
Bacteriodetes	9	7	-		-	
Proteobacteria						
α class	2	2	1	1	-	
β class	12	5	2	2	-	
γ class	1	1	-		-	
δ class	1	1	-		-	
Not assigned to class	3	2	2	2		
Firmicutes	13	9	39	15	5	4
Bacteria incertae sedis	1	1	-		-	
Unassigned	6	5	13	9	4	2
	62	46	57	29	43	6

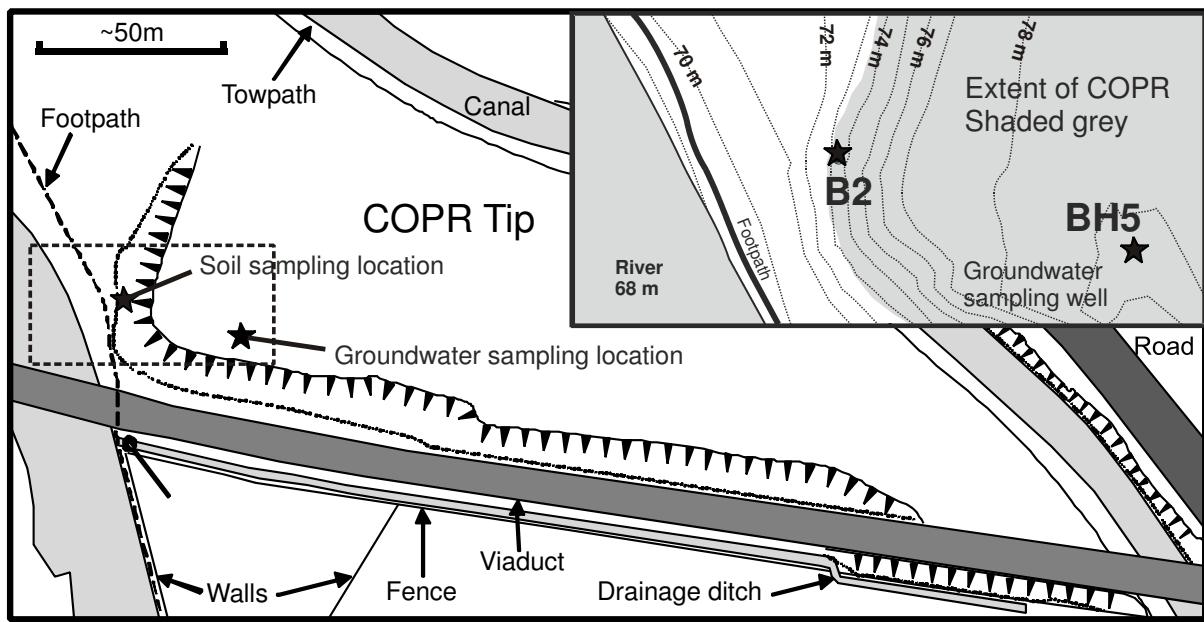


Figure 1. Sketch map of the site showing soil (B2) and water (BH5) sampling locations. Insert corresponds to dashed selection.

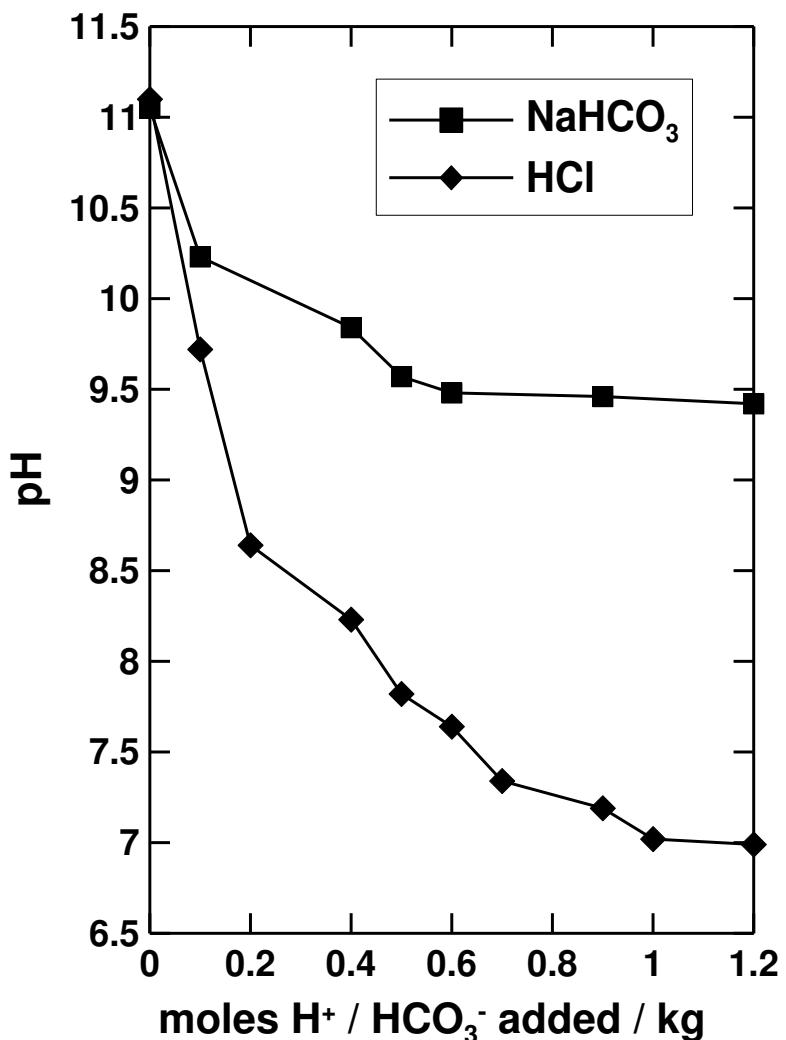


Figure 2. Titration curves obtained from the acid neutralisation test on freeze dried soil B2-310 n=1.

◆ = addition of HCl, ■ = HCO_3^- addition.

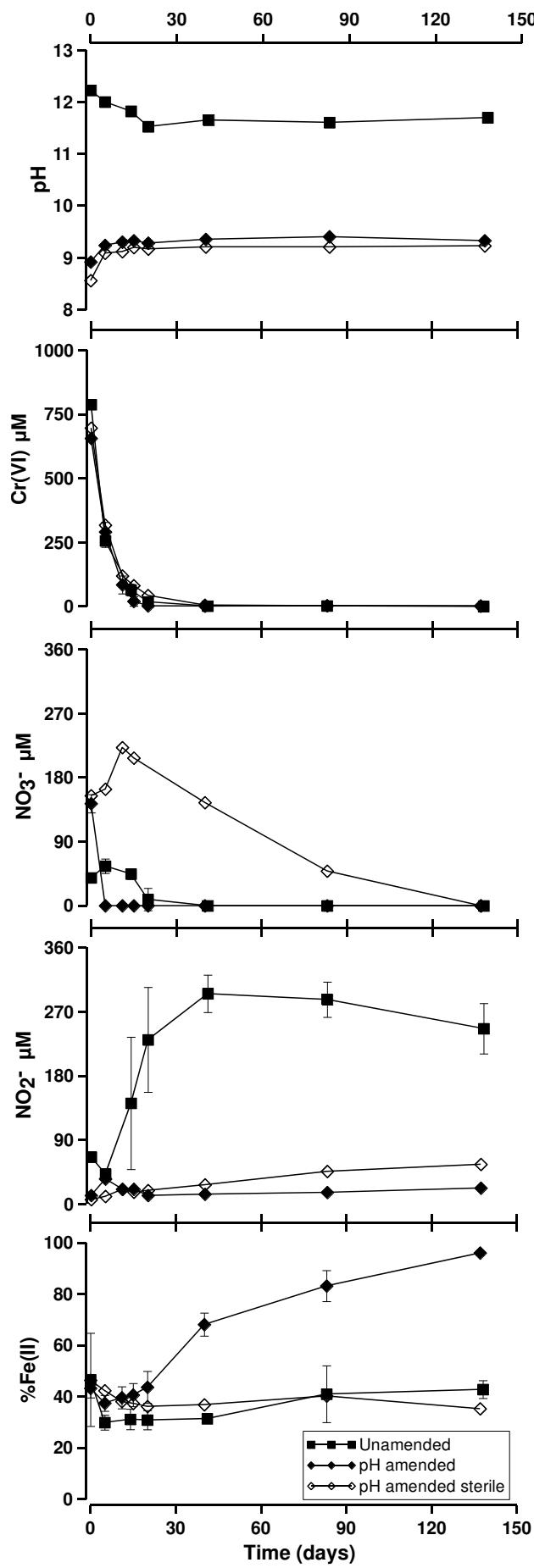


Figure 3. Geochemical response of the *pH amended* (\blacklozenge n=3), *unamended control* (■ n=3) and *sterile control* (\lozenge n=1) microcosms. Top to bottom; pH; pore water Cr(VI) concentration; pore water NO_3^-

concentration; pore water NO_2^- concentration; % of 0.5 N HCl extractable Fe as Fe(II) in soils. Error bars shown are one standard deviation from the mean of triplicate experiments.

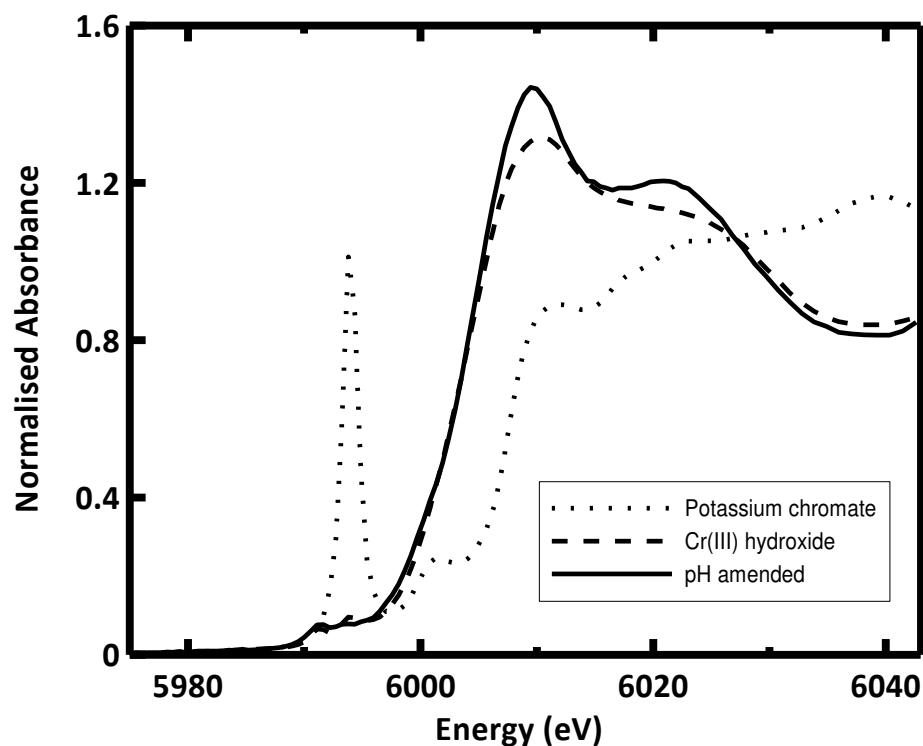


Figure 4. Normalised chromium K XANES spectra for soil from the pH 9 amended microcosms on day 153 and for the standards.

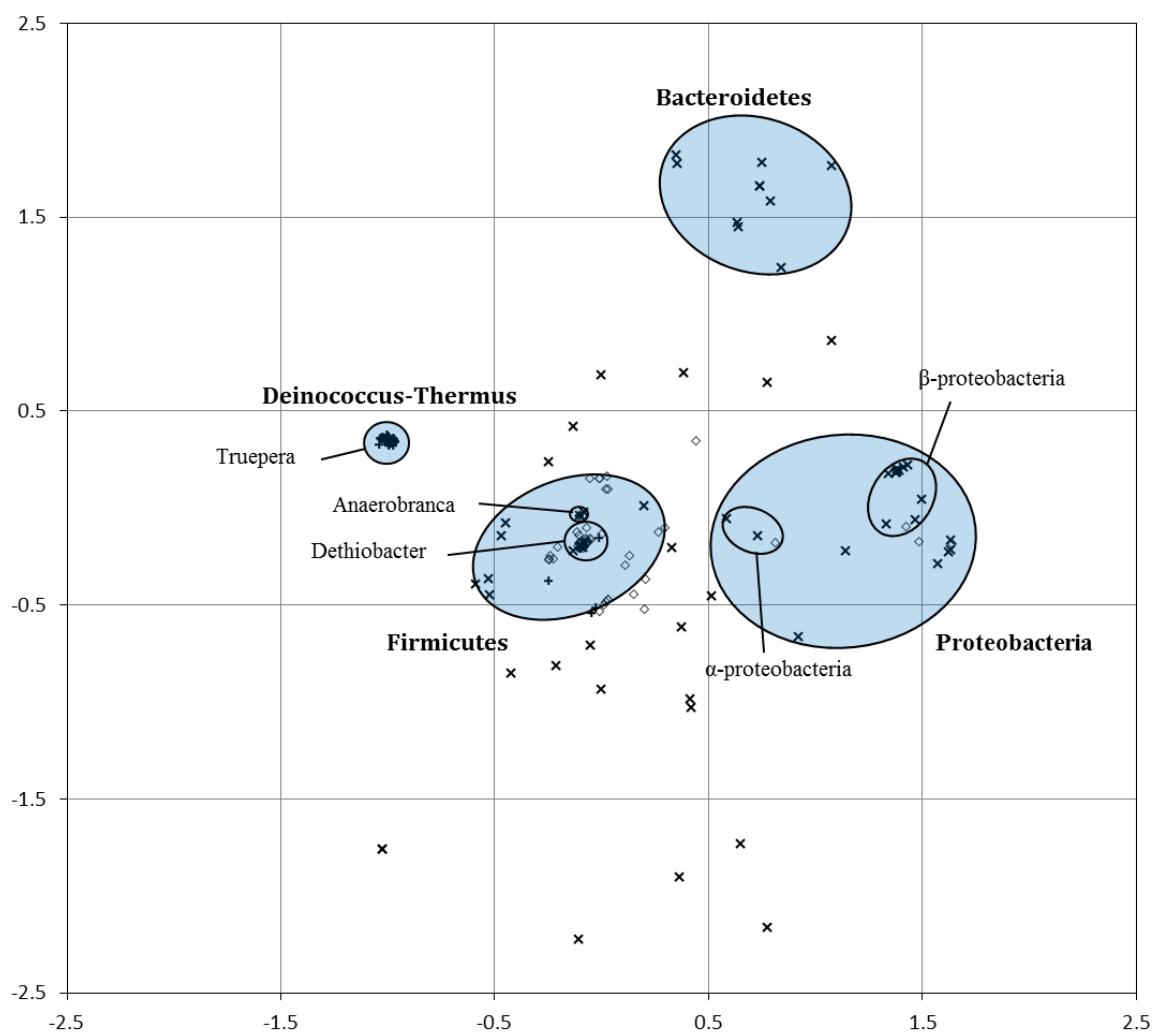


Figure 5. Two-dimensional configuration from the MDS analysis of the pair-wise sequence dissimilarity scores: (x) B2-310 population, (◇) pH amended microcosm, (+) unamended microcosm (distance scale within this Euclidean space is an arbitrary function of dissimilarity). The “stress” (a measure of lack of fit) associated with this two dimensional representation decreased marginally from 0.20 to 0.17 when the number of dimensions was increased to three, which suggest that two dimensions adequately represent the dissimilarities in the data.

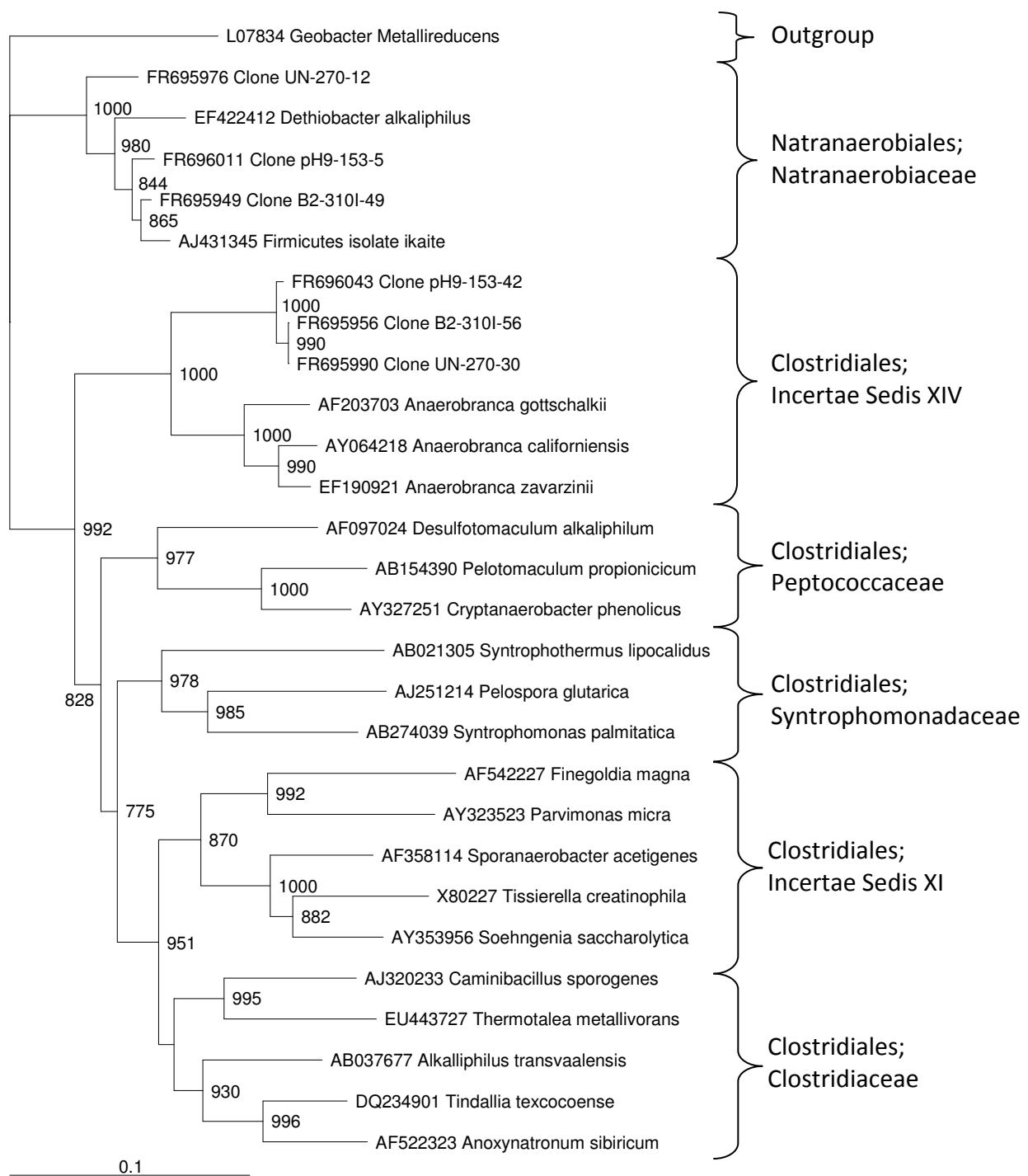


Figure 6. Phylogenetic tree showing the relationship between representative sequences from major OTU's recovered from each population and other members of the orders Clostridiales and Natranaerobiales of Firmicutes. *Geobacter metallireducens* (δ -proteobacteria) is included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.