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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 NO_3^- 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 Fe 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 Fe in hyperalkaline Cr contaminated soils could be enhanced by reducing the pH to a value optimal for many alkaliphilic bacteria. The addition of $NaHCO_3$ produced a well buffered system with a pH of ~9.3 and Fe reducing conditions developed within 1 month once complete denitrification had occurred. Fe(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.

1. Introduction

Disposal of Cr 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 (Weng et al., 2000; Deakin et al., 2001; Stewart et al., 2010). Chromium is extracted from its ore, chromite, by roasting 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 O_2 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 accounted for 40% of Cr 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 to 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(VI) can easily become mobile in water as the toxic and carcinogenic CrO_4^{2-} anion (James, 1994; Farmer et al., 2006; 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; Higgins et al., 1998; Geelhoed et al., 2002; 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 Fe(III) reduction in soils beneath (or adjacent to) COPR may be a viable method to control Cr(VI) leaching from the waste.

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Soil microorganisms can couple the oxidation of soil organic matter to the reduction of transition metals, such as Fe and Mn, 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, 1997; Zavarzina et al., 2006; Pollock et al., 2007). Numerous Fe-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* (Zavarzina et al., 2006; Pollock et al., 2007). During dissimilative reduction, bacteria transfer electrons from organic C 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 Fe 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 Cr, during dissimilatory metabolism. Microbial reduction of Cr(VI) has been reported in a number of Gram negative genera including *Desulfovibro* and *Shewanella*, and members of the Gram positive *Bacillus* and *Cellulomonas* genera (Romanenko and Koren'kov, 1977; Lovley, 1993a; Francis et al., 2000; 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, Cr-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 Fe reduction coupled to oxidation of soil organic matter (Lovley and Phillips, 1986b).

The stimulation of Fe 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), these harsh conditions may 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 $\sim$ 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 $\sim$ 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 Fe 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 microorganisms). 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.

2. Materials and methods

2.1. 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 1 m core sampler in March 2009 (sample locations are shown in Fig. 1). Water from within the COPR waste was collected at the same time from a nearby 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.

2.2. Sample characterisation

Soil pH was measured following the ASTM standard method (ASTM, 2006). The total organic C content of oven dried and HCl treated subsamples was measured using a Carlo-Erba 1106 elemental analyser (Schumacher, 2002). The acid neutralisation capacity was determined with both 1 M HCl and 1 M $NaHCO_3$. Freeze dried soil (10 g) was suspended in deionised water (100 mL) in a sealed flask stirred by magnetic flea (to limit CO_2 in-gassing). The titrating solution was added in increments (1 mL) and allowed to equilibrate (4 h).

2.3. Reduction microcosm experiments

A *pH amended* microcosm series was established in triplicate with corresponding sterile controls. Each microcosm consisted of 10 g of homogenised B2-310 soil and 100 mL of COPR water in a 120 mL glass serum bottle. After sealing these were buffered to pH 9 using 1 M $NaHCO_3$ solution, and the headspace purged with N_2. Sterile controls were prepared by autoclaving soil with a N_2-purged headspace (120 °C, 20 min) 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 3 mL soil slurry extracted. Samples were centrifuged (3 min, 16,000*g*) 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.

2.4. Geochemical methods

Eh and pH were measured using an Orion meter (pH calibrated at 7 and 10). Nitrate concentrations were determined by ion

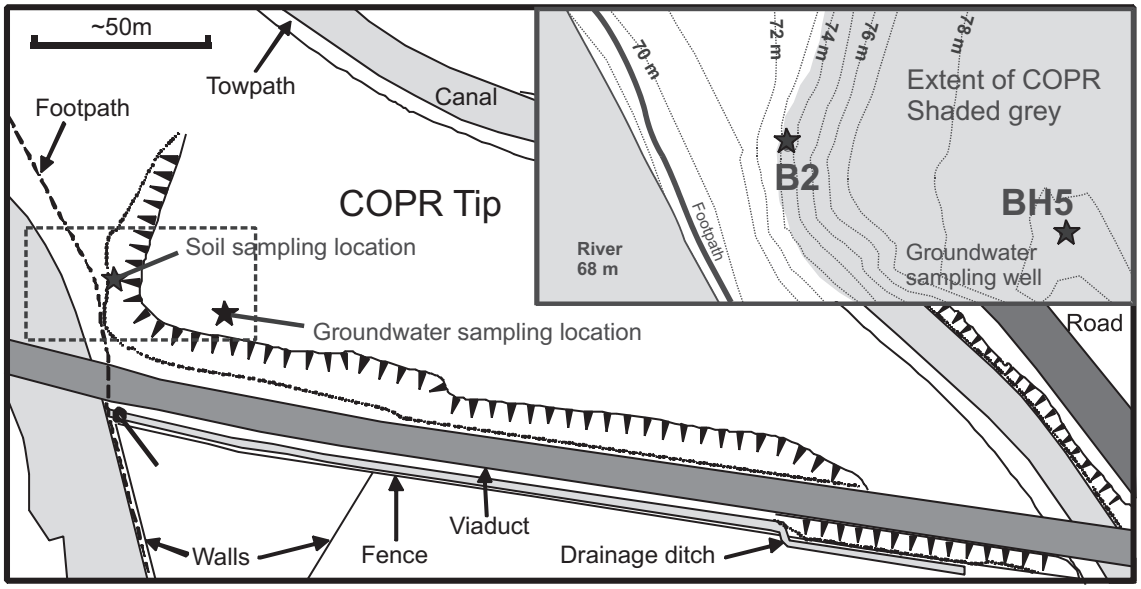

Fig. 1. Sketch map of the site showing soil (B2) and water (BH5) sampling locations. Insert corresponds to dashed selection.

chromatography on a Dionex DX-600 with an AS50 autosampler using a 2 mm AS16 analytical column, with suppressed conductivity detection and gradient elution to 15 mM KOH over 10 min. Samples were loaded in a random order to avoid systematic errors, and the column was flushed between samples with deionised water for 1.5 min. UV/VIS spectroscopy methods were used to determine aqueous Cr(VI) concentrations based on reaction with diphenycarbazide (USEPA, 1992) and aqueous NO_2^- concentrations following reaction with sulphanilaminde (SAN) and naphthylethylenediamine dihydrochloride (Harris and Mortimer, 2002). Iron was extracted from solids using 0.5 N HCl (a proxy for microbial available Fe, Weber et al., 2001; Burke et al., 2006), 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 and calibration standards were used regularly.

2.5. 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 $CrCl_3$ solution using 10 M NaOH (Saraswat and Vajpei, 1984). XANES spectra were summed and normalised using Athena v0.8.056 (see S.I. for details).

2.6. 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 *Escherichia 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 characterise 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).

2.7. 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 visualise 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.

3. Results

3.1. Sample recovery

Borehole B2 (Fig. 1) was located near the south-western edge of the COPR tip. It encountered a topsoil layer then entered COPR waste at 190 cm, grey sandy clay at 310 cm, and terminated in a gravel layer at 365 cm. 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 yr ago (Stewart et al., 2010).

3.2. 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 Fe was present as Fe(II). The major minerals present were quartz, kaolinite and albite. The Cr concentration was approximately 3400 mg kg^{-1}, which Cr K-edge XAS analysis showed was predominately present in the 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 0.5 N HCl acid-extractable Fe in the Fe(II) oxidation state can vary at this scale from 5% to 90%.

The water from within the COPR collected from BH5 (Fig. 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 mol H^+ per kg of soil (Fig. 2). The addition of $NaHCO_3^-$ produced a slower decline in pH, with 0.6 mol HCO_3^- 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 mol HCO_3^- per kg of soil.

3.3. 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 Fig. 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

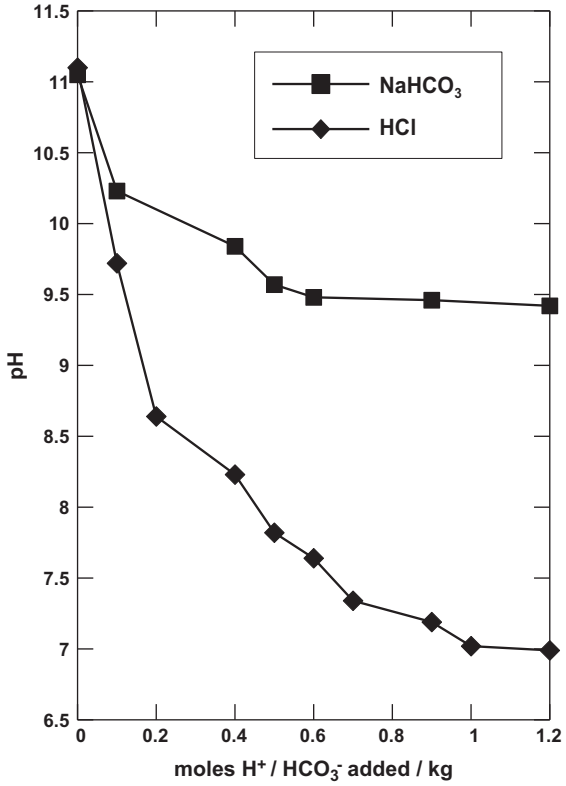

Fig. 2. Titration curves obtained from the acid neutralisation test on freeze dried soil B2-310 $n = 1$. ♦ = addition of HCl, ■ = HCO_3 addition.

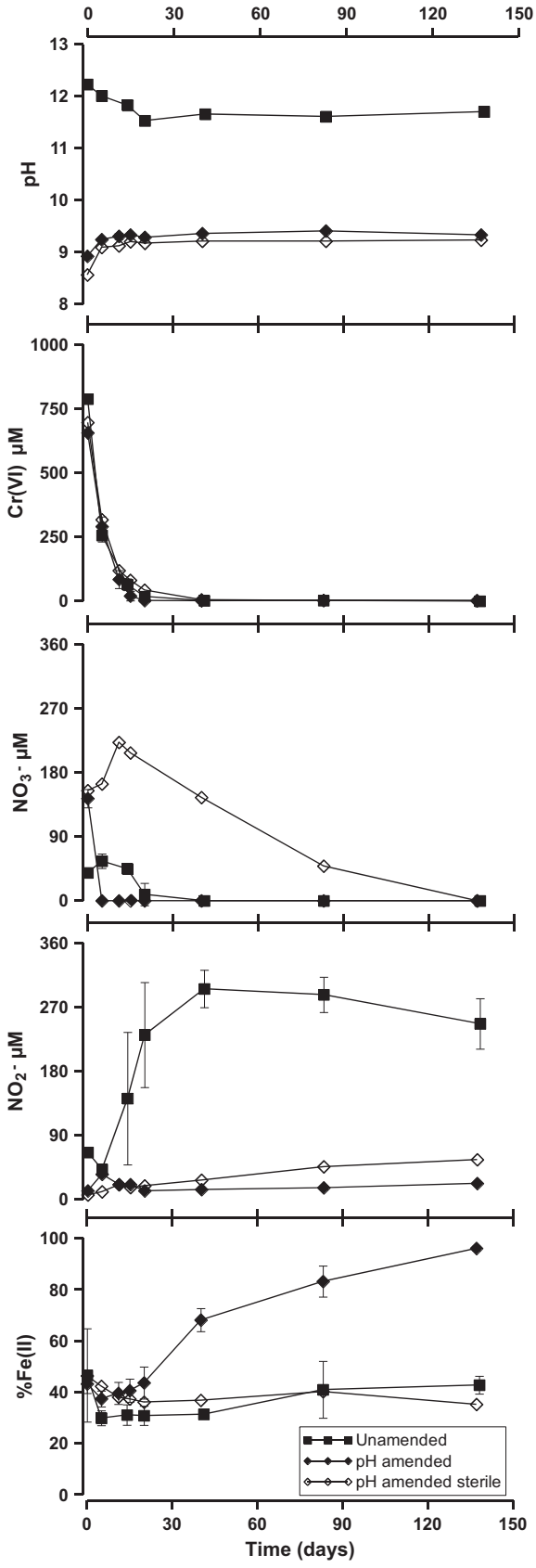

Fig. 3. Geochemical response of the *pH amended* (♦ $n = 3$), *unamended* control (■ $n = 3$) and sterile control (◇ $n = 1$) microcosms. Top to bottom; pH; pore water Cr(VI) concentration; pore water NO_3^- concentration; pore water NO_2^- concentration; % of 0.5 N HCl extractable Fe as Fe(II) in soils. Error bars shown are one standard deviation from the mean of triplicate experiments.

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 h) 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 NO_3^- 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 (NO_3^- was not detected in either backup control when they were sampled on day 137). Aqueous NO_2^- concentration in *pH amended* microcosms increased slightly over the first 5 days, before decreasing over the next 15 days. In contrast, NO_2^- 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 ± 2 → 296 ± 26 μmol L^{-1} by day 41 before decreasing steadily over the remainder of the test.

The percentage of 0.5 N HCl-extractable Fe 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 Fe present as Fe(II) increased steadily from 43% → 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.

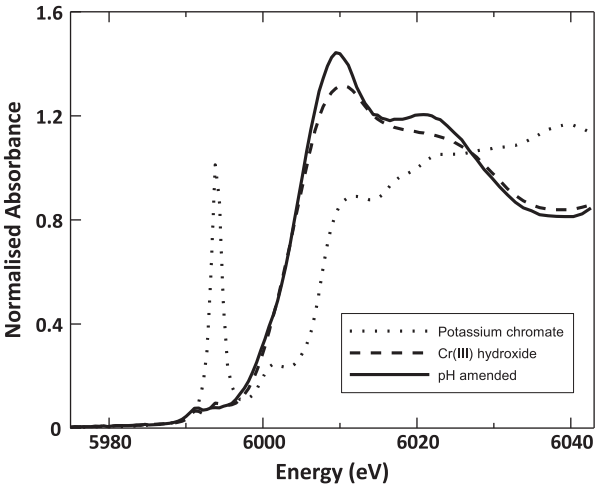

Fig. 4. Normalised chromium K XANES spectra for soil from the pH 9 amended microcosms on day 153 and for the standards.

3.4. X-ray absorption spectroscopy

The Cr XANES spectra from soil recovered from the pH 9 amended microcosms on day 153 (Fig. 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).

3.5. Microbial community analysis

A total of 62 rRNA gene sequences were obtained from B2-310. These were assigned to nine 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–S3 for full assignments).

Rarefaction analysis of the three populations (S.I. Fig. 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 (Fig. 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

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

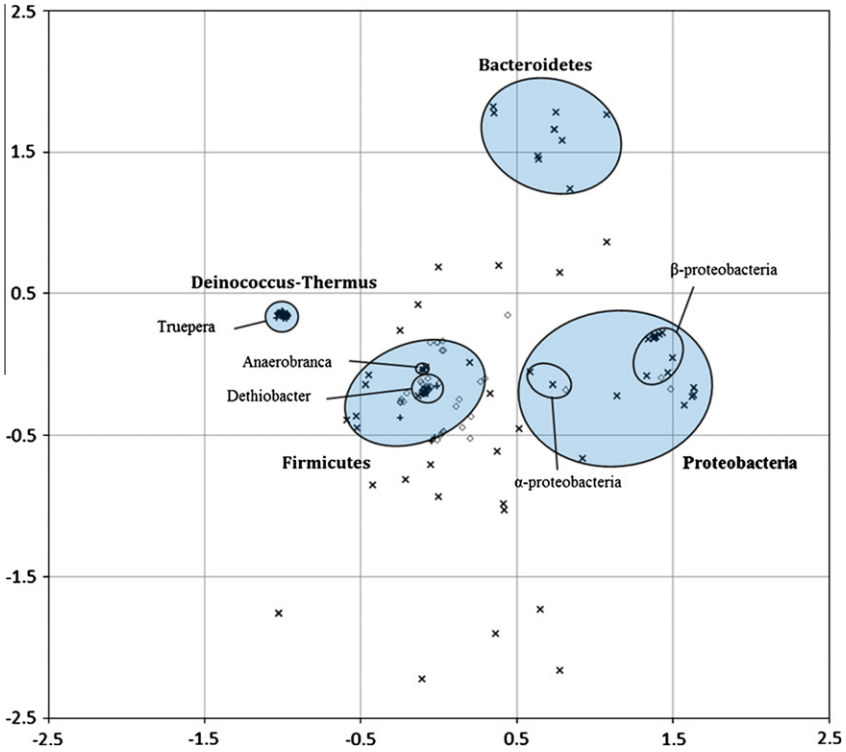

Fig. 5. Two-dimensional configuration from the MDS analysis of the pair-wise sequence dissimilarity scores: (×) B2-310 population, (◇) *pH amended* microcosm, (+) *unamended* microcosm (distance scale within this Euclidean space is an arbitrary function of dissimilarity). The "stress" (a measure of lack 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 dissimilaries in the data.

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 Fig. 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.

4. 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. Chromium(VI) bearing leachate from the COPR waste has probably been entering this soil for over 100 yr (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. Chromium(VI) readily reacts abiotically with Fe(II) to produce Cr(III) and Fe(III) (Lin, 2002). Therefore, the

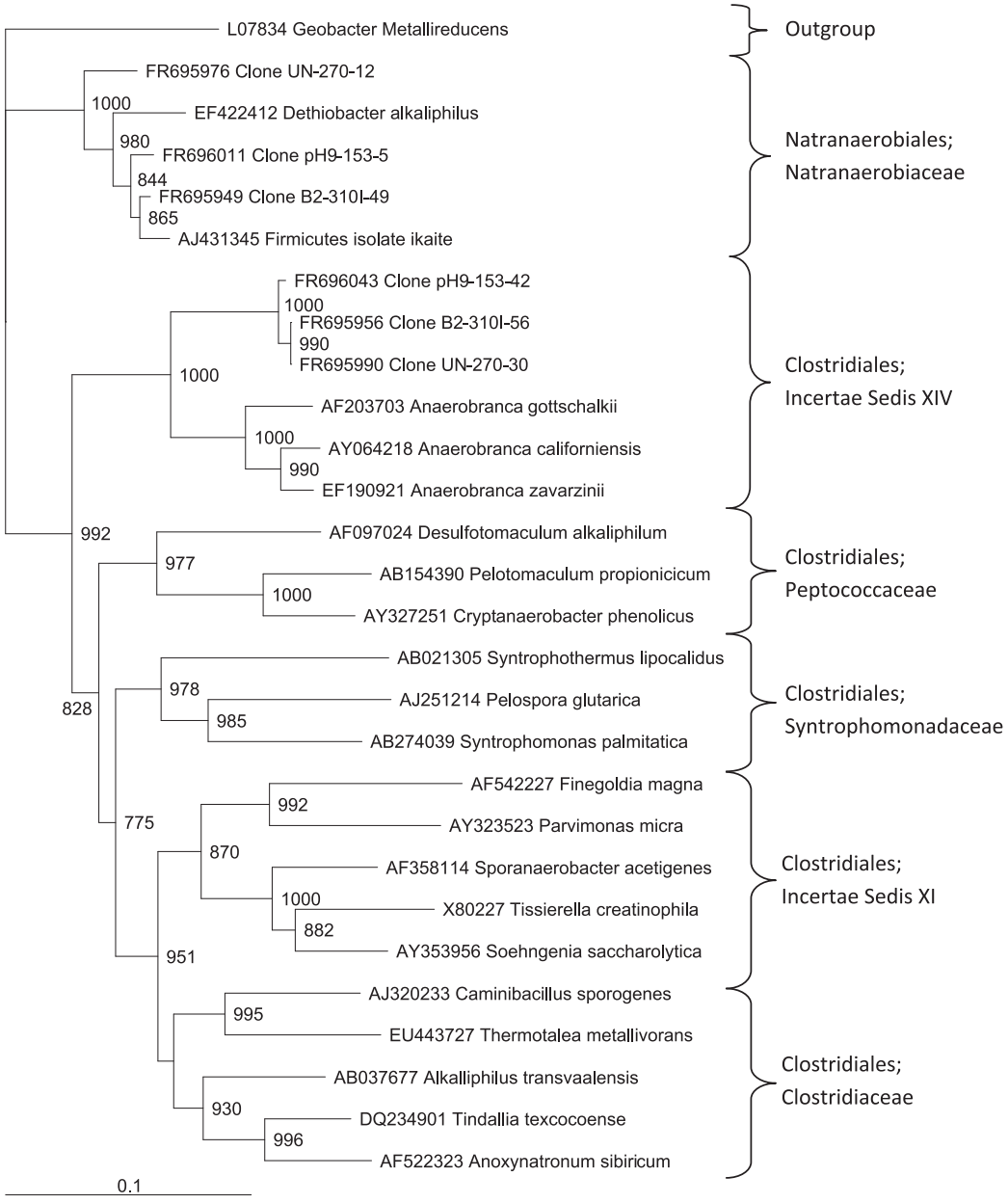

Fig. 6. Phylogenetic tree showing the relationship between representative sequences from major OTU's recovered from each population and other members of the orders Clostridiales and Natranaerobiales of Firmicutes. *Geobacter metallireducens* (δ-proteobacteria) is included as an out-group. The scale bar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

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 Fe was deemed present as Fe(II)). XANES spectra collected from soil from a pH-amended microcosm after Cr(VI) removal confirmed that soil-associated Cr was predominately present in the Cr(III) oxidation state. A small decrease in the initial percentage of acid-extractable Fe 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 NO_3^- 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 NO_2^- concentration increased slightly during NO_3^- removal but decreased again once the NO_3^- was gone. The removal of NO_3^- was then followed rapidly by an increase in the proportion of acid-extractable Fe present as Fe(II). The rate of NO_3^- removal in the active microcosms (in comparison with the control) and subsequent depletion of NO_3^- and Fe(III) suggests that the reactions are microbially mediated and part of a cascade of terminal electron-accepting processes. The gradual removal of NO_3^- in the *pH-amended* sterile control was most likely an abiotic process as NO_3^- was also removed from both backup controls (i.e. if NO_3^- 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 NO_3^- by Fe^{2+}(aq) is thermodynamically very favourable (even at high pH), but a direct reaction between these ions in solution is kinetically inhibited (Hansen et al., 1996; Ottley et al., 1997; Choe et al., 2004).

However, the reaction can be surface catalysed by transition metals and freshly formed Fe(III) precipitates (Postma, 1990; Ottley et al., 1997; Fanning, 2000). Iron(II) oxyhydroxides are still sparingly soluble at around pH9, where Fe^{2+} is the main aqueous species (Langmuir, 1997). It, therefore, seems reasonable that NO_3^- reduction by Fe^{2+}(aq) in a surface catalysed reaction was the mechanism by which NO_3^- 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 NO_2^- concentration. Subsequently the NO_2^- concentration decreased steadily over the remainder of the experiment, suggesting that NO_2^- reduction was following-on from NO_3^- reduction. 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., 2011a), and in such systems it has been observed that NO_3^- reduction to N_2 tends to lag behind NO_3^- reduction to NO_2^- (Glass and Silverstein, 1998). Furthermore, the abiotic reduction of NO_3^- 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 NO_3^- and NO_3^- 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 Fe 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 Fe reduction was the predominant biogeochemical process. Amendment of the pH to ~9 and the subsequent development of Fe-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 S cycle, and is capable of reducing elemental S (Sorokin et al., 2008), but to date no capacity for Fe 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 active 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 Fig. 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 (Prowe and Antranikian, 2001; Gorlenko et al., 2004; Kevbrin et al., 2008). Similarly *A. horikoshii* is an alkali-tolerant thermophilic anaerobe (Engle et al., 1995). *A. californiensis*, *A. gottschalkii* and *A. horikoshii* are able to reduce Fe and S 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 NO_2^- 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. Fig. S2) confirms this classification. The only *Truepera* species 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 C 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 C). 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 H_2 (Lovley, 1993a; Leschine, 1995). As processes such as dissimilative NO_3^- and Fe reduction require labile organic C (Gottschalk, 1986; Kim and Gadd, 2008) the soil must contain a 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 NO_3 reduction after 270 days may be due in part to loss of microbial diversity impacting the community's ability to breakdown soil organic matter.

5. Environmental implications

Soil recovered from beneath the COPR at this study site contained Fe in the Fe(II) oxidation state that was available for Cr(VI) reduction. The Fe(II) present 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 Fe 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 $NaHCO_3$ promoted microbial NO_3^- and Fe(III) 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 (Fig. 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 CO_3^{2-}/HCO_3^- buffered system will be better for bacterial growth.

6. 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 NO_3^- and Fe during the progression of microbial anoxia. Addition of $NaHCO_3$ 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 Fe 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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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apgeochem.2012.10.003.

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