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Effect of Microbially Induced Anoxia on Cr(VI) Mobility at a Site Contaminated with Hyperalkaline Residue from Chromite Ore Processing

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Abstract

This paper reports an investigation of microbially mediated Cr(VI) reduction in a hyper alkaline, chromium contaminated soil-water system representative of the conditions at a chromite ore processing residue (COPR) site. Soil from the former surface layer that has been buried beneath a COPR tip for over 100 years was shown to have an active microbial population despite a pH value of 10.5. This microbial population was able to reduce nitrate using an electron donor(s) that was probably derived from the soil organic matter. With the addition of acetate, nitrate reduction was followed in turn by removal of aqueous Cr(VI) from solution, and then iron reduction. Removal of ~300μM aqueous Cr(VI) from solution was microbially mediated, probably by reductive precipitation, and occured over a few months. Thus, in soil that has had time to acclimatize to the prevailing pH value and Cr(VI) concentration, microbially mediated Cr(VI) reduction can be stimulated at a pH of 10.5 on a time scale compatible with engineering intervention at COPR contaminated sites.

Keywords: anaerobe, alkaliphile, bacteria, chromate, contaminated land, COPR, iron-reduction, microbial-reduction

INTRODUCTION

Chromium is among the most extensively used transition metals in the chemical and metal alloy industries, including leather tanning, wood preservation, chrome metal finishing, and manufacture of dyes, paints, pigments, and stainless steel (Wang 2000; Morales-Barrera and Cristiani-Urbina 2008). In order to obtain chromium from chromite ($FeCr_2O_4$), the ore is roasted with an alkali-carbonate at 1150ºC, to oxidise the relatively insoluble Cr(III) to soluble chromate (Cr(VI)), which is then extracted with water as sodium chromate upon cooling. Lime (CaO) was traditionally added as a diluent to increase air penetration and provide sufficient O_2 for chromite oxidation in a practice known as the "high-lime" process (Farmer et al. 1999). Lime was replaced by cheaper alternatives of limestone ($CaCO_3$) and

dolomite ($CaMg(CO_3)_2$) around the turn of the 20th century, and this variant of the high-lime process remained the dominant method of chromium extraction until the early 1960's when it was superseded by lime free processes (Darrie 2001).

Economically developed nations no longer use the high-lime process to extract chromium, but until recently it still accounted for 40% of chromium production worldwide (Darrie 2001). Due to its inefficient use of raw materials the high-lime process produces up to 4 tonnes of waste per ton of product (Walawska and Kowalski 2000). Thus it is still responsible for producing large quantities of chromium containing waste (600,000 $t.yr^{-1}$ in 2001; Darrie 2001). This waste, known as chromite ore processing residue (COPR), is highly alkaline due to the calcium hydroxide (CaOH) produced from the limestone, and typically contains between 2-8% chromium (w/w) (Walawska and Kowalski 2000; Sreeram and Ramasami 2001; Geelhoed et al. 2003; Tinjum et al. 2008). Of this, up to 35% can be in the form of the toxic, carcinogenic and environmentally mobile chromate anion (CrO_4^{2-}) (James 1994; Farmer et al. 2006; Tinjum et al. 2008). As a result, water in contact with COPR has a characteristically high pH of 9-12 (Geelhoed et al. 2003; Stewart et al. 2007), and can contain up to 1.6 mM Cr(VI) as chromate (Farmer et al. 2002).

Remediation of legacy sites contaminated with COPR is challenging, particularly because these site are often in urban areas and date from times when COPR disposal was quite poorly managed (Stewart et al. 2007). Traditional "dig and dump" remediation strategies are not only financially costly due to the large volumes of waste involved, but also inadvisable due to the risk of forming Cr(VI) bearing dusts during large scale manipulation. Such an approach would create a pathway to human exposure, as Cr(VI) bearing dust is a confirmed human carcinogen through inhalation (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, required for fat and glucose metabolism, amino and nucleic acid synthesis, and correct insulin function (Richard and Bourg 1991; Pechova and Pavlata 2007). Also the Cr(III) cation is much less mobile in the subsurface environment than the CrO_4^{2-} anion as it 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; Lee et al. 2003; Han et al. 2006; Fonseca 2009). Thus the reduction of Cr(VI) to Cr(III) in-situ would significantly reduce the hazard posed by chromium contaminated groundwater.

The ability of indigenous soil microorganisms to couple organic matter oxidation to the reduction of transition metals, such as iron and manganese, during dissimilatory metabolism is well documented (Lovley 1993a). Where sufficient organic matter is available for

oxidation, progressively more anoxic conditions develop and a cascade of terminal-electron-accepting processes (TEAPs) occur in sequence (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).

Iron is by far the most abundant redox-active metal in the soils, and cycling between Fe(II) and Fe(III) is a prominent factor affecting other chemical processes in soils (Stucki et al. 2007). Fe(III) is relatively insoluble except in acidic solutions and precipitation usually proceeds via intermediates, $Fe_2(OH)_2^{4+}$ and ferrihydrite, which are metastable with regard to goethite (α-FeO(OH) and hematite (α-Fe_2O_3); goethite being favoured in alkaline conditions (Schwertmann et al. 1999; Cudennec and Lecerf 2006). Aqueous Fe(II) is stable in acidic and neutral conditions, but can precipitate as siderite ($FeCO_3$) if carbonate is present and will precipitate as $Fe(OH)_2$ at high pH values (Langmuir 1997). Fe(II)/Fe(III) cycling occurs naturally in soils particularly where there are periodic changes in water content (Stucki et al. 2007), and iron cycling can be important where there is a redox active contaminant flux (Lovley 1993a). For example Cr(VI) is readily reduced to Cr(III) by Fe(II) oxidation to Fe(III) (Richard and Bourg 1991). As Cr(III) can substitute for Fe(III) in many iron minerals, any Cr(VI) that is reduced by Fe(II) is likely to be incorporated into iron(III) oxyhydroxides (Fendorf 1995). Such metastable iron oxyhydroxides exhibit high bioavailability (Hansel et al. 2005) and thus Fe(II)/Fe(III) cycling continues.

A broad range of microbial taxa can grow optimally and robustly in high pH environments like those found at COPR disposal sites (Roadcap et al. 2006). 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). Similarly many microorganisms have demonstrated tolerance to Cr(VI) including *Pannonibacter phragmitetus*, which showed no evidence of cell degradation at 500 mg l^{-1} Cr(VI) (Chen and Hao 1998; Rehman et al. 2008; Zhu et al. 2008; Chai et al. 2009). As Cr(VI) is readily able to cross cell membranes by utilising the sulphate transport system (Cervantes et al. 2001), tolerance to Cr(VI) may indicate an evolutionary response to Cr(VI) toxicity.

Microbial reduction of Cr(VI) was first observed with *Pseudomonas dechromaticans* (Romanenko and Koren'kov 1977), and has since been reported in a number of Gram negative

genera including *Pseudomonas*, *Desulfovibro* and *Shewanella*, and members of the Gram positive *Bacillus* and *Cellulomonas* (Lovley 1993b; Francis et al. 2000; Sani et al. 2002; Sau et al. 2008). Direct microbial Cr(VI) reduction has been observed during aerobic (Bopp and Ehrlich 1988; Ishibashi et al. 1990) and anaerobic respiration (Suzuki et al. 1992; Neal et al. 2002; Daulton et al. 2007), but only a few studies have clearly demonstrated anaerobic growth dependent solely on the use of Cr(VI) as an electron acceptor (e.g. *Pantoea agglomerans*, Francis et al. 2000). Even fewer studies have demonstrated direct microbial Cr(VI) reduction at high pH (although notable examples are reported by VanEngelen et al. 2008; Zhu et al. 2008; Chai et al. 2009). 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, e.g. Fe(II) produced by respiration (Lloyd et al. 1998).

This paper reports an investigation of microbially mediated Cr(VI) reduction in hyper alkaline soils (pH >10) from a COPR contaminated site in the north of England. It uses a multidisciplinary approach to gain an understanding into microbially induced anoxia at high pH, the microbial communities that develop, and their influence on Cr(VI) geochemistry in closed systems.

MATERIALS AND METHODS

Site Description. The study site is in a glacial valley in-filled with alluvial deposits, which is located in the north of England (see Figure 1). COPR waste has been tipped against the valley side between a canal and a river (the canal follows the valley side and locally is ~7 metres above the level of the river). The waste tip is approximately 2.2 hectares in area with a relatively flat top surface ~1.5m above the canal bank and steep side-slopes down to the valley floor (Whittleston et al. 2007; Stewart et al. 2009). This landform first appeared on historical maps in the late 19th century. Currently there is thin soil cover on the waste with vegetation dominated by grasses and occasional small trees; however erosion has left the waste exposed on steeper slopes. A drainage ditch along the southern waste boundary frequently contains water that is alkaline, visibly yellow in colour, and has elevated Cr(VI) concentrations.

Site Sampling. Several exploratory boreholes were advanced in March 2007 using cable percussion drilling. The soil sample used in this study was taken from ~1m below the waste, at a depth 7 metres below ground level (the borehole location is shown in Figure 1). It consisted of grey silty clay that is representative of the alluvial soils that would have been the

surface layer prior to waste tipping. A water sample was collected from the leachate drainage ditch in February 2008 from a location close to where it enters the river. Samples were placed in sealed polythene containers at time of sampling and were stored at 4ºC in the dark within 4 hours of collection. Sample manipulations were kept to a minimum until they were required for experiments in March 2008. The soil sample was homogenised prior to use.

Sample Characterisation. X-ray powder diffraction (XRD) analysis of the alluvial soil (ground to < 75 μm) was performed on a Philips PW1050 Goniometer, and X-ray fluorescence (XRF) analysis was undertaken using a fused sample on a Philips PW2404 wavelength dispersive sequential X-ray spectrometer (data were corrected for loss on ignition). Approximately 25g of homogenised soil was oven dried at 105°C and disaggregated with a mortar and pestle for carbon content determination. A portion of each sample was pre-treated with 10% HCl to remove any carbonates present (Schumacher 2002). The total organic and inorganic carbon content of oven dried and HCl treated subsamples was measured using Carlo-Erba 1106 elemental analyser.

Reduction Microcosm Experiments. Microcosms were prepared using 10g of homogenised soil and 100 ml of ditch water in 120 ml glass serum bottles and sealed with butyl rubber stoppers and aluminium crimps. After sealing, the headspace was purged with nitrogen to displace oxygen present. Two different experimental conditions were established in triplicate microcosms. *Unamended* microcosms contained only the soil and ditch water. *Acetate amended* microcosms also contained sodium acetate to a final concentration of 20 mM. A control was prepared for each experiment in which the soil, sealed in the bottle with a nitrogen purged headspace, was heat sterilised at 120ºC for 20 minutes in an autoclave before filter sterilized ditch water and, in one case, sodium acetate were added.

The microcosms and controls were periodically sub-sampled for geochemical and microbiological analysis to produce a progressive time series. At each sample point, microcosms were shaken and 3 ml soil slurry extracted using aseptic technique with sterile syringes and needles (Burke et al. 2006). Samples were centrifuged (5 min, 16,000g) and soil and water were analysed for a range of redox indicators, Cr(VI) and microbiology.

Geochemical Methods. The pH was measured using an Orion bench top meter and calibrated electrodes (the pH electrode was calibrated between 4 and 10 using standard buffer solutions). The soil pH was measured using a 1:1 suspension in deionised water (ASTM 2006). Sulfate, nitrate and chloride 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. Standards covering the anticipated range of analyte concentrations were prepared with the addition of 25 μM Cr(VI) as potassium chromate. Between loading samples, the column was flushed with deionised water for 1.5 minutes.

Standard UV/VIS spectroscopy methods based on reactions with diphenycarbazide and ferrozine were used to determine aqueous Cr(VI) and Fe concentrations, respectively, using a Cecil CE3021 UV/VIS Spectrophotometer (USEPA 1992; Viollier et al. 2000). 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. Soil samples from the triplicate microcosms at a single time point (day 68) were combined (typically ~0.25 g of soil) and microbial DNA was extracted using a FastDNA spin kit (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´) where K = G or T, W = A or T (Lane et al. 1985). Each PCR reaction mixture contained 20 μl of purified DNA solution, GoTaq DNA polymerase (5 units), 1× PCR reaction buffer, $MgCl_2$ (1.5mM), PCR nucleotide mix (0.2 mM), T4 Gene 32 Protein (100 ng/μl) and 8f and 519r primers (0.6 μM each) in a final volume of 50 μl. The reaction mixtures were incubated at 95ºC for 2 min, and then cycled 30 times through three steps: denaturing (95ºC, 30 s), annealing (50ºC, 30s), primer extension (72ºC, 45 s). This was followed by a final extension step at 72ºC for 7min. The PCR products were purified using a QIAquick PCR Purification Kit. Amplification product sizes were 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 surfaced dressed with IPTG and X-gal (as per Stratagene

protocol) for blue-white colour screening. For each sample, 48 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 a QIAprep Spin miniprep kit (QIAGEN Ltd., UK) or PureYield Plasmid Miniprep System (Promega, UK) and sent for automated DNA sequencing on an ABI 3100*xl* Capillary Sequencer using the T7P primer. Sequences were analysed against the EMBL release nucleotide database in April 2009 using the NCBI-BLAST2 program (version 2.2.19 November 2009) and matched to known 16S rRNA gene sequences. Default BLAST parameters were used (match/mismatch scores 2, -3, open gap penalty 5, gap extension penalty 2). The nucleotide sequences described in this study were deposited in the GenBank database (accession numbers FN706451 - FN706510).

Phylogenetic Tree Building. The EMBOSS needle pairwise alignment program was used to assign similar gene sequences into clades based on sequence homology, using default parameters (open gap penalty 10, gap extension penalty 0.5). Selected sequences were then aligned with known bacterial 16S rRNA gene sequences from the EMBL database using the ClustalX software package (version 2.0.11), and a 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 Characterisation.

XRD and XRF analysis of the alluvial soil showed that the major mineral was quartz with small amounts of kaolinite and muscovite. The XRF analysis indicated a concentration of chromium of 3020 $mg.kg^{-1}$ in the solid phase (see Table 2). The soil had a pH of 10.5. The total organic carbon (TOC) and total inorganic carbon (TIC) of the soil were found to be 3.6 and 0.2%, respectively. Water from the ditch along the southern edge of the waste had a pH of 11.4, and a Cr(VI), nitrate and sulphate concentrations of 293 μM (15.2 $mg.l^{-1}$), 163 μM (10.1 $mg.l^{-1}$), and 3.29 mM (316 $mg.l^{-1}$), respectively.

Reduction Microcosm Experiments

The initial pH values of the *unamended* and *acetate-amended* microcosms were both 10.9, whereas the pH values of sterile controls were both 10.8. The active microcosm experiments had an initial aqueous Cr(VI), nitrate and sulphate concentration of 279 ± 2 μM, 96.5 ± 4.87

μM, and 3.01 ± 0.10 mM, respectively (see Figure 2). The sterile controls had slightly higher initial aqueous Cr(VI) and sulphate concentrations of 287 ± 4 μM and 3.26 ± 0.06 mM, respectively. The *acetate-amended* sterile control had an initial nitrate concentration of 89.7 ± 9 μM. The nitrate concentration of the *unamended* sterile control was not measured due to technical difficulties (there was insufficient sample to repeat measurement). Initially the percentage of the total 0.5 N HCl extractable iron present as Fe(II) was 13.7 ± 1.1% in the active experiments whereas it was 10.0 ± 0.6% the heat treated controls.

In the microbially active *unamended* microcosms the pH of the active microcosms decreased from 10.9 to 9.9 over 175 days of incubation, whereas the pH of the sterile control decreased from 10.8 to 10.4 in the same time period (see Figure 2). Nitrate removal from aqueous solution commenced shortly after the start of the test, with the concentration dropping by two-thirds by day 5, and was not detected on day 15. Over the test period, we noted very little change in aqueous Cr(VI) concentration in either the active *unamended* or the control microcosms. Similarly we noted little change in the amount of total Fe(II) extractable by 0.5 N HCl and no discernable change in aqueous sulphate concentration in either the active or the control microcosms (see Figure 2).

In the active *acetate-amended* microcosms the pH decreased from an initial value of 10.9 to a value of 10.1 on day 175, whereas the pH value of the sterile control decreased from 10.8 to 10.5. The trend in nitrate data was similar to that in the *unamended* microcosms, with nitrate removal commencing shortly after the start of the test and becoming undetectable by day 5. No nitrate removal was observed in the corresponding sterile control. The aqueous Cr(VI) concentration decreased in all three replicates once nitrate was below detection limits, but at different rates. In replicate II in which the aqueous chromate concentration decreased most rapidly, Cr(VI) was not detected on day 118. In replicate III where aqueous chromate concentration decreased least rapidly the concentration on day 175 was two-thirds of the initial value. No change in Cr(VI) concentration occurred in the corresponding controls. The trends in the proportion of the acid extractable iron present as Fe(II) also differed between the three replicates. In the early stages of all three *acetate-amended* microcosms about 20% of the 0.5 N HCl extractable iron was in the Fe(II) oxidation state, and this did not change significantly with time in replicates I and III. However in replicate II there was a significant increase in the proportion of the acid extractable iron present as Fe(II) shortly after Cr(VI) was completely removed from solution. In the *acetate-amended* sterile control (like the *unamended* sterile control), roughly 10% of the 0.5 N HCl extractable iron was present as Fe(II), which did not change with time. There was no discernable change in the aqueous

sulphate concentration in either the active *acetate-amended* microcosms or corresponding control.

Microbiological Community Analysis.

Geochemical analysis indicated aqueous chromate removal was underway by day 68 in the *acetate amended* microcosms, but the behaviour of the three replicate microcosms had not diverged significantly. Therefore, on day 68, we pooled soil from each of the three *unamended* microcosms into one combined sample and from each of the three *acetate-amended* microcosms into a second combined sample. These two combined samples were then used to compare the microbial communities in each with respect to chromate removal from the liquid phase. Thirty 16S rRNA gene sequences recovered from each of the two samples were analyzed.

Initially sequences were assigned to a phylum (or class in the case of proteobacteria) using the NCBI-BLAST2 program, based on >95% identity over a sequence length of >400 bp to a known sequence in the EMBL release database. However, less than a third of sequences could be assigned in this way, although many sequences were more than >95% homologous to sequences from unidentified bacteria recovered from alkaline environments. Sequences that were >95% homologous to the same sequence in the database were further analysed using EMBOSS and grouped based on >98% mutual homology. ClustalX analysis and neighbour joining tree construction of these groups indicated there were four distinct clades amongst the initially unidentified sequences, subsequently called clades A, B, C and E. Further ClustalX analysis and NJ tree construction using characteristic members of each clade was used to assign clade members to a phylum. Members of clade A have been thus assigned to the Comamonadaceae family of β-proteobacteria that appear to be most closely related to the genera Rhodoferax, Hydrogenophaga and Malikia (Figure 3). Clade B were members of the Flexibacteraceae family of Bacteroidetes that appeared to be most closely related to the genera Aquiflexum (Figure 4). Clade C were members of the Xanthomonadaceae family of γ-proteobacteria that appeared to be most closely related to the genera Lysobacter (Figure 5). Clade E were members of the Sphingomonadaceae family of α-proteobacteria that appeared to be most closely related to the genera Sphingomonas (Figure 6).

Of the thirty clones isolated from the *unamended* microcosms on day 68, 14 sequences (46%) were β-proteobacteria including 11 (36%) from clade A, 5 (17%) were α-proteobacteria including 4 (13%) from clade E, 5 (17%) were bacteroidetes including 3 (10%)

from clade B, 4 (13%) were γ-proteobacteria all from clade C (see Figure 7a). Thus β-proteobacteria are an important component of the bacterial population of the *unamended* microcosms on day 68 and, overall, two-thirds of sequences isolated were from one of four bacterial clades.

Of the thirty clones isolated from the *acetate-amended* microcosms on day 68, 28 (93%) were β-proteobacteria including 23 sequences (77%) from clade A, the remaining 2 sequences (7%) being unidentified (see Figure 7b). Thus β-proteobacteria, particularly those from clade A, dominated the bacterial population of the *acetate-amended* microcosms on day 68.

DISCUSSION

Currently there is a perched water table in the waste pile and downward seepage through the clayey former surface layer into the underlying alluvium where the water table is controlled by the river. Observation of the site over a period of four years suggests these conditions are typical of the site (Studds *pers. comm.*). Thus, alkaline, highly oxidising and oxygenated Cr(VI) containing leachate from the tip has been percolating through, and interacting with the former surface soil for over 100 years. Therefore any microorganisms in this soil have had a long time to adapt to the local geochemical environment.

As the leachate from the waste pile seeps downwards it will undergo geochemical changes as it interacts with both the soil and its microbial population. Flow through natural soils can vary greatly spatially and tends to follow preferential paths. These temporal and spatial variations in the flow, and the development of increasingly more reducing conditions down the flow-path, have resulted in the highly variable redox conditions in the clayey former surface soil beneath the waste. For example the percentage of 0.5 N HCl extractable Fe(II) in soils found beneath the waste ranges from less than 5% Fe(II) to more than 90% Fe(II) on a centimetre scale (Tilt 2009). This distribution probably reflects the balance between the rate of ingress of initially highly oxidising oxygenated Cr(VI)-containing groundwater, and the rate of in situ bioreduction at particular locations.

The initial solution composition in the microcosm experiments reflects the composition of the ditch water, which is a reasonable proxy for the waste leachate (leachate emerging from the waste pile is the main flux into the ditch). The percentage of 0.5 N HCl extractable solid phase iron as Fe(II) determined when microcosms were established was between 10-20 % Fe(II). Thus the initial conditions in the microcosms are representative of the more oxidised end of the range of conditions observed in-situ. Such starting material is likely to have low

numbers of obligate anaerobes, whose presence would be anticipated in the more reducing soils on site.

The initial behaviour of the two microcosm systems (*unamended* and *acetate-amended*) was similar. Nitrate was removed rapidly from solution (this occurred slightly more quickly in the *acetate-amended* system but the difference was small). There was no noticeable change in the nitrate concentration in *acetate-amended* sterile control. Thus it is inferred that nitrate removal from the active microcosms is likely to have been microbially mediated, and probably due to the action of nitrate reducing bacteria. It used to be a widely held belief that microbial nitrate reduction is optimal at pH 7 – 8 (Knowles 1982; Wang et al. 1995). However there is now ample evidence that microbial nitrate reduction can occur at high pH when the microbial community has adapted to the ambient pH (Glass and Silverstein 1998; Dhamole et al. 2008). Indeed the rate of nitrate reduction to nitrite can increase with increasing pH, although the time taken for complete denitrification at high pH tends to be unaffected as nitrite reduction to N_2 tends to lag behind nitrate reduction to nitrite in alkaline systems (Glass and Silverstein 1998). Also, as Fe(II) is present in these soils it is possible that nitrate dependant Fe(II) oxidation (Straub et al. 1996) processes may have contributed to nitrate reduction in these experiments, however, we did not observe a reduction in % Fe(II) concurrent with nitrate reduction, therefore, is not possible to report whether such reduction processes occurred in these experiments.

Once the nitrate was removed from solution the *unamended* system exhibited little further geochemical change. It is unclear from these experiments whether these microcosms had reached a long-term steady-state, or whether further microbially mediated reactions were merely slow in the absence of a readily metabolizable electron donor.

It is likely that bacterial reduction of nitrate in the *unamended* system was coupled to oxidation of soil organic matter (the soil contained 3.6% organic carbon and no exogenic carbon source was added). 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). The soil used in the microcosm experiments was covered with COPR waste for over 100 years, and thus it is likely that the labile organic components present prior to burial was already consumed by microorganisms, leaving behind the less labile components such as lignin and cellulose. Anaerobic respiration cannot be supported directly by these polymeric substrates (Kim and Gadd 2008), so nitrate reduction in the microcosm experiments suggests that the microbial diversity reported in

Figure 7a represents a community capable of the complete oxidation of complex organic matter. Thus it is likely that further microbially mediated geochemical reactions would have eventually followed in time, as they did in the *acetate-amended* microcosms.

In *acetate-amended* system Cr(VI) removal followed nitrate removal, however the three replicates responded at different rates and Cr(VI) removal only reached completion in one microcosm (replicate II). In this replicate there was a significant increase in the proportion of the acid extractable iron present as Fe(II) once Cr(VI) had been removed from solution. In the other two replicates the proportion of the acid extractable iron present as Fe(II) was generally higher than in the *unamended* microcosms or the sterile control, but there was no noticeable increasing trend with time. Comparison with the sterile control, which showed no major change in Cr(VI) concentration with time, suggests that Cr(VI) removal from solution is microbially mediated. Because iron reduction began after Cr(VI) removal ceased, the overall response of the *acetate-amended* system was indicative of a cascade of terminal respiratory processes, which occurred in the normal sequence expected during the progression of microbially induced anoxia (NABIR 2003).

Because Cr(VI) removal in the *acetate-amended* microcosms occurred as part of a redox cascade, it is likely that it occurred by reduction and precipitation since Cr(III) has very low solubility at high pH (Fendorf and Zasoski 1992; Fendorf 1995). However, it is not possible to determine whether Cr(VI) reduction was a direct enzymatic process, or indirect microbially mediated process involving other redox active species (e.g. Fe(III)/Fe(II) cycling). The slightly higher proportion of acid extractable iron in the form of Fe(II) during Cr(VI) reduction may be indicative of iron cycling and thus indicative of the latter mechanism. It is therefore speculated that Cr(VI) reduction in *acetate-amended* microcosms was mediated by reaction with Fe(II) from microbial Fe(III)-reduction. Accumulation of Fe(II) in solids, however, would not be expected in these microcosms until all Cr(VI) was removed from solution. The different response rates of the three replicates may indicate this was a rather marginal environment for bacteria and, as a result, the rate of response was sensitive to subtle differences in microbiology and geochemistry (e.g. micro-environments).

Addition of acetate to this soil-water system will have preferentially supported the growth of alkaliphilic Cr(VI) tolerant bacteria within the soil that can respire anaerobically on acetate (acetate cannot support fermentative growth). On day 68 these appear to have been predominantly β-proteobacteria, with a single group of closely related bacteria within the family Comamonadaceae dominating (clade A). At this time point Cr(VI) was being removed from all three replicates, probably by reductive precipitation associated with Fe(III)/Fe(II)

cycling. Thus it appears likely that members of clade A were able to couple acetate oxidation to iron reduction. At a pH value of 10.5 the coupling of acetate oxidation to the reduction of Fe(III) to Fe(II) is thermodynamically favourable (see Table 1), and thus can support energy metabolism by microorganisms. It has been observed that closely related members of the Comamonadaceae family can couple acetate oxidation to Fe(III) reduction (e.g. *Rhodoferax ferrireducens*; Finneran et al. 2003). However genera in the Comamonadaceae family are phenotypically highly diverse, even when they are phylogenetically closely related (Spring et al. 2005) so the apparent similarity to clade A is not evidence that clade A will have similar metabolism.

Over the period of observation the pH value of both sterile controls decreased slightly with time, reaching a value around 10.5, the measured pH of the soil sample, suggesting the ditch water pH value was chemically buffered by contact with the soil. The pH values of both active microcosms decreased to a value 0.5 pH units below the measured pH of the soil. This difference is small but was probably due to microbial activity (e.g. the release of metabolic products such as CO_2 by bacteria).

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 may nevertheless be significant that each of the four bacterial clades identified in this study appeared to be closely related to genera that are adapted to similar harsh environments. For example the sequences in clade A have ≥97% identity with sequence AM778004 found in a non-saline alkaline environment, and ≥96% identity to sequence AM884728 found in an alkaline, chromium contaminated soil from a COPR disposal site (Stewart et al. 2007). Both these alkali tolerant species are shown for comparative purposes on the phylogenetic tree constructed for β-proteobacteria (Figure 3). Sequences within clade B appeared to be closely related to members of the *Aquiflexum* genus (Figure 4) and had ≥94% identity to *Aquiflexum* clone EU283506 isolated from sediment from a brackish alkaline lake. Sequences within clade C (Figure 5) had ≥98% identity to sequence AM884695 isolated from an alkaline, chromium contaminated soil from a COPR disposal site (Stewart et al. 2007). Members of clade E appeared to be members of the *Sphingomonas* genus (Figure 6), which contains hardy species capable of uranium reduction in alkaline solutions (Nilgiriwala et al. 2008). More detailed investigation of these species may provide interesting insights into life in harsh environments.

The findings of this study will have a major impact on the long-term management of the COPR waste site from which the samples were obtained, and offer a potential solution to the

downward leaching of chromate at many other COPR legacy sites. Environmentally sound management of such sites is very unlikely to involve removal of the waste, as industrial scale excavation will almost certainly generate chromate bearing dusts that will act as a pathway to human exposure. Thus remediation of COPR disposal sites will almost always involve three elements:

- Better separation of the waste from the surface environment
- Measures to reduce water infiltration into the waste
- Measures to treat leachate emerging from the waste

The first two elements will usually involve placement of a capillary barrier and a low permeability cover layer over the waste. However such a capping layer is unlikely to reduce the rainwater influx to zero, and thus there will always be the risk that contaminated water from the waste will leach downwards and contaminate underlying water resources.

This study has shown that the microbial community in soil, given time, can adapt to life at high pH. If that community is provided with a suitable electron donor, then progressive anoxia develops and chromium accumulates in the soil by reduction and precipitation at the point in the redox cascade just before iron reduction becomes fully established. Creating such a reductive zone in the soil will act as a barrier to the migration of chromium, which should reduce the impact of the waste on the wider environment. It is not clear from the current study whether the residual organic matter still remaining in the former surface layer can support iron reduction, or if it now needs augmenting with an organic substrate such as acetate, however the widespread presence of Fe(II) and the amount of chromium that has accumulated in that soil layer is evidence that it has done so the past.

CONCLUSIONS

A former surface soil that has been buried beneath COPR tip for over 100 years has an active microbial population despite it having a pH value of 10.5. Without the addition of an exogenic electron donor this microbial population is able to reduce nitrate using an electron donor(s) that is probably derived from the soil organic matter. With the addition of acetate as a more readily available electron donor, Cr(VI) removal occurred after nitrate reduction, to be followed by iron reduction. It is proposed that Cr(VI) removal from solution was by microbially mediated reductive precipitation. This was either a direct enzymatic process with Cr(VI) being used as an electron acceptor, or more likely an indirect process involving an abiotic reaction with Fe(II) produced by microbial Fe(III) reduction.

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References

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.

Bopp, L. H. and H. L. Ehrlich (1988). "Chromate resistance and reduction in *pseudomonas-fluorescens* strain LB300." Archives of Microbiology **150**(5): 426-431.

Burke, I. T., C. Boothman, J. R. Lloyd, F. R. Livens, J. M. Charnock, J. M. McBeth, R. J. G. Mortimer and K. Morris (2006). "Reoxidation behavior of technetium, iron, and sulfur in estuarine sediments." Environmental Science & Technology **40**(11): 3529-3535.

Cervantes, C., J. Campos-Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J. C. Torres-Guzman and R. Moreno-Sanchez (2001). "Interactions of chromium with microorganisms and plants." Fems Microbiology Reviews **25**(3): 335-347.

Chai, L. Y., S. H. Huang, Z. H. Yang, B. Peng, Y. Huang and Y. H. Chen (2009). "Hexavalent chromium reduction by Pannonibacter phragmitetus BB isolated from soil under chromium-containing slag heap." Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering **44**(6): 615-622.

Chen, J. M. and O. J. Hao (1998). "Microbial chromium (VI) reduction." Critical Reviews in Environmental Science and Technology **28**(3): 219-251.

Cudennec, Y. and A. Lecerf (2006). "The transformation of ferrihydrite into goethite or hematite, revisited." Journal of Solid State Chemistry **179**(3): 716-722.

Darrie, G. (2001). "Commercial extraction technology and process waste disposal in the manufacture of chromium chemicals from ore." Environmental Geochemistry and Health **23**(3): 187-193.

Daulton, T. L., B. J. Little, J. Jones-Meehan, D. A. Blom and L. F. Allard (2007). "Microbial reduction of chromium from the hexavalent to divalent state." Geochimica Et Cosmochimica Acta **71**(3): 556-565.

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 And Biotechnology **151**(2-3): 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.

Farmer, J. G., M. C. Graham, R. P. Thomas, C. Licona-Manzur, C. Licona-Manzur, E. Paterson, C. D. Campbell, J. S. Geelhoed, D. G. Lumsdon, J. C. L. Meeussen, M. J. Roe, A. Conner, A. E. Fallick and R. J. F. Bewley (1999). "Assessment and modelling of the environmental chemistry and potential for remediative treatment of chromium-contaminated land." Environmental Geochemistry and Health **21**(4): 331-337.

Farmer, J. G., E. Paterson, R. J. F. Bewley, J. S. Geelhoed, S. Hillier, J. C. L. Meeussen, D. G. Lumsdon, R. P. Thomas and M. C. Graham (2006). "The implications of integrated assessment and modelling studies for the future remediation of chromite ore processing residue disposal sites." Science of the Total Environment **360**(1-3): 90-97.

Farmer, J. G., R. P. Thomas, M. C. Graham, J. S. Geelhoed, D. G. Lumsdon and E. Paterson (2002). "Chromium speciation and fractionation in ground and surface waters in the vicinity of chromite ore processing residue disposal sites." Journal of Environmental Monitoring **4**(2): 235-243.

Fendorf, S. E. (1995). "Surface-Reactions of Chromium in Soils and Waters." Geoderma **67**(1-2): 55-71.

Fendorf, S. E. and R. J. Zasoski (1992). "Chromium(III) oxidation by δ-MnO_2: 1. Characterization." Environmental Science & Technology **26**(1): 79-85.

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.

Fonseca, B. M., H. Quintelas, C. Teixeira, A. Tavares, T. (2009). "Retention of Cr(VI) and Pb(II) on a loamy sand soil: Kinetics, equilibria and breakthrough." Chemical Engineering Journal **152**(1): 212-219.

Francis, C. A., A. Y. Obraztsova and B. M. Tebo (2000). "Dissimilatory metal reduction by the facultative anaerobe Pantoea agglomerans SP1." Applied and Environmental Microbiology **66**(2): 543-548.

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.

Geelhoed, J. S., J. C. L. Meeussen, M. J. Roe, S. Hillier, R. P. Thomas, J. G. Farmer and E. Paterson (2003). "Chromium remediation or release? Effect of iron(II) sulfate addition on chromium(VI) leaching from columns of chromite ore processing residue." Environmental Science & Technology **37**(14): 3206-3213.

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.

Han, X., Y. S. Wong and N. F. Y. Tam (2006). "Surface complexation mechanism and modeling in Cr(III) biosorption by a microalgal isolate, Chlorella miniata." Journal of Colloid and Interface Science **303**(2): 365-371.

Hansel, C. M., S. G. Benner and S. Fendorf (2005). "Competing Fe(II)-induced mineralization pathways of ferrihydrite." Environmental Science & Technology **39**(18): 7147-7153.

Ishibashi, Y., C. Cervantes and S. Silver (1990). "Chromium reduction in *pseudomonas-putida*." Applied and Environmental Microbiology **56**(7): 2268-2270.

James, B. R. (1994). "Hexavalent Chromium Solubility and Reduction in Alkaline Soils Enriched with Chromite Ore Processing Residue." Journal of Environmental Quality **23**(2): 227-233.

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

Knowles, R. (1982). "Denitrification." Microbiological Reviews **46**(1): 43-70.

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.

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. Englewood Cliffs, NJ: Prentice Hall.".

Lee, T., H. Lim, Y. Lee and J. W. Park (2003). "Use of waste iron metal for removal of Cr(VI) from water." Chemosphere **53**(5): 479-485.

Lloyd, J. R., H. F. Nolting, V. A. Sole and K. Bosecker (1998). "Technetium reduction and precipitation by sulfate-reducing bacteria." Geomicrobiology Journal **15**(1): 45-58.

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

Lovley, D. R. (1993b). "Anaerobes into Heavy-Metal - Dissimilatory Metal Reduction in Anoxic Environments." Trends in Ecology & Evolution **8**(6): 213-217.

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.

Morales-Barrera, L. and E. Cristiani-Urbina (2008). "Hexavalent chromium removal by a Trichoderma inhamatum fungal strain isolated from tannery effluent." Water Air and Soil Pollution **187**(1-4): 327-336.

NABIR (2003). Bioremediation of metals and radionuclides: what it is and how it works. A NABIR primer, NABIR primer prepared for US Department of Energy**:** p.78.

Neal, A. L., K. Lowe, T. L. Daulton, J. Jones-Meehan and B. J. Little (2002). "Oxidation state of chromium associated with cell surfaces of Shewanella oneidensis during chromate reduction." Applied Surface Science **202**(3-4): 150-159.

Nilgiriwala, K. S., A. Alahari, A. S. Rao and S. K. Apte (2008). "Cloning and overexpression of alkaline phosphatase PhoK from Sphingomonas sp strain BSAR-1 for bioprecipitation of uranium from alkaline solutions." Applied and Environmental Microbiology **74**(17): 5516-5523.

Pechova, A. and L. Pavlata (2007). "Chromium as an essential nutrient: a review." Veterinarni Medicina **52**(1): 1-18.

Rai, D., B. M. Sass and D. A. Moore (1987). "Chromium(Iii) Hydrolysis Constants and Solubility of Chromium(Iii) Hydroxide." Inorganic Chemistry **26**(3): 345-349.

Rehman, A., A. Zahoor, B. Muneer and S. Hasnain (2008). "Chromium tolerance and reduction potential of a Bacillus sp.ev3 isolated from metal contaminated wastewater." Bulletin of Environmental Contamination and Toxicology **81**(1): 25-29.

Richard, F. C. and A. C. Bourg (1991). "Aqueous geochemistry of Cr: A review." Water Research **25**(7): 807-816.
Roadcap, G. S., R. A. Sanford, Q. S. Jin, J. R. Pardinas and C. M. Bethke (2006). "Extremely alkaline (pH > 12) ground water hosts diverse microbial community." Ground Water **44**(4): 511-517.
Romanenko, V. I. and V. N. Koren'kov (1977). "Pure culture of bacteria using chromates and bichromates as hydrogen acceptors during development under anaerobic conditions." Mikrobiologiia **46**(3): 414-7.
Sani, R. K., B. M. Peyton, W. A. Smith, W. A. Apel and J. N. Petersen (2002). "Dissimilatory reduction of Cr(VI), Fe(III), and U(VI) by Cellulomonas isolates." Applied Microbiology and Biotechnology **60**(1-2): 192-199.
Sau, G. B., S. Chatterjee, S. Sinha and S. K. Mukherjee (2008). "Isolation and Characterization of a Cr(VI) Reducing Bacillus firmus Strain from Industrial Effluents." Polish Journal of Microbiology **57**(4): 327-332.
Schumacher, B. A. (2002). Methods for the Determination of Total Organic Carbon (TOC) in Soils and Sediments. Las Vegas, United States Environmental Protection Agency.
Schwertmann, U., J. Friedl and H. Stanjek (1999). "From Fe(III) ions to ferrihydrite and then to hematite." Journal of Colloid and Interface Science **209**(1): 215-223.
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.
Sreeram, K. J. and T. Ramasami (2001). "Speciation and recovery of chromium from chromite ore processing residues." Journal of Environmental Monitoring **3**(5): 526-530.
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.
Straub, K. L., M. Benz, B. Schink and F. Widdel (1996). "Anaerobic, nitrate-dependent microbial oxidation of ferrous iron." Applied and Environmental Microbiology **62**(4): 1458-1460.
Stucki, J. W., K. Lee, B. A. Goodman and J. E. Kostka (2007). "Effects of in situ biostimulation on iron mineral speciation in a sub-surface soil." Geochimica et Cosmochimica Acta **71**(4): 835-843.
Studds, P. G. (pers. comm.). Director, Environment and Nature, Ramboll UK
Stumm, W. and J. J. Morgan (1996). Aquatic Geochemistry New York, John Wiley and Sons.
Suzuki, T., N. Miyata, H. Horitsu, K. Kawai, K. Takamizawa, Y. Tai and M. Okazaki (1992). "NAD(P)H-dependent chromium (VI) reductase of Pseudomonas ambigua G-1: a Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III)." Journal of Bacteriology **174**(16): 5340-5345.
Thauer, R. K., K. Jungermann and K. Decker (1977). "Energy conservation in chemotrophic anaerobic bacteria." Bacteriological Reviews **41**(1): 100-180.
Tilt, Z. (2009). The subsurface migration of Cr(VI) at a chromium waste tip in the North of England. School of Earth and Environment, University of Leeds. **MSc**.
Tinjum, J. M., C. H. Benson and T. B. Edil (2008). "Mobilization of Cr(VI) from chromite ore processing residue through acid treatment." Science of the Total Environment **391**(1): 13-25.
USEPA (1992). "SW-846 Manual: Method 7196a. Chromium hexavalent (colorimetric). Retrieved 6th Jan 2006."
USEPA (1998). "Toxicological review of hexavalent chromium." Available online at http://www.epa.gov/ncea/iris.
VanEngelen, M. R., B. M. Peyton, M. R. Mormile and H. C. Pinkart (2008). "Fe(III), Cr(VI), and Fe(III) mediated Cr(VI) reduction in alkaline media using a Halomonas isolate from Soap Lake, Washington." Biodegradation **19**(6): 841-850.
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.
Walawska, B. and Z. Kowalski (2000). "Model of technological alternatives of production of sodium chromate (VI) with the use of chromic wastes." Waste Management **20**(8): 711-723.
Wang, J. H., B. C. Baltzis and G. A. Lewandowski (1995). "Fundamental Denitrification Kinetic-Studies With Pseudomonas-Denitrificans." Biotechnology And Bioengineering **47**(1): 26-41.
Wang, Y. T. (2000). "In Lovley D.R. "Environmental Microbe-Metal Interactions" ASM Press." Chapter 10: Microbial Reduction of Chromate: 225-235.
Whittleston, R. A., D. V. Hughes-Berry, D. I. Stewart and I. T. Burke (2007). "A Natural In-situ Reactive Zone beneath a COPR Tip." 3rd International Symposium on Permeable Reactive Barriers and Reactive Zones, Rimini, Italy.

Zhu, W. J., Z. H. Yang, Z. M. Ma and L. Y. Chai (2008). "Reduction of high concentrations of chromate by Leucobacter sp CRB1 isolated from Changsha, China." World Journal of Microbiology & Biotechnology **24**(7): 991-996.

Table 1: Microbially significant half-reaction reduction potentials: Standard Reduction Potential, E^0, and redox potential, Eh, at pH 7 and 10.5 (at 25°C and atmospheric pressure).

Transformation	Reaction	E_0 (V)	Eh @ pH 7 (V)	Eh @ pH 10.5 (V)	Assumptions
O_2 Depletion$^+$	$O_2 + 4H^+ + 4e^- = 2H_20$	1.23	0.805	0.598	P_{O2} = 0.2 bar
Denitrification$^+$	$NO_3^- + 6H^+ + 5e^- = 1/2N_2 + 3H_2O$	1.24	0.713	0.464	$[NO_3^-]$ = 1 mmol L^{-1} P_{N2} = 0.8 bar
Cr reduction* Cr(VI) to Cr(III)	$CrO_4^{2-} + 5H^+ + 3e^- = Cr(OH)_3 + H_2O$	1.198	0.508	0.163	$[CrO_4^{2-}]$=250 μM
Mn reduction $^+$ Mn(III) to Mn(II)	$Mn_3O_4 + 2H^+ + 2H_2O + 2e^- = 3Mn(OH)_2$	0.480	0.066	-0.140	-
Fe reduction* Fe(III) to Fe(II)	$Fe(OH)_3 + H^+ + e^- = Fe(OH)_2 + H_2O$	0.257	-0.157	-0.364	-
Fe reduction$^+$ Fe(III) to Fe(II)	$Fe(OH)_3 + HCO_3^- + 2H^+ + e^- = FeCO_3 + 3H_2O$	1.078	-	-0.266 -0.321	$[HCO_3^-]$ = 20 mmol L^{-1} $[HCO_3^-]$ = 1 mmol L^{-1}
Sulfate reduction$^+$ S(VI) to S(-II)	$SO_4^{2-} + 10H^+ + 8e^- = H_2S + 4H_2O$	0.301	-0.217	-0.476	$[SO_4^{2-}] = [H_2S]$
Bicarbonate reduction to acetate$^\times$ C(VI) to C(0)	$2HCO_3^- + 9H^+ + 8e^- = CH_3COO^- + 4H_2O$	0.187	-0.292	-0.525	$[HCO_3^-] = [CH_3COO^-]$ = 20 mmol L^{-1}

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

Table 2: Major elements in fused samples measured by XRF (corrected for loss on ignition at 1000°C).

	SiO_2 %	Al_2O_3 %	CaO %	MgO %	Fe_2O_3 %	Cr_2O_3 %	TiO_2 %	Mn_3O_4 %	Na_2O %	K_2O %	SO_3 %	LOI %
Grey silty clay	71.41	9.73	2.29	0.35	3.47	0.45	0.56	0.12	0.56	1.56	0.03	9.30
Unweathered waste	3.61	4.27	40.29	5.85	7.04	4.93	0.05	0.07	n.d.	0.03	5.10	28.40

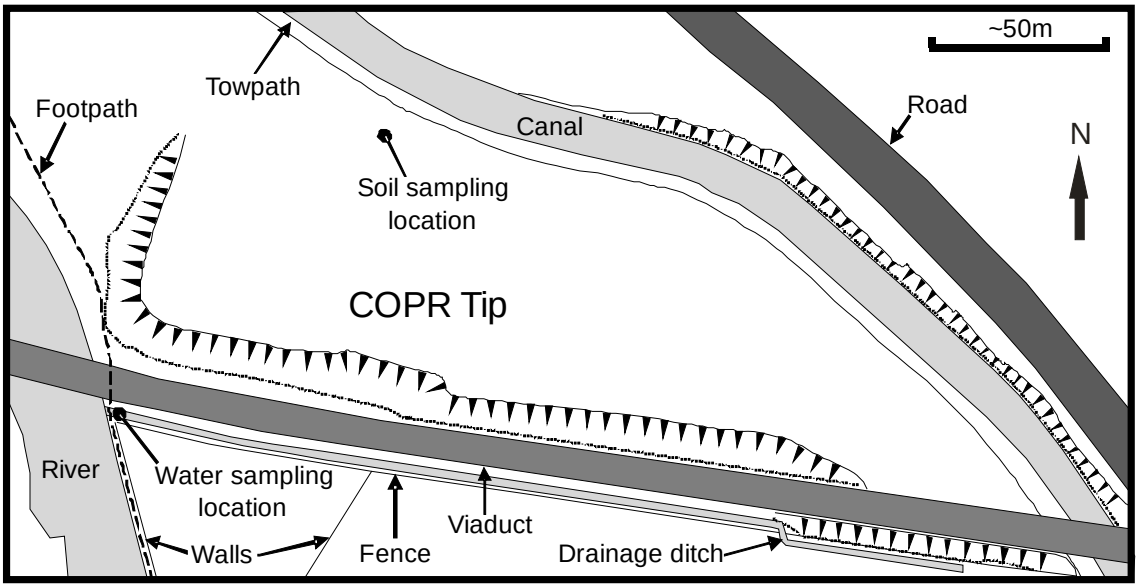


Figure 1. Sketch map of the site showing the sampling locations.

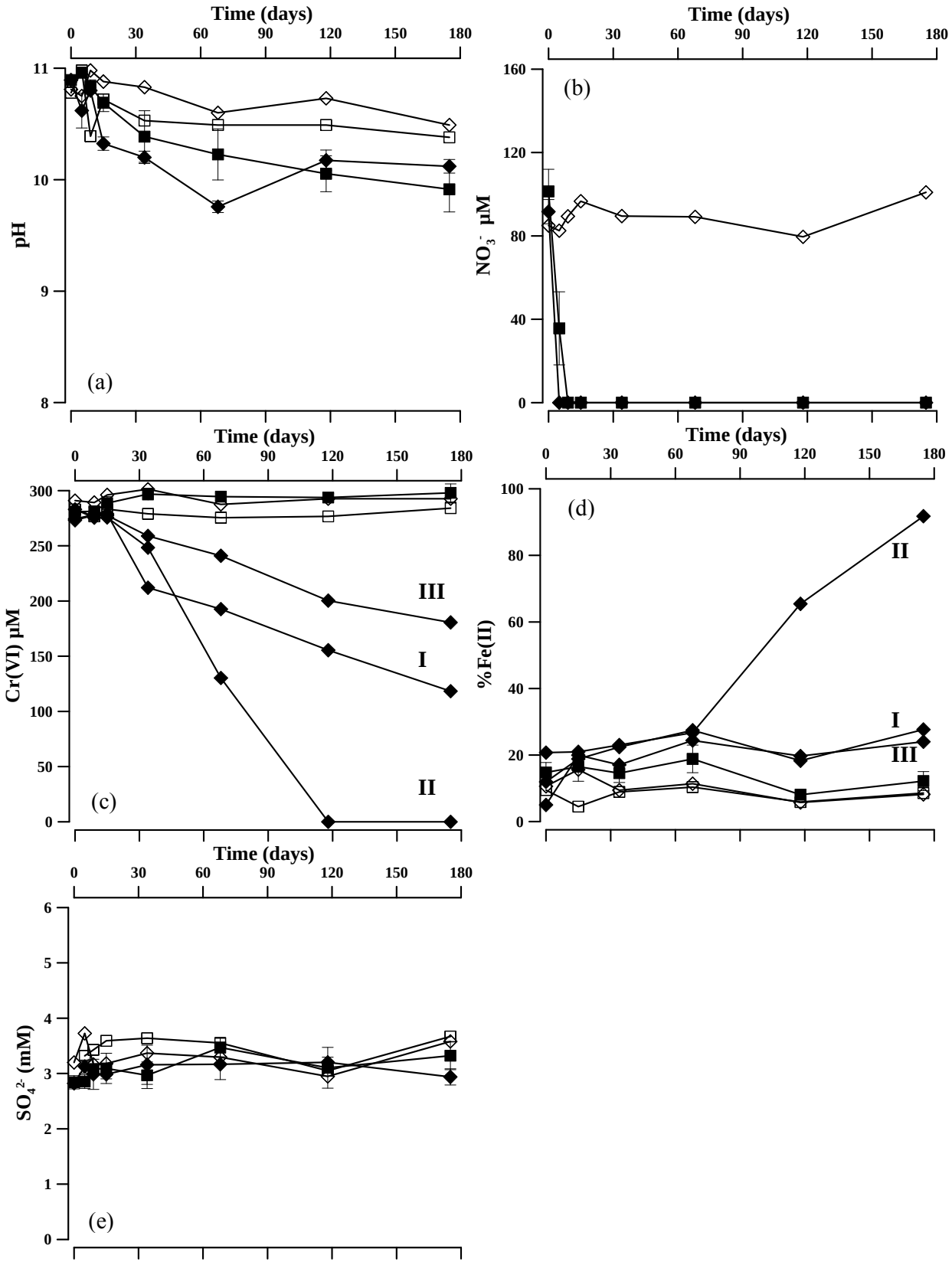


Figure 2. Geochemical response of the *unamended* (■) and *acetate-amended* (◆) microcosms: (a) pH, (b) porewater NO_3^- concentration, (c) porewater Cr(VI) concentration (d) % of 0.5 N HCl extractable Fe as Fe(II) in soils, (e) porewater SO_4^{2-} concentration. Response in individual *acetate-amended* microcosms from triplicate series (I-III) are shown in (c) and (d). Error bars shown are one standard deviation from the mean of triplicate experiments. NO_3^- and SO_4^{2-} data were corrected using Cl^- variability to account for instrument variability. Data from sterile controls are shown using open symbols.

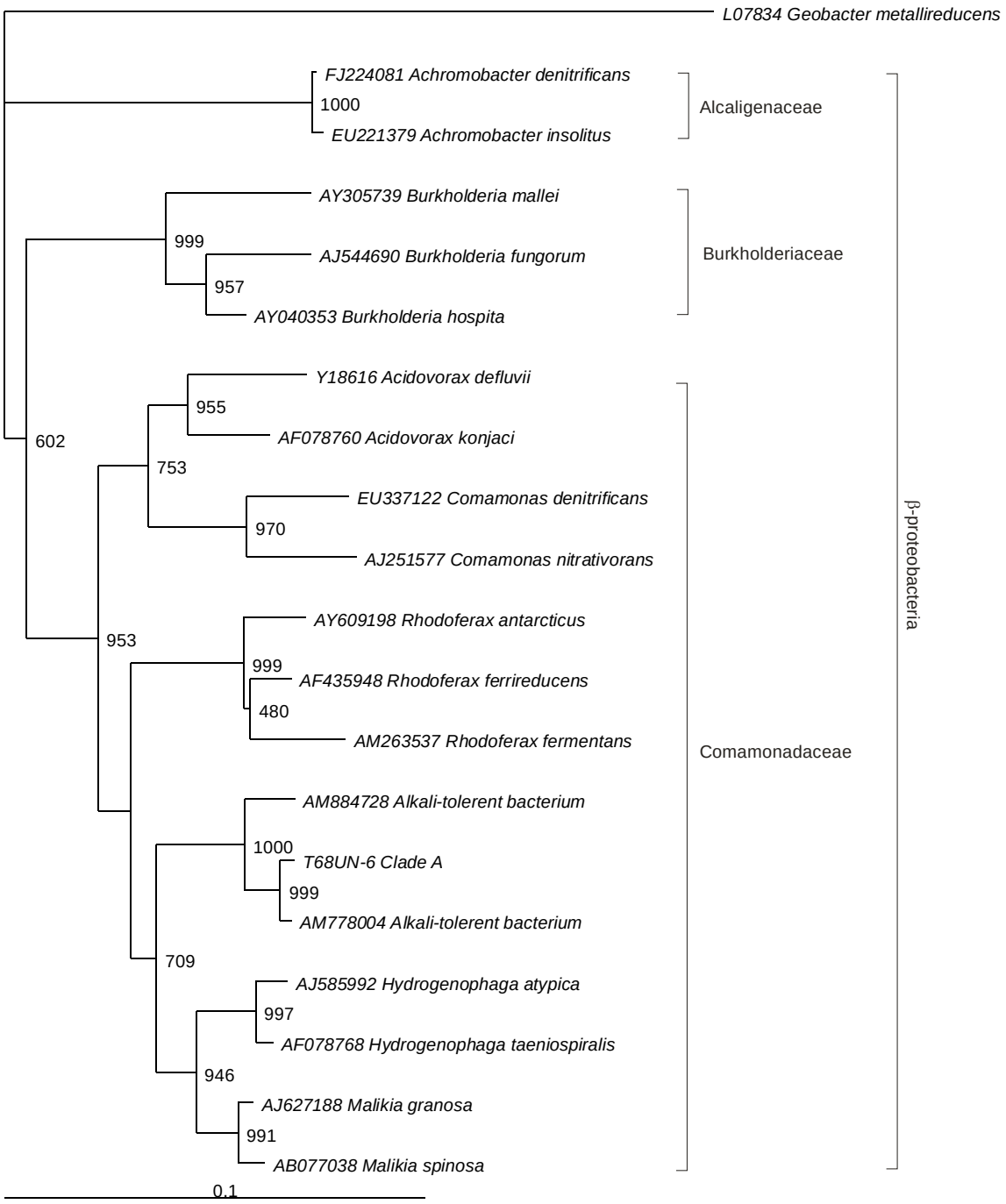


Figure 3: Phylogenetic tree showing the relationship between a representative sequence from clade A and 16S rRNA gene sequences of previously described bacteria. Geobacter metallireducens was included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.

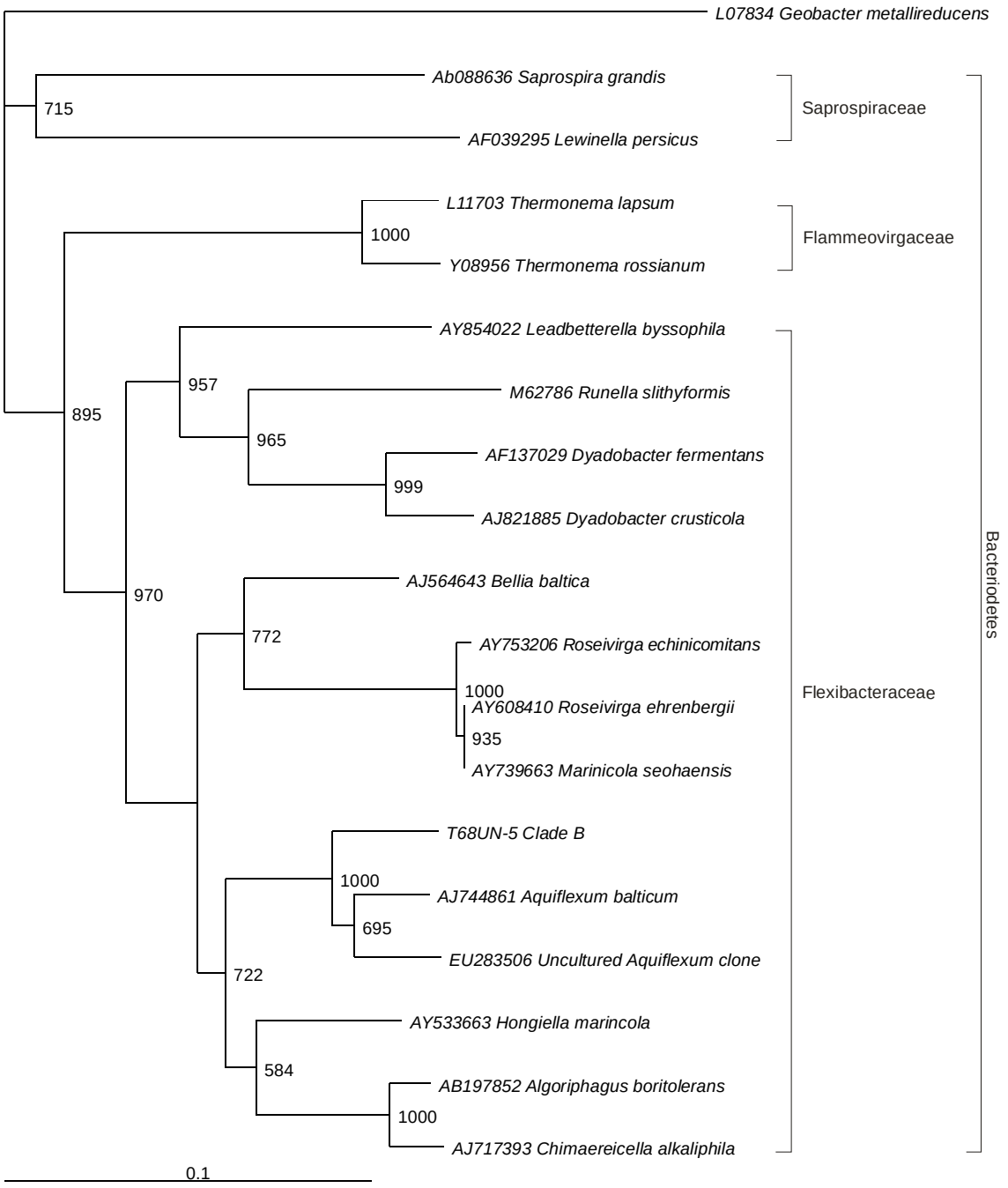


Figure 4: Phylogenetic tree showing the relationship between a representative sequence from clade B and 16S rRNA gene sequences of previously described bacteria. Geobacter metallireducens was included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.

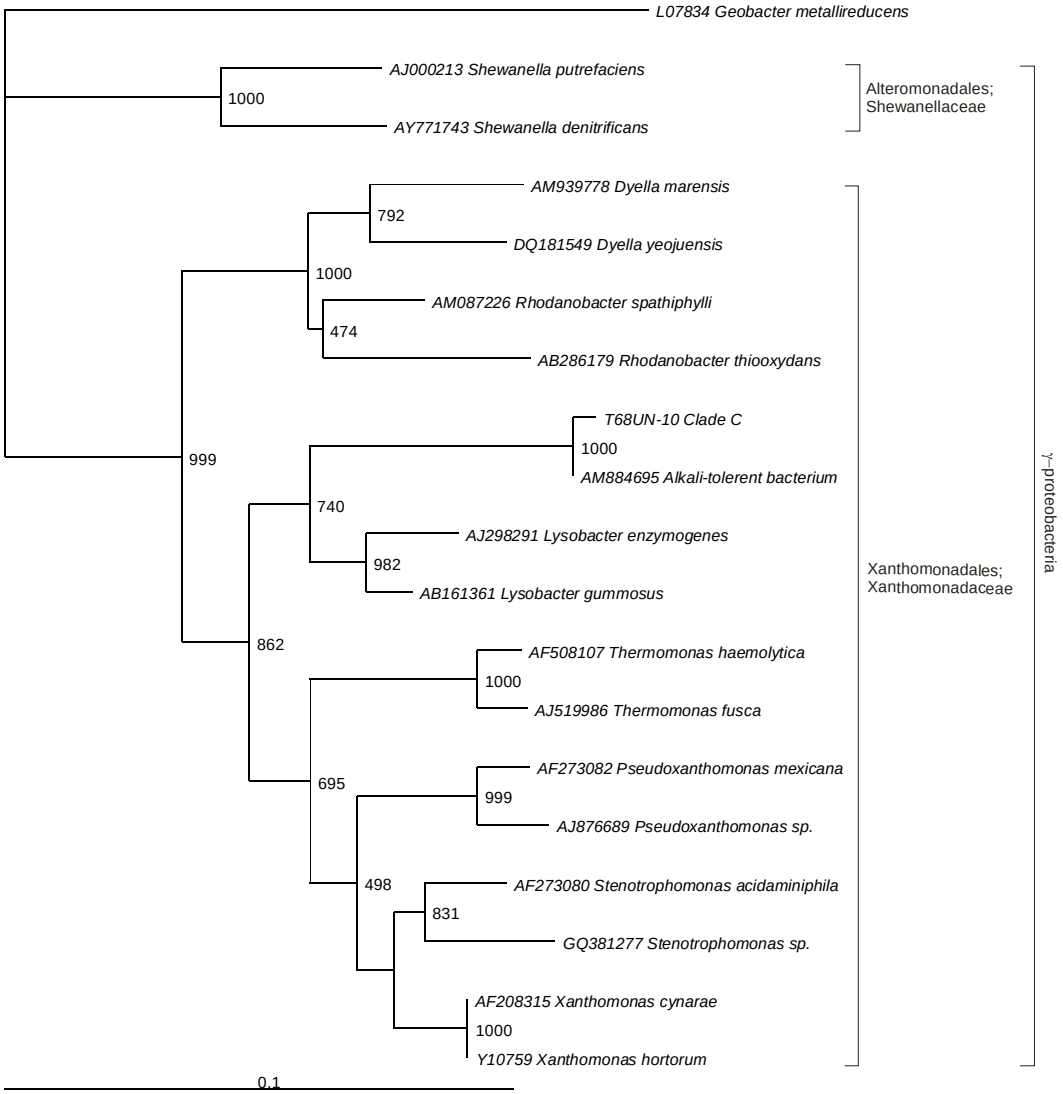


Figure 5: Phylogenetic tree showing the relationship between a representative sequence from clade C and 16S rRNA gene sequences of previously described bacteria. Geobacter metallireducens was included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.

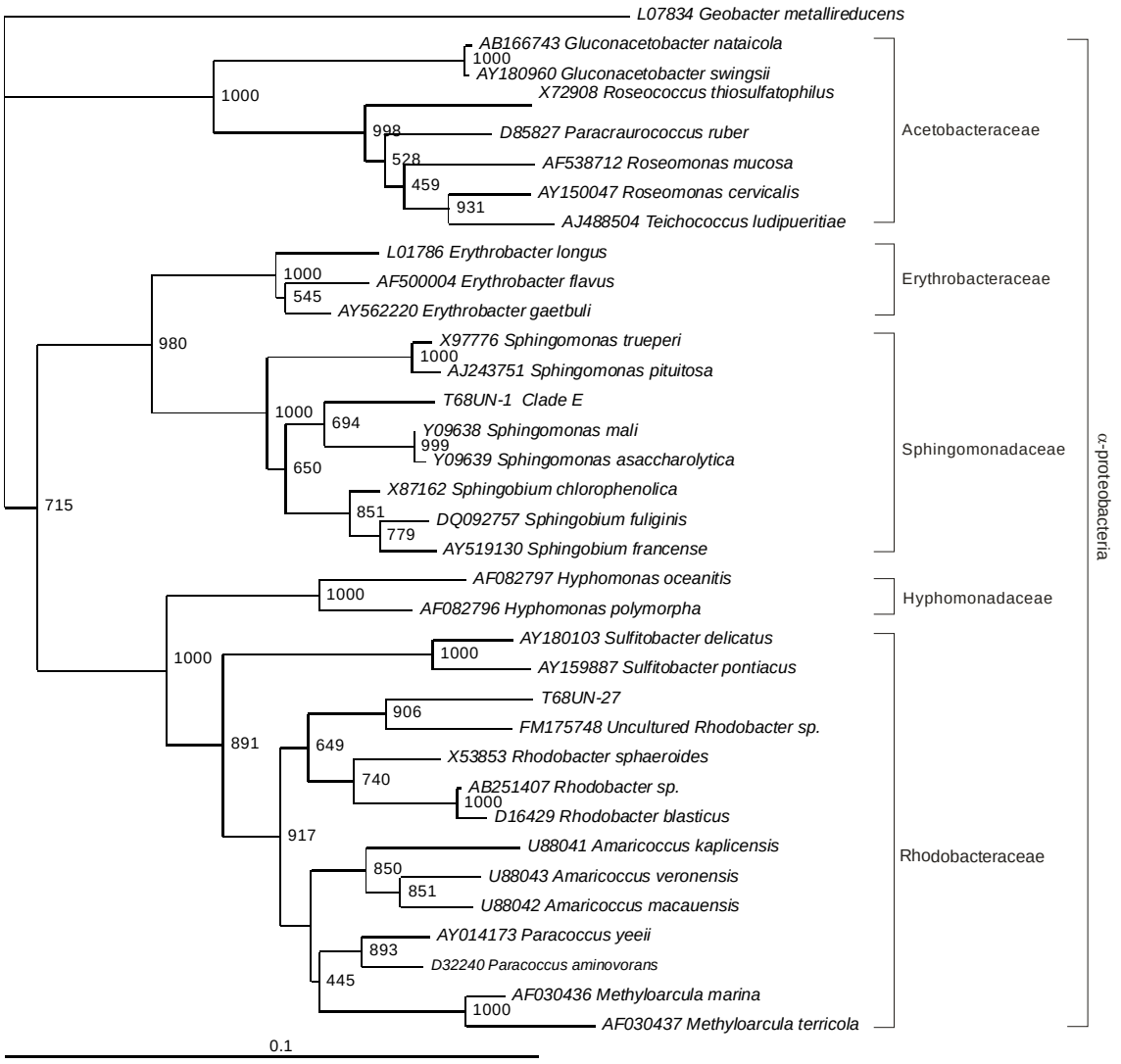
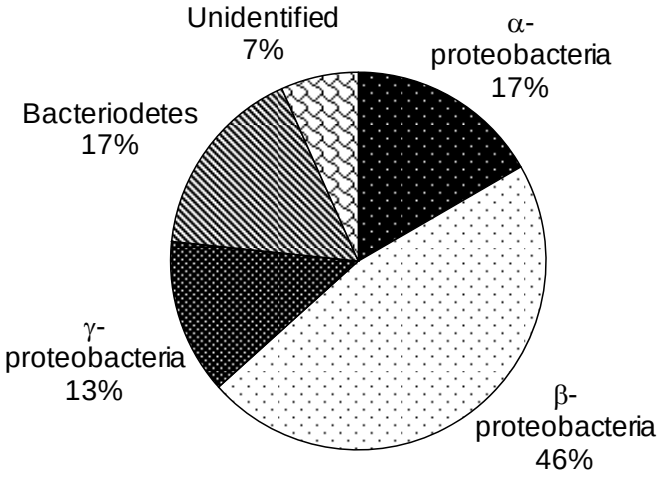
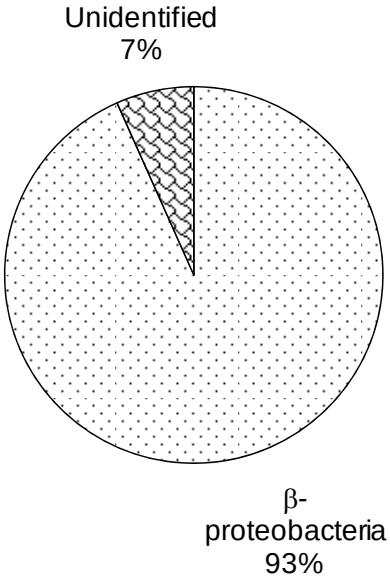


Figure 6: Phylogenetic tree showing the relationship between a representative sequence from clade E and 16S rRNA gene sequences of previously described bacteria. Geobacter metallireducens was included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.



(a)



(b)

Figure 7: Microbial communities of (a) the unamended microcosms (30 clones) and (b) the acetate-amended microcosms (30 clones) after incubation under anaerobic conditions for 68 days. Charts show phylogenetic affiliation of the 16S rRNA gene sequences