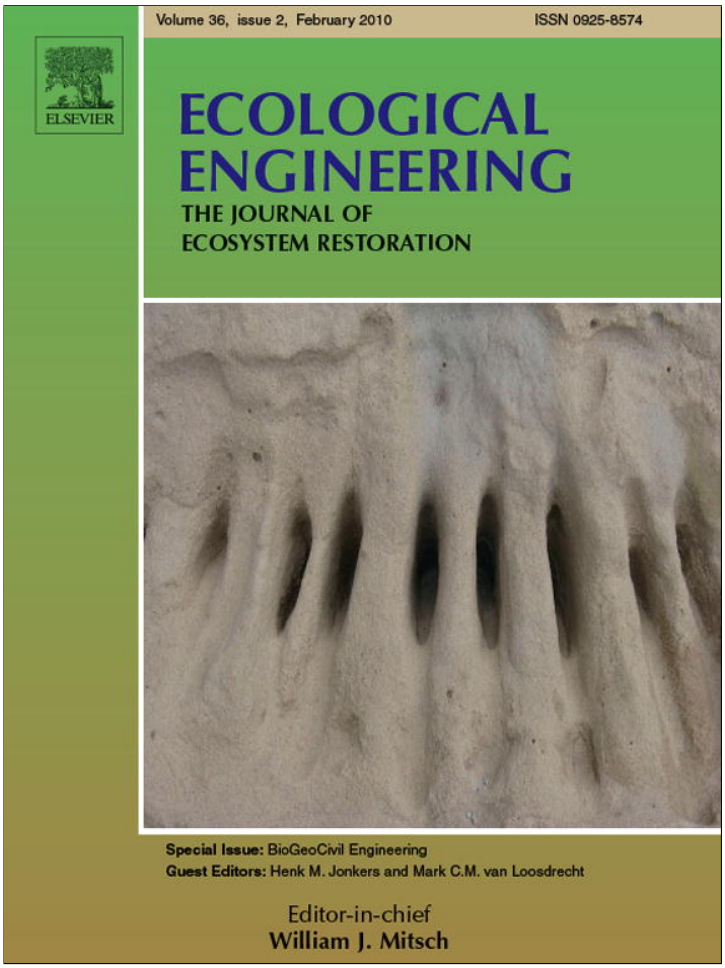

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Microbially mediated chromate reduction in soil contaminated by highly alkaline leachate from chromium containing waste

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ABSTRACT

This paper reports an investigation into the possible fate of Cr(VI) that is migrating downwards from a chromium ore processing residue (COPR) tip into the underlying soils. This waste was deposited at a site in the north of England more than 100 years ago and is currently a cause for environmental concern because groundwater emerging from the waste is alkaline, visibly yellow and has an elevated Cr(VI) concentration. Sandy clay from immediately beneath the waste (assumed to be the topsoil layer prior to waste tipping) contains between about 600 and 3000 mg kg^{-1} of Cr, and around 60% of 0.5 N HCl extractable iron was present as reduced Fe(II). DNA fragments from soil bacteria were extracted from this soil, and microcosm experiments where the pH was adjusted to more neutral values showed that it contains a viable bacterial population capable of iron-reduction. This sandy clay layer, despite a pH value of 10.5, appears to be acting as a natural reactive zone beneath the waste as it is accumulating chromium. It is thought that the mechanism of Cr(VI) reduction is most likely to be an abiotic reaction with the Fe(II) present in the soil, and that Fe(II) in the soil is being replenished by microbial iron-reduction (although the rate of replenishment is unknown).

1. Background

Poorly controlled landfilling of chromite ore processing residue (COPR) is a globally widespread problem (Darrie, 2001; Geelhoed et al., 2002), and locally such sites can be major point sources of pollution. The conventional engineering approach to most pollution problems is to remove the contaminant, possibly treat it, and store it in a better location. Such conventional approaches to COPR legacy sites would expend massive resources, particularly non-renewable energy sources, and in the short term would increase the exposure of both humans and the wider ecosystem to the waste. On the other hand, an ecological engineering approach attempts to restore ecosystems disturbed by environmental pollution or land disturbance by exploiting their self-designing capacity in the restoration process (Mitsch and Jørgensen, 2003; Jørgensen, 2006). So far such ecological engineering principles have been successfully applied to a variety of engineering problems: root reinforcement of soil slopes (Fan and Su, 2008), lake management (Jørgensen, 2006), the anaerobic treatment of municipal wastewater (Álvarez et al., 2008) and the restoration of mined lands (Bradshaw, 1997). This study reports an investigation into the role of microbial processes in the fate of Cr(VI) that migrates downwards from the waste into the underlying soils. It shows that a self-designed microbial ecosystem is acting to transform hazardous Cr(VI) species into less hazardous and less mobile Cr(III) species, and thus that ecological engineering principles can be exploited in the restoration of this site.

Chromite is an iron chromium oxide mineral, $FeCr_2O_4$, belonging to the spinel group. Magnesium is always present in variable amounts, substituting for Fe(II) (the end member, $MgCr_2O_4$, is called magnesiochromite), and aluminium and iron can substitute for the trivalent chromium (Darrie, 2001; Deakin, 2002; Guertin et al., 2005). Chromite ore is processed by roasting it with an alkali-carbonate at 1150 °C to oxidise the insoluble Cr(III) to soluble Cr(VI) which is then extracted with water upon cooling. Traditionally, limestone was added to the reaction mixture to improve air penetration, and this "high-lime" process was the only commercial method of chromium smelting in the UK up to the 1960s (Darrie, 2001). COPR from the high-lime process is highly alkaline and typically contains 2–6% total chromium by weight (Gemmell, 1973; Deakin et al., 2001a,b; Geelhoed et al., 2002). Much of that chromium is unreacted insoluble chromite ore (i.e. Cr(III)) but, as a result of oxidation during ore processing, up to 30% can be chromate (Cr(VI)) (Geelhoed et al., 2003). As a result, the pore water in abandoned waste piles can have a pH >12 and has been reported

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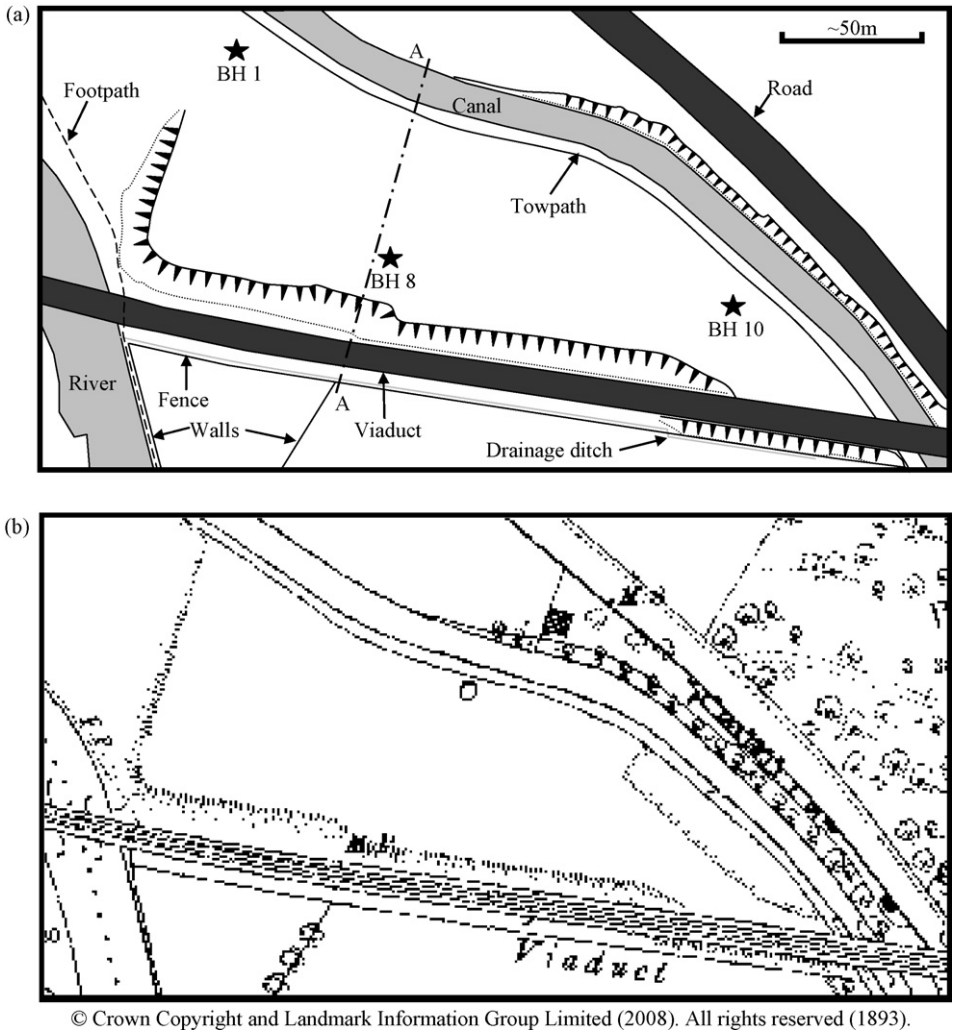

Fig. 1. (a) Sketch map of the site showing the sampling location and the position of cross-section AA; and (b) historic map showing site in 1893 (excerpt from National Grid 1:2500).

to contain up to 640 μM of chromate (Farmer et al., 1999; Deakin, 2002).

This paper describes the investigation of a site in the North of England where COPR has been deposited against a valley side close to a river, probably at the end of the 19th century. The site is causing environmental concern because the groundwater which is emerging from near the base of the waste pile is visibly yellow and has an elevated Cr(VI) concentration. Currently water from the site enters both the local groundwater and surface water systems.

2. Methods

2.1. Site description

The site is situated between a canal and a river that are about 150 m apart (see Fig. 1a). The canal is approximately 7 m above the river on the valley side. Waste has been tipped against the valley side between the river and canal covering an area of 1.8 ha. Ground level on the tip is about 1.5 m higher than the canal towpath, and the site is now covered with grass. Geological maps indicate that this is a glacial valley within the millstone grit series in-filled with alluvial deposits (silt, clay and sand). Historical maps show that this landform (called the "old tip") first appeared in the late 19th century (Fig. 1b), and has not been substantially altered since.

2.2. Soil and waste sampling and characterisation

A commercial ground investigation was undertaken in March 2007 to determine the hazard posed by the site and to evaluate the remedial options available to the site owners. This investigation included seven boreholes through, and six window sampler holes immediately adjacent to, the waste tip. The soil and waste samples used in this research project were taken from boreholes 1, 8 and 10 which were advanced using cable-percussion drilling (the locations of these boreholes are shown in Fig. 1a). Soils were transferred directly into sterile polythene containers, transported back to the laboratory, and stored in the dark at 4 °C until use. Two waste and two soil samples were subjected to analysis: weathered waste from a depth of 2–3 m in BH10, unweathered waste from a depth of 4–7 m in BH1, grey clay from a depth of 7 m in BH1 (~1 m below the waste), and brown clay from a depth of 10 m in BH8 (immediately below the waste). During the site investigation water samples were taken from the site drainage ditch close to where it enters the river (at the base of the viaduct) for use in microcosm incubations and from the canal for use in leaching experiments. Leaching tests and microcosms experiments were started within 6 months of the site investigation and all soil manipulation was kept to a minimum prior to use. A further water sample was taken from the site drainage ditch in June 2007 after a period of prolonged rainfall.

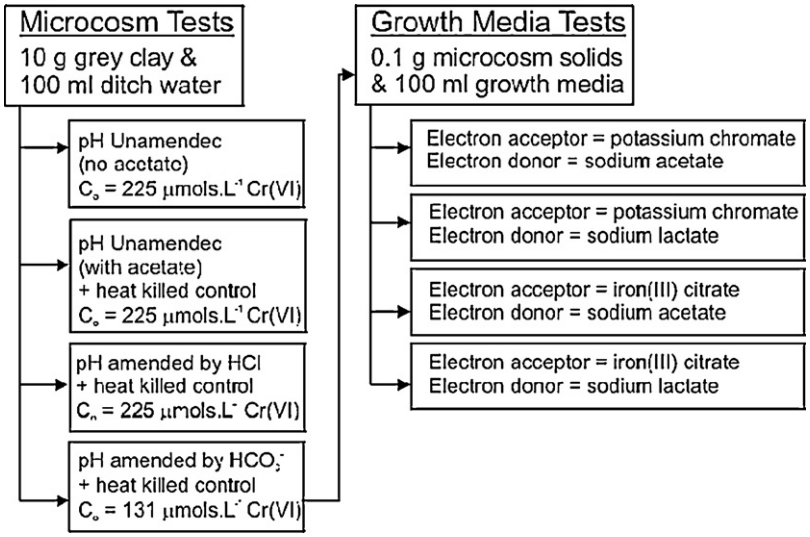

Fig. 2. Flow diagram showing relationship of microcosm and freshwater growth media experiments.

Soil and waste pH were measured in a 1:1 suspension in distilled water (ASTM, 2006). X-ray powder diffraction (XRD) analysis of soil and waste samples ground to <75 μm was undertaken on a Philips PW1050 Goniometer. X-ray fluorescence (XRF) analysis of fused soil and waste samples were undertaken on a Philips PW2404 wavelength dispersive sequential X-ray spectrometer and a Philips PW2440 sequential spectrometer, respectively (data were corrected for loss on ignition). Approximately 25 g of each soil was oven dried at 105 °C and disaggregated and homogenised with a mortar and pestle for carbon content determination. A portion of homogenised samples was pre-treated with 10% HCl to remove any carbonates present (Schumacher, 2002) and the carbon content of oven dried and HCl-treated sub-samples were measured using Carlo-Erba 1106 elemental analyser.

A sample of the unweathered waste was prepared for scanning electron microscope (SEM) analysis. It was dried at 105 °C, disaggregated with a spatula and sections were prepared by impregnation with Struers Epofix resins at a ratio of 15:1. Grinding and polishing was performed using a progressively finer grit of silicon carbide paper on a Struers RotoPol-35 using Kemet type OS (a hydrocarbon) as a lubricant. The polished section was imaged on a Philips XL30 environmental SEM at an acceleration voltage of 30 kV, in back scattered mode under normal vacuum, and analysed using an Oxford Instruments INCA 250 energy dispersive X-ray analysis (EDX) system. Semiquantitative EDX analysis was processed with INCA software, the matrix corrections being carried out with oxygen and the appropriate ZAF correction procedure applied to the elemental abundance data.

2.3. Sequential leaching tests

Sequential leaching tests were performed in order to assess the potential release of Cr(VI) from the weathered and unweathered waste found at the site. In triplicate tests, the waste material was suspended in canal water at a 1:1 ratio (w/w) and shaken on an orbital shaker at 150 rpm for a minimum of 24 h. After shaking the mixture was centrifuged at 8000 rpm, and the supernatant removed and analysed for Cr(VI), pH, total dissolved solids (TDS), Ca and sulphate as described below. Fresh water was then added to the leached samples and the cycle was repeated 20 times per sample.

2.4. Microcosm incubation experiments

Microcosm experiments were performed to assess the behaviour of the Cr(VI) contaminated site leachate when mixed with the soil found directly below the waste (a flow chart showing the experimental conditions is shown in Fig. 2). Microcosms were made up using 10 g of the grey clay and 100 ml of waste leachate from the site drainage ditch in 120 ml glass serum bottles, and were sealed with butyl rubber stoppers and aluminium crimps (no attempt was made to purge the small amount oxygen trapped in the headspace). Five microcosm experiments and two sterile controls were run using the ditch water sample obtained in March 2007. Two microcosms and a sterile control were run without pH adjustment (the *pH-unamended* microcosms), and had an average initial pH value of 11.0. Sodium acetate to a final concentration of 20 mM was added to one pH-unamended microcosm and the related control. Three repeat microcosms and a sterile control were amended with HCl to a final concentration of 30 mM and sodium acetate to a final concentration of 20 mM. These *HCl-amended* microcosms had an average initial pH value of 4.6, and the sterile control had a value of 4.2. Three repeat microcosm experiments and a sterile control were run using the ditch water sample obtained in June 2007. These were amended with $NaHCO_3$ solution and sodium acetate each to a final concentration of 20 mM. These *HCO_3^--amended* microcosms had an average initial pH value of 9.3, and the sterile control had a value of 9.2. The control microcosms were sterilised by heat treatment at 120 °C for a minimum of 20 min in an autoclave. All microcosm experiments and controls were incubated anaerobically at 21 °C in the dark. Microcosm experiments and controls were periodically sub-sampled for geochemical analysis to produce a progressive time series. During sampling soil microcosms were shaken and 3 ml soil slurry samples withdrawn using aseptic technique with sterile syringes and needles (Burke et al., 2006). Samples were centrifuged (5 min, 16,000 × *g*) and then pore waters and soils analysed for a range of redox indicators and Cr(VI) as below.

2.5. Freshwater growth media incubations

Soil was taken from the HCO_3^--amended microcosm experiments from a single bottle after 101 days incubation. Soil samples (0.1 g) were added to 100 ml of freshwater growth media at pH

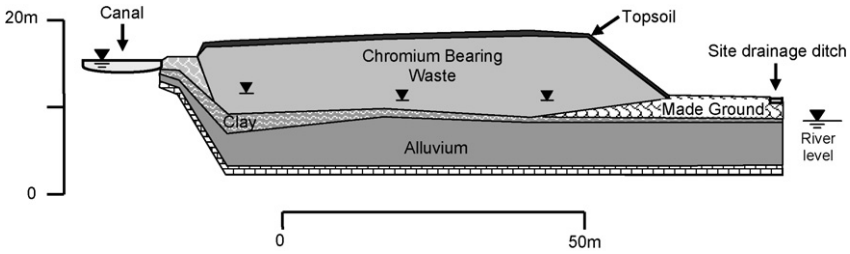

Fig. 3. Sketch diagram showing cross-section AA through the site (AA is shown in Fig. 1a).

7.3 in 120 ml glass serum bottles and sealed with butyl rubber stoppers and aluminium crimps. Each bottle also contained either 20 mM sodium acetate or 20 mM sodium lactate as the only electron donors, and either 10 mM iron(III) citrate or 250 μM potassium chromate as the only electron acceptor. Triplicate incubations were run for each electron donor/acceptor pairing.

2.6. Geochemical methods

Cr(VI) and total aqueous Fe were determined by standardised UV–vis spectroscopy methods (US-EPA, 1992; Viollier et al., 2000). Sulfate was determined by ion chromatography on a Dionex DX-500 with an AS14A analytical column, 1.0 mM sodium bicarbonate/8 mM sodium carbonate eluent at a flow rate of 1.2 ml/min, and PeakNet 5.11 software, except the measurements on the canal and March 2007 ditch water which were conducted using a turbidity method (Greenberg et al., 1992). Ca was determined by Atomic Absorption Spectroscopy on a Hitachi Z-5300. Fe(II) in solids was determined after extraction by 0.5 N HCl and reaction with Ferrozine™ (Lovley and Phillips, 1986). Standards were used regularly to check method quality and calibration linear regressions or quadratic fits normally produced *r*-squared values of 0.99 or better. Eh, pH and TDS readings were taken using Hanna or Orion bench-top meters and calibrated electrodes.

2.7. 16S rRNA gene sequencing

Microbial DNA was extracted from the grey clay (0.25 g) using a FastDNA spin kit and FastPREP instrument (Qbiogene, Inc.). DNA fragments in the size range 3 kb to ~20 kb were isolated on a 1% agarose "1×" Tris-borate-EDTA (TBE) gel stained with ethidium bromide for viewing under UV light (10× TBE solution supplied by Invitrogen Ltd., UK). The DNA was extracted from the gel using a QIAquick gel extraction kit (QIAGEN Ltd, UK); final elution was by 1/10th strength elution buffer (unless explicitly stated, the manufacturer's protocols supplied with all kits employed were followed precisely).

A fragment of the 16S rRNA gene of approximately ~500 bp was amplified by polymerase chain reaction (PCR) using broad-specificity bacterial primers in a Mastercycler gradient thermal cycler (Eppendorf, Germany). The DNA primers were 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Eden et al., 1991) and 519r (5′-GWATTACCGCGGCKGCTG-3′) (Lane et al., 1985). Each PCR reaction mixture contained 25 μl of purified DNA solution, 5 units of GoTaq DNA polymerase (Promega Corp., USA), 1× PCR reaction buffer, 1.5 mM $MgCl_2$ (already in the GoTaq reaction buffer), 0.2 mM PCR nucleotide mix (Promega Corp., USA), and 0.6 μM DNA primers in a final volume of 50 μl. The reaction mixtures were incubated at 94 °C for 4 min, and then cycled 30 times through three steps: denaturing (94 °C, 30 s), annealing (50 °C, 30 s), primer extension (72 °C, 60 s). This was followed by a final extension step at 72 °C for 7 min. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN Ltd, UK). 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 a standard cloning vector (p-GEM-T Easy supplied by Promega), transformed into super-competent *E. coli* cells (XL1-Blue from Stratagene), and colonies were grown on LB-agar plates containing ampicillin (100 μg ml^{-1}) surface dressed with IPTG and X-gal (as per the Stratagene protocol) for blue-white colour screening. Colonies containing the insert were re-streaked on LB-ampicillin agar plates, and 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) and sent for automated DNA sequencing (ABI 3100*xl* Capilliary Sequencer) using the T7P primer.

Sequences were analysed against the EMBL release nucleotide database in July 2007 using the NCBI-BLAST2 program and matched to known 16S rRNA gene sequences. Default settings were used for the BLAST parameters (match/mismatch scores 2, −3, open gap penalty 5, gap extension penalty 2). The nucleotide sequences described in this study are deposited in the GenBank database (accession numbers FM946038–FM946069).

2.8. Phylogenetic tree building

Gene sequences were aligned using the ClustalX software package and a phylogenetic tree constructed from the distance matrix by neighbour joining. Default alignment parameters were used for the alignment (gap opening penalty 15.0, gap extension penalty 6.66). Bootstrap analysis was performed with 1000 replicates. Phylograms were drawn using the TreeView software package.

3. Results

The ground investigation revealed that a typical vertical profile through the waste tip consists of about 0.5 m of topsoil overlying up to 8 m of alkaline chromium containing waste. Under the waste there is 6–8 m of brown slightly sandy slightly gravelly clay (the alluvium) with occasional gravel layers overlying siltstone/sandstone bedrock (millstone grit series). The bedrock elevation is fairly constant across the tip. Immediately under the waste there is a layer of soft dark brown clay that is up to 2.5 m thick and in places contains decayed vegetation (in borehole 1 the top 1.2 m of this layer was slightly grey in colour and friable in texture). Borehole logs and historical maps indicate that this was probably the original surface deposit, which may have been deposited from the river during over-bank flow. The upper horizons of the waste material are a grey to brown slightly clayey sand-sized material, whereas deeper horizons are a greenish yellow slightly clayey sand-sized material. Differences in the colour and composition (reported later) of these two materials are most likely consequences of the greater exposure of the near-surface waste to rainwater infiltration, so the upper and lower horizons will henceforth be referred to as weathered and unweathered waste, respectively. Groundwater

Table 1
Major elements in fused waste samples measured by XRF (corrected for loss on ignition at 1000 °C).

	SiO_2 (%)	Al_2O_3 (%)	Fe_2O_3 (%)	MgO (%)	CaO (%)	SO_3 (%)	Cr_2O_3 (%)	Mn_3O_4 (%)	LOI (%)
Weathered waste	4.14	5.33	5.91	4.11	26.77	12.50	3.70	0.05	37.00
Unweathered waste	3.61	4.27	7.04	5.85	40.29	5.10	4.93	0.07	28.40

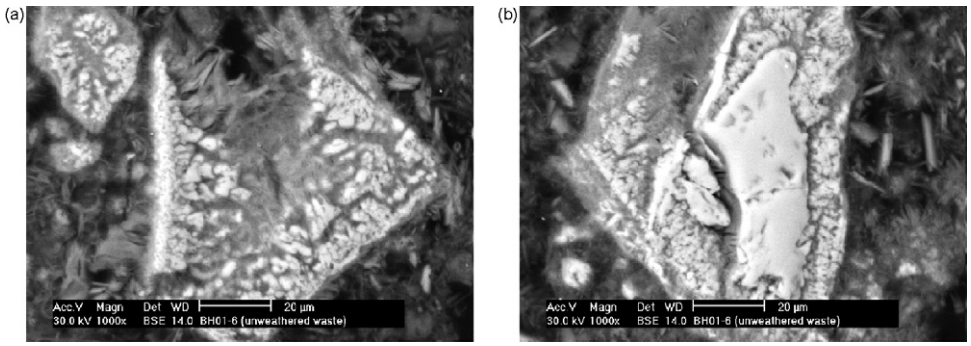

Fig. 4. Backscatter electron micrographs of the unweathered waste from BH01.

was encountered during drilling, rising to about 6 m below ground level within 20 min. A cross-section through the site is shown in Fig. 3.

The canal water sample had a pH of 7.11, a TDS of 87 mg L^{-1}, and no aqueous Cr(VI) was detected. The water sample taken from the site drainage ditch in March 2007 had a pH of 12.0, a Cr(VI) concentration of 225 μM (11.7 mg L^{-1}) and a TDS of 406 mg L^{-1}. The water sample taken from the site drainage ditch in June 2007 after a period of prolonged rainfall had a pH of 9.0, a Cr(VI) concentration of 131 μM (6.8 mg L^{-1}) and a TDS of 295 mg L^{-1}. The sulphate concentrations canal water and the March 2007 ditch water were 83 μM (8 mg L^{-1}) and 1.51 mM (145 mg L^{-1}), respectively.

3.1. Geochemical properties of the soil and waste samples

The chemical compositions of the weathered and unweathered waste (measured by XRF) are reported in Table 1. Calcium is the most abundant and sulphur the second most abundant constituent of the weathered waste (in a 3:1 molar ratio). Calcium is by far the most abundant constituent of the unweathered waste. The major crystalline phase in the weathered waste detected by XRD was calcite (calcium carbonate) with a minor quantity of quartz. The major crystalline phase detected in the unweathered waste was portlandite (calcium hydroxide).

Backscatter electron (BSE) micrographs of the unweathered waste are shown in Fig. 4. Particle morphology and image brightness were used to visually identify different phases within the waste (elements with a high atomic numbers have a high BSE luminescence). Most of the waste had a relatively dull appearance (similar to the background material in Fig. 4a and b). EDX analysis indicated that this was a calcium bearing phase with magnesium, and some sulphur. Particles with a bright appearance had two characteristic appearances. Most of the brighter particles exhibited a

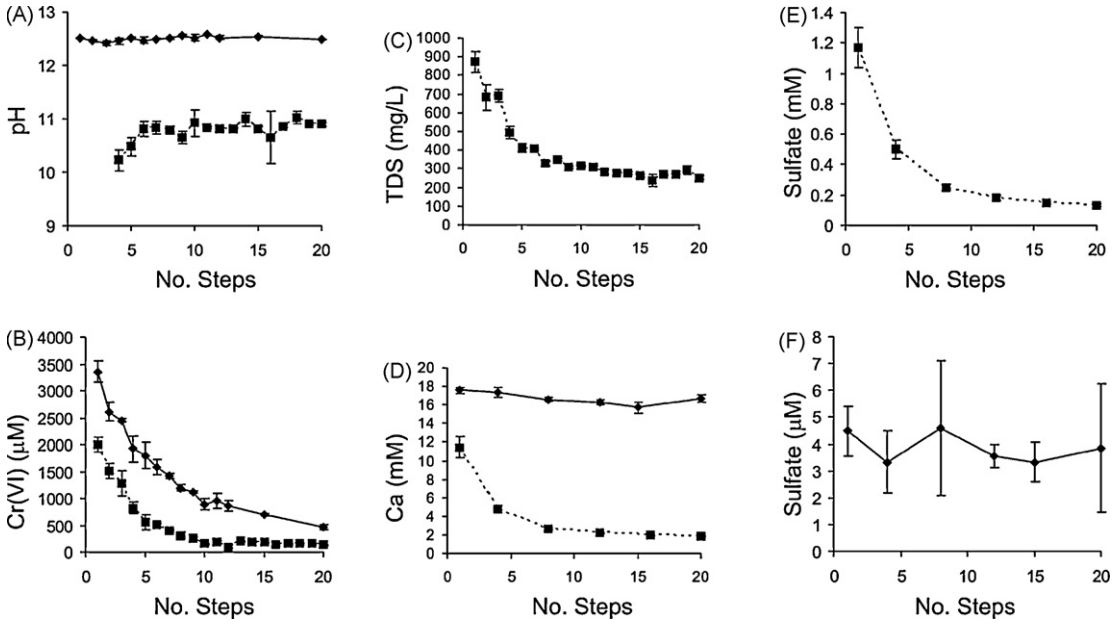

Fig. 5. Leaching properties of the chromium waste. Results for 1:1 solid:water leaches for unweathered (♦ – solid lines) and weathered (■ – dashed lines) wastes are shown. Error bars shown represent one standard deviation of triplicates.

Table 2
Leaching properties of the chromium waste.

	Predominant mineral phase (XRD analysis)	pH	Sequential 1:1 leaching tests with canal water							
			Step 1				Step 20			
			Cr(VI) (mM)	Ca^{2+} (mM)	SO_4^{2-} (mM)	TDS (mg L^{-1})	Cr(VI) (mM)	Ca^{2+} (mM)	SO_4^{2-} (mM)	TDS (mg L^{-1})
Weathered waste	Calcite	~11	1.98	11.25	1.17	870	0.13	1.88	0.13	250
Unweathered waste	Portlandite	12.5	3.37	17.50	0.004	–	0.46	16.75	0.004	–

Table 3
Properties of the clay from beneath the waste (XRF corrected for loss on ignition at 1100 °C).

	Mineralogy (XRD analysis)	pH	Major elemental composition, % (XRF analysis)							
			SiO_2	Al_2O_3	Fe_2O_3	CaO	K_2O	SO_3	Cr_2O_3	LOI
Grey clay	Quartz with traces of kaolinite and muscovite	10.5	71.41	9.73	3.47	2.29	1.56	0.22	0.44	9.30
Brown clay	Quartz with some kaolinite and muscovite	10.6	58.98	15.67	6.97	1.18	2.16	1.00	0.09	11.85

clear outline but were quite disrupted internally (Fig. 4a). EDX analysis of these particles must be treated with caution as continuous zones within the particles were smaller than area being analysed, but suggested that this was a calcium bearing phase, with some iron, magnesium and aluminium. A few bright particles appeared to be intact mineral particles (Fig. 4b). EDX analysis of one of these particles indicated that it contained chromium and magnesium in a roughly 2:1 molar ratio with smaller amounts of aluminium and iron.

In sequential 1:1 leaching tests on weathered waste with canal water (see Fig. 5 and Table 2), the pH of the supernatant was about 11, and the aqueous Cr(VI) concentration in the first step was about 2000 μM (103 mg L^{-1}) dropping to 1500 μM (79 mg L^{-1}) in the second step and decreasing steadily to just under 150 μM (7 mg L^{-1}) after 20 steps. In sequential 1:1 leaching tests on the unweathered waste the pH of the supernatant was 12.5, and the aqueous Cr(VI) concentration in the first step was almost 3400 μM (175 mg L^{-1}), dropping to about 2600 μM (136 mg L^{-1}) in the second step and decreasing steadily to just under 500 μM (24 mg L^{-1}) after 20 steps. TDS values are not reported for the unweathered waste as it is estimated from solution conductivity, which produces misleading values at very high pH (the hydroxyl anion has an effective electrophoretic mobility an order of magnitude higher than other anions).

XRD and XRF analysis of the grey clay recovered from BH1 (see Table 3) indicates the major mineral is quartz with small amounts of kaolinite and muscovite, and the XRF analysis indicates that there is 3020 mg kg^{-1} of chromium in the solid phase. The soil pH was 10.5 and 60 ± 20% of the acid extractable iron was Fe(II). A rough estimate of the total 0.5 N HCl extractable iron is approximately 2000 mg kg^{-1}, which can be considered as a proxy for the total amount of microbially available iron in soils (Lovley and Phillips, 1986). XRD and XRF of the brown clay recovered from BH8 indicates that the major mineral is also quartz with some kaolinite and muscovite (the brown clay has a higher clay content than the grey clay), and XRF analysis indicates that there is 630 mg kg^{-1} of chromium in the solid phase. The soil pH was 10.6. Elemental analysis of the grey and brown clays produced total organic carbon (TOC) values of 3.6% and 0.7%, respectively, and lower total inorganic carbon (TIC) values of 0.2% and 0.5%, respectively, indicating that the brown clay and, in particular, the grey clay contain significant amounts of soil organic matter.

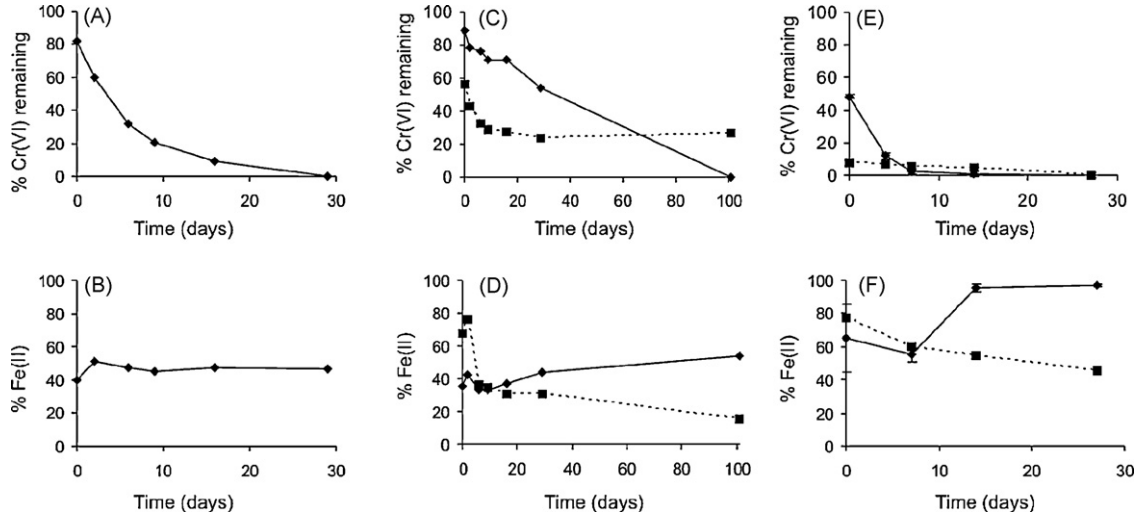

Fig. 6. Results of pH-amended and pH-unamended microcosms containing contaminated ditch water and soil from below the waste. Microbially active microcosms (♦ – solid lines) and heat-killed controls (■ – dashed lines) are shown. (A and B) show changes in Cr(VI) and Fe(II) in pH-unamended microcosm without acetate. (C and D) show changes in Cr(VI) and Fe(II) in pH-unamended microcosms with added sodium acetate. (E and F) show changes in Cr(VI) and Fe(II) in sodium bicarbonate pH-amended microcosms. Ditch water sample for microcosms shown in (A–D) taken in March 2007: C_o = 225 μM Cr(VI). Ditch water sample for microcosms shown in (E and F) taken in June 2007: C_o = 131 μM Cr(VI). Error bars shown in (E) and (F) represent one standard deviation of triplicates.

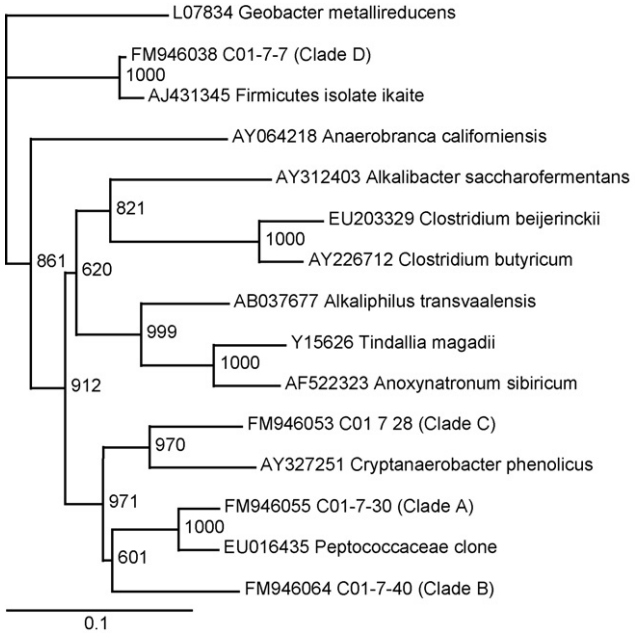

Fig. 7. Phylogenetic tree showing the relationship between representatives of four clades of bacterial sequences from the grey clay recovered from BH1 to 16S rRNA gene sequences of previously described bacteria. *Geobacter metallireducens* was included as an out-group. The scale bar corresponds to 0.1 nucleotide substitutions per site. Bootstrap values (from 1000 replications) are shown at branch points.

3.2. Microcosm incubations

The microcosm tests showed that when Cr(VI) contaminated ditch water is incubated with the grey clay from immediately below the waste that Cr(VI) was removed from solution in all experiments (see Fig. 6 and Table 4). In the HCl-amended microcosms (pH 4–5) complete Cr(VI) removal occurred rapidly on day 0. In the HCO_3^--amended microcosms (pH 8.5–9.5) about half the Cr(VI) was removed immediately and removal of Cr(VI) from solution was largely completed within 14 days (the initial Cr(VI) concentration in this test series was 40% lower than in the other microcosm experiments reported in Table 4). In these tests the proportion of acid extractable iron in the lower Fe(II) oxidation state increased with time. In the sterile control for the HCO_3^--amended microcosms most of the aqueous Cr(VI) was removed upon heat treatment, whereas the proportion of acid extractable Fe in the lower Fe(II) oxidation state decreased steadily with time.

In the pH-unamended microcosm without acetate (pH 10–11) about a fifth of the Cr(VI) was removed from solution immediately and the remaining Cr(VI) was removed over a period of 29 days. In this microcosm the extractable Fe(II) increased only slightly with time. In the pH-unamended microcosm containing acetate (pH 10–11) about a 10th of the Cr(VI) was removed immediately and the remaining Cr(VI) was removed from solution over a period of 101 days (it is inappropriate to ascribe too much importance to differences between the pH-unamended microcosms as only single replicates were conducted). Interestingly, however, a larger increase in extractable Fe(II) was observed over this longer duration test. In the pH-unamended control nearly 50% of the aqueous Cr(VI) was removed upon heat treatment, a further steady drop in aqueous Cr(VI) concentration occurred over time, and the proportion of acid extractable Fe in the lower Fe(II) oxidation state also decreased steadily with time.

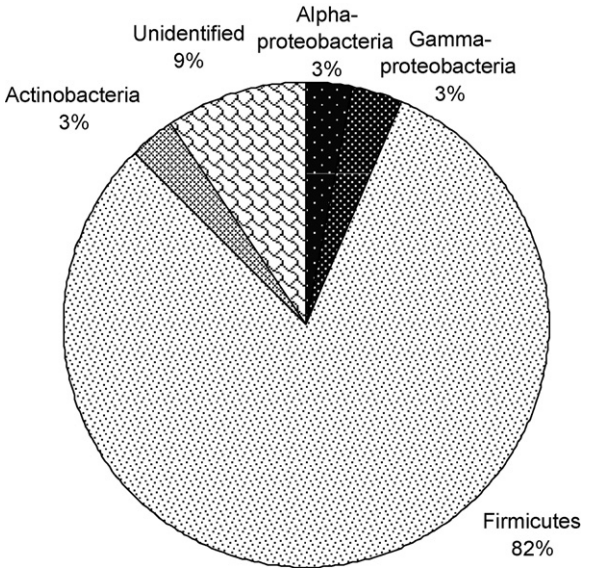

Fig. 8. Composition of the microbial community in the grey clay recovered from BH1 (32 clones).

3.3. Freshwater growth media incubations

All freshwater growth media incubations at pH 7.3 containing Fe(III) citrate with soil from the HCO_3^--amended microcosms scored positive for growth within 30 days (darkening of the media indicating conversion of Fe(III) to Fe(II); Collins et al., 1995). No growth was observed in media with potassium chromate as the only electron acceptor.

3.4. Microbiological community analysis

A total of 32 rRNA gene sequences were isolated from the grey clay. Blast analysis indicated that just 8 of these sequences had >95% sequence identity to a known sequence in the EMBL release database. This indicates that bacterial DNA can be recovered from the grey clay but, on its own, provides little information on the bacterial species present in this chromium contaminated highly alkaline environment. In fact the failure to match 16S rRNA gene sequences to sequences in the database may indicate that such environments have not been widely studied.

Initial ClustalX analysis and neighbour joining tree construction indicated that 25 of the sequences fell into four distinct clades. Clade A contained 15 sequences (including sequence C01-7-30), clade B contained four sequences (including C01-7-40), and clade C contained two sequences (including C01-7-28). Sequences in clades A, B and C had <95% identity to known sequences in the database. Four sequences (including C01-7-7) that had >96% identity to a firmicutes isolate were found to form a fourth clade (clade D). Fig. 7 has been constructed by further ClustalX analysis and neighbour joining tree construction where, for clarity of presentation, only a representative member of each clade was included in the analysis. The bacteria *Alkalibacter saccharofermentans*, *Alkaliphilus transvaalensis*, *Anaerobranca californiensis*, *Anoxynatronum sibiricum*, *Clostridium beijerinckii, Clostridium butyricum*, and *Tindallia magadii*, whose 16S rRNA gene sequences are used for comparison in the ClustalX analysis, are all anaerobic alkaliphiles in the order Clostridiales within the class Clostridia of the phylum Firmicutes (Kevbrin et al., 1998; Dobbin et al., 1999; Park et al., 2001; Takai et al., 2001; Garnova et al., 2003; Garnova et al., 2004; Gorlenko et al., 2004). *Cryptanaerobacter phenolicus* is a member of the family *Peptococcaceae* in the order Clostridiales. The δ–proteobacterium, *Geobacter metallireducens*, was included in the analysis as an outgroup. The phylogentic tree presented in Fig. 7 strongly suggests

Table 4
Results of pH-amended and pH-unamended microcosms containing contaminated ditch water and soil from below the waste.

Microcosm system	Time (d)	Cr(VI) (C/C_o)	%Fe(II)$_{(s)}$	Fe$_{(aq)}$ (μM)
HCl-amended @ pH 4–5[a]	0	n.d.	28 ± 7	360 ± 98
pH-unamended (no acetate) @ pH 10–11[a]	0	0.82	40	3.7
	29	n.d.	47	2.7
pH-unamended (with acetate) @ pH 10–11[a]	0	0.89	36	1.8
	101	n.d.	54	0.4
HCO_3^--amended @ pH 8.5–9.5[b]	0	0.48	54 ± 3	18 ± 1
	14	0.01	96 ± 2	18 ± 2

n.d. = not detected
[a] Made with March 2007 ditch water that contained 225 μmol L^{-1} Cr(VI) (used as C_o to normalise the data).
[b] Made with June 2007 ditch water that contained 131 μmol L^{-1} Cr(VI) (used as C_o to normalise the data).

that sequences in clades A, B and C, like clade D, belong to phylum Firmicutes, probably within the order Clostridiales. Thus it appears that members of the phylum Firmicutes may represent over 80% of the 16S rRNA gene sequences recovered from the grey clay (see Fig. 8).

4. Discussion

4.1. Waste composition

Electron microscopy has identified a mineral particle within the unweathered waste that contains chromium and magnesium in a roughly 2:1 molar ratio, along with smaller amounts of aluminium and iron. This composition is consistent with it being a particle of magnesiochromite (Guertin et al., 2005). Several other particles had an appearance and composition consistent with them being post-reaction relics of magnesiochromite. These particles are within a calcium, and to a lesser extent magnesium and sulphur, containing phase that XRD identified as predominantly calcium hydroxide (the leaching test results were consistent with calcium hydroxide controlling the pH value). Thus the chemical composition, mineralogy and leaching behaviour of the waste are consistent with it being the residue from producing chromate chemicals from chromite ore using the "high-lime" process (Darrie, 2001; Deakin et al., 2001a,b). Sulphur, which was extracted during the leaching tests as sulphate, is not an element usually found in the waste from the high-temperature chromite oxidation reaction, but a sulphate bearing waste is created in the purification stages of chromate chemical production which is often co-disposed with COPR (Gemmell, 1973).

Historical maps indicate that the waste was deposited on the site at the end of the 19th century, and is thus at least 100 years old. The high-lime process was the predominant commercial method producing chromium chemicals at that time (Darrie, 2001). At the time when the waste appeared on the maps there were two chemical works within a distance of less than 1 km of the site supplying chemicals to the textiles industries, and thus the production of chromium chemicals in the locality is quite probable.

COPR weathering consists of a cascade of dissolution and precipitation reactions, that starts with the calcium hydroxide and calcium aluminate phases in the fresh waste and tends towards carbonate minerals (Deakin et al., 2001a,b; Geelhoed et al., 2003; Moon et al., 2007). At any stage during this weathering cascade the phase undergoing dissolution controls the solution pH, and phases that are not soluble at the prevailing pH can precipitate out of solution. Thus hydrogarnet ($Ca_3Al_2(H_4O_4)_3$), hydrocalumite ($Ca_2Al(OH)_7{\cdot}3H_2O$) and, when sulphate is present, ettringite ($3CaO{\cdot}Al_2O_3{\cdot}3CaSO_4{\cdot}32H_2O$) are common weathering intermediates of COPR (Geelhoed et al., 2002).

The pH value and the elevated calcium concentration of the leachate from the unweathered waste indicate its pH value is probably being buffered by the dissolution of a calcium hydroxide (portlandite), and the most likely source for the chromate is calcium chromate co-precipitated in the calcium hydroxide (Deakin, 2002). The pH value and the elevated calcium and sulphate concentrations of the leachate from the weathered waste indicate its pH value is probably being buffered by the dissolution of a calcium alumina-sulphate phase such as ettringite (Cr-ettringite dissolves when the pH value is below 10.9; Terai et al., 2006). The chromate in the leachate is probably released by this ettringite (chromate, CrO_4^{2-}, and sulphate, SO_4^{2-}, are both tetrahedral divalent anions, so chromate can isomorphously substitute for sulphate in ettringite). At pH values around 11 calcium would readily precipitate with any carbonate in the leachate and this is probably the source of the calcite in the weathered waste.

The leaching tests on the unweathered waste are a guide to the composition of the pore water in the lower horizons of the waste pile (generally the unweathered waste underlies the weathered waste). Thus they are a guide to the composition of any groundwater that may be seeping downwards from the waste into the underlying soil. They also demonstrate that the waste will be slow to chemically stabilise in the environment, and has capacity to leach significantly elevated Cr(VI) concentrations after many pore water replacements. This may, in part, explain why the waste tip still poses a threat to the environment more than 100 years after it was created. The amount of unweathered waste still on site indicates that the waste tip will continue to pose a threat for the foreseeable future unless there is some form of remedial intervention.

The soil layers immediately beneath the waste have high pH values and elevated chromium contents. Consideration must be given to possibility that these are the results of mixing between the waste and the soil, either when the waste was tipped, or during soil sampling. However the grey clay sample, which has the highest chromium content, was recovered from nearly a metre below the waste/soil interface making widespread mixing unlikely. It is also unlikely that 'spot' contamination during sampling would result in the elevated soil pH value. As it has been clearly demonstrated that the grey clay can remove Cr(VI) from solution, the high pH values and elevated chromium contents of the grey and brown clay soils are most likely to be the result of pore water from the waste migrating downwards under a diffusive and/or advective gradient.

4.2. Mechanism of Cr(VI) removal from solution

The partial removal of Cr(VI) from solution on day 0 in all the microcosm experiments was probably the result of an abiotic reaction with the microbially available Fe(II) present in the soil (aqueous Cr(VI) reacts readily with Fe(II) and is reduced to insoluble Cr(III); Richard and Bourg, 1991; Stewart et al., 2007). The difference in

the amount of Cr(VI) that was removed immediately correlates roughly with the increasing solubility of sorbed Fe(II) at lower pH values. However, the source of this Fe(II) in the soil is, on first inspection, something of a mystery because, as a former near-surface layer, it would be expected that most of the amorphous iron would have originally been in the Fe(III) oxidation state prior to burial and prolonged exposure to aqueous Cr(VI) would be expected to readily oxidise any remnant Fe(II). In the HCO_3^--amended microcosms there was a significant increase in the proportion of acid extractable Fe as Fe(II) as time progressed and in the pH-unamended microcosms there was a small increase in the %Fe(II) over time which, taken together, are clear evidence that Fe(III) was being reduced within these alkaline microcosms. Both the HCO_3^--amended and the pH-unamended sterile controls exhibited steady decreases in the proportion of acid extractable Fe as Fe(II) with time. Thus it is inferred that the increase in acid extractable Fe(II) in these tests is most likely due to microbial iron(III)-reduction (Burke et al., 2005; Stewart et al., 2007). Furthermore, the growth media experiments inoculated from the HCO_3^--amended microcosms showed that a viable microbial consortia capable of Fe(III)-reduction could be extracted and grown. Thus it is reasonable to speculate that iron(III)-reduction is an on-going process in the soil immediately beneath the tip. This supposition is also supported by the existence of relatively high levels of organic matter in the former surface layers beneath the waste, particularly the grey clay, providing a carbon source to support bacterial growth.

An alternative explanation for the reduction of both Fe(III) and Cr(VI) in the microbially active microcosms is a reaction with reduced sulphur species produced by sulphate reducing bacteria (SRB). Certainly sulphate reducing bacteria have been found in the anoxic waters of naturally alkaline soda lakes (Scholten et al., 2005). However, while this study cannot eliminate this mechanism, it is thought less likely than that suggested above as none of the 16S rRNA gene sequences isolated from the grey clay were similar to known SRB 16S rRNA gene sequences.

4.3. Microbial community

A wide variety of bacteria grow optimally and robustly at pH values as high as 11 (Krulwich et al., 2001). Such alkaliphiles exhibit a broad range of physiology and metabolism (Jones et al., 1998). In the saturated subsurface environment, where there is adequate organic matter, conditions rapidly become anaerobic due to the activity of aerobic microorganisms. Depletion of oxygen then favours the proliferation of anaerobes including sulphate reducing bacteria, anaerobic fermenters and methanogens. Among the most frequently reported anaerobic alkaliphiles are fermentative Gram positive bacteria (Kevbrin et al., 1998; Dobbin et al., 1999; Park et al., 2001; Takai et al., 2001; Garnova et al., 2003; Garnova et al., 2004; Gorlenko et al., 2004).

Life at alkaline pH poses a number of challenges. Firstly the microbial cell wall must be stable and exteriorly facing enzymes must retain activity at this pH. Secondly, as most alkaliphiles have an optimum cytoplasmic pH for growth within 0.5 pH units of the optimum for most other bacteria (Krulwich, 2006), they must possess mechanisms for maintaining pH homeostasis. Thirdly they need to overcome the large proton counter-gradient and develop an electromotive driving force across the cytoplasmic membrane to energise solute transport, motility and adenosine triphosphate synthesis (ATP; the universal energy currency of cells). Many alkaliphiles use an Na^+ electrochemical gradient to maintain pH homeostasis, and to energize solute uptake and motility (Krulwich et al., 2001; Detkova and Pusheva, 2006). ATP can be synthesised by two mechanisms; respiration (which can be aerobic or anaerobic) and fermentation. During respiration protons are pumped from the cytoplasm across the membrane to generate a membrane potential that drives ATP synthesis (oxidative phosphorylation). In fermentation ATP is generated within the cytoplasm by enzyme-mediated reactions that transfer a phosphate group from the substrate to adenosine diphosphate to form ATP (substrate level phosphorylation) (Nelson and Cox, 2005). The means by which respiring alkaliphiles can generate a membrane potential when the external pH is higher that the cytoplasmic pH remains a topic of debate (Goto et al., 2005; Krulwich, 2006), however fermentative bacteria avoid the problem as they generate ATP within the cytoplasm (indeed it is an interesting possibility that very high pH may favour fermentative bacteria as they generate ATP inside the cell rather than from a proton gradient across the cell membrane). Many fermentative anaerobes can produce H_2 in addition to other fermentation products such as acetate, lactate, formate and ethanol (Gottschalk, 1986). H_2 evolution is principally a mechanism to optimise the thermodynamic efficiency of fermentation by providing an alternative pathway for reoxidising reduced coenzymes involved in fermentation. As an alternative optimisation strategy, some fermentative alkaliphiles in the order Clostridiales seem to maintain redox balance by dumping electrons externally to Fe(III) present in soils and sediments reducing it to Fe(II) (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). Dobbin et al. (1999) showed that the mechanism of Fe(III) reduction in *C. beirjerinckii* involved a membrane associated Fe(III) reductase.

The phylogenetic analysis of 16S rRNA extracted from soil bacteria indicated that 65% of the 16S rRNA gene sequences isolated from the grey clay were from bacteria in the order Clostridiales. These are all fermentative bacteria, and thus Fe(III) is probably being used as an electron sink to maintain redox balance during fermentative metabolism. However phylogenetic similarity of the 16S rRNA gene sequences isolated from grey clay to these alkaliphilic iron reducing bacteria should not be used to imply that they possess similar metabolic pathways, and elucidation of the species actually responsible for iron-reduction and the metabolic pathways involved, will require further work.

4.4. Engineering intervention

Two of the aims of any remedial engineering intervention at this site would be to prevent the chromate contaminated alkaline waste leachate from entering either the surface water or groundwater systems. The water emerging from the waste pile is likely to have at least two sources; water leaking from the canal, and rainwater. Both are likely to be contributing to the seepage, but the precise balance between these sources is unclear at this stage. However even if an impermeable capping layer is placed over the waste to reduce rainfall infiltration, this is unlikely to reduce the water flux to zero. It would be relatively easily to intercept any contaminated groundwater emerging from the tip and treat it. It will be less easy to prevent any water that enters the side of the waste from leaching downwards.

Fortuitously it appears that the former topsoil layer immediately beneath the waste is acting as a natural reactive zone as it is accumulating chromium. This natural barrier beneath this site will certainly reduce the impact of the waste on the wider environment. However its presence should not be taken for granted, as factors determining its long-term viability are unknown. For example an increase in the Cr(VI) flux may easily overwhelm its treatment capability, changes in the influent water may cause changes in the microbial population, and the available soil organic matter may

eventually be consumed, any of which may threaten its long-term survival.

5. Conclusions

The former top-soil layer beneath a 100-year-old COPR tip appears to be acting as a natural reactive zone preventing the downward movement of chromate leached from the waste. The mechanism is thought to be an abiotic reduction of Cr(VI) to Cr(III) by Fe(II) present in the soil. However it is thought that Fe(II) in the soil is being replenished by microbial iron(III) reduction despite the pH of 10.5.

Interestingly (Higgins et al., 1998) observed that an organic rich layer underlying COPR disposed at a site in New Jersey acted as a natural barrier to Cr(VI) migration. Although they did not directly demonstrate microbial activity, they inferred biological processes were important in this behaviour. Our study demonstrates that microbial Fe(III) reduction coupled to abiotic reduction of Cr(VI) is a likely mechanism. Together the two studies suggest that ecological engineering approaches to site remediation may be feasible where COPR waste has been deposited over organic rich layers.

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