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Publisher: Taylor & Francis

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Geomicrobiology Journal

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/ugmb20>

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Available online: 05 Mar 2012

To cite this article: Clare L. Thorpe, Gareth T. W. Law, Christopher Boothman, Jonathan R. Lloyd, Ian T. Burke & Katherine Morris (2012): The Synergistic Effects of High Nitrate Concentrations on Sediment Bioreduction, *Geomicrobiology Journal*, 29:5, 484-493

To link to this article: <http://dx.doi.org/10.1080/01490451.2011.581332>

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The Synergistic Effects of High Nitrate Concentrations on Sediment Bioreduction

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Groundwaters at nuclear sites can be characterized by low pH and high nitrate concentrations (10–100 mM). These conditions are challenging for bioremediation, often inhibiting microbial Fe(III)-reduction which can limit radionuclide migration. Here, sediment microcosms representative of the UK Sellafield site were used to study the influence of variable pH and nitrate concentrations on microbially-mediated TEAP (terminal electron accepting processes) progression. The rate of reduction through the terminal electron accepting cascade $\text{NO}_3^- > \text{NO}_2^- > \text{Mn(IV)/Fe(III)} > \text{SO}_4^{2-}$ at low pH (~ 5.5) was slower than that in bicarbonate buffered systems (pH ~ 7.0), but in the low pH systems, denitrification and associated pH buffering resulted in conditioning of the sediments for subsequent Fe(III) and sulfate-reduction. Under very high nitrate conditions (100 mM), bicarbonate buffering (pH ~ 7.0) was necessary for TEAP progression beyond denitrification and the reduction of 100 mM nitrate created alkaline conditions (pH 9.5). 16S rRNA gene analysis showed that close relatives of known nitrate reducers *Bacillus niacini* and *Ochrobactrum grignonense* dominated the microbial communities in this reduced sediment. In Fe(III)-reducing enrichment cultures from the 100 mM nitrate system, close relatives of the Fe(III)-reducing species *Alkaliphilus crotonatoxidans* and *Serratia liquifaciens* were observed. These results highlight that under certain conditions and contrary to expecta-

tions, denitrification may support bioreduction *via* pH conditioning for optimal metal reduction and radionuclide immobilization.

Keywords nitrate, bioreduction, iron reduction, radionuclides, microcosms

INTRODUCTION

The remediation of radioactively contaminated land in the UK is of immediate concern due to the ongoing decommissioning of British nuclear sites. Further, there is a need for solutions to existing contaminant problems prior to the possible onset of new nuclear power. At the Sellafield nuclear reprocessing site in Cumbria, mobile groundwater contaminant radionuclides include ⁹⁹Tc and ⁹⁰Sr, and groundwater co-contaminants include nitrate (from nitric acid), organic acids, and pH variance (BNFL 2003; McKenzie and Armstrong-Pope 2010).

Similar contamination issues have been documented at a range of US nuclear sites (e.g., Oak Ridge, TN (Edwards et al. 2007; Istok et al. 2004; Li and Krumholz 2008; McBeth et al. 2007), San Juan River, Shiprock, NM (Finneran et al. 2002), and Hanford, WA (Singleton et al. 2005)). A proposed *in situ* strategy to remediate contaminants at such sites is “biostimulation.” Here, an electron donor is added to the subsurface to stimulate the indigenous microbial community, promoting a cascade of terminal electron accepting processes (TEAPs) that favour radionuclide removal from groundwaters (Lloyd and Renshaw 2005; Lovley and Coates 1997).

This approach has been shown to reduce the mobility of redox-active radionuclides such as ⁹⁹Tc and U, *via* the reduction of soluble oxidized species (Tc(VII), U(VI)) to poorly-soluble reduced species (Tc(IV), U(IV)) (Edwards et al. 2007; Istok et al. 2004; Law et al. 2010; McBeth et al. 2007; Morris et al. 2008). It may also be possible for bioreduction to occur in sediments via natural attenuation without electron donor addition (Alvarez

Received 9 February 2011; accepted 7 April 2011.

We thank David Ashley (University of Leeds) and John Waters (University of Manchester) for help in data acquisition and Chris Hubbard and Sarah Wallace for help with sample collection. This work was funded by the Engineering and Physical Science Research Council (EPSRC) as part of the Decommissioning, Immobilization and Management of Nuclear Waste for Disposal (DIAMOND) consortium grant EP/F055412/1. We also acknowledge support of NERC grant NE/H007768/1.

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et al. 2006; Burke et al. 2010; Manaka et al. 2007). Regardless, in most cases, radionuclide reduction is associated with microbially mediated Fe(III) reduction (Lloyd 2003; Lloyd and Renshaw 2005).

As a consequence, the actions of Fe(III)-reducing bacteria and subsequent changes in Fe redox chemistry and Fe mineralogy are likely to play a key role in governing the mobility of redox-active radionuclides. Furthermore, changes in Fe mineralogy have the potential to affect the sorption and mobility of other (non-redox-active) radionuclides e.g., ^{137}Cs or ^{90}Sr (Chiang et al. 2010; Langley et al. 2009; Roden et al. 2002).

However, the comparatively low groundwater pH conditions and/or high nitrate concentrations that can characterize nuclear sites represent challenging bioremediation scenarios. Low pH decreases microbial diversity and metabolic function (Baker and Banfield 2003; Robinson et al. 2009) whilst nitrate is an energetically more favourable electron acceptor than Fe(III) (and redox active radionuclides) and thus can inhibit TEAP progression and reductive immobilization of radionuclides (DiChristina 1992).

Indeed, whilst some studies demonstrate microbially-mediated metal reduction in the presence of high nitrate (Madden et al. 2007), numerous sediment microcosm studies indicate that microbially mediated metal and radionuclide reduction does not commence until nitrate and nitrite are reduced (e.g. Burke et al. 2005; Edwards et al. 2007; Law et al. 2010; Li and Krumholz 2008; McBeth et al. 2007; Wilkins et al. 2010). Further, some biostimulation studies with low pH sediments have demonstrated that the extent of nitrate removal is strongly pH dependant, with NaHCO_3 or crushed lime amendment necessary to stimulate bioreduction and TEAP progression (Edwards et al. 2007; Michalsen et al. 2009; North et al. 2004).

Conversely, in field studies, dual denitrification and metal reduction was observed at low-pH (Istok et al. 2004) and in microcosm studies, denitrification and associated pH buffering (*via* OH^- and HCO_3^- production) has been shown to stimulate TEAP progression to metal reduction (Law et al. 2010). Clearly, the variable effects of low-pH and nitrate on electron flow warrant further study and here, electron flow in representative Sellafield sediments was studied under a range of environmentally relevant nitrate (0.4–100 mM), pH, and carbonate conditions.

EXPERIMENTAL

Sample Collection

Sediments representative of the Quaternary unconsolidated alluvial flood-plain deposits that underlie the UK Sellafield re-processing site (Law et al. 2010) were collected from the Calder Valley, Cumbria, during December 2008 (herein called Sellafield sediment). The sampling area was located ~ 2 km from the Sellafield site (Lat $54^\circ 26' 30$ N, Long $03^\circ 28' 09$ W). Sediments were transferred directly into sterile containers, sealed, and stored at 4°C . Experiments began within 6 months of field sampling.

Bioreduction Microcosms

Sediment microcosms (10 ± 0.1 g Sellafield sediment, 100 ± 1 ml groundwater) were prepared using a synthetic groundwater representative of the Sellafield region, with background nitrate present at ~ 0.4 mM (Law et al. 2010; Wilkins et al. 2007). The groundwater was manipulated to produce a range of treatments (Table 1). Bicarbonate-free systems with an initial pH of ~ 5.5 were prepared with 0.4, 2, 10, and 100 mM nitrate. Bicarbonate buffered systems with an initial pH of 6.8 were prepared with 0.4, 10, and 100 mM nitrate.

Sodium acetate was added as an electron donor in excess of extant available electron acceptors (14 mM for 0.4–10 mM nitrate treatments, and 70 mM for 100 mM nitrate treatments) and anoxic NaNO_3 was used as a NO_3^- source. The groundwater was sterilized by autoclaving (1 hour at 120°C), and then bubbled with filtered 80/20 N_2/CO_2 , and the pH set *via* dropwise addition of 0.5 M HCl or 1M NaOH. Sediment and sterile groundwaters were added to sterile 120 ml glass serum bottles (Wheaton Scientific, USA) using aseptic technique and sealed with butyl rubber stoppers.

All microcosms were then incubated anaerobically at 21°C in the dark for 80–230 days and each treatment was run in triplicate. Throughout the incubation, sediment slurry was periodically extracted under an O_2 -free Ar atmosphere using aseptic technique. The extracted sediment slurry was centrifuged (15,000 g; 10 minutes) to provide separate sediment and pore-water samples and ~ 0.5 g of sediment was stored at -80°C

TABLE 1
Initial composition of microcosm systems

System name	Amendment	Nitrate	pH
0.4 mM nitrate	None	0.4 mM NaNO_3	~ 5.5
2 mM nitrate	None	2 mM NaNO_3	~ 5.5
10 mM nitrate	None	10 mM NaNO_3	~ 5.5
100 mM nitrate	None	100 mM NaNO_3	~ 5.5
Bicarbonate buffered 0.4 mM nitrate	3 mM NaHCO_3 and OH^-	0.4 mM NaNO_3	6.8–7.0
Bicarbonate buffered 10 mM nitrate	3 mM NaHCO_3 and OH	10 mM NaNO_3	6.8–7.0
Bicarbonate buffered 100 mM nitrate	3 mM NaHCO_3 and OH	100 mM NaNO_3	6.8–7.0

TABLE 2
Details of Fe extraction series (Poulton and Canfield 2005)

Fraction	Extraction	pH	Time
Carbonate associated	1 M sodium acetate	4.5	24 hours
Easily reducible oxides	1 M hydroxylamine HCl in 25 % v/v acetic acid		48 hours
Reducible oxides	50 gL ⁻¹ sodium dithionite	4.8	2 hours
Magnetite	0.2 M ammonium oxalate	3.2	6 hours
Residual Fe	XRF	N/A	N/A

for microbiological characterization. Sediments from the initial and final time points of each treatment underwent a sequential extraction procedure to assess changes in Fe mineralogy during biostimulation (Poulton and Canfield 2005; Tessier et al. 1979). Sequential extractions procedures targeted: i) carbonate associated Fe; ii) easily reducible oxides; iii) reducible oxides; iv) magnetite; and, v) residual Fe (Table 2). These extractions comprised i) 1 M sodium acetate (pH 4.5); ii) 1 M hydroxylamine HCl; iii) sodium dithionite—sodium citrate (pH 4.8); iv) 0.2 M ammonium oxalate (pH 3.2); and, v) residual Fe was determined by XRF minus the extracted phases (Poulton and Canfield 2005). The sediment to solution ratio was 0.1 g in 10 ml (1:100) at each stage.

Geochemical Analyses

During microcosm sampling, total dissolved Fe, Mn(II), and NO₂⁻ concentrations were measured with standard UV-Vis spectroscopy methods on a Cecil CE 3021 spectrophotometer (Goto et al. 1997; Harris and Mortimer 2002; Viollier et al. 2000). Aqueous NO₃⁻, SO₄²⁻, and acetate were measured by ion chromatography (Dionex ICS-90) (Burke et al. 2005). Ammonium was measured by flow injection analysis (Dionex ICS-90) (Hall and Aller 1992). Total bioavailable Fe(III) and the proportion of extractable Fe(II) in the sediment was estimated by digestion of 0.1 g of sediment in 5 ml of 0.5 N HCl for 60 minutes followed by the ferrozine assay, with and without hydroxylammonium HCl (1.4M H₂NOH.HCl in 2M HCl) (Lovley and Phillips 1987; Stookey 1970; Viollier et al. 2000).

The pH and Eh were measured with an Orion 420A digital meter and calibrated electrodes. Standards were routinely used to check the reliability of all methods and calibration regressions had R² ≥ 0.99. The elemental composition and bulk mineralogy of the sediment were determined by XRF (Thermo ARL 9400 XRF) and XRD (Philips PW 1050 XRD).

Microbial Community Analysis

Selected samples from bicarbonate-free microcosms containing 0.4 and 10 mM nitrate and bicarbonate buffered microcosms containing 100 mM nitrate underwent PCR-based 16S

rRNA gene analysis. Additionally, sub-aliquots of sediment slurry from the 100 mM nitrate treatment were added (1:10 sediment/solution ratio) to an Fe(III)-citrate containing medium (Lovley and Phillips 1986) with 20 mM acetate or 0.2% (w/v) yeast extract as an electron donor, to make an enrichment culture to identify the microorganisms responsible for Fe(III) reduction at pH > 9. Enrichment cultures were incubated at 20° C for 4–5 weeks before further sub-aliquots were transferred (1:10 sediment/solution ratio) to fresh Fe(III)-citrate medium. This procedure was repeated 7 times and then finally 16S rRNA gene analysis was used to identify the species present in the final enrichment. XRD was used to analyse the mineralogical products of Fe(III) reduction in the enrichment systems.

Amplification of 16S rRNA Gene Sequences

DNA was extracted from samples using a PowerSoil DNA Isolation Kit (MO BIO, USA). Copies of the 16S rRNA gene (approximately 1490 b.p. fragment) was amplified from samples using the broad-specificity primers 8F (Eden et al. 1991) and 1492R (Lane et al. 1985). PCR reactions were performed in thin-walled tubes using a BioRad iCycler (BioRad, UK). The PCR amplification protocol used with the 8F and 1492R primers was: initial denaturation at 94°C for 4 minutes, melting at 94°C for 30 seconds, annealing at 57°C for 30 seconds, elongation at 72°C for 1 minute; 35 cycles, followed by a final extension step at 72°C for 10 minutes. The purity of the amplified products was determined by electrophoresis in a tris-acetate-EDTA (TAE) gel. DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, UK).

Cloning

PCR products were purified using a QIAquick PCR purification kit (Qiagen, UK) and ligated directly into a cloning vector containing topoisomerase I-charged vector arms (Agilent Technologies, UK) prior to transformation into *E. coli* competent cells expressing Cre recombinase (Agilent Technologies, UK). White transformants that grew on LB agar containing ampicillin and X-Gal were screened for an insert using PCR. Primers were complementary to the flanking regions of the PCR insertion site of the cloning vector. The PCR method used was: an initial denaturation at 94°C for 4 minutes, melting at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute; 35 cycles, followed by a final extension step at 72°C for 5 minutes. The resulting PCR products were purified using an ExoSap protocol, and 2 µl of ExoSap mix (0.058 µl Exonuclease I, 0.5 µl Shrimp Alkaline Phosphatase, and 1.442 µl QH₂O) was added to 5 µl of PCR product and incubated at 37°C for 30 minutes followed by 80°C for 15 minutes.

DNA Sequencing and Phylogenetic Analysis

Nucleotide sequences were determined by the dideoxynucleotide method. An ABI Prism BigDye Terminator Cycle Sequencing Kit was used in combination with an ABI Prism 877

Integrated Thermal Cycler and ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems, UK). Sequences (typically 900 base pairs in length) were analysed against the NCBI (USA) database using the BLAST program packages and matched to known 16S rRNA gene sequences.

RESULTS AND DISCUSSION

Sediment Characteristics

The mineral content of the sediment as sampled was dominated by quartz, feldspars (albite and microcline), and sheet silicates (muscovite and chlorite). The sediment had a high Si content (33.2 wt %) and contained Al (5.9%), Fe (4.2%), K (2.6%), Na (1.1%), Mg (<0.1 %), Ti (0.4%), Ca (0.14%), and Mn (<0.1%). The concentration of 0.5 N HCl extractable Fe in the sediment was 5.6 ± 0.5 mmol kg⁻¹ prior to incubation and the sediment pH was ~5.5.

Progressive Bioreduction in Bicarbonate-Free Systems

In bicarbonate-free systems with initially low pH (5.5) and with varying initial nitrate concentrations (Table 1), microbially mediated TEAP progression was monitored as bioreduction developed. Microbial activity was observed in all electron donor amended microcosms (Figure 1), whereas no biogeochemical changes were observed in sterile-controls (data not shown). Electron acceptor utilization was observed in the order NO₃⁻, NO₂, Mn(IV), Fe(III) and SO₄²⁻ as indicated by changes in the relevant biogeochemical indicators (Figure 1).

Furthermore, Eh decreased during TEAP progression and acetate was removed from porewaters (Table 3). As expected, the onset of microbially-mediated Mn(IV) and Fe(III) reduction was largely inhibited until nitrate and nitrite were removed *via* denitrification. The inhibition time was dependent on the initial nitrate concentration with 0.4, 2, and 10 mM nitrate removed by 14, 18 and 25 days, respectively, and with the onset of metal reduction indicated by increased Fe(II) in sediments, occurring immediately afterwards (Figure 1).

Interestingly, the rates of Mn(IV) and Fe(III) reduction were increased, compared to the low nitrate systems after nitrate had been removed from microcosms in the systems with higher nitrate additions. For example, in the 0.4, 2, and 10 mM nitrate systems, essentially complete Fe(III) reduction was seen at ~50 days despite the delay in onset of Fe(III) reduction in the 10 mM system compared to the lower nitrate concentration experiments. By contrast, the 100 mM nitrate, bicarbonate-free system appeared to be overwhelmed by the competing electron acceptor and although substantial nitrate reduction had occurred, 60 mM nitrate remained in solution after 230 days incubation and no evidence for Fe(III) reduction was observed (Figure 1).

Previous work has reported an increase in Fe(III) reduction rates in low pH sediments following nitrate reduction and attributed this to a rise in pH due to OH⁻ and HCO₃⁻ production during denitrification (Law et al. 2010). In this study the pH in bicarbonate-free systems with an initial pH of 5.5 and nitrate

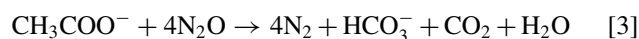
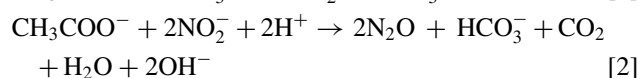
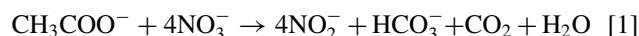
concentrations of 0.4, 2 and 10 mM, increased to pH 6.8, 7.0, and 7.5 respectively (Figure 1). Thus, the pH adjustment from pH ~5.5 to circumneutral caused by nitrate reduction apparently stimulated metal reduction in these sediments. This is consistent with the fact that the diversity and metabolic function of neutrophilic metal reducers is decreased at low pH (Lloyd 2003; Reardon et al. 2004; Fields et al. 2005; Edwards et al. 2007).

In these microcosms, reduction of even relatively low concentrations of nitrate (0.4 mM) were sufficient to increase pH to a region where Fe(III) reduction became viable. By contrast, in the bicarbonate-free system with 100 mM nitrate, nitrite accumulation in the microcosm was almost stoichiometric with respect to observed nitrate reduction. This implied that nitrite remained unreduced in this system which “stalled” at ~40% nitrate removal (Figure 1).

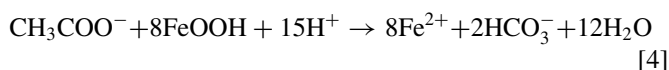
Several studies have demonstrated the increased toxicity of nitrite with decreasing pH and this is likely due to the presence of nitrous acid (HNO₂) at low pH entering the cell and interfering with the pH gradient across the cell membrane (Weon et al. 2002; Zhou et al. 2007; Zhou et al. 2010). Thus, stoichiometric accumulation of nitrite combined with the low initial pH (5.5) of sediments suggests that nitrite toxicity may be an issue for this system although, interestingly after extended (230 days) incubation, nitrate and nitrite levels did appear to fall and pH did rise (Figure 1).

Bioreduction Pathways

Calculations based on acetate consumption compared to nitrate reduction, combined with only a minor amount of ammonia being detected in the bioreduced microcosms suggest that denitrification to N₂ or N₂O is the dominant pathway for nitrate reduction in these systems (Table 3). Equations for the 5 electron transfer from NO₃⁻ to N₂ coupled to acetate oxidation show the production of OH⁻ during NO₂⁻ reduction to N₂O with HCO₃⁻ produced at all stages (Equations 1–3) and in agreement with the observed rise in pH in our systems.



Metal reduction then followed nitrate reduction with 0.5 N HCl extractable Fe(II) ingrowth to sediments observed followed by accumulation of Mn in porewaters (Figure 1). Although the pH rose most steeply during nitrate reduction, the pH in all microcosms continued to trend upwards during Fe(III) reduction consistent with continued consumption of H⁺ and release of HCO₃⁻ during Fe(III) oxide reduction coupled to acetate oxidation (Equation 4).



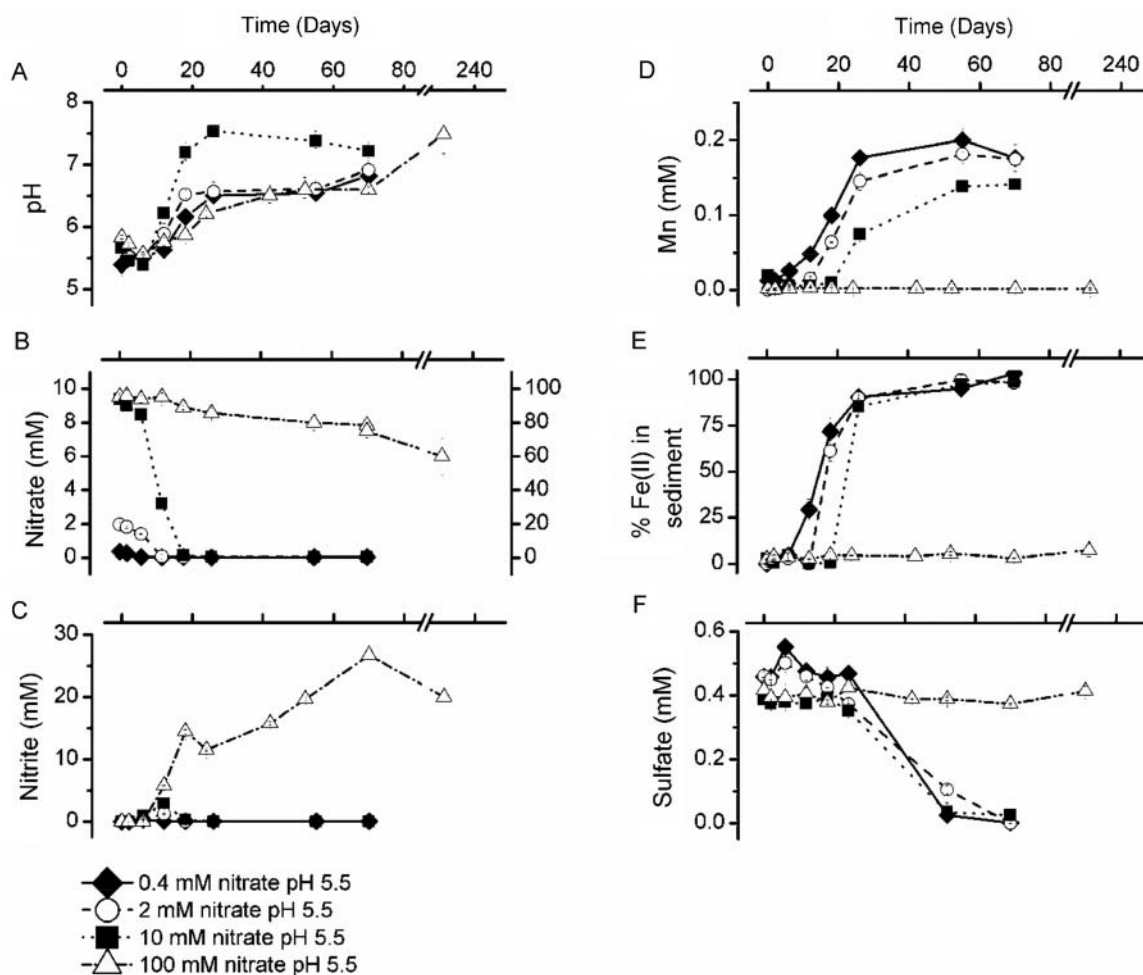
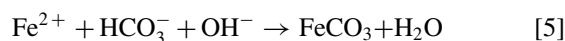


FIG. 1. Microcosm incubation time-series data (days 0-230). (A) pH, (B) NO_3^- , (C) NO_2^- , (D) porewater Mn, (E) 0.5 N HCl% extractable sedimentary Fe as Fe(II), (F) porewater SO_4^{2-} . Black diamonds = 0.4 mM nitrate system; unfilled circles = 2 mM nitrate system; black squares = 10 mM nitrate system; unfilled triangles = 100 mM nitrate system. Initial pH in all microcosms was ~ 5.5 . Error bars represent 1σ experimental uncertainty from triplicate microcosm experiments (where not visible error bars are within symbol size).

Interestingly, sequential extractions conducted on sediment from the bicarbonate buffered system with 10 mM nitrate before and after bioreduction suggest an increase in the “carbonate fraction” coupled to a reduction in the “easily reducible” fraction in the sediments after bioreduction (Figure 2). The final pH in these systems was between pH 7.5 and 8. This is consistent with observations that Fe^{2+} , alkalinity, and HCO_3^- all favour the formation of carbonate minerals such as siderite (Equation 5) (Coleman et al. 1993; Roden et al. 2002).



Microbial Community Analysis in Bicarbonate-Free Systems

The microbial ecology of the pH ~ 5.5 microcosms was assessed by 16S rRNA gene analysis at key points as bioreduction

progressed. Analysis of the oxic sediment revealed a diverse population with 11 different phyla and 59 distinct organisms detected in 73 clones. The clone library was dominated by species from the phylum *Acidobacteria* ($\sim 50\%$) with close relatives of *Bacillus* species present ($\sim 7\%$) (Figure 3). This is similar to past work with Sellafeld-type sediments where *Acidobacteria* also dominated the clone libraries (Law et al. 2010).

When the 0.4 mM nitrate system had undergone nitrate and Fe(III) reduction (at day 50) the microbial community had changed and comprised 11 different phyla and 71 distinct sequences from the 83 clones analysed. Members of *Clostridiales* now made up $\sim 17\%$ of the clone library and *Acidobacteria* only $\sim 21\%$ (Figure 3). Organisms affiliated with the *Clostridiales* order included close relatives of known Gram-positive metal-reducing species *Desulfosporosinus sp.* S8 and *Desulfobacterium metallireducens* (Robertson et al. 2000; Spring and Rozenzweig 2006), which have been isolated as key

TABLE 3
pH, Eh and acetate utilization data

System	pH		Eh		Acetate (mM)		NH ₄ ⁺ (mM) Max. in porewaters
	Initial	Final	Initial	Final	Utilized during nitrate reduction	Required for denitrification to N ₂	
0.4 mM nitrate	5.5	6.8	+187	-86	0.22 ± 0.01	0.25	—
2 mM nitrate	5.5	6.95	+240	-67	2.58 ± 0.06	1.25	—
10 mM nitrate	5.5	7.25	+273	-62	7.25 ± 0.32	6.25	<0.5
100 mM nitrate	5.5	6.5-8	+184	+166	17.3 ± 0.45*	62.2	—
Bicarbonate buffered 0.4 mM nitrate	7	7.2	+274	-57	1.63 ± 0.15	0.25	—
Bicarbonate buffered 10 mM nitrate	7	7.5	+274	-20	8.23 ± 0.32	6.25	<0.7
Bicarbonate buffered 100 mM nitrate	7	9.3	+286	50	86.4 ± 4.56	62.2	—

Errors are 1 σ of triplicate measurements. *reduced only ~40% of nitrate.

metal-reducing bacteria in high nitrate sediments at Oak Ridge, TN (Li and Krumholz 2008; Shelobolina et al. 2003).

Also present in the clone library were species of the known Fe(III)-reducing genus *Geobacter* and the known nitrate-reducing genus *Bacillus*. In contrast, when the bicarbonate-free 10 mM nitrate system had undergone nitrate and Fe(III) reduction (50 days), the diversity was very much reduced even compared to the 0.4 mM system at 50 days, and 87% of the

clone library (76 of 87 clones sequenced) comprised of close relatives (>99%) of *Bacillus niacini* (Figure 3).

Bacillus niacini has been shown to reduce nitrate to nitrite under anaerobic conditions (Nagel and Andreesson 1991) and close relatives have been identified in nitrate amended sediments at a uranium waste tailing site and in Sellafield-type sediments (Law et al. 2010; Selenska-Pobell and Geissler 2008). These results suggest a trend toward much lower microbial diversity

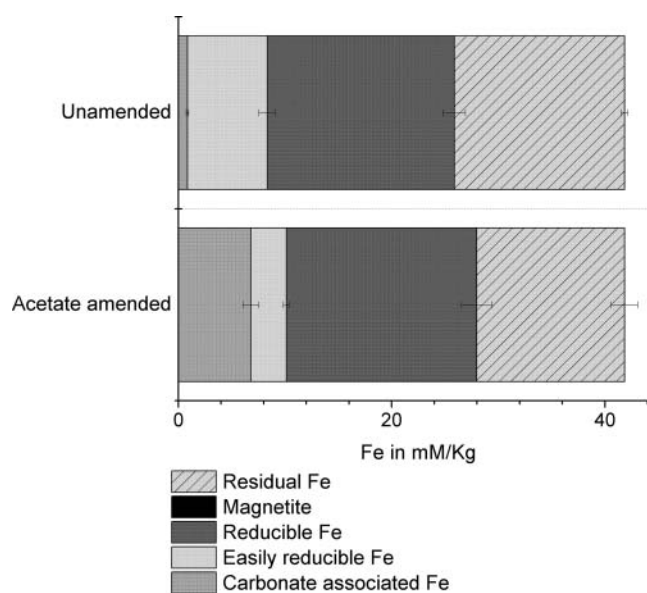


FIG. 2. Sequential extraction data comparing the Fe mineralogy of bicarbonate buffered 10 mM nitrate reduced sediments with that of oxic sediment. Dark grey = carbonate associated Fe; light grey = easily reducible Fe oxides; very dark grey = reducible oxides; black = magnetite; striped = residual Fe as determined by XRF.

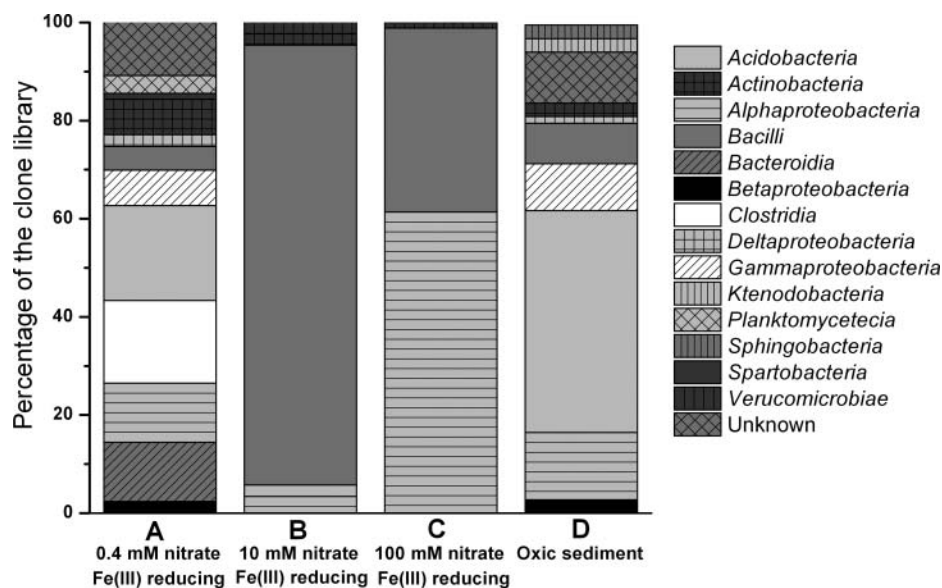


FIG. 3. Microbial community analysis of (A) Fe(III)-reducing bicarbonate-free sediment with 0.4 mM initial nitrate ($T = 50$), (B) Fe(III)-reducing bicarbonate-free sediment with 10 mM initial nitrate ($T = 50$), (C) Fe(III)-reducing bicarbonate-free sediment with 100 mM initial nitrate ($T = 70$) and (D) oxic sediment.

as initial nitrate concentrations increase, with a close relative (>99%) of *Bacillus niacini* suggested as a key, acid tolerant nitrate-reducing organism in systems with elevated nitrate, and with *Gram-positive* species potentially significant in mediating Fe(III) reduction.

Progressive Bioreduction in Bicarbonate Buffered Systems

When systems were buffered with bicarbonate to pH 7 to stimulate bioreduction, there was a general increase in the rate of bioreduction compared to the unbuffered pH 5.5 microcosms. For example, in the bicarbonate buffered 0.4 and 10 mM nitrate systems, extensive Fe(III)-reduction, indicated by ~100% 0.5 M HCl extractable Fe converted to Fe(II), was observed by 21 days compared to 50 days in the parallel unbuffered system (Figures 1 and 4). Interestingly, although the microbial community was unable to reduce 100 mM nitrate at pH 5.5 (Figure 1), when the pH was buffered to circumneutral prior to incubation, the system was able to facilitate complete reduction of 100 mM nitrate by 70 days and metal reduction commenced thereafter (Figure 4).

Nitrite in this system was transient and it is probable that the higher initial pH reduced the toxicity of nitrite in this system and thereby allowed nitrite metabolism to proceed (Zhou et al. 2010). Development of metal-reducing conditions in microcosms with very high nitrate is variable with some studies reporting development of Fe(III)-reduction in 100 mM nitrate, carbonate buffered experiments (Edwards et al. 2007), whilst other workers observed only partial reduction of 100 mM nitrate and no development of Fe(III)-reducing conditions (McBeth et al. 2007). Interestingly, in dynamic push-pull tests at the Field Research Centre in Oak Ridge Tennessee, electron

donor amendment and pH neutralization was needed to reduce > 100 mM nitrate (Istok et al. 2004; North et al. 2004). In the bicarbonate buffered experiments, pH increased from pH 7.0 to 7.2, 8.1 and 9.5 for systems with 0.4, 10 and 100 mM nitrate respectively, and as expected the onset of metal-reducing conditions was delayed as the initial nitrate concentration increased. For example, complete reduction of 0.5 N HCl extractable Fe(III) took 18, 25 and 230 days in the 0.4, 10 and 100 mM nitrate systems respectively (Figure 4). Indeed, the observation of Fe(III)-reduction in microcosms where pH was greater than 9 is interesting in terms of the microbial tolerance of the system across pH 5.5–9. Indeed, there are few published studies on metal reduction in alkaline sediments and the majority of available studies focus on halophillic species from alkaline soda lakes (Gorlenko et al. 2004; Pollock et al. 2007).

Only a few species including *Alkaliphilus metalireducens* and *Anaerobranca californiensis* have been isolated and shown to reduce Fe(III) above pH 9 (Gorlenko et al. 2004; Ye et al. 2004). More recently, Fe(III) reduction has been demonstrated in a highly contaminated, high pH chromium waste site in the UK (Stewart et al. 2010). In our study, sequence analyses of amplified 16S rRNA genes showed that during Fe(III) reduction after incubation for 70 days the bicarbonate buffered 100 mM nitrate system had a restricted clone library with only 5 different species detected in 88 clones.

The system was dominated by a close relative (>99% sequence homology) of *Ochrobactrum grignonense* strain c259 (59% of the clones) with a close relative (>99% sequence homology) of *Bacillus niacini* also significant at ~37% of the clone library (Figure 3). *Ochrobactrum grignonense* is

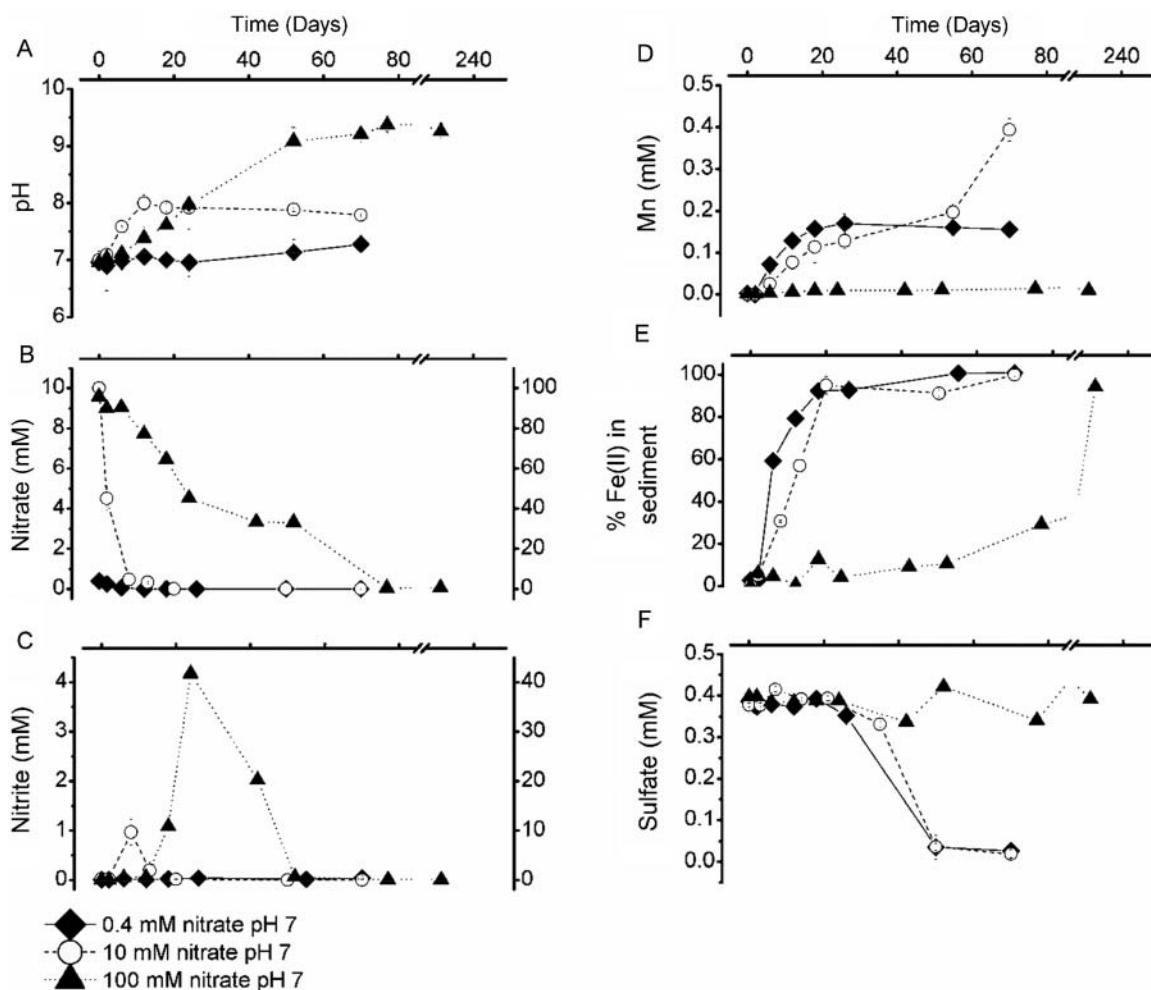


FIG. 4. Microcosm incubation time-series data (days 0–230). (A) pH, (B) NO_3^- , (C) NO_2^- , (D) porewater Mn, (E) 0.5 N HCl% extractable sedimentary Fe as Fe(II), (F) porewater Fe, (G) porewater SO_4^{2-} and (H) Eh. Black diamonds = bicarbonate buffered 0.4 mM nitrate system; unfilled circles = bicarbonate buffered 10 mM nitrate system; black triangles = bicarbonate buffered 100 mM nitrate system. The initial pH in all microcosms was ~ 7.0 . Error bars represent 1σ experimental uncertainty from triplicate microcosm experiments (where not visible error bars are within symbol size).

capable of denitrification and growth between pH 3–9 (Lebuhn et al. 2000) and some species of *Bacillus* are presumably alkali tolerant as they have been isolated from soda lakes at pH > 9 (Carrasco et al. 2007; Pollock et al. 2007).

Enrichment Cultures

In the 100 mM bicarbonate buffered system that had undergone bioreduction and was poised at pH 9.5, the sediment molecular ecology studies were dominated by close relatives of known nitrate-reducing microorganisms and thus the Fe(III)-reducing bacteria, which were obviously active, could not be identified unequivocally in the clone library. Therefore, in order to gain further insight into the alkali tolerant Fe(III)-reducing species that were active in these systems, enrichment cultures were established with medium containing Fe(III)-citrate as the sole electron acceptor at pH 9.5 and inoculated initially with

10% of the bioreduced 100 mM carbonated buffered sediment (see methods).

After seven enrichment subcultures (using 10% v/v inocula throughout), a sample was taken for molecular characterization. Here, 16S rRNA gene analysis revealed that a bacterium closely related (>99%) to *Alkaliphilus crotonatoxidans* made up 41% of the enrichment culture (37 of 91 clones) and a bacterium closely related (>99%) to *Serratia liquifaciens* made up a further 56% (51 of 91 clones) (Table 4).

Alkaliphilus crotonatoxidans is a strict anaerobe with a reported growth range of pH 5.5–9 (Cao et al. 2003), whereas *Serratia liquifaciens* is a facultative anaerobe and has not previously reported as alkali tolerant. Repeated subcultures of the enrichment consortium over several months show that the consortium is stable and capable of growth at pH >9, while facilitating Fe(III)-reduction in this high pH system.

TABLE 4

Phylogenetic affiliation of 16S rRNA gene sequences detected in the clone library from the Fe(III) reducing enrichment culture at pH 9.5

No. in Clone Library	Closest Matching Micro-organism [accession Number]	% Match	% Present	Phylogenetic Class
37	<i>Alkaliphilus crotonatoxidans</i> [AF467248]	99%	40.7%	Clostridia
51	<i>Serratia liquefaciens</i> [AJ306725]	99%	56%	Gammaproteobacteria
2	<i>Clostridium celerecrescens</i> clone IrT-JG1-12[AJ295659]	98%	2.2%	Clostridia
1	uncultured bacterium; 3BH-2FF [EU937958]	97%	1.1%	Betaproteobacteria

Implications for Bioremediation

This study highlights the sensitivity of nitrate and Fe(III)-reducing communities in Sellafield-type sediments to initial pH conditions. It was found that in these batch experiments, while low pH may inhibit the progression of TEAPs in nitrate amended systems, moderate nitrate concentrations up to 10 mM actually stimulated the development of metal-reducing conditions *via* production of OH⁻ and HCO₃⁻ during nitrate reduction and resultant pH amendment (Figure 1).

These observations are in contrast to similar studies with nitrate contaminated sediments from the Oak Ridge nuclear site where pH buffering with NaHCO₃ or crushed lime was necessary to stimulate bioreduction (Edwards et al. 2007; Michalsen et al. 2009; North et al. 2004). In our systems, we observed faster TEAP progression when microcosms were buffered to an initial pH of 7.0 with bicarbonate buffer compared to the naturally mildly acidic Sellafield material. Indeed, in our experiments very high (100 mM) nitrate was only fully reduced in bicarbonate buffered systems.

This information is useful in understanding pH amendment *via* bioreduction that may be occurring in high nitrate groundwaters, and may be beneficial in planning engineered bioreduction treatments in low pH environments although clearly there is a need for further lab and field scale studies on dynamic flow systems to constrain this potential further. Interestingly, although reduction of a pH 7 microcosm containing 100 mM nitrate lead to the development of a pH of 9.5 prior to metal reduction starting, the system appeared robust and progression to Fe(III) reduction occurred at these alkaline conditions.

Overall, the representative Sellafield sediments appear to support a diverse range of microorganisms which in batch experiments are capable of metal reduction between pH 6 and 9.5 provided there is sufficient electron donor to first deplete nitrate. Interestingly, a rise in pH during bioreduction may also benefit the removal of non redox active radionuclides such as ⁹⁰Sr which is less mobile at alkaline pH and is predicted to associate with carbonate phases above pH ~ 9 (Ferris and Roden 2000; Langley et al. 2009; Roden et al. 2002). This work highlights that biostimulation coupled to pH modification by denitrification is possible under constrained, batch experiment conditions and, contrary to initial expectations, may provide some potential for

enhanced removal of problematic radionuclides and contaminants at industrial sites.

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