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The synergistic effects of high nitrate concentrations on sediment bioreduction

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16   **Abstract**

17   Groundwaters at nuclear sites are often characterised by low pH and high nitrate

18   concentrations (10-100 mM). These conditions are challenging for bioremediation, often

19   inhibiting microbial Fe(III)-reduction that can limit radionuclide migration. Here, sediment

20   microcosms representative of the UK Sellafield site were used to study the influence of

21   variable pH and nitrate concentrations on microbially-mediated TEAP (terminal electron

22   accepting processes) progression. The rate of bioreduction at low pH (~ 5.5) was slower than

23   that in bicarbonate-amended systems (pH ~ 7.0), but in the low pH systems, denitrification

24   and associated pH buffering resulted in conditioning of the sediments for subsequent Fe(III)

25   and sulfate reduction. Under very high nitrate conditions (100 mM), bicarbonate amendment

26   (pH ~ 7.0) was necessary for TEAP progression beyond denitrification and the reduction of

27   100 mM nitrate created alkaline conditions (pH 9.5). 16S rRNA gene analysis showed that

28   close relatives of known nitrate reducers *Bacillus niacini* and *Ochrobactrum grignonense*

29   dominated the microbial communities. In the 100 mM nitrate system, close relatives of the

30   Fe(III)-reducing species *Alkaliphilus crotonatoxidans* and *Serratia liquifaciens* were

31   observed. These results highlight that denitrification can support bioreduction *via* pH

32   conditioning for optimal metal reduction and immobilization.

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Introduction

34

35   The remediation of radioactively contaminated land in the UK is of immediate concern

36   due to the ongoing decommissioning of the UK's nuclear sites. Further, there is a need for

37   solutions to existing contaminant problems prior to the onset of new nuclear power

38   generating programmes. At the Sellafield nuclear reprocessing site in Cumbria, mobile

39   groundwater contaminant radionuclides include ^{99}Tc and ^{90}Sr, and groundwater co-

40   contaminants include nitrate (from nitric acid), organic acids, and pH variance (BNFL 2003;

41   Sellafield Ltd 2008). Similar contamination issues have been documented at a range of US

42   nuclear sites (e.g., Oak Ridge, TN (Istok et al. 2004; Edwards et al. 2007; Li and Krumholz

43   2008), San Juan River, Shiprock, NM (Finneran et al. 2002), and Hanford, WA (Singleton et

44   al. 2005)). A proposed *in situ* strategy to remediate contaminants at such sites is

45   "biostimulation". Here, an electron donor is added to the subsurface to stimulate the

46   indigenous microbial community, promoting a cascade of terminal electron accepting

47   processes (TEAPs) that favour radionuclide removal from groundwaters (Lovley and Coates

48   1997; Lloyd and Renshaw 2005). This approach has been shown to reduce the mobility of

49   redox-active radionuclides such as ^{99}Tc and U, *via* the reduction of soluble oxic species

50   (Tc(VII), U(VI)) to poorly-soluble reduced species (Tc(IV), U(IV)) (Istok et al. 2004;

51   Edwards et al. 2007; Morris et al. 2008; Law et al. 2010). It may also be possible for

52   bioreduction to occur in sediments with an alternative supply of electron donor (Alvarez et al.

53   2006; Manaka et al. 2007; Burke et al. 2010). Regardless, in most cases, radionuclide

54   reduction is associated with microbially-mediated Fe(III) reduction (Lloyd 2003; Lloyd and

55   Renshaw 2005). As a consequence, the actions of Fe(III)-reducing bacteria, and subsequent

56   changes in Fe redox chemistry and Fe mineralogy, likely play a key role in governing

57   mobility of redox-active radionuclides. Furthermore, changes in Fe mineralogy have the

58 potential to affect the sorption and mobility of other (non redox-active) radionuclides e.g.,

59 ^{137}Cs or ^{90}Sr (Roden et al. 2002; Langley et al. 2009; Chiang et al. 2010).

60 However, the comparatively low groundwater pH conditions and/or high nitrate

61 concentrations that often characterise nuclear sites represent challenging bioremediation

62 scenarios. Low pH critically decreases microbial diversity and metabolic function (Madigan

63 and Martinko 2006; Robinson et al. 2009) whilst nitrate is an energetically more favourable

64 electron acceptor than Fe(III) (and redox active radionuclides) and thus can inhibit TEAP

65 progression and reductive immobilization of radionuclides (DiChristina 1992). Indeed,

66 numerous sediment microcosm studies indicate that microbially-mediated metal and

67 radionuclide reduction do not commence until nitrate and nitrite are completely reduced (e.g.

68 Burke et al. 2005; Edwards et al. 2007; McBeth et al. 2007; Li and Krumholz 2008; Law et

69 al. 2010; Wilkins et al. 2010). Further, some biostimulation studies with low pH sediments

70 have demonstrated that the pathways and extent of nitrate removal are strongly pH

71 dependant, with artificial $NaHCO_3$ or crushed lime amendment necessary to stimulate

72 bioreduction and TEAP progression (North et al. 2004; Edwards et al. 2007; Michalsen et al.

73 2009). Conversely, in field studies, dual denitrification and metal reduction was observed at

74 low-pH (Istok et al. 2004) and in microcosm studies, denitrification and associated pH

75 buffering (*via* OH^- and HCO_3^- production) stimulated TEAP progression to metal reduction

76 (Law et al. 2010). Clearly, the variable effects of low-pH and nitrate on electron flow

77 warrants further study. Consequently, here, electron flow in Sellafield nuclear site sediments

78 was studied under a range of environmentally relevant nitrate (0.4 – 100 mM), pH, and

79 carbonate conditions.

80

81 **Experimental Section**

82 *Sample Collection*

83   Sediments representative of the Quaternary unconsolidated alluvial flood-plain deposits

84   that underlie the UK Sellafield reprocessing site (Law et al. 2010) were collected from the

85   Calder Valley, Cumbria, during December 2008 (herein called Sellafield sediment). The

86   sampling area was located ~ 2 km from the Sellafield site (Lat 54°26'30 N, Long 03°28'09

87   W). Sediments were transferred directly into sterile containers, sealed, and stored at 4 ℃.

88   Experiments began within 6 months of field sampling.

89

90   ***Bioreduction Microcosms***

91   Sediment microcosms (10 ± 0.1 g Sellafield sediment, 100 ± 1 ml groundwater) were

92   prepared using a synthetic groundwater representative of the Sellafield region (Wilkins et al.

93   2007; Law et al. 2010) that was manipulated to produce a range of treatments (Table 1).

94   Bicarbonate unamended systems with an initial pH of 5.5 were prepared with 0.4, 2, 10, and

95   100 mM nitrate. Bicarbonate amended systems with an initial pH of 6.8 were prepared with

96   0.3, 10, and 100 mM nitrate. Sodium acetate was added as an electron donor in excess of

97   extant available electron acceptors (14 mM for 0.3 − 10 mM nitrate treatments, and 70 mM

98   for 100 mM nitrate treatments) and anoxic $NaNO_3$ was used as a NO_3^- source. Sediment and

99   sterile groundwaters were added to sterile 120 ml glass serum bottles (Wheaton Scientific,

100   USA) using aseptic technique and sealed with butyl rubber stoppers. All microcosms were

101   then incubated anaerobically at 21 ℃ in the dark for 80 - 230 days and each treatment was

102   run in triplicate. Throughout the incubation, sediment slurry was periodically extracted under

103   an O_2-free Ar atmosphere using aseptic technique. The sediment slurry was centrifuged

104   (15,000 g; 10 minutes) to provide separate sediment and porewater samples and a ~ 0.5 g

105   aliquot of untreated sediment was stored at - 80 ℃ for microbiological characterization.

106   Sediments from the initial and final time points of each treatment underwent a sequential

107   extraction procedure to assess changes in Fe mineralogy during biostimulation (Tessier et al.

108   1979; Poulton and Canfield 2005). Sequential extractions procedures targeted: i) carbonate

109   associated Fe, ii) easily reducible oxides, iii) reducible oxides, iv) magnetite and v) residual

110   Fe (Table 2).   These extractions comprised i) 1 M sodium acetate (pH 4.5), ii) 1 M

111   hydroxylamine HCl, iii) sodium dithionite - sodium citrate (pH 4.8), iv) 0.2 M ammonium

112   oxalate (pH 3.2), and v) residual Fe was determined by XRF minus the extracted phases

113   (Poulton and Canfield 2005).   The sediment to solution ratio was 0.1 g in 10 ml (1:100) at

114   each stage.

115

116   *Geochemical Analyses*

117   During microcosm sampling, total dissolved Fe, Mn(II), and NO_2^- concentrations were

118   measured with standard UV-vis spectroscopy methods on a Cecil CE 3021 spectrophotometer

119   (Goto et al. 1997; Viollier et al. 2000; Harris and Mortimer 2002).   Aqueous NO_3^-, SO_4^{2-}, and

120   acetate were measured by ion chromatography (Dionex ICS-90) (Burke et al. 2005).

121   Ammonium was measured by flow injection analysis (Dionex ICS-90; Hall and Aller 1992).

122   Total bioavailable Fe(III) and the proportion of extractable Fe(II) in the sediment was

123   estimated by digestion of 0.1 g of sediment in 5 ml of 0.5 N HCl for 60 minutes followed by

124   the ferrozine assay, with and without hydroxylamine hydrochloride (Stookey 1970; Lovley

125   and Phillips 1987).   The pH and Eh were measured with an Orion 420A digital meter and

126   calibrated electrodes.   Standards were routinely used to check the reliability of all methods

127   and calibration regressions had $R^2 \geq 0.99$.   The elemental composition and bulk mineralogy

128   of the sediment were determined by X-ray fluorescence (Thermo ARL 9400 XRF) and X-ray

129   diffraction (Philips PW 1050 XRD).

130

131   *Microbial community analysis*

7

132   Selected samples from bicarbonate unamended microcosms containing 0.4 and 10 mM

133   and bicarbonate amended microcosms containing 100 mM initial nitrate underwent PCR-

134   based 16S rRNA gene analysis. Additionally, sub-aliquots of sediment slurry from the

135   100 mM nitrate treatment were added (1:10 sediment/solution ratio) to an Fe(III)-citrate

136   medium (Lovley and Phillips 1986) with 20 mM acetate or 0.2% (w/v) yeast extract as an

137   electron donor, to make an enrichment culture to identify microorganisms responsible for

138   Fe(III) reduction at pH > 9. Enrichment cultures were incubated at 20 ^{o}C for 4 - 5 weeks

139   before further sub-aliquots were transferred (1:10 sediment/solution ratio) to fresh Fe(III)-

140   citrate medium. This procedure was repeated 7 times and then finally 16S rRNA gene

141   analysis was used to identify the species present. XRD was used to analyse the mineralogical

142   products of Fe(III) reduction in the enrichment systems.

143

144   *Amplification of 16S rRNA gene sequences*

145   DNA was extracted from samples using a PowerSoil DNA Isolation Kit (MO BIO, USA).

146   Copies of the 16S rRNA gene (approximately 1490 b.p. fragment) was amplified from

147   samples using the broad-specificity primers 8F (Eden et al. 1991) and 1492R (Lane et al.

148   1985). PCR reactions were performed in thin-walled tubes using a BioRad iCycler (BioRad,

149   UK). The PCR amplification protocol used with the 8F and 1492R primers was: initial

150   denaturation at 94 ºC for 4 minutes, melting at 94 ºC for 30 seconds, annealing at 57 ºC for

151   30 seconds, elongation at 72 ºC for 1 minute; 35 cycles, followed by a final extension step at

152   72 ºC for 10 minutes. The purity of the amplified products was determined by

153   electrophoresis in a Tris-borate-EDTA (TBE) gel. DNA was stained with ethidium bromide

154   and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, UK).

155

156   *Cloning*

157   PCR products were purified using a QIAquick PCR purification kit (Qiagen, UK) and

158   ligated directly into a cloning vector containing topoisomerase I-charged vector arms

159   (Agilent Technologies, UK) prior to transformation into *E. coli* competent cells expressing

160   Cre recombinase (Agilent Technologies, UK). White transformants that grew on LB agar

161   containing ampicillin and X-Gal were screened for an insert using PCR. Primers were

162   complementary to the flanking regions of the PCR insertion site of the cloning vector. The

163   PCR method was: an initial denaturation at 94 ℃ for 4 minutes, melting at 94 ℃ for

164   30 seconds, annealing at 55 ℃ for 30 seconds, extension at 72 ℃ for 1 minute; 35 cycles,

165   followed by a final extension step at 72 ℃ for 5 minutes. The resulting PCR products were

166   purified using an ExoSap protocol, and 2 µl of ExoSap mix (0.058 µl Exonuclease I, 0.5 µl

167   Shrimp Alkaline Phosphatase, and 1.442 µl QH_2O) was added to 5 µl of PCR product and

168   incubated at 37 ℃ for 30 minutes followed by 80 ℃ for 15 minutes.

169

170   *DNA sequencing and phylogenetic analysis*

171   Nucleotide sequences were determined by the dideoxynucleotide method. An ABI Prism

172   BigDye Terminator Cycle Sequencing Kit was used in combination with an ABI Prism 877

173   Integrated Thermal Cycler and ABI Prism 377 DNA Sequencer (Perkin Elmer Applied

174   Biosystems, UK). Sequences (typically 900 base pairs in length) were analysed against the

175   NCBI (USA) database using the BLAST program packages and matched to known 16S

176   rRNA gene sequences.

177

178   **Results and discussion**

179   *Sediment characteristics*

180   The mineral content of the sediment as sampled was dominated by quartz, feldspars (albite

181   and microcline), and sheet silicates (muscovite and chlorite). The sediment had a high Si

182   content (33.2 wt %) and contained Al (5.9 %), Fe (4.2 %), K (2.6 %), Na (1.1 %), Mg

183   (<0.1 %), Ti (0.4 %), Ca (0.14 %), and Mn (< 0.1 %).   The concentration of 0.5 N HCl

184   extractable Fe in the sediment was 5.6 ± 0.5 mmol kg^{-1} prior to incubation and the sediment

185   pH was ~ 5.5.

186

187   ***Progressive bioreduction in bicarbonate unamended systems***
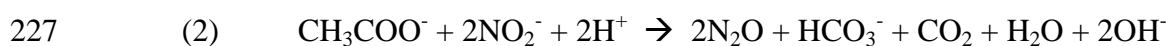
188   In bicarbonate unamended systems with initially mildly acidic pH, and with varying initial

189   nitrate   concentrations   microbially-mediated   TEAP   progression   was   monitored   as

190   bioreduction   developed   in   the   different   experimental   systems   (Table 1).   Microbial

191   metabolism was observed in all electron donor amended microcosms (Figure 1) whereas no

192   biogeochemical   changes   were   observed   in   sterile-controls   (data   not   shown).   Electron

193   acceptor utilisation was observed in the order $NO_3^- \geq Mn(IV) \geq Fe(III) \geq SO_4^{2-}$ as indicated

194   by ingrowth of the relevant biogeochemical indicators (Figure 1). Eh decreased during TEAP

195   progression and acetate was removed from porewaters (Table 3). As expected, the onset of

196   microbially-mediated Mn and Fe(III) reduction was inhibited until nitrate and nitrite were

197   removed from batch experiments *via* denitrification. The inhibition time was dependent on

198   the initial nitrate concentration with 0.4, 2, and 10 mM nitrate removed by 14, 18 and 25 days

199   respectively and the start of metal reduction occurring immediately afterwards (Figure 1).

200   Interestingly, the rates of Mn(IV) and Fe(III) reduction were increased after nitrate had been

201   removed from the microcosms with higher nitrate additions. For example, in the 0.4, 2, and

202   10 mM nitrate systems, essentially complete Fe(III) reduction was seen at the same time (50

203   days) despite the delay in onset of Fe(III) reduction observed in the 10 mM system compared

204   to the lower concentrations. By contrast, the 100 mM nitrate, bicarbonate unamended system

205   appeared to be overwhelmed by the competing electron acceptor and although substantial

206   nitrate reduction had occurred, 60 mM nitrate remained in solution after 230 days incubation

207 and no Fe(III) reduction was observed (Figure 1). Previous studies have reported an increase

208 in Fe(III) reduction rates in low pH sediments following nitrate reduction and attributed this

209 to a rise in pH due to OH^- and HCO_3^- production during denitrification (Law et al., 2010).

210 Here, the pH in bicarbonate unamended systems with an initial pH of 5.5 and nitrate

211 concentrations of 0.4 2 and 10 mM, increased to pH 6.8, 7.0, and 7.5 respectively (Figure 1).

212 Thus, the pH adjustment from pH ~5.5 to circumneutral pH conditions, caused by nitrate

213 reduction, apparently stimulates metal reduction in these sediments. This is consistent with

214 the fact that the diversity and metabolic function of neutraphilic metal reducers is decreased

215 at low pH (Lloyd 2003; Reardon et al. 2004; Fields et al. 2005; Edwards et al. 2007). In

216 these microcosms, even low concentrations of nitrate (0.4 mM) were sufficient to increase pH

217 to a region where Fe(III) reduction was viable.

218

219 ***Bioreduction pathways***

220   Calculations based on acetate consumption compared with nitrate reduction, combined

221 with only a minor amount of ammonia being detected in the bioreduced microcosms (Table

222 3) suggest that denitrification to N_2 or N_2O is the dominant pathway for nitrate reduction in

223 these systems. Equations for the 5 electron transfer from NO_3^- to N_2 coupled to acetate

224 oxidation show the production of OH^- during nitrite reduction to N_2O and HCO_3^- produced at

225 all stages (Equations 1-3) and in agreement with the observed rise in pH.

226   (1) $CH_3COO^- + 4NO_3^- \rightarrow 4NO_2^- + HCO_3^- + CO_2 + H_2O$

227   (2) $CH_3COO^- + 2NO_2^- + 2H^+ \rightarrow 2N_2O + HCO_3^- + CO_2 + H_2O + 2OH^-$

228   (3) $CH_3COO^- + 4N_2O \rightarrow 4N_2 + HCO_3^- + CO_2 + H_2O$

229 Metal reduction then followed nitrate reduction with % 0.5 N HCl extractable Fe(II) ingrowth

230 to sediments observed followed by an accumulation of Mn and Fe in porewaters (Figure 1).

231 Although initially associated with nitrate reduction, the pH in all microcosms continued to

232 trend upwards during Fe(III) reduction consistent with continued consumption of H^+ and

233 release of HCO_3^- during Fe(III) oxide reduction coupled to acetate oxidation (Equation 4).

234 (4) $CH_3COO^- + 8FeOOH + 15H^+ \rightarrow 8Fe^{2+} + 2HCO_3^- + 12H_2O$
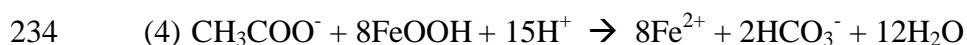
235 Interestingly, sequential extractions conducted on sediment from the bicarbonate amended

236 systems with 10 mM nitrate before and after bioreduction suggest an increase in the

237 "carbonate fraction" and a reduction in the "easily reducible" fraction in the sediments after

238 bioreduction (Figure 3). The final pH in these systems was between pH 7.5 and 8. This is

239 consistent with observations that Fe^{2+}, alkalinity and HCO_3^- all favour the formation of

240 siderite (Equation 5) (Coleman et al., 1993; Roden et al., 2002).

241 (5) $Fe^{2+} + HCO_3^- + OH^- \rightarrow FeCO_3 + H_2O$
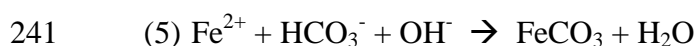
242

243 *Microbial community analysis in bicarbonate unamended systems*

244 The microbial ecology of the unamended, pH ~5.5 microcosms was assessed by 16S

245 rRNA gene analysis at key points as bioreduction progressed. Analysis of the oxic sediment

246 revealed a diverse population with 11 different phyla and 59 distinct organisms detected in 73

247 clones. The clone library was dominated by species from the phylum *Aciodobacteria* (~

248 50%) with close relatives of *Bacillus* species present (~7%) (Figure 4). This is similar to past

249 work with Sellafield-type sediments where *Acidobacteria* also dominated the clone libraries

250 prepared from oxic sediments (Law et al. 2010). When the 0.4 mM nitrate system had

251 undergone nitrate and Fe(III) reduction (at day 50) the microbial community had shifted and

252 comprised 11 different phyla and 71 distinct sequences from the 83 clones analysed.

253 Members of the *Clostridiales* now made up ~17 % of the clone library and *Acidobacteria*

254 only ~21 % (Figure 4). Organisms affiliated with the *Clostridiales* order included close

255 relatives of know Gram-positive metal-reducing species *Desulfosporoinus sp.* S8 and

256 *Desulfitobacterium metallireducens* (Robertson et al. 2000; Spring and Rozenzweig 2006)

257 and close relatives have been isolated as key metal-reducing bacteria in high nitrate

258 sediments at Oak Ridge, TN (Shelobolina et al. 2003; Li and Krumholz 2008). Also present

259 in the clone library were species of the known Fe(III)-reducing genus *Geobacter* and known

260 nitrate reducing genus *Bacillus*. When the bicarbonate unamended 10 mM nitrate system had

261 undergone nitrate and Fe(III) reduction (50 days), the diversity was very much reduced with

262 87 % of the clone library (76 of 87 clones sequenced) comprised of close relatives (> 99%) of

263 *Bacillus niacini* (Figure 4). *Bacillus niacini* has been shown to reduce nitrate to nitrite under

264 anaerobic conditions (Nagel and Andreeson 1991) and close relatives have been identified

265 previously in nitrate amended sediments at a uranium waste tailing site and in representative

266 Sellafield sediments (Selenska-Pobell and Geissler 2008; Law et al. 2010).   These results

267 suggest a reduced microbial diversity as nitrate concentrations increase with a close relative

268 (> 99%) of *Bacillus niacini* suggested as a key, acid tolerant nitrate-reducing organism in

269 these systems and with *Gram-positive* species potentially significant in mediating Fe(III)

270 reduction.

271

272 ***Progressive bioreduction in bicarbonate amended systems***

273 When systems were amended with bicarbonate to pH 7 to stimulate bioreduction, there was a

274 general increase in the rate of bioreduction compared to the unamended microcosms. For

275 example, in the bicarbonate amended 0.4 and 10 mM nitrate systems, extensive Fe(III)-

276 reduction, indicated by 100 % 0.5 M HCl extractable Fe converted to Fe(II), was observed by

277 21 days compared to 50 days in the parallel unamended system (Figures 1 and 2).

278 Interestingly, although the microbial community was unable to reduce 100 mM nitrate at pH

279 5.5 (Figure 1), when the pH was buffered to circumneutral prior to incubation, the system

280 was able to facilitate complete reduction of 100 mM nitrate by 70 days and metal reduction

281 commenced thereafter (Figure 2). Development of metal-reducing conditions in microcosms

282   with high nitrate is variable with some studies reporting development of Fe(III)-reduction in

283   100 mM nitrate, carbonate buffered experiments (Edwards et al, 2007) whilst other workers

284   observed only partial reduction of 100 mM nitrate and no development of Fe(III)-reducing

285   conditions (McBeth et al 2007). Interestingly, in dynamic push- pull tests at the Field

286   Research Centre in Oak Ridge Tennessee, electron donor amendment and pH neutralization

287   was needed to reduce >100 mM nitrate (Istok et al. 2004; North et al. 2004). In the

288   bicarbonate-amended experiments, pH increased from pH ~7.0 to ~7.2, ~8.1 and ~9.5 for

289   systems with 0.4, 10 and 100 mM nitrate, and as expected the onset of metal-reducing

290   conditions was delayed as the nitrate concentration increased. Indeed, reduction of 0.5 N

291   HCl extractable Fe(III) took 18, 25 and 230 days in the 0.4, 10 and 100 mM nitrate systems

292   respectively (Figure 2). The Fe(III)-reducing populations were therefore not inhibited

293   compared to the unamended, pH 5.5 systems. Indeed, the observation that in the 100 mM

294   nitrate system, Fe(III) reduction occurred at pH > 9 and following reduction of 100 mM

295   nitrate seems to be a novel observation. Indeed there there are few published studies on

296   metal reduction in alkaline sediments and the majority of available studies focus on

297   halophillic species from alkaline soda lakes (Gorlenko et al. 2004; Pollock et al. 2007).

298   Indeed, only a few species including *Alkaliphilus metalireducens* and *Anaerobranca*

299   *californiensis* have been isolated and shown to reduce Fe(III) above pH 9 (Gorlenko et al.

300   2004; Ye et al. 2004). More recently, Fe(III) reduction has been demonstrated in a highly

301   contaminated, high pH chromium waste site in the UK (Stewart et al. 2010). Sequence

302   analyses of amplified 16S rRNA genes showed that during Fe(III) reduction after incubation

303   for 70 days the bicarbonate amended 100 mM nitrate system had a restricted clone library

304   with only 5 different species detected in 88 clones. The system was dominated by a close

305   relative (> 99 % sequence homology) of *Ochrobactrum grignonense* strain c259 (59 % of the

306   clones) with a close relative (> 99 % sequence homology) of *Bacillus niacini* also significant

307 at ~ 37% of the clone library (Figure 4). *Ochrobactrum grignonense* is capable of

308 denitrification and growth between pH 3-9 (Lebuhn et al. 2000) and some species of *Bacillus*

309 are presumably alkali tolerant as they have been isolated from soda lakes at pH > 9 (Pollock

310 et al. 2007; Carrasco et al. 2007).

311

312 *Enrichment cultures*

313 In the 100 mM bicarbonate amended system that had undergone bioreduction and was

314 poised at pH 9.5, the molecular ecology studies were, not unsurprisingly, dominated by close

315 relatives of known nitrate-reducing microorganisms. Therefore, in order to gain further

316 insight into the alkali tolerant Fe(III)-reducing species that were active in these systems,

317 enrichment cultures were established with Fe(III)-citrate medium at pH 9.5 and inoculated

318 initially with 10 % of the bioreduced 100 mM carbonated amended sediment (see methods).

319 After seven enrichment subcultures (using 10% v/v inocula throughout), a sample was taken

320 for molecular ecology characterisation. Here, 16S rRNA gene analysis revealed that a

321 bacterium closely related (> 99%) to *Alkaliphilus crotonatoxidans* made up 41 % of the

322 enrichment culture (37 of 91 clones) and a bacterium closely related (> 99%) to *Serratia*

323 *liquifaciens* made up a further 56 % (51 of 91 clones) (Table 4). *Alkaliphilus*

324 *crotonatoxidans* is a strict anaerobe with a reported growth range of pH 5.5 - 9 (Cao et al.

325 2003) whereas *Serraitia liquifaciens* is a facultative anaerobe and has not previously reported

326 as alkali tolerant. Repeated subcultures of the enrichment consortium over several months

327 show that the consortium is stable and capable of growth at pH >9 while facilitating Fe(III)-

328 reduction in this high pH system.

329

330 **Implications for bioremediation**

331   This study highlights the sensitivity of nitrate and Fe(III)-reducing communities in

332   representative Sellafield sediments to initial pH conditions. It was found that while low pH

333   may inhibit the progression of TEAPs, in nitrate amended systems, nitrate concentrations up

334   to 10 mM actually stimulated the development of metal-reducing conditions *via* the release of

335   OH^- and HCO_3^- during nitrate reduction and resultant pH amendment to circumneutral

336   conditions (Figure 1). These observations are in contrast to similar studies with nitrate

337   contaminated sediments from the Oak Ridge nuclear facility where pH amendment with

338    $NaHCO_3$ or crushed lime to circumneutral conditions was necessary to stimulate bioreduction

339   (North et al. 2004; Edwards et al. 2007; Michalsen et al. 2009). In our systems, we observed

340   faster TEAP progression when our experiments were amended to an initial pH of 7.0 with

341   bicarbonate buffer compared to the unamended, naturally mildly acidic Sellafield material.

342   Indeed, in our experiments very high (100 mM) nitrate was only fully reduced in bicarbonate

343   amended systems.   This information is useful in understanding pH amendment *via*

344   bioreduction that may be occurring in high nitrate groundwaters, and may be beneficial in

345   planning engineered bioreduction treatments in low pH environments. Interestingly, although

346   reduction of a pH 7 microcosm containing 100 mM nitrate lead to the development of a pH of

347   9.5 prior to metal reduction starting, the system appeared robust and progression to Fe(III)

348   reduction occurred at these alkaline conditions.   Overall, the representative Sellafield

349   sediments appear to support a diverse range of microorganisms capable of metal reduction

350   between pH 6 and 9.5 provided there is sufficient electron donor to first deplete nitrate.   A

351   rise in pH during bioreduction may also benefit the removal of non redox active radionuclides

352   such as ^{90}Sr which is less mobile at alkaline pH and at pH 9.5 is predicted to form

353   associations with carbonate mineral phases (Ferris and Roden 2000; Roden et al. 2002;

354   Langley et al. 2009). This work highlights that biostimulation coupled with pH modification

355 by denitrification is possible under constrained conditions and may provide the enhanced

356 removal of problematic radionuclides and contaminants at industrial sites.

357

358 **Acknowledgments**

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365

366 **Figure legends**

367 *Figure 1.* Microcosm incubation time-series data (days 0-230). (A) pH, (B) NO_3^-, (C) NO_2^-,

368 (D) porewater Mn, (E) 0.5 N HCl % extractable sedimentary Fe as Fe(II), (F) porewater SO_4^{2-}

369 . Black diamonds = unamended 0.4 mM nitrate system; unfilled circles = unamended 2mM

370 nitrate system; black squares = unamended 10 mM nitrate system; unfilled triangles =

371 unamended 100 mM nitrate system. Initial pH in all microcosms was ~5.5. Error bars

372 represent 1σ experimental uncertainty from triplicate microcosm experiments (where not

373 visible error bars are within symbol size).

374

375 *Figure 2.* Microcosm incubation time-series data (days 0-230). (A) pH, (B) NO_3^-, (C) NO_2^-,

376 (D) porewater Mn, (E) 0.5 N HCl % extractable sedimentary Fe as Fe(II), (F) porewater Fe,

377 (G) porewater SO_4^{2-} and (H) Eh. Black diamonds = bicarbonate amended 0.4 mM nitrate

378 system; unfilled circles = bicarbonate buffered 10 mM nitrate system; black triangles =

379 bicarbonate amended 100 mM nitrate system. The initial pH in all microcosms was ~7.0.

380 Error bars represent 1σ experimental uncertainty from triplicate microcosm experiments

381 (where not visible error bars are within symbol size).

382

383 *Figure 3*. Sequential extraction data comparing the Fe mineralogy of bicarbonate amended

384 10 mM nitrate reduced sediments with that of non bioreduced oxic sediment. Dark grey =

385 carbonate associated Fe; light gray = easily reducible Fe oxides; very dark grey = reducible

386 oxides; black = magnetite; striped = residual Fe as determined by XRF.

387

388 *Figure 4.* Microbial community analysis of (A) Fe(III)- reducing bicarbonate unamended

389 sediment with 0.3 mM initial nitrate (T=50), (B) Fe(III)- reducing bicarbonate unamended

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390   sediment with 10 mM initial nitrate (T=50), (C) Fe(III)- reducing bicarbonate amended

391   sediment with 100 mM initial nitrate (T=70) and (D) unreduced oxic sediment.

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393 **Tables**
394
395 **Table 1**. Initial composition of microcosm systems

System name	Amendment	Nitrate	pH
Unamended 0.3 mM nitrate	None	0.4 mM NaNO$_3$	~5.5
Unamended 2 mM nitrate	None	2 mM NaNO$_3$	~5.5
Unamended 10 mM nitrate	None	10 mM NaNO$_3$	~5.5
Unamended 100 mM nitrate	None	100 mM NaNO$_3$	~5.5
Bicarbonate amended 0.3 mM nitrate	3 mM NaHCO$_3$ and OH$^-$	0.4 mM NaNO$_3$	6.8-7.0
Bicarbonate amended 10 mM nitrate	3 mM NaHCO$_3$ and OH	10 mM NaNO$_3$	6.8-7.0
Bicarbonate amended 100 mM nitrate	3 mM NaHCO$_3$ and OH	100 mM NaNO$_3$	6.8-7.0

396

397 **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

398

399 **Table 3.** pH, Eh and acetate utilisation data

System	pH		Eh		Acetate (mM)		NH$_4^+$ (mM)
	Initial	Final	Initial	Final	Utilised during nitrate reduction	Required for denitrification to N$_2$	Max. in porewaters
Unamended 0.4 mM nitrate	5.5	6.8	+187	-86	0.22 ± 0.01	0.25	
Unamended 2 mM nitrate	5.5	6.95	+240	-67	2.58 ± 0.06	1.25	
Unamended 10 mM nitrate	5.5	7.25	+273	-62	7.25 ± 0.32	6.25	<0.5
Unamended 100 mM nitrate	5.5	6.5-8	+184	+166	17.3 ± 0.45*	62.2	
Bicarbonate amended 0.4 mM nitrate	7	7.2	+274	-57	1.63 ± 0.15	0.25	
Bicarbonate amended 10 mM nitrate	7	7.5	+274	-20	8.23 ± 0.32	6.25	<0.7
Bicarbonate amended 100 mM nitrate	7	9.3	+286	50	86.4 ± 4.56	62.2	

400 Errors are 1σ of triplicate measurements. *reduced only ~40% of nitrate
401

402 **Table 4.** Phylogenetic affiliation of 16S rRNA gene sequences detected in the clone
403 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	Alkaliphilus crotonatoxidans [AF467248]	99%	40.7%	Clostridia
51	Serratia liquefaciens[AJ306725]	99%	56%	Gammaproteobacteria
2	Clostridium celerecrescens clone IrT-JG1-12[AJ295659]	98%	2.2%	Clostridia
1	uncultured bacterium; 3BH-2FF [EU937958]	97%	1.1%	Betaproteobacteria

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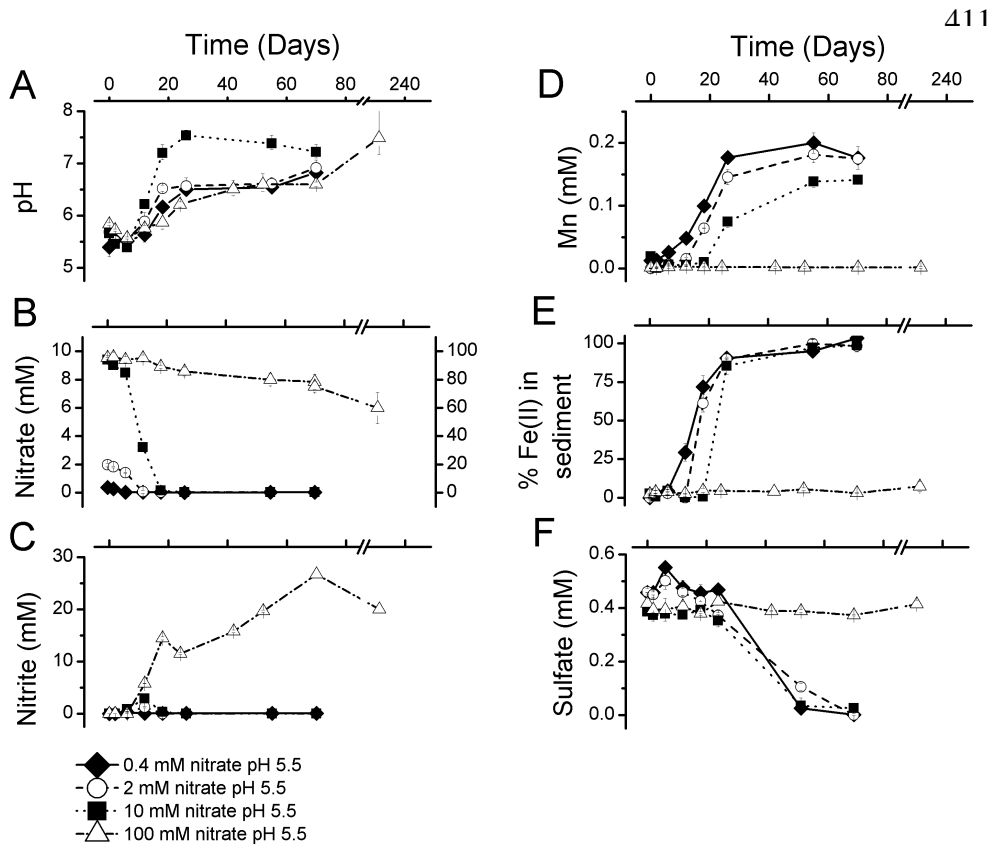
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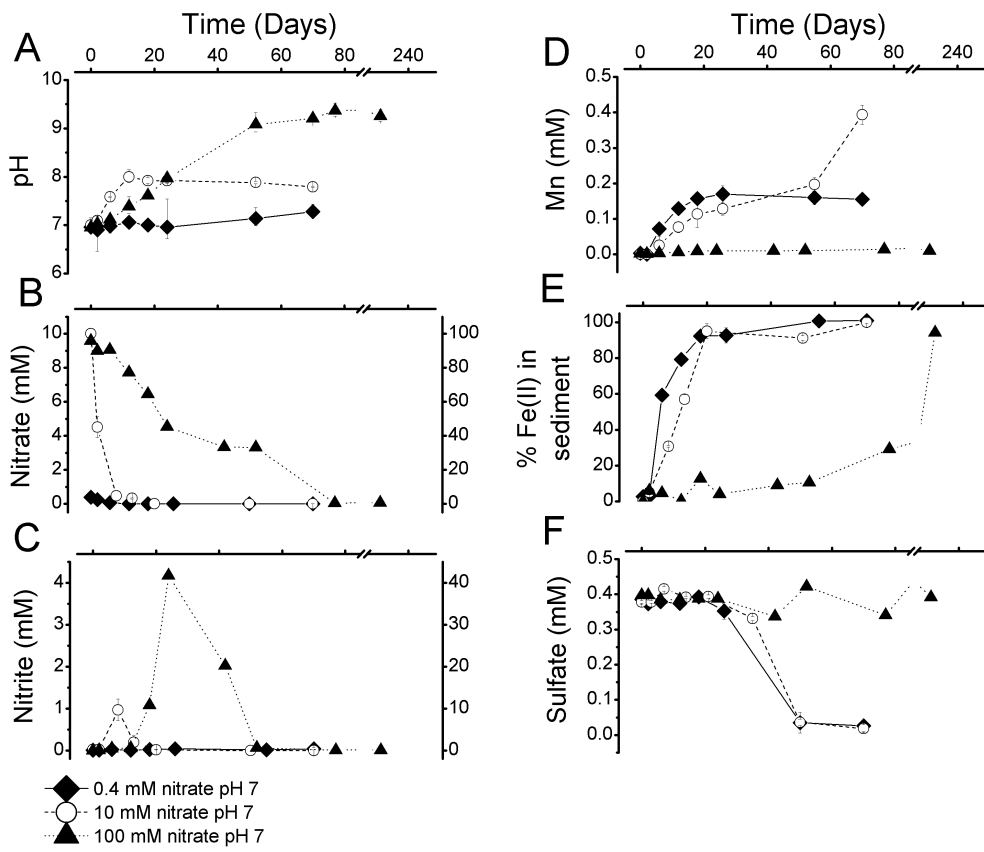20

410   Figure 1

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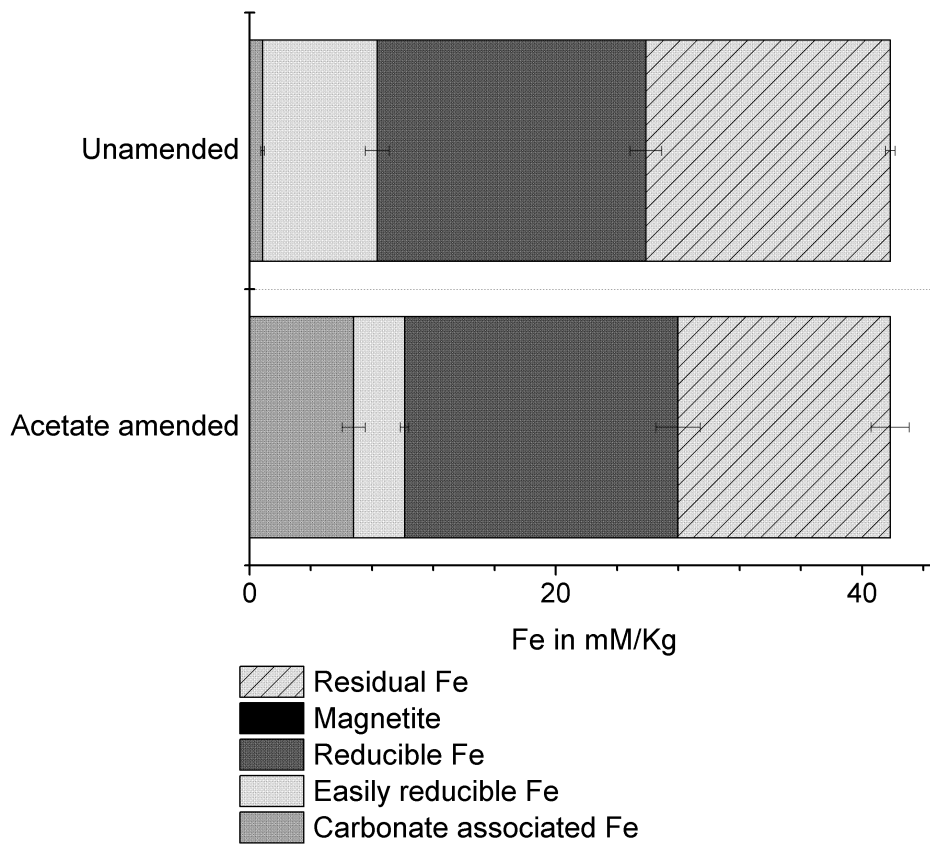21

Figure 2

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449   Figure 3

Residual Fe
Magnetite
Reducible Fe
Easily reducible Fe
Carbonate associated Fe

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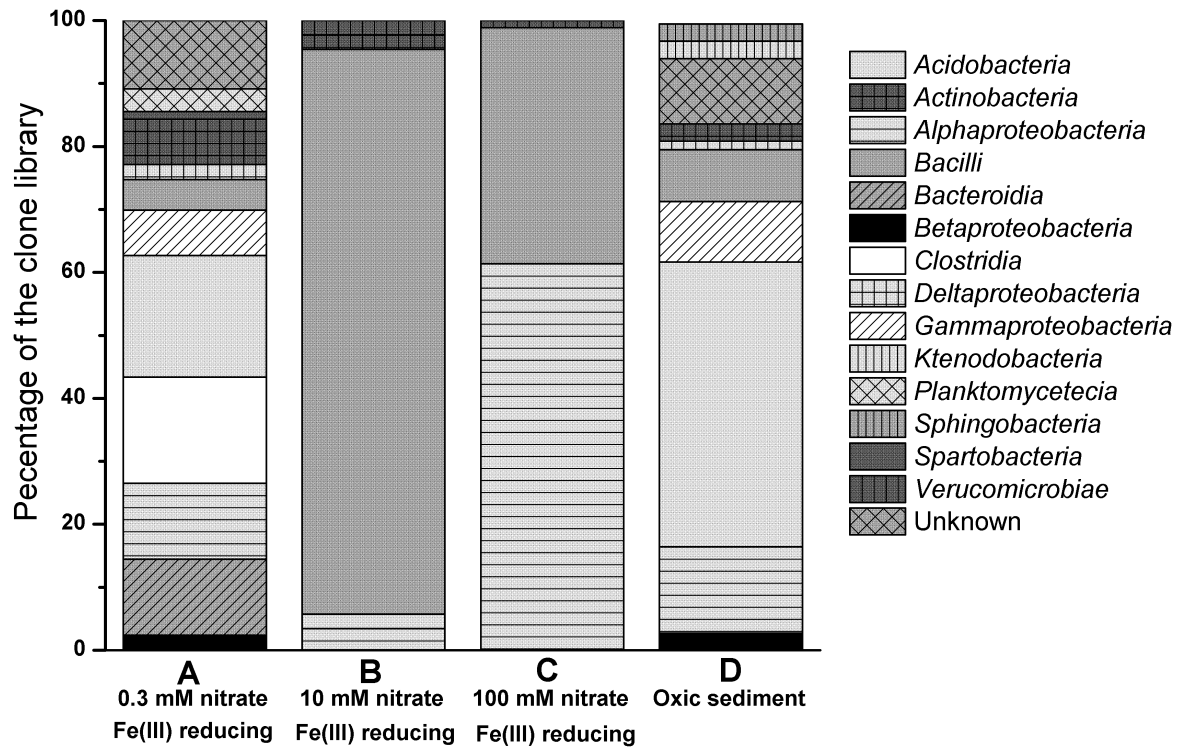
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Figure 4

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