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# Anaerobic redox cycling of iron by freshwater sediment microorganisms

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## Summary

The potential for microbially mediated anaerobic redox cycling of iron (Fe) was examined in a first-generation enrichment culture of freshwater wetland sediment microorganisms. Most probable number enumerations revealed the presence of significant populations of Fe(III)-reducing (approximately  $10^8$  cells ml<sup>-1</sup>) and Fe(II)-oxidizing, nitrate-reducing organisms (approximately  $10^5$  cells ml<sup>-1</sup>) in the freshwater sediment used to inoculate the enrichment cultures. Nitrate reduction commenced immediately following inoculation of acetate-containing (approximately 1 mM) medium with a small quantity (1% v/v) of wetland sediment, and resulted in the transient accumulation of NO<sub>2</sub><sup>-</sup> and production of a mixture of gaseous end-products (N<sub>2</sub>O and N<sub>2</sub>) and NH<sub>4</sub><sup>+</sup>. Fe(III) oxide (high surface area goethite) reduction took place after NO<sub>3</sub><sup>-</sup> was depleted and continued until all the acetate was utilized. Addition of NO<sub>3</sub><sup>-</sup> after Fe(III) reduction ceased resulted in the immediate oxidation of Fe(II) coupled to reduction of NO<sub>3</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup>. No significant NO<sub>2</sub><sup>-</sup> accumulation was observed during nitrate-dependent Fe(II) oxidation. No Fe(II) oxidation occurred in pasteurized controls. Microbial community structure in the enrichment was monitored by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified 16S rDNA and reverse transcription polymerase chain reaction-amplified 16S rRNA, as well as by construction of 16S rDNA clone libraries for four different time points during the experiment. Strong similarities in dominant members of the microbial community were observed

in the Fe(III) reduction and nitrate-dependent Fe(II) oxidation phases of the experiment, specifically the common presence of organisms closely related ( $\geq 95\%$  sequence similarity) to the genera *Geobacter* and *Dechloromonas*. These results indicate that the wetland sediments contained organisms such as *Geobacter* sp. which are capable of both dissimilatory Fe(III) reduction and oxidation of Fe(II) with reduction of NO<sub>3</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup>. Our findings suggest that microbially catalysed nitrate-dependent Fe(II) oxidation has the potential to contribute to a dynamic anaerobic Fe redox cycle in freshwater sediments.

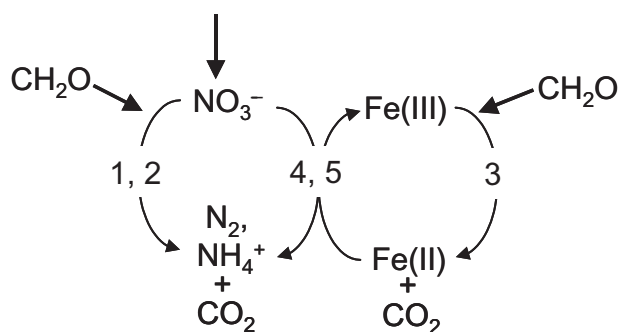
## Introduction

Iron (Fe)-bearing minerals are abundant in soil and sedimentary environments, where they exist predominantly as solid-phase minerals containing Fe in the ferrous [Fe(II)] and/or ferric [Fe(III)] oxidation state (Cornell and Schwertmann, 1996). Cycling between Fe(II) and Fe(III) (i.e. Fe redox cycling) can significantly affect the biogeochemistry of hydromorphic soils and sediments (VanBreemen, 1988; Stumm and Sulzberger, 1992; Davison, 1993; Roden *et al.*, 2004). Direct microbial (enzymatic) reduction coupled to oxidation of organic carbon and H<sub>2</sub> by dissimilatory iron-reducing bacteria (DIRB) is recognized as the dominant mechanism for Fe(III) oxide reduction in non-sulfidogenic anaerobic soils and sediments [see Lovley (1991; 2000) for review]. This process contributes to both natural and contaminant (hydrocarbon) organic carbon oxidation in sedimentary environments, and exerts a broad range of impacts on the behaviour of trace and contaminant metals and radionuclides (Lovley and Anderson, 2000).

When Fe(II) comes into contact with O<sub>2</sub> or other suitable oxidants, Fe(II) can be re-oxidized to Fe(III). The dominant role of microbial catalysis in Fe(II) oxidation in acidic environments (e.g. acid mine drainage and acid hot springs) is well-established (Brock and Gustafson, 1972; Singer and Stumm, 1972; Johnson *et al.*, 1993). In contrast, Fe(II) is subject to spontaneous chemical oxidation by dissolved O<sub>2</sub> at circumneutral pH (Davison and Seed, 1983; Millero *et al.*, 1987), and the quantitative role of microbial catalysis in Fe(II) oxidation by O<sub>2</sub> in circumneutral aerobic environments is still a matter of debate (Emerson, 2000; Emerson and Weiss, 2004; Roden *et al.*, 2004). A previously unrecognized potential for microbial

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**Fig. 1.** Potential Fe–N redox pathways in anoxic sediments: Organotrophic  $\text{NO}_3^-$  reduction to  $\text{N}_2$  (1) or to  $\text{NH}_4^+$  (2); organotrophic dissimilatory Fe(III) reduction (3); lithotrophic [Fe(II)-driven]  $\text{NO}_3^-$  reduction to  $\text{N}_2$  (4) or to  $\text{NH}_4^+$  (5). Thick lines denote external loading of  $\text{NO}_3^-$  and organic carbon ( $\text{CH}_2\text{O}$ ). Temporal variations in  $\text{NO}_3^-$  and  $\text{CH}_2\text{O}$  loading have the potential to cause temporal/spatial overlap of organotrophic and lithotrophic pathways (see text).

Fe redox cycling under anoxic conditions has been revealed through the recent discovery of nitrate-reducing microorganisms capable of enzymatic oxidation of Fe(II) [Straub *et al.* (1996; 2004); see Fig. 1]. In contrast to abiotic Fe(II) oxidation by  $\text{O}_2$ , the abiotic reaction of Fe(II) with  $\text{NO}_3^-$  is negligible under the temperature and aqueous geochemical conditions typical of natural soil and sedimentary environments (Weber *et al.*, 2001). Microorganisms capable of oxidizing Fe(II) with reduction of  $\text{NO}_3^-$  have been observed in several different freshwater sediments (Kluber and Conrad, 1998; Straub and Buchholz-Cleven, 1998; Caldwell *et al.*, 1999; Ratering and Schnell, 2000; Chaudhuri *et al.*, 2001; Hauck *et al.*, 2001; Finneran *et al.*, 2002; Senn and Hemond, 2002; Shelobolina *et al.*, 2003) as well as sewage sludge systems (Nielsen and Nielsen, 1998a,b).

The demonstrated potential for biological nitrate-dependent Fe(II) oxidation in a wide variety of natural systems suggests that this reaction may play a significant role in the coupling of Fe and N redox cycles in sedimentary environments. In addition, the recent demonstration of biological nitrate-dependent Fe(II) oxidation by a predominant environmental Fe(III)-reducing bacterium, *Geobacter metallireducens* (Finneran *et al.*, 2002), suggests that anaerobic Fe redox cycling could be catalysed by a single group of microorganisms. A tight coupling between Fe and N redox cycles in anaerobic sedimentary environments has significant implications for mechanisms of  $\text{NO}_3^-$  removal and the regeneration of reactive Fe(III) oxides in hydromorphic soils and sediments, as well as the transformation of various natural and contaminant organic and inorganic compounds.

Although the potential for enzymatic Fe(II) oxidation coupled to  $\text{NO}_3^-$  reduction has been well documented, the microbial communities associated with Fe–N redox cycling in natural environments are not yet well under-

stood. In this study, a first-generation enrichment culture of freshwater wetland sediment was subjected to a sequential shift in redox conditions [from organotrophic  $\text{NO}_3^-$  reduction, to organotrophic Fe(III) reduction, to lithotrophic nitrate-dependent Fe(II) oxidation] in order to explore the coupling between microbial N and Fe redox cycling in sediments. Changes in microbial community structure associated with redox shifts were monitored by denaturing gradient gel electrophoresis (DGGE) analysis of polymerase chain reaction (PCR)-amplified 16S rDNA and reverse transcription polymerase chain reaction (RT-PCR)-amplified 16S rRNA, and the phylogenetic association of organisms predominant in the culture was assessed through 16S rDNA clone libraries. A follow-up study evaluated the potential for *G. metallireducens* to catalyse anaerobic Fe redox cycling analogous to that observed in the enrichment culture.

## Results

### Most probable number (MPN) enumerations

Approximately  $10^5$  cells (ml wet sediment) $^{-1}$  of culturable (MPN assay) nitrate-dependent Fe(II)-oxidizing microorganisms were detected in Talladega Wetland surface sediment (Table 1). The abundance of culturable acetate-oxidizing [nitrate- and Fe(III)-reducing] microorganisms was approximately three orders of magnitude higher.

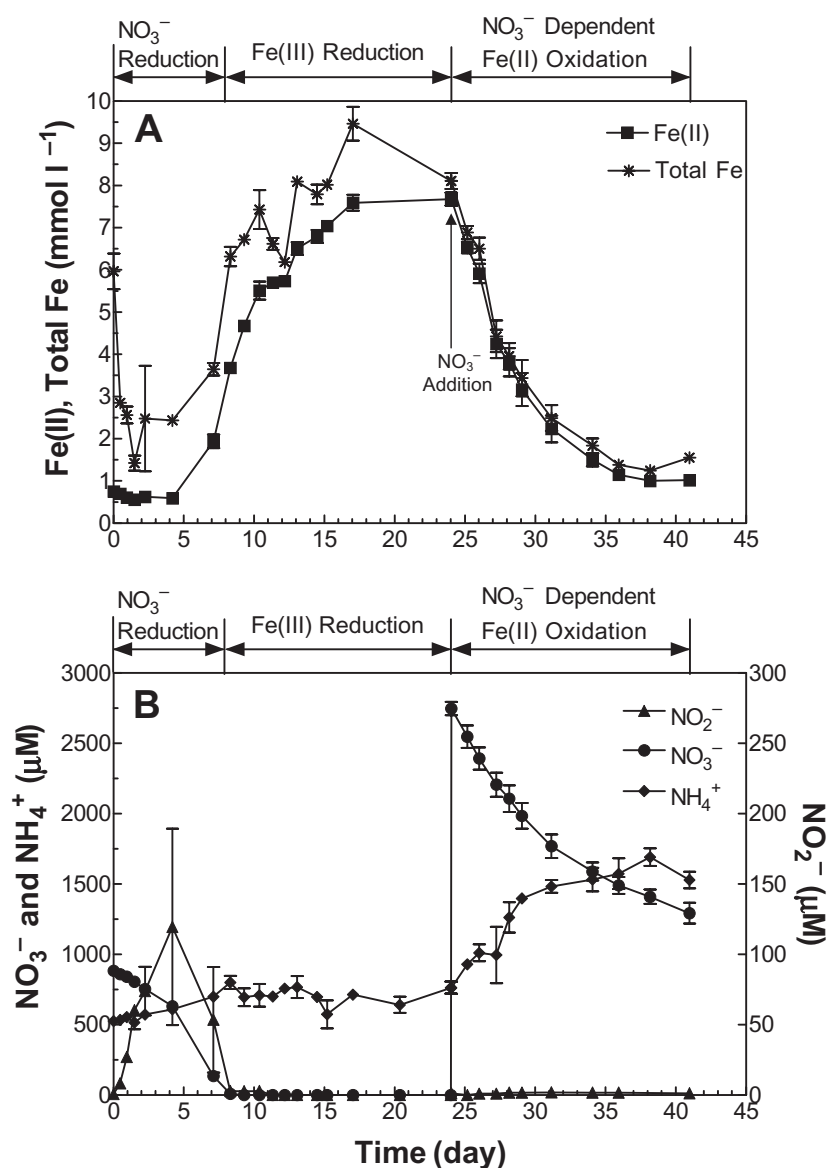
### Sequential nitrate reduction, Fe(III) reduction and nitrate-dependent Fe(II) oxidation in the sediment enrichment culture

Talladega Wetland sediment served as the inoculum (1% vol:vol) to artificial groundwater (AGW) containing 1 mM  $\text{NO}_3^-$ , 2 mM acetate and 50 mmol  $\text{l}^{-1}$  of synthetic high surface area goethite. Nitrate was consumed during the initial 7 days of incubation, resulting in transient accumulation of  $\text{NO}_2^-$  and production of approximately 0.2 mM  $\text{NH}_4^+$  (Fig. 2A and B). The molar ratio of  $\text{NH}_4^+$  produced to  $\text{NO}_3^-$  reduced (0.280,  $r^2 = 0.940$ ) was substantially lower than 1.0, which indicates that gaseous end-products such as  $\text{NO}$ ,  $\text{N}_2\text{O}$  and/or  $\text{N}_2$  (not measured in this study) were likely produced. A decrease in Fe(II) (0.75 mmol  $\text{l}^{-1}$  of Fe(II) was introduced with the sediment inoculum) of approximately 0.2 mmol  $\text{l}^{-1}$  occurred during the initial  $\text{NO}_3^-$

**Table 1.** MPN enumerations of nitrate-reducing, Fe(III)-reducing and nitrate-dependent Fe(II)-oxidizing microorganisms in Talladega Wetland surface sediments.

Culture conditions	MPN (cells $\text{ml}^{-1}$ )	95% confidence interval
Acetate + $\text{NO}_3^-$	$9.3 \times 10^7$	$2.1 \times 10^7$ – $2.7 \times 10^8$
Acetate + Fe(III)	$9.3 \times 10^7$	$2.1 \times 10^7$ – $2.7 \times 10^8$
Fe(II) + $\text{NO}_3^-$	$2.4 \times 10^5$	$4.8 \times 10^4$ – $9.6 \times 10^5$





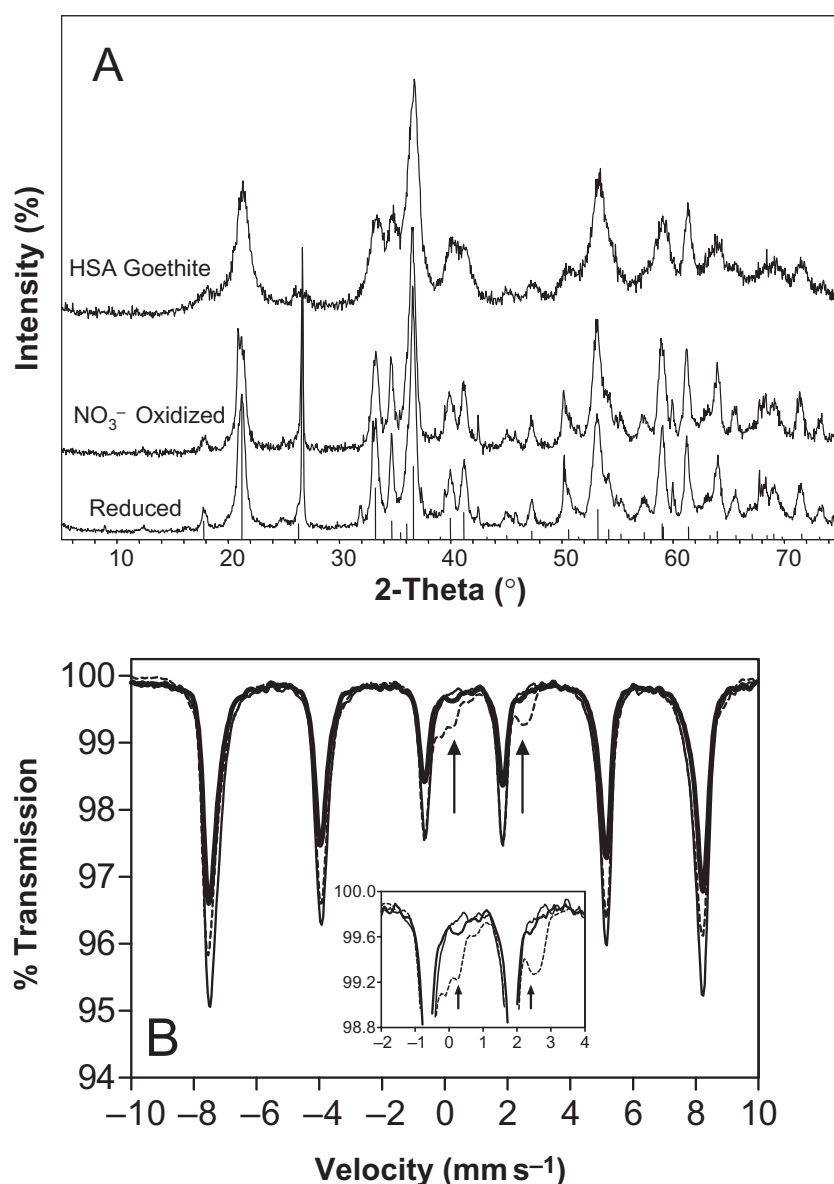
**Fig. 2.** Change in 0.5 M HCl-extractable Fe(II) and total Fe (A); and NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> (B) over time in the wetland sediment enrichment culture. Arrow denotes NO<sub>3</sub><sup>-</sup> amendment to induce nitrate-dependent Fe(II) oxidation. Error bars indicate standard error of triplicate cultures; bars not visible are smaller than symbol.

reduction phase. Because this loss of Fe(II) occurred during the period of transient NO<sub>2</sub><sup>-</sup> accumulation, it is possible that abiotic Fe(II) oxidation by NO<sub>2</sub><sup>-</sup> generated during organotrophic NO<sub>3</sub><sup>-</sup> reduction was responsible for this result. However, abiotic Fe(II) oxidation by NO<sub>3</sub><sup>-</sup> can be ruled out based on the results of pasteurized control cultures (see below).

Fe(III) reduction [Fe(II) accumulation] commenced once NO<sub>3</sub><sup>-</sup> decreased to below approximately 0.5 mM (Fig. 2A and B) and continued until acetate was depleted (data not shown), yielding 7.6 mmol l<sup>-1</sup> of 0.5 M HCl-extractable Fe(II) [equivalent to approximately 15% of the initial Fe(III) content of the slurry]. Approximately 35% of the HCl-extractable Fe(II) was present as dissolved Fe(II) at the end of the Fe(III) reduction phase. Reduction of the synthetic goethite resulted in an obvious colour change in

the mineral from gold-yellow to dark greenish-brown. Mixed Fe(II)–Fe(III) phases such as magnetite and/or green rust were not detected by X-ray diffraction (XRD) (Fig. 3A). However, comparison of a low-temperature (77K) Mössbauer spectra for the reduced goethite with that from a sample of microbially reduced (*Shewanella putrefaciens* in AQDS and HCO<sub>3</sub><sup>-</sup> containing medium) natural goethite (Kukkadapu *et al.*, 2001) indicated the presence of trace amounts of Fe(II) associated with green rust (Fig. 3B). The formation of only minor amounts of distinct Fe(II)-bearing mineral phases is consistent with other recent studies of the end-products of natural and synthetic goethite reduction by dissimilatory Fe(III)-reducing bacteria (Kukkadapu *et al.*, 2001; Zachara *et al.*, 2001). The vast majority of solid-associated Fe(II) was presumably sorbed and/or





**Fig. 3.** X-ray diffraction (A) and 77K Mössbauer (B) spectra of microbially reduced and nitrate-dependent oxidized HSA goethite from the sediment enrichment culture. The 'HSA goethite' spectrum in panel A is from a mineral preparation similar (but not identical) to the material used in the enrichment culture experiment; major peak lines for a reference goethite phase are shown at bottom. Thick and thin solid lines in panel B correspond to microbially reduced and nitrate-dependent oxidized HSA goethite, respectively, from the sediment enrichment culture. The dashed line shows results for microbially reduced natural goethite from Kukkadapu and colleagues (2001). Arrows in panel B point to an Fe(II) doublet (superimposed on the goethite sextet) that can be attributed to green rust (Kukkadapu *et al.*, 2001).

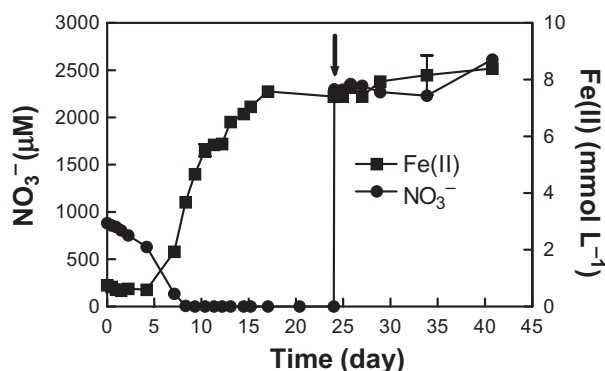
surface-precipitated on residual Fe(III) oxide surfaces (Zachara *et al.*, 2001; Roden and Urrutia, 2002).

Addition of NO<sub>3</sub><sup>-</sup> following cessation of Fe(III) reduction resulted in immediate oxidation of Fe(II) and consumption of NO<sub>3</sub><sup>-</sup> (Fig. 2A). Subcultures removed from the primary enrichment cultures and pasteurized prior to NO<sub>3</sub><sup>-</sup> re-addition showed no Fe(II) oxidation or NO<sub>3</sub><sup>-</sup> consumption (Fig. 4). Biological oxidation caused the microbially reduced goethite to change from greenish-brown back to its original goldish-yellow colour. Approximately 85% of 0.5 M HCl-extractable Fe(II) was oxidized within 15 days in live cultures. Total 0.5 M HCl-extractable Fe [Fe(II) + Fe(III)] decreased in parallel with HCl-extractable Fe(II) during nitrate-dependent Fe(II) oxidation (Fig. 2A), which suggests the production of crystalline Fe(III) oxide

phases not soluble in 0.5 M HCl. X-ray diffraction and Mössbauer spectra of the nitrate-oxidized material were virtually identical to those of the reduced mineral (Fig. 3), suggesting that goethite was likely reformed.

In contrast to the initial organotrophic NO<sub>3</sub><sup>-</sup> reduction phase of the experiment, nitrate-dependent Fe(II) oxidation did not result in the transient accumulation of NO<sub>2</sub><sup>-</sup> (< 2 µM). Significant accumulation of NH<sub>4</sub><sup>+</sup> (approximately 0.9 mM) took place during nitrate-dependent Fe(II) oxidation (Fig. 2B). The molar ratio of NO<sub>3</sub><sup>-</sup> reduced to Fe(II) oxidized (0.191,  $r^2 = 0.983$ ) was higher than the theoretical ratio for Fe(II) oxidation coupled to NO<sub>3</sub><sup>-</sup> reduction to NH<sub>4</sub><sup>+</sup> (0.125), which indicates that small quantities of end-products other than NH<sub>4</sub><sup>+</sup> (e.g. N<sub>2</sub>, NO, and/or N<sub>2</sub>O) were likely produced.





**Fig. 4.** Change in  $\text{NO}_3^-$  and 0.5 M HCl-extractable Fe(II) over time in pasteurized subsamples of the wetland sediment enrichment culture amended with approximately 2.5 mM  $\text{NO}_3^-$ . The arrow indicates time at which the enrichment culture was pasteurized and amended with  $\text{NO}_3^-$ . Error bars indicate standard error of triplicate cultures; bars not visible are smaller than symbol.

#### Changes in microbial community structure during Fe–N redox metabolism

Denaturing gradient gel electrophoresis analysis of PCR-amplified 16S rDNA and 16S rRNA RT-PCR products revealed a change in microbial community structure as redox conditions shifted from  $\text{NO}_3^-$  reduction to Fe(III) reduction (Fig. 5). The DGGE results must be interpreted as a preliminary fingerprint of microbial community structure, as repeated attempts to amplify bands excised from the denaturing gradient gel failed. Phylogenetic association of dominant members of the microbial community from each phase of the experiment was achieved via construction and sequencing of 16S rDNA clone libraries.

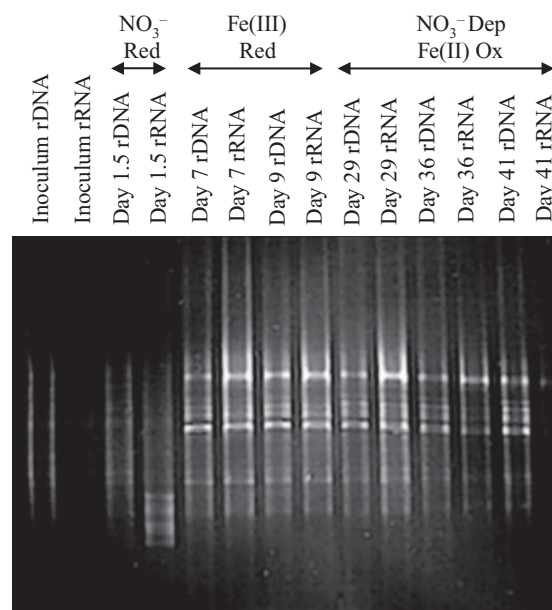
The clone libraries verified that a substantial change in community structure took place upon the shift from nitrate-reducing to Fe(III)-reducing conditions (Fig. 6A). One of the most significant changes was an increase in the number of clones associated with the beta subclass of the Proteobacteria (Betaproteobacteria), from approximately 3% under nitrate-reducing conditions to approximately 60% under Fe(III)-reducing conditions (Fig. 6A). *Dechloromonas* sp. was the most frequently identified (75%, Fig. 6C) phylogenetic group among the Betaproteobacteria clones. A significant fraction (25%) of clones from the Fe(III)-reducing phase were associated with the delta subclass of the Proteobacteria (Deltaproteobacteria) (Fig. 6A). Most of these Deltaproteobacteria clones (66%, Fig. 6B) were  $\geq 95\%$  similar to organisms from the genus *Geobacter*, a well-recognized group of dissimilatory Fe(III)-reducing bacteria (Lovley, 2002). This observation is consistent with the previous documentation of significant (approximately  $10^6 \text{ ml}^{-1}$ ) numbers of culturable acetate-oxidizing, Fe(III)-reducing *Geobacter* sp. in Talladega Wetland surface sediments (Coates *et al.*, 1996).

The 16S rDNA/rRNA fingerprints from the nitrate-

dependent Fe(II) oxidation phase were similar to those from the Fe(III) reduction phase (Fig. 5), which suggests that the microbial community active during Fe(III) reduction was also responsible for nitrate-dependent Fe(II) oxidation. The corresponding clone libraries revealed only a small shift in community structure. The frequency of Betaproteobacteria clones increased slightly from 60% to 65%, and the frequency of Deltaproteobacteria clones decreased slightly from 25% to 22% (Fig. 6A). Within the Betaproteobacteria, the frequency of clones identified as *Dechloromonas* sp. decreased (to 40%) and clones identified as *Azospira* sp. (11%), *Aquaspirillum* sp. (11%), *Aquabacterium* sp. (4%) appeared along with several clones (33%) that were not classified within the Betaproteobacteria (Fig. 6C).

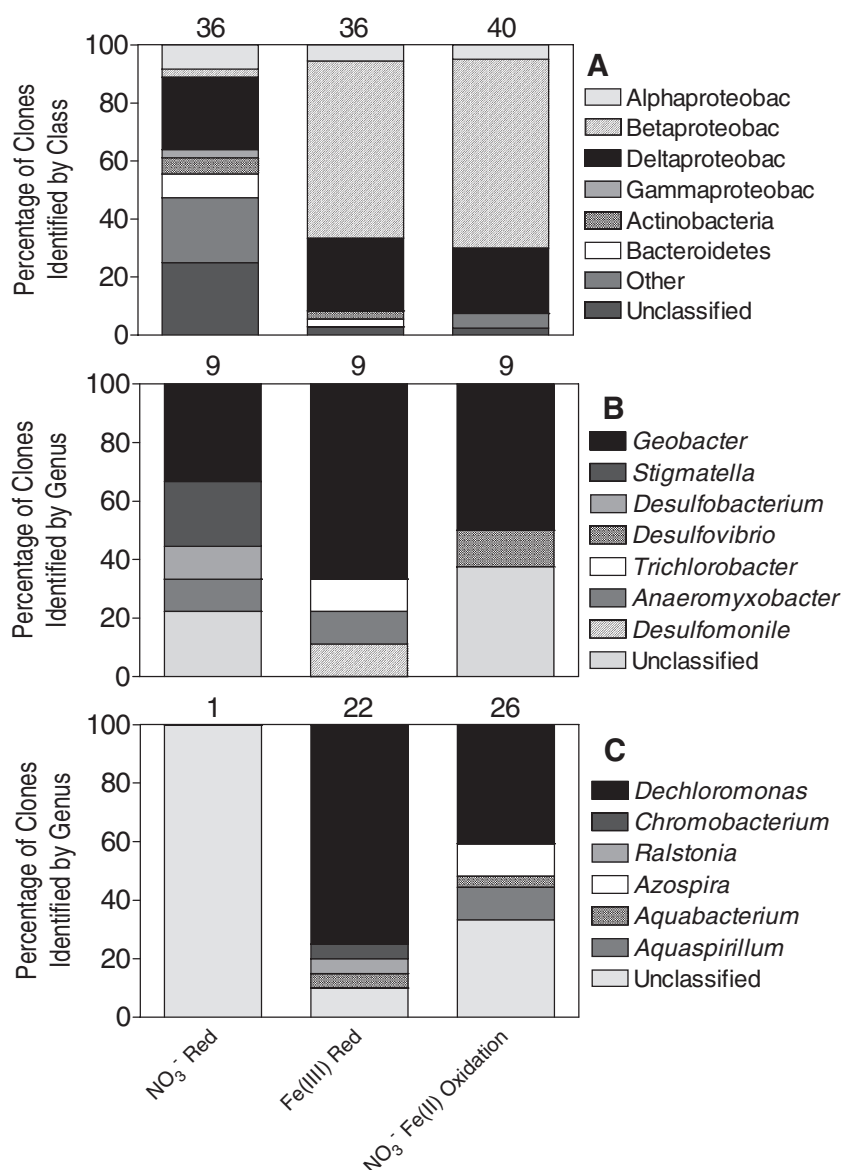
#### Sequential $\text{NO}_3^-$ Reduction, Fe(III) Reduction and nitrate-dependent Fe(II) oxidation by *G. metallireducens*

The potential for *G. metallireducens* to carry-out anaerobic Fe redox cycling was examined in an experiment analogous to the wetland sediment enrichment culture study.  $\text{NO}_3^-$  was reduced within the first few days of incubation, resulting in a transient accumulation of  $\text{NO}_2^-$  (61  $\mu\text{M}$ ) and stoichiometric production of  $\text{NH}_4^+$  (97% of added  $\text{NO}_3^-$ , Fig. 7). The presence of  $\text{NO}_3^-$  did not inhibit Fe(III) reduction, as evidenced by accumulation of Fe(II) concurrent with  $\text{NO}_3^-$  reduction. When  $\text{NO}_3^-$  was added upon cessa-



**Fig. 5.** DGGE analysis of PCR-amplified 16S rDNA and RT-PCR-amplified 16S rRNA from the enrichment culture. Time (day) corresponds to the x-axis in Fig. 2. 'Inoculum' refers to 16S rDNA and 16S rRNA extracted from the sediment used to inoculate the enrichment cultures.





**Fig. 6.** Relative proportions of 16S rRNA gene sequences in clone libraries from the enrichment culture.

A. Sequences within the domain Bacteria.

'Other' represents *Verrucomicrobiae*, *Deferribacteriales*, *Cytophaga*, *Planctomycetacia*, *Fusobacteria* and *Acidobacteria* combined.

B. Sequences with  $\geq 95\%$  sequence similarity to known genera within the Deltaproteobacteria.

C. Sequences with  $\geq 95\%$  sequence similarity to known genera within the Betaproteobacteria.

'Unclassified' represents sequences with  $< 95\%$  sequence similarity to known genera. Numbers above the bars indicate the total number of clones within a given group.

tion of Fe(III) reduction, 86% of 0.5 M HCl-extractable Fe(II) was oxidized within 4 days coupled to consumption of NO<sub>3</sub><sup>-</sup> and accumulation of NH<sub>4</sub><sup>+</sup> (Fig. 7). Fe(II) oxidation then continued at a slower rate, resulting in the consumption of virtually all (93%) of the 0.5 M HCl-extractable Fe(II). The molar ratios of NO<sub>3</sub><sup>-</sup> reduced to Fe(II) oxidized and NH<sub>4</sub><sup>+</sup> produced to Fe(II) consumed (0.175,  $r^2 = 0.989$  and 0.189,  $r^2 = 0.897$ , respectively) were slightly higher than the theoretical ratio for NO<sub>3</sub><sup>-</sup> reduction to NH<sub>4</sub><sup>+</sup> coupled to Fe(II) oxidation (0.125).

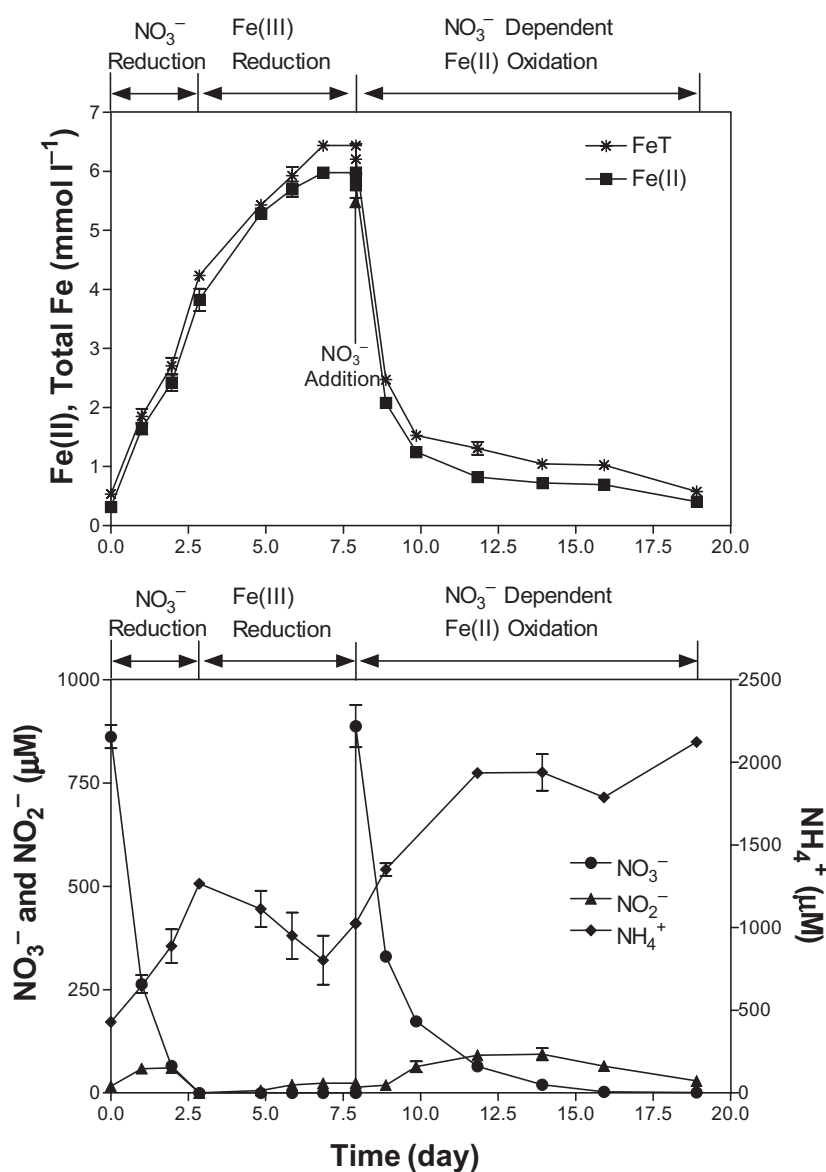
## Discussion

### Nitrate inhibition of Fe(III) reduction

Nitrate inhibited Fe(III) reduction in the sediment enrich-

ment culture (Fig. 2A), a result consistent with previous studies of the influence of NO<sub>3</sub><sup>-</sup> on Fe(III) reduction in pure and mixed cultures of Fe(III)-reducing bacteria (Obuekwe *et al.*, 1981; Sorensen, 1982; Jones *et al.*, 1983; DiChristina, 1992; Achtnich *et al.*, 1995; Finneran *et al.*, 2002; Cooper *et al.*, 2003). In contrast, Fe(III) was reduced simultaneously with NO<sub>3</sub><sup>-</sup> in the *G. metallireducens* cell suspension. The latter result may be attributed to the presence of a relatively high initial cell density (approximately 10<sup>8</sup> cells ml<sup>-1</sup>) and an excess of electron donor, which allowed both processes to occur together. Similar experiments using a 10-fold lower initial *G. metallireducens* cell density showed inhibition of Fe(III) reduction by NO<sub>3</sub><sup>-</sup> comparable to that observed in the wetland sediment enrichment culture.





**Fig. 7.** Change in 0.5 M HCl-extractable Fe(II), and total Fe (A) and NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> (B) over time in growth medium inoculated with nitrate-grown *G. metallireducens* cells. Arrow denotes the NO<sub>3</sub><sup>-</sup> to induce nitrate-dependent Fe(II) oxidation. Error bars indicate standard error of triplicate cultures; bars not visible are smaller than symbol.

#### Mechanism and end-products of nitrate-dependent Fe(II) oxidation

The addition of NO<sub>3</sub><sup>-</sup> following the cessation of Fe(III) reduction resulted in immediate and rapid oxidation of both aqueous and solid-phase Fe(II) and production of substantial quantities of NH<sub>4</sub><sup>+</sup> (Fig. 2). Abiotic oxidation of Fe(II) by gaseous end-products such as NO and N<sub>2</sub>O can be ruled out based on previous studies in which NO and N<sub>2</sub>O additions to soil slurries containing Fe(II) did not result in an increase in Fe(III) concentrations (Kluber and Conrad, 1998). Moreover, lack of significant Fe(II) oxidation in pasteurized cultures (Fig. 4) indicated that NO<sub>3</sub><sup>-</sup> did not abiotically oxidize the microbially reduced goethite. This result is consistent with previously reported results

in which abiotic oxidation of Fe(II) by NO<sub>3</sub><sup>-</sup> was not observed in suspensions of microbially reduced goethite or other Fe(III) oxide-bearing solids (Weber *et al.*, 2001).

Production of NH<sub>4</sub><sup>+</sup> as an end-product of biological nitrate-dependent Fe(II) oxidation has not been previously reported. To date, only studies of abiotic NO<sub>3</sub><sup>-</sup> reduction coupled to oxidation of green rust compounds have demonstrated NH<sub>4</sub><sup>+</sup> as a predominant end-product of nitrate-dependent Fe(II) oxidation (Hansen *et al.*, 1996; Hansen *et al.*, 2001). Green rust was not identified as a major product of microbial goethite reduction generated in this study nor in previous studies using similar culture conditions (Cooper *et al.*, 2000; Kukkadapu *et al.*, 2001; Zachara *et al.*, 2001). This result, together with the lack of Fe(II) oxidation in pasteurized controls, argues against



abiotic reaction of  $\text{NO}_3^-$  with green rust as a mechanism for  $\text{NH}_4^+$  production in the enrichment culture. A recent report documented green rust as a minor end-product of soluble Fe(II) ( $\text{FeCl}_2$ ) oxidation coupled to  $\text{NO}_3^-$  reduction by *Azospira suillum* (Chaudhuri *et al.*, 2001). These findings suggest the possibility that green rust compounds, generated during the initial stages of nitrate-dependent Fe(II) oxidation, served as a reductant for further (i.e. autocatalytic) abiotic reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$  in our experiments. However, Chaudhuri and colleagues (2001) reported that  $\text{NO}_3^-$  was stoichiometrically reduced to  $\text{N}_2$  under biological Fe(II)-oxidizing conditions, whereas stoichiometric reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$  is observed during abiotic oxidation of synthetic chloride green rust [ $\text{Fe(II)}_{\text{GR-C}}$ ] (Hansen *et al.*, 2001). These results argue against a green rust-mediated autocatalytic mechanism for abiotic Fe(II) oxidation in the experiments of Chaudhuri and colleagues (2001), further supporting an enzymatic Fe(II) oxidation mechanism. Together these previous observations reinforce the argument that the  $\text{NH}_4^+$  produced during nitrate-dependent Fe(II) oxidation in this study was the result of microbial (enzymatic) catalysis rather than abiotic reaction(s) with a green rust intermediate.

Oxidation of Fe(II) (soluble and solid-phase) coupled to  $\text{NO}_3^-$  reduction during the final phase of the sediment enrichment, as well as the pure culture study with *G. metallireducens* (Fig. 7), resulted in a decline in total 0.5 M HCl-extractable Fe in parallel with the decrease in Fe(II) (Fig. 2A). These results suggest that crystalline Fe(III) oxide phases were formed, in contrast to previous studies which indicated that nitrate-dependent Fe(II) oxidation resulted in the formation of poorly crystalline Fe(III) oxide (Straub *et al.*, 1996; 1998). These contrasting results may be attributed to the relatively low concentration of phosphate in the culture medium used in our experiments (0.05 mM, compared with 1.5 mM in the culture medium employed by Straub *et al.*, 1996, 1998): other studies with the Straub and colleagues (1996) enrichment culture (Weber, 2002) have shown that a decrease in phosphate from approximately 1.5–0.05 mM resulted in a progressive increase in the formation of crystalline (goethite, lepidocrocite) versus amorphous phases. These data suggest that in the absence of high concentrations of inhibitors of Fe(III) oxide crystallization such as phosphate (Cornell and Schwertmann, 1996), nitrate-dependent Fe(II) oxidation is likely to result in the production of predominantly crystalline Fe(III) oxide phases which are insoluble in dilute (0.5 M) HCl. Given that solid-associated Fe(II) was the dominant form of Fe(II) available for nitrate-dependent oxidation in the enrichment culture experiment, and that virtually all of the Fe(II) oxidized in this experiment was lost from the 0.5 M HCl-extractable pool, our results indicate that both aqueous and solid-phase Fe(II) are subject to conversion to crystalline Fe(III) oxides dur-

ing nitrate-dependent Fe(II) oxidation. The similarity of XRD and Mössbauer spectra for the reduced and nitrate-dependent oxidized goethite (Fig. 3) indicate that goethite phases with particle size and crystallinity nearly identical to that of the starting material were regenerated during nitrate-dependent oxidation.

#### *Microbial communities associated with Fe–N redox cycling*

Although previous studies have demonstrated that pure cultures of organotrophic denitrifying bacteria are capable of nitrate-dependent Fe(II) oxidation (Straub *et al.*, 1996; Benz *et al.*, 1998; Chaudhuri *et al.*, 2001), the majority of the organisms detected during nitrate-dependent Fe(II) oxidation in this study were more similar to those present during the Fe(III) reduction phase than in the organotrophic  $\text{NO}_3^-$  reduction phase (Figs 5 and 6). These results indicate that microorganisms capable of both Fe(III) reduction and nitrate-dependent Fe(II) oxidation were responsible for the Fe(II) oxidation activity observed in the latter stages of the experiment. The rapid onset of nitrate-dependent Fe(II) oxidation following Fe(III) reduction supports the idea that Fe(III)-reducing microorganisms which proliferated during the Fe(III) reduction phase of the experiment were responsible for subsequent nitrate-dependent Fe(II) oxidation. Organisms such as *G. metallireducens* (Lovley and Phillips, 1988) and other members of Geobacteraceae (Lovley, 2002), which are capable of organotrophic growth with either  $\text{NO}_3^-$  or Fe(III) as an electron acceptor, are logical candidates for such organisms.

In light of the recent observation by Finneran and colleagues (2002) that *G. metallireducens* can couple oxidation of soluble Fe(II) to reduction of  $\text{NO}_3^-$ , we examined whether *G. metallireducens* could catalyse nitrate-dependent oxidation of microbially reduced goethite under conditions analogous to the wetland sediment enrichment culture. The results provided clear evidence of such activity (compare Figs 2 and 7). The large proportion of *Geobacter* sp. 16S rDNA sequences in clone libraries from the nitrate-dependent Fe(II) oxidation phase of the experiment (Fig. 6B), the persistence (suggested by DGGE analysis; see Fig. 5) of a few key genotypes throughout the Fe(III) reduction and nitrate-dependent Fe(II) oxidation phases, and the production of  $\text{NH}_4^+$  as an end-product of nitrate-dependent Fe(II) oxidation sp., provides convincing evidence for the role of *Geobacter* in nitrate-dependent Fe(II) oxidation in the wetland sediment. It is thus far unknown whether *G. metallireducens* and other nitrate-reducing members of Geobacteraceae are able to conserve energy for growth/maintenance from nitrate-dependent Fe(II) oxidation.

The detection of significant numbers of 16S rDNA



clones associated with *Dechloromonas* sp. and other organisms from the Betaproteobacteria in libraries from the Fe(III)-reducing and nitrate-dependent Fe(II) oxidation phases in the enrichment culture (Fig. 6A and C) suggests the possibility that such organisms may have also been involved in anaerobic Fe–N redox cycling. This idea is supported by the fact that nitrate-dependent Fe(II) oxidation has been described within the Betaproteobacteria, specifically in *Dechloromonas* sp. and *Azospira* sp. (Bruce *et al.*, 1999; Chaudhuri *et al.*, 2001), as well as in the nitrate-dependent Fe(II)-oxidizing bacterium BRG2, which has 98% sequence identity with *Aquabacterium commune* (Buchholz-Cleven *et al.*, 1997). *Dechloromonas* sp., *Azospira* sp. and *Aquaspirillum* sp. have been described as denitrifiers which produce  $N_2$  as the reduced N product (Mahne and Tiedje, 1995; Chaudhuri *et al.*, 2001; Coates *et al.*, 2001). Given that chemical analysis of the wetland sediment enrichment identified  $NH_4^+$  as a primary product of nitrate-dependent Fe(II) oxidation, these bacteria were obviously not the only active members of the nitrate-dependent Fe(II)-oxidizing microbial community. The combined results of the DGGE and 16S rDNA clone library analyses suggest (but do not prove) that organisms related to *Dechloromonas* sp. were abundant during Fe(III) reduction as well as nitrate-dependent Fe(II) oxidation. Although dissimilatory Fe(III) reduction by *Dechloromonas* sp. has not been observed in pure culture, Fe(III) reduction by Betaproteobacteria has been identified in *Ferribacterium limneticum* (Cummings *et al.*, 1999), whose 16S rRNA gene sequence is 99% similar to that of *Dechloromonas aromatica* strain RCB (Coates *et al.*, 2001).

#### Biogeochemical significance

Oxidation of soluble and solid-phase Fe(II) coupled to  $NO_3^-$  reduction provides the potential for a tight coupling between N and Fe redox cycles in sedimentary environments (Straub *et al.*, 2001; 2004). Such coupling is likely to be particularly significant at the interface between  $NO_3^-$  and Fe(III) reduction zones in sediments influenced by periodic fluctuations in the inputs of organic carbon and oxidants (see Fig. 1). These fluctuations are common in shallow subsurface environments, which are typically very active hydrologically and support a rich and diverse microflora (Chapelle, 2001). When inputs of organic carbon are relatively high compared with  $NO_3^-$ , organotrophic  $NO_3^-$  reduction may exhaust available  $NO_3^-$ , thus allowing microbial Fe(III) reduction and associated production of aqueous Fe(II) and/or Fe(II)-bearing solid-phases to occur. During subsequent periods of reduced organic carbon loading, rates of  $NO_3^-$  re-supply may exceed rates of organotrophic  $NO_3^-$  reduction, resulting in the availability of nitrate for lithotrophic, nitrate-dependent Fe(II) oxida-

tion. In this way, reducing equivalents stored in the form of aqueous and/or Fe(II)-bearing solid-phases may serve as an significant source of energy for microbial metabolism during periods of reduced organic carbon input. Organisms such as *G. metallireducens* which are capable of switching between organotrophic nitrate and/or Fe(III) reduction and nitrate-dependent Fe(II) oxidation are likely to have a competitive advantage in such environments. Detailed studies of the response of sediment microbial communities to repeated fluctuations in organic carbon and  $NO_3^-$  loading are required to verify this hypothesis.

Nitrate-dependent Fe(II) oxidation has the potential to significantly influence patterns of organic and inorganic contaminant transformations in anaerobic soils and sediments through local consumption of  $NO_3^-$  and regeneration of Fe(III) oxides. Straub and colleagues (2004) recently described an example of the potential impact of Fe–N redox cycling on benzoate oxidation in anaerobic sediments. A co-culture consisting of Fe(III)-reducing (*Geobacter bremensis*) and a consortia of nitrate-dependent Fe(II)-oxidizing organisms (the stable enrichment culture described in Straub *et al.*, 1996) was able to oxidize benzoate coupled to Fe(III) oxide reduction through a process in which a relatively small amount of amorphous Fe(III) oxide was continuously recycled via nitrate-dependent Fe(II) oxidation. Such interactions expand the range of potential mechanisms by which natural and contaminant organic compounds may be oxidized in anaerobic soils and sediments. Recent examples of the influence of anaerobic Fe–N redox cycling on inorganic biogeochemical processes include the oxidation and coprecipitation of Fe(III) and As(V) in stratified lake waters (Senn and Hemond, 2002), and the oxidation of U(IV) by Fe(III) generated during nitrate-dependent Fe(II) oxidation in uranium-contaminated subsurface sediments (Finneran *et al.*, 2002). In addition, formation of relatively crystalline Fe(III) oxides during biological oxidation of aqueous and solid-phase Fe(II), as observed in this study, could contribute to the sequestration of metal-radionuclide contaminants by incorporation of the metal or radionuclide into the oxide lattice and/or physical envelopment of the contaminant(s). Lack and colleagues (2002) demonstrated the potential for immobilization of Co(II) and U(VI) in crystalline Fe(III) oxides formed during nitrate-dependent Fe(II) oxidation. Because crystalline Fe(III) oxides are not as available to microbial Fe(III) reduction as amorphous Fe(III) oxides, metal-radionuclide contaminants immobilized in crystalline biogenic Fe(III) oxides may be resistant to remobilization with the onset of Fe(III)-reducing conditions. Further studies of the influence of solid-phase Fe(II)–Fe(III) conversions on metal-radionuclide speciation are needed to determine the long-term effects of Fe–N redox cycling on the mobility of such contaminants in sediments.



## Experimental procedures

### Most probable number enumerations

The abundance of culturable acetate-oxidizing nitrate-reducing bacteria, acetate-oxidizing Fe(III)-reducing bacteria, and Fe(II)-oxidizing nitrate-reducing bacteria in freshwater wetland surface sediments were estimated using a three-tub MPN technique (Woomer, 1994). A freshwater wetland sediment core was collected from a small (15 ha) wetland located in the Talladega National Forest, Hale County, Alabama. Previous studies have documented the quantitative significance of microbial Fe(III) reduction and Fe redox cycling on sediment carbon metabolism and energy flow in this environment (Roden and Wetzel, 1996). Upon return to the laboratory, sediment from the upper 5 cm of the core was homogenized, sieved (1 mm), and placed under  $N_2$  at room temperature. Triplicate pressure tubes containing sterile, anaerobic ( $N_2:CO_2$ ; 90:10) AGW medium (10 mM PIPES, 2 mM  $NaHCO_3$ , 5 mM  $NH_4Cl$ , 0.5 mM  $KH_2PO_4$ , pH 6.8) were inoculated with serially diluted anaerobic, homogenized sediment. For enumeration of acetate-oxidizing,  $NO_3^-$ -reducing bacteria, tubes were amended with 5 mM  $NaNO_3$  and 10 mM Na-acetate from sterile, anaerobic stock solutions. Acetate-oxidizing, Fe(III)-reducing bacteria were enumerated in medium amended with 10 mmol  $l^{-1}$  of synthetic hydrous ferric oxide prior to autoclaving. The culture tubes were then amended with 10 mM Na-acetate and 2 mM  $FeCl_2$  (as a reducing agent) from sterile, anaerobic stock solutions. The medium for enumeration of nitrate-dependent Fe(II)-oxidizing bacteria was amended with 0.5 mM Na-acetate, 5 mM  $NO_3^-$  and 10 mM  $FeCl_2$  from sterile anaerobic stock solutions.

Pressure tubes were inoculated with 1-ml portions of the homogenized freshwater wetland sediment slurry. The tubes were then homogenized by vortexing, serially diluted, and incubated statically in the dark at 30°C for 10 weeks. Positive results for acetate-oxidizing nitrate reducers were determined checking for depletion of  $NO_3^-$  (to < 1 mM) by ion chromatography. Visual assessment of blackening of the medium, and formation of reddish-brown precipitates, was used to identify positive results for Fe(III) reducers and nitrate-dependent Fe(II) oxidizers respectively. MPN estimates were obtained using the Most Probable Number Calculator version 4.05 (©1996 Albert J. Klee, Risk Reduction Engineering Laboratory, United States Environmental Protection Agency, Cincinnati, Ohio, freeware available at <http://www.epa.gov/nerlcwww/other.htm>).

### Enrichment culture experiment

A PIPES-buffered (10 mM, pH 6.8) AGW medium was used for the wetland sediment enrichment culture experiment. The basal AGW contained 0.11 mM  $MgCl_2$ , 0.61 mM  $CaCl_2$  and 2 mM  $NaHCO_3$ , and was supplemented with 1 mM  $NaNO_3$ , 2 mM Na-acetate, 0.5 mM  $NH_4Cl$ , 0.05 mM  $KH_2PO_4$ , 0.1× of previously described (Lovley and Phillips, 1988) vitamin and trace element solutions, and 50 mmol  $l^{-1}$  of synthetic 'high surface area' (approximately 200  $m^2 g^{-1}$ ) goethite. The synthetic goethite was synthesized by slow air oxidation of  $FeCl_2$  in  $NaHCO_3$  buffer (Schwertmann and Cornell, 1991), and its surface area was determined by multipoint  $N_2$  (BET) adsorption (Micromeritics Model Gemini). Triplicate bottles of

medium were inoculated with 1% (vol:vol) of anaerobic sediment slurry immediately after collection and preparation as described above. Samples were collected over time for analysis of  $NO_3^-$ ,  $NO_2^-$ ,  $NH_4^+$ , acetate, Fe(II), total Fe and 16S rDNA/rRNA. Once  $NO_3^-$  and Fe(III) reduction ceased,  $NO_3^-$  (3 mM) was re-added to the enrichment cultures in an attempt to induce nitrate-dependent Fe(II) oxidation. Immediately prior to the 3 mM  $NO_3^-$  amendment, a 30-ml subculture was removed from each culture bottle, transferred into a sterile, anaerobic ( $N_2:CO_2$ ; 80:20) serum bottles, and pasteurized (80°C; 15 min) to produce killed controls. Killed controls were amended with  $NO_3^-$  (3 mM) following pasteurization. Live and pasteurized cultures were incubated statically in the dark at 30°C and homogenized prior to sampling.

### Anaerobic Fe redox cycling by *G. metallireducens*

*Geobacter metallireducens* (a gift from D. R. Lovley, University of Massachusetts Amherst) was grown in  $NaHCO_3$ -buffered medium (30 mM  $NaHCO_3$ , 10 mM  $NH_4Cl$ , 1 mM  $KH_2PO_4$ ) with acetate (10 mM) as the electron donor and nitrate (20 mM) as the electron acceptor. The growth medium was supplemented with 0.5 mM Fe(III)-citrate in order to provide the extra Fe required for sustained growth with nitrate as the electron acceptor (Senko and Stolz, 2001), as well as 1 mM ascorbic acid to reduce the accumulation of  $NO_2^-$  which can inhibit cell growth on nitrate (E. J. P. Phillips, US Geological Survey, pers. comm.). Cells were harvested by centrifugation (7000 g, 10 min) and washed twice with sterile, anaerobic  $NaHCO_3$  buffer. Triplicate 20-ml bottles of the AGW medium described above were inoculated with approximately  $10^8$  cells  $ml^{-1}$  of washed cells, and changes in  $NO_3^-$ ,  $NO_2^-$ ,  $NH_4^+$ , acetate, Fe(II) and total Fe were monitored over time. As in the case of the wetland sediment enrichment culture experiment,  $NO_3^-$  (approximately 1 mM) was added to the cultures once acetate was depleted and Fe(III) reduction had ceased.

### Chemical and spectroscopic analyses

Samples for analyses of Fe(II) and total Fe were collected and centrifuged (10 000 g) inside an anaerobic chamber, as previously described (Weber *et al.*, 2001). The supernatant was withdrawn from the pellet and immediately analysed for aqueous Fe(II) using Ferrozine (Stookey, 1970). The pellet was resuspended in 0.5 M HCl and allowed to extract overnight. Fe(II) and total Fe in the extract were determined as described in Roden and Lovley (1993). The concentration of 0.5 M HCl-extractable Fe(II) determined via pellet extractions, together with aqueous Fe(II) measurements, were summed to yield total Fe(II) concentrations (Weber *et al.*, 2001). Samples for  $NO_3^-$ ,  $NO_2^-$ ,  $NH_4^+$  and acetate were filtered through a 0.2- $\mu m$  nylon filter immediately after collection and exposed to  $O_2$  in order to rapidly oxidize Fe(II) (Weber *et al.*, 2001). The filtered samples were centrifuged and the supernatant withdrawn for  $NO_3^-$  and  $NO_2^-$  analyses. Samples collected for  $NH_4^+$  were filtered a second time prior to analysis.  $NO_3^-$  and acetate were determined by ion chromatography (IonPac® AS14 analytical column, Dionex DX-100 system, Dionex,



Sunnyvale, CA).  $\text{NO}_2^-$  and  $\text{NH}_4^+$  concentrations were determined colorimetrically (Wetzel and Likens, 1991) with detection limits of 0.01  $\mu\text{M}$  and 1  $\mu\text{M}$  respectively.

Powder XRD and low-temperature (77K) Mössbauer spectra of (i) microbially reduced goethite and (ii) microbially reduced goethite subjected to nitrate-dependent oxidation, were obtained as described in Kukkadapu and colleagues (2001, 2004). An XRD pattern was also obtained for a non-biologically transformed HSA goethite preparation similar (but not identical) to the material used for the enrichment culture experiment.

#### Nucleic acid extraction and purification

Samples for nucleic acid extraction were collected in sterile, diethyl pyrocarbonate (DEPC) treated polyethylene centrifuge tubes and maintained on ice until centrifugation. Collected samples were centrifuged (10 000 g, 20 min) and washed once with 10 mM RNase-free Tris Buffer (pH 7.8). The pellet was immediately frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until extraction of nucleic acids.

Nucleic acids were extracted using a modified method of Zhou and colleagues (1996) by grinding in liquid  $\text{N}_2$ , freeze-thawing and extended heating ( $60^\circ\text{C}$ ) in a high-salt extraction buffer containing hexadecylmethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS). CTAB was heated to  $60^\circ\text{C}$  prior to being added to the extraction buffer. Following centrifugation (6000 g, 10 min), the supernatant was removed from the pellet and nucleic acids were extracted in phenol:chloroform (1:1; vol:vol), followed by an additional chloroform extraction. Nucleic acids were precipitated in isopropanol, centrifuged (9000 g, 20 min), and resuspended in RNase-free Tris-EDTA (TE) buffer. The nucleic acid extracts were divided into two equal volumes and stored at  $-80^\circ\text{C}$  until DNA and RNA purification. All materials and solutions used during the nucleic acid extraction process were treated with DEPC prior to use, with the exception of solutions containing Tris which were prepared in DEPC-treated (denoted as RNase-free) deionized water.

Nucleic acid extracts used for amplification of 16S rDNA were purified by gel filtration using Sepharose CL-4B columns as described by Jackson *et al.* (Jackson *et al.*, 1997), precipitated with NaCl (0.2 M) in absolute ethanol overnight, and resuspended in TE buffer. DNA samples were stored at  $-80^\circ\text{C}$ . Nucleic acid extracts dedicated to RT-PCR amplification of 16S rRNA were purified by gel filtration using DEPC-treated Sephadex G-75 (Moran *et al.*, 1993), precipitated with RNase-free Na-Acetate (0.3 M) in absolute ethanol overnight and resuspended in RNase-free TE buffer. DNA was removed from RNA by treatment with DNase I (RNase-free; Roche Diagnostics Corporation) followed by a phenol:chloroform (1:1; vol:vol) extraction. RNA was concentrated by precipitating with RNase-free Na-Acetate (0.3 M) in absolute ethanol overnight and resuspended in RNase-free TE buffer. RNA extracts were stored at  $-80^\circ\text{C}$ .

#### Denaturing gradient gel electrophoresis

A fragment of the variable V3 region of 16S rDNA corresponding to positions 341–907 in *Escherichia coli* was PCR-amplified (Touchdown PCR) using the primers denoted as

341F, 5'-CCTACGGGAGGCAGCAG-3' and 907R, 5'-CCGTC AATTCCTTTTAGTTT-3' with a GC-Clamp added to 5' end of forward primer: 5'-CGCCCCGCGCGCGCGCGGGCGGG GCGGGGGCACGGGGGG-3' as described by Muyzer and colleagues (1995). Within the Domain Bacteria, these primers are universally conserved (Medlin *et al.*, 1988) and have thus been used to evaluate bacterial diversity in environmental samples (Muyzer *et al.*, 1993; Muyzer and Ramsing, 1995) as well as the diversity of anaerobic Fe(II)-oxidizing bacteria (Buchholz-Cleven *et al.*, 1997; Straub and Buchholz-Cleven, 1998). Products from 10 replicate PCR amplifications were combined, extracted in phenol:chloroform (1:1; vol:vol), precipitated with NaCl (0.2 M) in absolute ethanol overnight, and resuspended in TE buffer. Negative control PCR amplifications (no DNA added to reaction mix) were routinely conducted.

Reverse transcription of 16S rRNA was performed using SUPERScript™ II RNase H<sup>-</sup> Reverse Transcriptase (GibcoBRL®) as specified by the manufacturer, with 907R serving as the reverse primer. The resulting cDNA was PCR-amplified as described above. Negative reverse transcriptase reactions (reverse transcriptase absent from reaction mix) were routinely conducted and subsequently PCR-amplified as described above.

Denaturing gradient gel electrophoresis was conducted using a 6% acrylamide gel containing a 28–56% denaturing gradient (Muyzer and Ramsing, 1995). Polymerase chain reaction products of 16S rDNA and reverse transcribed 16S rRNA were applied directly to a 6% acrylamide gel in 0.5× TAE buffer. The gel was electrophoresed at  $60^\circ\text{C}$  at 200 V for 6 h using a Bio-Rad D Gene™ System (Bio-Rad Laboratories, Hercules, CA) (Muyzer *et al.*, 1993). After electrophoresis, the gel was stained with ethidium bromide (0.5 mg l<sup>-1</sup>) for 15 min and rinsed with deionized  $\text{H}_2\text{O}$  for 10 min prior to UV transillumination. Gel images were captured digitally.

Band intensity on DGGE gels was used to infer the relative abundance and metabolic activity of microorganisms present at different times during the enrichment culture experiment. This approach is based on the assumption that 16S rDNA abundance is directly related to density of the corresponding organism in the sample (Muyzer *et al.*, 1993; Bruggemann *et al.*, 2000), and that metabolically active cells possess greater 16S rRNA content than non-active cells (Kemp *et al.*, 1993). It is recognized, however, that correlation of metabolic activity with 16S rRNA content must be done with caution as regulation of metabolic activity and rRNA content may differ among various bacterial species (Wagner, 1994). In addition, the potential for inherent biases in PCR-based detection/quantification strategies is well recognized (VonWintzingerode *et al.*, 1997). Hence, changes in DGGE-banding patterns are interpreted as a first approximation of change in microbial community structure and metabolic activity in the enrichment culture samples.

#### Clone libraries

Amplification products for construction of 16S rDNA clone libraries were generated with primers corresponding to the positions 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), and 907R (described above) in *E. coli*. Polymerase chain reaction products were purified using QIAquick PCR Purification Kit



(QIAGEN #28106) according to manufacturer's instructions and stored at  $-20^{\circ}\text{C}$ . The pGem@-T vector System (Promega #A3600) was used according to manufacturer's instructions, with Top10 competent *E. coli* cells from Invitrogen, to generate clone libraries from PCR-amplified 16S rDNA from samples collected at days 0, 1.5, 9 and 36 of the enrichment culture experiment. Between 90 and 100 clones were picked from each sample transformation. Vector primers M13F1 and M13R1 were used to amplify cloned sequences. Sequences were concentrated by precipitation in 95% ethanol and NaCl (200 mM) overnight at  $-20^{\circ}\text{C}$  and resuspended, in TA buffer (10 mM, pH 7.6). Concentrated DNA was purified by electrophoresis using a low melt agarose gel (1.5%) in TAE running buffer. Bands in the gel were cut out, frozen overnight, and spun down at 10 000 *g* for 10 min. Supernatants were collected and used for sequencing. Clone sequences (44, 58, 53 and 62 for days 0, 1.5, 9 and 36, respectively) were obtained commercially from Macrogen (Seoul, South Korea) using T7F and SP6R primers. Each sequence was analysed by means of GenBank using BLAST (Altschul *et al.*, 1997) as well as the Ribosomal Database Project – II (Cole *et al.*, 2003) in order to identify the closest relative. Sequences which were identified as chimeric were discarded. A value of 95% 16S rDNA sequence identity was established as a conservative cut-off for assignment of genus-level phylogenetic affiliation (Gillis *et al.*, 2001). The final sets of sequences have been submitted to GenBank (Accession numbers DQ110012–DQ110129).

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