Microbially Catalyzed Nitrate-Dependent Oxidation of Biogenic Solid-Phase Fe(II) Compounds

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The potential for microbially catalyzed NO$_3^-$-dependent oxidation of solid-phase Fe(II) compounds was examined using a previously described autotrophic, denitrifying, Fe(II)-oxidizing enrichment culture. The following solid-phase Fe(II)-bearing minerals were considered: microbially reduced synthetic goethite, two different end products of microbially hydrous ferric oxide (HFO) reduction (biogenic Fe$_3$O$_4$ and biogenic FeCO$_3$), chemically precipitated FeCO$_3$, and two microbially reduced iron(II) oxide-rich subsoils. The microbially reduced goethite, subsoils, and chemically precipitated FeCO$_3$ were subject to rapid NO$_3^-$-dependent Fe(II) oxidation. Significant oxidation of biogenic Fe$_3$O$_4$ was observed. Very little biogenic FeCO$_3$ was oxidized. No reduction of NO$_3^-$ or oxidation of Fe(II) occurred in pasteurized cultures. The molar ratio of NO$_3^-$ to Fe(II) oxidized in cultures containing chemically precipitated FeCO$_3$ and one of the microbially reduced subsoils approximated the theoretical stoichiometry of 0.2:1. However, molar ratios obtained for oxidation of microbially reduced goethite, the other subsoil, and the HFO reduction end products did not agree with this theoretical value. These discrepancies may be related to heterotrophic NO$_3^-$ reduction coupled to oxidation of dead Fe(II)-reducing bacterial biomass. Our findings demonstrate that microbially catalyzed NO$_3^-$-dependent Fe(II) oxidation has the potential to significantly accelerate the oxidation of solid-phase Fe(II) compounds by oxidized N species. This process could have an important influence on the migration of contaminant metals and radionuclides in subsurface environments.

Introduction

Direct microbial catalysis is responsible for the majority of iron(III) oxidoreduction occurring in anoxic nonsulfidogenic natural systems (1–3). Enzymatic reduction of iron(III) oxides yields both soluble Fe(II) and a variety of solid-phase Fe(II) [Fe(II)(s)] compounds including minerals such as Fe$_3$(PO$_4$)$_2$, FeCO$_3$, and Fe$_3$O$_4$ as well as unspecified amorphous Fe(II) phases, including Fe(II) sorbed to iron(III) oxide surfaces and other minerals (2, 4–7).

Oxidation of Fe(II) produced by bacterial iron(III) oxide reduction may occur via several different abiotic and biotic pathways. In aerobic environments at circumneutral pH, the chemical oxidation of Fe(II) by O$_2$ is a rapid and potentially dominant process (8). However, recent studies indicate that microaerophilic Fe(II)-oxidizing bacteria may contribute significantly to Fe(II) oxidation at circumneutral pH (9–12). Oxidation of Fe(II) is not limited to aerobic environments. Several abiotic and biotic Fe(II) oxidation processes are operative under anaerobic conditions. Anoxic Fe(II) oxidation can occur through the activity of anaerobic phototrophic, purple, nonsulfur bacteria (13). Manganese(IV) abiotically oxidizes Fe(II) at circumneutral pH (14). It has also been suggested that Fe(II) may be oxidized by NO$_3^-$ in aerobic, sedimentary environments (10, 15–20). Abiotic reduction of NO$_3^-$ to NH$_4^+$ by Fe(II) at a circumneutral pH occurs at high temperatures (75 °C) (21) and in the presence of green rust (22). The presence of trace metals such as Cu$^{2+}$ (23–25) or crystalline iron oxide (lepidocrocite and goethite) surfaces (25) accelerates low-temperature reduction of NO$_3^-$ coupled to Fe(II) oxidation at pH values greater than 8.0. Postma (26) concluded that, at low pH ranges, Fe(III) precipitates formed during iron silicate dissolution may catalyze oxidation of Fe(II) by NO$_3^-$.

The relatively specialized conditions required (i.e., high temperature, pH, catalyst) for abiotic Fe(II) oxidation by NO$_3^-$ suggests that these reactions, with the exception of iron(III) oxide surface catalysts, may not be prevalent in typical natural sedimentary environments (26). Recently, denitrifying microorganisms capable of coupling Fe(II) oxidation to NO$_3^-$ reduction to N$_2$ at circumneutral pH, in some cases under strict anaerobic conditions, have been identified (11, 27). Such organisms have been detected in a variety of freshwater sediments (28) as well as sewage sludge systems (29, 30).

The occurrence of microbially catalyzed NO$_3^-$-dependent Fe(II) oxidation in a variety of natural systems suggests that this reaction may play a significant role in coupling the redox cycles of N and Fe in sedimentary environments. As opposed to abiotic NO$_3^-$-dependent Fe(II) oxidation reactions, this biotic process proceeds readily at relatively low temperatures and circumneutral pH and does not require specific catalysts. This process has important implications for both NO$_3^-$ removal and the formation of reactive iron(III) oxides in subsurface sediments. The latter process could significantly affect the migration of contaminant metals and radionuclides whose behavior is strongly influenced by sorption reactions at iron(III) oxide surfaces (31, 32). The impact of NO$_3^-$ on contaminant metal/radionuclide geochemistry may be particularly significant at U.S. Department of Energy sites where NO$_3^-$ is often present at high concentrations (33) as a result of its use in nuclear fuels reprocessing. Although competition between NO$_3^-$ and Fe(III)-reducing bacteria is likely to play a major role in the overall impact of NO$_3^-$ on Fe/metal contaminant biogeochemistry (34), NO$_3^-$-dependent Fe(II) oxidation may present an important secondary mechanism for retarding migration of metals (divalent cations with a high affinity for iron(III) oxides such as Zn$^{2+}$, Pb$^{2+}$, and Hg$^{2+}$) and radionuclides in subsurface environments, specifically in sedimentary environments where NO$_3^-$ enters zones of contaminant metal mobilization associated with bacterial iron(III) oxide reduction.

Although the capacity for microbially catalyzed NO$_3^-$-dependent oxidation of soluble Fe(II) is well-documented, it is currently unknown whether Fe(II)(s) compounds are subject to oxidation via this metabolism. This is an important consideration given that solid Fe(II) phases are the dominant...
TABLE 1. Characterization of Solid-Phase Fe(II) Compounds

<table>
<thead>
<tr>
<th>Fe(II) phase</th>
<th>Fe(II)HCl/total FeHCl</th>
<th>mineral phases present</th>
<th>surface area (m²/g)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>chemically precipitated FeCO₃</td>
<td>0.95</td>
<td>siderite</td>
<td>23.2</td>
<td>10.0</td>
</tr>
<tr>
<td>HC-70</td>
<td>1.0</td>
<td>quartz kaolinite goethite</td>
<td>28.7</td>
<td>1.2</td>
</tr>
<tr>
<td>CP-90</td>
<td>0.88</td>
<td>goethite quartz kaolinite</td>
<td>21.1</td>
<td>0.97</td>
</tr>
<tr>
<td>goethite</td>
<td>0.93</td>
<td>goethite siderite</td>
<td>32.3</td>
<td>2.7</td>
</tr>
<tr>
<td>biogenic FeO₄</td>
<td>0.44</td>
<td>magnetite goethite (trace)</td>
<td>32.9</td>
<td>3.5</td>
</tr>
<tr>
<td>biogenic FeCO₃</td>
<td>0.94</td>
<td>siderite goethite (trace)</td>
<td>3.01</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* Determined by 0.5 N HCl extraction.  † Solid-phase Fe mineral phases identified by XRD analyses (Phillips XRG 3100, Cu line source).  ‡ Surface area determined by BET N₂ adsorption (Micromeritics Model Gemini); values are averages of triplicate samples.  ‡ SD, standard deviation.

end products of bacterial iron(III) oxide reduction in soils and sediments. In this study, we examined the potential for microbially catalyzed NO₃⁻-dependent oxidation of several Fe(II)(s) compounds, analogous to reduced phases abundant in anaerobic, nonsulfidogenic sedimentary environments. The primary objective was to investigate the rate and extent to which solid-phase end products of microbial iron(III) oxide reduction can be oxidized by this microbial process.

Materials and Methods

**Biological NO₃⁻ and NO₂⁻ Dependent Fe(II) Oxidation.** The NO₃⁻-reducing, Fe(II)-oxidizing enrichment culture described by Straub et al. (27) was used to examine Fe(II)(s) oxidation coupled to NO₃⁻ reduction. Duplicate bottles of anaerobic, NaHCO₃-buffered (30 mM, pH 6.8) growth medium (27) were amended with various Fe(II)(s) compounds (see below). Initial concentrations of 0.5 M HCl-extractable Fe(II) in the cultures ranged from ca. 2 to 20 mmol L⁻¹. Very little aqueous Fe(II) was present in cultures amended with Fe(II)(s) compounds (≤0.24 mM). Approximately 55% of the Fe(II) remained soluble in cultures amended with 10 mM FeSO₄·7H₂O; the remainder was associated with Fe(II)(s) carbonate and Fe(II)(s) phosphate precipitates. NO₃⁻ reducing Fe(II)-bearing mineral slurries were amended with NO₃⁻ from sterile stock solutions to achieve concentrations of approximately 2.5–6 mM.

Duplicate cultures were inoculated (10% v/v) with the lithotrophic, denitrifying Fe(II)-oxidizing enrichment culture grown as described by Straub et al. (27). Duplicate Fe(II)(s) mineral slurries amended with a pasteurized (80 °C, 10 min) inoculum served as killed controls. Cultures were incubated statically in the dark at 30 °C. Samples collected were analyzed for Fe(II), total Fe, NO₃⁻, and NO₂⁻ (see below). N₂O was not measured in this study.

However, previous studies with the lithotrophic NO₃⁻-dependent Fe(II)-oxidizing enrichment culture have not observed the production of N₂O (27).

**Chemical Oxidation of Fe(II) by NO₂⁻.** Chemical oxidation of Fe(II)(s) compounds by NO₂⁻ was examined under conditions similar to those present in the biological Fe(II) oxidation experiments. NO₂⁻ was added from anaerobic, sterile stock solutions to Fe(II)(s) compounds to achieve a NO₂⁻:Fe(II) ratio of approximately 1:4. This ratio provided sufficient Fe(II) for peroxide complete reduction of NO₂⁻ to N₂. Samples were collected and analyzed for NO₂⁻, Fe(II), and total Fe as described below.

**Preparation and Characterization of Solid-Phase Fe(II) Compounds.** Microbially reduced synthetic goethite and two microbially reduced iron(III) oxide-rich subsoils (HC-70 and CP-90; 35) were generated by Shewanella algae strain BrY (ca. 10⁷ tryp tic soy broth-grown cells mL⁻¹) in NaHCO₃-buffered medium [pH 6.8, N₂:CO₂ (80:20) atmosphere] containing 4.4 mM NH₄Cl, 0.44 mM KH₂PO₄, 30 mM lactate, and vitamin and trace mineral solutions as previously described (36). Biogenic FeCO₃ was produced via the chemical precipitation of FeCO₃ (siderite) was prepared by combining 250 mM Na₂CO₃ and 250 mM FeCl₃ under anaerobic conditions. The precipitate was centrifuged under N₂ and washed three times with anaerobic, deionized H₂O. The precipitate was resuspended, dispersed into anaerobic sterile serum bottles, and pasteurized. Production of siderite was confirmed by XRD analysis. Approximately 70% of the Fe(II)(s) was recovered as solid-phase carbonate.

**Surface Area Analyses.** Triplicate samples of the Fe(II)-bearing minerals were collected and dried under a stream of N₂ for 48 h. Quantiﬁcation of Fe(II) and total Fe by 0.5 M HCl extraction and ferrozine analysis (see below) before and after drying indicated that the drying process did not cause oxidation of the Fe(II)-bearing minerals. The surface area of the minerals was analyzed by multipoint BET N₂ adsorption (Micromeritics Model Gemini).

**X-ray Diffraction.** Samples of microbially reduced iron(III) oxide minerals and chemically precipitated FeCO₃ were smeared onto petrographic slides and dried inside an anaerobic chamber for 48 h. To prevent oxidation of reduced Fe minerals, slides were then coated with ethyl cellulose dissolved in amyl acetate (8% w/v). Slides were stored...
anaerobically until XRD analyses on a Phillips XRG 3100 X-ray diffractometer with a Cu-line source.

**Chemical Analyses.** Samples for NO$_3^-$ and NO$_2^-$ were filtered through a 0.2-$\mu$m nylon filter and exposed to O$_2$, which rapidly oxidized Fe(II) and thereby prevented further reduction of NO$_3^-$ by Fe(II) (39). The filtered samples were centrifuged, and the supernatant was withdrawn for NO$_3^-$ and NO$_2^-$ analysis. NO$_3^-$ was determined by ion chromatography (IonPac AS14 analytical column, Dionex DX-100 system, Dionex Corp., Sunnyvale, CA). NO$_2^-$ was determined colorimetrically (40) with a detection limit of 0.01 $\mu$M.

The amount of Fe(II) and total Fe extractable by 0.5 M HCl was determined as previously described (4). The difference between total Fe and Fe(II) in 0.5 M HCl represents poorly crystalline Fe(II) minerals formed by NO$_3^-$-dependent Fe(II) oxidation. Crystalline iron(III) oxides (goethite and Fe(III) phases in subsoils) were not liberated by the 0.5 M HCl extraction. Because NO$_2^-$ spontaneously oxidizes Fe(II) at an acidic pH, Fe(II) determined by 0.5 M HCl extraction would be inaccurate if high concentrations of NO$_2^-$ were present. To avoid such artifactual Fe(II) loss, samples for analyses of Fe concentrations were also collected by centrifugation under anaerobic conditions. The supernatant was withdrawn, and 0.5 M HCl was added to the pellet. The pellet was resuspended in acid and allowed to extract overnight. Fe(II) and total Fe in the extract were then determined using ferrozine. Aqueous Fe(II) was determined by analyzing an aliquot of sample filtered through a 0.2-$\mu$m nylon filter with ferrozine. The concentrations 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. This method yielded results equal to the 0.5 M HCl-extractable Fe(II) content of whole culture samples (aqueous + solid phase). Fe(II)-bearing mineral slurries amended with NO$_3^-$ (chemical oxidation studies) and NO$_2^-$-reducing cultures containing biogenic FeO$_4^-$ and biogenic FeCO$_3^-$ were analyzed in this manner due to the substantial concentrations of NO$_2^-$.

**Data Presentation.** Data are presented in the form of a ratio of the amount of Fe(II) to the total amount of Fe liberated by 0.5 M HCl extraction [Fe(II)$_{HCl}$/total Fe$_{HCl}$]. This approach reduced data scatter resulting from difficulty in obtaining subsamples of uniform particle content from suspensions of aggregated solids. Systematic changes in the total Fe content of the HCl extracts were not observed, which verified that all of the Fe(III) formed during NO$_3^-$-dependent Fe(II) oxidation was recovered by the 0.5 M HCl extraction.

**Results**

**Microbially Catalyzed NO$_3^-$-Dependent Fe(II) Oxidation.** No significant NO$_3^-$ reduction or Fe(II) oxidation was observed in pasteurized control cultures (Figures 1–3). In contrast, rapid NO$_3^-$ dependent oxidation of the following Fe(II)(s) minerals was observed in cultures inoculated with the enrichement culture described by Straub et al. (27): chemically precipitated FeCO$_3^-$ (Figure 1B), HC-70 (Figure 2A), CP-90 (Figure 2B), and goethite (Figure 2C). The significant random data fluctuations observed in some of the control cultures (especially the microbially reduced goethite and CP-90 subsoil) resulted from difficulty in obtaining samples of uniform particle content from suspensions of highly aggregated solids. Aqueous Fe(II) concentrations did not increase in pasteurized cultures over the course of the experiment (data not shown); thus the microbially catalyzed Fe(II)(s) oxidation observed in these studies cannot simply be attributed to dissolution of Fe(II)-bearing solids. As observed by Straub et al. (27), Fe(II) was rapidly oxidized in cultures amended with FeSO$_4$ (Figure 1A). NO$_2^-$ concentrations did not exceed 15 $\mu$M in the above cultures. Significant oxidation of biogenic FeO$_4^-$ was observed (Figure 3A); a transient accumulation of NO$_2^-$ was observed in these

![Image](https://via.placeholder.com/150?text=FIGURE+1.+Biological+NO$_3^-$-dependent+oxidation+of+(A)+FeSO$_4$+(10 mM+Fe(II))+(B)+chemically+precipitated+FeCO$_3^-$(20 mmol+of+Fe(II)+L$^{-1}$).+

**FIGURE 1.** Biological NO$_3^-$-dependent oxidation of (A) FeSO$_4$ (10 mM Fe(II)) and (B) chemically precipitated FeCO$_3^-$ (20 mmol of Fe(II)) L$^{-1}$. (a) Fe(II)$_{HCl}$/total Fe$_{HCl}$ live culture; (b) Fe(II)$_{HCl}$/total Fe$_{HCl}$ pasteurized culture; (c) NO$_3^-$ live culture; (c) NO$_3^-$ pasteurized culture. Error bars indicate $\pm$ range of duplicate cultures; bars not visible are smaller than the symbol.

**FIGURE 3.** Chemical oxidation of (A) FeSO$_4$ (10 mM Fe(II)) and (B) chemically precipitated FeCO$_3^-$ (20 mmol of Fe(II)) L$^{-1}$.

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The molar ratio of NO\textsubscript{3}\textsuperscript{-} reduced to Fe(II) oxidized in the HC-70 subsoil cultures (0.26; \( r^2 = 0.859 \)) also approximated the stoichiometry of eq 3. However, the molar ratio of NO\textsubscript{3}\textsuperscript{-} consumed to Fe(II) oxidized in the CP-90 subsoil (0.83), goethite (1.21), and biogenic FeCO\textsubscript{3} (0.88) cultures did not agree with the theoretical stoichiometry. Whereas the molar ratios of NO\textsubscript{3}\textsuperscript{-} consumed:Fe(II) oxidized in the chemically precipitated FeCO\textsubscript{3}, FeSO\textsubscript{4}, and HC-70 cultures remained constant throughout the course of Fe(II) oxidation, the ratio of NO\textsubscript{3}\textsuperscript{-} to Fe(II) oxidized during oxidation of biogenic Fe\textsubscript{3}O\textsubscript{4} increased to 2.6 at 1.9 d, when NO\textsubscript{2}\textsuperscript{-} concentrations were 0.3 mM, and then decreased to 0.5 by the end of the study. This observation is consistent with organotrophic reduction of NO\textsubscript{3}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{-} followed by organotrophic NO\textsubscript{2}\textsuperscript{-} reduction and/or abiotic oxidation of Fe(II) by NO\textsubscript{2}\textsuperscript{-} (see below).

**Chemical Oxidation of Solid-Phase Fe(II)-Bearing Minerals by NO\textsubscript{2}\textsuperscript{-}**

To assess the potential significance of the abiotic oxidation of Fe(II)s by NO\textsubscript{2}\textsuperscript{-} produced as an intermediate or end product of NO\textsubscript{3}\textsuperscript{-} reduction, a series of chemical Fe(II) oxidation studies were conducted for these experiments, pseudo-first-order rate constants were calculated according to eq 1 to allow for comparison with analogous rate constants for biological NO\textsubscript{3}\textsuperscript{-}-dependent Fe(II) oxidation. However, we recognize that the oxidation of Fe(II) by NO\textsubscript{2}\textsuperscript{-} was not likely a first-order reaction at the concentrations of NO\textsubscript{2}\textsuperscript{-} and Fe(II) used. NO\textsubscript{2}\textsuperscript{-} oxidized Fe(II)s at an initially rapid rate (pseudo first-order rate constants of 2.9–9.1 d\textsuperscript{-1}) with the exception of biogenic and chemically precipitated FeCO\textsubscript{3}, for which no oxidation of Fe(II) or reduction of NO\textsubscript{2}\textsuperscript{-} was apparent after \( \sim 40 \) d (Table 3). Although an initially rapid rate of abiotic oxidation of Fe(II)s by NO\textsubscript{2}\textsuperscript{-} was observed, the fraction of Fe(II) oxidized by NO\textsubscript{2}\textsuperscript{-} (Table 3) was less than observed in live NO\textsubscript{3}\textsuperscript{-}-dependent Fe(II)s oxidation cultures (Table 2). The lack of complete Fe(II) oxidation was not due to exhaustion of NO\textsubscript{2}\textsuperscript{-}, since substantial quantities (\( \gtrsim 0.5 \) mM) remained at the end of each of the experiments. Molar ratios of NO\textsubscript{2}\textsuperscript{-} reduced to Fe(II) oxidized for Fe(II)s containing HC-70 (0.33), goethite (0.36), and biogenic Fe\textsubscript{3}O\textsubscript{4} (0.27) were in the range of the theoretical stoichiometries for reactions such as

\[
6\text{Fe}^{2+} + 2\text{NO}_2^- + 14\text{H}_2\text{O} \rightarrow 6\text{Fe(OH)}_3^+ + \text{N}_2 + 10\text{H}^+ \tag{4}
\]

\[
4\text{Fe}^{2+} + 2\text{NO}_2^- + 9\text{H}_2\text{O} \rightarrow 4\text{Fe(OH)}_3^+ + \text{N}_2\text{O} + 6\text{H}^+ \tag{5}
\]

The slurry amended with reduced CP-90 subsoil exhibited a molar ratio of NO\textsubscript{2}\textsuperscript{-} reduced to Fe(II) oxidized (0.10) less than the predicted molar ratio.

**Discussion**

The lack of significant NO\textsubscript{3}\textsuperscript{-} accumulation in NO\textsubscript{3}\textsuperscript{-}-reducing cultures containing FeSO\textsubscript{4}, chemically precipitated FeCO\textsubscript{3}, HC-70, CP-90, and goethite suggested that Fe(II) oxidation was coupled to direct reduction of NO\textsubscript{3}\textsuperscript{-} to N\textsubscript{2}. A kinetic model simulation of the FeSO\textsubscript{4} oxidation experiment was
TABLE 2. Biological NO$_3^-$-Dependent Oxidation of Fe(II) Compounds

<table>
<thead>
<tr>
<th>Fe(II) phase</th>
<th>Fe(II) (mmol L$^{-1}$)</th>
<th>NO$_3^-$ (mM)</th>
<th>time$^a$ (d)</th>
<th>% Fe(II) oxidized</th>
<th>molar ratio NO$_3^-$ consumed:Fe(II) oxidized</th>
<th>$r^2$</th>
<th>NO$_3^-$-dependent Fe(II) oxidation</th>
<th>$K^d$ (d$^{-1}$)</th>
<th>$R^2$</th>
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</thead>
<tbody>
<tr>
<td>FeSO$_4$</td>
<td>9.1</td>
<td>4.9</td>
<td>6</td>
<td>99</td>
<td>0.26$^a$ $\pm$ 0.01</td>
<td>0.996</td>
<td>0.46$^d$</td>
<td>0.995</td>
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</tr>
<tr>
<td>chemically precipitated FeCO$_3$</td>
<td>20.3</td>
<td>5.6</td>
<td>37</td>
<td>97</td>
<td>0.24$^a$ $\pm$ 0.01</td>
<td>0.980</td>
<td>0.17 $\pm$ 0.01</td>
<td>0.986</td>
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<tr>
<td>HC-70</td>
<td>8.1</td>
<td>2.4</td>
<td>8</td>
<td>87</td>
<td>0.26$^a$ $\pm$ 0.03</td>
<td>0.859</td>
<td>0.63 $\pm$ 0.07</td>
<td>0.973</td>
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</tr>
<tr>
<td>CP-90</td>
<td>5.2</td>
<td>2.4</td>
<td>11</td>
<td>49</td>
<td>0.83$^a$ $\pm$ 0.13</td>
<td>0.773</td>
<td>0.71 $\pm$ 0.11</td>
<td>0.855</td>
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<tr>
<td>goethite</td>
<td>2.0</td>
<td>3.0</td>
<td>8</td>
<td>80</td>
<td>1.21$^a$ $\pm$ 0.10</td>
<td>0.921</td>
<td>1.08 $\pm$ 0.23</td>
<td>0.916</td>
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<tr>
<td>biogenic Fe$_2$O$_4$</td>
<td>5.6</td>
<td>5.4</td>
<td>61</td>
<td>77</td>
<td>0.50$^a$ $\pm$ 0.10</td>
<td>0.746</td>
<td>0.29 $\pm$ 0.06</td>
<td>0.863</td>
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<tr>
<td>biogenic FeCO$_3$</td>
<td>18.7</td>
<td>6.1</td>
<td>61</td>
<td>6</td>
<td>0.88 $\pm$ 0.12</td>
<td>0.109</td>
<td>0.07 $\pm$ 0.03</td>
<td>0.573</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values represent the time when Fe(II) and NO$_3^-$ concentrations are no longer significantly changed. $^b$ Determined by linear least-squares regression analyses of NO$_3^-$ vs total Fe(II) data for live cultures. Error terms represent the standard error of the slope. An asterisk (*) indicates statistical significance ($p < 0.05$). $^c$ First-order rate constant (k) determined by nonlinear least-squares regression fitting (Prism GraphPad) of Fe(II) vs time to eq 1 in the text. Error terms represent the standard error of k. $^d$ Kinetics of this reaction did not follow a first-order rate equation. Half-life was determined by nonlinear least-squares regression fitting (Prism GraphPad) of Fe(II) vs time data to a sigmoidal variable slope equation and converting $t_{1/2}$ to k according to $k = \ln(2)/t_{1/2}$.

TABLE 3. Abiotic Oxidation of Fe(II) Compounds by NO$_2^-$

<table>
<thead>
<tr>
<th>Fe(II)-bearing minerals</th>
<th>Fe(II) (mmol L$^{-1}$)</th>
<th>NO$_2^-$ (mM)</th>
<th>time$^a$ (d)</th>
<th>% Fe(II) oxidized</th>
<th>molar ratio NO$_2^-$ consumed:Fe(II) oxidized</th>
<th>$r^2$</th>
<th>NO$_2^-$-dependent Fe(II) oxidation</th>
<th>$K^d$ (d$^{-1}$)</th>
<th>$R^2$</th>
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<tbody>
<tr>
<td>FeSO$_4$</td>
<td>12.1</td>
<td>3.0</td>
<td>38</td>
<td>76$^d$</td>
<td>0.38$^a$ $\pm$ 0.03</td>
<td>0.947</td>
<td>0.153</td>
<td>0.016</td>
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<tr>
<td>chemically precipitated FeCO$_3$</td>
<td>22.7</td>
<td>4.2</td>
<td>40$^a$</td>
<td>0</td>
<td>na$^a$</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>HC-70</td>
<td>7.9</td>
<td>2.2</td>
<td>7</td>
<td>32</td>
<td>0.19$^a$ $\pm$ 0.07</td>
<td>0.544</td>
<td>0.651 $\pm$ 0.23</td>
<td>0.720</td>
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<tr>
<td>CP-90</td>
<td>3.9</td>
<td>1.3</td>
<td>14</td>
<td>30</td>
<td>0.22 $\pm$ 0.18</td>
<td>0.178</td>
<td>0.229 $\pm$ 0.04</td>
<td>0.639</td>
<td></td>
</tr>
<tr>
<td>goethite</td>
<td>1.8</td>
<td>0.5</td>
<td>2</td>
<td>25</td>
<td>0.11$^a$ $\pm$ 0.05</td>
<td>0.404</td>
<td>0.275 $\pm$ 0.26</td>
<td>0.507</td>
<td></td>
</tr>
<tr>
<td>biogenic Fe$_2$O$_4$</td>
<td>4.8</td>
<td>1.2</td>
<td>21</td>
<td>57</td>
<td>0.27$^a$ $\pm$ 0.11</td>
<td>0.519</td>
<td>2.92 $\pm$ 1.25</td>
<td>0.566</td>
<td></td>
</tr>
<tr>
<td>biogenic FeCO$_3$</td>
<td>16.63</td>
<td>3.7</td>
<td>40$^a$</td>
<td>0</td>
<td>na$^a$</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Values represent the time when Fe(II) and NO$_2^-$ concentrations are no longer significantly changed. $^b$ Determined by linear least-squares regression analyses of NO$_2^-$ vs Fe(II) data. Error terms represent the standard error of the slope. An asterisk (*) indicates statistical significance ($p < 0.05$). $^c$ First-order rate constant (k) determined by nonlinear least-squares regression (Prism GraphPad) of Fe(II) vs time to eq 1 in the text. Error terms represent the standard error of k. $^d$ Complete oxidation of FeSO$_4$ was not observed in this experiment due to exhaustion of NO$_2^-$.

developed to assess the potential importance of NO$_3^-$, produced as an intermediate during NO$_2^-$ reduction, as an abiotic oxidant during NO$_3^-$-dependent Fe(II) oxidation (see Supporting Information). The model incorporated an experimentally determined rate constant for the reaction of FeSO$_4$ with NO$_2^-$ derived from the abiotic oxidation experiment reported in Table 3. Results of the simulation suggested that accumulation of NO$_2^-$ far in excess of that observed in the cultures would have occurred if enzymatic NO$_3^-$ reduction to NO$_2^-$ followed by abiotic oxidation of Fe(II) by NO$_2^-$ was the mechanism responsible for Fe(II) oxidation. It was not possible to conduct analogous simulations of the biogenic Fe(II)s oxidation experiments because of complexity introduced by the lack of complete abiogenic Fe(II) oxidation by NO$_2^-$.

However, the generally much lower degree of abiogenic Fe(II)s oxidation by NO$_2^-$ as compared to NO$_3^-$-dependent microbial activity (mean = 32 ± 27%, n = 6) emphasizes the role of direct enzymatic reduction of NO$_3^-$ to N$_2$ during Fe(II) oxidation. Particularly significant in this regard is the behavior of the chemically precipitated FeCO$_3$, which showed no reaction with NO$_2^-$ but was rapidly oxidized enzymatically with little or no accumulation of NO$_2^-$.

Molar ratios of NO$_3^-$ consumed:Fe(II) oxidized in experimental FeCO$_3$, FeSO$_4$, and HC-70 cultures approximated the theoretical stoichiometry of eq 3, suggesting that Fe(II)s was coupled to the reduction of NO$_3^-$ to N$_2$. However, molar ratios in goethite, CP-90, biogenic FeCO$_3$, and biogenic Fe$_2$O$_4$ cultures exceeded the theoretical stoichiometry. The reason for this disagreement is unclear. Other studies of biological NO$_3^-$-dependent Fe(II) oxidation have also observed molar ratios of NO$_3^-$ consumed to Fe(II) oxidized in excess of theoretical values (27, 30). A purified, lithotrophic, NO$_3^-$-reducing, Fe(II)-oxidizing culture (BrG2) consumed more NO$_3^-$ than theoretically predicted (27). The authors speculated that some unidentified N species may have formed a stable complex with iron, a phenomenon that could also have taken place in our cultures. An alternative explanation is that organisms in the enrichment culture reduced NO$_3^-$ organotrophically concurrent with Fe(II) oxidation, using dead cell biomass from the iron(III) oxide-reducing cultures as an energy source. NO$_3^-$-reducing Fe(II)-oxidizing microorganisms have the ability to utilize a variety of organic substrates (27). The ability of the enrichment culture used in our experiments to oxidize lactate and other substrates coupled to NO$_3^-$ reduction has been verified (K. Weber, unpublished data).

Heterotrophic NO$_2^-$ reduction provides an explanation for the transient accumulation of NO$_3^-$ in biogenic Fe$_2$O$_4$ cultures, given that the molar ratio of NO$_3^-$ reduced to Fe(II) oxidized (0.5) exceeded theoretical predictions. This process probably occurred in other cultures (e.g., those containing microbiobally reduced goethite and CP-90 subsoil) but for unknown reasons did not lead to a significant accumulation of NO$_3^-$.

The results of an attempt to observe biological NO$_2^-$ reduction coupled to oxidation of biogenic Fe$_2$O$_4$ supports the conclusion that heterotrophic NO$_2^-$ reduction was responsible for NO$_3^-$ accumulation. In this experiment, virtually no difference was observed between the amount of Fe(II) oxidized by live and pasteurized cultures, while a significantly greater amount of NO$_2^-$ was reduced in live cultures relative to pasteurized cultures (data not shown), i.e., additional reducing equivalents were obtained from sources other than Fe(II). However, it is important to note that the amount of biogenic Fe$_2$O$_4$ oxidized in NO$_3^-$-reducing cultures cannot be accounted for alone by abiogenic reaction with NO$_2^-$ of organotrophic origin. The extent of abiogenic Fe$_2$O$_4$
Although most of the Fe(II)(s) compounds used in this study did not occur under the culture conditions in this study. (half-life of NO3\textsuperscript{−} (Table 3). Hence, the 1 mmol Fe(II) L\textsuperscript{−}\textsuperscript{−} oxidation by NO\textsubscript{2}\textsuperscript{−} Fe\textsubscript{3}O\textsubscript{4} used in our experiments showed that 0.5 M HCl such HFO reduction end products. Analysis of the biogenic Fe\textsubscript{3}O\textsubscript{4} used in our experiments that 0.5 M non-HCl-extractable Fe(II)(s) was more slowly and/or less extensively oxidized than 0.5 M HCl-extractable Fe(II)(s). If so, then the data reported here could represent an over estimation of rate and extent of overall Fe(II)(s) oxidized. 

Very little of the Fe(II) in the biogenic FeCO\textsubscript{3} was oxidized. Heterotrophic NO\textsubscript{3}\textsuperscript{−} reduction was thus probably responsible for much of the NO\textsubscript{3}\textsuperscript{−} accumulation observed in these cultures. NO\textsubscript{3}\textsuperscript{−} did not chemically oxidize biogenic FeCO\textsubscript{3} (Table 3). Hence, the 1 mmol Fe(II) L\textsuperscript{−}\textsuperscript{−} that was oxidized in live biogenic FeCO\textsubscript{3} cultures was the result of biological catalysis. In contrast to the biogenic Fe\textsubscript{3}O\textsubscript{4}, 95% of the chemically precipitated FeCO\textsubscript{3} was oxidized at a rapid rate (Figure 1B, Table 2). The greater reactivity of chemically precipitated FeCO\textsubscript{3} to biological oxidation may be a result of the much greater (ca. 8-fold) surface area per unit mass (Table 2) available for microbial oxidation.

Environmental Significance. Most of the Fe(II)(s) phases examined were subject to rapid NO\textsubscript{3}\textsuperscript{−} dependent oxidation in the presence of active microbial metabolism. In contrast, no significant NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidation occurred in cultures containing heat-killed cells. Although a recent study demonstrated that Fe(II) associated with iron(III) oxide (goethite) surfaces was subject to abiotic oxidation by NO\textsubscript{3}\textsuperscript{−} (half-life of NO\textsubscript{3}\textsuperscript{−} for 10 g wet weight goethite was 1600 min; 25). However, significant abiotic oxidation of Fe(II)(s) by NO\textsubscript{3}\textsuperscript{−} did not occur under the culture conditions in this study. Although most of the Fe(II)(s) compounds used in this study were subject to significant chemical oxidation by NO\textsubscript{3}\textsuperscript{−}. NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidation was substantially more efficient than abiotic oxidation by NO\textsubscript{3}\textsuperscript{−}. In addition, only minor concentrations of NO\textsubscript{3}\textsuperscript{−} were formed during biological NO\textsubscript{3}\textsuperscript{−} dependent Fe(II)(s) oxidation, which indicated that Fe(II)(s) oxidation was coupled directly to NO\textsubscript{3}\textsuperscript{−} reduction to N\textsubscript{2}. These findings indicate that microbial activity has the potential to vastly accelerate the coupling of N and Fe redox cycles in sedimentary environments.

Microbially catalyzed NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidation could have a significant influence on the fate of NO\textsubscript{3}\textsuperscript{−} in subsurface environments, particularly environments with a limited supply of organic carbon. Inverse correlations between NO\textsubscript{3}\textsuperscript{−} and surface-bound Fe(II) in subsurface profiles have been observed (15, 18, 19). Although the authors have attributed this observation to the reduction of NO\textsubscript{3}\textsuperscript{−} by Fe(II) (15, 18, 19), whether NO\textsubscript{3}\textsuperscript{−} reduction was abiotically or biotically coupled to the oxidation of Fe(II) was not determined. A recent study concluded that microorganisms were not responsible for reduction of NO\textsubscript{3}\textsuperscript{−} and oxidation of Fe(II) in these environments (20). However, this study examined heterotrophic NO\textsubscript{3}\textsuperscript{−} reduction rates and quantified the abundance of aerobic heterotrophs; the potential for anaerobic NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidation was not examined. The identification of NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidizing microorganisms in a variety of environments (11, 28–30) suggests that these organisms could inhabit sediments investigated by Lind, Ernsten and colleagues. Biological NO\textsubscript{3}\textsuperscript{−} dependent Fe(II)(s) oxidation could provide an explanation for chemical profiles of NO\textsubscript{3}\textsuperscript{−} and Fe(II)(s) observed in these subsoil environments.

The formation of reactive iron(III) oxides, as a result of biological NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidation, has the potential to exert a major influence on the aqueous geochemistry of anaerobic soils and sediments. The mobility of contaminant metals and radionuclides is influenced strongly by adsorption to reactive iron(III) oxide surfaces (32). Recent studies in our laboratory indicate that adsorption of Zn to biogenic iron(III) oxide surfaces produced by microbial NO\textsubscript{3}\textsuperscript{−} dependent oxidation of Fe\textsubscript{2}O\textsubscript{3} is comparable to adsorption of Fe(II) to reactive iron(III) oxide surfaces, the potential also exists for trapping of contaminant metals via coprecipitation with iron(III) oxides formed during NO\textsubscript{3}\textsuperscript{−} dependent Fe(II) oxidation. Further studies examining the kinetics of NO\textsubscript{3}\textsuperscript{−} dependent oxidation of microbially reduced iron(III) oxides and the reactivity of the resulting end products are required to understand the impact that this process may exert on the fate of contaminant metals and radionuclides in sedimentary environments.

Acknowledgments

Thanks to Dr. Kristina L. Straub for providing the NO\textsubscript{3}\textsuperscript{−} dependent Fe(II)-oxidizing enrichment culture, Dr. Matilde Urrutia for assistance with surface area analyses, and Dr. D. Craig Cooper for XRD analyses. This research was supported by the Department of Energy, Natural and Accelerated Bioremediation Program, through Grant DE-FG02-97ER62482.

Supporting Information Available

A description of the development and results of the kinetic model simulation of the Fe\textsubscript{2}O\textsubscript{4} oxidation experiment (4 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

Supplementary Information – Kinetic modeling of nitrate-dependent oxidation of FeSO₄

The goal of the modeling exercise was to assess whether or not abiotic reaction of FeSO₄ with NO₂⁻ would be fast enough to prevent significant NO₂⁻ accumulation if enzymatic reduction of NO₃⁻ to NO₂⁻, followed by abiotic reduction of NO₂⁻ to N₂, was the mechanism responsible for NO₃⁻-dependent Fe(II) oxidation. This coupled mechanism is illustrated by the following reaction scheme:

\[ 2\text{Fe(II)} + \text{NO}_3^- \rightarrow 2\text{Fe(III)} + \text{NO}_2^- \quad \text{(enzymatic)} \]  
\[ n\text{Fe(II)} + \text{NO}_2^- \rightarrow n\text{Fe(III)} + \text{reduced N} \quad \text{(abiotic)} \]  

where \( n \) represents the ratio Fe(II) to NO₂⁻ consumption during the abiotic reaction between these two species (see below), and “reduced N” represents one or more N end-products with an oxidation state lower than +3.

The model simulates the time variation of NO₃⁻, NO₂⁻, and Fe(II) during NO₃⁻-dependent oxidation of FeSO₄ according to the above reaction scheme. The simulation is driven by the observed time course of NO₃⁻ consumption over time in the FeSO₄ cultures. The NO₃⁻ vs. time data (Fig. 1A in Weber et al., 2001; see also Suppl. Mat. Fig. 1C) data were fit by nonlinear least-squares regression to a sigmoidal function

\[ C(t) = C_{\text{min}} + (C_{\text{max}} - C_{\text{min}})/(1 + 10^{-(\log t_{1/2} - t)}) \]  

where \( C(t) \) is the NO₃⁻ concentration at time \( t \); \( C_{\text{min}} \) is the minimum concentration of NO₃⁻, observed at the end of the experiment; \( C_{\text{max}} \) is the maximum concentration of NO₃⁻, present at the start of the experiment; and \( t_{1/2} \) is time at which half of the total amount of NO₃⁻ consumed during the experiment has been utilized, i.e. the time at which \( C(t) = 0.5(C_{\text{max}} - C_{\text{min}}) \). This fitting function was not chosen on theoretical grounds, but rather was used because it provided
the most accurate empirical fit of the NO$_3^-$ vs time data. Instantaneous rates of NO$_3^-$ reduction were computed by evaluating the first derivative of equation 3 with respect to time:

$$R_{\text{NO}_3}(t) = \frac{dC(t)}{dt} = \frac{(C_{\text{max}} - C_{\text{min}})}{(1 + 10^{\gamma (\log t_{1/2} - t)})^{2} \times 10^{\gamma (\log t_{1/2} - t) \times \ln 10}}$$  \hspace{1cm} (4)

This rate term was incorporated into the following system of differential equations describing the rate of change of NO$_3^-$, NO$_2^-$, and Fe(II) concentration over time:

$$\frac{d[\text{NO}_3]}{dt} = -R_{\text{NO}_3}(t)$$  \hspace{1cm} (5)

$$\frac{d[\text{NO}_2]}{dt} = R_{\text{NO}_3}(t) - \frac{(1/n)k[\text{Fe(II)}]^{n}[\text{NO}_2]}{}}$$  \hspace{1cm} (6)

$$\frac{d[\text{Fe(II)}]}{dt} = -2R_{\text{NO}_3}(t) - \frac{k[\text{Fe(II)}]^{n}[\text{NO}_2]}{}}$$  \hspace{1cm} (7)

This set of equations assumes that enzymatic reduction of NO$_3^-$ coupled to Fe(II) oxidation leads to production of NO$_2^-$ according to a 1:2 ratio, and that consumption of NO$_2^-$ occurs solely via abiotic reaction with Fe(II) according to a mass action rate law of order $(1+n)$.  \hspace{1cm} (7)

The rate constant for FeSO$_4$ oxidation by NO$_2^-$ ($k$) was obtained from an experiment conducted in sterile growth medium. The composition of the medium was identical to that used for the biological NO$_3^-$-dependent Fe(II) oxidation experiments. The rate constant was estimated by numerically integrating (using a fifth-order Runge-Kutta algorithm with truncation error control, obtained from ref. 2) the following set of mass action expressions which describe the rates of Fe(II) and NO$_2^-$ consumption during abiotic FeSO$_4$ oxidation by NO$_2^-$ according to a $(1+n)$th order rate law:

$$\frac{d[\text{Fe(II)}]}{dt} = -k[\text{Fe(II)}]^{n}[\text{NO}_2]$$  \hspace{1cm} (8)

$$\frac{d[\text{NO}_2]}{dt} = -(1/n)k[\text{Fe(II)}]^{n}[\text{NO}_2]$$  \hspace{1cm} (9)

and varying the value of $k$ in order to obtain an approximate best-fit to the observed Fe(II) and NO$_2^-$ vs. time data (Suppl. Info., Fig. 1A). Initial Fe(II) and NO$_2^-$ concentrations were set equal to the average values measured at the start of the abiotic oxidation experiment, and the value of $n$
was obtained from the slope of a scatter plot of the observed Fe(II) vs. NO$_2^-$ concentrations during the experiment (Suppl. Info., Fig. 1B).

Using the values of $k$ and $n$ obtained from the abiotic oxidation experiment, the system of coupled equations 5-7 was integrated numerically over a six-day time period. Initial NO$_3^-$, NO$_2^-$, and Fe(II) concentrations were set equal to those measured at the start of the biotic NO$_3^-$-dependent FeSO$_4$ oxidation experiment (ca. 5, 0, and 9 mM, respectively). The results of the simulations (Suppl. Info. Fig. 1C) indicate that accumulation of NO$_2^-$ in excess of 2 mM would have occurred if the mechanism described by equations 1 and 2 was responsible for NO$_3^-$-dependent Fe(II) oxidation activity. In contrast, measured concentrations of NO$_2^-$ in the FeSO$_4$ cultures never exceeded 0.015 mM. The simulation also predicted much slower consumption of Fe(II) over time than was observed in the experiment. These disparities lead to the conclusion that direct enzymatic reduction of NO$_3^-$ to N$_2$ was responsible for FeSO$_4$ oxidation in the experiment shown in Fig. 1A in Weber et al., submitted.

**Literature Cited**


Suppl. Info. Fig. 1. Panel A: Abiotic oxidation of FeSO₄ by NO₂⁻ in sterile growth medium. Symbols represent measured concentrations in triplicate reactions systems; lines represent results of numerical integrations used to estimate the rate constant k (see text). Mean initial Fe(II) and NO₂⁻ concentrations were 12.1 and 2.75 mM, respectively. Panel B: [Fe(II)] vs. [NO₂⁻] during abiotic oxidation of FeSO₄ by NO₂⁻. Panel C: Results of simulation model. Symbols represent mean measured concentrations of Fe(II), NO₃⁻, and NO₂⁻ in the biological FeSO₄ oxidation experiment (Fig. 1A in Weber et al. 2001).