Kinetics of Competitive Reduction of Nitrate and Iron Oxides by *Aeromonas hydrophila* HS01

While the interaction between NO$_3^-$ and Fe reduction is well reported, it still lacks a detailed quantitative analysis of the properties and reactions responsible for the competitive reduction. In this study, the kinetics of the reduction of NO$_3^-$ and Fe oxides by *Aeromonas hydrophila* HS01 were investigated in an artificial groundwater medium. The presence of Fe oxides showed an inhibitory effect on NO$_3^-$ reduction to a varying extent, and vice versa, which was also evidenced by cyclic voltammetry analysis of Fe(II)/Fe(III). Higher Fe(III) availability and increased Fe oxide surface areas were more favorable for Fe reduction but resulted in a stronger inhibitory effect on NO$_3^-$ reduction. The inhibitory effect also decreased with increased hematite crystallinity. Based on the kinetics and the elementary reactions, we established a kinetic model suggesting that direct biotic NO$_3^-$ reduction by HS01 is the leading contribution, while the cell-associated Fe(II) produced by HS01 also reduced NO$_3^-$ but with a limited contribution because the reaction rates of NO$_3^-$ reduction by cell-associated Fe(II) are lower than that of NO$_3^-$ reduction by HS01. This detailed kinetics and modeling study provides useful information for understanding the interaction between NO$_3^-$ and Fe reduction in various subsurface environments.

Abbreviations: CV, cyclic voltammetry; DIRB, dissimilatory iron-reducing bacteria; IO, iron oxide; SCE, saturated calomel electrode; KRD, X-ray diffraction.

Iron, as the fourth most abundant element in the Earth’s crust, is essential to nearly all known organisms (Achtnich et al., 1995; Bonneville et al., 2004). The redox transformation of Fe plays a key role in the fate of contaminant metals (Bose et al., 2009; Borch et al., 2010; Li et al., 2012b) and organic compounds (Li et al., 2009a, 2010; Cao et al., 2012) in soils and sediments (Lovley, 2006; Kappler and Straub, 2005). Iron exists predominantly as insoluble, solid-phase Fe(III) minerals at pH values at or above a circumneutral pH (~pH 7) in most natural waters or sediments (Stumm and Morgan, 1996). The reduction of Fe(III) to Fe(II) under anoxic conditions is dominated by dissimilatory iron-reducing bacteria (DIRB), which can use Fe(III) oxides for growth as terminal electron acceptors coupled with the oxidation of organic matter (Lovley and Lonergan, 1990; Liu et al., 2011; Li et al., 2012a). So far, a wide phylogenetic diversity of microorganisms has been reported as being capable of dissipimilatory Fe(III) reduction (Lovley and Lonergan, 1990; Dobbin et al., 1996; Bose et al., 2009; Li et al., 2009b). In addition, the oxidation of organic electron donors can also be coupled with the reduction of many other electron acceptors such as NO$_3^-$ (Chakraborty et al., 2011; Liu et al., 2014; Zhang et al., 2014), humic substances (Jiang and Kappler, 2008; Li et al., 2013, 2014; Wu et al., 2014), and Mn minerals (Lovley et al., 2004) in natural environments. The coexistence of these ac-
ceptrons may lead to complicated interactions (e.g., inhibition and enhancement) during their reduction processes (Chithaisong and Conrad, 2000; Nevin et al., 2003; Borch et al., 2010).

Among the numerous reduction processes of the matrix solutions, NO$_3^-$ reduction plays a key role in the N cycle and has important implications for agriculture, the environment, and public health (Moreno-Vivián et al., 1999). While NO$_3^-$ reduction is mainly dominated by microbial enzyme reactions in natural environments (Cabello et al., 2004; Gonzalez et al., 2006), the abiogenic reduction of NO$_3^-$ also occurs under some extreme conditions (Buresh and Moraghan, 1976; Moraghan and Buresh, 1977; Petersen, 1979; Hansen et al., 1996; Otley et al., 1997). Recently, the Fe cycle has been proven to be coupled with NO$_3^-$ reduction processes in Fe-bearing soils and sediments (Nielsen and Nielsen, 1998; Weber et al., 2006). Microbial NO$_3^-$-dependent Fe(II) oxidation, including abiotic–biotic interactions, proceeds readily at low temperature and at circumneutral pH (Straub et al., 1996; Kappler et al., 2005; Chakraborty et al., 2011; Konhauser et al., 2011; Picardal, 2012). Nitrate reduction can also be catalyzed by some Fe(III)-reducing microorganisms, and the impact of Fe oxide on NO$_3^-$ reduction is complicated. On the one hand, the presence of Fe(III) oxides was reported to dramatically accelerate NO$_3^-$ reduction by a few Fe-reducing bacteria, and a mechanism for Fe oxide conduction band-mediated electron transfer from cells to NO$_3^-$ was supposed to account for such enhancement (Zhang et al., 2012; Liu et al., 2014). On the other hand, the presence of NO$_3^-$ has been reported to inhibit microbial goethite reduction and vice versa when NO$_3^-$ and goethite are serving as terminal electron acceptors simultaneously (Cooper et al., 2003).

The inhibition of microbial Fe(III) reduction by NO$_3^-$ has been demonstrated in previous studies (Obuekwe and Westlake, 1981; Sørensen, 1982; Sørensen and Thorling, 1991; DiChristina, 1992; Lee et al., 2000; Kappler et al., 2005); this can be mainly attributed to two possible reasons: (i) NO$_3^-$ is soluble and has a higher redox potential than Fe(III) oxide and can therefore be reduced more easily than poorly soluble Fe(III) oxides by microorganisms when NO$_3^-$ and Fe(III) oxide are present simultaneously in natural environments (Lovley et al., 2004); and (ii) Fe(III) reduction may be blocked by secondary Fe(III) mineral crusts around cells as a result of the abiogenic or biotic oxidation of biogenic Fe(II) sorbed to the cellular surface by NO$_2^-$ or NO$_3^-$ (Rodan and Urrutia, 1999; Kappler et al., 2005; Senko et al., 2005).

In addition to NO$_3^-$, there are many factors that influence the rate and extent of Fe oxide reduction by DIRB in the natural environment, including the crystalline structure and degree of Fe-bearing minerals (Li et al., 2012a), the presence of electron shuttles and secondary minerals (Liu et al., 2011), and adsorbed Fe(II) on the Fe(III) solid (Sørensen and Thorling, 1991) or cell surface (Urrutia et al., 1998; Liu et al., 2001). While many studies have reported how the properties of Fe oxides (e.g., surface areas, Fe availability, and crystalline degree) influence microbial Fe reduction (Rodan and Zachara, 1996; Roden, 2006; Liu et al., 2007; Jiang and Kappler, 2008), few studies have investigated in detail how these properties of Fe oxides affect microbial NO$_3^-$ reduction, particularly when the microorganisms can catalyze both Fe and NO$_3^-$ reduction simultaneously. Recently, Zhang et al. (2014) investigated the competitive reduction kinetics of NO$_3^-$ and Fe oxides by the DIRB _Shewanella putrefaciens_ 200 and found that the higher crystallinity of hematite resulted in decreased inhibition of NO$_3^-$ reduction. However, it is still not clear what kinds of reactions are responsible for the competition of microbial NO$_3^-$ and Fe reduction.

_Aeromonas hydrophila_ HS01, isolated from subterranean forest soil, has been identified as an Fe-reducing bacterium that can also reduce chlorinated organic compounds under anoxic conditions (Cao et al., 2010, 2012; Li et al., 2014). _Aeromonas hydrophila_ HS01 was found to be able to reduce NO$_3^-$ in the current study; therefore HS01 is a suitable strain to be used for investigating the competitive kinetics of microbial NO$_3^-$ and Fe reduction. Four types of Fe oxides (goethite, lepidocrocite, hematite, and maghemite) and hematite with different degrees of crystallinity were used as the Fe sources to reveal how the properties of Fe oxides (i.e., Fe(III) availability, surface area, and crystalline degree) affect microbial NO$_3^-$ reduction. After a detailed discussion on the roles of Fe-reducing bacteria, Fe oxide, and Fe(II) species in the competitive system of NO$_3^-$ and Fe reduction based on the kinetics and electrochemical evidence obtained, a model was established to illustrate the various key reactions responsible for the inhibition of NO$_3^-$ and Fe reduction. This study provides a more comprehensive understanding of the competitive mechanism between microbial Fe and N cycles in the natural environment.

**MATERIALS AND METHODS**

**Materials**

_Aeromonas hydrophila_ HS01 (Deposition no. CCTCCAB 209165), isolated from subterranean forest soil in our laboratory, has been reported as capable of Fe(III) reduction and DDT (1,1’-(2,2,2-trichloroethylidene)bis[4-chlorobenzene]) transformation under anoxic conditions (Cao et al., 2012). Goethite (α-FeOOH), lepidocrocite (γ-FeOOH), and hematite (α-Fe$_2$O$_3$) were synthesized according to previously described procedures (Li et al., 2009b): α-FeOOH was prepared by dissolving Fe(NO$_3$)$_3$·9H$_2$O in KOH; γ-FeOOH was synthesized by mixing FeCl$_2$·4H$_2$O, (CH$_2$)$_6$N$_4$, and NaNO$_2$ in deionized water; α-Fe$_2$O$_3$ was formed by sintering γ-FeOOH powder at 420°C for 2 h at a temperature increase rate of 2°C min$^{-1}$; and maghemite (γ-Fe$_2$O$_3$) was prepared using FeCl$_2$·(CH$_2$)$_6$N$_4$, and NaNO$_3$ (Wang et al., 2008). A series of α-Fe$_2$O$_3$ samples were synthesized at 300, 400, 500, 600, 700, and 800°C (Liu et al., 2011), identified here as Hem-300, Hem-400, Hem-500, Hem-600, Hem-700, and Hem-800, respectively. All of the Fe oxides were ground to pass through a 149-μm (100-mesh) sieve before use. Sodium nitrate (≥99.0%), NaNO$_3$ (≥99.5%), FeCl$_3$ (≥98.0%), and other analytical-grade chemicals were purchased from Guangzhou Chemical Co.
Batch Experimental Procedures

Strain HS01 was aerobically inoculated in a nutrient broth (consisting of 10 g L\(^{-1}\) peptone, 3 g L\(^{-1}\) beef extract, and 5 g L\(^{-1}\) NaCl at pH 7.2 ± 0.2) for 16 h in a shaker at 180 rpm and 30°C. Cells were harvested by centrifugation at 8000 \(\times g\) for 10 min at 4°C for three rounds using sterile NaHCO\(_3\)-buffered solution when the cells approached the exponential phase. To avoid any interference from other inorganic anions in NO\(_3\)\(^-\) detection, the NaHCO\(_3\)-buffered solution (30 mmol L\(^{-1}\), pH 6.8, with an atmosphere of 80:20 N\(_2\)/CO\(_2\)) consisted of only 7.0 mmol L\(^{-1}\) NaNO\(_3\) as an electron acceptor and 5 mmol L\(^{-1}\) glucose as an electron donor, to which the harvested HS01 cells with a final concentration of approximately 2 \(\times 10^7\) cells mL\(^{-1}\) were added.

Six batch treatments for NO\(_3\)\(^-\) reduction, including controls, were conducted: (i) HS01; (ii) HS01 + \(\alpha\)-FeOOH; (iii) HS01 + \(\gamma\)-FeOOH; (iv) HS01 + \(\alpha\)-Fe\(_2\)O\(_3\); (v) HS01 + \(\gamma\)-Fe\(_2\)O\(_3\); or Hem-300–800 with a solid Fe oxide content of 4.5 g L\(^{-1}\); (iii) 0.7 mmol L\(^{-1}\) Fe(II); (iv) 4.5 g L\(^{-1}\) \(\alpha\)-FeOOH; (v) 4.5 g L\(^{-1}\) \(\alpha\)-FeOOH + 0.7 mmol L\(^{-1}\) Fe(II); and (vi) HS01 + 0.7 mmol L\(^{-1}\) Fe(II). Standard anaerobic techniques were used throughout all experiments as previously described (Zhang et al., 2012). Inoculation and sampling were conducted with sterile syringes and needles. All trials were conducted in triplicate in 25.2-mL serum bottles with 5.2-mL headspaces, and the vials were incubated in a BACTRON Anaerobic/Environmental Chamber II (SHELLAB, Sheldon Manufacturing Inc.) at 30°C in the dark.

**Analytical Methods**

During a 14-d incubation period, triplicate bottles were used for chemical analysis at regular intervals (Days 1, 2, 4, 6, 10, and 14). The total Fe(II) [also known as HCl-extractable Fe(II)] was determined as previously described (Li et al., 2010). The analysis of NO\(_3\)\(^-\) and NO\(_2\)\(^-\) was performed by ion chromatography (DionexICS-90) with an ion column (IonPac AS14A 4 \(\times\) 250 mm). A mobile phase consisting of 8.0 mmol L\(^{-1}\) Na\(_2\)CO\(_3\) and 1.0 mmol L\(^{-1}\) NaHCO\(_3\) solutions was operated at a flow rate of 1.0 mL min\(^{-1}\). Before injection, sample solutions were always filtered through a 0.22-\(\mu\)m membrane filter. Cyclic voltammetry (CV) was performed in a conventional three-electrode electrochemical cell using a CHI 660C potentiostat (Chenhua Co. Ltd.). A glass C electrode (2 mm in diameter) was used as the working electrode, with a saturated calomel electrode (SCE) and a Pt wire (0.5 mm in diameter) as the reference and counter electrodes, respectively. Unless mentioned otherwise, all the reported potentials refer to SCE. The CV measurements were performed with the suspended reaction solutions taken from the batch kinetic studies and conducted under an 80:20 N\(_2\)/CO\(_2\) atmosphere at 25°C at a scan rate of 50 mV s\(^{-1}\). The surface area of the Fe oxides was measured by the Brunauer–Emmett–Teller method using a Coulter SA-3100, in which N\(_2\) adsorption at 77 K was applied, and a Carlo Erba sorptometer was used. The X-ray diffraction (XRD) patterns of the Fe(III) oxides were recorded on a Rigaku D/Max-IIIA diffractometer at room temperature, operating at 30 kV and 30 mA, using Cu K\(\alpha\) radiation (wavelength \(\lambda = 0.15418\) nm). The phases were identified by comparing diffraction patterns with those on the standard powder XRD cards compiled by the Joint Committee on Powder Diffraction Standards (Schwertmann and Cornell, 1991). Because the intensity of the main peaks characterized by XRD can properly reflect the crystallinity of the materials tested (Liu et al., 2013), the intensity at 2\(\theta\) = 33.2° as the dominant peak of hematite can be used to quantitatively represent the degree of crystallinity of hematite synthesized at different temperatures.

**Kinetics Calculations**

To clearly illustrate the effects of Fe oxides on the NO\(_3\)\(^-\) reduction, the pseudo-first-order rate constant (\(k\)) of NO\(_3\)\(^-\) reduction was calculated according to

\[
C_{NO_3^{-}} = C_{NO_3^{-0}} \exp(-kt) \quad [1]
\]

where \(C_{NO_3^{-0}}\) is the initial NO\(_3\)\(^-\) concentration and \(C_{NO_3^{-}}\) is the NO\(_3\)\(^-\) concentration at the reaction time (\(t\)).

The zero-order rate (\(r\)) of Fe reduction was calculated as

\[
C_{Fe(II)} = rt \quad [2]
\]

where \(C_{Fe(II)}\) is the Fe(II) concentration at the reaction time (\(t\)).

**Statistical Analysis**

Statistical analyses of the experimental data were performed using SPSS 16.0 software. Student’s \(t\)-test with \(P < 0.05\) was used for evaluating the statistically significant differences of Fe(II) concentrations between treatments in the absence and presence of NO\(_3\)\(^-\) at each sampling interval. Differences of NO\(_3\)\(^-\) and Fe(III) reduction rates were determined by one-way analyses of variance (ANOVA) on ranks followed by Duncan’s test. Significant differences (\(P < 0.05\)) between treatments were determined. A correlation analysis was conducted by Pearson correlation with a significance level of \(P < 0.05\) (two-tailed).

**RESULTS AND DISCUSSION**

**Inhibition of Nitrate Reduction by Iron Oxides**

Batch experiments were first undertaken to examine the reduction of NO\(_3\)\(^-\) by HS01 in the absence and presence of Fe oxides. The results shown in Fig. 1a reveal that while HS01 alone could completely reduce NO\(_3\)\(^-\) within 4 d, the presence of Fe oxides slowed down the rate of NO\(_3\)\(^-\) reduction. The pseudo-first-order rate constants (\(k\)) in Table 1 show that NO\(_3\)\(^-\) reduction by HS01 was inhibited markedly in the presence of \(\alpha\)-FeOOH, \(\gamma\)-FeOOH, and \(\alpha\)-Fe\(_2\)O\(_3\); whereas \(\gamma\)-Fe\(_2\)O\(_3\) showed a weak inhibitory effect, which is similar to our previous study (Zhang et al., 2014). Because the experiments were performed under non-growth conditions, i.e., in a medium lacking phosphate and essential trace elements and vitamins, it is unlikely that assimilatory NO\(_3\)\(^-\) reduction played an important role in the removal of NO\(_3\)\(^-\). Nitrite, as the major reduced intermediate, increased gradually with time to Day 10 and then decreased from Day 10 to Day 14 in the HS01 treatment (Fig. 1b), suggesting that the
Inhibition of Iron Oxide Reduction by Nitrate

As shown in Fig. 2, biogenic Fe(II) formation was observed in all inoculated vials in the absence of NO$_3^-$, and the HCl-extractable Fe(II) concentrations gradually increased with time. The zero-order reaction rates ($r$) of Fe(II) formation from different Fe oxides in the absence of NO$_3^-$ were ranked as γ-FeOOH > α-FeOOH > α-Fe$_2$O$_3$ > γ-Fe$_2$O$_3$ (Table 1). Iron(II) formation in the presence of NO$_3^-$ was significantly lower than in the absence of NO$_3^-$ ($P < 0.05$), confirming that the addition of NO$_3^-$ strongly inhibited the reduction of Fe oxide by HS01. The redox potential of NO$_3^-$/NO$_2^-$ is 0.43 V at pH 7, which is higher than that for Fe(III) on the surface of Fe oxides, e.g., 0.229 V for α-FeOOH/Fe(II) (Zhang et al., 2012; Liu et al., 2014). Therefore, NO$_3^-$ is a more competitive electron acceptor and can more readily be reduced than Fe(III) on the surface of Fe oxide (Lovley et al., 2004).

The rate and extent of Fe reduction by microorganisms is strongly related to the properties of Fe oxides, such as Fe(III) availability, surface area, and crystallinity, as reported previously (Bonneville et al., 2004; Yan et al., 2008; Liu et al., 2011; Li et al., 2012a). The different extents of inhibition of microbial NO$_3^-$ reduction by Fe oxides (Fig. 1) are probably associated with the properties of different Fe oxides as well. Particular consideration is given below to the effects of these Fe oxide properties on the inhibition of microbial NO$_3^-$ reduction.

**Effects of Iron Oxide Properties**

**Effect of Iron(III) Availability from Iron Oxides**

The availability of Fe(III) oxide is correlated with its solubility, which appears to be the rate-controlling factor in biotic Fe(III) reduction by *S. putrefaciens* 200R (Bonneville et al., 2004). Hydroxylamine-extractable Fe(III) can reflect the available Fe(III) in different Fe oxides, as it is considered to be a selective indicator of the availability of microbially reducible Fe(III) in Fe oxides (Lovley and Phillips, 1987; Li et al., 2012a). Experimental results showed that 100, 5.57, 12.8, and 0.904 mmol L$^{-1}$ of Fe(III) were extracted by hydroxylamine hydrochloride from 100 mmol L$^{-1}$ of α-FeOOH, γ-FeOOH, α-Fe$_2$O$_3$, and γ-Fe$_2$O$_3$, respectively. As shown in Fig. 3a, the Fe(III) reduction rates by HS01 were positively correlated with the available Fe(III) from different Fe oxides in the absence ($r = 0.960$, $P = 0.04$) and presence ($r = 0.963$, $P = 0.037$) of NO$_3^-$, though the Fe(III) reduction rate by HS01 alone was much higher than in the presence of NO$_3^-$; however, the NO$_3^-$ reduction rates by HS01 in the presence of Fe oxides were negatively correlated ($r = -0.689$, $P = 0.198$) with the available Fe(III) of different Fe oxides, indicating that a higher Fe(III) availability of an Fe oxide resulted in a stronger inhibitory effect on microbial NO$_3^-$ reduction. Similar results in the microbial reduction of

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**Table 1. The zero-order rates ($r$) of Fe(II) formation, the pseudo-first-order rate constants ($k$), and the contribution parameters ($\chi$) of NO$_3^-$ reduction in the presence of different Fe oxides.**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>With NO$_3^-$</th>
<th>Without NO$_3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$ mmol L$^{-1}$ d$^{-1}$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>HS01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HS01 + α-FeOOH</td>
<td>0.003 ± 0.001 b</td>
<td>0.873</td>
</tr>
<tr>
<td>HS01 + γ-FeOOH</td>
<td>0.002 ± 0.002 b</td>
<td>0.776</td>
</tr>
<tr>
<td>HS01 + α-Fe$_2$O$_3$</td>
<td>0.000 ± 0.000 b</td>
<td>0.624</td>
</tr>
<tr>
<td>HS01 + γ-Fe$_2$O$_3$</td>
<td>0.000 ± 0.000 b</td>
<td>0.703</td>
</tr>
</tbody>
</table>

† Means ± standard deviations. Different lowercase letters indicate significant differences (ANOVA, Duncan’s test, $P < 0.05$) among the treatments.
Fe(III)-oxide-containing soil materials by *Shewanella alga* BrY have been reported previously (Roden and Zachara, 1996; Bonneville et al., 2004). Differences in the Fe(III) availability of Fe oxides may arise from variations in the specific surface area and crystallinity of Fe oxides (Bonneville et al., 2004), the effects of which on microbial Fe and NO$_3^-$ reduction are discussed below.

**Effect of Iron Oxide Surface Area**

The surface areas of the Fe(III) oxides are ranked as follows: $\alpha$-FeOOH (121 m$^2$ g$^{-1}$) > $\gamma$-FeOOH (115 m$^2$ g$^{-1}$) > $\alpha$-$\text{Fe}_2\text{O}_3$ (29.4 m$^2$ g$^{-1}$) > $\gamma$-$\text{Fe}_2\text{O}_3$ (14.4 m$^2$ g$^{-1}$). The linear correlation, with a significance level at $P < 0.05$ (two-tailed), between surface area and Fe reduction rate in the absence and presence of NO$_3^-$ is depicted in Fig. 3b, with results showing that the Fe reduction rates increased with increasing surface areas of the Fe oxides. Both the availability of Fe(III) oxides and the concentration of surface sites for microbial contact are important factors controlling the bioreduction rate of Fe(III) oxides and are positively correlated with the surface area (Bonneville et al., 2004; Yan et al., 2008). The lowest Fe(III) reduction rate in the present study, regardless of the presence of NO$_3^-$, was that of $\gamma$-$\text{Fe}_2\text{O}_3$, which is due to its having the lowest available Fe(III) and surface area. However, the NO$_3^-$ reduction rate constants decreased with increasing Fe oxide surface area, suggesting that a higher Fe oxide surface area causes a higher inhibitory effect on microbial NO$_3^-$ reduction. Therefore, the Fe(III) availability and surface area of Fe oxide did play an important role in the competitive reduction of NO$_3^-$ and Fe oxides by HS01.

**Effect of Hematite Crystallinity**

To clearly illustrate the effect of the degree of crystallinity on NO$_3^-$ and Fe(III) reduction by HS01, additional HS01 NO$_3^-$ reduction experiments were conducted in the presence of hematite with different degrees of crystallinity with XRD patterns in Fig. 4a, as well as Fe(III) reduction in the absence and presence of NO$_3^-$ (Fig. 4b). Nitrate reduction by HS01 was inhibited in the presence of hematite with different crystallinities (Fig. 4b); the pseudo-first-order rate constants of NO$_3^-$ reduction in Table 2 confirmed that a higher crystallinity resulted in less inhibition of NO$_3^-$ reduction. On the other hand, the rates of Fe(II) formation in Table 2 showed that the extent of Fe(II) formation by HS01 in the presence of hematite decreased with an increase in the crystallinity of the hematite. Both the NO$_3^-$ reduction rates and Fe(II) formation rates are plotted against the peak intensities at $2\theta = 33.2^\circ$ of the XRD patterns of hematite in Fig. 4c. It is apparent that the crystallinity of the Fe oxides was negatively correlated ($r = -0.628$, $P = 0.182$) with Fe reduction but
positively correlated ($r = 0.991, P = 0.00$) with NO$_3^-$ reduction by HS01, which is consistent with our previous study (Zhang et al., 2014). Iron oxide with a higher crystallinity has a more stable crystal structure and lower surface area, causing difficulties in Fe reduction by microorganisms, such as a weakening of the inhibitory effects of the Fe oxide on NO$_3^-$ reduction, which was also indicated by the results of NO$_3^-$ reduction vs. available Fe(III) in Fig. 3a. These results suggested that the crystallinity of Fe oxide may be another critical factor controlling the competitive reduction of NO$_3^-$ and Fe oxide by HS01.

**Electrochemical Behavior**

Cyclic voltammetry was performed to identify whether any redox species such as Fe(II)/Fe(III) were produced during the reduction of Fe(III) by HS01 because the redox peaks of Fe(II)/Fe(III) derived from CV curves can reflect the extent of microbial reduction of Fe oxides (Li et al., 2010). The CV of three treatments, Fe oxides (IOs), HS01 + IOs, and HS01 + IOs + NO$_3^-$, was measured for the four types of Fe oxides after 14 d of incubation (Fig. 5). While no obvious redox peak was observed in the tested system containing Fe oxide alone, a pair of peaks was observed at approximately 0.16 and −0.14 V vs. SCE in the HS01 + IOs system, which represent the oxidized peak of Fe(II) and the reduced peak of Fe(III), respectively. In addition, the peak intensities of Fe(II) and Fe(III) for the different Fe oxides showed the same ranking order as that obtained for the kinetics of Fe reduction by HS01 without NO$_3^-$ (Table 1), indicating that the peak intensities of the redox couple Fe(II)/Fe(III) can represent the capacity for Fe reduction by HS01 of the different Fe oxides. However, hardly any Fe(II)/Fe(III) peak was observed in the CV curves of the HS01 + IOs + NO$_3^-$ system, which further confirms that the presence of NO$_3^-$ inhibited Fe reduction by HS01.

**Role of Biogenic Iron(II) during Competitive Reduction of Iron and Nitrate**

Because several abiotic and biotic Fe(II) oxidation processes coupled to NO$_3^-$ reduction are operative under anoxic conditions (Hansen et al., 1996; Straub et al., 1996; Orteley et al., 1997), it is necessary to illustrate the role of biogenic Fe(II) species during NO$_3^-$ reduction by HS01. Additional treatments, including

Table 2. The zero-order rates ($r$) of Fe(II) formation, the pseudo-first-order rate constants ($k$), and the contribution parameters ($\chi$) of NO$_3^-$ reduction in the presence of hematite with different crystallinities (synthesized at 300–800°C).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>$2\theta = 33.2^\circ$</th>
<th>Fe(II) formation</th>
<th>NO$_3^-$ reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$R^2$</td>
<td>$r$</td>
</tr>
<tr>
<td></td>
<td>mmol L$^{-1}$ d$^{-1}$</td>
<td>mmol L$^{-1}$ d$^{-1}$</td>
<td>mmol L$^{-1}$ d$^{-1}$</td>
</tr>
<tr>
<td>HS01</td>
<td>0.005 ± 0.002</td>
<td>0.796</td>
<td>0.233 ± 0.068</td>
</tr>
<tr>
<td>HS01 + Hem-300</td>
<td>0.022 ± 0.001</td>
<td>0.854</td>
<td>0.025 ± 0.008</td>
</tr>
<tr>
<td>HS01 + Hem-400</td>
<td>0.016 ± 0.004</td>
<td>0.906</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>HS01 + Hem-500</td>
<td>0.000 ± 0.000</td>
<td>0.883</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>HS01 + Hem-600</td>
<td>0.000 ± 0.000</td>
<td>0.759</td>
<td>0.002 ± 0.001</td>
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<tr>
<td>HS01 + Hem-700</td>
<td>0.000 ± 0.000</td>
<td>0.770</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>HS01 + Hem-800</td>
<td>0.000 ± 0.000</td>
<td>0.712</td>
<td>0.002 ± 0.001</td>
</tr>
</tbody>
</table>

† Means ± standard deviations. Different lowercase letters indicate significant differences (ANOVA, Duncan's test, $P < 0.05$) among the treatments.
Fe(II), α-FeOOH, Fe(II) + α-FeOOH, and HS01 + Fe(II), were further conducted to investigate the potential reactions between Fe(II) and NO$_3^-$ . The results in Fig. 6 show that neither NO$_3^-$ reduction nor NO$_2^-$ formation was observed in the controls (i.e., Fe(II), α-FeOOH, and Fe(II) + α-FeOOH) after 14 d of incubation, suggesting that NO$_3^-$ cannot be chemically reduced by the free Fe(II) ions or Fe(II) adsorbed on Fe oxide in the tested system. Reportedly, the abiotic processes of Fe(II) oxidation coupled to NO$_3^-$ reduction occurred only under extreme conditions (e.g., elevated temperatures and acidic or alkaline conditions) or in the presence of catalysts (Buresh and Moraghan, 1976; Moraghan and Buresh, 1977; Petersen, 1979; Hansen et al., 1996).

The NO$_3^-$ reduction rate by HS01 was found to be slowed down in the presence of Fe(II) in the control of HS01 + Fe(II) (Fig. 6a), which is consistent with previous studies (Coby and Picardal, 2005; Zhang et al., 2014). In addition, while a pair of remarkable Fe(II)/Fe(III) peaks was observed in the cyclic voltammograms of the HS01 + Fe(II) system, the peak intensities decreased in the presence of NO$_3^-$ after 14 d (Fig. 7), which confirmed that Fe(II) oxidation occurred in the HS01 + Fe(II) + NO$_3^-$ system. It has been shown that cell-associated Fe(II) can react with NO$_3^-$ to form an Fe (hydr)oxide coating that may be responsible for the observed inhibition of NO$_3^-$ reduction (Coby and Picardal, 2005). However, complete NO$_3^-$ reduction, which was still observed in the HS01 + Fe(II) system, is different from the incomplete NO$_3^-$ reduction in most of the HS01 + IOs systems (Fig. 1a). Because the maximum Fe(II) produced by HS01 in the Fe reduction experiments was less than the tested concentration of Fe(II) (0.7 mmol L$^{-1}$) used in the control, the inhibition of NO$_3^-$ reduction in the HS01 + IOs + NO$_3^-$ system is probably partially due to the cell-associated Fe(II) produced by HS01.

**Kinetic Model of Competitive Reactions between Iron and Nitrate Reduction**

Based on the aforementioned results, the competition between NO$_3^-$ and Fe reduction by HS01 is confirmed by both the NO$_3^-$ and Fe(III) reduction kinetics and the electrochemical evidence of Fe(II)/Fe(III), which includes biotic and/or abiotic reduction. To reveal the competition mechanism in the complicated interactive system of HS01 + IOs + NO$_3^-$, a model was tentatively established to illustrate the key reactions taking place. The NO$_3^-$ reduction is divided into two parts, wherein one part is shown as Reaction 1 (R$_1$), representing the biotic NO$_3^-$ reduction process catalyzed directly by HS01, and the other part is shown as Reactions 2 and 3 (R$_2$–R$_3$), representing the biotic–abiotic NO$_3^-$ reduction processes catalyzed by the Fe(II) produced by HS01 (Table 3).

The concentration of residual and consumed NO$_3^-$ can be calculated according to the first-order-rate kinetics for R$_1$ as in Eq. [3] and [4], respectively:

Fig. 5. Cyclic voltammograms obtained on a glass C electrode under different reaction conditions after 14 d of operation. Treatments were Fe oxides, HS01 + Fe oxides and HS01 + Fe oxides + NO$_3^-$ . The initial substrate concentrations were 7 mmol L$^{-1}$ NO$_3^-$, 2 $\times$ 10$^7$ cells mL$^{-1}$ HS01, 5 mmol L$^{-1}$ glucose, and 4.5 g L$^{-1}$ Fe oxides. The scan rate was 50 mV s$^{-1}$.

Fig. 6. Nitrate reduction in abiotic–biotic controls including Fe(II), α-FeOOH, Fe(II) + α-FeOOH, HS01, and HS01 + Fe(II). The initial substrate concentrations were 7 mmol L$^{-1}$ NO$_3^-$, 0.7 mmol L$^{-1}$ Fe(II), 2 $\times$ 10$^7$ cells mL$^{-1}$ HS01, 5 mmol L$^{-1}$ glucose, and 4.5 g L$^{-1}$ α-FeOOH. Error bars represent standard deviation from the mean (n = 3).
The NO$_3^-$ reduced through $R_1$ and $R_2$–$R_3$ can be determined according to Eq. [4] and [8], respectively. To clarify the contribution of the biotic process ($R_1$) and the biotic–abiotic interactive process ($R_2$–$R_3$) to the overall NO$_3^-$ reduction in the HS01 + IOs + NO$_3^-$ system, the relative contribution of $R_1$ is defined as a parameter ($\chi$, $0 < \chi \leq 1$), while the relative contribution of $R_2$–$R_3$ should be $(1 - \chi)$. The concentrations of consumed and residual NO$_3^-$ in the HS01 + IOs + NO$_3^-$ system ($R_1$–$R_3$) can be calculated, respectively, as

$$C_{\text{NO}_3^-,\text{overall}} = \chi C_{\text{NO}_3^-,R_1} + (1 - \chi) C_{\text{NO}_3^-,R_2-R_3}$$

$$= \chi C_{\text{NO}_3^-,0} \left[ 1 - \exp(-k_{r1}t) \right]$$

$$+ \frac{1}{2} r_{r1} \left[ 1 - \exp(-k_{r1}t) \right]$$

$$C_{\text{NO}_3^-,\text{overall}} = C_{\text{NO}_3^-,0} - \chi C_{\text{NO}_3^-,0} \left[ 1 - \exp(-k_{r1}t) \right]$$

$$- \frac{1}{2} r_{r1} \left[ 1 - \exp(-k_{r1}t) \right]$$

To obtain the value of the ratio $\chi$, calculations can be performed using Eq. [10] with the experimental data available. For example, the NO$_3^-$ reduction rate in reaction $R_1 (k_1)$ and the Fe(III) reduction rate in reaction $R_2 (r_2)$ are shown in Tables 1 and 2. The NO$_3^-$ reduction rate by cell-associated Fe(II) in reaction $R_3 (k_3)$ can be obtained based on the NO$_3^-$ reduction kinetics of the HS01 + Fe(II) system in Fig. 6. The fitting results in Fig. 8a and Table 1 show that the $\chi$ values of the treatments with different Fe oxides can be ranked in the order: $\gamma$-FeOOH < $\alpha$-FeOOH < $\alpha$-Fe$_2$O$_3$ < $\gamma$-Fe$_2$O$_3$, suggesting that with increasing Fe(III) availability and surface area, the contribution of direct reduction by HS01 decreased while the contribution of the reduction by the cell-associated Fe(II) increased. The experimental NO$_3^-$ reduction data in the HS01 + hematite + NO$_3^-$ system can be fitted using Eq. [10], yielding the results shown in Fig. 8b, and the $\chi$ values obtained after fitting are shown in Table 2 as well. The gradual increase in $\chi$ values with increasing crystallinity of the Fe oxides in the HS01 + hematite + NO$_3^-$ system indicates that the biotic process ($R_1$) eventually plays a predominant role and that the contribution of the biotic–abiotic processes ($R_2$–$R_3$) weakens as the crystallinity of Fe oxides increases. The inhibition of NO$_3^-$ reduction by Fe oxide in this study is attributed to two reasons: (i) the presence of Fe oxide, which acts as a competitive electron acceptor, and (ii) the slow reaction between cell-associated Fe(II) and NO$_3^-$ ($R_3$) because $k_2 > k_1$ and the low rate of Fe(III) reduction by HS01 is a rate-limiting process during $R_2$–$R_3$.

**Table 3. Biotic–abiotic reactions in the system HS01 + Fe oxides + NO$_3^-$.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reaction rate (constant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3^-$ + 2e$^- \rightarrow \text{HSO}_4^+$ + glucose $\rightarrow$ NO$_2^-$</td>
<td>$k_1$</td>
</tr>
<tr>
<td>Fe(II) + e$^- \rightarrow \text{HSO}_4^+$ + glucose $\rightarrow$ Fe(II)</td>
<td>$r_2$</td>
</tr>
<tr>
<td>NO$_3^-$ + cell-Fe(II) + H$^+$ $\rightarrow$ NO$_2^-$ + cell-Fe(III) + H$_2$O</td>
<td>$k_3$</td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

All of the tested Fe oxides showed an inhibitory effect on the reduction of NO$_3^-$ by HS01 to different extents; the reduction of Fe by HS01 was also inhibited by the presence of NO$_3^-$ . The extent of NO$_3^-$ reduction inhibition by Fe oxide increased with increasing Fe(III)
availability and Fe oxide surface area and decreased with increasing Fe oxide crystallinity. The mutual inhibition between Fe and NO$_3^-$ reduction was mainly attributed to the competition between NO$_3^-$ and Fe oxide when acting as electron acceptors. The simple kinetic model established in this study demonstrates that the inhibition of NO$_3^-$ reduction by Fe oxide can also be attributed to the slower rate of the biotic–abiotic reduction of NO$_3^-$ by cell-associated Fe(II) produced by HS01 compared with that of direct reduction of NO$_3^-$ by HS01. The results obtained from this study are helpful for understanding the contributions of Fe-reducing bacteria, Fe oxide, and Fe(II) species in the competitive reduction of NO$_3^-$ and Fe oxide in natural subsurface environments.

**NOMENCLATURE**

- $C_{NO_3,R_1}$: conc. of residual NO$_3^-$ in $R_1$ (mmol L$^{-1}$)
- $C_{NO_3,0}$: initial conc. of NO$_3^-$ (mmol L$^{-1}$)
- $C_{NO_3,r,R_1}$: conc. of consumed NO$_3^-$ in $R_1$ (mmol L$^{-1}$)
- $k_1$: pseudo first-order rate constant in $R_1$ (d$^{-1}$)
- $t$: reaction time (d)
- $C_{Fe(II),R_2}$: conc. of generated Fe(II) in $R_2$ (mmol L$^{-1}$)
- $r_2$: Fe(II) generation rate (mmol L$^{-1}$ d$^{-1}$)
- $C_{Fe(II),R_3}$: conc. of residual Fe(II) in $R_3$ (mmol L$^{-1}$)
- $k_3$: conc. of consumed Fe(II) in $R_3$ (mmol L$^{-1}$)
- $C_{Fe(II),r,R_3}$: conc. of first-order rate constant in $R_3$ (d$^{-1}$)
- $C_{NO_3,r,R_3}$: conc. of consumed NO$_3^-$ in $R_3$ (mmol L$^{-1}$)
- $C_{NO_3,overall}$: conc. of consumed NO$_3^-$ in mixed system (mmol L$^{-1}$)
- $C_{NO_3,overall}$: conc. of residual NO$_3^-$ in mixed system (mmol L$^{-1}$)

$\chi$: relative contribution of $R_1$

$1 - \chi$: relative contribution of $R_2 - R_3$

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**REFERENCES**


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