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Enhancement of Hematite Bioreduction by Natural Organic Matter

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The effects of natural organic matter (NOM), ferrozine, and AQDS (anthraquinone-2,6-disulfonate) on the reduction of hematite (α-Fe_2O_3) by *Shewanella putrefaciens* CN32 were studied. It has been proposed that NOM enhances the reduction of Fe(III) by means of electron shuttling or by Fe(II) complexation. Previously both mechanisms were studied separately using "functional analogues" (AQDS for electron shuttling and ferrozine for complexation) and are presently compared with seven different NOMs. AQDS enhanced hematite reduction within the first 24 h of incubation, and this had been ascribed to electron shuttling. Most of the NOMs enhanced hematite reduction after 1 day of incubation indicating that these materials could also serve as electron shuttles. The effect of ferrozine was linear with concentration, and all of the NOMs exhibited this behavior. Fe(II) complexation only enhanced hematite reduction after sufficient Fe(II) had accumulated in the system. Fe(II) complexation appeared to alleviate a suppression of the hematite reduction rate caused by accumulation of Fe(II) in the system. Addition of Fe(II) to the hematite suspension, prior to inoculation with CN32, significantly inhibited hematite reduction and greatly diminished the effects of all of the organic materials, although some enhancement was observed due to addition of anthroquinone-2,6-disulfonate. These results demonstrate that NOM can enhance iron reduction by electron shuttling and by complexation mechanisms.

Introduction

The microbial reduction of iron(III) is recognized as an important process for in situ bioremediation due to the abundance of Fe(III) and the capacity of dissimilatory iron reducing bacteria (DIRB) to immobilize or degrade a wide variety of contaminants (*1*−*5*). Dissimilatory iron(III) reduction is also an important process in the biogeochemistry of hydric soils (*6*). Wetlands are important from regulatory, ecological, and sometimes engineering standpoints and can be in part defined by the presence of redoximorphic features that are due to microbial iron and manganese reduction (*6*). Natural organic matter (NOM) has been shown to enhance Fe(III) reduction in laboratory systems (*7*−*12*). Two mechanisms have been proposed to explain this enhancement: electron shuttling and Fe(II) complexation (*7*, *13*−*15*).

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In the context of this work, electron shuttling is the transfer of electrons from the DIRB to the ferric (oxyhydr)oxide surface via a soluble, biologically reducible compound. The electron shuttling compound must have a redox potential that is sufficiently low to reduce the Fe(III) mineral and yet high enough so that the compound can be reoxidized and recycled. This effect has been demonstrated repeatedly with the quinone, anthroquinone-2,6-disulfonate (AQDS), a proposed humic acid analogue (*9*, *13*, *14*, *16*−*18*). AQDS enhances the rate of Fe(III) reduction by both *Shewanella* spp. and *Geobacter* spp. with a variety of Fe(III) sources (*9*, *13*, *14*, *17*, *18*). In the case of *Shewanella putrefaciens* CN32, AQDS is rapidly reduced to anthrohydroquinone-2,6-disulfonate (AH_2-DS) using hydrogen as the electron donor (*19*). It has been proposed that NOM acts in a manner analogous to AQDS, with quinone moieties involved in the electron transfer (*11*, *13*). In a study of electron transfer from several types of biologically reduced NOM to ferric citrate, quinone content, as quantified via electron spin resonance (ESR) spectroscopy, was correlated with Fe(II) production (*11*). Quinone content has been used as a measure of the electron shuttling capacity of NOM in terms of electrons transferred per unit mass of NOM, but other, as yet unidentified, factors may influence the electron shuttling capacity and reactivity of NOMs.

It is important to note that AQDS is proposed to mimic NOM in only one very specific manner, namely serving to transfer electrons from a microorganism to Fe(III). It is therefore a "functional analog" in this respect only, and by no means a structural analogue. Indeed, NOM may possess functional groups other than quinones which are equally or more important in influencing iron reduction. Aromaticity, acidity, and other chemical characteristics may also determine effectiveness of NOMs as stimulants of iron reduction. Additionally, NOM macromolecules are far larger than AQDS, which will greatly influence their relative diffusivities and possibly the "ease" with which proper stereochemical interactions may take place for electron transfer.

Complexation of Fe(III) and Fe(II) also have been shown to enhance DIRB-mediated reduction of several Fe(III) sources. Enhanced bioreduction due to complexation of solid-phase Fe(III) has been demonstrated using synthetic chelators (*15*, *20*−*24*), naturally produced compounds such as siderophores (*25*) and NOM (*7*, *14*, *15*). NOM can also complex Fe(II) (*7*).

Reduction of solid-phase Fe(III) slows when there is accumulation of dissolved Fe(II) (*26*). Addition of the Fe(II)-specific chelator ferrozine has been shown to increase the extent of hematite reduction in direct proportion to its Fe(II) complexation capacity (*7*, *14*). This same mechanism of enhancement has been attributed to NOM (*14*). The capacity for NOM to complex Fe(II) can be estimated from its acidity at the experimental pH (*27*).

There is disagreement regarding the mechanism by which ferrozine (or other Fe(II) complexing agents) can enhance the reduction of solid phase Fe(III). The inhibitory effect of Fe(II) accumulation in Fe(III)-reducing systems has been previously attributed to a mechanism termed "passivation" (*22*, *28*). The buildup of Fe(II) at the cell-oxide interface may also inhibit Fe(III) reduction via a mass transfer linked thermodynamic mechanism, and ferrozine might dissolve or increase transport of Fe(II) from this interface. Also, the accumulation of Fe(II) in solution or at the oxide surface can limit the rate of reaction by decreasing the thermodynamic driving force or even by making further oxide reduction thermodynamically unfavorable. Complexation of Fe(II) would decrease the chemical potential of Fe^{2+}, thus increasing

TABLE 1. Characteristics of NOM and Their Enhancement of Hematite Reduction

NOM	source	organic radical content (spins $g^{-1} \times 10^{17}$)	acidity at pH = 6.8 (mequiv g^{-1})	aromaticity (%)	carbon content (%)	slope (enhancement factor) (mmol Fe(II) mg^{-1} NOM)
soil HA	terrestrial	12.9	4.03	50	58.1	1.36×10^{-3} (1.61×10^{-3})[b]
Georgetown NOM	aquatic	6.67	3.36[a]	30 (ca.)	48.3	6.37×10^{-4}
Summit Hill HA	terrestrial	5.38	3.20	30	54.0	1.07×10^{-4} (9.68×10^{-4})[b]
Leonardite HA	terrestrial	3.12	3.78	58	63.8	1.30×10^{-3}
Suwannee River HA	aquatic	1.15	3.97	37	52.6	7.97×10^{-4}
Suwannee River FA	aquatic	0.54	5.55	24	53.0	5.07×10^{-4}
Suwannee River NOM	aquatic	0.54 (ca.)	4.08	24 (ca.)	48.8	1.61×10^{-4} (3.94×10^{-4})[b]

[a] Acidity reported for pH 6.6. [b] Raw data, i.e., not corrected for Fe(II) production in the abiotic controls, in all other cases no Fe(II) was detected in abiotic controls.

the driving force for the reduction of solid-phase Fe(III). These mechanisms are related, since decreasing the activity of Fe^{2+} at the solid/water or cell/solid interfaces would influence the diffusive transport of Fe(II).

The objectives of the present study were (1) to determine the effects of several well-characterized NOMs on the biological reduction of hematite (α-Fe_2O_3) and (2) to determine whether the increased bioreduction of Fe(III) was due to electron shuttling and/or Fe(II) complexation. Acidity and electron spin resonance (ESR) spectroscopy data were used as the indices of Fe(II) complexation and electron shuttling capacity, respectively.

Experimental Section

Microorganism and Culture Conditions. *Shewanella putrefaciens* strain CN32 was grown aerobically on tryptic soy broth without dextrose (Difco) at 20 °C. Cells were harvested by centrifugation (3510 x *g*, 10 min, 15 °C) from a 16-h-old culture (late log-decreasing growth phase). Cells were washed three times in 50 mM PIPES plus 30 μM phosphate buffer (hereafter referred to as PIPES-phosphate buffer, pH 6.8) with the final wash made with deoxygenated solution. This buffer was used for all experiments. Cell pellets were resuspended in deoxygenated PIPES-phosphate buffer in an anaerobic chamber (Coy) under a N_2:H_2 (ca. 97.5:2.5) atmosphere and cell density was determined by absorbance at 420 nm.

Hematite. An iron oxide powder was obtained from J. T. Baker and identified by X-ray diffraction and Mössbauer spectroscopy to be hematite (α-Fe_2O_3) of greater than 99% purity. The hematite powder had an average particle diameter of ca. 300 nm and a specific surface area of 9.04 m^2 g^{-1} (measured by 5-point N_2−BET). Hematite was heated to 550 °C in air overnight before use to remove any organic carbon. Hematite was added to the PIPES-phosphate buffer at least 48 h prior to any experiment to allow for hydration.

Bioreduction Experiment Preparation. The bioreduction experiments were run in crimp-sealed (Teflon faced butyl rubber stoppers) amber serum bottles (10 mL) containing 5 mL of medium. All preparations were performed in an anaerobic chamber. The test medium contained PIPES-phosphate, 2.0 g L^{-1} hematite (25 mM as Fe), and was inoculated to achieve a final cell density of 10^8 cells mL^{-1}. Sealed bottles were incubated at 20 °C on orbital shakers, outside of the anaerobic chamber. Unamended biotic controls containing only the inoculated basic test medium (i.e. no NOM or other amendment) were run for all experiments. All treatments and controls were run at least in triplicate. Uninoculated controls for each amendment were incubated in quintuplicate for 5 days. Serum bottles were sacrificed for Fe(II) analyses after 5 days of incubation or earlier in some experiments. Previous experiments with and without (30 μM of phosphate) established that phosphate had no effect on the amount of biogenic Fe(II) produced, or the fraction in which that Fe(II) was recovered, under the present experimental conditions (*7*). The pH of all experiments never varied outside the range of 6.6−7.2.

Variable Mixing Speed Experiments. Nongrowth bioreduction cultures were incubated under different mixing speeds to study the effect of mass transfer on hematite bioreduction. The experimental systems used conditions identical to the standard (unamended) bioreduction experiments except that the cultures were incubated in 250 mL media bottles rather than crimp sealed serum vials. Two cultures were mixed on a magnetic mixer at two speeds (400 and 1200 rpm) and one was incubated statically. Bottles were periodically transferred to the glovebox and sampled for Fe(II). A triplicate set of bottles was sampled at 1, 5, and 19 days to allow a statistical comparison between the mixing regimes. When tightly sealed, these bottles were found to be impermeable to oxygen.

Functional Analogue Amendments. Ferrozine (J. T. Baker), a specific Fe(II) chelator, was added to experimental systems as a dry powder (*29*). AQDS ($E^0_w = -184$ mV, pH 7) (Aldrich, Milwaukee, WI) was added volumetrically from a filtered (0.1 μm), aerated, concentrated stock solution (*30*).

NOMs. Georgetown NOM (GNOM) was provided courtesy of Dr. Baohua Gu (Oak Ridge National Laboratory) and is a well characterized, salt-free, freeze-dried material that was originally collected from a wetland pond in Georgetown, SC (*31*). The acidity of GNOM at pH 6.6 in 10 mM NaCl was 6.98 mequiv g^{-1} C (B. Gu, personal communication). The metal binding capacity of GNOM was equivalent to 3.49 mmol Fe(II) g^{-1} C at pH 6.6, assuming 2 acid equiv per Fe(II) ion (*27*). Six other NOMs from the International Humic Substances Society (IHSS) were also tested: Suwannee River NOM (SRNOM), Suwannee River fulvic acid (SRFA), Suwannee River humic acid (SRHA), Leonardite humic acid (LHA), Summit Hill humic acid (SHHA), and soil humic acid (SHA). ESR data for all of these materials were supplied by IHSS. Proton titration data (NOM at 400 mg L^{-1} in 0.1 M NaCl) for all of these materials were provided courtesy of Dr. M. Perdue (Georgia Institute of Technology, personal communication). Aromaticity values for the NOMs were determined from ^{13}C NMR spectra from a published report for the IHSS materials (*32*) and from personal communication (B. Gu) for GNOM. Acidity, organic radical content, percent carbon, aromaticity, and source (i.e., terrestrial vs aquatic) data for these materials are given in Table 1.

The "enhancement" factor for each NOM was defined as the slope of the linear regression for acid extractable Fe(II) produced after 5-day incubation-vs-NOM concentration (mmoles Fe(II) mg^{-1} NOM, Table 1). Abiotic controls that contained SRNOM, SHA, and SHHA produced Fe(II) (data not shown), and the values in Figures 3 and 5 and Table 1 were corrected to show only the incremental Fe(II) production that was ascribed to biological activity.

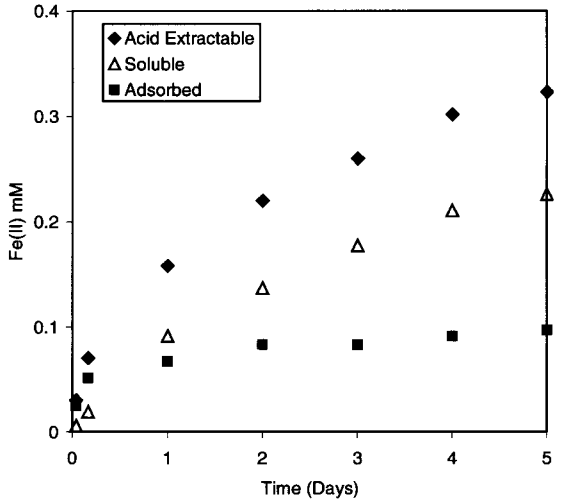

FIGURE 1. Acid extractable, soluble, and adsorbed Fe(II) as a function of time in unamended biotic control. Culture mixed at 400 rpm, see Figure 2 for complete data.

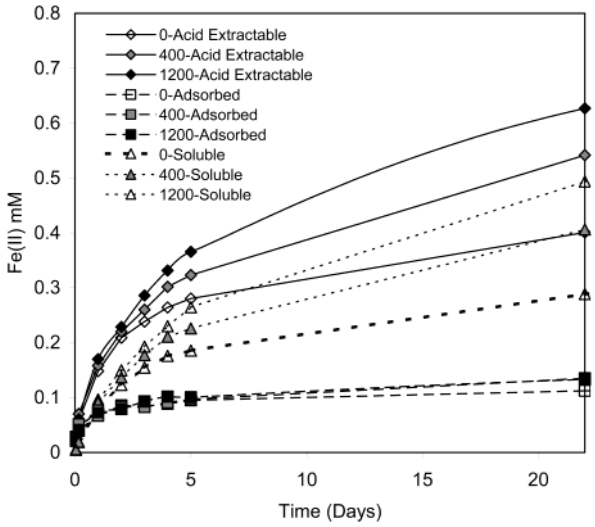

FIGURE 2. Acid extractable, soluble, and adsorbed Fe(II) as a function of time in unamended biotic experiments mixed at different speeds. The numbers in the legend indicated the speed in RPM of the magnetic mixer. Zero indicates a static bottle.

Fe(II) Preloading Experiment. In an effort to isolate the electron shuttling function of NOM, an experiment was conducted in which systems were spiked with Fe(II), added from an anaerobic $FeCl_2$ stock solution prior to inoculation, to a final concentration of 0.79 mM. The objective was to effectively exhaust the complexation capacity of the NOMs by providing Fe(II) significantly in excess of their estimated complexation capacity, thus allowing the NOMs to enhance Fe(III) reduction only via electron shuttling. The concentrations of NOM in each system were calculated to yield an estimated Fe(II) complexation capacity of 0.27 mM based on acidity at pH 6.8 (or pH 6.6 for GNOM). The concentrations of each NOM used were (mg L^{-1}) as follows: SRFA, 96.9; SRNOM, 131.6; SHA, 133.5; SRHA, 135.5; LHA, 142.3; GNOM, 159.0; SHHA, 168.2. The specific order of addition and equilibration times were as follows: hematite + buffer (48 h), $FeCl_2$ (24 h), cells (5 min), and finally NOM. The point at which the NOM was added was considered the start of the experiment. AQDS (50 μM) was included as a treatment to evaluate the role of the added Fe(II) on the effectiveness of a quinone known to serve as an electron shuttle. For unamended biotic controls the combined hematite plus biomass Fe(II) adsorption capacity was estimated to be approximately 0.18 mM. Triplicate samples were sacrificed and analyzed after 5-day incubation.

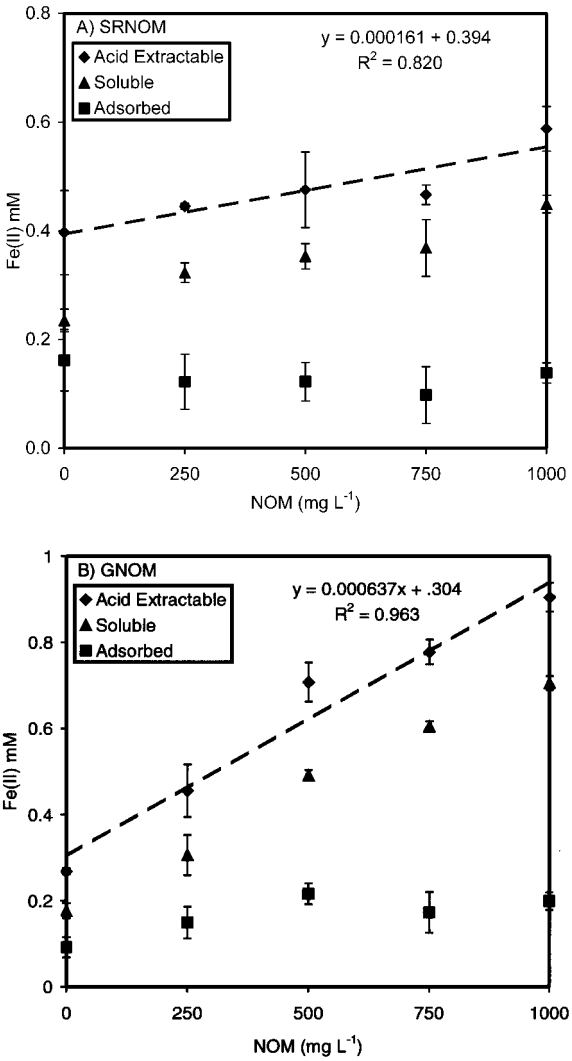

FIGURE 3. Acid extractable, soluble, and adsorbed Fe(II) after 5 days as a function of NOM concentration for (A) Suwannee River NOM and (B) Georgetown NOM. Values are means of three replicates (± standard deviation). Abiotic Fe(II) production (control experiments) have been subtracted. Dashed line is the linear regression of corrected acid extractable Fe(II) vs. NOM; the regression equation and R^2 value are displayed above the line. Reproduced with permission from *Environ. Sci. Technol.* 2002, *36*, 1939−1946. Copyright 2002 American Chemical Society.

Analytical Techniques. Fe(II) was reported as soluble, acid extractable, and adsorbed. Soluble was defined as that fraction of Fe(II) which passed through a 0.1 μm filter. Acid extractable was that Fe(II) which passed through a 0.1 μm filter after ca. 16 h extraction in 0.5 N HCl. Adsorbed Fe(II) was the acid extractable minus the soluble fraction. Samples from each system were filtered (0.1 μm), and an aliquot of the filtrate was added to 5 mL of ferrozine reagent (1.96 mM ferrozine in 50 mM HEPES, pH 8.0) in the anaerobic chamber. Solution pH was determined by combination electrode on the remaining filtrate in the anaerobic chamber. After at least 10 min, samples were removed, the absorbance (562 nm) was determined using a Shimadzu UV/Vis-1601 spectrophotometer, and the result was converted to concentration of soluble Fe(II). Acid extractable Fe(II) was determined by adding a 1 mL aliquot from the serum bottle to 4 mL of 0.625 N HCl to achieve a final normality of 0.5 N. The solution was then removed from the anaerobic chamber and allowed to mix overnight (ca. 16 h). The samples were then filtered (0.1

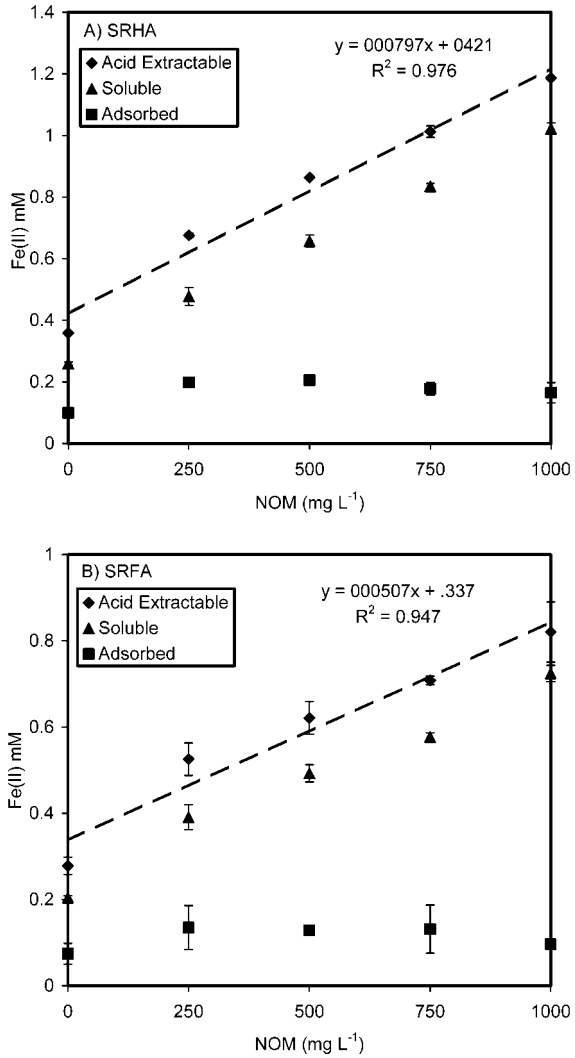

FIGURE 4. Acid extractable, soluble, and adsorbed Fe(II) after 5 days as a function of NOM concentration for (A) Suwannee River humic acid and (B) Suwannee River fulvic acid. Values are means of three replicates (± standard deviation). Abiotic Fe(II) production (control experiments) has been subtracted. Dashed line is the linear regression of corrected acid extractable Fe(II)-vs-NOM; the regression equation and R^2 value are displayed above the line.

μm), and the filtrate was analyzed as described above for soluble Fe(II). Adsorbed Fe(II) was calculated as the difference between soluble and acid extractable Fe(II).

Results

The rate of hematite reduction in the absence of amendments was greatest within the first 24 h of a 5-day incubation period (Figure 1). The decrease in acid extractable Fe(II) production coincided with approaching the maximum adsorbed Fe(II) concentration, which approached a stable value of about 0.08 mM within 24 h. When corrected for sorption by the bacteria (*26*) this corresponded to 1.9 Fe(II) sorbed per nm^2, which is in agreement with previous measurements of between 1.2 and 3.8 maximum adsorbed Fe(II) per nm^2 of ferric oxide surface (*33*). Adsorbed Fe(II) was relatively constant after 24 h, and almost all subsequently generated Fe(II) was soluble.

Increased mixing in unamended systems increased Fe(II) production (Figure 2). This trend continued to the latest sampling point (85 days, not shown). The adsorbed Fe(II) concentration was consistent among the different mixing conditions with all nearly all Fe(II) generated after the first 24 h occurring as soluble Fe(II). In addition to the single

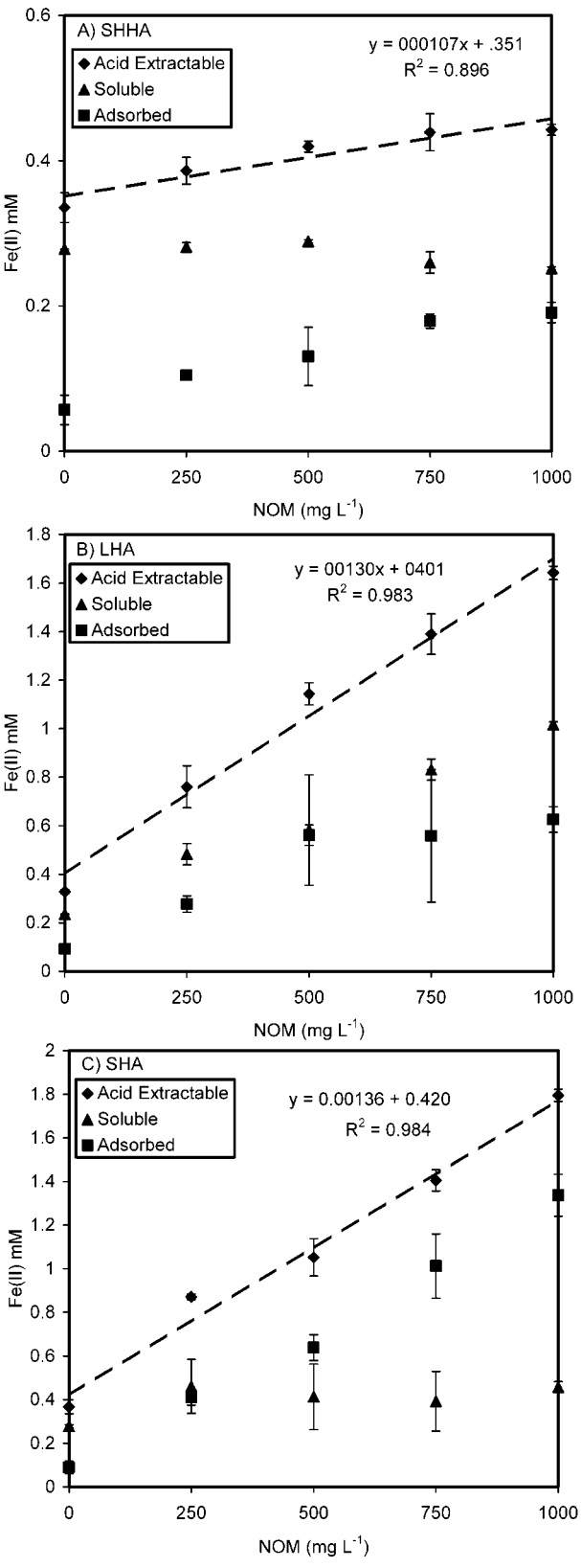

FIGURE 5. Acid extractable, soluble, and adsorbed Fe(II) after 5 days as a function of NOM concentration, (A) Summit Hill humic acid, (B) Leonardite humic acid, and (C) soil humic acid, after 5-day incubation. Values are means of three replicates (± standard deviation). Abiotic Fe(II) production (control experiments) have been subtracted. Dashed line is the linear regression of corrected acid extractable Fe(II)-vs-NOM; the regression equation and R^2 value are displayed above the line.

reactor experiments shown in Figure 2 an identical experiment was conducted with triplicate reactors but less intensive sampling (samples taken at 1, 5, and 19 days). The triplicate bottles allowed for statistical comparison of the three mixing regimes. Acid extractable Fe(II) from each mixing speed was compared against the other two speeds for each time point (all pairings for a given time were tested) using Student's *t*-test ($\alpha = 0.05$). The following pairs were found to be significantly different (numbers indicate RPM of mixing): day 1, 0 vs 400, 0 vs 1200; day 5, 0 vs 1200; day 19, 0 vs 400, 0 vs 1200, and 400 vs 1200. In the 1-day samples the mixed systems were statistically separable from nonmixed samples and by day 19 all three conditions were statistically separable. The day 5, 400 rpm sample was not separable from either the 0 or 1200 rpm samples due to a relatively higher variance [coefficients of variance (as %) for 0, 400, and 1200 were as follows: 0.47, 11.6, and 5.54 respectively].

SRNOM and GNOM are "whole" NOMs, i.e., they represent the entire NOM content of the source waters and were not separated into NOM fractions, such as humic or fulvic acids. Both SRNOM and GNOM enhanced the bioreduction of hematite in 5-day samples (Figure 3) compared to no-NOM biotic controls (the *y*-intercepts). The relationship of acid extractable Fe(II) versus NOM concentration was approximately linear in both cases. GNOM (Figure 3b) enhanced hematite reduction approximately four times more than SRNOM (Figure 3a). There was slightly more sorbed Fe(II) for the GNOM treatments (compared to either SRNOM or the no-NOM control), but the maximum adsorbed Fe(II) for either GNOM or SRNOM was consistent with previous measurements for adsorption onto hematite (*33*) and onto CN32 (*26*). GNOM has a much higher organic radical content (ca. 12 times greater) but lower acidity (ca. 20% lower) than SRNOM.

The two fractions of SRNOM exhibited different abilities to enhance hematite reduction (Figure 4). The general trend of acid extractable Fe(II) versus NOM concentration was similar to the whole NOMs. The humic acid fraction (SRHA) of SRNOM enhanced hematite reduction more than the fulvic acid fraction (SRFA, Figure 4). Both the humic and the fulvic fractions enhanced hematite bioreduction more than the SRNOM parent material (Figures 3a and 4). SRHA has more than twice as much organic radical content as SRFA, and only 72% of the acidity of SRFA. SRNOM is largely fulvic acid (>90%) so the difference in slope between SRNOM (Figure 3b) and SRHA (Figure 4a) was unexpected. Adsorbed Fe(II) was within the range that was expected based on cell and hematite adsorption experiments. As with the whole NOMs, the majority of the Fe(II) generated beyond the unamended biotic control was recovered as soluble Fe(II) (Figure 4).

The three soil humic acids all enhanced hematite reduction in direct proportion to their concentration (Figure 5, Table 1). SHHA (Figure 5a) enhanced hematite reduction the least, whereas LHA (Figure 5b) and SHA (Figure 5c) enhanced hematite reduction more than all of the other NOMs. All three soil humic acids have relatively high organic radical contents, but organic radical content did not correspond directly to enhancement as determined from the slopes of the acid extractable Fe(II)-vs-NOM concentration regressions (Table 1). Furthermore, acidities of these materials differed much less than their effectiveness in enhancing hematite reduction. All three soil humic acids increased adsorbed Fe(II) over unamended biotic controls (Figure 5) to a greater extent than the aquatic materials (Figures 3 and 4). The soil humic acids resulted in sorbed Fe(II) that was greater than would be predicted using maximum sorption onto hematite (*33*) and CN32 (*26*). For SHA, "sorbed" Fe(II) was approximately 10 times the predicted "maximum sorption" but less than the maximum complexation capacity of the added SHA. Humic acids are operationally defined as materials that precipitate when acidic functional groups are neutralized by H^+ or by other cations, and it is possible that the soil humic acids were precipitated due to the high Fe(II) concentrations.

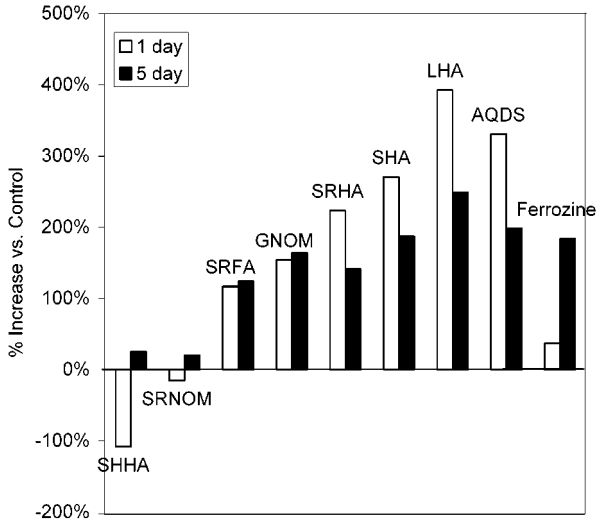

FIGURE 6. Enhancement of Fe(II) production relative to biotic controls for 500 mg L^{-1} NOM, 1.47 mM ferrozine, or 50 μM AQDS and after 1 and 5 days. Abiotic Fe(II) production due to NOM has been subtracted.

NOMs (500 mg/L), AQDS (50 μM), and ferrozine (1.47 mM) were tested in 1- and 5-day samples, and the cumulative production of Fe(II) was compared to unamended biotic controls (Figure 6). Ferrozine was previously shown to enhance iron reduction at this concentration while not being degraded or serving as a substrate for cell growth (*7*). All of the NOMs, except SRNOM and SHHA, produced more Fe(II) than the unamended biotic control after 1 day of incubation. SRNOM and SHHA also gave the smallest enhancement after 5 days (Figure 6). When expressed as a percentage increase over the unamended biotic control many of the 1-day samples exhibited more enhancement than the same concentration in 5-day samples. The ranking of these materials in terms of enhancing hematite reduction did not match their ranking by organic radical content.

In an attempt to resolve the electron shuttling function of the NOMs, experimental systems were "preloaded" with 0.79 mM Fe(II) prior to NOM additions. Each NOM was added to achieve an estimated complexation capacity of approximately 0.27 mM Fe(II). The approximately 3:1 ratio of Fe(II) to NOM complexation capacity was used to limit the influence of complexation (either by the NOM or the cell and oxide surfaces) on the generation of Fe(II) during the experiment. The NOMs then could only enhance hematite reduction via electron shuttling as they possessed essentially no unused complexation capacity. The cell and oxide surfaces combined had a complexation capacity of ca. 0.18 mM Fe(II). The results of 5-day incubations showed that there was less than 50% of the normal hematite reduction in the unamended biotic control. None of the NOMs significantly (two-tailed *t*-test, $\alpha = 0.05$) enhanced hematite reduction (Figure 7). The AQDS-amended system with Fe(II) preloading yielded more than twice as much biogenic Fe(II) as the unamended biotic control (Figure 7) but still produced less than in nonpreloaded systems (0.357 mM (preloaded) vs 1.27 mM in nonpre-loaded unamended biotic control after 5 days of incubation).

Discussion

To interpret how NOM enhances hematite reduction it is important to consider what actually controls the rate of

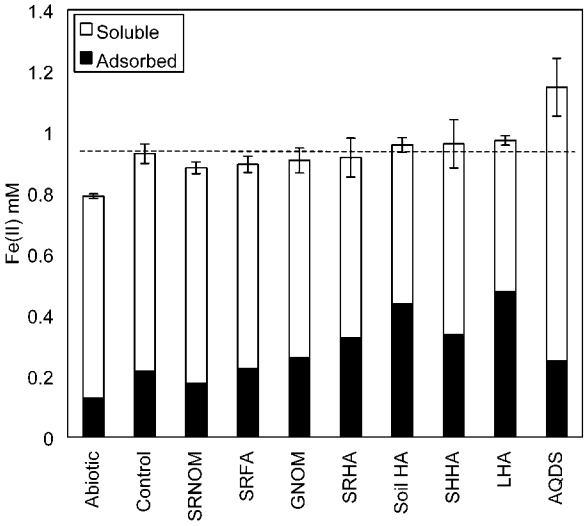

FIGURE 7. Acid extractable Fe(II) after 5 day incubation in systems "preloaded" with 0.79 mM Fe(II). All NOM concentrations were added to achieve a complexation capacity of 0.27 mM Fe(II). Values are means of three replicates (± standard deviation).

hematite reduction in its absence. It appears that the factors initially (up to ca. 24 h) controlling the rate of hematite reduction differ from those later in the incubation period. The coincidence of the slowing of hematite reduction (i.e., inhibition) with the accumulation of adsorbed Fe(II) in the unamended biotic controls is consistent with adsorbed Fe-(II) interfering with subsequent hematite reduction (Figure 1). The precise mechanism by which Fe(II) interferes with hematite reduction is unknown. Alteration of the hematite surface, physical blocking of hematite surface sites, sorption of Fe(II) to bacteria, changes in the thermodynamics of the solution-solid interface, or mass transfer limitations (either from or to hematite or cells) may all play a role. While the mechanism is unknown, results from these studies and others clearly indicate that Fe(II) inhibits further hematite reduction (Figures 1, 2, and 7) (*7*, *14*, *26*, *34*).

It appears that hematite bioreduction in the present study may be initially under kinetic control (the reaction rate is limited by the slowest step in a series of chemical reactions which constitute the overall reaction) and then transitions to mass transfer control (the chemical reaction kinetics are not limiting but instead transfer of a product or reactant is limiting the observed reaction rate). While under kinetic control the rate of reaction is limited by the slowest step in the process of hydrogen oxidation to the dissolution of the Fe(II) from the hematite crystal lattice. Experiments using Fe(III)-citrate as the terminal electron acceptor in lieu of hematite (but in all other ways identical to the present experiments) support a much greater rate of iron reduction than hematite (data not shown). Indeed, with Fe(III)-citrate the rate of iron reduction is more than 50 times that observed even in the 1200 rpm mixed hematite suspension. The Fe-(III)-citrate reduction data indicate that hydrogen transfer to the organism is far faster than required to meet the demand during hematite bioreduction, eliminating hydrogen transfer as the rate-limiting process during hematite bioreduciton. The increase in Fe(II) production with increasing mixing speed is indicative of a mass transfer controlled process (Figure 2) (*35*). The required high rate of hydrogen transport during Fe(III)-citrate bioreduction and the increasing disparity in Fe(II) production between the different mixing regimes over time strongly support a limitation by a reaction product, e.g. Fe(II) rather than a reactant, e.g. molecular hydrogen. Additionally, this is consistent with the correlation between the slowing of Fe(II) production and the attainment of nearly maximal adsorbed Fe(II) (Figures 1 and 2). The activity of Fe^{2+} at the surface might remain low until the sorptive capacity of the "high-affinity", "strong", or type 1 surface sites (which represent only a fraction of the cation binding sites when using a two-site sorption model) is saturated (*36*). As Fe(II) production continues, Fe(II) may accumulate to an interfacial (cell-hematite-solution interface) concentration that slows further Fe(III) reduction. This interfacial region can be modeled as a stagnant film in which all transport to and from the bulk solution is via diffusion. The thickness of this stagnant film is a function of the fluid environment, with film thickness decreasing with increasing mixing intensity or flow rate. Initially, the cell and hematite surfaces serve as sinks for biogenic Fe(II). Gradually, when these surfaces are effectively saturated the system reaches its apparent Fe(II) "sorption" capacity and hematite reduction begins to slow (Figures 1and 2).

The mechanism of this inhibition is not understood but may be thermodynamic (*34*). Hematite reduction with H_2 as the electron donor is not a very favorable process and is calculated to be unfavorable if high μM quantities of Fe(II) accumulate in the medium (ca. 0.229 mM "free" $Fe(II)_{aq}$ yields a net free energy of zero at $T = 20$ °C, pH 6.8). Diffusion of Fe(II) away from the cell-hematite interface may be insufficient to keep Fe(II) below the level required to remain a thermodynamically favorable process. It is hypothesized that under these conditions the diffusional flux of Fe(II) may limit the rate of Fe(III) reduction, resulting in a system that is under thermodynamic control (as a consequence of slow mass transfer) rather than kinetic control. In other words, further iron reduction is inhibited due to an unfavorable thermodynamic situation that arises as a consequence of relatively slow (compared to the rate of reaction that is possible without Fe(II) accumulation) movement of Fe(II) (product) away from the site of reaction. This is not the sole possible explanation of why mass transfer could be limiting in the present study. Mass transfer may also be influencing the rate at which Fe(II) leaves the hematite surface rather than, or in addition, to the rate at which electrons are passed from the cells to the hematite.

If the above conceptualization of initial kinetic control followed by mass transfer control (which may be due to thermodynamics) is accurate, one can more easily interpret the role of Fe(II) complexants and electron shuttles in solid-phase Fe(III) reduction. Electron shuttling should be most effective when the system is under kinetic control as it serves to increase bioreduction kinetics (*9*−*13*). Electron shuttles may also allow Fe(III) reduction to occur in microenvironments distant from the cell where thermodynamic conditions may be more favorable. Conversely their effectiveness would be expected to be limited in a system where the rate of solid phase Fe(III) reduction was controlled by mass transport of either the electron donor to the cell or the reaction products away from the cell-Fe(III) oxide interface. If Fe(II) transport away from the interface was limiting the rate of solid phase Fe(III) reduction, then Fe(II) complexants would be expected to increase Fe(III) reduction, by lowering the concentration of "free" (uncomplexed) Fe(II) in the bulk solution (i.e., not in the stagnant film/diffusion layer) thus increasing the diffusional flux by increasing the driving force for diffusion into the bulk solution. Fe(II) complexation within the diffusion layer would decrease the free Fe(II) concentration, potentially effecting dissolution. If we consider that these two mechanisms (electron shuttling and Fe(II) complexation) were operative in the present experimental systems, then we would expect electron shuttling to be most effective early in the experiments and Fe(II) complexation to be more effective after mass transfer has begun to control the rate of hematite reduction. Given this conceptual model of the experimental system one may now better evaluate the role of NOM in enhancing hematite reduction.

Complexation of metals by NOM is a well documented phenomenon (*27*, *37*−*42*). The similarity of 5-day acid extractable Fe(II)-vs-NOM concentration relationships to 5-day acid extractable Fe(II)-vs-ferrozine concentration relationships (*7*, *14*) indicate that Fe(II) complexation may be occurring (Figures 3−5). Further, a dialysis experiment using GNOM confirmed that it was capable of complexing Fe(II) (*7*). The increase in adsorbed Fe(II) in the presence of the soil humic acids is possible additional evidence of Fe(II) complexation, in this case the additional adsorbed Fe(II) occurring as an adsorbed (or nonfilterable) NOM-Fe(II) complex (Figure 5). The two humic acids (SHA and LHA) which produced the most adsorbed Fe(II) were also the most effective NOMs with respect to the enhancement of hematite reduction (Table 1, Figure 5). This suggests that perhaps adsorbed NOM may be very effective at enhancing hematite reduction by Fe(II) complexation.

Previous research on hematite bioreduction has demonstrated that electron shuttling by AQDS enhanced the rate of reduction during the first 24 h of incubation, while Fe(II) complexation by ferrozine did not affect the initial rate of reduction but did enhance the 5-day extent of reduction (indicating that the rate between day 1 and 5 was higher than that of the unamended biotic control) (*7*, *14*). Kinetic studies have further shown that AQDS is most effective in stimulating hematite reduction within the first several hours of incubation. NOM enhancement of hematite reduction was effective in 1-day samples in most cases (Figure 6). These results indicate that a mechanism in addition to, or other than, Fe(II) complexation is occurring in these experimental systems. The rapid enhancement of Fe(III) reduction by NOM in the absence of another exogenous electron shuttling compound indicates that NOMs are likely enhancing hematite reduction by electron shuttling. This is supported by previous research which has shown that DIRB can use NOM as electron acceptors and that reduced NOM can transfer electrons to Fe(III) (*11*).

The lack of electron shuttling in the Fe(II) "preloading" experiment (Figure 7), designed to test electron shuttling separately from complexation, contradicts our interpretation of the results of the 1-day NOM amended systems (Figure 6). The addition of Fe(II) to the experimental systems prior to inoculation may have inhibited Fe(III) reduction by decreasing the overall thermodynamic driving force for hematite reduction. Additionally, it may have inhibited hematite reduction via Fe(II) sorption; however, the molecular basis for this mechanism of inhibition is unknown (*22*, *26*, *28*). Regardless of the mechanism, the Fe(II) preloaded systems further confirmed that Fe(II) inhibits hematite reduction, the unamended biotic control produced only ca. 0.14 mM Fe(II) where ca. 0.36 mM was expected from nonpreloaded unamended biotic controls. AQDS was still effective at enhancing hematite reduction under these conditions, although its enhancement was not as great as observed in nonpreloaded systems (Figure 6). The Fe(II) preloading data indicate that Fe(II) interfered with electron shuttling by NOM. The NOM concentrations used in this experiment were low relative to the other experiments in this study (all less than 170 mg L^{-1}). However, due to the general effectiveness of the NOM amendments, detectable enhancements were expected in most cases. The interference of Fe(II) with electron shuttling may be due to decreased electron shuttling by Fe(II)-NOM complexes or precipitation of NOM in the presence of elevated Fe(II) levels. NOM precipitation by Ca^{2+} was tested under similar solution conditions (500 mg/L NOM, 1.0 mM $CaCl_2$, 50 mM PIPES-phophsate buffer, pH = 6.8) and found to occur only with the three soil humic acids and then only resulting in a 7−11% decrease in soluble NOM (as determined by A_{254}). The means by which Fe(II) interferes with electron shuttling by NOM remains unresolved.

Attempts to correlate the enhancement of hematite bioreduction (5-day data) with either acidity or organic content radical, by Pearson's correlation and single and multiple linear regressions, resulted in no significant correlation ($p > 0.05$ for Pearson's correlation and r^2 less than 0.26 for all linear regression). A better correlation ($r^2 = 0.81$) existed between aromaticity and relative enhancement of iron reduction. The lack of a good correlation with either acidity or organic radical content parameter indicates that either these two indices employed are not representative of their proposed functions, the response to these variables is nonlinear, another mechanism of enhancement is dominant, or an important characteristic of the NOM remains and needs to be included in the analyses. The better correlation with aromaticity is more difficult to interpret mechanistically but could indicate that an NOM's tendency to adsorb to hematite is important or aromaticity is a better overall indicator of functional groups important for enhancing hematite bioreduction in the present study. Significant contributions by multiple mechanisms may partially explain the poor correlations observed between Fe(II) production and acidity and organic radical content of the NOMs employed in this study.

The results of this study demonstrate that a variety of NOMs can be highly effective at enhancing hematite reduction by *Shewanella putrefaciens* CN32, albeit at very high NOM concentrations. The nature of this enhancement exhibits characteristics of electron shuttling and Fe(II) complexation. Such a conclusion is consistent with previous research, although these mechanisms have generally been considered separately in most studies and electron shuttling has been presumed to be most significant. The apparent inhibition of NOM-mediated electron shuttling by Fe(II) was observed although the mechanism of this inhibition is unclear. Additionally, further evidence is presented that supports the concept of Fe(II) inhibition of hematite reduction.

Environmental Implications. Dissimilatory iron reduction is an important process in controlling contaminant fate and has been proposed for the bioremediation of various types of wastes (*1*−*5*). Dissimilatory iron reduction may result in a number of critical reactions with contaminants including redox changes, displacement of bound contaminants, and formation of immobile phases. Understanding what limits Fe(III) reduction and how it may be stimulated are important for improving bioremediation. The addition of NOM may represent an innocuous means of stimulating in situ dissimilatory iron reduction. However, the mechanism(s) of this enhancement must be understood to make intelligent selections of appropriate materials. Understanding how NOM and other stimulants function is critical for formulating effective bioremediation strategies. This research showed that NOM appears to stimulate bioreduction by at least two mechanisms, i.e., electron shuttling and Fe(II) complexation. Ferric iron reduction and its interactions with NOM may also be critical in understanding iron and manganese biogeochemistry in wetland soils, which has regulatory, ecological, and engineering implications (*6*).

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