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Enhancement of Biological Reduction of Hematite by Electron Shuttling and Fe(II) Complexation

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Natural organic matter (NOM) enhancement of the biological reduction of hematite (α-Fe_2O_3) by the dissimilatory iron-reducing bacterium *Shewanella putrefaciens* strain CN32 was investigated under nongrowth conditions designed to minimize precipitation of biogenic Fe(II). Hydrogen served as the electron donor. Anthraquinone-2,6-disulfonate (AQDS), methyl viologen, and methylene blue [quinones with an E_w^0 (pH 7) of 0.011 V or less], ferrozine [a strong Fe(II) complexing agent], and characterized aquatic NOM (Georgetown NOM or Suwannee River fulvic acid) enhanced bioreduction in 5-day experiments whereas 1,4-benzoquinone (E_w^0 value = 0.280 V) did not. A linear relationship existed between total Fe(II) produced and concentrations of ferrozine or NOM but not quinones, except in the case of methylene blue. Such a linear relationship between Fe(II) and methylene blue concentrations could be due to the systems being far undersaturated with respect to methylene blue or the loss of the thermodynamic driving force. A constant concentration of AQDS and variable concentrations of ferrozine produced a linear relationship between total Fe(II) produced and the concentration of ferrozine. Enhancement effects of both AQDS and ferrozine were additive. NOM may serve as both an electron shuttle and an Fe(II) complexant; however, the concentration dependence of hematite reduction with NOM was more similar to ferrozine than quinones. NOM likely enhances hematite reduction initially by electron shuttling and then further by Fe(II) complexation, which prevents Fe(II) sorption to hematite and cell surfaces.

Introduction

Dissimilatory iron(III) reduction is a process of major importance in the biogeochemistry of non-sulfidogenic sediments. The abundance of ferric minerals in the subsurface and their affinity for contaminants through adsorption has made solid-phase iron reduction a subject of importance with respect to understanding the biogeochemical dynamics of groundwater contaminants. The fate of both organic and inorganic contaminants has been found to be influenced by microbial iron reduction (*1*−*4*). In addition, natural organic matter (NOM) has been demonstrated to participate in oxidation−reduction reactions with both organic and inorganic contaminants under anoxic conditions (*5*−*7*).

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Mechanisms for the enhancement of in situ dissimilatory iron reduction are important for improving the bioremediation potential of this process. The three basic strategies are (i) eliminate the need for cell−oxide contact, (ii) alleviate Fe(II) inhibition, and (iii) make Fe(III) more bioavailable or bioreducible. These three basic strategies are illustrated in Figure 1 as electron shuttling, Fe(II) complexation, and Fe(III) complexation, respectively. NOM has been proposed to enhance iron reduction by either electron shuttling (*8*) or ferrous iron complexation (*9*). Ferric iron complexation has been previously shown to enhance iron reduction possibly either through formation of a surface complex or by increasing Fe(III) bioavailability (*10*−*13*). Chelators such as NTA and EDTA may also complex Fe(II) and thereby enhance iron reduction by at least two parallel mechanisms (*9*) rendering experimental data interpretation more difficult than with a highly specific complexant. The first two mechanisms are the focus of this paper and will be discussed in more detail.

The processes that control microbial iron reduction in the natural environment are not clearly understood. The fate of biogenic Fe(II) has been considered crucial to regulation of the rate and extent of iron reduction (*9*, *10*, *14*−*17*). It has been proposed that Fe(II) adsorption to ferric oxide and microbial cell surfaces "passivates" these surfaces and inhibits further iron-reducing activity (*14*, *18*). Ferric oxide bioreduction experiments are designed to produce substantial quantities of Fe(II), yet the secondary reactions of Fe(II) with system components can be very complex and ultimately regulate bioreduction. Secondary reactions of Fe(II) may include aqueous Fe(II) complexation, adsorption to the oxide, and precipitation of ferrous minerals [e.g., $FeCO_3$ in HCO_3^--buffered systems, $Fe_3(PO_4)_2 \cdot 8H_2O$ in systems with phosphate, and Fe_3O_4] (*19*). In addition, ferric oxide reduction produces substantial alkalinity, influencing the solubility of Fe(II). Many previous studies have included media components, either as buffers or as nutrients that influenced the results. The present experiments were designed to minimize system complexity to better isolate the effects of the "functional analogues" (synthetic compounds with one of the proposed functions of NOM) and NOMs.

The presence of exogenous soluble redox active compounds that can be biologically reduced and interact by electron transfer to solid-phase ferric minerals has been proposed as an important means to enhance iron reduction (*8*, *20*−*22*). These "electron shuttle" compounds may serve to increase the rate and extent of reduction by alleviating the need for direct contact between the cell and the oxide surface (*20*, *23*). Arnold et al. (*11*) hypothesize shuttling of electrons across the periplasmic space by "... soluble cytochromes or other molecules of biological origin ..." to ferric oxides. In the work of Arnold et al. (*11*), it was found that direct cell−oxide contact was required for ferric iron reduction, even in the presence of nitrilotriacetic acid (NTA) (Figure 1). The term electron shuttle in this paper refers exclusively to exogenous compounds that do not require direct cell−oxide contact in order to mediate iron reduction (Figure 1). Low molecular weight electron shuttles could also gain access to surface sites excluded from bacteria such as micropores or clay interlayers (*24*). Quinone-containing compounds, such as humic and fulvic acids, have been proposed to be naturally occurring electron shuttling compounds that are capable of abiotically reducing ferric iron (*8*, *22*).

The enhancement of ferric iron bioreduction by NOM has been previously reported (*8*, *15*, *21*). This is potentially significant in controlling the rate and extent of iron reduction

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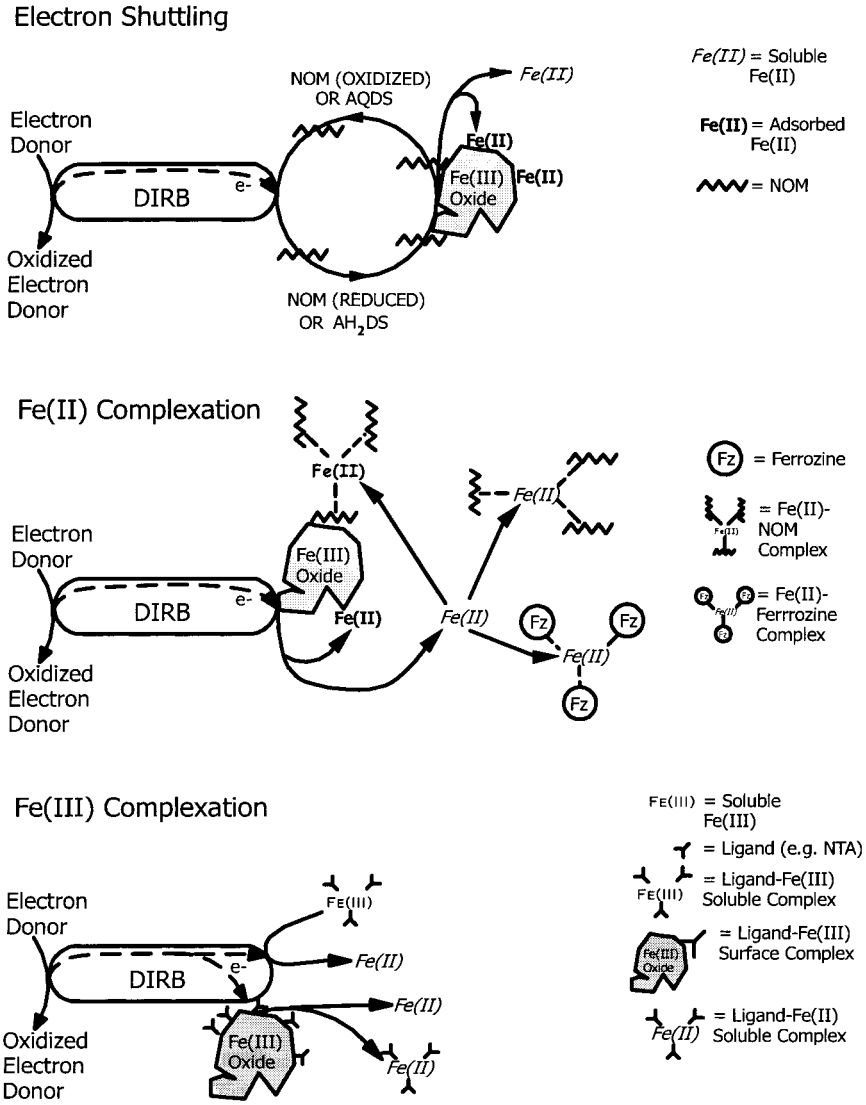

FIGURE 1. Proposed mechanisms for the enhancement of iron reduction by DIRB, electron shuttling, Fe(II) complexation, and Fe(III) complexation. DIRB, dissimilatory iron-reducing bacteria; NOM, natural organic matter; AQDS, anthroquinone-2,6-disulfonate; AH_2DS, hydroquinone (reduced) form of AQDS. The enhancement of iron reduction by electron shuttling by NOM has been attributed to the first two mechanisms. Electron shuttling alleviates the need for cell−oxide contact, effectively increasing the surface area available for reduction. It may also simply be faster than direct reduction. Fe(II) complexation alleviates an observed inhibitory effect of Fe(II) accumulation. Ferric iron complexation may function by either increasing the soluble (and more bioavailable) ferric iron concentration or promoting enhanced reduction of a ligand−Fe(III) complex. For a discussion of how the latter mechanism might function, the reader is referred to the work or Arnold et al. (*10*, *11*).

under relevant environmental conditions. The mechanism of this enhancement has been hypothesized to be either complexation of Fe(II) (*9*) or electron shuttling between the cells and the iron oxide surface (Figure 1) (*8*). The relative importance of these mechanisms may be dictated by a variety of environmental and experimental factors. The goal of the present study was to clarify the relative contribution of these two proposed functions in experiments employing two compounds serving as separate functional analogues of NOM, each of which mimics only one of the two proposed functions of NOM. The selected compounds ferrozine [an Fe(II) chelating compound; *25*] and anthraquinone-2,6-disulfonate (AQDS, a soluble electron shuttle) have been demonstrated to enhance dissimilatory iron reduction (*19*, *20*, *23*, *26*, *27*). However, previous studies with nonspecific chlelators and AQDS involved different organisms, culture conditions, ferric iron sources, incubation periods, and other factors that make direct comparison of the two mechanisms difficult. The present study allows a direct comparison of these enhancement mechanisms under identical chemical and microbial conditions. The effects of these compounds and purified, characterized NOMs were compared in order to evaluate which mechanism of enhancement is predominant with the NOMs tested.

Experimental Section

Microorganism and Culture Conditions. The dissimilatory iron-reducing bacterium (DIRB) *Shewanella putrefaciens* strain CN32 was provided courtesy of Dr. David Balkwill (Subsurface Microbial Culture Collection, Florida State University). This organism was originally isolated from a subsurface core sample (250 m beneath the surface) obtained from the Morrison Formation in northwestern New Mexico (*28*). All cells used were grown aerobically on tryptic soy broth without dextrose (Difco) at 20 °C. The cells for the inoculation of each experiment were harvested by centrifugation (3510*g*, 10 min, 15 °C) from a 16-h-old culture (late log-decreasing growth phase). The cells were washed three

TABLE 1. Reduction Potentials of Quinones and Ferric Oxides

half-reaction[a]	E^0 (V)	E' (V)[b]	source
$^1/_2Q + H^+ + e^- \leftrightarrow {}^1/_2HQ$	+0.693	+0.228	*34*
$^1/_2MB + H^+ + e^- \leftrightarrow {}^1/_2HMB$	+0.424	−0.041	*34*
$^1/_2AQDS + H^+ + e^- \leftrightarrow {}^1/_2AH_2DS$	+0.23	−0.235	*35*
$^1/_2MV + H^+ + e^- \leftrightarrow {}^1/_2HMV$	−0.017	−0.482	*34*
$^1/_2Fe_2O_3 + 3H^+ + e^- \leftrightarrow Fe^{2+} + {}^3/_2H_2O$	+0.66	−0.19	*36*
$H^+ + e^- \leftrightarrow {}^1/_2H_2(g)$	−0.0	−0.358	*36*

[a] Q and HQ, 1,4-benzoquinone and reduced form; MB and HMB, methylene blue and reduced form; MV and HMV, methyl viologen and reduced form; AQDS and AH_2DS, anthraquinone-2,6-disulfonate and reduced form; Fe_2O_3, hematite. [b] E' is determined for the following conditions: log $[H^+] = -6.8$, [HQ] = 50 μM, [Q] = 0.50 μM, $[Fe^{2+}]$ = 1 μM, partial pressure of $H_2(g)$ = 0.025 atm, 25 °C, activity coefficients = 1.

times in 50 mM PIPES plus 30 μM phosphate buffer (pH 6.8) with the final wash made with deoxygenated solution. Cell pellets were resuspended in 7−20 mL of deoxygenated PIPES−phosphate buffer in an anaerobic chamber (Coy) under a N_2:H_2 (ca. 97.5:2.5) atmosphere, and the cell density was determined by absorbance at 420 nm.

Iron Oxide. 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 had an average particle diameter of ca. 300 nm and a specific surface area of 9.02 $m^2\ g^{-1}$ (measured by five-point N_2-BET). Electrophoretic mobility and proton titrations were used to estimate the zero point of charge to be at pH 8.5. Hematite was heated to 550 °C in air overnight before use to remove any organic carbon. Hematite was added to the buffer at least 48 h prior to any experiment to allow for hydration.

Bioreduction Experiment Preparation. Test systems for bioreduction experiments consisted of crimp-sealed (Teflon-faced butyl rubber stoppers) amber serum bottles (10 mL nominal volume, ca. 15 mL actual volume) containing 5 or 10 mL of medium. Sealed serum bottles were incubated at 20 °C on orbital shakers outside of the anaerobic chamber. All preparations were performed in an anaerobic chamber. The basic test medium contained 50 mM PIPES plus 30 μM phosphate buffer and 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}. Phosphate was included to ensure adequate phosphorus for microbial energetic needs, although supplementation proved unnecessary. Parallel to all treatments, unamended biotic controls were run that contained only the inoculated basic test medium. All treatments and controls were run in at least triplicate. Uninoculated abiotic controls for each amendment type were incubated in quintuplicate for 5 days. In all experiments, serum bottles were sacrificed for iron analyses after 5 days of incubation and, in some experiments, after 1 day of incubation. Bacterial viability remained greater than 99% after 5 days of incubation as measured by direct counts using the LIVE/DEAD *Bac*light viability stain (Molecular Probes, Eugene, OR).

Functional Analogue Amendments. Ferrozine (J. T. Baker), a specific Fe(II) chelator that forms a 3:1 ferrozine:Fe(II) complex with an absorption maximum at ca. 562 nm (*25*), was added to experimental systems as a dry powder. Ferrozine was found to be stable in the bioreduction experiments neither serving as a substrate for microbial growth or being degraded after 5 days of incubation. Four quinones (Aldrich, Milwaukee, WI) of varying reducing potentials were each tested (Table 1). All quinones were added volumetrically from filtered (0.1 μm) concentrated stock solutions. All stock solutions were rendered anaerobic before addition to the experimental solutions.

Natural Organic Materials. Suwannee River fulvic acid (SRFA) is a well-characterized aquatic NOM available from the International Humic Substance Society (IHSS). SRFA was collected from the Suwannee River near Fargo, GA. SRFA is 53.0 and 43.9 wt % C and O, respectively. GNOM was provided courtesy of Dr. Baohua Gu (Oak Ridge National Laboratory) and is a well-characterized, salt-free, freeze-dried natural organic material that was originally collected from a wetland pond in Georgetown, SC. GNOM is 48.3 and 42.1 wt % C and O, respectively. FTIR and NMR spectra of GNOM display characteristics typical of aquatic and soil fulvic acids with relatively low aromatic C content but relatively high aliphatic, alcoholic, and carbohydrate C content as compared to soil humic acids (*29*).

The metal binding capacities of these materials were estimated from their acidities at or very near the experimental pH values. One mole of acidity was considered to be capable of binding 0.5 mol of Fe(II) (*30*). SRFA has an acidity of 10.46 mequiv (g of C)$^{-1}$ at pH 6.8 (M. Perdue, personal communication), resulting in an estimated Fe(II) complexation capacity of 5.23 mmol of Fe(II) (g of C)$^{-1}$. The acidity of GNOM at pH 6.6 in 10 mM NaCl was 7.0 mequiv (g of C)$^{-1}$ (B. Gu, personal communication). The metal binding capacity of GNOM was equivalent to 3.49 mmol of Fe(II) (g of C)$^{-1}$ at pH 6.6.

Organic radical content as measured by electron spin resonance spectroscopy (ESR) was used as a measure of electron shuttling capacity of the two NOMs tested. ESR data (spins g^{-1}) were found to correlate well with the quantity of Fe(III) reduced by a given mass of reduced NOM presumably through the reaction with quinone moieties (*22*). SRFA has a relatively low organic radical content (5.4×10^{16} spins g^{-1}) as compared to soil humic acids whereas GNOM has a much higher value (6.67×10^{17} spins g^{-1}), which is similar to that reported for soil humic acids. The ESR data for SRFA were supplied by the International Humic Substance Society, and the datum for GNOM was provided by Dr. J. Chen from Oak Ridge National Laboratory (personal communication).

Analytical Techniques. Ferrous iron was reported as soluble and acid extractable. 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, and the absorbance (562 nm) was determined with a Shimadzu UV/Vis-1601 spectrophotometer. The result was corrected for dilution, converted to Fe(II) by comparison with standards, and reported as 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 μm), and the filtrate was analyzed for Fe(II) as above. Adsorbed Fe(II) was calculated as the difference between soluble and acid-extractable Fe(II).

Results

Phosphorus. Phosphate was present in the medium at a low concentrations (30 μM). In the presence of phosphate, Fe(II) may precipitate as the mineral vivianite, $Fe_3(PO_4)_2{\cdot}8H_2O$. The maximum amount of vivianite that could precipitate in the present experimental systems would account for the loss of 20 μM Fe(II) from solution. Additionally, the adsorption of phosphate to hematite may influence adsorption of Fe(II) to hematite (*31*). To evaluate the effect of phosphate in these test systems quintuplicate cultures with and without 30 μM phosphate were tested in 1- and 5-day experiments. There was no detectable statistical difference between the two

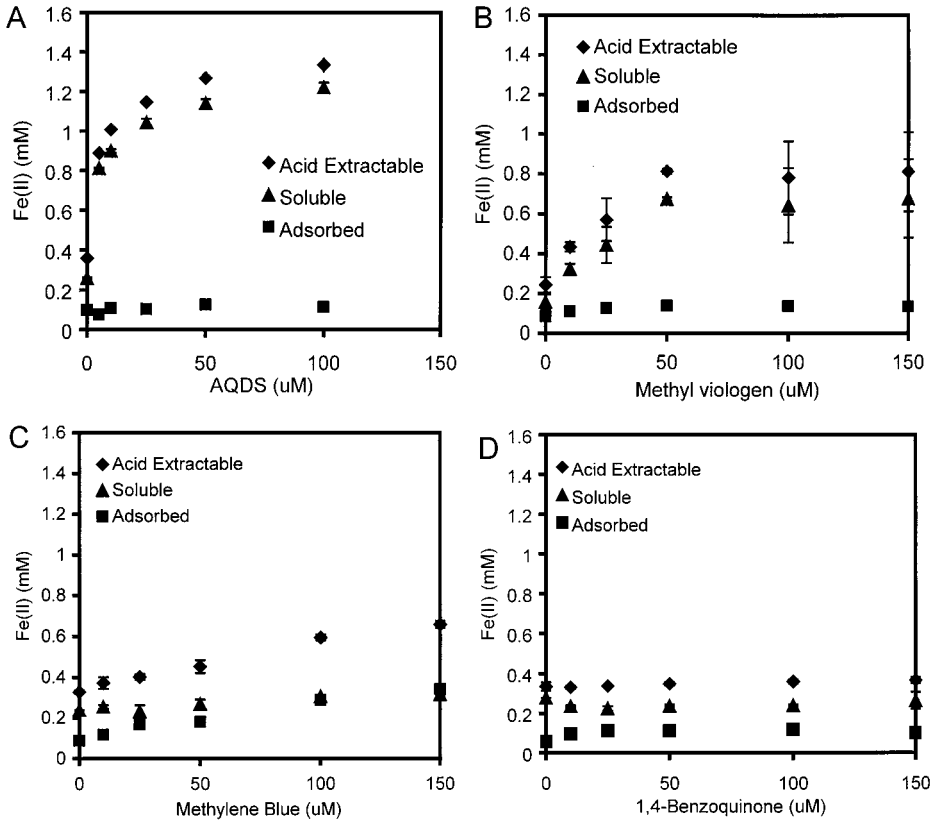

FIGURE 2. Acid-extractable, soluble, and adsorbed Fe(II) as a function of quinone concentration of (A) AQDS, (B) methyl viologen, (C) methylene blue, and (D) 1,4-benzoquinone after 5 days of incubation. *Shewanella putrefaciens* CN32 was used (10^8 mL^{-1}) under nongrowth conditions. Values are means of three replicates (± standard deviation). Final pH of all samples was between 6.6 and 7.2.

treatments, indicating that the phosphate played no significant role in these experiments.

Quinones. The three quinones having the lowest reducing potentials (AQDS, methyl viologen, and methylene blue) were able to enhance hematite reduction. AQDS increased the 5-day extent of hematite bioreduction by more than 3-fold as compared to unamended biotic controls (Figure 2A). Methyl viologen and methylene blue also significantly enhanced hematite reduction, albeit to a lesser extent than AQDS (Figure 2B,C). 1,4-Benzoquinone, which had the highest redox potential of the quinones tested, did not significantly increase hematite reduction over unamended biotic controls and did not affect the adsorption of biogenic Fe(II) at any concentration tested (Figure 2D). Ferrous production as a function of quinone concentration in the AQDS and methyl viologen systems did not increase as a linear function of concentration. Incremental enhancement [millimoles of Fe(II) per micromole of AQDS] of Fe(II) production diminished with increasing AQDS concentrations, although some increase was noted even between the highest concentrations (50 and 100 μM) tested (Figure 2A). Methyl viologen exhibited behavior similar to AQDS; however, the enhancement of hematite reduction was identical at the concentrations from 50 to 150 μM (Figure 2B). In contrast, methylene blue enhanced hematite reduction in direct proportion to its concentration (Figure 2C). Quinone-amended systems produced no increase in adsorbed Fe(II), with all Fe(II) greater than that present in unamended biotic controls being recovered in soluble form (Figure 2A–C). The increase in primarily soluble Fe(II) is expected based upon the sorption characteristics of the combined cell–hematite system. Adsorbed Fe(II) in unamended biotic control experiments consistently became asymptotic [versus soluble Fe(II)] at an equivalent adsorbed concentration of ca. 0.1 mM. None of the quinones reduced hematite in uninoculated abiotic controls (data not shown).

Ferrozine. Ferrozine did not enhance hematite bioreduction after 1 day of incubation, although it prevented adsorption of biogenic Fe(II) to cell and hematite surfaces (Figure 3A). All concentrations of ferrozine tested significantly increased the extent of hematite bioreduction after 5 days when compared to unamended biotic controls (Figure 3B). In contrast to results with the two most effective quinones, Fe(II) production in 5-day ferrozine-amended cultures was linearly dependent on the concentration of ferrozine (Figure 3B). Ferrozine was somewhat less effective in 5-day cultures than predicted based upon its published Fe(II) complexation capacity (*25*) with the difference between actual and predicted Fe(II) increasing at higher ferrozine concentrations (Figure 3B). In all cases, agreement between the predicted and the actual Fe(II) concentrations for 5-day samples was greater than 80%. Unlike 1-day samples, 5-day unamended biotic control and ferrozine-amended cultures had similar levels of detectable adsorbed Fe(II) (Figure 3B). Ferrozine did not reduce hematite in the uninoculated abiotic controls (data not shown).

Combined Ferrozine and AQDS. Combinations of AQDS and ferrozine were used to evaluate the relative importance of electron shuttling and Fe(II) complexation as parallel mechanisms of enhancing iron reduction. The extent of Fe(II) production in 5-day cultures with a fixed concentration of AQDS (50 μM) and variable ferrozine concentrations was linearly dependent on the concentration of ferrozine (Figure 4), similar to findings for the ferrozine-only amended cultures (Figure 3). In contrast to the ferrozine-only cultures (Figure 3A), combined AQDS–ferrozine systems were effective at enhancing hematite reduction after only 1 day (Figure 4A). AQDS alone effectively enhanced hematite reduction after

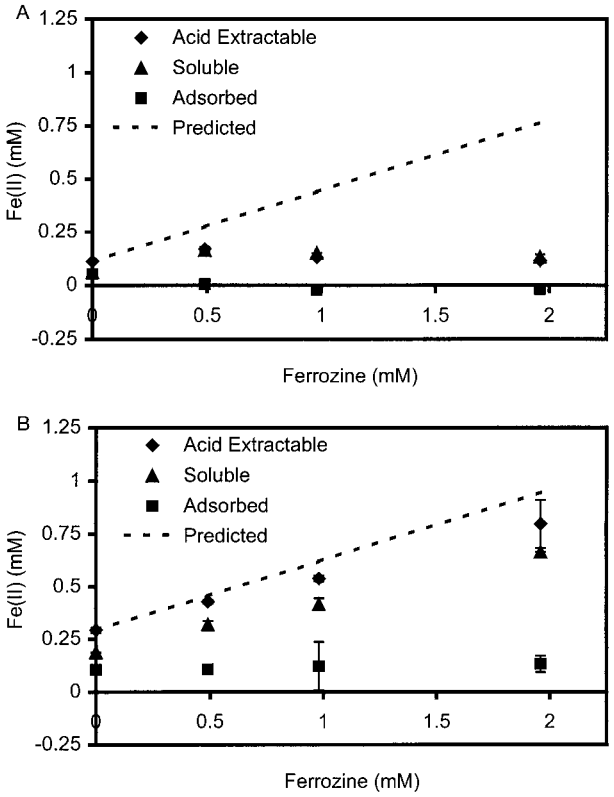

FIGURE 3. Acid-extractable, soluble, and adsorbed Fe(II) as a function of ferrozine concentration after (A) 1 and (B) 5 days of incubation. *Shewanella putrefaciens* CN32 was used (10^8 mL^{-1}) under nongrowth conditions. Values are means of three replicates (± standard deviation). Predicted lines are based upon ferrozine-free biotic control plus a complexation capacity of 0.3333 mol of Fe(II)/mol of ferrozine. Predictions are of acid-extractable Fe(II). Final pH of all samples was between 6.6 and 7.2.

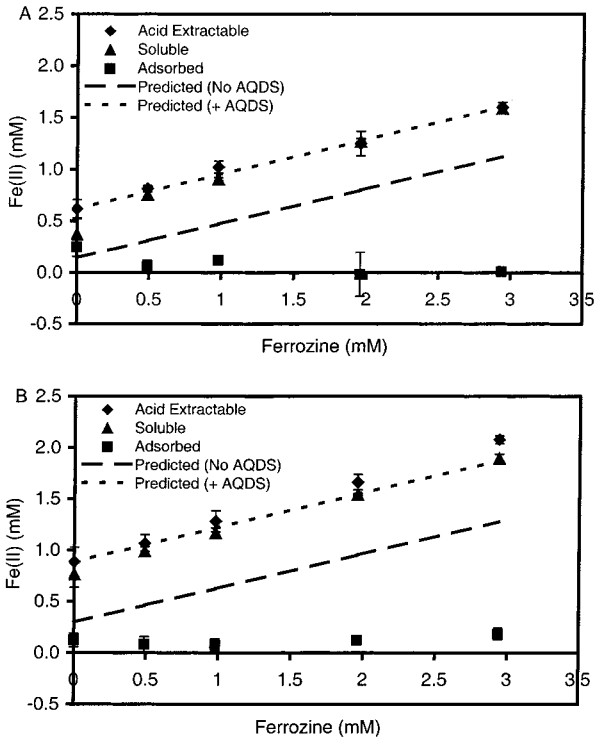

FIGURE 4. Acid extractable, soluble, and adsorbed Fe(II) as a function of ferrozine concentration in systems with 50 μM AQDS: (A) 1 day incubation and (B) 5 day incubation. *Shewanella putrefaciens* CN32 was used (10^8 mL^{-1}) under nongrowth conditions. Values are means of three replicates (± standard deviation). Predicted lines are based upon appropriate (ferrozine free) controls plus a complexation capacity of 0.3333 mol Fe(II) per mol of ferrozine. Predicted (no AQDS) is extrapolated from the AQDS free control. Predicted (+ AQDS) is extrapolated from the 50 μM AQDS, ferrozine free control. Predictions are of acid extractable Fe(II). Fe(II) values (mM) for the unamended (ferrozine and AQDS free) controls (not shown) were as follows: 1 day, acid extractable = 0.14, soluble = 0.063, adsorbed = 0.080; 5 day, acid extractable = 0.30, soluble = 0.02, adsorbed = 0.098. Final pH of all samples was between 6.6 and 7.2.

1 day as is evidenced by the difference between the AQDS-only and the unamended biotic controls (Figure 4A). As in the variable concentration AQDS experiment (Figure 2A), AQDS increased hematite reduction over unamended biotic controls (Figure 4). Predicted enhancements of hematite reduction calculated from the Fe(II) complexation capacity of ferrozine and extrapolated from the AQDS-only and unamended biotic controls clearly agreed with the prediction based upon the AQDS-only control plus predicted Fe(II) complexation capacity in both 1- and 5-day samples (Figure 4). This agreement indicates that the effects of ferrozine and AQDS were additive, i.e., no single mechanism dominated enhancement. Ferrous iron was recovered almost exclusively in soluble form in 1-day samples whereas some adsorbed Fe(II) was present in 5-day samples (Figure 4). Hematite reduction continued after the first day of the experiment as evidenced by the higher Fe(II) concentrations in 5-day samples relative to 1-day samples. The increase in hematite reduction between days 1 and 5 represented only a small portion of the total hematite reduction, indicating that most of the enhancement due to AQDS occurred during the first day of incubation. Further hematite reduction was possible even though the Fe(II) complexation capacity of the ferrozine appeared to be exhausted after the first day of the experiment.

The effect of variable AQDS concentration in the presence of constant ferrozine (1.34 mM) was also evaluated. AQDS was highly effective at enhancing hematite reduction after 1 and 5 days of incubation (Figure 5). One-day samples showed hematite reduction well in excess of that predicted based solely upon the Fe(II) complexation capacity of ferrozine, indicating once again that electron shuttling was effective within the first day of incubation. As was observed in the ferrozine-only cultures (Figure 3A), 1-day ferrozine-only (AQDS-free) controls showed minimal enhancement of hematite reduction relative to unamended (ferrozine and AQDS-free) biotic controls (Figure 5A). Five-day samples exhibited a significant enhancement in hematite reduction by ferrozine alone (Figure 5B). As in the previous experiment, 1-day samples containing ferrozine revealed little or no detectable adsorbed Fe(II) while 5-day samples demonstrated adsorbed ferrous levels similar to the ferrozine- and AQDS-free biotic controls (Figures 4 and 5). Both 1- and 5-day samples at all AQDS concentrations (with ferrozine present) produced very similar levels of enhancement, in contrast to the AQDS-only experiment (Figure 2A).

Natural Organic Materials. Two NOMs were tested in order to compare concentration-dependent effects on hematite reduction with that of the functional analogues. Both GNOM (Figure 6A) and SRFA (Figure 6B) enhanced hematite reduction in 5-day samples with ferrous production being generally linear with NOM concentration. Linear regressions of the total Fe(II) versus NOM concentrations for GNOM and SRFA had slopes of 0.637 and 0.507 mmol of Fe(II) (g of NOM)$^{-1}$ and r^2 values of 0.963 and 0.948, respectively. GNOM appeared to be more effective than SRFA at identical concentrations, but the slopes were not significantly different based upon their 95% confidence intervals. Adsorbed Fe(II) was not significantly affected by the presence of either NOM

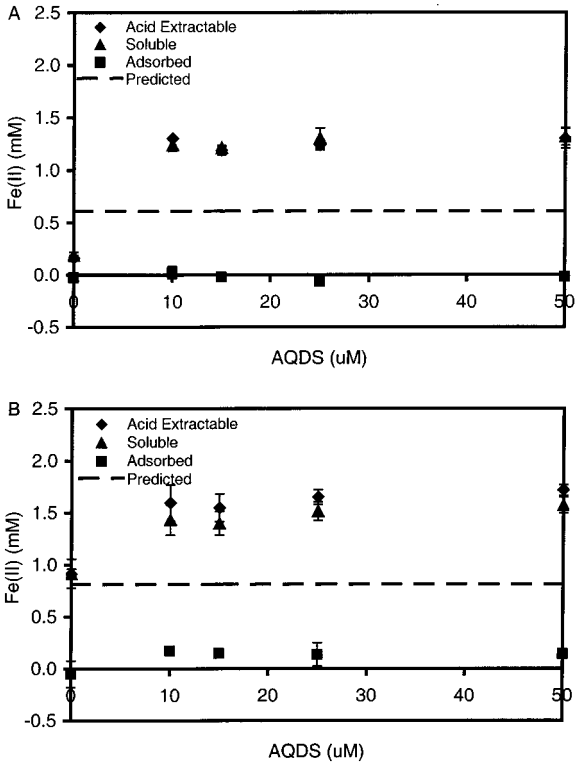

FIGURE 5. Acid extractable, soluble, and adsorbed Fe(II) as a function of AQDS concentration in systems with 1.47 mM ferrozine: (A) 1 day incubation and (B) 5 day incubation. *Shewanella putrefaciens* CN32 was used (10^8 mL^{-1}) under nongrowth conditions. Values are means of three replicates (± standard deviation). Predicted lines are based upon unamended (no ferrozine or AQDS) control plus a complexation capacity of 0.3333 mol Fe(II) per mol of ferrozine. Predictions are of acid extractable Fe(II). Fe(II) values (mM) for the unamended (ferrozine and AQDS free) controls (not shown) were as follows: 1 day, acid extractable = 0.12, soluble = 0.085, adsorbed = 0.034; 5 day, acid extractable = 0.32, soluble = 0.21, adsorbed = 0.11. Final pH of all samples was between 6.6 and 7.2.

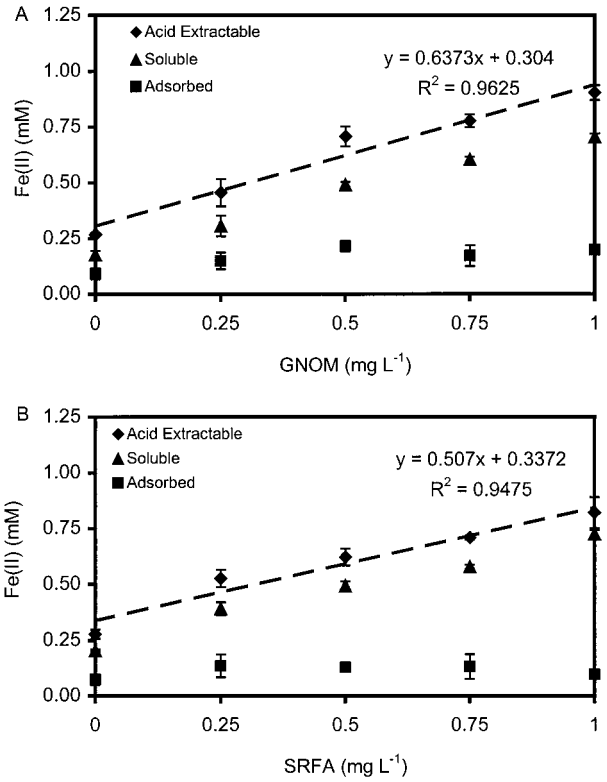

FIGURE 6. Acid-extractable, soluble, and adsorbed Fe(II) as a function of NOM concentration with (A) Georgetown NOM and (B) Suwannee River fulvic acid after 5 days of incubation. *Shewanella putrefaciens* CN32 was used (10^8 mL^{-1}) under nongrowth conditions. Values are means of three replicates (± standard deviation). Final pH of all samples was between 6.6 and 7.2.

with virtually all additional Fe(II) (versus the unamended biotic control) appearing in the soluble fraction. Neither NOM generated Fe(II) in uninoculated abiotic controls (data not shown). A dialysis experiment confirmed that GNOM was capable of complexing Fe(II) as evidenced by a significantly ($p < 0.00001$, two-tailed t-test) increased Fe(II) concentration in a compartment containing GNOM versus the bulk medium when separated by a 500 molecular weight cutoff membrane (data not shown).

Discussion

In this study, enhancement of hematite reduction was demonstrated to occur in the presence of both an Fe(II) complexing agent (ferrozine) and three electron shuttling compounds (AQDS, methyl viologen, and methylene blue), indicating that both complexation of Fe(II) (*9*) and electron shuttling between the cells and the iron oxide surface (*8*) are possible (Figure 1).

Fe(II) Complexation. A variety of complexing agents (e.g., citrate, EDTA, NTA) have been shown to enhance the bioreduction of solid-phase ferric iron (*9*−*12*, *14*). When nonspecific complexing agents capable of both significant Fe(III) and Fe(II) complexation are employed, enhancement of iron reduction may be due to at least two mechanisms, namely, dissolution of solid-phase ferric iron with subsequent reduction of soluble complexed ferric iron (*1*, *9*, *10*, *12*), and complexation of Fe(II) preventing its accumulation on cell and/or oxide surfaces (Figure 1) (*9*).

The enhancement of hematite reduction by ferrozine appeared to be directly related to its Fe(II) complexation capacity. The theoretical Fe(II) complexation capacity of ferrozine is close to the slope of the total Fe(II) versus ferrozine concentration in Figure 3B [slope 0.255 mmol of Fe(II) (mmol of ferrozine)$^{-1}$, $r^2 = 0.999$). Abiotic controls produced no detectable Fe(II).

The increase in Fe(II) production by ferrozine in 5-day samples as compared to 1-day samples indicates that complexation of Fe(II) is highly effective in promoting increased hematite reduction under the conditions employed (Figure 3). These results indicate that the adsorption of Fe(II) onto either cell or oxide surfaces or the accumulation of free Fe(II) in solution reduces the rate and extent of bioreduction within the time scale of these experiments. The lack of enhancement in 1-day samples with ferrozine as the only amendment indicates that ferrozine only effects the system after sufficient Fe(II) has been generated. When significant Fe(II) was produced in systems containing AQDS and ferrozine, ferrozine enhanced hematite reduction within 1 day (Figures 3A and 4A). The enhancement of hematite reduction by ferrozine in 1-day AQDS- and ferrozine-amended systems indicates that the effectiveness of ferrozine occurs in the presence of elevated ferrous concentrations regardless of the length of the culture incubation period.

Ferrous iron inhibition of solid-phase iron bioreduction has been previously modeled (*15*). Solid-phase ferric iron (geothite) bioreduction was kinetically represented as a first-order reaction with respect to free surface sites. The accumulation of adsorbed Fe(II) is considered to block ferric iron surface sites, resulting in a decrease in the rate of bioreduction. Researchers have shown that prevention of the sorption of Fe(II) to the ferric iron surface by aqueous or solid-phase complexants (*9*, *24*) or advective transport of

Fe(II) out of the system (*16*) can significantly enhance the rate and extent of solid-phase iron reduction. The results of the present study are in agreement with the previously proposed mechanism for inhibition of solid-phase iron reduction by Fe(II) sorption (*10*, *18*).

GNOM and SRFA appear to complex Fe(II) according to the similarity of the results obtained with ferrozine and these NOMs. The linear relationship between NOM concentration and Fe(II) production may be reflective of an Fe(II) complexation similar to that occurring in the ferrozine experiments. Since the total amount of metal complexation capacity by NOM can be estimated from its total acidity (*30*), acidity at or near the experimental pH was used to estimate the Fe(II) complexation capacity of each NOM. The slopes of the total Fe(II) vs NOM concentration curves given previously are less than those predicted based upon acidity [actual vs predicted: 0.637 vs 1.69 mmol of Fe(II) (g of GNOM)$^{-1}$ and 0.507 vs 2.77 mmol of Fe(II) (g of SRFA)$^{-1}$]. In fact, GNOM, which has less acidity than SRFA, was slightly (although not statistically significantly) more effective in enhancing hematite reduction. In addition, GNOM has a greater organic radical content than SRFA, suggesting that electron shuttling may be important. Either acidity-based estimates are inappropriate for estimating Fe(II) complexation capacities in some cases or Fe(II) complexation cannot be the sole mechanism responsible for the observed enhancement of hematite reduction.

Electron Shuttling. AQDS can mediate the bacterial reduction of noncrystalline hydrous ferric oxides (*8*, *19*) and crystalline ferric oxides such as hematite and goethite (*20*, *21*). Microbially reduced AQDS can abiotically reduce Fe(III) with the regeneration of AQDS. Electron shuttling by small amounts (e.g., micromolar quantities) of redox active materials has been proposed to have a significant effect on solid-phase iron bioreduction because the NOM, like AQDS, may be regenerated through a catalytic cycle (*21*, *23*). Quinone moieties have been proposed to be important electron-accepting groups for the microbial reduction of humic substances (*8*, *21*−*23*). Electron spin resonance (ESR) spectroscopy has been used to measure the organic radical content of humic and fulvic substances and quinones (*22*). Compared to soil humic acids with high organic radical content (*22*), SRFA has a relatively low organic radical content (5.4×10^{16} spins g^{-1} measured by ESR). In contrast, GNOM has a much higher organic radical content (6.67×10^{17} spins g^{-1}) comparable with soil humic acids. Thus, the two NOMs employed should vary greatly in their ability to shuttle electrons. GNOM, which has more than an order of magnitude more organic radicals per unit mass than SRFA, was only 26% (statistically insignificant based upon the 95% confidence intervals of the slopes of lines shown in Figure 6) more effective in enhancing hematite reduction. It is therefore clear that a simple proportional relationship did not exist between organic radical content and hematite reduction enhancement for the NOMs employed in the present study.

The thermodynamic feasibility of the reduction of hematite by reduced quinones is strongly controlled by the chemistry of the system. Two critical variables that may change as a function of time are the hydroquione/quinone concentration ratio (QH_2/Q) and the activity of free, aqueous Fe(II). The half-cell potentials (E') shown for a set of conditions which might occur in the present experiments are given in Table 1. Under the conditions specified, methyl viologen and AQDS are the only quinones of the four tested capable of reducing hematite (Table 1), yet experimentally methylene blue enhanced hematite reduction (Figure 2C). The reduction of hematite by the hydroquinone species of methylene blue is favorable ($E = 0.0214$ V) if Fe(II) $= 1 \times 10^{-9}$ M and the QH_2/Q ratio is 100:1. If the Fe(II) accumulates to 1×10^{-8} M, then the ratio QH_2/Q must be increased to 10,000:1 to maintain the same thermodynamic driving force. This calculation illustrates that the systems is 10 times more sensitive to the Fe(II) concentration than to the QH_2/Q ratio, which is evident from the reaction stoichiometry. It is important to note that the ferrous iron activity represents only free Fe(II), i.e., not that which is complexed or sorbed (Figure 1). Any Fe(II) complexants or sorbents could therefore help to maintain the thermodynamic driving force for quinone-mediated hematite reduction, thus presenting a role for complexation in enhancing electron shuttling.

The nonlinear concentration dependence of Fe(II) on both AQDS and methyl viologen activities are in contrast to the highly linear relationships exhibited by ferrozine and the NOMs. In a previous study, the authors observed that at higher concentrations (greater than 200 μM) AQDS was actually less effective than at lower concentrations (*32*). The "weakest" of the electron shuttles, methylene blue, however had a linear concentration dependence indicating that the linearity of the Fe(II)−amendment concentration relationship is not a clear indicator of the mechanism of enhancement. One possible explanation for this linear relationship could be the system was far undersaturated (from a kinetic perspective) with respect to methylene blue. Such an explanation is consistent with the catalytic nature of quinones and the results observed for AQDS and methyl viologen. Methylene blue may also have only been capable of enhancing iron reduction for a brief time due to the thermodynamic issues discussed above. This may have resulted in linear concentration relationship rather than a curve that reflects a strong kinetic influence. Quinone reduction kinetic data would be required for a further explanation of these relationships. The disparate behavior between the two most potent electron shuttling compounds (AQDS and methyl viologen) and NOM casts doubt upon electron shuttling as the predominant mechanism of iron reduction enhancement by NOM in this study.

Comparison of Mechanisms. Electron shuttling and Fe(II) complexation appear to be complimentary mechanisms of iron reduction enhancement based upon experiments employing both analogues. When ferrozine and AQDS were employed simultaneously, ferrozine controlled the 5-day extent of bioreduction while AQDS strongly influenced the initial (first 24 h) rate. The 5-day extent of bioreduction was not solely that predicted based upon the Fe(II) complexation capacity of ferrozine but rather that of the AQDS-containing systems plus that predicted based upon the ferrozine concentration. It therefore appears that ferrozine functions equally well in the presence and the absence of AQDS.

When variable AQDS concentrations were tested at a fixed ferrozine concentration, all AQDS concentrations produced essentially identical levels of enhancement (Figure 5). When AQDS alone was added, this was not the case (Figure 2A). The reason for this is not known but may be related to the lack of adsorbed Fe(II) in the 1-day samples. Reduced AQDS (AH_2DS) accumulates rapidly in experiments where it is added to the standard experimental conditions at a final concentration of 50 μM (data not shown). This indicates that the transfer of electrons from the AH_2DS to hematite is probably rate limiting, not the bioreduction of the quinone. Adsorbed Fe(II) may interfere with the reaction between AH_2DS and ferric iron in hematite. In such a situation increased AH_2DS concentrations may more effectively compete with Fe(II) for hematite surface sites than lower concentrations of AH_2DS. This constraint or competition may be relaxed by the action of ferrozine to keep Fe(II) from adsorbing until after the ferrozine has been saturated with ferrous iron. This situation therefore represents a condition where low concentrations of electron shuttling compounds might be made more effective by the presence of Fe(II) complexing agents.

The above observations are consistent with surface passivation (*15*) or free Fe(II) concentration decreasing the rate of hematite bioreduction. Since both analogues were equally functional in isolation and combination, it is feasible that NOM may function both as iron complexing and electron shuttling agents. If this is the case, the 5-day extent of bioreduction may be initially accelerated by electron shuttling and then further enhanced by Fe(II) complexation, producing a linear concentration dependence exactly as was observed in the experiment with a fixed AQDS concentration and variable ferrozine concentrations (Figure 4). In the case of NOM, both electron shuttling and Fe(II) complexation capacities would increase simultaneously with increasing NOM concentration. The relatively high concentrations of NOM used could represent conditions where excess NOM quinone content had little or no effect on iron reduction as was noted for high concentrations of AQDS and methyl viologen. It is, therefore, possible that both functions contributed to the overall enhancement of hematite reduction, and further work is underway to examine these mechanisms with a variety of NOM. It is important to remember that *S. putrefaciens* CN32 is serving as a model organism in this study and may differ fundamentally from other iron-reducing bacteria such as *Geobacter*, some *Desulfovibrio* species, and *Deinococcus*. Factors such as the requirement for a chelated Fe(III) form or the presence of an electron shuttling compound (*33*) or the ability to synthesize siderophores or electron shuttling compounds may significantly impact the importance of NOM in enhancing iron reduction by different organisms.

Environmental Implications. Dissimilatory iron(III) reduction is a potentially important process in controlling contaminant fate. It has the potential for being particularly useful in the remediation of metals and radionuclides and reducible organics such as chlorinated aliphatic and nitroaromatic compounds. Means for stimulating iron(III) reduction will be useful in enhancing bioremediation processes. The mechanisms evaluated in this paper represent two of the proposed mechanisms by which NOM may enhance iron(III) reduction. Understanding how NOM and other stimulants function is critical for formulating effective bioremediation strategies.

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