Effects of Humic Substances and Quinones at Low Concentrations on Ferrihydrite Reduction by *Geobacter metallireducens*

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Humic substances (HS) and quinones can accelerate dissimilatory Fe(III) reduction by electron shuttling between microorganisms and poorly soluble iron(III) (hydr)oxides. The mechanism of electron shuttling for HS is not fully understood, but it is suggested that the most important redox-active components in HS are also quinones. Here we studied the influence of HS and different quinones at low concentrations on ferrihydrite reduction by *Geobacter metallireducens*. The aquatic HS used were humic and fulvic acids (HA and FA) isolated from groundwater of a deep aquifer in Gorleben (Niedersachsen, Germany). HA stimulated iron reduction stronger than FA down to total HA concentrations as low as 1 mg/L. The quinones studied showed large differences: some had strong accelerating effects, whereas others showed only small effects, no effects, or even inhibitory effects on the kinetics of iron reduction. We found that the redox potentials of the most active quinones fall in a narrow range of −137 to −225 mV vs NHE at pH 7. These results give evidence that the kinetic of microbial iron reduction mediated by electron shuttles is mainly controlled by thermodynamic parameters, i.e., by the redox potential of the shuttle compound, rather than by the proportion of dissolved vs adsorbed compound.

Introduction

Microbial dissimilatory Fe(III) reduction of poorly soluble iron (hydr)oxides (often abundant in aquatic sediments or aquifers) is an important process to degrade natural and anthropogenic organic substances in anoxic groundwater (*1*). In this process, electrons from organic electron donors can be transferred to poorly soluble ferric (hydr)oxides (e.g., ferrihydrite, goethite, hematite) by different mechanisms: (a) electron transfer by direct contact between the microorganisms and the iron (hydr)oxide maybe even facilitated by so-called "bacterial nanowires" produced by some microorganisms (*2*−*4*), (b) indirect electron transfer to the iron (hydr)oxide by a redox-active organic electron shuttle or a Fe(III) chelator produced and excreted by the microorganisms (*5*−*11*), (c) indirect electron transfer by redox-active humic substances (HS) or other organic compounds acting also as electron shuttles (*12*−*16*), and (d) Fe(III)-HS complexes increasing the availability of Fe(III) for the microorganisms (*9*).

It is only poorly understood what type of molecules or fractions of HS are responsible for their electron shuttling properties. In the pioneering work of Lovley et al. in 1996 (*12*) similar stimulation was found during microbial Fe(III) reduction of poorly crystalline Fe(III) oxide by using soil humic acid (HA, 2 g/L) from the International Humic Substances Society (IHSS), Aldrich HA (2 g/L), or AQDS (9,10-anthraquinone-2,6-disulfonate, 100 μM) as electron shuttle. Furthermore, different types or fractions of HS (fulvic acid (FA) and HA) showed different reducing properties with Fe(III) citrate as electron acceptor. Bound iron in HS, once reduced, could not transfer electrons to Fe(III) (hydr)oxide and it was suggested that other electron-accepting moieties, such as quinones, may be the important electron-accepting and shuttling molecular groups in HS (*17*). Electron spin resonance (ESR) measurements demonstrated that HS with higher electron-accepting capacity also contain higher free radical contents and that the ESR spectra were consistent with semiquinones as the main organic radicals (*18*). These results provide direct evidence that organic radicals in HS, which are primarily generated from quinone groups, were microbially reduced to hydroquinones and that these hydroquinones were oxidized by the ferric (hydr)oxide again to the corresponding semiquinones, and finally to quinones. One the other side Struyk and Sposito in 2001 (*19*) stated that quinone groups, determined by measurements of the free radical content of HS, only represented a small part of the observed electron equivalents which were transferred during reduction of Fe(III) or reduction of I_2 by HS. Chen et al. in 2003 (*20*) found different influences of three natural organic matter (NOM) fractions on the microbial Fe(III) reduction but these differences were not attributed to different quinone contents in the NOM fractions because no correlation of the NOM oxidation capacity (equivalent to its Fe(III)-reducing capacity) with the measured free radical concentrations in the NOM fractions was found. Instead the differences between these NOM fractions were explained by different structural and functional properties of the three fractions: soil HA showed at neutral pH the highest redox reactivity followed by the polyphenolic NOM fraction and the carbohydrate NOM fraction. Recently, Ratasuk and Nanny, 2007 (*21*), also found unknown redox-active compounds with nonquinone structure as well as relatively high contents of quinones with different redox properties supporting the hypothesis that quinones are important redox-active molecules at least in some types of HS. Unfortunately only a few studies with quinones, other redox-active compounds, or HS at low concentrations are known (*15, 16*). However, in most previous studies, HS were added in vast excess out of the range of ecological relevant HS concentrations (usually 0.1 to some hundred mg/L) making it impossible to properly define the status of dissolved and adsorbed HS.

Here, we have investigated the effects of aquatic FA, HA, and quinones with different chemical structures and redox potentials on the kinetics of microbial ferrihydrite reduction by *Geobacter metallireducens* at environmentally relevant low concentrations. *Geobacter metallireducens* is a strictly anaerobic microorganism, does not release electron shuttles or Fe(III) chelators (*22*), and is an important reducer of ferric

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TABLE 1. Characteristic Data Together with the Different Reducing Capacities and Normalized Reaction Rates of the Aquatic FA and HA Used As Mediators in Dissimilatory Iron Reduction of Ferrihydrite by *Geobacter metallireducens*

humic substance	C, H, O, N, S and Fe content ± SD [%][a]	number-average molecular size/mass M_n [Da][a]	reducing capacity vs potassium ferricyanide ± SD [mequ/g C][b]	reducing capacity vs ferrihydrite [mequ/g C][c]	normalized reaction rate (HS concentration [μM] in brackets)
FA: Fulvic acid (Gohy-573 FA)	54.1 ± 0.1 (C) 4.23 ± 0.08 (H) 38.94 ± 0.04 (O) 1.38 ± 0.02 (N) 1.32 ± 0.01 (S) 0.00238 ± 0.00005 (Fe)	470[d]	2.0 ± 0.5 (native) 2.2 ± 0.5 (microbial)	0.27 (native) 0.33 (chemical) electro-chemical: 0.30 (−178 mV) 0.29 (−328 mV) 0.29 (−478 mV)	1 (2.1) 1.2 (5.1) 1.4 (10.7) 1.5 (21.3) 1.6 (42.6) 1.8 (107) 1.9 (107) 2.5 (213) 2.4 (426) 3.2 (1065)
HA: Humic acid (Gohy-573 HA)	59.3 ± 0.1 (C) 4.57 ± 0.02 (H) 32.1 ± 0.1 (O) 2.01 ± 0.06 (N) 2.02 ± 0.09 (S) 0.055 ± 0.005 (Fe)	990[d]	2.7 ± 0.1 (native) 3.1 ± 0.5 (microbial)	0.21 (native) 0.46 (chemical) electro-chemical: 0.33 (−178 mV) 0.41 (−328 mV) 0.38 (−478 mV)	1.7 (1) 2 (2) 1.9 (5.1) 2.3 (10.1) 2.1 (20.2) 2.5 (50.5)

[a] Data from ref *27* and partly rounded. 1 Da (Dalton) corresponds to a molar mass of 1 g/mol. [b] Microbial reducing capacities were obtained after reduction of the native HS with *Shewanella oneidensis* MR-1. [c] For the electrochemically reduced HS the respective redox potential at pH 7 vs NHE (normal hydrogen electrode) during reduction is given. [d] M_n determined by asymmetrical flow field-flow fractionation (AFFFF).

iron and HS in anaerobic sedimentary environments (*23, 24*). The main purposes of this investigation were first, to detect the lowest possible concentrations of HS at which electron shuttling effects could be observed. This value is important because some groundwaters have very low HS concentrations (around 1 mg/L or lower) and it would be interesting to know if iron reduction in these ground waters may be potentially accelerated by electron shuttling of HS. Second, we wanted to examine if adsorbed HS and quinones exhibit different electron shuttling activities compared to dissolved HS, and third, to investigate the impact of the redox potential of the used quinones on their electron shuttling function in microbial iron reduction.

Materials and Methods

Humic Substances. The natural aquatic HS used for the experiments were extracted from 6000 L of groundwater pumped from a deep borehole (Gohy-573) located in the Gorleben aquifer above the Gorleben salt dome in Niedersachsen, Germany (*25*−*27*). This borehole has an inflow of groundwater from Präelster sediments in the depth range 134−137 m. The groundwater is dark brown due to an elevated dissolved organic carbon (DOC) content of 97.2 mg C/L with the main DOC components being HA (60 mg C/L) and FA (17 mg C/L) (*25, 26*). The HS in this groundwater was enriched in the field by reverse osmosis and fractionated in the laboratory into HA and FA by the XAD-8 method (*28*). The HA and FA fractions were purified and characterized by elemental composition (C, H, O, N, S), inorganic constituents, proton exchange capacities, size, and mass distribution (*27*). Additionally, in the present study the reduction capacities for these HA and FA were determined (Table 1). The main reasons for the use of these HS in our study were that both HS originate from a deep anoxic groundwater source, were well characterized by their molecular size and mass, and were available as highly purified compounds.

Reducing Capacity of HS. The reducing capacities of native, chemically (Pd/H_2), electrochemically, or microbially (*Shewanella oneidensis* strain MR-1) (*15*) reduced HS (in 50 mM phosphate buffer at pH 7) were determined in triplicate setups after overnight reaction with potassium ferricyanide ($K_3[Fe(CN)_6]$) (*14, 15*) or with ferrihydrite (0.4 mL of 5 mM ferrihydrite + 0.8 mL of 0.5 g/L HS) in the dark and on a rotary shaker (200 rpm). The electrochemical reduction of HS was carried out at different redox potentials with an electrochemical cell specified in ref *29*.

Quinones and Solubility Experiments. All quinones used (Figure 1A and Table 2) were purchased from Sigma-Aldrich (Taufkirchen, Germany) in analytical grade. Quinone solubility experiments were carried out to not exceed the maximal solubility in shuttle experiments and only work with totally dissolved compounds. For solubility experiments up to 16 μmol of the respective quinones were added to 100 mL of bicarbonate-buffered (30 mM) fresh water medium (*30*), flushed with a N_2/CO_2 (80/20, v/v) gas mixture, and equilibrated 3 days in the dark at room temperature by overhead rotation. After centrifugation (30 min, 2200*g*), followed in some cases by filtration (0.45 μm), the obtained supernatants were sterile filtered (0.2 μm) with a syringe in sterile 120 mL serum bottles, which were amended with butyl stoppers and filled with a N_2/CO_2 (80/20, v/v) gas mixture before use. The first 1−2 mL of sample passing through the filter was discarded. The quinone concentrations (up to 0.16 mM) of these obtained stock solutions were finally determined by dissolved organic carbon (DOC) analysis.

Dissolved Organic Carbon (DOC) Analysis. The DOC concentrations were measured with a total organic carbon analyzer TOC-V CHP/CPN (Shimadzu, Kyoto, Japan) after internal acidification of 2−5 aliquots of the samples with 2 M HCl.

Other Chemicals. All other chemicals used were at least of reagent grade quality and used as delivered from commercial sources (Aldrich, Sigma-Aldrich, Fluka). All aqueous solutions were prepared with high purity water (Milli-QPLUS, Millipore, Bedford, MA).

Ferrihydrite and Sorption Experiments. Suspensions of ferrihydrite (400 mM) were prepared after ref *31* by hydrolysis of $FeCl_3 \cdot 6H_2O$ following the 2-line ferrihydrite synthesis. The ferrihydrite precipitate was extensively washed with water, sterilized by autoclaving (30 min, 121 °C), and stored at 4 °C in the dark before use. X-ray diffraction (XRD) powder analysis of a freeze-dried sample of the prepared ferrihydrite indicated

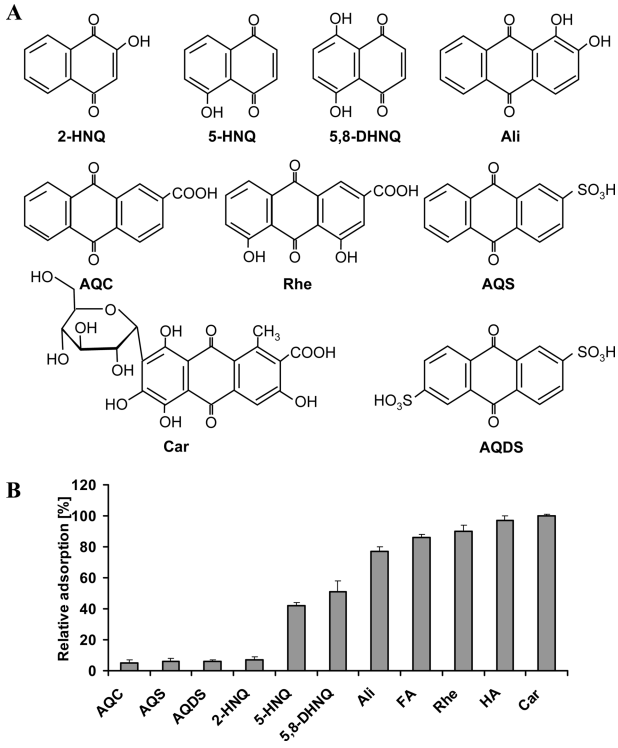

FIGURE 1. (A) Chemical structures of the quinones tested as electron-shuttles in ferrihydrite reduction by *Geobacter metallireducens*. (B) Estimated relative adsorption of HS and various quinones at low concentrations to ferrihydrite (40 mM) in bicarbonate-buffered fresh water medium (27 °C, pH ~7). The error bars indicate standard deviations from multiple DOC measurements (see Supporting Information, Table S1).

a poorly crystalline product containing mainly ferrihydrite but also some hematite and goethite (these iron minerals were probably generated during the autoclaving).

For batch sorption experiments, FA, HA, or the respective quinones were added in different concentrations to bicarbonate-buffered (30 mM) fresh water medium (*30*) amended with 40 mM ferrihydrite and flushed with a N_2/CO_2 (80/20, v/v) gas mixture (pH of the medium ~7). After daily shaking and at least 5 days equilibration time at 27 °C in the dark, the suspensions were centrifuged (2200*g*, 30 min), the DOC concentrations of the supernatants were measured, and the relative adsorption (in % of the added compound in mg C/L) of the different substances on ferrihydrite were determined.

Microorganisms and Cell Suspension Experiments. *Geobacter metallireducens* (DSM 7210) was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ), Braunschweig, Germany. For the respective experiments *Geobacter metallireducens* cells were grown in 2 × 500 mL serum bottles at 27 °C in the dark with 10 mM acetate as electron donor and 50 mM ferric citrate as electron acceptor in bicarbonate-buffered (30 mM) fresh water medium containing 1 g/L NaCl, 0.4 g/L $MgCl_2 \cdot 6H_2O$, 0.2 g/L KH_2PO4, 0.25 g/L NH_4Cl, 0.5 g/L KCl, and 0.15 g/L $CaCl_2 \cdot 2H_2O$ (*30*). The medium was amended with 1 mL/L trace-element solution SL 10 (*32*), 1 mL/L selenite-wolframate solution (*30*), 0.5 mL/L of 7-vitamine solution (*33*), 3.3 mL/L 0.3 M $FeCl_2$ solution, and 10 mL/L 1 mM cAMP (adenosine-3′,5′-cyclophosphoric acid) solution and flushed with a N_2/CO_2 (80/20, v/v) gas mixture; the pH of the final fresh water medium was ~7. The cells were harvested after 4 or 5 days in the late exponential growth phase by anoxic centrifugation (30 min at 2800*g*). The obtained cell pellets were suspended in 2 × 500 mL of fresh water medium and centrifuged again as before. Finally, the obtained pellets were pooled and suspended in 80 mL of fresh water medium resulting in a cell density of approximately 3×10^9 cells/mL. For the cell suspension experiments, 120 mL serum bottles with 45 mL of fresh water medium and 5 mL of 400 mM ferrihydrite suspension were amended with the respective HS or quinone concentration with a syringe through the butyl stopper and equilibrated at least 5 days at 27 °C in the dark before adding 10 mM acetate and 5 mL of fresh cell concentrate. In each experimental setup two AQDS (0.1−100 μM) amended samples served as positive control and two samples, also amended with AQDS but immediately autoclaved after adding the cell concentrate (30 min at 121 °C), served as negative control. The work was carried out in an anoxic glovebox under a N_2/H_2 (90/10, v/v) atmosphere, if necessary. The assays were stored at 27 °C in the dark and Fe(II) concentrations were measured daily to monitor the iron reduction.

Fe(II) Analysis. Fe(II) was measured in triplicate by the ferrozine method (*34*) after adding 0.1 mL of sample to 0.9 mL of 1 M HCl, shaking overnight, and centrifugation (15 min at 12,100*g*). By this method the analyzed Fe(II) corresponded to the total produced Fe(II) (dissolved and adsorbed). Dissolved Fe(III) and quinones did not interfere with the method.

Results and Discussion

Reducing Capacity of HS. Reducing capacities of FA and HA under different reducing conditions were determined to study their capacity and reducing potential at different redox potentials to understand differences in their electron shuttling activity. The reducing capacities (determined by titration with potassium ferricyanide) of native and microbially reduced HA (2.7 and 3.1 mequiv/g C (mequ/g C), respectively) were slightly higher than those of FA (2.0 and 2.2 mequ/g C, respectively) (Table 1). Taking into account for our HS the molecular size/mass of 470 Da (FA) and 990 Da (HA) (Table 1), determined by asymmetrical flow field-flow fractionation (AFFFF) (*35*), we calculated reducing capacities of 1.6 equ/mol (native) and 1.8 equ/mol (microbially reduced) for HA and 0.5 equ/mol (native) and 0.6 equ/mol (microbially reduced) for FA. These obtained reducing capacities refer to the amount of redox active groups which are oxidized by potassium ferricyanide ($E_0' = 430$ mV), are probably 1 order of magnitude higher than those determined by titration with ferric citrate (*36*), and may therefore be used only as "maximal reducing capacities" with respect to electron shuttling. Whether these values refers to the maximal redox active groups available to microorganisms is not exactly known and has to be investigated in the future. The much lower reducing capacities of native and chemically reduced HA (0.21 and 0.46 mequ/g C, respectively) and FA (0.27 and 0.33 mequ/g C, respectively) determined by reaction with ferrihydrite, which has a much less favorable redox potential for electron transfer than ferricyanide/ferric citrate, support this statement. Related to the molecular weight again higher values for the reducing capacities of native and chemically reduced HA (122 and 272 mequ/mol, respectively) than for FA (68 and 85 mequ/mol, respectively) were calculated. In contrast to FA, HA showed a significant increase of the reducing capacity after electrochemical reduction at redox potentials of −178 and −328 mV (at pH 7 vs NHE (normal hydrogen electrode)). As described below, this range of redox potentials is close to the redox potentials of most active quinones used as electron shuttles. The reason for the increase in ferrihydrite reduction rate of FA at higher concentrations (see below) may be that also FA has some redox active groups in the redox potential range −150 to −250 mV but these were not clearly detectable by our method due to significantly lower concentrations than in HA. If the FA concentrations in the experiments increase, also the

TABLE 2. Characteristic Data and Normalized Reaction Rates of the Quinones Tested for Electron Shuttling in Dissimilatory Iron Reduction of Ferrihydrite by *Geobacter metallireducens*

quinone	chemical formula	molar mass	redox potential E_0' (at pH 7 vs NHE[a]) [mV]	normalized reaction rate (quinone concentration [μM] in brackets)
2-HNQ: 2-hydroxy-1,4-naphthoquinone (Lawsone)	$C_{10}H_6O_3$	174.15	−137[b]	1.9 (1.6) 3.4 (16)
5-HNQ: 5-hydroxy-1,4-naphthoquinone (Juglon)	$C_{10}H_6O_3$	174.15	−3[b]	1.3 (0.8) 0.4 (8)
5,8-DHNQ: 5,8-dihydroxy-1,4-naphthoquinone (Naphthazarin)	$C_{10}H_6O_4$	190.15	−50[b]	1.3 (0.19) 1.4 (1.9)
Ali: 1,2-dihydroxy-9,10-anthraquinone (Alizarin)	$C_{14}H_8O_4$	240.21	−344[c]	1 (0.1) 1.1 (0.5) 0.9 (2)
AQC: 9,10-anthraquinone-2-carboxylic acid	$C_{15}H_8O_4$	252.22	−254[c]	1 (0.1) 0.8 (1) 0.1 (10)
Rhe: 9,10-dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracene carboxylic acid (Rhein)	$C_{15}H_8O_6$	284.22	−270[d]	1.1 (0.1) 0.4 (0.5) 0.2 (2)
Car: 7-α-D-glucopyranosyl-9,10-dihydro-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-2-anthracene carboxylic acid (Carminic acid)	$C_{22}H_{20}O_{13}$	492.39	−500[e]	1 (0.1) 1 (1) 1.1 (10)
AQS: 9,10-anthraquinone-2-sulfonic acid	$C_{14}H_8O_5S$	288.28	−225[b]	4.0 (0.95) 4.5 (9.5)
AQDS: 9,10-anthraquinone-2,6-disulfonic acid	$C_{14}H_8O_8S_2$	368.34	−184[b]	1.9 (0.1) 2.4 (0.3) 3.9 (1) 6.9 (10) 8.9 (100)

[a] Normal hydrogen electrode. [b] Ref *42* and references therein. [c] Ref *45*, and Figure 5 in ref *46*. [d] Ref *47*. [e] Converted to NHE from a measured redox potential of Car related to SCE (standard calomel electrode) (*48*).

concentrations of these redox active functional groups increase together with an increase in ferrihydrite reduction rates.

Adsorption of FA, HA, and Quinones on Ferrihydrite. Ferrihydrite is a nanocrystalline iron(III) (hydr)oxide ($Fe_{10}O_{14}(OH)_2$), frequently found in near-surface environments, with high sorption capacity and a structure only determined very recently (*37*). The concentration of the electron-shuttling compound in solution depends on the total concentration of the compound added and on the fraction of the adsorbed compound. If only the dissolved fraction is acting as electron shuttle then we would expect to see higher electron-shuttling activity by compounds with higher fractions of dissolved compounds or vice versa with lower fractions of adsorbed compounds. Therefore adsorption experiments with FA, HA, and different quinones were carried out to estimate the adsorption properties of these compounds at low concentrations. The results showed large differences of the relative adsorption (in % of the added compound in mg C/L) of the different compounds to ferrihydrite at DOC concentrations between 2 and 23 mg/L (Figure 1B; Supporting Information, Table S1). Sorption was in equilibrium after 5 days (Figure S1) and concentrations were far away from saturation as indicated by the linear range of the sorption isotherm (Figure S2). Low relative adsorption was found for AQC, AQS, AQDS, and 2-HNQ, stronger relative adsorption was found for 5-HNQ and 5,8-DHNQ, and high relative adsorption was found for Ali, FA, Rhe, HA, and Car (see Figure 1A for structures). Molecules with more functional groups seem to adsorb generally stronger to ferrihydrite but no significant correlation of the normalized reaction rates with the relative adsorption of the different substances was found. This suggests that parameters other than the fraction of dissolved or adsorbed compound have a stronger influence on the electron-shuttling property of HS and quinones. Sorption of HS and quinones to cell surfaces does not matter due to the some magnitudes lower surface area of the cells compared to the surface area provided by the ferrihydrite.

Iron Reduction Experiments with Cell Suspensions of *Geobacter metallireducens*. In all iron reduction experiments carried out with active cell suspensions, ferrihydrite was reduced with initially fast reaction rates decreasing over time (Figure 2A). The reaction kinetics for the different compounds showed some differences from those in Figure 2A. In contrast to O'Loughlin (*16*), who found in some cases long lag phases in growth experiments with different quinones, we did not find any long lag phases. Generally, during the first days we observed the highest reaction rates (so-called "initial iron reduction rates"). The plateaus in Fe(II) concentrations of some assays that were reached after about 100 h may represent final Fe(II) concentrations of the experiments, probably when the free energy $\Delta G_0'$ reaches the limit for ATP production ($\Delta G_0'$ −20 kJ/mol (*38*); $\Delta G_0'$ −22.7 kJ/mol (*39*)) (see below in "Thermodynamic Considerations"). Iron reduction was accelerated by AQDS and other quinones as reported earlier (*12, 13*). The initial iron reduction rates (*39*) were first estimated from the kinetic data of the first days by linear regression of the Fe(II) formation rates. For better comparison of the individual experiments the rates were normalized to the initial iron reduction rates of experiments with ferrihydrite and cells only. This so-called "normalized reaction rate" is dimensionless because it is only the ratio between the two rates with the same dimension (e.g., produced Fe(II)/hour). It should be noted that our precultures were grown with dissolved ferric citrate and might perhaps grow and metabolize slightly differently from cells pregrown

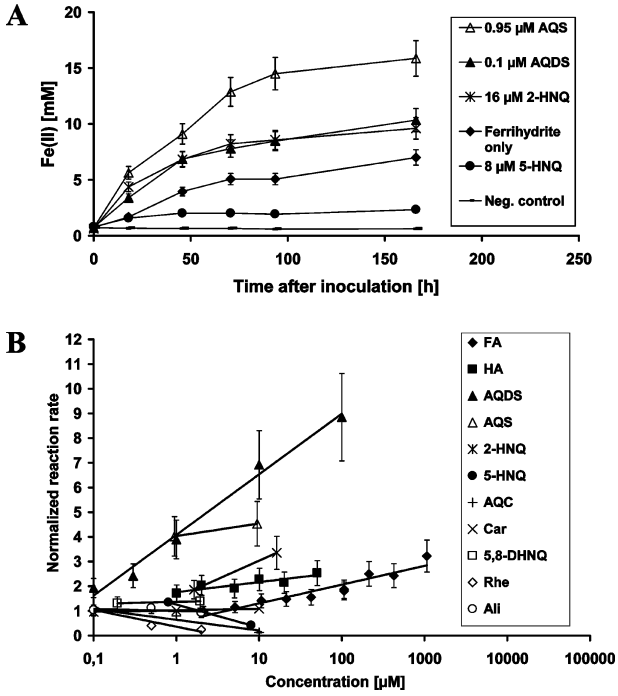

FIGURE 2. (A) Example for the kinetics of ferrihydrite reduction by *Geobacter metallireducens* with selected naphthoquinones, AQS, and AQDS added at low concentrations (0.1−16 μM). Only 6 of 14 samples are shown for clarity. Fe(II) corresponded to the totally produced Fe(II) (dissolved and adsorbed), AQDS served as positive control, and the tag "Ferrihydrite only" stands for an assay with ferrihydrite and *Geobacter metallireducens* cells but without quinones. The error bars correspond to an estimated relative standard deviation of the measured Fe(II) concentration of 10%. (B) Ferrihydrite reduction rates by *Geobacter metallireducens* with FA, HA, or different quinones added. The "normalized reaction rate" represents the ratio of the initial iron reduction rate with HS or quinones added divided by the initial iron reduction rate with ferrihydrite only. The error bars correspond to an estimated relative standard deviation for the normalized reaction rate of 20%.

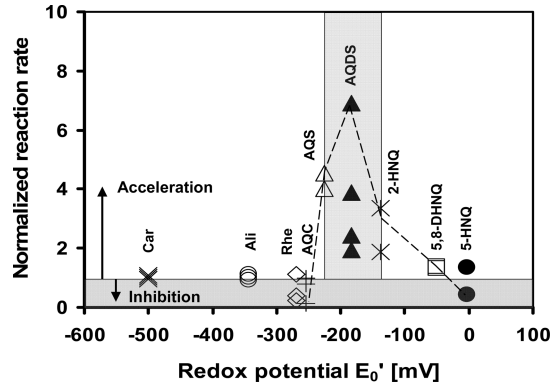

FIGURE 3. Normalized reaction rates of ferrihydrite reduction by *Geobacter metallireducens* vs redox potentials (E_0' at pH 7 vs NHE) of various quinones in the concentration range 0.1−16 μM. The arrows indicate increasing acceleration or inhibition of iron reduction with increasing concentrations of quinones added. The dotted line corresponds to a quinone concentration of 10 μM of the respective compounds and is plotted for comparison of different quinones at equal concentrations. The normalized reaction rates for 10 μM quinone were partly estimated from their logarithmic concentration dependency (Figure 2B).

with solid ferric (hydr)oxides due to a different protein expression profile of the pregrown cells.

Both FA and HA showed positive effects on the kinetics of iron reduction and their normalized reaction rates seemed to be linearly correlated with the logarithm of the HS concentration (Figure 2B). FA showed significant stimulation of Fe(III) reduction down to total FA concentrations of 5 mg/L (= 2.7 mg C/L or ~11 μM) corresponding to dissolved FA concentrations of only 0.61 mg/L (= 0.33 mg C/L or ~1.3 μM). HA accelerated iron reduction more strongly than FA at comparable concentrations and showed a significant positive kinetic effect down to a total HA concentration as low as 1 mg/L (= 0.59 mg C/L or ~1 μM) corresponding to dissolved HA concentrations of only 0.025 mg/L (= 0.015 mg C/L or ~0.025 μM). The stronger kinetic effect of HA compared to FA can be explained by the higher reducing capacity of HA especially in the optimal range of redox potentials (see below). The results showed also that FA and HA stimulate iron reduction by *Geobacter metallireducens* at ecologically relevant low dissolved concentrations below 1 mg C/L often found in groundwater. These concentrations are lower than the required dissolved HS concentrations (5−10 mg C/L) for electron shuttling recently published (*15, 16*). Reasons for the relatively high HS values necessary for electron shuttling in these studies might be the use of other types of HS (Pahokee Peat HA; Suwannee River FA, HA, NOM) and/or different microorganisms (*Shewanella oneidensis* MR-1; *Shewanella putrefaciens* CN32). Unlike *Geobacter metallireducens*, the microorganism *Shewanella oneidensis* MR-1 and other *Shewanella* species are able to produce flavins (*8, 9*) as electron shuttles or a Fe(III)-solubilizing ligand (*6, 11*) and therefore higher concentrations of HS may be necessary to observe additionally stimulating effects on microbial iron reduction.

For the various quinones studied as electron shuttles we found large differences in stimulating iron reduction (Figure 2B). Some quinones showed strong accelerating effects (AQDS, AQS, and 2-HNQ at all concentrations investigated (0.1−100 μM)), whereas others showed only small effects (5-HNQ at a concentration of 0.8 μM and 5,8-DHNQ at concentrations of 0.19 and 1.9 μM). No significant (AQC and Rhe at concentrations of 0.1 μM, Ali and Car at all concentrations studied (0.1−10 μM)) or even inhibitory effects on the kinetics of iron reduction were observed for 5-HNQ at concentrations of 8 μM, AQC at concentrations of 1 and 10 μM, and Rhe at concentrations of 0.5 and 2 μM. Our results with different quinones did not confirm in all cases the results of O'Loughlin (*16*) probably due to different experimental conditions. Instead of using *Geobacter metallireducens* as iron reducer and acetate as electron donor in our case, this author was using *Shewanella oneidensis* as iron reducer and formate as electron donor. Due to the lower redox potential of the CO_2/formate couple (E_0' −432 mV (*40*)) in comparison to the CO_2/acetate couple (E_0' −290 mV (*40*)) enough free energy for ATP production may be gained for the microorganism also in cases of quinones with lower redox potentials. Like HS, AQDS also showed a linear correlation of the normalized reaction rates with the logarithm of the concentration. The inhibitory effect of some of the investigated quinones might have been caused by antibiotic properties of these quinone compounds (*41*). Furthermore it is interesting to note that the results of experiments with three different concentrations of the quinones Car and Ali, respectively, showed no concentration dependency on the normalized reaction rate (Figures 2B and 3). Both quinones adsorbed strongly on ferrihydrite (Figure 1B), but should have theoretically no or only minor electron shuttling activity due to their very low redox potentials. These results support the assumption that passivation of ferrihydrite by surface coverage plays only a minor role under our conditions.

Thermodynamic Considerations. By plotting the normalized reaction rates of the different quinones vs their

respective redox potential E_0' (determined at pH 7 vs NHE) (Figure 3) we found that the quinones with the most positive kinetic effects have E_0' values of −137 mV (2-HNQ), −184 mV (AQDS), and −225 mV (AQS), respectively (ref *42* and references therein). These results may give evidence that the kinetics of iron reduction mediated by electron shuttles is controlled by the redox potential of the electron shuttle and the free energy ($\Delta G_0'$) in the two reaction steps. This means that in the first electron transfer from the microorganism to the electron shuttle the difference in $\Delta G_0'$ between the CO_2/acetate redox couple (E_0' −290 mV (*40*)) and the used quinone/hydroquinone redox couple should be at least as negative as −20 kJ/mol (*38*) to allow ATP synthesis by electron transport phosphorylation in bacterial cells. A comparable value of consistent excess of free energy (−22.7 kJ/mol) was also evaluated for the end states of bacterial reduction of goethite (*39*). From our data the calculated $\Delta G_0'$ values under standard conditions are −50.2 kJ/mol (AQS), −81.8 kJ/mol (AQDS), or −118.1 kJ/mol (2-HNQ) and therefore are all more negative than the minimum value of −20 kJ/mol necessary for ATP production. In the second step (electron transfer to the insoluble iron(III) (hydr)oxide) the difference between the respective quinone/hydroquinone redox couple and the ferrihydrite/Fe(II) redox couple (E_0' +100 mV in bicarbonate-buffered solutions (*43*)) should be as negative as possible to allow for this rate-limiting step (*15*) the highest possible driving force for the electron transfer. This would produce the highest iron reaction rates from a thermodynamic point of view. The calculated $\Delta G_0'$ values for the second reaction step are −62.7 kJ/mol (AQS), −54.8 kJ/mol (AQDS), or −45.7 kJ/mol (2-HNQ) for the respective quinone/hydroquinone redox couple and in a similar range as in the first reaction step. Recently, Marsili et al. (*8*) and von Canstein et al. (*9*) found E_0' values of −219 to −208 mV for flavin mononucleotide and riboflavin excreted by *Shewanella* species which fall in the same range of redox potentials as our most redox-active quinones.

HS are suggested to contain a large number of so far unknown redox-active molecules with redox potentials distributed over a broad range of redox potentials (−300 to +400 mV (*14*); −0.9 to +1.0 V (*44*)). We found that a part of these molecules may have redox-potentials also in the range of −225 to −137 mV found for the most active electron shuttles with quinone structures and these molecules may determine the electron-shuttling properties of the different HS to a large extent. According to our findings electron shuttling activities of HS or quinones are mainly controlled by the redox potentials of the molecules active in electron shuttling rather than by the adsorption properties of the different compounds on the iron mineral ferrihydrite.

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Supporting Information Available

Relative adsorption of HS and quinones on ferrihydrite (Table S1); kinetic of FA adsorption on ferrihydrite (Figure S1); adsorption isotherm of FA on ferrihydrite (Figure S2). This information is available free of charge via the Internet at http://pubs.acs.org.

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