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Kinetics of Microbial and Chemical Reduction of Humic Substances: Implications for Electron Shuttling

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Humic substances (HS) are redox-active natural organic compounds and serve as electron shuttles between microorganisms and iron(III) minerals. Here we demonstrate that electron shuttling is possible only at concentrations of dissolved HS of at least 5−10 mg C/L. Although such concentrations can be found in many rivers, lakes, and even in some aquifers there are also many marine and freshwater systems with DOC <5 mg C/L where consequently electron shuttling is not expected to happen. We found that in the case of HS concentrations which do not limit electron shuttling, *Geobacter sulfurreducens* transfers electrons to HS at least 27 times faster than to Fe(III)hydroxide. Microbially reduced HS transfer electrons to ferrihydrite at least 7 times faster than cells thereby first demonstrating that microbial mineral reduction via HS significantly accelerates Fe(III) mineral reduction and second that electron transfer from reduced HS to Fe(III) minerals represents the rate-limiting step in microbial Fe(III) mineral reduction via HS. Microbial reduction of HS transfers as many electrons to HS as chemical reduction with H_2 indicating that all redox-active functional groups that can be reduced at a redox potential of −418 mV (E_h^0 of H_2/H^+ redox couple at pH 7) can also be reduced by microorganisms.

Introduction

Humic substances (HS) are a chemically heterogeneous class of polymeric organic compounds and constitute the major organic fraction in soils (*1, 2*). Many microorganisms, e.g., iron-reducing, sulfate-reducing, and some fermenting bacteria, are able to use HS as an electron acceptor for anaerobic oxidation of organic and inorganic electron donors (*3*−*7*). Quinones were suggested to function as the main electron accepting moieties in HS (*8, 9*). Reduced HS (containing the reduced form of quinones, i.e., hydroquinones) can transfer electrons to dissolved and solid-phase Fe(III). As the humic compounds get reoxidized during this redox process and therefore are able to take up electrons from bacteria again, HS can function as recyclable electron shuttles between bacteria and Fe(III) minerals (Figure 1) (*3, 7*). Besides increasing iron(III) mineral reduction rates (*10*), electron shuttling via dissolved and diffusible HS also allows electron transfer from microbial cells to spatially distant electron acceptors that are not directly accessible.

HS concentrations in groundwater (aquifers) and surface water (rivers and lakes) range from 0.1 mg C/L to several hundred mg C/L (*2*). However, concentrations of HS (with approximately 50 weight % C) typically used thus far in laboratory experiments studying microbial electron shuttling were 1000 mg/L (*7*) or even 2000 mg/L (*3*), and thus much higher than the concentrations typically observed in nature. Whether the processes observed in these laboratory experiments using artificially high HS concentrations are applicable to environmental systems remains unclear. This is particularly true as it is unclear whether there is a minimum concentration of dissolved HS required for electrons shuttling from microorganisms to Fe(III) minerals. At very low total concentrations of HS most, if not all, humic compounds are expected to sorb to the iron mineral surfaces (*11*). As a consequence, no stimulation of iron reduction via electron shuttling in terms of bridging the distance between cells and Fe(III) minerals is possible due to the lack of dissolved and diffusible HS. However, even in the presence of low concentrations of dissolved HS, iron reduction is not necessarily stimulated. First, if electron shuttling happens by electron transfer from microbial cells to iron(III) minerals by electron hopping through a sequence of dissolved HS molecules present between the cells and the minerals, a minimum concentration of HS is necessary otherwise the distance between the HS molecules is too large for electron transfer. Second, if the electron shuttling effect relies on diffusion of the reduced/oxidized humic molecules between iron(III) minerals and cells, a very low concentration of HS would allow transfer of only a few electrons per time and therefore would lead to no significant stimulation. Which of these two mechanisms (or whether a combination of both) is indeed responsible for stimulation of microbial iron(III) reduction is unknown, as well as it is not known whether sorbed HS can help to transfer electrons to the minerals or, in contrast, prevent electron transfer by blocking mineral surface sites.

In any case, in order to stimulate microbial reduction of iron(III) (hydr)oxides, both microbial reduction of HS and chemical reduction of iron(III) by the microbially reduced HS have to be faster than direct microbial reduction of iron(III) minerals. The electron transfer rates from microorganisms to dissolved HS molecules and from HS to Fe(III) minerals compared to the direct electron transfer from microorganisms to a mineral surface has not yet been determined quantitatively.

It has been demonstrated that *Geobacter* spp. are the predominant organisms that emerge when dissimilatory metal reduction is stimulated in subsurface environments by addition of various electron donors and/or electron shuttling compounds (*4*). *Geobacter* species are of particular interest because of their capability of transferring electrons both to Fe(III) and to quinone moieties present in HS (*3, 4, 8, 12*). Additionally, several *Shewanella* strains were also shown to transfer electrons to natural organic matter or to anthraquinone-2,6-disulphonate (AQDS), a model compound for quinone moieties in HS (*13, 14*). *Geobacter sulfurreducens* and *Shewanella oneidensis* strain MR-1 were therefore chosen in our study as model strains to reduce HS.

Depending on how many redox-active functional groups in HS are reduced or oxidized, HS possess a certain capacity to take up electrons by the oxidized functional groups (electron accepting capacity) or to release electrons from reduced functional groups to an electron acceptor with a more positive redox potential, e.g., Fe(III) or organic contaminants (reducing capacity) (*15*−*18*). In many studies,

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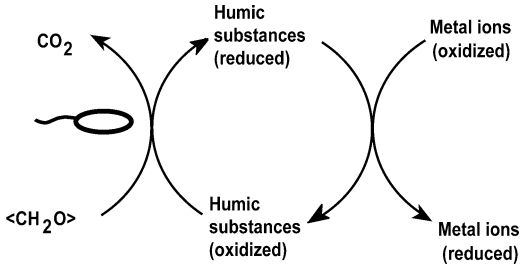

FIGURE 1. Scheme illustrating electron shuttling by humic substances (HS). HS can transfer electrons from microbial oxidation of electron donors (e.g., organic compounds but also inorganic compounds such as H_2) to metal ions such as Fe(III).

researchers used chemically reduced instead of microbially reduced humic compounds to investigate redox properties of HS. Whether chemical reduction of aquatic HS and commercially available HS reduces the same functional groups as microbial reduction of the same HS molecules is currently unknown.

From the knowledge gaps outlined above, we defined the following objectives for this study: (i) to quantify the kinetics of microbial HS reduction and the kinetics of (chemical) Fe(III) mineral reduction by reduced HS and to compare these rates to direct microbial Fe(III) mineral reduction, (ii) to determine the minimum concentration of HS necessary for stimulation of microbial iron(III) mineral reduction, and (iii) to compare microbial vs chemical reduction of aquatic and commercially available HS.

Materials and Methods

Source of Humic Substances. Suwannee River humic and fulvic acid (SRHA, SRFA) and Pahokee Peat humic acid (PPHA) were purchased from the International Humic Substances Society (IHSS). Aldrich humic acid was purchased from Aldrich.

Preparation of Humic Substance Solutions. HS were added to phosphate buffer (50 mM, pH 7) in concentrations of 250−2500 mg C/L and diluted to 2−250 mg DOC/L. The preparations were agitated for 1 h at 200 rpm at 25 °C, filtered and at the same time sterilized (0.22 μm, mixed cellulose ester membrane). To determine the DOC, aliquots of the filtered HS preparations were analyzed with a TOC analyzer (Elementar Analysensystem). If necessary, the HS solutions were deoxygenated 3 times (each time 2 min vacuum, 2 min N_2-flushing) leading to negligible concentrations of O_2 as indicated by control bottles without HS to which Fe(II) was added that visually showed no Fe(II) oxidation (not shown). In order to avoid photochemical reactions, all HS solutions were stored in the dark.

Determination of Microbial Reduction Rates of HS and Ferrihydrite in Cell Suspension Experiments. *Geobacter sulfurreducens* was cultured as previously described (*19*) and harvested in the exponential growth phase by anoxic centrifugation (10 min, 8000 rpm). To reduce HS under anoxic conditions, cells were resuspended (0.3 mg protein per mL, determined by the Bradford assay) in 0.5 mg/mL filtered solutions of either humic or fulvic acid (50 mM phosphate buffer, pH 7, flushed with N_2) at 30 °C and incubated in the dark. Acetate was added (10 mM) as electron donor. Samples were taken at the starting and end points (3 h) of HS reduction to determine the redox state of the HS (i.e., the reducing capacity meaning the amount of electrons that can be transferred to Fe(III); see below). The DOC content was quantified after filtration to calculate the reducing capacity per DOC.

For quantification of rates for direct reduction of ferrihydrite by *Geobacter sulfurreducens* at surface-area-limited or cell-limited conditions, either cells (0.003−0.3 mg protein/mL) were inoculated with 5 mM ferrihydrite or cells with 0.3 mg protein/mL were inoculated with 0.5, 2.5, or 5 mM ferrihydrite (50 mM phosphate buffer, pH 7, flushed with N_2) and acetate (10 mM) as electron donor. Ferrihydrite was prepared according to Cornell and Schwertmann (*20*) and identified by X-ray diffraction (not shown).

To determine whether significant cell lysis happened during the cell suspension experiments, aliquots of *Geobacter sulfurreducens* cell suspensions (0.3 mg protein/mL) were filtered (0.22 μm; mixed cellulose ester membrane) at the beginning and end of the experiment and DOC (see above) and reducing capacities of the filtrates (see below) were quantified. To determine whether the cells stored significant amounts of electron donor (or organic intermediates) during growth, we decreased the ratio of electron donor to acceptor from 1:1 to 1:2 (by using 40 or 80 mM fumarate) in the cultures that were used for the cell suspension experiments. Additionally, we aimed to deplete the cells in internally stored electron donor by preincubating the cells for 1 h with 10 mM fumarate instead of washing the cells with plain phosphate buffer.

Determination of Minimum Concentrations of Dissolved HS Necessary for Electron Shuttling. *Shewanella oneidensis* strain MR-1 was grown aerobically under oxygen-limited conditions in LB medium (*21*) (30 °C, 14 h), harvested by centrifugation (10 min, 8000 rpm) in the early stationary phase, and then washed twice with anoxic LML medium (*21*). For the quantification of stimulation of microbial iron(III) hydroxide reduction by dissolved HS, the washed cells were resuspended (cell density approximately 10^{10} cells/mL corresponding to 0.3 mg protein/mL) in suspensions of peat humic acids (0, 2, 5, 7, 10, 24, 49, 98 mg carbon/L) and ferrihydrite (1 mM) in LML medium. Aliquots were taken at different time points, acidified with 1 M HCl, and the amount of reduced iron [Fe(II)] was quantified spectrophotometrically (ferrozine assay (*22*)). The rates of microbial iron reduction were plotted vs DOC determined from parallel experiments without cells.

Chemical Reduction of HS. HS were chemically reduced by H_2 with a Pd catalyst (palladium-coated alumina pellets, 0.5% Pd, Merck) as described previously (*5, 7*).

Determination of the Redox State of HS. The redox state of native (nonreduced) and reduced HS was determined by measuring their reducing capacity, i.e., the amount of electrons which were transferred to $K_3[Fe(CN)_6]$ by the HS and their electron accepting capacity (the amount of electrons that can be taken up) as described previously (*5, 7*).

Determination of Rates of Ferrihydrite Reduction by Reduced HS. Peat humic acids (0.5 mg/mL filtered solutions in 50 mM pH 7 phosphate buffer, flushed with N_2) were reduced by *Geobacter sulfurreducens* under the same conditions as used for determination of microbial reduction rates of HS (see above). Reduced humic acids were mixed with ferrihydrite and the production of Fe(II) was followed spectrophotometrically (ferrozine assay).

Results and Discussion

Rates of Microbial Reduction of HS and Iron(III) (Hydr)oxides. The reducing capacities of HS incubated with *Geobacter sulfurreducens* increased rapidly within the first minutes of incubation at a rate of approximately 617 μmol electrons transferred min^{-1} (g protein)$^{-1}$ (Figure 2A).The maximum reduction rate was determined from the first two data points (measured for the first 2 min) and most of the HS present in the assay were reduced within the first 5 min. This experiment also revealed that even native

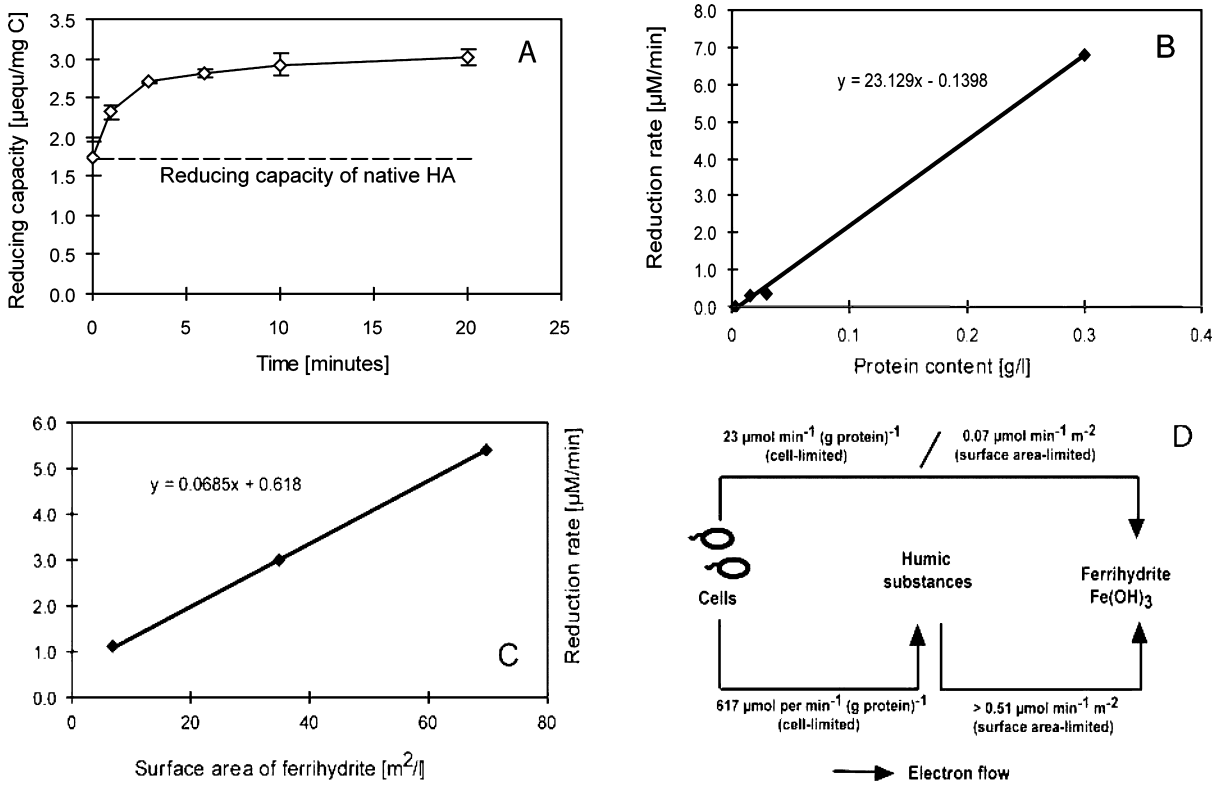

FIGURE 2. (A) Microbial reduction of Aldrich humic acids by *Geobacter sulfurreducens* cell suspensions. The reduction is followed over time as reducing capacity of the humic acids, i.e., the amount of electrons that can be transferred from the reduced humic acids to dissolved iron(III) present in form of ferricyanide $K_3[Fe(CN)_6]$ with an E_0' of +430 mV. Error bars give standard deviations (SD) calculated from three parallels. The dashed line shows that even the native (nonreduced) humic substances can reduce iron(III) to a certain extent indicating that native HS have an inherent reducing capacity (see also Figure 4). (B) Rates for microbial ferrihydrite reduction by *Geobacter sulfurreducens* under cell-limited conditions with a final protein content ranging from 0.003 to 0.3 mg/mL (ferrihydrite 5 mM). Acetate was added (10 mM) as electron donor. (C) Rates of microbial ferrihydrite reduction under surface area-limited conditions with a protein content of 0.3 mg/mL and 0.5, 2.5, and 5 mM ferrihydrite. Acetate was added (10 mM) as electron donor. (D) Rates of microbial reduction of ferrihydrite by *Geobacter sulfurreducens* cell suspensions under either cell-limited or ferrihydrite surface-area-limited conditions compared to the rate of microbial HS reduction and the rate of ferrihydrite reduction by microbially reduced HS. All experiments were done at non-HS-limiting conditions of 0.5 mg/mL.

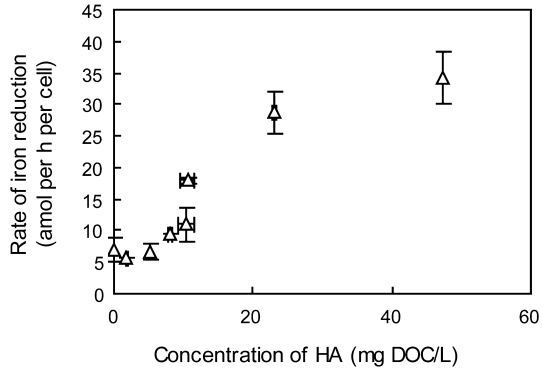

FIGURE 3. Rates of reduction of ferrihydrite by *Shewanella oneidensis* MR-1 in the presence of different concentrations of dissolved PPHA. The PPHA concentration is given in mg of dissolved organic carbon (DOC) per L. Error bars give standard deviations (SD) calculated from 2 parallels.

(nonreduced) HS have already an inherent reducing capacity (see also section on chemical and microbial reduction of HS).

To determine whether iron(III)-reducing bacteria reduce dissolved HS faster than poorly soluble iron(III) (hydr)oxides, we compared the rates of microbial HS reduction to microbial reduction of poorly crystalline Fe(III) (hydr)oxide (ferrihydrite). Since Fe(III) (hydr)oxides are solid-phase electron acceptors with a defined surface area and thus with a limited number of surface sites available for microbial electron transfer, in microbial reduction assays either the cell number or the mineral surface area can be limiting. Assays are considered to be cell-limited when reduction rates are linearly correlated with the cell number as it is the case in our assays with 5 mM ferrihydrite and 0.003, 0.015, 0.03, and 0.3 g protein per L (Figure 2B). On the other hand, assays are considered to be surface-area-limited when reduction rates are linearly correlated with the amount of iron mineral (and thus surface area) as it is the case in the assays with 0.3 g protein per L and 0.5, 2.5, and 5 mM ferrihydrite (Figure 2C).

First we compared per cell rates of HS reduction (assays with an excess of HS) to mineral reduction experiments that contained an excess of available surface area (providing maximum mineral reduction rates per cell). In cell suspension experiments of direct microbial iron(III) mineral reduction by *Geobacter sulfurreducens*, we measured that ferrihydrite was reduced at a rate of 23 μmol electrons transferred min^{-1} (g protein)$^{-1}$ (Figure 2B). This means that on a per cell basis (in the presence of excess electron acceptor) electrons are transferred to dissolved HS approximately 27 times faster than to solid Fe(III) (617 μmol electrons transferred min^{-1} (g protein)$^{-1}$ for HS vs 23 μmol electrons transferred min^{-1} (g protein)$^{-1}$ for Fe(III). While our study was done with *Geobacter sulfurreducens*, previous studies with *Shewanella alga* strain BrY showed that in such cell-limited ferrihydrite reduction assays without electron shuttles, approximately 29 μmol electrons are transferred directly to ferrihydrite per min per g protein

(calculated from approximately 5 μmol electrons L^{-1} min^{-1} for 0.5 $\times$ 10^9 cells/mL given by Roden and Zachara (*23*)), a rate similar to that we determined for *Geobacter sulfurreducens*.

For electron shuttling, not only the microbial reduction of HS but also the subsequent reduction of iron(III) minerals by microbially reduced HS has to be faster than the direct microbial reduction of iron(III) minerals. In order to compare maximum rates of iron(III) mineral reduction by HS (per m^2 mineral surface area) to maximum rates of direct mineral reduction by microorganisms (per m^2 mineral surface area), a defined (limited) mineral surface area has to be present. In experiments with reduced HS and 5 mM iron(III) hydroxide (ferrihydrite) we found that reduced HS reacted almost instantaneously with ferrihydrite: already two minutes after mixing microbially reduced HS with 5 mM ferrihydrite more than 135 μM Fe(III) was reduced to Fe(II). The reaction was even too fast to determine an accurate initial rate for this process. However, since about 135 μM Fe(II) were produced from 5 mM ferrihydrite within 2 min, the rates have to be faster than 0.51 μmol electrons per min per m^2 surface area (calculated with a surface area of ferrihydrite of 250 m^2/g given in Cornell and Schwertmann (*20*) (Figure 2D)). This rate of reduction of ferrihydrite by HS can now be compared to direct ferrihydrite reduction by *Geobacter sulfurreducens* in assays with a limited mineral surface area. In our experiments, we determined that approximately 0.07 μmol electrons were transferred from the cells per min per m^2 ferrihydrite in setups with ranges of 0.5 to 5 mM ferrihydrite and a protein content of 0.3 mg/mL (Figure 2 C). This rate is significantly lower than the number of electrons transferred from reduced HS to ferrihydrite in a purely abiotic reaction (0.51 μmol electrons per min per m^2 surface area, see above).

Since microbial reduction of HS is about 27 times faster than direct microbial reduction of Fe(III) minerals and since chemical electron transfer from microbially reduced HS to Fe(III) minerals is about 7 times faster than direct microbial reduction of Fe(III) minerals, the overall rate of electron transfer from microorganisms to Fe(III) minerals via HS is approximately 7 times faster than the direct electron transfer rate from the cells to Fe(III) minerals. Additionally, we can conclude that the chemical step of electron transfer from reduced HS to Fe(III) minerals and not microbial electron transfer to HS is the rate-limiting step in microbial reduction of iron(III) minerals via humic substance electron shuttling.

Dependence of Microbial Electron Shuttling on Dissolved HS Concentration. Besides faster rates of microbial HS reduction and reduction of iron(III) minerals by reduced HS compared to rates of direct microbial iron(III) reduction, an additional requirement for electron shuttling is the presence of dissolved HS in concentrations that exceed sorption capacity of the mineral surfaces (*11*) and provide dissolved HS molecules. A minimum level of dissolved HS is necessary, either to provide enough HS molecules to allow electron hopping from the cells to the iron(III) minerals through a sequence of dissolved HS molecules, or (if the distance between the humic molecules is too large for direct electron hopping) to stimulate electron transfer by efficient diffusion of HS molecules between the cells and minerals.

Since in aquatic environments dissolved HS concentrations cover a broad range from 0.1 mg C/L in groundwater to several hundred mg C/L in surface water (*2*), the concentration dependence of electron shuttling has to be known in order to evaluate the environmental relevance of this process in particular in low-DOC environments. We could show that concentrations of dissolved HS <5 mg DOC/L do not stimulate microbial ferrihydrite reduction by *Shewanella oneidensis* MR-1 (Figure 3) suggesting that below 5 mg DOC/L efficient shuttling either via electron hopping or via diffusion is not possible. However, when the concentrations of dissolved HS were increased from about 5 to 25 mg DOC/L, the rates of microbial ferrihydrite reduction were directly proportional to the concentrations of HS. There was almost no significant further increase of iron(III) reduction rates when the concentrations of HS exceeded 25 mg DOC/L suggesting that at these concentrations enough HS for efficient electron transfer, via electron hopping or diffusion, are available.

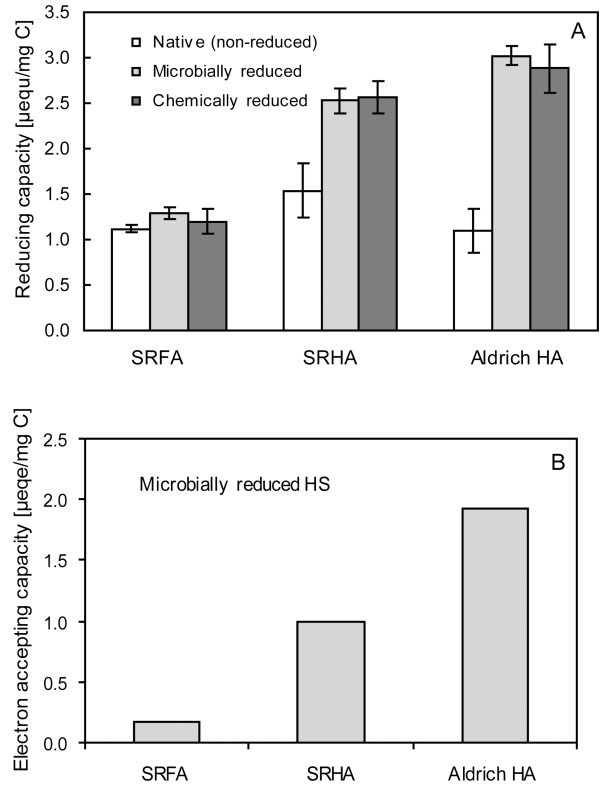

FIGURE 4. (A) Reducing capacities of microbially (grey bar) and chemically (black bar) reduced IHSS Suwannee River fulvic and humic acids (SRHA and SRFA) and Aldrich humic acids compared to nonreduced (native) (white bar) preparations of the same HS. All HS preparations (0.5 mg/mL) were filtered prior to the experiments. The electron accepting capacities (B) were calculated as difference of the reducing capacities of the reduced and native preparations. Standard deviations were calculated from at least 3 independent experiments with three parallels each and triplicate measurements of each parallel sample.

These results suggest that stimulation of iron(III) mineral reduction takes place mainly in environmental systems with HS concentrations of at least several mg DOC per liter. Examples for such environments are, besides surface water, sediment porewater, or soil porewater, also aquifers that receive recharge from organically rich waters or that have been in contact with sediments rich in organic matter.

As described in the section above, stimulation of iron(III) mineral reduction depends on the rates of the respective electron transfer steps. Quantification of the rates of enzymatically catalyzed electron transfer from microorganisms to HS and from reduced HS to iron(III) (hydr)oxides (chemical reaction) yielded an acceleration of about 7 times (Figure 2D). This expected acceleration corresponds well to the stimulation of iron(III) mineral reduction observed in our experiment (Figure 3) where the maximum rates of ferrihydrite reduction in the presence

of saturating concentrations of HS increased about 7-fold from approximately 5 pmol h^{-1} $cell^{-1}$ (in the absence of HS) to about 35 pmol h^{-1} $cell^{-1}$ (approximately 50 mg DOC/L).

Exclusion of Artifacts by Cell Lysis in Electron Shuttling Assays with Dense Cell Suspensions. Experiments evaluating the minimum concentrations of HS necessary for electron shuttling (Figure 3), and determination of reducing capacity of microbially reduced HS (Figure 4), as well as experiments quantifying the HS reduction rates (Figure 2), were all done in cell suspension assays containing a very high number of cells per volume (usually about 10^{10} cells/mL). A potential concern is the lysis of microbial cells potentially releasing organic redox-active compounds and influencing the electron shuttling experiments. Both the DOC and reducing capacity of aliquots of cell suspensions filtered at the beginning and at the end of the experiment showed negligible values close to zero and did not increase within 3 h (Supporting Information, Table S1). This indicates that during our experiments, the cells did not release significant amounts of organic compounds and therefore did not influence the reducing capacities of the incubated HS. We conclude that no significant cell lysis happened during our cell suspension experiment.

Source of Electrons for HS Reduction in Cell Suspension Experiments. Since our cell suspension experiments were aimed to determine the maximum rates of HS reduction, we used fresh and very active cells harvested from exponential growth phase in cultures grown under substrate-rich conditions. Unexpectedly, even cell suspensions without addition of electron donor showed reduction of humic acids in the same range as obtained in the presence of the electron donor (Supporting Information, Table S2). Initially, we assumed that this is due to the storage of significant amounts of electron donor or organic intermediates in the cells. However, decreasing the ratio of electron donor to acceptor in the growing cultures (expected to favor complete oxidation of the organic substrate and preventing carbon storage) did not impede humic acid reduction by the harvested cells in *Geobacter sulfurreducens* cell suspension experiments done in the absence of an added electron donor (Supporting Information, Table S2). Additionally, even after preincubating the harvested cells with fumarate (in order to deplete the stored organic carbon by its oxidation and concurrent reduction of the fumarate to succinate) in cell suspension experiments the cells still transferred the same amounts of electrons to HS (Table S2). Since we ruled out potential artifacts stemming from lysing cells (see above), we conclude that the cells either stored electron equivalents that can not be easily depleted or the cells oxidized small organic molecules (such as amino acids or sugar molecules) released from the structurally heterogeneous HS. It is well-known that humic acids contain a significant amount of labile amino acids (*24*) that could potentially be released and used by microorganisms. We believe that this is a relevant process that has to be considered in similar types of experiments with HS.

Comparison of Microbial and Chemical Reduction of Reference and Commercially Available HS. In earlier studies investigating redox properties of HS, HS were reduced chemically and the obtained data were used to interpret and understand microbially catalyzed redox reactions of HS (*5, 17, 25*). However, it is unclear whether chemical and microbial processes reduce the same functional groups in HS. Therefore in this study, we compared the extent of chemical to that of microbial reduction of both aquatic HS purchased from the IHSS and commercially available humic acids. Additionally, these experiments allow evaluating whether commercial humic acids can be used as representatives for natural humic compounds participating in biogeochemical processes.

Our results show that all humic compounds investigated were able to transfer a significant amount of electrons to Fe(III) provided in form of ferricyanide with the relevant redox couple (E_0') of $[Fe(CN)_6]3^-/[Fe(CN)_6]4^-$ at +430 mV before and after chemical or microbial reduction (Figure 4A). Electron transfer to ferricyanide (and other Fe(III) compounds such as ferric citrate and even ferric hydroxide) even without reduction despite storage of the humic compounds under oxic conditions in the laboratory was described earlier (*7, 17, 18, 26*) and can be interpreted 2-fold. Either these humic compounds contain reducing equivalents that are stable against oxidation with O_2 or after incubation with ferricyanide (or other Fe(III) compounds) the humic molecules change their 3-D structure, e.g., by complexation reactions with Fe(III) or by ligand-exchange reactions with cyanide from the ferricyanide. The 3-D structure change could expose previously unexposed redox-active functional groups rendering them accessible for the interaction with iron(III). Such an iron-dependent structure change during quantification of the redox properties of the humic substances could be avoided by using a noncomplexing electron acceptor such as molecular O_2 instead of Fe(III). Since all experiments were performed in the dark, we can rule out light-induced redox reactions.

For both IHSS and Aldrich humic acids, chemical as well as microbial reduction transferred a significant amount of electrons to the organic molecules indicated by the higher reducing capacities of the reduced vs the nonreduced (native) humic acids (Figure 4A). In contrast, the reduction of IHSS fulvic acids did not increase their reducing capacity significantly. Obviously, most of the redox active functional groups in the fulvic acids were already reduced as can be seen from the significant amount of electrons that was transferred by the native fulvic acids to iron(III). This is obvious when calculating the electron accepting capacities from the reducing capacities (as difference between reduced and nonreduced HS): SRFA showed a much lower electron accepting capacity compared to SRHA and Aldrich HA (Figure 4B). A lower electron accepting capacity of fulvic acids was described already by other authors (*3, 8*) and is probably due to the lower content of redox-active functional groups, e.g., quinones, (as indicated by data provided for the fulvic and humic acids by the IHSS).

When chemical and microbial reduction of aquatic HS and commercially available humic acids were compared, we found for all HS investigated that chemical reduction by H_2/Pd leads to reducing capacities similar to those of microbial reduction with approx 1 μeq/mg C for SRFA and 2.5−30 μequ/mg C for SRHA and Aldrich HA (Figure 4A). For peat and soil HS a similar behavior was described recently also by Peretyazhko and Sposito using not a pure strain of microorganisms but rather an indigenous population of soil microorganisms for microbial reduction (*18*). Considering the different redox potential (at pH 7) of the two different electron donors used in these experiments, $E_h^0 = -418$ mV for $2H^+/H_2$ (chemical reduction) and $E_h^0 = -0.28$ V for CO_2/acetate (microbial reduction), we can conclude that most redox-active functional groups in HS that are reduced chemically by H_2 are also bioreducible. We therefore conclude that chemically reduced HS can be used as representatives of microbially reduced natural humic substances. This has to be kept in mind, for example, when studying the effect of reduced humic compounds on pollutant transformation (*4, 17, 27*).

Aldrich humic acids are cheap and commercially available. They are highly humified and altered and contain large

amounts of inorganic impurities (*28*). Because of Aldrich humic acid's high humification degree and high aromatic content, one might expect a high content of redox-active aromatic constituents. However, in our experiments we observed that both microbial and chemical reduction led to reducing and electron accepting capacities in the same range as were obtained for IHSS humic acids. Reasons for that could be either the fact that they also contain a lot of impurities or that some of the aromatic constituents in Aldrich HA can not accept electrons (e.g., because these aromatic constituents are not quinones). From our data it seems that at least with regard to reducing and electron accepting capacities, Aldrich humic acids can be used as a model for humic acids from the environment (but not with regard to their chemical structure which is different from natural HS since Aldrich humic acids represent highly humified and altered organic compounds). Additionally, it has to be kept in mind that the amount of electrons accepted and transferred does not give any information about the "quality" of the electrons, i.e., the redox potential at which the electrons are accepted by or released from the HA.

Environmental Implications. Due to their reactivity and ubiquity in the environment, HS have the potential to greatly influence biogeochemical processes, and in particular, the electron flow and pollutant transformation in both terrestrial and aquatic environments. It was demonstrated that HS can be reduced by a variety of microorganisms such as fermenting, iron(III)-reducing, sulfate-reducing, and even methanogenic microorganisms (*3*−*7*), probably leading to the presence of reduced HS in many aquatic and terrestrial environments. And indeed, analysis of HS redox state in a freshwater sediment indicated that in the anoxic part of the sediment all HS were reduced to a significant extent (*7*). Since a variety of organic and inorganic pollutants such as chlorinated hydrocarbons and toxic metals (e.g., Cr(VI) and As(V)) can be reduced by reduced HS (*17, 29*−*32*), microbial transformation of HS can significantly influence the reductive transformation of pollutants in the environment. Additionally, Fe(II) associated with Fe(III) minerals is an effective reductant that was shown to reductively transform a variety of organic pollutants (*33*). Therefore, in environments that contain significant amounts of humic material and iron minerals, HS-stimulated, microbial reductive dissolution of Fe(III) (hydr)oxides that leads to the formation of mineral-bound, reactive Fe(II) species has also the potential to stimulate reductive transformations of pollutants.

Quantification of the kinetics of microbial HS reduction, a comparison to direct Fe(III) mineral reduction, and the determination of minimum concentrations of HS necessary for stimulation of microbial iron(III) (hydr)oxide reduction suggest that electron shuttling via HS can stimulate microbial electron transfer to solid-phase electron acceptors only in environments with at least 5−10 mg DOC/L. This therefore implies that electron shuttling via HS will not play a significant role in very DOC-poor rivers, lakes, and aquifers. Whether bacteria that are able to catalyze HS reduction are indeed present in environments that contain more than 5−10 mg DOC/L, which enzymes are responsible for the microbial reduction of the HS molecules, and whether solid-phase and mineral-sorbed HS also play a role in electron transfer remains to be answered in future experiments.

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Supporting Information Available

Reducing capacities and DOC of filtered *Geobacter sulfurreducens* cell suspensions (Table S1); reducing capacities of Aldrich HA after incubation with *G. sulfurreducens* (Table S2). This information is available free of charge via the Internet at http://pubs.acs.org.

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