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Rates and Extent of Reduction of Fe(III) Compounds and O_2 by Humic Substances

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Humic substances (HS) can be reduced by microorganisms and oxidized by electron acceptors such as Fe(III) or O_2. However, redox reactions between HS and highly crystalline Fe(III) minerals and O_2 have not yet been quantified. We therefore determined the rates and extent of goethite and hematite reduction by HS in comparison to those of dissolved and poorly crystalline Fe(III) compounds and O_2. Although nonreduced HS transferred significant amounts of electrons only to dissolved Fe(III) citrate and ferrihydrite, reduced HS additionally reduced goethite and hematite. The extent of reduction depended on the redox potentials of the Fe(III) compounds. Fewer electrons were transferred from HS to O_2 than to Fe(III) despite the more positive redox potential of the O_2/H_2O redox couple. Reoxidation of reduced HS by O_2 took place within minutes and yielded reoxidized HS that were still more reduced than nonreduced HS, indicating that some reduced moieties in HS are protected from reoxidation by O_2. Our data suggests (i) reduction of crystalline Fe(III) minerals by reduced HS has to be considered in the environmental electron transfer network, (ii) exposure of reduced HS to O_2 does not reoxidize HS completely within short time frames, and therefore, (iii) HS electron shuttling to Fe(III) can occur even in the presence of O_2.

Introduction

Humic substances (HS) are polymeric and polydisperse organic compounds present in almost all terrestrial and aquatic environments (*1*). They are redox-active, with the most important redox-active moieties in HS molecules being functional groups such as sulfuryl or phenolic groups and, in particular, quinoid moieties (*2*−*5*). Additionally, aromatic structures (*6*) and complexed iron were suggested to be involved in HS redox processes (*3, 4*).

Microorganisms capable of a variety of metabolic pathways such as fermentation, methanogenesis, and Fe(III) or SO_4^{2-} reduction are able to use HS as terminal electron acceptors (*7*−*10*). After reduction, electrons can subsequently be transferred from reduced HS to other electron acceptors with more positive redox potentials (E_h), for example, to solid-phase electron acceptors such as poorly soluble Fe(III) mineral phases to which direct microbial access is limited. Microorganisms can therefore overcome the limited bioavailability of these solid-phase electron acceptors, and many microorganisms with various metabolic capabilities can indirectly reduce such electron acceptors even at a distance from the cells. This electron shuttling process can contribute significantly to microbial electron transfer and in particular to Fe(III) reduction in natural environments (*7, 11*). HS can be reduced not only by microbes but also by electrochemical and chemical processes (*10, 12*). It has been shown that microbial and chemical reduction with H_2/Pd reduce HS to a similar extent (*9, 13*).

To date, laboratory experiments investigating abiotic redox reactions between HS and Fe(III) compounds have used predominantly poorly crystalline Fe(III) hydroxides ($Fe(OH)_3$) or even dissolved Fe(III) compounds such as ferricyanide $[Fe(CN)_6]^{3-}$, $FeCl_3$, or ferric citrate (*2, 6*−*8, 10*−*16*). However, these dissolved iron complexes are not present in nature at the concentrations used in these experiments. Additionally, their positive E_h values are substantially more favorable for reduction by HS compared to Fe(III) minerals whose E_h values become more negative with increasing crystallinity. In many soils and sediments, highly crystalline Fe(III) minerals such as goethite (α-FeOOH) or hematite (α-Fe_2O_3) represent a significant fraction of the Fe(III) compounds present. Even a modest reduction of these minerals can lead to a release of surface-bound toxic trace metals or the formation of highly reactive Fe(II) species adsorbed onto Fe(III) mineral surfaces, which can than participate in further redox reactions (*17*). The kinetics and extent of abiotic redox reactions between HS and higher crystalline iron(III) minerals have not yet been quantified.

At oxic−anoxic interfaces, oxygen (O_2) is expected to represent the main oxidant for reduced HS. As for crystalline Fe(III) minerals, the thermodynamics and kinetics of redox reactions between HS and O_2 have not been quantitatively followed. Wang and Newman recently showed that ferric (hydr)oxide reduction by redox-active antibiotics (phenazines), which also function as electron shuttles, can be inhibited by O_2, which consequently serves as a terminal electron acceptor in place of Fe(III) (*18*). It is unknown whether the reaction of reduced HS with the four electron acceptor O_2 yields HS molecules with the same redox state as oxidation with one electron-accepting Fe(III) compounds, in particular because the reduction of O_2 produces O_2 radicals as intermediates (*19*), which can potentially react with the HS molecules.

In order to fully assess the contribution of electron shuttling via HS within the electron transfer network in natural environments, it is necessary to understand HS reduction by bacteria as well as electron transfer processes from HS to electron acceptors such as O_2 and other environmentally relevant Fe(III) mineral phases like goethite and hematite. The aim of this study therefore was to quantify electron transfer from reduced and nonreduced HS to a variety of Fe(III) minerals (2-line ferrihydrite, goethite, and hematite) in comparison to dissolved Fe(III) citrate and O_2. These electron acceptors cover a wide range of $E_h^{0\prime}$ values from −287 mV to ∼0 mV for Fe(III) minerals, +372 mV for Fe(III) citrate (*20*), and +810 mV for O_2 (*19*) (Table S1 of the Supporting Information), allowing a comparison of the extent and kinetics of electron transfer with E_h of the electron acceptor. Finally, we quantitatively compared the abilities of Fe(III) and O_2 to reoxidize reduced HS.

Materials and Methods

Source of HS and Preparation of HS Solutions. Pahokee peat humic acid reference (PPHA) was purchased from the International Humic Substance Society (IHSS). Aldrich humic acid (AHA) was purchased from Aldrich. Nonreduced humic acid (HA) stock solutions (0.5 g L^{-1}) were prepared in phosphate buffer (50 mM, pH 7) according to Jiang and

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Kappler (diluted to 0.3 g L^{-1} in the experiments) (*9*). HA solutions were reduced chemically with H_2/Pd as described previously (*11*) and filtered (cellulose acetate, 0.22 μm, Fisher Scientific, Germany). Solubility of HA solutions in 50 mM phosphate buffer was ~100% for PPHA and ~70% for AHA. HA solutions were kept in the dark for most times of the experiments except for certain procedures such as sampling and aeration experiments (see below). In an independent experiment, electron spin resonance (ESR) measurements showed no significant differences in the ESR spectra of reduced PPHA solutions before and after exposure to light (not shown), suggesting that the short exposure time and low light intensity in our experiments did not induce any organic radical formation that could have influenced the subsequent redox processes of the HA with O_2.

Preparation of Fe(III) Compounds. Ferric citrate ($FeC_6H_5O_7 \cdot H_2O$, 0.5 M) was dissolved in boiling Millipore water followed by adjustment to pH 7 with 10 M NaOH. After filtration (see above) the solution was autoclaved and stored under N_2 at room temperature in the dark. Ferrihydrite ($Fe(OH)_3$) was prepared according to Cornell and Schwertmann (*21*) by neutralization of a 200 mM $Fe(NO_3)_3$ solution with 1 M KOH (final pH 7.5). The end product was identified as 2-line ferrihydrite by X-ray diffraction (XRD, data not shown). Goethite (α-FeOOH, Bayferrox 920Z, Figure S3A of the Supporting Information) was purchased from LANXESS (Leverkusen, Germany). α-FeOOH was purified by extraction with the reducing agent hydroxylamine hydrochloride (HAHC) for 30 min at 50 °C in order to remove poorly crystalline and thus more soluble Fe(III) (*22*). Results obtained with the HAHC-extracted mineral were similar to those determined with the nonextracted α-FeOOH (not shown). Therefore, the purification step was simplified by washing of all minerals three times with Millipore water. After the last washing, the minerals were air-dried and stored at room temperature in the dark until further use. Hematite (α-Fe_2O_3, Figure S3B of the Supporting Information) was tempered (1 h heating up, 2 h at 900 °C) from magnetite (Fe_3O_4, Bayoxide E 8710). Purity of all minerals was verified by XRD measurements. Minerals were suspended in Millipore water to a concentration of 5 mM, which was diluted to 1.7 mM in the experiments. For long-term experiments (>1 week) with goethite and hematite, all laboratory equipment and mineral suspensions were autoclaved (121 °C, ~2 bar) or sterilized in an oven at 180 °C for 4 h. XRD measurements after autoclaving showed that goethite did not change its mineralogical identity. Mineral changes of hematite due to autoclaving were not expected (*21*). Surface area of the Fe (III) minerals was determined via BET analysis with N_2 (Gemini 2375 surface area analyzer).

Quantification of Reducing Capacities and Reduction Rates of HS using Different Fe(III) Compounds as Electron Acceptors. For the quantification of their reducing capacities (RC, amount of electrons transferred from HS to an electron acceptor; here, Fe(III) compounds), nonreduced and reduced HA solutions were incubated with Fe(III) compounds. Anoxic aqueous solutions (Fe(III) citrate) or suspensions (Fe(III) minerals) were added to nonreduced HA solutions in an anoxic and H_2-free glovebox (MBraun Unilab glovebox with 100% N_2, two separate integrated purification systems for H_2O and O_2 and continuous positive pressure of N_2). Control experiments were performed in parallel with HA and water (without an Fe(III) compound) or with the corresponding Fe(III) compound with phosphate buffer only (without HA), in order to quantify any Fe(II) leaching from HA or present in the Fe(III) compounds.

The bottles were closed with O_2 tight butyl rubber stoppers, which were boiled several times in deionized water to remove any possibly reactive contaminations (e.g., H_2S). The bottles were then incubated outside of the glovebox on an overhead shaker (10 rpm) in the dark at room temperature, with the exception of experiments with Fe(III) citrate, which were conducted completely in the glovebox because of short incubation periods (1 h). Samples from all setups were taken under sterile conditions at several time intervals, and rates of Fe(II) formation as well as HA adsorption onto mineral surfaces were determined. Total Fe(II) (adsorbed and dissolved) was determined by the spectrophotometric ferrozine assay (*23*). Dissolved organic carbon (DOC) was quantified using a DOC analyzer (Elementar High TOC, Hanau, Germany). Because of the careful exclusion of O_2 in our experiments, the amount of Fe(II) produced equals the amount of electrons transferred from HA to Fe(III) and can be used as a quantitative measure for HA electron transfer.

RCs were normalized to mass of dissolved HA in grams present in the experiments. Experiments were terminated when no further increase in Fe(II) concentration could be detected. After completion of long-term experiments, we tested representative setups for the presence of microorganisms, using a fluorescent dye, i.e., DAPI (4′,6-diamidino-2-phenylindol) and microscopy. Microbial cells were not detected in any bottles tested. For comparison of RCs with E_h values of the Fe(III) compounds, we used the amount of transferred electrons at the last sampling point of the reaction. Reduction rates were calculated by linear regression of the initial slope of Fe(III) reduction.

Quantification of O_2 Reduction by HS. An O_2 electrode (OX1LP dissolved O_2 package, Qubit Systems, Ontario, Canada) was used to quantify electron transfer from AHA and PPHA to O_2. Electron transfer was followed over time via quantification of O_2 consumption (for more details see Figure S1 of the Supporting Information).

Quantification of HS Reducing Capacities after Reoxidation by O_2 (Aeration Experiment). Reduced and nonreduced IHSS Pahokee peat humic acid (PPHA) solutions were prepared in several parallel vials. Three vials containing nonreduced and three vials containing reduced HA were used directly to quantify the RC of nonreduced and reduced PPHA. A series of vials containing reduced HA were opened synchronously and exposed to air in the light under vigorous stirring in order to maximize aeration (O_2 concentration was not limiting throughout the experiment), and reoxidation of the reduced HA. At certain time points (after 1, 5, 15, and 60 min of aeration), three of the reoxidized vials were closed simultaneously and immediately deoxygenated by flushing with N_2 to stop the reoxidation process. When reoxidation of the last sample (60 min) was finished, RCs of all samples were quantified. To this end, samples were incubated with Fe(III) citrate for 1 h, followed by a photometric ferrozine assay for quantification of Fe(II) production (*23*). A detailed method description of the quantification of RCs using Fe(III) compounds as electron acceptors is given above.

Results and Discussion

Rates and Extent of Fe(III) Reduction by HS. In the past, abiotic redox reactions between Fe(III) and HS were typically assessed using only poorly crystalline or dissolved Fe(III) compounds. In order to evaluate the significance of HS redox processes involving other environmentally relevant Fe(III) phases, we quantified electron transfer from HS to goethite and hematite in comparison to poorly crystalline ferrihydrite and Fe(III) citrate.

Nonreduced PPHA transferred significant amounts of electrons only to ferrihydrite [58 ± 5 μeq (g HA)$^{-1}$] and to dissolved Fe(III) citrate [53 ± 3 μeq (g HA)$^{-1}$] but not to goethite or to hematite (Figure 1). Reduced PPHA, however, transferred more electrons to ferrihydrite and Fe(III) citrate, and in contrast to nonreduced PPHA, they also reduced goethite and hematite. The fact that Fe(II) was detected in setups with reduced PPHA and goethite and hematite but not with nonreduced PPHA suggests that reduction of the

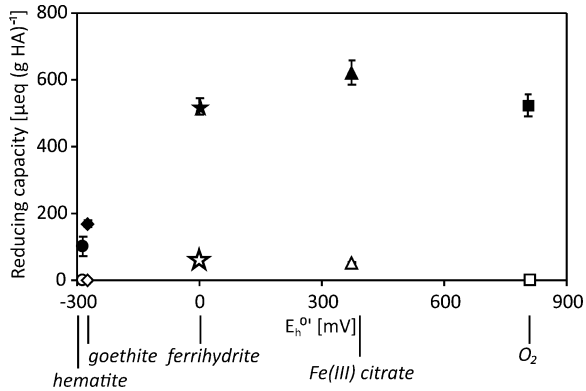

FIGURE 1. Reducing capacities, i.e., amount of electrons transferred from HS to Fe(III) and O_2, of nonreduced (open symbols) and reduced IHSS Pahokee peat HA (closed symbols) are plotted against standard redox potentials of the electron acceptors at pH 7 and 25 °C. Standard deviations are calculated from three independent replicates. $E_h^{0\prime}$ values used were +372 mV for Fe(III) citrate, 0 mV for ferrihydrite, −274 mV for goethite, −287 mV for hematite (*20*), and +810 mV for O_2 (*19*).

(solid) Fe(III) minerals and not reduction of Fe(III) solubilized from these minerals by HS or phosphate buffer occurred because nonreduced PPHA were shown to be able to reduce dissolved Fe(III), e.g., present in form of Fe(III) citrate. The extent of Fe(III) reduction by reduced PPHA increased in the order of hematite < goethite < ferrihydrite < Fe(III) citrate with 102 ± 29 μeq and 169 ± 10 μeq electrons transferred per gram of HA to hematite and goethite, respectively, and even more electrons being transferred to ferrihydrite [521 ± 25 μeq (g HA)$^{-1}$] and dissolved Fe(III) citrate [621 ± 36 μeq (g HA)$^{-1}$]. In all of the experiments, the electron acceptor (Fe(III)) was in excess relative to the electron donor (HA) and at maximum, about 12% of the initially present Fe(III) was reduced to Fe(II). The RC values measured in our experiments were in a similar range as RCs quantified by Ratasuk and Nanny (*16*) who give values up to about 400 μeq (g HA)$^{-1}$ for reduction of Fe(III) citrate by PPHA. Different experimental conditions (pH 8, 300 mM phosphate buffer) may be the reason for their slightly lower values. It is known that in systems with high ionic strength, HS can change their spatial structure resulting in smaller particle sizes (*24*), which might lead to decreased reactivity due to a change in accessibility of redox-active functional groups. Since their phosphate buffer concentration was 6-fold higher than in our experiments, this could be an explanation for the observed differences in redox activities.

In order to quantify any Fe(II) leaching from PPHA, they were incubated with Millipore water instead of Fe(III) compounds. We observed a difference in the amount of Fe(II) leached from reduced and nonreduced HA with more Fe(II) leaching out of reduced HA (Figure 2). During reduction, some structural Fe(III) present in the HA obviously was reduced to Fe(II) either directly by Pd/H_2 or indirectly via reduced functional groups in HA. Fe(II) is bound less strongly in complexes with organic ligands than Fe(III) and can therefore be leached more easily (*25*). Since the concentrations of Fe(II) quantified in the presence of nonreduced HA, goethite, and hematite are in the range of the concentrations of Fe(II) leached from PPHA alone (Figure 2), we conclude that nonreduced HA did not transfer significant amounts of electrons to goethite and hematite.

In order to see whether the reduction of crystalline Fe(III) minerals could also be performed by HA other than PPHA, we incubated AHA with goethite and hematite. We also observed reduction of both minerals by reduced AHA, however, to a lower extent compared to reduction by PPHA

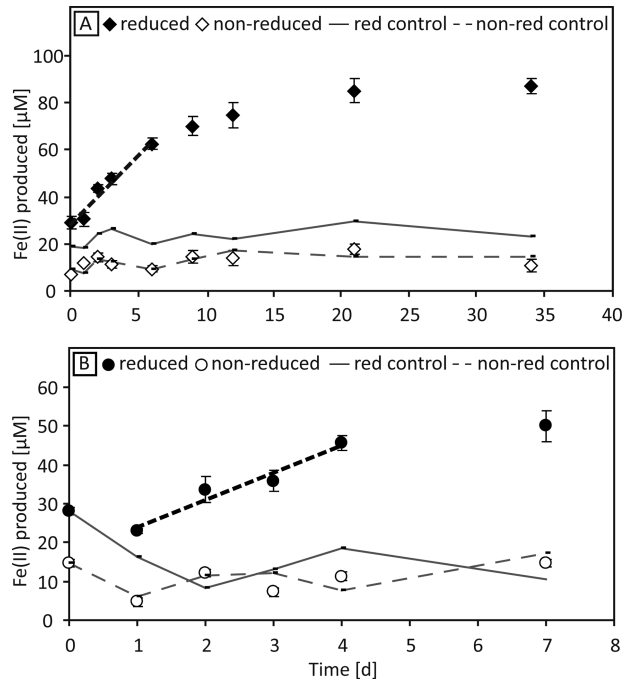

FIGURE 2. Kinetics of reduction of goethite (A) and hematite (B) by nonreduced (open symbols) and reduced (closed symbols) IHSS Pahokee peat HA. Standard deviations were calculated from three independent parallels. Solid and dashed lines represent Fe(II) from control experiments, where reduced (solid line) and nonreduced PPHA (dashed line) were incubated with water only instead of Fe(III) to quantify Fe(II) leaching from HA. The thick black dashed line represents the linear regression from which reduction rates were calculated. Note the different scales on the axes of both diagrams.

with a maximum of 55−60 μeq (g HA)$^{-1}$ for goethite and hematite within 29 days (after subtraction of Fe(II) leached from the reduced AHA themselves). Fe(II) leaching from reduced AHA was significant because of the high content of structural iron present in AHA with values approximately 3−4-fold higher than that measured for PPHA. Nonreduced AHA transferred negligible amounts of electrons to goethite and hematite.

Besides electron transfer and leaching processes, cation exchange reactions could also have been responsible for the detection of Fe(II). Fe(III) ions from the Fe(III) electron acceptors could have released Fe(II) (and other possibly redox-active cations) from HA by cation exchange potentially leading to a slight overestimation of RCs. However, we assume this process did not significantly influence our results because Fe(III) citrate is expected to lead to a higher extent of cation exchange than ferrihydrite (due to the presence of the high concentration of dissolved Fe(III) in the case of ferric citrate compared to the Fe(III) mineral ferrihydrite), but RCs of nonreduced PPHA with both electron acceptors are almost the same. Additionally, other redox-active metal impurities such as Cr(VI) or Cu(II) could have been reduced by H_2/Pd and could have affected the measured RCs ascribed to HS. However, because of the harsh isolation and purification conditions of PPHA (including a hydrofluoric acid treatment), we believe that the concentration of such trace metals is low, and their contribution to PPHA redox activity is minor.

Reduction rates also varied for the different Fe(III) compounds indicated by the differences in production of Fe(II) over time. Immediately after mixing either Fe(III) citrate or ferrihydrite with PPHA solution, Fe(II) formation reached a plateau, followed by only a small increase in Fe(II) concentration over the next minutes (Fe(III) citrate) or hours (ferrihydrite). For Fe(III) citrate, Fe(II) was therefore quanti-

fied after 1 h, when Fe(III) reduction was complete. Fe(II) production from ferrihydrite reduction was measured after 24 h. The kinetics for the reduction of Fe(III) citrate and ferrihydrite were too fast to determine initial reduction rates accurately. In contrast, reduction of hematite and goethite was completed after one week and three weeks, respectively (Figure 2). For goethite and hematite, we quantified HA mass-normalized initial (maximum) reduction rates of 17.5 μmol (d g HA)$^{-1}$ and 21.1 μmol (d g HA)$^{-1}$, respectively (Figure 2). Calculating reduction rates normalized to the surface area of the corresponding minerals yielded values of 4.3 μmol (d m^2)$^{-1}$ for goethite and 11.7 μmol (d m^2)$^{-1}$ for hematite. Goethite reduction was found to occur slower than hematite reduction.

Redox Potentials of Fe(III) and HS and Their Influence on Electron Transfer. As can be observed in Figure 1, the extent of electron transfer from reduced PPHA to Fe(III) compounds depended on the crystallinity and solubility and therefore on the E_h of the electron acceptor. With increasing crystallinity the solubility of an Fe(III) mineral decreases (*26*). This in turn influences the redox potential of a mineral and consequently, as expected, more electrons were transferred from reduced PPHA to Fe(III) compounds with increasing solubility. In contrast to the extent of reduction, reduction rates did not only depend on the E_h of the Fe(III) compounds used as electron acceptor. As expected due to their positive E_h values, Fe(III) citrate and ferrihydrite reacted very fast. However, goethite reduction by PPHA occurred slower than hematite reduction and the system took longer to reach equilibrium although goethite has a more positive and thus more favorable E_h for reduction. This is in contrast to the study of Bauer et al. (*27*) who showed that thermodynamics as well as reduction kinetics are linked to the E_h of the electron acceptor. However, except for ferrihydrite, these authors carried out experiments only with dissolved Fe(III) complexes. In our experiments using crystalline goethite and hematite as Fe(III) phases, surface processes influencing electron transfer from HA might have taken place and could potentially explain our results (see next section).

Drawing conclusions about the effects of mineral redox potentials on reduction rates/extent is nontrivial since E_h values of Fe(III) minerals are not well-defined. Furthermore, it is unclear to which extent the bulk E_h of the mineral represents the E_h relevant for the processes occurring at the mineral surface. However, even if our experimentally determined RCs cannot be attributed to defined Fe(III) mineral redox potentials, a clear tendency (i.e., increasing extent of reduction of Fe(III) compound with increasingly positive E_h) becomes obvious.

Describing the redox potentials of HS is even more complex. The E_h of HS is a composite redox potential of different overlaying E_h values due to the presence of a number of redox-active functional groups in HS, each one having its own characteristic E_h. Although the E_h values for HS are not easily determined experimentally, a variety of redox potentials can be found in the literature. E_h values given for HS at pH 7 extend from −200 mV to +500 mV (*28*−*31*). More specific values such as +700 mV (pH 0) or +778 mV (pH 5) have also been suggested using a variety of oxidants (*3, 32*). However, some of these E_h values were determined by titrations either in organic solvent-systems or using oxidants with a very high E_h, e.g. iodine with an $E_h^{0\prime}$ of up to +985 mV (*3*), which are not representative of environmentally relevant oxidants.

Our results show that even in HA that were not reduced chemically before the experiments, some reduced functional groups were present transferring electrons to Fe(III) citrate and to ferrihydrite. These intrinsically reduced functional groups were not oxidized by O_2 during storage of the HA. Thus we conclude that their E_h was negative enough to reduce the Fe(III) compounds with very positive E_h values, (Fe(III) citrate and ferrihydrite) but not negative enough to reduce goethite and hematite.

Influence of Mineral Surface Processes on Fe(III) Mineral Reduction by HS. Besides the redox potentials of Fe(III) compounds and HS, specific interactions of the surface of Fe(III) minerals with HS molecules and Fe(II) produced during the electron transfer are expected to influence electron transfer processes. HS have a strong affinity to Fe(III) mineral surfaces (*33, 34*). In our experiments, we observed sorption of HA to the surfaces of all three Fe(III) minerals (ferrihydrite, goethite, and hematite). We found no differences in sorption behavior between nonreduced and reduced HA (Figure S2 of the Supporting Information). This suggests that the lower RCs of nonreduced HA are not caused by a limited contact area between HA and Fe(III) minerals but in fact by the difference in the amount of electrons available in nonreduced HA versus reduced HA. The amount of HA sorbed also did not change significantly during Fe(III) reduction (Figure S2 of the Supporting Information). In addition to HS, Fe(II) sorbs onto Fe(III) mineral surfaces. This can lead to mineral transformation (*35*) and to formation of reactive Fe(II) species (*36*) or secondary minerals, which were shown to passivate the surfaces of Fe(III) minerals during microbial Fe(III) reduction (*17*). These surface processes can modify the mineral surfaces and thus influence HS electron transfer.

The influence of the mineral surface properties on HS electron transfer also becomes obvious from the biphasic behavior of goethite reduction (Figure 2A). This indicates that Fe(III) mineral reduction might be controlled either by HA fractions or Fe(III) mineral surface sites of different reactivity (e.g., one highly reactive and one less reactive fraction). However, since electron transfer to Fe(III) citrate was essentially instantaneous and the reoxidation by O_2 also was very rapid, this suggests that there are not more or less reactive functional groups in HS but instead the reactivity was controlled by the goethite surface. A similar two-phasic reaction behavior was also described by Royer et al. (*37*), who performed experiments following bioreduction of a highly crystalline Fe(III) mineral using NOM as electron shuttles. They also detected two phases during Fe(III) mineral reduction and suggested the first phase to be under kinetic control (limited by the dissolution of Fe(II) from the crystal lattice), while the second phase might be controlled by a different factor, possibly a thermodynamic mechanism (caused, for example, by high concentrations of Fe(II) accumulating at the mineral surface, leading to unfavorable thermodynamic conditions for further reduction). Surface processes could also explain that despite the lower E_h of hematite its reduction rate was faster than the rate of goethite reduction. Since we observed no changes in the amount of HA sorbed during Fe(III) mineral reduction (Figure S2 of the Supporting Information), it is plausible that no mineral transformation or secondary mineral formation took place or that the surface properties (with regard to HA sorption) of the new mineral phases were very similar to the original mineral surface but sufficiently different to influence goethite and hematite reduction rates. These surface remodeling processes could explain the slower reduction rate in the goethite system and the extended time period needed to reach equilibrium compared to hematite (Figure 2) despite its more favorable redox potential for reduction in our experiments. In other studies, it was shown that hematite solubility increases with increasing hydration times, leading to solubilities comparable to goethite or even hydrous ferric oxides (*38*). However, we assume this process did not play a significant role in our experiments because of the following two reasons. First, incubation times in our experiments were much shorter compared to their study. Second, we detected no Fe(II) formation after incubation of hematite with nonreduced HA, suggesting the absence of dissolved Fe(III)

since the presence of dissolved Fe(III) should have led to detectable RCs of nonreduced HA (as was the case in the Fe(III) citrate experiments).

Reoxidation of Reduced HA by O_2. Oxygen plays a crucial role for redox processes at oxic−anoxic interfaces in the environment. To date, the kinetics and extent of redox reactions between O_2 and HS have not been quantified. It is unknown whether the oxidation of HS by O_2 yields the same HS redox state as the oxidation of HS by Fe(III). We therefore quantified the extent of HS oxidation by O_2 and compared these results to oxidation of reduced HS by Fe(III).

When we added reduced AHA or PPHA solution to O_2-saturated water or phosphate buffer, O_2 was consumed rapidly, and the maximum O_2 consumption was reached within approximately 3 min (data not shown). This implied rapid reoxidation of reduced functional groups in HA by O_2. In contrast, nonreduced HA did not consume any O_2, suggesting that no electron transfer from nonreduced HA to O_2 occurred. This was anticipated because the HA used for these experiments were stored in dried form under O_2 for several months. Therefore, all redox-active groups that can be oxidized by O_2 probably had reacted with O_2 already. However, it is known that when nonreduced HS stored in a dry state under oxic conditions for several months or even years (e.g., commercially available IHSS or Aldrich HS) are incubated with certain Fe(III) compounds, Fe(II) can be detected (Figure 1 and refs *9, 12)*. This suggests either the persistence of some reduced HS functional groups that cannot be oxidized by O_2 or the presence of cation exchange effects (although we believe that the latter are of minor importance, see above). We can also conclude that no detectable amounts of O_2 were absorbed by HA from the fact that the addition of nonreduced HA solutions yielded no consumption of O_2. There was also no significant difference in the results whether the HA solutions were added to O_2-saturated water or phosphate buffer.

The RCs of PPHA and AHA solutions (at different HA concentrations), using O_2 as an electron acceptor on a per gram of dissolved HA basis, yielded values in a very narrow range between 475 ± 76 (1 g L^{-1}) and 571 ± 90 (0.5 g L^{-1}) μeq (g HA)$^{-1}$ for AHA (Figure 3A). For PPHA, RC values ranged from 497 ± 29 (1 g L^{-1}) to 581 ± 43 (2 g L^{-1}) μeq (g HA)$^{-1}$ (Figure 3A). This showed that the RC of reduced HA was independent from HA concentrations. It is known that with varying concentrations of dissolved HS the spatial structure of HS changes (*39*) and, therefore, potentially also the exposure of redox-active functional groups and HS redox activity. However, from our results, we conclude there were either no changes in the HS spatial structure in the concentration range tested (0.5−2 g L^{-1}) or that the changes did not lead to changes in HA redox activity.

Reducing Capacities of Reduced HS after Reoxidation with O_2. In order to assess the redox state of reduced HA after reoxidation by O_2, we incubated reduced, nonreduced, and O_2-reoxidized PPHA with Fe(III) citrate. While the RCs of reduced HA (before reoxidation) using Fe(III) citrate as the terminal electron acceptor yielded around 650 μeq (g HA)$^{-1}$, after exposure to air for one minute reduction capacities of the HA decreased to around 220 μeq (g HA)$^{-1}$ and did not decrease further within 1 h of exposure to O_2 (Figure 3B). Obviously, the reoxidation process reached stable values almost immediately after aeration, demonstrating the sensitivity of reduced HA toward reoxidation by O_2.

However, the reduced and then reoxidized HA solutions transferred almost 4 times as many electrons to Fe(III) citrate than the ones that were not reduced before (nonreduced HA) but were stored under O_2 for several months [61 μeq (g HA)$^{-1}$]. This shows that O_2 was not able to fully reoxidize chemically reduced HS within the time frame of our experiment. While some reduced functional groups obviously

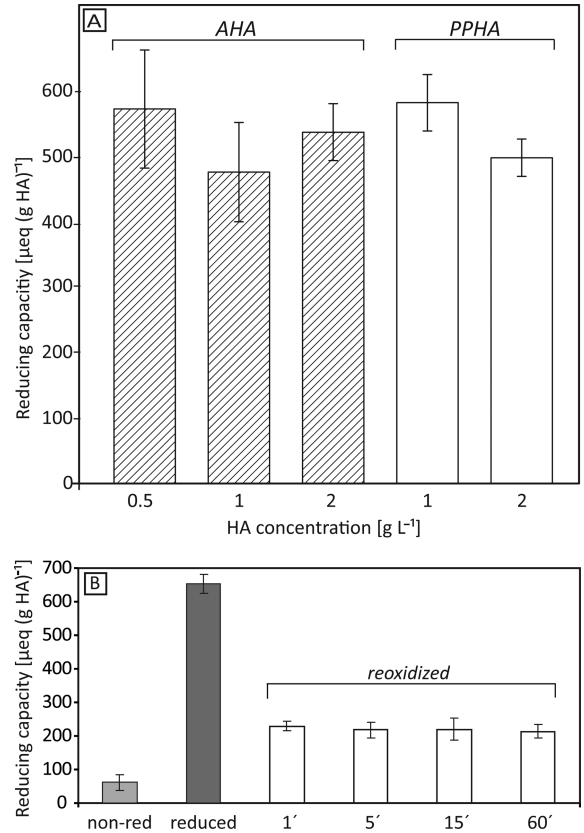

FIGURE 3. (A) Reducing capacities of reduced Aldrich HA (AHA, hashed bars) and IHSS Pahokee peat HA (PPHA, white bars) determined at different HA concentrations (0.5, 1, and 2 g L^{-1} for AHA, 1 and 2 g L^{-1} for PPHA), using O_2 as electron acceptor. RCs were calculated from at least two parallels. (B) Reducing capacities of nonreduced (light gray), reduced (dark gray), and reoxidized (white) PPHA determined with Fe(III) citrate as the terminal electron acceptor. Numbers (i.e., 1′, 5′, etc.) indicate minutes for which reduced HS solutions were exposed to air for reoxidation. Experiment was performed in triplicate. All reducing capacities (in A and B) are normalized to a per gram of dissolved HA basis.

are oxidized instantaneously, other reduced functional moieties remained reduced in the presence of O_2 (at least within 1 h of O_2 exposure) and were then able to transfer their electrons to Fe(III) citrate. It is expected that the nonreduced and reoxidized HS reveal similar redox properties since they were both oxidized by the same oxidant (O_2). However, the first difference between these two oxidized HA fractions is that exposure times vary from hours to months or even years. This could indicate different fractions of redox-active functional groups in humics: one that is rapidly (almost instantaneously) reoxidized by O_2 and a second that is reoxidized more slowly, maybe within months and years. Second, the nonreduced fraction was stored and exposed to O_2 as a dry powder until immediately before dissolution and further processing within the experiment. In contrast, the O_2-reoxidized fraction was first dissolved, then reduced, and then exposed to O_2 in solution. Therefore, we hypothesize that during dissolution of dried HS followed by reduction and reoxidation by O_2 or Fe(III) structural rearrangements in the spatial structure of the HA may have taken place as suggested previously (*40*). These modifications may potentially lead to differences in exposure and thus reactivity of reoxidized HS functional groups. As a consequence, some reduced moieties that would be available for oxidation by O_2 in a dried state could be protected from reoxidation in the HS structure when HA are in a dissolved state. Support for this hypothesis comes from a previous study in which the

existence of cage-like, pseudomicellar structures in HS was shown to protect fluorophores from quenching by bromide (*41*).

In order to serve as an environmentally relevant electron shuttle, it is essential that HS can undergo a reduction−reoxidation cycle several times. Therefore, no irreversible alterations of HS molecules must take place, and their ability to shuttle electrons has to be sustainably preserved. Ratasuk and Nanny (*16*) showed for a variety of commercially available HS that their RCs always yielded the same values after each of 5 cycles of reoxidation (with air) and chemical reduction (H_2/Pd), suggesting that both processes are completely reversible. Combining their and our results, we hypothesize that the first chemical reduction step after dissolution followed by reoxidation changes the spatial structure of HA molecules and their redox properties slightly compared to the nonreduced state. After this first change, further redox reactions are then reversible.

Comparison between HS Oxidation by O_2 and Fe(III). On average only about $530 \pm 55\,\mu$eq $(g\,HS)^{-1}$ were transferred to O_2 by PPHA—much less than expected according to the E_h of the O_2/H_2O redox couple, which is significantly more positive than those of all of the Fe(III) compounds (Figure 1). A potential reason for the higher RC values in the experiments using Fe(III) as an electron acceptor compared to those of O_2 could be cation exchange reactions in experiments with Fe(III) that could lead to a release of Fe(II) from the PPHA, a process that is not possible when using O_2. This could have led to an overestimation of RC when using Fe(III) as the electron acceptor. However, as discussed above, we believe that these processes did not play a significant role in our experimental system. A better explanation for the lower RC measured for O_2 than for the Fe(III) phases could be that the complete reduction of O_2 to H_2O consists of four single electron transfer steps producing intermediate species with varying reactivity. The first electron transfer step has a rather negative $E_h^{0\prime}$ of −160 V and yields the hyperoxide radical, $O_2^{-\bullet}$ (*19*). The low E_h of this first step in the reduction chain is probably one reason why O_2 is not a good oxidant for quinone moieties in HS (*42, 43*). From this initial step, further reduction to H_2O is facilitated due to the more positive E_h values of the following reaction steps (*19*). In addition to this thermodynamic explanation for the low reactivity of O_2 toward HS, kinetic arguments also suggest unfavorable redox reactions of O_2 with HS. Molecular O_2, which is in a triplet state, has two unpaired electrons and is therefore not a very good oxidant when reacting with organic molecules that are in a singlet state (*43*).

Numerous reactive oxygen species (ROS) are produced during reduction of O_2 to H_2O (e.g., $^1O_2$, $O_2^{-\bullet}$, O_2^{2-}, $OH^\bullet$, and H_2O_2) (*19, 44*) which can easily react with HS molecules. It is possible that of all potential reactions mainly the initial electron transfer step to O_2 (yielding the $O_2^{-\bullet}$ radical) is mediated by HS. This initial reaction is then followed by a reaction chain driven by ROS that can lead to humic radical formation. Evidence for such reactions potentially comes from the high concentrations of radicals measured in O_2-exposed HS (*2*). However, there are no indications that the ROS formed lead to any polymerization or degradation processes of the HS molecules affecting their redox activity. Ratasuk and Nanny showed that after several cycles of chemical reduction and subsequent reoxidation under air a variety of commercially available HS yielded unchanged RCs as mentioned above (*16*).

Environmental Implications. Our results show that the rate and extent of electron transfer from HS to Fe(III) compounds is strongly influenced by the identity of the Fe(III) compound. Reduced HS are not only able to reduce dissolved or poorly crystalline Fe(III) compounds but also highly crystalline Fe(III) minerals. Goethite and hematite showed slower reduction rates and smaller extent of reduction than dissolved and poorly crystalline Fe(III) compounds. However, the reducing power of HS also depends on the biogeochemical conditions present in a natural system, e.g., on the ratio of reduced to oxidized HS. When only a few microbial cells deliver reducing equivalents to HS and the ratio of reduced to oxidized HS is low, Fe(III) reduction might cease rapidly. However, when many metabolically active (HS-reducing) microbial cells are present that constantly produce reduced HS by electron transfer (keeping the ratio of reduced to oxidized HS high), the E_h of HS would be negative enough to sustainably reduce even crystalline Fe(III) phases. Consequently, HS can be important players in the redox cascade, particularly in electron donor- and microbe-rich anoxic environments and even in environments where highly crystalline Fe(III) minerals predominate.

For oxidation of HS by O_2, only very short contact times (seconds or minutes) are necessary. However, reoxidation of reduced HS by O_2 does not necessarily prevent HS from further electron transfer to other electron acceptors. Depending on the identity of the subsequent electron acceptor (e.g., dissolved Fe(III) complexes), there are still reduced functional groups present in the HS molecules that can transfer electrons even after reoxidation of reduced HS by O_2. At the same time, this means that in contrast to microbial metabolism, where aerobic respiration provides the highest energy gains for microbes, O_2 is not the strongest or most favorable oxidant for reduced HS. This suggests that electron shuttling via HS to Fe(III) might be possible even in the presence of O_2, for example, under conditions of rapidly fluctuating redox conditions such as near surface sediments or microbial mats. During the day when oxygenic photosynthesis is active, anoxic layers where HS reduction has taken place during the night become oxygenated. While O_2 could rapidly oxidize dissolved Fe^{2+}, the reduced HA could at least partially maintain their reduced redox state and then react with the Fe(III) formed by aerobic Fe(II) oxidation. Similar scenarios could be envisioned in soils during wetting and drying events.

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Supporting Information Available

Detailed experimental setups and measurements using an O_2-electrode (Figure S1), quantification of HA adsorption to Fe(III) minerals over the duration of Fe(III) reduction experiments (Figure S2), redox potential calculations of Fe(III) compounds (Table S1), and SEM images showing particle sizes and morphology of crystalline Fe(III) minerals (Figure S3).

This material is available free of charge via the Internet at http://pubs.acs.org.

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