

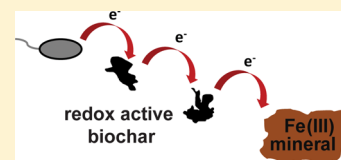
Biochar as an Electron Shuttle between Bacteria and Fe(III) Minerals

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S Supporting Information

ABSTRACT: Biochar influences soil fertility, N₂O emissions, and atmospheric CO₂ budgets, and because of its quinone and aromatic structures, it is redox-active. Here we demonstrate that biochar concentrations of 5 and 10 g L⁻¹ stimulate both the rate and the extent of microbial reduction of the Fe(III) oxyhydroxide mineral ferrihydrite (15 mM) by *Shewanella oneidensis* MR-1, while lower biochar concentrations (0.5 and 1 g L⁻¹) have a negative effect on ferrihydrite reduction. Control experiments showed that biochar particles and not biochar-derived water-soluble organic compounds are responsible for the stimulating and inhibiting effect. We also found that biochar changed the mineral product of ferrihydrite reduction from magnetite (Fe₃O₄) to siderite (FeCO₃). Our study suggests that biochar can influence soil biogeochemistry not only indirectly by changing the soil structure and chemistry but also by directly mediating electron transfer processes, i.e., by functioning as an electron shuttle.



INTRODUCTION

Biochar is a carbon-rich solid produced by thermal decomposition of biomass under a limited oxygen supply at ≤ 700 °C. Amendment of soils with biochar was used ~ 1000 years ago by Amazonian Indians for enhancing soil fertility; these soils are commonly known by the term Terra Preta (de Indio).¹ In the past years, biochar has attracted attention because of its potential to store carbon that would otherwise be mineralized to CO₂, thus influencing Earth's climate.^{2–4} Although biochar addition also has the potential to contaminate soils because of its polycyclic aromatic hydrocarbon and metal content, additional positive effects of soil biochar amendment such as increases in soil fertility and plant growth^{5–7} and decreases in N₂O emissions^{8–10} have been demonstrated.

These biochar effects are caused by its sorption capacity,^{11,12} pH,^{9,13} stability against biodegradation,^{3,4} and the resulting consequences for soil water holding capacity, pH, bulk density, nutrient availability,^{5,6,14,15} and thus the microbial community composition and activity.^{10,16,17} However, recently, a new but potentially significant property of biochar has been revealed. It has been demonstrated that biochar contains aromatic and quinone structures,^{18–21} biochar is redox-active,²² and biochar can participate in environmentally relevant abiotic redox reactions.^{23–27} Electrochemical analyses of biochars showed that depending on the origin of the organic material used for biochar production and the charring temperature, biochars can take up and release several hundred micromoles of electrons per gram of biochar.²²

The fact that biochar can accept and donate electrons could have significant consequences for biogeochemical cycles in soils. Dissolved and particulate soil organic matter (humic substances) can be reduced by a variety of microorganisms with various metabolisms^{28–34} and can transfer the electrons in an abiotic reaction to other electron acceptors such as Fe(III) minerals and molecular oxygen.^{22,28,33,35–38} However, it is currently unclear whether biochar can be reduced by

microorganisms and whether the electrons accepted by biochar can be further transferred to other electron acceptors, in particular to Fe(III) minerals. A contribution of biochar as an electron shuttle in denitrification and interspecies electron transfer has been suggested^{9,39} but has not been experimentally verified. The main goals of this study therefore were to determine whether the presence of biochar can influence the rate and extent of microbial ferrihydrite reduction and the identity of the mineral products formed during ferrihydrite reduction.

MATERIALS AND METHODS

Biochar. Biochar was produced by sieving residues of wood chip production via slow pyrolysis (620 °C) by Swiss Biochar (Belmont-sur-Lausanne, Switzerland). It carries the European Biochar Certificate (<http://www.european-biochar.org/en>) and is part of the COST Action-TD1107 biochar ring trial. Biochar properties are listed in Table S1 of the Supporting Information. The biochar was powdered to a particle size of 0.1–0.3 μm , although the analysis of particle volume (in percent) versus particle size also revealed a significant fraction of 3–30 μm particles (Figure S1 of the Supporting Information).

Microorganisms and Cultivation. *Shewanella oneidensis* MR-1 was cultivated in LB medium.⁴⁰ After incubation for 16 h, 100 mL of the culture was centrifuged (7197g for 5 min), and the cell pellet was washed four times with anoxic bicarbonate buffer (30 mM, pH 7) and suspended at a density of 2×10^{10} cells mL⁻¹ in the same buffer.

Ferrihydrite and Biochar Suspensions and Leachates. Ferrihydrite was synthesized as described previously.⁴⁰ Anoxic

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biochar suspensions were prepared by deoxygenating powdered biochar overnight in the evacuated vacuum chamber of an anoxic glovebox (100% N₂). Degassed biochar was suspended in anoxic Millipore water in the glovebox to final concentrations of 10, 20, 100, and 200 g L⁻¹. To disperse the biochar and remove the remaining oxygen, the suspensions were treated 10 min with an ultrasonic probe (150W, Sonopuls GM2200, Bandelin electronic, Berlin, Germany). Afterward, the biochar suspensions were degassed (1 min under vacuum), flushed with N₂ (3 min), and sterilized by being autoclaved (120 °C for 20 min). The leachate was autoclaved to maintain consistency with the experiments using particulate biochar that also had been autoclaved.

Biochar leachates were prepared by centrifuging biochar suspensions (10, 20, 100, and 200 g L⁻¹) through centrifuge filters (0.22 μm, Costar-Spin-X Centrifuge-Tube) inside an anoxic glovebox (5 min at 12045g). The leachate, which might contain some <0.22 μm biochar particles and/or colloids, was sterilized by being autoclaved (120 °C for 20 min).

Setup of Ferrihydrite Reduction Experiments. Cell suspension experiments were set up in triplicate in 16 mL tubes with 10 mL of 30 mM bicarbonate buffer (pH 7), ferrihydrite (15 mM), and sodium lactate (30 mM); 500 μL of the biochar suspensions (final concentration of 0.5, 1, 5, or 10 g L⁻¹), 500 μL of the biochar leachates, 800 μL of anthraquinone-2,6-disulfonic acid (AQDS, final concentration of 100 μM), or anoxic water as a control was added. To determine the potential toxic effects of the biochar, biochar (0.5, 1, 5, or 10 g L⁻¹) was added to an additional series containing 100 μM AQDS. All experiments, except abiotic controls, were amended with 2 × 10⁹ *S. oneidensis* MR-1 cells mL⁻¹. To quantify total Fe(III) and total Fe(II), 100 μL of the sample was withdrawn at different time points over 72 h, extracted with 900 μL of 1 M HCl (1 h), and centrifuged (0.22 μm centrifuge filters). Control experiments showed that all minerals that formed, including biogenic magnetite, could be dissolved in 1 M HCl.

Analytical Methods. Fe(II) and Fe(III) were quantified using the ferrozine assay.^{40,41} Fastest initial reduction rates [millimolar Fe(II) per hour] and corresponding standard deviations were determined by linear regression from the steepest slopes of the reduction curves for the three individual replicates (Figures S4 and S5 of the Supporting Information). Iron mineralogy was analyzed using X-ray diffraction (μXRD) and Mössbauer spectroscopy.^{42,43} The biochar reducing capacity [electrons that could be transferred to Fe(III)] was determined by the ferric citrate assay.³⁶ The biochar particle size was determined by laser diffraction (Malvern Mastersizer2000, Hydro2000S dispersion unit). Ferrihydrite and biochar aggregates were visualized via confocal laser-scanning microscopy (Leica TCS SPE system, Leica Microsystems, Wetzlar, Germany).

RESULTS AND DISCUSSION

Rates and Extents of Microbial Ferrihydrite Reduction in the Absence or Presence of Biochar. When *S. oneidensis* MR-1, a known Fe(III) mineral-reducing bacterium, was incubated with 15 mM ferrihydrite and lactate, we observed 58 ± 0.7% of Fe(II) formation within 72 h (Figure 1A). The addition of the known electron shuttle AQDS led to an initial rate of Fe(III) reduction (1.46 ± 0.42 mM/h) higher than the rate of 0.54 ± 0.15 mM/h without AQDS but to a similar extent of Fe(II) formation (59 ± 0.9%) as observed without AQDS. Stimulation of microbial Fe(III) reduction by AQDS

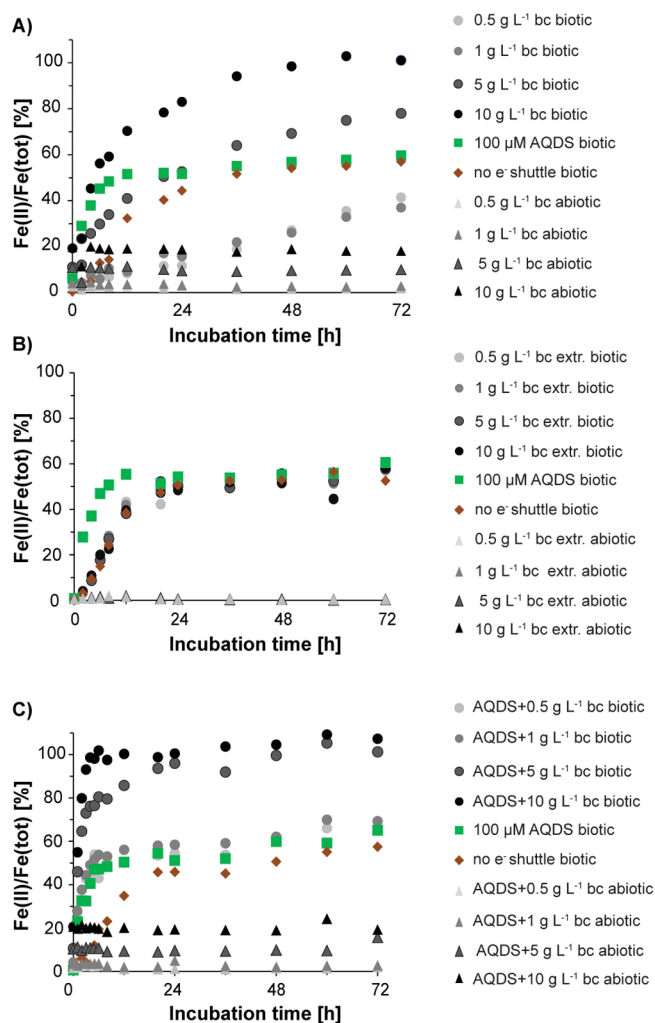


Figure 1. Reduction of 15 mM ferrihydrite by *S. oneidensis* MR-1. Formation of Fe(II) in tubes amended with (A) biochar (0.5, 1, 5, and 10 g L⁻¹) and (B) biochar leachates (extr.) at concentrations present in the tubes with 0.5, 1, 5, and 10 g L⁻¹ biochar. For comparison, results with AQDS as a known electron shuttle are shown (100 μM). (C) Formation of Fe(II) in AQDS–ferrihydrite setups amended with biochar (0.5, 1, 5, and 10 g L⁻¹). Data for sterile setups (no MR-1 cells added) are shown as triangles. Representative data sets for each setup are shown here to facilitate comparison. Full data sets of triplicate microbial experiments and duplicate abiotic controls are shown in Figure S4 of the Supporting Information.

via electron shuttling has been described previously.^{28,44–46} When biochar was added at concentrations of 5 and 10 g L⁻¹ to MR-1 inoculated with ferrihydrite and lactate, we observed not only a faster Fe(III) reduction rate (0.87 ± 0.19 and 1.49 ± 0.23 mM/h, respectively) compared to that of ferrihydrite, lactate, and MR-1 only but also a higher extent of ferrihydrite reduction (77 ± 1.2 and 103 ± 1.5%, respectively). In contrast, tubes with biochar concentrations of 0.5 and 1 g L⁻¹ showed rates and extents of reduction (0.12 ± 0.03 and 0.26 ± 0.09 mM/h and 40 ± 1.7 and 38 ± 1.1%, respectively) lower than those of the tubes with AQDS and rates and extents even lower than those of the tubes without an electron shuttle, suggesting an inhibiting effect of these two lower biochar concentrations on microbial Fe(III) mineral reduction.

To determine whether biochar particles themselves or water-soluble organic compounds released from the biochar are

responsible for the stimulation and inhibition effects, we incubated ferrihydrite with MR-1 and added biochar leachates at the same concentrations as in the biochar setups (Figure 1B). At all leachate concentrations tested, we did not see a stimulating or inhibiting effect compared to the effect in the tubes containing only ferrihydrite and MR-1. Abiotic setups showed that the leachates had no significant reducing capacity and electron transfer by the leachates to Fe(III) was negligible. These results suggest that the biochar particles and not dissolved compounds that can be extracted with water from biochar particles are responsible for the observed effects on microbial ferrihydrite reduction.

To determine whether the biochar particles cause any toxic or harmful effects to the cells, thus negatively influencing their metabolic activity and explaining our observations at 0.5 and 1 g L⁻¹ biochar, we incubated MR-1 with ferrihydrite, lactate, and AQDS and amended these setups with different biochar concentrations (Figure 1C). We found that 0.5 and 1 g L⁻¹ biochar did not change Fe(III) reduction rates and extents, thus ruling out harmful effects of the biochar particles for the cells' electron transfer capabilities. However, at high biochar concentrations (5 and 10 g L⁻¹), we even saw a stimulating effect, suggesting that the combination of solid-state redox-active biochar with dissolved redox-active AQDS further facilitates electron transfer between cells and Fe(III) minerals.

Electron Shuttling by Biochar. Electron transfer from Fe(III)-reducing bacteria to Fe(III) minerals depends on the accessibility of Fe(III) to the cells. It has been demonstrated that direct contact and electron transfer via outer membrane cytochromes or nanowires, electron shuttling via redox-active organic and inorganic molecules, or even Fe(III) solubilization via chelators is an efficient strategy for transferring electrons from cells to Fe(III).^{47,48} In our non-growth cell suspension experiments, during which the synthesis of cellularly derived electron shuttles, nanowires, and Fe(III) chelators should be limited if not completely prevented (although nutrients from lysed cells might facilitate low levels of biosynthesis), we observed that addition of redox-active biochar particles at concentrations of 5 and 10 g L⁻¹ stimulated electron transfer. Abiotic controls with biochar and ferrihydrite showed that even in the absence of cells the biochar contained electrons that can be transferred to Fe(III) [~ 10 and $\sim 18\%$ Fe(II) formation for 5 and 10 g L⁻¹ biochar setups, respectively (Figure 1A and Figure S2 of the Supporting Information)]. However, in the presence of cells and 5 and 10 g L⁻¹ biochar, microbial reduction rates and extents increased significantly beyond the additive values of biochar with ferrihydrite and cells with ferrihydrite, respectively. This suggests that biochar itself can stimulate electron transfer by functioning as an electron acceptor for *S. oneidensis* MR-1 and by transferring electrons from microbially reduced biochar to the Fe(III) mineral ferrihydrite. A similar stimulation of microbial ferrihydrite reduction by redox-active particulate compounds has been described for solid-phase humic substances³³ and for redox-active/conductive iron minerals.⁴⁹ For biochar, it has also been demonstrated that it can be reduced electrochemically. Depending on the biochar feedstock and charring temperature, biochar can store several hundred micromoles of electrons per gram of biochar.²² These values of electron uptake capacities are in a range similar to that observed for humic substances,^{36,50} further supporting the potential role of biochar as an electron shuttle between Fe(III)-reducing bacteria and Fe(III) minerals.

Effects of Biochar on Ferrihydrite Reduction by *S. oneidensis* MR-1. The data obtained in this study suggest that biochar can function as an electron shuttle in a manner similar to that demonstrated previously for other redox-active compounds such as humic substances, sulfur compounds, and even minerals. Changes in ferrihydrite reduction rates depend on the electron shuttle concentration as observed here for biochar (Figure 1A) and have been reported previously for dissolved humic substances.⁴⁰ In the study by Amstaeetter et al.,⁴⁰ the inhibitory effect of low concentrations of humic substances has been attributed to sorption of humic substances to ferrihydrite, leading to negatively charged patches at the otherwise positively charged ferrihydrite surface and thus to ferrihydrite aggregation and a lowering of microbial accessibility. In contrast, at higher concentrations of humic substances, the minimal concentration necessary for effective electron shuttling is reached³⁵ and ferrihydrite aggregation is overcome by complete coverage of ferrihydrite by negatively charged humic substances, leading to repulsion of individual ferrihydrite particles and better accessibility to microorganisms.⁴⁰ To determine whether addition of biochar leads to similar aggregation and flocculation processes, we analyzed ferrihydrite suspensions in the presence of biochar. However, we did not find evidence of aggregation of ferrihydrite in the presence of low or high biochar concentrations (Figure S3 of the Supporting Information). Because our control experiments (Figure 1B,C) did not show any toxic effects of the biochar particles or the organic compounds leached from biochar, the reasons for the decrease in the extent of ferrihydrite reduction at biochar concentrations of 0.5 and 1 g L⁻¹ remain unknown. Although we performed our experiments under nongrowth conditions, MR-1 might still produce and release small amounts of electron shuttles that could facilitate ferrihydrite reduction in the absence of biochar or AQDS but that are sorbed onto biochar at low concentrations.

However, the visualization of the ferrihydrite–biochar aggregates also showed that biochar particles are closely associated with ferrihydrite. This suggests that the stimulation of ferrihydrite reduction by biochar is probably not due to more efficient electron transfer facilitated by diffusion of biochar particles between cells and ferrihydrite but rather by electron hopping among cells, biochar, and ferrihydrite. Considering the rough surface of biochar particles as seen in electron micrographs,^{51,52} the distances between cells and biochar particles as well as between the biochar and ferrihydrite particles in most cases are probably larger than the 20 Å maximal distance required for direct electron transfer.⁵³ This explains why we observed the highest Fe(III) reduction rate in the presence of a combination of biochar and AQDS because AQDS could bridge the gaps between cells and biochar or between biochar and ferrihydrite.

Implications of Biochar Electron Shuttling for Iron Mineralogy and Biogeochemical Cycles in the Environment. In addition to the effects on the rates and extents of Fe(III) reduction, we also observed a change in iron mineralogy during microbial ferrihydrite reduction in the presence of biochar. Analysis by XRD (Figure 2) and Mössbauer spectroscopy (data not shown) revealed that magnetite (Fe₃O₄) is the main mineral phase formed by MR-1 in the presence of AQDS within the first 25 h. After 72 h also, some siderite (FeCO₃) that originates either from direct precipitation of Fe(II) with bicarbonate or from further reduction of magnetite was detected. However, in the presence of biochar

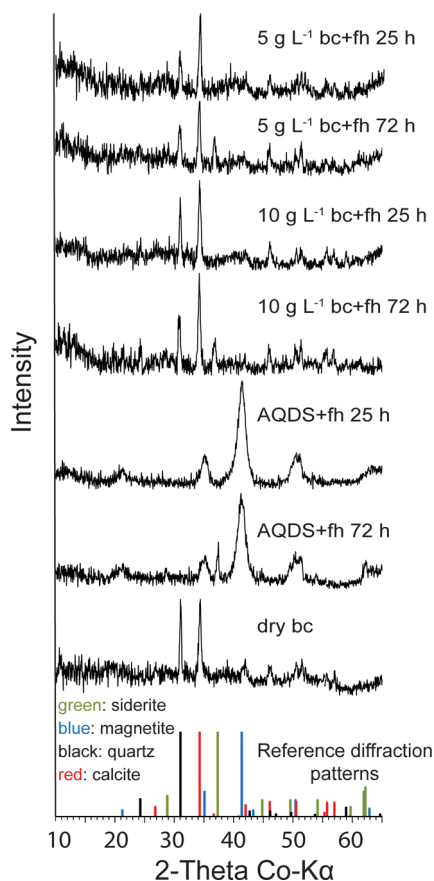


Figure 2. XRD data for minerals produced by microbial reduction of 15 mM ferrihydrite after 25 and 72 h in the presence of 5 and 10 g L⁻¹ biochar compared to ferrihydrite reduced in the presence of AQDS. For comparison, XRD data of the biochar itself are shown. Note that on the basis of the XRD results the biochar contains calcite and quartz. X-ray diffractograms for ferrihydrite and the biogenic minerals produced from ferrihydrite in the absence of shuttles have been published previously.^{40,45,62}

(both 5 and 10 g L⁻¹), no magnetite was observed but the Fe(II) produced precipitated as siderite. A similar change in mineralogy from magnetite to siderite during ferrihydrite reduction has been observed for experiments with humic substances as the electron shuttle^{45,46,54} and could be due to either faster reduction rates, sorption of Fe(II) to biochar, or sorption of the organics to the ferrihydrite surface. All three processes can prevent solid-state conversion of ferrihydrite and crystallization as the mixed-valent Fe(II)–Fe(III) mineral magnetite.^{40,46,55} Consequently, the Fe(II) formed during ferrihydrite reduction precipitates directly with the carbonate from the bicarbonate buffer forming siderite. Furthermore, sorption of Fe(II) onto biochar instead of ferrihydrite could also lead to an increased extent of Fe(III) reduction (in addition to biochar electron shuttling) by preventing ferrihydrite surface passivation.

In summary, our results suggest that the addition of biochar to soils can influence soil biogeochemistry not only by changing soil physicochemical properties but potentially also by directly mediating electron transfer processes, i.e., by functioning as an electron shuttle. The changing soil mineralogy, which is dependent on the presence of other ions such as bicarbonate or phosphate, can lead to differences in the sorption of nutrients and trace metals and to changes in soil mineral

reactivity generally influencing both microbial processes and pollutant transformation reactions.^{56,57} The redox activity and electron shuttling capacity of biochar also must be considered when applying biochar for remediation of metal-contaminated sites because biochar could influence the fate of metals such as Cr and As not only by sorption as suggested previously^{58,59} but also by undergoing redox reactions with these metal ions. Finally, we think that the electrical conductivity of biochar particles can have similar consequences for long-range electron transfer in soils as recently observed for sedimentary “cable” bacteria,^{60,61} i.e., the bridging of different redox zones over millimeter and even centimeter distances via electron transfer through conductive biochar–mineral–aggregate networks. However, future studies have to confirm whether electron shuttling via biochar particles, as suggested previously,^{9,39} occurs in soils and not only in laboratory experiments with biochar, ferrihydrite, and bacterial cells. Empirically how “field aging” will affect the long-term electron shuttling capability of biochars remains to be evaluated.

■ ASSOCIATED CONTENT

Supporting Information

Additional material as mentioned in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

A.K. and M.L.W. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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