Nanosized Iron Oxide Colloids Strongly Enhance Microbial Iron Reduction

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Microbial iron reduction is considered to be a significant subsurface process. The rate-limiting bioavailability of the insoluble iron oxyhydroxides, however, is a topic for debate. Surface area and mineral structure are recognized as crucial parameters for microbial reduction rates of bulk, macroaggregate iron minerals. However, a significant fraction of iron oxide minerals in the subsurface is supposed to be present as nanosized colloids. We therefore studied the role of colloidal iron oxides in microbial iron reduction. In batch growth experiments with Geobacter sulfurreducens, colloids of ferrihydride (hydrodynamic diameter, 336 nm), hematite (123 nm), goethite (157 nm), and akaganéite (64 nm) were added as electron acceptors. The colloidal iron oxides were reduced up to 2 orders of magnitude more rapidly (up to 1,255 pmol h⁻¹ cell⁻¹) than bulk macroaggregates of the same iron phases (6 to 70 pmol h⁻¹ cell⁻¹). The increased reactivity was not only due to the large surface areas of the colloidal aggregates but also was due to a higher reactivity per unit surface. We hypothesize that this can be attributed to the high bioavailability of the nanosized aggregates and their colloidal suspension. Furthermore, a strong enhancement of reduction rates of bulk ferrihydrite was observed when nanosized ferrihydrite aggregates were added.

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Dissimilatory iron reduction is an important anaerobic respiration process in anoxic subsurface environments. However, the reactivity of ferric iron is mostly limited by the reduction kinetics of the poorly soluble, extracellular iron minerals. Electron transfer from microorganisms to iron oxides can occur via direct contact or by electron shuttling compounds (46). Transport of the electron shuttle between the redox partners is then assumed to occur via diffusion. For example, humic substances can serve as natural electron shuttles that can be reduced by microorganisms and subsequently chemically oxidized by the ferric oxide (18). Shewanella oneidensis excretes a flavin to stimulate hematite reduction, functioning in a similar manner (27). As another option, formation of conductive pili serving as nanowires was described as a possible way of transferring electrons to the oxide surface (15, 34). Nevertheless, direct attachment has been recognized as a major mode of accessing iron oxides as electron acceptors (12). Direct transfer between microbial outer membrane reductases and the ferric minerals, however, requires close contact of less than 14 Å between the terminal iron reductase on the cell surface and the iron oxide molecule at the mineral surface (19, 25), limiting the rates of electron transfer between cell and mineral.

Several parameters have been discussed in this context as being decisive for the bioavailability and reactivity of iron oxides, such as, e.g., the mineral surface area (8, 41). Larger surface areas have been shown to be accompanied by higher initial reduction rates. Another parameter that might determine reactivity is the low solubility of ferric iron in water at neutral pH (20). Low solubility entails high crystallinity, which reduces reaction rates (4). Therefore, crystalline bulk iron phases such as goethite or hematite (9) are poorly reducible by microorganisms, in contrast to amorphous ferrihydrite (41). Naturally, well crystalline minerals have lower surface areas, and the effects of surface area and solubility cannot be distinguished sharply. Cell density, initial oxide and substrate concentrations, and ferrous iron adsorbed to the bulk mineral surface were also reported to control microbial reduction rates by exhibiting mutual saturation behavior in Michaelis-Menten-type kinetics (3, 22, 40).

The latter studies also considered particle sizes, a parameter that has often been overlooked so far. All concepts mentioned above generally assumed a bulk state of the electron-accepting iron oxide. Indeed, iron oxides used in microbiological experiments appear mainly as coarse, flocculating macroaggregates, visible to the naked eye as sludge-like precipitates. In nature, however, nanosized iron oxides are abundant (32, 45) and play a vital role in many biogeochemical processes (2, 16, 28). Such nanoparticles may appear in stable colloidal suspension, even if aggregated as a stable cluster of multiple particles (13). Ferric oxide particles can appear in colloidal suspensions of different aggregate sizes and densities.

Different particle aggregate sizes might influence the bioavailability of iron oxides in microbial reduction. Nanosized aggregates appearing in colloidal suspensions might be spatially more accessible for microorganisms than large aggregates flocculating as bulk phases. Therefore, the present study aims at assessing the reactivity and putative role of aggregate sizes of iron oxides in dissimilatory iron reduction. A set of ferrihy-
drite, hematite, goethite, and akaganite colloids was compared to their respective noncolloidal bulk phases to evaluate this effect.

MATERIALS AND METHODS

Microorganisms and cultivation. *Geobacter sulfurreducens* DSMZ 12127 (7) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was cultivated using standard anaerobic techniques at 30°C in the dark. It was grown in a medium consisting of 1.0 g liter⁻¹ NaCl, 0.4 g liter⁻¹ MgCl₂ · 6H₂O, 0.2 g liter⁻¹ KH₂PO₄, 0.25 g liter⁻¹ NH₄Cl, 0.5 g liter⁻¹ KCl, and 0.15 g liter⁻¹ CaCl₂ · 2H₂O. The medium was supplemented with trace elements, selenite-tungsten, and vitamins solutions. Ten μM Na₂SO₄ was added as a sulfur source. The medium was buffered with 10 mM NaHCO₃ at pH 6.8. Sodium acetate (10 mM) was added as the sole energy and carbon source. Precultures were grown with 50 mM technical-grade ferric citrate (pH 7.0) as an electron acceptor. If not otherwise stated, all chemicals (Sigma, Deisenhofen, Germany) were at least American Chemical Society grade.

Microbial reduction experiments. Experiments were performed in 60-ml medium batch incubations, initiated in 100-ml glass serum bottles sealed with butyl rubber stoppers and flushed with 20/80% CO₂/ N₂. The specific colloidal or bulk iron oxide aggregates and the bacterial cell suspension were added to the medium via anoxic syringes and at specific amounts to obtain comparable initial ferric iron (~9.6 mM) and surface area (~110 m²/liter) concentrations. Colloidal iron oxides were applied at lower concentrations of 1.4 mM, since these materials would otherwise coagulate after inoculation with the bacterial cell suspension. Colloids stayed stable during the entire reaction. Abiotic controls were performed by adding the cell suspension through a 0.22-μm filter, thereby retaining the bacteria. Immediately after inoculation, the first samples for Fe²⁺ analysis were withdrawn. Then, the experimental bottles were shaken at 300 rpm at 30°C in the dark.

Cell suspension. To ensure colloidal stability, an anoxic low-salt medium was used for the batch cell suspension experiments throughout the study. The culti- 
vation medium described above was modified to 0.1 g liter⁻¹ NaCl, 4 mg liter⁻¹ MgCl₂, 6H₂O, 25 mg liter⁻¹ NH₄Cl, 50 mg liter⁻¹ KCl, and 15 mg liter⁻¹ CaCl₂ · 2H₂O. The medium was buffered with 10 mM Tris-HCl (pH 6.8) instead of NaHCO₃. All other ingredients remained unchanged.

After growth to the late exponential phase, 3 liters of preculture was harvested by centrifugation for 20 min at 2,000 × g at 20°C (Avanti J-E centrifuge with JA-10 rotor; Beckman-Coulter, California). The cell pellet was resuspended in 100 ml fresh low-salt medium in an anoxic glove box (O₂ < 3 ppm). Remaining Fe²⁺ in the cell suspension was <2 fmol cell⁻¹. These cell suspensions were immediately added to the reduction experiments in a 1:10 ratio and yielded final cell densities of ~2.1 × 10⁸ cells/ml in all experiments. For the normalization of reduction rates, flow cytometry was applied to mea- sure cell numbers of the cell suspensions for each individual experiment using an LSRII cell analyzer (Becton Dickinson Bioscience, Franklin Lakes, NJ). Para- formaldehyde-fixed cells from the cell suspensions were stained by SYBR green I nucleic acid stain (Molecular Probes, Eugene, OR), diluted in 0.22-μm-filtered Dulbecco’s phosphate-buffered saline, and counted at a wavelength of 510 nm in a Trucount bead (Becton Dickinson) calibrated measurements.

Preparation of bulk and nano-sized iron oxide aggregates. Nanosized ferrhydrite and hematite aggregates were synthesized according to the methods of Leibl et al. (21) and Madden and Hochella (26). Colloidal goethite was synthe- sized according to the method of Anschutz and Penn (1), and colloidal akaga- neite was synthesized by following the procedure of Gonsalves et al. (14). At 4°C in the dark, nano-sized iron oxide aggregates remained in stable colloidal sus- pension for >3 months.

Bulk 6-line ferrhydrite was synthesized according to the method of Lovley and Phillips (24). Bulk goethite and hematite were obtained from Sigma-Aldrich (Deisenhofen, Germany). Bulk iron oxide macroaggregates were aged for 6 weeks at room temperature to achieve maximum coagulation.

All colloidal iron oxides were synthesized in <3 months’ proximity to the experiments to avoid Ostwald ripening.

After synthesis, iron oxides were cleared of remaining ions from the prepara- tion. Ferrhydrite colloids were centrifuged (60 min, 6,000 × g at 4°C, Avanti J-E with rotor JA-10; Beckman-Coulter) and resuspended in Milli-Q water until the total iron content in the supernatant dropped below 1 mg/l. All other colloids were dialyzed using Spectra/Por dialysis tubes with a molecular mass cut-off 10 Kda (Carl Roth, Karlsruhe, Germany). Dialysis occurred against Milli-Q Water at pH 4.0 (adjusted with 0.5 M HCl) and finally against pure Milli-Q Water until the conductivity of fresh dialysis medium did not further increase. If needed, residual large particles were removed by 0.45-μm filtration. All iron oxides were stored under an anaerobic atmosphere for 24 h to remove oxygen, sealed in airtight glass bottles, and stored at 4°C in the dark. Autoclaving was omitted to avoid changes in crystal structures.

Aggregate size measurement. Dynamic light scattering (DLS) was applied to measure hydrodynamic diameter sizes of the water-dispersed and diluted colloidi- dal samples, using a ZetaSizer Nano ZS and an HPSS 5001 system (Malvern Instruments, Worcestershire, United Kingdom) with low-volume folded capillary sizing cuvettes. At least triplicate measurements of 30 s each were done. Eighty-nm calibration latex beads were measured to confirm the accuracy of the method. The autocorrelation function was inverted using the CONTIN software program (Provencher).

Aggregate size was measured in deionized water. Additionally, size was mea- sured in the low-salt medium applied in the cell suspension experiments after 288 h of incubation. Here, particle sizes remained ≤600 nm (data not shown). Bulk iron oxides may not be assessed by DLS due to fast sedimentation of the large particles. Bulk particles, present as large, flocculated aggregates, were measured directly in aqueous suspension. By this method, an aggregate size assessment comparable to the DLS measurement environment could be performed, and such effects of sample drying as aggregate coagulation or decay could be avoided. We used a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Jena, Germany), measuring 100 particles each at 1,600- to 320-fold magnification against a metered calibration slide, with an assumed error of 30%.

Crystallographic structure and surface measurements. X-ray diffraction (XRD) measurement for crystallographic structure analysis and surface measurements was performed with aliquots which were rapidly frozen using liquid N₂, lyophilized, and stored in a desiccator.

XRD patterns were obtained using a Philips PW 1050 diffractometer (Phillips, Eindhoven, The Netherlands) equipped with a diffracted-beam graphite mono- chromatizer and a Bruker AXS microdiffractometer equipped with a General Area Detection Diffraction System (GADDS) detector. CoKα X-ray radiation was applied. Random powder specimens were measured from 5° to 80° 2θ in steps of 0.02° 2θ, with a counting time of 5 s for each increment. The specific surface area of the freeze-dried sample material was determined using N₂ physisorption at 77.35 K. The calculations were performed from 11-point isotherms according to the Brunauer-Emmett-Teller (BET) equation (6). A Quantachrome Autosorb 1 analyzer (Syosset, NY) was used to perform the analysis. Before the measurement, sample material was degassed under vacuum at 30 to 35°C overnight. This mild treatment was chosen in order to prevent structural changes in the dried iron oxides due to heating. All samples were measured at least two times with a reproducible standard deviation of less than 1% (except for bulk hematite [2.5%]) of the obtained specific surface area. An Al₂O₃ bead standard with a specific surface area of 79.8 ± 0.4 m²/g was applied for external calibration. All measured values of the standard fell within the 95% confidence interval.

Iron analysis. Iron was measured using the ferrozine assay (42). Aliquots of 0.2 ml (each) were withdrawn from the experiment, diluted 1:10 in 1 M HCl, and shaken for 4-400 rpm for 10 min. All adsorbed Fe²⁺ from iron oxide surfaces. Total iron measurements were treated with 10% (vol/vol) hydroxyl- amine-HCl for a complete reduction of all Fe(III) to Fe²⁺. Subsequently, aliqu- ots from this aqueous ferrous iron preparation were diluted with ferrozine, incubated for 10 min, and shaken for 30 s. Absorbance at 560 nm was measured using a Wallace 1420 Viktor³ plate reader (Perkin Elmer, MA).

Rate calculations. The obtained raw ferrous iron data were first normalized to the individual cell densities of each experiment. By this method, we could exclude slight variations in the preculture cell density. However, surface-normalized reduction rates as given in Table 1 are derived from the initial reduction rates, which were calculated by linear regression of the Fe²⁺ increase versus time. Since colloidal reduction proceeded rapidly and was dependent on the initial Fe(III) concentration, the end point of the regression analysis was set to a point in time when 50% of the maximum Fe²⁺ concentration had been produced and involved at least 4 to 6 data points. For bulk ferric iron reduction, a linear regression was calculated over the entire time span of the experiment, since bulk iron oxides did not reach a degree of 50% reduction. Error bars were calculated by an error propagation including the standard errors of the triplicate iron measurements and of multiple cell counts and standard deviations of the tripli- cate individual experiments.

RESULTS AND DISCUSSION

Synthesis of iron oxide aggregates. Synthesis of iron oxides yielded stable, monodisperse colloidal suspensions. The iden-
TABLE 1. Bulk and colloidal iron oxide aggregates and their respective reduction rates

<table>
<thead>
<tr>
<th>Iron oxide (specified by XRD)</th>
<th>State</th>
<th>Aggregate diam (nm)</th>
<th>BET specific surface area (m²/g)</th>
<th>Avg net iron reduction rate× (µmol/h)</th>
<th>Avg cell normalized net iron reduction rate× (pmol/h cell⁻¹)</th>
<th>Surface area-normalized reduction rate (nmol/m² h⁻¹)</th>
<th>Surface area and cell density-normalized rate (pmol liter/m² h⁻¹ 10⁶ cells⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrihydrite</td>
<td>Colloidal</td>
<td>336 ± 40</td>
<td>275 ± 0.6</td>
<td>244.5 ± 84.5</td>
<td>1,255 ± 406</td>
<td>3,292 ± 992</td>
<td>1,563 ± 471</td>
</tr>
<tr>
<td>Hematite</td>
<td>Colloidal</td>
<td>123 ± 4</td>
<td>127 ± 1.1</td>
<td>11.6 ± 5.6</td>
<td>175 ± 84</td>
<td>397 ± 180</td>
<td>189 ± 86</td>
</tr>
<tr>
<td>Akaganeite</td>
<td>Colloidal</td>
<td>157 ± 3</td>
<td>219 ± 0.3</td>
<td>20.4 ± 6.5</td>
<td>156 ± 92</td>
<td>417 ± 136</td>
<td>198 ± 65</td>
</tr>
<tr>
<td>Goethite</td>
<td>Colloidal</td>
<td>64 ± 3</td>
<td>136 ± 0.5</td>
<td>13.9 ± 84.5</td>
<td>77 ± 17</td>
<td>1,349 ± 169</td>
<td>641 ± 80</td>
</tr>
<tr>
<td>Ferrihydrite</td>
<td>Bulk</td>
<td>70,200 ± 30%</td>
<td>169 ± 0.3</td>
<td>4.7 ± 0.7</td>
<td>74 ± 47</td>
<td>37 ± 17</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>Hematite</td>
<td>Bulk</td>
<td>27,600 ± 30%</td>
<td>37 ± 0.9</td>
<td>12.4 ± 6.2</td>
<td>59 ± 34</td>
<td>89 ± 12</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>Goethite</td>
<td>Bulk</td>
<td>11,900 ± 30%</td>
<td>11 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>6 ± 3</td>
<td>8 ± 2</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

× Values are means ± propagated SE. Conditions were pH 6.8 and 30°C, ambient pressure, with initial ferric iron concentrations of ~1.4 mM (colloids) and ~9.6 mM (bulk), 10 mM sodium acetate as an electron donor, and ~2.1 × 10⁴ cells/liter.

b Provided in the supplemental material.

c Rates were dependent on the initial ferric iron concentration (see Results and Discussion and the supplemental material).

d Calculated by linear regression from data presented in Fig. 1.

e Calculated by linear regression from data presented in Fig. 1.

ties of the respective phases were confirmed by XRD (see the supplemental material), and surface areas lay within a range of 100 to 275 m² g⁻¹ for the colloids as determined by BET N₂ physisorption (Table 1). The initial colloidal aggregate diameters ranged from 64 to 336 nm as confirmed by dynamic light scattering techniques (DLS).

Particle aggregation is of concern when characterizing iron oxides. Single-crystal particles most often undergo aggregation or cluster formation (13). Ferrihydrite is a natural nanophase (17, 29), mostly being present in nano- to microparticulate aggregates which may remain in stable colloidal suspension. Typically, such small particles coagulate immediately and produce a gel, whereas the BET surface area remains more or less the same (41).

Our colloidal ferrihydrite aggregates stayed in stable suspension with constant aggregate sizes, while bulk ferrihydrite macroaggregates occurred as sludge-like precipitate with visible flocculation. The same characteristic behavior was also observed for hematite and goethite. The overall aggregate size as indicated by DLS and microscopy consisted of two size classes: small particles in the nanosize range, which remained in stable colloidal suspension, and larger macroaggregates well above the nanosize range, which readily settled. Flocculated macroaggregates showed sizes in the range of several microns, but since aggregates of both fractions were examined in aqueous suspension (see Materials and Methods) and disturbances like freeze-drying were omitted, we assumed the measured aggregate sizes realistically reflected the aggregate size in our experiments. We observed that sample preparation for electron microscopy via freeze-drying or fixation to grids easily changes the aggregation state of iron oxides (see the supplemental material).

Highly reactive nanosized iron oxide colloids. When nanosized ferrihydrite colloidal aggregates were applied as electron acceptors in cell suspensions experiments with G. sulfurreducens, rapid reduction occurred relative to the bulk phase (Fig. 1a). The initial reduction kinetic was 600 to 1,255 pmol h⁻¹ cell⁻¹ ferrous iron production. This was up to ~30 times faster than reduction of macroaggregated ferrihydrite, with 41 to 153 pmol h⁻¹ cell⁻¹. The soluble electron acceptor ferric citrate was reduced only 9 times faster than the nanosized ferrihydrite colloids (data not shown), indicating that these aggregates are reduced with kinetics comparable to those for dissolved iron complexes.
The observed effect of enhanced reduction kinetics for nanosized aggregates was similar for hematite (Fig. 1b), goethite, and akaganeite (Fig. 1c), with maximum rates of 50 to 336 pmol h$^{-1}$ cell$^{-1}$. The maximum reduction rates of the colloidal aggregates (Table 1) were to some extent dependent on the initial ferric iron concentrations (see the supplemental material), tentatively indicating a Michaelis-Menten kinetic of the reaction similar to that for dissolved iron complexes. Macroaggregate maximum reduction rates were ~10 to ~100 times lower and ranged from 9 to 105 pmol h$^{-1}$ cell$^{-1}$ only (Table 1).

Abiotic control experiments clearly showed no increase in ferrous iron for bulk and nanosized iron oxides (data not shown), thereby ruling out abiotic iron reduction effects.

High degree of colloid reduction. Surprisingly, colloidal aggregates of ferrihydrite, goethite, and akaganeite (Fig. 1c), with maximum rates of 50 to 336 pmol h$^{-1}$ cell$^{-1}$. The maximum reduction rates of the colloidal aggregates (Table 1) were to some extent dependent on the initial ferric iron concentrations (see the supplemental material), tentatively indicating a Michaelis-Menten kinetic of the reaction similar to that for dissolved iron complexes. Macroaggregate maximum reduction rates were ~10 to ~100 times lower and ranged from 9 to 105 pmol h$^{-1}$ cell$^{-1}$ only (Table 1).

Abiotic control experiments clearly showed no increase in ferrous iron for bulk and nanosized iron oxides (data not shown), thereby ruling out abiotic iron reduction effects.

High degree of colloid reduction. Surprisingly, colloidal aggregates of ferrihydrite, goethite, and akaganeite were almost totally reduced, to extents of 78 to 100% (Fig. 1). In contrast, macroaggregated ferrihydrite, hematite, and goethite were reduced to a degree of only 6 to 24% over the time course of the experiment. So the limiting of bulk iron oxide reactivity by surface passivation via ferrous iron (11, 35) or by the high aggregation density was not occurring during colloid reduction. An exception was nanohematite, which was reduced only to a degree of 23 to 39%. During the microbial incubation of nanohematite, visible coagulation occurred, probably limiting further reduction.

The high degree of reduction observed in our study is probably a result of the complete suspension of the colloidal iron oxide aggregates and demonstrates the possibility of a high bioavailability of colloidal iron oxides. This is striking, since for nanoparticulate aggregates, as used in our study, surface passivation should have even more impact than for discrete particles (47). That study also reported a degree of reduction of up to 17% for hematite nanoparticle reduction, and only a slight degree of reduction was observed when applying Shewanella instead of Geobacter (5). The latter study used noncolloidal iron oxides, indicating that the state of aggregation and suspension had an impact on reactivity. Earlier studies already had indicated an influence of aggregation on reduction rates (41).

Surface-normalized reduction rates. The surface area-normalized reaction rates of the aggregates used in this study were dependent on the aggregate size and the colloidal suspension (Fig. 2). Nanosized, colloidal aggregates clearly showed a higher reactivity per unit surface than noncolloidal macroaggregates. The surface-normalized rates of 397 ± 80 nmol/m$^2$ h ferrous iron production for our nanosized hematite aggregates lay slightly below the range observed by Yan et al. of 837 to 2,280 nmol/m$^2$ h for monocrystalline hematite nanoparticles in a size range of 10 to 50 nm and hydrogen as an electron donor (47). Rates of 2.4 to 22.26 nmol/m$^2$ h$^{-1}$ were reported by Roden and Zachara (41), which corresponds to the reduction rates of the macroaggregates used in our study (Table 1).

Minor impact of crystallinity in colloidal aggregates. To some extent, crystallinity has an impact on iron oxide reactivity of bulk phases (33, 36, 37). Abiotic dissolution rates, with ascorbic acid as the reducing agent, decreased in the order ferrihydrite > lepidocrocite > goethite > hematite (33). However, more crystalline mineral phases, such as akaganeite and goethite, when present as nanosized, colloidal aggregate, appeared to be reactive in a way similar to that of, e.g., colloidal ferrihydrite and hematite aggregates in our experiments (Fig. 2). Hence, at a sufficiently small aggregate size, the crystal structure of the ferric mineral probably became of minor importance in determining reduction rates of ferric nanoparticles compared to those of bulk macroaggregates.

Catalytic effect. Apart from the high reactivity of the nanosized, colloidal iron oxide aggregates as electron acceptors, we also observed a stimulation of bulk ferric oxide reduction upon combining bulk and colloidal iron oxides (Fig. 3). By adding a catalytic amount of nanosized ferrihydrite aggregates (3.3 fmol cell$^{-1}$) to a ~10-times-larger volume of bulk ferrihydrite mac-

FIG. 2. Surface-normalized maximum reaction rates for colloidal hematite (▲), colloidal ferrihydrite (△), colloidal akaganeite (○), colloidal goethite (■), bulk goethite (■), bulk hematite (▼), and bulk ferrihydrite (▲).

FIG. 3. Catalytic effect of a small amount of colloidal ferrihydrite added to a large volume of bulk, macroaggregate ferrihydrite. Colloidal 336-nm ferrihydrite alone was added at 0.86 mM (2.9 fmol cell$^{-1}$; n = 3) (○). Bulk ferrihydrite alone was added at 3.52 mM (33.1 fmol cell$^{-1}$; n = 1) (●). In the combination, bulk and colloidal ferrihydrite were added at a ~10:1 ratio (bulk over colloidal) at an initial Fe(III) concentration of 2.71 mM total (25.9 fmol cell$^{-1}$; n = 3) (●).
roaggregates (21.6 and 25.9 fmol cell\(^{-1}\)), 85% of the bulk mineral was reduced within 100 h. This was 3 to 4 times more than the maximum reduction detected for bulk ferrihydrite alone, which was reduced to only 25% within the time frame of the experiment. Clearly, the nanosized aggregates had a catalytic effect and the bulk, relatively inert iron oxides became readily available to the microorganisms. The maximum reduction rates amounted to 2,400 pmol h\(^{-1}\) cell\(^{-1}\) and were higher than nanoparticle iron reduction rates only (maximum, 1,255 pmol h\(^{-1}\) cell\(^{-1}\)) or a bulk ferrihydrite reduction rate of 41 to 153 pmol h\(^{-1}\) cell\(^{-1}\). The data show that addition of colloidal ferrihydrite aggregates to bulk ferrihydrite phases enhanced the kinetics of the reduction of bulk ferrihydrite macroaggregates by a factor of \(\sim 30\).

We speculate that the small amount of nanosized ferrihydrite can act as an electron shuttle between the bulk iron oxide and the cell surface, as has been previously proposed (44). Apart from serving as an electron acceptor itself, nanosized iron oxide aggregations can theoretically deliver electrons to the bulk ferric oxide phase and be reoxidized. This might function via a mechanism similar to that proposed for organic electron shuttles, such as humic acids, by diffusion between the cell and the ferric oxide surface (18). However, previous studies of iron bound to humics argue against such a mechanism (23). Instead, the extensive adsorption of colloidal aggregates onto the macroaggregates might produce a microbiologically accessible surface layer, activating the surface by conducting electrons to the interior of the bulk mineral. An alternative explanation for the high reactivity observed upon combining bulk and nanosized ferrihydrite aggregates might be the scavenging of aqueous Fe\(^{3+}\) by the large surface of the colloidal material. This would diminish or clear the inhibitory effect of ferrous iron adsorption and concomitant passivation of bulk iron oxide surfaces (39). With the high cell densities used, binding of Fe\(^{2+}\) to cell surfaces might also have helped in relieving oxide passivation. Still, this effect would also have been in charge during bulk oxide reduction.

Furthermore, the reaction mechanisms in this mixed ferric-ferrous system are complex and may involve phase transformation (31). Yet the existing data cannot reveal which mechanism is occurring, and further research is needed to investigate the impact of particle-particle and particle-cell aggregation.

**Model for iron oxide aggregate reactivity.** Figure 2 shows that the differences in the surface-normalized reactivities within the various colloidal aggregate species are only minor and do not follow a size-dependent relationship. Even among monocristalline nanoparticles of <100 nm, no clear size-reactivity relationship was discovered (5, 47). Instead, in our experiments we observed a size dependency of the reduction rate when comparing colloidal, nanosized aggregates to bulk macroaggregates; the difference in the determined biotic reactivity might thus be credited to the suspended state and, therefore, the high spatial bioaccessibility of the colloidal iron oxide aggregate. It has been shown before that the iron oxide-microorganism contact area has an impact on reduction rates (3). Colloids constantly undergo Brownian movement, probably increasing their potential for capturing electrons from the cell surface by higher numbers of contact events with reactive sites at the microbial cell surface, although there is also a potential for long-term attachment of nanoparticles to iron-reducing cells (3). Another aspect might be that colloidal particles have the potential to fully cover the bacterial cell following a Langmuir isotherm (3), as opposed to bulk ferric iron phases, which probably cannot cover the cell due to a spatial limitation. The high spatial accessibility of nanosized iron oxide colloids contrasts with the dense macroaggregation of the bulk iron oxides applied in this study. We speculate that the inner surfaces of these aggregates are barely accessible for microbial contact, drastically limiting rates and degrees of reduction. Therefore, apart from the surface area, the degree of aggregation, the aggregate stability, and putatively the size of the monocrystalline particles within such aggregates also can be considered to have an impact on the reduction rates. This clearly has to be investigated. One recent study already clearly highlighted the impact of the iron oxide aggregation state on the reactivity in microbial iron reduction (10).

All of these models assume a direct electron transfer from cell surfaces toward the applied iron oxides. Although some iron-reducing bacteria produce electron shuttles on their own (27), no evidence is available for the production of an electron shuttle compound by *Geobacter*. Other electron shuttling compounds, such as, e.g., humics or anthraquinone-2,6-disulfonate, were not added to our experiments, and the formation of nanowires in a shaken culture bottle within hours seems rather unlikely.

**Environmental implications.** In our study, the two clusters of particles differ in size by 2 orders of magnitude. This approach reveals the impact of aggregate particle size and density on a scale which is environmentally relevant, since iron oxides occur mostly as bulk aggregate or coating in the environment or as mobile nanoparticle. The subtle differences among nanoparticle reactivity at particle sizes of <100 nm contribute to the understanding of the mechanism governing microbial reduction of nanoparticles and ferric iron in general, but our study highlights the difference between suspended, nanosized and large, flocculating particle aggregates. We consider this to be of environmental relevance; nevertheless, further research should focus on a broader particle and aggregate size and density spectrum of specific minerals.

In summary, we suggest expanding the model proposed by Roden and Zachara (41), in which iron oxide reactivity in microbial reduction is determined by surface area alone, by inclusion of aggregate size as one controlling parameter. If the size of iron oxide aggregates falls below a critical size that allows a homogenous, stable dispersion and a quasi-“dissolved” state of the particles, the reactivity is greatly enhanced, and in competition with a bulk ferric oxide phase, suspended, nanosized particles will be reduced much faster. The high reactivity observed in our study would enforce the possibility of close-distance iron redox cycling, as proposed in reference 38. In this model, Fe\(^{3+}\) from microbial iron reduction in anoxic zones of an aquifer gets reoxidized in neighboring zones with O\(_2\) or nitrate. Reoxidation might take place chemically at oxic microhabitats or oxic zones close to a capillary fringe or might be done biologically by ferrous iron-oxidizing, aerobic nitrate-reducing bacteria. This again would generate nanoparticles or nanosized aggregates (43). They would then incidentally diffuse back to anoxic zones and again serve as electron acceptors. Our results tentatively support the assumption that iron...
oxide nanoparticles are feasible agents linking iron-reducing and iron-oxidizing zones in this concept, especially when they are highly mobile due to colloidal suspension.

Given the high abundance of nanosized iron oxides in the environment (45) and the widespread ability among bacteria to reduce iron (46), our results indicate that the reactivity of iron oxides in the environment might have been underestimated so far. Therefore, our results add a biological aspect to the larger picture of the geochemical significance of nanoparticles for the environment (16, 30).

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**REFERENCES**