Iron phase transformations resulting from the respiration of *Shewanella putrefaciens* on a mixed mineral phase

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**Abstract.** The initial Fe(III) minerals and the secondary mineralization products of *Shewanella putrefaciens* CN32 grown in the presence of dissolved phosphate and a commercial Fe(III) oxide, nominally nanoparticulate lepidocrocite, were determined using XRD and XAFS. The starting material was transformed by the bacteria from a reddish brown, rust colour mineral to a dark green phase over 90 days. Acid extraction of the bioreduced solids with 0.75 M HCl recovered 83% of the total iron as Fe(II), leaving a solid, acid-resistant phase. The latter was identified as nanoparticulate hematite by EXAFS. Subsequently, the starting Fe(III) phase was determined to be a mixture of 60% lepidocrocite, 26% ferrihydrite, and 14% hematite, using linear combination EXAFS analysis. For the acid-extractable phase, XANES and EXAFS indicated a predominantly Fe(II) valence state and a spectrum consistent with a mixture of brucite-type minerals (e.g., green rust or ferrous hydroxide) and siderite. The observed transformations suggest that in this mixed-mineral system, lepidocrocite and ferrihydrite are readily reducible to green rust and siderite, whereas hematite is less amenable to bacterial reduction. This study also demonstrates the utility of XAFS spectroscopy in the quantitative characterization of dissimilatory metal transformations, particularly in complex systems such as nanoparticulate minerals in hydrated mineral-bacteria assemblages.

1. Introduction
Dissimilatory iron reducing bacteria (DIRB) are a group of microorganisms that can utilize Fe(III) minerals as terminal electron acceptors for anaerobic respiration [1]. The resulting Fe(II)/Fe(III) transformations are studied extensively to understand bacterial metabolism, but there is also significant interest in using reduced bacterial products for *in situ* bioremediation. For instance, the reduction of soluble U(VI) to the relatively insoluble U(IV) is considered a promising immobilization strategy for subsurface uranium plumes. The reactivity of Fe(II) with respect to U(VI) reduction can depend strongly on Fe(II) speciation [2], and studies have shown that different Fe(II) minerals exhibit varying redox reactivity [3]. Therefore, it is important to be able to identify the secondary mineralization products resulting from DIRB respiring on Fe(III) oxides and the factors that lead to the formation of reactive Fe(II) phases. Although there are many reports characterizing the products of DIRB respiration on single phase iron oxides (e.g., [4] and references inside), there are limited reports on the differential ability of DIRB to utilize a defined mixture of Fe(III) mineral phases or on the biomineralization products in the presence of phosphate. Environmental systems consist of multiple mineral phases that are often nanoparticulate, making characterization by laboratory x-ray diffraction (XRD) or electron microscopy difficult. In addition, some dominant Fe(III) phases such as ferrihydrite have only broad XRD features that cannot be used.
for identification, especially in cases where particle size is small. In this study, we have used Fe K-edge x-ray absorption spectroscopy (XANES and EXAFS) to characterize the initial minerals and the bio-mineralization products during the growth of *Shewanella putrefaciens* strain CN32 in the presence of a commercial Fe(III) oxide, nominally a nanoparticulate lepidocrocite. The preliminary stages of this work identified the presence of additional Fe(III) phases in the parent material, which allowed us to draw conclusions about the dynamics of Fe during anaerobic respiration of *S. putrefaciens* CN32 using a nanoparticulate, mixed Fe(III) oxide phase as the terminal electron acceptor.

2. Materials and methods

2.1.1. System composition, experimental conditions, and solution phase characterization

The iron oxide—nominally a nanoparticulate lepidocrocite (γ-FeOOH)—used in this study was generously provided by Rockwood Pigments in the form of an aqueous slurry. Defined mineral medium[5]—containing 80 mM Fe(III) and 5 mM phosphate—was placed in serum bottles. The serum bottles were sealed with aluminum crimp caps and deoxygenated by sparging with sterile Ar. The suspensions were inoculated with *S. putrefaciens* CN32, (cultured in tryptic soy broth for 24 hr, harvested, and rinsed twice in sterile medium without Fe(III) or e- donor) at an initial cell density of 5 × 10^9 cells mL^-1. The suspensions were placed on a roller drum and incubated at 30 °C in the dark. Samples of the suspensions were collected over time using sterile syringes. The reduction of the Fe(III) oxides was monitored by using the ferrozine assay to measure the total Fe(II) content of 0.75 M HCl extractions of the suspensions [5]. Whenever possible, sample collection and processing were conducted in a glove box containing an anoxic atmosphere (95% N₂ with 5% H₂).

2.1.2. X-ray absorption spectroscopy characterization (XANES and EXAFS)

The solid and solution fractions of the suspensions were separated by filtration and the hydrated filter cakes were sealed in polycarbonate film. Strict anoxic conditions were maintained during sample manipulations, transport, and x-ray measurements. X-ray absorption fine structure spectroscopy (XAFS) spectra were collected at the MRCAT bending magnet station, sector 10-BM at the Advanced Photon Source near Chicago, IL. Measurements on well-characterized crystalline Fe(II) and Fe(III) oxyhydroxide minerals were used as standards in the analysis. Data reduction was performed using the program Autobk[6]. Linear combination (LC) EXAFS analysis was performed using the program SIXpack [7].

3. Results and discussion

3.1. Observed evolution of the system

Over the course of 90 days following inoculation, the starting material changed from a reddish brown rust colour to a dark green phase, concomitant with total Fe(II) concentration reaching steady state. Acid extraction of the final phase recovered 83% of the total iron added as Fe(II), leaving a solid, acid-resistant phase. No colour transformation or Fe(II) production was observed in an abiotic (sterile) control. The rapid dissolution kinetics of the green coloured phase (<30 sec) suggests the presence of a pure ferrous phase (e.g., ferrous hydroxide, carbonate, or phosphate) or a green rust phase (a mixed Fe(II)/Fe(III) brucite mineral), but not magnetite. The remaining acid-resistant phase was bright red.

3.2. Characterization of the reduced solid phase

The Fourier transformed EXAFS data from the bright-red fraction remaining after acid extraction of the bioreduced phase is shown on Fig. 1A. Comparison to spectra taken from single phase Fe minerals identified it as nanoparticulate hematite, as evidenced by the smaller amplitude of the peaks around 3.5 Å, resulting from the reduced average Fe-Fe coordination of the surface Fe atoms. The Fourier transformed EXAFS data from the green-coloured bioreduced phase is shown on Fig. 1B. The spectrum indicates a local atomic environment similar to that of Fe in green rust; however, the
XANES spectrum (not shown) and the Fe(II) content analysis were consistent with a more reduced Fe phase. Linear combination (LC) analysis (Fig. 2A) produced a best fit with 63% green rust, 18% Fe(OH)₂, and 24% siderite (ferrous carbonate). Ferrous phosphate (vivianite) was not consistent with the observed spectrum. It should be noted that the spectral mixture of Fe(OH)₂ and siderite could be indicative of the presence of ferrous hydroxycarbonate. The local atomic environment of Fe atoms in ferrous hydroxycarbonate is that of Cu in malachite (Cu₂(OH)₂CO₃), consisting of edge sharing Me octahedra (as found in Fe(OH)₂ or green rust) which are bound at the corners to carbonate groups (as found in siderite); however, we lacked a spectrum of the crystalline mineral to compare.

3.3. Characterization of the parent Fe(III) material
The parent Fe(III) material was characterized at the beginning of the experiment using a Rigaku MiniFlex x-ray diffractometer (Ni-filtered Cu Kα radiation, scanned between 5° and 80° 2θ at a speed of 2.5° 2θ min⁻¹). The diffractogram on Fig. 3 shows three broad, very low intensity peaks, that can be attributed to lepidocrocite; however, assignment of such broad features is ambiguous. Hematite peaks could not be observed. The ambiguity of the XRD data and the unexpected results from the analysis of the bioreduced products prompted a more detailed characterization of the parent material. Figure 2B shows the result of the LC analysis of the EXAFS spectrum from the parent material. The best fit (as judged by the addition of all fractions to 1 and the lowest R-factor) was obtained by a spectral mixture of 60% lepidocrocite, 26% ferrihydrite, and 14% hematite (Fig. 2B).

3.4. Discussion
It is generally accepted that the reactivity of a mineral increases with decreasing particle size, both because of the larger surface area on a mass basis and because of the increased strain at the surface. It is therefore important to accurately identify small-sized reactive phases and their transformations. In this study, we show that laboratory-based XRD analysis may not be effective in detecting nanoparticulate iron minerals in multi-phase hydrated systems. On the other hand, it is possible to use linear combination EXAFS analysis to identify and quantify Fe nanoparticulate minerals that are
utilized or formed during iron reduction. In combination, EXAFS, XRD, and Fe(II)/Fe(total) content analyses can be powerful tools in tracking dissimilatory iron transformations.

The multiple Fe(III) phases detected in the parent material allowed us to study bacterial respiration in the presence of several minerals. Preferential utilization of lepidocrocite and ferrihydrite is observed, whereas hematite appears recalcitrant over the time period of the experiment. This result is consistent with the relative rates of Fe(III) reduction observed in systems with single phase minerals [4, 8]. The usually observed biomineralization product with lepidocrocite or ferrihydrite as the electron acceptor is magnetite (e.g., [9]). However, in the presence of phosphate green rust has been observed [8]. Recent work by our group shows that increasing the concentration of phosphate or other oxyanions beyond a “tipping point” causes a change in the biomineralization products from magnetite to green rust [10]. The transformation mechanism of lepidocrocite or ferrihydrite during DIR is considered to be reductive dissolution of the parent material and reprecipitation of the end product. The reason for the observation of green rust in our systems vs. magnetite in phosphate-free systems could be different reduction and dissolution kinetics of the nanoparticulate phase relative to the bulk phases; in addition, adsorption of the reduced Fe(II) to the more slowly dissolving iron phases could also lead to different reprecipitation dynamics. Alternatively, the presence or absence of dissolved phosphate may be the determining factor. The mechanisms by which oxyanions such as phosphate influence the formation of green rust instead of magnetite remain unclear and will be studied in future work.

4. Conclusions

We have shown that linear combination EXAFS analysis can enable the identification and quantification of mixed-phase, nanoparticulate Fe minerals during dissimilatory iron reduction. The application of such analysis to the parent material and the secondary mineralization products formed as a consequence of S. putrefaciens CN32 respiring on a mineral mixture of lepidocrocite, ferrihydrite, and hematite allowed us to discern differences in the reduction kinetics of the different phases. Lepidocrocite and ferrihydrite showed faster reduction kinetics in this mixed system, whereas hematite was recalcitrant and remained in the system after steady state Fe(II) production was reached. The primary secondary mineralization product in our experimental system was green rust rather than magnetite, the latter being commonly observed in phosphate-free systems. The mechanisms of these transformations will be investigated in future work.

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5. References

[1] D. R. Lovley, D. E. Holmes, and K. P. Nevin, in Advances in Microbial Physiology, Vol. 49, Vol. 49, Academic Press Ltd, London, 2004, p. 219.
[2] M. I. Boyanov, E. J. O'Loughlin, E. E. Roden, J. B. Fein, and K. M. Kemner, Geochimica Et Cosmochimica Acta 71 (2007) 1898.
[3] M. Elsner, R. P. Schwarzenbach, and S. B. Haderlein, Environmental Science & Technology 38 (2004) 799.
[4] C. M. Hansel, S. G. Benner, P. Nico, and S. Fendorf, Geochimica Et Cosmochimica Acta 68 (2004) 3217.
[5] E. J. O’Loughlin, Environmental Science & Technology 42 (2008) 6876.
[6] M. Newville, P. Livins, Y. Yacoby, J. J. Rehr, and E. A. Stern, Physical Review B 47 (1993) 14126.
[7] S. M. Webb, Physica Scripta T115 (2005) 1011.
[8] J. K. Fredrickson, J. M. Zachara, D. W. Kennedy, H. L. Dong, T. C. Onstott, N. W. Hinman, and S. M. Li, Geochimica Et Cosmochimica Acta 62 (1998) 3239.
[9] R. S. Cutting, V. S. Coker, J. W. Felllowes, J. R. Lloyd, and D. J. Vaughan, Geochimica et Cosmochimica Acta 73 (2009) 4004.
[10] E. J. O’Loughlin, C. Gorski, M. M. Scherer, M. I. Boyanov, R. E. Cook, and K. M. Kemner, Environmental Science and Technology (in review)