

Electronic Annex EA-1

DETAILS ON ANALYTICAL METHODS AND EXPERIMENTAL DESIGN

“Experimental constraints on Fe isotope fractionation during magnetite and Fe carbonate formation coupled to dissimilatory hydrous ferric oxide reduction”

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Fe Isotope Analysis

Isotopic analysis followed the methods used in Skulan et al. (2002) and Beard et al. (2003a). A standard-sample-standard approach was used to correct for instrumental mass bias. Our standard ion-exchange methods provide excellent purification from cations such as Ca (e.g., Beard and Johnson, 2004b), which is essential to assure that the mass bias corrections calculated from standards are applicable to bracketed samples (Albarède and Beard, 2004). Iron isotope compositions were determined using a GV Instruments *IsoProbe* that utilizes a collision cell to remove Ar interferences, where a mixture of hydrogen and argon removed all ArN and ArO interferences on the Fe mass spectrum, as well as essentially all ArOH species. There was no evidence for polyatomic cation isobars such as $^{40}\text{Ca}^{14}\text{N}$ or ^{54}Cr (isobaric with ^{54}Fe), $^{40}\text{Ca}^{16}\text{O}$ (isobaric with ^{56}Fe), or $^{40}\text{Ca}^{16}\text{OH}$ (isobaric with ^{57}Fe).

Over a one-year period, analysis of three Fe solution standards gave the following values: UW J-M Fe: $\delta^{56}\text{Fe} = +0.25 \pm 0.05$ ‰ (n=47), $\delta^{57}\text{Fe} = +0.39 \pm 0.07$ ‰; UW HPS Fe: $\delta^{56}\text{Fe} = +0.49 \pm 0.05$ ‰, $\delta^{57}\text{Fe} = +0.74 \pm 0.07$ ‰ (n=52); IRMM-14 Fe: $\delta^{56}\text{Fe} = -0.09 \pm 0.05$ ‰, $\delta^{57}\text{Fe} = -0.11 \pm 0.07$ ‰ (n=54), where all uncertainties are 1σ external standard deviations. External reproducibility for the data presented here may be assessed from 99 replicate analyses. Sixty analyses were duplicated through analysis of the same Fe solution (after ion-exchange chromatography) in different analytical sessions, and the duplicates have an average reproducibility of ± 0.06 ‰, where 85 % of the duplicates fell within 0.10 ‰ of the first analysis. Thirty-one samples were processed through the entire procedure, including ion-exchange separations, and the average reproducibility for these duplicates was also ± 0.06 ‰, where 90 % of the duplicates fell within ± 0.12 ‰ of the first analysis. Eight aqueous Fe(II) samples were re-analyzed 6 months after initial collection to test for stability of the solutions in terms of possible oxidation and precipitation of the anaerobically-collected samples, and the average reproducibility was ± 0.10 ‰.

Comparison between laboratories may be made through the IRMM-14 Fe standard, which has a $\delta^{56}\text{Fe}$ value of -0.09 ± 0.05 ‰ (Beard et al., 2003a). Note that some labs report Fe isotope variations in ϵ notation (parts per 10,000) relative to an assumed $\epsilon^{56}\text{Fe}$ value of zero for the IRMM-14 standard (e.g., Zhu et al., 2001); the $\epsilon^{56}\text{Fe}$ values reported by these labs may be converted to equivalent $\delta^{56}\text{Fe}$ values as reported here by dividing by 10 and adding 0.09 ‰. It is important to note that Fe isotope data reported in terms of $^{57}\text{Fe}/^{54}\text{Fe}$ ratios will appear to be 1.5 times larger than if reported in terms of $^{56}\text{Fe}/^{54}\text{Fe}$ ratios, although both approaches describe the same isotopic variability on a ‰/mass basis.

Experiment 1: Biogenic ferrous carbonate mineral production by *Shewanella putrefaciens*

S. putrefaciens strain CN32 was cultivated aerobically in tryptic soy broth at 37 °C. After 16 hr of aerobic growth, cells were collected by centrifugation (7000 x g, 10 min), washed in anaerobic bicarbonate buffer, and re-suspended to a density of ca. 10^9 cells mL⁻¹. The washed cells were used to inoculate (final cell density ca. 10^8 mL⁻¹) bicarbonate-buffered growth medium (30 mM NaHCO₃, 0.5 mM KH₂PO₄, 10 mM NH₄Cl, plus vitamins and trace elements) containing 10 mM sodium lactate as an electron donor and 10 mmol L⁻¹ of freshly synthesized HFO. The HFO was produced by adjusting the pH of 0.4M FeCl₃·6H₂O to 7.0 with 1M NaOH, after which the oxide precipitated was washed by centrifugation with distilled H₂O until the Cl⁻ concentration was < 1 mM (Lovley and Phillips, 1986).

The culture medium also contained 100 g L⁻¹ of quartz sand (Sigma Chemicals), which promoted carbonate mineral precipitation and prevented the adherence of mineral precipitates to the bottom of the culture bottles. Experiment 1A did not contain Ca, but in Experiment 1B, 10 mM CaCl₂·2H₂O was added in order to induce formation of Ca-bearing ferrous carbonate precipitates. Previous studies showed that ankerite and siderite are the dominant carbonate mineral end-products of HFO reduction by strain CN32 in the presence and absence of 10 mM Ca, respectively (Roden et al., 2002). No detectable amounts of CaCO₃ minerals precipitated in either the inoculated experiments or the abiotic controls, as determined by XRD spectra, despite the fact that under the conditions of these experiments, CaCO₃ was always over-saturated (Roden et al., 2002).

After 485 d of incubation at 30 °C, the solid-phase end-products of HFO reduction in Experiment 1 were collected by centrifugation, washed with sterile anaerobic Pipes buffer, and dried under a stream of O₂-free N₂. The aqueous phase of the cultures was filtered through a 0.2 µm diameter syringe filter and acidified with HNO₃ (1% final concentration). Solid run products were characterized using XRD (bulk material), as well as SEM imaging. Iron and Ca contents of the total solid products were obtained using Atomic Absorption spectroscopy with a graphite furnace. Chemical compositions were determined for individual crystals using EDS spectra obtained during SEM imaging.

Experiments 2 and 3: Biogenic magnetite production by *Geobacter sulfurreducens* and *Shewanella algae*

S. algae and *G. sulfurreducens* were cultivated in anaerobic (80% N₂, 20% CO₂ headspace) bicarbonate-buffered growth medium (30 mM NaHCO₃, 4.4 mM KH₂PO₄, 28 mM NH₄Cl, plus vitamins and trace elements, as described in Lovley and Phillips, 1988), containing 20 mM sodium lactate (*S. algae*) or 20 mM sodium acetate (*G. sulfurreducens*) as the electron donor, and sodium fumarate (20 mM) as the electron acceptor. After 3-4 d of growth at 30 °C, cells were harvested by centrifugation (7000 × g, 10 min), washed in sterile, anaerobic bicarbonate buffer, and re-suspended to a density of ca. 10⁹ cells mL⁻¹. Thirteen replicate 50-mL bottles of Pipes-buffered (10 mM, pH 6.8) HFO (50 mmol L⁻¹) medium were inoculated with ca. 10⁷ cells mL⁻¹ of washed cells that were initially grown in fumarate. Pipes-buffered rather than bicarbonate-buffered medium was used in this experiment in order to favor magnetite production over siderite precipitation during HFO reduction. The 10-fold lower cell density relative to Experiments 1, 4, 5, and 6 was designed to slow down the overall rate of HFO biotransformation in order to observe more clearly temporal patterns in Fe isotope fraction associated with phase conversions, as well as assess any kinetic effects on Fe isotope fractionation. Single culture bottles for each species were sacrificed (no pasteurization) at various times during a 291-d incubation period. The solid and aqueous phases were separated by centrifugation (7000 × g, 10 min), and the pellet was stored frozen as a wet paste under N₂ prior to wet-chemical, isotopic, XRD, and SEM analysis as described above. The total solid was analyzed for each bottle, and partial dissolutions of the total solid in 0.5 M HCl was done for each bottle to monitor HFO conversion. In addition, partial dissolutions in weak HCl were done for three time samples for each species.

Experiment 4: Biogenic magnetite and siderite production by *Geobacter sulfurreducens*

G. sulfurreducens was grown as in Experiment 2 and washed cells were used to inoculate (final cell density ca. 10⁸ mL⁻¹) bicarbonate-buffered growth medium (30 mM NaHCO₃, 1 mM KH₂PO₄, 10 mM NH₄Cl, plus vitamins and trace elements) containing 20 mM sodium acetate and 50 mmol L⁻¹ of freshly synthesized HFO (see above). Single 50 mL bottles were sacrificed after 0, 4, 11, 22, or 164 d of incubation. The bottles sacrificed at 0, 4, 11, and 22 d were pasteurized at 80 °C in a water bath for 15 min; the bottle taken at 164 d was not pasteurized, nor were samples in the other experiments. SEM imaging of control experiments with and without pasteurization did not show significant differences in crystallinity of the solid products, suggesting that re-crystallization during pasteurization was minimal.

Visual inspection indicated extensive conversion of HFO to magnetite after ca. 4 d of incubation. Time-zero controls and uninoculated cultures showed no HFO phase conversion after 164 d, as shown visually and in ferric and ferrous iron assays by *Ferrozine*. The magnetic precipitates were separated (inside an anaerobic chamber) from the aqueous phase by decanting the liquid into a beaker while holding the culture bottles up to a strong magnet. Virtually all of the mineral precipitates in the cultures were held in place by the magnet, and there was no visual evidence for the presence of residual HFO in the decanted liquid. However, ferric and ferrous iron contents determined on the magnetic concentrates indicated the presence of significant quantities of unreacted HFO up through 22 d (Table 6). The magnetic precipitates were washed twice with sterile, anaerobic Pipes buffer (10 mM, pH 6.7), here again using a magnet to separate the precipitates from the liquid phase. The washed precipitates were dried under a stream of O₂-free N₂ and stored anaerobically.

SEM and TEM imaging, as well as partial and total HCl extractions were used to assess the heterogeneity of the solid phase components and the extent of reaction of HFO to magnetite. These extractions included rinsing the solid in 0.1 M HCl, partial extraction in 0.5 M HCl, and total extraction in 3 M HCl, followed by determination of ferric/ferrous ratios in the extractable components using *Ferrozine* assays, following the approach of Fredrickson et al. (1998). The solid run products were further characterized for crystal structure by XRD on bulk products, as well as electron diffraction measurements on individual or groups of crystals using TEM. Chemical compositions for individual crystals were obtained using EDS spectra during SEM analysis.

Experiments 5 and 6: Biogenic magnetite and siderite production by *Geobacter sulfurreducens*

The culture medium (bicarbonate-buffered) for these experiments was identical to that employed in Experiment 4 with *G. sulfurreducens*, with the exception that the NTA/trace element solution was omitted from Experiment 6. Two sets of duplicate 100-mL bottles (0 and 1), with NTA (Experiment 5) and without NTA (Experiment 6), were inoculated with ca. 10⁸ cells mL⁻¹ of washed acetate/fumarate-grown cells. In contrast to previous experiments in which whole bottles of inoculated culture medium were sacrificed over time, in this experiment the same culture bottles were subsampled periodically over time for wet-chemical, isotopic, XRD, and SEM/TEM analysis as described above. Separate 10-mL subsamples were collected for separation of magnetic vs. nonmagnetic solids, which was achieved by immersing a Teflon-coated magnetic stir bar retriever into the subsample, and then transferring the magnetic solids to a vial containing 5 mL of 6M HCl, in which the solids rapidly dissolved. The stir bar retriever was rinsed thoroughly between separations. The residual non-magnetic solids were dissolved in 0.5M HCl, and the quantity of Fe in magnetic vs. nonmagnetic separates was determined with *Ferrozine*. Sufficient solid and solution Fe was obtained from the original duplicate bottles (0 and 1) for Experiment 5 (NTA-bearing) through 27 d, and for Experiment 6 (NTA-absent) through the end of the entire run at 90 d; for Experiment 5, a third reserve bottle (2) was required for samples taken at 57, 90, and 113 d, and therefore a discontinuity in the reduction progress and Fe isotope compositions is possible. In addition to analysis of the total and magnetic solid, partial dissolution of solids for Experiment 5 was done using weak HCl and HAc.