Facultative Fe(III) and Mn(IV) Reducer Diversity

A report on the independent project of:

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Introduction.

Not all organisms have the luxury of using dioxygen as an electron acceptor. Many other oxidized compounds such as nitrate, Mn(IV) oxides, Fe(III) oxides, sulfate and CO_2 also serve as terminal electron acceptors during microbial respiration (Canfield & Thamdrup 2009). These processes have long been recognized as important drivers of biogeochemical cycles (Falkowski et al. 2008). Microbial reduction of transition metals in particular has been studied for decades for its implications for trace metal availability, geochemical processes, bioremediation, and energy production (Nealson & Saffarini 1994; Anderson et al. 2003; Stobbe et al. 1999). Despite a large interest in the diversity and physiology of organisms that reduce oxidized transition metals, the molecular underpinnings and conferred advantage of fermentative metal reduction are still not well understood.

Several bacterial taxa that reduce manganese oxides (generally MnO_2) and iron oxides (including iron oxides, iron hydroxides, and iron oxyhydroxides—hereafter referred to as Fe oxides or Fe_2O_3) have been discovered to grow through fermentation of various types (Lovley 1987). The reduction of these metal oxides is not critical to the organisms' growth, however (Lovley 2006). These organisms facultatively resort to fermentation for energy and can do so in the absence of Fe(III) or Mn(IV), the presence of which does not often enhance growth rate (Lovley 2006). However, reduction of oxidized metal species does favor the formation of more oxidized fermentation products, suggesting that the metals serve as an electron sink in the reoxidation of redox moieties involved in fermentation (Benz, Schink & Brune 1998). Suspecting this role of oxidized transition metals in microbial fermentative metabolism, several experiments can be constructed to test the predicted mechanisms by which this reduction occurs, which in turn can support or reject the hypothesis that metal reduction recycles important redox molecules during fermentation.

This miniature project explored the enrichment, isolation, and initial characterization of facultative fermenting Mn(IV) and Fe(III) reducers. Inoculum from environmental freshwater sediment samples was plated onto MnO_2-containing freshwater plates and grown aerobically to try to isolate fermentative Mn(IV) reducers that grew in the hypothesized anaerobic interiors of colonies. Inoculum from environmental freshwater, brackish and marine sediment samples was added to anaerobic bottles containing Fe_2O_3 and one of two carbon sources to document iron reduction and fermentation product formation over time. These bottles were passaged to purify, and eventually their contents were plated to isolate pure cultures. The benefit of isolating and identifying individual colonies is the development of a system with which to characterize important parameters and limits on their ability to reduce Fe(III) and Mn(IV). These parameters might include metal oxide concentration, carbon source and concentration, the availability of exogenous electron shuttles such as humic acids, and distance from oxidized metals. Ideally, mutant libraries could be created of organisms incapable of reducing Mn(IV) or Fe(III) oxides, where a simple screen would be clearing of MnO_2 wells or turning Fe_2O_3 lawns from a rusty brown to a blackish color. While several of these parameters possibly influencing metal reduction were explored here, more time than the allotted three weeks post-inoculation would be necessary to more fully explore the mechanism behind fermentative Fe(III) and Mn(IV) reduction.

Methods.

Sampling. 50 ml Falcon tubes each of estuarine, freshwater and marine sediments were collected in Woods Hole, MA on July 30th, 2014 at high tide between 1500 and 1700 hours. The estuarine sample was taken from 8 m into a pond formed by Trunk River just prior to opening out into Vineyard Strait. Sediments were sandy and likely anoxic, as inferred from pockets of gas (likely methane or hydrogen sulfide) bubbling up from below the sediment surface; interstitial water had a pH of 7, salinity 4.8% w/v, and density 1.004 g/cm^3, thus resembling freshwater more strongly than seawater. The freshwater sample was taken from submerged sediments at School Street Marsh on School St. just north of Maury Lane 5 m upstream from the embankment at the edge of School St. The sediments were dark-colored, likely rich in organics, and smelled of sulfide. The marine sample was taken from Stoney Beach, near the intersection of Bar Neck Rd. and Gosnold Rd., at a depth of 1.5 m. These sediments were predominantly sandy with some green microbial coloration on top that was avoided when sampling. Samples were stored at room temperature in the dark in 50 ml Falcon tubes.

Treatments and enrichment protocols. *MnO_2 reducers*. In order to enrich for Mn oxide reducers, solid freshwater media (FWB) was prepared with the alterations of initially using only 950 ml DI H_2O and adding 21.7 ml of 2.3 M MnO_2 slurry (final concentration 50 mM) to the media after autoclaving and just prior to pouring plates, as well as including the Bacto agar. One medium was prepared without MnO_2 and pipetted while warm into plates in 15 ml aliquots. FWB media with MnO_2 was pipetted in 7.5 ml aliquots over the FWB layer once it was cooled to make MnO_2 pie plates. A third layer was poured over the cooled MnO_2 layer in 7.5 ml aliquots to make MnO_2 sandwich plates. School Street Marsh sediments and their ~1 cm overlying water were shaken by hand, allowed to settle, and 0.5 ml of the supernatant removed to use as inoculum. The supernatant was taken to 1 ml with 1X FW Base and then a dilution series up to $1/10^5$ was prepared. 50 µl of each dilution (six total) were spread-plated on the MnO_2 plates and incubated at 34 ^{0}C. Clear colonies were struck onto new plates and ability to clear MnO_2 layer (turning it from gray with black mineral particles to clear) was used as an indicator of Mn(IV) reduction. Five Mn(IV) reducers were isolated in total, all of which could additionally survive on and clear the Mn oxides when grown on the sandwich plates. Isolates were preserved by flooding 24 h plates with 1X FWB, adding 750 µl of cell suspension to a 2 ml cryo-tube, and then adding 750 µl of 50% glycerol solution and storing at -4 ^{0}C.

Fe_2O_3 reducers. 250 ml serum bottles were autoclaved, 50 ml of either degassed FWA or SW medium was added to each bottle, and then bottles were individually degassed with 80:20 N_2/CO_2. Bottles received 5 ml of either 1 M acetate + 5 ml of 1 M dextrose or just 5 ml of 1 M dextrose (making each carbon source 10 mM) and 0.8 g of PCIO (final concentration of Fe_2O_3 being 10 mM). In the anaerobic chamber, pea-sized inocula from the appropriate sampling site were added to each vial as described in Table 1. Enrichments were left to grow in the dark at 30 ^{0}C. Turbidity, production of bubbles and darkening of iron oxides were interpreted as signs of microbial growth. Once cultures became milky, they were passaged into media identical to their original bottles.

Bottle ID	1	2	3	4	5	6
Sample Site	Trunk River	Trunk River	School St. Marsh	School St. Marsh	Stoney Beach	Stoney Beach
Sample Type	Estuarine	Estuarine	Freshwater	Freshwater	Marine	Marine
Medium	SW	FWA	FWA	FWA	SW	SW
Carbon Source	Dextrose	Dextrose	Dextrose	Dex. + Acetate	Dextrose	Dex. + Acetate

Table 1: The six Fe_2O_3 reducer enrichment treatments used. SW and FWA media are described in Appendix 1. Dextrose and acetate were each added at 10 mM concentration to the bottles, in equal molarity with Fe_2O_3, 0.8 g of which was added to each bottle.

After 8 days, the most recent culture of each treatment was spread plated onto FWA plates with the appropriate salinity and carbon source in the anaerobic chamber. Solid FWA media was degassed with 80:20 N_2/CO_2 prior to pouring in the anaerobic chamber. Colonies that appeared over Fe oxide clearings were struck onto new plates after 24 h of growth. 32 h later, single colonies were picked for PCR and potential 16S sequencing.

Fe(II) and Fe(III) quantification. The ferrozine assay (Stookey (1970), amended by AK in 2003 in Woods Hole) was used to colorimetrically determine ferrous and total iron content in Fe oxide reducer enrichments. Dissolved and adsorbed Fe(II) was assessed by adding 0.1 ml of sample to 0.9 ml 0.5 M HCl, mixing, and filtering. After 2 min, 0.1 ml of the filtered solution was transferred to 0.9 ml of 1 M HCl in disposable cuvettes and 1 ml ferrozine solution (0.1% ferrozine, w/v, in 50% w/v ammonium acetate) was added. Solutions were mixed, left standing 10 min, then measured for absorbance at 562 nm. $FeSO_4$ standards were prepared at 50, 100, 250 and 500 μM concentrations and treated with the same procedure. Total iron, or Fe(II) + Fe(III), was assessed by adding to 0.9 ml 6M HCl 0.1 ml of sample and mixing. After 24 h, samples were passed through 0.4 μm syringe filters into disposable cuvettes filled with 0.9 ml 1 M hydroxylamine hydrochloride (HAHC, 10% w/v in 1 M HCl) and mixed. 1 ml of ferrozine solution was then added, the solutions left to sit 10 min, and then the absorbance was read at 562 nm. Where necessary, samples were diluted with DI H_2O to fall within the absorbance range covered by the standard curve. 1 M HCl was used to blank the Fe(II) readings and 1 M HAHC was used to blank the total iron readings on the spectrophotometer.

Chemical profiles. Oxygen profiles for a MnO_2 reducing isolate were created using a Unisense O_2 probe. All measurements were taken using ambient oxygen as a baseline, as the instrument tended to drift its baseline from one profile to the next. Profiles were taken from several centimeters above the petri dish rim to ~1..5 mm into the agar. Profiles were obtained for a single round white colony, the cleared agar 1 cm from any colonies, and an adjacent colony 1 h later after letting the plate sit with the lid on to determine whether the novel profile was a product of microbial activity.

16S sequencing. Single colonies were picked and boiled for 10 min at 90 ^{0}C in 20 µl nuclease-free water. 2 µl of each boiled sample were added to PCR tubes containing a reaction mix of 12.5 µl Promega Go-Taq Green 2X Mix, 2.0 µl 16S_8F (15 pM), 2.0 µl 16S_1391R (15 pM), and 6.5 µl nuclease-free water. PCRs were run with initial denaturation set at 2 min at 95 ^{0}C followed by 30 repeats of denaturation (30 sec, 95 ^{0}C), annealing (30 sec, 46 ^{0}C), and extension (1.5 min, 72 ^{0}C), then a 10 min final extension at 72 ^{0}C. Bands were visualized on 1% agarose gels using a 1 kb ladder and run at 120 V. Purified bands were submitted for Sanger sequencing, but results were unavailable at the time of this report.

Imaging. Samples from the iron oxide reducer enrichments were viewed using Zeiss confocal microscopes and a scanning electron microscope (SEM) that had energy dispersive X-ray spectroscopy (EDS) capabilities. Manganese oxide reducing colonies were viewed using a Zeiss dissecting microscope.

Results and Discussion.

MnO_2 reducers. Five colonies were isolated that completely cleared Mn oxides on the pie plates (Fig. 1). While each of these isolates grew on the sandwich plates and mineral clearing appeared to occur, the layers of agar prevented clear views and accurate assessments of the presence or absence of MnO_2 in the middle layer.

Because these enrichments were performed under aerobic conditions, the manganese oxide clearings—assumed to represent successful microbial reduction of these minerals—suggested that the interiors of the colonies experienced an anaerobic environment. Because O_2 is a more favorable terminal electron acceptor than Mn(IV) and respiration yields more energy than fermentation, Mn(IV) reduction should only occur in environments that are low in oxygen. In order to test this hypothesis and determine whether fermentation or respiration were the major metabolic pathway on these colonies' interior, chemical profiles were taken vertically over the colonies and surrounding agar for a white isolate (Figs 1a, 2). pH could not be determined due to the microsensor being broken.

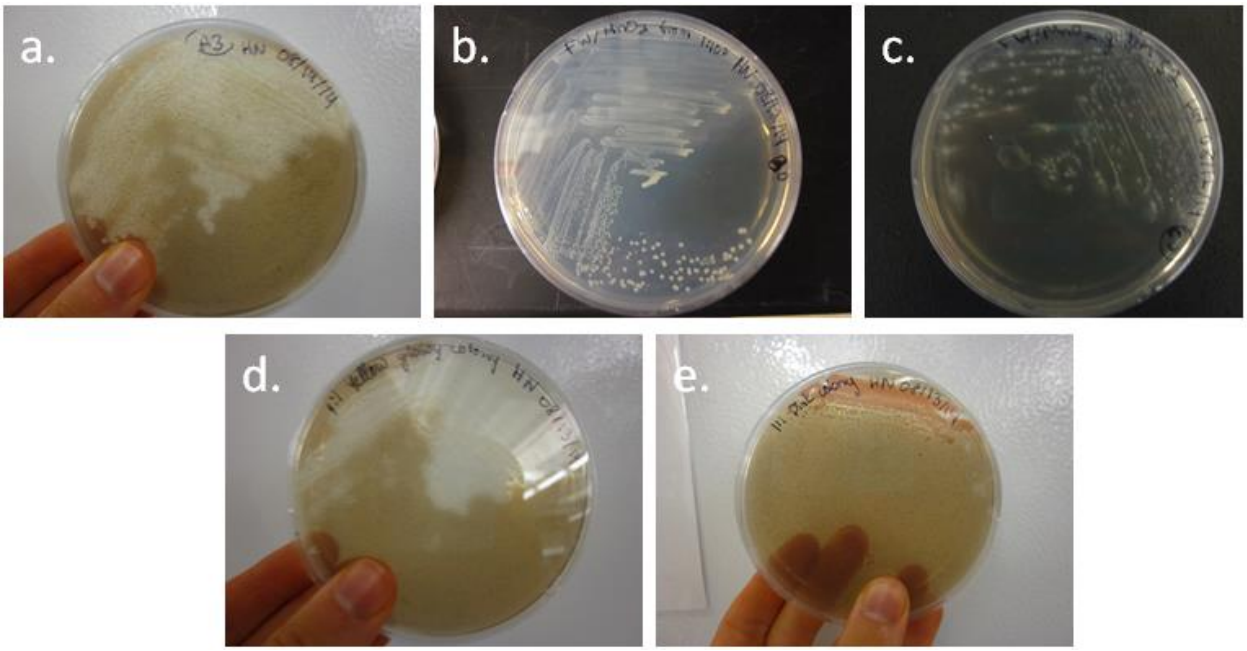

Figure 1: Five isolates of Mn(IV) reducers. All isolates were re-struck at least once and cleared the MnO_2 particles around or, for the isolate in (c), directly beneath their colonies. Isolates shown in panels a, b, d and e also grew on and cleared MnO_2 when grown on MnO_2-agar sandwich plates overnight.

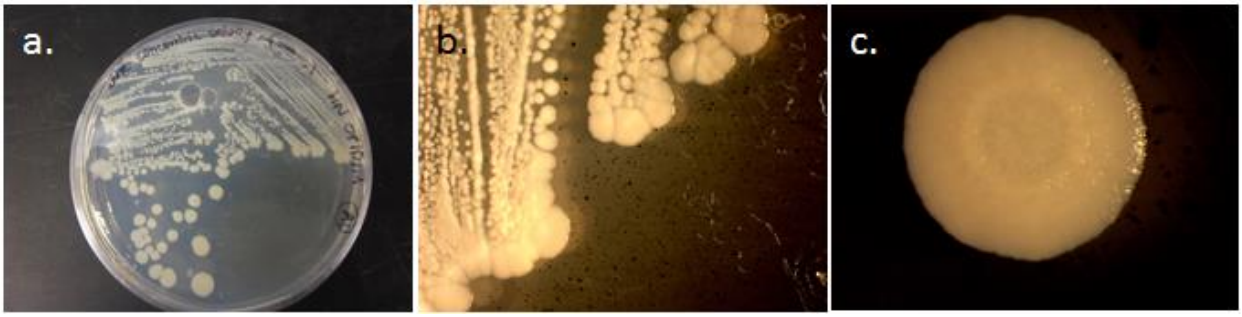

Figure 2: Colonies of the white isolate that cleared Mn(IV) oxides and was measured for O_2 (Fig. 3). The single colony pictured in (c) is the colony for which the oxygen profile in 3a. was taken. On the same plate (a), the front at which MnO_2 occurs is seen in (b) as a lighter area near the colonies with fewer black MnO_2 particles.

The oxygen profile over the colony showed a surprising increase in O_2 relative to ambient partial pressures once the probe descended below the height of the petri dish rim (Fig. 3a). Oxygen was then depleted descending into the colony, but not below ambient levels. Moving into the agar, the oxygen level once again rose. To test whether this phenomenon was directly related to the colony, an identical profile was taken over Mn oxide-cleared agar (Fig. 3c). This profile looked as one might expect for a plate of respiring organisms: oxygen levels slowly declined as the probe was lowered towards the agar, then more uniformly and sharply declined going into the agar (Fig. 3c). However, when the profile over a colony was repeated 1 h later,

the O_2 levels resembled those seen in the profile over the cleared agar (Fig. 3b). Four hours after letting the plate sit unmoved with the lid on, the oxygen profile resembled that seen in Fig. 3c. The time-dependent oxygen profile coupled with the oxygen supersaturation seen immediately above and below the colony in Fig. 3a suggest that the microbes were either directly producing O_2 or else modifying their medium in a way that evolved O_2.

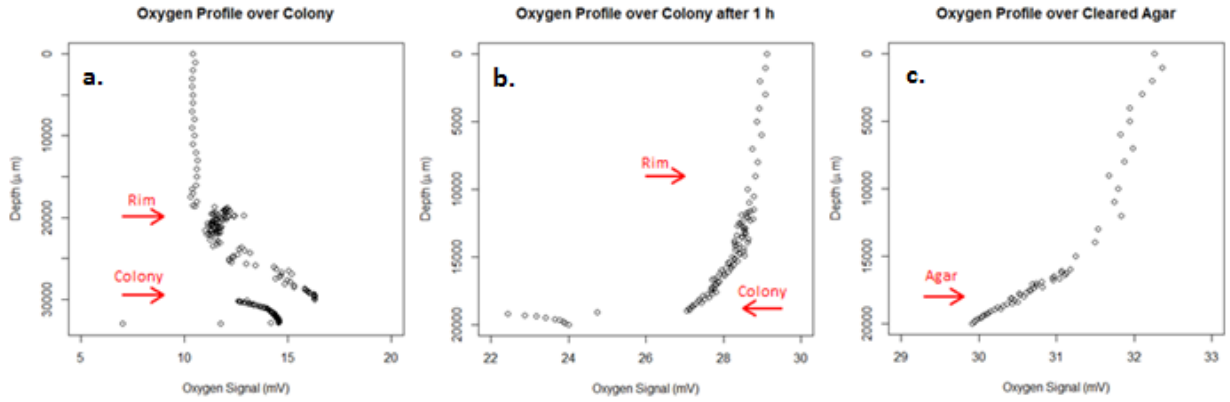

Figure 3: Oxygen profiles over MnO_2 pie plate with white, circular Mn(IV) reducing isolate. Oxygen increases from ambient levels around colony within 20 min of opening the dish (a), whereas oxygen levels continually decline over MnO_2 clearings in the agar (b) or over a colony after 1 h of the dish being opened (c). Axes are NOT identical.

To test whether these organisms were producing O_2 using catalase, a hydrogen peroxide assay was performed. 30 µl of Triton X-100 were used to suspend a colony from the plate being profiled. 30 µl 30% w/v hydrogen peroxide were added to the tube and mixed. Production of O_2 bubbles would indicate catalase activity. While the suspension had bubbles, the bubbles were large and immobile. The bubbles were likely due to Triton X-100 because they resembled the bubbles present in a cell-less control. Alternatively, the MnO_2 or reduced Mn(II) compounds could be interacting with media- or biologically-derived chemicals to evolve oxygen. Potential reactions were not explored further due to time limitations. These unexplained results serve as a reminder that microbiological processes cannot be understood except in the context of the organisms' surrounding chemical environment, which in the case of aerobic manganese reducers is poorly understood.

Fe_2O_3 reducers. *Growth in liquid medium.* One day after inoculation, bottles 2, 3 and 4 had developed milky white (3) and milky white-yellow (2 and 4) turbidity. These same bottles had many bubbles which were large (3) or small (2 and 4), indicating the production of a gas. No change in PCIO was observed for these bottles. No changes were observed in the other enrichments. Two days after inoculation, bottles 1-4 had developed chalk colored turbidity. All bottles had bubbles, which were large for the seawater enrichments (up to 1 cm diameter) and small (<1 mm diameter) for all the other enrichments. The iron oxides from bottles 3 and 4 developed dark brown to black coloration that indicated ferrous iron oxides were present. Three days after inoculation, all bottles had developed chalky white to white-yellow turbidity but only

3 and 4 had dark colored solids. Four days after inoculation, half of the solids in bottle 2 had become dark brown while the other bottles' iron oxides remained unchanged. Turbidity in bottle 1 had declined. By five days after inoculation, all solids except for those in bottle 6 had developed some dark brown to black coloration. Turbidity and bubbles were unchanged. Botle 5 was dropped and broken this day and therefore does not appear in any figures or quantitative data. The bottles appeared this way going into six and seven days after inoculation. Finally, on day eight after inoculation, the iron oxides in bottle 6 had begun to darken (Fig. 4).

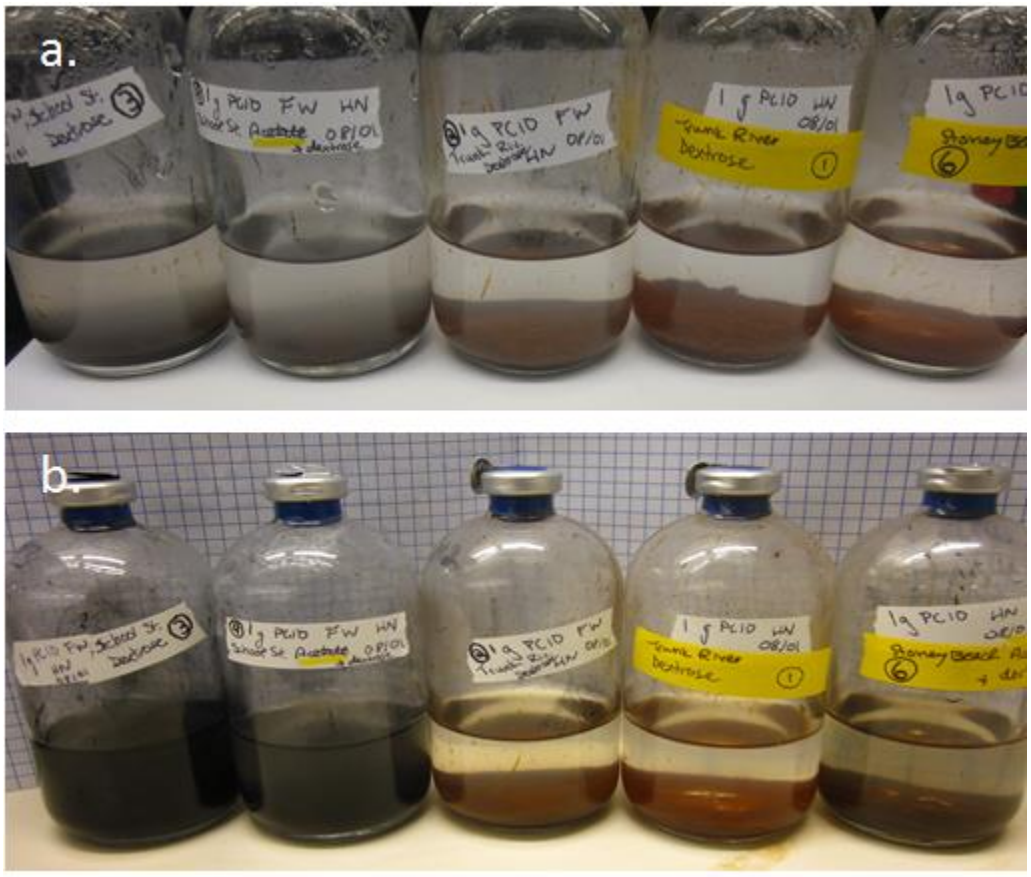

Figure 4: Enrichment bottles imaged after 24 h without perturbation or mixing six days (a) and nine days (b) after inoculation. Samples ordered from left to right are treatments 3, 4, 2, 1, and 6.

Microscope images confirmed the presence of black minerals in the enrichments inoculated with School St. and Trunk River sediments but not for the enrichment inoculated from Stoney Beach sediments (Fig. 5). School St. enrichments developed more organized crystals that tended to orient themselves in a particular direction and contained higher concentrations of the black mineral that is possibly magnetite (Fig. 5a,b). However, magnet experiments did not succeed in reorienting iron oxides or in attracting potentially magnetotactic bacteria. In general, no significant difference in metal oxidation state (*i.e.* the color of the solid) was observed visually for freshwater enrichments using acetate versus dextrose carbon sources.

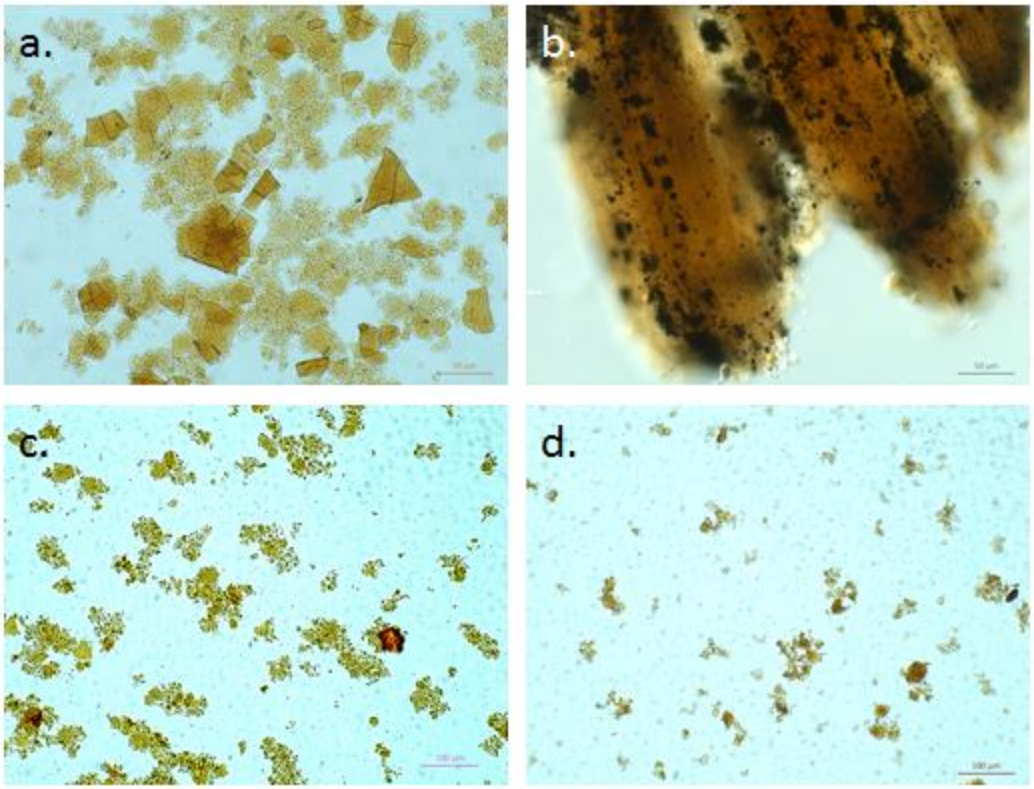

Figure 5: Iron oxides from School St. enrichments (a, b), a Trunk River enrichment (c), and the Stoney Beach enrichment (d) nine days after inoculation. Black particles, possibly magnetite, are visible amongst the rust colored particles in panels a, b and c. No magnetite is present in the Stoney Beach iron oxides.

Interpreting turbidity as evidence of cell growth and black solids as Fe(II) compounds, the freshwater enrichments from freshwater inoculum grew the fastest, followed by the freshwater enrichments of estuarine inoculum and then the marine enrichment. This pattern reflects the baseline distribution of Fe(III) reducers in freshwater versus non-extreme marine environments that is found in the literature (Lovley 2006). However, a low number of studies has likely introduced bias in our understanding of which environments are likely to harbor Fe(III) oxide reducers. Furthermore, the chalky turbidity may be due to $Fe(OH)_2$ formation rather than cells. Similarly, the black solids could have been either FeO or the product of reactions with either $Fe(OH)_2$ or FeO that result in the formation of magnetite, an Fe(II,III) oxide mineral with the chemical formula Fe_3O_4. Visual methods therefore could not definitively determine the progress of cellular growth in this enrichment process.

Cells were present in all enrichments. Freshwater enrichments using both acetate and dextrose carbon sources produced rod-shaped organisms, including subterminal endospore-formers in the School St. enrichments (Fig. 6a,b). Trunk River enrichments had a mixture of shortened rods (the majority) and rounded cells (Fig. 6c,d). Stoney Beach enrichments had predominantly round cells (Fig. 6e,f). Cells in all enrichments appeared to be evenly distributed with respect to Fe oxide particles. This suggests that the method of Fe(III) reduction does not

require attachment; however, rapid adsorption-desorption coupled with metal reduction cannot be ruled out.

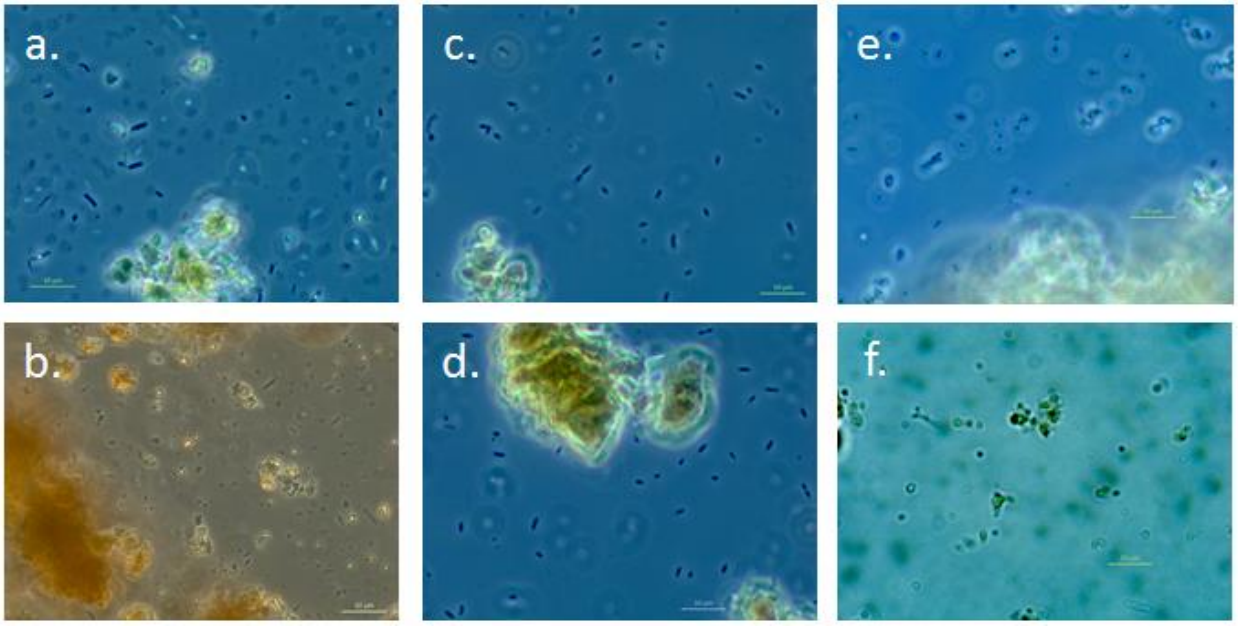

Figure 6: The organisms found in the School St. enrichments (a & b), the Trunk River enrichments (c & d), and the Stoney Beach enrichment (e & f). No cultures were pure and cells appeared evenly distributed with respect to the mineral particles.

Iron oxide composition. PCIO had a very smooth, angular crystal structure (Fig. 7a). Presumably reduced iron oxides had more amorphous structures in addition to extremely angular crystals (Fig. 7b). Iron oxides had on average similar Fe:O content between the original PCIO crystals and the darker solids (Fig. 7c,d). However, in addition to minerals containing between a 1:2 and 1:3 ratio of iron to oxygen, the enrichments that formed darker solids also had a reduced iron oxide with an Fe:O ratio near 1:1 (Fig. 8). This supports the use of dark coloration as an indicator of ferrous compounds. Elements such as S, N, P and C were not present in large abundance on the minerals. Cells were not associated with the minerals. While not conclusive, this supports the use of an electron shuttle to reduce Fe(III).

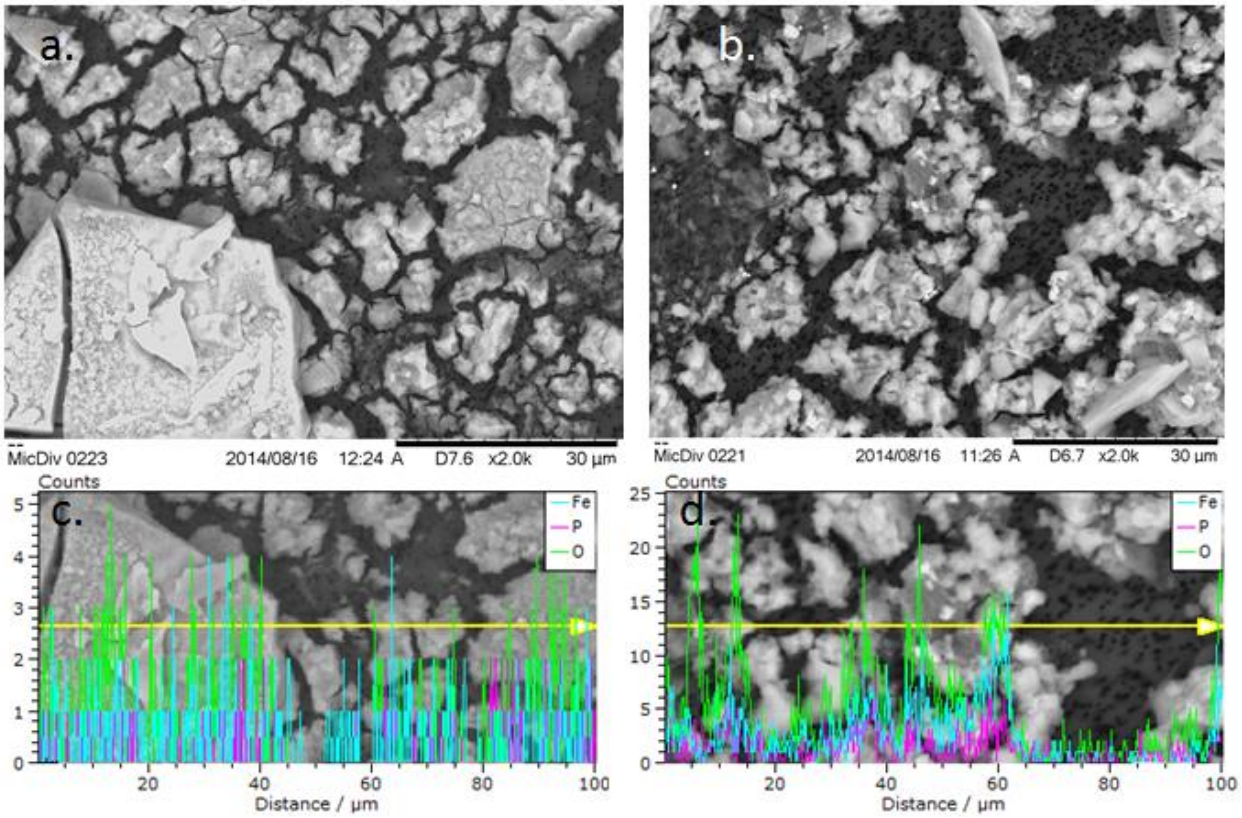

Figure 7: Poorly crystalline iron(III) oxides (a) and iron(II) oxides (b) viewed by SEM with transects depicting Fe, P and O content in the Fe(III) (c) and Fe(II,III) minerals (d), analyzed using EDS. PCIO was taken from an uninoculated control jar. Reduced iron oxides were taken from the School St. + dextrose enrichment 10 days after inoculation.

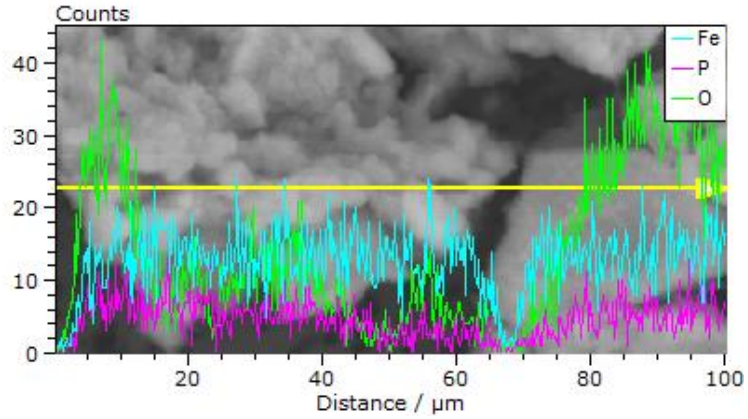

Figure 8: Transect along minerals from School St. + dextrose enrichment. Fe:O ratio varies between particles, meaning that Fe_2O_3 reduction occurred but was not complete despite completely black (to the unaided eye) solids.

Conclusions.

Manganese(IV) oxide reducers were successfully isolated. While acid production and chemical reduction of the MnO_2 cannot be ruled out, results from a pilot study by C. Salgado suggest that a pH of 1-2 would need to be achieved before MnO_2 mineral is chemically reduced. Thus, it seems reasonable that we isolated MnO_2 reducers. Furthermore, these organisms reduce Mn(IV) from a distance. This implicates a shuttle or long-distance transmission method such as an electron wire. Future studies could identify distance limits on MnO_2 reduction by these isolates and compare these distances to diffusion gradients of endogenous electron shuttles and exportable redox-active moieties. Transposon mutagenesis and screening for organisms that do not clear MnO_2 media accompanied by arbitrary PCR would identify genes responsible for this phenotype and possibly elucidate a pathway for fermentative reduction of Mn(IV) compounds.

Iron(III) reducers were cultured in communities in liquid medium and transferred to solid medium to obtain isolated colonies. While individual isolates that are amenable to laboratory experiments should be found to facilitate studies of the mechanism of fermentative Fe(III) reduction (which would resemble the experiments described for the Mn(IV) reducers), other ecological and energetics questions could be explored within the multi-species cultures. For instance, time series of qPCR for specific organisms accompanied with GC and HPLC measurements might identify organisms present and their metabolisms over the succession of the microbial community from highly abundant carbon and Fe(III) sources to less abundant substrates and possibly lower pH conditions.

Suffice it to say, these enrichments and few characterizations of the metal oxides and organisms merely probed the surface of defining and explaining the diversity of facultative fermenting reducers of Fe(III) and Mn(IV). Further steps have been suggested. It now rests in the hands of the reader to not let this topic disappear from the literature for decades.

Acknowledgements. Special thanks to A. Bose for help with the anaerobic cultures, A. Stacy for help prepping the ferrozine assay, C. salgado and D. Newman for suggestions on isolating MnO_2 reducers, and the Microbial Diversity class of 2014 for being generally helpful. Thanks to NSF, the University of Michigan, and the William Randolph Hearst Educational Endowment for funding this educational experience.

Appendix 1: Media Preparation.

Freshwater Medium A (FWA, for Fe_2O_3 reducers). Freshwater liquid medium was prepared by adding to 900 ml deionized (DI) water 10 ml of 100X Freshwater Base (final concentration 17.1 mM NaCl, 1.97 mM $MgCl_2 \cdot 6H_2O$, 0.68 mM $CaCl_2 \cdot 2H_2O$, 6.71 mM KCl), 10 ml of 100X NH_4Cl (final concentration 10 mM), 2.5 g $NaHCO_3$ (final concentration 29.8 mM), 0.6 g of NaH_2PO_4 (final concentration 4.2 mM), 1 ml 1M Na_2SO_4 (final concentration 1 mM), 1 ml 1000X Mineral Stock Solution, 15 ml 1M MOPS (pH 7.2), the trace metals $AlK(SO_4)_2$ and $MgSO_4$ at 0.001 g and 0.614 g, respectively, and 50 ml of 1M carbon source stock solution (final concentration 50 mM). The media was autoclaved, 1 ml of 1000X Vitamin Stock was added, and the bottle was degassed while warm with N_1/CO_2 at a ratio of 80:20. For solid medium, 15 g of Bacto agar were added prior to autoclaving.

Seawater Medium (SW). 930 ml of 1X Salt Water Base (Appendix 1) were dispensed. To this were added 2.50 g $NaHCO_3$ (final concentration 29.8 mM), 10 ml 100X K_2HPO_4/KH_2PO_4 buffer (final concentration 1 mM), 10 ml 100X NH_4Cl (final concentration 10 mM), 1 ml 1000X Mineral Stock Solution, the trace metals $AlK(SO_4)_2$ and $MgSO_4$ at 0.001 g and 0.614 g, respectively, and 50 ml 1 M carbon source. The solution was then autoclaved, after which 1 ml of 1000X Vitamin Stock Solution was added with sterile technique through a 0.2 μm syringe filter. For solid medium, 15 g of Bacto agar were added prior to autoclaving.

Freshwater Medium B (FWB, for MnO_2 reducers). Added into 970 ml DI 10 ml of 100X Freshwater Base (final concentration 17.1 mM NaCl, 1.97 mM $MgCl_2 \cdot 6H_2O$, 0.68 mM $CaCl_2 \cdot 2H_2O$, 6.71 mM KCl), 10 ml of 100X NH_4Cl (final concentration 10 mM), 0.6 g of NaH_2PO_4 (final concentration 4.2 mM), 1 ml 1M Na_2SO_4 (final concentration 1 mM), 1 ml 1000X Mineral Stock Solution, 15 ml 1M MOPS (pH 7.2), and 50 ml of 1M carbon source stock solution (final concentration 50 mM). For solid media, added 15 g Bacto agar. Autoclaved. With sterile technique, added 1 ml 1000X Vitamin Stock Solution through a 0.2 μm syringe filter.

Carbon Stock Solutions. Anaerobic dextrose and acetate carbon stocks were prepared by dissolving 3.6 g and 1.64 g of each respectively in 20 ml of DI water and then adding these to autoclaved anoxic serum vials through 0.2 μm syringe filters, then replacing the headspace with 80:20 N_2/CO_2. Aerobic dextrose stock was made by adding 36.03 g dextrose to 200 ml of autoclaved DI H_2O.

1000X Mineral Stock Solution. Per L of DI H_2O, the following chemicals were added: 20 mM HCl (20 mM), 2100 mg $FeSO_4 \cdot 7H_2O$ (7.5 mM), 30 mg H_3BO_3 (0.48 mM), 100 mg $MnCl_2 \cdot 4H_2O$ (0.5 mM), 190 mg $CoCl_2 \cdot 6H_2O$ (6.8 mM), 24 mg $NiCl_2 \cdot 6H_2O$ (1.0 mM), 2 mg $CuCl_2 \cdot 2H_2O$ (12 μM), 144 mg $ZnSO_4 \cdot 7H_2O$ (0.5 mM), 36 mg $Na_2MoO_4 \cdot 2H_2O$ (0.15 mM), 3 mg $NaVO_3$ (25 μM), 3 mg $Na_2WO_4 \cdot 2H_2O$ (9 μM), 6 mg $Na_2SeO_3 \cdot 5H_2O$ (23 μM).

1000X Vitamin Solution. Per L of DI H_2O, the following chemicals were added: 1000 ml 10 mM MOPS, pH 7.2 (10 mM), 100 mg Riboflavin (0.1 mg/ml), 30 mg Biotin (0.03 mg/ml), 100 mg Thiamine HCl (0.1 mg/ml), 100 mg L-Ascorbic acid (0.1 mg/ml), 100 mg d-Ca-pantothenate (0.1 mg/ml), 100 mg Folic acid (0.1 mg/ml), 100 mg Nicotinic acid (0.1 mg/ml), 100 mg 4-aminobenzoic acid (0.1 mg/ml), 100 mg pyridoxine HCl (0.1 mg/ml), 100 mg Lipoic acid (0.1 mg/ml), 100 mg NAD (0.1 mg/ml), 100 mg Thiamine pyrophosphate (0.1 mg/ml), 10 mg Cyanocobalamin (0.01 mg/ml). Solution was titrated dropwise with 5 M NaOH until dissolved then filter sterilized using a 0.2 μm filter and refrigerated in the dark.

Iron Oxides. Poorly crystalline iron oxides (PCIO) were prepared as described in Lovley (2006) with several modifications. 27.05 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 250 ml DI H_2O and then brought up to just below pH 7 using 1 M NaOH dropwise while stirring. The slurry was left to stir for 30 min and then centrifuged at 5000 rpm for 15 min. The slurry was then washed with DI H_2O and spun down at 5000 rpm for 15 min, which step was repeated three times. Iron oxides were stored on the bench top at room temperature in fresh DI H_2O after the final wash step.

Manganese Oxides. MnO_2 was prepared as described in Lovley (2006). 250 ml of 30 mM $MnCl_2$ were slowly added to 250 ml of solution with 80 mM NaOH and 20 mM $KMnO_4$ and

the precipitate was centrifuged down at 5000 rpm for 15 min, the supernatant removed, and then washed with DI H_2O three times using the same spin cycle. After the final wash, the solid was suspended in 50 ml of DI H_2O as a concentration of ~2.3 M and stored on the bench top at room temperature.

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