Microbially catalyzed manganese cycling in freshwater aquatic ecosystems

Alexander S. Bradley* & William D. Leavitt*

1Harvard University, Department of Organismic & Evolutionary Biology, Cambridge, MA 02138

*These authors contributed equally to this work.

(Received July 30, 2008; accepted in unrevised form June 14, 2008)

Manganese plays a major role in biogeochemical redox cycling, but remains understudied in aquatic environments. Environmental V6 tag pyrosequencing of sediment from a manganese-enriched freshwater lake (Ashumet Pond, Massachusetts) revealed that one-quarter of all sequences could not be assigned to any known phylogenetic group. Group 8 Acidobacteria, previously known only to be anaerobes, were abundant in the aerobic zone, along with Nitrosomonas. Beta- and Delta-proteobacteria were abundant in both aerobic and anaerobic samples. Attempts to culture manganese-cycling bacteria under a variety of conditions produced isolates that seemingly produce crystalline manganese on plates. Manganese-oxidizing phototrophs were not isolated, but turbidity developed in enrichments targeting these organisms. These results suggest that manganese-cycling microbes are present in the Bacterial community of Ashumet Pond, and may play an important biogeochemical role.

Key Words: manganese biogeochemistry, pyrosequencing, CARD-FISH, SEM

1. Introduction

Manganese is the 12th most abundant element in the Earth’s crust and manganese oxide minerals commonly occur in marine and terrestrial environments, but the role of manganese in biogeochemical cycling remains relatively understudied. Biogeochemists often consider the manganese cycle as an afterthought to the more well-understood iron cycle. This is despite the fact that Mn(IV) is an energetically superior electron acceptor in comparison to Fe(III). Both transition metals share certain aspects of their biogeochemistry. The reduced forms of both iron and manganese are very soluble in water, while the oxidized forms are insoluble, and both metals share the property that abiotic oxidation rates increase with pH. However, at circumneutral pH the abiotic oxidation rate of manganese is very slow in the

Correspondance to:
ASB (bradley@fas.harvard.edu)
WDL (wleavitt@fas.harvard.edu)
absence of catalysis, while the oxidation of reduced iron is comparatively fast. The consequence of the kinetic rates is that reduced iron is more limited than manganese in oxygenated natural waters. The sluggishness of abiotic manganese oxidation also suggests that precipitation of manganese oxides in nature may often be catalyzed by microbial activity.

Iron exists in two ionic forms: Fe(II) and Fe(III), and correspondingly its oxidation consists of the transfer of a single electron. This distinguishes it from the oxidation of Mn(II) to Mn(IV), which is a two-electron transfer. Traditionally the two-electron oxidation of manganese was considered the only one of importance, although recent work by Trouwborst et al. (2006) has demonstrated that Mn(III) can be stabilized in suboxic water columns, and that one-electron transfers involving manganese may be more important than had previously been known.

1.1 Aerobic Mn oxidation
Molecular oxygen can be an electron acceptor for Mn(II). The general equation can be written as the following:

\[ \text{Mn}^{2+} + \frac{1}{2} \text{O}_2 \rightarrow \text{MnO}_2 + 2 \text{H}^+ \] (Tebo, 2005)

Biological oxidation of manganese is assumed to proceed via a pair of one-electron transfers, and in some cases is mediated by a family of enzymes related to multi-copper oxidases (MCOs) enzyme (Tebo, 2005). The ability to carry out this process exists in a wide range of Bacteria, including Firmicutes, Actinobacteria, and the \( \alpha \), \( \beta \), and \( \gamma \)-Proteobacteria (Tebo, 2005; Hansel et al., 2006), and Fungi. The biological role of manganese oxidation remains enigmatic: despite its thermodynamic favorability, there is no good evidence linking manganese oxidation to energy conservation. A range of biological roles for manganese oxidation have been proposed, including superoxide scavenging and electron acceptor storage (Tebo, 2005). It has even been suggested that manganese oxidation does not have any biological role, and exists only as an ‘accidental’ biproduct of the oxidation of other metals, such as iron (Edwards et al., 2003). Catabolic use of reduced manganese remains a pathway ‘missing’ from nature.

1.2 Mn oxidation with other electron acceptors
The redox couple for the Mn(III)/Mn(II) pair has an \( E' \) of +1.18 V, indicating that Mn(III) is an excellent electron acceptor, but that Mn(II) a poor electron donor. This situation is altered if Mn(II) is complexed to bicarbonate, shifting its midpoint potential to +0.61 V. This potential is sufficient for Mn(II)-bicarbonate to be an electron donor coupled to reduction of oxygen to water or nitrate to dinitrogen. It does not allow coupling of Mn(II) oxidation to reduction of sulfate, Fe(III), \( \text{S}^0 \), or nitrate reduction to ammonium. The potential to oxidize Mn(II) with nitrate suggests a potentially viable physiology that remains undescribed.

Geochemical pore water profiles from anoxic marine sediments indicate tight spatial couplings between manganese and nitrogen redox cycling (2-4). Yet, to-date no isolated microorganism is capable of directly linking these reactions for energy metabolism: anaerobic ammonia oxidation coupled to manganese oxide reduction (AmOxMnRed) or manganese oxidation by nitrate reduction (MnOxNORed) (Table 1).

1.3 Potential for chemolithoautotrophy with Mn as an electron donor
Reduced manganese, even coupled to bicarbonate, does not have a reduction potential sufficient to directly reduce inorganic carbon. However, if organisms exist that can conserve energy by the oxidation of Mn(II) coupled to oxygen or nitrate, that energy could be used to generate reducing power for carbon fixation. Alternatively, Mn
could conceivably be used as an electron donor to a photosystem, which would in turn generate reduction potential for carbon fixation, in a manner similar to phototrophic Fe oxidizers (Ehrenreich et al., 1994). All attempts to isolate a phototrophic manganese-oxidizing organism have failed.

An examination of the thermodynamics allows speculation about the type of metabolism that a hypothetical manganese oxidizing phototroph might employ. The midpoint potential of free Mn(II) can not donate electrons to either a Type I or Type II reaction center, which have midpoint potentials of approximately +0.45 V and +1.12 V respectively. Bicarbonate-complexed Mn(II) should be able to donate electrons to a type II reaction center, but not to a type I reaction center. Many previous attempts to isolate phototrophic manganese oxidizers have employed DMCU to inhibit cyanobacterial growth. DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a potent inhibitor of photosystem II, and is often added to enrichments of anaerobic photrophs, which typically use photosystem I only. If a phototrophic manganese oxidizer is ever successfully obtained, we predict that it will use a Type II reaction center. Successful enrichments for these organisms will probably not employ DCMU.

Phototrophic iron oxidation has been used to explain the deposition of massive banded iron formations that accumulated from ~3.8 - 1.8 billion years ago. Much of this deposition occurred before the rise of atmospheric oxygen, and potentially before the evolution of oxygenic photosynthesis. However, there is no corresponding depositional record of manganese oxides. The oldest known manganese deposit is the Kalahari Manganese Field in South Africa, which is deposited coincident with a large rise in atmospheric oxygen concentrations ~2.2 billion years ago. The lack of older deposits has been used as one line of argument to suggest that phototrophic manganese oxidation never evolved (Kirshvink et al., 2008).

1.4 Mn-oxides as electron acceptors
Mn(IV) in manganese oxides is a strong electron acceptor, with an Eo’ that varies by mineral phase but is near +0.55 for the redox pair with Mn(II). This suggests that Mn(IV) could be coupled to the oxidation of organic carbon, as well as many reduced substrates such as ammonium. As is the case for ferric minerals, oxidized manganese compounds are insoluble, and organisms utilizing them must have a means to either transfer oxidized manganese to the interior of the cell (perhaps with a siderophore-like compound), or shuttle electrons to the cellular exterior either chemically (via an electron shuttle) or via a ‘nanowire’ pilin.

1.5 Ashumet Pond
Ashumet Pond in Mashpee, Massachusetts is well-suited to an investigation of manganese-cycling microorganisms. This pond is a glacial kettle pond located in the outwash sediments that make up the western part of Cape Cod. Ashumet Pond is located southeast of the Massachusetts Military Reservation, where disposal of treated sewage onto sand beds from 1936-1995 created a plume of contamination that entered the relatively permeable glacial outwash sediments (McCobb et al., 1999). This plume intersects Ashumet Pond at its western edge, near a small inlet known as Fishermans Cove (Figure 1).

The sewage plume continues to infiltrate the Pond today, although dumping ceased in 1995. The reduced groundwater in the plume carries high amounts of dissolved iron, manganese, phosphorus, ammonium, and other compounds. As of 1999, manganese concentrations reached concentrations of up to 10.3 mg/l (187 micromolar), while nitrate and ammonium were each near 6 mg/l (~100, and ~300 micromolar, respectively). A zone of active iron precipitation can be seen along the western
Figure 1: A) Three sampling sites on the western shore of Ashumet Pond. The red oval denotes the region where active iron precipitation is visible. Plume enters from the Massachusetts Military Reservation, to the northwest. B) Contour map of manganese concentrations, as of 1999 (USGS). C) Contour map of nitrate concentrations as of 1999 (USGS)
shore from aerial photos (Figure 1), and inspection of the rocks along this shore shows that many of the rocks are covered in a dark mineral crust, consistent with the properties of manganese oxides (Figure 2).

High concentrations of reduced manganese, along with active manganese oxide crusts, and the presence of nitrate and ammonium suggests that oxidation of dissolved manganese by oxygen and nitrate are both feasible microbial processes in this setting. Ammonium oxidation by precipitated manganese oxides is also conceivably important.

2. Approach
To address these gaps in our biogeochemical understanding of the manganese cycle we enriched for microbes catalyzing manganese redox chemistry reactions under static batch and continuous-flow conditions, utilizing selective conditions for aerobic heterotrophic, anaerobic chemolithoautotrophs and anaerobic photolithoautotrophs.

2.1 Batch Enrichment
Batch enrichment cultures were set up to enrich for aerobic, microaerophilic, and phototrophic manganese oxidizers, Culture and media conditions are specified in the methods section.

2.2 Continuous-Flow Enrichment
Static batch culture enrichment techniques work well for aerobic and rapidly growing microorganisms. However, for more slowly growing microorganisms, continuous flow enrichment may be a better technique, as they better maintain consistent conditions in the culture. Figure 3 shows a schematic of our continuous-flow setup.

2.3 Environmental pyrosequencing
In order to assess the potential for the existence of manganese cycling microbes in the environment, two samples – one aerobic and one anaerobic – were selected for environmental V6 tag sequencing. These samples were selected from the top and bottom of a core sampled at Site 3.

3. Results and Discussion
3.1 Aerobic Plates
Enrichments on aerobic plates all showed numerous colonies in the first three days. A small proportion of the colonies (two on succinate plates, and one on pyruvate plate) showed evidence of manganese oxide precipitation after six days. Figure 4 shows these colonies, with the development of manganese oxide crystals. In strain AB27, development of what appears to be a hexagonal crystal structure occurs in the center of the colony (Figure 4a), but close microscopy of this strain does not show any evidence of manganese oxide precipitation on individual cells (Figure 4c). Strains AB31 (Figure 4b) and AB 32 (Figure 4d) seem to show development of amorphous manganese oxides within the colonies.

3.2 Phototrophic enrichments
After 20 days of enrichment, one phototroph enrichment, inoculated from Site 3, had developed turbidity of a pale yellow hue (Figure 5). The corresponding dark control and the enrichment from the same inoculum in culture media containing DCMU lacked turbidity. Due to the presence of sediment from the inoculum assessing
Figure 3: Schematic of flow-through enrichment setup. Three media containers, containing reduced manganese, ammonium, and nitrate respectively are pumped via peristaltic pumps into incubators. Incubator A contains manganese oxide and is supplied with ammonium. Incubator B is supplied with reduced manganese and nitrate. Enrichments A and B are incubated in the dark. Incubator C is supplied with reduced manganese and incubated in the light. Effluent from all incubators are pooled and flow into a common waste container (left).
the culture bottle for the presence of precipitated manganese oxides was not possible. Microscopic examination of the enrichment compared to the dark control confirmed an increase in cell number in the light enrichment.

3.3 Microaerophilic enrichments
All of the microaerophilic enrichments developed turbidity, which was localized at specific depths from the overlay top. The enrichment using bicarbonate as the sole carbon source developed turbidity in a zone at a depth of 45-50 mm, succinate turbidity occurred in a thick zone from the top of the overlay extending to 39 mm and pyruvate turbidity occurred as discrete colonies from the top of the overlay to a depth of 32 mm. The development of turbidity near the top of the agar in the pyruvate and succinate tubes corresponded with a rapid decrease in the oxygen concentration at depths of 3 – 4 mm (Figure 6). This pattern may indicate that cultures in the tubes containing pyruvate and succinate are simply heterotrophs – and that the presence of the manganese plug is incidental to their growth. Oxygen concentrations drop to the lowest level in the succinate tube, and increase slightly towards the bottom of the tube in both the pyruvate and succinate tubes. This may indicate that succinate is the most readily accessible electron donor, and that all the tubes have residual oxygen at the tube bottoms (from pouring tubes on an aerobic benchtop).

In contrast, in the bicarbonate enrichment oxygen was depleted rapidly at a depth range of 23-28 mm, well above the region of formation of a turbid colony. In the absence of any readily oxidizable organic substrate in the bicarbonate tube, oxygen permeated much more deeply. The reductant

Figure 4: Isolates of putative manganese-oxidizing microbes. A) Strain AB27, B) Strain AB31, C) micrograph of Strain AB27, D) Strain AB32
responsible for its rapid decrease in concentration over the zone between 23-38 mm depth is not constrained, but reduced manganese diffusing up from the bottom plug is a likely candidate. This may be biologically catalyzed by small colonies that were not visually observed. Furthermore the lack of an organic substrate may imply that the turbid culture developed at 45-50 mm depth is a chemolithoautotroph that can function under microaerophilic conditions (~50 μmol/l O₂).

3.4 V6-tag rDNA sequencing

Ashumet Pond core tops (aerobic iron-oxide zone at sediment-water interface) and 10cmbs (anaerobic black sandy sediment) (Figure 7) were sequenced for the variable region 6 (V6) of the rDNA gene. Core tops (T) yielded a total of 44,633 reads from two separate initial tag PCR reactions (18,499 and 26,134 reads from T07 and T15, respectively), and core bottom (B) yielded total of 31565 tags (11,058 and 20,507 from B08 and B16, respectively). An exhaustive plot of all Bacterial tags detected, resolved to the phylum level, is shown in Figure 8.

Unidentified Bacterial V6 sequences clearly dominate both samples, lending weight to the notion that most microbial species remain unknown to science, and that the vast majority of those known remain uncultured (e.g., the ‘Rare Biosphere’ (Sogin et al. 2007) and ‘Unknown Majority’ (Whitman et al. 1998)).

Though one may perform numerous analyses of such community data, including overlaying with key environmental parameters, such as nitrate, nitrite, ammonia, nitrous oxide, manganese (II) and (III/IV) concentrations and availabilities; we
Figure 7: Core from Site 3 at Ashumet pond, showing red oxidized sediment from core top at left and black reduced sediment from core bottom at right. Oxidized and reduced sediments were the source of DNA for pyrosequencing analysis.
Figure 8: A) Abundances of V6 tags of selected Bacterial taxa in the top (oxic) and bottom (anoxic) sections of Ashumet Pond core from Site 3, B) Abudances of Proteobacterial taxa from top and bottom of Ashumet Pond core.
were limited in our analyses, and will thus focus on three points of interest regarding top versus bottom community comparisons. First, the Acidobacteria group 8 are clearly the most abundant member of this group in both the aerobic and anaerobic zones. Interestingly, only two members of this group are in pure culture, *Geothrix fermentans* and *Holophaga foetida*, and both are strict anaerobes (Barnes et al. 2005). The fact that we detect them at or near the oxic sediment-water interface indicates potential for novel physiology within this group; however, we cannot directly correlate physiology to phylogeny with a mere 60 base pairs of sequence data. Interestingly, both *Geothrix* and *Holophaga* sequences and isolates are found in Uranium contaminated groundwater aquifers (Barnes et al. 2005), indicating heavy-metal and radio-tolerance mechanisms in the Acidobacteria. Other key features to this dataset are the overall high abundance of Delta- and Beta-Proteobacteria (Figure 8) in both core top and bottom samples. Lastly, Nitrosomonas sp. make up a large proportion of the Bacterial community in the top sediment (Figure 8), and are completely dominated by a single tag sequence, most closely related to *Magnetobacterium* sp., groups known to respire nitirite to nitrate (Hovanec et al. 1998).

To more thoroughly understand the key differences in microbial population structure, diversity, richness and smoothness these two datasets must be run through programs such as DOTUR and SONS (Schloss and Handlesmen, 2005). These analyses coupled with geochemical measurement's of preserved water samples will allow us to draw more substantial conclusions regarding the roles of various phylotypes abundance and community structure to biogeochemical processes occurring in Ashumet Pond.

### 3.5 Relative Abundance of Microbial Groups from Flowing Enrichments

Each of the six continuous flow enrichment conditions yielded somewhat different bacterial communities (Table 1). In sharp contrast, Archaea were present in vanishingly small abundance—detected by the Arch915 CARD-FISH probe—though the School Street Marsh Mn(II) with nitrate yielded 0.1%. This dearth of detectable Archaea may well be due to the fact that the Arch915 probe does not completely cover all Archaeal groups (Amann and Fuchs, 2008); alternatively, the selective conditions imposed may have favored bacterial populations capable of Manganese and Nitrogen cycling at mildly acidic conditions (see V6 sections). Despite the limited nature of the CARD-FISH counts completed here, it is apparent that Beta-Proteobacteria play a significant role in all of the six enriched conditions (Figure 9); however, we were never able to account for the entire population visualized from each enrichment, and therefore must assume that the approximately 25% unresolved V6-Bacterial sequences.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target Group</th>
<th>ASH Mn(II)+NO3</th>
<th>SSM Mn(II)+NO3</th>
<th>ASH NH4+MnO2</th>
<th>SSM NH4+MnO2</th>
<th>Mn(II)+A</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>non</td>
<td>negative</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Eubi-iii</td>
<td>Eubacteria</td>
<td>99.90%</td>
<td>99.90%</td>
<td>99.90%</td>
<td>99.90%</td>
<td>99.90%</td>
<td>99.90%</td>
</tr>
<tr>
<td>Arc915</td>
<td>Archaea (90% coverage)</td>
<td>nd</td>
<td>0.10%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Alfa968</td>
<td>alpha-proteobacteria</td>
<td>10.00%</td>
<td>0.53%</td>
<td>0.46%</td>
<td>0.64%</td>
<td>0.28%</td>
<td>10%</td>
</tr>
<tr>
<td>Beta42a+Compet</td>
<td>beta-proteobacteria</td>
<td>3.87%</td>
<td>7.72%</td>
<td>5.90%</td>
<td>3.86%</td>
<td>44.80%</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Delta495a/b/c</td>
<td>Delta-proteobacteria</td>
<td>3.36%</td>
<td>5.38%</td>
<td>4.47%</td>
<td>16.47%</td>
<td>1.08%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Gam42a+Compet</td>
<td>Gamma-proteobacteria</td>
<td>3.29%</td>
<td>95%</td>
<td>5.36%</td>
<td>0.10%</td>
<td>nd</td>
<td>7.30%</td>
</tr>
<tr>
<td>Roseo</td>
<td>Roseobacterium</td>
<td>&lt;0.01%</td>
<td>nd</td>
<td>&lt;0.01%</td>
<td>&lt;0.01%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>GS352</td>
<td>greek sulfur bacteria</td>
<td>&lt;0.01%</td>
<td>&lt;0.001%</td>
<td>nd</td>
<td>&lt;0.1%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Anox/Bs</td>
<td>Anamox bacteria</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NISO190</td>
<td>Nitrosomonas sp.</td>
<td>nd</td>
<td>nd</td>
<td>0.01%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>NISO1225</td>
<td>Nitrosoccus sp.</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd=none detected ND=not determined all values averaged for probe/DAPI stained cells

*Table 1: CARD-FISH counts Archaea and various Bacterial groups from different continuous-flow enrichment treatments. Counts for each probe were normalized to total cells detected per filter piece by DAPI counted cells.*
Figure 9a: CARD-FISH hybridizations with either the Beta42a+Competitor or EubI-III probes, labelled with Fitzie and counterstained with DAPI. (A) long filamentous chains of Beta-proteobacteria found in high abundance in a number of the MnOxAmOx continuous flow enrichments, (B) diverse cell morphologies of Eubacteria from School Street Marsh manganese oxidizing continuous flow phototroph enrichment; (C) lower abundance Beta-proteobacteria from Ashumnet pond continuous flow enrichment containing Mn(II) and nitrate; (D) Beta-proteobacteria from School Street Marsh ammonia oxidizing manganese oxide-reducer flow enrichment.
Figure 9b: Clockwise from top left, DAPI-stained bacterial cells attached to autoflorescent Eukaryotic algae; bacterial cells attached to poorly crystalline iron or manganese oxides; inset, F430-filter of Eukaryotic algae; large (approximately 50 micrometer) Eukaryotic cell entwined in bacterial filaments and rod-shaped biofilm formers; (bottom-left) diverse morphotypes attached to mineral autoflorescent mineral; (inset left) DAPI-stained cells attached to poorly-crystalline metal oxide. The majority of DAPI-stained cells present on all slides from flowing enrichments occurred attached to particulate metal oxides.
3.6 Microbial Communities on Mineral Surfaces: Using Microbes on Minerals to indicate Manganese Oxide cycling

SEM images from environmentally derived biofilms (slide incubations) and continuous-flow enrichment incubations (six condition sets) yielded the images seen in Figure 10. Though we cannot draw direct correlations based on these images, it appears that metal oxide formation and potentially cycling is occurring as a result of bacterial attachment. As a result we may assume that if these attached populations are capable of dissimilatory metal reduction or oxidation, it is occurring in these sediments and our continuous flow enrichment apparatus. Furthermore, V6-tag sequence results indicate a high abundance of known metal cycling groups within the Delta- (e.g. Geobacteracae and Desulfobacteracae) Gamma-Proteobacteria (*Shewanella* sp.) play a key role in these visibly organic poor, iron and manganese rich pore waters and sediments.

4. Conclusions

Cultivation-independent V6 pyrosequencing is a powerful technique with which to uncover the phylogenetic diversity in an environment. While this technique can inform us about the overall structure and complexity of a microbial community, at present and in the near future many sequences are likely to be novel. This powerful tool opens a new window into microbial diversity. Classic cultivation techniques remain as important and useful methods for understanding the function of a fraction of the organisms in the environment. These can potentially be used as model organisms to better understand the linkage between the microbial world and biogeochemical cycles. Even in six weeks!

Methods

Community Analysis of Ashumet Sediments by 16S rDNA V6-tag pyrosequencing

Push cores from Ashumet Pond sediments were taken on 11 July 2008, covering the iron-oxide (rust colored) water-sediment interface, to the underlying reduced (black, sandy) sediments, 20 cm below the sediment surface, at site 3 (Figure 1). Cores were closed with butyl-rubber stoppers, sealed with Scotch-33+ electrical tape (3M Corp.) and stored on dry ice for return to the lab; samples were placed at -80°C for until further processing. Approximately 10 cm³ from core top (T) and bottom (B) were aseptically removed from the center of the core, so as to prevent vertical cross-contamination, homogenized, and extracted for DNA in eight approximately 1 cm³ replicates each, using the MoBio PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). Final environmental DNA (eDNA) extracts were combine on the kit spin columns into 2-replicates per sample (four initial extracts combine per Top i/ii or Bottom i/ii), and eluted in 30 µl sterile Tris-EDTA pH 8.1. eDNA extracts were quantified on the NanoDrop1000 (Thermo Scientific, Wilmington, Delaware USA), checked for size by gel electrophoresis on 2% agarose gels, and integrity by Bacterial 16S rDNA PCR with primers 8F-1492R in a 25 cycle reaction (anneal at 48°C).

Pyrosequencing of the 16S rDNA V6 region was performed at the Josephine Bay Paul Center (JBPC) at the Marine Biological Laboratory (MBL) using the Genome Sequencer FLX™ System (454 Life Sciences, Roche, Branford, CT). Subsequent sequence analysis, GHAST, and data deposition to the VAMPS database were also carried out at the JBPC.

Rarefaction analysis of both samples are shown below in Figure S1.

---

**Figure S1:** Rarefaction curves for V6 tags at the 0.03 similarity cutoff for the top (T) oxidzed sample, and bottom (B) reduced sample.
Figure 10: (A, B) filamentous bacteria attached to glass-slide, incubated in Ashumet Pond at the sediment-water interface for 5 days (B) non-contiguous filament of rod-shaped bacteria attached via pillin-like structures; (C) slide incubated in School Street Marsh Mn(II) + nitrate continuous flow chamber, for 3-days under flow, 3-days static; (D) slide from Ashumet Pond Mn(II) + nitrate, a well-defined metal oxide likely growing off the surface of ~7 micrometercoccus.
Enrichment-Isolation Strategy: Continuous Flow

Bioreactor & Static Batch Incubations

To enrich for microbial populations co-cycling Mn and N in both reductive and oxidative directions (Table [PROBE TABLE]), Figure Mn+N CYCLEs we setup six continuous-flow bioreactors (Figure [SCHEMATIC]), and each of the six reactors with 1X K-trace metals, 1X ε/2 vitamin solution (MD2008 course Handbook, originally from The Prokaryotes), MOPS buffered Freshwater Base medium (per 1L MilliQ water: 0.3 g NH₄Cl, 0.5 g KH₂PO₄, 0.4 g MgCl₂, 0.1 g CaCl₂) adjusted to pH 6.0, autoclaved then supplemented further with filter-sterilized 5mM NaHCO₃, and one of the following selective electron donor-acceptor couples: 10mM MnCl₂ + 10mM Nitrilotriacetic Acid (NTA; to chelate Mn(II)) with KNO₃; 6mM NH₄Cl with approximately 1mM MnO₂ (0.08g per 30 mL culture, crystalline brown powder, mineral phase unknown, from Sigma), both incubated in the dark; and lastly, 10mM MnCl₂ +10mM NTA with light (incandescent 60W bulb, at approximately 10cm distance). About 1 g Ashumet Pond (Mashpee, MA) or School Street Marsh (Woods Hole, MA) surface and anoxic sediments were combine and used as inocula for the three enrichment conditions outlined above in the MnOxAmOx-flow system. To maintain anaerobic conditions and well-mixed reactors, the 200mL elbow flasks were sealed with butyl-rubber stoppers and continuously purged with N₂:CO₂ (90:10 v/v). Flowing enrichments were allowed to run for 14-days at room temperature, then allowed to sit static for 3-day, after which 1mL samples were transferred 1:10 to the appropriate medium in Balch sets for DAPI and Fitzie (teramide) labelled cells.

Continuous Flow (from Filters)

After 10-days of continuous enrichment and 3-days static, 100ul from each flow-through apparatus were collected and fixed in 1% Formaldehyde for 1h at room temperature, filtered onto 35mm diameter white polycarbonate 0.22 micrometer membranes (Millipore, Billerica, MA), dehydrated with 1:1 EtOH:1XPBS, then completely with 100% EtOH, dried before Critical Point Drying, and subsequent Ag/Pl sputter coating. Slides incubated in flow-through enrichments were left for 4 days (2-flowing, 2-static) and fixed and washed similar to environmental slides. All coated samples were stored under desiccant at room temperature until imaging (within 96h, environmental samples within 24h or coating). CARD-FISH (multiple probe approach)

In Situ

Slides were recovered as described above and prepared for CARD-FISH by sectioning fixed slides with a diamond pen. Slide slices from aerobic and anaerobic sediments were hybridized with the Eubacterial and Non probes as described in the MD2008 Course handbook and previously (Amann et al. (1990) AEM 56: 1919-1925). Slides were imaged on a Zeiss Imager MR.1 using the AxioCam digital camera under appropriate filter sets for DAPI and Fitzie (teramide) labelled cells.

Biofilm-Slide Colonization & Scanning Electron Microscopy (SEM)

Sterile glass slide were incubated in Ashumet pond for 5 days at the sediment-water interface either horizontally or vertically (half-in half-out of the reduced underlying reduced zone); samples were removed directly to 2% glutaraldehyde in 1X PBS and placed on ice for 24h or at room temp for 1h. Samples were then washed three times in 1XPBS, dehydrated step-wise by 10 min incubations, on ice, of 50, 70, 85, 95, then 3-times 100% ethanol. Slides were not allowed to dry before Critical Point Drying, and subsequent Ag/Pl sputter coating. Slides incubated in flow-through enrichments were left for 4 days (2-flowing, 2-static) and fixed and washed similar to environmental slides. All coated samples were stored under desiccant at room temperature until imaging (within 96h, environmental samples within 24h or coating). CARD-FISH (multiple probe approach)
Acknowledgements
First and foremost we would like to thank course directors Tom Schmidt and Bill Metcalf, for planning and executing a phenomenal inquiry-based course, and to those who made it all possible, the dauntless teaching staff Amy Apprill, Arpita Bose, Evelyn Marschall, Clegg Waldron, David Walsh, Dagmar Woebken, Verity Johnson, and especially Stephanie A. Eichorst; and to the visiting faculty Dianne Newman, Jorg Overmann, and Rolf Thauer, as well as the numerous excellent lecturers and symposium speakers. Thanks also to the MD2008 class, who maintained professional, scholarship and good nature through this onslaught of Science, disorientation, frustration, hilarity, and moments of Bliss on the SEM. Finally, none of this work would be possible without the resources of the MBL Imaging Facility and the expertise of Louis Kerr, and certainly our funding sources: the Gordon and Betty Moore Foundation, equipment from Zeiss, 454 support by the Bay Paul Center and the Mitchell Sogin and the Josephine Bay Paul Center. ASB and WDL acknowledge the Milton L. Shifman Endowed Scholarship for critical financial support. And finally, to our advisors Chris Marx (ASB) and Peter Girguis (WDL), for financial and other encouragement to take this time at the MBL. Schmidt happens.

References: (in no particular order)