Exploring Anaerobic Microbial Communities Capable of Oxidizing Manganese

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ABSTRACT

The metabolic pathway of oxygenic photosynthesis is fueled by sunlight and the oxidation of water; oxygen is produced as a waste product. Before the rise of oxygenic phototrophs 2.3 billion years ago, microbes utilized anoxygenic photosynthesis to form organic carbon compounds using electron donors such as iron [Fe(II)] and reduced sulfur compounds. Anoxygenic phototrophs can be found in modern environments like meromictic lakes. We studied enrichment cultures of microorganisms driven by anoxygenic photosynthesis to determine whether and how they use another possible electron donor, manganese [Mn(II)]. Cultures reduced with sulfide [H₂S] and enriched with Mn(II) as a potential electron donor oxidized Mn(II) and formed various manganese oxide minerals in the presence of light. To further characterize these anaerobic microbial communities and the role of manganese, we grew the cultures on different concentrations of Mn(II) and H₂S and isolated individual microbes. We characterized the composition of the microbial communities by extracting copies of a specific gene, 16S rRNA, which is highly conserved and a standard species-identifying marker. Communities grew only in the presence of light and obligately anaerobic green sulfur bacteria from the phylum Chlorobi were the only phototrophic organisms. From these communities, a species of Chlorobi identified as *Chlorobium limicola* was isolated on an agar medium enriched with H₂S as the electron donor and characterized by sequencing nearly the entire 16S rRNA gene and a species of *Desulfomicrobium* identified as *Desulfomicrobium baculatum* was isolated on an agar medium enriched with sulfate. We found a robust microbial community capable of oxidizing Mn(II) in the absence of molecular oxygen. These communities are driven by anoxygenic photosynthesis and dependent on the concentration of sulfur sources, like sulfide. Optimal conditions for anaerobic oxidation of Mn(II) suggest this process occurs in source environment.
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INTRODUCTION

Aerobic and Anaerobic Manganese Oxidation

The process of manganese oxidation is commonly thought to occur only in the presence of oxygen, yet, manganese oxides have been found in geological layers from before the rise of atmospheric oxygen (Post, 1999; Schröder et al., 2011). Manganese is the twelfth most abundant element on our planet and the third most common transition metal in Earth’s crust (Post, 1999). This element can be found within cells in manganese catalases and in the water-oxidizing center of photosystem II in chloroplasts (Dismukes et al., 2001). Manganese can form oxide and bicarbonate compounds in different redox states (Morgan, 2005). Mn(III) and Mn(IV) are insoluble whereas Mn(II) is soluble in water (Morgan, 2005). Manganese is delivered from Earth’s interior in the Mn(II) redox state and is oxidized when exposed to the atmospheric oxygen (Stumm and Morgan, 2012). When Mn(II) is oxidized by atmospheric oxygen and other reactive oxygen species, it forms one of thirty known stable manganese oxides or hydroxides, in the Mn(III) or Mn(IV) redox states (Post, 1999). Microbes catalyze this process and oxidize manganese almost a million times faster than abiotic processes, but only when oxygen and reactive oxygen species are present (Davies and Morgan, 1989; Diem and Stumm, 1984; Hastings and Emerson, 1986; Tebo et al., 2004).

Anaerobic oxidation of manganese has not been previously observed in living bacteria. If manganese oxidation can occur only in the presence of oxygen, manganese oxides should not be observed in the geological record before the evolution of oxygenic photosynthesis around 2.35 billion years ago (Schröder et al., 2011). Yet, manganese oxides have been identified in photic paleoenvironments from the Koegas Subgroup in South Africa dated to 2.415 billion years ago,
before the rise of atmospheric oxygen (Schröder et al., 2011). This geological evidence suggests manganese can be oxidized in absence of molecular oxygen.

**Anoxyogenic Photosynthesis and Manganese Oxidation**

Some stages in the evolution of oxygenic photosynthesis are hypothesized to have included anaerobic manganese oxidation (Dismukes et al., 2001). Cyanobacteria and plants use oxygenic photosynthesis wherein water is oxidized and oxygen is produced as a by-product. Before the rise of oxygen in Earth’s atmosphere organisms harnessed the power of the sun by anoxygenic photosynthesis [see Box 1]. These microbes utilize alternative electron donors that were more easily oxidized than water. The mechanisms of anoxygenic photosynthesis have been observed in species from six bacterial phyla: *Chlorobi, Chloroflexi, Proteobacteria, Firmicutes, Acidobacteria*, and *Gemmatimonadetes* (Bryant et al., 2007; Bryant and Liu, 2013; Madigan and Jung, 2009; Overmann, 2008; Sattley and Blankenship, 2010; Zeng et al., 2014). Bacteria from these phyla are known to oxidize a variety of compounds: some *Proteobacteria* such as *Rhodobacterium* utilize reduced iron, some *Chlorobi* use reduced sulfur, while other anoxygenic phototrophs oxidize arsenic, nitrite, hydrogen, or even organic compounds (Ehrenreich and Widdel, 1994; Griffin et al., 2007; Hanada, 2016; Kulp et al., 2008; Widdel et al., 1993). Manganese has been proposed as another possible electron donor in anaerobic environments, but this metabolism has never been observed in modern organisms (Dismukes et al., 2001). The presence of manganese oxides in the geological record prior to the rise of oxygen and the evolution of oxygenic photosynthesis also supports the hypothesis of manganese as an electron donor (Schröder et al., 2011). Anaerobic manganese oxidation, however, has been observed neither in a singular anoxygenic phototroph nor a community of microbes.
**Box 1: What is the difference between oxygenic and anoxygenic photosynthesis?**

Often when talking about photosynthesis, people assume oxygenic photosynthesis is being discussed. This is the type of the photosynthesis that occurs in the chloroplasts of plants and cyanobacteria where water is oxidized and oxygen is produced. Oxygenic photosynthesis produces 18 times more ATP than its predecessor, anoxygenic photosynthesis, which is likely why it became the more prominent type of photosynthesis on Earth once it evolved (Dismukes et al., 2001). In anoxygenic photosynthesis, water is not oxidized and thus oxygen is not produced as a by-product; organisms capable of this kind of photosynthesis are limited to a few phyla within the kingdom Bacteria (Hanada, 2016). Anoxygenic phototrophs each have one reaction center of which there are a few varieties but oxygenic phototrophs, in contrast, have two reaction centers, or photosystems, that are highly conserved (Dismukes et al., 2001; Hanada, 2016). Because of this difference in number of photosystems, the movement of electrons through the electron transport chain differs between oxygenic and anoxygenic photosynthesis: four electrons from two molecules of water pass through oxygenic photosynthesis in a Z-pattern and one electron from a variety of electron donors cycles through anoxygenic photosynthesis (Fig. 1) (Dismukes et al., 2001; Hanada, 2016). While oxygenic photosynthesis only has one possible electron donor, the list of electron donors in anoxygenic photosynthesis has not been fully elucidated.
Figure 1. Comparative timeline of Mn oxide formation and evolution of oxygenic photosynthesis. Timeline stretches from beginning of Earth 4.5 billion years ago (bya) to current time. Period names indicated above. Arrows indicate significant events in the evolution of photosynthesis. Figure adapted from Fischer et al. (2016).
Modern Environments Where Anaerobic Manganese Oxidation Could Occur

If modern bacteria are capable of anaerobic manganese oxidation and that process relies on anoxygenic photosynthesis, such microbial communities would require a body of water where part of the photic zone is anoxic. Such environments include salt marshes, thick microbial mats, and meromictic lakes (Cohen et al., 1986; Havig et al., 2015; Pierson et al., 1987). Because these modern environments are rich in sulfide and low in oxygen, meromictic, or permanently stratified lakes can be used as an analog to similar conditions during the Proterozoic and Archaean periods (Canfield, 1998; Crowe et al., 2008; Reinhard et al., 2009) [see Box 2]. The biochemistry of meromictic lakes across the globe has been well studied (Baatar et al., 2016; Brunskill and Ludlam, 1969; Crowe et al., 2008; Havig et al., 2015; Jones et al., 2011; Lehours et al., 2005; Savvichev et al., 2017; Schouten et al., 2001; Tonolla et al., 2003). The unique chemical gradients in these lakes are maintained by microbial communities, particularly in the chemocline which divides the upper, oxic and lower, anoxic layers [see Box 2]. The chemocline of meromictic lakes tends to be rich in purple sulfur bacteria or green sulfur bacteria, anoxygenic phototrophs known to oxidize sulfide among other compounds (Baatar et al., 2016; Crowe et al., 2008; Jones et al., 2011; Lehours et al., 2007; Tonolla et al., 2003). Some of these lakes, such as Lake Matano, Indonesia, are also rich in manganese, particularly within the chemocline (Crowe et al., 2008; Jones et al., 2011).

Another meromictic lake rich in manganese is Green Lake in Fayetteville, New York, the microbial ecology of which was previously characterized by Brunskill and Culver and the Klepac-Ceraj lab (1969; personal communication with V. Klepac-Ceraj). The geochemistry of
**Box 2: An explanation of meromictic lakes, in three parts**

A meromictic lake, also known as a permanently stratified lake, contains an upper oxic layer exposed to oxygen and a lower anoxic layer devoid of molecular oxygen; the zone which divides these two layers and is characterized by steep chemical gradients is called a chemocline (Fig. 2). Meromictic lakes can be found across the world from India to Russia to Upstate New York (Baatar et al., 2016; Havig et al., 2015; Savvichev et al., 2017). These lakes can form because of a supply of denser water from an underground source, the accumulation of decomposing organic material in bottom layers, biogeochemical cycling, and substantial depth of the lake basin (Hakala, 2004). In the case of Green Lake in Fayetteville, NY, it is supplied with sulfate-rich water from beneath which helps maintain its distinct layers (Brunskill and Ludlam, 1969). Due to their unusual anoxic chemical conditions, the meromictic lakes are home to a wide variety of anaerobic and photosynthetic bacteria.
Figure 2. Green Lake, Fayetteville, NY, example of sulfate-rich meromictic lake containing manganese. (A) Location of Green Lake in the U.S. and U.S. Geological Survey map of Green Lake. Yellow star indicates deepest point. (B) Cross-section of Green Lake showing specifics of its layers where the mixolimnion is the oxic layer and the monimolimnion is the anoxic layer. Figure designed by Vanja Klepac-Ceraj and used with her permission.
this lake has been well researched (Brunskill and Culver, 1969; Brunskill and Harriss, 1969; Brunskill and Ludlam, 1969; Havig et al., 2015). The top oxic layer extends from 0 to 15 meters, the bottom anoxic layer extends below 21 meters, and a 6 meter chemocline divides the two (Brunskill and Harriss, 1969; Havig et al., 2015). The chemocline of Fayetteville Green Lake is maintained by the supply of sulfate-rich groundwater and communities of anoxygenic phototrophs (Havig et al., 2015). The chemocline contains peak concentrations of iron at about 3 µM and manganese at about 60 µM compared to other depths of the lake (Havig et al., 2015). The population of bacteria is the densest within the chemocline and includes cyanobacteria around 15 meters, purple sulfur bacteria between 19 and 21 meters, and green sulfur bacteria around 21 meters (Havig et al., 2015). This microbial plate cycles reduced and oxidized forms of iron and manganese (Havig et al., 2015). The peak of soluble Mn(II) occurs at the same depth as the dense layer of green sulfur bacteria (Havig et al., 2015). Bacteria from all depths can fall to the bottom of Green Lake such that the sediment contains most of the microbial diversity present in the lake. Because of the overlap in manganese and iron concentrations and the microbial plate in the chemocline, it has been hypothesized that the bacterial community reduces and oxidizes the redox states of manganese and iron, with the oxidation of manganese typically attributed to the presence of oxygen at the top of the chemocline (Havig et al., 2015).

**Experimental Summary**

To characterize the process of Mn oxide formation in anaerobic microbial communities, we enriched Green Lake sediment samples with Mn(II) and observed the production of geochemical signals, the growth of the biofilm, and the composition of the community. We further investigated the properties of these microbial communities by further enriching microbes.
from the enrichments. We analyzed the community composition and biogeochemical transformations in cultures over the course of a month to understand how these microbial communities changed over time, and if this altered the formation of Mn oxides. We primarily explored the importance of sulfide and manganese in the formation of Mn oxides by cultivating microbial communities at different ratios of manganese to sulfide, as well as in enrichments with no sulfur source at all. We found our communities were able to oxidize Mn(II) in anoxic conditions in the presence of light. The only phototroph present in these enriched cultures was a species of green sulfur bacteria. The absence of light or the absence of a sulfur source decreased the abundance of similar phyla and inhibited Mn oxidation. This suggests that the process of anaerobic manganese oxidation is driven by anoxygenic photosynthesis and sulfur cycling.
METHODS

Manganese Enrichment Cultures

Sediment samples from Green Lake in Fayetteville, NY were grown anaerobically at RT in Green Lake (GL) medium. Green Lake (GL) medium was prepared using the recipe described below to reflect the major ion concentrations in the lake as reported by Brunskill and Ludlam (1969). Vitamin solution (1 mL) and trace element solution SL-10 (1 mL) were added to the basal medium. The basal GL medium contained KH$_2$PO$_4$ 13.6 mg/L, NH$_4$Cl 0.3 g/L, KCl 67.1 mg/L, and NaHCO$_3$ 9 g/L. Trace metal solution SL-10 was composed of HCl (25%; 7.7 M) 10 mL, FeCl$_2$ x 4 H$_2$O 1.5 g/L, ZnCl$_2$ 70 mg/L, MnCl$_2$ x 4 H$_2$O 100 mg/L, H$_3$BO$_3$ 6 mg/L, CoCl$_2$ x 6 H$_2$O 190 mg/L, CuCl$_2$ x 2 H$_2$O 2 mg/L, Na$_2$MoO$_4$ x 2 H$_2$O 31 mg/L. The vitamin solution contained biotin 2 mg/L, folic acid 2 mg/L, pyridoxine-HCl 10 mg/L, thiamine-HCl x 2 H$_2$O 5 mg/L, riboflavin 5 mg/L, nicotinic acid 5 mg/L, D-Ca-pantothenate 5 mg/L, vitamin B12 0.1 mg/L, p-aminobenzoic acid 5 mg/L, lipoic acid 5 mg/L. The final pH of the medium was 7.2.

The atmospheric conditions were 80%:20% N$_2$:CO$_2$. All electron acceptors and carbon sources were sterilized in anaerobic 1 M solutions and added to the sterilized medium to give final concentrations as specified for particular experiments below. Ascorbate or sulfide were added to scavenge any remaining oxygen. All media were flushed with a mix of 80%:20% N$_2$:CO$_2$.

Cultures were grown in a 12-hour light/dark cycle for 14 days at RT.

Longitudinal Analysis of Mn(II) Oxidation in Enrichment Cultures

All cultures were grown in 10 mL serum bottles, in media prepared as described above and inoculated with 1 µl sample of starting cultures. To understand how these Mn-oxidizing
cultures change over time, enrichments were grown in three different experimental conditions: 
(1) 1 mM Mn(II), 0.05 mM H₂S, grown in 12-hour light/dark cycle; (2) 1 mM Mn(II), 0.05 mM 
H₂S, grown in the dark; (3) 0 mM Mn(II), 0.05 mM H₂S, grown in 12-hour light/dark cycle. 
Experimental condition (2) controlled for the effect of light on the microbial community and 
condition (3) controlled for the effect of Mn(II) on the microbial community. Each condition also 
included sterile controls to detect any contamination that may have occurred in preparing the 
cultures and understand chemical changes that occurred in the absence of microbes. Cultures 
were harvested in an anaerobic hood at time 0, 2, and 4 weeks after inoculation. All conditions 
were grown in triplicate for each time point. 

The liquid phase of each sample was sampled and used to determine sulfide 
concentration, alkalinity, pH, and anion concentrations. Sulfide concentrations were determined 
by reacting the sample with zinc acetate and determining concentration through absorption 
(Beinert, 1983). Alkalinity was determined by titration with 0.02 M sulfuric acid and pH was 
measured using pH strips in the anaerobic hood. Anion concentration was characterized by ionic 
chromatography. This method separates ions based on their affinity. The solid phases of the 
microbial samples were characterized using Scanning Electron Microscopy (SEM) and 
Transmission Electron Microscopy (TEM) for imaging, X-Ray Powder Diffraction (XRD) for 
mineral identification, and X-Ray Photoelectron Spectroscopy (XPS) to determine the redox 
state of manganese. A 200 μL sample of biofilm from each enrichment was taken for DNA 
extraction and sequence analyses (described below).
Analysis of Mn(II) Oxidation in the Presence of Different Ratios of Mn(II)/H₂S

The initial microbial communities and cultures were prepared similarly as in the longitudinal analysis. We designed this experiment to determine the optimal conditions for Mn(II) oxidation. Enrichments were grown in eight experimental conditions: (1) Low Mn: 0.1 mM Mn(II), 0.05 mM H₂S; (2) Medium Mn/Low H₂S: 1.0 mM Mn(II), 0.05 mM H₂S; (3) High Mn: 5.0 mM Mn(II), 0.05 mM H₂S; (4) Medium H₂S: 1.0 mM Mn(II), 0.25 mM H₂S; (5) High H₂S: 1.0 mM Mn(II), 1.0 mM H₂S; (6) No Sulfide, Sulfate: 1.0 mM Mn(II), 1.0 mM sulfate; (7) No Sulfide, Ascorbate: 1.0 mM Mn(II), 1.0 mM ascorbate; (8) No Sulfide, Titanium: 1.0 mM Mn(II), 1.0 mM titanium. Each condition also included sterile controls. All conditions were grown in triplicate. All cultures were grown in the presence of a light cycle as described above.

Cultures were harvested four weeks after inoculation. Sulfide and manganese concentrations, pH, and alkalinity were characterized as described above. The solid phase of the microbial samples was characterized similarly to above. A 200 μL sample of biofilm from each enrichment was taken for DNA extraction and sequence analyses (described below).

DNA Extraction and 16S rRNA Gene Sequencing

For each biofilm sample, 0.2-1 ml of sample was harvested in anaerobic hood (5%:5%:90% H₂:CO₂:N₂) and spun down into a pellet. Total DNA was extracted from samples using the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA) according to manufacturer’s instructions and eluted in 60 µl C6 solution. Upon extraction, DNA was quantified using NanoDrop (Thermo Scientific, Inc., Wilmington, DE, USA). The extracted samples were stored at -80°C until they were sent to Argonne National Lab on dry ice for sequencing.
The community composition was characterized using 16S rRNA gene amplicon paired-end sequencing on the MiSeq Illumina platform. Briefly, V4 region of the 16S rRNA gene (515F-806R) from each sample was amplified using the bacterial-specific primers 515F (5’- GTGCCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) using PCR conditions described in Caporaso et al. (2011). After amplifications, the PCR amplicons were quantified using PicoGreen (Invitrogen, Carlsbad, CA) and pooled in equal concentrations to a single tube. This pool was cleaned up using UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA) and quantified using the Qubit (Invitrogen, Carlsbad, CA). The pooled samples were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA) as described in Caporaso et al. (2011).

Bioinformatic Analysis

Paired end reads were joined in QIIME v.1.9.0 using join_paired_ends.py script (Caporaso et al., 2010). Reads that did not pass the quality filtering or that did not assemble were discarded from the subsequent analyses. Non-chimeric sequences greater than 250 base-pairs in length were assigned to a taxonomy using open reference OTU picking at 97% sequence identity. Each cluster was assigned taxonomy by BLAST analysis using the GreenGenes reference database (McDonald et al., 2012). Chloroplast and mitochondrial sequences were removed. Data was normalized.

To compare relative abundances of phyla under different experimental conditions, OTU tables were compiled and triplicates averaged. Beta diversity was compared by calculating weighted Unifrac in QIIME and visualized on principle coordinate analysis plots made in R with the ggplot2 package. Statistical differences between the relative abundances of taxa of interest
were analyzed with unpaired t-test for initial manganese enrichment cultures and ANOVA and Tukey HSD for later experiments (significant if p<0.05).

**Further Enrichments and Isolation of Bacterial Species**

Biofilm samples from Mn-oxidizing cultures were used to inoculate GL medium with 1.5% agar in Hungate tubes. The medium was flushed with 80%: 20% N\textsubscript{2}:CO\textsubscript{2} and then autoclaved. The tubes were moved to a 65°C water bath and reduced by the addition of Na\textsubscript{2}S to a final concentration of 0.05 mM. A tube was inoculated with 1 ml of biofilm at 42 °C and serially diluted. The inoculated tubes were immediately moved to ice to solidify. The cultures were grown in a 12-hour light/dark cycle for 2 to 3 weeks. After isolated colonies grew, they were excised from the agar under the stream of 80%: 20% N\textsubscript{2}:CO\textsubscript{2} gas and transferred to sterile liquid GL medium in 10 ml serum bottles. To further enrich microbes that can grow under these conditions, the process was repeated 2-3 times. To isolate anoxygenic phototrophs, we used shake tubes containing 0.05 mM sulfide with and without added carbon sources in the form of 1 mM yeast and 1 mM acetate. A different set of shake tubes containing 1 mM Mn and 0.01 mM sulfide were used to determine what isolates preferred these conditions. Finally, one last round of shake tubes containing 1 mM sulfate and x mM pyruvate was used to isolate sulfate-reducing bacteria.

**Isolate Identification by Sanger Sequencing**

For each isolate sample, 3 ml of biofilm were harvested in anaerobic hood (5%:5%:90% H\textsubscript{2}:CO\textsubscript{2}:N\textsubscript{2}) and spun down into a pellet. The genomic DNA of selected bacterial isolates was extracted by suspending a colony in polymerase chain reaction (PCR)-grade water and freezing for 20 min at -80°C and then thawing. A 1465 base pair (bp) sequence of the 16S rRNA gene
was PCR-amplified from genomic DNA (10-30 ng). Mastermix reagents consisted of 200 μM dNTP mix, 1x buffer standard \textit{Taq} reaction buffer, 0.2 μM 27F primer (AGA GTT TGA TCC TGG CTC AG), 0.2 μM 1492R primer (ACG GCT ACC TTG TTA CGA CTT) (IDT Integrated DNA Technologies, Inc., Coralville, IA) (29), and 1.25 units/50 μl \textit{Taq} DNA polymerase (New England BioLabs). After initial denaturation step (3 min at 95 °C), samples underwent 30 cycles of denaturation (30 sec at 95°C), primer annealing (25-30 sec at 50 °C), and primer extension (1.5 min at 72 °C), followed by a final extension (10 min at 72 °C). Amplicons were checked on 1.5% agarose gel using gel electrophoresis and quantified on the NanoDrop 2000 UV-Vis spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Inc., Wilmington, DE).

Excess TAQ polymerase, primer and nucleotides were precipitated from the amplicon solution by adding 1μL USB ExoSap-iT reagent (Affymetrix, Inc., Santa Clara, CA) to 9μL of amplicon. Purified products were identified via Sanger sequencing of the 16S rRNA gene in both directions using two separate reactions, one with 27F primer (5’-AGAGTTTGATCCTGGCTCAG-3’) and the other one with 1492R primer and assembled to get a nearly full-length 16S rRNA gene (GeneWiz, Madison, WI, USA). The resulting gene sequences were identified to the species level through nucleotide BLAST on GeneBank (Benson et al., 2013).
RESULTS

Characterization of Initial Mn(II) Enrichment Cultures

To investigate microbiologically-mediated anaerobic manganese oxidation, we inoculated sediment samples from the meromictic Green Lake, Fayetteville, New York in anaerobic minimal GL medium enriched with 1 mM Mn(II) and reduced by 0.05 mM Na₂S. The atmosphere contained 80% N₂ and 20% CO₂, making CO₂ the only exogenous source of carbon. To determine the importance of anoxygenic photosynthesis in anaerobic manganese oxidation, enrichments were either grown in a 12-hour light/dark cycle or in serum bottles that were shielded from the light by aluminum foil. Biofilms grew more in light conditions, as determined by sight and crystal violet measurements (Fig. 3). This shows that photoautotrophic microbes drive the increase in biomass of the entire microbial community.

To compare the composition of the anaerobic microbial communities, DNA was extracted from biofilm samples of exploratory enrichments and relative abundance of phyla determined through 16S rRNA gene amplicon paired-end sequencing on the MiSeq Illumina platform. The 16S rRNA gene is a highly conserved gene and is considered a gold standard for assigning taxonomic identification to bacteria. Enrichments lacking Mn(II) were included to observe differences in the microbial community and Mn oxide formation. We used DCMU in some cultures to inhibit photosystem II in oxygenic photosynthesis and prevent the production of oxygen which could oxidize manganese. In the enrichments without Mn(II), Desulfovibrio, Clostridiaceae, Christensenellaceae, and Marinilabaceae spp. were more abundant than in enrichments containing Mn(II) (average relative abundances of respective taxa - 5.31%:1.74%,...
Figure 3. Growth of anaerobic microbial cultures from Green Lake sediment in light or dark conditions. Microbial samples grown in cultures with GL medium enriched with 1 mM Mn(II) in (A) 12-hour light cycle or (B) dark. The headspace in each bottle contained 80% N\textsubscript{2} and 20% CO\textsubscript{2} and the medium is saturated with respect to CaCO\textsubscript{3} at pH = 7.2. Oxygen concentrations in the cultures were determined by luminescence detectors sensitive to nanomolar levels of O\textsubscript{2}. 
Figure 4. Relative abundance (%) of family and genus-level taxa within the phyla Bacteroidetes, Chlorobi, Firmicutes, Proteobacteria, and Spirochaetes in enrichment cultures. Identification of bacteria was obtained through 16S rRNA gene amplicon paired-end sequencing on the MiSeq Illumina. Taxa with abundance >1% were plotted on the graph.
4.04%:0.96%, 5.45%:2.06%, 17.64%:6.41%) but not significantly (Unpaired t-tests, df = 6, p >0.05) (Fig. 4). In contrast, in the Mn(II) enrichments Geobacter, Paludibacter, and unidentified species of Bacteroidetes were more abundant in enrichments that lacked Mn(II) (average relative abundances of respective taxa - 10.31%:2.90%, 2.31%:1.77%, 14.40%:2.64%) but only the abundance of the unidentified species of Bacteroidetes differed significantly (Unpaired t-test, t = -8.703, df = 6, p = 0.0001) (Fig. 4). Bacteria from the genus Desulfomicrobium appeared in all enrichments at an average relative abundance of 8% (Fig. 4). Across all conditions, we did not detect oxygenic phototrophs such as cyanobacteria (Fig. 4). Luminescence detectors sensitive to nanomolar levels of oxygen confirmed that no oxygen was introduced to the system (Fig. 3). The only identifiable phototrophs were from the genus Chlorobium. These microbes were often the most abundant taxon in the community (Fig. 4).

**Mn Oxide Formation in Initial Mn(II) Enrichment Cultures**

To determine if these anaerobic microbial communities oxidize Mn(II), biofilm samples were analyzed after two weeks of growth by scanning electron microscopy (SEM), focused ion beam (FIB), and transmission electron microscopy (TEM). Mineral content was analyzed by energy dispersive X-ray spectroscopy (EDAX) and high resolution transmission spectroscopy (HRTEM) with selected area electron diffraction (SAED). In microbial cultures incubated in 12-hour light/dark cycle, minerals containing oxidized Mn(II) nucleate around cell surfaces and are closely associated with the microorganisms at oxygen concentrations smaller than 2 nM (Fig. 5 C). Some cells are loosely attached to minerals (Fig. 5 A,B). Most of the precipitates are nanocrystalline oxides containing Mn and Ca and often occur in clusters (Fig. 5 B). In the light-shielded cultures, rod-shaped non-photosynthetic cells dominated, photosynthetic
Figure 5. Mn oxidation in anaerobic microbial cultures incubated in the presence and absence of light. Scanning electron microscopy (SEM) of microbial communities. (A-C) Cultures incubated in the light. (D-F) Cultures incubated in the dark. (A) Cultures after two weeks of growth. Focused ion beam (FIB) cross section (B, E) of biofilms shows interior biofilm layers. Energy dispersive X-ray spectroscopy (EDAX) shows Mn and Ca minerals in light cultures and Ca minerals in dark [data not shown]. Transmission electron microscopy (TEM) at 80 kV of light (C) and dark (F) after two weeks of growth.
organisms of Chlorobi species were degraded and minerals were mostly absent (Fig. 5 D, E, F). Powder X-ray Diffraction (XRD) of the microbial cultures incubated in light cycle with Mn(II) shows peaks that can be indexed to CaMnO$_3$. X-ray Photoelectron Spectroscopy (XPS) confirmed the redox state of the manganese formed in the microbial cultures. The Mn2p XPS spectra of the dark culture exhibits two major peaks at binding energies of 640 eV and 552.2 eV corresponding to Mn2p2/3 and Mn2p1/2 respectively in agreement with other reports on Mn(II) phases of MnO (Oku et al., 1975; Baggetto et al., 2013). In the light culture, the Mn2p peak shifted to a high-energy side and the intense satellite peak characteristic of Mn(II) diminishes with redox states of Mn(III) and Mn(IV). The oxidation of Mn in photosynthetic anoxygenic cultures that contain Chlorobi, occurred only in the presence of light and low sulfide concentrations between 20 and 50 µM.

**Mn(II) Oxidation Over Time**

To characterize how these anaerobic microbial communities change over time and under different conditions of light or Mn(II), we observed the cultures over the course of four weeks with or without Mn(II) and in the presence or absence of light and compared the microbial community compositions across the conditions and over time. The beta diversity of these microbial communities became more distinct over time (Fig. 6). Light or dark conditions contributed to a greater difference in beta diversity than Mn(II) (Fig. 6). There were not large differences in alpha or beta diversity of cultures grown in light with or without Mn (Fig. 6). The most abundant taxon across all conditions (30-60%) was classified as a *Chlorobium* spp (Fig. 7). In the presence of light and Mn(II), the abundances of *Chlorobium, Paludibacter*, and *Acholeplasma* spp. increased (average relative abundances of respective taxa- 35%:56%, 3%:5%,
Figure 6. Principal component analyses of the diversity in microbial communities grown under different conditions. Light, Mn: 12-hr light cycle with 1 mM Mn(II); Light, No Mn: 12-hr light cycle, no Mn(II); Dark, Mn: dark, 1 mM Mn(II). Darker colors show amples from older cultures. All cultures were inoculated with the inoculum from Mn-oxidizing cultures grown in the presence of light.
Figure 7. Abundances of taxa under different conditions. Average relative abundances of taxa (cultures grown in triplicates) after four weeks of growth. V4 region of the 16S rRNA gene sequenced on Illumina MiSeq platform. Abundances greater than 1% shown.
Cultures grown in light had significantly more *Chlorobium* (ANOVA, $F = 23.4521$, df factor = 5, df error = 12, $p < 0.0001$; Tukey’s HSD, $p < 0.01$) (Fig. 7). In the dark, *Geobacter*, *Clostridium*, and *Acetobacterium* increased in abundance (average relative abundances of respective taxa- 9.5%:16.5%, 0.4%:4%, 0.6%:4%) (Fig. 7).

**Optimal Conditions for Mn(II) Oxidation**

To determine optimal Mn(II) oxidation conditions, cultures were grown in the presence of different combinations of Mn and sulfide concentrations. Cultures enriched on Mn(II) but without sulfide were included to control for the role of sulfide as a reducing agent in the medium. The absence of any sulfur source produced the most different microbial communities when comparing beta diversity (Fig. 8). In the absence of sulfide or sulfate, the relative abundance of *Chlorobium* decreased (Fig. 9). The abundance of the phyla *Clostridium_sensu_stricto*, *Clostridium_XIVb*, *Paludibacter*, and *Acholeplasma* increased (Fig. 9). High concentrations of sulfide or the absence of a sulfur source inhibited Mn oxidation (Fig. 10). At the highest concentration of Mn(II), Mn oxidation was also inhibited (Fig. 10). The optimal conditions for Mn(II) oxidation from those tested appear to be 1 mM Mn(II) and 0.25 mM sulfide (Fig. 10).

**Enrichment and Identification of Bacterial Species in Mn(II)-Oxidizing Cultures**

To determine if individual organisms are capable of Mn oxidation and to characterize individual members of the microbial community, samples from enrichment cultures were transferred to anaerobic solid GL medium containing sulfide, Mn(II), and/or sulfate. Individual colonies were cut out of the agar and then transferred two to four times to liquid GL medium that...
Figure 8. Principal component analysis of microbial communities grown at various concentrations of Mn and H₂S. Cultures inoculated with samples from Mn-oxidizing cultures and grown in GL medium in 12-hour light cycle. Cultures grown on a range of Mn(II) and H₂S concentrations.
Figure 9. Differences in relative abundance of phyla across varying concentrations of Mn(II), sulfide, and other reducing agents. Average relative abundance of each condition in triplicate shown. V4 region of the 16S rRNA gene sequenced on Illumina MiSeq platform. Abundance over 2% shown.
Figure 10. Minerals that form in the presence of different concentrations of Mn(II), sulfide, and other reducing agents (XRD). Samples of biofilm were analyzed using X-Ray Powder Diffraction. From top: 1 mM Mn, 0.05 mM S; 1 mM Mn, 1 mM S; 1 mM Mn, 0.25 mM S; 1 mM titanium; 1 mM ascorbate (Not all samples shown).
contained the same concentrations of manganese, sulfide or sulfate (Fig. 11). The species of each isolate was identified by sequencing 16S rRNA gene using two separate sequencing reactions, one with 27F and the other with 1492R primer and assembling the two reads to near-full length 16S rRNA gene. The 26 colonies that grew in the presence of high sulfide, with or without added organic compounds to accelerate growth, or enriched on 1 mM Mn(II) and 0.01 mM sulfide were identified as *C. limicola* strain Pfenning 1630, *C. limicola* strain Pfenning 1830, and *Chlorobium phaeobacteroides* strain Glu (Table 1). The four colonies isolated in the presence of 1 mM sulfate and x mM pyruvate were all identified as *Desulfomicrobium baculatum* strain DSM 1742 (Table 1).

Although the 16S rRNA sequences appeared clean, genome analyses of colonies that grew on sulfide-rich and Mn-rich media showed that the three *Chlorobium*-containing cultures were not pure (data not shown). All samples did contain a *Chlorobium spp.* that was identified as *C. limicola*. The sample enriched on 1 mM Mn also contained *Acholeplasma equifetale* and *Geobacter lovleyi* (personal communication with Anna Farrell-Sherman). The sample enriched with sulfide and organic compounds contained a plethora of species including *Acholeplasma axanthum* and *Desulfomicrobium baculatum* (personal communication with Anna Farrell-Sherman). The sample enriched with sulfide alone contained similar additional species: *Acholeplasma equifetale* and *Desulfomicrobium baculatum* (personal communication with Anna Farrell-Sherman). This suggests *Chlorobium* tends to grow in association with other bacteria and involved in a network of close interactions.
Conclusions

Through multiple experiments, communities of anaerobic microbes originally from Green Lake, Fayetteville, NY, have been shown to oxidize Mn(II). This process seems to be driven by anoxygenic photosynthesis because manganese oxidation does not occur in the absence of light. The cycling of sulfur also appears to play a key role in these Mn-oxidizing microbial communities because Mn oxidation does not occur in the absence of sulfide or sulfate. The accumulation of elemental sulfur in cultures that lack Mn also suggests that sulfide is likely the principal electron donor for *Chlorobium* under our experimental conditions. The microbial communities capable of anaerobic Mn-oxidation are robust and include species from the genera *Chlorobium, Geobacter,* and *Desulfomicrobium.* Colonies from these communities were identified either as 100% identical on the 16S rRNA gene level as three *Chlorobium* species: *C. limicola* strain Pfenning 1630, *C. limicola* strain Pfenning 1830, and *Chlorobium phaeobacteroides* strain Glu; or as *Desulfomicrobium baculatum* strain DSM 1742. Whole genome analysis found these colonies grew in association with various species including *Acholeplasma axanthum, Acholeplasma equifetale, Desulfomicrobium baculatum,* and *Geobacter lovleyi.* These species are possibly involved in anaerobic Mn oxidation.
**Figure 11. Isolation of colonies from Mn-enriched cultures.** Samples from Mn(II)-enriched cultures grown in anaerobic agar shake tubes (A). Colonies that grew in the solid medium (B) were transferred two to four times to liquid cultures with the same concentrations of Mn(II) and sulfide (C-E).
Table 1. Identification of colonies grown on solid medium inoculated with Mn-oxidizing cultures. We investigated the following four chemical conditions: (1) 0.05 mM sulfide (n = 4); (2) 0.05 mM sulfide, 1 mM yeast, 1 mM acetate (n = 9); (3) 1 mM Mn, 0.01 mM sulfide (n = 13); (4) 1 mM sulfate, x mM pyruvate (n = 4). N/A indicates no data on base pair (% identity).

<table>
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<th>Mn(II)</th>
<th>Sulfide</th>
<th>Sulfate</th>
<th>Yeast Extract</th>
<th>Pyruvate</th>
<th>Base Pairs (% Identity)</th>
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DISCUSSION

To explore the process of anaerobic manganese oxidation, a previously uncharacterized process, we grew enrichment cultures initially inoculated with sediment from Green Lake and then investigated the role of light and different concentrations of Mn(II), sulfide, and other reducing agents on the community composition and chemical cycling. We found a robust microbial community capable of oxidizing Mn(II) in the absence of molecular oxygen. These communities are driven by anoxygenic photosynthesis and dependent on the concentration of sulfur sources, like sulfide.

Experimental Design Biases

Sediment was used as the first inoculum rather than a sample from the chemocline of Green Lake because it was not known what microbes were capable of anaerobic Mn oxidation. The sediment theoretically offered the greatest diversity of microbes because of bacteria descending from upper layers (Brunskill and Harriss, 1969; Thompson et al., 1990; Thompson et al., 1997). In Green Lake, a seasonal bloom of cyanobacterial Synechococcus spp. leads to the precipitation of calcium carbonate, which brings bacteria towards the sediment (Thompson et al., 1990; Thompson et al., 1997). The inoculum was grown in a minimal medium without complex carbon sources to promote a community around autotrophs. Biofilm growth under light and dark conditions supported this prediction and showed that the phototroph at the base of this community was Chlorobium (Fig. 3, 4). The absence of any oxygenic phototrophs, even in cultures without DCMU or ascorbate, allowed us to replicate the microbial community without added DCMU and with sulfide as a reducing agent rather than a carbon compound (Fig. 4).
The absence of light appeared to have the greatest effect on the beta diversity of the Mn-oxidizing microbial communities, more so than the absence of Mn(II) (Fig. 6). In the time course experiment comparing cultures grown in light or dark and with or without Mn(II), the dark cultures were inoculated with the starting culture most closely located on the PCoA plot (Fig. 6). But this starting culture was also used as an inoculum for some of the cultures grown in light conditions with Mn(II), indicating that the community moved further from the starting culture under light conditions (Fig. 6).

The communities did not change substantially over time and this may have occurred for a few reasons. First, we did not remove dead cells so DNA extractions may have included copies of the 16S rRNA gene from bacteria not surviving in the cultures. Second, the inoculum may have been too large for elastic changes in the microbial community over 4 weeks. This could be remedied by decreasing the inoculum size as well as standardizing the starting culture used for each replicate. Despite these biases, we observed communities becoming more unique over time, triplicates clustering together, and light conditions clustering (Fig. 6). The abundance of *Chlorobium*, *Paludibacter*, and *Acholeplasma* decreased and *Geobacter* and *Clostridium* increased under dark conditions suggesting their abundances are linked to anoxygenic photosynthesis (Fig. 7).

**Processes and Species Linked to Anaerobic Mn Oxidation**

Mn oxidation was only observed under light conditions, suggesting that it is driven by anoxygenic photosynthesis (Fig. 5). If Mn oxides were able to form abiotically or in the absence of light in the enrichment medium, they would have been observed under dark conditions. Only calcium minerals, likely precipitated from the CaCO$_2$ present in the medium, were found in the
enrichment cultures grown under dark conditions (Fig. 4). Light, and therefore anoxygenic photosynthesis, seems to be crucial to the oxidation of manganese in these anaerobic microbial communities. Although it has been hypothesized that Mn(II) may be used as an electron donor in anoxygenic photosynthesis, the reduction potential of bacteriochlorophyll a, the photosynthetic pigment in purple and green bacteria like Chlorobium, is too low to oxidize Mn(II) (Dismukes et al., 2001). It is more likely that Chlorobium fuels the cohort of bacteria necessary for the anaerobic oxidation of manganese.

The phyla associated with these anaerobic Mn-oxidizing communities are Chlorobium, Desulfomicrobium, Paludibacter and Geobacter which can catalyze a system of related reactions (Figs. 4, 7, 9). Chlorobium is known to oxidize sulfur compounds including iron and manganese sulfides (Borrego and Garcia-Gil, 1995; Figueras et al., 1997; Heising et al., 1999). Desulfomicrobium sp. are known to reduce sulfate and Fe(III) using H₂ and organic compounds as electron donors (Langendijk et al., 2001; Lovley and Phillips, 1994). Desulfomicrobium sp. have also been shown to oxidize elemental sulfur to sulfate when coupled to the reduction of Mn(IV) (Lovley and Phillips, 1994). Paludibacter sp. are strict anaerobes that produce propionate through fermentation (Qiu et al., 2014; Sánchez-Andrea et al., 2014; Ueki et al., 2006). Geobacter sp. are capable of oxidizing hydrogen, sulfur compounds, and organic compounds and coupling that to the reduction of metals like Fe(III) and Mn(IV) (Caccavo et al., 1994; Lovley et al., 1993). Metagenome analysis of Mn-oxidizing communities has identified the species of some of these phyla as Chlorobium limicola, Desulfomicrobium baculatum, and Geobacter lovelyi (personal communication with Vanja Klepac-Ceraj). This cohort of bacteria can cycle sulfur compounds, produce Mn(II), and exchange organic compounds.
The importance of sulfur cycling in the formation of Mn oxides was underscored by the differences under varying conditions of sulfide concentrations (Fig. 10). The lack of a sulfur source resulted in the greatest difference in beta diversity for those replicates grown in different Mn(II) and sulfide concentrations (Fig. 8). In contrast to dark conditions, the abundance of *Paludibacter* and *Acholeplasma*, along with *Clostridium* increased and *Geobacter* decreased without a sulfur source (Fig. 10). Without a sulfur source, Mn oxidation was inhibited suggesting sulfur cycling is related to manganese oxidation (Fig. 10). Both a lack of light and sulfur source resulted in a decrease in the relative abundance of *Chlorobium* (Fig. 9). It is unclear whether the other changes in the community are a result of the particular conditions of those enrichments, the decreased abundance of *Chlorobium*, or both. Because of the central role of *Chlorobium* as the only photoautotroph, the decreased growth under dark conditions, and decrease of Mn oxidation under dark conditions or without sulfur suggests *Chlorobium* is significantly linked to manganese oxidation and the phyla which fluctuate most around the abundance of *Chlorobium* may also be involved in the process.

The connection of these taxa is further strengthened when looking at the isolate cultures. Isolates grown on sulfide-rich or Mn(II)-rich shake tubes were identified through 16S rRNA sequencing as *Chlorobium limicola* and those grown on sulfate-rich shake tubes were identified as *Desulfomicrobium baculatum* (Fig. 11, Table 1). These species match those found in the enrichment cultures (Figs. 4, 7, 9). When investigating the genome of the *Chlorobium* isolates, we found they were not actually fully isolated despite the clean 16S rRNA sequence. In the sample enriched with Mn, *Acholeplasma equifetale* and *Geobacter lovleyi* accompanied the most prevalent *C. limicola* (personal communication with Anna Farrell-Sherman). In Green Lake, the
reduction of Mn(IV) and oxidation of reduced sulfur compounds has been hypothesized so it is possible that the reverse may also occur (Havig et al., 2015). *Chlorobium* has also been known to pair with other bacteria to oxidize other compounds, like a *Geospirillum* sp. to oxidize ferrous iron (Heising et al., 1999). Aerobic Mn oxidation is also known to be a multi-step process (Tebo et al., 2005; Wariishi et al., 1992). The close association of *C. limicola* with other taxa suggests that anaerobic manganese oxidation and sulfur cycling are interconnected processes.

**Optimization of Anaerobic Mn Oxidation**

Mn oxide formation was highest in cultures enriched with 1 mM Mn and 0.05 mM S (Fig. 10). These concentrations are very similar to those found in Green Lake at 21 m: 0.08 mM Mn and 0.04 mM S (Havig et al., 2015). *Chlorobium* is found around 21 m, at the bottom of the chemocline beneath communities of algae and cyanobacteria and purple sulfur bacteria (Havig et al., 2015). This suggests anaerobic Mn oxidation may be occurring in Green Lake. In other Mn-rich lakes like Lake Matano in Indonesia, manganese is oxidized in the oxic layer, reduced in the chemocline, and then precipitates out as insoluble manganese oxides (Jones et al., 2011). Mn(II) peaks at 100 m with a concentration of 20 µM (Jones et al., 2011). Green sulfur bacteria can be found in Lake Matano at least 15 m below, between 115 and 125 m (Crowe et al., 2008). Although the green sulfur bacteria in Lake Matano do not overlap with Mn(II) as in Green Lake, Lake Matano is sulfide-poor and it seems that sulfide may be crucial to anaerobic manganese oxidation (Crowe et al., 2008). Anaerobic Mn oxidation will continue to be studied using bacteria from Green Lake. Further research will be conducted to determine if Mn oxide formation can be observed *in situ* in the chemocline of Green Lake.
Future Work

Ongoing research is investigating the spatial relationship of *Chlorobium* and *Desulfomicrobium* in fixed biofilm samples through fluorescence *in situ* hybridization (FISH). While we observed Mn oxides forming immediately around cells, the bacterial species could not be identified through morphology (Fig. 5). Through FISH, which labels bacteria with a 16S rRNA-based oligomer probe, the spatial relationships of the bacteria within these Mn-oxidizing cultures could be visualized. Further isolation of different bacterial species in combination with FISH could help establish a minimum community required for Mn oxidation. Isolation techniques would need to be optimized by extracting colonies from agar more precisely using a dissecting microscope. Such experiments could also be informed by the determination of 0.25 mM sulfide and 1.0 mM Mn(II) as optimized conditions for Mn oxidation (Fig. 10).

A robust community of anaerobic microbes has been shown to oxidize manganese in the absence of molecular oxygen. This community includes *Chlorobium, Desulfomicrobium, Paludibacter, Acholeplasma,* and *Geobacter.* Further research will investigate the individual roles of these and other prominent bacterial species in these Mn-oxidizing communities in order to determine how anaerobic Mn(II) oxidation occurs and the major processes and community members are involved.
REFERENCES


