The Role of Bacteria in the Cycling of Manganese in Rifle, CO

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The Role of Bacteria in the Cycling of Manganese in Rifle, CO

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Introduction

This study investigated the role of bacteria in the cycling of manganese in the groundwater and sediments at Rifle, Colorado. It is thermodynamically favorable for Mn, a common element in the Rifle sediments, to be oxidized from a soluble Mn(II) form in the water to a Mn(IV) oxide mineral coating in the sediments. However, this reaction occurs very slowly (Tebo et al., 2005). We propose that bacteria present in the water and sediments commonly act as a catalyst in the reaction of Mn(II) to Mn(IV), producing highly reactive Mn(IV) oxide minerals. This study isolated and identified manganese oxidizing bacteria from Rifle.

Understanding more about these bacteria is important because once manganese (IV) oxide minerals are formed, they can bind toxic metals and remove them from water, thus improving water quality (Villalobos et al., 2005). Mn(IV) oxides may also react with natural organic matter in the water and sediments, producing low molecular weight organic compounds that can then be used as substrates for microbial growth (Sunda and Kieber, 1994). These are just two examples of environmental processes that can be affected by the activity of bacteria that cycle manganese.

Rifle is a former uranium and vanadium milling site as well as a former U.S. Department of Energy research site. High concentrations of soluble U(VI) persisted at the Rifle site, and bioremediation was used as a strategy to reduce mobile U(VI) to immobile U(IV) (DOE 1999). This strategy relied on injecting organic carbon into the subsurface to stimulate the growth of microbial communities with the ability to reduce U(VI). Extensive research has been conducted on the subsurface geochemistry and microbiology at Rifle, but many questions remain about how redox active elements such as manganese participate in numerous geochemical reactions that control the water quality and the transformation of organic carbon.

Background Information on the Rifle Field Site

Rifle, CO is located along the Colorado River in Garfield County and was the site of a uranium and vanadium mill from 1924-1958. The tailings remained at the Rifle site until 1996 when the Department of Energy began a remediation program, and the mill tailings and other contaminated radioactive material were moved to the Estes Gulch disposal cell 9 miles north of Rifle (DOE 1999). Uranium is mobile in the oxidized U(VI) state, and immobile in the reduced U(IV) state. High concentrations of U(VI) persisted at the old Rifle site, and between 2002 and 2006 organic carbon was injected into the aquifer to study processes related to the bioremediation of U(VI) (Williams et al., 2011). The organic carbon provided an energy source for many different kinds of microbes, including bacteria that can oxidize manganese which are the focus of this study.
Figure 1: This image shows the location of Rifle relative to Denver and Colorado Springs.
The Rifle site sits atop a perched aquifer composed of poorly sorted alluvium delivered by the Colorado River. These floodplain deposits are Quaternary in age. Underlying this aquifer is the Wasatch formation which acts as an aquitard. The Wasatch formation is composed of clays and silty-clays and is Tertiary in age. (DOE 1999). Recharge to the aquifer comes from subsurface inflow from north of the site due to regional rain fall and snow melt as well as from the inflow of the Colorado River. Gypsum is abundant near Rifle, and the dissolution of gypsum can cause the recharge to the aquifer to be enriched in sulfate (Williams et al., 2011).

Uranium and vanadium were mined from Triassic and Jurassic sandstones northeast of Rifle from 1925-1926. The host rocks for the uranium and vanadium are the Triassic Chinle formation, the Triassic-Jurassic Glen Canyon sandstone, and the Jurassic Entrada Sandstone (Chenoweth, 1982). The primary vanadium minerals are micaeous silicates and montroseite, a vanadium oxide. The mines in this area produced over 47 million pounds of vanadium oxide ($V_2O_5$) and uranium oxide ($U_3O_8$).
Figure 3: Geologic map of Rifle, CO (DOE 1999).
Background information on manganese and dissolved organic carbon

Manganese is a critical element in many systems, and electron transfers between Mn species are coupled to reactions that affect the carbon, nitrogen, sulfur, oxygen, and iron cycles (Webb et al., 2005). Mn is essential for life, and the speciation of Mn is important for microbial life as well as for the cycling of nutrients. The Mn redox cycle is intimately connected to microbial activity. The oxidation of Mn(II) to Mn(IV) is thermodynamically favorable but slow under most environmental conditions. This oxidation reaction is almost always catalyzed by microbes, and most Mn oxides in the environment are thought to be of biogenic origin (Webb et al., 2005). Mn oxides are some of the strongest oxidants in the environment and can impact the distribution of many trace elements and contaminants as well as act as terminal electron acceptors for bacterial respiration (Tebo et al., 2005). Understanding the cycling of manganese is critical for unraveling a number of biogeochemical processes.

Bacteria have a variety of reasons to oxidize Mn(II), although the core function and enzymatic pathways involved in bacterial Mn-oxidation are still not well known. Mn is required as a trace element for many cellular functions, and bacteria that oxidize Mn(II) to Mn oxides can protect themselves from UV radiation, predation, viral attack, and heavy metal toxicity (Tebo et al., 2005).

Most bacteria also require organic carbon as an energy source for growth, and dissolved organic matter is crucial in a number of biogeochemical processes. Dissolved organic matter is comprised of many different high to low molecular weight organic compounds, and these components behave differently with respect to solubility and reactivity (Aiken, 2014). Dissolved organic matter is critical in providing organic carbon for microbial metabolism, and the bioavailability of dissolved organic matter for microbial metabolism depends on factors such as molecular size, structure, reactivity (Sunda and Kieber, 1994). If the microbes responsible for the reduction of uranium at Rifle are pervasive in the subsurface, as previous studies suggest, then a natural source of electron donors are needed. This source of natural electron donors has been attributed to dissolved organic matter (DOE, 2011).

Little is known about what percentage of the DOC at Rifle is labile and likely to participate in microbially mediated chemical reactions in the aquifer (DOE, 2011). According to historic data, the dissolved organic carbon (DOC) levels in Rifle vary from 2-5 mg/L (DOE, 2011). These concentrations of DOC are similar to the concentrations of DOC in the Colorado River and in a seep north of the Rifle site.

If biogenic manganese oxides in Rifle are participating in redox reactions with DOC, then lower molecular weight organic compounds may be produced. Microbes could then potentially directly metabolize these lower molecular weight organic compounds (Sunda and Kieber, 1994). Bacteria, manganese oxides, and DOC would all then be closely connected and intimately involved in several biogeochemical cycles.
Materials and Methods

A field trip was taken to Rifle, Colorado in August of 2013 to collect rocks and groundwater to use for culturing bacteria capable of oxidizing Mn(II) to Mn(IV). Cobbles from the Colorado River were put into plastic zip lock bags and stored with ice in a cooler. Groundwater from the Rifle site was pumped from two different wells, FP-102 and CD-01. Water flows downslope from well FP-102, a “background well” to well CD-01. Well CD-01 has been used in prior acetate injection experiments (Williams et al., 2011). Groundwater was pumped using peristaltic pumps attached to plastic tubing into 50 mL falcon tubes and also placed in the cooler. Plastic tubing from well FP-102 was also sampled. This tubing had been in the well since May of 2013 and was encrusted in brown material. Approximately 4 inches of the tubing was cut and placed into a falcon tube with groundwater that came from the same well. The tubing sample was also stored in the cooler.

Figure 4: This map displays the site boundary and well locations at the Rifle Integrated Field Research Challenge Site. Well FP-102 is circled in red and an arrow indicates the approximate location of well CD-01.

Upon return to Boulder, the samples from Rifle were tested for the presence of manganese (III/IV) oxides using the dye Leucoberbelin Blue (LBB). Upon contact with manganese oxides, LBB turns blue due to a redox reaction with the oxidized Mn. The intensity of the blue color corresponds to the concentrations of manganese oxides and this assay can be
used as a qualitative test or quantitative colorimetry assay (Tebo et al., 2007). Samples that tested positive for manganese oxides in the presence of LBB were considered possible sources for culturing manganese oxidizing bacteria. Figures 5 and 6 shows samples from Rifle that were used for culturing manganese oxidizing bacteria.

Media in which to culture manganese oxidizing bacteria were then prepared. A freshwater medium designed to mimic Rifle groundwater was prepared based on the freshwater medium recipe from Lovley and Philips (1998). The pH of the medium was measured and adjusted if it was less than 6 or greater than 8. The medium was then separated into two separate volumes and different carbon sources were added to each volume of freshwater media. These solutions were then sterilized in an autoclave. After autoclaving, 0.1 ml of pre-sterilized MnCl₂ and 1 ml of a trace element solution were added to 1 liter of sterile media. This media was autoclaved and poured into petri dishes and allowed to solidify. The petri dishes were then inoculated with rock scrapings, Rifle groundwater, and scrapings from the Rifle well tubing. These plates were sealed with parafilm and stored in the dark at room temperature. Table 1 gives the recipe for the medium used to mimic Rifle groundwater.

<table>
<thead>
<tr>
<th>Salts added per 1 liter of Deionized Water</th>
<th>Carbon Amendment A per 1 Liter media</th>
<th>Carbon Amendment B per 1 Liter media</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>NaCH₃COOH</td>
<td>Glucose</td>
</tr>
<tr>
<td>1g</td>
<td>2.7g</td>
<td>0.1g</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>Agar</td>
<td>Peptone</td>
</tr>
<tr>
<td>0.1g</td>
<td>15g</td>
<td>0.1g</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td>Yeast Extract</td>
</tr>
<tr>
<td>0.1g</td>
<td></td>
<td>0.1g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td></td>
<td>Agar</td>
</tr>
<tr>
<td>1.5g</td>
<td></td>
<td>15g</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Single Colonies of bacteria were then successively picked and moved to new plates until the colonies were isolated in pure culture. Over time, the colonies were also spot tested for manganese oxides with LBB. Once colonies of bacteria were isolated from several different samples, the next goal was to extract their genomic DNA. First, greater cell mass of the bacteria was needed. The colonies of bacteria were placed in sterile 5 mL tubes with the Rifle groundwater media and shaken at room temperature for several days. Once turbidity in the tubes was visible, the tubes were put in the centrifuge at 7500 rpm for 10 minutes. This was repeated until a pellet was visible. A DNeasy kit was then used to extract genomic DNA from the pellets of bacteria, and 100 microliters of cleaned gDNA was stored at -20°C. Figures 7 and 8 show colonies of Mn-oxidizing bacteria isolated in pure culture in petri dishes.
A polymerase chain reaction was then performed to amplify the DNA. This method uses thermal cycling to copy DNA many times. At 95 °C, the two strands of the DNA double helix are broken apart. The temperature is then dropped to 50 °C. This is the annealing stage, and DNA binds to the primer which is a short piece of DNA. In the third thermal stage, the temperature is brought to 72 °C and extension occurs. The DNA polymerase used in this reaction comes from *Thermus aquaticus*, an organism that lives in the hot springs of Yellowstone. This cycling repeats 30 times over the course of several hours. In one PCR reaction, the primers 27F and 1492R were used, and in a second reaction using the same genomic DNA, universal primers were used. Universal primers are based on sequences that may be invariant among genes of prokaryotes and eukaryotes (Marchesi et al., 1998). These universal primers can then amplify the DNA of bacteria as well as fungi.

To test whether the PCR reaction was successful, an electrophoresis DNA gel was prepared. This method uses an electric current to make DNA migrate through a gel. A 0.8% gel was prepared from Syber stain and agarose. A 1X TAE buffer was pour over the gel. The PCR products were mixed with a dye and then injected into wells in the gel. A current of 150 mA was then run through the gel for 45 minutes which caused the DNA to move through the gel. This migration separates bands of DNA that are a specific length (i.e. based on size). The length of the bands can then be compared to a standard to determine if the PCR reaction amplified the DNA. The standard used was a 1Kb ladder.

Once the success of the PCR reaction was confirmed via the electrophoresis DNA gel, the PCR product was cleaned using the Purelink PCR purification kit. This was necessary because PCR product is saline with excess primers, dNTPs, Taq, and magnesium. The PCR product was then sent to the company SeqWright for the sequencing of DNA. Using the software Sequencher, the DNA sequences from the forward and reverse primers were
combined. This consensus was then entered into the website Nucleotide BLAST, and organisms that matched the DNA sequence were returned (Altschul et al., 1997).

The second phase of this project investigated the dissolved organic matter in Rifle and how manganese oxides and dissolved organic matter interact. Six liters of groundwater from the wells FP-102 and CD-01 was collected in Rifle on January 20th and filtered with 0.45um Geotech dispos-a-filters. The filters were rinsed with 1 liter of DI water prior to filtration. The water was pumped into amber bottles, placed in a cooler, and shipped to the USGS in Boulder.

UV 254 measurements were made on the Rifle whole water samples on a Hewlett-Packard 8453 UV-Vis with deionized water used as the blank. Prior to measurement, samples were taken out of the refrigerator and allowed to warm to room temperature. A cuvette with a 1.0 cm path length was used. Dissolved organic carbon measurements were made on an OI Analytical Model 700 TOC analyzer (Aiken et al., 1992). SUVA 254 values were calculated by dividing the UV 254 measurement by the DOC concentration. SUVA is the UV absorbance normalized for the DOC concentration and can be indicative of dissolved aromatic (Weishaar et al., 2003). Higher SUVA values tend to correspond to a greater concentration of dissolved aromatic carbon. Iron concentrations in the whole water samples were measured using Hach Ferrover iron reagent powder pillows. Fluorescence spectroscopy measurements were taken using a Horiba Jobin Yvon Aqualog. A quartz cell with a 1.0 cm path length was used and deionized water was used as the blank.

The whole water samples were fractionated using a two column array of XAD-8 and XAD-4 resins in series. The pH of the whole water samples were first brought to a pH of 1.97 using phosphoric acid. At this pH, the NOM becomes hydrophobic enough to leave solution and sorb to the XAD-8 resin in the column. After the columns have been rinsed with phosphoric acid, the water sample is pumped through the column. This effluent consists of hydrophobic neutral acids. The column is then back eluted with NaOH in order to concentrate the hydrophobic organic acids (HPOA). The effluent from the XAD-8 columns is then loaded onto the XAD-4 columns. After the effluent from the XAD-4 columns has been collected, the columns are back eluted with NaOH. This concentrates transphillic organic acids (TPIA) (Aiken et al., 1992).

Synthetic manganese oxides were then reacted with the whole water sample from well FP-102. 2 mL of 0.05M synthetic manganese oxides were added to 120mL of Rifle groundwater. A complete protocol for preparing synthetic manganese oxides can be found in Villalobos et al. (2003). The water and manganese oxides were placed in a 125mL amber bottle and stored at room temperature in a dark cupboard for two weeks. As controls, 2 mL of 0.05M MnO₂ were added to 120mL of deionized water and 2mL of deionized water were added to 120 mL of the whole water sample. These controls were also stored in amber bottles at room temperature in the dark. UV 254, dissolved organic carbon, and fluorescence measurements were also taken on these samples.
Results

Bacteria with the ability to oxidize Mn(II) to Mn(IV) were isolated from a Colorado River rock and the tubing from well FP-102. Over the course of several weeks, these isolates developed a brown color and were flagged for testing for the presence of manganese oxides with the LBB spot test. Manganese oxides oxidize LBB, and this reaction produces a blue color. The LBB method is used quantitatively with a spectrographic assay. In this assay, potassium permanganate was used to prepare a standard curve, where one mol of KMnO$_4$ oxidizes five mols of LBB (Tebo, B.M. et al., 2007). Isolates from the Colorado River rock and well tubing tested positive for the presence of manganese oxides with the LBB spot test. The figures below shows the blue color that formed when these isolates were spot tested with LBB.

![Figure 9](image9.jpg) Figure 9: This image shows the blue color that formed when the isolate from the Colorado River rock was spot tested with LBB.

![Figure 10](image10.jpg) Figure 10: This image shows the blue color that formed when the isolate from the groundwater tubing was spot tested with LBB.

![Figure 11](image11.jpg) Figure 11: This image shows the blue color that formed when synthetic manganese oxides were spot tested with LBB.

After successful DNA extraction and PCR amplification using bacterial primers, cleaned PCR products from the three known manganese oxidizing bacteria was sent to SeqWright for DNA sequencing. The assembled DNA sequences for these organisms were 789 base pairs. The sequences obtained were matched against previously published sequences available in the National Center for Biological Information using the BLAST programs (Altschul et al., 1997). The BLAST programs are frequently used to search DNA databases for sequence similarities. Sequences for these three organisms were all 99% similar to various strains of *Pseudomonas*. The most closely related strains of *Pseudomonas* include *Pseudomonas moraviensis*, *Pseudomonas fluorescens*, *Pseudomonas mandelli*, and *Pseudomonas monteilii*.

Dissolved Organic Matter Results

6 Liters of Rifle groundwater were shipped to the USGS and analyzed for DOC concentration, UV absorbance, and iron. This water came from well FP-102 and well CD-01.
Well FP-102 samples water moving onto the Rifle site from the regional aquifer, and well CD-01 samples water that was in the middle of a previous organic carbon amendment experiment. Table 2 summarizes the results of the UV-Vis, OI 700 carbon analyzer, and Hach FerroVer measurements for UV absorbance, DOC concentration and iron concentration. Groundwater from well CD-01 had a slightly higher concentration of DOC than water from well FP-102. The two samples had the same specific UV absorbance and similar concentrations of iron. The DOC was determined to be 39% hydrophobic acids (HPOA) and 20% transphillic acids (TPIA) in well CD-01 and 32% HPOA and 17% TPIA in well FP-102. The HPOA fraction generally contains more chromophores than other fractions of DOC and absorbs light more strongly (Aiken 1992). HPOA is also the most aromatic fraction of DOC and tends to be more reactive with respect to metals. SUVA is correlated with the aromaticity of DOC and can be an indicator of dissolved aromatic carbon content (Weishaar et al., 2003). The TPIA fraction is composed of lower molecular weight compounds and tends to be less reactive with respect to metals (Aiken 1992).

Table 2: This table shows the results of the UV, DOC, SUVA, and iron analyses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>UV absorbance, 254 nm</th>
<th>DOC Mg/L</th>
<th>SUVA 254</th>
<th>Total Iron mg/L</th>
<th>HPOA SUVA</th>
<th>HPOA %</th>
<th>TPIA SUVA</th>
<th>TPIA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-01</td>
<td>0.069</td>
<td>4.7</td>
<td>1.5</td>
<td>0.02</td>
<td>2.0</td>
<td>39%</td>
<td>1.6</td>
<td>20%</td>
</tr>
<tr>
<td>FP-102</td>
<td>0.056</td>
<td>3.7</td>
<td>1.5</td>
<td>0.03</td>
<td>2.0</td>
<td>32%</td>
<td>1.7</td>
<td>17%</td>
</tr>
</tbody>
</table>

Fluorescence spectroscopy was also used to analyze DOC in Rifle. There is widespread interest in using fluorescence spectroscopy to study dissolved organic carbon. Making fluorescence measurements on samples is relatively straight-forward, and fluorescence spectroscopy is an attractive method to characterize DOC. Often 3 dimensional excitation-emission (EEMs) matrices are used to analyze different characteristics of DOC, such as the different components present in a sample (Korak et al., 2003). The fluorescence properties of model organic compounds have been well characterized. If it is assumed that natural DOC samples behave similarly to model compounds, then fluorescence can be considered a useful tool for characterizing natural DOC. Based on this assumption, changes in spectral properties like intensity, peak width, and the wavelength of maximum intensity can be interpreted as changes in the components of DOC. Changes in spectral properties can also then be attributed to microbial activity. If bacteria consume DOC or produce minerals that then react with DOC, some change in the spectral properties of the DOC would be expected.

Visually inspecting EEMs and “peak picking” is a common qualitative technique for characterizing DOC. Other statistically based methods are also used to assess fluorescence data,
including parallel-factor analysis (Stedmon and Bro, 2008) and principle component analysis (Hall and Kenny, 2007). These methods assume the linear behavior of the components of a spectrum. The figure below illustrates commonly referenced peaks for the technique of peak picking.

![Figure 12: These are two EEMs from river water samples. Primary fluorescence peaks are labeled with the letters A, C, T, B, and M. Peaks A and C correspond to humic substances. The white area in the upper left of each EEM is where scatter has been removed. Fluorescence intensities are in Raman units (Fellman et al., 2010).](image)

Table 3: This table describes commonly observed natural fluorescence peaks of DOC (Fellman et al., 2010).

<table>
<thead>
<tr>
<th>Component</th>
<th>Excitation and emission maxima (nm)</th>
<th>Peak name</th>
<th>Probable sources*</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine-like</td>
<td>ex 270–275, em 304–312</td>
<td>B1, γ</td>
<td>T, A, M</td>
<td>Amino acids, free or bound in proteins, fluorescence resembles free tyrosine, may indicate more degraded peptide material</td>
</tr>
<tr>
<td>Tryptophan-like</td>
<td>ex 270–280 (&lt;240), em 330–368</td>
<td>B‡, T1, δ</td>
<td>T, A, M</td>
<td>Amino acids, free or bound in proteins, fluorescence resembles free tryptophan, may indicate intact proteins or less degraded peptide material</td>
</tr>
<tr>
<td>Ultraviolet A (UVA) humic-like</td>
<td>ex 290–325 (&lt;250), em 370–430</td>
<td>M‡, β</td>
<td>T, A, M</td>
<td>Low molecular weight, common in marine environments associated with biological activity but can be found in wastewater, wetland, and agricultural environments</td>
</tr>
<tr>
<td>UVC humic-like</td>
<td>ex &lt;260, em 448–480</td>
<td>A‡, Α, αζ</td>
<td>T</td>
<td>High molecular weight and aromatic humic, widespread, but highest in wetlands and forested environments</td>
</tr>
<tr>
<td>UVC humic-like</td>
<td>ex 320–360, em 420–460</td>
<td>C‡, C', αζ</td>
<td>T</td>
<td>High-molecular-weight humic, widespread, but highest in wetlands and forested environments</td>
</tr>
<tr>
<td>Unknown</td>
<td>280/370</td>
<td>N‡</td>
<td>A</td>
<td>Very labile, associated with freshly produced DOM</td>
</tr>
</tbody>
</table>

* T, terrestrial plant or soil organic matter; A, autochthonous production; M, microbial processing; U, unknown.
† Coble et al. (1990).
‡ Coble et al. (1998).
ζ Parlanti et al. (2000).
A small number of molecules that make up DOC fluoresce. Organic fluorophores in soil and aquatic environments are usually aromatic molecules. Because of this, there are usually more fluorophores present in the HPOA fraction of DOC than in the whole water sample. Samples that have higher SUVA values generally tend to have a greater fraction of HPOA and then should also have a greater proportion of fluorophores than samples with lower SUVA values (Aiken, in press). When interpreting EEMS from natural DOC samples, it is important to consider that DOC fluorescence can rarely be explained as simply the sum of present fluorophores. Intramolecular interactions in natural samples can have a great impact on fluorescence behavior (Korak et al., 2014). Therefore, similarity in fluorescence behavior between model compounds and natural DOC samples is not necessarily indicative of the presence of these compounds in the natural sample of DOC (Aiken, in press).

The EEMS of the whole water samples from Rifle were normalized per milligram of DOC, and compared using the peak-picking technique. Using the peak-picking technique, and commonly referenced peak and region locations for components of EEMs, these EEMS indicate the presence of humic-like fluorophores in Rifle groundwater (Fellman et al., 2010). The scale of Raman units indicates that the sample from well CD-01 fluoresced slightly more intensely than the sample from well FP-102. This may be due to the slightly greater fraction of HPOA present in the DOC in well CD-01 than well FP-102. The HPOA fraction of DOC generally contains more fluorophores than other fractions of DOC.

Figure 13: EEM of whole water sample from well CD-01, normalized per milligram of DOC. This EEM has been corrected for inner-filter effects.
Groundwater from well FP-102 was then reacted with synthetic manganese oxides in the dark at room temperature for 19 days. A control of groundwater from well FP-102 was also stored in the same conditions. UV, DOC, and fluorescence measurements were then made on these samples. The table below summarizes the results of these measurements.

Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>UV absorbance, 254 nm</th>
<th>DOC mg/L</th>
<th>SUVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP-102 groundwater Control</td>
<td>0.055</td>
<td>3.3</td>
<td>1.6</td>
</tr>
<tr>
<td>FP-102 groundwater reacted with MnO₂</td>
<td>0.046</td>
<td>3.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Figure 15: EEM of whole water sample from well FP-102, measured on March 7, 2014 and normalized per milligram of DOC. This EEM has been corrected for inner-filter effects.

Figure 16: EEM of whole water sample from well FP-102 that had been reacted with synthetic manganese oxides, measured on March 7, 2014. This EEM has been corrected for inner-filter effects.
There is no significant difference in the fluorescence properties between the two samples after the 19 days of contact or between the sample from well FP-102 was measured on January 22nd.

Discussion

Manganese oxidizing Bacteria

Field samples from Rifle including groundwater, soil, rocks, surface water from the Colorado River, and rocks from the Colorado River all tested positive for the presence of manganese oxides with the LBB spot test. Given the widespread distribution of field samples from Rifle that tested positive for manganese oxides, manganese oxidation is clearly an ongoing process at Rifle. The blue color that formed when the isolates from the Colorado River rock and groundwater tubing were spot tested with LBB indicates the accumulation of manganese oxides by these bacteria. Therefore, it can be claimed that these bacteria are manganese oxidizers. The sequences of these bacteria were all 99% similar to several strains of *Pseudomonas*, so it can also be claimed that there are manganese oxidizing strains of *Pseudomonas* present at Rifle.

*Pseudomonas* is a well-documented manganese oxidizer (Villalobos et al., 2003), and strains of *Pseudomonas* are prevalent in freshwater and soil environments. *Pseudomonas fluorescens*, one of the closely related strains to the isolates from Rifle, is also a documented manganese oxidizer (Nogueira et al., 2007). *Pseudomonas fluorescens* was shown to influence manganese uptake in soybean plants. *Pseudomonas moraviensis* and *Pseudomonas protegens*, both strains that are closely related to the isolates from Rifle, may also be associated with plant health in agricultural areas (Ramette et al., 2011).

*Pseudomonas putida* has been studied extensively for its ability to oxidize manganese. This strain of *Pseudomonas* is representative of freshwater environments, and produces the poorly crystalline manganese oxide mineral birnessite (Viallalobos et al., 2005). *P. Putida* oxidizes manganese outside of the cell and produces a biofilm. This biofilm enmeshes both the bacterial cells and the manganese oxide minerals they produce. The crystal structure of birnessite has hexagonal symmetry, and the oxidation state of manganese is is 3.90 +/- 0.05 (Toner et al., 2006). The minerals produced by *P. Putida* also have more surface area compared to non-biogenic manganese oxide minerals.

The difference in crystal structure between biogenic and non-biogenic manganese oxides is important in the reaction of biogenic manganese oxides with metals. Because of their small particle sizes and malleable crystal structures, biogenic manganese oxides are highly
reactive with toxic trace metals including lead, zinc, cadmium, cobalt, and nickel. For example, there is a 10 fold greater retention of lead in biogenic manganese oxides than in synthetic manganese oxides (Villalobos et al., 2005). Other studies have shown that the presence of biogenic manganese oxides is an important control on the cycling of zinc in freshwater environments (Toner et al., 2006). The ability of biogenic manganese oxides to scavenge toxic metals has been well documented, and these minerals may have a great impact on water quality. The presence of biogenic manganese oxides in Rifle may be an important factor in the cycling of toxic metals.

Dissolved Organic Matter

The UV and DOC measurements made on Rifle groundwater in January are consistent with historic UV and DOC data from Rifle and the Colorado River. Water from well CD-01 had a DOC concentration of 4.7 mg/L and the water from well FP-102 had a DOC of 3.7 mg/L. Historic DOC data from the DOE (2011) reports that the DOC in Rifle tends to vary between 2-5 mg/L. An unpublished study by Davis et al. shows similar results. The DOC and SUVA values from wells FP-102 and CD-01 are low compared to environments that have a lot of moisture (Spencer et al., 2013). In general, the drivers of DOC inputs to the environment are wetlands. In arid places like western Colorado, where there are few wetlands and little precipitation, there is less loading of DOC into the environment (Spencer et al., 2013). It follows then that the groundwater samples from Rifle would have low SUVA values and low DOC measurements. Historic UV and DOC data from the Colorado River at sites in Utah also show generally low levels of DOC. Inputs of natural organic matter to the Rifle aquifer include inflow from the Colorado River, recharge by rainfall and snowmelt, in situ plant degradation, and in situ microbial activity.
Table 5: This table shows that several wells that were sampled in March 2013 by Davis et al. (unpublished) have similar UV, DOC, and SUVA values to the UV, DOC, and SUVA values measured in January 2014. There are some wells in this table that have unusually high SUVA values, such as CO-02-5, 743-2, 743-3, and LQ-112-5. Perhaps this is due to the manipulation of the groundwater chemistry that has taken place at Rifle.

<table>
<thead>
<tr>
<th>Sample Well</th>
<th>Sample Date</th>
<th>UV Absorbance, 254nm</th>
<th>DOC [mg/L C]</th>
<th>SUVA, 254nm</th>
<th>Total Iron [mg/L]</th>
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<tr>
<td>742-2</td>
<td>3/20/2013 0:00</td>
<td>0.041</td>
<td>2.1</td>
<td>1.9</td>
<td>0.02</td>
</tr>
<tr>
<td>742-3</td>
<td>3/20/2013 0:00</td>
<td>0.050</td>
<td>2.2</td>
<td>2.3</td>
<td>0.03</td>
</tr>
<tr>
<td>743-2</td>
<td>3/21/2013</td>
<td>0.216</td>
<td>4.2</td>
<td>5.1</td>
<td>0.13</td>
</tr>
<tr>
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<td>4.3</td>
<td>4.0</td>
<td>0.09</td>
</tr>
<tr>
<td>744-2</td>
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<td>0.101</td>
<td>3.9</td>
<td>2.6</td>
<td>0.52</td>
</tr>
<tr>
<td>744-3</td>
<td>3/20/2013 0:00</td>
<td>0.063</td>
<td>3.5</td>
<td>1.8</td>
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<td>4.9</td>
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<tr>
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<td>4.3</td>
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<tr>
<td>LQ-112-5</td>
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<td>1.3</td>
<td>0.07</td>
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<td>0.035</td>
<td>2.3</td>
<td>1.5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Based on fluorescence spectroscopy, there does not appear to be a significant difference in the DOC from well CD-01 to well FP-102. There is also no significant difference in the EEMs from the groundwater from well FP-102 and the groundwater that was reacted with the synthetic manganese oxides. After being stored at room temperature in the dark for 19 days, the DOC in the groundwater decreased from 3.7 mg/L to 3.3 mg/L. The groundwater that was stored in the same conditions and reacted with synthetic manganese oxides had a DOC value of 3.2 mg/L. The synthetic manganese oxides did not appear to react with the DOC in the Rifle groundwater in any significant way over this time period.
Conclusion

The manganese oxidizing bacteria *Pseudomonas* are present in the soils, groundwater and rock surfaces at Rifle and produce manganese oxides in the field and in the lab. These bacteria were cultured from well tubing from the Rifle site as well as from a rock taken from the Colorado River. These bacteria may then be widespread throughout the Rifle site. To further confirm the presence of manganese oxide in the field samples, future work would include X-Ray Diffraction analyses. X-ray diffraction measurements could also be made on the Mn-oxides produced in the lab by *Pseudomonas* for comparison.

Rifle groundwater was analyzed, and based on the DOC, UV, SUVA, and fluorescence data from the groundwater from wells CD-01 and FP-102, there may be some small variation in the DOC in the groundwater from a well that has been previously injected with acetate (CD-01) versus a background well (FP-102). The groundwater from well CD-01 had 1 mg/L more of DOC than the groundwater from well FP-102 and a slightly higher fraction of HPOA. However, these wells were only sampled one time. To determine if there is any statistically relevant difference in the groundwater chemistry, more samples would need to be analyzed across the hydrograph.

Whether the biogenic manganese oxides play any role in reactions with DOC in Rifle groundwater is unclear. The reaction of Rifle groundwater with synthetic manganese oxides produced no change in the groundwater DOC properties. It is worth noting that during this experiment, the manganese oxides settled to the bottom of the bottle of groundwater. Perhaps some sort of agitation or circulation of the manganese oxides would induce a greater extent of reaction with the DOC. This experiment could be repeated using an agitation method and an increased length of time. Future work could include high-performance liquid chromatography (HPLC) to detect small changes in low molecular weight organic compounds in the water samples. This method would be more sensitive to small changes in organic compounds that do not affect the bulk DOC properties. However, employing this method was beyond the scope of this study.

It has been well documented that biogenic manganese oxides are far more reactive with respect to metals than synthetic manganese oxides are. The crystal structure of biogenic manganese oxides has more surface area and more sites available to participate in reactions with metals than synthetic manganese oxides. Because of their differences in crystal structure, biogenic manganese oxides may also be more reactive with DOC than synthetic manganese oxides are. Biogenic manganese oxides have also been shown to react with high molecular weight humic substances to produce low molecular weight organic compounds (Sunda and Keiber, 1994). Although the synthetic manganese oxides did not react with the Rifle groundwater in a measureable way, it might be worthwhile to design an experiment to react biogenic manganese oxides with Rifle DOC. The lack of reaction between synthetic manganese...
oxides and Rifle groundwater could be attributed to the crystal structure of the synthetic manganese oxides. The biogenic manganese oxides may behave differently in the presence of Rifle DOC due to their malleable crystal structure. This experiment would need to design a way to separate the bacterial cells from the manganese oxides. The biogenic manganese oxides produced by the *Pseudomonas* cultured from the Rifle field samples were very closely associated with the cells and hard to separate, as shown in figure 17.

![Image of bacterial cells and biogenic manganese oxides](image)

Figure 17: Image of bacterial cells and biogenic manganese oxides taken on a Zeiss Axiocam fluorescence microscope. The dark areas show the manganese oxide minerals and the lighter areas show the biofilm and free bacteria cells.

Microbially catalyzed manganese oxidation is clearly occurring at Rifle. Strains of the manganese oxidizing bacteria *Pseudomonas* are widespread throughout the site. The manganese oxide minerals produced by these bacteria may exert an important control on the cycling of toxic metals at Rifle. Given their small particle size and large surface area, these biogenic manganese oxides may also be reactive with respect to dissolved organic carbon, although further study in this area is needed. In conclusion, microbes, manganese oxide minerals, and the cycling of metals in Rifle, Colorado are intimately associated.
Works Cited


