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Mn Oxide Biogenesis and Metal Sequestration in the Presence of Co (II) and Cu (II) By *Bacillus* SG-1 Bacterial Spores

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Abstract

Mn Oxide Biogenesis and Metal Sequestration in the Presence of Co (II) and Cu (II) by *Bacillus* SG-1 Bacterial Spores. Nader Bayat (University of California at Berkeley, Berkeley, CA 94720) JOHN BARGAR and SAMUEL WEBB (Stanford Linear Accelerator Laboratory, Menlo Park, CA 94025).

Mn oxides play an important role in degrading contaminants and cycling nutrients in soils and natural waters. The process in which Mn (II) oxidizes to form MnO_x is slow; however, *Bacillus* SG-1 bacterial spores can catalyze the process and allow it to proceed up to five orders of magnitude faster. This experiment explored the affects of co-ion metal concentrations on Biogenic Mn oxide production and their ability to sequester metal cations. Spore solutions were prepared with different ratios of Metal (II): Mn (II) added over a three-week period; this was done separately for Co (II) and Cu (II). The copper solutions were analyzed with ICP/AES to check for the amount of copper and manganese left in solution after biogenic MnO_x production. ICP/AES was used to analyze the ratio of Co: Mn in spores where Co was the co-ion metal. Observations showed very little dissolved Cu and Mn exist in solutions with low copper concentrations, but a large amount of Cu and Mn were left in solutions where higher Cu concentrations were used. This shows that high Cu concentration inhibits biogenic Mn oxide production and Cu sequestration. For the experiments with Co as the co-ion metal, it was observed that the ratio of Co: Mn in the spores is relatively similar to the ratios added; however, an exception to this rule was experiments where high concentrations of Co were used. The inconsistency in Co: Mn ratios at high Co concentrations showed that high Co concentrations also inhibit biogenic Mn oxide production.

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Introduction:

Biogenic Mn oxides play an important role in degrading contaminants and cycling nutrients in soils and natural waters. They can degrade toxic organic contaminants, including aromatic hydrocarbons. Furthermore, Mn oxides have high sorptive capacities for metal ions and can oxidize a variety of inorganics such as Cr (III), Co (II), and hydrogen Sulfide (Tebo et al., 1984). Their high sorptive capacities allow them to concentrate metals such as Co, Ni, Cu, and Zn up to several percent (Manceau et al., 1986). This is particularly important in waters contaminated with heavy metals where living organisms can form inorganic compounds leading to metal scavenging and pollution attenuation; this process is commonly know as biomineralization (Marble et al. 1999) The catalytic and degradative capacities of Mn oxides have lead to their use in bioremediation technologies and heterogeneous catalysis, and Mn (II) oxidation is important in industry, agriculture, and natural water treatment (Tebo et al., 1997).

Mn oxide nanoparticles and grain coatings are ubiquitous in natural waters and are believed to be dominantly of microbial origin (Tebo et al., 1984). Exploring the mechanisms by which bacteria, especially microbes, oxidize Mn (II) in the presence of other metal ions is fundamental to understanding the environmental cycling of essential and toxic constituents in natural waters (Tebo, 1991). Most of the Mn oxides found in natural waters are of the form MnO_x, where manganese has an average oxidation state between +3 and +4. Mn (IV) is thermodynamically more favorable than Mn (II) in aqueous solution and near neutral pH; however, the transformation from Mn (II) to Mn (IV) is kinetically slow. Certain bacterial spores, such as *Bacillus* SG-1, have been observed to catalyze the reaction from Mn (II) to Mn (IV) and allow it to proceed up to five orders of magnitude faster. The exact catalytic mechanism by which the redox reaction occurs on the surface of these bacterial spores is still unknown. The affects of co-

ion metal concentrations on the rate of biogenic Mn oxide production and the later sequestration of metals by the Mn oxides are also a very active area of research. The ability of bacteria to catalyze Mn oxide production is of great importance to those who wish to use biogenic oxides for projects such as the bioremediation of sites contaminated with heavy metals. The bacteria can quickly help the formation of biogenic Mn oxides, which can sequester the toxic chemicals and prevent drainage into water supplies and agricultural lands.

This research project studied the affects of co-ion metal concentrations on the formation of biogenic Mn oxides close to the surface of *Bacillus* SG-1 bacterial spores during active biomineralization. Two questions were examined and answered by this research. First, the affects of cobalt (II) concentrations on the formation of biogenic Mn oxides were analyzed. In addition, this project explored the sorptive capacity of biogenic Mn oxides in solutions of differing Cu (II) concentrations. Co (II) and Cu (II) metal ions were chosen for this project because they are found in all natural water sources and thus affect the formation of Mn oxides in the environment. The bacterial spores of marine *Bacillus*, strain SG-1, were chosen because the metabolic inactivity of the spores allow the characterization of the oxidation process and pathways in a simplified system without the interference of metabolic byproducts. The total amount of Mn oxide generated and the amount of metal ion sequestered on these oxides were analyzed by Inductively Coupled Plasma/Atomic Emission spectroscopy (ICP/AES); these were compared to the actual ratios of Mn (II) : Metal (II) added. Dr. Samuel Webb had already prepared the biogenic Mn oxides in the presence of Co (II). These Mn oxides were digested with acid and the resulting solutions were analyzed by the ICP/AES to check for the amount of Mn oxide formed and Co sequestered. The Mn oxides were prepared in the presence of Cu (II) and ICP/AES was

preformed on the solutions (not the spores) to check for Mn and Cu remaining in solution. The copper spores will be analyzed later by Dr. Webb at Argonne National Laboratories and SSRL.

This research is part of a broader project, which is aimed at understanding the local structures of Cu (II), Co (II), and Pb (II) sequestered in Mn-oxides produced by *Bacillus* SG-1 bacteria spores during active biomineralization. In addition to the affects of different metal concentrations on Mn oxide formation and metal sequestration, future studies will use X-ray Absorption Spectroscopy (XAS) and X-ray diffraction (XRD) to study the molecular mechanisms in which Mn oxidizing bacteria transform Mn (II) to Mn (IV). Furthermore, the environmental influence of contaminant and nutrient sequestration and degradation will be analyzed in the future.

Materials and Methods

Eight 250 mL plastic centrifuge tubes, three 250 mL erlenmeyer flasks, four 500 mL erlenmeyer flasks, and a 1000 mL erlenmeyer flask were washed and rinsed with borax soup and tap water. Then, ~ 5 grams of ascorbic acid was added to each flask which was then diluted and allowed to soak overnight in milli-q water, obtained from a Millipore water filtration system. This acidic solution ensured that all undissolved metal complexes in the flask were dissolved and subsequently washed off; thus avoiding future metal readings that were not supposed to be part of the project.

The *Bacillus* SG-1 bacteria spores were taken out of the fridge and allowed to melt for 45 minutes. The spores were then sonicated for 15 minutes in an Aquasonic model 150 HT sonicator that uses sonic waves to homogenize none uniform solutions. 0.5032 g of the spore suspension was placed in a 10 mL falcon tube and diluted with 9.6714 grams of milli-q water.

This sample was later analyzed by ICP/AES to check for the presence of any Mn, Cu, or Co content already on the spores prior to the start of any experiment. Any amount of metal found on these spore samples was to be subtracted from the total amount of metal after the experiment. The rest of the spores were be used in the experiment with Cu (II) as the co-ion metal. Dr. Webb had previously preformed the biogenic Mn oxide synthesis in the presence of Co (II). The product of his experiment was also analyzed using ICP/AES to check for Mn oxide production and subsequent Co (II) sequestration.

It is desirable that the pH of all solution be about the same throughout the entire experiment since different pH values may affect the production of Mn oxides and metal sequestration. In addition, most natural waters (ex. sea water) have a pH of ~7.5; therefore, adjusting the pH to 7.5 can provide a more realistic environment for our experiment. A one molar solution of HEPES, a common biological buffer, was prepared and the pH was adjusted to 7.503.

10 mM $MnCl_2$ was prepared from $MnCl_2 * 4\ H_2O$. In addition, 0.1 M, 5mM, and 0.5 mM $CuCl_2$ solutions were prepared from $CuCl_2 * 2\ H_2O$.

HEPES, spores, Cu (II) and Mn (II) were added to the erlenmeyer flasks according to the measurements indicated in table 1. The ratio of Cu (II): Mn (II) added is shown in table 2. Cu (II) and Mn (II) solutions of the indicated amounts were added to each flasks three times a day for three weeks. The only exception to the addition was Cu1m that received its Copper addition only once a day due to the very large amount that was being added. The additions were made in order to keep the different metal concentrations in each tube at the same level throughout the experiment as MnO_x formed and copper was sequestered. The solutions were

shaken 24 hours a day (with the exception of when additions were made) on a New Brunswick Scientific Shaker Classic Series at a rate of 50X a minute. Dr. Webb had previously used this process for the biogenic Mn oxide synthesis in the presence of Co (II).

Preparation of Biogenic Mn oxide in the presence of Cu (II)

After the three weeks, the shaker was turned off and the content in the bottles allowed to settle down. A ten mL portion of each solution was taken out of each flask and acidified with ~0.25 mL of concentrated nitric acid and ~0.5 mL of 0.1 M ascorbic acid. The acid was added to solutions in order to ensure all Mn and Cu complexes were dissolved and capable of detection by ICP/AES. This analysis showed the ratio of Mn (II): Cu (II) in the solutions. These results were compared to the initial Mn (II): Cu (II) ratios added to look for trends or inconsistencies. The rest of the solutions were added to 250 mL centrifuge tubes and centrifuged at 7500 rpm at 4°C for 20 minutes in a Sorvall super T21 Centrifuge. The supernatant was removed and the remaining solid was packed inside small plastic vials by the use of the Sorvall Biofuge Fresco centrifuge at 10000 rpm, 4°C, and 5minutes. The final products in the vials were the bacterial spores along with the produced biogenic Mn oxides and sequestered copper. Dr. Sam Webb will later analyze these products using EXAFS, XRD, and XAS.

Preparing spores containing biogenic Mn oxides and sequestered Co for ICP/AES analysis

The procedure followed to prepare the biogenic spores in the presence of copper was earlier used by Dr. Webb to produce the spores in the presence of Co (II). The Co (II): Mn (II) ratios added to each of Dr. Webb's solutions is shown in table 3. The spores containing the Mn oxides and sequestered Co were placed inside 10 mL plastic vials and diluted to 10mL. ~0.25 grams of HNO_3, and ~0.5 grams of ascorbic acid were added so that all metal contents were dissolved and

in solution for later ICP/AES. All solutions were then filtered through syringe filters and placed in new, labeled tubes to be taken for ICP/AES analysis. All this was done only for the Co samples of Dr. Webb. He will later analyze the copper spores. The ICP/AES analysis of the Co solutions showed the ratio of Mn and Co on the spores.

Preparation of Standards for ICP/AES analysis

Cu (II), Mn (II), and Co (II) solutions of concentration 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM, 50 μM, and 100 μM were made from $CuCl_2 * 2H_2O$, $MnCl_2 * 4H_2O$ and, $CoCl_2 * 6H_2O$. Solutions containing (All in the same solution) 500 uM and 100 μM of Cu (II), Co (II), and Mn (II) were also prepared. In addition, a 10 mL sample of 92.5% water, 5.0% ascorbic acid, and 2.5 % of HNO_3 was also prepared for ICP/AES analysis to check for metal contamination in our milli-q water and acids.

ICP/AES analysis

The ICP/AES used for our analysis was a TJA Solutions IRIS Advantag#89A and is located in the Soil Chemistry division of Stanford University. The detection limit for this machine for Cu, Co, and Mn is ~ 0.3 – 4 ng/mL. In atomic emission spectroscopy, a sample solution is introduced into the core of an inductively coupled argon plasma (ICP) at a temperature of approximately 8000 °C. At this temperature all elements become thermally excited and emit light at their characteristic wavelengths. This light is collected by the spectrometer and passes through a diffraction grating that serves to resolve the light into a spectrum of its constituent wavelengths. Within the spectrometer, this diffracted light is then collected by wavelength and amplified to yield an intensity measurement that can be converted to an elemental concentration by comparison with calibration standards (Harris, 1999).

Results

ICP/AES analysis of copper containing solutions

The analysis of our standards along with the solution (not spores) of the samples where biomineralization of biogenic Mn oxides occurred at different concentrations of copper allowed calculation of the amount of Cu and Mn left in solution after the biomineralization process. Table 4 shows the amounts of copper and manganese left in the solutions of different copper concentrations. All solutions, with the exception of cu1m and cu100μ, have only very small amounts of cupper and manganese in the solution phase.

ICP/AES analysis of biogenic Mn oxides and sequestered Co

Table 5 shows the amounts of Co and Mn found sorbed, the original ratios of Co (II): Mn (II) added, and the final ratio of Co: Mn found surrounding the spores. The original unreacted spores and acid solutions showed no sign of Co, Cu, or Mn.

Discussion

Solutions derived from the biogenesis of Mn oxides in the presence of differing amounts of copper showed that, for the most part, manganese was taken out of solution and incorporated within the biogenic Mn oxide. This process also sequestered most of the Cu (II) out of the solution; the major exceptions to this rule were the Cu100μ and cu1m. In these two solutions, the high Cu concentration inhibited Mn (II) oxidation. In addition, most of the copper in these two latter samples was converted to $Cu(OH)_2$ which has a solubility of less than $5 * 10^{-8}$ moles/liter under a pH of 7.5. The blue color of these two solutions is attributed to the $Cu(OH)_2$ solid. Excess copper probably poisons Mn oxidation by blocking the sites where the biomineralization is catalyzed. A conclusion derived from our results would be that small changes in

environmental copper concentrations would not have a major impact on the formation of biogenic Mn oxides and the later sequestration of copper; increases in copper concentration become a major problem as soon as the solubility limits of copper are reached.

The ICP/AES analysis of the samples containing the biogenic Mn oxides formed in the presence of cobalt showed that sorbed Co: Mn parallels the initial ratio of Co: Mn for all solutions except solutions D and E where the ratio of Co: Mn added were 10:1 and 100:1. These two solutions showed a dramatic inhibition of Mn oxidation and Co sequestration. The high amounts of Co poisoned Mn (II) oxidation, which in turn allowed less cobalt to be sequestered. As seen in the copper, the experiment points towards the belief that excess concentration of Co will inhibit biogenic Mn oxide production usually catalyzed by the *Bacillus*, strain SG-1 bacterial spores.

Other interesting observations in the cobalt data were the apparent switch in sorbed Co: Mn ratios between experiments H and J (initial ratio of 1:200 and 1:100) and experiments C and B (initial ratio of 1:2 and 1 to 1). The data showed that the sorbed Co: Mn ratio in experiment H was ~ 1:100 and 1:164 in sample J. Furthermore, experiment C had a sorbed ratio of 1:1.047 while experiment B had a ratio of 1:1.74. At this time, we cannot determine an absolute reason for the reversal in the ratios. Further tests, perhaps using the same procedures, should be done to confirm that this reversal is not due to some random experimental error.

No previous experiments similar to these have been previously reported in literature. Experiments have been done by Alain Manceau and Ewen Silvester on the affects of cobalt metal ion concentration on the metal sequestration abilities of synthetically produced MnO_x. The difference between our experiments was that we were investigating biogenic Mn oxide synthesis

and metal sequestration in the presence of Co and Cu with the metal concentrations held constant by numerous fixed additions over a three-week period of time. The Manceau experiments looked at the ability of synthetically and chemically produced Mn oxides to sequester Co at three different Co concentrations. Although their results also pointed out that at high metal concentrations Mn oxides can not sequester metal as effectively, the fact that their experiment used chemically and synthetically produced Mn oxides makes a comparison difficult with our experiments where biogenic Mn oxides were studied.

Dr. Sam Webb will be performing further analysis, such as EXAFS, XRD, and XAS, on our samples. His analysis will provide a better picture for the mechanisms in which Mn oxides form; therefore, he may be able to further explain our experimental results.

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Figures and Tables

Table 1: The concentration and amount of HEPES, spores, Mn (II) and Cu (II) added to the different erlenmeyer flasks.

Cu (II) + spores				Cu Solution Added		Mn Solution Added		HEPES	Spores
Experiment	Volume (mL)	Mn Added (μM)	Cu Added (μM)	Volume (mL)	Stock Concentration	Volume (mL)	Stock Concentration	Volume (mL) ~1 M	Volume (mL)
Cu1m	150	10	1000	1.5	0.1M	0.15	10 mM	3.0	2.4
Cu100u	150	10	100	0.15	0.1 M	0.15	10 mM	3.0	2.4
Cu20u	150	10	20	0.6	5mM	0.15	10 mM	3.0	2.4
Cu10u	300	10	10	0.6	5 mM	0.30	10 mM	6.0	4.8
Cu1u	400	10	1	0.08	5 mM	0.40	10 mM	8.0	6.4
Cu500n	400	10	0.5	0.4	500 μM	0.40	10 mM	8.0	6.4
Cu100n	400	10	0.1	0.08	500 μM	0.40	10 mM	8.0	6.4
Cu50n	800	10	.05	0.08	500 μM	0..80	10 mM	16.0	12.8

Table 2: The ratio of Cu (II): Mn (II) added to the solutions

Experiment	Cu (II): Mn (II) Ratio added to each Experiment
Cu1m	100: 1
Cu100u	10: 1
Cu20u	2: 1
Cu10u	1:1
Cu1u	1:10
Cu500n	1:20
Cu100n	1:100
Cu50n	1:200

Table 3: The ratio of Cu (II): Mn (II) added to the solutions

Experiment	Co (II): Mn (II) Ratio added to each Experiment
E	100:1
D	10:1
C	2:1
B	1:1
A	1: 10
K	1:20
J	1:100
H	1:200
N	No Cobalt

Table 4: The amount of copper and Manganese left in solution after biomineralization

Experiment	Copper Left in solution (ng)	Mn Left in Solution (ng)
Cu50n	1.639	1.166
Cu100n	0	0
Cu500n	0.1437	0
Cu1μ	4.256	5.397
Cu10μ	39.44	0
Cu20μ	47.79	21.15
Cu100μ	1646	252.6
Cu1m	10890	386.5

Table 5: Amounts of sorbed Co and Mn (absorbed or in solid phase), the original ratios of Co (II): Mn (II) added, and the final ratio of Co: Mn found surrounding the spores

Experiment	Cobalt Sorbed (ng)	Manganese Sorbed (ng)	Original Co (II): Mn (II) ratio added	Final Co: Mn ratio found surrounding the spores
N	0.1158	2501	No cobalt	0
H	41.5	4155	1 to 200	1 to 100.12
J	20.52	3381	1 to 100	1 to 164.66
K	213.2	4080	1 to 20	1 to 19.13
A	315.6	3940	1 to 10	1 to 12.48
C	680.6	712.9	1 to 2	1 to 1.047
B	876.5	1523	1 to 1	1 to1.74
D	640.8	881.2	10 to 1	1.34 to 1
E	853.8	606.6	100 to1	1 to 0.71