Published in final edited form as:

Geochim Cosmochim Acta. 2013 January 15; 101: 1–11. doi:10.1016/j.gca.2012.10.008.

The effect of Ca^{2+} ions and ionic strength on Mn(II) oxidation by spores of the marine *Bacillus* sp. SG-1

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Abstract

Manganese(IV) oxides, believed to form primarily through microbial activities, are extremely important mineral phases in marine environments where they scavenge a variety of trace elements and thereby control their distributions. The presence of various ions common in seawater are known to influence Mn oxide mineralogy yet little is known about the effect of these ions on the kinetics of bacterial Mn(II) oxidation and Mn oxide formation. We examined factors affecting bacterial Mn(II) oxidation by spores of the marine *Bacillus* sp. strain SG-1 in natural and artificial seawater of varying ionic conditions. Ca^{2+} concentration dramatically affected Mn(II) oxidation, while Mg^{2+} , Sr^{2+} , K^{+} , Na^{+} and NO_3^{-} ions had no effect. The rate of Mn(II) oxidation at 10mM Ca^{2+} (seawater composition) was four or five times that without Ca^{2+} . The relationship between Ca^{2+} content and oxidation rate demonstrates that the equilibrium constant is small (on the order of 0.1) and the binding coefficient is 0.5. The pH optimum for Mn(II) oxidation changed depending on the amount of Ca^{2+} present, suggesting that Ca^{2+} exerts a direct effect on the enzyme perhaps as a stabilizing bridge between polypeptide components.

We also examined the effect of varying concentrations of NaCl or KNO_3 (0 mM – 2000 mM) on the kinetics of Mn(II) oxidation in solutions containing 10 mM Ca^{2+} . Mn(II) oxidation was unaffected by changes in ionic strength (I) below 0.2, but it was inhibited by increasing salt concentrations above this value. Our results suggest that the critical coagulation concentration is around 200 mM of salt (I = ca. 0.2), and that the ionic strength of seawater (I > 0.2) accelerates the precipitation of Mn oxides around the spores. Under these conditions, the aggregation of Mn oxides reduces the supply of dissolved O_2 and/or Mn^{2+} and inhibits the Mn(II) → Mn(III) step controlling the enzymatic oxidation of Mn(II). Our results suggest that the hardness and ionic strength of the aquatic environment at circumneutral pH strongly influences the rate of biologically mediated Mn(II) oxidation.

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1. INTRODUCTION

In aquatic environments, manganese exists as Mn(II) in solution, while Mn(III, IV) (oxyhydr)oxides are found as insoluble particulates (collectively referred to as Mn oxides). Mn oxides, among the strongest common chemical oxidants except oxygen (Langmuir, 1997), play an important role in the environment and promote the oxidation of a variety of inorganic species including Cr(III), Co(II), and hydrogen sulfide, and organic compounds such as humic substances and aromatic hydrocarbons (Huang, 1991; Post, 1999). Mn oxides also serve as electron acceptors for the oxidation of hydrogen or organic matter by anaerobic bacteria, and they scavenge a variety of metals on their highly charged surfaces.

Currently, microbes are thought to be the primary source of Mn oxides in most environments (e.g., Nealson et al., 1988). Microbes increase the rate of Mn(II) oxidation by at least 3–5 orders of magnitude over abiotic surface-catalyzed Mn(II) oxidation (Hasting and Emerson, 1986; Nealson et al., 1988; Tebo, 1991; Tebo et al., 1997). Thus, Mn(II)-oxidizing bacteria have a tremendous impact on the geochemical cycling of Mn and consequently on the cycling of other elements (e.g., Francis and Tebo, 1999; Brouwers et al., 2000; Tebo et al., 2004).

So far the Mn(II)-oxidizing proteins of several different model organisms have been identified: MnxG in *Bacillus* sp. strain SG-1; MofA in *Leptothrix discophora* strain SS-1 and MoxA in *Pedomicrobium* sp. ACM 3067 (van Waasbergen et al., 1996; Corstjens et al., 1997; Francis and Tebo, 1999; Brouwers et al., 2000; Francis and Tebo, 2001, 2002; Tebo et al., 2005; Ridge et al., 2007). Although they have never been purified, based on genetic studies, these enzymes are thought to be multicopper oxidase (MCO)-like proteins. This family of enzymes uses multiple Cu atoms as cofactors in coupling the one-electron oxidation of substrates (previously, only organics and Fe(II)) to the reduction of O₂ to H₂O (Solomon et al., 1996).

A previous report (Brouwers et al., 1999) of the MCO, CumA, catalyzing Mn(II) oxidation in *Pseudomonas putida* strain GB-1 has recently been disproved (Geszvain and Tebo, 2010). The mechanism of Mn(II) oxidation in *P. putida* GB-1 is unknown. *Aurantimonas manganoxydans* strain SI85-9A1 possess a Ca²⁺-binding heme peroxidase which oxidizes Mn(II) (Anderson et al., 2009) while in *Roseobacter* strain AzwK-3b enzymatic production of superoxide has been implicated in Mn(II) oxidation (Learman et al., 2011a).

Spore-forming *Bacillus* species may be a large component of the total colony-forming bacteria in many environments (Bonde, 1981; Lee and Tebo, 1994). The extensive phylogenetic diversity of marine, Mn(II)-oxidizing *Bacillus* spores has been recognized (Francis and Tebo, 2002), e.g., in Guaymas Basin hydrothermal sediments and plumes (Dick et al., 2006, 2009, 2010). The marine Mn(II)-oxidizing *Bacillus* sp. strain SG-1 was isolated from sandy marine sediments in La Jolla, CA (Nealson and Ford, 1980). Dormant spores of this organism, not the vegetative cells, enzymatically catalyze the oxidation of soluble Mn(II) to highly insoluble Mn(III, IV) oxide precipitates on the spore surface (e.g., Nealson and Ford, 1980; Tebo and He, 1999). The Mn(II)-oxidizing enzyme is located in the exosporium, the outermost layer of the spore coat (Francis et al., 2002). Studies of the

isolated exosporium and the partially purified the Mn(II) oxidizing enzyme from SG-1 spores suggested that MnxG catalyzes two sequential one-electron oxidations from Mn(II) to Mn(III) and from Mn(III) to Mn(IV), a novel type of reaction for a MCO (Webb et al., 2005; Dick et al., 2008). Although biochemical analysis of the Mn(II) oxidase has not yet been possible, SG-1 is still a convenient model organism for enzymatic Mn(II) oxidation.

Following biological oxidation, Mn has been found in a variety of forms including poorly crystalline or amorphous manganese oxide (-MnO_2 or vernadite), triclinic Na birnessite, todorokite, buserite (hydrated phyllomanganate), hausmannite (Mn_3O_4), manganite (-MnOOH), and feitknechtite ($\beta\text{-MnOOH}$) (e.g., Burns and Burns, 1979; Tebo et al., 2004). Bargar et al. (2000), using X-ray absorption near edge spectroscopy (XANES), demonstrated that the primary product of Mn(II) oxidation by SG-1 spores in buffered 50 mM NaCl solutions was -MnO_2 which formed within minutes of the onset of the reaction; there was no evidence of any Mn(II,III) solid phase intermediates. Further, experiments at both low (10 μM) and high (1 mM) Mn(II) concentrations demonstrated the formation of buserite and feitknechtite, respectively, as secondary oxidation products catalyzed by the surface of the biogenic -MnO_2 -like Mn oxide (Bargar et al. 2005). As reviewed by Spiro et al. (2010), these recent investigations proved that some Mn(II) oxidation products found in early bacterial studies (Hastings and Emerson, 1986; Mann et al., 1988; Mandernack et al., 1995a), e.g., hausmannite and manganite, are the products of abiotic oxidation of Mn(II), most likely resulting from autocatalytic oxidation of adsorbed Mn(II) on the surface of a freshly precipitated bacteriogenic Mn(IV) oxide. Abiotic Mn(II) oxidation generates Mn(III) or mixed Mn(II,III) solid phase intermediates, which subsequently disproportionate to Mn(II)(aq) and Mn(IV) oxides (Hem and Lind, 1983; Murray et al., 1985).

If the properties of the Mn(II) oxidizing enzymes are consistent with known MCO proteins, Mn(III) should be the initial reaction product following enzymatic action. Indeed, Webb et al. (2005a) used pyrophosphate, a compound that forms a strong, stable complex with Mn(III) (Kostka et al., 1995), to trap Mn(III) during Mn(II) oxidation by *Bacillus* SG-1 spores. However, Mn(III) was found only as a transient soluble intermediate; with time, the Mn(III) was also enzymatically converted to Mn(IV) oxide (Webb et al., 2005a). Since Mn(III) is not usually observed during Mn(II) oxidation (Bargar et al., 2000), this suggests that Mn(II) to Mn(IV) oxidation occurs via two sequential one-electron oxidation reactions and that oxidation of Mn(II) to Mn(III) is the rate limiting step. Additionally, the overall rate of Mn(II) oxidation is controlled by the transfer of oxygen to Mn(II) from both water molecules and dissolved oxygen in seawater (Mandernack et al., 1995b). Thus, Mn(II) oxidation appears affected by both the rate of oxygen incorporation from H_2O as well as atmospheric O_2 .

An increased understanding of bacterial Mn(II) oxidation, including enzymatic mechanisms, reaction kinetics and Mn oxide products is essential for the application of this natural technology to projects such as the remediation of contaminated ground water (Francis and Tebo, 2002). Although a variety of studies have investigated the effects of environmental parameters such as temperature, pH, salinity and some metals on Mn(II) oxidation by SG-1 spores, very little is known about the kinetics of Mn(II) oxidation under these conditions. In this study, we examined the overall rate of Mn(II) oxidation by spores of the marine *Bacillus*

sp. strain SG-1 as a function of concentration and ionic strength over time and over a pH range from 7.6 to 8.0 at temperatures of 22–24°C under conditions of 100% air saturation. Interestingly, Ca^{2+} ions dramatically affected rates of Mn(II) oxidation in artificial seawater. Additionally, we show that ionic strength differentially affects the two steps of the overall process, i.e., the Mn(II) to Mn(III) and Mn(III) to Mn(IV) reactions.

2. MATERIAL AND METHODS

2.1. Preparation of artificial seawater for incubation experiments

All glassware used in this study were acid washed for >1 day in 2 M HCl and rinsed with milli-Q water. The following reagents were used to make the artificial seawater (ASW) and variations thereof: NaCl, Na_2SO_4 , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, KCl, and $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$. The compositions of the ASW variants are based on the ionic strength and general chemical concentration of natural seawater [468 mM Na^+ , 53 mM Mg^{2+} , 10 mM Ca^{2+} , 0.09 mM Sr^{2+} , 10 mM K^+ , 546 mM Cl^- , 28 mM SO_4^{2-}] (Millero, 1996). The ionic strength of natural seawater is approximately 0.70 M. Filter sterilized HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer was added to a final concentration of 2mM, and the pH was adjusted to 7.5 – 7.9 using NaOH. After the addition of sterile MnCl_2 , the total mass of the ASW solution was adjusted to 500 g with milli-Q water. The initial Mn(II) concentration was approximately 30 μM or 50 μM for the incubation experiments.

Natural seawater (NSW) was collected from near-shore seawater off the pier of the Scripps Institution of Oceanography, aged for several months at 4°C to reduce the amount of organic matter and filtered through a 0.22 μm Millipore MF membrane. NSW was also amended with filter-sterilized HEPES, the pH adjusted to 7.6–8.0, and spiked with MnCl_2 to near 30 μM or 50 μM . HEPES is a non-chelating buffer useful for studies with metal ions with a negligible stability constant for Mn ions (Good et al., 1966; Lambeth et al., 1992) and thus should not significantly affect the speciation of Mn(II) in this study. The pKa value of HEPES buffer at 20°C is 7.55, and the oxidation of Mn(II) is not expected to cause a shift in pH greater than – 0.1 during all experiments.

2.2. Preparation of spores of *Bacillus* sp. strain SG-1

Spores of the marine *Bacillus* sp. strain were purified based on a modification of published procedures (Rosson and Nealson, 1982). Briefly, *Bacillus* sp. strain SG-1 was maintained on agar plates. It was grown on K medium (0.5 g·L⁻¹ yeast extract, 2 g·L⁻¹ peptone, 10 mM HEPES autoclaved buffer, 100 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ in 75% strength natural seawater). 1L batch cultures were grown in 2L flasks with constant shaking at room temperature for 6 days. The sporulated mature culture was centrifuged at 4°C, and the pellet was rinsed with Milli-Q water and centrifuged for spore purification. The pellet was resuspended and lysed at 4°C overnight in 100 mL of 10 mM Tris/10 mM EDTA containing 3 mg of phenylmethylsulfonyl fluoride (PMSF) and 5 mg of lysozyme. The spores were centrifuged and washed with 100 mL 1 M NaCl/10 mM Tris/10 mM EDTA/3mg PMSF, followed by 0.15 M NaCl/10 mM Tris/10 mM EDTA/3mg PMSF and 0.2 M ascorbate. The pellets were centrifuged and leached with 100 mL of 0.1% SDS at room temperature overnight and centrifuged and washed at least 5 times with milli-Q water. The spores were aliquoted to microcentrifuge

tubes, and the weights of the wet spore pellets were recorded. The purified spores were stored at -20°C until use. Prior to use, the spores were slowly thawed on ice and thoroughly homogenized using a tissue homogenizer.

2.3. Kinetic experiments and measurement of Mn(II) concentration

For Mn(II) oxidation experiments, 400 mL of the indicated pH-adjusted ASW variations or NSW was amended with Mn(II) and placed in acid-washed 500 mL Erlenmeyer (Pyrex) flasks. The mouth of the flask was sealed with Saran wrap during the incubation; we designate these incubations semi-closed systems. The amount of molecular O_2 consumed during the incubation is negligible compared with the air-volume of the vacancy in the 500-mL flasks. Thus, we consider the redox conditions of these incubations to be 100% air saturation. We also performed continuous sparging incubations in which air was supplied which was passed through a gas-washing bottle filled with milli-Q water to prevent evaporation. In order to examine reactions in the absence of carbon dioxide, a second gas-washing bottle filled with a sodium hydroxide (NaOH) solution was put in line before the milli-Q water bottle to trap the CO_2 .

The pH values of the media were checked and readjusted if necessary before beginning the experiments. The Mn(II) concentration was measured before and just after the addition of spores using the formaldoxime colorimetric technique (Brewer and Spencer, 1971) or by ICP-AES (Seiko Co. SPS7700). Replication of Mn(II) concentrations over $10\text{ }\mu\text{M}$ was within 1–2% relative standard deviation in both methods.

Thawed spores were resuspended in 15 mL milli-Q water and homogenized. The spore suspension was pipetted into weighed 400-mL solutions, and the number of spores in each flask was determined microscopically using a Petroff-Hauser counting chamber. The final spore concentrations were between 6×10^9 to 8.2×10^9 spores/L. The rate of Mn(II) removal (particulate Mn formation) was monitored by removing 1 mL aliquots at the indicated intervals, filtering through $0.2\text{ }\mu\text{m}$ disc-syringe HT Tuffryn filters (polysulfone) to remove cells and oxidized Mn, and measuring the dissolved Mn(II) concentration. All incubation experiments were done at room temperature ($22\text{--}24^{\circ}\text{C}$) under static conditions.

Even under static conditions, the diffusion of Mn(II) to the spore surface is not rate limiting for Mn(II) oxidation. In preliminary studies we measured the decrease in dissolved Mn concentration under different dynamic and static culture conditions and no significant differences among the five mixing modes in the initial stages are observed ($0.517 \pm 0.042\text{ }\mu\text{M/h}$; Table 1). The observed decrease in dissolved Mn concentration is entirely attributable to Mn(II) adsorption on spores and subsequent Mn(II) oxidation. After the initial period of Mn(II) binding, the observed constant decrease in Mn(II) concentration is approximately equal to the biological Mn(II) oxidation rate. The sampling times and Mn(II) concentrations for these experiments are indicated in the figures.

3. RESULTS AND DISCUSSION

3.1. Effect of cation composition of ASW on Mn(II) oxidation rates

We examined Mn(II) oxidation rates under differing cation concentrations (Table 2). The rates of Mn(II) removal were linear over the initial time course of the experiment and gradually increased as Mn(II) concentration decreased to below 15 μM ; this change was likely due to autocatalytic oxidation on the surface of MnOx (Diem and Stumm, 1984). Interestingly, Ca^{2+} concentration had the greatest effect on Mn(II) oxidation, with the most rapid oxidation occurring in ASW solutions with 10 mM Ca^{2+} ($0.523 \pm 0.038 \mu\text{M/h}$), followed by NSW ($0.375, 0.388 \mu\text{M/h}$) and ASW with 2 mM Ca^{2+} ($0.279 \pm 0.013 \mu\text{M/h}$); oxidation rates were slowest in ASW without Ca^{2+} ($0.102 \pm 0.011 \mu\text{M/h}$). Variations in both K^{+} and Mg^{2+} concentrations did not affect the rate of Mn(II) oxidation. Although NSW contains 10 mM Ca^{2+} , the rate of Mn(II) oxidation was substantially slower in NSW than in ASW containing 10 mM Ca^{2+} . While this may arise from differences in ionic strength between NSW ($I=0.68$) and ASW ($I=0.63\text{--}0.70$), it is likely that some other component of filtered NSW inhibits Mn(II) oxidation. Another experiment (Table 3) also shows the rate of Mn(II) removal in filtered NSW ($0.240 \pm 0.004 \mu\text{M/h}$) was slower than in ASW ($0.323 \pm 0.006 \mu\text{M/h}$). Moreover, the rate of Mn(II) removal in unfiltered NSW ($0.194 \pm 0.003 \mu\text{M/h}$) was slower than in filtered NSW ($0.240 \pm 0.004 \mu\text{M/h}$). It is possible that organic matter in seawater can react with Mn oxides (Sunda and Kieber, 1994) giving the appearance that Mn(II) oxidation is inhibited due to competitive oxidation. Alternatively, organic matter could form a complex with a soluble Mn(III) intermediate, thus inhibiting full Mn oxide formation (Parker et al., 2004, 2007; Webb et al., 2005a). Other trace elements could also be present in seawater that inhibit Mn(II) oxidation in other ways (e.g., competition for Mn(II) binding). It is also possible that photochemical reduction of Mn(IV) oxides by sunlight to form soluble Mn(II) in the presence of organic reductants (e.g., humic substances) was occurring. To test this we performed an experiment comparing a flask fully covered with aluminum foil with unshielded glass flasks incubated on the lab bench for 48 hours after spore-addition. The results show that the known effect of light on dissolution of Mn oxides (Kwon et al., 2009) is negligible in this experiment (Table 4).

We further investigated whether Na^{+} or K^{+} affected Mn(II) oxidation rates by varying Na^{+} and K^{+} concentration (0 – 1000 mM) in ASW (Table 5). Despite these variations, no differences in the Mn(II) oxidation rates were seen ($0.369 \pm 0.016 \mu\text{M/h}$; Table 5). Variations in Sr^{+} concentrations (0–1.35 mM) in the above ASW, also had no significant differences in rates of Mn(II) oxidation ($0.336 \pm 0.005 \mu\text{M/h}$; Table 3).

3.2. Effect of anion composition and pH

The above data indicate that, among the cations tested, only Ca^{2+} concentration affects the rate of Mn(II) oxidation in ASW. To examine whether varying anion concentrations changed the rate of Mn(II) oxidation, we tested effects of Cl^{-} , NO_3^{-} , CO_3^{2-} and OH^{-} (pH) concentrations. For Cl^{-} , we varied the concentration of Cl^{-} and NO_3^{-} in ASW from 0 to 1000 mM while maintaining a constant ionic strength (ionic strength = 1.005; $\text{Cl}^{-} + \text{NO}_3^{-} = 1000 \text{ mM}$). No significant differences were seen in the rate of Mn(II) oxidation under these different conditions ($0.880 \pm 0.041 \mu\text{M/h}$; Table 6).

The $[\text{MnCO}_3]/[\text{Mn}^{2+}]$ ratio varies as a function of pH and could affect Mn(II) oxidation rates. The calculated manganese carbonate concentration decreases dramatically with decreasing pH (Zhang et al., 2002). We examined the effect of the addition of various amounts (0–2.5 mM) of Na_2CO_3 on Mn(II) oxidation at pH 8; no effect was observed ($0.245 \pm 0.011 \mu\text{M/h}$; Table 4).

Finally, we examined the effect of pH on Mn(II) oxidation in the presence and absence of Ca^{2+} . In the absence of Ca^{2+} , the rate of Mn(II) oxidation in 0.54 M $\text{NaCl}/0.03 \text{ M Na}_2\text{SO}_4$ was measured over a pH range from 6.45 to 8.14 at 24°C , sparged with CO_2 -scrubbed-air, and amended with 8.2×10^9 spores/L. The Mn(II) concentration was monitored for 50 h prior to the addition of spores, and, because the Mn(II) concentration decreased, the pH 8.6 sample was not followed further. Under most conditions tested, the rate of Mn(II) oxidation remained constant (Fig. 1). Overall, the Mn(II) oxidation rate was positively correlated with the root of $[\text{OH}^-]$ in the pH range 7.0–8.14.

We also examined the rate of Mn(II) oxidation under different pH values in ASW with Ca^{2+} (2 mM and 10 mM Ca^{2+}) and 4×10^9 spores/L (Fig. 2). For comparison, the results in Ca^{2+} free ASW are also shown in Fig. 2. The maximum Mn(II) oxidation rates were at pH 7.6 and pH 7.1 in the presence of 10 mM and 2 mM Ca^{2+} , respectively. The observed pH optimum of 7.5 for 10 mM Ca^{2+} is very close to the reported values of pH 7.3–7.5 for *L. discophora* strain SS1 (Boogerd and de Vrind, 1987; Adams and Ghiorse, 1987; Zhang et al., 2002) and higher than the pH optimum of ~ 7 for the partially purified Mn^{2+} -oxidizing factor of *Pseudomonas putida* strain GB-1 (Okazaki et al., 1997).

As mentioned below, Ca^{2+} profoundly affects the kinetics and rate of Mn(II) oxidation. Increasing Ca^{2+} from 2 mM to 10 mM shifted the pH optimum of the reaction from 7.1 to 7.5. These data suggest that Ca^{2+} is affecting the activity of the enzyme and that Ca^{2+} plays an important biochemical role in Mn(II) oxidation by *Bacillus* sp. strain SG-1.

3.3. The effect of Ca^{2+} on Mn(II)-oxidizing activity

We examined the effect of Ca^{2+} on the oxidation of Mn(II) by SG-1 more fully by varying the Ca^{2+} concentration from 0 to 20 mM in ASW while maintaining a constant ionic strength of 0.66 with Mg^{2+} at pH 7.8 under CO_2 -scrubbed-air with 6.0×10^9 spores/L at 24°C . At concentrations above 20 mM, Ca^{2+} precipitates out of solution. As seen in Figure 3, Ca^{2+} dramatically affects the kinetics of Mn(II) oxidation. These results were replicated in a second experiment at pH 7.95 with 7.0×10^9 spores/L.

Figure 4 summarizes the relationship between $[\text{Ca}^{2+}]$ and the rate of Mn(II) oxidation. Based on this data we propose a model wherein Ca^{2+} acts as a modulator for the SG-1 Mn oxidase (presumably MnxG), converting it from an inactive form to the active form following binding:

where ' $\leftrightarrow$ ' indicates a reversible reaction and 'n' represents the number of Ca^{2+} ions bound per enzyme. The equilibrium constant, K, for the binding reaction is defined as:

$$K = \frac{[\text{nCa}^{2+} - \text{MnxG}]}{([\text{MnxG}][\text{Ca}^{2+}]^n)}.$$

We can write:

$$c = 1 / \{1 + 1 / (K[\text{Ca}^{2+}]^n)\}$$

where c equals the binding coefficient ($0 \leq c \leq 1$) and

$$V = cV^* + (1 - c)V_{\text{free}}$$

where V is the measured Mn(II) oxidation rate, V^* represents the maximum Mn(II) oxidation rate with Ca^{2+} present, and V_{free} is the minimum velocity in the absence of Ca^{2+} .

The results of three separate experiments with slightly different concentrations of spores gave similar results (Fig. 4). In this figure, the closed diamonds are data from the experiment shown in Table 2, the closed squares are data from the experiment shown in Figure 3, and the closed circles are the results of a replicate experiment for which the time course data is not shown. Since there are two variables, K and V^* , it is impossible to accurately estimate these values from the regression curves. Although we have no information about the number of Ca^{2+} ions per Mn(II)-oxidizing enzyme, assuming $n = 0.5$ provides the best fit to our data (Fig. 4). Ca^{2+} is implicated in ternary structure stabilization in several proteins (Bailey and Ollis, 1986), e.g., the metallo-oxo cluster in photosystem II (Yocum, 2008). Calcium also binds to ceruloplasmin, the MCO most homologous to MnxG, and increases its thermostability (Musci et al., 1996). It is plausible that Ca^{2+} can serve as a stabilizing bridge analogous to a disulfide bond by forming ionic bonds with two different amino acid residues (Solomon et al., 1996).

Incidentally, the study on surface charge properties of and Cu(II) adsorption by SG-1 spores also indicates that one Cu(II) ion shares two surface sites on SG-1 spores (He and Tebo, 1998). In the absence of Ca^{2+} , the V_{free} values were 0.054 $\mu\text{M/h}$, 0.092 $\mu\text{M/h}$ and 0.113 $\mu\text{M/h}$ for three different spore concentrations. The Mn(II) oxidation rate (maximum velocity) of the Ca^{2+} bound Mn oxidase is about two and half orders of magnitude higher than that of the Ca^{2+} -free enzyme assuming $n = 0.5$.

3.4. Effects of ionic strength on Mn(II) oxidation

The influence of salinity on biological Mn(II) oxidation has not been reported, but several studies examined the role of salinity on abiotic Fe(II) and Cu(I) oxidation rates in seawater (e.g., Millero, 1985). In both cases, the "activated complex theory" accurately predicted the effect of ionic strength on reaction rates. This theory assumes that an activated metal complex is formed in the rate-limiting step and the equation (Brezonik, 1993) is expressed as follow:

$$\log(k) = \log(k_0) + 1.02 Z_A Z_B (I^{0.5} / (1 + I^{0.5})) \quad (4)$$

where k is the actual rate constant, k_0 is the rate constant at zero ionic strength, Z_A and Z_B are the electrostatic charges on the reacting species, and I is the ionic strength of the solution. The kinetic electrolyte effect depends on charge ($Z_A Z_B$) and ionic strength (I).

To investigate the effect of salinity on enzymatic Mn(II) oxidation, time course measurements of Mn(II) oxidation by SG-1 spores at constant Ca^{2+} concentration (10 mM) and varying concentrations of NaCl (0 mM–2000 mM) were performed at pH 7.6–7.8 with CO_2 -scrubbed-air and 6.3×10^9 spores/L at 22°C. As seen in Figure 5, the rate of biological Mn(II) oxidation was relatively constant in solutions containing 0 to 200 mM NaCl. Between 200 and 2000 mM, however, the rate of Mn(II) oxidation slowed. When the calculated rates of Mn(II) oxidation are plotted against the ionic strength, it is clearly seen that the rate constant decreases with increasing ionic strength. To confirm that these results were dependent on ionic strength rather than NaCl specifically, we varied the concentration of KNO_3 from 0 to 2000 mM under similar conditions and obtained similar results (Fig. 6). In both the NaCl and KNO_3 data, two regression lines with different slopes are plotted and they cross at ca. 0.2 M ionic strength. The obvious inflection of the two regression lines cannot be explained by the “activated complex theory” and the significant effect of ionic strength on Mn (II) precipitation rates in seawater-like solutions ($I > 0.2$ M) requires another explanation. Ionic strength of 0.2 M is equivalent to ca. 0.31 of $(I / (1 + I))$ at the inflection point in Figure 6.

A new technology for oxidation state mapping with high spatial resolution revealed a heterogeneous distribution of Mn-species among different spores. A substantial amount of Mn(III) was found around most spores; notable quantities of Mn(II), but only small amounts of Mn(IV), were also observed (Pecker et al., 2003). Given the length of the incubation period combined with the high concentration of Mn(II), it is likely that the Mn(III) detected in that work was due to the formation of solid phase Mn(III)-bearing minerals on the surface of the initial Mn(IV) bio-oxide as opposed to an enzyme-bound Mn(III) intermediate (Tebo et al., 2004).

After enzymatic Mn(II) $\rightarrow$ Mn(IV) oxidation, it is believed that the subsequent release, hydrolysis and polymerization of Mn(IV) should be followed by the production of X-ray amorphous Mn oxide. The primary solid biogenic product following this reaction is similar to -MnO_2 , which has a Mn oxidation state between 3.7 and 4.0, and is a highly reactive catalyst for further Mn(II) oxidation reactions (Bargar et al., 2000, 2005; Learman et al., 2011b). Reaction of Mn(II) with the primary biogenic oxide produced by SG-1 results in the production of the abiotic secondary products, feitknechtite or buserite in 10 μM or 1 mM Mn(II)-containing HEPES-buffered 50 mM NaCl solutions (Bargar et al., 2005). Webb et al. (2005b) demonstrated that the presence of Ca in these products confer greater stability to these bacteriogenic phyllo-manganate bioxides. Diverse secondary reaction products can form depending on the solution conditions, but likely explain the salt-effect observed in Figure 6.

It is well known that the collision efficiencies for colloidal particle interactions strongly depend on the ionic composition of the medium as well as the surface charge of the colloids. The phenomenon is accounted for by Derjaguin-Landau-Verwey-Overbeek (DLVO) theory combining the effects of the van der Waals attraction and the electrostatic repulsion due to double-layer interaction (Elimelech et al., 1995). At low ionic strength, the particles are very stable due to electrostatic repulsion between the positively charged colloids of Mn oxides. With increasing ionic strength, the surface charge is increasingly screened, resulting in increased collision efficiencies and aggregation or deposition. The ability of a particular electrolyte to cause flocculation can be expressed in terms of its critical coagulation concentration (Elimelech et al., 1995). In this study, the critical coagulation concentrations for Mn oxide colloids are around 200 mM of NaCl or KNO₃ ($I = ca. 0.2$) without agitation.

Limitations in the mass transport of dissolved O₂ and/or Mn²⁺ to the spore surface was suggested from a study examining the oxidation state mapping of Mn precipitation by SG-1 spores (Pecher et al., 2003). Our results are likely induced by the flocculation of Mn(III)-bearing secondary reaction products around the spores in solution of MnCl₂ with 75% seawater ($I = 0.5$) without agitation resulting in significant decrease in the Mn precipitation rate in solutions of higher ionic strength ($I > ca. 0.2$; NaCl or KNO₃ $> ca. 200$ mM; Figure 6). Under these conditions the diffusion of dissolved O₂ and/or Mn²⁺ which depends on the viscosity, temperature and molecular radius, may also be limited.

The inorganic auto-catalytic reaction at the Mn oxide surface surrounding the spore could also affect the Mn removal rate, but this effect is likely negligible except for later time points. Thus, we conclude that the aggregation of Mn oxide products induces the salt-effect observed in Figure 6. It remains unclear whether the supply of dissolved O₂ or Mn²⁺ to the spore surface decreases with time and ultimately whether these changes effect the oxidation rate of the Mn(II) $\rightarrow$ Mn(III) step in high ionic strength solutions. This point is a topic of a future report.

3. CONCLUSION

This study is the first to systematically report a dramatic effect of Ca²⁺ concentration on the rate of bacterially mediated Mn(II) oxidation. The observed changes in pH maximum suggests that Ca²⁺ binds to the MnxG enzyme on the spore coat of the marine *Bacillus* sp. SG-1 and affects the conformation and activity of the enzyme. This conclusion is supported by the well-recognized role of Ca²⁺ in other MCOs (Musci et al., 1996; Bento et al., 2007), e.g., ceruloplasmin, the MCO most homologous to MnxG (van Waasbergen et al., 1996). We calculated that the equilibrium constant for this reaction is on the order of 0.1, and the binding coefficient is 0.5. The Mn(II) oxidation rate of the Ca²⁺ bound Mn oxidase is about two and half orders of magnitude higher than that of the Ca²⁺-free enzyme. However, further work is required to confirm the equilibrium constant and the binding coefficient determined in this study.

Many studies have clearly documented that Cu stimulates the Mn(II) oxidizing activity of Mn-oxidizing bacteria (van Waasbergen et al., 1996; Corstjens et al., 1997; Brouwers et al., 1999; Francis and Tebo, 1999; Brouwers et al., 2000; Francis and Tebo, 2001, 2002), and,

consequently, we did not examine the effect on Cu on the SG-1 spores in this study. In contrast, little work has characterized the role Ca^{2+} in metal oxidation in general, let alone Mn(II) oxidation. In oxygenic photosynthesis, calcium is required for efficient Mn oxidation, which is a key step leading to formation of O_2 from H_2O in photosystem II (Yocum, 2008). In this system, the addition of Sr^{2+} is capable of restoring activity, but at a level less than about 50% of the rate obtained with added Ca^{2+} . In contrast, Sr^{2+} (and Mg^{2+}) ions had no effect on spores of the marine *Bacillus* sp. strain SG-1.

Recently, a new type of manganese-oxidizing enzyme was identified in two alphaproteobacteria, “*Aurantimonas manganoxydans*” strain SI85-9A1 and *Erythrobacter* sp. strain SD-21 (Johnson and Tebo, 2008; Anderson et al., 2009). This enzyme is a type of animal heme peroxidase and contains hemolysin-type calcium binding domains. The addition of both Ca^{2+} ions and H_2O_2 to the enriched protein from *Aurantimonas* increased manganese oxidation activity 5.9-fold and the putative Ca^{2+} binding heme peroxidase has tentatively been named MopA (Anderson et al., 2009). This work demonstrates the ability of Ca^{2+} to modulate the activity of diverse Mn(II)-oxidizing enzymes including heme peroxidases and MCOs.

Our results also demonstrate the relationship between ionic strength and bacterial Mn(II) oxidation by spores of the marine *Bacillus* sp. SG-1. In higher ionic strength solutions ($I > 0.2$) such as seawater ($I = \text{ca. } 0.7$), the increased salt concentration inhibits the rate of Mn(II) oxidation. In contrast, in lower ionic strength solutions ($I < 0.2$) changes in salt concentration did not affect the rate of Mn(II) oxidation. At ionic strengths greater than the critical coagulation concentration ($I = \text{ca. } 0.2$), the coagulation and precipitation of Mn oxides around the spore would be accelerated and Mn(II) oxidation is inhibited possibly limiting the supply of dissolved O_2 and/or Mn^{2+} . Further work is required to determine the precise mechanism of this phenomenon. The effect of salt on microbial Mn(II) oxidation must be considered when examining aquatic Mn cycles, particularly in brackish waters.

In three experiments (e.g., Table 2), we observed significant differences in the rates of Mn(II) oxidation between natural seawater and artificial seawater, despite similar Ca^{2+} content and ionic strength. Dissolved organic matter in natural seawater may explain these differences (Sunda and Kieber, 1994), but this needs to be examined in greater detail. As discussed above, we focused on the effects of Ca^{2+} ions and ionic strength on Mn(II) oxidation by spores of the marine *Bacillus* sp. SG-1. Other factors (e.g., spore concentration, initial Mn(II) concentration, temperature, oxygen concentration, and pH) influencing bacterial Mn(II) oxidation by the SG-1 spores are currently under examination. In conclusion, our results suggest that the ionic composition and strength of natural waters can profoundly affect Mn cycles in aquatic environments.

Acknowledgments

We are grateful to Margo Haygood for her kind encouragement, to Carolyn Sheehan for her assistance, to Anna Obraztsova for the microbiological instruction to Kazuhiro Toyoda, and to the Tebo and Haygood laboratory members for their kind support. We also thank two anonymous reviewers for their helpful comments and criticisms. Most experiments were performed during Toyoda's stay at Scripps Institution of Oceanography-UCSD as a visiting scientist supported by MEXT overseas Scholarship of Japan. This research was supported by the National Institute of Environmental Health Sciences Superfund Basic Research Program (Grant # ES10337 to the University of

California San Diego) and a Collaborative Research Activities in Environmental Molecular Sciences (CRAEMS) grant (CHE 0089208) from the National Science Foundation.

References

- Adams LF, Ghiorse WC. Characterization of extracellular Mn(II) oxidizing activity and isolation of and Mn(II) oxidizing protein from *Leptothrix discophora* SS1. *J Bacteriol.* 1987; 169:1279–1285. [PubMed: 3818545]
- Anderson R, Johnson HA, Caputo N, Davis RE, Torpey JW, Tebo BM. Mn(II) oxidation is catalyzed by heme peroxidases in “*Aurantimonas manganoydans*” strain SI85-9A1 and *Erythrobacter* sp. strain SD-21. *Appl Environ Microbiol.* 2009; 75:4130–4138. [PubMed: 19411418]
- Bailey, EJ., Ollis, FD. *Biochemical Engineering Fundamentals*. McGraw-Hill; 1986.
- Bargar JR, Tebo BM, Villinsk JE. In situ characterization of Mn(II) oxidation by spores of the marine *Bacillus* sp. strain SG-1. *Geochim Cosmochim Acta.* 2000; 64:2737–2749.
- Bargar JR, Tebo BM, Bergmann U, Web SM, Glatzel P, Chiu V, Villalobos M. Biotic and Abiotic Products of Mn(II) Oxidation by Spores of the Marine *Bacillus* sp. strain SG-1. *Am Miner.* 2005; 90:143–154.
- Bento I, Peixoto C, Zaitsev N, Lindley PF. Ceruloplasmin revisited: structural and functional roles of various metal cation-binding sites. *Acta Cryst.* 2007; D63:240–248.
- Bonde, GJ. *Bacillus* from marine habitats: allocation to phenotypes established by numerical techniques. In: Berkeley, RCW., Goodfellow, M., editors. *The aerobic endospore-forming bacteria: classification and identification*. Academic Press; New York: 1981. p. 181–215.
- Boogerd FC, de Vrind JPM. Manganese oxidation by *Leptothrix discophora*. *J Bacteriol.* 1987; 169:489–494. [PubMed: 3804969]
- Brewer PG, Spencer DW. Colorimetric determination of manganese in anoxic waters. *Limnol Oceanogr.* 1971; 16:107–112.
- Brezonik, PL. *Chemical Kinetics and Process Dynamics in Aquatic Systems*. Lewis Publishers; Tokyo: 1993.
- Brouwers G-J, de Vrind JPM, Corstjens PLAM, Cornelis P, Baysse C, de Vrind-de Jong EW. CumA, a gene encoding a multicopper oxidase, is involved in Mn²⁺-oxidization in *Pseudomonas putida* GB-1. *Appl Environ Microbiol.* 1999; 65:1762–1768. [PubMed: 10103278]
- Brouwers G-J, Vijgenboom E, Corstjens PLAM, de Vrind JPM, de Vrind-de Jong EW. Bacterial Mn(II) oxidizing systems and multicopper oxidases: An overview of mechanisms and functions. *Geomicrobiol J.* 2000; 17:1–24.
- Burns, RG., Burns, VM. Manganese oxides. In: Burns, RG., editor. *Marine Minerals*. Mineralogical Society of America; 1979. p. 1–46.
- Corstjens PLAM, de Vrind JPM, Goosen T, de Vrind-de Jong EW. Identification and molecular analysis of the *Leptothrix discophora* SS-1 *mofA* gene, a gene putatively encoding a manganese oxidizing protein with copper domains. *Geomicrobiol J.* 1997; 14:91–108.
- Dick GJ, Lee YE, Tebo BM. Manganese(II)-oxidizing *Bacillus* spores in Guaymas Basin hydrothermal sediments and plumes. *Appl Environ Microbiol.* 2006; 72:3184–3190. [PubMed: 16672456]
- Dick GJ, Torpey JW, Beveridge TJ, Tebo BM. Direct identification of a bacterial manganese(II) oxidase, the multicopper oxidase MnxG, from spores of several different marine *Bacillus* species. *Appl Environ Microbiol.* 2008; 74:1527–1534. [PubMed: 18165363]
- Dick GJ, Clement DG, Webb SM, Fodrie FJ, Bargar JR, Tebo BM. Enzymatic microbial Mn(II) oxidation and Mn biooxide production in the Guaymas Basin deep-sea hydrothermal plume. *Geochim Cosmochim Acta.* 2009; 73:6517–6530.
- Dick GJ, Tebo BM. Microbial diversity and biogeochemistry of the Guaymas Basin hydrothermal plume. *Environ Microbiol.* 2010; 12:1334–1347. [PubMed: 20192971]
- Diem D, Stumm W. Is dissolved Mn²⁺ being oxidized by O₂ in the absence of Mn-bacteria or surface catalysts? *Geochim Cosmochim Acta.* 1984; 48:1571–1573.
- Elimelech, M., Jia, X., Gregory, J., Williams, R. *Particle Deposition & Aggregation: Measurement, Modelling and Simulation*. Butterworth-Heinemann Ltd; Stoneham: 1995.

- Francis CA, Tebo BM. Marine *Bacillus* spores as catalysts for oxidative precipitation and sorption of metals. *J Mol Microbiol Biotechnol*. 1999; 1:71–78. [PubMed: 10941787]
- Francis CA, Tebo BM. cumA multicopper oxidase genes from diverse Mn(II)-oxidizing and non-Mn(II)-oxidizing *Pseudomonas* strains. *Appl Environ Microbiol*. 2001; 67:4272–4278. [PubMed: 11526033]
- Francis CA, Tebo BM. Enzymatic Manganese(II) Oxidation by Metabolically Dormant Spores of Diverse *Bacillus* Species. *J Mol Biotechnol*. 2002; 1:71–78.
- Francis CA, Casciotti KL, Tebo BM. Localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, MnxG, to the exosporium of the marine *Bacillus* sp. strain SG-1. *Arch Microbiol*. 2002; 178:450–456. [PubMed: 12420165]
- Gammons CH, Sewars TM. Stability of manganese (II) chloride complexes from 25 to 300°C. *Geochim Cosmochim Acta*. 1996; 60:4295–4311.
- Geszvain K, Tebo BM. Identification of a Two-Component Regulatory Pathway Essential for Mn(II) Oxidation in *Pseudomonas putida* GB-1. *Appl Environ Microbiol*. 2100; 76:1224–1231.
- Good N, Winget D, Winter W, Connolly TN, Izawa S, Singh RMM. Hydrogen ion buffers for biological research. *Biochemistry*. 1966; 5:467–477. [PubMed: 5942950]
- Hastings D, Emerson S. Oxidation of manganese by spores of a marine *Bacillus*: kinetic and thermodynamic considerations. *Geochim Cosmochim Acta*. 1986; 50:1819–1824.
- He LM, Tebo BM. Surface characterization of and Cu(II) adsorption by spores of a marine *Bacillus*. *Appl Environ Microbiol*. 1998; 64:1123–1129. [PubMed: 16349517]
- Huang, PM. Kinetics of redox reactions on manganese oxides and its impact on environmental quality. In: Sparks, DL., Suarez, DL., editors. *Rates of Soil Chemical Processes*. Soil Science Society of America, Inc; Madison: 1991. p. 191-230.
- Johnson HA, Tebo BM. In vitro studies indicate a quinone is involved in bacterial. *Arch Microbiol*. 2008; 189:59–69. [PubMed: 17673976]
- Kostka JE, Luther GW III, Nealson KH. Chemical and biological reduction of Mn (III)-pyrophosphate complexes: Potential importance of dissolved Mn (III) as an environmental oxidant. *Geochim Cosmochim Acta*. 1995; 59:885–894.
- Kwon KD, Refson K, Sposito G. On the role of Mn(IV) vacancies in the photoreductive dissolution of hexagonal birnessite. *Geochim Cosmochim Acta*. 2009; 73:4142–4150.
- Langmuir, D. *Aqueous environmental geochemistry*. Prentice Hall; Tokyo: 1997.
- Lambeth DO, Muhonen WW, Jacoby GH, Ray PD. Factors affecting the manganese and iron activation of the phosphoenolpyruvate carboxykinase isozymes from rabbit. *Biochim Biophys Acta*. 1992; 1156:85–89. [PubMed: 1472544]
- Learman DR, Voelker BM, Vazquez-Rodriguez AI, Hansel CM. Formation of manganese oxides by bacterially generated superoxide. *Nature Geosci*. 2011a; 4:95–95.
- Learman DR, Wankel SD, Webb SM, Martinez N, Madden AS, Hansel CM. Coupled biotic-abiotic Mn(II) oxidation pathway mediates the formation and structural evolution of biogenic Mn oxides. *Geochimica et Cosmochimica Acta*. 2011b; 75:6048–6063.
- Nealson KH, Ford J. Surface enhancement of bacterial manganese oxidation: Implications for aquatic environments. *Geomicrobiol J*. 1980; 2:21–37.
- Nealson KH, Tebo BM, Rosson RA. Occurrence and mechanisms of microbial oxidation of manganese. *Adv Appl Microbiol*. 1988; 33:279–318.
- Mandernack KW, Post J, Tebo BM. Manganese mineral formation by bacterial spores of the marine *Bacillus* strain SG-1: Evidence for the direct oxidation of Mn(II) to Mn(IV). *Geochim Cosmochim Acta*. 1995a; 59:4393–4408.
- Mandernack KW, Forgel ML, Tebo BM, Usui A. Oxygen isotope analyses of chemically and microbially produced manganese oxides and manganates. *Geochim Cosmochim Acta*. 1995b; 59:4409–4425.
- Mann S, Sparks NHC, Scott GHE, deVrind-deJong EW. Oxidation of manganese and formation of Mn₃O₄ (hausmannite) by spore coats of a marine *Bacillus* sp. *Appl Environ Microbiol*. 1988; 54:2140–2143. [PubMed: 16347723]

- Millero FJ. The effect of ionic interactions on the oxidation of metals in natural waters. *Geochim Cosmochim Acta*. 1985; 49:547–553.
- Millero, FJ. Marine Science Series. 2. CRC Pr; Tokyo: 1996. Chemical Oceanography.
- Murray JW, Dillard JG, Giovanoli R, Moers H, Stumm W. Oxidation of Mn(II): Initial mineralogy, oxidation state and aging. *Geochim Cosmochim Acta*. 1985; 49:463–470.
- Musci G, di Patti MCB, Petruzzelli R, Giartosio A, Calabrese L. Divalent cation binding to ceruloplasmin. *BioMetals*. 1996; 9:66–72. [PubMed: 8574094]
- Okazaki M, Sugita T, Shimizu M, Ohode Y, Iwamoto K, de Vrind-de Jon EW, de Vrind JPM, Corstjens PLAM. Partial purification and characterization of manganese-oxidizing factors of *Pseudomonas fluorescens* GB-1. *Appl Environ Microbiol*. 1997; 63:4793–4799. [PubMed: 9406397]
- Parker DL, Spiro G, Tebo BM. Manganese(III) binding to a pyoverdine siderophore produced by a manganese(II)-oxidizing bacterium. *Geochim Cosmochim Acta*. 2004; 68:4809–4820.
- Parker DL, Morita T, Mozafarzadeh ML, Verity R, McCarthy JK, Tebo BM. Interrelationships of MnO₂ precipitation, siderophore-Mn(III) complex formation, siderophore degradation, and iron limitation in Mn(II)-oxidizing bacterial cultures. *Geochimica et Cosmochimica Acta*. 2007; 71:5672–5683.
- Pecher K, McCubbery D, Kneedler E, Rothe J, Bargar J, Meigs G, Cox L, Nealson K, Tonner B. Quantitative charge state analysis of manganese biominerals in aqueous suspension using scanning transmission X-ray microscopy (STXM). *Geochim Cosmochim Acta*. 2003; 67:1089–98.
- Post JE. Manganese oxide minerals: crystal structures and economic and environmental significance. *Proc Natl Acad Sci USA*. 1999; 96:3447–3454. [PubMed: 10097056]
- Ridge JP, Lin M, Larsen EI, Fegan M, McEwan AG, Sly LI. A multicopper oxidase is essential for manganese oxidation and laccase-like activity in *Pedomicrobium* sp. *ACM* 3067. *Environ Microbiol*. 2007; 9:944–53. [PubMed: 17359266]
- Rosson RA, Nealson KH. Manganese binding and oxidation by spores of a marine *Bacillus*. *J Bacteriol*. 1982; 151:1027–1034. [PubMed: 6212577]
- Solomon EI, Sundaram UM, Machonkin TE. Multicopper oxidases and oxygenases. *Chem Rev*. 1996; 96:2563–2605. [PubMed: 11848837]
- Spiro TG, Bargar JR, Sposito G, Tebo BM. Bacteriogenic manganese oxides. *Acc Chem Res*. 2010; 42:2–9.
- Stumm, W., Morgan, JJ. Aquatic Chemistry. John Wiley and Sons; New York: 1996.
- Sunda WD, Kieber DJ. Oxidation of humic substances by manganese oxides yields low molecular-weight organic substrates. *Nature*. 1994; 367:62–64.
- Tebo BM, Emerson S. Microbial manganese (II) oxidation in the marine environment: a quantitative study. *Biogeochemistry*. 1986; 2:149–161.
- Tebo, BM., Ghiorse, WC., Van Waasbergen, LG., Siering, PL., Caspi, R. Bacterially Mediated Mineral Formation: Insights into Manganese(II) Oxidation from Molecular Genetic and Biochemical Studies. In: Banfield, JF., Nealson, KH., editors. *Geomicrobiology: Interactions Between Microbes and Minerals*. Vol. 35. Mineralogical Society of America; Washington, D.C: 1997. p. 226-266. Rev. Miner
- Tebo, BM., He, LM. Microbially mediated oxidative precipitation reactions. In: Sparks, DL., Grundl, TJ., editors. *Mineral-Water Interfacial Reactions Kinetics and Mechanisms*. American Chemical Society; Washington, D.C: 1999. p. 393-414. ACS Symposium Series 715
- Tebo BM, Bargar JR, Clement B, Dick G, Murray KJ, Parker D, Verity R, Webb S. Manganese biooxides: properties and mechanisms of formation. *Ann Rev Earth and Planet Sci*. 2004; 32:287–328.
- Tebo BM, Johnson HA, McCarthy JK, Templeton AS. Geomicrobiology of manganese(II) oxidation. *Trends Microbiol*. 2005; 13:421–428. [PubMed: 16054815]
- van Waasbergen LG, Hildebrand M, Tebo BM. Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine *Bacillus* sp. strain SG- 1. *J Bacteriol*. 1996; 178:3517–3530. [PubMed: 8655549]
- Villalobos M, Bargar J, Toner B, Sposito G. Characterization of the manganese oxide produced by *Pseudomonas putida* strain MnB1. *Geochim Cosmochim Acta*. 2003; 67:2649–2662.

- Webb SM, Dick GJ, Bargar JR, Tebo BM. Evidence for the presence of Mn(III) intermediates in the bacterial oxidation of Mn(II). *Proc Natl Acad Sci USA*. 2005a; 102:5558–5563. [PubMed: 15800042]
- Webb SM, Tebo BM, Bargar JR. Structural influences of sodium and calcium ions on biogenic manganese oxides produced by the marine *Bacillus* sp., strain SG-1. *Geomicrobiol J*. 2005b; 22:181–193.
- Yocum CF. The calcium and chloride requirements of the O₂ evolving complex. *Coord Chem Rev*. 2008; 252:296–305.
- Zhang J, Lion LW, Nealson YM, Shuler ML, Ghiorse WC. Kinetics of Mn(II) oxidation by *Leptothrix discophora* SS1. *Geochim Cosmochim Acta*. 2002; 65:773–781.

Fig. 1.

The effect of pH on Mn(II) oxidation. Solutions of ASW with 2 mM HEPES buffer and 8.2×10^9 spores/L were adjusted to the indicated pH and incubated at 24°C under CO₂-scrubbed-air bubbling.

Fig. 2.

Variation in the rate of Mn(II) oxidation under different pH and Ca^{2+} values. Solutions of ASW with the indicated Ca^{2+} concentrations and 2 mM HEPES were incubated as follows: Ca^{2+} -free solutions were treated as in Figure 1; ASW with 2 mM Ca^{2+} was maintained at 24°C with bubbling of CO_2 -scrubbed-air, 8.2×10^9 spores/L, and initial $[\text{Mn}] = \text{ca. } 47 \mu\text{M}$; ASW with 10 mM Ca^{2+} was maintained at 24°C with bubbling of CO_2 -scrubbed-air, 12.0×10^9 spores/L, and initial $[\text{Mn}] = \text{ca. } 52 \mu\text{M}$. Error bars represent one standard error (SE) of the rate measurements.

Fig. 3.

Mn(II) oxidation in solutions containing differing concentrations of Ca²⁺. Solutions of ASW containing the indicated concentrations of Ca²⁺ were adjusted to a total ionic strength with Mg²⁺. ASW also contained 2 mM HEPES adjusted to pH 7.8 and incubated with CO₂-scrubbed-air and 6.0×10^9 spores/L at 24°C.

Fig. 4.

The effect of Ca²⁺ content on Mn(II) oxidation rates from three experiments. The presented working curves are from the tentative estimation of the half binding site of the spores with $K = 0.1 \text{ (M}^{-1}\text{)}$. The three closed diamonds are data from the experiment shown in Table 1; The seven closed square are data from the experiment in Figure 3; The seven closed circles are data from a third experiment. The figures of 0.054 μM/h, 0.092 μM/h and 0.113 μM/h are the calculated Mn(II) oxidation rates of SG-1 incubated without Ca²⁺ for each experimental set. The Mn(II) oxidation rate of the Ca-bound enzyme (V*) is higher than that of the Ca-free enzyme binding by two and half orders of magnitude. Error bars represent one standard error (SE) of the rate measurements.

Fig. 5.

Changes in the oxidation rate of Mn(II) under varying concentrations of NaCl. Solutions of ASW containing the indicated concentrations of NaCl, 2 mM HEPES, and 10 mM CaCl_2 were maintained at pH 7.6–7.8 and incubated under CO_2 -scrubbed-air with 6.3×10^9 spores/L at 22°C.

Fig. 6.

Change in the oxidation rate of Mn(II) as a function of ionic strength (I) from the data of Figure 5 and an identical experiment using a KNO_3 containing solution. The closed circles represent Mn(II) removal in a NaCl containing solution (Fig. 5) and the closed squares are data obtained using a KNO_3 containing solution. The logarithm of the rate was a linear function of the abscissa ($I / (I + 1)$) with slopes of -3 ($I > 0.2$) and -0.31 ($I < 0.2$) in NaCl solution and -1.5 ($I > 0.2$) and -0.10 ($I < 0.2$) in KNO_3 solution. Changes in the slope around 200 mM of NaCl or KNO_3 with 10 mM CaCl_2 cannot be explained by “activated complex theory” and it may indicate alterations in the mechanism of the rate-limiting reaction around 0.2 M ionic strength. Error bars represent one standard error (SE) of the rate measurements.

Table 1

The effect of mixing mode on Mn(II) oxidation rate of 8.2×10^9 spores/L at 23–24°C, with constant Ca^{2+} (10 mM), Mg^{2+} (53 mM), Na^+ (474 mM), Cl^- (540 mM) and SO_4^{2-} (30 mM) concentrations. The initial $[\text{Mn}] = \text{ca. } 30 \mu\text{M}$.

<u>Mixing mode</u>	<u>Stirring</u>	<u>Shaking (150 rpm)</u>	<u>Shaking (70 rpm)</u>	<u>Bubbling</u>	<u>Static</u>
pH	7.5	7.5	7.5	7.3	7.5
Ox. rate ($\mu\text{M/h}$)	0.474	0.478	0.542	0.526	0.578
SE ($\mu\text{M/h}$)	---	---	0.010	0.001	---

Ox. rate: Mn(II) oxidation rate; SE: standard error of the rate.

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The effect of cation concentration on Mn(II) oxidation at 8.2×10^9 spores/L, 24°C, with shaking (150 rpm). The initial [Mn] = ca. 34 μ M.

ASW = artificial seawater; NSW: filtered natural seawater with a little addition of di.HCl for pH adjustment;

IS: ionic strength; Ox. rate: Mn(II) oxidation rate; SE: standard error of the rate.

Table 3

The effect of organic matter and Sr concentration on Mn(II) oxidation rate of 7.0×10^9 spores/L, at pH 7.9–8.0 and 23–24°C, with constant Ca^{2+} (10 mM), Mg^{2+} (53 mM) and SO_4^{2-} (28 mM) concentrations. The initial $[\text{Mn}] = 33\text{--}34 \mu\text{M}$.

Static mode	Unfiltered NSW#	Filtered NSW#	ASW	ASW	ASW	ASW
Sr^{2+} (mM)	0.09 *	0.09 *	0	0.45	0.90	1.35
NO_3^- (mM)	0 *	0 *	0	0.45	0.90	1.35
Na^+ (mM)	468 *	468 *	487	487	487	487
K^+ (mM)	10 *	10 *	0	0	0	0
Cl^- (mM)	546 *	546 *	558	558	558	558
Ox. rate ($\mu\text{M}/\text{h}$)	0.194	0.240	0.323	0.335	0.332	0.341
SE ($\mu\text{M}/\text{h}$)	0.003	0.004	0.006	0.002	0.003	0.007

Unfiltered and filtered NSW: natural seawater before and after through a 0.22 μM Millipore MF membrane.

* Literature values from Millero (1996);

Ox. rate: Mn(II) oxidation rate; SE: standard error of the rate.

Table 4

The effect of light and CO_3^{2-} on Mn(II) oxidation rate in ASW of 15×10^9 spores/L at 23–24°C, with keeping Ca^{2+} , Mg^{2+} and K^+ = 0 mM; Na^+ = 600–605 mM; Cl^- = 540 mM; and SO_4^{2-} = 30 mM. The initial $[\text{Mn}]$ = ca. 30 μM .

Solution type	ASW	ASW	ASW	ASW	ASW	NSW*
Exposure mode	in dark [#]	in light ^{\$}	in light ^{\$}	in light ^{\$}	in light ^{\$}	in light ^{\$}
CO_3^{2-} (mM)	0.0	0.0	1.0	2.5	---	---
pH	7.7	7.7	7.7	7.8	8.0	8.0
Ox. rate ($\mu\text{M/h}$)	0.246	0.235	0.245	0.257	1.000	1.000
SE ($\mu\text{M/h}$)	0.008	0.004	0.007	0.016	---	---

NSW*: filtered natural seawater with a little addition of dil HCl of 15×10^9 spores/L at 23–24°C.
 in dark[#]: in a flask fully covered with aluminum foil next to the flasks of this experimental run on lab-bench.
 in light^{\$}: in a unshielded glass flask next to the covered flask during 48 hours after spore-addition.
 Ox. rate: Mn(II) oxidation rate; SE: standard error of the rate.

Table 5

The effect of K^+ or Na^+ contents on Mn(II) oxidation rate in ASW of 4.9×10^9 spores/L at 23–24°C and pH = 7.5, with constant Ca^{2+} (10 mM), K^+ + Na^+ (1000 mM), Cl^- (1020 mM), and SO_4^{2-} (0 mM) concentrations. The initial $[Mn] = \text{ca. } 47 \mu\text{M}$.

Na^+ (mM)	0	5	20	50	100	200	500	1000
K^+ (mM)	1000	995	980	950	900	800	500	0
Ox. rate ($\mu\text{M/h}$)	0.391	0.337	0.381	0.366	0.373	0.368	0.373	0.364
SE ($\mu\text{M/h}$)	0.027	0.012	0.033	0.041	0.021	0.024	0.021	0.015

Ox. rate: Mn(II) oxidation rate; SE: standard error of the rate.

Table 6

The effect of Cl^- or NO_3^- contents on Mn(II) oxidation rate in ASW of 7.9×10^9 spores/L at 23–24°C and pH = 7.5, $\text{Ca}^{2+} = 10$ mM, $\text{Na}^+ = 980$ mM, $\text{Cl}^- + \text{NO}_3^- = 1000$ mM, $\text{K}^+ = 0$ mM and $\text{SO}_4^{2-} = 0$ mM. The initial $[\text{Mn}] = \text{ca. } 49 \mu\text{M}$.

Cl^- (mM)	1	5	20	100	200	500	1000
NO_3^- (mM)	999	995	980	900	800	500	0
Ox. rate ($\mu\text{M/h}$)	0.937	0.893	0.919	0.826	0.867	0.836	0.882
SE ($\mu\text{M/h}$)	0.097	0.097	0.124	0.138	0.079	0.058	0.084

Ox. rate: Mn(II) oxidation rate; SE: standard error of the rate.