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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Biological Mn(II) oxidation in freshwater and marine systems: new perspectives on  
reactants, mechanisms and microbial catalysts of Mn cycling in the environment

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Brian Gregory Clement

Committee in charge:

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Kit Pogliano

2006

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Chair

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2006

# **DEDICATION**

For Mom

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My subsequent success at SIO reflects the support and guidance I received from my advisor, Brad Tebo. The great diversity of field and laboratory research I experienced as a student at SIO was due to Brad's expertise and funding acumen, his well-deserved reputation as an excellent collaborator, and the strong laboratory roster he maintained. He provided opportunities to study geomicrobiology in a variety of settings which helped me develop a multi-national network of collaborators and friends. Brad took the time to teach me the analytical, experimental and presentation skills necessary to accomplish my scientific goals and at the same time gave me room and encouragement to work through difficult patches in my own way.

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BM Tebo, JR Bargar, **BG Clement**, GJ Dick, KJ Murray, D Parker, R Verity and SM Webb. 2004. Biogenic manganese oxides: Properties and mechanisms of formation. *Annu. Rev. Earth Planet. Sci.* 32:287-328

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## AWARDS

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## PRESENTATIONS

BG Clement, GJ Dick, AE Peetz, YE Lee and BM Tebo. 2006. Cultivation and Cultivation-independent Identification of a Dominant, Mn(II)-oxidizing *Pseudoalteromonas* strain from the Black Sea Suboxic Zone. Contributed talk. American Society of Limnology and Oceanography Ocean Sciences Meeting, Honolulu.

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BG Clement and BM Tebo. 2005. Microbes don't model: A 'tale' from the Black Sea. Contributed Talk. Astrobiology Graduate Conference, Scripps Institution of Oceanography, La Jolla.

BG Clement and BM Tebo. 2004. Detection limits on our imagination: Oxygen is the oxidant of Mn(II) in the Black Sea Suboxic Layer. Contributed Talk. Southern California Geobiology Symposium, California Institute of Technology, Pasadena

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- BG Clement and BM Tebo. 2001. Bioremediation of Acid Mine Drainage: Mn(II) Oxidation at Low Oxygen Concentrations by Diverse Bacteria from a Freshwater Stream. Contributed Talk. San Diego Microbiology Group, Annual Symposium, La Jolla, CA
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- MD Braid, LM Nicholas, BG Clement, JB Kitner, MN Brolaski, RJ Venugopal and CL Kitts. 1999. Testing the UltraClean® soil DNA purification kit on a diverse

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BG Clement and CL Kitts. 1998. PCR primer selection for microbial community analysis by 16s rDNA terminal restriction fragment patterns. Contributed Poster. International Society for Microbial Ecology, 8th International Symposium, Amsterdam, The Netherlands LE Kehl,

BG Clement and CL Kitts. 1998. Reproducibility and discriminatory power of 16S rDNA terminal restriction fragment pattern analyses of bacterial community diversity. Contributed Poster. International Society for Microbial Ecology, 8th International Symposium, Amsterdam, The Netherlands

BG Clement and CL Kitts. 1997. Genetic diversity of microbial populations in *Odocoileus hemionus* feces. Contributed Poster. American Society for Microbiology, General Meeting, Miami, FL



## **ABSTRACT OF THE DISSERTATION**

Biological Mn(II) oxidation in freshwater and marine systems: new perspectives on reactants, mechanisms and microbial catalysts of Mn cycling in the environment

by

Brian Gregory Clement

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2006

Bradley M. Tebo, Chair

This research characterized the Mn(II) oxidizing bacterial populations in a freshwater creek and a stratified marine basin, determined their response to O<sub>2</sub> concentrations and examined mechanisms that produce oxidized Mn in situ. As introduced in Chapter I, biogenic Mn oxides are reactive, ubiquitous minerals that adsorb and/or oxidize metals, inorganics and organics, thereby influencing the surrounding chemistry. Chapter II describes a diverse group of Mn(II) oxidizing bacteria from Pinal Creek, an Arizona stream impacted by mine pollution that is naturally attenuated by biogenic Mn oxides. In mixed Pinal Creek cultures, biogenic Mn oxides were produced preferentially at low O<sub>2</sub> concentration in close association with Mn oxide reducing organisms. Chapter III details the low O<sub>2</sub> concentrations required for biologic Mn(II) oxidation in the Black Sea, a stratified marine basin where Mn oxides play a large role in chemical interactions. Mn oxide production was observed at O<sub>2</sub> concentrations below the typical detection limits of 3-5 μM, at rates

more rapid than predicted by biogeochemical models. Chapter IV reports cultivation-independent confirmation that Mn(II)-oxidizing *Pseudoalteromonas* spp. isolated from the Black Sea can be abundant within the Mn oxide rich layer. These organisms both oxidize Mn(II) and produce the Mn(III)-binding siderophore desferrioxamine E. Chapter V illustrates that current knowledge about dissolved, oxidized Mn in natural systems is incomplete and describes an assay for Mn(III)-complex production that, when applied in the Black Sea indicated that direct and indirect mechanisms for oxidizing Mn(II) may co-occur. Finally, Chapter VI details open questions in and novel opportunities for bacterial Mn(II) oxidation research.

# CHAPTER I

## **Introduction: Biological Mn(II) oxidation in the environment**

Manganese is an interesting transition metal from biological and geochemical viewpoints due to its many chemical interactions in natural systems and the primary role of biological catalysis in altering its oxidation state and form. The Earth's second-most abundant transition metal behind Fe, Mn occurs in three different oxidation states in aerated environments at near-neutral pH (Post, 1999). The reduced form, Mn(II), occurs in the environment as a soluble cation, while the oxidized forms, Mn(III/IV), occur as mineral oxides and, potentially, as ligand-stabilized solutes (Kostka et al., 1995; Stumm and Morgan, 1996). The redox reactions that transform Mn are catalyzed largely by microorganisms through both direct and indirect action (Burdige, 1993; Burdige and Nealson, 1986; Emerson et al., 1982; Tebo et al., 2004). Biological oxidation of soluble Mn(II) produces an insoluble Mn(IV) oxide mineral phase analogous to a mineral sponge that greatly influences the surrounding environment by adsorbing and/or oxidizing a wide range of metals, inorganics and organics (Goldberg, 1954; Tebo et al., 2004). Thus, microbial Mn(II) oxidation is a determinant of elemental cycling and pollutant transport in environments where large quantities of Mn exist. This introduction provides a brief background of the geochemistry, occurrence, and microbiology of bacterial Mn oxide formation and describes how this dissertation contributes to the general knowledge about microbial Mn(II) oxidation in the environment. Readers looking for a comprehensive review of biogenic Mn oxides will find the information herein helpful but should also read the recent review by Tebo and coworkers (2004).

Mn oxides in the environment are largely thought to result from microbial Mn(II) oxidation (Tebo et al., 2004). While Mn oxides are thermodynamically favored in aerated systems at near-neutral pH, the chemical oxidation of Mn(II) by O<sub>2</sub> proceeds slowly (Morgan, 2005; Stumm and Morgan, 1996). Microorganisms have been shown to increase the rate of Mn(II) oxidation by orders of magnitude over chemical rates alone at hydrothermal vents, in freshwater lakes and streams, in stratified fjords and in stratified marine basins (Aguilar and Nealson, 1998; Chapnick et al., 1982; Cowen et al., 1990; Emerson et al., 1982; Harvey and Fuller, 1998; Mandernack and Tebo, 1993; Marble et al., 1999; Tebo, 1991; Tebo and Emerson, 1986). In fact, Mn(II)-oxidizing microorganisms appear to be primary catalysts of Mn(II) oxidation wherever Mn oxides are prevalent.

Cultivated Mn(II)-oxidizing bacteria (MnOB) are found in phyla Firmicutes, Proteobacteria and Actinobacteria, but not in phylogenetically distinct groups, even within a given class or genus (Ehrlich, 1999; Nealson et al., 1988; Tebo et al., 2004). The described MnOB are all heterotrophs and grow aerobically. Biogenic Mn oxides are generally produced by MnOB as vegetative cells in the late exponential phase of growth or by otherwise metabolically dormant spores of the genus *Bacillus* (Tebo et al., 2004). While the reaction is thermodynamically favorable and, thus, potentially energy-generating, bacteria have not yet been shown to conserve energy for growth from Mn(II) oxidation (Tebo et al., 1997). A number of other hypotheses have been put forward regarding the functional benefits of Mn(II) oxidation, including protection from viruses, predators, toxins and photodegradation, but all remain difficult to directly test (for an extended discussion of this, see Tebo et al., 1997). Instead, recent

research into Mn(II) oxidizing bacteria has focused on the enzymatic mechanisms as a means of determining how, and thus possibly why, bacteria make Mn oxides.

To date, genetic efforts have identified similar multicopper oxidase (MCO) genes required for Mn(II) oxidation in all of the isolates to which these methods have been applied (Brouwers et al., 1999; Corstjens et al., 1997; Francis and Tebo, 2001; Francis and Tebo, 2002; vanWaasbergen et al., 1996). Experiments with wild-type *Bacillus* sp. SG-1 and mutants incapable of Mn(II) oxidation indicated that the *mnxG* gene product catalyzes Mn oxide formation via a Mn(III) intermediate (Webb et al., 2005a). This is consistent with the single-electron oxidation mechanism of well-characterized MCOs but indicates that more than one MCO or a modified MCO with multiple active sites may be required for Mn oxide production (Brouwers et al., 2000). Indeed, the *mnx* operon in *Bacillus* spp. contains additional Cu binding sites, one of which is in *mnxG* (vanWaasbergen et al., 1996; G. Dick, personal communication). This may indicate an additional catalytic site that could explain the successive electron removals by a single MCO. In contrast to *Bacillus* spp., the *Pseudomonas putida* genome appears to contain genes for two MCOs of interest in Mn(II) oxidation (J. McCarthy, pers. comm.). The first, *cumA*, is well-characterized as a required gene for Mn(II) oxidation and contains four Cu-binding domains, similar to most MCOs (Brouwers et al., 1999; Francis and Tebo, 2001). The second, unnamed gene was revealed by genome sequencing of a related strain (KT2440) and is more similar to *mnxG* from *Bacillus* spp. than *cumA*, except that it contains only four Cu-binding domains (J. McCarthy, personal communication). Thus, it seems possible that *Pseudomonas putida* also catalyzes successive single-electron oxidations of Mn

species, although no experimental evidence is yet available. These observations are important in identifying or implicating an intermediate step in which Mn(III) is produced and in laying the groundwork for determining where the electrons from Mn end up and which intermediate steps (such as an electron transport chain) they pass through.

While the intermediate steps of Mn(II) oxidation are just beginning to be revealed, the end products have been well-characterized by fine-scale x-ray methods. Biogenic Mn oxides appear to be exclusively poorly crystalline, layered minerals composed of Mn(IV) oxides, or  $\delta\text{MnO}_2$  (Bargar et al., 2005; Villalobos et al., 2003; Webb et al., 2005b). These initial products undergo alteration to other Mn minerals depending upon the Mn(II) content, ionic strength, pH and Eh of the surrounding solution (Bargar et al., 2005). Thus, biogenic oxides in the environment can incorporate Mn(III) and/or Mn(II) and exhibit mean oxidation states  $< 4$ . This prevents simple identification of biogenic Mn oxides by mineralogy alone. Instead, biological formation of Mn oxides in the environment is usually assessed by dissolved Mn removal rate measurements with and without biological poisons.

Despite careful early work to demonstrate their efficacy, poisons remain somewhat controversial as they may be suspected of altering solution chemistry in activity assays. Rosson and coworkers (1984) demonstrated that sodium azide had relatively little effect on solution chemistry in samples from a fjord, but recent similar work in particle rich freshwater gave contrasting results (Rosson et al., 1984; Shiller and Stephens, 2005). Regardless of the efficacy of poisons in specific systems, the biological nature of Mn(II) oxidation is apparent simply from the reaction rates

(Morgan, 2005). Chemical rates under environmental conditions are orders of magnitude slower than Mn(II) oxidation rates observed in a variety of environments (summarized in Tebo, 1991). Such rapid rates alone imply catalysis by some non-chemical entity and, when combined with little or no Mn(II) oxidation in poisoned incubations and the existence of Mn(II) oxidizing bacteria, illustrate the importance of biogenic Mn oxides in the environment.

Biogenic Mn oxides are found widely, occurring in both soils and aquatic systems, and are generally prevalent near interfaces between oxidizing and reducing zones—where Mn(II) and potential oxidants coincide. Within soil or sediments, these interface zones may be exceedingly thin—millimeters or less—because O<sub>2</sub> penetration is limited to molecular diffusion by the soil/sediment matrix. At hydrothermal vents, the opposite situation occurs; rapid, turbulent mixing between buoyant, reduced hydrothermal fluids containing Mn(II) and O<sub>2</sub>-laden seawater promotes Mn oxide formation in large, transient plumes. Within stratified waters, moderately slow chemical transport by eddy diffusion or advection can spread the redox interface over centimeters to meters. In such stratified sites, Mn oxides are prevalent within defined layers, often where little O<sub>2</sub> is apparent.

While the presence of Mn oxides is unquestioned at most sites with measurable quantities of particulate Mn, the majority of Mn measurements in natural systems are operational, not determinative, with respect to oxidation state. The most widely used operational separation of Mn species, and that used throughout this dissertation, is filtration of natural waters through a 0.2 or 0.4 μm pore-size membrane (cf. (Konovalov et al., 2003; Lewis and Landing, 1991; Tebo, 1991)). The particulate Mn



collected on the membrane is often described as Mn oxide or  $MnO_x$ , where  $1 < x < 2$ , and dissolved Mn in the filtrate is treated as Mn(II). Experimental evidence indicates that particulate Mn is largely oxidized Mn (Anschutz et al., 2005; Mandernack et al., 1995; Murray et al., 1984; Tipping et al., 1985). In contrast, little work has been done on determining the oxidation state of dissolved Mn. This is primarily due to the instability of aqueous Mn(III) and insolubility of Mn(IV), leading to the simplified view that all dissolved Mn is Mn(II) and all Mn(III/IV) is found as insoluble oxides.

Trivalent Mn disproportionates to Mn(II) and Mn(IV) in aqueous solution unless stabilized by a ligand (Stumm and Morgan, 1996). A number of studies have implicated or predicted the occurrence of ligand-bound Mn(III) in natural settings, but measurements remain difficult (Kostka et al., 1995; Luther et al., 1994; Stone, 1987). Using voltammetry in Chesapeake Bay, Luther and coworkers found evidence for a ligand-bound, dissolved Mn phase that they speculated could be comprised of Mn(III) bound to dissolved organic substances (Luther et al., 1994). More recently, the interactions of siderophores, a class of biogenic metal ligands, and Mn(III) have been explored in relation to Mn(II) oxidation. Parker and coworkers discovered that the bacterial siderophore pyoverdine bound and stabilized Mn(III) in solution with stability constants in excess of those for Fe(III) (Parker et al., 2004). While intriguing with regards to Mn oxide production by cells in vitro and Mn-siderophore interactions, pyoverdine detection relied on spectrophotometry and fluorometry methods that are of limited use with complex, dilute natural samples.

More applicable to environmental samples is the electrochemically-active complex of Mn(III) and desferrioxamine B (DFOB). The Mn(III)-DFOB complex is

well-known as a superoxide dismutase analog, but more recent work has focused on the abiotic air oxidation of Mn(II) and the dissolution of manganite, a Mn(III) bearing mineral, promoted by DFOB. Recent work (Trouwborst, 2006) undertaken in the Black Sea made use of DFOB to trap naturally occurring Mn(III). The resulting Mn(III)-DFOB complex was then detected by voltammetry, indicating that significant fractions of dissolved Mn in the Black Sea suboxic zone were Mn(III), not reduced Mn(II). Given that dissolved Mn(III) has a redox potential similar to O<sub>2</sub>, the occurrence of this chemical species may promote novel, unrecognized redox reactions where previously only unreactive Mn(II) was known to exist (Kostka et al., 1995).

In contrast to the unknowns surrounding dissolved Mn, Mn oxides are known greatly affect local chemical conditions. Biogenic Mn oxides are typically poorly crystalline and have high-surface area to mass ratios—features which facilitate the availability of surface reaction sites (e.g. Villalobos et al., 2003). Such features, combined with negative surface charge at circumneutral pH and strong oxidizing potential make them potent oxidizing and adsorbing agents (Tebo et al., 2004). Two examples that illustrate this are pollutant transport in Pinal Creek, Arizona and the control of vertical sulfide flux in the Black Sea.

Pinal Creek, the study site explored in Chapter II, is a perennial stream in Arizona that has been polluted by mining activities above the “head of flow” or where the underground water table is forced to the surface by the basin geological structure (Brown et al., 1998). The Cu mine waste responsible for the pollution contains a large fraction of sulfide bearing minerals which, upon air oxidation, acidified holding ponds on the mine site. An acidic plume then penetrated into the water table, dissolving

alluvial minerals along the way to produce an underground plume laden with Cu, Ni, Zn and Mn (Brown et al., 1998). The plume travels downhill and eventually mixes into the creek surface water through the sediments. This mixing zone, called the hyporheic zone, contains large quantities of Mn oxides (Fuller and Harvey, 2000; Lind and Hem, 1993). The Mn oxides in Pinal Creek have adsorbed a significant fraction of metal pollution throughout the creek reaches where the pollutant plume mixes into the surface water, lowering the overall metal content of the surface water (Fuller and Harvey, 2000). Previous research indicates that the Mn oxides in the hyporheic zone are biogenic, by virtue of their formation rates and their saturation kinetics with respect to O<sub>2</sub> concentration (Marble et al., 1999).

Chapter II explores the diversity of cultivable MnOB from Pinal Creek, the influence of O<sub>2</sub> on growth and Mn(II) oxidation by bacterial populations from the creek and examines the bacterial diversity associated with Mn oxides produced at low O<sub>2</sub> concentrations in mixed cultures. The MnOB cultivated from Pinal Creek were from all three bacterial phyla known to contain Mn(II) oxidizing species and included  $\alpha$ -proteobacteria,  $\gamma$ -proteobacteria, *Bacillus* spp. and an *Arthrobacter* spp. Ecologically, this diversity seems to indicate a variety of niches for MnOB in Pinal Creek or a benefit to producing Mn oxides in the presence of metal pollutants. When grown in mixed-cultures containing an O<sub>2</sub> gradient, MnOB appeared to prefer low O<sub>2</sub> concentrations and would oxidize first at the oxic/anoxic boundary within the gradient. One such Mn oxide associated population was sampled using DNA-based methods and revealed the close association of  $\gamma$ - and  $\beta$ -proteobacteria. This, combined with observation that Mn oxides produced in mixed cultures would be reduced by Mn oxide

reducing organisms when the culture became anoxic, illustrates one of the complications of using Pinal Creek as a natural laboratory for biological Mn(II) oxidation.

Pinal Creek is characterized by water flow patterns that vary on timescales of days to weeks due to changing hydrologic conditions, riparian growth and remediation efforts upstream. As a result, there are undoubtedly a multitude of microbial niches with respect to just O<sub>2</sub> supply in any one sample. Thus, despite high Mn levels (nearly 1 mM at times) and copious Mn oxides in Pinal Creek, it is difficult to connect a pattern seen in the laboratory with one seen in situ—e.g. a role for a specific organism under certain conditions—because the conditions are quite variable. In its favor, Pinal Creek is a unique environment where the overall activity of MnOB is clearly important to limiting pollutant flow but correlating environmental conditions with microbial activity is perhaps done best under more stable conditions such as those found in stratified water columns.

In the Black Sea, the study site for chapters 3-5, a 5-40 m thick suboxic layer or zone occurs, generally defined by O<sub>2</sub> and sulfide concentrations below detection levels (Murray et al., 1995). Within the suboxic zone, reactants with redox potentials between oxygen and sulfate play important roles in elemental cycling and the transfer of oxidizing power to depth. These reactions create a vertical hierarchy of redox-active compounds within the suboxic layer, including nitrate, nitrite, particulate Mn, dissolved Mn, and ammonium. Biogenic Mn oxides formed within the suboxic zone are key to controlling the upward flux of sulfide in a system where little O<sub>2</sub> appears to reach the anoxic interface. Reduced Mn(II) that diffuses into the suboxic zone is

oxidized biologically to solid phase Mn oxides, which then sink at a rate that outstrips diffusional processes. The sinking Mn oxides then oxidize the upward flux of sulfide at the bottom of the suboxic zone. In this way, the Mn phase and oxidation state change catalyzed by microbes facilitates the rapid transport of oxidizing power to depth. Interestingly, a number of studies have noted that microbial Mn(II) oxidation seems to occur in the absence of O<sub>2</sub> or in regions with little O<sub>2</sub> supply (Konovalov et al., 2004; Konovalov et al., 2001; Murray et al., 1995; Oguz et al., 2001).

The O<sub>2</sub> supply discrepancy was the motivation for the work described in Chapter III. Biological Mn(II) oxidation in the Black Sea was demonstrated by Tebo (1991) during the 1988 R/V *Knorr* expeditions; the activity was found to be inhibited by poisons and enhanced by O<sub>2</sub> additions. Subsequent geochemical models could not reconcile the rates predicted by those experiments with the O<sub>2</sub> quantities available in the suboxic zone (Konovalov et al., 2004; Murray et al., 1995; Oguz et al., 2001). This led to the hypothesis that alternate electron acceptors such as nitrate, nitrite and iodate were also being utilized by bacteria for Mn(II) oxidation in the suboxic zone.

Attempting to address this, Schippers and coworkers observed no stimulation of Mn oxide formation under anoxic conditions using Black Sea water amended with nitrate, nitrite and iodate (Schippers et al., 2005). They concluded that lateral O<sub>2</sub> flux was responsible for Mn oxide production in the Black Sea, a conclusion supported by the observations reported by Konovalov and coworkers (2003) of lateral O<sub>2</sub> plumes generated in the Bosphorus region. Chapter III reports the rate of Mn oxide formation at different sites in the Black Sea in response to different O<sub>2</sub> concentrations. The results indicate that microbial Mn(II) oxidation in the Black Sea is extremely sensitive

to O<sub>2</sub> concentration and follows Michaelis-Menten-like enzyme kinetics. Therefore, traditional, pseudo first-order rate constants used to model the geochemistry of the Black Sea underestimate the microbial response to O<sub>2</sub> in terms of Mn oxide formation. Indeed, the microbial populations in the Black Sea produce Mn oxides rapidly at O<sub>2</sub> concentrations that are currently difficult or impossible to measure, making rate predictions difficult.

In this way, Chapter III serves to illustrate how important facets of microbial Mn(II) oxidation in the environment can be misunderstood from geochemical data. The bulk of our knowledge about microbial Mn(II) oxidation comes from independent laboratory and field investigations. Rarely have the two been connected and Chapters IV and V illustrate both promising progress and entrenched difficulties in making connections between the microbiology and geochemistry of Mn(II) oxidation in the environment.

As mentioned above, the described MnOB are relatively diverse, hailing from three divergent phyla. This diversity, combined with little sequence similarity on the DNA level among the MCO genes required for Mn(II) oxidation prevents, at this time, cultivation independent identification of MnOB in environmental samples (Tebo et al., 2004). While direct links remain problematic, the Black Sea suboxic zone offers a unique opportunity to correlate bacterial populations with discrete layers where processes of interest occur. To this end, Chapter IV describes the results of cultivation-independent (i.e. DNA-based) approaches to describing the bacterial populations associated with Mn oxide rich layers on the 2001 and 2005 Black Sea cruises and their connection to cultivated MnOB from the 2003 cruise.

Cultivation-independent approaches indicated that the Mn oxide maximum was dominated by *Pseudoalteromonas* spp. during the 2001 cruise, but not during the 2005 cruise. The 2005 samples showed little *Pseudoalteromonas* presence which seemed to correlate with a vertically ill-defined Mn oxide maximum. More interestingly, a direct link was made between the *Pseudoalteromonas* sequences from 2001 and isolates obtained in a separate effort by members of the Tebo lab during the 2003 Black Sea cruise.

In 2003, seven Mn(II)-oxidizing *Pseudoalteromonas*-like isolates were recovered, four of which matched the most abundant sequence recovered by DNA-based methods from the 2001 sample. Two *Pseudoalteromonas* isolates were found to produce a siderophore, desferrioxamine E. As noted above, recent results suggest that siderophores may play a role in stabilizing or producing Mn(III) in natural systems. The Black Sea *Pseudoalteromonas* isolates and sequences link a group of organisms capable of oxidizing Mn(II) with one that is dominant in the suboxic zone Mn oxide maximum. The *Pseudoalteromonas* isolates that make DFOE and oxidize Mn(II) are particularly compelling, as Chapter V presents evidence consistent with Mn(III) formation in the presence of DFO.

Mn(III), if present in the Black Sea suboxic zone in significant quantity, would present an unexamined reservoir of both oxidizing and reducing power. Given that Mn(III) has been detected as an intermediate in Mn oxide production by cultivated organisms, Mn(II) oxidation in the presence of Mn(III)-binding molecules seems a likely source for Mn(III) in suboxic environments. Chapter V reports initial efforts to

separate Mn(III) from Mn(II) in seawater and apply that separation method to detecting Mn(III) production with the radioisotope  $^{54}\text{Mn}$ .

The method, based on solid phase extraction of Mn(III)-DFOB complexes with commercially available columns, separated dissolved, reduced Mn(II) from dissolved, oxidized Mn—assumed to be Mn(III)-DFOB due the known properties of the complex (Faulkner et al., 1994). Application at sea with water from the Black Sea suboxic zone and using  $^{54}\text{Mn}$  produced equivocal results. Under the aerated conditions used to observe rapid Mn(II) oxidation rates, poisoned controls produced large quantities of dissolved, oxidized Mn when incubated with free DFOB. While un-poisoned incubations produced more dissolved, oxidized Mn, consistent with a biological role in production, it remains unclear which pathway(s) were operative. DFOB additions may have trapped an Mn(III) intermediate during the enzymatic oxidation of Mn(II), dissolved new, biogenic Mn oxides or catalyzed the con-proportionation of Mn(II) and Mn(IV) oxides to Mn(III)-DFOB. Still, Chapter V presents the intriguing possibility that microbial Mn(II) oxidation in situ is analogous to that observed in vitro and that the products of this reaction in situ are both Mn(III) complexes and Mn oxide minerals.

Finally, Chapter VI provides a brief, forward-looking summary of the results presented in Chapters II through V and details directions in which research might logically proceed from this dissertation, particularly in the upcoming era of environmental genomics and proteomics. Microbial Mn(II) oxidation is not a process which yields answers readily and yet it greatly affects the environments in which it occurs. This dichotomy is, in my opinion, due to the ubiquity of and multiple benefits



potentially conferred by Mn oxide production; no one site or organism can answer all our questions nor suffice as a singular model. Thus, as this dissertation presents, microbial Mn(II) oxidation provides ample opportunities to investigate many facets of geobiology—from biological diversity to organic-inorganic interactions to in situ kinetics.

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## **CHAPTER II**

### **Diverse bacterial populations involved in Mn(II) oxidation at Pinal Creek, Arizona**

## Abstract

Pinal Creek, a perennial freshwater stream in Arizona, is impacted a subterranean acid mine plume that injects Mn, Ni, Zn, and Co into the surface water. Within the creek and its hyporheic zone (where surface and ground water mix), microbial Mn(II) oxidation produces Mn oxides that adsorb and/or oxidize pollutants, thereby attenuating the mine plume. To better understand the Mn(II)-oxidizing microbial populations involved, plate culture, gradient culture and DNA-based methods were utilized. Plate cultures produced twelve bacterial isolates from five genera, including one *Pseudomonas*, five *Bosea*, one *Arthrobacter* and three *Bacillus* which oxidized Mn(II) in pure culture as well as an *Arthrobacter* that was induced to oxidize Mn(II) only when in contact with a non-oxidizing *Flavobacterium*. Microbes from creek sediments were grown in gradient cultures that simulated in situ, depth-dependent O<sub>2</sub> gradients and produced Mn oxide bands at low O<sub>2</sub> concentration. A 16S rRNA gene clone library indicated that both  $\gamma$ - and  $\beta$ -proteobacteria were associated a Mn oxide band from a gradient culture. In gradient cultures capped to prevent air exchange, Mn oxide bands were reductively dissolved by anaerobic activity. These data indicate that Mn oxidizing and reducing bacteria live in close association within Pinal Creek sediments, Mn(II) oxidation in Pinal Creek is catalyzed by numerous bacterial species from phylogenetically distant lineages and the process is spatially constrained by O<sub>2</sub> concentrations and bacterial interactions.



## Introduction

Pinal Creek is a perennial stream in central Arizona polluted by Cu mining upgradient from the head of flow (Figure 2.1). Acidic mine drainage percolating into the groundwater dissolves alluvial minerals and the resulting pH ~5 groundwater plume contains high concentrations of Mn, Zn, Ni and Co (Brown et al., 1998; Fuller and Harvey, 2000; Lind and Hem, 1993). The plume travels down-gradient underground and into Pinal Creek surface water via the hyporheic zone, a 15-30 cm deep subsurface region where oxic surface water and anoxic, metal-laden groundwater mix (Figures 2.1 and 2.2). This mixing gives rise to sharp gradients of dissolved oxygen and metals in the hyporheic zone. Within the hyporheic zone, dissolved Mn(II) is oxidized resulting in large Mn oxide deposits (Lind and Hem, 1993). Mass balance experiments have shown that large quantities of soluble metals passing through the hyporheic zone are adsorbed and/or oxidatively precipitated by the Mn oxides. In this way, Mn oxides have provided significant natural attenuation of the metal-laden mine plume (Fuller and Harvey, 2000). The processes that promote Mn oxide deposition in Pinal Creek are of particular interest because the creek drains into the Salt River, a water supply for Phoenix, AZ.

As has been demonstrated in disparate systems such as the Black Sea and hydrothermal vents, previous Pinal Creek studies indicate that Mn(II) oxidation is largely biologically mediated in situ. Mn(II) removal, the sum of Mn(II) oxidation and Mn(II) sorption to oxide surfaces, was inhibited by sodium azide and increased

asymptotically to oxygen concentration (Harvey and Fuller, 1998). Both results indicate that bacteria are likely important players in Mn(II) oxidation: azide is a useful for discriminating biotic and abiotic Mn(II) oxidation and the response to oxygen was typical for a catalyst (enzyme) mediated reaction (Emerson et al., 1982; Rosson et al., 1984). Furthermore, microscopic observations have shown that numerous prokaryotic morphotypes are associated with Mn oxides from the surfaces within Pinal Creek (Robbins and Corley, 2005).

Numerous bacteria are known to produce solid-phase Mn(III/IV) oxides but their importance in any particular environment remains unclear, save for the *Leptothrix* spp. commonly found in Fe and Mn oxide-rich freshwater surface films (Siering and Ghiorse, 1997a; Siering and Ghiorse, 1997b). While microscopy has revealed structures resembling *Leptothrix* spp. holdfasts associated with surficial Mn oxides in Pinal Creek (Robbins and Corley, 2005), holdfast morphology alone is insufficient for confirming the presence and importance of any particular Mn(II) oxidizing bacterial species. In general, many methods for identifying in situ, and quantifying and isolating particular functional groups of bacteria are inadequate for use with Mn(II)-oxidizing bacteria.

The known Mn(II)-oxidizing bacteria (MnOB) are found within the distantly related phyla Proteobacteria, Actinobacteria and Firmicutes (Tebo et al., 2004). Within each of these phyla, Mn(II) oxidation is not monophyletic; thus, even in a single phylum, MnOB share no phylogenetic traits amenable to identification with gene probes targeting ribosomal or other ubiquitous genes. The known functional genes, specifically a type of multicopper oxidase (MCO) shown to be required for

Mn(II) oxidation in all three phyla containing MnOB, are unsuitable targets for gene probes or amplification methods (Tebo et al., 2004). These MCO genes share little homology beyond their short Cu-binding sites, and only the amino acid sequence is conserved within those sites, not the DNA sequence (Francis and Tebo, 2001; Francis and Tebo, 2002). Finally, Mn oxide production has not been conclusively shown to confer specific benefits, preventing selective culture of MnOB. Thus, the known MnOB have been first isolated on media containing Mn(II) and then characterized *in vitro*.

This study was undertaken to determine the identity and diversity of bacteria associated with Mn(II) oxidation in hyporheic regions of Pinal Creek with both traditional isolation methods and methods that simulate *in situ* conditions more accurately than plate culture.

## Materials and Methods

### Media

Spread plates for initial isolations and gradient cultures were made with M medium plus Artificial Creek Water (ACW). ACW consisted of 10.4 mM Ca<sup>2+</sup>, 6.3 mM Mg<sup>2+</sup>, 3.7 mM Na<sup>+</sup>, 0.14 mM K<sup>+</sup>, 16.3 mM SO<sub>4</sub><sup>2-</sup>, 2.24 mM Cl<sup>-</sup>, and 2.0 mM HCO<sub>3</sub><sup>-</sup>. M<sub>ACW</sub> was ACW supplemented with 0.005% yeast extract (Difco, Detroit MI), 0.005% bacto-peptone (Difco), 20 mM HEPES buffer (pH 7.75) and 1mM KHCO<sub>3</sub>. Gradient overlays were made with M<sub>ACW</sub> and 0.25% (wt/vol) low melting point agarose (Fisher Scientific, Pittsburgh PA). Gradient plugs and spread plate M<sub>ACW</sub>+Mn contained 1.5% noble agar (Difco) and, respectively, 1.0 and 0.1 mM MnCl<sub>2</sub>. *Leptothrix* medium was made as previously described (Boogerd and De Vrind, 1987). Luria-Bertani (LB) broth contained 5 g yeast extract and 10 g NaCl per liter deionized water.

### Samples and Culture Conditions

Sample locations and characteristics are summarized in Table 1 and Figure 2.1. Pinal Creek sediments were collected in sterile plastic tubes and kept on ice or refrigerated until inoculated. Initial 1:10 sediment dilutions (wt/vol) were made in filter-sterilized (0.2 μm) ACW with 0.01% Tween 80 (Fisher Scientific, Tustin, CA) to disperse attached cells. Gradients were inoculated with 100 μl aliquots of 1:100 dilutions. Further serial dilutions were made in ACW and spread in 100 ul aliquots on M<sub>ACW</sub> plates. Plates and gradients were incubated at 18 °C and observed regularly for

the appearance of dark brown-black Mn oxides. Colonies with visible Mn oxides were transferred onto  $M_{ACW}$  initially to obtain isolated, pure colonies. Subsequent transfers were made on *Leptothrix* medium which promoted more rapid growth.

Gradient cultures were constructed in two ways. Overlay gradients were made by pouring 1.2 ml sterile, molten  $M_{ACW}+Mn$  “plugs” into 15 ml screw-cap culture tubes (cf. Figure 2.3). The tubes were then sparged with  $N_2$  or incubated in an anaerobic chamber with loosened caps for > 2 days. Molten  $M_{ACW}$  was then cooled to ~30 °C and 11.8 ml was mixed with 100  $\mu$ l of inoculum (see above) and poured over the plugs. The gradients were then sparged with  $N_2$  or incubated anaerobically with loosened caps for 2-3 days to remove residual oxygen. Thereafter, gradients were incubated at 18 °C with loosened caps under atmospheric or 1%  $O_2$ . High [ $O_2$ ] control gradients were made without  $N_2$  sparging or anaerobic incubation and some were bubbled with air through a cotton-plugged pipette prior to solidifying.

Stab-inoculated gradient cultures were constructed by pouring 40 ml of molten  $M_{ACW}$  or *Leptothrix* medium with 100  $\mu$ M Mn(II) and 1.5% agar into sterile 50 ml tubes. The tubes were allowed to solidify and incubated for >3 days in an anaerobic atmosphere to remove oxygen. The cultures were then inoculated directly into the center of the agar by injecting 100  $\mu$ l of culture or environmental inoculum using a 75 mm needle and a 1 ml syringe. This resulted in a central column of inoculated medium about 70 mm deep within each tube (Figure 2.4).

Isolates (except P9-2) were checked for Mn(II) oxidation in liquid using duplicate 10 ml *Leptothrix* cultures and incubated at 20-25 °C with constant shaking. Another set of liquid cultures was made with 500  $\mu$ l Lept in 5 ml tubes. Four tubes

per isolate were used, two of which had ~1 ml of 1mm glass beads. These were incubated at 18 °C without shaking. Additionally, some strains were grown in LB broth under full aeration (shaking) to facilitate DNA isolation in the absence of Mn oxides.

### **DNA Isolations**

Isolated colonies were scraped from plates or pelleted by centrifugation from LB broth and suspended in either 100 µl sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) or a lysis tube from an UltraClean™ Soil DNA Kit (MoBio, Solana Beach, CA). DNA was obtained via the Soil DNA kit as suggested by the manufacturer except that DNA was eluted with TE. Cells suspended in TE were boiled for 10 min, then centrifuged 10 min at 20k x g to pellet cellular debris. The supernatant was transferred to a clean tube.

Oxide bands from gradient cultures were removed with a syringe and diluted ~10-fold in ACW. This suspension was centrifuged 10 min. at 20k x g to pellet cells and the supernatant was decanted to remove agarose and reduce the total volume. DNA was isolated from the pellet by boiling lysis in TE as above.

### **Polymerase Chain Reaction (PCR) and Cloning**

PCRs were carried out in 25 µl and consisted of 2 µl DNA samples in the 1X reaction buffer (Boehringer-Mannheim; Indianapolis IN), 1.5 mM MgCl<sub>2</sub>, 0.25 mM each dNTP (all Gibco, Gaithersburg, MD), 0.5 U Taq DNA polymerase (Boehringer-Mannheim) and 0.1 µM each primer. Primers 27f (5' AGA GTT TGT TCM TGG CTC AG 3') and 1492r (5' TAC GGY TAC CTT GTT ACG ACT T 3') targeted a ~1400 bp region of the 16S rRNA gene homologous to positions 8-1492 in *E. coli* K-

12. Thermal cycling consisted of an initial 5 min., 94 °C denaturation followed by 30 cycles of 1 min., 94 °C; 1 min 50 °C, 2 min. 72 °C. Final extensions were carried out at 72 °C for 7 min.

Resulting PCR products were used to create a clone library via a TA-Topo kit in conjunction with a pCR2.1 vector and blue/white screening as directed by the manufacturer (Invitrogen, La Jolla, CA). White colonies were picked and suspended in 10 µl sterile de-ionized water. Reagents were added directly to these tubes for colony PCRs with M13 primers (Invitrogen) targeted to the vector.

### **Sequence Analyses**

DNA sequencing was performed as directed by the manufacturer (Applied Biosystems, Foster City, CA) for one-quarter volume reactions with 16S rRNA gene-specific primers and 1-4 µl of the M13 PCR product using a 373-Stretch autosequencer (Applied Biosystems). Individual reads were edited and assembled into contiguous sequences using Sequencher software (Gene Codes, Ann Arbor, MI).

Cloned sequences from the Mn oxide bands in gradient cultures (band clone or PCBC sequences hereafter) were screened for anomalous sequences (chimeras and poor assemblies) using the Mallard software package available from Cardiff University's School of Biosciences (<http://www.cf.ac.uk/biosi/research/biosoft/Mallard/index.html>). Mallard applies the Pintail sequence comparison algorithm to an entire library of sequences (Ashelford et al., 2005). Briefly, clone library sequences and an E. coli 16S rRNA gene sequence (Genbank accession U00096) were aligned using ClustalW software via the European Bioinformatics Institute online toolset ([www.ebi.ac.uk](http://www.ebi.ac.uk)). Aligned sequences were

analyzed by Mallard and all sequences reported as anomalous at a 95% cut-off were further analyzed using Pintail to compare them to high-scoring matches reported by BLAST (Altschul et al., 1990). Sequences that appeared anomalous when compared to closely-allied BLAST matches were removed from further analyses.

Non-chimeric PCBC and isolate sequences were aligned to selected reference sequences using the NAST alignment algorithm at greengenes.lbl.gov. Seaview software was used to edit NAST alignments by hand, delete gaps common to all sequences and identify well-conserved sites for phylogenetic tree reconstruction (Galtier et al., 1996). Conserved sites were identified by using Seaview's consensus sequence function with gaps allowed and a threshold setting of 51%. The resulting mask contained 400 positions which were present and identical in at least 51% of the sequences compared. These sites were used to construct a phylogenetic tree using PhyloWin software with the Neighbor-Joining method, Jukes and Cantor corrected distances and 500 bootstrap replicates (Galtier et al., 1996).

## **Results**

### **Gradient cultures**

Two to four days after exposing overlay gradient cultures from environmental inocula to air, one or more bands of dense growth developed in the semi-solid agar. Within 7 to 14 days, a Mn oxide band consistently developed 0.5-1.2 cm below the surface of the agar (e.g. Figure 2.3). Oxide bands in gradients poured and incubated without an oxygen removal step developed deeper in the culture than in gradients purged with N<sub>2</sub> or initially incubated anaerobically. Bubbling air through the molten agar immediately after inoculation also increased the depth of the oxide band relative



to gradients from which oxygen was removed (data not shown). Under a 1% O<sub>2</sub> atmosphere, Mn oxide bands occurred only at the agar surface while identical cultures incubated under air (~21% O<sub>2</sub>) produced an oxide band 0.7 cm below the agar surface (Figure 2.4).

Stability of the oxide bands created by environmental inocula varied. In some cases, extended incubation resulted in Mn oxide deposits accumulating above the band (occasionally up to the agar surface) while the oxide bands in other gradients remained narrow (e.g. Figure 2.3). Mn oxide bands were not observed to move downward, nor did additional bands form beneath the original band, even after extended incubation (months to years). The Mn oxide band in the P1a gradient sampled for producing a clone library (below) disappeared over a period of ~1 month after the screw cap was tightened and this observation was repeated with other environmental inocula.

Gradient cultures inoculated with pure cultures of Mn(II) oxidizing strains exhibited a different pattern of Mn oxide bands than those inoculated with environmental (mixed) samples. Pure cultures initially oxidized Mn(II) anywhere from 0.5-3 cm below the agar surface. Over time, Mn oxides were formed both above and below the initial zone of oxidation in all strains tested, sometimes with discrete banding patterns.

### **Band Clones**

Seventeen unique Proteobacteria sequences were obtained from partial sequencing of 22 inserts in the gradient band clone (sequences labeled PCBC) library (Table 3). Three anomalous sequences were identified by Mallard and Pintail analyses: clones PCBC-11 and PCBC-24 were chimeras comprised, respectively, of

*Pseudomonas/Rheinheimera*-like sequence and *Pseudomonas/Aeromonas*-like sequences. Clone PCBC-40 appeared to be a truncated *Pseudomonas* sequence. The remaining 14 unique sequences most closely matched established sequences from the  $\beta$ - (8 sequences) and  $\gamma$ -proteobacteria (6 sequences). Sequences from the  $\gamma$ -proteobacteria dominated the library, comprising 60% of the 19 clones. Two unique sequences, represented by PCBC-06 and PCBC-12, appeared 3 and 2 times, respectively and were closely related to the  $\gamma$ -proteobacterial genus *Rheinheimera* (Figure 2.6). The genus *Pseudomonas* produced three unique sequences from five clones with PCBC-10 appearing once and both PCBC-15 and PCBC-18 appearing twice each. The  $\beta$ -proteobacteria sequences comprised 40% of the library and no  $\beta$ -proteobacterial sequence appeared more than once.

### **Isolates**

Samples that produced bacterial colonies associated with Mn oxides on the first round of spread plates are summarized in Table 1. Isolates were named by the origin of the sample (Figure 2.1B) and numbered in series, e.g. the first isolate from site PC2 was PC2-1. Letters after the appropriate number were used to designate multiple samples from a single site (e.g. PC1a) or isolates that were later split into multiple distinct strains (e.g. P6-1a). Most colonies associated with Mn oxides either did not oxidize Mn(II) or survive in subsequent transfers. Ultimately, 12 isolates from sediment samples were maintained. Ten isolates oxidized Mn(II) in pure culture, one (PC6-3a) oxidized sporadically except in a consortium with the twelfth isolate, the non-Mn(II)-oxidizing PC6-3m (see consortium results below). When inoculated into the agar, the PC1b and PC2 isolates oxidized Mn(II) first in the agar, then on the agar

surface. Few isolates grew or oxidized Mn(II) well in liquid Lept medium under constant shaking. All strains tested except PC6-1a grew in liquid Lept medium when not shaking (Table 2). Mn(II) oxidation, although limited to a few isolates, was enhanced relative to constant shaking except for the PC6-3a/m consortium. The presence of additional surface area (glass beads) had no apparent effect on growth or Mn(II) oxidation (data not shown). All isolates grew and oxidized Mn(II) in axenic gradient cultures as described above. Six unique 16s rRNA gene sequences from three phyla were observed among the 12 isolates (Figure 2.6). Colony/cell morphology, Mn(II) oxidation characteristics and 16S sequence analyses did not agree in some cases. The isolates PC1b-2a, PC2-1, PC2-2, PC2-5, PC2-7 were identical by 16S sequence, yet PC1b-2a did not oxidize Mn(II) in liquid culture (Table 2). Likewise, isolates PC6-1a and PC6-1b were identical by 16S rDNA sequence but PC6-1b oxidized Mn(II) significantly slower on plates and PC6-1a did not grow in liquid culture.

### **Consortium**

The PC6-3a/m consortium consisted of isolate PC6-3m (related to *Flavobacterium* spp.), which grew as a thin, marginally visible swarming layer and the *Arthrobacter* isolate, PC6-3a, which produced Mn oxides only when in contact with PC6-3m. During routine cultivation, a third colony type was observed on one plate oxidizing Mn(II) rapidly in isolation from PC6-3m. This organism was isolated and designated PC6-3c. The 16S rRNA gene sequences of PC6-3c and PC6-3a were identical despite differences in colony morphology and Mn(II) oxidation. PC6-3c colonies were waxy and oxidized Mn(II) in the absence of PC6-3m, while PC6-3a

colonies were mucoid and generally required contact with PC6-3m cells to produce significant Mn oxides (Figure 2.7). Occasionally, portions of PC6-3a colonies would oxidize Mn(II) in axenic culture producing a sectoring or pie chart appearance unlike the colony-wide Mn oxide deposition seen on consortium plates. In liquid *Leptothrix* medium, PC6-3c grew and oxidized more rapidly than any other strain in this study. The PC6-3a/m consortium also grew and oxidized Mn(II) relatively well in liquid, but axenic liquid cultures of PC6-3a and PC6-3m failed to produce Mn oxides.

## Discussion

Bacterial isolates are often obtained from plate cultures that fail to mimic the in situ conditions of the environment of interest. To address this, previous studies have employed gradient cultures that provide opposing gradients of electron donor and acceptor in a semi-solid medium which allows bacterial migration to regions with preferred redox potentials (Dalsgaard and Bak, 1992; Nelson and Jannasch, 1983). This is particularly appropriate for investigating the hyporheic zone, a system in which gradients of electron donor (Mn(II)) and acceptor (O<sub>2</sub>) occur naturally (Figure 2.2). In this study, gradient cultures designed to mimic the chemical gradients across the hyporheic zone were used in combination with spread plates to, respectively, characterize the microbial populations associated with Mn(II) oxidation and isolate Mn(II) oxidizing bacteria from Pinal Creek.

Gradient cultures were utilized in two ways. First to assess MnOB population responses to variable [O<sub>2</sub>] and, second, to characterize the phylogenetic diversity associated with the Mn(II)-oxidation zone. Mn(II) oxidation below the surface of gradient or agar “deep” cultures has been reported previously (Mulder, 1964). Mn(II) oxidation is generally feasible wherever O<sub>2</sub> and Mn(II) coexist. In Pinal Creek, forced mixing driven by the intersection of surface and ground water rapidly mixes anoxic ground water into oxic surface water. Therefore, large quantities of Mn(II) are available throughout a range of [O<sub>2</sub>] in and above the hyporheic zone. Since the free energy liberated by Mn(II) oxidation (i.e.  $\Delta G$ ) increases with increasing

concentrations of Mn(II), O<sub>2</sub> and OH<sup>-</sup>, the most favorable environment for Mn(II) oxidation would be high oxygen surface waters and MnOB in Pinal Creek might well find high oxygen concentrations most favorable (Tebo et al., 1997).

This expectation was not supported by gradient culture results. The initial, overlay gradient cultures all produced bands of Mn oxide 0.5-1 cm deep in the culture, where oxygen was assumed to be low (Figure 2.3). While this could be interpreted as confirmation that MnOB prefer low [O<sub>2</sub>], the supply of reduced carbon (in this case yeast extract) was initially homogeneous. As O<sub>2</sub> penetrated the culture, aerobic consumption of the yeast extract likely created an opposing gradient of carbon. This could have localized the majority of growth deep in the culture merely because the O<sub>2</sub> and yeast extract gradients allowed aerobic heterotrophy and rapid growth only in a narrow overlap region; not, as often assumed, because the organisms living there prefer micro-oxic conditions.

To address this fundamental problem with overlay-type gradient cultures, two complimentary methods were utilized. First, identical overlay gradient cultures were incubated under different [O<sub>2</sub>] exposure, revealing that growth and Mn oxide band depths did indeed correlate with initial [O<sub>2</sub>] availability in the culture. Removal of O<sub>2</sub> by anaerobic incubation or N<sub>2</sub> sparging resulted in delayed O<sub>2</sub> penetration, moving the growth location upward. Incubation under different O<sub>2</sub> concentrations affected the location of growth in a similar fashion (Figure 2.4). Thus, there was some minimum [O<sub>2</sub>] required to establish a growth band of such density that an equilibrium between subsequent carbon and O<sub>2</sub> diffusion was created and a visible growth band appeared. While not much improved over standard overlay gradient cultures, this method

demonstrated the role of  $O_2$  as a master variable for the location of microbial growth, and thus, Mn(II) oxidation.

The second approach provided confirmation that Pinal Creek MnOB prefer low  $[O_2]$ . Stab inoculations of anoxic agar deeps containing Mn(II) resulted in MnOB growth at depth in horizontal bands (Figure 2.5). The stab method allows the carbon source to diffuse into the growth regions both vertically and horizontally, while  $O_2$  diffusion is only occurring vertically. Thus, the observed horizontal growth bands were in response to  $O_2$  gradients alone. The growth and Mn oxide band locations were exclusively deep in the agar, indicating a preference among MnOB for low  $O_2$  conditions. In situ, these responses to  $O_2$  supply and concentration (controlled by hydrologic exchange rates) would be reflected in variations in the location of active Mn oxide deposition in Pinal Creek (see Fig. 2, ref (Harvey and Fuller, 1998)).

Another issue relevant to the hyporheic zone in Pinal Creek, is cyclic oxidation and reduction of Mn. In aquatic systems harboring strong chemical gradients and oxic/anoxic interfaces the largest concentration of Mn oxides in a system occurs just above the interface (Aguilar and Nealson, 1998; Emerson et al., 1979; Emerson et al., 1982; Spencer and Brewer, 1971). This phenomenon is due to the relatively slow flux, i.e. at or near the rate of oxidation, of soluble Mn(II) from reducing zones below. When diffused into an oxic environment, Mn(II) is rapidly oxidized by MnOB to insoluble Mn(III/IV) oxides and sinks to the anoxic region below where it can be reduced again by Mn(III/IV) reducing bacteria (Aguilar and Nealson, 1998; Emerson et al., 1979; Emerson et al., 1982; Spencer and Brewer, 1971).

Pinal Creek is similar to oxic/anoxic interfaces in aquatic systems in that Mn(II) is supplied from an anoxic region below and O<sub>2</sub> is mixed in from above; however, the sediments prevent downward flux of particulate Mn oxides. The gradient cultures are the same: Particulate Mn oxides cannot move from where they were formed and are very stable over time. However, the hyporheic zone is subject to changes in hydrologic transport over time that may move the anoxic boundary, exposing Mn oxides to Mn(III/IV) reducing bacteria and releasing the bound metal pollutants back into the groundwater. The potential for Mn(II) oxidation and reduction in the same environment was present in the inocula used in this study, indicating the presence and growth of anaerobic organisms despite repeated exposure to air during sampling and subsequent handling. When the discrete Mn oxide band in a P1a gradient culture was partially removed to create a clone library, the screw cap was inadvertently, but fortuitously, tightened. Approximately one month later, the remaining Mn oxides had disappeared, implying bacterial Mn(III/IV) reduction in the absence of gas exchange. This was later observed in other Pinal Creek gradient cultures, indicating that the long term stability of Mn oxides requires oxic conditions. Moreover, this indicates that the benefits of pollutant adsorption by Mn oxides can likely be reversed by simple flow rate changes in the creek that create anoxic conditions in Mn oxide rich regions.

Concordant with closely associated aerobic and anaerobic processes, the band clone sequences revealed the presence of groups closely associated with both known Mn(II)-oxidizing bacteria and anaerobic, metal-reducing bacteria. As noted in the introduction to this chapter, taxonomic affiliation is not sufficient to establish Mn(II)



oxidizing capability; the same is true for metal oxide reduction. Still, the phylogenetic affiliation of Mn oxide band clone sequences, summarized in Figure 2.6 and Table 3, suggests the potential for both aerobic and anaerobic metabolisms.

The  $\gamma$ -proteobacteria clones were closely related, by 16S gene sequence, to three primary groups; the nutritionally diverse *Pseudomonas*, the aquatic heterotrophic genus *Aeromonas* and a newly described halotolerant, non-fermenting genus, *Rheinheimera* (Brettar et al., 2002). The *Pseudomonas* clones, as a group, represent 5 of the 11  $\gamma$ -proteobacterial sequences. While Mn(II) oxidizing *Pseudomonas* are well known, the band clone sequences do not closely match any described Mn(II) oxidizers including the *Pseudomonas* sp. isolated in this study (see below). The sequences PCBC-06 and -12 also represent five of the 11  $\gamma$ -proteobacteria and are affiliated with the *Rheinheimera* group, but sequences currently assigned to the genus are widely divergent in origin, including the Baltic Sea, the deep Pacific Ocean and “soil aggregates” (Brettar et al., 2002; Romanenko et al., 2003).

The  $\beta$ -proteobacterial sequences also weren't indicative of any particular metabolic capacity, although a possible theme might be the anaerobic degradation of complex carbon structures. The closest affiliates of PCBC-22 and -32 were, respectively, *Dechloromonas* spp. and *Sterolibacterium* spp. *Dechloromonas* spp. are known to degrade such molecules as benzene and toluene in conjunction with denitrification, while *Sterolibacterium* spp. couple denitrification to cholesterol consumption (Chakraborty and Coates, 2005; Chakraborty et al., 2005; Tarlera and Denner, 2003). While these species are not direct metal reducers, their metabolic byproducts (low molecular weight, organic acids and/or low pH) might reduce Mn

oxides indirectly. Band clone PCBC-08 was unique in its lack of similarity to any previously reported sequence; both BLAST searches and the RDP's Sequence Match tool found the highest similarity among the  $\alpha$ -proteobacteria (Table 3) while treeing analyses with global gap removal (Figure 2.6) or pairwise removal (data not shown) placed the sequence closest to the  $\beta$ -proteobacteria (note the location of *A. tumefaciens* and PCBC-08, Figure 2.6). This raises the possibility that PCBC-08 is a chimera. While Mallard and Pintail (see methods) analyses did not characterize it as anomalous, the underlying algorithm becomes less effective as phylogenetic distance increases—sequences without close relatives are difficult to confirm as genuine or anomalous (ref). The remaining  $\beta$ -proteobacterial sequences were not amenable to treeing analysis due to their short length and poor coverage, but RDP's Sequence Match placed them in diverse genera, including *Zoogloea*, *Azoarcus*, *Azospira*, *Aquaspirillum* and *Sterolibacterium*.

Another important feature of the clone library, especially in reference to the Mn(II) oxidizing isolates obtained from Pinal Creek (Table 2), was the absence of Gram-positive sequences. The lack of *Bacillus* sp. or other Gram positive sequence types in the clone library may indicate inefficient cell lysis during DNA isolation or reflect the diversity of organisms present in the original sample. Additionally, Mn(II) oxidizing organisms, spore-formers or otherwise, may not dominate at the gradient culture interface sampled. Phase-bright spores could be seen in the gradient band populations by microscopy, but they were a minority of the microbial morphotypes present (data not shown). Ultimately, the band clones serve to illustrate the existence of multiple metabolic strategies in and around the Mn oxide band—the zone of Mn(II)

oxidation is not monospecific but comprises a diverse community of, at the least, Proteobacteria. As such, it remains impossible to link any one clone to Mn(II) oxidation without cultivating an identical MnOB in pure culture.

In terms of phyla, the diversity of Mn(II) oxidizing bacteria isolated from both Pinal Creek water and sediments was greater than that seen in the cultivation-independent clone library. All three bacterial phyla that contain Mn(II) oxidizing species, Actinobacteria, Firmicutes and Proteobacteria, were represented (Tebo et al., 2004).

Isolate PC1b-1a, a *Pseudomonas* sp., was the most prolific Mn(II) oxidizer in the isolate collection. PC1b-1a, also called PCP2 in an earlier paper (Francis and Tebo, 2001), is most closely related to *Pseudomonas* sp. KI, a strain, according to the Genbank submission (accession AJ278107) associated with N fixation (Figure 2.6). Mn oxide deposition did not occur just prior to the cessation of colony growth as it did in all other strains. Instead, P1b-1a oxidized Mn(II) just behind the edge of expanding colonies.

Isolate PC1b-2a and the four MnOB from sample PC2 were all small rods and displayed identical colony morphologies (Table 2). After 1-2 weeks of growth, Mn oxides both coated the colonies and formed in halos 2-4 mm around the colonies, in the agar. Sequence analyses confirmed that P1b-2a, PC2-2, PC2-5 and PC2-7 comprise a single species (>99% sequence identity) most closely related to the  $\alpha$ -proteobacterial genus *Bosea*. Closely related and more completely described are the *Bradyrhizobia*, N-fixing microaerophiles known to colonize plant roots but not known to oxidize Mn(II) (Madigan et al., 1997). Mn(II) oxidation is known among other  $\alpha$ -

proteobacteria, including marine isolates affiliated with the genera *Aurantimonas*, *Erythrobacter* and *Roseobacter* as well as the freshwater genus *Pedomicrobium* (Hansel and Francis, 2006; Sly et al., 1990; Tebo et al., 2004). Interestingly, both *Aurantimonas* sp. SI85-91 (Y. Rivera-Espinoza, H.A. Johnson, B.M. Tebo, unpublished data) and the *Roseobacter*-like isolate AzwK-3b deposit Mn oxides at some distance from cells and colonies (Hansel and Francis, 2006). A mechanism specific to  $\alpha$ -proteobacteria is not indicated by the available genetic data; database searches reveal both *Pedomicrobium* and *Aurantimonas* sp. SI85-91 possess MCOs similar to those seen in MnOB that accumulate Mn oxides on the outer cell or spore surface (G. Dick, pers. comm.).

The *Bacillus* isolates PC6-1a and PC6-1b were first transferred as a single strain, then separated when differences in oxidation became apparent. Sequence analyses indicated that both were strains of a single *Bacillus* sp. (Table 2), despite different Mn(II) oxidation patterns. These isolates were most closely related to a *Bacillus* sp. isolated from alkaline waters (strain CV53, (Tiago et al., 2004)) and did not group with the three major Mn(II) oxidizing *Bacillus* lineages, represented by strains MB-7, SG-1 and PL-12 (Figure 2.6). Like previously isolated Mn(II)-oxidizing *Bacillus* sp., these strains oxidized as otherwise dormant spores. PC6-1a produced dark black Mn oxides rapidly and colony-wide after sporulation while PC6-1b oxidized more slowly and sporadically among different colonies to a light brown color. The cellular morphology of these isolates was identical at various phases of growth and, given that many Mn(II) oxidizing isolates lose the capability after a few transfers, PC6-1b is likely a poorly-oxidizing mutant of PC6-1a. Isolate PC9-2 also

oxidized Mn(II) only as a spore and was identified as a *Bacillus* sp. closely related to *Bacillus firmus* but failed to group closely with the known Mn(II) oxidizers (Figure 2.6).

Most remarkable among all the isolates was the isolation of a Mn(II) oxidizing consortium that repeated the fifty-year old findings of Bromfield and Skerman (Bromfield and Skerman, 1950). Bromfield and Skerman's consortium consisted of a *Arthrobacter* sp. (formerly *Corynebacterium*) and a *Flavobacterium* sp. (formerly *Chromobacterium*) isolated from Australian soil (Bromfield and Skerman, 1950; Madigan et al., 1997). The consortium isolated from Pinal Creek consisted of an *Arthrobacter* sp. (PC6-3a) and a *Flavobacterium* sp. (isolate PC6-3m, Table 2, Figure 2.7). In a 1956 manuscript, Bromfield reported sporadic oxidation by pure cultures of his *Arthrobacter* sp., similar to the sectoring behavior reported here of isolate PC6-3a (Bromfield, 1956). Eventually, Bromfield isolated a second strain of the *Arthrobacter* sp., designated strain B, that oxidized Mn(II) in pure culture (Bromfield, 1956; Bromfield, 1974). Bromfield established through biochemical tests that strain B differed only in its ability to oxidize manganese from the original strain (Bromfield, 1956). As noted in the results, *Arthrobacter* sp. PC6-3c was isolated from apparently pure cultures of PC6-3a after it was observed to oxidize Mn(II) in the absence of the *Flavobacterium* sp. PC6-3M. The environmental relevance of Mn(II) oxidizing consortia is not clear; the isolation of a putative mutant that oxidizes Mn in the absence of the inducing strain in both this study and previous work half a world away argues for some selective pressure regulating Mn(II) oxidation in situ that is not

present in vitro—i.e. in situ, Mn(II) oxidation is not required or beneficial without the inducing organism.

## Conclusions

While this study was broad, some conclusions can be drawn about the population of Mn(II) oxidizing bacteria in Pinal Creek: (i) the location of bacterial Mn(II) oxidation depends greatly on O<sub>2</sub> penetration, (ii) regions rich in Mn oxide deposits harbor bacterial populations with the potential to both produce and consume Mn oxides, (iii) in situ maintenance of Mn oxides, and, by extension, any adsorbed pollutants, requires oxic conditions, (iv) the MnOB population is diverse, with representatives of all bacterial groups known to oxidize Mn(II), (v) Pinal Creek MnOB populations thrive in low oxygen environments and (vi) cell-cell interactions between strains from Pinal Creek can stimulate Mn(II) oxidation.

Complex systems like the hyporheic zone in Pinal Creek present challenges to those who seek to manage and predict the distribution of pollutants. This study has shown that the Mn(II) oxidation processes in Pinal Creek are dynamic with respect to environmental conditions and likely catalyzed by a number of different MnOB in different modes: as spores, as vegetative cells and in response to other organisms. Predicting outcomes with regards to Mn oxide-catalyzed pollutant adsorption and oxidation over long periods will require special attention to the location and duration of oxic conditions throughout Pinal Creek's hyporheic zone. Understanding the specific biological mechanisms involved would benefit from comparative studies between the diverse MnOB indigenous to the watershed and the existing model MnOB with which basic Mn(II) oxidation mechanisms are slowly being revealed.

Table 2.1 Sample Summary

<b>Sample</b>	<b>Date</b>	<b>Characteristics</b>	<b>Mn(II) Oxidizing Colonies</b>	<b>Mn(II) Oxidizing Isolates</b>
P1a	11-4-99	Mn oxide coated algal/cyanobacterial nodules and sediment crust	+	-
P1b	11-4-99	Mn oxide coated algal/cyanobacterial nodules	+	+
P2	11-4-99	Mn oxide coated algal mat and sediment crust	+	+
P3a	11-4-99	Thin Mn oxide crust above gravel	+	-
P3b	11-4-99	Gravel under Mn oxide crust without obvious Mn oxides	-	-
P4	11-4-99	Friable Mn oxide crust	+	-
P5	11-4-99	Mn oxides associated with dead grass	-	-
P6	11-4-99	Mn oxides associated with live plant roots	+	+
P7	11-4-99	Mn oxide crust	-	-
P8	3-23-00	Moss and sediment	+	-
P9	3-23-00	Speedwell plant with Mn oxides on roots	+	+



Table 2.2 Pinal Creek isolate characterization.

Isolate*	Putative Identification by 16S rDNA Sequence†	Solid Media		Liquid Medium with Shaking		Liquid Medium no Shaking	
		Growth	Mn(II) Oxidation	Growth	Mn(II) Oxidation	Growth	Mn(II) Oxidation
PC1b-1a	<i>Pseudomonas</i> sp. (1442 bp)	++++	++++	++	-	++	+
PC1b-2a	<i>Bosea</i> sp. (1354 bp)	+++	++	+	-	+	-
PC2-1	ND	+++	++	++	-	+	-
PC2-2	ND	+++	++	+	-	+	-
PC2-5	<i>Bosea</i> sp. (1354 bp)	+++	++	++	-	+	+
PC2-7	<i>Bosea</i> sp. (1345 bp)	+++	++	++	-	+	+
PC6-1a	<i>Bacillus</i> sp. (1428 bp)	++	+++	-	-	-	-
PC6-1b	<i>Bacillus</i> sp. (1428 bp)	++	++	-	-	+	+
PC6-3a	<i>Arthrobacter crystallopoietes</i> P6-3A (1421 bp)	++++	+ / +++‡	+++	+ / +++‡	+++	- / +‡
PC6-3c	<i>Arthrobacter crystallopoietes</i> P6-3C (1182 bp)	++++	+++	+++	+++	+++	++
PC6-3m	<i>Flavobacterium</i> sp. (1341 bp)	++++	-	+++	-	ND	ND
PC9-2	<i>Bacillus</i> sp. (1120 bp)	+++	+++	ND	ND	ND	ND

\* Isolates are grouped by common colony and/or cell morphology.

† Determined by the highest BLAST match score over the analyzed sequence length (in parentheses).

‡ Scores for P6-3a in pure culture in contact with P6-3m colonies. The consortium oxidized reliably and over the entire surface

of P6-3a colonies. In pure culture, P6-3a oxidized sporadically in patches of some colonies (see discussion).

ND = not done

Table 2.3 Band clone 16S gene matches

Clone	Sequence Length (bp)	Percent of Library	Class	Representative RDP Match	RDP Match Score
BC-01	1431	7%	$\gamma$	<i>Aeromonas enteropelogenes</i> (T)	0.935
BC-03	780	7%	$\beta$	<i>Zoogloea resiniphila</i> (T)	0.687
BC-06	1408	21%	$\gamma$	<i>Rheinheimera baltica</i>	0.849
BC-08	1422	7%	$\alpha$	<i>Agrobacterium tumefaciens</i>	0.644
BC-09	626	7%	$\beta$	<i>Azoarcus evansii</i> (T)	0.611
BC-10	1423	7%	$\gamma$	<i>Pseudomonas pseudoalcaligenes</i>	0.909
BC-12	1124	14%	$\gamma$	<i>Rheinheimera baltica</i>	0.819
BC-13	606	7%	$\beta$	<i>Sterolibacterium denitrificans</i> (T)	0.840
BC-15	1427	14%	$\gamma$	<i>Pseudomonas anguilliseptica</i>	0.958
BC-18	1053	14%	$\gamma$	<i>Pseudomonas fluorescens</i>	0.954
BC-19	604	7%	$\beta$	<i>Aquaspirillum metamorphum</i> (T)	0.865
BC-21	472	7%	$\beta$	<i>Azospira oryzae</i> (T)	0.871
BC-22	1002	7%	$\beta$	<i>Dechloromonas denitrificans</i>	0.890
BC-32	799	7%	$\beta$	<i>Sterolibacterium denitrificans</i> (T)	0.709

Shaded sequences were of insufficient coverage to include in a phylogenetic tree (Figure 2.6). Representative matches were chosen from the twenty highest match scores based on match sequence length and the quantity of characterization information available for each match strain. Uncultivated or poorly characterized top match sequence are not reported here.

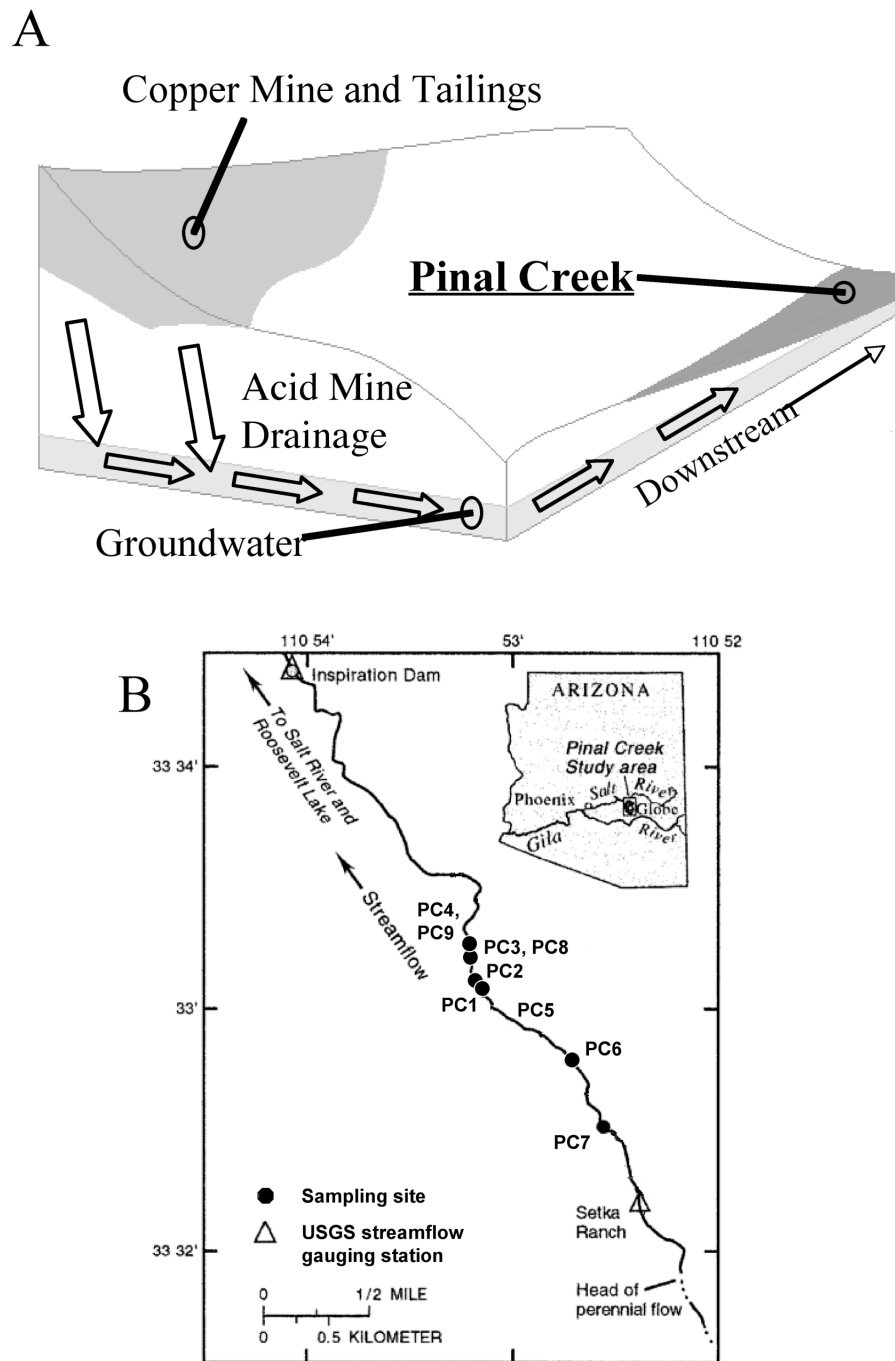


Figure 2.1 Pinal Creek study site. (A) Diagram of the subsurface acid mine drainage process that has contaminated Pinal Creek. (B) Pinal Creek location and sample site map. See Table 1 for sample characteristics.

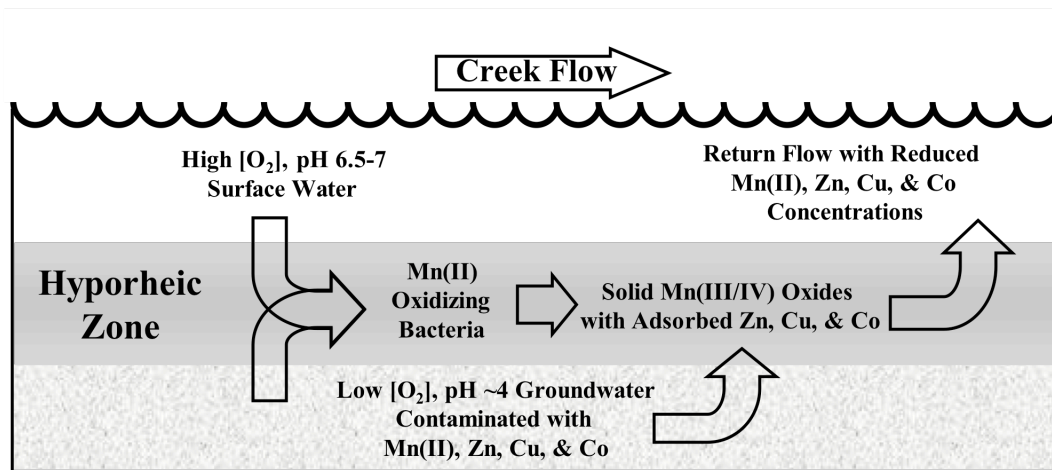


Figure 2.2 Hyporheic zone schematic highlighting hydrologic transport processes and bacterial Mn(II) oxidation in Pinal Creek. The hyporheic zone is generally 10-15 cm in depth. Modified from Harvey and Fuller, 1998.

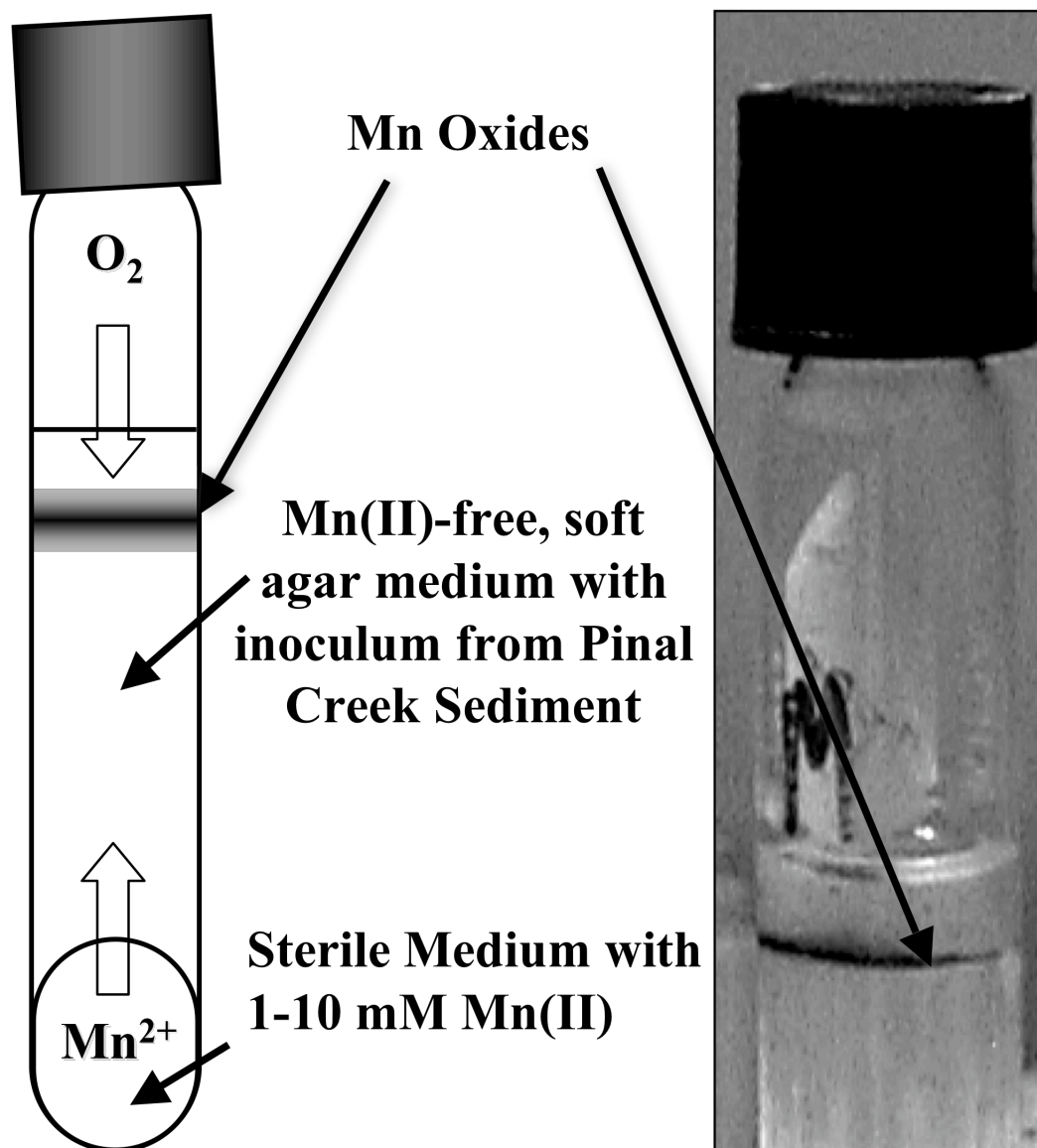


Figure 2.3 Cartoon and photo of overlay-type gradient cultures and Mn oxide band formation.

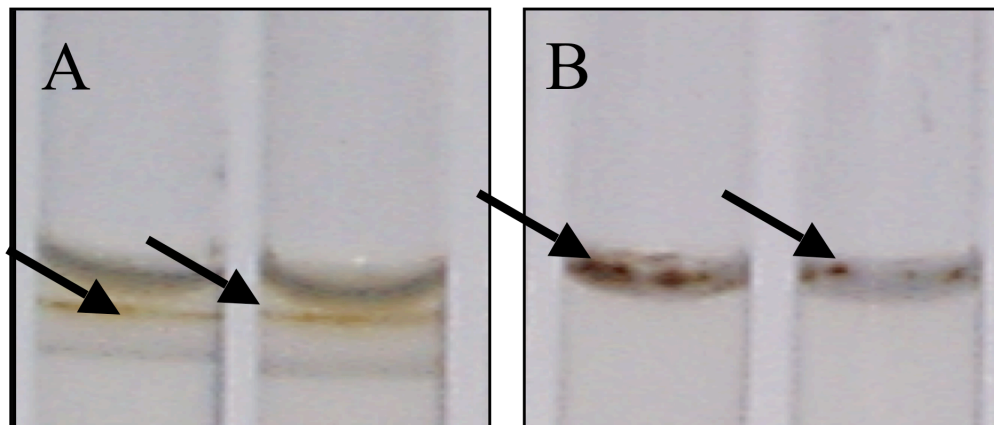


Figure 2.4 Mn oxide band depth in gradient cultures exposed to different  $O_2$  concentrations; . 21%  $O_2$  (A) and 1% (B) .

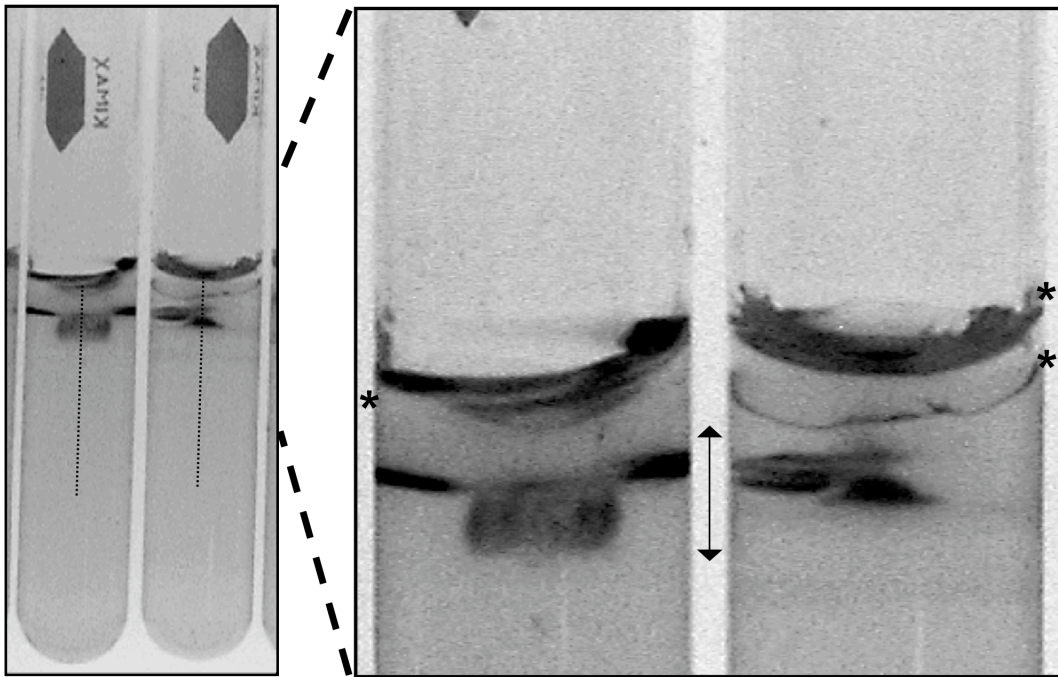


Figure 2.5 Stab-inoculated gradient cultures. Dotted lin in left panel indicates approximate inoculation region. Magnified right panel shows Mn oxide bands (indicated by double arrow) extending horizontally from inoculation region. \*Darkened regions at agar surface are photographic artifacts due to refraction of light through the agar surface.

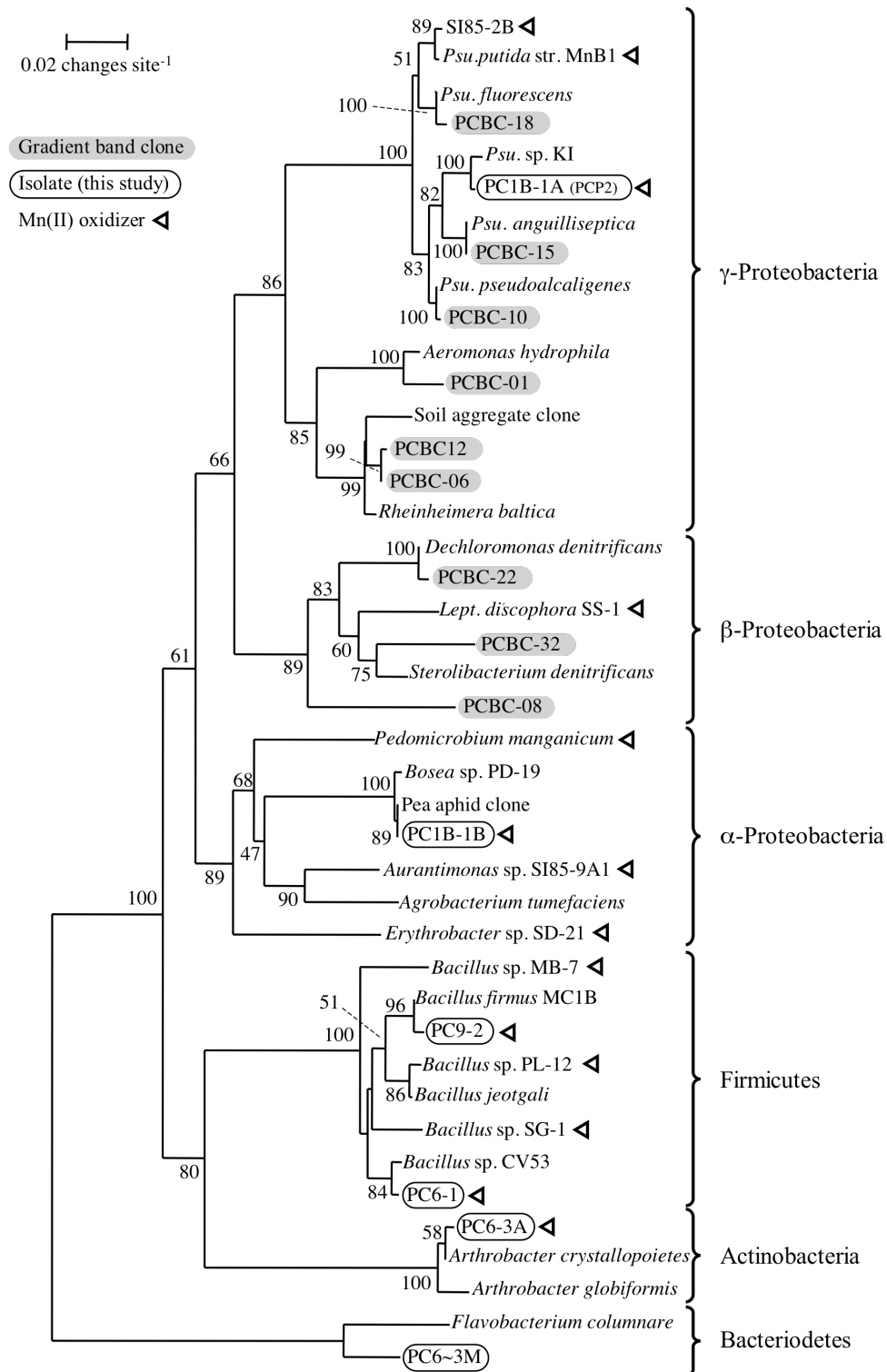


Figure 2.6 Neighbor joining phylogenetic tree of band clone and isolate sequences created with Jukes and Cantor corrected distances using 400 informative sites (global gap replacement) and 500 bootstrap replicates. Bootstrap values <50 were omitted.



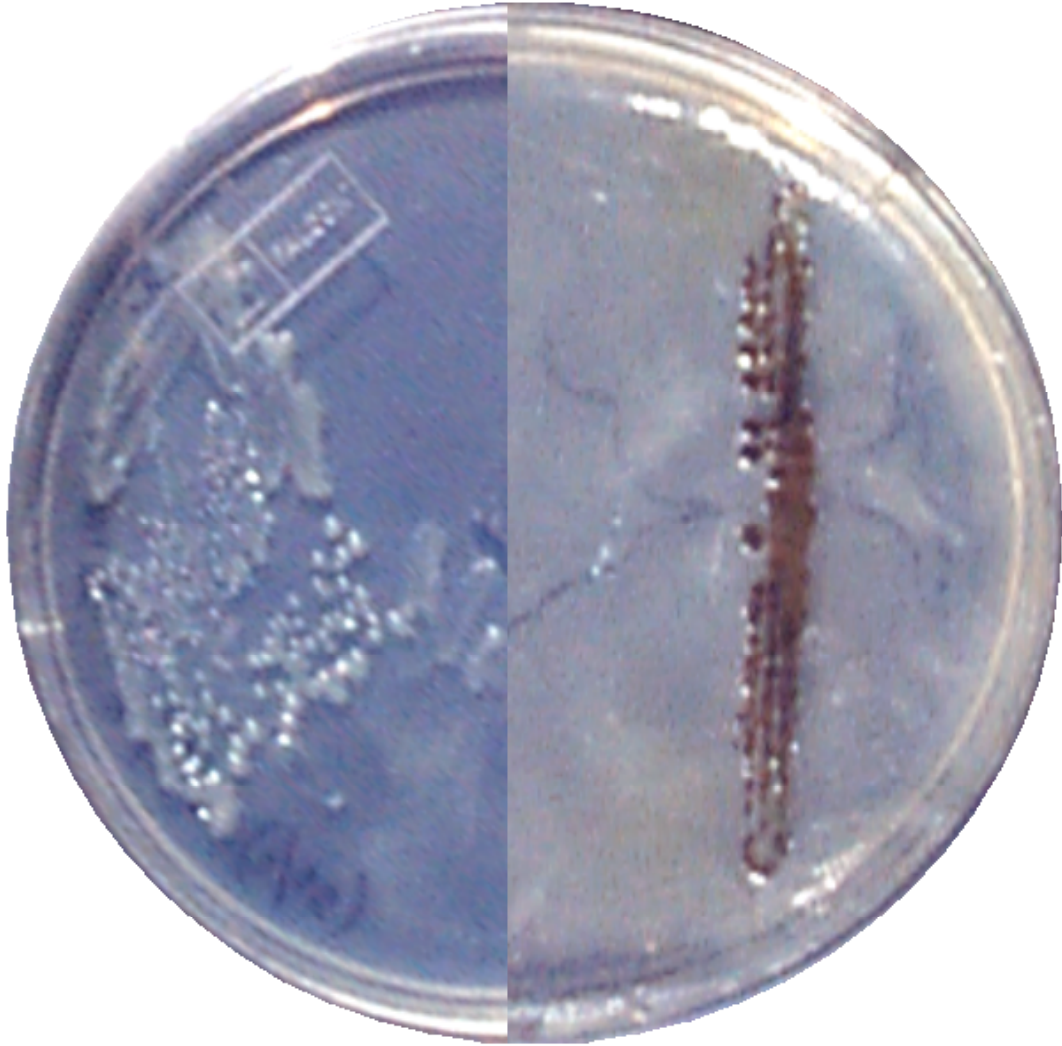


Figure 2.7 Composite picture of *Arthrobacter* sp. PC6-3A and *Flavobacterium* sp. PC6-3M consortium. Left half: PC6-3A in pure culture with no Mn oxides apparent. Right half: consortium showing PC6-3A colonies coated in black/brown Mn oxides in contact with the yellow sheen of PC6-3M swarming cells.

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## **CHAPTER III**

### **Rapid, oxygen-dependent microbial Mn(II) oxidation in the Black Sea suboxic zone at sub-micromolar oxygen concentrations**

## Abstract

Microbial Mn(II) oxidation kinetics in response to oxygen concentration were assessed in suboxic zone water at six sites throughout the Black Sea. Mn(II) oxidation rates increased asymptotically with increasing oxygen concentration, consistent with Michaelis-Menten enzyme kinetics. The environmental half-saturation constant,  $K_E$ , of Mn(II) removal (oxidation) varied from 0.30-10.5  $\mu\text{M}$  dissolved oxygen while the maximal environmental rate,  $V_{E-\text{max}}$ , ranged from 4-50  $\text{nM h}^{-1}$ . These parameters varied spatially and temporally, consistent with a diverse population of enzymes catalyzing Mn oxide production in the Black Sea. Coastally-influenced sites produced lower  $K_E$  and higher  $V_{E-\text{max}}$  constants relative to the Western and Eastern Gyre sites. In the Bosphorus region, the Mn(II) residence time calculated using  $K_E$  and  $V_{E-\text{max}}$  with 0.1  $\mu\text{M}$  oxygen was 4.5 days, 20-fold less than previous estimates. Our results (i) indicate that rapid Mn(II) oxidation in the Black Sea's suboxic zone is stimulated by oxygen concentrations well below the 3-5  $\mu\text{M}$  reliably detected by current oceanographic methods, (ii) suggest the existence of multiple, diverse Mn(II)-oxidizing enzymes, (iii) are consistent with shorter residence times than previously calculated for Mn(II) in the suboxic zone and (iv) cast further doubt on the existence of proposed reactions coupling Mn oxide production to electron acceptors other than oxygen.

## Introduction

The Black Sea is well known as the world's largest permanently stratified marine basin and an excellent study site for suboxic marine geochemistry (Murray et al., 1995). Due to a strong salinity gradient, vertical mixing is largely limited to molecular and eddy diffusion and anoxic, sulfidic conditions prevail below 65-180 m throughout the Black Sea (Konovalov et al., 2003; Konovalov and Murray, 2001; Murray et al., 1989). Just above the anoxic waters, a 5-40 m thick suboxic zone (SOZ) occurs where  $O_2$  concentrations are  $\leq 5 \mu\text{M}$  and sulfide is not detectable (Konovalov et al., 2003; Murray et al., 1989). This gap between oxic and sulfidic layers is maintained, in part, by the reduction-oxidation (redox) cycle of manganese (Konovalov et al., 2003; Tebo, 1991).

While Mn is typically found at low nanomolar concentrations in the world's oceans, total Mn concentrations in the Black Sea reach  $8 \mu\text{mol l}^{-1}$  just below the SOZ and up to  $2 \mu\text{mol l}^{-1}$  of particulate Mn(III/IV) oxides are found within the SOZ (Bruland, 1983; Konovalov et al., 2003; Lewis and Landing, 1991; Tebo, 1991). Particulate Mn(III/IV) oxides, hereafter referred to as Mn oxides, are produced by microbial oxidation of dissolved Mn(II) within the SOZ (Tebo, 1991). The Mn oxides then sink into the anoxic waters below where abiotic reactions with sulfide produce oxidized S species and Mn(II) (Konovalov et al., 2003; Oguz et al., 2001). Since the sinking Mn oxide flux outpaces diffusional fluxes of dissolved oxidants (e.g.  $O_2$  and nitrate) it both caps the upward sulfide flux and widens the gap (i.e. the SOZ) between oxic and anoxic waters. Thus, Mn is an important cyclic link in the movement of



oxidizing power from oxic to anoxic waters and a central factor in maintaining the SOZ.

A fundamental problem with this simple model of Mn redox cycling in the Black Sea is that vertical O<sub>2</sub> flux into the SOZ is insufficient to explain the quantity of Mn oxides observed (Konovalov et al., 2004; Lewis and Landing, 1991; Murray et al., 1995; Oguz et al., 2001). Combined with similar observations in marine sediments, this led to the hypothesis that Mn oxide production could be biologically coupled to nitrate reduction in lieu of oxygen reduction (Aller, 1990; Aller et al., 1998; Luther et al., 1997; Murray et al., 1995; Schulz et al., 1994; Shaw et al., 1990). Indeed, a Black Sea geochemical model invoking this hypothetical, anaerobic biological reaction reproduced observed SOZ chemical profiles better than those with Mn(II) oxidation coupled to O<sub>2</sub> reduction alone (Oguz et al., 2001). Recently, Schippers and coworkers found no experimental evidence for Mn(II) oxidation coupled to nitrate, nitrite or iodate reduction in the Black Sea (Schippers et al., 2005). We came to the same conclusion after performing similar experiments in the Black Sea with nitrate and nitrite (data not shown).

At least two alternative interpretations of available Black Sea data sets can explain the presence of Mn oxides in the absence of anoxic Mn(II) oxidation. The first, cited by Schippers and coworkers, is that vertical models fail to accurately describe O<sub>2</sub> and Mn oxide transport in the Black Sea (Schippers et al., 2005). In 1991, Tebo proposed that Mn oxides were primarily produced near the coast and advected into the gyres along isopycnals (Tebo, 1991) (Lewis and Landing, 1991). Lateral advection is consistent with the higher Mn oxide concentrations and production rates

near the coast as well as evidence from cesium isotopes, chemical profiles and temperature anomalies that indicate O<sub>2</sub>-laden Mediterranean waters are injected into suboxic and, possibly, anoxic layers near the Bosphorus (Buesseler et al., 1991; Konovalov et al., 2003; Ozsoy et al., 1993). Additionally, Lewis and Landing (1991) calculated that lateral advection of undetectable O<sub>2</sub> (i.e.  $\leq 3 \mu\text{M}$ ) could supply enough oxidizing potential to account for Mn oxide concentrations in the interior regions. To date, the injection and lateral advection hypotheses suffer, with regards to Mn oxide production, from a lack of quantitative data on the seasonality and magnitude of lateral O<sub>2</sub> transport in the Black Sea and the fraction of O<sub>2</sub> used for Mn(II) oxidation. Nonetheless, it could explain how sufficient O<sub>2</sub> is supplied at the proper depths to explain Mn oxide distributions.

A second alternative explanation for the absence of anoxic Mn(II) oxidation is that the modeling parameters do not adequately describe a biological response to changing reactant concentrations. Microbial Mn(II) oxidation is largely enigmatic because the biological benefit, the biochemical mechanism(s) and the organisms catalyzing the reaction in the environment remain unknown (Tebo et al., 2004). The available evidence implicates enzymes as the catalytic agent in Mn(II) oxidation. Multiple cultivated bacteria require a multicopper oxidase, a type of metallo-enzyme, to oxidize Mn(II). Metallo-enzyme inhibitors, including cyanide and azide, have been shown to inhibit Mn(II) oxidation in both the environment and laboratory cultures (Rosson et al., 1984; Tebo, 1991; Tebo et al., 2004). While there is no evidence to link cultivated Mn(II) oxidizers and/or multicopper oxidases to Mn oxide production in the environment, enzyme kinetics might describe microbial Mn(II) oxidation more

accurately than single rate constants due to the biological nature of the process. Tebo and Emerson (1986) found a Michaelis-Menten kinetic approach described the microbial population response to different Mn(II) concentrations in Saanich Inlet, a seasonally-stratified fjord where Mn oxides are prevalent at the oxic/anoxic interface.

To date, such kinetic considerations have not been incorporated into biogeochemical models of microbial Mn(II) oxidation in the Black Sea. Mn(II) oxidation rates in the Black Sea were originally determined at O<sub>2</sub> concentrations between 7.5 and 11.5 μM (Tebo, 1991). Rate constants were then calculated according to the equation for autocatalytic Mn(II) oxidation by Mn oxides to facilitate a direct comparison with chemical (non-biological) rates (Tebo, 1991). Subsequently, these constants were adapted for use in similar rate equations for modeling the biogeochemistry of the Black Sea (Murray et al., 1995; Oguz et al., 2001). While these models failed to explain SOZ chemical profiles with only O<sub>2</sub>-dependent Mn(II) oxidation, the equations did not consider enzymes obeying simple Michaelis-Menten kinetics. Thus, it remains to be seen if chemical rate constants provide robust estimates of biological Mn(II) oxidation at O<sub>2</sub> concentrations below those utilized in the original experiments. Interestingly, Schippers and coworkers reported rapid Mn(II) oxidation at 0.5 μM O<sub>2</sub> and calculated that only 0.2 μM O<sub>2</sub> was required to oxidize the Mn(II) present in their incubations (Schippers et al., 2005). This result is intriguing as it indicates rapid Mn(II) removal with very little O<sub>2</sub>, but even the most recent model of the sensor they used is not suited to O<sub>2</sub> measurements < ~6 μM (i.e. 2% of saturation, see [www.seabird.com](http://www.seabird.com)).

As part of the 2003 R/V Knorr cruises in the Black Sea, we sought to address the discrepancy between models that require anoxic, nitrate-dependent Mn(II) oxidation and empirical work indicating that only O<sub>2</sub> stimulates Mn(II) oxidation. To accomplish this, we determined Mn(II) oxidation rates in suboxic waters at a range of O<sub>2</sub> concentrations to determine the kinetic parameters governing the O<sub>2</sub>-dependence of Mn oxide production.

## Methods

### Stations and samples

We sampled seven stations on legs two (voyage 172-8) and three (voyage 172-9) of the 2003 R/V Knorr cruise in the Black Sea (Table 3.1, Figure 1.1). Samples were collected from the particulate Mn maximum or light transmission minimum (a proxy for the particulate Mn maximum) at each site via Niskin bottles on a CTD-equipped rosette. The collection bottle (2.5 l) was filled from the Niskin bottle slowly to prevent air entrainment and transferred immediately to the isotope lab for sparging.

### Gassing and aliquoting

Water samples were sparged (bubbled) with air or N<sub>2</sub> gas supplemented with CO<sub>2</sub> (for pH maintenance) to achieve air-saturated or anoxic conditions prior to use. The anoxic gas stream was passed through a saturated sodium sulfite solution upstream of the samples to remove residual O<sub>2</sub>. Approximately 500 ml from each sample was sparged with air and CO<sub>2</sub> while the remaining 2 l were sparged with N<sub>2</sub> and CO<sub>2</sub> for >1h. During this procedure, the water was monitored with a Fisher Scientific portable pH meter and a Ross Combination pH electrode that had been equilibrated with seawater; the CO<sub>2</sub> flow rate was controlled using a gas proportioner to maintain a pH of 7.6 +/- 0.1.

To create a variety of low O<sub>2</sub> conditions, triplicate (duplicate at the Central and Eastern Gyre sites) 40 ml mixtures were made in 60 ml serum bottles using different ratios of oxic and anoxic water; six or seven O<sub>2</sub> concentrations (hereafter simply [O<sub>2</sub>]) were tested in each experiment. Anoxic water was added to each bottle first and sparged through a needle with N<sub>2</sub> and CO<sub>2</sub> in the same proportions that stabilized the

pH. After 4 minutes, anoxic (no O<sub>2</sub> added) controls were sealed. For low [O<sub>2</sub>] incubations, the sparging needle was retracted such that anoxic gas was flushing only the headspace. Oxidic water was then added and the bottles were quickly capped with a butyl septum and aluminum crimp seal. O<sub>2</sub>-saturated incubations were made with 40 ml oxidic water under an air headspace and sealed as above. Water temperatures during handling were 16 ± 2° C.

The [O<sub>2</sub>] in air-sparged (oxidic) water was calculated using a fitting equation for the dependence of O<sub>2</sub> solubility on salinity and temperature (Benson and Krause, 1984). This value was used to calculate the O<sub>2</sub> quantity added to each serum bottle at the ambient lab temperature of 16° C. The final [O<sub>2</sub>] for each ratio was calculated, assuming equilibration between the headspace and liquid, using Henry's law and the incubation temperature of 8° C. Five or six [O<sub>2</sub>] between 0.23 and 325 μM were utilized at each site. Note that during equilibration from the 16° C handling temperature to the 8° C incubation temperature, O<sub>2</sub> solubility increased—the reported [O<sub>2</sub>] for incubations below saturation are thus an upper limit.

#### **<sup>54</sup>Mn oxidation assay**

Using a syringe and needle, 0.4 μCi from an anoxic stock of <sup>54</sup>MnCl<sub>2</sub> (carrier free, NEN Life Sciences, Boston) was added to each serum bottle through the septum and mixed by shaking. Poisoned controls were created by the addition of sodium azide (0.1% final) prior to isotope addition. Samples were incubated in the dark at 8 ± 2° C in a water bath for 5-7 hours.

Sample analysis largely followed the procedures of Tebo, 1991. To estimate the total <sup>54</sup>Mn activity, two 1 ml subsamples were taken at the end of the incubation

period. The remaining 38 ml and a 5 ml rinse of 0.2  $\mu\text{m}$  filtered seawater was vacuum filtered through a 0.2  $\mu\text{m}$  MF filter (Fisher Scientific, Hampton, NH). A 3 ml subsample of the resulting filtrate was collected for counting. Filters and “total” subsamples were brought up to 3 ml volume in 0.1 % hydroxylamine hydrochloride to solubilize Mn oxides by reduction to  $\text{Mn}^{2+}$  and eliminate geometry differences between samples. The filters and subsamples were counted at sea on a Wallac (Perkin-Elmer, Wellesley, MA) gamma counter.

$^{54}\text{Mn}(\text{II})$  removal was calculated as the number of counts per minute trapped on the filter relative to the total counts per minute in the sample. Counting efficiency was not explicitly calculated and was assumed to be equal for all samples due to the ionic state of the isotope and similar matrices (aqueous solutions of hydroxylamine or seawater). Recovery was defined as the sum of the counts per minute from the filter and filtrate divided by the total counts per minute in the sample. The fraction of  $^{54}\text{Mn}$  collected on the filter was multiplied by the dissolved Mn concentration to determine the quantity of Mn removed as a proxy for oxidation (Tebo, 1991). Dissolved and particulate Mn concentrations were determined at each site using the formaldoxime method (Brewer and Spencer, 1971) on filters (as above) and filtrates from samples of the same Niskin bottle or depth used for kinetic determinations, on the same cast when possible.

To determine if a particular  $[\text{O}_2]$  stimulated  $^{54}\text{Mn}(\text{II})$  removal, mean removal rates from triplicates were tested for significance ( $P \leq 0.001$ ) against the no  $\text{O}_2$  added control using the Student's t test. Experiments with the Central and Eastern Gyre

water were not analyzed for significant stimulation—the t-test offers little discrimination between means with only duplicate samples.

### **Estimates of kinetic parameters**

Kinetic parameters similar to the Michaelis-Menten parameters  $K_M$  and  $V_{Max}$  (see Results and Discussion) were estimated after triplicate  $^{54}\text{Mn(II)}$  removal rates were Q-tested to remove outliers. The mean values of remaining replicates were fit to a rectangular hyperbola equation (see Equation 1, below) by KaleidaGraph software using the Levenberg-Marquardt algorithm.



## Results and Discussion

Given that nitrate, nitrite and iodate do not stimulate Mn oxide production under anoxic conditions, undetected  $[O_2]$  seems the most likely oxidant of Mn(II) in the SOZ (Schippers et al., 2005). However, assessing microbial responses at undetectable  $[O_2]$  is complicated by the same technical and handling problems that limit  $O_2$  measurement. For this reason, we adopted an approach wherein  $O_2$  was actively removed from our seawater samples by sparging with anoxic gas. Air-saturated seawater was then added in various amounts to deliver a known amount of  $O_2$  and permit  $^{54}\text{Mn(II)}$  removal determinations at 10-fold lower  $[O_2]$  than can be measured by sensors or voltammetry. In combination with anoxic and/or poisoned controls, this approach revealed rapid microbial Mn(II) oxidation at remarkably low  $[O_2]$  (Table 3.1, Figure 3.3).

### Control Experiments

The potential confounding factors in determining microbial Mn(II) oxidation rates using the radioactive tracer  $^{54}\text{Mn}$  in environmental samples were addressed in detail by Tebo and Emerson (1986). Briefly, they found that (1)  $^{54}\text{Mn}$  largely behaves as an ideal tracer, (2) cellular uptake of Mn(II) is constrained by total cellular volume and thus not a significant source of particulate Mn, (3) Mn oxide reduction in anoxic controls was not apparent and (4) the respiratory inhibitory sodium azide did not affect the abiotic binding of  $^{54}\text{Mn}$  to particles (Tebo and Emerson, 1986). We are obliged to review this last point as it has been questioned by recent work. Shiller and coworkers (2005) reported that azide desorbs Mn(II) from particle surfaces and is therefore a

poor control for distinguishing biological from chemical Mn(II) removal/oxidation (Shiller and Stephens, 2005).

We utilized sodium azide (0.1% final concentration) to determine if  $^{54}\text{Mn(II)}$  removal observed in no  $\text{O}_2$  added controls was due to biological activity and, therefore,  $\text{O}_2$  contamination. If azide desorbed  $\text{Mn}^{2+}$  from particles over the course of these experiments, less  $^{54}\text{Mn}$  removal (i.e. exchange) would have been observed in the poisoned control than in the live control. If  $\text{O}_2$  contamination occurred, biological catalysis would have increased  $^{54}\text{Mn(II)}$  removal in the live no  $\text{O}_2$  added control, relative to the poisoned control. Instead, no differences were observed between poisoned and live no  $\text{O}_2$  added incubations in all five experiments with azide controls (Figure 3.2). Therefore, the most likely explanation for  $^{54}\text{Mn(II)}$  removal in the poisoned and live no  $\text{O}_2$  added controls is equilibrium exchange with isotopically stable Mn(II) on particle surfaces, as seen in previous marine and laboratory work (Emerson et al., 1982; Rosson and Neelson, 1982; Rosson et al., 1984; Tebo, 1991; Tebo and Emerson, 1985; Tebo and Emerson, 1986; Tebo et al., 1984). More importantly, these results indicate that the no  $\text{O}_2$  added controls were indeed anoxic.

### **Oxygen addition experiments**

Oxygen addition stimulated  $^{54}\text{Mn(II)}$  removal in all experiments. In the five experiments with triplicate incubations, stimulation of  $^{54}\text{Mn(II)}$  removal was significant at  $[\text{O}_2] \leq 2.3 \mu\text{M}$  ( $P \leq 0.001$ , see Table 3.1).  $^{54}\text{Mn(II)}$  removal was positively correlated with  $\text{O}_2$  concentration but not described by a linear relationship. Instead,  $^{54}\text{Mn(II)}$  removal approached a maximum with increasing  $\text{O}_2$ , consistent with saturation or Michaelis-Menten enzyme kinetics (Figure 3.3). Simple saturation

kinetics are described by a rectangular hyperbola of the form

$$v = \frac{s \cdot V_{\max}}{s + K_M} \quad (1)$$

where  $v$  is the observed rate (or velocity),  $s$  is the concentration of the limiting substrate,  $V_{\max}$  is the predicted rate at infinite  $s$ , and  $K_M$  is the  $s$  required for a  $v$  of 0.5  $V_{\max}$ . While the experiments presented here resulted in Michaelis-Menten-like curves, they are fundamentally distinct from ideal Michaelis-Menten experiments.

Several assumptions, required to apply the Michaelis-Menten equation, specify the conditions under which Michaelis-Menten assays must be performed. In order to isolate the effects of  $O_2$  concentration under otherwise environmentally-relevant conditions, each experiment presented here necessarily violates these conditions in three ways: (i) The samples contain a heterogeneous mixture of organisms and enzymes that likely utilize  $O_2$  for purposes unrelated to Mn(II) oxidation, such as carbon oxidation in heterotrophic metabolism, forcing the Mn(II) oxidases present to compete for  $O_2$ . (ii) The dissolved Mn(II) concentration (0.5-2  $\mu\text{M}$ ) may have been low enough to result in Mn(II) limitation. Substrate limitation is usually evident at  $\leq 10$  times the  $K_M$ ; reported  $K_M$  values for  $\text{Mn}^{2+}$  in Saanich Inlet were 0.2-0.9  $\mu\text{M}$  (Tebo and Emerson, 1986). (iii) Due to  $O_2$  utilization,  $[O_2]$  was likely dropping during the incubation. These effects all likely resulted in lower  $^{54}\text{Mn}$  removal at a given  $[O_2]$  than would occur in an ideal Michaelis-Menten kinetics experiment.

In addition to the factors described above, previous numerical simulations of Michaelis-Menten kinetics applied to complex microbial populations have

demonstrated that  $K_M$  values observed in heterogeneous microbial populations are not a simple mean of  $K_M$  values for the individual enzymes present (Williams, 1973). Instead, Michaelis-Menten kinetics applied to environmental samples results in environment-specific parameters, which we call here the environmental half-saturation constant,  $K_E$  and the environmental rate maximum,  $V_{E-max}$ . The distinction between  $K_E / K_M$  and  $V_{Max} / V_{E-max}$  can be illustrated. Following Williams,

$$\frac{s \cdot V_{E-max}}{s + K_E} \approx v = \sum_{i=1}^n \frac{s \cdot V_{max}^i}{s + K_M^i} \quad (2)$$

where the observed rate  $v$  is the sum of the Michaelis-Menten equations for each different enzyme, in terms of  $K_M$  and  $V_{Max}$ , that catalyzes the reaction of interest (Williams, 1973). This summation does not produce a perfect, two-parameter rectangular hyperbola (Williams, 1973), but the resulting curve can be fit to one, resulting in the parameter estimates,  $K_E$  and  $V_{E-max}$ .

Data from seven experiments at six stations fit Equation 2 with  $R^2$  values  $>0.8$  (Table 3.1).  $K_E$  values were  $\leq 0.87 \mu\text{M O}_2$  at the Bosphorus Plume, Sakarya Eddy, Batumi Eddy and Central Gyre stations. The Western Gyre and Eastern Gyre produced  $K_E$  values between 1.2 and 11  $\mu\text{M O}_2$ . Estimated  $V_{E-max}$  values varied over a smaller range, from 4 to 50  $\text{nM Mn h}^{-1}$  and did not correlate with  $K_E$  values. Typically, the Michaelis-Menten parameters  $K_M$  and  $V_{Max}$  for two different enzymes catalyzing the same reaction by the same mechanism are positively correlated. In environmental experiments where enzyme type and abundance are unknown, no such correlation is expected (Williams, 1973). Instead, the parameters  $K_E$  and  $V_{E-max}$  reflect different properties of each sample.

The maximal observed rate,  $V_{E\text{-max}}$ , is sensitive to both enzyme abundance and activity (velocity). Variation in  $V_{E\text{-max}}$  can thus be explained by differences in Mn(II) oxidizer abundance, Mn(II) oxidase enzyme abundance per organism and catalytic rate differences in the population of enzymes present. Essentially,  $V_{E\text{-max}}$  is a measure of Mn(II) oxidizing capacity in the enzyme/microbial population sampled. Observed  $V_{E\text{-max}}$  values, 4-50 nM Mn(II) h<sup>-1</sup>, were in the same range reported for unlimited O<sub>2</sub> (≥50% saturation) in previous Black Sea work (Schippers et al., 2005; Tebo, 1991).

The half-saturation constant,  $K_E$ , is a measure of enzyme-substrate affinity and determines the shape of the response hyperbola, independent of the magnitude of  $V_{E\text{-max}}$ . Therefore,  $K_E$  values are independent of total microbial population size but greatly affected by the relative abundance of each unique (in terms of  $K_M$ ) enzyme (Williams, 1973). The range in  $K_E$  observed across all experiments indicates that multiple Mn(II)-oxidizing enzymes with different  $K_M$  values coexist in the Black Sea. Since the  $K_E$  is a population measure, it can be viewed as an upper limit on the  $K_M$  for one or more Mn(II)-oxidizing enzymes present. Moreover, these enzymes catalyze Mn oxide production quite rapidly at [O<sub>2</sub>] well below reliable detection limits of 3-5 μM.

Taken as a whole, the kinetic parameters,  $V_{E\text{-max}}$  and  $K_M$ , observed in the Black Sea indicate that Mn(II) oxidation may occur at unexpectedly rapid rates in regions where O<sub>2</sub> is apparently absent. However, the range in these parameters clearly indicate that both the capacity for Mn oxide production and the effects of [O<sub>2</sub>] vary in space and time.

### **Spatial and temporal kinetic variability**

The Bosphorus Region and the Sakarya Eddy sites had low  $K_E$  and high to moderate  $V_{E-max}$  values, indicative of an abundant and/or active Mn(II)-oxidizing microbial population expressing enzymes responsive to low  $[O_2]$ . These low  $K_E$  values are somewhat paradoxical as this region is subjected to intrusions of  $O_2$ -laden water from the Bosphorus plume at the sampled densities (Konovalov et al., 2003). A large  $O_2$  supply might be expected to favor populations with higher  $K_E$  values relative to interior regions that experience less lateral  $O_2$  flux. However, no  $O_2$  plumes were observed during sampling (data not shown) so it remains unknown whether the sampled populations had been subjected to a recent  $O_2$  plume. Alternatively, the growth of organisms able to rapidly exploit small quantities of  $O_2$  could be favored at these sites, possibly due to undetectable plumes of  $O_2$ -containing water or a decayed  $O_2$  plume, analogous to the larger, readily apparent plumes.

The Central Gyre and Batumi Eddy sites were similar to the Bosphorus and Sakarya sites, exhibiting  $K_E$  values  $<1\mu M$  and moderate to high  $V_{E-max}$  values. While not influenced directly by the Bosphorus plume, the surface circulation patterns in the Black Sea are such that both sites are directly influenced by meso-scale eddy processes along the coast (Oguz et al., 1993). These features may inject  $O_2$  or other growth substrates (e.g. from sediments or coastal runoff) at depth, stimulating microbial activity. Alternatively, the microbial populations responsible for rapid Mn(II) oxidation rates and high Mn oxide levels near the Bosphorus may be advected into these regions along a short path, relative to that required for transport into the

interior gyres. Rapid transport of these organisms may import a large Mn(II)-oxidizing capacity into the Central Gyre and Batumi Eddy regions.

Samples from the Eastern and Western Gyre sites had the lowest  $V_{E-max}$  and highest  $K_E$  ( $1.2^{-1} \mu M O_2$ ) values observed. These two anti-cyclonic interior gyres are isolated from coastal influence by circulation patterns and, relative to coastally influenced sites, are more stable (Konovalov et al., 2003; Oguz et al., 1993; Ozsoy et al., 1993). As indicated by the kinetic parameters (Table 3.1), Microbial Mn(II) oxidation in the interior gyres is less intense and stimulated only at higher  $[O_2]$ . While possibly representing remnant coastal populations with degraded Mn(II)-oxidizing capacity, the interior gyre Mn(II) oxidizing populations may simply be distinct from coastal populations.

The Western Gyre was sampled twice for this work and exhibited temporal variability in Mn(II) oxidation parameters. Two samples collected and assayed 4 days apart exhibited almost a 10-fold range in  $K_E$ , a five-fold range in  $V_{E-max}$  values, and a two-fold difference in particulate Mn abundance (Table 3.1). The predicted residence time for dissolved Mn at  $1 \mu M O_2$  was 44 and 77 days in the first and second sample, respectively (cf. Figure 3.4). Given the general circulation pattern in the Black Sea and the variability in Mn oxide abundance, the observed differences between the two western gyre samples may depend on the history of the sampled water parcels. Small differences in nutrient availability (including  $O_2$ ) along the path to the gyre may result in microbial populations with unique kinetic parameters for Mn(II) oxidation.

### **Mn(II) residence time**

The observed  $K_E$  and  $V_{E-max}$  values at the coastal and central gyre sites predict rapid rates of Mn(II) oxidation at extremely low  $O_2$  concentrations, relative to previous efforts. During the same expeditions on which the Black Sea SOZ was first recognized, in situ rates of Mn(II) oxidation were determined at  $[O_2]$  concentrations  $\geq 7.5\mu M$ , well above the 0-3  $\mu M$  (i.e. at or below detection) found in the suboxic waters where Mn oxides are prevalent (Konovalov et al., 2003; Tebo, 1991). Oguz and coworkers subsequently utilized Tebo's 1991 data to produce a second-order rate equation

$$v = k[Mn^{2+}][O_2] \quad (3)$$

with  $k$  equal to 0.01-0.1  $\mu M^{-1} d^{-1}$  (Oguz et al., 2001). As noted in the introduction, the resulting geochemical model could not reproduce the observed vertical patterns of particulate Mn distribution with only  $O_2$ -stimulated Mn(II) oxidation. However, the rate constant utilized in that model does not always agree with the saturation kinetic approach used here, especially at low  $O_2$  concentrations at sites exhibiting rapid Mn(II) oxidation.

Residence times predicted using the Oguz et al. constants and our  $K_E$  and  $V_{E-max}$  diverge widely at four of six sites when  $O_2 < 1 \mu M$  (Figure 3.4). This discrepancy is not surprising because the original rate constants were determined at  $O_2$  concentrations above most of the  $K_E$  values observed in this work. Such rate constants can only model hyperbolic responses accurately over their pseudo-linear range, i.e. at or below the  $K_E$ . Indeed, the rates predicted by Oguz and coworkers (i.e. Equation 3) agree well with data from the western and eastern gyre stations where  $K_E > 1 \mu M$  (Figure 3.4).



However, our residence times at sites with coastal influence are potentially much lower than predicted by Equation 3.

At sites where the rapid  $^{54}\text{Mn(II)}$  removal was observed, the Bosphorus, Sakarya Eddy, Central Gyre and Batumi Eddy stations, our kinetic approach predicts much shorter residence times for Mn(II) than the Oguz et al. calculations (Figure 3.4). Such short residence times,  $\leq 10$  days at  $0.1 \mu\text{M O}_2$ , indicate that rapid Mn(II) oxidation occurs at coastal SOZ sites when no  $\text{O}_2$  is apparent. Moreover, our  $[\text{O}_2]$ -dependent residence time estimates are likely upper limits. As noted above, our reported  $\text{O}_2$  additions are likely upper limits due to temperature and consumption considerations. Additionally, the samples all endured extensive handling prior to incubation with  $^{54}\text{Mn(II)}$ . Thus, low  $[\text{O}_2]$  stimulates Mn oxide production to a much greater degree than previously calculated and potentially more than reported here.

## Conclusions

Microbial Mn(II) oxidation rates throughout the Black Sea are governed by biochemical parameters that vary spatially and temporally. These parameters,  $K_E$  and  $V_{E\text{-max}}$ , are consistent with enzymatic responses and thus emphasize the biological nature of Mn(II) oxidation in the Black Sea SOZ. Mn(II) oxidation in the SOZ is stimulated by  $\text{O}_2$  concentrations well below current detection limits at rates in excess of those predicted by previous biogeochemical models. Such rapid rates at undetectable  $\text{O}_2$  concentrations eliminate the need to invoke anoxic Mn(II) oxidation to explain Mn oxide distributions in suboxic regions.

Our findings have implications for understanding and modeling microbial redox processes associated with chemical gradients in the environment. (i)

Undetected chemical gradients may support observable processes. In the marine environment,  $O_2$  is undetectable below  $3 \mu\text{M}$  with typical methods, yet this is enough  $O_2$  to create up to  $6 \mu\text{mol l}^{-1}$  Mn oxide, approximately 60-fold higher than the detection limit for Mn oxides in 50 ml of seawater. (ii) When enzymes are the catalysts of interest, saturation kinetic experiments are more useful than single rate constants for predicting microbial responses along substrate (reactant) gradients. Earlier Mn(II) oxidation rates in the Black Sea were in the zero-order range of the kinetic curves reported here and thus gave little indication of the magnitude of  $[O_2]$  dependence although it was clear that low  $[O_2]$  stimulated the process significantly. (iii) Biogeochemical models may be improved by incorporating kinetic parameters or linear rate constants that are determined within the linear response range, i.e. at substrate levels below the  $K_E$ . Incorporating Michaelis-Menten kinetics into biogeochemical models of multiple constituents may prove complex, but assessing the kinetic response in advance can inform the choice of constants and equations.

The variability observed in kinetic parameters that likely stems from spatial and temporal variability in microbial populations presents another challenge to modeling. Future efforts to parameterize the Mn redox cycle in the Black Sea would be improved by robust, time-course determinations of Mn(II) oxidation at  $\leq 0.5 \mu\text{M } O_2$  to provide single rate constants compatible with previous models. Additional improvement could be gained by repeated estimation of such constants in vertical profiles at both coastal and gyre sites to determine the range of variation. Finally, methods for determining  $[O_2] \geq 0.1 \mu\text{M}$  *in situ* would greatly broaden our understanding of redox cycling in the Black Sea and other suboxic environments.

Table 3.1 Summary of sample characteristics and kinetic results

Map ID	Location	Date	Depth (m)	$\sigma_T$	Dissolved [Mn] ( $\mu\text{M}$ )	Particulate [Mn] ( $\mu\text{M}$ )	Minimum Stimulating $[\text{O}_2]$ ( $\mu\text{M}$ )*	$V_{E-\text{max}}$ ( $\text{nM h}^{-1}$ )	$K_E$ ( $\mu\text{M O}_2$ )	$R^2$
1	Bosporus Region	9 May 2003	85	16.00	0.939	0.040	0.23	50	0.30	0.8050
2	Sakarya Eddy	13 May 2003	145	15.91	0.495	0.710	0.46	16	0.39	0.9774
3	Western Gyre A	7 May 2003	67	16.00	1.250	0.019	2.31	4	1.16	0.9697
4	Western Gyre B	11 May 2003	68	16.00	0.939	0.040	0.23	12	9.18	0.9869
5	Central Gyre	29 April 2003	73	15.96	1.104 <sup>†</sup>	0.033 <sup>†</sup>	nc	14	0.87	0.9950
6	Eastern Gyre	1 May 2003	99	15.90	1.400	0.011	nc	4	10.5	0.9753
7	Batumi Eddy	4 May 2003	107	15.98	1.498	0.018	0.23	31	0.56	0.9926

\*Minimum  $\text{O}_2$  concentration at which  $^{54}\text{Mn}$  removal was significantly ( $P \leq 0.001$ ) greater than that observed with no added  $\text{O}_2$ .

<sup>†</sup> Mn data taken from different bottle on the same cast at 70 m and  $\sigma_T = 15.93$

nc, Not calculated; duplicate incubations performed on these samples prevented meaningful significance testing—triplicate incubations were performed with all other samples

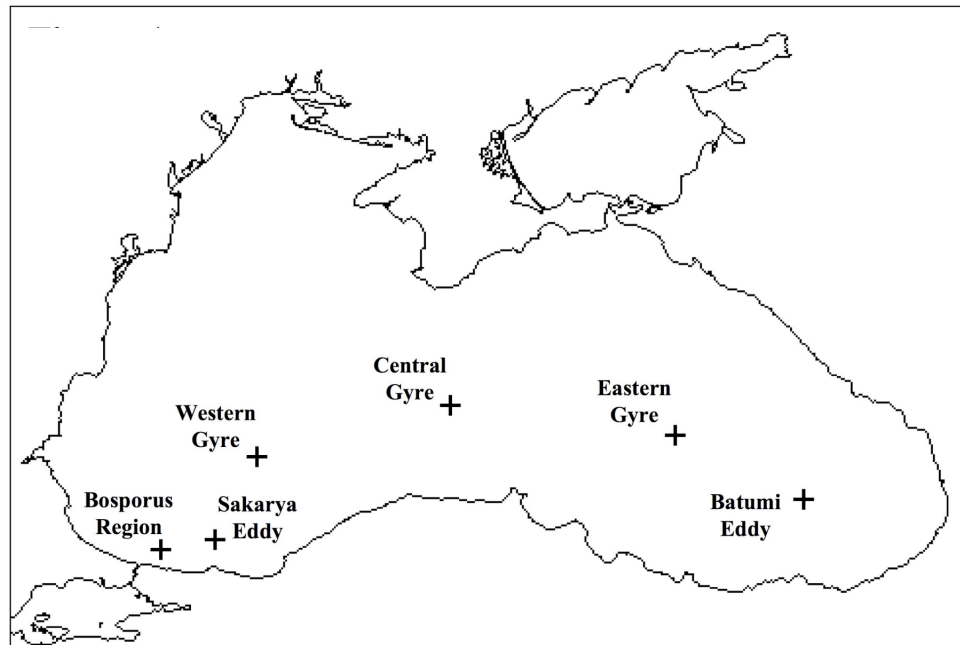


Figure 3.1 Sampling locations for  $K_E$  and  $V_{E-Max}$  determinations. Site names follow surface current features identified in Oguz et al., 1993. The Western and Eastern gyres are, relative to the Central Gyre, disconnected from coastal influence by slow transport.

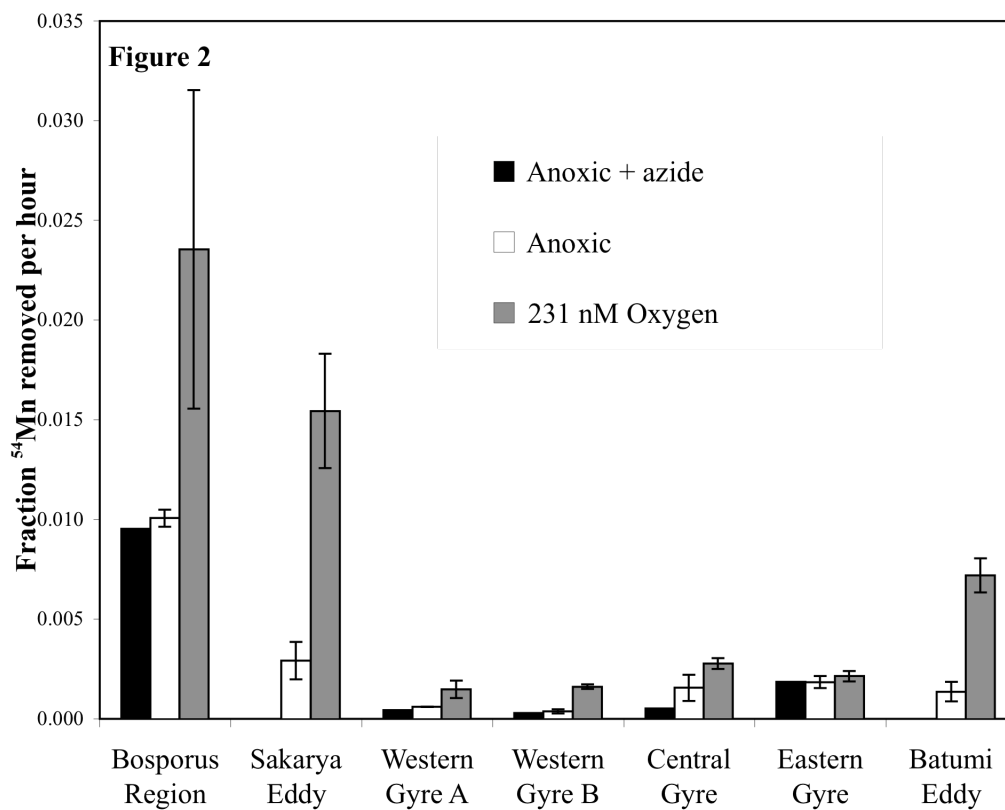


Figure 3.2 Comparison of anoxic, anoxic + azide (poisoned) and low  $[O_2]$  incubations. Error bars represent one standard deviation from duplicate or triplicate incubations. Poisoned incubations were not performed at the Sakarya and Batumi Eddy sites.

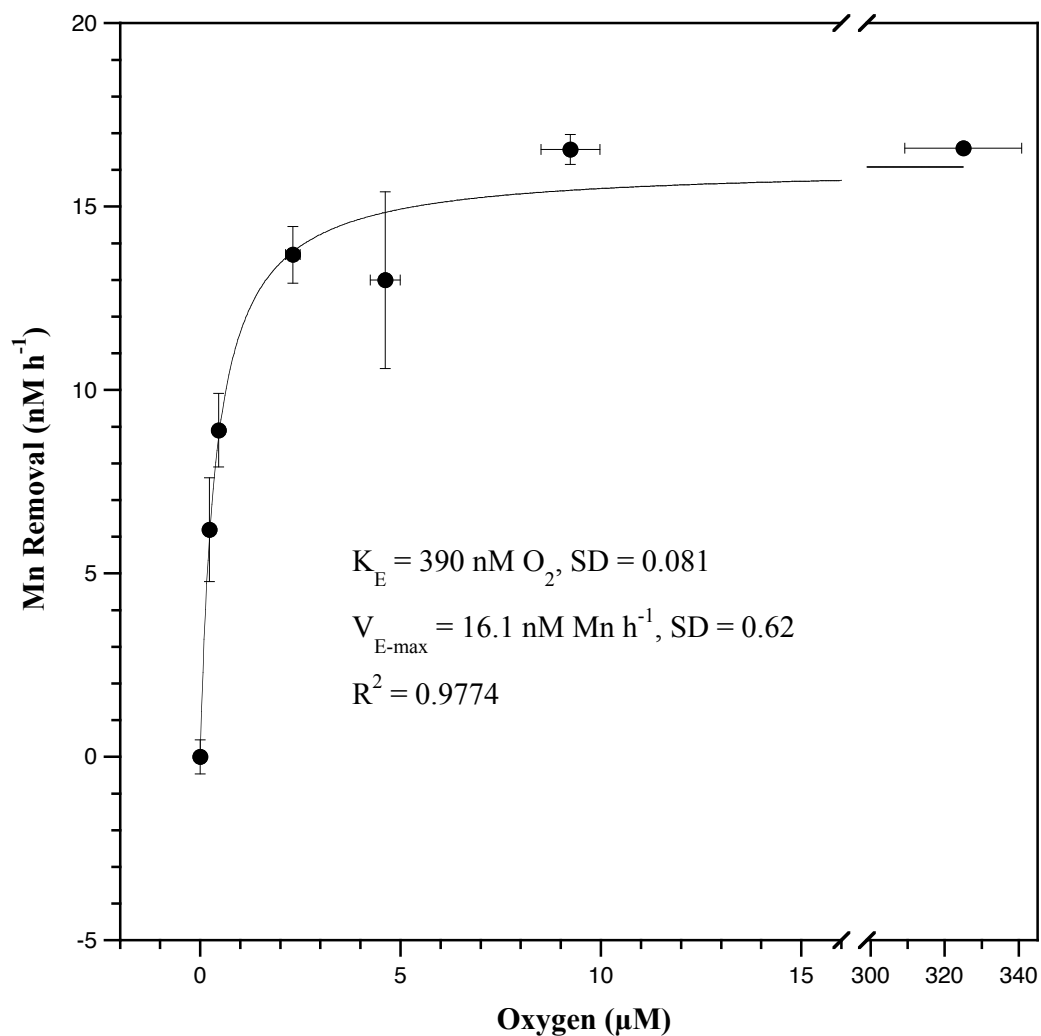


Figure 3.3 Rectangular hyperbola fit to data from Sakarya Eddy and resulting parameters  $K_E$  and  $V_{E\text{-max}}$ . Error bars represent one standard deviation from triplicate incubations (y-axis) and uncertainty in  $[\text{O}_2]$  (x-axis) equal to  $\pm 2^\circ \text{C}$  variation in temperature during handling and incubation. SD = standard deviation calculated for each fitting parameter.

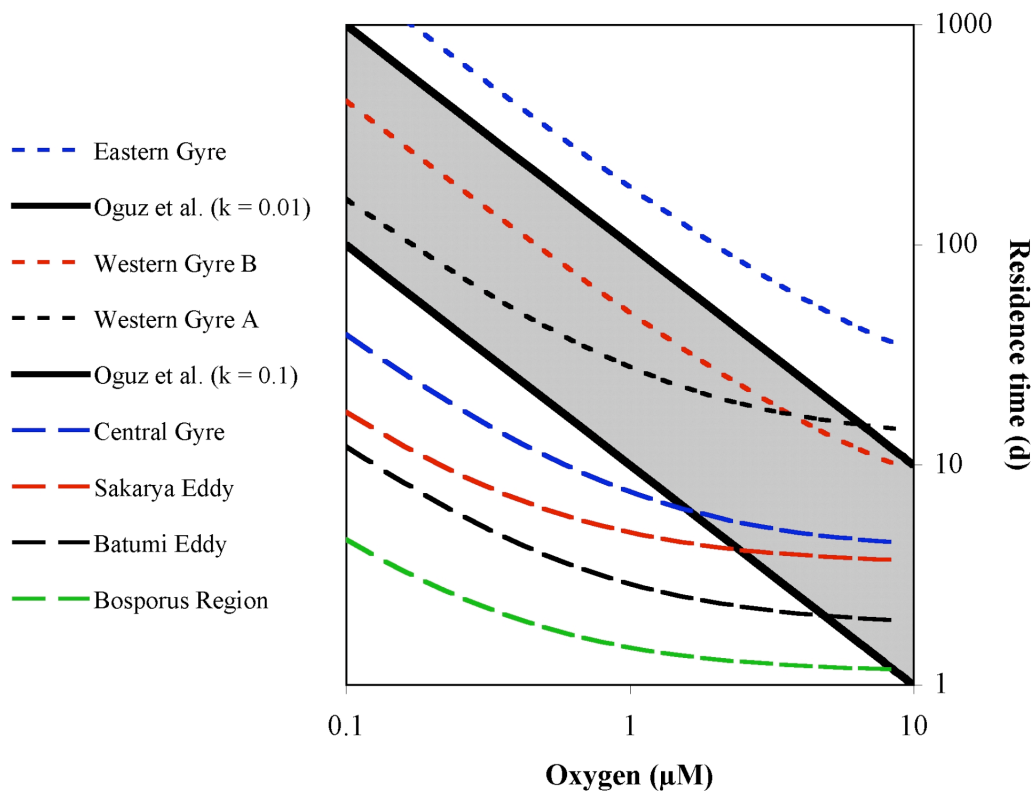


Figure 3.4 Predicted dissolved Mn residence times as a function of  $[O_2]$  utilizing  $K_E/V_{E-max}$  data (this work) and the rate constants in Oguz et al., 2001. The shaded area corresponds to the range modeled by Oguz et al. Note the divergence at low  $[O_2]$  between residence times calculated using the Oguz et al. model and kinetic data from four coastally influenced sites (this study—large dashes).

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## CHAPTER IV

**Cultivation-dependent and cultivation-independent identification of  
abundant, Mn(II)-oxidizing, siderophore-producing  
*Pseudoalteromonas* spp. from the Black Sea suboxic zone**

## Abstract

In the Black Sea suboxic zone, microbial Mn(II) oxidation produces particulate Mn(III/IV) oxides that sink into anoxic water and abiotically oxidize sulfide. The process controls, in part, chemical dynamics between oxic and anoxic regimes. In this work, a rigorous DNA extraction protocol was developed, a 16s rRNA gene clone library of suboxic zone bacteria was created and the resulting sequences compared to Mn(II) oxidizing isolates from the Black Sea. Proteobacterial sequences dominated the library, with small contributions from the phyla Actinobacteria and Planctomycetes and “SBR1093”. *Pseudoalteromonas* was the dominant genus, representing 91 of 138 sequences. The remaining sequences were more evenly distributed across diverse genera including *Pseudomonas*, nitrite-oxidizing *Nitrospina* and sulfide-oxidizing *Arcobacter*. The dominant clone, BS01-003, represented over 41% of the clone library and was >99% identical to the Mn(II)-oxidizing isolate BS03-23. Two *Pseudoalteromonas* spp. from the Black Sea were shown to produce the Mn-binding siderophore desferrioxamine E, which is suspected of binding and stabilizing Mn(III) in the suboxic zone. Thus, the Black Sea bacterial population at the Mn oxide-sulfide interface is, at times, dominated by *Pseudoalteromonas* strains, some of which are identical or closely related to cultivable Mn(II)-oxidizing strains and may be linked to production of Mn(III)-binding siderophores.

## Introduction

The Black Sea is well-known as an excellent study site for reduction and oxidation (redox) processes (Murray et al., 1995; Oguz et al., 2001). Stratified conditions occur year round below the winter mixed layer (~40 m depth) and anoxia prevails at relatively shallow depths (~90-200 m) throughout the basin (Stanev et al., 2004). The transitional layer from oxic to anoxic conditions, which occurs on millimeter scales in ocean sediments, occurs over meters in the Black Sea. This layer, known as the suboxic zone (SOZ), is defined by the O<sub>2</sub> concentrations < 5 μM and sulfide < 1 μM (Murray et al., 1989).

The Black Sea SOZ is generally 10 to 40 m thick, with the thickest regions occurring in the central gyres (Murray et al., 1995; Oguz et al., 2001). A variety of major environmental redox cycles—oxygen, nitrate, nitrite, metal and sulfate reduction and sulfide, Mn and ammonia oxidation— are catalyzed in a predictable vertical sequence, both biologically and abiotically, in or near the SOZ (Figure 4.1). Since these chemical strata occur over the span of meters, the SOZ is an excellent site to explore processes that occur on un-resolvable scales in other environments.

One unique feature of the Black Sea SOZ is the prevalence of Mn cycling. Typical oceanic concentrations of Mn are in nM range while in the Black Sea concentrations of ~8 μM occur just below the SOZ (Bruland, 1983; Landing and Bruland, 1987; Lewis and Landing, 1991). In the SOZ, Mn occurs in both dissolved and solid-phase forms (Lewis and Landing, 1991; Tebo, 1991). Mn cycling in the SOZ begins with O<sub>2</sub>-dependent oxidation of dissolved Mn(II) to solid-phase

Mn(III/IV) oxides, which sink to the suboxic/anoxic interface. There, the Mn oxides are reduced abiotically to Mn(II) by sulfide, which is oxidized to sulfur (Konovalov et al., 2004; Konovalov et al., 2001). The newly-reduced Mn(II) is then subject to diffusion transport such that some returns to be oxidized in the SOZ again. Thus, Mn plays a cyclic role in maintaining the SOZ by linking O<sub>2</sub> and sulfide consumption without overlap between the two and creating a defined region where other electron acceptor/donor couples can be exploited by microbes.

Mn(II) oxidation in the Black Sea is biologically catalyzed: oxidation rates observed in the Black Sea are orders of magnitude more rapid than chemical oxidation rate constants predict and this activity is inhibited by biological poisons (Tebo, 1991; Tebo et al., 1991). Numerous Mn(II)-oxidizing bacteria (MnOB) have been isolated from both fresh- and seawater environments and Mn oxide production by these organisms is also faster than abiotic rates and sensitive to biological poisons (Tebo et al., 2004). However, no MnOB have been linked to Mn(II) oxidation in the Black Sea.

The MnOB are a phylogenetically indistinguishable group, as they are affiliated with three different phyla, Proteobacteria, Firmicutes and Actinobacteria (Tebo et al., 2004). Even within a given phylum, the MnOB are not monophyletic—within the Proteobacteria, the species that oxidize Mn(II) are spread among three different classes, the  $\alpha$ -,  $\beta$ - and  $\gamma$ -proteobacteria. This wide-but-diffuse distribution of MnOB throughout unrelated bacterial clades prevents cultivation-independent surveys from linking Mn(II) oxidation to organisms found in situ.

Without knowledge of the indigenous MnOB, our understanding of Mn(II) oxidation in the Black Sea is limited to activity and chemical measurements. While known to be biologically-catalyzed, little is known about the mechanism, the functional role or the parameters that induce Mn(II) oxidation in the Black Sea. This is true of nearly all sites where Mn(II) oxidation is prevalent—only in freshwater surface films and at the Loihi seamount have specific MnOB, *Leptothrix* and *Pseudoalteromonas* spp., respectively, been identified as numerically abundant and thus, likely important in Mn oxide production in their respective environments (Ghiorse and Chapnick, 1983; Moyer et al., 1995; Siering and Ghiorse, 1997; Templeton et al., 2005).

The need for MnOB isolates and data on their abundance in situ is highlighted by recent research demonstrating that bacterial siderophores, previously thought to be specific ligands for Fe(III), both promote Mn(II) oxidation and bind the resulting oxidation product, Mn(III), in a stable aqueous complex (Duckworth and Sposito, 2005; Parker et al., 2004). Previously, it was thought that dissolved Mn in natural waters was reduced, i.e. Mn(II), that biogenic Mn oxides were produced enzymatically and that any Mn(III) in solution would rapidly disproportionate to Mn(II) and Mn(IV). Siderophore-mediated processes complicate this scenario. Dissolved Mn may contain both reduced and oxidized Mn, while Mn oxides may be the product of direct Mn(II) oxidation or result from spontaneous disproportionation of Mn(III) upon degradation, biologically catalyzed or not, of Mn(III)-siderophore complexes. Interestingly, at least one MnOB, *Pseudomonas putida* MnB1, can both enzymatically oxidize Mn(II) to Mn oxides and produce Mn(III)-binding siderophores (Parker et al., 2004). Thus, isolating

MnOB and determining their abundance in the Black Sea SOZ are important first steps to describing the microbial pathways that lead to production of Mn oxides in the environment.

This research was undertaken with the primary goal of characterizing the microbial population associated with the Mn oxide-rich layer through cultivation-independent 16 ribosomal RNA gene surveys. Over the course of three Black Sea cruises and in collaboration with other members of the Tebo lab, a fortuitous link was made between abundant *Pseudoalteromonas* spp., their Mn(II)-oxidizing capacity and siderophore production.

## **Methods**

### **Cruises and sampling**

Water samples were obtained from the standard western cyclonic gyre site in the Black Sea in May-June 2001 and April-May 2003 aboard the R/V Knorr and in March-April 2005 aboard the R/V Endeavor (Figure 4.2). Sampling was accomplished with 24-place rosettes equipped with Niskin bottles and SeaBird CTD sensors. Samples were located relative to water density (i.e.  $\sigma_T$ ) instead of depth because stratification in the Black Sea limits diapycnal mixing and processes therefore occur on diagnostic density surfaces, irrespective of depth.

Manganese measurements were made using the formaldoxime method after filtration of 50 ml seawater through 0.2  $\mu\text{m}$  polyethersulfone filters (Brewer and Spencer, 1971). This method operationally defines Mn as particulate and dissolved fractions and does not discriminate by oxidation state. The particulate Mn fraction is



composed of Mn(III/IV) oxides and adsorbed Mn(II) while the dissolved fraction is composed of Mn(II) and, potentially, soluble Mn(III)-ligand complexes.

Cells for DNA and culture isolation were collected on 142 mm diameter, 0.2  $\mu\text{m}$  pore-size polycarbonate filters. Water was pressure-filtered under  $\text{N}_2$  gas directly from Niskin bottles on deck using custom-built clamps to keep the bottle closed and quick disconnect valves to introduce gas. Pressure was maintained at or below 1 atmosphere throughout filtration. Filters for DNA isolation were preserved in plastic freezer bags by adding 3 ml RNAlater (Ambion, Austin, TX), incubating for 24 h at 4  $^\circ\text{C}$  and then freezing at  $< -20^\circ\text{C}$ . Filters were returned to the lab by air freight, on dry ice, either from Istanbul, Turkey, immediately after each cruise or from a U.S.A. port upon the ship's return.

### **Culture media and growth conditions**

Isolates were obtained during the 2003 cruise by G. Dick and maintained at Scripps Institution of Oceanography by Y. Lee. Initial culturing was done on spread plates containing  $M_{\text{BSW}}$  or  $K_{\text{BSW}}$  medium inoculated with serially-diluted Black Sea water or particulates filtered from Mn oxide-rich layers.  $M_{\text{BSW}}$  medium consisted of 1 liter Black Sea water amended with 0.005% yeast extract and bacto peptone, 2 mM  $\text{KHCO}_3$ , 10 mM HEPES buffer (pH 7.8), 100  $\mu\text{M}$   $\text{MnCl}_2$  and 15 g Noble agar.  $K_{\text{BSW}}$  medium consisted of 1 liter Black Sea water plus 0.5 g yeast extract, 2.0 g bacto peptone, 20 mM HEPES buffer (pH 7.8), 100  $\mu\text{M}$   $\text{MnCl}_2$  and, for plates, 15 g Noble agar. Mn(II) oxidation was confirmed by testing colonies with a 0.04% solution of the dye leucoberbelin blue (LBB). LBB becomes brilliant blue in reaction with oxidized

Mn (Krumbein and Altman, 1973). Once isolated, strains were maintained on standard K medium, identical to K<sub>BSW</sub> except for the substitution of Black Sea water with 750 ml filtered, aged natural seawater and 250 ml deionized water.

Isolates were grown in liquid K medium to test for siderophore production by the CAS shuttle assay as previously described (Schwyn and Neilands, 1987). Isolates were also tested for siderophores on ASG/CAS plates (Haygood et al., 1993).

### **Spore preparations**

*Bacillus* sp. SG-1 was grown for 6-8 days in liquid K medium on a rotary shaker, until the majority of cells in the culture had sporulated. Spores were collected by centrifugation, vegetative cells were lysed and cell debris were removed as previously described (Dick et al., 2006). The resulting spore preparation was kept in 18 MΩ H<sub>2</sub>O at 4° C for up to a week. Just prior to use, spore aggregates were disrupted by low-pressure passage through a French pressure cell.

### **DNA isolations**

Up to 200 mg filter membrane or 200 µl of dilute spore preparation was placed in a 2.0 ml screw-cap tube containing 200 mg each of 0.1, 0.5 and 2.0 mm zirconium/glass beads (Biospec, Bartlesville, OK) and 580 µl lysis solution: 300 mM EDTA, 300 mM NaCl and 300 mM Tris buffer (pH 7.5). Immediately after the addition of 70 µl of fresh, 15% sodium dodecyl sulfate and 35 µl of 1 M dithiothreitol in 0.01 M sodium acetate, each tube was vortex mixed and incubated at 70 ° C for 30 minutes. Samples were then cooled to < 40 ° C, amended with 14 µl of 5% lysozyme solution and incubated at 37 ° C for 20 minutes. The samples were then processed on

a FastPrep beat-beating machine (Qbiogene, Irvine, CA) for 45 seconds at setting 6.5. Cell debris was collected by centrifugation for 1 minute at 14,000 x g and 500  $\mu$ l supernatant was transferred to a new tube containing 75  $\mu$ l of 2 M potassium chloride. Tubes were vortex mixed, incubated on ice for 5 minutes. Insoluble potassium-dodecyl sulfate-protein complexes were then pelleted by centrifugation for 5 minutes at 14,000 x g. The resulting supernatant was then added to a Montage PCR clean-up unit (Millipore, Billerica, MA) in 400  $\mu$ l aliquots and centrifuged for 15 minutes at 1,000 x g. The filtrates were discarded and the process repeated until the entire sample had been passed through the filter unit; the final supernatant aliquot was brought up to 400  $\mu$ l after transfer with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). After the entire sample had been passed through the filter (typically two rounds of 400  $\mu$ l), 400  $\mu$ l TE buffer was added and centrifuged through as above to further wash the DNA on the filter.

The Montage filter unit was then inverted into a fresh collection tube and 40  $\mu$ l TE buffer was added to the filtrate side. DNA was collected by centrifuging this TE aliquot back through the filter for 15 minutes at 1,000 x g. The resulting DNA solution was transferred to a 1.5 ml screw-cap tube and frozen for later use.

For comparison to commercially available methods, two kits for extracting DNA from soil and environmental samples were used to extract DNA from 200  $\mu$ l of the same spore preparation. Both the Qbiogene FastDna kit (Kit Q; Qbiogene) and the MoBio SoilDNA kit (Kit M; MoBio, Carlsbad, CA) were used as suggested by the manufacturers.

### **DNA quantification, amplification and Cloning**

DNA solution concentrations were determined by the PicoGreen assay (Invitrogen, La Jolla, CA) in a 200  $\mu$ l assay according to the manufacturer's protocol and subsequently diluted to 1 ng per  $\mu$ l in TE buffer. 16S ribosomal RNA genes were amplified by PCR using 10 ng DNA in 25  $\mu$ l reactions consisting of the 1X reaction buffer (Boehringer-Mannheim; Indianapolis IN), 1.5 mM MgCl<sub>2</sub>, 0.25 mM each dNTP (all Gibco, Gaithersburg, MD), 0.5 U Taq DNA polymerase (Boehringer-Mannheim), 0.1  $\mu$ M each primer and an additional 0.8 mM MgCl<sub>2</sub> to account for the EDTA in the sample buffer. Primers 27f (5' AGA GTT TGT TCM TGG CTC AG 3') and 1492r (5' TAC GGY TAC CTT GTT ACG ACT T 3') targeted a ~1400 bp region of the 16S rRNA gene homologous to positions 8-1492 in *E. coli*. Thermal cycling consisted of an initial 5 min., 94 °C denaturation followed by 30 cycles of 1 min., 94 °C; 1 min 50 °C, 2 min. 72 °C. Final extensions were carried out at 72 °C for 7 min.

Clone libraries were created with a TA-Topo kit in conjunction with a pCR2.1 vector and blue/white screening as specified by the manufacturer (Invitrogen). 192 white colonies were picked with pipette tips and suspended in 10  $\mu$ l sterile H<sub>2</sub>O, each pipette tip was immediately used to inoculate each picked colony into 1 ml liquid culture in LB supplemented with 25  $\mu$ g/ml kanamycin. Liquid cultures were grown overnight at 37° C, supplemented with glycerol to a final concentration of 10% and archived at -80° C.

Reagents were added directly to the colony-water suspensions for PCR with primers (M13, Invitrogen) targeted to the vector. PCR conditions were identical to the

original amplification without the additional MgCl<sub>2</sub>. Amplification products were cleaned using Montage 96-well plates, suspended in H<sub>2</sub>O and frozen.

Sequencing was performed using BigDye 3.1 reaction mix (Applied Biosystems, Foster City, CA) in 10 µl reactions with 4 µl sample and 1 µM primer. All samples were first sequenced with the 338f universal primer (5' ACT CCT ACG GGA GGC AGC 3') to de-replicate the library. Unique inserts were then fully sequenced using primers 536r (5' GTA TTA CCG CGG CTG CTG G 3') 27f and 1492r.

Sequence assembly was performed using Sequencher software (Gene Codes, Ann Arbor, MI) and screened for anomalous sequences (chimeras and poor assemblies) using the Mallard software package available from Cardiff University's School of Biosciences (<http://www.cf.ac.uk/biosi/research/biosoft/Mallard/index.html>). Mallard applies the Pintail sequence comparison algorithm to an entire library of sequences (Ashelford et al., 2005). All sequences reported by Mallard as anomalous at a 95% cut-off were further analyzed using Pintail to compare them to a high-scoring BLAST match from a cultivated species (Altschul et al., 1990). Sequences that appeared anomalous when compared to closely-allied BLAST matches were removed from further analyses.

Clone and isolate sequences were aligned to selected reference sequences using the NAST alignment algorithm at [greengenes.lbl.gov](http://greengenes.lbl.gov). Seaview software was used to edit NAST alignments manually and delete gaps common to all sequences (Galtier et al., 1996). The resulting aligned sequences were used to construct a

phylogenetic tree using PhyloWin software with the Neighbor-Joining method, Jukes and Cantor corrected distances, pairwise gap replacement and 500 bootstrap replicates (Galtier et al., 1996).

### **Quantitative PCR**

Quantitative PCR was performed on a Stratagene MX3000p thermal cycler with Brilliant<sup>®</sup> SYBR<sup>®</sup> Green qPCR reagents according the manufacturer's instructions (Stratagene, La Jolla, CA) and using the primers and temperatures specified previously for determining *Pseudoalteromonas* abundance (Skovhus et al., 2004). Standard curves consisted of  $10^2$  to  $10^7$  copies of PCR-amplified 16S genes from isolate BS03-23 that were cleaned and quantified as above. Copy number was calculated assuming a gene length of 1500 bp and  $650 \text{ g DNA mol}^{-1} \text{ bp}^{-1}$ . All standards and samples (1 ng DNA each) were amplified in triplicate. Cycle thresholds were picked by the analysis software and checked manually to assure that the exponential phase of amplification was utilized.

### **Mass spectrometry**

Spent medium from strains BS03-44 and -45 was filter-sterilized and sent to the Butler laboratory at the University of California Santa Barbara. Further sample preparation and mass spectrometry analyses were kindly performed by A. Tincu as previously described (Martinez et al., 2001). Briefly, organic compounds were extracted from spent medium by solid-phase extraction using C-18 Sep Pack columns (Waters, Milford, MA). The organic phase was eluted with methanol and resuspended 1:5:1 (sample: methanol: 1% formic acid in water). Mass/charge ratios of positively charged ions were determined using electrospray ionization. Tandem MS was

performed on m/z peaks corresponding to known siderophores to confirm identifications by fragmentation pattern.

## **Results**

### **DNA isolation**

*Bacillus* sp. SG-1 spores were utilized to test the efficacy of the described DNA isolation protocol because they represent a difficult-to-lyse target cell and, in terms of Mn(II) oxidation, potentially active catalysts in the environment. Significantly more DNA from SG-1 spores was harvested with the described protocol than with either commercial kit tested (Figure 4.3). Yield calculations gave wide ranges; spore clumps limited the accuracy of visual counting by phase-contrast microscopy. Assuming 5 fg DNA spore<sup>-1</sup>, and scaling for the volume of sample actually recovered post-lysis, lysis efficiency estimates ranged from 6 to 26% for the CMF protocol, 4 to 17% for Kit Q, 3 to 12% for Kit M and 1 to 3% for the TE buffer.

### **Clone library**

The 2001 clone library was created from a 10 liter water sample collected in the Mn oxide maximum ( $\sigma_T = 16.00$ ) of the Western Gyre (Figure 2). The library yielded 181 inserts in the correct size range (~1300-1600 bp) from 192 picked transformants. Mallard and Pintail analyses (see methods) identified 42 of the 181 inserts (23.2%) as chimeric or anomalous. The remaining 138 inserts contained 40 sequences that were unique at a similarity threshold of 99%. The abundance of each unique sequence in the library is detailed in the upper left panel of Figure 4.4.

Insert sequences most closely related to known  $\gamma$ -proteobacteria dominated the library, with 116 inserts and 21 unique sequences. Sequences similar to the  $\gamma$ -

proteobacterial genus *Pseudoalteromonas* were most abundant, accounting for 91 inserts, or 66% of the library. One *Pseudoalteromonas*-like sequence, BS01-003, accounted for 41% of the library. Other  $\gamma$ -proteobacterial sequences included 14 *Pseudomonas*-like (6.5%), 8 *Marinobacter*-like (3.6%), 1 each similar to *Photobacterium*, *Acinetobacter* and *Methylobacter* (0.7% each), and 8 inserts that matched described sequences only distantly. Two of these unclassified sequences branched within a group that includes the genus *Legionella* while the remainder, including a unique sequence (BS01-045) that appeared 4 times in the library, had no significant similarity to any described genus.

The remainder of the clone library consisted of sequences appearing once or twice in the library and similar to  $\alpha$ -,  $\epsilon$ -, and  $\delta$ -proteobacteria classes, the candidate genus *Microthrix*, the phylum Planctomycetes, and “SBR1093”, a phylum-level group isolated from a sequencing batch reactor. An additional two inserts, BS01-104 and -043 were most similar to a group of sequences recovered from various environments that have no cultivated affiliates, apparently as deep as the phylum level.

### **qPCR**

To assess the abundance of *Pseudoalteromonas*-like sequences, a kinetic or quantitative PCR (qPCR) approach was used in conjunction with a depth (density) profile of the SOZ. The general method determines the abundance of specific target sequences in a DNA sample by monitoring the rate at which targets are amplified by PCR. The PCR product abundance is measured by fluorescence, and the reaction cycle at which this fluorescence reaches a threshold level is compared to that of known standards. Quantitative PCR with primers specific for *Pseudoalteromonas* spp.



(Skovhus et al., 2004) produced a linear response between  $10^2$  and  $10^7$  control targets (Figure 4.7). Efficiency (calculated as  $E = 10^{-1/s} - 1$  where  $s$  is the slope of the standard curve) was estimated at 86% with the specific primer set. Primers designed to amplify the same region of the 16S rRNA gene in all bacteria (i.e. “universal”) amplified with an apparent efficiency of 115% but low sensitivity resulting in all experimental samples having gene copy content  $>10^7$  per ng population DNA (pDNA), higher than the highest copy number standard. Given that the 1 ng pDNA added to each PCR is equal to  $9.3 \times 10^5$  Mbp of DNA, a 16S gene content of  $>10^7$  would imply 10 copies per Mbp, or about 40 copies per 4 Mbp genome. This over-estimation of gene content is likely due to the one-base mismatch between the universal primer Univ907RC and the *Pseudoalteromonas* sequence that depressed the amplification efficiency of the standard curve, relative to the pDNA that contained a variety of sequences perfectly matched to the universal primer. Thus, the results of the *Pseudoalteromonas*-specific primers are reported as gene copies per ng pDNA.

While not ideal since the absolute population size per ng DNA may be different in each sample, the results from replicate DNA isolations in Figure 4.8 indicate that replication at both the DNA isolation step and the DNA quantitation steps was reliable. Estimated *Pseudoalteromonas* gene abundance in the seven samples tested was between 300 and 400,000 copies ng pDNA<sup>-1</sup>. The 2001 samples from  $\sigma_T = 15.95$  and 16.00 both exceeded 10,000 *Pseudoalteromonas* gene copies ng pDNA<sup>-1</sup>, while all 2005 samples were below 2300 copies ng pDNA<sup>-1</sup>. Variation within triplicate samples, expressed as one standard deviation, is indicated by the error bars in

Figure 4.8 and was generally low. Replicate DNA extractions (a and b samples) from the 2005 filters had similar *Pseudoalteromonas* gene abundance.

### **Mn(II) oxidizing isolates**

*Pseudoalteromonas* isolates and data regarding additional Mn(II)-oxidizing isolates from the 2003 Black Sea cruise were kindly provided by fellow Tebo lab members G. Dick and Y. Lee. In total, Dick and Lee maintained 53 Black Sea strains in pure culture that produced visible Mn oxides or oxidized the Mn oxide-specific dye LBB (Table 1). Sequence analyses of the PCR-amplified 16S rRNA genes from these strains indicated that three genera, *Bacillus*, *Pseudomonas* and *Pseudoalteromonas* and 9 species (i.e. 16S rRNA gene sequences with >99% similarity) were represented in the isolate library (Table 1). Notable differences in growth rate and Mn oxide production were apparent among *Pseudoalteromonas* strains both between and within the three species (Table 2). All strains produced colonies that reacted with LBB, indicating the presence of oxidized Mn (i.e. +3 or +4 oxidation states) despite the absence of visible Mn oxide production by strains BS03-24 and -44.

Only strains BS03-23 and BS03-24 grew on the glycerol-based CAS medium, producing 4-5 mm orange halos, indicative of siderophore production. All strains grew robustly in liquid K medium; filtered, spent medium was checked for CAS activity. Spent medium from strains BS03-44 and -45 reacted with CAS dye (Table 2). Solid phase extraction followed by mass spectrometry revealed peaks corresponding to the siderophore desferrioxamine E (DFOE+H<sup>+</sup>, m/z = 599.32) in spent medium from both strains (Figure 4.9). The mass spectrum contained two additional ions with m/z 1 and 2 mass units greater than expected and peaks consistent with Fe-DFOE

complexes ( $653 < m/z < 655$ ). Mn-DFOE complexes were not observed, possibly due to the formic acid in the sample solution—Mn-DFO complexes are not stable below pH 6.0 (Duckworth and Sposito, 2005).

## **Discussion**

Identifying the microorganisms responsible for specific biogeochemical processes in the environment is an important first step to defining, monitoring and predicting processes of interest in situ. In the Black Sea, microbial Mn(II) oxidation promotes vertical oxidant transfer and, thus is a key part of the biogeochemical processes that promote chemical stratification. Understanding the mechanism and magnitude of Mn(II) oxidation requires knowledge of the reactants, products and the occurrence and prevalence of the microbes catalyzing the process. The cultivation-based and cultivation-independent approaches utilized in this work established a link between Mn(II) oxidation, siderophore production and bacteria abundant in the SOZ.

### **DNA extraction**

Analyses based on microbial pDNA can only provide unbiased results when cell lysis and pDNA recovery are unbiased. Characterizing microbial populations with Mn(II) oxidizing activity presents an additional challenge to DNA isolation because spores cannot be discounted as inactive, and therefore unimportant, members of the population. Since Mn(II) oxidation by *Bacillus* spp. spores is well-documented (Francis and Tebo, 2002) and spores are generally considered a difficult microbial cell type to lyse, a DNA isolation protocol designed to lyse spores was tested prior to isolating DNA from Black Sea samples.

The chemical-mechanical-filtration (CMF) DNA isolation, is a hybrid of chemical disruption protocols demonstrated to permeate spore coats, mechanical lysis (bead beating) and filtration through a molecular weight cutoff membrane to purify the final DNA solution. Chemical disruption was accomplished by the addition of a reductant, dithiothreitol, that disrupts protein disulfide bonds, a protein denaturant, sodium dodecyl sulfate and lysozyme, an enzyme that degrades the peptidoglycan structural component of the spore coat (Fischer et al., 1995). Subsequent mechanical lysis was performed with three size classes of zirconium-silicate beads to disrupt spores and spore aggregates of different sizes. The DNA purification steps that followed lysis were performed with 60% of the original lysis volume (commercial kits generally utilize ~50% or less), minimizing DNA lost in the pelleted cell debris and beads. Proteins were removed by the addition of potassium to precipitate insoluble potassium dodecyl sulfate-protein adducts (Trask et al., 1984). The DNA solution was further purified by filtration on molecular weight filters with a cut-off of ~100 kDa.

The CMF method nearly doubled the DNA quantity recovered from identical *Bacillus* sp. SG-1 spore solutions, relative to the best of the two commercial “Soil DNA” kits (Figure 4.3). (The DNA in the TE buffer solution control is likely due to mother cell remnants carried over during the spore preparation.) Since the exact chemistry and methods are not published for either kit, it is difficult to determine why the kits were less effective than the described method, other than the fact that only 40 and 50%, respectively, of the original lysis volume is eventually recovered by Kits M and Q. While both kits utilize a detergent (i.e. a solution prone to foaming) that can be precipitated in a similar fashion to the KDS-protein adduct used in the CMF method,

neither utilizes any pungent chemicals (e.g. reductants like DTT or cysteine) nor any enzymes requiring specific temperature incubations. Kit M utilizes crushed mineral gravel for lysis while Kit Q uses beads similar to those utilized by the method described in this work. While the beads used in Kit Q and the CMF method offer a defined range of sizes, the crushed mineral gravel is generally the same size, providing little range of ‘projectiles’ for shearing a range of cell aggregate sizes. The purification methods of both kits appear similar; following protein precipitation, a concentrated salt solution is added to the sample and introduced to a white mineral similar to silica gel. Such silica-based methods are common around the molecular biology laboratory, and generally lauded not for their recovery but speed and ease of use. The molecular weight cutoff membrane used in the CMF method is less rapid, but the DNA stays in solution throughout the process, potentially facilitating greater recovery.

The key points of this method are that it (i) directly targets spore coat proteins and peptidoglycan, (ii) disrupts aggregates and lyses spores with the use of specific size beads and (iii) purifies DNA without the use of silica gel. Given the CMF method’s relative efficiency with spores, it is expected that other cell types with less protein and/or peptidoglycan per cell would also be lysed.

The March, 2005 Black Sea samples (utilized for qPCR in this study) produced between 0.51 and 0.63  $\mu\text{g}$  DNA per isolation. Each isolation was from one-quarter of a filter, or approximately 2.5 liters of Black Sea water. Assuming 2.5 fg DNA cell<sup>-1</sup> in seawater and between 2 and 6 x 10<sup>5</sup> cells ml<sup>-1</sup> in the Black Sea SOZ, this equates to an estimated yield of 13 to 50% (Button and Robertson, 2001; Sorokin et al., 1995).

Such yields are reasonable, given that some DNA is lost in the method because no more than 60% of the original solution is actually harvested. Additionally, 10 l is an upper bound for volume filtered as the Niskin bottles were often sampled by other researchers ahead of filtration for this work. Additionally, Niskin bottles do not contain or dispense precise volumes.

### **Clone library**

Establishing a robust DNA isolation method facilitated the construction of a clone library from cells collected in the Black Sea SOZ. This is not the first clone library created from the Black Sea SOZ; 16S sequences and terminal restriction fragment length polymorphisms (TRFLPs) from DNA collected over multiple depths during the 1988 Black Sea cruises were recently published (Vetriani et al., 2003). Unfortunately, that work was not resolved with respect to the geochemical parameters that make the Black Sea a useful study site. In fact, the sampling station(s) was/were not specified, only the depth of each collection. This is problematic because processes in the Black Sea are density, not depth, specific—diagnostic chemical characteristics, e.g. the onset of sulfide, occur at varying depths throughout the basin, but usually at or near the same density surface. Thus, the Vetriani et al. study (2003) is a useful survey of microbial sequences found in the Black Sea water column but offers little resolution as to where, or what processes are associated with which sequences.

The library in this study was generated from cells collected in the Western Central Gyre at a density of  $\sigma_T = 16.00$  where Mn, ammonia and sulfide oxidation and nitrate and nitrite reduction all coincide (Figure 4.1). Generally, the phylotypes identified in the library were consistent with those found by Vetriani et al. (2003),

across a range of depths (Figures 4.5, 4.6). Most interesting were the numerically dominant *Pseudoalteromonas*-like sequences, one of which proved identical to an Mn(II)-oxidizing species isolated two years later on a separate cruise (Figure 4.4, 4.5). Other phylotypes of note within the library included those similar to organisms known to catalyze suboxic redox processes and sequences related to uncultivated clades, for which little or no metabolic data are available. The latter phylotypes will be discussed first, followed by the *Pseudoalteromonas* spp.

In terms of numerical dominance, *Pseudomonas*-like sequences were second only to the *Pseudoalteromonas*-like sequences (Figure 4.4). The genus *Pseudomonas* is characterized by diverse, heterotrophic metabolisms including such SOZ processes as aerobic heterotrophy, denitrification and Mn(II) oxidation. Thus, *Pseudomonas* spp. may be well suited to SOZ conditions and data from the clone and isolate libraries indicate they are numerically important and potentially involved in Mn(II) oxidation.

Other  $\gamma$ -Proteobacteria of note include the three *Marinobacter*-like sequences and the three sequences, BS01-119, -103 and -045, associated with a deeply branching group without cultivated relatives (Figure 4.5). The described *Marinobacter* spp. include Mn(II)-oxidizing strains and siderophore producers, and are, like *Pseudomonas* spp., generally metabolically diverse (Kersters et al., 2003; Martinez et al., 2000; Templeton et al., 2005). The most enigmatic sequence that grouped with the  $\gamma$ -proteobacteria was BS01-045. This sequence shared little homology with publicly available sequences (<87%) yet was abundant, appearing four times in the library. Its nearest neighbors in the clone library, BS01-119 and -103, were affiliated

with clones obtained from sulfide incubators, suggesting they may be involved in sulfide or sulfur oxidation, an active process at the density sampled.

As also reported by Vetriani et al. (2003),  $\alpha$ -proteobacteria sequences related to the SAR-11/*Pelagibacter* clade were recovered, but unlike those libraries, additional  $\alpha$ -proteobacterial clades were present (Figure 4.6). Five sequences distinct from the *Pelagibacter* group were recovered that grouped with deep Pacific Ocean clones, clones recovered from basalt surfaces at the Juan de Fuca trench and a clone from the Arabian Sea, which, like the Black Sea, harbors suboxic waters. As a group, these  $\alpha$ -proteobacterial sequences appear to be associated with either oligotrophic or micro-oxic conditions but not closely related to the known Mn(II)-oxidizing  $\alpha$ -proteobacterial species (cf. Tebo et al., 2004).

The  $\epsilon$ -proteobacteria were represented by only one sequence in the library, BS01-057, which was most closely related to the genus *Arcobacter* (Figure 4.6). Vetriani et al. (2003) recovered a distantly-related sequence from 217 meters depth and Thamdrup et al. reported that *Arcobacter*-like organisms from Black Sea shelf sediments (i.e. beneath oxic waters) dominated enrichments of Mn oxide-reducing bacteria (Thamdrup et al., 2000). Candidatus *Arcobacter sulfidicus* is a marine autotroph known to oxidize sulfide under microaerobic conditions and utilize the reverse tricarboxylic cycle to fix CO<sub>2</sub> (Hugler et al., 2005; Wirsen et al., 2002). Since the Mn oxide and sulfide profiles overlap within the same density interval sampled, this raises the possibility that such organisms might couple sulfide oxidation to Mn oxide reduction, a process currently believed to be abiotic (Burdige and Nealson, 1986; Luther et al., 1991; Yao and Millero, 1993). While a biological role in coupled



Mn-S cycling is speculative, micro-oxic Fe(II) oxidation at circumneutral pH is known to be biologically-catalyzed despite the speed with which abiotic Fe(II) oxidation proceeds under those conditions (Edwards et al., 2004). Speculation aside, the *Arcobacter*-like BS01-057 highlights the numerous electron acceptor/donor couples that may be biologically utilized in the Black Sea SOZ.

Another group known to catalyze anaerobic metal and sulfur cycling are the  $\delta$ -proteobacteria (Kersters et al., 2003). Five sequences that grouped with  $\delta$ -proteobacteria appeared in the clone library, including two that branched deeply, BS01-025 and -162 (Figure 4.6). Clone sequences BS01-063 and -106 were more closely related to described sequences, although only those from seawater samples. The only  $\delta$ -proteobacteria-like clone sequence suggestive of some role in the SOZ was BS01-023, which grouped with the lithotrophic nitrite-oxidizing genus, *Nitrospina* (Watson, 1971). Nitrite maxima are typically observed at the upper onset of nitrate and again at the base of the nitrate profile in the SOZ (Murray et al., 2005). In previous work, the upper nitrite maxima was identified as the most active region of chemosynthesis related to nitrite oxidation, but this activity was also observed in the SOZ (Sorokin et al., 1995). This raises the possibility that the *Nitrospina*-like species identified here may couple nitrite oxidation to extremely low oxygen levels as Mn(II) oxidizers do (see Chapter III), or to an alternate electron acceptor such as Mn oxides. While, organisms are not yet known to couple Mn oxide reduction to nitrite oxidation, the chemical reaction has been demonstrated—in contrast to the reverse reaction—Mn(II) oxidation coupled to nitrate reduction—which is often proposed to link suboxic

Mn and N cycling (Luther and Popp, 2002; Luther et al., 1997; Murray et al., 1995; Oguz et al., 2001).

Another feature of the N cycle within the SOZ is the overall loss of fixed, inorganic nitrogen species (ammonium, nitrite and nitrate) to gaseous N<sub>2</sub> (Codispoti et al., 1991; Murray et al., 1995; Oguz et al., 2001). Anaerobic ammonia oxidation (anammox) is known to be a fixed-nitrogen loss process in the SOZ and has been linked to anammox bacteria from the order Planctomycetales (Kuypers et al., 2003). While no sequences closely related to the Kuypers et al. (2003) sequences were recovered in the clone library described here, one clone sequence, BS01-079, was somewhat similar to a sequence from Vetriani et al. (2003) and most closely matched to sequences from phylum Planctomycetes (Figure 4.6). A recent dedicated survey of Planctomycete diversity in the Black Sea recovered Planctomycetes-like sequences from throughout the water column but these sequences were not similar to BS01-079 (Kirkpatrick et al., 2006). It remains to be seen if the Planctomycetes spp. in the Black Sea are all capable of anammox reactions but anammox seems restricted to the interface of nitrite and ammonium in the SOZ (Kuypers et al., 2003).

In addition to sequences with some similarity to described, cultivated organisms, three sequences from deeply branching groups without cultivated relatives were recovered. Two of these, BS01-104 and -043, clustered with a group of sequences recovered from marine environments, including the Black and Arabian seas (Figure 4.6). The remaining sequence, BS01-081, was affiliated with the SBR1093 group, a phylum-level group first identified in a sequencing batch reactor (Hugenholtz et al., 2001). While identifying any potential role for these bacteria in the SOZ is not

currently possible, the environment is a stable and unique environment relative to open ocean systems. Thus it is not particularly surprising to recover novel sequences from an environment so dissimilar from those which are more frequently sampled.

More surprising, given the numerous *Bacillus* strains isolated by Dick and Lee (see results) and a DNA isolation protocol directed at spores, was the absence of sequences from the phylum Firmicutes. While this may reflect the difficulty of lysing spores, it could also reflect a low abundance of *Bacillus* spp. in the original sample. The latter explanation is somewhat more likely on two counts. First, the heat treatments employed in isolating the *Bacillus* strains (G. Dick, pers. comm.) enriched for spore formers, especially in samples taken from ~8° C water. Second, Gram-positive phyla appeared once in the library—clone BS01-081 grouped with environmental sequences from the phylum Actinobacteria (Figure 4.6). Thus, absent any evidence of abundant *Bacillus* spp. in the Mn oxide rich layer of the SOZ, the Proteobacteria, especially the numerically dominant *Pseudoalteromonas* spp. are the most compelling possibility for a major role in Mn(II) oxidation in situ.

*Pseudoalteromonas* is a ubiquitous marine genus, containing numerous heterotrophic, psychrotolerant and halotolerant isolates (Ivanova et al., 2004; Mikhailov et al., 2002). Often associated with surfaces, they were first identified as Mn(II) oxidizers by Templeton et al. (2005) on fresh basalts near the Hawaiian undersea volcano, Loihi. These Mn(II)-oxidizing isolates matched (>99%) a *Pseudoalteromonas* sequence recovered from a microbial mat at Loihi years earlier (Moyer et al., 1995). The Loihi sequences are not the nearest matches to any of the

*Pseudoalteromonas* isolates or clone sequences from the Black Sea, but are >96% similar, as are most sequences classified within the genus.

The Black Sea *Pseudoalteromonas* isolates varied in their Mn(II) oxidizing activity, both between species (isolates >99% identical by 16S gene sequence) and between strains (Table 4.2). The two strains without visible Mn oxide production, BS03-22 and -44 were both LBB and CAS-positive, confirming the production of, respectively, oxidized Mn (i.e. redox state above +2) and Fe(III)-binding ligands such as a siderophore. Indeed, mass spectrometry revealed the presence of the cyclic siderophore deferrioxamine E in spent media from one of these isolates, BS03-44, (Figure 4.9) and its sister strain, BS03-45.

Siderophores are known to stabilize Mn(III) in solution, preventing disproportionation to Mn(II) and Mn(IV) (Duckworth and Sposito, 2005; Parker et al., 2004). This aqueous, oxidized Mn phase may be a redox buffer in the SOZ, similar to nitrite in its capacity to be oxidized or reduced. As noted in Chapter V, Mn(III) is both produced and maintained in the Black Sea SOZ, and preliminary evidence indicates that a Mn(III)-deferrioxamine E complex can be detected there (Luther, pers. comm.).

The cultivation-independent clone library confirmed the numerical dominance of a Mn(II)-oxidizing *Pseudoalteromonas* in the Mn oxide maximum. Clone BS01-003 was identical over 1473 bp to the isolate BS03-23 and this sequence represented four of the seven *Pseudoalteromonas* isolates and 41% of the clone library inserts. The remaining Mn(II)-oxidizing *Pseudoalteromonas* isolates (BS03-22 and -44) were not identical to any clone sequences but grouped in a strongly-supported clade with

clones BS01-002, -020 and -156. Vetriani et al. (2003) reported that *Pseudoalteromonas*-like fragments in terminal restriction fragment length polymorphism patterns were the most prevalent fragments in the upper water column of the Black Sea and recovered *Pseudoalteromonas*-like sequences in clone libraries from a number of depths (Figure 4.4). The results reported here are similar but it remains unclear if the “upper water column” described in that work included the SOZ.

The numerical dominance of the *Pseudoalteromonas* group in the clone library, combined with the confirmed capacity of Black Sea *Pseudoalteromonas* isolates to oxidize Mn(II) and produce siderophores known to interact with Mn(III) are strongly indicative of an important role in SOZ Mn(II) oxidation. Still, in vitro activity does not assure in situ activity; these strains may opportunistically oxidize Mn(II) merely in response to cultivation conditions. While no current means exists for identifying in situ Mn(II)-oxidizing activity by single species or cells, in situ abundance can be correlated with suboxic chemical profiles.

The numerical dominance seen in the 2001 sample was not apparent in samples taken four years later, but was correlated with Mn oxide abundance. Quantitative PCR results suggest that the 2001 samples from  $\sigma_T = 15.95$  and 16.00 had the highest abundance of *Pseudoalteromonas* 16S genes and that there was a distinct difference between the two despite being taken from depths just 3 m apart (Figure 4.8). In contrast, all five densities sampled in 2005 had relatively low numbers of *Pseudoalteromonas*. particulate and dissolved Mn profiles suggest that water column conditions were markedly different when each sample was taken.

In the March 2005 sample, the particulate Mn (i.e. Mn oxide) profile is indistinct, possibly the result of winter mixing (Figure 4.10). In the May 2001 sample, the particulate Mn maximum is distinct and is 5-fold greater than the peak concentrations in the 2005 sample. Particulate Mn was not observed below the onset of dissolved Mn in 2001, while the 2005 sample appears to have a deep particulate Mn peak. While generally considered a stable, stratified basin, the Black Sea exhibits seasonal mixing in the upper water column that modifies the temperature and current structure (Oguz and Besiktepe, 1999). How these processes affect Mn cycling remains to be seen; the broad particulate Mn distribution seen in 2005 may indicate vertical mixing of the Mn oxide maximum or slower Mn(II) oxidation during the winter months. Ultimately, the relative differences in gene abundance and particulate Mn between the 2001 and 2005 samples are consistent with a link between *Pseudoalteromonas* spp. and Mn(II) oxidation in the Black Sea.

## Conclusions

The clone library described in this study highlights the dominance of Proteobacteria within the Black Sea SOZ while revealing a diverse set of potential metabolic strategies involving S, N and Mn cycling. While the Black Sea's SOZ is renowned for its redox stratification, the sequence diversity of the narrow layer sampled clearly indicates that a single depth hosts multiple processes. In the future, environmental genomics and proteomics studies may provide greater confidence in assigning functional roles to uncultivated organisms in the Black Sea.

In the meantime, cultivation remains the best means for confirming the metabolic potential of microbes in the environment. The isolates kindly provided by Dick and Lee for this work have linked the presence and abundance of *Pseudoalteromonas* spp. with recently discovered processes in the Black Sea. Both the production of Mn oxides and Mn(III)-binding ligands are critical to the maintenance of the Black Sea's SOZ and, potentially, other suboxic waters. *Pseudoalteromonas* spp represent a dominant phylotype that is both associated with prevalent biogeochemical processes in situ and capable of catalyzing those processes.

Table 4.1 Mn(II) oxidizing bacteria from the 2003 Black Sea cruise, isolated by G. Dick and Y. Lee. *Bacillus* isolates are shown primarily for reference and are discussed in Dick et al., in prep.

Genus	Isolates	Species
<i>Bacillus</i>	32	4
<i>Pseudomonas</i>	14	2
<i>Pseudoalteromonas</i>	7	3



Table 4.2 Genotypic grouping and phenotypic characteristics of *Pseudoalteromonas* isolates from the 2003 Black Sea Cruise. LBB reaction tests for oxidized Mn production, either Mn(III) or Mn(IV) gives a positive result; CAS assay tests for the production of Fe-binding ligands, e.g. siderophores.

16S rRNA species	Geno- Isolate	Growth (30 d)	Visible Mn oxide	LBB reaction (+/-)	CAS Plate Assay	Liquid CAS Assay
BS03-22	BS03-22	+++	++	+	-	-
BS03-23	BS03-23	+++	+++	+	+	-
	BS03-24	++	-	+	+	-
	BS03-25	++	++	+	-	-
	BS03-26	++	+	+	-	-
BS03-44	BS03-44	+++	-	+	-	+
	BS03-45	+++	++	+	-	+

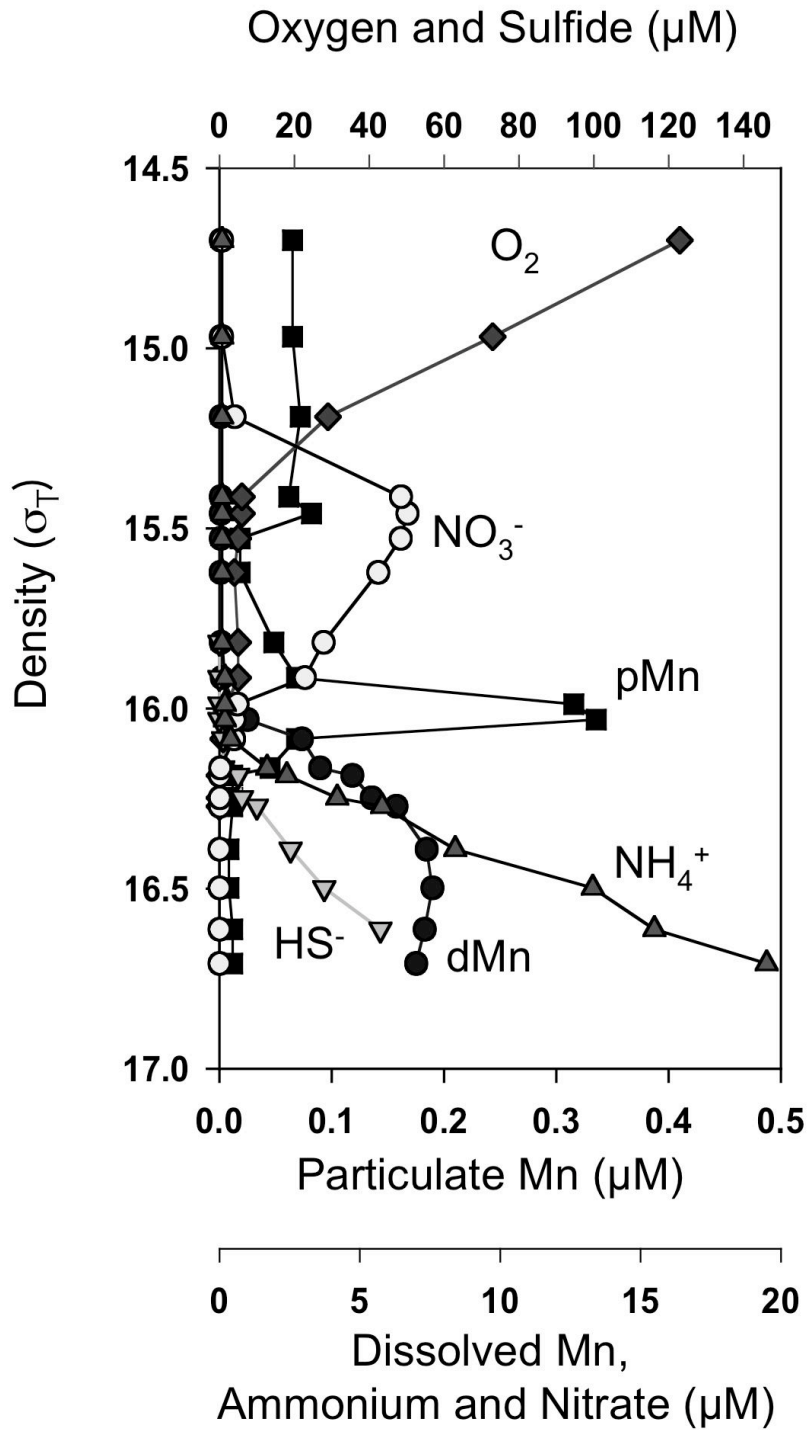


Figure 4.1 Typical chemical profiles in the Black Sea suboxic zone. Note the particulate Mn peak at  $\sigma_T = 16.0$ . Note: Nitrate data provided by J. Murray; ammonium and sulfide data by S. Konovalov and A. Romanov.

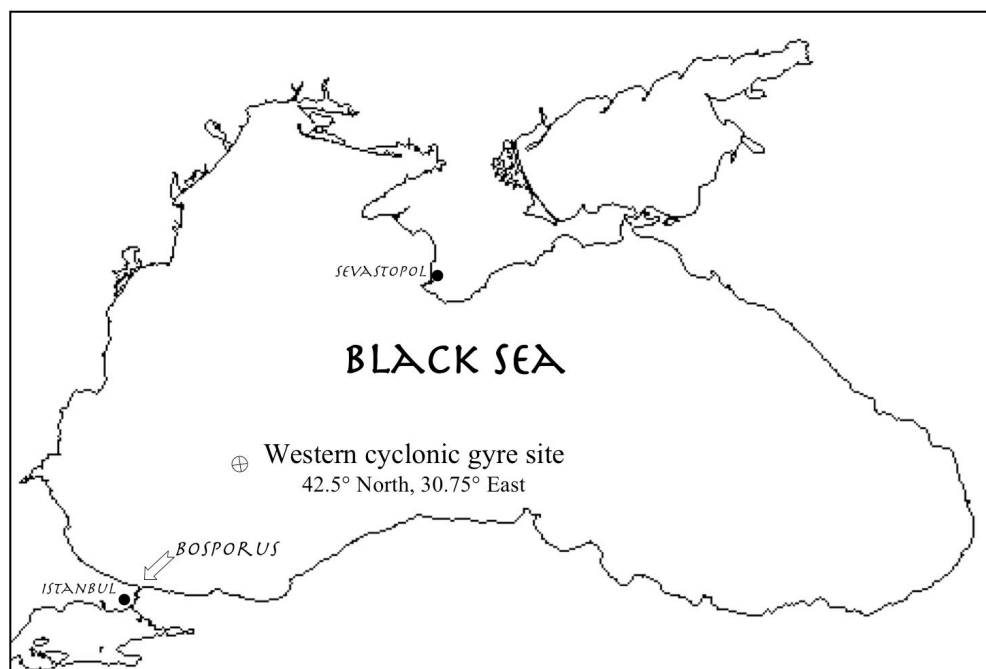


Figure 4.2 Population DNA sampling locations in 2001 and 2005

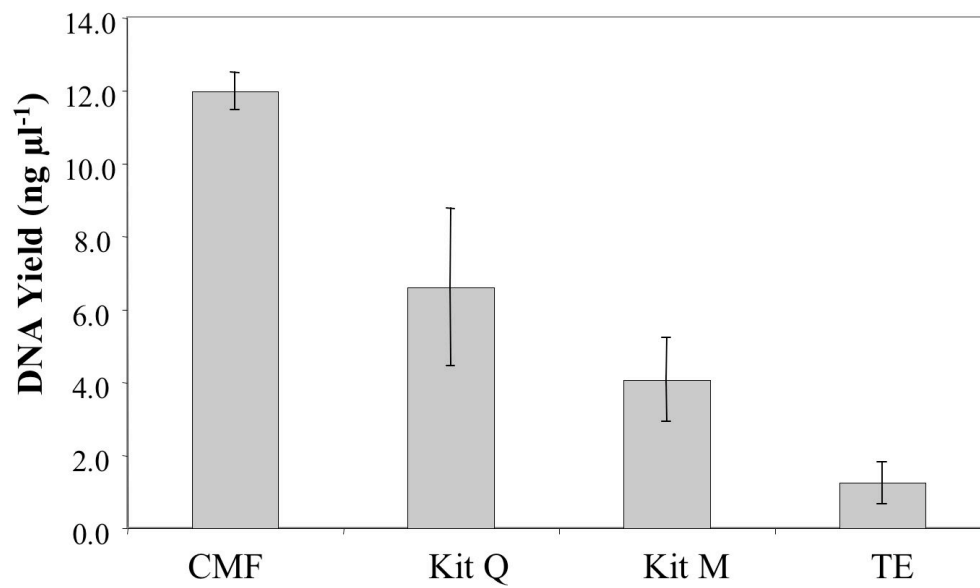


Figure 4.3 DNA yields from *Bacillus* sp. SG-1 spores using different methods. Error bars are equivalent to one standard deviation of duplicate or triplicate samples.

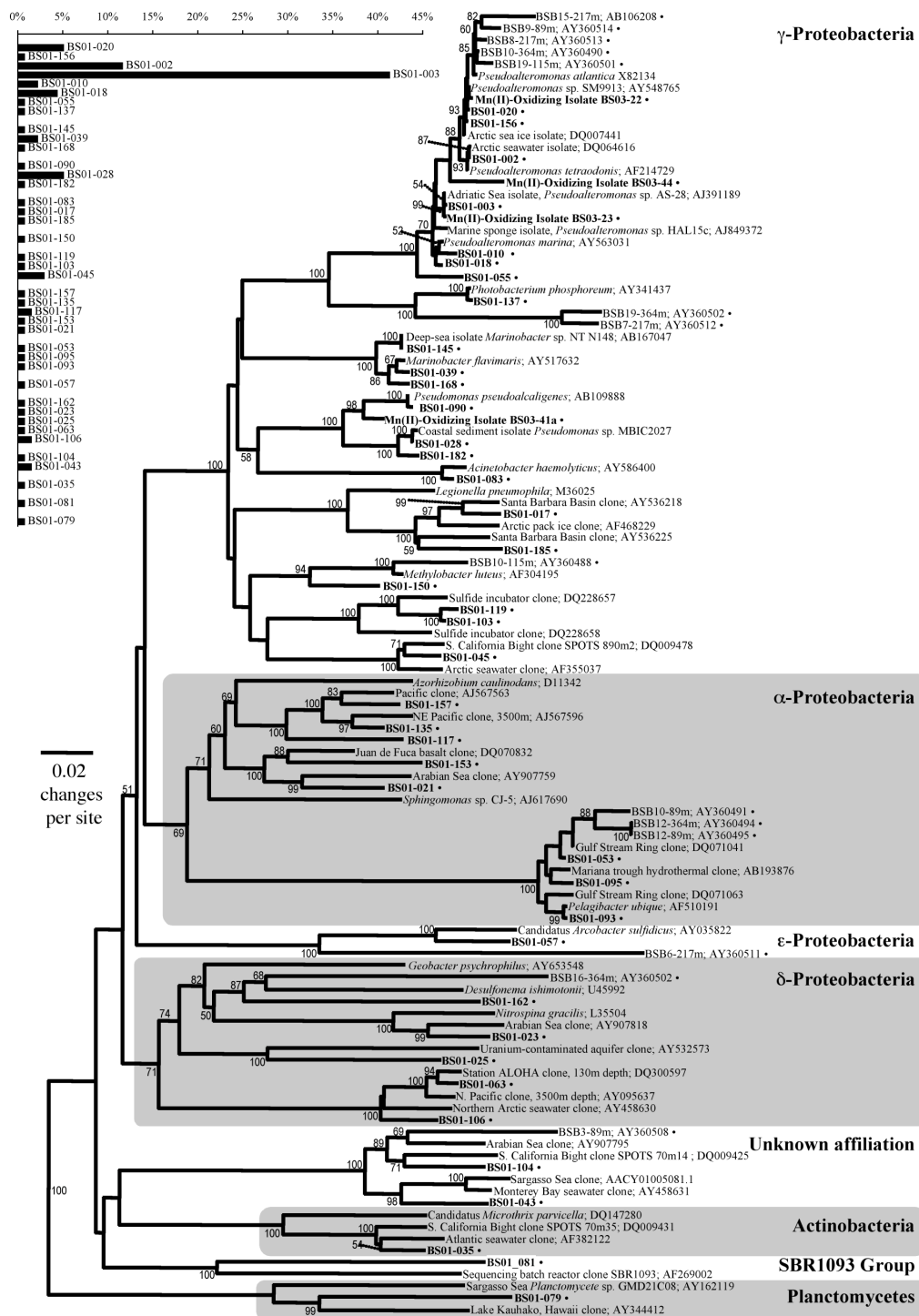


Figure 4.4 Neighbor-joining phylogenetic tree. Sequences from the Black Sea are indicated by • symbols; sequences from this study are in bold. Bootstrap values are percent branch occurrence in 500 replicates. Upper left panel shows relative abundance of individual sequences in the library. Magnified tree sections are shown in Figures 4.5 and 4.6.

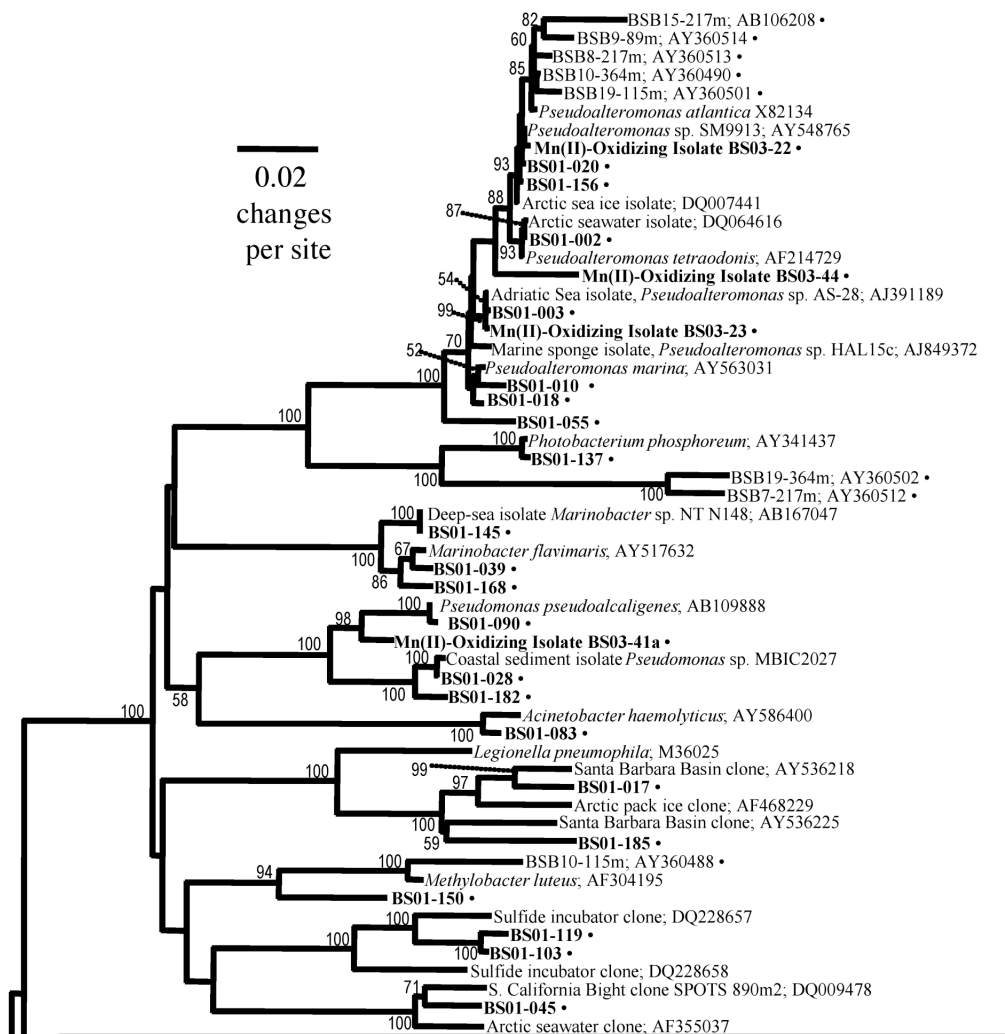


Figure 4.5 Detail of phylogenetic tree in Figure 4.4 showing  $\gamma$ -proteobacteria. Sequences from the Black Sea are indicated by • symbols; sequences from this study are in **bold**. Bootstrap values are percent branch occurrence in 500 replicates.

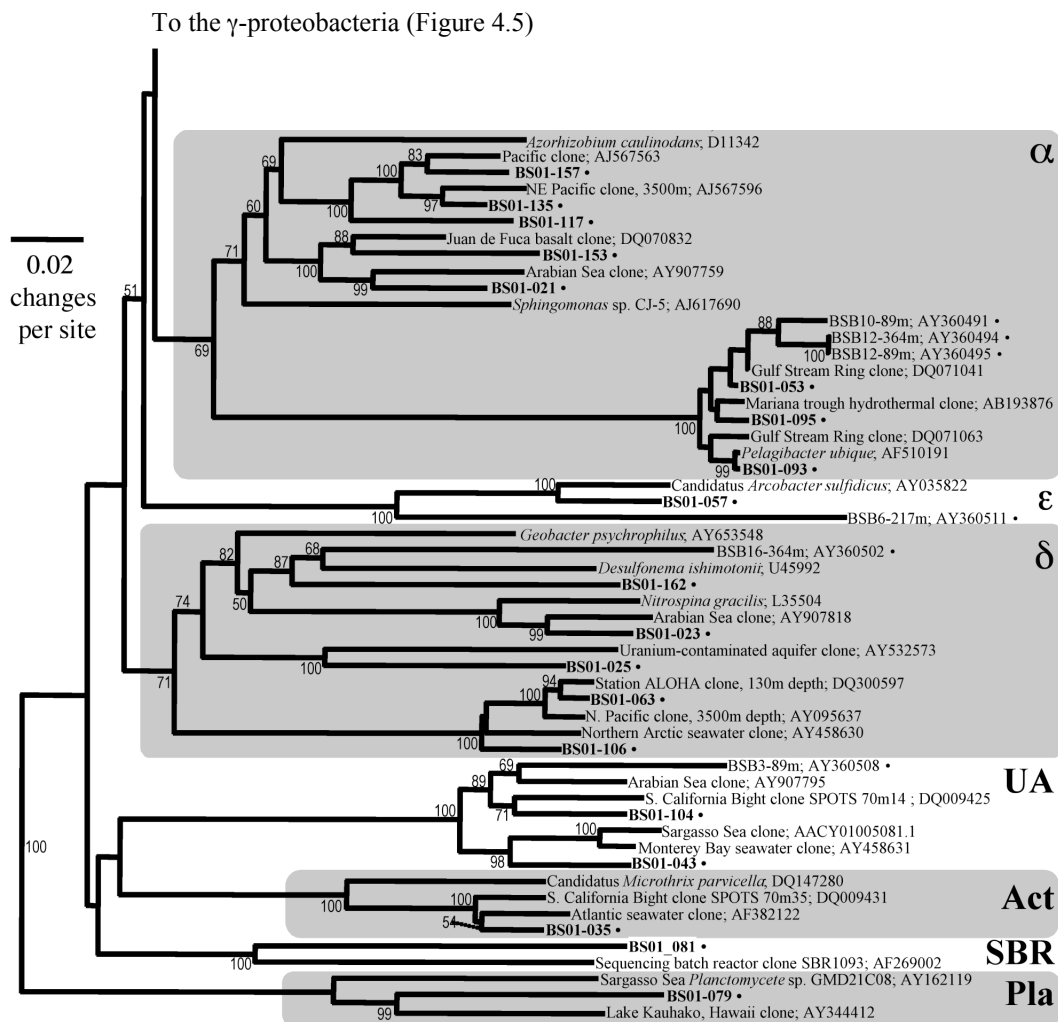


Figure 4.6 Detail of lower section of the phylogenetic tree in Figure 4.4, with branch leading to  $\gamma$ -proteobacteria (Figure 4.5) shown for clarity. Sequences from the Black Sea are indicated by • symbols; sequences from this study are in **bold**. Bootstrap values are percent branch occurrence in 500 replicates. Proteobacterial classes are indicated by Greek letters, remaining group names are abbreviated: UA, unknown affiliation; Act, Actinobacteria; SBR, SBR1093 Group; Pla, Planctomycetes.

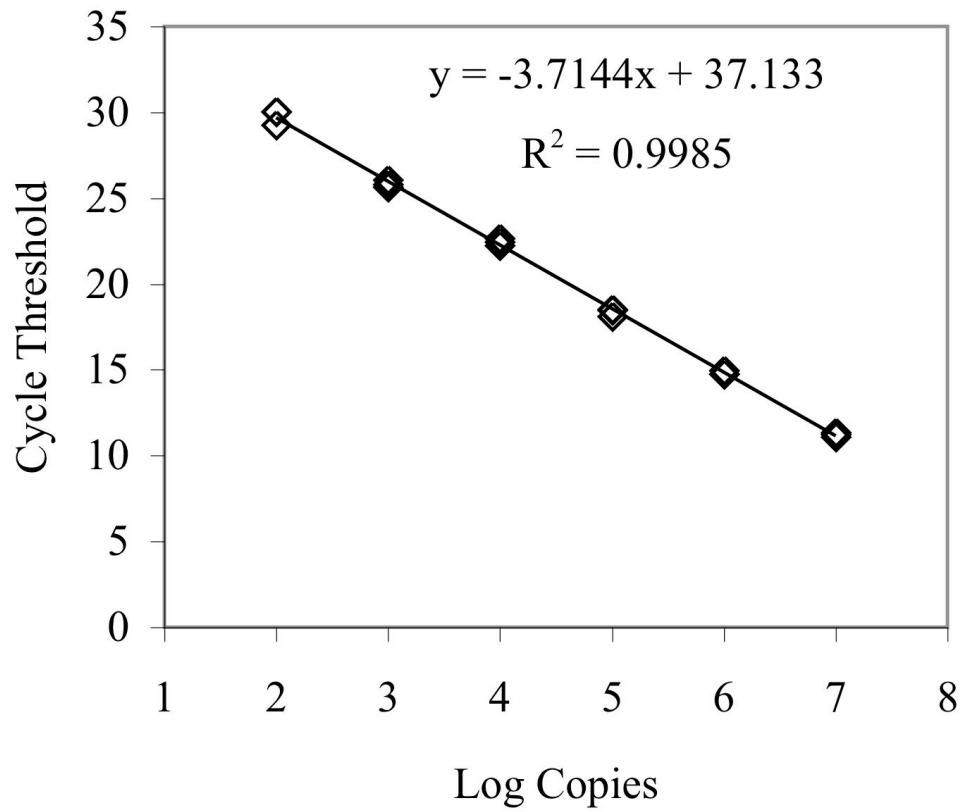


Figure 4.7 Quantitative PCR standard curve with primers targeting *Pseudoalteromonas* 16S ribosomal RNA genes. Triplicate values are shown for each 10-fold dilution of *Pseudoalteromonas* DNA.



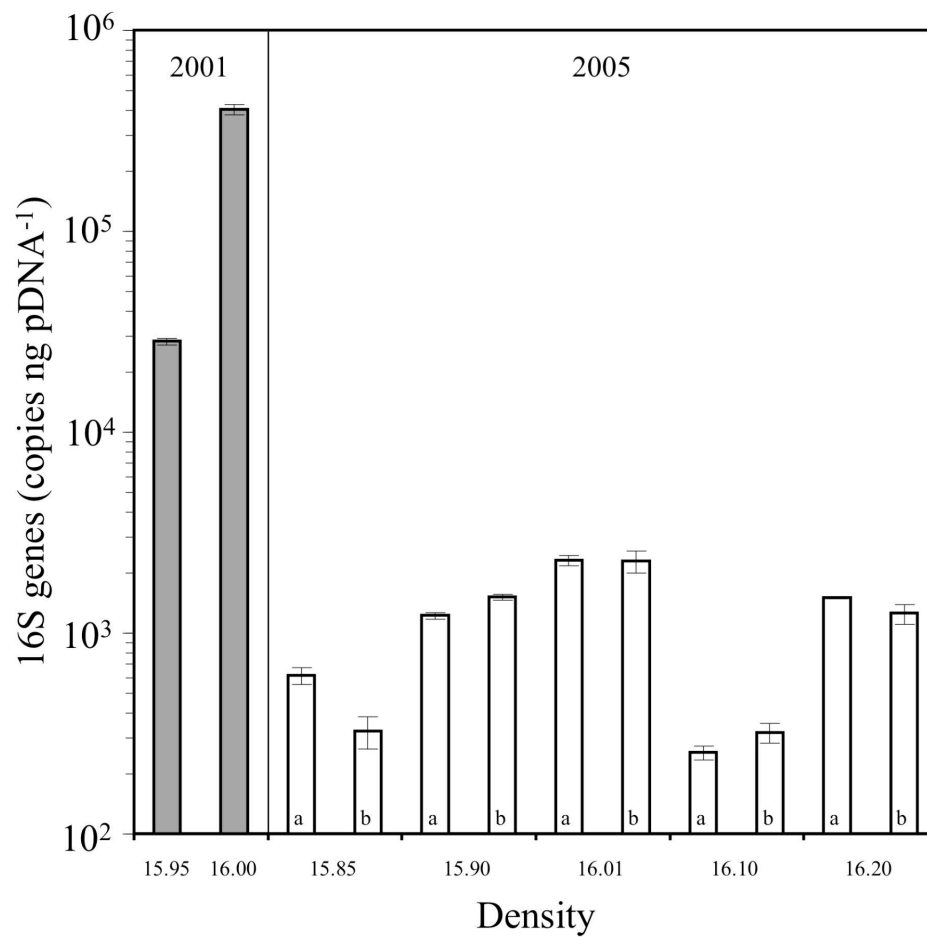


Figure 4.8 *Pseudoalteromonas* spp. gene abundance determined by qPCR. Note log scale on y-axis. Error bars represent one standard deviation. Designations (a) and (b) refer to replicate DNA extractions from the same filter membrane used to collect cells.

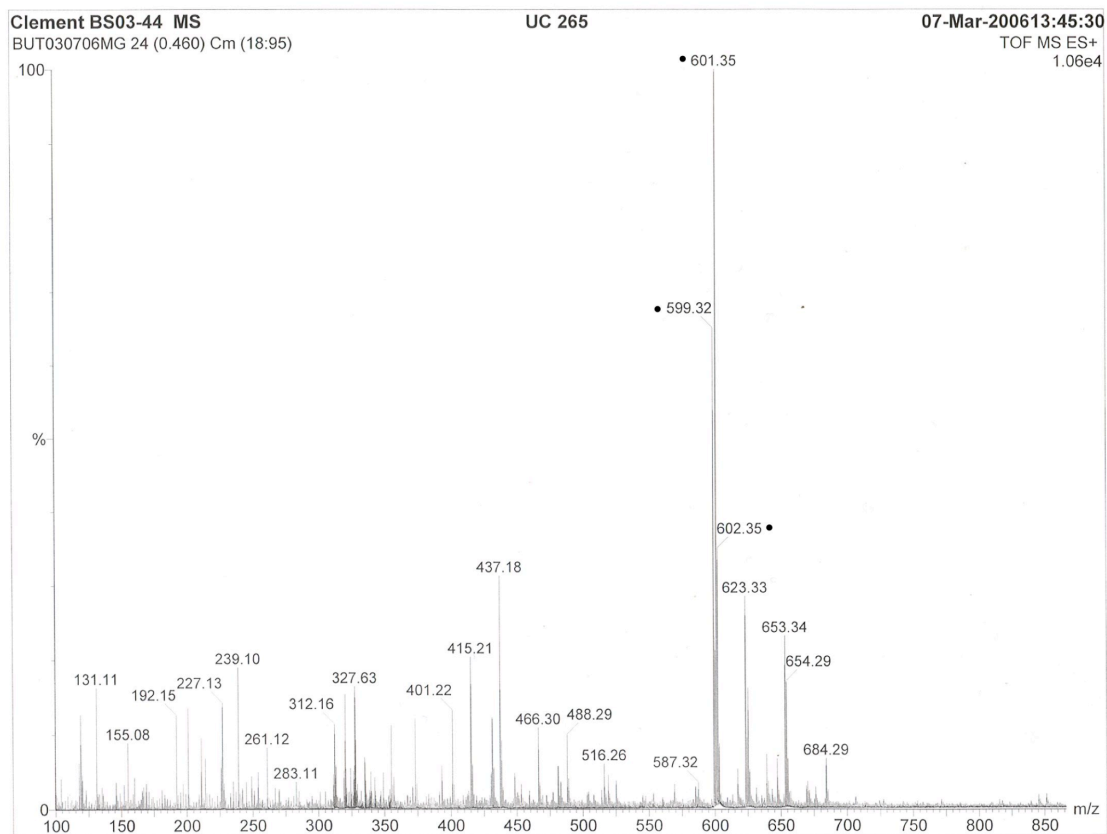


Figure 4.9 Mass spectrum of CAS-positive spent medium from *Pseudoalteromonas*-like isolate BS03-44. Symbols (•) indicate peaks consistent with desferrioxamine E.

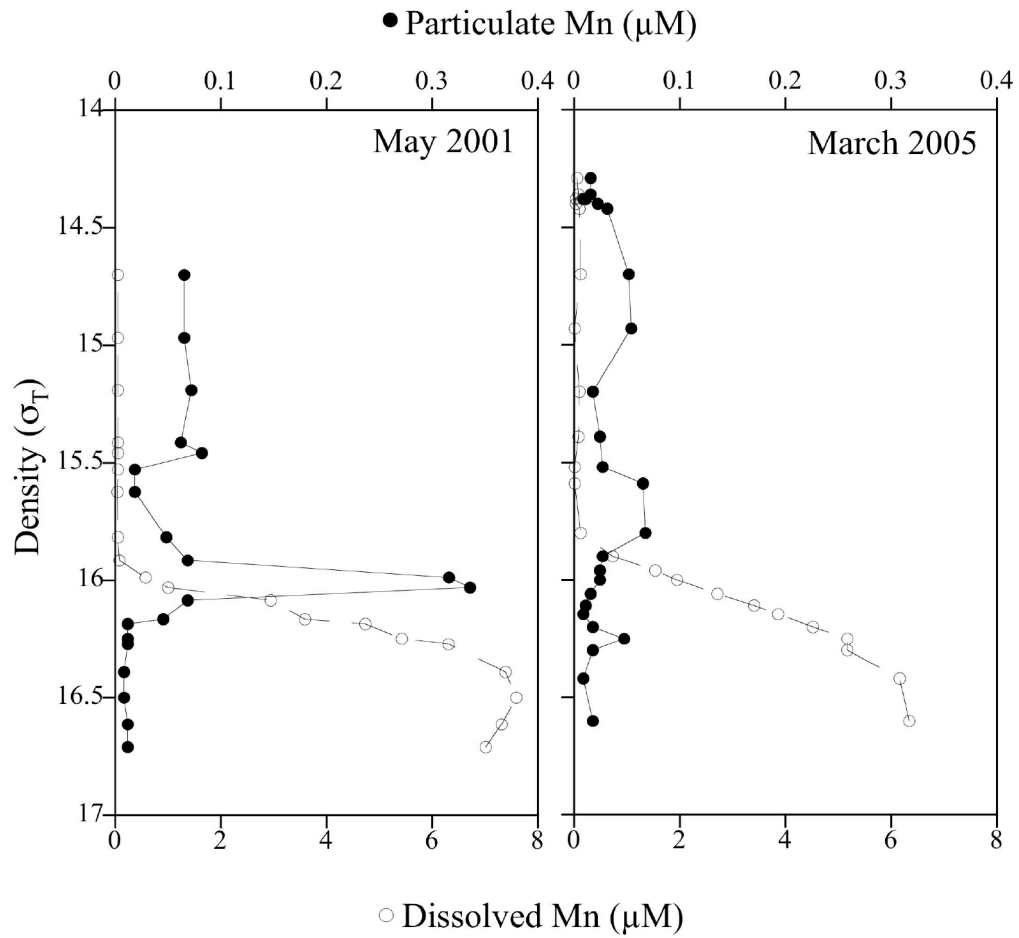


Figure 4.10 Density-resolved Mn profiles for sample sites. Dissolved Mn (open symbols) and particulate (closed symbols) Mn were measured within four (2001) or two (2005) days of cell collection.

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## **CHAPTER V**

### **Biological production of dissolved, oxidized Mn in the suboxic zone of the Black Sea**

## Abstract

Microbial Mn oxide production via Mn(II) oxidation is a key reaction in suboxic waters, such as those found in the Black Sea. Recently, Mn(III) was identified as an intermediate in Mn oxide production by a *Bacillus* sp., revealing the stepwise mechanism of the two electron oxidation of Mn(II). In natural waters, Mn(III) is unstable in aqueous solution and disproportionates to Mn(II) and Mn oxide. However, some siderophores bind and stabilize Mn(III) in solution, producing a dissolved, oxidized Mn species, or dMn(III/IV). Mn(III)-ligand complexes have recently been observed in the suboxic zone of the Black Sea, prompting questions about their mechanism of formation and role in suboxic chemistry. To assess Mn(III) production mechanisms, a method to trap and separate radiolabeled dMn(III/IV) bound to the siderophore desferrioxamine B was developed and applied in the Black Sea. Dissolved Mn(III/IV) production was observed in the Black Sea suboxic zone, by both biological Mn(II) oxidation and abiotic oxidation catalyzed by the siderophore. These results are consistent with previous work characterizing the chemistry of Mn-siderophore interactions and the biological mechanism of Mn(II) oxidation. Thus, dMn(III/IV) production in the suboxic zone of the Black Sea is likely controlled by the abundance of ligands and enhanced by biological Mn(II) oxidation in the presence of those ligands.

## Introduction

Microbial Mn oxidation has been observed since the early 1900s but fundamental questions about the function and benefits of Mn oxide production remain unanswered (Brouwers et al., 2000; Tebo et al., 1997). Since Mn(II) oxidation to Mn(III/IV) oxides is energetically favorable when coupled to O<sub>2</sub> reduction, researchers have speculated that microbes may conserve energy from this reaction for growth (Ehrlich and Salerno, 1990; Kepkay and Nealson, 1987). However, growth on Mn(II) and O<sub>2</sub> alone has not been demonstrated; all cultivated Mn(II)-oxidizing bacteria are heterotrophic and an entire subgroup oxidizes Mn(II) as otherwise dormant spores (Tebo et al., 2004; Tebo et al., 1997). Thus, demonstrating energy conservation, particularly in the presence of reduced carbon substrates, requires establishing the pathways, if any, of electron transfer from Mn(II) to O<sub>2</sub>, the number of electrons passed along per Mn(II) oxidized and the resulting ATP production (Nealson et al., 1988). A logical starting point in this effort is a mechanistic understanding of microbial Mn(II) oxidation.

Proteins involved in catalyzing Mn(II) oxidation were identified in the 1990s, when similar enzymes in dissimilar organisms were linked to Mn oxide production. Genetic methods demonstrated that the phylogenetically-distinct Mn(II)-oxidizing organisms *Pseudomonas putida* GB-1, *Leptothrix discophora* SS-1 and *Bacillus* sp. SG-1, all required similar multicopper oxidases (MCOs) for Mn(II) oxidation (Brouwers et al., 1999; Corstjens et al., 1997; vanWaasbergen et al., 1996). While the Mn(II)-oxidizing MCOs have proven recalcitrant to purification and, thus, in vitro

mechanistic analyses, the general properties of MCOs offered clues to how Mn(II) is oxidized.

The described MCOs, which oxidize organic (phenolic) substrates or Fe(II), oxidize their substrate via single electron transfer events (Brouwers et al., 2000). Initially, this seemed incompatible with Mn oxide production as the mineral phase produced by bacteria is exclusively Mn(IV)—a two-electron oxidation of Mn(II) (Bargar et al., 2005). The intermediate oxidation state, Mn(III), predicted to result from the first single-electron oxidation was believed to be problematic as it would represent a second substrate for the MCO and is unstable in aqueous solution (Stumm and Morgan, 1996).

In 2005, Webb and coworkers found an Mn(III) intermediate in Mn(II) oxidation by pyrophosphate trapping (Webb et al., 2005). Utilizing mutant strains, they demonstrated that the *Bacillus* sp SG-1 MCO, MnxG, was required for oxidation of both Mn(II) and Mn(III). The *mnx* operon, while producing a protein (MnxG) similar to the putative Mn(II)-oxidizing MCOs in other strains, appears to code for two additional copper binding domains (G. Dick, personal communication). This might be indicative of a greater substrate range, (e.g. Mn(II) and Mn(III)) in the *Bacillus* MCO than in the more typical, four Cu-domain Proteobacterial MCOs involved in Mn(II) oxidation (Brouwers et al., 1999; Corstjens et al., 1997). More importantly, the existence of an Mn(III) intermediate in Mn(II) oxidation that can be bound by ligands in solution implied that metal-binding ligands might complex Mn(III) during biological Mn(II) oxidation in the environment.

Geochemists have long suspected that Mn(III)-organic complexes occur in marine and estuarine systems because organic acids common in these systems can chelate metals (Kostka et al., 1995; Luther et al., 1994). One common type of metal chelating ligands in marine systems are siderophores, biogenic molecules typically described as Fe(III)-ligands for biological Fe acquisition. Parker and coworkers (2004) found that the *Pseudomonas* siderophore, pyoverdine, bound and stabilized Mn(III) in solution. That work that echoed the earlier findings of Faulkner and Fridovich (1994) who produced stable Mn(III) complexes with the microbial siderophore desferrioxamine B (DFOB). Such interactions appear to be important in regions where biological Mn(II) oxidation produces particulate Mn oxides.

Using cyclic voltammetry to analyze Mn(III) bound by added DFOB, Trouwborst (2006) observed Mn(III) in the Black Sea and Chesapeake Bay at or near oxic/anoxic interfaces where biogenic Mn oxide production is prevalent. The O<sub>2</sub> concentrations in such interface zones are < 5 μM, yet the abiotic formation of Mn(III)-DFOB requires O<sub>2</sub> (Duckworth and Sposito, 2005b). Thus, in the low O<sub>2</sub> regions where biological Mn(II) oxidation is prevalent, biological catalysis might play a role in the formation of dissolved Mn(III)-complexes.

The Black Sea provides an excellent model system for observing redox transformations at low O<sub>2</sub> concentrations. Due to strongly stratified conditions, vertical transport is limited to diffusion and a strong redox gradient exists below the surface mixed layer (< 40 m). Within this redox gradient exists a thick (10-40 m) layer where oxygen and sulfide are absent (Murray et al., 1995; Murray et al., 1989). This suboxic zone (SOZ) exists year round in the Black Sea and hosts a vertical

sequence of redox reactions involving N, Mn and S. Mn is abundant in the Black Sea; total Mn peaks at  $\sim 8 \mu\text{M}$  10-20 meters below the top of the sulfide gradient and up to  $2 \mu\text{M}$  particulate Mn has been observed within the suboxic zone (Konovalov et al., 2003).

Redox cycling in and below the SOZ between soluble, reduced Mn and solid-phase Mn oxides provides an important link between oxic and anoxic waters. Essentially, reduced Mn(II) diffuses up from the anoxic zone and is oxidized by microbes in the suboxic zone to particulate Mn(III/IV) oxides (Oguz et al., 2001; Tebo, 1991). The oxidized, solid-phase Mn then sinks into the anoxic, sulfidic waters below where it is reduced by sulfide, producing dissolved, reduced Mn and oxidized S species (Murray et al., 1995; Oguz et al., 2001).

Mn(II) oxidation in the Black Sea is catalyzed biologically with the most rapid rates being found just above the suboxic/anoxic boundary (Tebo, 1991). The process is enhanced by  $\text{O}_2$  additions, as also shown in Chapter III, and is orders of magnitude more rapid than chemical oxidation alone. The rapid rates of Mn redox reactions combined with stratified waters that allow high-resolution sampling of specific redox strata make the Black Sea an excellent site to observe Mn(III)-ligand complexes in the environment and examine if microbial Mn(II) oxidation enhances Mn(III)-ligand complex production in situ. This study was undertaken to develop a sensitive radiotracer method for measuring Mn(III) production rates in natural waters and apply that method to examine the mechanisms and products of biological Mn(II) oxidation in the Black Sea.

## Methods

### Samples

Sample locations and properties are summarized in Figure 5.1. Water was collected from the R/V *Endeavor* using PVC Niskin bottles attached to a 24-place rosette. A SeaBird CTD sensor package was used to determine rosette depth and water properties including density as  $\sigma_T$ . Dissolved and particulate Mn was determined by the formaldoxime method using 0.2  $\mu\text{m}$  pore-size polyethersulfone membranes for separation (Brewer and Spencer, 1971).

### Mn(III)-desferrioxamine complex

The Mn(III)-DFOB complex was made by mixing dry Mn(III) acetate into a HEPES-buffered solution of DFOB at a ratio of 1:2. The resulting solution was filtered to remove any particulate Mn. The total Mn concentration was measured by formaldoxime and the green, Mn(III)-DFOB complex was measured by absorbance at 310 nm,  $\epsilon=2050 \text{ M}^{-1} \text{ cm}^{-1}$  (Faulkner et al., 1994). Yields of Mn(III)-DFOB obtained by adding dry Mn(III)-acetate to aqueous DFOB were typically 60% of the total Mn. Stock solutions  $\text{MnCl}_2$  (in water) and Mn(III)-DFOB (in buffer) were further diluted to desired concentrations in artificial seawater solution buffered with 10 mM HEPES, pH 7.7. Artificial seawater consisted of 0.1 M  $\text{MgSO}_4$ , 0.02 M  $\text{CaCl}_2$ , 0.6 M NaCl and 0.02 KCl.

### Separation protocol

Either C-18 Sep Packs or HLB Oasis columns (Waters, Milford, MA) solid-phase extraction (SPE) columns were used for all experiments in this work. Solutions

were drawn slowly through the columns by hand with syringes when using stable isotopes and by vacuum using a valved manifold when using  $^{54}\text{Mn}$ . As suggested by the manufacturer, C-18 columns were kept wet by maintaining the meniscus at or above the upper frit; samples run on HLB columns were collected in their entirety. All columns were pre-wet with 2 ml methanol and washed with 2 ml 10 mM HEPES, pH 7.7. For stable isotope work, 2 ml samples were passed through the columns, followed by a 2 ml wash with 10 mM HEPES, pH 7.7. Compounds bound to the column were eluted in either 50% methanol with 10 mM HEPES (pH 7.7) or in 100% methanol.

#### $^{54}\text{Mn}$ assays

For  $^{54}\text{Mn}$  assays, the general protocol followed by Tebo (1991) was modified. Black Sea water was obtained from the suboxic zone and sparged with air and  $\text{CO}_2$  gas. Gas ratios were controlled with a gas proportioner to poise sample pH at 7.6 +/- 0.1. Samples were sparged for 1 hour or until the pH stabilized, whichever was longer. The sample was then aliquoted in 50 ml volumes into 75 ml serum bottles. Two types of incubations were used, one to detect Mn(III) complexed during the assay to naturally occurring ligands (natural ligand assay) and one to detect and trap Mn(III) in a Mn(III)-DFOB complex (trapping assay). Trapping assay incubations were amended with 25  $\mu\text{M}$  DFOB before incubation. Both incubation types were paired with poisoned controls that were amended with 0.1% final concentration of sodium azide. All incubations were sealed immediately after addition of 1  $\mu\text{Ci}$  of  $^{54}\text{Mn}(\text{II})$  and incubated for 7-9 hours in an 8° C water bath.



After incubation, 2 x 1 ml samples were taken for total  $^{54}\text{Mn}$  before particulate and dissolved  $^{54}\text{Mn}$  fractions were separated by filtration through 0.2  $\mu\text{m}$  polyethersulfone membranes. Natural ligand assay filtrates were then amended with 25  $\mu\text{M}$  DFOB. Filtrate subsamples (10 ml) were drawn through pre-wet and washed HLB columns, followed by a 2 ml wash with 10 mM HEPES—the combined post-extraction sample and wash, i.e. the aqueous phase, was collected. Column-bound compounds were eluted with 3 ml methanol. Prior to counting, the total and filter samples were brought up to 3 ml total volume with 0.1% hydroxylamine to dissolve particulate Mn and equalize counting geometry. A total of 6 samples—2 total and 1 each of the filter, the filtrate, the aqueous phase and the elution, all 3 ml each—from each incubation were counted at sea on a Wallac  $\gamma$ -counter. The resulting counts were expressed as a fraction of the average total  $^{54}\text{Mn}$  for each set of triplicate incubations. These fractions were then multiplied, assuming  $^{54}\text{Mn}(\text{II})$  behaves as an ideal tracer, by the in situ dissolved Mn concentration to determine the absolute quantity of Mn in each subsample.

Isotopic exchange controls were performed by washing the column with 10  $\mu\text{M}$  Mn(II) in 10 mM HEPES instead of the buffer alone. Reduction controls consisted of pre-reduction of the filtrate by addition of 0.1% hydroxylamine prior to adding the sample to the SPE column.

## Results

### General Method

Solid phase extraction (SPE) separated aqueous Mn(II) from Mn(III)-DFOB with Mn(II) passing through the SPE column while the Mn(III)-DFOB was retained. The Mn(III)-DFOB was then recovered by elution semi-quantitatively with 50 or 100% methanol (Figure 5.2). Absorbance measurements at 310 nm were compromised by a contaminant from the columns that remained after pre-washing with methanol, thus Mn(III)-DFOB measured directly in the methanol elution appeared to be higher than the formaldoxime measurement (Figure 5.3). For this reason, recovery was calculated as initial Mn(III)-DFOB (absorbance at 310 nm) divided by the total Mn measured in the elution step by the formaldoxime method. Nearly 100% recovery of the Mn(III)-DFOB complex was achieved with HLB columns using 100% methanol for the elution step and this method was used at sea (Figure 5.3).

### <sup>54</sup>Mn Method

The radioisotope method was more sensitive than the formaldoxime method, allowing resolution of differences in <sup>54</sup>Mn partitioning between the particulate, dissolved-unbound and column-bound fractions of 2.5% (1 standard deviation, cf. Table 5.1). Column-bound <sup>54</sup>Mn did not exchange with isotopically stable Mn(II) but was eluted by 0.1% hydroxylamine (Figure 5.4). The majority of <sup>54</sup>Mn was recovered in all assays; the sum of all fractions was calculated to contain between 92 and 105% of the added radiolabel (cf. Figure 5.4).

### **Experimental incubations**

Dissolved and particulate Mn concentrations were significantly different in the two SOZ samples tested, due to their density differences and distance from the Mn oxide rich waters near the Bosphorus plume (Konovalov et al., 2003; Tebo, 1991). In general, Mn(II) oxidation activity was greater at Station 4, closer to the Bosphorus and deeper in the SOZ, than at Station 3 (Figure 5.5). Production of natural Mn(III)-ligand complexes was similar in poisoned and un-poisoned controls. The natural ligand assay at Station 3 exhibited a small increase in Mn(III)-L production relative to the poisoned control while the poisoned and live natural ligand incubations at Station 4 were nearly identical (Figure 5.5). Particulate Mn production in the natural ligand assays was over 50 nM h<sup>-1</sup> at Station 4 but less than 2 nM h<sup>-1</sup> at Station 3.

More than half of the particulate Mn production was complexed as Mn(III)-DFOB in the trapping assay. The Mn(III)-DFOB rate at Station 4 was 76 nM h<sup>-1</sup> and 6.4 nM h<sup>-1</sup> at Station 3. Poisoned controls also produced large quantities of Mn(III)-DFOB in the trapping assays, while producing little or no particulate Mn. Combined Mn(III)-DFOB and particulate Mn production was greater in the trapping assays than in the natural ligand assays, by more than double in the case of Station 3.

## Discussion

### General Method

In developing a method for measuring in situ activity, specificity and sensitivity are critical. In situ methods must be specific for the compound of interest so that it can be measured in a complex mixture of solutes with enough sensitivity to reveal its presence at environmentally relevant concentrations. The method developed here had excellent specificity for dissolved, oxidized Mn over Mn(II), reasonable recovery efficiency and high sensitivity when used with  $^{54}\text{Mn}$ .

Specificity determinations were hampered by a column contaminant that interfered with measuring the Mn(III)-DFOB complex directly in column fractions by absorbance. Instead, the initial absorbance at 310 nm, measured in the absence of the column contaminant, was compared to the total Mn measured in the elution fraction (Figure 5.3). While this approach could not confirm that the Mn eluted from the column was Mn(III), it did confirm that Mn eluted from the column did not exceed the quantity of Mn(III)-DFOB placed on the column (Figure 5.3). This, combined with the absence of measurable Mn in the methanol elution when only Mn(II) was added to the initial solution (Figure 5.2), is consistent with separation of Mn(III)-DFOB from Mn(II).

Results from  $^{54}\text{Mn}$  experiments also indicate the method was specific for oxidized Mn. In the presence of DFOB,  $^{54}\text{Mn}$  bound to the column not removed by exchange with isotopically-stable Mn(II) added in the aqueous wash (Figure 5.4). If  $^{54}\text{Mn(II)}$  were bound by the column (either alone or in a complex with DFOB),

isotopic exchange between the radiolabel and the 10  $\mu\text{M}$  of stable Mn(II) would have raised recovery in the unbound, aqueous fraction and reduced it in the methanol elution. Instead, the stable Mn(II) wash had no effect (Figure 5.4). Adding a reductant, 0.1% hydroxylamine, to the filtrate prior to separation on the column eliminated  $^{54}\text{Mn}$  binding completely. This indicates that reduced Mn, i.e. Mn(II), did not bind to the sorbent and that binding observed in the standard procedure was due to the presence of an oxidized Mn form.

Mass balance calculations performed for total Mn in both the stable and radioactive isotope assays indicated that Mn recovery was acceptable; in all cases greater than 92% of the initial Mn was recovered. Initial stable isotope control experiments raised the possibility that some Mn, particularly Mn(III)-DFOB, was lost in processing (Figure 5.2). However, initial Mn(III)-DFOB was comparable to total Mn in the elution when 100% methanol was utilized for the elution step. Mass balance deviations in the  $^{54}\text{Mn}$  assays were likely associated with sample volume lost in the tubing and valves of the vacuum manifold as some values exceeded 100% and no radioactivity was detected in the columns after use (data not shown).

Sensitivity was excellent with the  $^{54}\text{Mn}$  assay. Triplicate determinations of particulate Mn and Mn(III)-L formation had standard deviations that represented <2.5% of the mean value (cf. Figure 5.5, Table 5.1). This equates to an error of 0.5 nM h<sup>-1</sup> at Station 3 and 1.4 nM h<sup>-1</sup> at Station 4. The stable isotope method is limited in its application to environmental analyses by the formadoxime method's sensitivity limit of  $\sim 0.5 \mu\text{M}$  Mn.

In summary, the data indicate that Mn bound to the sorbent during SPE is oxidized Mn, the recovery of Mn(III)-DFOB is greater than 60% and the sensitivity of the  $^{54}\text{Mn}$  method is on the order of a few nM Mn h<sup>-1</sup>. Due to the large concentrations of DFOB used in experiments with Black Sea water, the stability of the Mn(III) complex (cf. Duckworth and Sposito, 2005b) and its behavior in the stable isotope assays, the dissolved, oxidized  $^{54}\text{Mn}$  separated in the assay is likely to be  $^{54}\text{Mn(III)-DFOB}$ ; however Mn(III/IV) bound to other ligands and colloidal forms of Mn(IV) oxide might also be separated by this method. Therefore, the Mn eluted by methanol in the natural ligand and trapping assays is referred to hereafter as dissolved (i.e. < 0.2  $\mu\text{m}$ ) Mn(III/IV) or dMn(III/IV).

#### **Dissolved Mn(III/IV) production**

Mn(II)-oxidizing activity was observed at both stations sampled (Figure 5.5). The particulate Mn production portion of the natural ligand assays is comparable, methodologically, to previous work and those results were consistent with previous data (Tebo, 1991). Although only two samples are reported here, depth in the suboxic zone and proximity to the southwest coast were correlated with high Mn oxide production rates, as in previous determinations (Chapter III; Tebo, 1991; Konovalov, 2003). At the station closest to the coast, Station 4, water was sampled from a density of  $\sigma_T = 16.01$  and rates were close to the maximum reported for the Black Sea. Further from the coast, at Station 3, and higher in the water column at  $\sigma_T = 15.89$ , rates were close to the low end for samples taken below  $\sigma_T = 15.85$  (Chapter III; Tebo, 1991). Mn oxide production activity in both samples was inhibited by poison (0.1% azide), as found in previous work.

Production of dMn(III/IV) was slow in natural ligand assays and not inhibited by poison. This might indicate a problem with exchanging  $^{54}\text{Mn}$  between natural ligands and the added DFOB. However, Trouwborst (2006) reported that additions of DFOB bound all the Mn(III) present in Black Sea samples within 30 seconds and, in the natural ligand assay, filtered (cell- and Mn oxide-free) samples were incubated with DFOB for 5 to 10 minutes.

Instead of exchange problems between the natural and added ligands, the free natural ligand pool may have been small, leaving little capacity to bind or oxidize the  $^{54}\text{Mn}$  tracer. Since the poison additions didn't affect the quantity of tracer bound by ligands present in SOZ water, the mechanism of formation was likely abiotic. Siderophores, and possibly other ligands, promote oxidation of Mn(II) to Mn(III), dependent on pH and the availability of suitable oxidants (Duckworth and Sposito, 2005b; Faulkner et al., 1994). One might expect the pool of natural ligands capable of Mn(II) oxidation and Mn(III) binding to be completely complexed in the Mn(II)-rich SOZ, however, the described assays were completed with saturating levels of  $\text{O}_2$  in order to observe processes that may occur more slowly at the low  $\text{O}_2$  levels in the SOZ. Thus the balance between Mn(III/IV)-ligand complex residence times and ligand-promoted Mn(II) oxidation rates at in situ  $\text{O}_2$  concentrations may favor a small fraction of free ligand.

While these ideas are consistent with the natural ligand assay results, they are not conclusive. Over the ~8 h time course of these experiments, little Mn(II) was moved into the dMn(III/IV) pool and the production observed was likely due to abiotic

oxidation by free ligands. This could be due to a small total ligand pool, a small pool of free ligand or a small potential for dissolved, oxidized Mn production.

The trapping assays revealed that the potential for dissolved, oxidized Mn production by abiotic and biological Mn(II) oxidation in the Black Sea SOZ is likely ligand-limited. Incubation with DFOB produced some dissolved, oxidized Mn abiotically and significantly more in the presence of biological Mn(II) oxidation (Figure 5.5). As previously noted, Mn(II) oxidation by DFOB in the presence of air has been reported by Duckworth and Sposito (2005b) and is consistent with the poisoned trapping assay results.

One alternative explanation for these results is that the first step in biological Mn(II) oxidation is insensitive to azide. However, Webb and coworkers (2005), did not find any activity in azide-poisoned spore coat preparations. Also, little oxidized Mn formation—particulate or dissolved—was seen in the poisoned natural ligand assays. Therefore, the best explanation for dissolved Mn(III/IV) production in the poisoned trapping assay incubations is abiotic Mn(II) oxidation by DFOB to Mn(III)-DFOB.

The abiotic production rate of dMn(III/IV) in the trapping assay was exceeded by the biologically-catalyzed (i.e. un-poisoned) rate (Figure 5.5, Table 5.1). Comparing the overall amount of biological Mn(II) oxidation in the natural ligand and trapping assays indicates that the rates were similar, within the summed error (Table 5.1). This, in turn, indicates that dMn(III/IV) was produced at the expense of particulate Mn oxides in the trapping assays. Two potential pathways for production of dMn(III/IV) in the presence of DFOB and Mn(II)-oxidizing cells are rapid, DFOB-



promoted dissolution of freshly made biogenic Mn oxides and trapping of an Mn(III) intermediate by DFOB during biological Mn(II) oxidation.

Mn oxide dissolution has been reported for the Mn(III) mineral manganite (Duckworth and Sposito, 2005a). Unlike the dissolution of Mn(IV) minerals, the formation of Mn(III)-DFOB from manganite does not require a Mn reduction step. Biogenic Mn oxides are generally believed to be exclusively Mn(IV) upon formation and altered over time to minerals of lower average oxidation state, generally by the auto-oxidation of dissolved Mn(II) to Mn(III), which is incorporated into the mineral phase (Bargar et al., 2005). While it seems thermodynamically unlikely that DFOB can both oxidize Mn(II) and reduce Mn(IV), the siderophore could enhance auto-oxidation of Mn(II) by Mn oxides by rapidly removing any Mn(III) that formed at the oxide surface. This mechanism could explain the relative loss freshly formed of particulate  $^{54}\text{Mn(IV)}$  by con-proportionation of one  $^{54}\text{Mn(II)}$  and one biogenic  $^{54}\text{MnO}_2$  into two  $^{54}\text{Mn(III)-DFOB}$ . The stoichiometry expected for this reaction would be such that the biological (un-poisoned minus poisoned) production of Mn(III)-DFOB would be twice the particulate Mn formation in the natural ligand assay. Data from Station 3 are consistent with this stoichiometry—addition of DFOB lowered biological particulate Mn production by 6.4 nM and raised Mn(III)-DFOB production by 19.6 nM (Table 5.1). In contrast, the particulate Mn production at Station 4 was lowered by 15.6 nM while the Mn(III)-DFOB production increased only 8.6 nM.

Another potential explanation for the difference between the trapping and natural ligand assays is that DFOB trapped an Mn(III) intermediate during microbial Mn(II) oxidation in a manner similar to that reported for pyrophosphate (Webb et al.,

2005). While the presence of an Mn(III) intermediate in Mn(II) oxidation has only been shown for spores from the genus *Bacillus*, its occurrence in other Mn(II)-oxidizing organisms and exposure to the extracellular environment is consistent with maximizing the energy yield from the reaction. Cells that oxidize Mn(II) during growth or early stationary phase (e.g. *Pseudomonas* spp.) could maximize the proton gradient produced by Mn(II) oxidation by segregating the two one-electron oxidation events. Completing the first step of Mn(II) oxidation in or near the inner membrane would allow O<sub>2</sub> reduction to occur within the cell, thus consuming protons during the production of H<sub>2</sub>O. The second step, from Mn(III) to Mn(IV), generates protons, likely by hydrolysis of water to form MnO<sub>2</sub>. This step would be most advantageous to catalyze in the periplasm or outside the cell altogether in order to create a maximal proton gradient (and thus ATP production) from Mn oxidation. Given that the end product is a solid-phase mineral, this step probably happens outside the cell, meaning that the Mn(III) intermediate would be transported to an extracellular enzyme and thus exposed to ligands in solution. For example, *Pseudomonas putida* KT2440 has recently been shown to possess two multicopper oxidases involved in Mn oxidation (J. McCarthy, personal communication). Thus, two segregated, sequential single-electron oxidations of Mn are feasible in *P. putida*, and the Mn(III) intermediate may be exposed to the extracellular environment during Mn(IV) formation, where DFOB could trap it in a stable complex.

Ultimately, the data presented here cannot discriminate the production of dMn(III/IV) by Mn(IV) oxide dissolution from trapping of an Mn(III) intermediate during biogenic Mn oxide formation. Regardless, dMn(III/IV) formation is stimulated

by microbial Mn(II) oxidation in the presence of Mn(III)-stabilizing ligands. This has important implications for chemical transformations within the Black Sea SOZ and for understanding mechanisms of microbial Mn cycling. The environmental implications have been reviewed by Trouwborst (2006), as well as Duckworth and Sposito (2005b), and are summarized here.

The maintenance of the suboxic zone and our understanding of oxidant transfer by Mn cycling within it are based on the characterization of dissolved Mn and particulate Mn as Mn(II) and Mn(III/IV) oxides. The potential for previously unseen oxidizing equivalents to be cycled into anoxic waters via dMn(III/IV) complexes and/or colloids adds another facet to understanding sulfide flux and consumption in the SOZ. Dissolved Mn(III/IV) in the SOZ is transported by diffusion, not sinking flux, raising the possibility that oxidized Mn is also being transported up into oxic waters, where its fate is unclear. Whether these complexes are created via biological Mn(II) oxidation at natural ligand concentrations remains to be seen, but dMn(III/IV) is clearly an alternate product to Mn(IV) oxides.

Perhaps this is the most interesting feature of the work presented here—that oxidized Mn can be siphoned out of Mn oxide production by biologically-produced ligands. The thermodynamics and kinetics of the Mn(III)-DFOB complex formation are very different than those of Mn oxide formation—as evidenced by air oxidation rates (Duckworth and Sposito, 2005b; Faulkner et al., 1994). Interactions between the N and Mn cycle that are suggested by vertical oxidant profiles in the SOZ, but lacking in experimental evidence, may produce or consume dMn(III/IV). As an example, biological nitrate reduction coupled to Mn(II) oxidation has been cited as a reaction

necessary to explain SOZ chemical profiles (Murray et al., 1995; Oguz et al., 2001). The first step of this reaction, nitrate reduction to nitrite, is thermodynamically unfavorable when coupled to the production of Mn oxides and experimental evidence suggests this reaction does not occur in the Black Sea (Schippers et al., 2005). The rapid formation kinetics of the Mn(III)-DFOB complex from its free constituents and its remarkable stability may allow the coupling of Mn(II) oxidation to electron acceptors other than O<sub>2</sub>, such as nitrate.

At the other end of the redox cycle, dMn(III/IV) may provide excellent substrates for reduction, relative to solid-phase Mn oxides, as microbes would not require a surface interaction to access an electron acceptor and could transport it into the periplasm or cell. The stability of the natural complexes may, again, alter the reaction dynamics such that redox coupling of dMn(III/IV) to a reductant such as ammonium or sulfide requires biological catalysis, thus opening an energetically rewarding niche in suboxic systems.

Finally, with regard to siderophores, biogenic Mn oxide production may be repressed by siderophore production via trapping and Mn(II) oxidation to Mn(III) may be catalyzed abiotically. The eventual fate of Mn(III)-siderophore complexes remains an open question—experimental evidence indicates that such complexes are quite stable (Duckworth and Sposito, 2005b). Still, upon their eventual degradation, disproportionation of the free Mn(III) could produce solid phase oxides completely in the absence of enzymatic catalysis.

In conclusion, the microbial Mn cycle in suboxic waters is more complex than previously envisioned. Dissolved Mn(III/IV) production by biogenic ligands and

microbial catalysis implies that novel biological reactions may yet be discovered linking the Mn cycle to other elemental cycles.

Table 5.1  $^{54}\text{Mn}$  partitioning due to biological activity. Particulate Mn and Mn(III)-DFOB are reported as the difference between triplicate incubations with and without the biological poison sodium azide. Errors are summed standard deviations. Total oxidized Mn is the sum of particulate and Mn(III)-DFOB, with summed errors.

Station	Assay	Particulate Mn (nM)	Mn(III)-DFOB (nM)	Total Oxidized Mn (nM)
3	Natural Ligand	12.8 $\pm$ 3.6	2.0 $\pm$ 2.0	14.8 $\pm$ 5.5
	Trapping	6.2 $\pm$ 0.7	19.6 $\pm$ 5.6	25.8 $\pm$ 6.3
4	Natural Ligand	19.2 $\pm$ 2.5	-0.3 $\pm$ 0.8	18.9 $\pm$ 3.3
	Trapping	3.6 $\pm$ 0.6	8.6 $\pm$ 4.1	12.3 $\pm$ 4.7

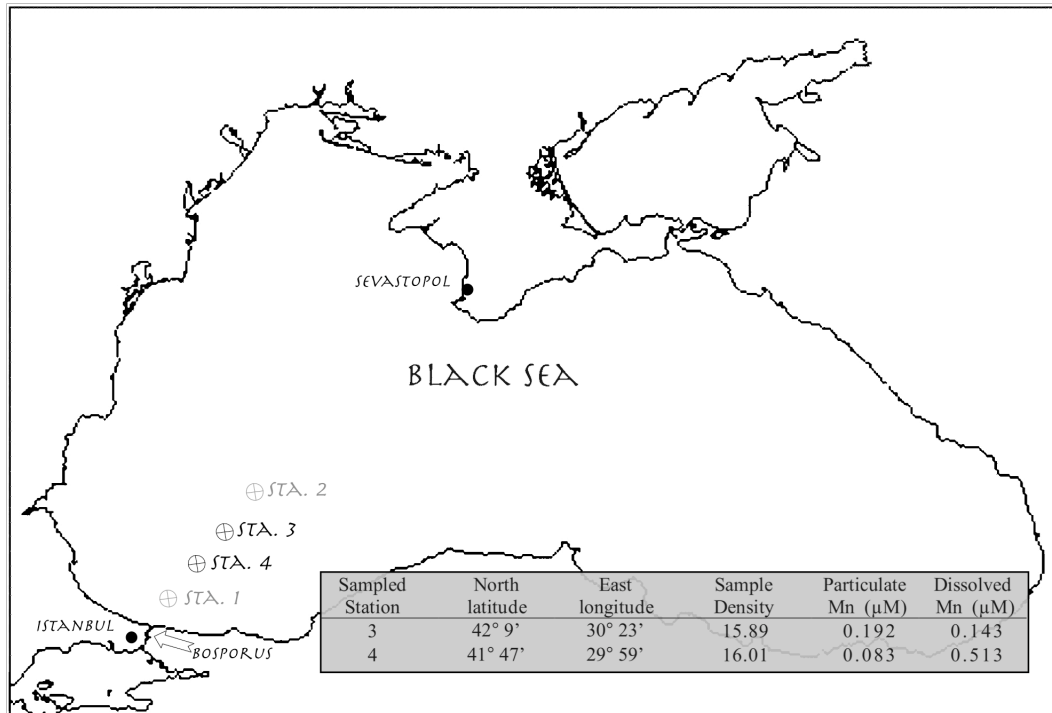


Figure 5.1 Sample locations and water parameters.

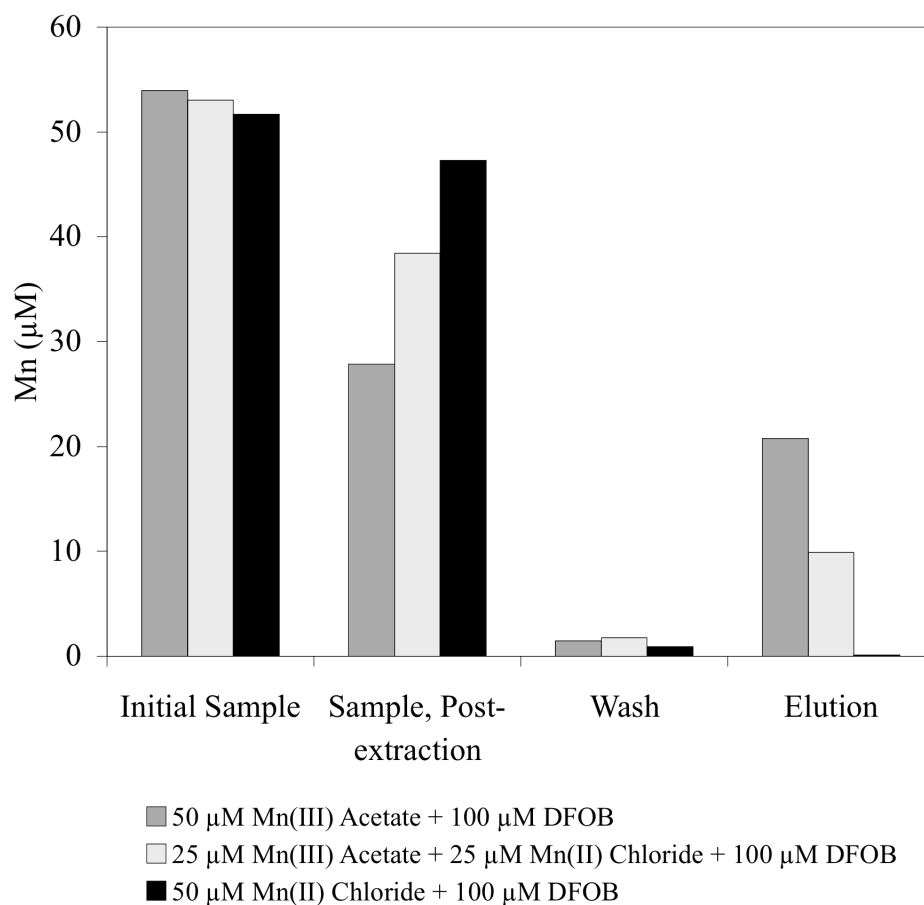


Figure 5.2 Fractionation of Mn during separation on HLB columns, as measured by formaldoxime. Sample conditions are listed in the legend; not all Mn(III) added was stabilized in Mn(III)-DFOB complexes.



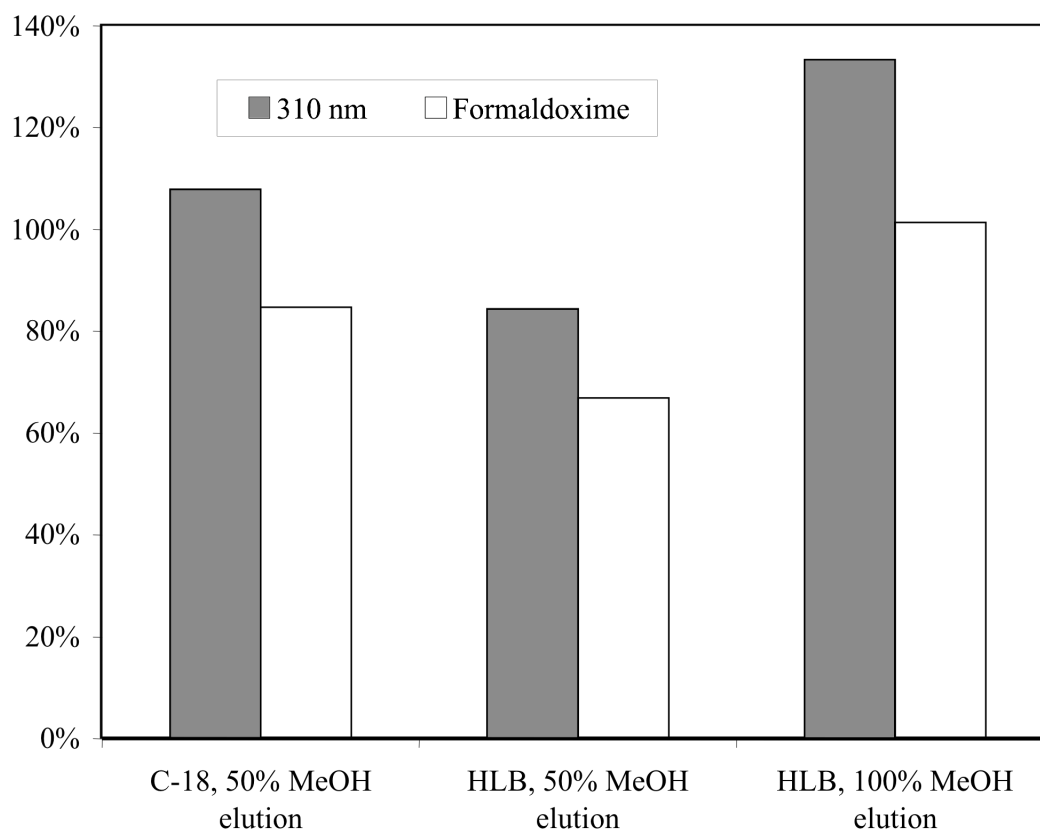


Figure 5.3 Relative recovery of Mn(III)-DFOB complex from different SPE columns using 50% or 100% methanol elutions. Legend indicates measurement method; 310 nm is the absorbance maximum of the Mn(III)-DFOB complex and formaldoxime is a measurement of total Mn. Note that the complex concentration was greater than the total after elution due to a contaminant that co-eluted from the column.

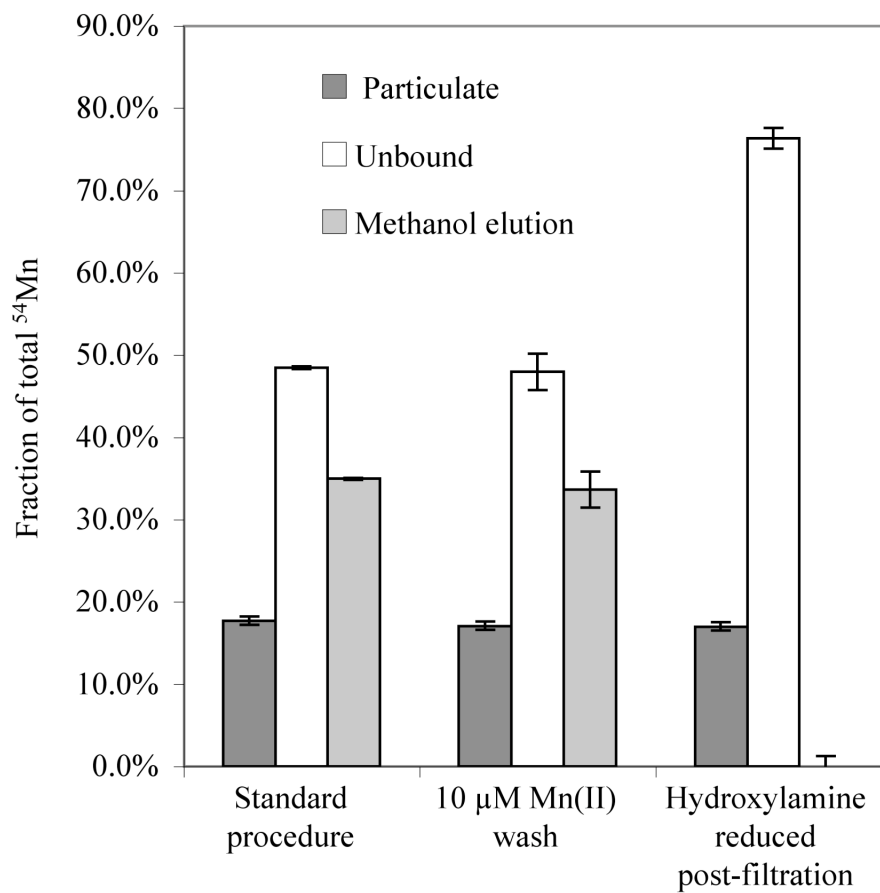


Figure 5.4  $^{54}\text{Mn}$  control experiment with Black Sea water plus 25  $\mu\text{M}$  DFOB. The  $^{54}\text{Mn}$  fraction eluted by methanol could not be exchanged with Mn(II) but was eliminated by reduction to Mn(II) by the addition of a reductant, 0.1% hydroxylamine. This behavior is consistent with specific binding of Mn(III)-DFOB to the column.

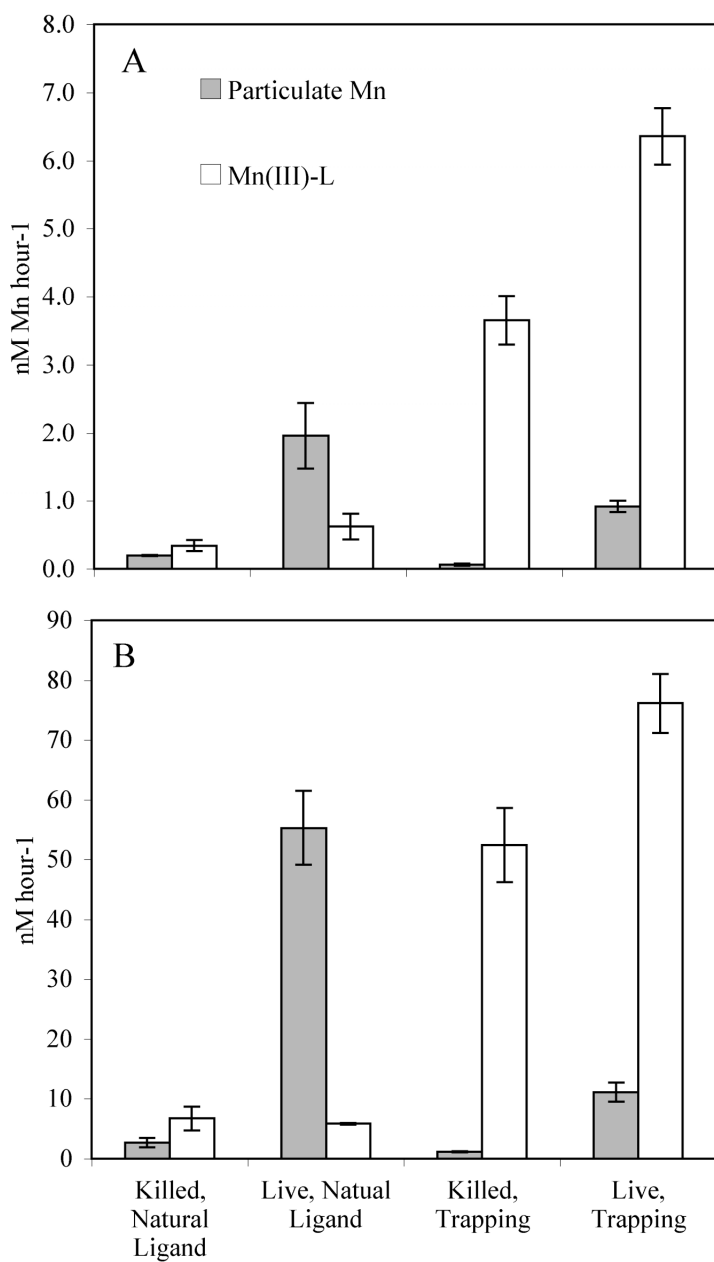


Figure 5.5 Particulate and Mn(III)-DFOB production at (A) station 3 and (B) station 4. Note different y-axis scales. Killed and live refer to poisoned (0.1% sodium azide) and un-poisoned incubations. Natural ligand and trapping refer to addition of DFOB prior to incubation (trapping) or after filtration (natural ligand)—see text for details.

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## **CHAPTER VI**

**Bacterial Mn(II) oxidation in the environment:**

**Open questions and novel research opportunities**

## Overview

This dissertation presents the results of diverse approaches to studying Mn(II) oxidation by microbes in the Black Sea and Pinal Creek, Arizona. While there are themes connecting the experimental chapters, specific additional data would serve to strengthen those connections. The two study sites are similar in the prevalence of biogenic Mn oxides but very different in terms of accessibility and stability. The Black Sea is difficult to access, requiring an oceanic or regional class ship and a full complement of researchers to completely characterize the water column. These constraints are balanced by a remarkably stable environment (aside from the occasionally tempestuous surface conditions) in which to assess spatial correlations between chemistry and biology. Located in central Arizona, Pinal Creek is more accessible, requiring only ground transportation, but does not present stable, accessible gradients. The gradients of O<sub>2</sub> and Mn(II) are clearly present, but the variable nature of the creek's sediment composition, hydrology and riparian growth make accessing them difficult. Such complications are in the nature of microbiological investigations—the spatial and concentration scales of interest are often near or below current analysis and/or sampling thresholds. Thus, it is not surprising that engaging questions are one result of the studies presented here. This chapter will briefly discuss both open questions and unique opportunities suggested by this dissertation.



## Open questions

The work described in Chapters II and III shares a theme of low  $O_2$  concentration as a determinant of microbial Mn(II) oxidation. In Pinal Creek cultures, low  $O_2$  was preferred by Mn(II)-oxidizing bacteria (MnOB) grown in  $O_2$  gradients. In work completed in the Black Sea, nanomolar  $O_2$  was sufficient to catalyze Mn(II) oxidation at surprisingly high rates. As noted in Chapter I, Pinal Creek studies are complicated by shifting water flow and plant growth on short time scales and there is little reason to suspect that any one microbial niche receives a constant supply of  $O_2$  or Mn(II). Still, the MnOB populations appear to prefer low  $O_2$  concentrations, possibly on the order of those that stimulate Mn(II) oxidation in the Black Sea (Chapter III). One potential reason for this that remains unexplored is the proximity of MnOB in both systems to anoxic conditions and anaerobic bacteria. This proximity may simply be due to the high availability of Mn(II) at the oxic/anoxic interface. However, consortia of microaerobic Mn(II) oxidizers and obligate or facultative anaerobes that reduce Mn are certainly possible. Both parties in such a consortium might rapidly consume the products of the other with the aerobes protecting the anaerobes from  $O_2$  exposure while simultaneously providing an electron acceptor in Mn oxides.

Oxygen also influences MnOB in pure culture, but the data are unclear as to how comparable the effects are to mixed cultures and natural populations. As briefly noted in Chapter II, Pinal Creek Mn(II)-oxidizing isolates formed discrete bands of Mn oxide like the mixed cultures but these bands did not remain stable. Instead, the Mn oxidation zone often expanded in either direction within  $O_2$  gradients and

secondary oxide bands often appeared. The Black Sea *Pseudoalteromonas* strains discussed in Chapter IV also grew and oxidized Mn(II) in gradient cultures but did so at the surface of the tube, where the O<sub>2</sub> supply was high (data not shown). Kinetic experiments similar to the  $K_E/V_{E-Max}$  determinations reported in Chapter III might clarify the relationship between O<sub>2</sub> and Mn(II) oxidation by assorted isolates, but only in the case of *Pseudoalteromonas* spp. is there any indication that MnOB cultivated in these studies are an important component of in situ microbial populations. It remains to be seen if the O<sub>2</sub>-dependent  $K_M$  and  $V_{Max}$  values of cultivated MnOB reflect those seen in the Black Sea or other environments.

The *Pseudoalteromonas* spp. also present a preliminary link between Chapters IV and V. Siderophore production by marine bacteria is common but the production of the siderophore desferrioxamine E by Black Sea MnOB is intriguing. Voltammetry measurements in the suboxic zone are consistent with the presence of Mn(III)-DFO complexes (G. Luther, pers. comm.) and cultivation results suggest that the *Pseudoalteromonas* isolates were producing oxidized Mn without always producing solid-phase oxides. Given that Mn(III) production is apparently catalyzed by the addition of DFOB to Black Sea suboxic zone water (Chapter V), it is possible that biological Mn(II) oxidation may proceed by either direct enzymatic activity or indirectly through siderophore production. The fate of Mn(III)-siderophore complexes in the Black Sea remains unknown and the production of Mn(III), in complexes or otherwise, by *Pseudoalteromonas* spp. has yet to be shown. The latter should be revealed by modified mass spectrometry protocols that limit or prevent degradation of

any Mn(III)-DFOE to Mn(II) and free DFOE. The former is more difficult to assess simply because so many fates are possible for a Mn(III)-siderophore complex.

The existence of Mn(III)-siderophore complexes in suboxic zones is an exciting possibility from a microbiological perspective. As noted in Chapters I and III, there have been some difficulties modeling the vertical flow of oxidants into the anoxic waters of the Black Sea. Lateral O<sub>2</sub> supply remains a poorly-constrained solution to this problem and our knowledge about Mn oxidation state is similarly limited. Since Mn measurements are operational in nature, dissolved or colloidal oxidized Mn could be an important part of an unseen oxidant pool, as Mn passing through filters is treated exclusively as Mn(II) by the published models (Murray et al., 1995; Oguz et al., 2001). If significant, this dissolved pool of oxidized Mn may also participate, with or without biological catalysis, in novel reactions such as anaerobic nitrification and/or chemo-denitrification. Additional interactions that should be examined are Fe availability in a system where high levels of Mn potentially interact with siderophores and the products of Mn-organic complex degradation, which might include Mn oxides via disproportionation of Mn(III). Finally, the siderophores themselves may, if abundant, represent a source of dissolved, fixed carbon and nitrogen in the SOZ with implications for the fate of both siderophores and siderophore-bound metals.

Another point made by this dissertation is that the diversity of microbes associated with Mn oxides in natural systems is poorly resolved. The results present in three recent papers (Kirkpatrick et al., 2006; Lin et al., 2006; Vetriani et al., 2003) and Chapter IV all reveal shortcomings in using PCR-based methods for determining

which microbes are responsible for specific processes, even in the well-stratified and well-resolved environment of the Black Sea suboxic zone. This is partly due to the wide density/depth distribution of some organisms like the Planctomyces and the diverse capabilities of abundant groups such as the  $\gamma$ -proteobacteria that were numerically dominant in my clone library (Chapter IV) and in previous clone library, hybridization and population fingerprinting studies (Kirkpatrick et al., 2006; Lin et al., 2006; Vetriani et al., 2003). Organisms or taxonomic groups that are distributed without respect to specific chemical profiles are difficult to assign to, or correlate with, a particular biogeochemical process. Conversely, organisms that appear to have some depth specificity promote speculation about their role when they are closely related to metabolically versatile organisms (e.g. *Pseudomonas* spp.). These difficulties in assigning environmental functions to phylotypes detected in situ highlights opportunities currently arising from new capabilities.

## **Novel research opportunities**

The two study sites discussed in this dissertation will offer unique opportunities in the near future as new technologies present additional options for researchers. Environmental genomics, sometimes referred to as eco- or meta-genomics could offer insights into Mn(II) oxidation in Pinal Creek and the Black Sea. Essentially, environmental genomics refers to sequencing vast amounts of DNA recovered from the system of interest without a PCR step. This eliminates the problem of prior sequence conservation knowledge, which is currently required for PCR primer design. In this way, examining the environmental diversity of multicopper oxidase genes similar to those associated with Mn(II) oxidation would become tractable (see Chapter I). Sequence from a large fraction of the DNA present, would permit functional genes to be linked to genes, such as rRNA genes, that would permit phylogenetic identification. Still, the discovery of MCO genes similar to those required for Mn(II) oxidation would not be indicative of activity.

Environmental proteomics, the determination of the amino acid sequences of proteins isolated from the environment, would largely solve this problem. Identification of expressed proteins, linked to genes and particular phylotypes by environmental genomics would be a relatively robust indication of activity in situ. Many technical and funding challenges remain for such an approach; an acid mine site that has been well-characterized by genomic and proteomic methods contains relatively low complexity because it contains only a few phylotypes (Ram et al., 2005; Tyson et al., 2004). To effectively analyze an environment like the Black Sea or Pinal

Creek, significantly more protein and DNA sequencing would be required, but technological advances may facilitate this, at a relatively low price, in the future.

Another sequencing approach that would offer insights into Mn(II) oxidation in the Black Sea is genomic sequencing of Mn(II)-oxidizing *Pseudoalteromonas* spp. As noted in Chapter IV, Mn(II) oxidizing *Pseudoalteromonas* strains have been isolated from Loihi seamount, where weathering of fresh basalts produces Fe and Mn oxide crusts (Templeton et al., 2005). Genomic comparisons of the Black Sea and Loihi strains would likely be revealing with respect to siderophore synthesis and multicopper oxidase genes. It is entirely possible that the *Pseudoalteromonas* strains oxidize Mn solely by producing DFOE and not through direct enzymatic action.

Along those lines, it seems that the *Pseudoalteromonas* sp., BS03-23 is an excellent candidate for in vitro analyses including mutagenesis and transcriptional profiling. Discoveries made regarding which genes are involved in Mn(II) oxidation and siderophore synthesis as well as the parameters induce their expression would have immediate application in the Black Sea and other environments where siderophores may interact with the Mn redox cycle.

An immediate approach which might pay dividends with fresh Black Sea samples would be fluorescence in situ hybridization (FISH) targeting *Pseudoalteromonas* spp. in samples from vertical and horizontal transects of the suboxic zone. This would mitigate biases associated with DNA extraction and unequal gene amplification inherent to the quantitative PCR approach used in Chapter IV and allow correlations between Mn profiles and *Pseudoalteromonas* abundance. Interestingly, a FISH study of the Black Sea has recently been published and  $\gamma$ -

proteobacteria, which contains the genus *Pseudoalteromonas*, appear to be most abundant group, relative to the total prokaryotic population, at or near the Mn oxide maximum (Lin et al., 2006).

A wholly different question that is not often discussed with regard to the Black Sea is the mobility of prokaryotes across the density gradients that constrain chemical mixing. Planktonic marine prokaryotes have been observed moving in nutrient gradients at rates of  $\sim 100 \mu\text{m s}^{-1}$  (Mitchell et al., 1995). Such a speed would allow a microbe to change its position one meter in less than 3 hours—a significant distance in suboxic waters with dramatic differences in chemical concentrations over as little as a meter. While marine prokaryotes rarely move in a straight line for periods  $> 1$  s, rapid top speeds might permit behavior like that of *Thioploca* spp. which migrate in sedimentary redox gradients in order to access spatially-separated electron acceptors and donors (Huettel et al., 1996). Evidence for this type of behavior in the Black Sea would be of great interest as it would represent microbe-forced mixing of an otherwise diffusion-based system. With regard to Mn redox cycling, one can imagine that Mn(II)-oxidizing organisms might be encrusting themselves with solid minerals in order to increase their density and migrate to reducing zones to access some otherwise-limiting substance, such as ammonium.

Novel opportunities with regard to microbial Mn(II) oxidation also remain in Pinal Creek where Mn oxide coatings are ubiquitous. Scanning electron micrographs of these coatings indicate they contain microbial shapes, much the way a polysaccharide matrix contains microbes in a biofilm (data not shown). A number of observations of mixed mineral precipitation in and around natural biofilms have been

made and their adsorption capacities have been correlated with metal oxide abundance (Nelson et al., 1999). However, not much is known about the lifestyle of the biofilm organisms and whether the mineral precipitates are a beneficial strategy. The production of low molecular weight carbon compounds from otherwise refractory humic acids catalyzed by Mn oxides (Sunda and Kieber, 1994) is particularly interesting with regard to Mn oxide coated biofilms. Mn oxide coatings might play a role in accessing carbon by acting as a non-specific oxidase upon complex, large molecular weight carbon compounds that come in contact with the mineral biofilm. Testing this hypothesis is likely a dissertation unto itself but Pinal Creek offers ample research opportunities due to the prevalence of Mn oxide coatings.



## Summary

This dissertation presents the view that low O<sub>2</sub> concentration is a controlling factor in determining the location and rate of Mn oxide production in the environment. In Pinal Creek, it appears that a diverse set of organisms catalyzes Mn oxide formation, while similar reactions in the Black Sea may be dominated by a small number of genera or species. While open questions remain as to the spatial and temporal importance of the organisms identified in this work, the *Pseudoalteromonas* spp. in particular provide a starting point for further detailed investigations of Mn(II) oxidation mechanisms in vitro and in situ. These organisms serve to illustrate how the complexity implied by previous experiments regarding Mn speciation and mechanisms of biogenic Mn oxide formation in vitro is truly important in the environment. Siderophores, Mn(II) oxidizing organisms and unseen oxidants such as nanomolar O<sub>2</sub> and Mn(III) are important features of environments where Mn oxides are produced.

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