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ASSESSMENT OF MICROBIAL COMMUNITIES IN HIGH ALTITUDE WETLANDS DISTURBED BY IRON AND MANGANESE

A Thesis by KRISTIN M. JOHNSON

Submitted to the Graduate School Appalachian State University In partial fulfillment of the requirement for the degree of MASTER OF SCIENCE

August 2009 Department of Biology

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ABSTRACT

ASSESSMENT OF MICROBIAL COMMUNITIES IN HIGH ALTITUDE WETLANDS DISTURBED BY IRON AND MANGANESE (August 2009)

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Chairperson: Ece Karatan

The Sorrento wetland is a high altitude wetland that has been disturbed due to anthropogenic activity. It has been previously established that this disturbance has led to several Fe and Mn seeps that contribute to high levels of Fe and Mn in stream and wetland water and sediment. While these high metal concentrations have led to decreases in the diversity of the benthic macroinvertebrate community, it is still unknown the extent to which these high metal concentrations have effected the microbial community. Four methods were chosen to characterize the microbial community at the site of the seeps, an upstream reference site, and a downstream wetland site. Enrichment and dilution cultures isolated several bacteria and fungi capable of oxidizing Mn from seep and wetland sites, including *Bacillus* spp., *Pseudomonas* spp., and *Leptothrix* sp. Most probable number assays indicated that seep and wetland sites contained higher numbers of Mn oxidizing heterotrophic microorganisms compared with the upstream reference site. Light microscopy and fluorescent in situ hybridization images indicated an abundance of Fe and Mn oxidizing *Leptothrix* spp. and *Gallionella ferruginea* in seep and wetland sites. Analysis of a 16S rRNA clonal library indicated an incredibly diverse community present at seep sites,

iv

including 62 genetically diverse phylotypes and only five dominant community members. Results indicate that increased metal concentrations are enriching for Fe and Mn oxidizing bacteria, that coexist within a diverse microbial community.

TABLE OF CONTENTS

Page
1 ago

Abstract	iv
Acknowledgements	vii
List of Tables	ix
List of Figures	xi
Introduction	1
Methods	
Results	37
Discussion	63
Conclusion	72
References	73
Appendix A	78
Appendix B	
Vita	86

ACKNOWLEDGEMENTS

I would like to thank my advisor, Ece Karatan, for all of her patience, help, and support these past two years. Whether it was lab techniques, grant or thesis writing, or last minute recommendation letters, you have been an incredible mentor and advisor and I'm so fortunate to have been a member of the Karatan lab.

I would also like to thank my committee member, Michael Windelspecht, for all of his assistance, especially in the writing and editing processes. I also appreciate all of the teaching opportunities you provided or had some role in providing. Thank you!

I would also like to thank my committee member, John Walker, for all of his assistance in the writing and editing processes.

I would also like to thank Dr. Suzanna Bräuer for all of her help with this project. This project really came together because of all of your added expertise and we couldn't have done it without you. Thank you for all of the supplies, advice, protocols, encouragement, ideas, company on wetland excursions, etc. Thank you so very, very much!!

I would also like to thank Dr. Guichuan Hou for assistance with the light and confocal microscopy portions of my research.

I would also like to thank Dr. Mary Connell for allowing me to use her equipment on a regular basis and providing advice on numerous molecular concerns. Also, thank you Jim, for always managing to find me exactly what I needed and where it was located. The members of the Zerucha lab also deserve a big thanks for sharing supplies and equipment on several occasions, many times saving my research!

Thank you also to Tracy and Betsy, the brains behind many of the operations I take for granted. You have both brightened my day many times.

I would also like to thank Dr. Dass for all of his support and mentorship the past several years, both in my undergraduate and graduate careers.

Thank you also to my friends and lab mates. Becca, Zach, Lindsay, Marcus, Josiah, Nick, and Alex, I appreciate your friendship, company, and advise over the past few years and thank you for keeping our lab such a great place to spend a majority of my waking hours.

Thank you also to the Office of Student Research and Graduate School Student Association for providing necessary funding on several occasions.

Finally, I owe a huge thanks to my husband, Brad. Thank you for putting up with me these past two years and putting up without me these past few months. You have really been my rock in all of this and I couldn't have finished this thesis without your support.

LIST OF TABLES

Table 1. Soil sediment and water Fe and Mn concentrations for the Sorrento wetland16
Table 2. Composition of hybridization buffers used to achieve different stringencies25
Table 3. List of probe sequences and tags used in FISH
Table 4. Composition of washing buffers used to achieve different stringencies
Table 5. List of primer sequences used in Polymerase Chain Reaction (PCR) amplification
Table 6. Tabular depiction of 96 well growth plates used for Most Probable Number(MPN) assays
Table 7. Soil and water Fe and Mn concentrations for the Sorrento stream
Table 8. Water quality indicators for the Sorrento stream
Table 9. Identification and description of Mn oxidizing bacterial isolates
Table 10. Identification and description of Mn oxidizing and non-Mn oxidizing fungal isolates
Table 11. Summary of MPNs found in the Sorrento wetland and stream

LIST OF FIGURES

Figure 1. Depiction of Fe cycling in freshwater circumneutral wetland ecosystems2
Figure 2. Depiction of Mn cycling in freshwater circumneutral wetland ecosystems3
Figure 3. Photograph of wetland from Sorrento Drive15
Figure 4. Depiction of the Sorrento wetland16
Figure 5. Photograph of rust colored seeps within S0 site leading to the Sorrento wetland
Figure 6. Close up photograph of surface sheen found at W site in the Sorrento wetland
Figure 7. The Sorrento wetland18
Figure 8. Photograph of wooly orange biomats present at the S0 site
Figure 9. Photograph of orange floc covering submerged grasses at S0 site20
Figure 10. Photograph of upstream reference site
Figure 11. Gel electrophoresis image of 1500 bp band corresponding to 16S rRNA gene
Figure 12. Gel electrophoresis image of 16S rRNA insert
Figure 13. Morphotype 1 from S0 seep sample taken in February of 2009
Figure 14. Morphotype 2 from S0 seep sample taken in June of 200840
Figure 15. SR reference site from February 200941
Figure 16. FISH image from S0 site using LD-1 probe labeled with Cy542
Figure 17. FISH image from SR site using LD-1 probe labeled with Cy542
Figure 18. FISH image from S0 site using EUB338 probe labeled with FITC43

Figure 19. FISH image from SR site using EUB338 probe labeled with FITC	.44
Figure 20. FISH image from S0 site using nonEUB338 probe labeled with FITC	.45
Figure 21. FISH image from S0 site using nonEUB338 probe labeled with Cy5	.45
Figure 22a-h. Images of bacterial isolates	.47
Figure 23. Comparison of total culturable heterotrophic microorganisms versus culturable heterotrophic Mn (II) oxidizers	.51
Figure 24a-l. RFLP images generated from 16S rRNA clonal library	.52

Introduction

Wetlands and Their Importance in Ecosystems

Wetlands are transitional zones between terrestrial and aquatic systems, characterized by shallow water overlying the surface a majority of the year. As a result, the soil is waterlogged, and plants and animals are adapted to survive in a waterlogged state (1, 2). Wetlands can vary extensively and differ according to their formation characteristics, including hydrology, landscape position, degree of colonization, and degree and type of disturbance (1). Riparian wetlands are those found in lower-lying bottomlands, alongside a stream. The water has a directional flow with a well-defined input water source and output water source (2).

Wetland ecosystems perform many vital functions in an environment. They provide a habitat for organisms; act as a filter and sink for pollutants and nutrients; store, transport, and promote sediment settling from surface run off and flood waters; regulate ground water levels; and act as a buffer between developed urban areas and natural areas (1-10). It has been increasingly noted that wetlands also act as important sinks for carbon (11). It is estimated that wetlands contribute up to 71% of terrestrial carbon stores (12). One of the most important functions that wetlands provide is to promote nutrient and metal cycling, contributing especially to iron (Fe) and manganese (Mn) cycling (1-10).

Fe in wetlands exists in predominantly two valence states, oxidized trivalent Fe (III) and reduced divalent Fe (II). At circumneutral pH, Fe (II) readily oxidizes to Fe (III), but there are several environmental factors that can effect the direction of this redox reaction. Because reduced Fe (II) is soluble at circumnetrual pH, it can be dissolved in the water and transported downstream. It can also act as an electron donor in anaerobic soils, forming Fe sulfides. Another fate is for reduced Fe (II) to be oxidized, either abiotically from oxygen released by plant rhizospheres and the water-air interface, or biotically from Fe oxidizing bacteria. The results of Fe (II) oxidation are typically poorly ordered Fe (III) hydroxides. These Fe (III) hydroxides are metabolized by Fe reducing bacteria present in aerobic zones of the soil, re-forming reduced Fe (II). Fe (III) hydroxides can couple with and bind organic matter, especially humic subtances, forming stable complexes (*13*). Figure 1 depicts Fe cycling in freshwater circumneutral wetland ecosystems.



Figure 1. Depiction of Fe cycling in freshwater circumneutral wetland ecosystems (13).Mn can exist in several valence states in wetlands, including Mn (II), Mn (III) (14),and Mn (IV). Mn (II) present in wetlands can have several fates. Because Mn (II) is readily

soluble at neutral pH, it can become dissolved in the watershed and transported elsewhere. Reduced Mn can also be oxidized, either from oxygen released from plant rhizospheres, or by Mn oxidizing bacteria present in soil or water (*13*). At circumneutral pH and at the low temperatures often found in wetlands, Mn oxidation from Mn (II) to Mn (III) or Mn (IV) is thermodynamically favored, but still proceeds at a fairly low rate (*15*). The presence of Mn oxidizing bacteria can speed up this rate five fold (*15-17*). Mn hydroxides are the product of Mn oxidation. These Mn hydroxides in turn form stable complexes with organic matter (*13*). Figure 2 depicts Mn cycling in freshwater circumneutral wetland ecosystems.



Figure 2. Depiction of Mn cycling in freshwater circumneutral wetland ecosystems (13).

Wetland cycling of Fe and Mn are heavily dependent on the Fe and Mn oxidizing and reducing bacteria that reside there. The following two sections will concentrate on microorganisms associated with Fe and Mn oxidation, as well as the mechanisms by which they occur.

Microbial Fe Oxidation: Microbes and Mechanisms

There are several different environments, both acidic and neutral in pH, known to harbor Fe oxidizing microorganisms. These environments all contain a constant source of Fe (II) and have low oxygen levels (18-20). In freshwater habitats, these environments include surface waters, upper layer sediments, and the rhizosphere of plant roots (18, 21). Iron oxidizing bacteria are also found extensively in subterranean environments including groundwater, water wells, water distribution systems and water treatment systems (18, 21). These microorganisms have also been found in caves, tunnels, hot springs, and mine shafts (18, 21).

Gallionella ferruginea was the first Fe oxidizing bacteria to be successfully isolated and identified in 1957 (22). It was enriched from Fe seeps along both a California beach and a New Jersey spring. *G. ferruginea* requires the presence of Fe (II) to grow and was originally though to be chemolithotrophic, metabolizing only Fe(II) for energy. It is now thought to be mixotrophic, as it has been shown to use carbon dioxide, glucose, fructose, and sucrose as a carbon sources (18, 22, 23). *Gallionella* spp. require a specific oxygen gradient; they will not grow in conditions that are too reducing or too oxidizing (24). *G. ferruginea* has also been isolated in acid mine drainage waters in Carnoules, France (25), microbial mats at the bottom of a thermal vent in Crater Lake, Oregon (26) and Fe-rich seeps along a California stream (27). *Gallionella* spp. are most noted for their distinct morphology consisting of long branching twisted stalks coated with ferric Fe oxides (18, 22, 24-27) extending from the bean-shaped bacterium. Iron oxidation in *Gallionella* spp. is thought to be associated with these stalks. In the absence of stalks, Fe oxidation and precipitation is slowed significantly (28, 29).

Leptothrix spp. are another commonly found Fe oxidizing bacterial genus, first described in1912 by Schwers (30), although it was observed hundreds of years earlier (31). Leptothrix is strictly aerobic and chemoheterotrophic and can even grow in the absence of Fe. The possibility does exist that it is mixotrophic, but to date no evidence exists proving this idea (31). Leptothrix is unique in its ability to enclose itself in an Fe and Mn oxide encrusted sheath (31). Although Leptothrix spp. have the ability to oxidize both Fe and Mn, it is believed that the Fe and Mn oxides present on the sheath are the result of abiotic oxidation (13). Leptothrix has been isolated in a variety of habitats and can often be found at sites with circumneutral pH, a constant source of reduced Fe and Mn, and an oxygen gradient (31, 32), including marine environments (33), bottom lake sediments (34, 35), microbial mats at the bottom of thermal vents (26), sewage sludge (36), wells and groundwaters (37), and wetlands (18, 32). This genus tends to form thick wooly flocs and carpets that range in color from orange dark brown, depending on the extent of Fe and Mn oxidation occurring (31, 38, 39).

Ferroglobus, *Siderocapsa*, *Sideroxydans*, *Naumanniella*, and *Rhodobacter* are other bacterial genera capable of oxidizing Fe in circumneutral conditions (*13, 40*) and exist in a range of environments including hydrothermal vents, freshwater and marine environments, soil, and the rhizosphere of plants (*18, 40-42*).

Acidophilic Fe oxidizers include the genera *Thiobacillus, Ferrobacillus, Sulfobacillus, Ferroplasma,* and *Sulfolobus.* These bacteria are autotrophic and are capable of using various inorganic compounds to obtain energy, including Fe (II), sulfides and sulfur, and hydrogen. Carbon dioxide acts as their carbon source and ammonia acts as their nitrogen source. All strains of these bacteria grow in acidic environments with pH<2 and

are often found in conjunction with acid mine drainage from mining sites and acidic peat bogs (13, 28, 40).

Iron is becoming recognized as playing a central role in the metabolism of a variety of microorganisms by serving as an electron donor for chemolithotrophic growth or as an electron acceptor under anaerobic or microaerophillic conditions (18). At neutral pH, abiotic Fe oxidation occurs rapidly, so microorganisms gaining energy from Fe oxidation will be in competition for this rate-limiting resource. This renders the entire idea of strictly chemolithotrophic Fe oxidation at neutral pH a controversial idea, although it has been reported in several instances (28, 35, 43). At acidic pH, Fe oxidation will not proceed abiotically and is due entirely to microorganisms present. However, the energy yield is minimal and large amounts of Fe must be oxidized in order to sustain growth (13, 28).

Evidence of chemolithotrophic bacteria that metabolize ferrous Fe as the sole source of energy have been reported, although most Fe oxidizing microorganisms studied are mixotrophic, meaning they can utilize Fe as an electron donor and other organic compounds for carbon sources (*18, 22, 40*). It is thought that the reason for Fe oxidation in mixotrophic or heterotrophic bacteria is to avoid encrustation by other Fe (II) metabolizing cells, which would prevent substrate intake and could cause cell death (*24*).

The exact pathway involved in microbial Fe oxidation is not clear and varying models are described in the literature. It is agreed that the initial oxidation of Fe (II) to Fe (III) occurs at the outer membrane of the cell, as was shown to be the case with the acidophilic Fe oxidizing bacteria *Thiobacillus ferroxidans* (13, 24, 44, 45). The electron is then transferred to a rusticyanin, a copper containing protein, and from there to a c-type cytochrome located within the periplasm of the cell, forming water through the action of

cytochrome oxidase (13, 44, 45). In the neutrophilic *Leptothrix* spp. an Fe (II)-oxidizing protein was found in spent culture media, suggesting that the Fe (II) oxidizing compounds are actively secreted by the cell (46). Recent studies indicate cytochrome 579 as being a key enzyme involved in microbial Fe oxidation in acidic environments. Red in color, it is found near the bacterial cell surface and is thought to act as a transfer protein, moving electrons obtained from Fe (II) oxidation for use in other metabolic activities (45).

Microbial Mn Oxidation: Microbes and Mechanisms

A diverse array of phylogenetic taxa including fungi and bacteria, and possibly even archae are capable of oxidizing soluble Mn (II), producing insoluble Mn (IV) oxides. Manganese oxidizing prokaryotes are ubiquitous in nature, having been found in a range of environments including hydrothermal vent deposits and plumes (47), hot springs (48), water pipes (49), hyporheic zones of streams, rivers, lakes, and wetlands (34, 50-55), caves (56), and soils (35, 57)

Most research involving Mn oxidation focuses on bacteria, as they have been thought to be the major Mn oxidizers in nature (17, 58). These Mn oxidizing bacteria include species and strains within the genera *Bacillus*, *Leptothrix*, *Pseudomonas*, *Arthrobacter*, *Pedomicrobium*, *Erythrobacter*, *Streptomyces*, *Bacteriodetes*, *Deinococcus*, *Chlamydiae*, *Caldimonas*, and *Nitrosomonas* (17, 59). Of these various bacteria, several have proven to be of great importance in laboratory work involving the mechanisms of Mn oxidation, including *Leptothrix discophora* SS-1, *Bacillus* spp. strain SG1, *Pseudomonas putida* MnB1, *Pseudomonas putida* GB-1, and *Pedomicrobium* sp. ACM 3067 (17, 58).

Although the specific mechanisms for Mn (II) oxidation in bacteria has yet to be fully described, much information has been gleaned from studies investigating this process. It is known that Mn (II) is oxidized to Mn (IV), although recent studies have provided evidence of a temporary Mn (III) intermediate between the oxidation of Mn (II) to Mn (IV) (14, 15, 60). Through studies involving transposon mutagenesis, over 18 genes from nine different operons have been identified as having some role in Mn oxidation (17). The mofA gene isolated from Leptothrix discophora SS-1 has been shown to encode a protein showing similarity to the multicopper oxidase family of proteins and also contains a signal sequence destining it for extracellular secretion (16). The mnxG gene isolated from Bacillus spp. strain SG-1, the cumA gene isolated from Pseudomonas putida GB-1, and the moxA gene isolated from *Pedomicrobium* sp. have been shown to encode for similar proteins also showing similarity to the multicopper oxidase family of proteins. The addition of reduced copper (Cu (II)) to growth cultures of all three bacterial strains has also been shown to stimulate Mn (II) oxidation, suggesting the role of multicopper oxidases in Mn oxide production, although it is not clear if this enzyme directly catalyzes Mn oxidation (16, 17, 61). Recent studies have shown that there are additional cofactors required for Mn oxidation, such as quinones. These quinones may represent cofactors for a new class of Mn oxidizing enzymes (61).

Little is known about why these prokaryotes oxidize Mn (17, 50). Several hypotheses have arisen concerning this occurrence. Even though the oxidation of Mn (II) to Mn (IV) is thermodynamically favored (17) and sheathed Mn oxidizing bacteria have been found in nutrient-lacking arctic lakes (62), there is no substantial evidence that Mn oxidizing bacteria derive their energy in this manner (17). To date, no bacterial species has been

known to show autotrophic growth on Mn (II) plates (15). It has been suggested by some that metal oxidation is simply an evolutionary holdover that once contributed to survival, but now has no physiological relevance (17). However, there are numerous benefits to Mn oxidation. By coating themselves with Mn oxides, bacteria have added resistance to environmental concerns such as ultraviolet radiation, reactive oxygen species, viral attack, and predation (15, 17, 63). Recent studies have shown that Mn oxides have high sorption capacities for heavy metals such as nickel (Ni), zinc (Zn), Cu, cobalt (Co), Mn, lead (Pb), and cadmium (Cd) (15, 17, 37, 58, 63, 64), which are known to inhibit microbial growth (58). Mn oxides are also known to degrade humic substances such as humic acids into low molecular weight compounds such as pyruvate that bacteria can then metabolize (15, 17, 58).

Other ideas consider the role of Mn oxidation in cellular functioning within bacterial cells. Mn is a trace nutrient and is needed for a variety of cellular processes (17). Mn is required for the activation of such enzymes as DNA polymerase and phosphoenolpyruvate carboxykinase. Mn oxides are suspected to be used by bacteria as terminal electron acceptors in anoxic or microaerophillic environments such as sediment and soil (15, 17, 58).

Alterations in Fe and Mn Cycling

Healthy Fe and Mn cycling is closely linked to the presence and roles of Fe and Mn oxidizing microorganisms, but several other factors can also cause fluctuations in metal cycling in freshwater systems, including the hydrology and geology of the area, the type and presence of vegetation, and anthropogenic disturbance.

Hydrology is considered to be one of the predominant factors controlling wetland processes and characteristics, including metal concentrations, redox status, pH, nutrient cycling, and community composition (9, 65-67). Seasonal hydrological variations can change the magnitude and persistence of anaerobic conditions, affecting any redox-sensitive minerals, including Fe (II) and Mn (II) (67). Not only do these variations directly affect metal cycling by altering groundwater and surface water flow, they have indirect affects as well. Hydrology affects microbial communities by changing the aerobic/anaerobic interface, which alters the distribution and abundance of key microbial nutrient cyclers (65, 66).

Geological conditions also affect metal cycling. Water that filters through granite based soils and rock tends to have low pH and low conductivity, meaning there are few dissolved inorganic compounds such as salts and other minerals. Low pH and low conductivity will prevent the abiotic oxidation of Fe and Mn precipitates (*67*). Water that filters through clay soils will have higher conductivity and many dissolved salts and minerals, causing the oxidation and precipitation of Fe and Mn (*67*). Also because the major source of Fe and Mn to ecosystems is the weathering of indigenous rock, the abundance of Fe and Mn in the surrounding rock has direct impacts on Fe and Mn cycling in local soils (*13*).

Vegetation abundance and type also affect concentrations of metals in wetland ecosystems. A study comparing concentrations of metals, including iron, of coastal wetland soils found that the presence of any vegetation contributes significantly to Fe cycling. This study is in agreement with numerous other studies suggesting that the rhizosphere of plants in wetland soils contributes to Fe cycling, due to oxygen release from the root system and

from Fe-oxidizing and Fe-reducing microbial communities associated with the root system (65, 68-72).

Anthropogenic disturbance also contributes to changes in Fe and Mn cycling in wetlands, especially by changing the hydrology and vegetation presence, further influencing metal cycling as mentioned previously. The type of disturbance varies with the specific location of the wetland, but is typically due to anthropogenic influence in the form of road construction, coal mining, urban and rural development, and agriculture (4, 73). Urbanization and agriculture have both been shown to increase nutrient loading and retention of nitrogen, phosphorus, and Fe in surrounding riparian wetland ecosystems (7-9), degrading water quality. Drainage of wetlands is also a common problem in agricultural areas (12). Urbanization has also been shown to increase habitat fragmentation and decrease water quality through chemical runoff (1, 7). Coal mining can cause extremely acidic runoff into wetland ecosystems, drastically lowering pH levels (74). Runoff from coal mining typically contains high concentrations of heavy metals including Pb, cesium (Cs), arsenic (As), Cu, Fe, Mn, and Zn that are sequestered in the surrounding freshwater environments (74-76), further degrading water quality.

Previous Research on Wetland Bioindicators and Microbes

Across the country, wetlands are becoming increasingly endangered ecosystems. Anthropogenic influence that alters the Fe and Mn cycling of wetlands can cause the ecosystems to become damaged and unhealthy. Therefore, wetland and stream research frequently investigates bioindicators of watershed ecosystem health. While benthic macroinvertebrates are most often used as bioindicators (*73*), other indicators have also been evaluated and found useful, such as vascular plant and avian diversity (77). Benthic macroinvertebrates are most commonly used as bioindicators of watershed quality because of their sensitivity to changes in water quality (73, 78-80). Numerous studies have shown that with an increase in nutrient concentrations or metal concentrations, there are decreases in macroinvertebrate feeding, growth, reproduction, and ultimately increases in macroinvertebrate mortality (73, 78-81). Several studies have indicated that increases in Fe and Mn concentrations can also lead to decreases in macroinvertebrate diversity (73, 81).

There has been relatively little work done on the relationship between bacterial community structure and watershed health and function. This is most likely attributed to the fact that wetland bacterial communities are highly complex in nature and to fully characterize a bacterial system, all organisms need to be counted and identified, a feat that proves to be time and resource intensive (*82*). Also, a vast majority of microorganisms are unable to be isolated and cultured under traditional laboratory settings and must be analyzed through culture independent techniques (*3, 83, 84*). Various molecular techniques have been adapted for this purpose. The most common approach for assessing bacterial diversity has been to create 16 S rRNA gene libraries and then analyze these libraries using a variety of approaches including Restriction Fragment Length Polymorphism (RFLP) and Terminal Restriction Fragment Length Polymorphism (RFLP) and sequencing (*25, 84-88*). Another common method for accessing bacterial community diversity has been Fluorescent In-Situ Hybridization (FISH), where oligonucleotide probes with fluorescent tags are used to determine the presence or absence of specific targeted bacteria. These fluorescent tags can then be visualized with a laser scanning confocal microscope (*88-92*). These methods can

give a snapshot of the microbial diversity present at a given time and location in an ecosystem.

Studies investigating the relationship between freshwater wetland health and the corresponding composition of the microbial communities are limited in number, and results are inconclusive. Environments studied often have increased concentrations of both Fe and Mn. Several studies have shown that the actual diversity of microbial communities does not change with increased Fe and Mn levels, but community composition can change (18, 81). Another study looking at the microbial assembly of constructed wetlands suggested that the diversity and composition seems to be randomly assembled and unaffected by nutrient or mineral inputs or vegetation type or presence (93). In contrast, other studies have shown that there is a strong correlation between increased Fe and Mn concentrations and decreases in microbial diversity (25, 94) and that wetland microbial communities, especially those rich in Fe and Mn, tend to be dominated by Leptothrix, Gallionella, Pseudomonas, Bacillus, Naumaniella, and Siderocapsa (13, 70, 74, 82, 92, 95, 96), all of which are Fe and Mn oxidizing bacteria that coexist in a biofilm rather than in a free-living state (97, 98). However, it has been suggested that different microbial communities respond differently to increasing metal levels, which may provide an explanation for the contradictory findings mentioned previously. While some communities present in Fe and Mn rich environments might be enriched for metal oxidizing taxa, others may not. Thus, it is not expected that all metal rich environments will be dominated by metal oxidizing microorganisms (18). It is possible that there are temporal changes in the microbial communities in response to increasing Fe and Mn concentrations but as communities acclimate and adjust over time, they become more similar to those of undisturbed environments (66). In this case,

community composition would be affected by how soon sampling was done after the disturbance.

Description of the Sorrento Wetland

While the southeastern states, including North Carolina, contain only 25% of the land mass in the continental US, they contain 47% of the nation's wetland ecosystems (7). Across the country, including northwestern North Carolina, wetlands are becoming widely recognized as endangered ecosystems. During the last 200 years, approximately half of the wetlands in the contiguous United States have been lost. Up to 98% of these are forested inland wetlands (12). It is estimated that in the southern Appalachian wetland system alone, only 300 ha of wetlands remain out of the original 2000 ha that once existed (4). Of the remaining 300 ha, many have been classified as disturbed. Because many wetlands, especially those located in mountainous areas, often lie in the flatter valley bottoms, they are more susceptible to development and more likely to be disturbed (73, 99). Our study involves a disturbed high altitude riparian wetland that has been previously characterized (73, 81).

The Sorrento wetland is fed by a mountain tributary that flows down the mountain, passing through two impoundments and crossing under Sorrento Drive (in Blowing Rock, NC) before reaching the wetland. Figure 3 is a photograph of the Sorrento Wetland and surrounding physical features. Note the presence of cattails in the background, delineating the wetland.



Figure 3. Photograph of wetland from Sorrento Dr. Note the presence of cattails and grasses, characteristic of wetland ecosystems.

Beginning at the point furthest upstream, site SR is an upstream reference site located across Sorrento Drive and approximately 500 m upstream from the Fe and Mn seeps. Site R is located below Sorrento Drive at the effluent of the second impoundment and receives water from Site SR and from several other smaller ponds located nearby. It is still approximately 25 m above the Fe and Mn seeps. Site S0 is located in the stream at the site of the first Fe and Mn seep. Site S25 is located 25 m downstream from site S0. Site S50 is 50 m downstream from site S0 and also downstream of a second and smaller Fe and Mn seep. Site W is located after the stream broadens and flows into the wetland itself. The land surrounding both the stream and wetland has been disturbed by several attempts at development in the last several years (73). Figure 4 is a schematic drawing of the Sorrento wetland and stream, including site names and locations.



Figure 4. Depiction of the Sorrento wetland. Site names are written in red and arrows indicate the direction of water flow. Site SR is an upstream reference site, Site R is located at the lower of two retention ponds, Site S0 is at the first of two Fe and Mn seeps, S25 and S50 are 25 and 50 meters respectively downstream of Site S0, and Site W is within the actual wetland.

This wetland has been found to have extremely high levels of Fe and Mn (see Table

1), both exceeding the Environmental Protection Agency (EPA) published limits and North

Carolina Department of Water Quality Standards (NCDWQ) known to sustain aquatic life

(69, 73, 81).

Table 1. Soil sediment and water Fe and Mn concentrations for the Sorrento wetland. Measurements were taken in February 2006. North Carolina Department of Water Quality Standards (NCDWQ) are provided for comparison. (73, 81).

		Sediment		W	ater
Date	Sample Site	Fe (mg/L)	Mn (mg/L)	Fe mg/L)	Mn (mg/L)
February	SO	539.0	382.0	5.780	0.350
2006	SR	52.0	83.0	0.323	0.015
	NCDWQ	-		1.00	0.20

There is also an abundance of rust colored seeps (see Figure 5) and oily appearing sheens that shatter upon touch, often as sociated with Mn oxidizing bacteria (*39*), at several points along the stream and within the wetland (Figures 5-7). There are also orange flocculent masses present at the soil water interface (Figure 8).



Figure 5. Photograph of rust colored seeps at S0 site, leading to the Sorrento wetland. These seeps are often associated with Fe and Mn oxidizing bacteria such as *Leptothrix* sp. and *Gallionella* sp.



Figure 6. Close up photograph of surface sheen found at W site in the Sorrento wetland.



Figure 7. The Sorrento wetland. Note the presence of orange biomass and oily sheen on water surface.



Figure 8. Photograph of wooly orange biomats present at the S0 site. These biomats are indicative of *Leptothrix* spp. and other Fe and Mn oxidizing bacteria. Such biomats often exist at the soil water interface within the stream.

Rocks within the streambed at and downstream of the seeps are coated with a thick orange layer of sediment, sometimes measuring up to two centimeters in thickness. Thick wooly flocs resembling orange carpet are also present at various locations along the banks of the stream attached to weeds and debris within the water and can extend up to several centimeters in length (Figure 9). None of the aforementioned features are present at the locations upstream of the seeps (Figure 10).



Figure 9. Photograph of orange floc covering submerged grasses at S0 site stream.



Figure 10. Photograph of upstream reference site. Note the lack of orange discoloration or submerged flocculent masses.

Purpose of Study and Hypothesis

The Sorrento wetland contains high concentrations of Fe and Mn, as well as Zn, while all other commonly used water quality indicators are within accepted range. Previous research on the Sorrento wetland has concluded that the increased Fe and Mn concentrations led to a decrease in macroinvertebrate diversity (73), but had no effect on microbial diversity. Furthermore, previously constructed 16 Sverdberg (16S) rRNA clonal libraries found no commonly recognized metal oxidizing bacteria dominating in stream or wetland sites, suggesting that the oxidation of Fe and Mn in the Sorrento wetland is not biological in nature (*81*). However, because only twenty clones from each site were sequenced and no library was constructed from the actual site of the seep within the Sorrento wetland, more work was needed to make the conclusion that metal oxidation is biologically independent.

My goal for this study is to further investigate the microbial community present in the Sorrento wetland to determine if Fe and Mn oxidation is biologically independent. I hypothesized that because of the physical characteristics of the wetland, including high metal concentrations, orange flocculent growth, and oily surface sheens, the high metal concentrations are enriching for Fe and Mn oxidizing bacteria, especially those of the genus *Leptothrix*, which will dominate the microbial community. I tested my hypothesis using a combination of four different methods. Light and FISH microscopy determined the presence or absence of *Leptothrix* spp., and other morphologically distinct Fe and Mn oxidizing bacteria, at various locations along the stream, wetland, and upstream reference sites. A 16S rRNA clonal library was constructed for the site of the seep and analyzed through RFLP and sequencing to determine dominant community members. Traditional culturing methods including enrichment plates and dilution series were used in an attempt to

grow up and isolate various Mn oxidizing microorganisms. Most Probable Number (MPN) assays were done to determine the relative abundance of culturable Mn oxidizing microorganisms compared to total culturable heterotrophic microorganisms for various sites along the stream, wetland, and upstream reference sites. The combination of these methods allowed for a more thorough picture of the microbial community and the role that microorganisms may play in Fe and Mn oxidation to be obtained.

Methods

Water Quality Indicators

Temperature, pH, and dissolved oxygen (DO) were measured using a LaMotte #54183 water chemistry kit (Chestertown, MD). Water samples were collected during the fall of 2007 at sites SR and SO by submerging the mouth of provided sample bottles in the midpoint of the stream channel until the flasks were full. Water Quality Services Laboratory in Banner Elk, NC, quantified the concentrations of Fe and Mn.

Light and Fluorescent In Situ Hybridization Microscopy

Light microscopy was used to identify morphologically distinct Fe and Mn oxidizing bacteria. Samples were collected from wetland, stream, and upstream reference sites by submerging a sterile Eppendorf TM tube into the water and using an ethanol sterilized spatula to guide the sample into the tube when necessary. Samples were transported immediately back to the lab. Slides were prepared by sterilely pipetting 40-50 µL of sample on to clean microscope slides. A coverslip was applied and edges were sealed with Maybelline ® Express Finish Clear Nail Polish (New York). Slides were viewed using a Meiji MX light microscope (Santa Clara, CA) and images were taken using a SONY HD 6.1 Megapixel Handycam (New York, NY) with Martin Microscope Company MM9 Adapter (Easley, SC).

My method for the FISH portion for the study was as follows. Microscope slides were sterilized with ethanol and placed within the flow of water in the wetland, stream, and

at the upstream reference site. Slides were anchored in a plastic tube rack sterilized with ethanol. After two weeks, slides were removed with ethanol-sterilized tweezers and immediately transported individually in sterile 50 mL conical tubes (NuncTM, Rochester, NY) back to the lab. Samples of water and sediment were also collected from the wetland, stream, and upstream reference sites by submerging a sterile EppendorfTM tube into the water and using an ethanol-sterilized spatula to guide sample into the tube. Samples of oily surface sheen were collected from the wetland by submerging a sterile microscope slide beneath the water surface and lifting up to adhere the surface sheen onto the slide. Slides were placed in a sterile 50 mL conical tube (NuncTM). Samples were transported immediately back to lab. Samples were homogenized for each site, with the exception of surface sheen slides from the wetland, and 50-100 µL were applied to sterile microscope slides and left to dehydrate for several minutes. All slides were heat-fixed by passing over a flame for 1-2 seconds. The microscope slides were fixed using the protocol described by Osborn et al. (100) for gram-negative bacteria. Slides were incubated in 50 mL conical tubes (NuncTM) containing ice-cold 4% paraformaldehyde solution for 6 hours at 4 °C. After 6 hours, slides were removed with sterile tweezers and briefly rinsed with 1 mL of icecold 1X Phosphate Buffered Saline (PBS) solution. Slides were then placed in sterile handcrafted plastic bins created to secure slides independent of one another. Bins were flooded with 1X PBS solution and shaken by hand for several seconds. This was done 2-3 times in succession. Fixed slides were resuspended in ice-cold 1X PBS solution and icecold 96% ethanol in a 1:1 volume ratio in 50 mL conical tubes (NuncTM). Samples fixed were stored at -20°C for up to three weeks before hybridization. Slides were hybridized using an amended protocol described by Osborn et al. (100) and Siering and Ghiorse (101).

Slides were removed from 50 mL conical tubes (NuncTM) using ethanol sterilized tweezers and placed on a sterile plastic micropipette tip lid for 15 minutes at 46°C. Slides were incubated sequentially for three minutes each in 50% ethanol, 80% ethanol, and 96% ethanol. Slides were dried an additional five to ten minutes at 46°C until dry. One mL of freshly prepared hybridization buffer ranging from 15-25 % stringency was prepared for each slide (see Table 2). A mixture of 10 μ L hybridization buffer and 1.5 μ L 50 μ M fluorescently tagged oligonucleotide probe was added to each slide (see Table 3 for probe descriptions).

Table 2. Composition of hybridization buffers used to achieve different stringencies. All volumes are in μ L and the final volume is always 1.0 mL.

Hybridization Stringency	10 %	15 %	20%	25 %	30%
5 M NaCl	180	180	180	180	180
1 M Tris-HCl, pH 9.5	20	20	20	20	20
Deionized, distilled water	699	659	599	549	499
Formaldehyde	100	150	200	250	300
10 % sodium dodecyl sulfate (SDS)	1	1	1	1	1

Table 3. List of probe sequences and tags used in FISH. Probes were constructed by MWG/Operon (Huntsville, AL).

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Probe	Sequence	Fluorescent	Expected Target	Reference
Name		Tag		
LD-1	5'-CTC TGC CGC	5'Cy5	Leptothrix discophora,	(36, 92,
	ACT CCA GCT-3'		Sphaerotilus natans	102)
PS-1	5'-GAT TGC TCC	5'Cy5	Leptothrix discophora	(92, 102)
	TCT ACC GT-3'			
EUB338	5'-GCT GCC TCC	5'FITC	Most Eubacteria	(102, 103)
	CGT AGG AGT-3'			
nonEUB338	5'-ACT CCT ACG	5'FITC	NA, serves as negative	(102, 103)
	GGA GGC AGC-3'		control for FITC	
nonEUB338	5'-ACT CCT ACG	5'Cy5	NA, serves as negative	(102, 103)
	GGA GGC AGC-3'	-	control for Cy5	

Slides were then placed in sterile 50 mL conical tubes (NuncTM) containing the

remaining hybridization buffer and lined with lightweight tissue paper (VWR, West Chester,
PA) and incubated horizontally for 8 hours at 46°C. Slides were rinsed with washing buffer corresponding to the stringency of hybridization buffer used (see Table 4) and incubated for 15 minutes at 48°C. Slides were cooled by dipping in ice-cold sterile water for 3 seconds and air dried in a sterile plastic micropipette tip lid and covered with aluminum foil to prevent exposure to light. Once dried, a Fluoromount antifadent (Sigma Aldrich) and glass coverslip were applied and slides were left to dry an additional three hours. Once dry, slides were viewed immediately or within 24 hours using a Zeiss LSM 510 Laser Scanning Microscope (Thornwood, NY).

Table 4. Composition of washing buffer used to achieve different stringencies. Volumes are in mL and the final volume is 50 mL.

Washing Buffer Stringency	10%	15%	20%	25 %	30
5 M NaCl	4.50	3.18	2.15	1.49	1.02
1 M Tris-HCl, pH 9.5	1	1	1	1	1
0.5 M ethylenediaminetetraacetic acid	0	0	0.5	0.5	0.5
(EDTA)					
Deionized, distilled water	44.5	45.82	46.35	250	47.01

Isolation of Mn Oxidizing Microorganisms

To enrich for and identify Mn oxidizing bacteria samples of soil, water, and biofilm sediment were collected from sites SR, SO, and W sites in sterile 1.5 mL EppendorfTM tubes (Westbury, NY) using an ethanol-sterilized spatula when needed. Samples were immediately transported back to the lab for dilution and plating. Dilutions of 1:10, 1:100, 1:1000 and 1:5000 were made with soil and biofilm sediment supernatant and water samples and plated on solid MnO1, MnO2, Fe/Mn, PTYP, and LB/Mn plates (see Appendix A for details). Plates were incubated at 27°C in the dark until colonies appeared, usually within 2-12 days. Colonies were restreaked for isolation on the corresponding media they were found growing on.

Isolated colonies were then tested for Mn oxidation by transferring a colony onto filter paper using a sterile toothpick and flushing with 20 µL 0.04 % Leucoberbelin Blue (Sigma Aldrich, St. Louis, MO) (LBB) (a redox indicator that is oxidized by Mn (III) to produce a bright blue color change (104)). Positive reactions formed a bright blue precipitate. Negative reactions formed no color change or precipitate. Bacteria that were found to be Mn oxidizing were grown up in liquid media corresponding to the agar on which they were originally isolated, at room temperature in a shaking incubator for 1 week or until turbid. Gram stains were performed on each isolated colony using standard procedures. DNA was extracted using Qiagen DNeasy ® Tissue Kit (Valencia, CA) following the protocol for gram-positive or gram-negative bacteria, depending on gram staining results. Resulting genomic DNA extract was used in a Polymerase Chain Reaction (PCR) reaction to amplify the 16S rRNA gene using universal bacterial primers. Each PCR amplification mixture contained 0.25 µL Crimson Taq (New England Biolabs, Ipswich, MA), 10 µL Crimson Tag buffer (New England Biolabs), 1 µL each primer (50 µM 1492R and 27F universal bacterial primers (see Table 5)), 1 μ L 10 mM dNTP, 1 μ L template, and distilled deionized water brought reactions to 50 µL. 16 S rRNA gene amplification reactions were cycled in an Eppendorf TM Mastercycler using an initial denaturation step of 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 53°C for 60 seconds, and 68°C for 90 seconds, followed by an extension step of 68°C for 5 minutes. PCR products were purified using Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI) following manufacturer's protocol and sent to Cornell University Life Sciences Core Laboratories Center (Ithaca, NY) for sequencing.

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Primer Name	Sequence	Expected Target	Reference
27F	5'-AGAGTTTGATCMTGGCTCAG-3'	All bacteria	(105)
1492R	5'-TACGGHTACCTTGTTACGACTT-3'	All bacteria	(105)
ITS1 F	5'-TCCGTAGGTGAACCTGCGG-3'	All fungus	(106)
ITS4 R	5'-TCCTCCGCTTATTGATATGC-3'	All fungus	(106)
M13 F	5'-TGTAAAACGACGGCCAGT-3'	TOPO 2.1 vector	
M13 R	5'-CAGGAAACAGCTATGACC-3'	TOPO 2.1 vector	

 Table 5. List of primer sequences used in PCR amplification.
 Primers were constructed

 by MWG/Operon.
 Primers were constructed

To enrich for and identify Mn oxidizing fungi, samples of soil, water, and biofilm sediment were collected from sites SR, S0, and W sites in sterile 1.5 mL EppendorfTM tubes (Westbury, NY) using an ethanol sterilized spatula when needed. Samples were immediately transported back to the lab for dilution and plating. Dilutions of 1:10, 1:100, 1:1000 and 1:5000 were made with soil and biofilm sediment supernatant and water samples and plated on solid MnO1, MnO2, Fe/Mn, and LB/Mn plates (see Appendix A for details). Plates were incubated at 27°C in the dark until colonies appeared, usually within 2-12 days. Colonies were streaked for isolation on the corresponding media they were found growing on (Table 10). Once isolated colonies were achieved, they were tested for Mn oxidation by streaking a colony onto filter paper using a sterile toothpick and flushing with 20 µL 0.04 % LBB. Positive reactions formed a bright blue precipitate. Negative reactions formed no color change or precipitate. Colonies that were filamentous in appearance under microscopic analysis and would not amplify with universal bacterial primers were considered fungal colonies. Mn oxidizing fungi were grown up in liquid media corresponding to the media they were found growing on, at room temperature in a shaking incubator for 1 week or until turbid. DNA was extracted using UltraClean Soil DNA Kit (Mobio, Carlsbad, CA). Resulting genomic DNA extract was used in a PCR reaction to amplify the ITS variable region using fungal specific primers. Each PCR amplification

mixture contained 0.25 µL Crimson Taq (New England Biolabs), 10 µL Crimson Taq buffer (New England Biolabs), 1 µL each primer (50 µM ITS1F and ITS4 universal fungal primers (see Table 5), 1 µL 10 mM dNTP, 1 µL template, and distilled deionized water brought reactions to 50 µL. ITS region amplification reactions were cycled in an Eppendorf TM Mastercycler using an initial denaturation step of 94°C for 3 minutes, followed by 40 cycles of 94°C for 60 seconds, 62°C for 60 seconds, and 74°C for 90 seconds, followed by an extension step of 68°C for 10 minutes. Fungal isolates F2 and F3 did not produce PCR products and were thus not able to be sequenced or identified. More work will be done in the future to identify these fungal isolates as they are likely contributors to the microbial community in the Sorrento wetland. PCR products were purified using Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI) following manufacturer's protocol and sent to Cornell University Life Sciences Core Laboratories Center for sequencing.

Most Probable Number Assays

To determine the abundance and quantity of culturable heterotrophic Mn oxidizing microorganisms compared to the total culturable heterotrophic microorganisms present, MPN assays were performed. Soil, sediment, and water samples were collected from sites SO, SR, and W in sterile 50 mL conical tubes (NUNC TM) using a spatula sterilized in ethanol when needed. Samples were immediately transported back to the lab for dilution. Serial dilutions initially ranging from 10⁻¹ to 10⁻¹⁰ were created in sterile Corning ® Costar ® 96 well cell culture plates (Lowell, MA) with eight replicates each (Table 6). It was noticed during enrichment culturing that different types of media selected for completely different microorganisms. Two different types of media were used, including MnOB and

LB/Mn, to attempt to culture a diversity of heterotrophic and Mn oxidizing microbes with various metabolic needs.

		Dilutions													
	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10-6	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
LBB	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10-6	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10 ⁻⁶	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10 ⁻⁶	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10 ⁻⁶	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
INT	10 ⁻¹	10 ⁻²	10-3	10-4	10 ⁻⁵	10 ⁻⁶	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			
	10 ⁻¹	10 ⁻²	10-3	10-4	10-5	10-6	10-7	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	+	-			

Ta	able () . '	Tabula	r de	piction	of 96	well	growth	plates	used	for	MPN	assays.
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Initial results indicated growth of both heterotrophic and Mn oxidizing bacteria in dilutions up to 10^{-9} so subsequent MPN plates had dilutions ranging from 10^{-4} to 10^{-13} . 200 uL of liquid media was added to each well and 20 µL of sample innoculum was added to the first column and mixed by pipetting up and down. 20 µL of dilution was added to subsequent wells creating serial dilutions until the maximum dilution was reached. One column was inoculated with Mn oxidizing *Pseudomonas* sp. to serve as a positive control and another column was left without sample innoculum to serve as a negative control. Plates were incubated at 27° C in the dark for three to four weeks. After incubation, 50 µL of 0.04% LBB was added to each well in four rows and incubated 24 hours to assess for Mn oxidation and 50 µL 0.3% iodonitrotetrazolium chloride (INT) (Sigma Aldrich) (a redox indicator that is reduced in the presence of dehydrogenase to produce a bright red color) was added to each well in four separate rows and incubated 24 hours to assess for heterotrophic metabolism of any bacteria. Colorimetric results were applied to a downloadable MPN calculator (*107*) to determine the relative number of culturable heterotrophic microorganisms compared to those that oxidize manganese. 16S rRNA Clonal Library Construction and Restriction Fragment Length Polymorphism Analysis

To determine the diversity present and dominant members of the bacterial community at the site of the seeps in the Sorrento wetland, a 16S rRNA clonal library was constructed and analyzed. Samples were taken from the SR, SO, W, S25, and S50 sites with the initial intention being to create clonal libraries for multiple sites within the Sorrento wetland. Samples were collected by submerging a sterile 50 mL conical tube (NUNC TM) into the water and scooping up soil and water. All samples were capped and immediately transported to the lab for DNA extraction.

Initially, DNA extraction was done using UltraClean Soil DNA Kit (Mobio) for soil water interface samples and rock sediment samples, following manufacturer's protocol and DNA extraction of submerged floc samples was done using Qiagen DNeasy ® Tissue Extraction Kit following the protocol for gram-positive bacteria. However, in early troubleshooting attempts, numerous problems have surfaced regarding the method for obtaining the genomic material to create the 16S rRNA libraries. DNA extraction using the UltraClean Soil DNA Kit resulted in DNA concentrations that are too low or too dirty to successfully amplify via PCR. The isolated DNA also contains high levels of PCR inhibitors, including humic acids and Fe and Mn precipitates, preventing successful amplification. Pre-lysis washing with ethylenediaminetetraacetic acid (EDTA) (*108*) and aluminum sulfate (*109*) to remove humic substances, ammonium oxalate to remove Fe precipitates (*86*), and Chelex (Biorad, Hercules, CA) bead washing to remove interfering ions were attempted with limited success. Disinhibitors such as Bovine Serum Albumin (BSA) (Fischer Scientific, Waltham, MA), Tween 20 (Calbiochem, San Diego, CA), and

dimethyl sulfoxide (DMSO) (Pierce, Rockford, IL) were added to PCR reactions, with no success. Other methods attempted included obtaining samples from soil seeps, root mass, floc material, and water. These all resulted in no success. PowerClean Soil DNA Kit (Mobio, Carlsbad, California) was also used with no success.

Because of this lack of success, the initial goal to create 16S rRNA clonal libraries for all sites, including reference sites, retention pond sites, wetland sites, and several different stream sites was amended slightly. Only samples obtained from the soil water interface, taken from the initial site of the seeps, using Mobio UltraClean Soil DNA Kit, are included in the results portion of this study, as it was the only sample able to be amplified.

Extracted DNA was then used to amplify 16S rRNA genes using universal bacteria specific primers 27F and 1492R (see Table 5) using the following protocol. Each PCR amplification mixture contained 5 μL Taq buffer (New England Biolabs), 2 μL MgCl₂, 1.5 μL dNTP (10 nM), 1 μL each of 27F and 1492R primers (50 nM), and 0.5 μL Taq polymerase (New England Biolabs) and enough water to bring the reaction to 50 uL. The insert was amplified using an Eppendorf TM Mastercycler using an initial denaturation step of 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 72°C for 2 minutes, and an extension step of 68°C for 10 minutes. The presence of the 16S rRNA gene was checked through gel electrophoresis using a 1 % agarose gel stained with ethidium bromide and then visualized using UV light. A 2log ladder (New England Biolabs) was used as a size marker. All reactions containing a single band roughly 1500 bp in size, corresponding to the 16S rRNA gene were considered successful reactions. Figure 11 shows a gel electrophoresis image of a 1500 bp band corresponding to the 16S rRNA gene isolated from S0 site.



Figure 11. Gel electrophoresis image of 1500 bp band corresponding to the 16S rRNA gene. Lane 1 shows a positive control generated from *Vibrio cholera* strain 249 genomic DNA, Lane 2 shows a negative control, Lane 3 shows the 1500 bp 16S rRNA band obtained from the S0 site genomic material, and Lane 4 shows a 2 log size marker. The arrow indicates a 1500 bp band.

Five PCR reactions containing the 16S rRNA gene were pooled to eliminate PCR bias and purified through gel extraction using Promega Wizard SV Gel and PCR Clean-Up System following manufacturer's protocols. The purified products were then cloned into a TOPO 2.1 vector using Invitrogen TOPO TA Cloning ® Kit (Carlsbad, CA). The ligation reaction was purified using Millipore Durapore ® 0.1 µm VVPP Membrane Filters (Billerica, MA) to remove residual salts before transformation. Products were transformed into *Escherichia coli* Top Ten electrocompetent cells. Transformants were plated onto Luria-Bertani (LB) agar plates containing 1µL/mL ampicillin and 40µL 5-bromo-4-chloro-

3-indolyl-b-D-galactopyranoside (X-Gal) (Gold Biotechnology, St. Louis, MO) and incubated overnight at 37°C. Ampicillin resistance and blue white colony screening indicated those transformants containing the cloned insert. White colonies were chosen from each plate with a toothpick and were replated on a separate LB-ampicillin plate containing X-Gal for a second round of blue white colony screening. Between 100 and 120 colonies from the transformation were chosen for replating. Replated clones were incubated overnight at 37°C. Clones were then grown up overnight in 2 mL LB containing 1µL/mL ampicillin in a shaking incubator at 37°C. Plasmids were isolated and extracted using the alkaline lysis method (Sambrook and Russell). Two mL of overnight growth was added to sterile 1.5 mL Eppendorf tubes and centrifuged at 13.2 X 1000 rpm for 30 seconds in an Eppendorf Centrifuge 5415 D. Liquid medium was removed by vacuum aspiration. The pellet was resuspended in 100 µL of ice-cold Alkaline Lysis I solution (see Appendix A) by vortexing vigorously. 200 µL Alkaline Lysis II solution (see Appendix A) was added to each suspension and tubes were capped and inverted several times to mix. Immediately 150 µL Alkaline Lysis III solution (see Appendix A) was added and tubes were capped and inverted several times to mix. Tubes were centrifuged at 4°C at 13.2 X 1000 rpm for 5 minutes. Supernatant was removed and transferred to sterile 1.5 mL Eppendorf tubes. Two volumes of 96% ethanol were added to precipitate nucleic acids. The mixture was vortexed and left to stand at room temperature for 2 minutes. Tubes were then centrifuged at 4°C at 13.2 X 1000 rpm for 5 minutes. Supernatant was removed by vacuum aspiration. One mL of 70% ethanol was added and each tube was mixed by inverting several times. DNA was recovered by centrifuging at 4°C at 13.2 X 1000 rpm for 2 minutes. Residual ethanol was removed by vacuum aspiration. Tubes were left open at room temperature for 30-60

minutes until all ethanol was evaporated. Recovered DNA was dissolved in 45 μ L Tris-EDTA (TE) buffer containing 20 μ g/mL Rnase (Sigma Aldrich). Plasmids were run on a 0.5% agarose gel to verify existence. Plasmids containing the 1500 bp insert were diluted 1:50 and PCR amplified using M13 forward and reverse primers, which have binding sites on the TOPO 2.1 vector flanking the insert. Each PCR amplification mixture contained 5 μ L Taq buffer (New England Biolabs), 2 μ L MgCl₂, 1.5 μ L dNTP (10 nM), 1 μ L each of M13F and M13R primers (50 nM), and 0.5 μ L Taq polymerase (New England Biolabs) and enough water to bring the reaction to 50 μ L. The insert was amplified using an Eppendorf TM Mastercycler using an initial denaturation step of 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 72°C for 2 minutes, and an extension step of 68°C for 10 minutes. Eight μ L of each reaction were run on a 0.5 % agarose gel to verify product insert size. Figure 12 is a gel electrophoresis image depicting results of PCR amplification of the 1500 bp 16S rRNA gene insert.



Figure 12. Gel electrophoresis image of 16S rRNA insert. The first lane contains a negative control. Lanes 1-8 depict inserts amplified from different clones. The last lane contains a 100 bp size marker. Note that lanes 3 and 5 contain multiple bands so they were not analyzed further.

PCR products from clones containing the insert were restricted with Rsa1 and Msp1 restriction enzymes (Promega) overnight and separated on a 4% agarose gel to create RFLP profiles. Each different pattern created is specific for each species of bacteria present in the initial sample. These profiles were analyzed and categorized numerically to determine the number of different microbial populations present. Dominant phylotypes were those that occurred more than once. Clones corresponding to dominant phylotypes were grown up overnight in 2 mL LB containing 1µL/mL ampicillin. Plasmids were isolated using Promega PureYield TM Plasmid Miniprep System following manufacturer's protocol. Plasmids were diluted 1:50 and PCR amplified using M13 forward and reverse primers. Each PCR amplification mixture contained 5 µL Taq buffer (New England Biolabs), 2 µL MgCl₂, 1.5 µL dNTP (10 nM), 1 µL each of M13F and M13R primers (50 nM), and 0.5 µL Taq polymerase (New England Biolabs) and enough water to bring the reaction to 50 µL. The insert was amplified using an EppendorfTM Mastercycler using an initial denaturation step of 94°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 72°C for 2 minutes, and an extension step of 68°C for 10 minutes. Eight µL of each reaction were run on a 1% agarose gel to verify product existence and size. PCR products were PCR purified using Promega Wizard ® SV Gel and PCR Clean-Up System. Purified PCR products were sent to Cornell University (Ontario, Canada) for sequencing using M13 Forward primers.

Results

Characterization of the Sorrento Wetland Using Water Quality Indicators

To verify that the Sorrento wetland still has the same parameters and characteristics as when it was last described 2 years ago (73, 81), water quality parameters were taken in October of 2008, including Fe and Mn concentrations (Table 7) and basic stream water indicators (Table 8).

Table 7 shows Fe and Mn concentrations for the Sorrento wetland stream water in February of 2006 and October of 2008. Most notable are Fe and Mn concentrations for the SO site for February 2006 and October 2008 exceeding standards set by NCDWQ. Mn concentrations for the SR site for October 2008 also exceed standards set by NCDWQ, although by a small amount. Results indicate that the Sorrento stream water still contains high concentrations of Fe and Mn and that Fe and Mn concentrations are higher at seep sites than at the upstream reference site, therefore site SR is still a suitable reference site for this study.

Table 7. Soil sediment and water Fe and Mn concentrations for the Sorrento stream.
Measurements were taken in October 2008. Measurements from February 2006 and
NCDWQ standards are provided for comparison (73, 81).

		Sedi	ment	Water				
Date	Sample Site	Fe (mg/L)	Mn (mg/L)	Fe mg/L)	Mn (mg/L)			
February	SO	539.0	382.0	5.780	0.350			
2006	SR	52.0	83.0	0.323	0.015			
October	SO	-	-	5.432	0.315			
2008	SR	-	-	0.011	0.260			
	NCDWQ	-	-	1.00	0.20			

Table 8 shows water quality indicators for the Sorrento stream in February of 2006 and October of 2008. Alkalinity levels were notably low when tested in October of 2008, indicating the lack of ability of the stream to withstand changes in pH. This is typical for streams in western North Carolina, due to the underlying granite bedrock that leaches few acid-neutralizing compounds (*110*). All other indicators are within the normal range. Results indicate that aside from alkalinity, all water quality indicators for the Sorrento wetland are still considered healthy.

Table 8. Water quality indicators for Sorrento stream. Measurements were taken in October 2008. Measurements from February 2006 and NCDWQ standards are provided for comparison (73, 81)

Date	Sample	Dissolved	pH	Temperature	Conductivity	Alkalinity
	Site	Oxygen		(°C)	(mS/cm)	(mg/L
		(ppm)				CaCO ₃)
February	SO	7.41	6.25	13.91	49.63	-
2006	SR	7.93	6.65	10.87	34.33	-
October	SO	7.8	6.3	7.0	- 1	20.0
2008	SR	9.0	6.5	7.0	-	18.0
	NCDWQ	>5.0	6-8	-		100

Analysis of Microbial Communities Using Light and Fluorescent In Situ Hybridization Microscopy

In order to determine the presence or absence of Fe and Mn oxidizing bacteria light microscopy and FISH analysis were employed. Light microscopy from slides incubated in SR, S0, and W sites revealed two distinct morphotypes of bacteria with characteristics of *Gallionella ferruginea* and *Leptothrix* spp. Neither morphotypes were present at upstream reference sites.

Morphotype 1 consisted of long twisted helical stalks, roughly 100-200 μ m in length and believed to be encrusted with Fe and Mn oxides (see Figure 13). Morphotype 1 displays characteristics similar to *Gallionella ferruginea*, a common Fe oxidizing bacteria often found in association with Fe rich aquatic environments (18, 22, 24-27). This morphotype dominated during winter months in both wetland and seep sites, although it was found in all seasons. It occurred in higher density in the wetland site compared with the seep site.



Figure 13. Morphotype 1 from S0 seep sample taken in February of 2009. Note the distinct helical structure, characteristic of *Gallionella ferruginea*.

Morphotype 2 consists of a dense tangle of long filamentous sheaths, ranging from $50 \ \mu\text{m}$ to $300 \ \mu\text{m}$ in length (see Figure 14). Note in the bottom right corner of Figure 14 (see arrow) a sheath thought to be encrusted with Fe and Mn oxides. These are all characteristics of *Leptothrix* spp., a common Fe and Mn oxidizing bacteria found in Fe and Mn rich aquatic environments. Morphotype 2 dominated in late spring to early fall months in both wetland and seep sites, although it was found in all seasons. An example of Morphotype 2 is also visible in the bottom right corner of Figure 13, taken in February of 2009. It appeared to be of higher density in the seep site compared with wetland sites and was also longer in length (roughly twice as long) in the seep site compared with wetland sites and sites (data not shown). Interesting to note is that the majority of examples of Morphotype 2

appear to be empty sheaths, void of any cells. This is also indicative of *Leptothrix* spp., which can have as few as 7% of all sheaths containing live cells (18, 111).



Figure 14. Morphotype 2 from S0 seep sample taken in June of 2009. Note the distinct long filamentous sheaths, characteristic of *Leptothrix* spp.

Figure 15 shows light microscope images from the upstream reference site. Neither

Morphotype 1 nor 2 were present at the SR site during the course of this study.



Figure 15. SR reference site from February 2009. Note the lack of Morphotypes 1 or 2.

Figures 16-18 show FISH images using probe LD-1, which targets *Leptothrix* spp., labeled with Cy5 fluorescent tags. Probe LD-1 was hybridized at 30% stringency for 8 hours for all sites. All images were taken using a Zeiss LSM 510 Laser Scanning Microscope using an Helium Neon 633 nm laser and LSM 510 software. Figure 16 shows a FISH image from a slide incubated in the S0 site, using probe LD-1 labeled with Cy5. Bacteria belonging to the genus *Leptothrix* fluoresce red. The first panel shows filamentous bacteria measuring approximately 40-100 μm in length, presumed to be *Leptothrix discophora*. Note that not all of the filaments are fluorescing, indicating a number of empty sheaths devoid of actual bacteria.



Figure 16. FISH image from S0 site using LD-1 probes labeled with Cy5. The first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the two.

Figure 17 shows a FISH image from a slide incubated in the SR site using probe LD-1 labeled with Cy5. The first panel shows no fluorescent image, similar to all slides obtained from the SR site. This indicates that *Leptothrix discophora* is not likely present in the reference site upstream from the wetland. DIC images show cocci bacteria, as well as a large diatom.



Figure 17. FISH image from SR site using LD-1 probes labeled with Cy5. The first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the two.

Figures 18 and 19 show FISH images using probe EUB338, targeting all eubacteria, labeled with FITC. Probe EUB338 with FITC was hybridized at 15% stringency for 8 hours. All images were taken using a Zeiss LSM 510 Laser Scanning Microscope using an Argon 488 nm laser and LSM 510 software. All eubacteria will fluoresce green. Figure 18 shows a FISH image from a slide incubated in the S0 site using FITC labeled EUB338 probes. Cocci, rod shaped, and long filamentous bacteria are present in the S0 site. Several larger spherical cocci bacteria are present at various locations on the slide. Based on morphology, these spherical cocci bacteria could belong to the genus *Siderocapsa*, a Fe and Mn oxidizing bacteria. *Siderocapsa* are identified based on the presence of several morphological traits including a large spherical shape, a capsule of varying thickness, and the ability to deposit Mn and Fe oxides on that capsule, similar to those found in Figure 18.



Figure 18. FISH image from S0 site using EUB338 probes labeled with FITC. The first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the two.

Figure 19 shows a FISH image from a slide incubated in the SR site using FITC labeled EUB338 probes. Cocci are present in the SR site. A long filamentous morphotype appears in the bottom right corner of the DIC image of Figure 19, although it does not fluoresce, indicating it could possibly be fungal in nature rather than bacterial, or that it too

is an empty sheath belonging to the *Leptothrix* spp, although it appears to have a branched structure, indicating otherwise.



Figure 19. FISH image from SR site using EUB338 probes labeled with FITC. The first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the two.

Figures 20 and 21 show FISH images using probe nonEUB338 labeled with FITC or Cy5, respectively. Probe nonEUB338 was hybridized at 15% for 8 hours. All images were taken using a Zeiss LSM 510 Laser Scanning Microscope and LSM 510 software. Because nonEUB338 is a nonsense probe, it should not bind to any bacteria, serving as a negative control for the reagents used in the hybridization process and for the attached fluorescent labels. Little to no fluorescence is expected in the following images. For all images, the first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the first two panels.

Figure 20 shows a FISH image from a slide incubated in the S0 site using FITC labeled nonEUB338 probes. Note the abundance of long filamentous bacteria in the DIC image, none of which show fluorescence at 488 nm. Negative controls using nonEUB338

FITC labeled probes were also performed for the SR site (data not shown) and no slides showed fluorescence, indicating the specificity of FITC labeled probes to their target.



Figure 20. FISH image from S0 site using nonEUB338 probes labeled with FITC to serve as a negative control. The first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the two.

Figure 21 shows a FISH image from the S0 site using nonEUB338 probes labeled

with Cy5. Numerous rod and cocci bacteria appear in the DIC image but none show

fluorescence at 633 nm. Negative controls using nonEUB338 Cy5 labeled probes were also

performed for SR site (data not shown) and no slides showed fluorescence, indicating

specificity of Cy5 labeled probes to their target.



Figure 21. FISH image from S0 site using nonEUB338 probes labeled with Cy5 to serve as a negative control. The first panel shows fluorescence, the second panel is a DIC image showing no fluorescence, and the third panel is an overlay of the two.

Isolation and Identification of Mn Oxidizing Microorganisms from the Sorrento Wetland

Preliminary data from Sorrento wetland show filamentous bacteria growing in both the seeps along the streambed and within the wetland (see Figure 7), consistent with Fe and Mn oxidizing bacteria such as *Leptothrix* spp and *Gallionella* spp. Water, soil, and biofilm cultures used as inocular on Fe/Mn and MnO1 solid media (see Appendix A) resulted in several dark brown, black, and orange colonies, consistent with Mn and Fe oxidizing bacteria, such as *Leptothrix* spp. Further testing with 0.04% LBB (Sigma Aldrich) gave both positive and negative results, indicating that at least some of the microorganisms growing on the plates were capable of oxidizing Mn. This prompted for the enrichment and isolation of possible Fe and Mn oxidizing microorganisms from seep and wetland sites. A total of eight Mn oxidizing bacteria, seven non-Mn oxidizing bacteria, and five fungi capable of oxidizing Mn were isolated from floc material and water from the S0 seep site.

Of the seven Mn oxidizing bacterial isolates that were able to be sequenced, sequence identities found that three belonged to the genus *Bacillus* and three belonged to the genus *Pseudomonas*, and one belonged to the genus *Leptothrix*. Both *Bacillus* and *Pseudomonas* are known Mn oxidizers and are often found in environmental samples. Members of the genus *Leptothrix* are Fe and Mn oxidizers, often found in wetlands and environments rich in Fe and Mn. All isolates except for isolate B2 tested LBB positive as soon as colonies appeared on plates. Isolate B2 required three to four weeks before it tested LBB positive. Spores of the genera *Bacillus* have been shown to directly oxidize Mn (II) to Mn (IV) (*112*, *113*), so the delay in Mn oxidation for isolate B2 is likely due to a delay in spore production. The 16S rRNA gene from isolate B3 was not able to be PCR amplified and therefore was not identified. Table 9 shows sequence identity information for each

isolate, as well the sample location and growth medium and dilution factor required to obtain pure isolates. Cell morphologies are also described. Figures 22a-h show images of each isolate. Figure 22c shows isolate B2 after it turned brown in color and tested LBB positive.



Figures 22a-h. Images of bacterial isolates. From 14a to 14h are SObA, B1, B2, B3, B5, B6, B7, and B8. Images are not to scale.

 Table 9. Identification and description of Mn oxidizing bacterial isolates.
 All isolates tested LBB positive, indicating Mn oxidizing ability.

	Gram	-/+	ı		-	+	+					+			ı			+			+				
	Cell Morphology		Dark brown round	colonies, <1 mm, thick Mn	oxide crust, rod shaped	Dark tan round colonies, 5 mm	White filamentous	colonies, 4-5 mm, Mn	oxidation is delayed 3-4	weeks, rapidly spreading,	rod shaped	Dark brown filamentous,	rapidly spreading, 2-4 mm,	During cound of aniapou	Brown round colonics with	darker center, 1 mm, rod	shaped	Dark orange filamentous	colonies, 3-4 mm, rapidly	spreading, rod shaped	Brown colonies, <1 mm,	thick Mn oxide crust, rod	shaped	Brown colonies, 1-2 mm,	lack distinct edge
	Growth	Medium	Solid MnO1	/	Y Services	Solid Fe/Mn	Solid	MnO1				Solid	Mn01	0.114	Solid	MnOl		Solid	MnO1		Solid	MnO1		Solid	MnO1
	Dilution	Growth Factor	1:1000			1:100	1:100					1:1000		1.1000	1:1000			1:1000			1:1000			1:1000	
	Sample	Location	S0 seep	water		S0 floc	S0 floc					S0 floc		CO US	SU HOC			S0 floc			S0 floc			S0 floc	
	Maximum	Sequence Identity Similarity	96%			95%	93%					NA	,	1000	0/1/6			96%			92%			%06	
y.	Sequence identity		Pseudomonas sp.			Bacillus sp.	Bacillus sp.	1				Unknown identity			Pseudomonas sp.			Pseudomonas sp.			Bacillus sp.			Leptothrix sp.	
dizing abilit	B	Name	S0bA			B1	B2					B3			69 CH			B6			B7			B8	

Of the five fungal isolates, only isolates F1 and F5 tested LBB positive throughout growth and culturing. Isolates F2, F3, and F4 began LBB positive and gradually lost Mn oxidizing ability over the course of several days to several weeks. Table 13 shows sequence identity information (when available) for each fungal isolate as well as sample location and growth medium required for isolation. Cell morphology is also described. The ITS regions from fungal isolates F3 and F4 was not able to be PCR amplified and therefore these isolates were not identified. The ITS regions from fungal isolates F1, F2, and F5 were able to be PCR amplified, but were unable to be sequenced due to budgetary constraints. More work will be done in the future to identify these fungal isolates as they likely contributors to the microbial community in the Sorrento wetland.

isolates				
ID Name	LBB	Sample	Growth	Description
	Status	Location	Medium	P. A.
F1	+	S0 floc	Fe/Mn	Dark brown matte colonies, very large and
		in redaints		flat
F2	+/-	S0 floc	MnO1	White, turning brownish gray, round, raised,
				fuzzy appearance
F3	+/-	S0 floc	MnO1	White, turning black, round slightly raised
				colonies
F4	+/-	S0 floc	MnO1	White, turning brown, round flat colonies,
				with concentric rings
F5	+	S0 floc	MnO1	Darker green, rapidly covering plate, large
				round spores

 Table 10. Identification and description of Mn oxidizing and non Mn oxidizing fungal isolates

Quantification of Culturable Mn-Oxidizing Microorganisms Through Most Probable

Number Assay

In order to determine the relative abundance and quantity of culturable Mn oxidizing heterotrophic microorganisms in the Sorrento wetland and stream compared to the culturable heterotrophic microorganisms, MPN Assays were performed. Two different types of media were used with differing nutrient and Mn concentrations, to best assess for differences in heterotrophic and Mn oxidizing microbial metabolic needs.

Initial MPN results indicated culturable Mn oxidizing heterotrophic growth exceeding 2.4 x 10-7 cells/mL from site S0 and over 1.1 x 10-5 from site SR. However, for results to be trustworthy, positive results must eventually taper off as the dilution increases and this was not achieved for these MPN plates, nor could similarly high numbers be replicated in future attempts. Therefore, numerical results from that sampling are not included in this analysis.

Overall MPN data indicated higher ratios of heterotrophic compared to Mn oxidizing heterotrophic bacteria in all sites tested. These ratios were highest in S0 and W sites. Most notable is that site SR cultivated no Mn oxidizing heterotrophic microrganisms in either culture medium tested. Only sites S0 and W were able to enrich for Mn oxidizing heterotrophic microorganisms. LB/Mn culture media was unable to enrich for Mn oxidizing heterotrophic bacteria in any site, possibly due to competition from rapidly growing heterotrophic bacteria in the nutrient rich medium. Results are summarized in Table 11 and include the MPN (cells per given mL sample innoculum) and upper and lower 95% confidence intervals. Because Sites S0 and SR co ntained no Mn oxidizing heterotrophic activity for the LB/Mn media and Site SR contained no Mn oxidizing heterotrophic activity for the MnO1 media, the MPN calculator scored it as <23 cells/mL and no confidence intervals were given.

Table 11. Summary of MPNs found in the Sorrento wetland and stream. MPN data represents only culturable heterotrophic microorganisms and includes the upper and lower 95% confidence intervals (CI).

		Heterotro	phic microor	ganisms	Mn Oxidizing Heterotrophic						
			microorganisms								
Culture	Site	95% CI	MPN	95 % CI	95% CI	MPN	95% CI				
Media		lower	(cells/mL)	upper	lower	(cells/mL)	upper				
LB/Mn	SO	540	1600	4700	-	<23	-				
	SR	730	2100	6300	-	<23	-				
	S0	360	1100	3600	3.6	25	180				
MnOB	SR	890	2700	8100	-	<23	-				
	W	1700	6200	22000	78	230	680				

Figure 23 compares MPN data between sites for the MnO1 media only since it was the only media that showed signs of having Mn oxidizing heterotrophs present. Results indicate that numbers of heterotrophic microorganisms are similar between different sites. However, only sites S0 and W contained Mn oxidizing heterotrophic activity, whereas site SR did not.



Figure 23. Comparison of total culturable heterotrophic microorganisms versus culturable heterotrophic Mn (II) oxidizers at various sampling locations in the Sorrento wetland, including the Sorrento upstream reference site (SR), the Sorrento seep (S0) and the Sorrento wetland (W). Data shown is for MnO1 media only. The Y axis is displayed in a logrithmic scale and represents the most probable number of cells in the sample per mL of starting material.

Analysis of Bacterial Community through 16S rRNA Clonal Library Construction and Restriction Fragment Length Polymorphism Analysis

A 16S rRNA clonal library was constructed and analyzed using RFLP in order to determine the bacterial diversity at the Fe and Mn seep and identify dominant bacterial community members. 16S rRNA gene inserts from 68 plasmids were analyzed and 62 different phylotypes were classified. Figures 24a-h are gel images of phylotypes generated through RFLP analysis. Each lane is labeled with the plasmid identification and a 100 bp size ladder flanks either side of each gel image. Only bands greater than 100 bp were used to determine phylotypes.



Figures 24 a. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 b. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 c. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 d. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 e. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 f. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 g. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 h. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 i. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 j. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.



Figures 24 k. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.


Figures 24 I. Gel electrophoresis images of RFLP patterns generated from digestion of clonal libraries. Lanes are labeled with plasmid number and are flanked on each side by a 100 bp size marker (M). Only bands greater than 100 bp were counted in RFLP analysis.

Of these 62 different phylotypes, there were 5 that were identified as dominant, meaning they appeared more than once. Plasmids corresponding to these five phylotypes are being sequenced and identified. Phylotypes 1, 6, 36, and 49 accounted for 2.94 % each of the community. Phylotype 4 accounted for 4.41% of the community. The remaining 83.83% of the bacterial community is comprised of genetically diverse individuals. Due to budgetary constraints, only one sequence corresponding to Phylotype 1 could be sequenced. It showed 80% similarity to *Methylobacter*, a gram-negative methanotrophic bacterium, often found in soils and aquatic environments. More work will be done at a future date to identify all dominant phylotypes within the 16S rRNA clonal library.

Discussion

Microbial community ecology is the study of how all populations of microorganisms within a community interact with each other. Studies involving microbial communities are important, as many behavioral characteristics of microbes are only present in a community context (*114*). Microbes may exist in complex symbioses and grow only in the presence of other microbes (*115*) and functions of entire microbial populations may be lost when not in a community setting (*116*). To fully characterize microbial communities, such as those present in the Sorrento wetland, all species present would need to be identified and counted. My research used four different methods in an attempt to accomplish this, including culture-based analyses: enrichment cultures and isolation, and MPN assays; and culture independent analyses: light and FISH microscopy, and RFLP analysis of a 16S rRNA clonal library.

Traditionally to characterize microbial communities, researchers would rely almost entirely on cultivation studies using enrichment cultures and dilution gradients. While enrichment cultures can give detailed information on what organisms are present and dilution gradients can give estimates of relative abundance, neither method can fully identify and enumerate all species present (*82*). This is because only 1-2% of all estimated microbial populations present are capable of being cultured in a laboratory setting (*89, 117, 118*). Because of this "great plate count anomaly" (*119*), it is unlikely that the 19 microorganisms isolated from the wetland represent even a fraction of the actual microorganisms present. Furthermore, even though the microorganisms present were isolated from dilutions upwards of 1:1000, it is unlikely that they represent even dominant members of the actual

community. They represent only the most dominant members of the culturable community. Of the seven identified Mn oxidizing bacteria isolated in the Sorrento wetland and stream, most belonged to members of the genera *Pseudomonas* or *Bacillus*. Containing over 230 and 48 (*120*) species respectively, these fast growing genera are ubiquitous, occurring in high numbers in soil and water samples, so their isolation is not surprising.

Mn and Fe oxidizing genera such as *Leptothrix* and *Gallionella* require a specific oxygen and metal gradient (21, 111), so it is common for *Leptothrix* and *Gallionella* to be identified through morphology but not through isolation cultures (21), so it is surprising then that one of the Mn oxidizing isolates was identified as belonging to the genus *Leptothrix*, even despite the fact that microscopy indicated an abundance of *Leptothrix* sp. in the Sorrento wetland. The 16S rRNA gene insert from isolate B8 showed 90% similarity to *Leptothrix mobilis*. *L. mobilis* are gram negative, rod shaped bacteria, and do not contain sheaths in pure culture (38), consistent with the characteristics found in isolate B8.

It is interesting that despite obtaining high concentrations of DNA from isolates B3, F3 and F4, they were still unable to be amplified via PCR. This is likely due to the presence of PCR inhibitors, including humic substances, mostly in the form of humic acids, and Fe and Mn oxides. Humic acids have the same long and helical shape and negative charge as DNA, so they are often co-purified along with genomic DNA during the extraction process (*109, 121*). Metals, such as Fe and Mn often interfere with PCR reactions by chelating negatively charged molecules such as DNA, preventing the binding of primers to the genomic template (*35*). These inhibitors must be removed in order for DNA to be amplified through PCR. Removal of humic substances and metals such as Fe and Mn often includes washes with harsh buffers and detergents and can result in cell damage and the loss of

subsequent DNA (*109*). Many studies get around the problem of inhibitors in PCR based studies by diluting DNA template samples, therefore diluting out the inhibitors (*35, 122*). Isolates B3, F3 and F4 were diluted several thousand fold, but still would not amplify, likely attributed to the DNA template being diluted out as well. Additives such as DMSO and Tween 20 were also added in an attempt to increase the likelihood of amplification, but no success was had. More work will be done on these isolates in an attempt to identify them through morphology, metabolic testing, or other genomic DNA extraction methods, as they are likely Mn oxidizing contributers of the wetland community.

Overall MPN data indicated higher ratios of heterotrophic compared to Mn oxidizing heterotrophic bacteria in SR sites than in S0 or W sites. MPN results from LB/Mn media contained no Mn oxidizing bacteria, even in S0 and W samples. This is typical of MPN results generated using nutrient rich LB media (*123*). It is likely that the nutrient rich media selects for faster growing non-Mn oxidizing bacteria or that Mn oxidizing microbes are adapted to nutrient-poor conditions and are inhibited by the rich media. Interestingly, site SR did not contain any Mn oxidizing microorganisms in either medium tested. Only sites S0 and W contained Mn oxidizing microorganisms and suggested cell counts of Mn oxidizing heterotrophs ranged from 3.6×10^3 to 2.2×10^4 cells/mL. This suggests that the increase in metal concentrations at sites S0 and W might actually enrich and select for Mn oxidizing heterotrophs in metal rich environments have cell counts ranging from 3×10^1 in the Columbia River Estuary (*124*) to 1×10^4 in a freshwater lake (*50*), so my data is well within the established and expected ranges. To date, omy study represents the first data regarding quantification of Mn oxidizing bacteria in wetland ecosystems, so it is unknown

how my numbers compare to other metal-rich or pristine wetlands. However, results from culture-based MPN assays are based only on what is capable of growing in culture, so it is possible that there are dominant community members, including Mn oxidizing microorganisms that are not accounted for in this assay. It is also important to note that INT indicates metabolic activity. When microorganisms encounter any environmental stressors, such as culturing media and conditions, they can enter a dormant state where they are no longer metabolically active (118) and therefore would not react with INT to cause a color change, biasing heterotrophic growth counts. MPN data was generated from samples taken from flocculent growth existing at the soil water interface within S0 and W sites and from sediment in the SR sites, because SR sites had no flocculent growth. It would be interesting to repeat the MPN assay for a number of different locations within the Sorrento wetland and upstream reference sites, including water, sediment, soil, rhizosphere, and Fe and Mn oxide deposits coating rocks and plants (S0 and W sites only), during multiple seasons during the year. This would give a better and more thorough overall picture of the abundance of heterotrophic and Mn oxidizing heterotrophic microorganisms within the Sorrento wetland because it would take into account all temporal and seasonal variations that might exist.

Because of all of the discrepancies and problems associated with assessing microbial community diversity through culturing and dilution methods, it is essential to incorporate other methods to fully characterize community diversity. My research employed two other methods to accomplish this, light and FISH microscopy and RFLP analysis of a 16S rRNA clonal library.

Results from the light microscopy showed a seasonal abundance of two different morphotypes in the S0 and W sites, thought to be Fe and Mn oxidizing *Leptothrix* spp. and

Gallionella ferruginea. Both morphotypes occurred in extremely high numbers, dominating microscopy images, but did not occur in the SR site. FISH analysis also showed Leptothrix spp. present in S0 and W sites, but not in the SR site. However, FISH images from the S0 and W sites showed far fewer numbers of *Leptothrix* bacteria when compared with light microscopy results. This could be for several reasons. It is well known that Leptothrix spp. are common inhabitants of freshwater wetlands, especially those containing high concentrations of Fe and Mn. However, most sheaths found in wetlands are devoid of cells; sometimes as few as 7% of all sheaths contain viable cells (18, 111). Because FISH depends on the presence of several thousand rRNA target molecules to obtain a strong signal (89), it is likely that because of the lack of viable metabolically active cells, FISH signals would be weaker or nonexistent. Another reason for the discrepancy between FISH results and light microscopy results is that humic acids have been shown to interfere with nucleic acid hybridizations, especially when they're present in strong concentrations (125). Humic substances remain in ecosystems as stable complexes bound to Fe and Mn hydroxides. Because of the abundance of Fe and Mn hydroxides in the wetland, it is likely also that humic substances are present in high concentrations as well. Preliminary spectroscopy results indicated high concentrations of humic substances absorbing at 230 nm (results not shown) in all sample sites tested. This could also lead to weak or nonexistent FISH signals.

Also interesting are the seasonal fluctuations in abundant morphotypes. Emperical observations from light microscopy indicated that *Leptothrix* spp. dominated in late spring and summer months, while *Gallionella ferruginea* dominated in cooler late fall and winter months. Fe and Mn concentrations are known to fluctuate seasonally, due especially to seasonal hydrological variations. These variations could alter the magnitude and persistence

of redox gradients, affecting metals such as Fe and Mn (67). Because *Leptothrix* spp. and *Gallionella* spp. both require specific oxygen and redox gradients (21, 111), these seasonal variations would affect them also, causing seasonal blooms and declines in populations. It is also possible that both *Leptothrix* spp. and *Gallionella* spp. are abundantly present in seep and wetland sites year round and only their temporal location changes seasonally, in keeping with their preferred redox gradient. This would also give the appearance of seasonal blooms and declines, unless a number of different locations within each sample site were viewed.

FISH images using the universal eubacterial EUB338 probe labeled with FITC showed an abundance of several morphotypes in S0 and W sample sites, including large spherical cocci, that appeared to dominate the slide image, especially in the S0 site. None of the bacterial isolates had cocci bacteria, nor do individuals within the genera Leptothrix or Gallionella, so it is likely that these morphotypes belong to a bacterium not yet identified by this study. Based on morphology, these bacteria could belong to the genus *Siderocapsa*. Siderocapsa are Fe and Mn oxidizing bacteria that are commonly found in wetlands and soils containing high metal concentrations. They are typically identified based on the presence of several morphological traits including a large spherical shape, a capsule of varying thickness, and the ability to deposit Mn and Fe oxides on that capsule. They also have the ability to adhere to and colonize glass slides (126). The cocci bacteria in the S0 and W sites fit this description, but it would be premature to assign their identity based solely on their morphology. Recent studies have indicated that morphology alone may no longer be sufficient in identifying Siderocapsa spp. Molecular comparison of 16S rRNA genes from a metal rich environmental isolate fitting the morphological description of Siderocapsa was found to actually have closer identity with Pseudomonas spp. and were

reassigned to that genus (55), which proves an interesting finding in reference to our study, since two of our isolates belonged to the genus *Pseudomonas*. For these reasons, it is possible that *Siderocapsa* maintains a presence in the wetland, but more work would be needed to confirm this identification, possibly through the design of FISH probes specific for 16S rRNA sequences from *Siderocapsa* spp. and *Pseudomonas* spp.

FISH images resulted mostly from slides that were incubated in the Sorrento wetland and upstream reference sites. This was done to select for *Leptothrix* spp. since they have the ability to readily adhere to glass and other smooth surfaces (*111*) to form biofilms. This could cause a bias because not all bacteria can adhere to smooth surfaces or form biofilms as easily. However, FISH results were also obtained by collecting samples from the Sorrento wetland and applying them directly to glass slides for fixation. Results did not appear to vary between these two methods (data not shown), so it is unlikely that the bias is large enough to adversely affects results.

Analysis of the 16S rRNA clonal library generated from S0 floc material showed a diverse community, with 62 different phylotypes generated from 67 clones. The five dominant phylotypes comprised only 17% of the community and the remaining 83% is composed of unique and diverse populations of bacteria, a percentage that is substantially higher than other environments that are considered to harbor very diverse communities including Mn-rich cave environments (*56*), metal-rich lake sediment (*35, 127*), and soil (*128*).

Several considerations must be addressed with reference to RFLP analysis of the 16S rRNA library. Gene inserts corresponding to dominate phylotypes were not sequenced. Without sequence identities from these bacteria, little information can be gleaned from such

studies. Sequence identities could determine genus and species, which could in turn determine Mn and Fe oxidizing capabilities, as well as other important roles in the community. Because of this, more work will be done in an attempt to identify those bacteria corresponding to dominant phylotypes.

Another consideration is that different populations of bacteria have different genome sizes and copy numbers of the 16S rRNA gene. This means that larger genomes containing more gene copies will have a greater chance of amplifying during the PCR process used to construct the 16S rRNA clonal library and resulting products will contain a greater percentage of those representatives than actually exists in the initial sample (*129-131*). Several studies have argued that because of this, RFLP analysis from 16S rRNA libraries created from PCR amplification cannot be trusted to provide completely accurate quantitative analysis of members of microbial communities (*129, 132*).

Another consideration is that the number of members in your community is based on the number of clones chosen for restriction digestion. A total of 67 clones were analyzed and found to contain five dominant community members. To fully enumerate the estimated 4000 bacterial populations present in a single gram of soil, several thousand clones would have to be digested or sequenced (*128*). Therefore it is unlikely that I exhausted the extent of the bacterial community, or that the five dominant community members found in this study are the only dominant populations present in the community.

Previous studies investigating trends in microbial community diversity in Fe and Mn rich environments have shown conflicting results. While most studies show that a strong correlation exists between increased metal concentrations and a decrease in diversity (*13*, 70, 74, 82, 92, 95), a select few have reported no decrease in diversity with increased Fe and

Mn concentrations (18, 127), including the previous study investigating microbial diversity in the Sorrento wetland (81). The discrepancy in results from similar environments leads to the suggestion that different microbial communities do respond differently to increasing metal levels. Most studies do agree however, that while diversity may or may not change, community composition does change, typically favoring *Leptothrix*, *Gallionella*, *Siderocapsa*, *Naumaniella*, *Hyphomicrobium*, *Pseudomonas*, and *Bacillus* (27, 35, 56, 94). Culturing and isolations from my study resulted in the enrichment of many *Pseudomonas* and *Bacillus* spp., and a relative of *Leptothrix* sp. from seep sites, while both light and FISH microscopy identified *Leptothrix*, *Gallionella*, and possibly *Siderocapsa*. This demonstrates the disparity that can often arise between culture-based and culture-independent analyses. However, neither *Leptothrix*, *Gallionella*, nor *Siderocapsa* were identified in upstream reference sites, suggesting that community compostion did change with increased metal concentrations.

Conclusion

Results from this study have shown a very diverse community of microorganisms coexisting in the Sorrento wetland. Culture and enrichment results showed the presence of several bacteria and fungus that are capable of oxidizing Mn (II). MPN data indicated an increase in Mn oxidizing heterotrophic bacteria in the Sorrento wetland compared with upstream reference sites. Light and FISH microscopy indicated Fe and Mn oxidizing bacteria, including *Leptothrix* and *Gallionella ferruginea* present in the Sorrento wetland, but not in upstream reference sites. 16S rRNA results indicated a very diverse community with few dominant community members. Based on my data I can conclude that the increase in Fe and Mn oxidizing bacteria. It is probable that these Fe and Mn oxidizing bacteria are at least partially responsible for the deposits of Fe and Mn oxides that are present throughout the Sorrento wetland, refuting previous claims that these oxides are likely abiotic in nature. More work is needed to determine if the diversity and composition of the community, especially in terms of Fe and Mn oxidizers, might change seasonally or temporally.

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APPENDIX A Recipes and Reagents

Alkaline Lysis I Solution (133)

50 mM glucose 25 mM Tris-Cl, pH 8.0 10 mM EDTA, pH 8.0

Alkaline Lysis II Solution (133)

0.2 N NaOH 1% (w/v) Sodium Doecyl Sulfate

Alkaline Lysis III Solution per 100 mL (133)

60.0 mL 5 M potassium acetate 11.5 mL glacial acetic acid

Manganese Oxidizing Medium I (MnO1) per liter, pH 7-7.2 (134)

10mL Major Metal Solution 1mL Trace Metal Solution 100 mg yeast extract 20 mL 1M HEPES 0.5 g 3% FAC 2.0 mL vitamin solution 1.0 mL 1 M MnCl₂ 5.0 mL 10% Casamino acid

Major Metal Solution per liter

12 g NaCl 1.2 g KCl 5.0 g MgCl₂[.]6H₂O 1.0 g KH₂PO₄ 2.0 g NH₄Cl 1.0 g CaCl[.]2H₂O

Trace Metal Solution per liter

155 mg CoCl₂·6H₂0 155 mg ZnCl₂ 50 mg boric acid 20 mg NiCl₂·6H₂0 10 mg Na₂Mo₄·2H₂0 100 mg MnSO₄·4H₂0 3 g MgSO₄·7H₂0 100 mg CaCl₂·2H₂0 10 mg CuSO₄·5H₂0 180 mg AlK(SO₄)₂·12H₂0

Manganese Oxidizing Medium II (MnO2) per liter, pH 7-7.2 (34)

0.5 g (NH₄)SO₄ 0.5 g NaNO₃ 0.5 g K₂HPO₄ 0.5 g MgSO₄·7H₂O 52.1 mL 1M citric acid 2 g sucrose 1 g tryptone 4.7 g MnSO₄·5H₂O 18 g agar (for solid media)

Luria Broth (LB) per liter

10 g tryptone 5 g yeast extract 10 g NaCl 15 g agar

Manganese Luria Broth (LB/Mn) per liter

10 g tryptone 5 g yeast extract 10 g NaCl 15 g agar 1.0 mL 1 M MnCl₂

Iron and Manganese Oxidizing Medium (Fe/Mn) per liter, pH 7-7.2 (135)

5 g peptone 0.15 g ferric ammonium citrate 0.20 g MgSO₄·7H₂O 0.05 g CaCl₂ 0.05 g MnSO₄·H₂O 0.01 g FeCl₃·6H₂O 12 g agar (for solid media)

PTYP medium per liter, pH 7-7.2 (101)

0.25 g peptone
0.25 g tryptic soy broth
0.5 g yeast extract
0.6 g MgSO₄
0.07 g CaCl₂
2.38 g HEPES
adjust pH to 7.2 with NaOH

APPENDIX B DNA Sequence Information

Each DNA sequence below represents partial sequences from Mn oxidizing bacterial

isolates generated from the S0 seep site and from plasmid DNA isolated from clone

members of the 16S rRNA clonal library. Isolate or plasmid name and primers used for

sequencing are provided for each sequence.

Sequence results for Mn oxidizing bacterial isolate SObA using 27 Forward primers

AGAGGTACANACCATGCAGTTCGAGCGGATGANAAGAGCTTGCTNNCNNNTTC AGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGGANAAC GTTTCGAAANGNACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGAC CTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT TGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTT CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTNTAAATTAATACTCTGCN GTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCG GTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT AGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGGCTCAACCTGGGAACTGCAT TCAAAACTGACNNACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCG GTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGA CTGATACTGACACTGAGGTGCGAAAGCGTGGGGGGGGGAGCAAACAGGATTAGATACC CTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCT TAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCNCAAGGT TAAAACTCAAATTGAATTGACGGGGGGCCCGCACAAGCGGTGGANCATGTGGTTT TATTTCNAAANCAACGCGAANAACCTTANCNGGNCTTGACTTCCAATGAAACTT TCNAAAAATGAATGGTGCCTTCGGGANCTTNGAAACAGGGCTGCATGGTTNTC TCNNCTCCTTTCCTGAAATNTTGGGTTAATTCCGTAAAAACCCNACCCTTNNCTT ATTTCCANCCNTTNTGGGGGGCCCTTTAAGGAACTGCCGGTANAAACNGANGAAG **GGGNATACNTNCTTCT**

Sequence results for Mn oxidizing bacterial isolate SObA using 1492 Reverse primers

GNCAACTNGAGATACTCAAANNTACCGTCCTCCCGAAGGATTAGACTAGCTANT TCTGGTGCAACCCACNTCCCATGGGTGGTGACGGGCGGNGTGTACAAGGCCCGG GAACGTATTCACCGCNACATTCTGATTCGNGATTACTAGCGATTCCGACTTCAC ACAGCNCGAGTTGCATNACTGCGATCCGGACTACAATCGGTTTTATGGGATTAG CTCCACCTCGCGGCTTGGCAACCCTCTGNACCGACCATTGTAGCNCGTGTGTAG CCCAAGCCGTAAGGNCNATGATGACTTGACGTCATCNCCNCCTTCCTCNGGTTT GTCACCGGNNGTCTCCTTAGAGTGCCCACCATNACGTGCTGGTNACTAAGGACA AGGGTTGCNCTCGTTACGGGACTTAACCNAACATCTCANGACACGAGCTGACNA CAGCCATGCAGCANNTGTCTCNNTGTTCCCGAANGCACCANTCNATCTNTGGAN AGTTCATTGGATGTCAAGGCCTGGTANGGTTCTTCNCGTTGCTTCAAANTAAAC CACATGCTCCACCGCTTGTGCGGNCCCCCGTCAATTCATTTGAGTTTTAACCTTG CGGNCGTACTCCCCANGCGGACAACTTAATGCGTTAACTGCGCCACTAAGATCT CAAGGCTCCCAACGGNTAGTTGACATCNTTTACGGNGTGCACTACCAGGGTATC NAATCCTGTTTGCTCCCCACGCTTTCGCANCTNAGTGTCGGTANNAANCCAGGN GGNCACCTTCNGCCACNGGTGTTCCTTCATATATCTACNCTTTTCACCGCTACAC ANGAAATTTCCACCNCCCNTNTACCATACTCTANTTTGGACAGTTTTTGAATGCA NTTCCCAGGTTGAACCCCGGNGAATTCATTNTCCANGNTNACAAACCACCTACG GGNNGCTNTGNNNCATTAANTNCAATAACNNTTGCCCCCCCNNTTTANCGCNGC TNTAGNNNAAAATNAGNNGGNGTTTTTNGGNNANANGANAAAGTGCANATNNT TAGTTANANCCTTCCCCAATAAAGGGGTTTNANCCAAAAACCTNCTCNNACCCC GGTTGCNGGANAAGTTTGGCATTNACAAAATCCC

Sequence results for Mn oxidizing bacterial isolate B1 using 27 Forward primers

TNAGGNAGNCGCAGCNTAATCATGCAAGTCGAGCGAACAGCAGAAGGAGCTTG TAGTTTGGGATAACTCCGGGAAACCGGGGGCTAATACCGAATAATCTATTTNACT TCATGGTGAAATACTGAAAGACGGTTTCGGCTGTCGCTATAAGATGGGCCCGCG GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCG ACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC GGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAAC GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAA CAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGG CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA TTATTGGGCGTAAAGCGCGCGCGCGGGCGGGTCCTTTAAGTCTGATGTGAAAGCCCA CGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGGACTTGAGTGCAGANGAGG AAAGTGGAATTCCAAGTGTANCGNTGAAATGCTTANAGATTTGGAGGAAACAC CANTGGCGAAAGGCGACTTTCTGGNTNTNTAACTNACNCTGAGGNGCGAAAAN CTTGGGNANCAAACCAGANTTATATACCCTGGNAGTCNCACGCCCGTAAAACN ATGAANTGNTAAGTGNTTAGGGGGGTTTCCGNCCTCTTAANNGCTTGCANCCTNA NTNCNTTAAANCCNTCCCCCTGGGGGGGGGGTNCCNGTTNNNAAAATTGAAACCTN CTAAANAANTTNACTGGGTGNCCCCNTCNATNNGGNGNAANCATTNTTGTTTTA TTGTTTGANNANNTGNGTTTTTCCCTTCGTGGTANANNNNTNTAACNATTTNGCC NCC

Sequence results for Mn oxidizing isolate B2 using 1492 Reverse primers

TTNGTAGNGNANNTACTTNNATGGCCCCACAGNGCCCGCACCAGACTAAAAAA ATANNNNNCTCTCNNGNCCCGTTGGGGGGGGTGTTGTACAAGGCCCGGGAACGTAT TCNCCGCGGCATGCGTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCG AGTTGCAGCCTANGGNTCCGAACTGAGAACGGTTTTATGAGATTAGCTCCACCT CGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCA TAAGGGGCATGATGATTTGACGTCNTCCCCACCTTCCTCCGGTTTGTCACCGGCN GTCACCTTAGAGTGCCCAACTTAGTGATGGCAACTAAGATCAAGGGTTGCGCTC GTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC ACCTGTCACTCTGCTCCCGAAGGAGAAGCTCTATCTCTAGAGTTTTCAGAGGAT GTCAAGACCTGGTANGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACC GCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCC CAGGCGGAGTGCTTAATGCGTNAACTTCNGCACTAAAGGGCGGAAACCCTCTAA CACTTAGCNCTCNTCGTTTTACGGCGTGGACTNCCAGGGTTATCTANNTCCTGTT TGCTCCCCACGCTTTTCGCGCCTTCAGTGTCANTTACNGACCAGAAAGTCGCCTT NCGCCCCTGGTGTTTCCTNCAANATCTCTACGCNATTTNNNCCGGCTACACATG GNANTTCCACNTTTGCCNTCTTCTGCACTTCANGTCATCCCCAGNTTTCCCANTG GNCCCCTCCCGCGGGTGGANGCCGGTGGGGCTTTNCNCACTCNANACTTTTAAN GAANCCCCCCTTGCGGNGCGNNTTTTCCNCCCCANNAANTTCCGGGGGANAACGC TTGNCCNCCTTNCGGNTTNCCCCNGNGGNTGCNNGGCNCGGTANTTTAGTCGGG GGGNTTTTNNTGGTTTNNGTTNCCNNCCNAGGGNNCCCGCCTTNNTTCNACNNN GCNCNTNNTTNTTNCCCNNAANNCACCNNNTTTTT

Sequence results for Mn oxidizing bacterial isolate B5 using 27 Forward primers

ATANGCACATATCTTGCANGGTCGNGCGGATGAAGANAGCTTGCTCTCTGATTC ANCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGGACAAC GTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGAC CTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGG TAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCT TCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGC AATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC GCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCG CGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTG CATTCAAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGT AGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACC TACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAG CTCTTAGTGGCGCANCTAACGCATTAAGTTGACCGCCTGGGGGAGTACGGCCGCA AGGGTTAAAAACTCAAATGAATTTGACGGGGCCCCCCACAAGCNGGTGGAGCA TGTGGTTTAATTCNAANNCACCCGAANAACCTTACCAGGCCTTGANATCCAAT

GAACTTTCCANANATGGATTGGTGCCTTTNGGAAACTTTGAAANAGGGGCTGCA TGGGCTTGTCCTCACCTCCGNTNCTGAAATTTNGGNTTANTNCNNTAACAANCC CAACCNTTNTCTTTATTNNCACCCCNTTTNGGGGGGNCNNCAAGGAAAC

Sequence results for Mn oxidizing bacterial isolate B6 using 27 Forward primers

NTNACNAGGGCTACCNTNCNNCNNCAATGGTTAGACTAGCTACTTCTGGTGCAA CCCACTTCCCAATGGATGTGACGGGGGGGGGTGTGTACAAGGCCCGGGAACGTATTC ACCGCGACATTCTGATTCGCGATTACTAGCGATTCCGACTTCACGCAGTCGA GTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCG CGGCTTGGCAACCCTCTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCGT AAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG CAGTCTCCTTAGAGTGCCCACCNTAACGTGCTGGTAACTAAGGACAAGGGTTGC GCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG CAGCACCTGTCTCAATGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCA TTGGATGTCAAGGCCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC TCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT ACTCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAGAGCTCAA GGCTCCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA TCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTC GCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACA **GGNAAATTCCACCACCCTCTACCATACTCTAGCTTGTCAGTTTTGAATGCAGTTC** CCAGGTTGAGCCCGGGGGCTTTCACATCCAACTTAACAAACCACCTACGCGCGCT TTACGCCCAGTAATTCNAATNAACGCTTGCACCCTCTGTATTACCGCGGCT GCTGGCAAAANTNAGCCGGNNNNTTATTCTGTCGGTAACGTCAAATTNNNAANT ATTAANTCTANAACCCNTTCCCCCCAACTTAAAGNGGTTTANAATCGAAAANCT TTTTCNANCNCCGGNANGGNTGGANAGNNTTNCCCCATTG

Sequence results for Mn oxidizing bacterial isolate B7 using 1492 Reverse primers

TGTTCCTCCACATCTCTACGCATNTCACCGCTACACGTGGAATTCCGCTTTCCTT TCTGTACTCAAGTCCCCANTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTT CACATCAAANTTAAAGGACNGCCTGCGCNCGCTTTACGCCCNATATTTCTGAAA ACNCTTGGCCNCTACANTTACCGGGCTGCTGNAACTAATTANCCNTGGCTTTNT GGGTAGNACCTTCAGGNACGGNANTATCGGNATTGTCTTCCTAACAAANAGTTA AACCGNAGCTCTCCTACNCGNNTGTCTAAATTCNCTTGGGAATCAAAGTGCCCN AAAATGGGCNGTNATCATTGCANNCCTNCAGGGTAACNCNNCCTGGANGNACN CAAACTAGC

Sequence results for Mn oxidizing bacterial isolate B8 using 1492 Reverse primers

CNANAGANATNNCCCTCCTTGCGGTTAGGCTAACTACTTCTGNCAGAACCCGCT CCCATGGATGTGACGGGNGGTGTGTACAANACCCGGGAACGTATTCACCGCGG CAAGCTGATCCGCGATTACTANCGATTCCGACTTCACGCAGATCGAGTTGCAGA CTGCGATCCGGACTACGACCGGTTTTCTGGGATTGGCTCCCCCTCGCGGGTTGGC AGCCCTCTGTACCGGCCATTGTATGACNTGTGTAGNCCTACCCATAAGGGCCAT GANGACCTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCATTAG AGTGCCCTTTCGTAGCAACTAATGACAAGGGTTGCGCTCGTTGCGGGACTTAAC CCAACATCTCACGACACAAGCTGACGACGGCCATGCAGCACCTGTGTCTAGGTT AGGTTTTTCGCGTTGCATCGAATTAAACCACATCATCCACCGCTTGTGCGGGTCC CCGTCAATTCCTTTGAGTTTCAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTT CACGCGTTAGCTACGTTACTGAGAAGAAACCCTCCCAACAACCATTTGACATCG TTTAGGGCNTGNACTACCAGGNTATCTAATCCTGTTTGCTCCCCACGCTTTCNTG CATGAGCGTCAGTACAGGTCCAGGGAACTGCCTTCGCCATCGGTGTTCCTCCGC ATATCTACGCATTTCACTGCTACACGCGGANTTCCATCCCCCTCTACCGTACTCT ANCCATACAGTCACAAATGCAGTTCCCAGGTTGAGCCNGGGGATTTCACATCTG TCTTATATAACCGCCTGCGCACGCTTTNACGNCCAGTAATTCCGATTAACNCTTG CACCCTACGTATTACCGCGNCTGCTGCCCGTAATANGCNGGNGCTTATTCTTNA AGNACCGTCATCCTCCCAGGGTATNACCCCCAAAAAATTTTTTCCCTGAAAAANN GGNTNANANCCCNAAGNCCTTNTTCCCCCNNCNGNATGGCTGGAAANGNTTGC CCNATTGTCNAAAATTCCCATGCTGGCCCCNGGAGAATNNGGNCGGNTCCAATC CCNATGGGTGGGNTCCTNNCAAANANATAGANGNCNCTTGNNGGCTTNCCCCN NAAAATTATTNAATGG

Sequence results for dominant phylotype P1, plasmid A40 using M13 Reverse primers

NAANCGAAAGNCAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGT GGATGGATATCTGCAGAATTNNCCCGTTGGTTACCTTGATTACGACTTCACCCCA GTTCATGAATCACAAAGTGGTNGAGCGCCCTCCCGAAGGTNAGACTACCCAC TTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGA ACGTATTCACCGCGACATTCTGATTCGCGATTACTAGCGATTCCGACCTCATGGA GTCGAGTTGCAGACTCCAATCCGGACTAAGATCGGCTTTCTGGGATTTGCTTACT CTCGCAAGTTCGCTGCCCTCTGTACCGACCATTGTAGCACGTGTGTANCCCTNCC

CATAAGGGCNNTGATGACTTGANGTCGTCCCCANCTTCCTCCGGTTTATCACCG GCAGTCTCCCTAGAGTTCCCGACATTACTCGCTGGCAACTAAGGATAAGGGTTG TGCAGCACCTGTNTCAGAGTTCCCGAAGGTNNTCTACTATCTCTAACAGATTCTC TGGATGTCAAGGGCTAGGTAAGGTTCTTCGCGNTGNATCGTAATTAAANNACAT GNNCCACNGCTTGTGCGGGCCCCNGNCAATTNATTTGAGTTTTTAANCTTTGCC GGCCNGANTCCNCANGNCGGTCAACTTTAATGNNTTATCTGCGCCANTTAATCC NGTTATATAGGACCAACGTGNTTAGCTTGAACNTCGGTTTACGGCCNTGGGAAT ACCCANGNGTAATCNNAATCCCTGNTTGGNTACCCANGNCTTTGNACNANTACG NTNANTTTGNAGTTNCCTNNAGTCNCCCTTTNGNCCNCNNGGNGTNCCTTNCNN ATANTCTANNAAATTTNACNGNTGNTACCTNAAAATTNCNNNTNTCCTNTAANN AANTNCAATTGCGNANGGTTTCAAATGAGTTNCCAAGGTNGAANCCCNNGGTTT TTANGTANNNATTTNANNNANTCNCTATACCCNTCNTTTANCNNTANAAATTNN AAAAANNCTTCNCCTCNCCGTTTTATNNNNAGNTGGCGAGANGAANNTTATCCC GNNTTCTNTNNNAGGGAANNNTAAAANCCAGGGNTTTNGACTTGTTNTTTCC NCCNTNGANTGGTTNTANACTAAAGGCNNCTGTCCCNTCTGNNTNGGNGACNNC NTTTANCCTTGTNAGNNCCCCGGGGGGNNCTTANNNTCGNNNNNTNAATACTTGG

Kristin Michelle Johnson was born in Siler City, NC on May 23, 1983. She attended Pinecrest High School in Southern Pines, NC where she graduated in 2001. She then attended Appalachian State University where she planned on majoring in Elementary Education. One semester in, she realized her mistake and graduated in August of 2006 with a Bachelor's Degree in Biology, concentrating in secondary education. After taking a oneyear sabatical to ride camels in India and hike glaciers in New Zealand with her husband, she returned to Appalachian State University in the fall of 2007 to pursue a Master's Degree in Biology.

Kristin will commence employment at Appalachian State University in August of 2009 as a science education instructor. She lives with her husband Brad, and their dog Jack Straw.

VITA