TRACE METAL BIOAVAILABILITY IN TWO UNIQUE SOIL SYSTEMS

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It is widely understood that soil microbial communities control nutrient cycling, trace metal biogeochemistry and bioavailability in both aquatic and terrestrial systems, but the underlying mechanisms are not entirely clear. The objective of this work was to describe the microbial community composition, dynamics, and mechanisms controlling heavy metal biogeochemistry in the rhizosphere of purple willow (*Salix purpurea* L.) growing in the heavy metal affected circum-neutral histosols of Elba, NY, as well as to explore microbial metal mobilization in the African dust deposited on Trinidad and Tobago, West Indies.

After extensive site characterization examining both spatial and temporal biogeochemistry of sulfur (S), zinc (Zn), cadmium (Cd), and copper (Cu) at the Elba site, I focused on the composition of the willow rhizosphere microbial community and specific activities leading to enhanced metal mobilization. Cloning of the 16S rRNA gene revealed a diverse and uniquely adapted microbial consortium present in the *S. purpurea* rhizosphere included members of *Acidobacteriales, Actinomycetales, Planctomycetales, Verrucomicrobiales, Myxococcales, Sphingobacteriales,* as well as members of *Alpha-*, *Beta-*, and *Gammaproteobacteria*. Culture-based techniques yielded a large proportion of isolates belonging to *Bacillales,* but also revealed members of *Actinomycetales, Xanthomonadales, Pseudomonadales,* and *Burkholderiales* to be contributing to heavy metal mobilization via siderophore production.

In Trinidad and Tobago, I utilized the African dust microbial culture collection of the University of the West Indies to quantify the proportion of organisms capable of producing siderophores and thereby releasing bioavailable iron (Fe) into downwind ecosystems. I found that roughly 65% of organisms collected during African dust events produced siderophores, and all classes of siderophores were detected.

The proportion of siderophore-producing isolates in Elba and in African dust was similar, and greater than in other reported soil systems. Increased siderophore production could be a result of heavy-metal stress; in the soils of Elba, Zn, Cd, Cu are inherently high, while in the African dust, typically Cu and molybdenum (Mo) are enriched. In these unique and disparate soil systems, microbial siderophore production appears to be shared mechanism for selective heavy metal mobilization or immobilization, demonstrating a powerful means by which soil microorganisms may impact biogeochemistry around the world.

BIOGRAPHICAL SKETCH

Tarah Shay Sullivan was born in College Station, TX, on June 3, 1979. Growing up in the rural backwoods of the "Big Thicket" of East Texas gave her a deep appreciation for the complexities of the natural world. Through involvement with Girl Scouts and 4-H, rodeo and the family cattle-ranch, she gained a profound admiration and curiosity for the environment which would serve as a springboard to her career in environmental sciences.

She graduated Cum Laude with a bachelor's degree in Rangeland Ecosystem Science at Colorado State University in 2002, as an undergraduate officer in the Society for Range Management. At that time, she was offered a fellowship for a master's degree with Dr. Mary Stromberger, on a project examining the long-term effects of biosolids land applications on soil microbial communities and the associated grassland communities of the short-grass steppe, which she completed December, 2004. In January 2005, Tarah began her PhD work at Cornell University in the laboratory of Dr. Janice Thies in Crop and Soil Sciences.

During her time at Cornell, she served as the President for the Soil and Crop Sciences Graduate Student Association for three years, she was awarded the Andrew Mellon Small Grant Award, as well as Outstanding Graduate Teacher in the Field of Soil & Crop Sciences, The MacDonald/Musgrave Award, the Graduate Student Award for Excellence, 3rd Place Graduate Poster Contest at the Annual SSSA Conference (2008), Fulbright Fellowship in Aeromicrobiology to Trinidad & Tobago, and the U.S. EPA STAR Fellowship in Environmental Microbiology.

During the spring semester of 2009 she also taught an undergraduate course in Enivronmental Microbiology at Wells College in Aurora, NY. Upon completion of

her degree in May 2010, Tarah will work as a postdoctoral associate in the laboratory of Dr. Chris Schadt at Oak Ridge National Laboratory in Oak Ridge, TN.

To my husband, my family, my friends...

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CHAPTER 1

TRACE METALS: CONTAMINATION AND REMEDIATION

"*These toxicants may be derived from mining operations, refining ores, sludge spread on land, fly ash from incinerators, the processing of radioactive materials, metal plating, or the manufacture of electrical equipment, paints, alloys, batteries, pesticides, or preservatives. The substances of concern include ionic lead, chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, gold, silver, copper, and nickel, as well as nitrate and cyanide*."

Alexander, M. (1999).

Trace metals that are essential micronutrients required for plant growth include boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), molybdenum (Mo), and zinc (Zn) (Marschner, 2003). These elements are biologically indispensible, but only necessary in very small quantities. When concentrations of trace elements in the environment are high enough, many trace elements, including the micronutrients, can become toxic to microorganisms, plants, animals, and even humans. The most toxic trace elements are those without a biological requirement, such as cadmium (Cd), mercury (Hg), and lead (Pb), but even Cu, cobalt (Co), and nickel (Ni), can be toxic to varying degrees in higher plants and animals (McBride, 1994).

Heavy metal contamination of the biosphere is a significant environmental problem on the rise since the industrial revolution (Nriagu, 1979). In contrast to many organic pollutants also on the rise in the biosphere, heavy metals are elements rather than compounds, which means they cannot be destroyed, nor do they degrade. Metal- and metalloid-containing molecules or ions may only be modified, immobilized, or detoxified. Thus, with regard to persistence, heavy metals "never go away" (Crosby, 1998).

In response to the growing need to address heavy metal contamination of soils, numerous mechanical, physical and chemical remediation technologies have been developed, including soil washing or flushing, soil vapor extraction, solidification, stabilization, vitrification, thermal desorption, encapsulation, and excavation (Hamby, 1996; Khan et al., 2004; Mulligan et al., 2001). Unfortunately, these techniques are typically expensive, often labor intensive, and frequently leave the soil highly disturbed and the land useless for further activities, such as plant growth (Marques et al., 2009). Phytoremediation, however, takes advantage of the naturally occurring processes by which plants and their associated rhizosphere microorganisms sequester, detoxify or immobilize heavy metals and other pollutants consequently "cleaning" not only contaminated soils but water as well (Pilon-Smits, 2005).

Traditionally, phytoremediation utilizes one or more of the 450 identified species of "hyperaccumulator" plants, or those plants capable of accumulating as much as 0.1 – 1% of the metal in their above-ground tissues (Maestri et al., 2010). Hyperaccumulator plants have largely been the focus of phytoextraction technology development for the past 20 years. Unfortunately, in the northern temperate zone, phytoextraction by hyperaccumulators is limited by the small, herbaceous nature of these plants, resulting in very slow metal extraction from soil and often requiring many cropping cycles (Baker and Brooks, 1989). More recently, willows (*Salix* spp.) have begun to gather more attention due to moderate metal accumulation rates coupled with substantial biomass production, leading to significant metal removal from soils and sediments over a relatively short period of time (Kuffner et al., 2010; Pulford and Watson, 2003; Unterbrunner et al., 2007).

In order for any type of plant to be able to effectively remediate heavy metals, they must not only be in contact with the pollutants, but also be able to take them up. Consequently, bioavailability is of utmost importance and rhizosphere microbial

communities have been identified as a key factor in phytoremediation success (Khan, 2005; Kidd et al., 2009; Lucy et al., 2004; Nascimento and Xing, 2006; Pilon-Smits, 2005; Pulford and Watson, 2003). Additionally, soil microbial communities primarily control nutrient cycling, trace metal biogeochemistry and bioavailability in both aquatic and terrestrial systems (Atlas and Bartha, 1998; Sylvia et al., 2005).

The microbial communities controlling these processes have been fairly well characterized in certain extreme environments such as Iron Mountain, CA (Edwards et al., 2000), or in the rhizosphere of a few specific hyperaccumulator plants such as *Thlaspi caerulescens* (Whiting et al., 2001) or *Sedum alfredii* (Xin-Xian et al., 2009). However, the community composition, dynamics, and mechanisms controlling heavy metal biogeochemistry in heavy metal affected circum-neutral soils and in the rhizosphere of *Salix* spp. are not well characterized.

Likewise, several studies have sought to characterize the composition of the microbial community of the African dust (AD) deposited in various locations within the Caribbean (Kellogg and Griffin, 2006; Kellogg et al., 2004). While certain studies have implicated microbial communities in AD impacts on human and coral reef health (Griffin et al., 2003; Prospero et al., 2008; Shinn et al., 2000), others have specifically implicated iron (Fe) deposition with AD in detrimental ecological effects (Muhs et al., 2007; Walsh et al., 2006). However, no study to date has attempted to draw a mechanistic link between the microbial communities of African dust over the Caribbean and Fe bioavailability in the dust.

Here, I attempt to address the topics of microbial community composition, dynamics, and mechanisms controlling heavy metal biogeochemistry in the metalliferous peat soils of Elba, NY, and to further explore microbial metal mobilization in the African dust deposited on Trinidad and Tobago, West Indies.

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CHAPTER 2

METALLIFEROUS PEAT BIOGEOCHEMISTRY: FIELD-SCALE HETEROGENEITY OF S, ZN, CD, AND CU IMPACTS PHYTOAVAILABILITY AND SOIL MICROBIAL COMMUNITY.

2.1 ABSTRACT

Because Zn, Cd, and Cu are chalcophilic heavy-metals, understanding the mechanisms that control biological oxidation of sulfur-metal compounds will lead to a better understanding of their cycling and availability, resulting in more effective remediation strategies for soils contaminated with these metals. In this study, we evaluate field-scale spatial variability in soil chemical parameters and relationships with the soil microbial community in the Elba muck soils of Western NY. In May, 2005, soil samples were collected along a series of transects in order to capture spatial heterogeneity across the site at a resolution relevant to the local agricultural stakeholders (approximate field dimensions: 36 x 90 m). Soils were collected at two depths, 0-15 and 15-30 cm, and analyzed for % H_20, pH, total, as well as 0.01 *M* $CaCl_2$-extractable S, Zn, Cd, and Cu. Soil Bacteria and Archaea community structures were characterized by terminal restriction fragment length polymorphism (T-RFLP) analysis. Field hedgerow willow (*Salix purpurea* L.) tissues were also analyzed for total S, Zn, Cd, and Cu. Spatial variability across the field was vast within all measured parameters excluding soil pH. Total soil S, extractable soil Cu and Zn exerted the greatest influence on the structure of the soil microbial communities, and willows were particularly sensitive to bioavailable fractions of soil heavy metals. Our results demonstrate the intricate link between soil chemistry and soil microbial community dynamics over a field scale and emphasize the importance of scale

considerations in developing more effective remediation strategies for Zn, Cd, and Cu contaminated sites.

2.2 INTRODUCTION

Zinc (Zn), cadmium (Cd), and copper (Cu) are major environmental pollutants in soils that can lead to phytotoxicity, reduces diversity of soil microorganisms, suppression of soil microbial processes, bioaccumulation in higher organisms, or toxicant movement into local water sources (Chandler and Brookes, 1991; Kelly et al., 2003; Khan, 2005; Simona et al., 2004). Because these elements are chalcophilic and, therefore, often found in association with sulfur (S), soil redox potential, organic matter content, and pH, as well as microbial activity largely dictate the mobility and potential toxicity of these metals (McBride, 1994).

A geological formation of Lockport Dolomite enriched in Zn and Cd underlies the wetlands near Elba in western New York and has historically undergone geochemical exchanges with the overlying peat soils. This has resulted in circum-neutral peats that contain high levels of Zn, Cd, and S (Cannon, 1955; Martínez et al., 2002). In the agricultural regions surrounding Elba, vegetable crop phytotoxicity was first observed in the early 1940's when the wetlands were initially drained for agricultural purposes (Cannon, 1955) and severe crop phytotoxicity due to high soil concentrations of Zn and Cd has persisted in the decades since those early studies (Martínez et al., 2002; Martínez et al., 2007). While Zn and Cd at this site are of natural origins and have percolated upwards through the soil profile, these peat soils have also become contaminated with Cu from fungicides and pesticides used by growers periodically for over 60 years (McBride, 2005, personal communication). Copper was therefore included in this study as another chalcophile with surface depositional sources rather than underlying geological sources, as for Zn and Cd, in

order to assess the differences in metal-behavior and spatial patterns resulting from source position.

Each spring for more than 60 years, the growers in the region drain their fields to prepare for planting and each fall after harvest the fields again become saturated due to the natural hydrology of the system. This has resulted in aerobic soil conditions during the growing season, which mobilizes heavy metals and sulfur through oxidation of organic matter and metal sulfides originally present in the peat. As the fields become flooded and the peats turn anaerobic in the winter months, conditions once again each year favor the formation of metal sulfides and result in the immobilization of heavy metals deep in the soil profile (Martínez et al., 2006; Martínez et al., 2007; McBride et al., 2005).

McBride (2005) and Martínez (2007) both found that vertical heterogeneity of the distribution of heavy metals through the profile of the Elba muck soils is considerable, in addition to the large chemical and biological differences in the soils between the winter months (November through April) and the growing season when the soils are annually drained (May through October). It is clear from these previous studies that S biogeochemistry in these unique peat soils largely dictates the mobility of these metals within the soil profile, and consequently the toxicity of heavy metals to crop plants. Understanding soil microbial community structure dynamics with regard to the prevailing environmental gradients will yield greater insight into the underlying processes at work. However, field-scale spatial variability at the site has yet to be fully explored.

In this study, we evaluate field-scale variability in soil chemical parameters and their relationships with the soil microbial community structure in the Elba, NY, muck soils. These naturally metalliferous peat soils provide a unique environment to examine the relationships of the microbial community with heavy metals and organic

matter under variable redox conditions. Results may be broadly useful in designing fine-scale resolution bioremediation and bioaugmentation strategies for heavy metal contaminated sites.

2.3 METHODS

Field site and sample collection

This study was conducted in the spring of 2005 on a long-term (~60 y) agricultural field near Elba, NY, located in Orleans County at 78°6'W, 43°9'N; elevation at the site is 273 m and does not vary. The specific field used in this study was chosen due to evidence from previous research of anomalously high Cd, Zn, and S levels (Martínez et al., 2002; McBride et al., 2005; Qureshi et al., 2003) as well as zones of visibly evident Zn phytotoxicity observed interspersed with agriculturally productive areas. However, at the time the study was conducted, crop growth was not sufficient for plant tissue collection and therefore the only plant tissues collected were those of the hedgerow willows (*Salix purpurea* L.) at the western edge of the field (Figure 2.1).

These soils, classified as Palms Muck (Natural Resources Conservation Services, http://websoilsurvey.nrcs.usda.gov/app/WebSoilSurvey.aspx), become waterlogged each winter and growers use drainage tiles with pump stations to lower the water table in preparation for tillage each spring. These peat soils have been reported to contain roughly 80% organic material at all depths, with total soil S ranging from around 4,000 to over 9,500 mg kg^{-1}, total soil Zn ranging from roughly 87 to over 15,000 mg kg^{-1}, and total soil Cd from 0.55 to over 80 mg kg^{-1} (Martínez et al., 2002) exhibiting a high, but as yet unquantified level of spatial variability.

The field was sampled on a rectangular grid pattern in order to capture spatial variability across the site (Figure 2.1). The number of sampling locations necessary

was determined based on previous research conducted on the site (Martínez et al., 2002; McBride et al., 2005) and visual observations of crop phytotoxicity during past growing seasons.

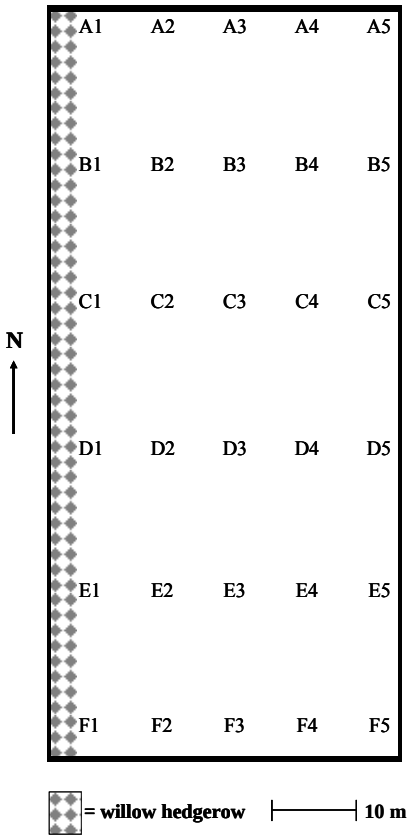

Figure 2.1 Diagram of the sampling regime used across the agricultural field site in Elba, NY. In May 2005, soils were collected at all 30 sampling points shown, while *Salix purpurea*, L. tissues were sampled only from the most western point along each transect.

Soil from six transects, at intervals roughly 18 m apart, was sampled at five points across the field from west to east approximately 9 m apart (Figure 2.1). Each transect was delineated north to south by the letters A through F; sampling points from west to east were delineated by the numbers 1 through 5 and each was located precisely by a Garmin ***e***-Trex® GPS unit. This design resulted in 30 soil sampling points with 6 corresponding willow hedgerow sampling points located at the western-most point on each transect (Figure 2.1).

In May, 2005, soil samples as well as hedgerow willow leaf tissue samples were collected along each transect. Three soil cores were collected at each sampling point and composited by depth (0-15 and 15-30 cm); sampling depth was determined by the rooting depth of the historically grown crop at the site (onion). The soil sampling auger was rinsed with a 5% bleach solution between sampling locations to wash off excess soil and prevent cross contamination. Each composite sample was stored on ice in a Ziploc® bag in the dark until transport to the laboratory, where soils were further analyzed.

Chemical analyses

Within 24 h of sample collection, gravimetric water content, bioavailable fraction extraction, and extractions for total elemental analysis were performed. Zn, Cd, and Cu ionic activity, and consequently bioavailability to plants, is highly susceptible to changes in soil solution pH and ionic strength. Therefore, chemical extraction of soils for determination of the bioavailable fraction of each element, was performed on 10 g of each field-sample composite in 50 ml of 0.01 M $CaCl_2$ which only minimally alters the soil pH and soil solution ionic strength (Houba et al., 1990), as compared to other common, more aggressive, soil extraction procedures. Soil extractions for total elemental analysis were performed with 0.5 g sub-samples from field composite samples using microwave assisted nitric acid digestion (EPA 3051). All soil extractions were performed in triplicate and each extract was analyzed for S, Zn, Cd, and Cu on an inductively coupled plasma (ICP) spectrometer (SPECTRO Analytical Instruments, Germany) at the Cornell Nutrient Analysis Laboratory (Ithaca, NY).

The first sampling point along each transect (on the western edge of the field) was located in the willow hedgerow; these soil samples were within the willow rooting

zone and corresponding *Salix purpurea* L. tissue samples were collected. At each hedgerow sampling location, three willow individuals were sampled in exactly the same manner. New leaf tissue growth of the same size and growth stage was sampled across the field. *S. purpurea* tissues (in duplicate) were digested using a heated $Mg(NO_3)_2$ dry ashing (Greweling, 1976) rather than a standard acid digestion method due to the fact that quantitative measurements of leaf S were desired. A fraction of sample S is lost by volatilization if standard dry-ash methods are employed. Willow tissue digests were analyzed for S, Zn, Cd, and Cu using an ICP spectrometer (SPECTRO Analytical Instruments, Germany).

Microbial community analyses

Microbial community composition was characterized by terminal restriction fragment length polymorphism (T-RFLP) analysis (Marsh, 1999). Extractions for soil microbial DNA were performed on 0.25 g of each sample using in the MoBio PowerSoil™ DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Three µl of each extracted DNA sample was then quantified against a calf thymus DNA standard curve in an 4X ethidium bromide (EtBr) solution using an EC3 Imaging System (UVP Bioimaging Systems, UVP LLC, Upland CA) and Quantity One™ (BioRad, Hercules, CA) software. DNA extracts were diluted with nuclease-free water to approximately 3-5 ng μl^{-1}.

Bacterial DNA was then amplified by polymerase chain reaction (PCR) using the fluorescently labeled forward primer 27f (5′-[6FAM] AGA GTT TGA TCC TGG CTC AG-3′) and the unlabeled reverse primer 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Integrated DNA Technologies, Coralville, IA) (Moeseneder et al., 1999). Duplicate reactions prepared from each sample were amplified using a MJ Research thermal cycler PTC 100 (MJ Research, Waltham, MA) and the following program: 5

min at 94°C, followed by 27 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Reaction volumes for each 50 µl reaction were as follows: 0.50 µl of 5U *Taq* polymerase (Applied Biosystems, Foster City, CA), 5.0 µl of the 10x PCR buffer supplied with the enzyme, 4.0 µl of 25 m*M* $MgCl_2$, 1.0 µl of 10 m*M* deoxy-nucleotide triphosphates (dNTPs), 0.5 µl of 10 µg μl^{-1} bovine serum albumin (BSA), 0.5 µl of each primer at 10 µ*M*, 33.0 µl nuclease free water (Promega, Madison, WI) and 5 µl of DNA template at 3-5 ng μl^{-1}.

Archaeal DNA was amplified by (PCR) using the fluorescently labeled forward primer Ar109f (5′-[6FAM] ACG/T GCT CAG TAA CAC GT-3′) and the unlabeled reverse primer Ar912r (5′-CTC CCC CGC CAA TTC CTT TA-3′) (Integrated DNA Technologies, Coralville, IA) (Leuders 2000). Duplicate reactions of each sample were amplified using a MJ Research thermal cycler PTC 200 (Waltham, MA) as follows: initial denaturation at 94°C for 5 min; 27 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 72°C for 45 s; and a final extension step at 72°C for 10 min. Reaction volumes and concentrations stated above were employed, but without of BSA added.

Restriction enzyme digests for each sample contained 1.25 µl *Sau96* I enzyme (New England Biolabs, Ipswich, MA), 2.5 µl of the 10x buffer supplied with the enzyme, 0.25 ul of BSA at 10 µg μl^{-1}, 8.50 µl nuclease-free water and 12.5 µl of amplified sample DNA. Restriction digestion was carried out in a MJ Research PTC 200 thermal cycler at 37°C for 4.5 h with a final step of 70°C for 15 min to stop the reaction. Complete digestion of the DNA was verified by inspecting digested products run on a 1.5% agarose gel stained with EtBr and visualized using an EC3 Imaging System (UVP Bioimaging Systems). Digested DNA was purified, lyophilized, and resuspended in a 10 µl mix containing 9.85 µl of formamide and 0.15 µl of LIZ 500 size standard (Applied Biosystems, Foster City, CA). Terminal fragment-size analysis

was performed using a 3730XL ABI electrophoretic capillary sequencer (Applied Biosystems) in conjunction with the Genemapper Software (Applied Biosystems) at Cornell University's Life Sciences Core Laboratories Center, Ithaca, NY.

Data analyses

Spatial trends were assessed by using GIS mapping software package Manifold System 6.50 (CDA International Ltd., 2005) which employs data kriging between sampling points to create surface interpolations and allow visual interpretations of spatial trends. Soil microbial T-RFLP pattern discrimination was determined by non-metric multidimensional scaling (NMS) analysis applied using the Jaccard distance measure and the Medium Autopilot mode in PC-Ord (MjMSoftware Design, Gleneden Beach, OR). This mode specifies: (i) Maximum number of iterations=200, (ii) Instability criterion=0.0001, (iii) Starting number of axes=4, (iv) Number of real runs=15, and (v) Number of randomized runs=30. Unlike other commonly used ordination techniques (i.e., principal components analysis), NMS does not assume a linear relationship among ecological variables (Culman et al., 2008). This analysis allows virtually any distance measure to be used in the construction of the similarity matrix and is currently considered the most appropriate analysis for T-RFLP data sets (Rees et al., 2004). Linear regression of soil chemical parameters against each other and against each microbial community ordination axis was performed with α=0.10 significance level (MINITAB ® 15.1.30.0).

2.4 RESULTS AND DISCUSSION

Soil chemistry

A clear spatial pattern was observed across the site in soil pH, total and $CaCl_2$-extractable soil Zn, and total soil S (Figure 2.2), however soil pH values and

concentrations of total and $CaCl_2$-extractable S, Zn, Cd, and Cu did not differ significantly between the surface (0-15 cm) and subsurface (15-30 cm) soils (Table 2.1). A high degree of variability in all measured variables excluding soil pH was observed at both soil depths (Table 2.1).

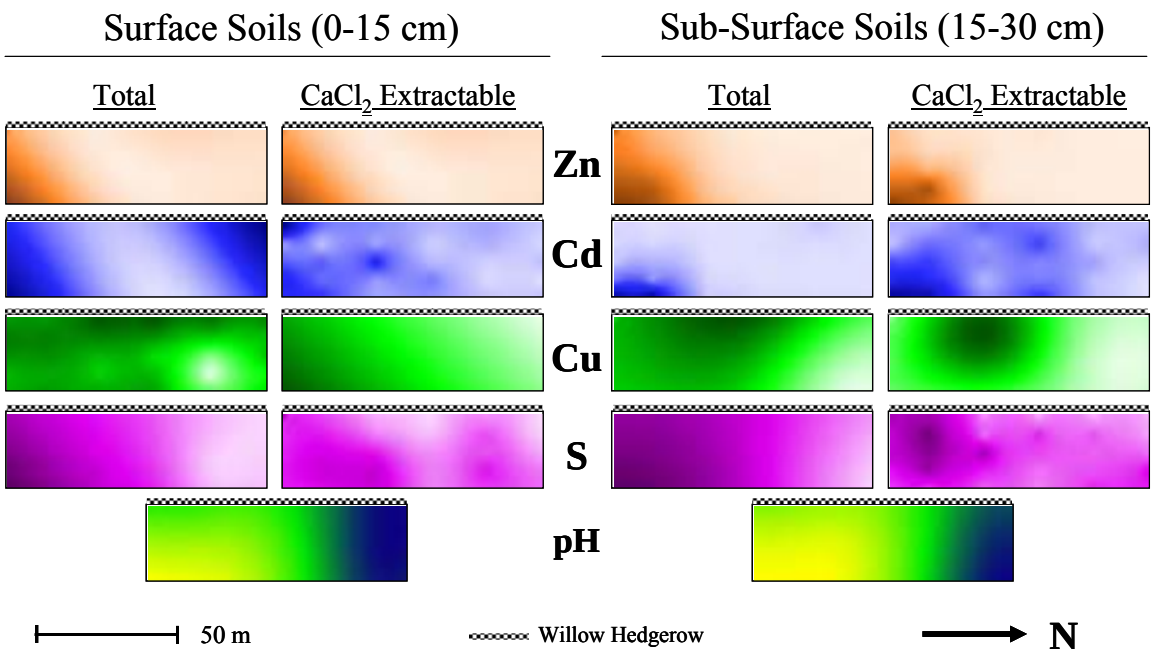

Figure 2.2 Spatial interpolations of soil chemical variables at the Elba, NY, field site in May, 2005, with the presence of the willow hedgerow indicated on the western edge of the site. Each map represents the sampling grid plane (36 m x 90 m) soil chemical data points kriged across the research site to create a surface visually displaying spatial trends in soil chemistry at a given depth. Darker colors represent higher values while lighter colors represent lower values. The precise range of values for each element and soil pH is given in Table 2.1.

While mean values across the site for Zn, Cd, and Cu are not extraordinary and could fall within the range of worldwide means (McBride, 1994), individual locations within the field exhibit high and potentially phytotoxic concentrations of all three metals, similar to those levels found at anthropogenically contaminated sites (Hinojosa et al., 2005), which results in significant heterogeneity across the site not captured in a single value, such as the mean.

Table 2.1 May, 2005, minimum, maximum, and mean values of soil chemical variables given reflect the high degree of spatial variability across the field site near Elba, NY. Standard deviations across the field are given in parentheses where appropriate, and values below the detection limits are indicated with <det.

Surface Soils (0-15 cm)

	Total		**$CaCl_2$ Extractable**		**Extractable Fraction**	
	range	mean	range	mean	range	mean
	---------- mg kg^{-1} dry weight ----------				---------- % ----------	
pH	5.5 - 7.5	6.4 (± 0.6)	-	-	-	-
S	2400 - 8400	5200 (± 1500)	5.1 - 25	14 (± 5.7)	0.10 - 0.66	0.28 (± 0.13)
Zn	49 - 910	220 (± 220)	<det - 23	3.3 (± 5.4)	0.0 - 2.5	0.96 (± 0.77)
Cd	<det - 0.53	0.08 (± 0.13)	<det - 0.03	0.007 (± 0.006)	0.2 - 77	16 (± 23)
Cu	3.9 - 250	180 (± 51)	<det - 0.38	0.12 (± 0.11)	0.0 - 0.23	0.06 (± 0.06)

Subsurface Soils (15-30 cm)

	Total		**$CaCl_2$ Extractable**		**Extractable Percentage**	
	range	mean	range	mean	range	mean
	---------- mg kg^{-1} dry weight ----------				---------- % ----------	
pH	6.0 - 8.0	6.8 (± 0.8)	-	-	-	-
S	150 - 8900	4700 (± 2700)	6.0 - 45	21 (± 11)	0.12 - 16	1.5 (± 3.2)
Zn	24 - 910	230 (± 290)	<det - 38	5.5 (± 11)	0 - 24	3.1 (± 5.8)
Cd	<det - 1.7	0.14 (± 0.41)	<det - 0.02	0.006 (± 0.006)	1.2 - 98	19 (± 32)
Cu	7.9 - 240	130 (± 69)	<det - 0.84	0.10 (± 0.17)	0 - 0.45	0.06 (± 0.09)

While total soil Zn content largely predicted the bioavailable soil Zn fraction ($r = 0.97$, $p < 0.0001$), soil pH and total S content also appear to be major soil variables driving biogeochemistry at the site. At the northern edge of the field, soil pH values were generally over 7.0, while at the southern end of the field they ranged just below 6.0 (Table 2.2).

Table 2.1 May, 2005, soil chemical data from 6 transects sampled across the field site near Elba, NY. Transects were located north to south across the site with transect "A" at the northern-most location and transect "F" at the southern-most location. Mean values, averaged across the five points within each transect, are given with standard deviation within each transect shown in parentheses.

Surface Soils (0-15 cm)

Sampling Transect	pH	Total				$CaCl_2$ Extractable			
		S	Zn	Cd	Cu	S	Zn	Cd	Cu
		mg kg^{-1} soil dry weight							
A	7.1 (± 0.2)	3700 (± 830)	110 (± 10)	0.18 (± 0.17)	170 (± 41)	10 (± 3.8)	0.14 (± 0.18)	0.002 (± 0.001)	0.04 (± 0.07)
B	7.2 (± 0.3)	3500 (± 730)	100 (± 33)	0.04 (± 0.09)	120 (± 91)	15 (± 9.1)	0.12 (± 0.09)	0.003 (± 0.002)	0.03 (± 0.03)
C	6.1 (± 0.2)	5300 (± 1200)	100 (± 27)	<0.10 (NA)	190 (± 42)	9.9 (± 3.2)	0.73 (± 0.33)	0.005 (± 0.003)	0.11 (± 0.10)
D	6.0 (± 0.2)	5400 (± 330)	110 (± 22)	0.02 (± 0.05)	190 (± 38)	16 (± 6.9)	1.4 (± 0.34)	0.008 (± 0.005)	0.14 (± 0.13)
E	5.9 (± 0.3)	6100 (± 680)	330 (± 210)	0.10 (± 0.09)	200 (± 19)	17 (± 3.7)	5.8 (± 4.1)	0.010 (± 0.005)	0.16 (± 0.10)
F	5.8 (± 0.3)	7100 (± 1100)	580 (± 250)	0.16 (± 0.21)	190 (± 22)	16 (± 3.2)	11 (± 8.0)	0.016 (± 0.009)	0.20 (± 0.11)

Subsurface Soils (15-30 cm)

Sampling Transect	pH	Total				$CaCl_2$ Extractable			
		S	Zn	Cd	Cu	S	Zn	Cd	Cu
		mg kg^{-1} soil dry weight							
A	7.8 (± 0.2)	1200 (± 670)	64 (± 29)	<0.10 (NA)	44 (± 27)	18 (± 10)	<0.10 (NA)	0.001 (± 0.001)	<0.10 (NA)
B	7.7 (± 0.4)	1600 (± 1800)	81 (± 43)	0.027 (± 0.06)	71 (± 98)	18 (± 7.2)	0.02 (± 0.03)	0.002 (± 0.001)	0.02 (± 0.03)
C	6.5 (± 0.1)	5300 (± 520)	88 (± 4.0)	0.006 (± 0.01)	170 (± 30)	16 (± 9.3)	0.78 (± 0.61)	0.008 (± 0.003)	0.12 (± 0.06)
D	6.3 (± 0.1)	5800 (± 380)	101 (± 17)	<0.10 (NA)	170 (± 34)	19 (± 16)	1.1 (± 0.45)	0.005 (± 0.002)	0.25 (± 0.34)
E	6.2 (± 0.2)	6900 (± 1400)	760 (± 820)	0.391 (± 0.71)	170 (± 26)	33 (± 13)	14 (± 16)	0.011 (± 0.006)	0.15 (± 0.15)
F	6.1 (± 0.3)	7300 (± 930)	915 (± 490)	0.388 (± 0.69)	150 (± 25)	24 (± 6.6)	18 (± 13)	0.010 (± 0.008)	0.08 (± 0.08)

Mean pH within the willow rooting zone along the hedgerow was, however, buffered at pH 6.7 (± 0.5) and did not exhibit the same level of variability as was observed across the field. Values for bioavailable $CaCl_2$-extractable soil Zn, Cd, and Cu concentrations were each inversely correlated with soil pH (Zn: r = -0.61; Cd: r = -0.55; Cu: r = -0.67; $p < 0.0001$ in all cases), as was expected due to the fact that the solubility threshold of the mobile ion for each of these metals is around pH 6; above pH 6, solubility is limited by strong complexation with organic matter and adsorption on mineral surfaces, but below pH 6.0 these elements become much more soluble and subsequently, bioavailable. This was most apparent in the spatial interpolation of bioavailable Zn (Figure 2.1), where the lowest bioavailable Zn (<0.10 mg kg^{-1}) existed at the northwest corner of the field and the highest bioavailable Zn (23 mg kg^{-1} in surface soils and 38 mg kg^{-1} in subsurface soils) was found at the southeast corner of the field. Higher pH at the northwest section of the field was associated visually with the slightly shallower depth of organic soils over the underlying calcareous marl. The organic soil layer became deeper toward the southeast corner of the field, where a subsequently lower surface soil pH was also measured.

Soil bioavailable S did not exhibit the clear spatial trend that was observed in soil pH and Zn, but ranged from 1.45 mg kg^{-1} to 21.45 mg kg^{-1} in a somewhat erratic manner across the field, and was only weakly correlated with bioavailable Zn (r = 0.31, $p = 0.09$). The lack of spatial pattern in extractable soil S is likely due to the fact that roughly 45% of the sulfur in these soils is in the chemically reduced state of sulfides and thiols (Martínez et al., 2002). Soil total S concentrations displayed a similar pattern to those of total soil Zn and Cu with significant positive correlations to both (r = 0.73, and 0.43, respectively; $p < 0.0001$ in both cases). The 0.01 *M* $CaCl_2$ extraction procedure and subsequent ICP analysis used to assess bioavailable S in this study only extracts labile forms of S, primarily sulfate (SO_4), which is apparently only

a small fraction of total S in these soils. The somewhat random spatial pattern of bioavailable S suggests that reduced forms of sulfur are oxidized to a small and highly variable degree, probably by both biological and chemical processes. This process of ZnS oxidation, however, may not be a strongly abiotic chemical process. Recent work on oxidation kinetics of synthetic Zn sulfides containing Cd by Barrett and McBride (2007) revealed that ZnS is relatively stable kinetically to abiotic chemical oxidation under laboratory conditions. These data suggest that biological metal sulfide oxidation in these soils is likely significant.

Willow uptake

In response to the north-south trend of increasing total and bioavailable soil Zn, hedgerow willow tissue Zn concentrations (Table 2.3) were lowest at the north end of the hedgerow (120 mg kg^{-1}) and highest at the south end of the hedgerow (410 mg kg^{-1}).

Table 2.3 Plant tissue concentrations of S, Zn, Cd, and Cu determined by $Mg(NO_3)_2$ digest and subsequent ICP analysis. Values below the detection limits are given are represented with <det.

Willow (*Salix purpurea* L.) Leaf Tissues				
Sampling Location	**S**	**Zn**	**Cd**	**Cu**
	----------mg g^{-1} dry weight----------			
A	2700	120	1.0	12
B	2500	150	1.4	17
C	1900	130	1.2	7.7
D	2300	230	0.86	7.4
E	1600	200	<det	4.1
F	2200	410	<det	4.1

Conversely, willow tissue concentrations of Cd and Cu both declined from north to south, with Cu concentrations declining as much as three-fold from transect A to transect F. This trend, despite increasing concentrations of soil bioavailable Cu, may suggest ionic competition at the root-soil-interface between Zn and Cu, with excessively high Zn^{2+} concentrations in soil solution competing with Cu^{+2} for root uptake and transport (Blaylock and Huang, 2000; Fritioff and Greger, 2006).

Willow tissue S content, however, showed no clear spatial trend, but rather an erratic distribution across the field with no correlation to total or bioavailable S in surface or subsurface soils. Conversely, willow tissue S content exhibited a strong positive correlation with rooting zone soil total Cd content ($r = 0.95$, $p = 0.004$), and may simply be an indicator of phytochelatins within the plant being utilized in response to Cd and Cu stress (Keltjens and van Beusichem, 1998). The variability in willow leaf S concentrations in comparison with the soil Cd concentrations point to an S-based phytochelatin or glutathione mechanism for heavy-metal detoxification within the plant (Bloem et al., 2005).

When higher plants become exposed, either internally or externally, to high concentrations of toxic heavy metals, such as Cd, cellular responses include the synthesis of polypeptides high in cysteine content, also called metallothioneins or more specifically phytochelatins (Marschner, 2003; Rauser, 1990). These phytochelatins bind the metals into reduced sulfur complexes via thiol coordination, which subsequently detoxifies the metals (Grill et al., 1987). However, due to the fact that the majority of the sulfur in these soils is complexed in non-available forms (Martínez et al., 2002) understanding the soil microbial communities and their mineralization of organic and reduced S may be key critical to understanding S bioavailability in these soils (Kertesz et al., 2007).

Microbial community composition and soil biogeochemistry

In addition to impacts on overall site variability and plant metal uptake, soil total S content and pH also exhibited significant influence over soil Bacterial community T-RFLP patterns (Figure 2.3). The strong influence of soil pH over the composition of the Bacterial community is seen on both the first axis ($r = 0.54$, $p < 0.001$) and the second axis ($r = 0.32$, $p < 0.001$) which is consistent with a wide array of previous studies.

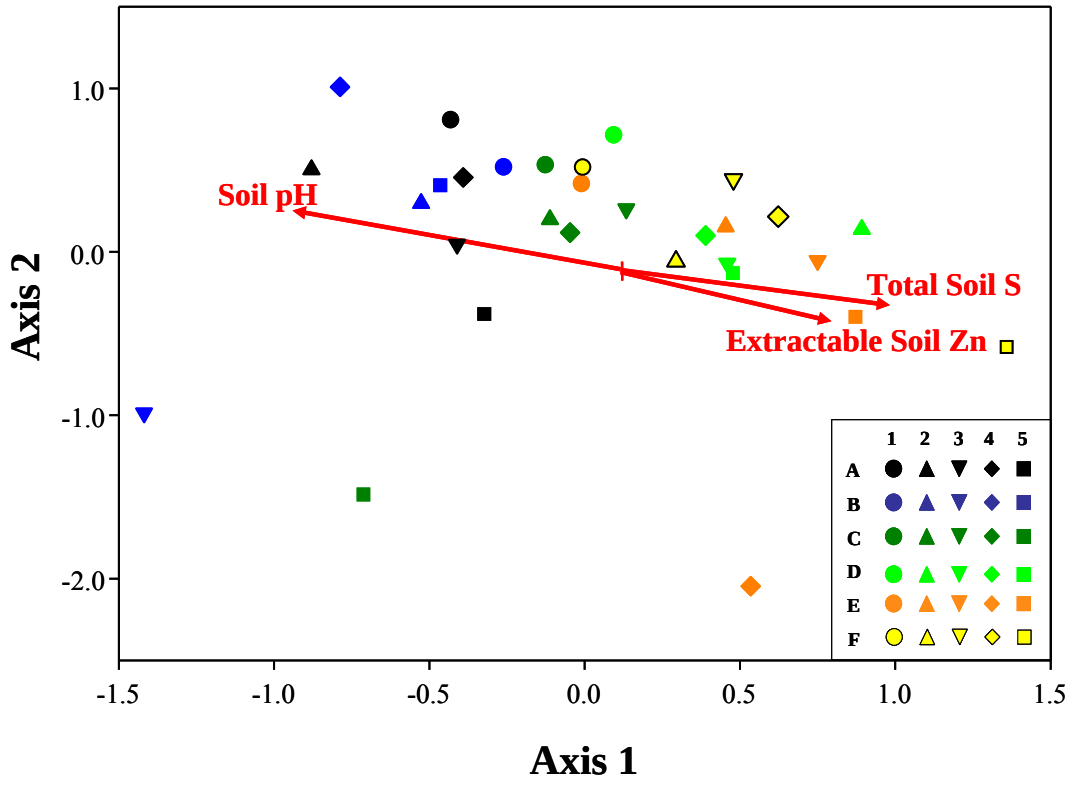

Figure 2.3 Non-metric multidimensional scaling (NMS) analysis of T-RFLP patterns for Bacteria from May, 2005, soils sampled along transects across the site in Elba, NY. Red lines display joint-plot vectors of the indicated soil chemical variables with r^2 values > 0.35. Ordination final stress = 13.33.

Soil pH alone has been shown to exert a significant influence over soil microbial communities from small scales (Baker et al., 2009; Becker et al., 2006; Nicol et al., 2008) to global scale analyses (Fierer and Jackson, 2006; Fierer et al., 2009). However, the direct effects of soil pH change combined with the indirect, but

powerful, effects that soil pH exerts over heavy metal soil chemistry and bioavailability (Eggleton and Thomas, 2004) influence the microbial community as is clearly exhibited in this study.

Soil bioavailable Zn was also significantly inversely correlated with soil pH, and consequently exhibited significant correlation with the first axis of the bacterial community ordination patterns ($r = -0.75$, $p < 0.001$) (Figure 2.3). These data are consistent with those from a recent study by Davis et al. (2004) in which the authors examined soil microbial community tolerance with regard to a pollution-induced Zn gradient. They determined that a given soil microbial community will shift with increased exposure to environmental Zn because sensitive species gradually die out and only tolerant species remain. This hypothesis was supported by our data (Figures 2.2 & 2.3) where we found clearly different soil microbial community patterns associated with differing levels of soil pH and extractable Zn.

In a study by Gremion et al. (2004) molecular and functional assays were combined to assess heavy metal contamination and phytoremediation impacts on the soil microbial community. They found that each of the three methods employed (denaturing gradient gel eletrophoresis, community substrate utilization patterns, and potential ammonia oxidation activity) reflected significant shifts of the microbial community due to the presence of heavy metals. The heavy metal contamination effect was greater than rhizosphere effect, except with regard to the substrate utilization assay. However, the role of rhizosphere microorganisms in the co-cycling of S with the chalcophilic metals Zn, Cd, and Cu in this naturally metalliferous peat soil system should not be underestimated because soil total S content ($r = 0.76$, $p < 0.0001$) was also strongly correlated with the first axis of the bacterial community ordination.

Relationships between the measured soil chemical variables with the Archaeal community composition ordination patterns were less clear than those with the bacterial community composition. The only significant correlation of soil chemical variable with Archaeal community composition existed between the second axis of the ordination pattern and soil extractable Cu concentrations ($r = 0.61$, $p < 0.001$) (Figure 2.4).

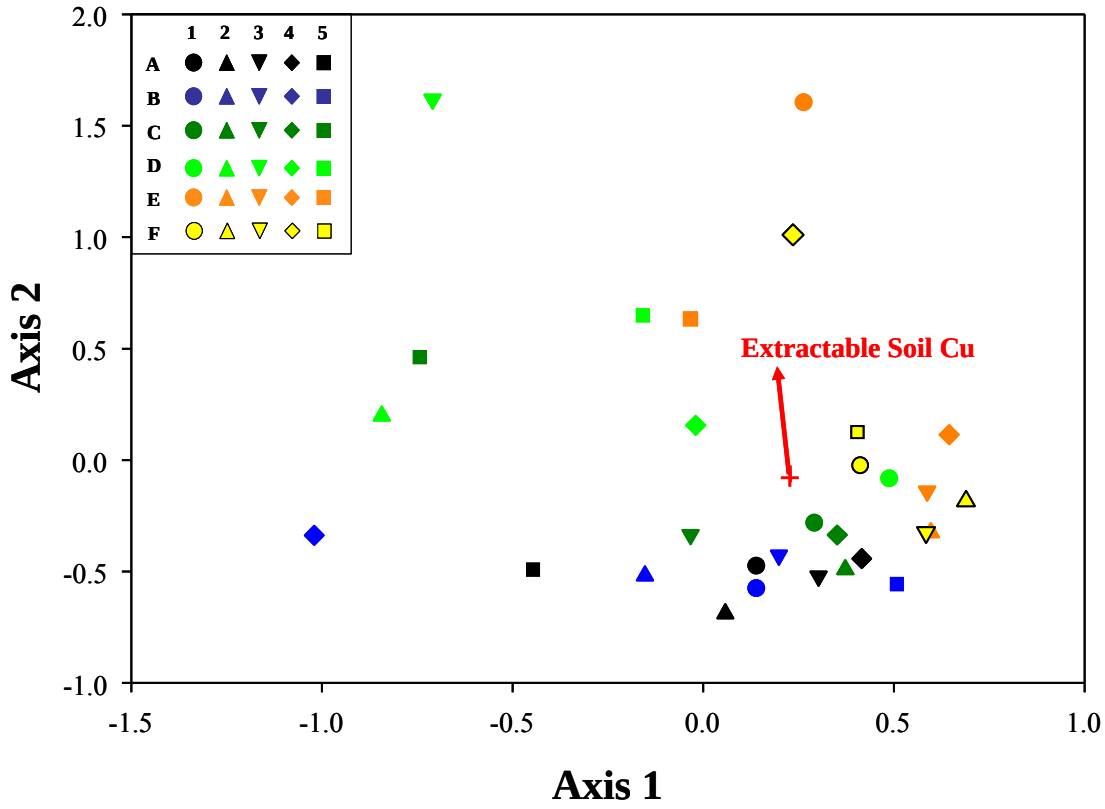

Figure 2.4 Non-metric multidimensional scaling (NMS) analysis of T-RFLP patterns for Archaea from May, 2005, soils sampled along transects across the site in Elba, NY. Red lines display joint-plot vectors of soil chemical parameters with r^2 values >0.25. Ordination final stress = 11.89.

However, soil extractable Cu was also significantly correlated with soil pH ($r = -0.67$, $p < 0.001$) such that as extractable Cu increased across the site, soil pH correspondingly decreased, which means pH may also be a factor in the observed Archaeal community composition shift. The differences in Archaeal community

fingerprints of the high pH (low Cu) soils to those of the lower pH (higher Cu) soils represents a distinct community shift at a field scale of under 100 m, and may be in contrast to the work of Sliwinski et al. (2004). In their study, they found very little within-site variability in community PCR-single-stranded-conformation-polymorphism patterns with regard to soil pH. Conversely, a recent study by Hansel et al. (2008) found significant response within the Archaeal community to soil carbon (C) availability, water content, and pH, which are consistent with the results of the present study. The discrepancy between the results of Sliwinski study and the results of Hansel and this current study may potentially be explained by the use of a different molecular fingerprinting method.

Archaeal community composition ordination patterns exhibited no significant correlations to soil Zn content, which is in contrast to the work of Macdonald et al. (2007) who observed a clear response of the soil Archaeal community to long term exposure to Zn (in sewage sludge) using a similar fingerprinting technique. It is possible that the effects of Cu override the effects of Zn on the Archaeal community when a more recent study by the same research group (Macdonald et al., 2008) is considered. They found that the soil Archaeal community response was dose-dependent, specifically with regard to Cu levels, and observed a similar response to a soil Cu gradient as the response we observed in our study (Macdonald et al., 2008).

Conclusions

This field site represents a unique ecological niche, not unlike long-term heavy metal contamination sites (Sprocati et al., 2006), where historically high concentrations of heavy metals have likely produced unusual microbial biodiversity as a result of chronic environmental stress and adaptive responses to these harsh conditions. Despite the fact that spatial variability is an inherent characteristic of soils,

it nonetheless has significant functional implications, particularly when the activities of soil microorganisms are considered (Franklin and Mills, 2009).

In this study, we investigated the relationships between the biogeochemistry of these unique metalliferous peat ecosystems with their indigenous bacterial and archaeal communities. We demonstrated a high degree of spatial variability at the field-level scale and highlighted its importance in understanding heavy metal biogeochemistry and bioavailability. Soil biochemistry at the site was largely driven by soil pH, which determined the bioavailable fraction of heavy metals in the soil solution. Soil pH as well as total S, extractable Zn and Cu, all also impacted the soil microbial community fingerprint patterns.

We conclude that the soil microbial community likely maintains a reciprocal relationship with these soil chemical variables, potentially both impacted by, as well as impacting soil pH, total S, extractable Zn and Cu. While Cu and Zn may serve as a selective force through toxicity effects, increased soil S levels could cultivate S-loving species capable of a variety of S-based biogeochemical transformations. In that regard, S-cycling and heavy metal bioavailability may be inextricably linked in these soils. Future research efforts at this site will emphasize specific functional mechanisms within the soil microbial community which may potentially mobilize heavy metals, such as metal-sulfide oxidation.

2.5 ACKNOWLEDGMENTS

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CHAPTER 3

NATURALLY METALLIFEROUS PEAT RHIZOSPHERE MICROBIAL COMMUNITIES INVOLVED IN ZN UPTAKE IN HEDGEROW WILLOWS (*SALIX PURPUREA*, L.)

3.1 ABSTRACT

On numerous occasions, rhizosphere microbial activities have been identified as the key factor in metal phytoavailability to various plant species and in the phytoremediation of metal contaminated sites. For soil bioremediation efforts in heavy metal contaminated areas, microbes adapted to tolerate high concentrations of metals are required. This study was a field survey undertaken to examine rhizosphere microbial community composition and biogeochemistry of soils associated with Zn accumulation by indigenous willows (*Salix purpurea* L.) in the naturally metalliferous peat soils located near Elba, NY. Soil and willow samples were collected from seven points, at intervals 18 m apart, along the willow hedgerow. Soil and willow tissues were collected and analyzed at four times during the growing season. Soil Bacteria community composition was characterized by terminal restriction fragment length polymorphism (T-RFLP) analysis and a 16S clone library was created from the rhizosphere soils associated with the greatest accumulation of soil and willow tissue Zn. Bacterial community composition was clearly correlated with variations in soil S and soil Zn levels, as well as willow tissue Zn. The cloned sequences revealed comparable phylogenetic associations to those found at other heavy metal-contaminated sites, and were dominated by *Acidobacteriaceae* (32%), followed by *α*- and β-*Proteobacteria* (19% and 12%, respectively) and to a lesser extent, *Actinobacteria*, *Sphingobacteriales, Gemmatimonadales, Planctomycetales,* and *Gammaproteobacteria.* Results of this study showed diverse microbial populations in

both rhizosphere and bulk soils of these naturally metalliferous peat soils near with community composition highly correlated to soil pH and bioavailable Zn content. Next steps with this community analysis include understanding specific mechanisms involved in heavy metal mobilization and detoxification.

3.2 INTRODUCTION

Heavy metal contamination of soils poses a significant hazard to human, plant, and animal health in addition to reducing or eliminating the functional capacity of a given ecosystem. Increasingly, contamination of soils with toxic trace metals is a result of anthropogenic activities typically related to mining, industrial emissions and wastes, land application of sewage sludge, fertilizer, and pesticide use (Nascimento and Xing, 2006). While zinc (Zn) is essential for normal plant growth and development and is required in several metabolic processes (Marschner, 2003) at higher concentrations, Zn will retard plant growth and development (Warne et al., 2008) and can eventually result in total crop mortality (Cannon, 1955).

The hazards to human, animal, and plant health associated with heavy metal soil contamination and the high cost to remove and replace polluted soil have lead to the development of alternative technologies to recover the degraded land (Wenzel, 2009; Wenzel et al., 1999) such as phytoremediation: a bioremediation technology involving plants to remediate polluted soils and to facilitate improvement of soil structure and health. Phytoextraction is a type of phytoremediation in which the plants are used to extract metals from soils and to transport and concentrate them in above-ground biomass and harvestable parts (Nascimento and Xing, 2006; Nowack et al., 2006). However, in many soil environments, trace metals like Zn exist in recalcitrant forms, not readily available for plant uptake and not conducive to phytoremediation strategies, such as phytoextraction (Wenzel, 2009). Inherent edaphic and biological

properties of the soil determine mobilization and transport of metals, therefore bioavailability of heavy metals in soil is a critical factor in effective phytoremediation (Göhre & Paszkowski, 2006). Extant chemical, mineralogical, and microbial properties of the rhizosphere have been identified recently as key components needed to not only understand heavy metal movement and transport, but in human health and ecological risk assessment strategies as well (Courchesne et al., 2008).

On numerous occasions, rhizosphere microbial activities have been identified as the key factor in metal phytoavailability to various plant species (Jin et al., 2006; Kuffner et al., 2008; Lucy et al., 2004; Rroço et al., 2003; Xin-Xian et al., 2009) and in phytoremediation of metal contaminated sites (He et al., 2010a; Whiting et al., 2001). Rhizosphere microorganisms are capable of enhancing metal bioavailability and phytoremediation through numerous activities, such as siderophore production (Joshi et al., 2006; Neilands, 1995; Oliveira et al., 2006), bioleaching of metal-sulfide minerals (Bosecker, 1997; Fowler and Crundwell, 1999) and increased plant growth and metal uptake through mycorrhizal symbioses (Göhre and Paszkowski, 2006). In recent reviews on phytoextraction, Nascimento and Xing (2006) and Wenzel (2009) highlighted the gaps in current scientific knowledge and plainly stated that understanding these complex rhizosphere interactions is key to future phytoremediation success. The authors felt that emphasis should be placed on characterizing bioavailable heavy metal pools and the dynamics of plant-microbe consortia capable of mobilizing metals/metalloids in the rhizosphere.

Elucidating soil microbial community structure and composition is a necessary first step to begin to understand the complexities of specific functions such as nutrient cycling, sulfide oxidation and changes in heavy metal bioavailability (Tiedje et al., 1999). Because biogeochemical processes in soil environments largely dictate the mobility, and consequently the toxicity of these heavy metals to plants, understanding

soil microbial community compositional dynamics with regard to environmental heavy metal gradients and plant metal uptake over time will yield greater insight into the underlying processes at work.

The current study was a field survey undertaken to examine Zn accumulation by indigenous willows (*Salix purpurea* L.) and associated rhizosphere microbial communities in a naturally metalliferous peat soil located near Elba, NY. Our objective was to determine the extent to which rhizosphere biochemical and microbiological relationships may be correlated with willow leaf-tissue metal content over the course of the growing season in 2006. Soil microbial populations at the Elba site have potentially been exposed to anomalously high levels of metals for hundreds to thousands of years (Cannon, 1955; Martínez et al., 2002), providing ample opportunity for natural selection of tolerant communities, as well as for specific microorganisms to adapt and develop mechanisms which allow them to become superior competitors under these unique conditions.

3.3 METHODS

Field site and sample collection

This study was conducted in the 2006 growing season on a long-term (~65 y) agricultural field near Elba, NY, located in Orleans County at 78°6'W, 43°9'N; elevation at the site is 273 m and does not vary. The specific field used in this study was chosen due to evidence from previous research of anomalously high Zn and S levels (Martínez et al., 2002; McBride et al., 2005; Qureshi et al., 2003) as well as zones of visibly evident Zn phytotoxicity observed interspersed with agriculturally productive areas. Extensive characterization of the heavy metal and pH heterogeneity across the site was described previously by Sullivan (Chapter 2).

These soils, classified as Palms Muck (Natural Resources Conservation Services, http://websoilsurvey.nrcs.usda.gov/app/WebSoilSurvey.aspx), become waterlogged each winter and growers use drainage tiles with pump stations to lower the water table in preparation for planting each spring. Soils at this site have been reported to contain roughly 80% organic material at all depths, with total soil S ranging from around 4000 to over 9,500 mg kg^{-1} with total soil Zn ranging from 87 to over 15,000 mg kg^{-1} (Martínez et al., 2002) and exhibiting a high level of spatial variability (see Chapter 2). The mean frost-free period at the site is 145 d, with a mean annual precipitation of 90.4 cm, and a mean annual temperature of 9.1°C (Batavia Station, Northeast Regional Climate Center, http://www.nrcc.cornell.edu/ccd.html).

Soil and willow samples were collected from seven points, at intervals 18 m apart along the willow (*Salix purpurea* L.) hedgerow at the western edge of the agricultural field as described in Chapter 2, and each collection point was delineated north to south by the letters A, B, C, D, E, F, and G. At each of the seven sampling points, rhizosphere soil, bulk soil, and willow tissues were collected. Rhizosphere soils were operationally defined as the soil adhering to and in close proximity (within 5 cm) to willow roots. Rhizosphere soils were obtained by first scraping aside debris at the soil surface near the base of willows within the hedgerow, then a hand-spade was used to sample soil from the top 20 cm of the willow rooting zone (0-20 cm). Bulk soils were obtained from unplanted soils precisely 1 m to the east of each rhizosphere soil sample. The sampling spade was washed with a 5% bleach solution then rinsed with a 70% ethanol solution and allowed to dry before the next soil sample was taken. Soil samples were then stored in Ziploc® bags on ice in the dark for transport to the laboratory (< 5 h) where soils were partitioned and various analyses conducted.

Willow tissues were collected by obtaining only new-growth leaves of the same size and growth stage at each sampling point along the field. Triplicate soil and willow tissue samples were collected at each point and composited, which resulted in 14 soil and seven willow tissue samples at each of four sampling times during the 2006 growing season: May 8 (T_1), May 24 (T_2), June 12 (T_3), and June 26 (T_4). This sampling design resulted in a total of 56 soil samples and 28 corresponding willow tissue samples across the season.

Soil and willow chemical analyses

Within 24 h of sample collection, gravimetric water content, soil slurry pH (H_2O), and soil metal extractions were performed. Zn ionic activity, and consequently bioavailability to plants, is highly susceptible to changes in soil solution pH and ionic strength. Therefore, chemical extraction of soils to determine the bioavailable fraction of each element, was performed on 10 g of each field-sample composite in 50 ml of 0.01 *M* $CaCl_2$ which only minimally alters the soil pH and soil solution ionic strength, as compared to other common, more aggressive, soil extraction procedures (Houba et al., 1990). Willow leaf tissues were ground to a fine dust using a ceramic mortar and pestle, and subsequently digested (in triplicate) by heated $Mg(NO_3)_2$ dry ashing (Greweling, 1976). All soil extractions and willow tissue digestions were performed in triplicate. Soil extracts and willow tissue digests were analyzed for S and Zn on an inductively coupled plasma (ICP) spectrometer (SPECTRO Analytical Instruments, Germany) by the Cornell Nutrient Analysis Laboratory (Cornell University, Ithaca, NY).

Microbial community profiling

Microbial community composition over the growing season was characterized by terminal restriction fragment length polymorphism (T-RFLP) analysis (Marsh, 1999). Extractions for soil microbial DNA were performed on 0.25 g of each sample using in the MoBio PowerSoil™ DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Extracted DNA was then quantified against a calf thymus DNA standard curve in an ethidium bromide (EtBr) solution using an EC3 Imaging System (UVP Bioimaging Systems, UVP LLC, Upland CA) and Quantity One™ (BioRad, Hercules, CA) software. DNA extracts were diluted with nuclease-free water to approximately 3-5 ng μl^{-1}.

Bacterial 16S rRNA genes were targeted for amplification by polymerase chain reaction (PCR) using the fluorescently labeled forward primer 27f (5′-[6FAM] AGA GTT TGA TCC TGG CTC AG-3′) and the unlabeled reverse primer 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Integrated DNA Technologies, Coralville, IA) (Moeseneder et al., 1999). Duplicate reactions of each sample were amplified using a MJ Research thermal cycler PTC 100 (MJ Research, Waltham, MA) and the following program: 5 min at 94°C, followed by 27 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Reaction volumes for each 50 µl reaction were as follows: 0.50 µl of 5U *Taq* polymerase (Applied Biosystems, Foster City, CA), 5.0 µl of the 10x PCR buffer supplied with the enzyme, 4.0 µl of 25 m*M* $MgCl_2$, 1.0 µl of 10 m*M* deoxy-nucleotide triphosphates (dNTPs), 0.5 µl of 10 µg μl^{-1} bovine serum albumin (BSA), 0.5 µl of each primer at 10 µ*M*, 33.0 µl nuclease free water (Promega, Madison, WI) and 5 µl of DNA template at 3-5 ng μl^{-1}.

Restriction enzyme digests of PCR amplified 16S rRNA gene amplicons from sampled soils were prepared per sample containing 1.25 µl *Sau96* I enzyme (New England Biolabs, Ipswich, MA), 2.5 µl of the 10x buffer supplied with the enzyme,

0.25 ul of BSA at 10 µg μl^{-1}, 8.50 µl nuclease-free water and 12.5 µl of amplified sample DNA. Restriction digestion was carried out in a MJ Research PTC 200 thermal cycler at 37°C for 4.5 h with a final step of 70°C for 15 min to stop the reaction. Complete digestion of the DNA was verified by inspecting digested products run on a 1.5% agarose gel stained with EtBr and visualized using an EC3 Imaging System (UVP Bioimaging Systems). Digested DNA was purified, lyophilized, and resuspended in a 10 µl mix containing 9.85 µl of formamide and 0.15 µl of LIZ 500 size standard (Applied Biosystems, Foster City, CA). Terminal fragment-size analysis was performed using an Applied Biosystems Automated 3730XL DNA Analyzer (Applied Biosystems) in conjunction with the Genemapper Software (Applied Biosystems) at Cornell University's Life Science Core Laboratories Center, Ithaca, NY.

Cloning and sequence analysis

The hedgerow location and sampling date on which willow Zn accumulation was highest was point G at T_4. The 16S rRNA gene PCR products amplified from the rhizosphere soils sampled at point G during T_4 were inserted into pCR®4-TOPO® vectors and transformed into One Shot® TOP10 chemically competent *Escherichia coli* cells using the TOPO TA Cloning kit for Sequencing (Invitrogen, Carlsbad, CA). Putative positive colonies were selected using kanamycin resistance reactions. Plasmid DNA was extracted using the QIAprep® Miniprep kit (Qiagen, Germantown, MD). Approximately 90 clones were sequenced using plasmid primers T3 and T7, ABI Big Dye 3.1 kit, and the Applied Biosystems Automated 3730XL DNA Analyzer (Applied Biosystems) at Cornell University's Life Science Core Laboratories Center, Ithaca, NY.

Sequences were combined using the CAP3 Sequence Assembly Program (Huang and Madan, 1999). Chimeric sequences were identified using the CHIMERA_CHECK program of the Ribosomal Database Project (RDP-II; (Cole et al., 2003) and sequences that appeared chimeric were excluded from further analyses. This resulted in 75 putative 16S rRNA gene sequences, for which similarity searches were performed using BLAST (Altschul et al., 1990). Sequences were edited and aligned using MEGA software package version 4 (Tamura et al., 2007). Terminal restriction fragment (T-RF) size of each clone was determined *in silico* using REBASE software version 910 (Roberts et al., 2010), specifying the use of *Sau96*I endonuclease for each theoretical digest. Soil T-RFLP peaks from all points along the hedgerow throughout the growing season were compared to the *in silico* T-RF sizes of each clone, and the most abundant cloned T-RF from each major phylogenetic group was determined by presence/absence of the T-RF.

Data analyses

For all analyses, only T-RFs of 50 to 500 bp in length were included. For soil microbial community T-RFLP analysis, peak sizes that differed by 3 bp were considered unique (peaks binned up), and pattern discrimination was determined by application of non-metric multidimensional scaling (NMS) analysis on presence/absence data of all T-RFs that appeared in at least 2 samples. NMS was applied using the Jaccard distance measure and the Medium Autopilot mode in PC-Ord (MjMSoftware Design, Gleneden Beach, OR). This mode specifies: (i) Maximum number of iterations=200, (ii) Instability criterion=0.0001, (iii) Starting number of axes=4, (iv) Number of real runs=15, and (v) Number of randomized runs=30. Unlike other commonly used ordination techniques (i.e., principal components analysis), NMS does not assume a linear relationship among ecological

variables (Culman et al., 2008). This analysis allows virtually any distance measure to be used in the construction of the similarity matrix and is currently considered the most appropriate analysis for environmental T-RFLP data sets (Rees et al., 2004). Sample means of soil chemical variables were not stationary, therefore raw data was detrended by linear regression carried out in homoscedastic sample subgroups using R2.10. Residuals from linear regressions of raw data were used in site correlation analyses and joint-plot vector analysis for NMS. Correlation analysis of soil chemical variable residuals against each other and willow tissue concentrations was performed with α=0.10 significance level (MINITAB ® 15.1.30.0).

Accession numbers

The 16S rRNA gene sequences were submitted to the GenBank database under accession numbers GU983304 to GU983378.

3.4 RESULTS

Soil chemistry and willow tissue concentrations

A natural environmental gradient from north to south along the hedgerow was apparent in all measured soil and willow variables (Figures 3.1-3.3). Soil pH showed a generally decreasing trend from north to south along the hedgerow (Figure 3.1), with the highest values observed at the north end of the hedgerow at point A (average pH >7.0) and the lowest values observed further south through the points D, E, F, and G (5.8-6.3).

After detrending the raw soil chemical data to account for spatial autocorrelations, there was no significant correlation between soil bioavailable Zn and soil pH within any given sampling time (Table 3.1). However, over the season, soil pH in bulk and rhizosphere soils increased, as much as an entire pH unit at point C,

but not less than 0.2 pH units at all points along the hedgerow. Conversely, average bioavailable soil Zn increased more than two orders of magnitude from point A (0.05 mg kg^{-1}) to point G (5.32 mg kg^{-1}) (Figure 3.1).

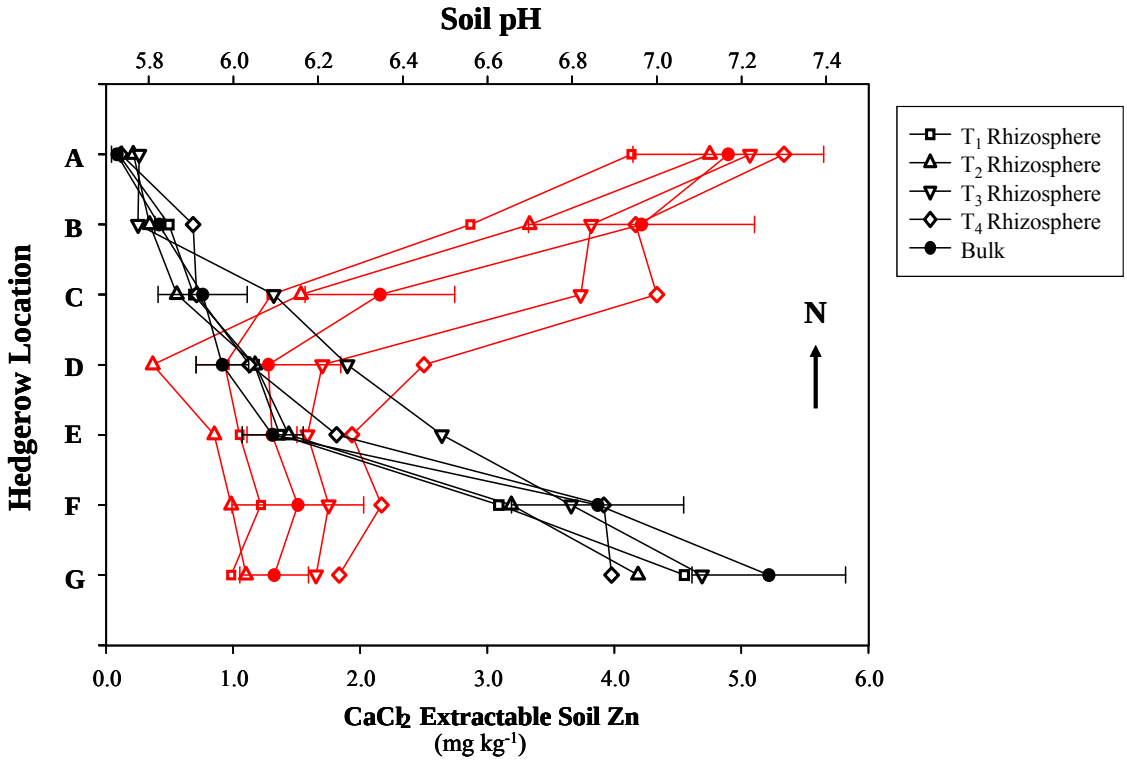

Figure 3.1 Soil pH (top axis – red symbols) and bioavailable Zn concentrations in soil (bottom axis – black symbols) at each location along the willow hedgerow through the growing season at the field site near Elba, NY.

Table 3.1 Pearson correlation coefficients (r) for detrended residuals of each indicated predictor and response variable. Only rhizosphere soil values were used in correlation analyses for willow tissue concentrations. NS indicates a value for which p>0.20 and therefore not significant.

		T_1		T_2		T_3		T_4		All Timepoints	
Predictor	**Response**	**Bulk**	**Rhiz**	**Bulk**	**Rhiz**	**Bulk**	**Rhiz**	**Bulk**	**Rhiz**	**Bulk**	**Rhiz**
Soil pH	*Soil Zn*	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.30
Soil pH	*Soil S*	-0.61	NS	NS	NS	NS	NS	NS	0.59	-0.50	0.15
Soil Zn	*Soil S*	NS	0.69	NS	NS	0.56	NS	0.65	NS	0.39	NS
Soil Zn	*Willow Zn*	-	NS	-	NS	-	0.58	-	NS	-	0.31

This resulted in a weak negative correlation between rhizosphere soil pH and bioavailable Zn over the course of the season, despite no apparent relationship between these two detrended variables along the hedgerow within each sampling time (Table 3.1). An additional north-to-south soil gradient was observed with regard to rhizosphere available Zn (Zn^{+2}) and S (SO_4^{-2}) despite a high level of variability in soil S concentrations (Figure 3.2).

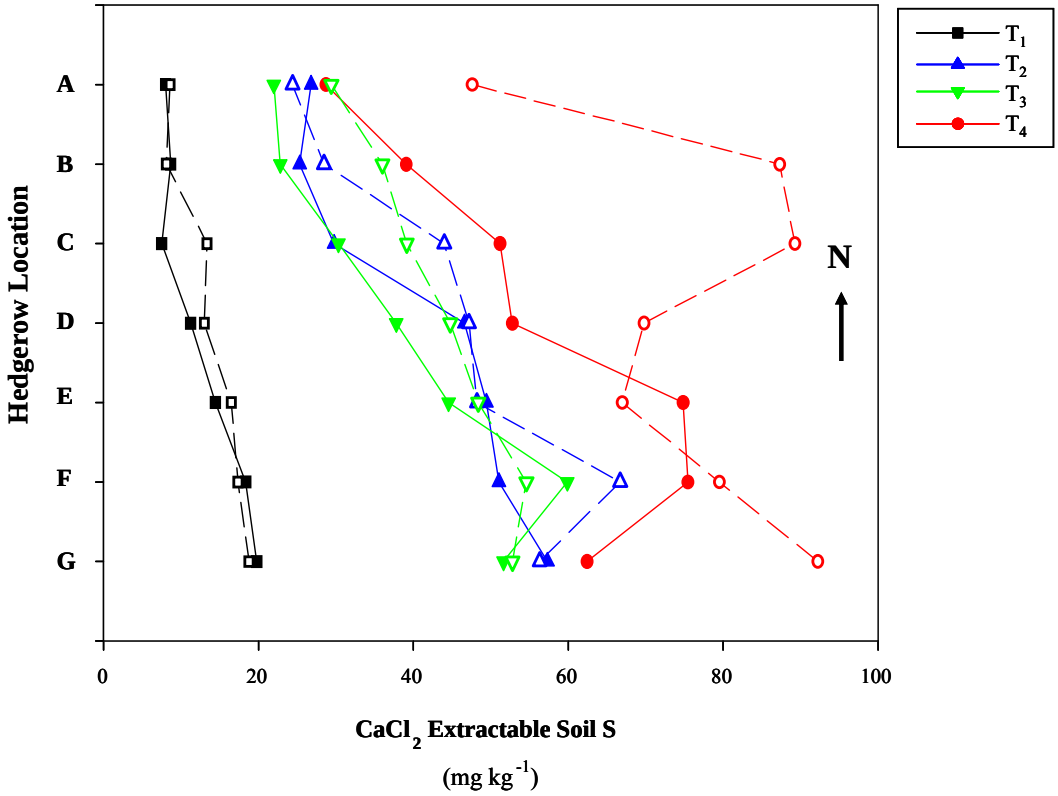

Figure 3.2 2006 rhizosphere and bulk soil bioavailable S concentrations at each location along the willow hedgerow throughout the growing season at the field site in Elba, NY. Closed symbols with solid lines represent bulk soils and open symbols with dashed lines represent rhizosphere soils.

Rhizosphere concentrations of soil S at T_4 exhibited a marked increase over all other sampling times, particularly at points B, C, and G, where concentrations increased roughly 5-fold over the course of the growing season. As soil S and soil pH increased in the rhizosphere over the season, a positive correlation was observed between the detrended residuals of these variables for the whole dataset (Table 3.1).

However, correlations of rhizosphere soil pH and soil S within each time point were not significant. The fact that soil pH was positively correlated with rhizosphere S while inversely correlated to bulk soil S over all sampling times (Table 3.1) suggests significantly different processes in the rhizosphere and bulk soils acting over the course of the growing season.

None of the measured soil chemical variables were strongly correlated to willow Zn accumulation over the season (data not shown). However, soil bioavailable Zn concentrations were weakly correlated with willow Zn levels over the season (Table 3.1). Willow Zn increased an order of magnitude from north to south along the hedgerow from T_1 at point A (96.5 mg kg^{-1}) to the highest value at T_4, point G (970 mg kg^{-1}), which far exceeded crop phytotoxicity levels and easily rivaled Zn levels found in many "hyperaccumulator" plant species (Figure 3.3).

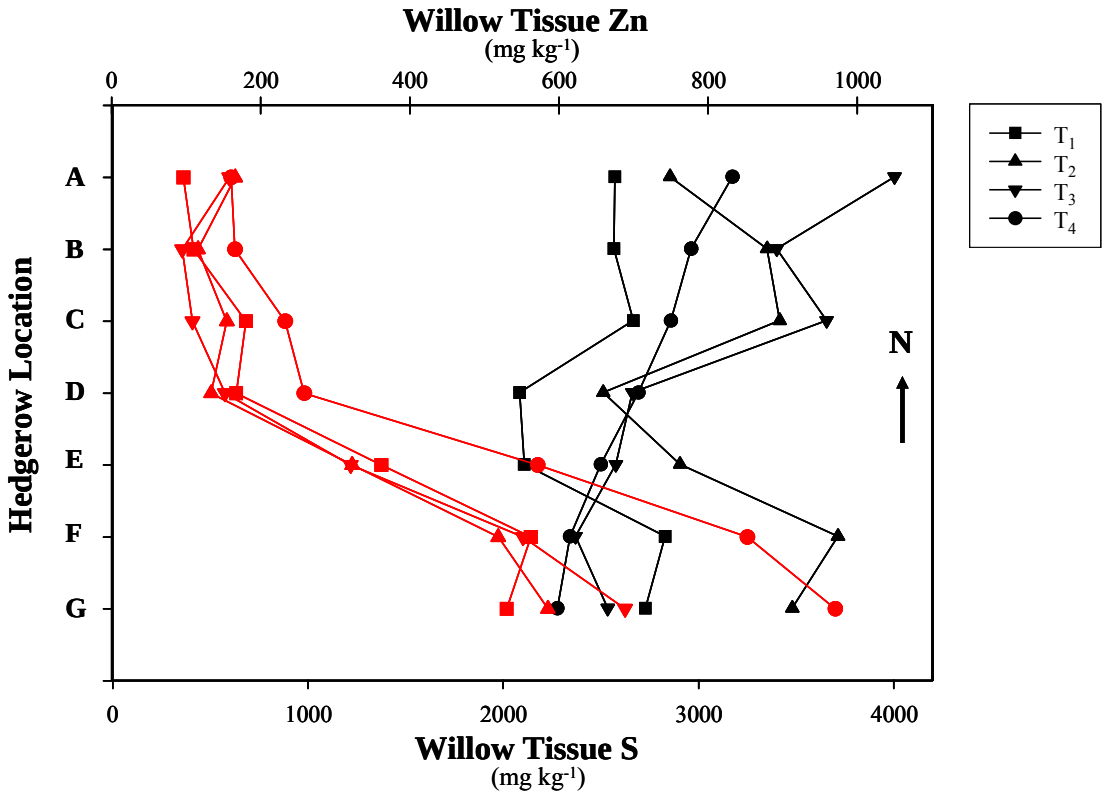

Figure 3.3 2006 willow leaf tissue Zn (top axis – red symbols) and S (bottom axis – black symbols) at each location along the willow hedgerow through the growing season at the field site in Elba, NY.

Microbial terminal restriction fragment length (T-RFLP) community profiles

NMS analysis of bulk and rhizosphere microbial community fingerprints at all hedgerow points and sampling times revealed great community fingerprint similarity, reflected in the lack of distinctive community groups separate from any other group (Figure 3.4). Despite a strong spatial gradient in soil pH and bioavailable Zn at the site, detrending soil chemical variables to account for spatial autocorrleations revealed the only soil chemical variable residual which was clearly related to bacterial community patterns was soil SO_4^{-2} (r^2 >0.45) (Figure 3.4).

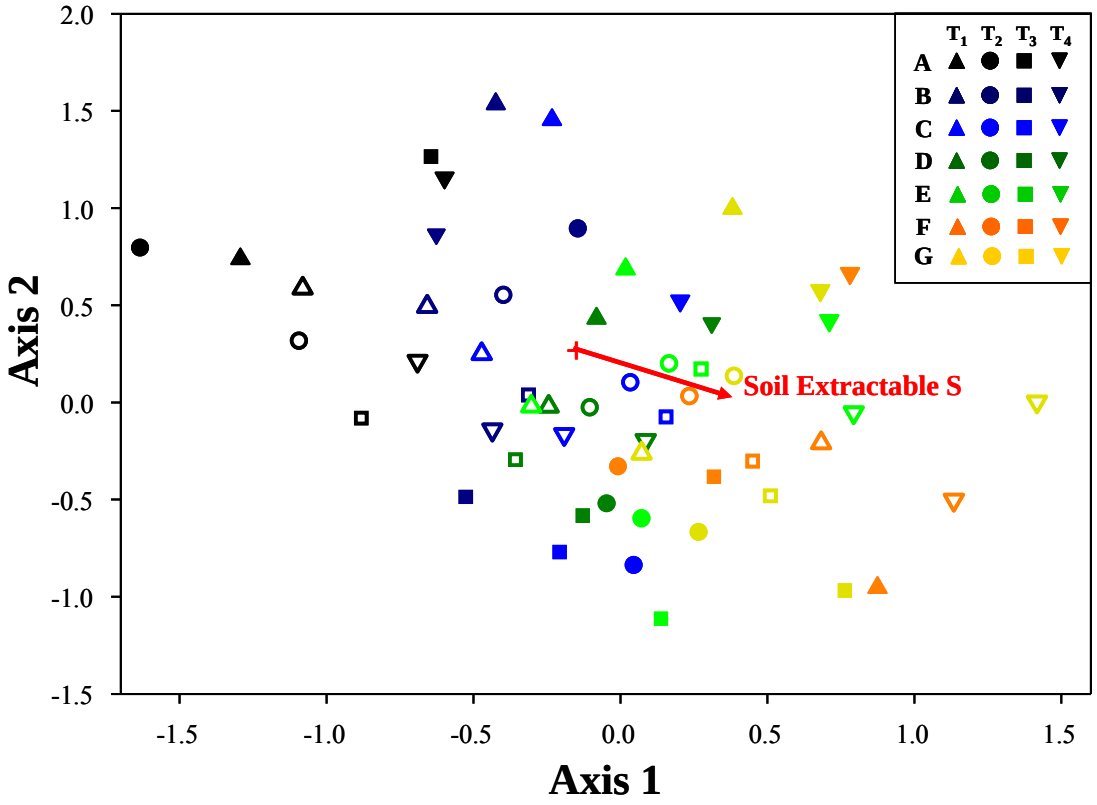

Figure 3.4 Non-metric multidimensional scaling (NMS) analysis of bulk (closed symbols) and rhizosphere (open symbols) bacterial communities at each point along the willow hedgerow through the course of the growing season at the field site in Elba, NY. Red lines display joint-plot vectors of detrended residuals of each soil chemical variable with r^2 values >0.25. Ordination final stress = 17.5.

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This resulted in communities from soils with higher S and consequently higher pH and lower Zn, generally exhibiting more negative values on Axis 1. Conversely, communities of lower S, lower pH and higher Zn, appeared with more positive values on Axis 1. Also, communities from rhizosphere soils showed less variability on Axis 2, than did bulk soil communities, resulting in a clustering effect of rhizosphere microbial community patterns with regard to Axis 2 (Figure 3.4).

Analysis of soil community composition profiles strictly from within each sampling time revealed a great deal of variability throughout the season, not only with regard to eachother, but also with regard to each of the measured soil chemical variables (Figure 3.5). At the first sampling time (T_1, Figure 3.5a), soil S was positively correlated with community patterns on Axis 2 and revealed the effect of this soil gradient at the site on the bacterial community patter. However, at the second sampling time (T_2, Figure 3.5b) a high degree of ordination stress (stress = 31.3) and lack of correlation of soil chemical variables indicated a high degree of variability and instability in the community patterns at that time. By the third sampling time (T_3, Figure 3.5c) the soil Zn gradient at the site dominated the bacterial community patterns. Despite a significant correlation of soil Zn with willow tissue Zn at T_3, willow tissue Zn was not correlated with soil microbial community patterns until the final sampling time (T_4, Figure 3.5d). Similarly to the observed patterns during T_1, at T_4, soil S again showed significant correlation with soil microbial community patterns, in addition to willow tissue Zn content. Also at T_4, the rhizosphere community of soils at point G exhibited the greatest divergence from their associated bulk soil communities.

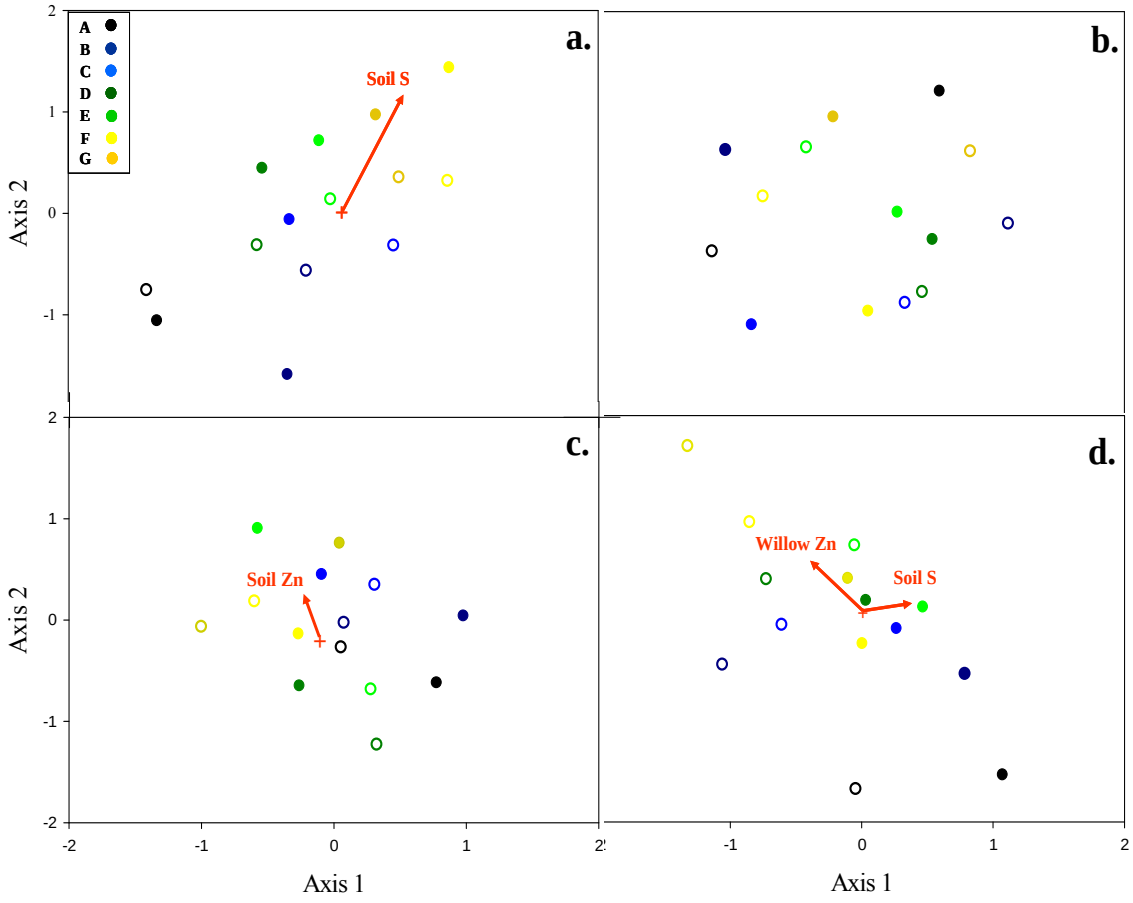

Figure 3.5 - Non-metric multidimensional scaling (NMS) analysis of bulk (closed symbols) and rhizosphere (open symbols) bacterial communities at each point along the willow hedgerow at T_1 (a. final stress = 10.65), T_2 (b. final stress = 31.3), T_3 (c. final stress = 8.2), and T_4 (d. final stress = 10.3). Red arrows display joint-plot vectors of detrended soil chemical variable residuals with r^2 values >0.25.

Clone phylogeny and T-RF distributions

The clone library from rhizosphere soil of point G, T_4, was dominated by *Acidobacteria* (32% of 75 clones sequenced), followed by *Alpha*- and *Betaproteobacteria* (19% and 12%, respectively) and by a lesser extent, *Actinobacteria*, *Sphingobacteriales, Gemmatimonadales, Planctomycetales,* and *Gammaproteobacteria* (Table 3.2).

Table 3.2 Rhizosphere soil 16S rRNA gene clone phylogenetic affiliations from soils sampled at point G during T_4 at the field site in Elba, NY. The most abundant T-RF sizes are given with corresponding representative clone IDs, accession numbers, and closest BLAST matches.

Phylogenetic Group	No. of Clones in Group	T-RF Sizes (bp)	Most Abundant TRFs	Representative Clone	Closest Match in GenBank (accession #)
Acidobacteriaceae, GP1	5	183, 185, 214, 225, 281	214	GU983336	*Acidobacteria* bacterium Ellin7137 (AY673303.1)
Acidobacteriaceae, GP2	1	158	158	GU983346	Uncultured lava cave *Acidobacteria* clone HAVOmat24 (EF032753.1)
Acidobacteriaceae, GP3	8	167, 209, 251, 254, 271, 273, 274, 276	209	GU983332	Uncultured *Acidobacteria* clone EB1129 (AY395448.1)
			271	GU983347	Uncultured soil *Acidobacteriales* clone Plot22-2F12 (EU665032.1)
Acidobacteriaceae, GP4	6	439, 440, 443, 717, 684	440	GU983330	Uncultured cotton rhizosphere clone 3y-44 (FJ444710.1)
Acidobacteriaceae, GP6	4	178, 194, 206, 311	178	GU983368	Uncultured *Acidobacteriales* DGGE band Plot21-2B7 (EU192959.1)
Actinobacteria, Rubrobacterales	5	140, 167, 175, 223, 289	175	GU983364	Uncultured soil *Rubrobacteraceae* clone Elev_16S_737 (EF019474.1)
Actinobacteria, Actinomycetales	1	183	183	GU983305	*Leifsonia sp* . S24526 (D84638.2)
Gemmatimonadales	3	106, 113, 133	133	GU983359	Uncultured *Gemmatimonadetes* clone AKYG1838 (AY921994.1)
Planctomycetales	3	135, 189, 223	189	GU983333	*Isophaera sp* . (X81958.1)
Verrucomicrobiales	2	196, 290	196	GU983373	Uncultured *Verrucomicrobia* soil clone 61LR-D8 (FJ822645.1)
Alphaproteobacteria, Rhizobiales	7	66, 122, 132, 153, 154, 143	143	GU983356	*Rhodomicrobium vannielii* strain DSM 162 (FN666247.1)
Alphaproteobacteria, Others	7	102, 135, 145, 162, 165, 167, 311	311	GU983371	*Stella vacuolata* strain DSM 5901 (NR_025583.1)
Betaproteobacteria	9	149, 151, 159, 164, 174, 189, 266, 287	164	GU983337	Uncultured *Burkholderiaceae* soil bacterium clone (EF019630)
Gammaproteobacteria	3	262, 287, 292	287	GU983323	Uncultured gamma proteobacterium clone AKYH1000 (AY922087.1)
Deltaproteobacteria	2	208, 212	212	GU983334	*Chondromyces pediculatus* , strain Cm p17 (AJ233940.1)
Sphingobacteriales	5	200, 201, 202, 213, 234	202	GU983360	*Flavisolibacter ginsengisoli* , strain Gsoil 643 (AB267477.1)
Cyanobacteria	1	243	243	GU983308	Uncultured marine cyanobacterium clone pItb-vmat-79 (AB294971.1)

Amongst all points along the willow hedgerow at all sampling times, T-RFs associated with clones in each phylogenetic group appeared more often in low pH soils than high pH soils (56% and 44%, respectively), due to the fact that the cloned sequences were obtained from a low pH soil sample. The T-RFs associated with clones of specific phylogenetic groups exhibited differential distributions within soil community T-RFLP patterns between soils with a higher pH (5.8 – 6.3) and those of lower pH (6.4 – 7.5) (Fig 3.6). Of the 17 representative clone T-RFs shown in Figure 3.6, all but five were more abundant in the low pH soils. Those that were more abundant in high pH soils were associated with the *Rubrobacterales* (T-RF:175), *Actinomycetales* (183), *Planctomycetales* (189), *Sphingobacteriales* (202), and *Acidobacteriale* (214).

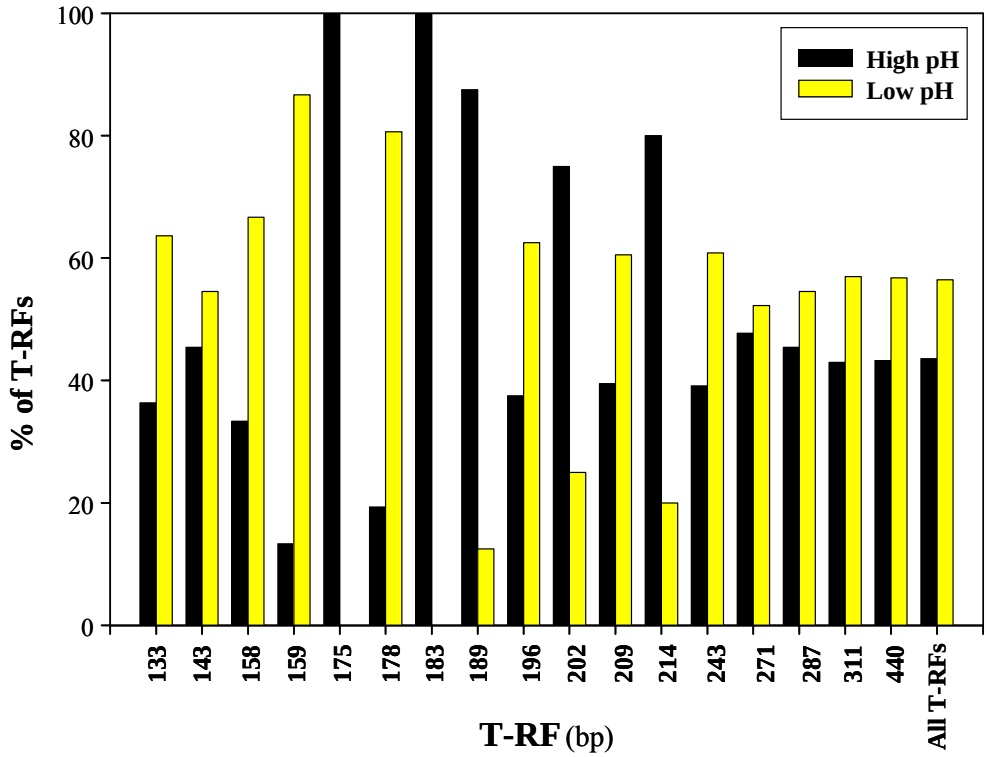

Figure 3.6 Comparison of cloned 16S rRNA gene T-RF presence/absence profiles within soils sampled from low pH (5.8-6.3) (n = 30) and high pH (6.4-7.5) (n = 26) through the growing season at the field site in Elba, NY. Percent of T-RFs was determined as the proportion of each cloned T-RF presence across total soil T-RF patterns.

3.5 DISCUSSION

The site studied is unique because of the presence of total and bioavailable soil Zn levels naturally rivaling those of many highly contaminated sites, including mine tailings and smelter zones (Cannon, 1955; Davis et al., 2004; Martínez et al., 2007; Rastogi et al., 2009; Wang et al., 2007; Zhang et al., 2007). High Zn concentrations have selected for a resistant soil microbial community associated with the indigenous willows. This allowed us to observe metal/microbe interactions in the rhizosphere and subsequent correlations with willow uptake. Additionally, the natural soil Zn gradient at the site made it possible to begin to discern the interactive effects of soil pH, soil Zn concentrations, and rhizosphere microbial community composition on willow Zn uptake.

A cursory glance at the raw data would lead one to believe that soil pH is the primary driving factor at this site with regard to bioavailable soil Zn concentrations as well as willow Zn uptake, e.g., as soil pH decreased across the site, soil bioavailable Zn and willow tissue Zn concentrations increased. This would be predicted based on Zn binding and complexation with organic matter and S-compounds at pH > 6.0; Zn availability in the soil solution increases dramatically as pH decreases (McBride, 1994). However, after detrending the raw data to account for spatial autocorrelations, we find that soil pH and soil Zn are only weakly correlated in the rhizosphere soils. Therefore, two additional aspects of the data set must be considered with regard to soil pH at the site when examining willow Zn uptake.

First, at any given sampling time, soil pH at the southern-most points along the hedgerow (E,F, and G) remained somewhat constant, while soil bioavailable Zn and willow tissue Zn increased dramatically along those same points, particularly on the final sampling date (T_4). Second, soil pH across the site increased over the season, while soil bioavailable Zn did not exhibit a corresponding decrease, and willow tissue

Zn concentrations continued to rise not only through time, but also moving south along the hedgerow. These data suggest another factor may be driving the differences in soil and willow Zn concentrations in soils at the southern-most points along the hedgerow, rather than being strictly a response to changes in soil pH.

Because Zn is chalcophilic, we must then consider the next major biogeochemical driver at the site: sulfur. Sulfur levels at the site are high and extremely heterogeneous (Chapter 2). Previous work at this site showed that a large fraction of the Zn in these soils is bound in the most reduced S-complexes (Martínez et al., 2006; Martínez et al., 2002; Martínez et al., 2007). As long as Zn is bound in reduced S complexes, it is not mobile and consequently unavailable for willow uptake. However, through the growing season as the soils drain and surface soils become more aerobic, these reduced Zn-S-complexes are oxidized and Zn as Zn^{+2} and S as SO_4^{-2} are released into the soil solution (McBride, 1994; McBride et al., 2005).

Additionally, recent work by Barrett and McBride (2007) on ZnS and CdS oxidation kinetics revealed two important facts relevant to the current study. First, that ZnS is extremely stable and virtually insoluble under axenic conditions, even at pH <5.0. And second, that when it does occur, ZnS oxidation, particularly mixed Zn/Cd sulfides, can result in an increase in solution pH by as much as 1.5 pH units. This is notable considering the substantial and self-perpetuating decrease in pH observed in pyrite (FeS_2) oxidation (Edwards et al., 2000), which is often considered the model process for metal sulfide oxidation and dissolution of sulfide minerals.

At the final sampling time (T_4), concentrations of soil S displayed marked increases at every point along the hedgerow, particularly in the rhizosphere. As soil S increased through the growing season, soil pH did as well, which resulted in the observed correlation in soil pH and soil S. Of note, rhizosphere soils at T_4 exhibited a marked increase in S, uncorrelated with soil pH.

These data could be suggestive of a rhizosphere microbial community controlling not only S biogeochemistry at the site, but that of soil and willow Zn concentrations as well, as indicated by the correlation of soil and willow tissue Zn with shifts in soil microbial community composition at T_3 and T_4. The effect of soil pH on microbial community composition has been documented in several previous studies from small spatial scales (Baker et al., 2009; Becker et al., 2006; Nicol et al., 2008) to a global scale (Fierer and Jackson, 2006; Fierer et al., 2009). However, detrended soil pH residuals did not correlate to the bacterial community composition patterns in our study and the effects of pH could be dominated by the effects of heavy metals and S at the field site in Elba, NY. Only a few studies have reported on the concomitant microbial community response to both decreased pH and increased heavy metal bioavailability.

Davis et al. (2004) examined the metabolic potential of soil microbial communities after decades of exposure to a pollution-induced Zn gradient and found a "hump-backed" response. The number of substrates utilized by the soil microbial community increased under increasing Zn concentrations up to 2,000 mg kg^{-1}. The authors concluded that a given soil microbial community will shift with increased exposure to environmental Zn due to the fact that sensitive species gradually die out and only tolerant species remain. Additionally, Wang et al. (2007) also observed significant differences in bacterial and actinomycete community composition in soils near a copper smelter in China, where levels of Zn and Cu decreased and soil pH increased with distance from the smelter.

The clones from the highest Zn soils (point G, T_4), reveal comparable phylogenetic associations to those found at heavy metal-contaminated sites reported in recent studies. Zhang et al. (2007) examined bacterial diversity at different depths in an abandoned mine tailings heap with high levels of Zn and Pb by means of 16S clone

libraries. Similarly to the current study, sample pH range was circum-neutral (7.03-7.86) and their clone library was dominated (57% of clones sequenced) by *Acidobacteria*. They also cloned sequences from a diversity of phylogenetic genera, including *Actinobacteria*, and *Gemmatimonadetes*. The authors concluded these phyla, particularly the *Acidobacteria*, had excellent adaptation capabilities for coping with heavy metal stress (Zhang et al., 2007). These observations are supported our results wherein clones were affilitated with every group of *Acidobacteria,* except GP5.

In a recent study, Rastogi et al. (2009) examined subsurface prokaryotic diversity in a former gold mine in South Dakota where a variety of toxic heavy metals were present, but total Zn concentrations and sample pH were very similar to those of the soils used in our study (Chapter 2). The majority of the clones (~80%) from the gold mine were affiliated with *Proteobacteria*, but similarly to our study, they also had large numbers of clones that were classified as *Acidobacteria,* and a few clones that represented *Planctomycetes* and *Actinobacteria*. Several cloned sequences were also affiliated with *Gemmatimonadetes* and *Verrucomicrobia* under these high-metal, circum-neutral conditions.

While overall abundance of T-RFs in the current study did not differ greatly between soils of high pH and soils of low pH, the two clone T-RFs affiliated with both the *Actinobacteria*, namely T-RF 175 (*Rubrobacterales)* and T-RF 183 (*Actinomycetales*) only appeared in soils of pH > 7.0 where available Zn dropped below 0.30 mg kg^{-1}(data not shown). The association of these T-RFs with higher pH soils would support the previously mentioned work of Wang et al. (2007) in which the Shannon diversity index for the *Actinomycete* community nearly doubled as Zn concentrations dropped and pH increased. These data suggest Actinobacterial communites are particularly susceptible to changes in soil pH and Zn content.

In summary, spatial and temporal variability are inherent characteristics of soils and have significant functional implications, particularly when the activities of soil microorganisms are considered (Franklin and Mills, 2009). Results of the present study showed diverse microbial populations in both rhizosphere and bulk soils of the naturally metalliferous peat soils near Elba, NY, with community composition primarily correlated to soil S and Zn content. The microbial communities in these metalliferous soils inhabit a unique ecological niche, not unlike long-term metal contaminated field sites where historically high concentrations of heavy metals have likely produced unusual microbial biodiversity as a result of chronic environmental pressure and adaptive responses to these harsh conditions. For soil bioremediation efforts in heavy metal contaminated areas to be successful, microbes adapted to such higher ranges of heavy metals are required (Haferburg and Kothe, 2007).

Our study has provided insight into these uniquely adapted rhizosphere microbial communities as a first step in understanding *Salix* spp. rhizosphere interactions and processes contributing to heavy metal uptake and accumulation over the growing season. Next steps with this community include understanding specific mechanisms involved in heavy metal mobilization and detoxification.

3.6 ACKNOWLEDGEMENTS

We gratefully acknowledge the Thies and Buckley laboratory members at Cornell University for their assistance with analyses and data management. The help of Dr. Peter Burholz was invaluable with regard to spatial analyses. This research was supported in part by NSF Award No. EAR-0311934, EPA STAR Award No. FP916841, and the Cornell University Department of Crop and Soil Sciences Scholarship.

CHAPTER 4

SIDEROPHORE PRODUCTION BY METALLIFEROUS PEAT MICROORGANISMS PRODUCES DIFFERENTIAL EFFECTS ON CD, CU, FE, NI, MN, MO, AND ZN SOLUBILITY IN ORGANIC SOILS.

4.1 ABSTRACT

Phytoextraction is an environmentally sustainable remediation technology aimed at removing toxic elements from the soil through plant uptake. However, little is known about the rhizosphere factors supporting the *Salix* spp. metal accumulation processes. The importance of metal resistant, siderophore-producing bacteria living in the rhizosphere has been highlighted as a means to not only provide essential nutrients to plants, but also reduce the deleterious effects of metal contamination. The objective of this study was to characterize culturable bacteria associated with Zn-accumulating *Salix purpurea* in naturally metalliferous peat soils with regard to their capacity to promote heavy metal phytoextraction via siderophore production. Of the 300 isolates tested for siderophore production, 184 (61%) produced at least one class of siderophore as determined by a modified CAS assay. The producer organisms selected for mobilization experiments were affiliated with the *Actinomycetales* (19% of isolates), *Beta-* and *Gammaproteobacteria* (5% and 19%, respectively), and *Bacillaceae* (57%), and closest BLAST matches were often with plant growth promoting rhizobacterial strains. *Actinomycetales* isolates significantly increased Cd, Cu, Ni, Mn, Mo, and Zn mobility in soil extraction assays, while most *Bacillaceae* isolates immobilized the same elements or did not differ from the control. We conclude that isolates BC4505, DE3508, FG4507, BC4401 and BC4402 could be used to improve *Salix purpurea* phytoextraction efficiency of Zn, Cd, Cu, Ni, and Mo in

organic soils. Further investigations focused on bioaugmenation should first address the plant growth promotion and metal accumulation effects of each of these organisms in the field, before contemplating inoculant production.

4.2 INTRODUCTION

Phytoextraction is an environmentally sustainable and economically feasible remediation technology used to remove trace and toxic elements from the soil through uptake by and accumulation in plants (Kidd et al., 2009). The plant biomass can be harvested, thereby removing the metals from the site. The success of phytoextraction is determined by the concentration of heavy metals in plant biomass, therefore it also depends on: 1) biomass production of the relevant plant species; and 2) availability and accessibility of contaminants in the rhizosphere allowing plant uptake and storage (Vangronsveld et al., 2009).

Metal hyperaccumulation capability has been identified in over 450 higher plant species (Maestri et al., 2010) and is often exploited in phytoextraction-based remediation strategies. However, most hyperaccumulator plant species occurring in the northern temperate zone are small herbaceous plants in the *Brassicaceae* (Baker and Brooks, 1989); thus, metal extraction efficiency is limited because of their typically low biomass production (Angle et al., 2001; Blaylock and Huang, 2000). Recently, a number willows (*Salix* spp.) have been recognized as ideal extractor plants because they take up relatively high concentrations of metals and accumulate them in harvestable leaf tissues and while simultaneously producing massive quantities of leaf biomass (Pulford and Watson, 2003; Unterbrunner et al., 2007).

Many studies have been conducted to determine both direct and indirect effets of rhizosphere microbial populations on phytoextraction success using a variety of hyperaccumulator plants (Khan, 2005), but currently, little is known about the

environmental factors that affect the *Salix* spp. Zn accumulation processes. In a recent study, Kuffner et al. (2010) found that specific members of the rhizosphere microbial populations associated with *Salix caprea* were capable of either increasing metal mobility and, consequently, *Salix* uptake, or decreasing metal mobility and *Salix* metal uptake, depending on the organisms' plant-growth promoting characteristics. One member of the genus *Microbacterium* significantly increased willow Zn and Cd uptake, and was identified as a potential candidate for improving phytoextraction efficienty of *Salix caprea*.

Phytoextraction may be enhanced by any number of plant growth promoting rhizosphere microbial processes (Wenzel, 2009), and studies of herbaceous heavy metal accumulator plants indicate rhizosphere bacteria contribute to heavy metal uptake and tolerance (de Souza et al., 1999; He et al., 2010a; He et al., 2010b; Whiting et al., 2001). The underlying mechanisms are not yet fully understood, but in a recent review, Rajkumar et al. (2010), asserts that siderophore production could be involved in detoxification, tolerance, and mobilization of metals simultaneously (Rajkumar et al., 2010).

Microbial siderophores are low molecular weight organic ligands with high affinity for ferric ions in the environment (Chincholkar et al., 2007; Neilands, 1995). However, many siderophores released by microorganisms have been found to bind a variety of metal ions in addition to Fe (Bellenger et al., 2007; Braud et al., 2009a; Wichard et al., 2008; Zawadzka et al., 2007).

The importance of metal-resistant, siderophore-producing bacteria in the rhizosphere has been highlighted as a means to not only provide essential nutrients to plants, but also reduce the deleterious effects of metal contamination on cell function (Dimkpa et al., 2009; Dimkpa et al., 2008; He et al., 2010b). Increases in trace metal bioavailability and uptake by plants caused by microbial siderophores may enhance

the effectiveness of phytoremediation strategies in contaminated soils (Rajkumar et al., 2010; Rajkumar et al., 2009).

The objective of this study was to characterize culturable bacteria associated with Zn-accumulating *Salix purpurea* in naturally metalliferous soils with regard to their capacity to promote heavy metal phytoextraction via siderophore production. We compared organisms that produced different classes of siderophores and originated from soils with differing levels of heavy metals to gain an insight into microbial rhizosphere metal mobilization mechanisms.

4.3 METHODS

Field site and sample collection

Salix purpurea L. shrubs growing along the hedgerow of a naturally metalliferous peat agricultural field, described previously (Chapter 2), were sampled in October, 2009. The site is a long-term (~65 y) agricultural field near Elba, NY, located in Orleans County at 78°6'W, 43°9'N; elevation at the site is 273 m and does not vary. The specific field used in this study was chosen due to evidence from previous research of anomalously high soil Zn concentrations (Martínez et al., 2002; McBride et al., 2005; Qureshi et al., 2003) as well as zones of visibly evident Zn phytotoxicity observed interspersed with agriculturally productive areas and adjacent to apparently unaffected willow hedgerows.

Soils at the site are classified as Palms Muck (Natural Resources Conservation Services, http://websoilsurvey.nrcs.usda.gov/app/WebSoilSurvey.aspx) and contain roughly 80% organic material at all depths, with total soil S content ranging from around 4,000 to over 9,500 mg kg^{-1} and total soil Zn content ranging from roughly 80 to over 15,000 mg kg^{-1} (Martínez et al., 2002) and exhibiting a high level of spatial variability. The mean frost-free period at the site is 145 d, with a mean annual

precipitation of 90.4 cm, and a mean annual temperature of 9.1°C (Batavia Station, Northeast Regional Climate Center, http://www.nrcc.cornell.edu/ccd.html).

When this area was converted from a natural wetland to agricultural production (circa 1950) growers installed hedgerows consisting primarily of naturalized willow species, *S. purpurea,* along the length of each field, from North to South, to help retain the highly friable organic soil and keep it from wind erosion. These willows have been shown to accumulate heavy metals in their leaf tissues far exceeding the levels causing toxicity in most plants (Chapters 2 and 3). We sampled the rhizosphere microbial communities of these willows in order to gain insight into possible microbial metal tolerance or detoxification mechanisms in the rhizosphere.

Soil and willow tissue samples were collected at three points, 25 m apart, along the willow hedgerow; sites were delineated from north to south: BC, DE, and FG (Table 4.1). Rhizosphere soils were operationally defined as the soil adhering and in close proximity (within 5 cm) to willow roots, and were obtained by first scraping aside debris at the soil surface near the base of willows within the hedgerow. Then a hand-spade was used to extract soil from the top 20 cm of the willow rooting zone. The sampling spade was washed with a 5% bleach solution between sampling points, rinsed with a 70% ethanol solution and allowed to dry before the next soil sample was taken. Willow tissues were collected from the willow trees directly associated with each rhizosphere soil sample by obtaining only new-growth leaves of the same size and growth stage at each sampling point along the field.

One additional soil sample was taken at a random point (denoted as ‘X’ in Table 4.1) from the bulk (unvegetated) soil approximately 2 m to the east of the willow hedgerow and roughly adjacent to rhizosphere sampling point DE.

Table 4.1 Rhizosphere soil chemical variables and associated willow leaf tissue elemental concentrations for locations where soils were sampled to obtain and isolate siderophore-producing rhizosphere microorganisms. Replicate means are given with standard deviations in parentheses (n = 3).

Rhizosphere Soils

Sampling Point	pH	Cd	Co	Cu	Fe	Ni	Mn	Mo	Mg	Zn
		mg kg^{-1}								
BC	6.1 (0.02)	1.08 (0.02)	3.13 (0.24)	285 (10.2)	10,600 (530)	8.11 (0.4)	910 (65)	1.47 (0.05)	2,300 (140)	170 (7.9)
DE	5.4 (0.01)	0.90 (0.04)	2.49 (0.25)	343 (24.2)	9,400 (750)	7.63 (0.6)	1,100 (86)	1.80 (0.26)	1,700 (140)	210 (14)
FG	6.1 (0.05)	1.16 (0.14)	2.21 (0.13)	283 (29.7)	8,300 (760)	6.84 (0.3)	880 (90)	1.78 (0.45)	1,900 (145)	720 (72)
X	6.3 (0.01)	0.84 (0.01)	1.77 (0.07)	250 (0.5)	6,700 (120)	5.67 (0.15)	760 (17)	<det	1,300 (5.7)	330 (1.7)

Willow Leaf Tissues

Sampling Point	Cd	Co	Cu	Fe	Ni	Mn	Mo	Mg	Zn
	mg kg^{-1}								
BC	1.46 (0.17)	<det	7.74 (1.01)	44.5 (12.0)	1.29 (0.54)	10.5 (1.71)	<det	960 (200)	150 (17)
DE	1.73 (0.07)	<det	7.16 (0.65)	52.6 (12.1)	1.09 (0.26)	14.8 (1.70)	<det	860 (130)	300 (27)
FG	1.01 (0.08)	<det	5.79 (0.59)	24.2 (4.54)	0.94 (0.17)	15.7 (2.05)	<det	1700 (350)	1200 (90)

Two kg of soil were collected for later use in metal mobilization experiments. Soil samples were stored in Ziploc® bags, willow samples were stored in brown-paper sacks, and all samples were immediately placed on ice and transported within four hours to the laboratory where various analyses took place. Within 24 h of sample collection, each soil sample was aseptically homogenized, sieved to 2 mm, partitioned, and stored at 4°C for further chemical and biological analyses.

Chemical analyses

Gravimetric water content was determined on duplicate 10 g subsamples, oven-dried for seven days at 65°C. Soil slurry pH was determined on triplicate 5 g subsamples, each diluted in 15 mL diH_2O and pH was measured using a standard pH meter (VWR, model 8100; West Chester, PA). Willow leaf tissues and soils from each sampling location were dried and ground to a fine dust using a ceramic mortar and pestle, and 0.5 g subsamples were subsequently digested by heated nitric acid (EPA3051). All soil and willow tissue digests were performed in triplicate. Soil extracts and willow tissue digests were analyzed for concentrations of Cd, Co, Cu, Fe, Ni, Mn, Mo, Mg, and Zn on an inductively coupled plasma (ICP) spectrometer (SPECTRO Analytical Instruments, Germany) by the Cornell Nutrient Analysis Laboratory (Cornell University, Ithaca, NY).

Isolation of rhizosphere bacteria

Within 24 h of sample collection, soil serial dilutions were conducted in triplicate using 10 g of soil diluted into 90 ml sterile 0.5 X BupH phosphate buffered saline (Thermo Scientific, Rockford, IL). Precisely 0.10 ml of each 10^{-6} dilution was plated in triplicate onto Fe-deprived Difco™ trypic soy agar (BD, Becton, Dickinson and Company, Sparks, MD)(1994). Plates were incubated for six days at 32°C in the

dark, at which point colonies which appeared morphologically distinct were streaked for isolation onto a fresh deferrated trypic soy agar plate for analysis of siderophore production.

Siderophore production

For siderophore production, isolates obtained from rhizosphere serial dilutions were cultivated by spot inoculation, under iron deprivation, and incubated at 32°C for 24-48 h. To achieve iron deprivation culture media was treated with $MgCO_3$ and all glass materials were soaked in HCl to remove traces of Fe. according to Cox (1994). After initial incubation, a modified chrome azurol-S medium (Schwyn and Nielands, 1987) was overlaid according to the methods described by Pérez-Miranda et al. (2007). Briefly, an overlay was applied to each petri plated on which an isolate was cultured and color changes indicated siderophore production by the cultured organism. A liter of the overlay was prepared as follows: Chrome azurol S (CAS), 60.5 mg; hexadecyltrimethyl ammonium bromide (HDTMA), 72.9 mg; Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 30.24 g; and 1 m$MFeCl_3 \cdot 6H_2O$ in 10 m*M* HCl 10 mL. Agarose (0.9%, w/v) was used as the gelling agent.

Siderophore production was detected after 5 mL (60 mm diameter Petri dish) of the overlay was applied to each agar plate containing cultivated microorganisms to be tested for siderophore production. After a maximum period of 168 h, a change in the color of the overlaid medium was observed exclusively surrounding siderophore-producing microorganisms. A color change from blue to purple, as described in the traditional CAS assay, indicates production of catechol siderophores, while a change from blue to orange indicates hydroxamate production and from blue to completely clear indicates production of carboxylate-siderophores (Pérez-Miranda et al., 2007). Each isolate was tested for siderophore production in triplicate, and a total of 300

isolates were tested as described. Control plates of each type of uninoculated medium, with the CAS overlay, were incubated under the same conditions described above with no color change observed after up to 30 days of incubation. The 27 isolates producing the highest quantities of siderophore (as estimated by the diameter of the color change zone around each colony, which is considered a rough proxy for quantity and solubility of siderophores produced) were selected for soil extraction assays.

Soil extractions with microbial metabolites

Each of the selected siderophore-producing isolates was inoculated into 150 mL of 0.5 X deferrated Bacto™ trypic soy broth (dTSB) (BD, Becton, Dickson and Company) and incubated on a rotary shaker at 32°C in the dark for 120 h, at which point cells were centrifuged into a pellet and the supernatant was filtered to 0.2 μm with Nalgene® Disposable bottle-top cellulose filter units (Nalgene, Rochester, NY). Soil used in extractions with microbial metabolites was collected from point X (bulk soil; see Table 4.1) and sterilized by autoclaving at 121°C, 15 psi, for 2 h on each of three subsequent days, then oven dried at 65°C for 7 days. Filtered supernatant from each siderophore-producing isolate was then used to extract duplicate soil samples by adding 50 mL of supernatant to 5 g of soil and shaking at high speed for 1 h. Triplicate control extractions were performed using 50 mL fresh (uninoculated) 0.5 X dTSB. Each soil slurry was subsequently twice-filtered through Whatman #42 cellulose filters and filtrates were analyzed on an inductively coupled plasma (ICP) spectrometer (SPECTRO Analytical Instruments) for Cd, Co, Cu, Fe, Ni, Mn, Mg, Mo, and Zn by the Cornell Nutrient Analysis Laboratory (Ithaca, NY).

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from each isolate by use of the Puregene ® Yeast/Bac kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Extracted DNA was then quantified against a calf thymus DNA standard curve in an ethidium bromide (EtBr) solution using an EC3 Imaging System (UVP Bioimaging Systems, UVP LLC, Upland CA) and Quantity One™ software (Applied Biosystems). DNA extracts were diluted with nuclease-free water to approximately 3-5 ng μl^{-1}.

Bacterial DNA was amplified by polymerase chain reaction (PCR) using the forward primer 27f (5′- AGA GTT TGA TCC TGG CTC AG-3′) and the reverse primer 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Integrated DNA Technologies, Coralville, IA) (Moeseneder et al., 1999). Duplicate reactions of each sample were amplified using a MJ Research thermal cycler PTC 100 (MJ Research, Waltham, MA) and the following program: 5 min at 94°C, followed by 27 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Reaction volumes for each 50 µl reaction were as follows: 0.50 µl of 5U *Taq* polymerase (Applied Biosystems, Foster City, CA), 5.0 µl of the 10x PCR buffer supplied with the enzyme, 4.0 µl of 25 m*M* $MgCl_2$, 1.0 µl of 10 µ*M* deoxy-nucleotide triphosphates (dNTPs), 0.5 µl of 10 µg μl^{-1} bovine serum albumin (BSA), 0.5 µl of each primer at 10 µ*M*, 33.0 µl nuclease free water (Promega, Madison, WI) and 5 µl of DNA template at 3-5 ng μl^{-1}.

PCR products for the 25 isolates selected were then prepared for sequencing with ExoSAP-IT ® (USB, Affymetrix, Cleveland, OH) and submitted for sequence analysis using the 27f and 1492r primers and the ABI Big Dye 3.1 kit in conjunction with the Applied Biosystems Automated 3730XL DNA Analyzer (Applied Biosystems) at Cornell University's Life Sciences Core Laboratories Center, Ithaca, NY. Forward and reverse sequences were combined using the CAP3 Sequence

Assembly Program (Huang and Madan, 1999) and total sequence length varied from 1295 to 1400 bp depending on sequence quality. Similarity searches were performed using BLAST (Altschul et al., 1990).

Accession numbers

The 16S rRNA gene sequences obtained from siderophore-producing isolates were submitted to the GenBank database under accession numbers HM161879 through HM161904.

4.4 RESULTS AND DISCUSSION

Of the 300 isolates tested for siderophore production (100 from each of the three sampling locations indicated in Table 4.1), 61% produced at least one class of siderophore and there was no significant difference in this proportion between sampling sites (data not shown). One-hundred percent of the siderophore-producing organisms used in soil extractions were able to either significantly mobilize or immobilize at least one of the measured metals when soil was shaken with filtrates of their culture medium (Figure 4.1).

The pH of sterile 0.5 X dTSB was 7.0 and, similarly to the observations of Kuffner et al. (2010), culture medium pH rose during the growth of all tested strains to values between 7.5 and 8.5 and was not correlated to metal mobility in the soil extracts (data not shown). The 21 siderophore-producing organisms chosen for mobilization experiments were affiliated with the *Bacillales* (57% of isolates), *Actinomycetales* (19%), *Xanthomonadales* (14%), *Pseudomonadales* (5%), and *Burkholderiales* (5%) (Table 4.2).

Table 4.2 Rhizosphere isolates' phylogenetic affiliation and class of siderophore produced.

Order	Isolate*	Siderophore	Accession Number	Closest Identified Relative (accession #)	Identitiy (%)
Actinomycetales					
	BC4505	carboxylate	HM161885	*Micrococcus luteus* strain U114 (FJ440960)	95
	DE3508	catechol	HM161887	*Streptomyces ryensis* (AB184517)	99
	FG4507	catechol	HM161888	*Streptomyces canus* strain cfcc3164 (FJ792576)	100
	BC4508	catechol	HM161880	*Rhodococcus wratislaviensis* (FM999002)	99
Proteobacteria					
	FG4405	carboxylate	HM161893	*Pseudomonas resinovorans* (AB021373)	97
	FG4011	hydroxamate	HM161883	*Stenotrophomonas maltophilia* PVAS8 (GU130531)	99
	BC5409	carboxylate	HM161881	*Variovorax paradoxus* strain B57 (EU169160)	98
	FG4412	hydroxamate	HM161882	*Stenotrophomonas rhizophila* Gd2T (GU391467)	99
	FG4409	catechol	HM161884	*Stenotrophomonas* sp. KMM 1417 (AB306289)	99
Bacillales					
	BC4401	carboxylate	HM161889	*Bacillus pumilus* strain 1352 (GU726861)	100
	BC5410	catechol	HM161894	*Bacillus altitudinis* strain IMAU80219 (GU125636)	100
	BC4402	carboxylate	HM161890	*Bacillus pumilus* strain Qtx-10 (GU201862)	99
	FG4501	carboxylate	HM161891	*Bacillus subtilis* strain 2J-2 (FJ493046)	100
	BC5401	catechol	HM161899	*Bacillus* sp. B2-9 (FJ528076)	99
	BC5407	catechol	HM161896	*Bacillus subtilis* strain N-6 (GQ452909)	99
	BC5404	catechol	HM161900	*Bacillus pumilus* strain GBSC66 (GU568205)	99
	FG4410	catechol	HM161892	*Bacillus* sp. 3LF 48P (FN666893)	99
	FG3501	catechol	HM161897	Bacilli bacterium JW22.2a (FN556567)	99
	FG3503	carboxylate	HM161902	*Lysinibacillus fusiformis* (AB245423)	99
	DE3406	catechol	HM161901	*Bacillus safensis* strain Zw-22-2 (GU201861)	99
	FG4413	catechol	HM161898	*Bacillus pumilus* strain HS6 (GU323367)	99

* The first two letters in the strain nomenclature corresponds to the location from which the organism was sampled.

The diversity of siderophore producing bacteria has been widely documented, particularly in members of the *Actinomycetales* and *Pseudomonadales*. In the current study, members of the *Actinomycetale,s* including isolates BC4508, BC4505, DE3508, and FG4507, each had differential impacts on metal mobilization from the metalliferous peat soils (Figure 4.1). Several members of the genus *Rhodococcus* have been identified as rhizosphere siderophore-producing bacteria (Belimov et al., 2005; Dhungana et al., 2007), but the *Rhodococcus wratislaviensis* sequence to which BC4508 was most closely related was described in a study in which the organism was used to degrade s-triazine (Grenni et al., 2009).

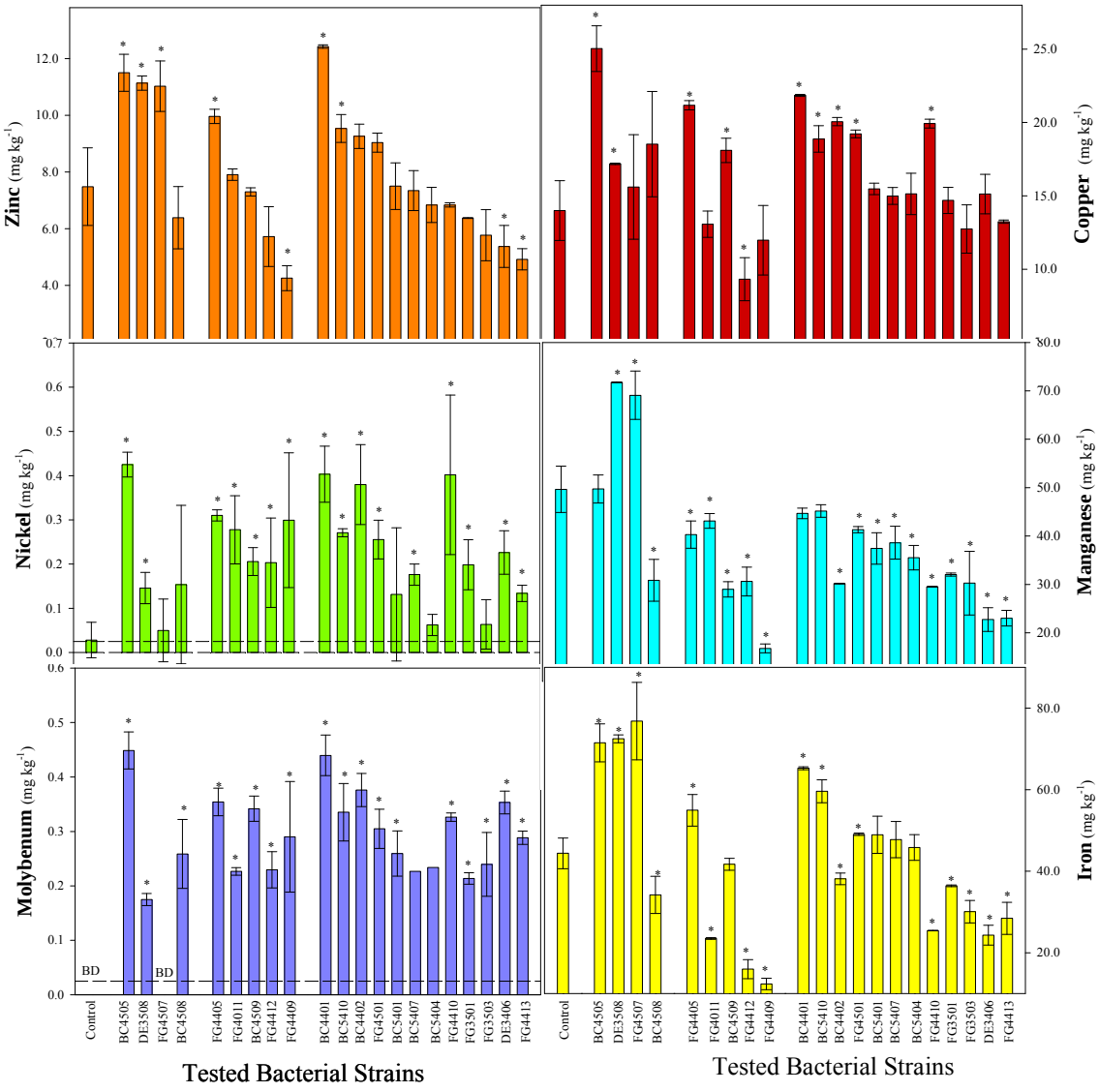

Figure 4.1 Metal mobilization by the microbial metabolites of each tested siderophore-producing bacterial strain. Dashed lines represent detection limits where relevant. Where the concentration of metal extracted was below the detection limit for that metal 'BD' is displayed. (*) indicates a significant difference from the control, as measured by standard error.

Other studies on *R. wratislaviensis* deomonstrate its ability to biodegrade atrazine (Behki et al., 1993) and polyaromatic hydrocarbons (Pizzul et al., 2006), which clearly demonstrates the applicability and versatility of this organism for environmental remediation applications. Unfortunately, the metabolites of BC4508

extracted only as much Cu, Ni, and Zn as did the control, with slightly decreased solubility of Fe and Mn and increased solubility of Mo (Figure 4.1).

Isolate BC4508 did not stand out amongst the organisms tested in metal mobilization, but the willow hedgerow from which these rhizosphere organisms were obtained was directly adjacent to areas of intensely managed agricultural fields where pesticides are applied regularly. While BC4508 may not be the most aggressive metal mobilizing or immobilizing rhizosphere organism, it could be providing a crucial pesticide degradation service in the willow rhizosphere.

Isolate BC4505 had <97% gene sequence identity to any described bacteria and could potentially represent a novel bacterium (Table 4.2). However, the closest identified relative to BC4505 was a strain of *Micrococcus luteus* that was isolated from a marine system in the search for new antimicrobial drugs (Penesyan et al., 2009). The first report of *M. luteus* producing siderophores was made recently by Cabaj and Kosakowska (2009) in which they isolated the organism from brackish waters of the southern Baltic Sea. *Micrococcus luteus* has also been isolated from rhizosphere soils, is known to associate with ectomycorrhizal fungi in the rhizosphere, and was recently used in experiments to examine the effects of co-inoculation of *M. luteus* with *Hebeloma crustuliniforme* on Zn and Cd uptake by *Salix viminalis x caprea* (Zimmer et al., 2009).

Co-inoculation of *M. luteus* with the mycorrhizal fungus increased willow Cd and Zn accumulation in shoots up to 53% over use of the fungus alone. The authors concluded that the bacterium enhanced ectomycorrhizal colonization and subsequent growth of the willows. They suggested that bacterial support of ectomycorrhizal fungi by species such as *M. luteus* may be a valuable way to improve the remediation success in metal contaminated soils using willows (Zimmer et al., 2009). While there is no direct evidence for mycorrhizal association in the current study, metabolites of

BC4505 significantly increased Cu, Fe, Ni, Mo, and Zn mobilization (Figure 4.1). These data suggest this organism should be a candidate for field trials to bioaugment metal phytoextraction with willows.

Members of the genus *Streptomyces* have long been touted for their capacity to produce a wide array of antibiotics and antimicrobials as well as a number of siderophores (Rajkumar et al., 2010) and have been reported to promote plant growth and enhance Cd uptake (Dimkpa et al., 2009). Not surprisingly, isolates DE3508 and FG4507, that were most closely related to strains of *Streptomyces ryensis* and *S. canus*, respectively, were the only two organisms tested whose metabolites were able to mobilize Cd above the control (data not shown). The metabolites of DE3508 also significantly increased mobility of Cu, Fe, Ni, Mo, and Zn (Figure 4.1). While the metabolites of isolate FG4507 significantly increased Zn mobility, they did not mobilize Ni and actually immobilized Mo resulting in concentrations below the detection limit. Despite the closely related phylogeny of these two organisms producing the same class of siderophore, metal mobilization was different between the two strains and highlights the potentially vast differences between siderophore production that exists between closely related organisms. Further chemical and molecular characterization of the siderophores produced by these two isolates may elucidate their specific binding properties, which would explain their metal mobilization differences.

16S rRNA gene sequence similarity of isolates BC5409, FG4405, FG4409, FG4011, and FG4412 indicated they are all members of the phylum *Proteobacteria* and each had a unique effect on the mobilization of heavy metals (Table 4.2, Figure 4.1). Isolate BC5409 and FG4405 performed similarly with regard to the metal mobilization capacity of their metabolites. Isolate BC5409 was most closely related to a strain of *Variovorax paradoxus* (98% identity) and FG4405 was most closely related

to *Pseudomonas resinovorans* (97% identity). Highly Cd-tolerant *V. paradoxus* has been identified previously in the rhizosphere of *Brassica juncea* where it aided plant growth (Belimov et al., 2005), as well as in the rhizosphere of *Alyssum murale* growing in high Ni serpentine soils where the organism exhibited tolerance to multiple heavy metals (Abou-Shanab et al., 2007). A closely related strain was recently used to oxidize and remove arsenic from mine drainage water (Battaglia-Brunet et al., 2006), and other members of the genus have been identified that produce siderophores in the Zn an Cd-contaminated rhizosphere soils associated with *Salix caprea* (Kuffner et al., 2010).

The *Variovorax* spp. examined by Kuffner et al. (2010) were found to decrease Zn and Cd mobility, while the metabolites of isolate BC5409 in our study did not. However, Cu, Ni, and Mo mobility were all increased significantly while only Mn mobility was reduced by the metabolites of both isolate BC5409 and isolate FG4405. Relatively low sequence identity of isolate FG4405 to *Pseudomonas resinovorans* suggests that the isolate may be a different strain, but members of the genus *Pseudomonas* have been identified in the rhizosphere of a variety of metal-accumulating plants, and are known for their ability to produce siderophores, promote plant growth, confer metal resistance and improve metal uptake (Braud et al., 2009b; Carrillo-Castaneda et al., 2003, 2005; Rodriguez et al., 2008; Sinha and Mukherjee, 2008; Tripathi et al., 2005). Our data suggest that isolates BC5409 and FG4405 could be considered for use in bioaugmentation of phytoextraction of Cu, Ni, and Mo and could potentially aid in rhizosphere detoxification of Mn.

Isolates FG4409, FG4011, FG4412 were all most closely related to members of the genus *Stenotrophomonas* and despite CAS indication of different classes of siderophores produced, metabolites of all three exhibited roughly the same trend with regard to metal extraction efficiency (Table 4.2, Figure 4.1). Metabolites of FG4409

and FG4011 reduced Cu mobility, but only those of FG4412 significantly immobilized Cu below that of the control. All three organisms significantly immobilized Fe to the lowest levels observed in the experiment. Ni and Mo were each significantly mobilized by these three organisms, but FG4409 and FG4412 immobilized Mn while only FG4409 significantly immobilized Zn.

Members of the genus *Stenotrophomonas* have recently been identified as producing siderophores in the rhizosphere of tobacco (Tian et al., 2009) and Indian mustard (Belimov et al., 2005). Of note, isolate FG4011 was most closely related to *S. maltophilia*, and while this species is a known common and evolving human pathogen (Chhibber et al., 2008), various strains have been isolated from soils and used in selinite bioremediation (Lampis et al., 2009) as well as exhibiting unique nematotoxic activity against plant-parasitic nematodes (Huang et al., 2009). So, while isolate FG4011 may not significantly alter the mobility of toxic metals, it could be conferring resistance to disease-causing nematodes in the rhizosphere.

The majority of the siderophore-producing isolates selected for metal mobilization experiments in the current study were most closely related to various members of the order *Bacillales*. While many members of the genus *Bacillus*, particularly *B. subtilis* and *B. pumilus*, are well known as plant growth promoting rhizobacteria (PGPR) (Khan, 2005; Zhuang et al., 2007), siderophore production is not commonly listed amongst their PGPR characteristics. However, a few recent studies have reported siderophore production by bacilli in the rhizosphere. Tian et al.(2009) conducted the most intensive survey of rhizosphere siderophore production and genetic diversity of siderophore-producing organisms to date and the genera *Bacillus* and *Rhodococcus* together constituted only 1.7% of the total isolates btested. The study by Kuffner et al. (2010) on willow rhizobacteria associated with Zn and Cd accumulation did not report a single rhizosphere isolate from the genus *Bacillus*. In

the studies by Sheng et al. (2008) and Barzanti et al. (2007) siderophore-producing *Bacillus* spp. isolates were used to stimulate plant growth and increase Pb and Ni mobilization and plant accumulation in each study, respectively.

In the current study, the isolates affiliated with the phylum *Bacillales* generally mobilized Cu, Ni, and Mo and immobilized Mn (Figure 4.1). The metabolites of BC4401 and BC4402 significantly increased the mobility of all measured metals, with the exception of Mn, and were the only two organisms whose metabolites increased the mobility of cobalt (Co) above the control (data not shown). These data, in light of the established PGPR characteristics of most *B. pumilis* strains, would strongly support further investigation and field trials using these two isolates for potential bioaugmentation of phytoextraction with willow in heavy metal contaminated areas.

Conclusions

For enhanced phytoextraction employing microbial inocula (e.g., bioaugmentation), microbes adapted to high concentrations of heavy metals are required (Haferburg and Kothe, 2007). Our case study is unique because of soil Zn and Cu concentrations naturally rivaling those of many contaminated sites including mine tailings and smelter zones (Cannon, 1955; Davis et al., 2004; Martínez et al., 2007; Rastogi et al., 2009; Wang et al., 2007; Zhang et al., 2007).

Long-term exposure to these elevated concentrations of heavy metals has likely selected for a tolerant or resistant soil microbial community associated with the indigenous willows. Several recent studies have found heavy metal exposure to have a stimulating effect on siderophore production in various bacteria (Braud et al., 2006; Dimkpa et al., 2008; Sinha and Mukherjee, 2008). The willow tissue elemental concentrations of Cd and Zn at sampling point FG meet or exceed concentrations found in the associated rhizosphere soil, as well as the average content in plant tissues

(Maestri et al., 2010). Willow Cd and Zn accumulation likely represent an accumulation of these metals from continued supply in the rhizosphere soil solution over the growing season and could result from *in situ* siderophore production by the associated rhizosphere bacteria.

The current study revealed a rhizosphere culturable bacterial composition somewhat dissimilar to those reported for willows growing in other high-metal soils, with a greater proportion of *Bacillales* and relatively few *Proteobacteria* isolated and sequenced. However, rhizosphere interactions are clearly species- and environment-specific. The culturable rhizosphere microbial community of *Salix purpurea* presented here reflects all the selective pressures of the environment including extreme annual wetting and drying cycles, cold and warm cycles, extremely rich organic soils and root exudates specific to this willow species under heavy metal stress.

Even so, high-metal habitats such as the metalliferous soils near Elba, NY, have the potential to provide a source of naturally selected, metal tolerant bacterial communities in the rhizosphere that have developed mechanisms allowing them to become superior competitors under these unique conditions. Isolates BC4505, DE3508, FG4507, BC4401 and BC4402 showed high potential for mobilizing metals in our extraction assays. Kuffner et al. (2010) compared metal mobilization assays, such as those employed in the current study, to greenhouse trials where willow plantlets were inoculated with each isolate and found that the metal mobilization assays were reliable predictors of greenhouse trial results. However, the data strongly suggest isolates BC4505, DE3508, FG4507, BC4401 and BC4402 could be employed for improving phytoextraction efficiency of Zn, Cd, Cu, Ni, and Mo in *Salix purpurea* in circun-neutral histosols. Further investigations focused on bioaugmenation should characterize the various possible plant growth promotion and metal accumulation effects of each of these organisms.

4.5 ACKNOWLEDGEMENTS

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CHAPTER 5

SIDEROPHORE PRODUCTION OF AFRICAN DUST MICROORGANISMS OVER TRINIDAD & TOBAGO

5.1 ABSTRACT

Iron (Fe) deposition has been implicated in a variety of environmental impacts of African dust on downwind terrestrial and marine ecosystems throughout the Caribbean. However, the most abundant form of Fe in African dust is the ferric ion (Fe^{III}) which is not bioavailable. The objective of this study was to determine to what degree African dust microorganisms, collected in Trinidad & Tobago, are capable of producing siderophores, effectively mobilizing bioavailable Fe in the environment. Aerosol samples were collected for microbial analyses during African dust conditions in source and downwind sites. Microbial community fingerprints, obtained by means of terminal restriction fragment length polymorphism (T-RFLP) analysis, were compared amongst aerosol samples as well as possible local sources of aerosolized microorganisms (sea water and soils). Ordination of the fingerprint data revealed similarities between aerosols from the source region and the aerosols and soils of downwind regions. Aerosol isolates were screened for siderophore production using a modified CAS assay. Twenty-five percent of isolates tested that were sampled under non-dust conditions and 65% of African dust isolates produced at least one type of siderophore; and, amongst African dust isolates, all known classes of siderophores were produced. These data support African dust microorganism siderophore production as a viable mechanism by which Fe bioavailability is increased in downwind locations.

5.2 INTRODUCTION

North Africa is by far the single greatest source of aerosolized soils and particulate matter ("dust") transported globally, resulting in hundreds of millions of tons of African dust carried on tradewinds across the Atlantic and to the Americas and the Caribbean annually (Moulin et al., 1997). Changing climate, escalating anthropogenic disturbance, and intense and prolonged droughts through recent decades have resulted in rising levels of dust being blown from northern Africa to the Caribbean (Prospero and Lamb, 2003). Concomitant transport of pollutants, metals, (Garrison et al., 2006) and viable microorganisms (Griffin et al., 2003; Prospero et al., 2005) is a cause for concern over downwind air quality and possible impacts on human health and ecosystems (Garrison et al., 2003; Griffin et al., 2001; Prospero, 1999; Prospero et al., 2008; Shinn et al., 2000; Walsh et al., 2006).

Iron (Fe) deposition from Saharan dust events has been implicated in red-tide blooms (Lenes et al., 2008; Walsh et al., 2006) and promoting microbial diseases on coral reefs (Garrison et al., 2003; Hayes et al., 2001). Iron availability is known to play a vital role in virulence and pathogenesis in bacterial and fungal diseases of humans and other organisms (Sritharan, 2000). However the majority of the Fe in African dust (up to 99%) has been reported as relatively unavailable or not biologically accessible (Schroth et al., 2009).

Most microorganisms need iron for a variety of functions including energy production (reduction of oxygen for synthesis of ATP, reduction of DNA precursors, formation of heme) and many other essential purposes. Biological demand for Fe in solution by living cells is approximately 10^{-6} *M*. However, free ferric (Fe^{III}) ion concentrations under aerobic conditions, at biological pH are rarely greater than 10^{-18} *M* (Budzikiewicz, 2004). The aerobic atmosphere of the earth rapidly oxidizes elemental Fe and the soluble ferrous (Fe^{II}) ion to the highly stable, almost insoluble,

and biologically inaccessible ferric ion. Microbes infecting humans or animals are faced with a similar Fe-limited situation within the bloodstream, where transferrin has a very high affinity for the metal and is typically only about one-third saturated (Weinberg, 2009). Such environmental constraints combined with said biological demands have resulted in many aerobic and facultatively aerobic microorganisms secreting specific molecules that can effectively scavenge Fe from the environment (Chincholkar et al., 2007; Schwyn and Nielands, 1987).

These special molecules, termed siderophores, are low molecular weight organic ligands with high affinity for ferric ions in the environment (Neilands, 1995). Microbial siderophores are excreted into the environment and bind Fe outside the cell for later uptake through specific cell membrane receptors (Budzikiewicz, 2005). Recent studies on siderophore cross-utilization have established that many organisms are capable of siderophore uptake and utilization regardless of their own ability to biosynthesize these molecules (Indiragandhi et al., 2008; Joshi et al., 2006; Khan et al., 2006). Additionally, the presence of exogenous siderophores may positively stimulate indigenous organisms to increase Fe uptake and synthesize their own native siderophore (Guan et al., 2001). Therefore, siderophore production by microbes associated with African dust could drastically alter Fe bioavailability and, consequently, marine and terrestrial biogeochemistry in downwind locations.

The primary objective of this study was to determine to what degree microorganisms collected under African dust conditions at downwind locations (Trinidad and Tobago) are capable of producing siderophores, effectively converting insoluble Fe^{III} into biologically available Fe^{II} in their environment. Increased levels of bioavailable Fe^{II} may serve as a mechanism for adverse ecological impacts in the downwind region (for sampling locations, see Table 5.1). Elemental analyses of aerosols from a source site (Mali) and downwind sites were compared with published

values of specific rare-earth ratios and results supported the African origin of aerosols collected in downwind locations (Garrison, unpublished data). Additionally, whole-community molecular fingerprints of aerosols and several possible sources of aerosol organisms were compared to evaluate community similarities/dissimilarities and further support the purported origin of the African dust isolates. African dust microbial siderophore production was assessed using a modified CAS overlay assay on isolates collected via impaction on agar petri plates during dust events from 2006 through 2008.

Table 5.1 Detailed sampling site location descriptions.

Sampling Site	Collection Location	Sample Type(s)	Sampling Period	Latitude	Longitude	Elevation
Galera Point, Trinidad	Lighthouse pinnacle	Aerosol	Nov '06 - July '08	10.8° N	59.9° W	70 m
Galera Point, Trinidad	Toco, windward coast	Soils & sea water	April - July '08	10.8° N	59.9° W	0 - 40 m
Flagstaff Hill, Tobago	Windward coast	Aerosol, soils, & sea water	June '08	11.3° N	60.5° W	329 m
Bamako, Mali	Escarpment above Niger River Valley	Aerosol	April '08	12.7° N	8.0° W	554 m

5.5 METHODS

Samples were collected from the windward coasts of Trinidad (November, 2006 through July, 2008) and Tobago (June 2008) and from an escarpment in a source region in Mali (April, 2008; Table 5.1). The locations of sampling sites were carefully chosen to minimize the possibility of impacts from local sources on aerosol collection. Aerosols in the downwind locations (Trinidad and Tobago) were collected only when the wind was from the east or southeast and Saharan dust was present in the atmosphere. The presence of Saharan dust was confirmed by: 1) forecasts by the Navy Aerosol Analysis and Prediction System models accompanied by a reduction in atmospheric visibility; 2) the presence of reddish-brown particles on filters; 3) high Fe

content of particles; and, 4) for 2008 Tobago and Mali samples only, La-Sc-Th abundance ratios fell within the range of atmospheric African dust (Garrison et al., 2010, accepted; Muhs et al., 2007).

Community fingerprinting: sample collection

Samples for microbial community fingerprinting of aerosols in Tobago and Mali (Table 5.1) were collected in 2008 by high- volume, brushless blower motors (2008 Mercury, 110v/12 amp or 220v/6amp); 200 – 500 m^3 air was filtered through 90 mm glass microfiber filters (Whatman GF/A; 0.7 micron) that had been individually wrapped in Al foil packets, sealed and autoclaved prior to sampling. During sampling, each filter was held in Teflon 90 mm filter-holders that had been pre-rinsed with ethanol. After sampling, each filter was aseptically resealed in its original Al foil packet, placed in a sterile Ziploc ® bag and frozen at -20°C for shipment to the U.S. for DNA extraction.

Soil samples were collected from unvegetated, undisturbed locations in the immediate vicinity of aerosol collection points on non-dust days; five soil samples were collected in Trinidad and four in Tobago. A standard soil spade, rinsed with a 70% ethanol solution before sampling and between samples, was used to aseptically collect approximately 10 g of soil was placed into a sterile Whirl-pac ® bag. Each sample bag was sealed and transported on ice to the laboratory where the samples were frozen and held at -20°C until DNA was extracted.

Approximately 2 L of sea water was collected approximately 10 m off the windward coast near each aerosol sampling location at Trinidad and at Tobago during non-dust conditions. Sea water samples were aseptically filtered through a 0.22 μm microcellulose filter, which was frozen at -20°C for storage until DNA extraction.

DNA extraction

Soil extractions for whole community microbial DNA were performed on 0.25 g of each soil sample using the MoBio PowerSoil™ DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Roughly one quarter of each sample filter was used for DNA extraction using the method described by Miller et al. (1999).This method was modified to include Lysing Matrix E (BioMedicals) bead lysis tubes and MaXtract High Density gel tubes (Qiagen) in the first two steps of the extraction protocol.

Extracted DNA was then quantified against a calf thymus DNA standard curve in an ethidium bromide (EtBr) solution using an EC3 Imaging System (UVP Bioimaging Systems, UVP LLC, Upland, CA) and Quantity One™ software (Applied Biosystems, Foster City, CA). DNA extracts were diluted with nuclease-free water to approximately 3-5 ng μl^{-1} and immediately frozen at -20°C until subsequent analysis.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

Bacterial 16S rRNA genes were targeted for amplification from environmental DNA extracts by polymerase chain reaction (PCR) using the fluorescently labeled forward primer 27f (5′-[6FAM] AGA GTT TGA TCC TGG CTC AG-3′) and the unlabeled reverse primer 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Integrated DNA Technologies, Coralville, IA) (Moeseneder et al., 1999). Duplicate reactions of each sample were amplified using a MJ Research thermal cycler PTC 100 (MJ Research, Waltham, MA) and the following program: 5 min at 94°C, followed by 27 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Reaction volumes for each 50 µl reaction were as follows: 0.50 µl of 5U *Taq* polymerase (Applied Biosystems), 5.0 µl of the 10x PCR buffer supplied with the enzyme, 4.0 µl of 25 m*M* $MgCl_2$, 1.0 µl of 10 µ*M* deoxy-nucleotide

triphosphates (dNTPs), 0.5 µl of 10 µg µl^{-1} bovine serum albumin (BSA), 0.5 µl of each primer at 10 µ*M*, 33.0 µl nuclease free water (Promega, Madison, WI) and 5 µl of DNA template at 3-5 ng µl^{-1}.

Archaeal DNA was then amplified by PCR using the fluorescently labeled forward primer Ar109f (5′-[6FAM] ACG/T GCT CAG TAA CAC GT-3′) and the unlabeled reverse primer Ar912r (5′-CTC CCC CGC CAA TTC CTT TA-3′) (Integrated DNA Technologies, Coralville, IA) (Lueders and Friedrich, 2000). Duplicate 50 µl reactions of each sample were amplified using a MJ Research thermal cycler PTC 200 (Waltham, MA) as follows: initial denaturation at 94°C for 5 min; 27 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 72°C for 45 s; and a final extension step at 72°C for 10 min. Reaction volumes and concentrations stated above were employed, without the use of BSA.

The fungal internal transcribed spacer (ITS) region from sample DNA was amplified by PCR using the fluorescently labeled forward primer ITS1Ff (50-[6FAM] CTT GGT CAT TTA GAG GAA GTA A-30) and the unlabeled reverse primer ITS4r (50-TCC TCC GCT TAT TGA TAT GC-30) (Integrated DNA Technologies) (Bruns et al., 1991). Duplicate reactions of each sample were amplified using an MJ Research PTC 100 thermal cycler and the following program: 5 min at 94 °C; followed by 30 cycles of 94 °C for 30 s, 51 °C for 45 s, and 72 °C for 45 s; and a final extension step at 72 °C for 10 min. Volumes and concentrations for each 50 µl reaction were as follows: 1.0 µl of 5U µl^{-1} *Taq* polymerase (Applied Biosystems), 5.0 µl of the 10x PCR buffer supplied with the enzyme, 6.0 µl of 25 m*M* $MgCl_2$, 3.0 µl of 10 m*M* dNTPs, 0.5 µl of BSA at 10 µg µl^{-1}, 1.0 µl of each primers at 10 µ*M*, 27.5 µl nuclease-free water (Promega) and 5 µl of DNA template at 3-5 ng µl^{-1}.

Restriction enzyme digests of Bacterial, Archaeal, and fungal amplicons were prepared per sample containing 1.25 µl Sau96I enzyme (New England Biolabs,

Ipswich, MA), 2.5 µl of the 10x buffer supplied with the enzyme, 0.25 ul of BSA at 10 µg μl^{-1}, 8.50 µl nuclease-free water and 12.5 µl of amplified sample DNA at 25 ng μl^{-1}. Restriction digestion was carried out in a MJ Research PTC 200 thermal cycler at 37°C for 4.5 h with a final step of 70°C for 15 min to stop the reaction. Complete digestion of the DNA was verified by inspecting digested products run on a 1.5% agarose gel stained with EtBr and visualized using an EC3 Imaging System (UVP Bioimaging Systems, UVP LLC, Upland, CA). Digested DNA was purified, lyophilized, and resuspended in a 10 µl mix containing 9.85 µl of formamide and 0.15 µl of LIZ 500 size standard (Applied Biosystems). Terminal fragment-size analysis was performed using an Applied Biosystems Automated 3730XL DNA Analyzer (Applied Biosystems) in conjunction with the Genemapper Software (Applied Biosystems) at Cornell University's Life Sciences Core Laboratories Center, Ithaca, NY.

While bacterial community rRNA gene amplification was successful across all sample types, Archaeal and fungal gene amplification was only successful in subsets of the environmental samples. With successful bacterial amplification, a variety of successful positive and negative controls, multiple optimization techniques were employed and repeated attempts yielded identical results. Therefore, in samples where amplification was not successful for either fungi or Archaea, it is likely that the respective group made little or no contribution to the microbial community of that particular sample type. By concatenating all three sets of T-RF data (Bacteria, Archaea, and fungi) for each individual sample, this difficulty was minimized and a comprehensive community fingerprint was achieved and analyzed as such.

Siderophore production: sample collection

African dust samples used to assess siderophore production were collected during sporadic dust events from November 2006 through July 2008 from the top of the lighthouse at the most northeastern tip of Trinidad (Galera Point, Toco; Table 5.1). For each sampling date, NOAA HYSPLIT (Hybrid Single Particle Lagrangian Integrated Trajectory Model) 4.9 air mass back-trajectories were calculated to support assumptions of air mass origins and $PM_{2.5}$ intensity was measured using a TSI DustTrak™ 8520 Aerosol Particulate Monitor. Aerosol samples were collected by vacuum impaction of 100 - 400 L air (28.3 L min^{-1}) using a pre-sterilized portable BioStage impactor (SKC Inc., Eighty Four, PA) containing sterile media (R2A, Yeast Extract Agar, Potato Dextrose Agar, or Bacto-Agar) in standard 90 mm diameter petri plates. Plates were then transported, for a maximum of two hours, on ice to The University of the West Indies in St. Augustine where they were incubated in the dark at 32°C. Colonies were characterized morphologically according to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994) as either bacteria or fungi, each given an individual identifier and subsequently isolated on fresh plates of the same media type on which they were originally collected. Molecular identification of these isolates is ongoing at the University of the West Indies.

O-CAS assay

For siderophore production, isolates obtained from aerosol impaction were cultivated by spot inoculation on the media described previously, under iron deprivation, at 32°C for 24-72 h. To achieve iron deprivation, each culture medium and all glass materials were treated according to Cox (1994). After initial incubation, a modified chrome azurol-S medium (Schwyn and Nielands, 1987) was utilized as an overlay to the culture according to the methods described by Pérez-Miranda et al.

(2007). Briefly, an overlay was applied to each cultured isolate and color changes indicated siderophore production by the culture. A liter of the overlay medium was prepared as follows: Chrome azurol S (CAS), 60.5 mg; hexadecyltrimethyl ammonium bromide (HDTMA), 72.9 mg; Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 30.24 g; and 1 mM$FeCl_3 \cdot 6H_2O$ in 10 mMHCl 10 mL. Agarose (0.9%, w/v) was used as the gelling agent.

Siderophore detection was achieved after 5 mL (60 mm Petri dish) of this medium was overlaid onto each agar plate containing a cultivated microorganism to be tested for siderophore production. After a maximum period of 14 d, a change in color in the overlaid medium was observed exclusively surrounding producer microorganisms. A color change, from blue to purple as described in the traditional CAS assay indicates production of catechol-type siderophores, while a change from blue to orange indicates hydroxamate production and from blue to completely clear indicates production of carboxylate-siderophores (Pérez-Miranda et al., 2007). Each isolate was tested for siderophore production in triplicate, and a total of 392 aerosol isolates were tested as described. Control plates of each type of uninoculated medium, with CAS overlay, were incubated under the same conditions described above with no color change observed up to 21 d.

Data Analysis

For community fingerprint analysis, only terminal restriction fragments (T-RFs) of 50 to 500 bp in length were included. Peak sizes that differed by 3 bp were considered unique (peaks binned up), and pattern discrimination was determined by application of variance/covariance centered Principal Components Analysis in PC-Ord (MjMSoftware Design, Gleneden Beach, OR) performed on concatenated T-RF presence or absence data for Bacteria, fungi, and Archaea.

Linear regression analysis and Pearson correlation coefficients between atmospheric concentrations of $PM_{2.5}$ and the proportion of siderophores produced were performed with α=0.10 significance level (MINITAB ® 15.1.30.0).

5.3 RESULTS

Community fingerprinting

Principal components analysis (PCA) of concatenated T-RFLP data sets explained 44.2% of the variability in the data and revealed a distinct community composition of the sea water samples apart from the communities of the aerosols or local soils of Trinidad and Tobago (TT) (Figure 5.1).

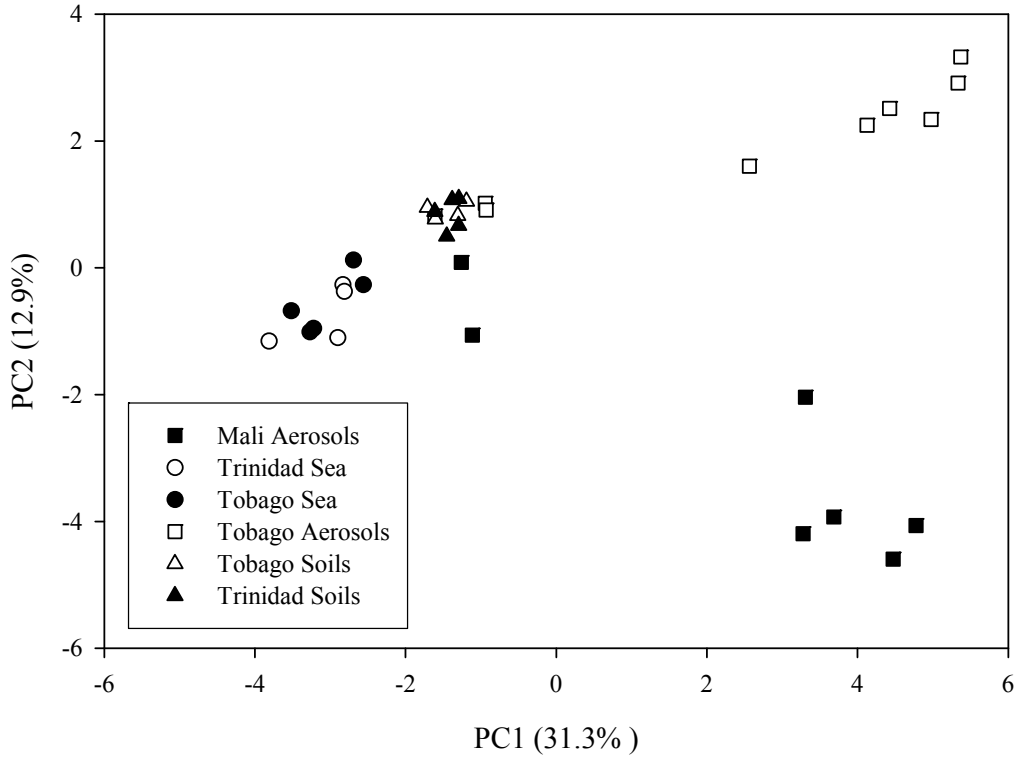

Figure 5.1 Principal components analysis ordination plot of the bacterial, fungal, and archaeal T-RFLP data consisting of the presence/absence of the terminal restriction fragments. Samples were collected in 2008 and origins include local soils and sea water (disputed sources of aerosolized microorganisms), as well as aerosols from Tobago and the source region, Mali.

On the first principal component (PC1), where the majority of the variability in the data was explained (31.3%), sea water microbial community fingerprints yielded negative values and did not share overlap with the communities of the aerosols or local soils. Soil microbial communities sampled from both Trinidad and Tobago from a variety of unvegetated locations on non-dust days revealed a tight cluster with regard to both axes and all other sample types. In contrast, microbial community fingerprints of aerosols collected from an escarpment above the Niger River Valley in Bamako, Mali, as well as aerosols collected from Flagstaff Hill on the windward coast of Tobago during African dust events revealed great diversity in their microbial community composition as well as partial overlap on each axis with the communities of local soils in TT.

Siderophore production

Bacterial and fungal isolates (n = 392) from aerosol samples collected at Galera Point were screened for siderophore production using the O-CAS assay and all known classes of siderophores were detected (Figure 5.2). Of the bacterial isolates tested, 53% (115 of 219) produced siderophores and 90% of those produced carboxylates (Figure 5.3). Amongst the bacteria producing siderophores, only two isolates produced catechol siderophores and 10 isolates produced hydroxamates. In contrast, there were fewer total fungal isolates screened (173), but a greater proportion of these produced siderophores (76%). Fungal siderophore production was more evenly distributed amongst the various types of siderophores; 34 fungi produced catechols, 26 produced hydroxamates, and 72 produced carboxylates.

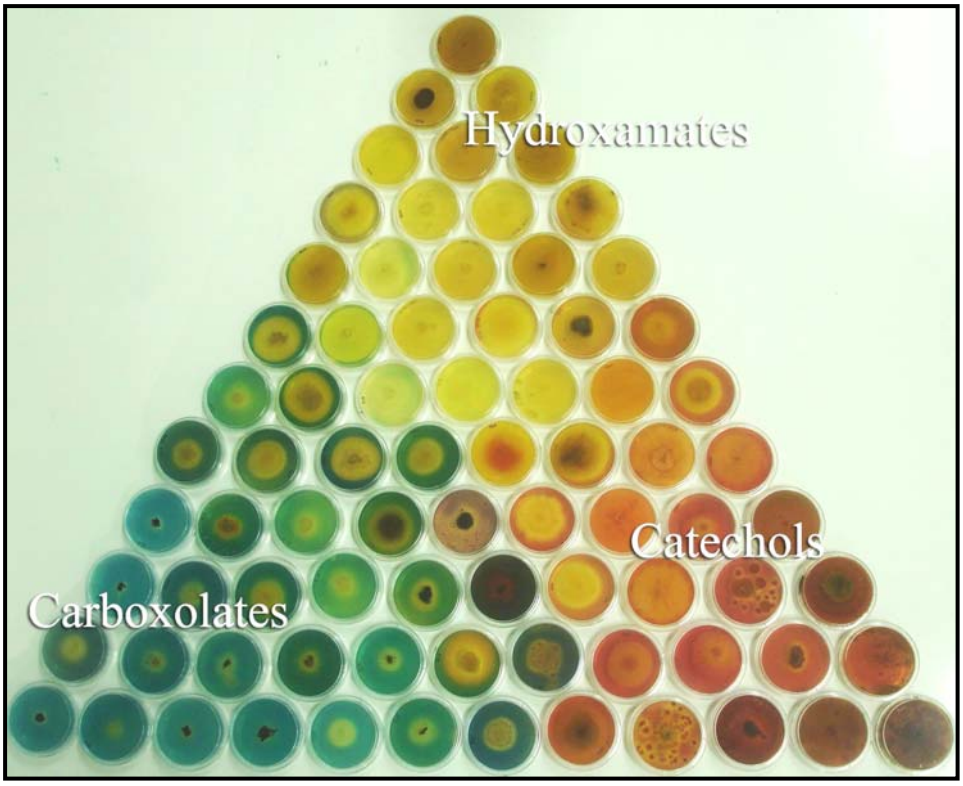

Figure 5.2 Siderophores produced by African dust microbial isolates, where each isolate is shown here on a separate petri plate with CAS overlay having changed to the color indicating the class siderophore(s) produced by the isolate.

NOAA HYSPLIT (Hybrid Single Particle Lagrangian Integrated Trajectory Model) 4.9 air mass back-trajectories supported the assertion that 320 of the tested isolates were collected under African dust conditions and 72 isolates were collected under non-African dust conditions when air masses appear to have originated from more local sources including the U.S., Canada, or various regions of South America. Only 25% (18) of the isolates tested from non-dust conditions produced siderophores, while 65% (208) of the African dust isolates produced siderophores.

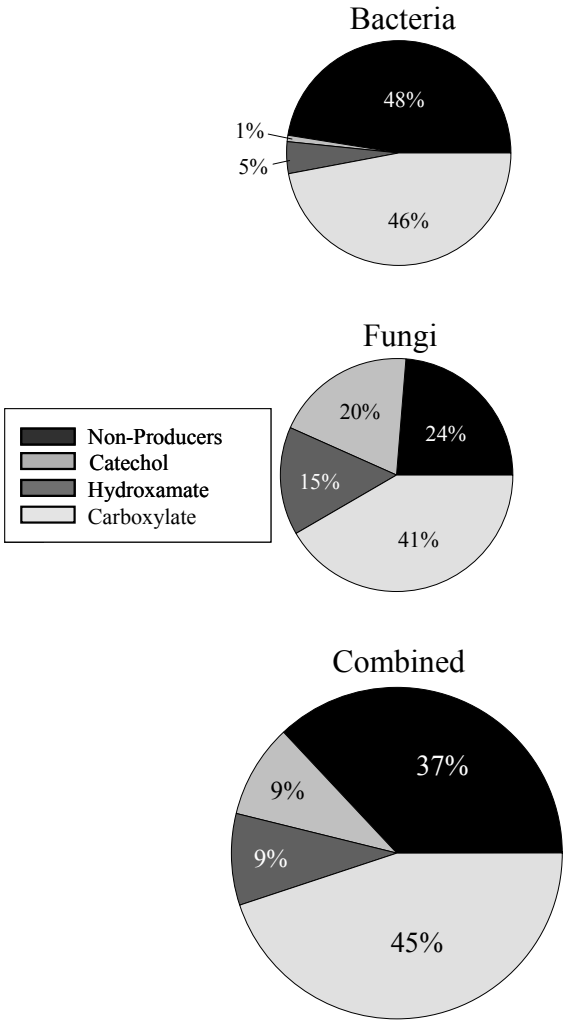

Figure 5.3 Overall siderophore production of dust culture collection isolates

Amongst the 320 isolates obtained when conditions indicated African dust over TT, the proportion of siderophore producing isolates was further analyzed according to the concentration of the particles with aerodynamic diameter of 2.5 μm or less ($PM_{2.5}$) in the atmosphere at the time the aerosols were collected (Figure 5.4). Regression analysis revealed a significant positive relationship between quantity of atmospheric African dust ($PM_{2.5}$) and proportions of isolates that produced any

siderophore (r = 0.78, p = 0.022) as well as those that produced specifically carboxylate siderophores (r = 0.77, p = 0.025).

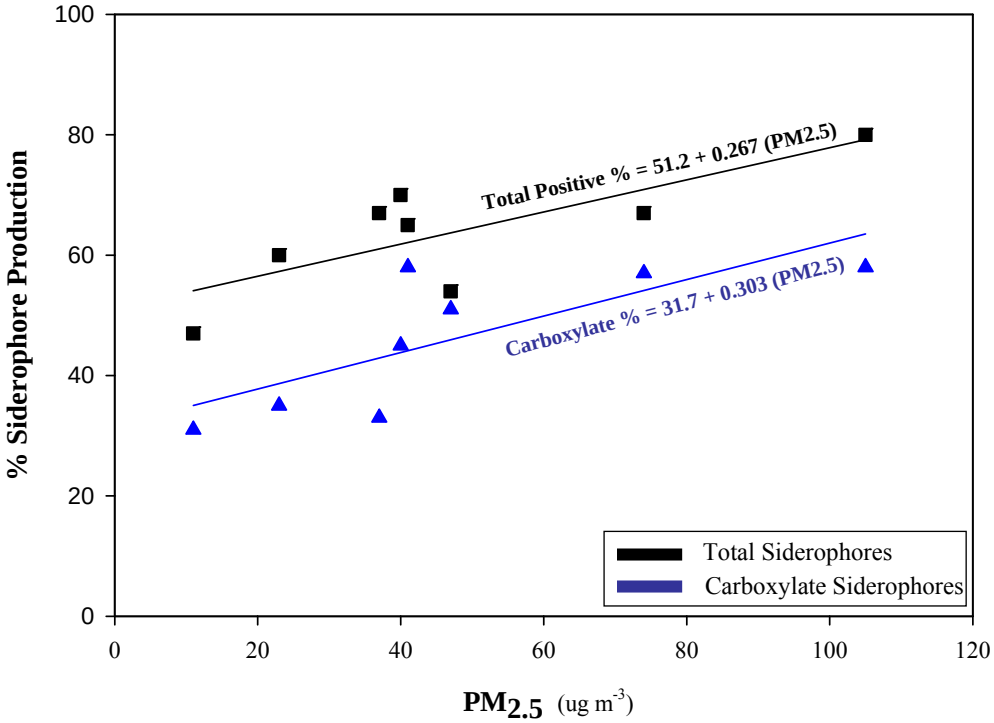

Figure 5.4 Proportion of African dust isolates positive for siderophore production during events of increasing intensity, measured by $PM_{2.5}$ particulate density.

5.4 DISCUSSION

It is not possible currently to conclusively discern a microbe's origin over the distances and under travel conditions such as those addressed in the current study. Rather than establishing aerosol origins, the forensics-style whole-community DNA extraction and fingerprinting method employed in the current study provided 80-170 resolved characters (T-RFs) for each sample and provided a glimpse of the microbial community biogeography.

Had we only to compare the aerosol communities collected in Tobago with the local soils and sea water communities, we might easily conclude that sea spray made little contribution but local soils had been aerosolized and clearly contributed to the

overall community patterns of the sampled aerosols in Tobago. The harsh conditions of life in the atmosphere might provide enough selective pressure to yield some differences between the communities sampled directly from soil and those that had been aerosolized, also explaining the great differences between most of the aerosol samples with local soils.

However, the community fingerprint data from aerosolized microbial communities collected in the source region yield valuable insights beyond those of the selective pressures of these three vastly different environments. The community composition of two samples collected in Bamako, Mali, on different days (10th and 14th April, 2008; Fig 5.1) revealed strikingly similar community composition to the local soils of TT as well as the purported African dust aerosols collected in Tobago. Since it is highly unlikely that the soils of Trinidad and Tobago contaminated aerosols collected thousands of miles away and two months earlier, these data most likely reflect the centuries-old effect of the phenomenon of African dust transport and resulting contributions to both the mineral and biological properties of local soils and ecosystems.

Importantly, however, a variety of environmental and anthropogenic disturbances over the last few decades have resulted in rapidly increasing quantities of dust being blown from northern Africa to the Caribbean (Prospero and Lamb, 2003). Therefore a better understanding of the microbial communities transported in African dust and their potential impact on biogeochemistry in downwind locations has become critical.

Microbial siderophore production is well established in a variety of environments from soils, to oceans, and even within the human body in the event of microbial infections (Chincholkar et al., 2007). However the types of siderophores produced and the levels of production vary greatly between systems, between species,

and require more study in virtually every environment. The CAS assay we used in the current study to assess siderophore production is based on the cultivable portion of the microbial community, and several studies have reported on the numbers and identities of cultured African dust microorganisms collected at downwind Caribbean locations (Garrison et al., 2006; Griffin et al., 2001; Griffin et al., 2003; Kellogg and Griffin, 2006; Prospero et al., 2005).

Kellogg et al. (2004) were even able to identify 19 bacterial isolates from dust events collected from an African source region of the same genus and species as isolates collected from dust events in the Caribbean. A large percentage (38-40%) of the identified organisms was related to the genera *Bacillus* and *Micrococcus*, with others from *Rhodococcus*, *Pseudomonas*, and *Streptomyces*. Fungi identified in these studies included primarily the genera *Cladosporium* and *Aspergillus* as well as a few others (Griffin et al., 2001; Kellogg et al., 2004).

Of particular interest in light of the previous studies are the recently established siderophore production capabilities of each of these genera. Various members of the *Bacillus* and *Micrococcus* genera have been reported to produce either catechol or hydroxamate siderophores (Cabaj and Kosakowska, 2009; Patel et al., 2010), while a member of *Rhodococcus* was actually found to produce both catechol and hydroxamate as a single mixed-ligand siderophore (Dhungana et al., 2007). Hydroxamate production has also been also recognized in members of the genera *Streptomyces* (Dimkpa et al., 2009; Dimkpa et al., 2008) and *Pseudomonas* (Braud et al., 2009a). However, members of the genus *Pseudomonas* have also been reported to produce a variety of carboxylate siderophores (Rodriguez et al., 2008). Fungi, including the genera *Cladosporium* and *Aspergillus* (Winkelmann, 2007), have been reported to produce polycarboxylates as well as catechols and hydroxamates (Singh et al., 2008).

Known spore-forming organisms, such as those in the genus *Bacillus* and various types of fungi, are most often reported to produce catechol or hydroxamate siderophores (Cabaj and Kosakowska, 2009; Singh et al., 2008; Vala, 2006), both of which showed no apparent relationship to increasing dust levels ($PM_{2.5}$; data not shown). However, as density of African dust in the atmosphere increased, a corresponding increase in carboxylate production was observed in the current study (Figure 5.4). Increased carboxylate production could suggest a greater proportion of non-spore forming organisms and a greater diversity of organisms transported overall as dust density increased possibly providing a shielding effect from UV and desiccation effects during transport.

In the current study, the culture collection of aerosol microorganisms belonging to The University of the West Indies was screened for siderophore production and the majority of isolates collected during African dust events (65%) produced at least one type of siderophore (Figure 5.2). Few studies have screened the cultivable fraction of a given microbial community for siderophore production, and most involve rhizosphere bacterial communities which may or may not prove useful for comparison to the African dust isolates.

A recent study by Chaiharn et al. (2009) was directed towards discovery of plant growth promoting rhizobacteria (PGPR) and surveyed soil bacteria in the rhizosphere of rice plants in northern Thailand for siderophore production. Only 48 (22%) of 216 bacterial isolates were reported to produce siderophores, three produced catechols and 45 produced hydroxamates. In a similar study by Khamna et al. (2009), 445 actinomycetes isolated from the rhizosphere of 16 different medicinal plants were screened for siderophore production; only 45 isolates (27.5%) produced siderophores. Even considering the bacterial dust isolates only, 53% tested positive for siderophore production, which is roughly twice the number found in these previous studies.

Fungal isolates from African dust exhibited a greater capacity for siderophore production than did the bacterial isolates, with roughly 76% producing siderophores. These findings are in general agreement with the findings of the most recent studies on fungal siderophore production. Vala et al. (2000) screened 13 marine fungi, Machuca et al. (2007) screened 5 soil fungi, and Baakza et al. (2004) screened 20 fungi (10 marine and 10 soil fungi). Of 38 fungi tested in these three studies, 34 (89%) produced siderophores and 100% of the fungi from soil were producers.

Of note in the work by Baakza et al. (2004) was a comparison of the quantity of siderophore produced by fungal isolates of the same genus and species from terrestrial and marine habitats. They found that fungi of terrestrial origin produced much greater quantities than those of marine origin, in some cases as much as 8.5-fold more. This represents almost an order of magnitude difference between the siderophore production levels of a single fungus based on its origins. The authors ascribed this difference to the mobile ambient environment of marine ecosystems, but the implications in light of the current study could be significant.

Not only do a greater proportion of African dust microorganisms produce siderophores than microbial populations from other environments, but due to their purported terrestrial origins, they may also be likely to produce greater quantities than organisms present in downwind marine ecosystems. Siderophore quantity produced was not measured in the current study; however many of the tested organisms created a color change in the entire overlay (rather than a small radius around the colony), which indicated high levels of output and would be consistent with the study by Baakza et al. (2004).

High levels of siderophore production by microbes isolated from African dust were observed in the current study. This may suggest that various types of stress experienced by organisms during dust transport over thousands of miles (e.g., UV

exposure, desiccation, freeze-thaw cycles, or heavy metal exposure) could trigger a greater proportion of the community to produce siderophores via alternative sigma factors (Boor, 2006; Stockwell and Loper, 2005). This would be supported by the observations of increasing siderophore production as the quantity of atmospheric dust increased (Figure 5.3), likely a result of the increased levels of heavy metals transported within the dust (Garrison et al., unpublished data). Heavy metal exposure often triggers alternative sigma factors, as well as siderophore production (He et al., 2010a; He et al., 2010b).

He et al. (2010b) screened a rhizosphere bacterial community that had been chronically exposed to high concentrations of heavy metals, specifically copper (Cu). Under elevated heavy metals concentrations, eight of the 13 bacterial isolates (62%) produced siderophores. Levels of extractable Cu in those soils ranged from 28 to 74 mg kg^{-1}, while levels of Cu in the African dust aerosols collected over Trinidad & Tobago were somewhat similar and ranged from 26 to 40 mg kg^{-1} (Garrison et al., 2010, accepted). Thus, stress during transport may have induced siderophore production in the African dust microorganisms, and it becomes important to consider that the same alternative sigma factors could also contribute to expression of virulence-based genes that could enhance the infective ability of opportunistic pathogens contained in the dust (Fang et al., 1994). However, that is a subject beyond the scope of the current research and will not be discussed further here.

Interest in African dust and research on its elemental composition, mineralogy, the geologic processes causing it, its transport, global deposition, optical properties, atmospheric chemistry, and particle size distribution have all increased rapidly over the last decade (Garrison et al., 2003). It is clear that Fe and other heavy metals play important roles in the ecological impacts of African dust deposition in downwind locations. However, our understanding of the mechanisms altering Fe bioavailability

and consequently impacting biogeochemical cycles has been limited. Results of this study suggest siderophore production as a viable mechanism by which African dust microorganisms may be capable of enhancing Fe bioavailability, which may have implications for human health, and marine and terrestrial ecology.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 6

OVERARCHING CONCLUSIONS

Microbial siderophore production appears to be a shared mechanism for metal mobilization between these two vastly different soil environments of the metalliferous peat soils near Elba, NY, and the African dust transported across the Atlantic to the Caribbean island-nation of Trinidad & Tobago. The fairly high levels of siderophore production observed in both locations are likely due to heavy metal exposure, proven to stimulate microbial siderophore production.

A variety of studies have examined the composition and phylogeny of the microorganisms being transported in African dust over the Caribbean, while other studies report adverse environmental impacts created by Fe deposition. The work included here was ground-breaking in that no previous study has attempted to elucidate a possible microbial mechanism for metal mobilization within a microbial community in transported dust.

At the site in Elba, NY, long-term exposure to elevated heavy metals has likely selected for a tolerant soil microbial community associated with indigenous willows and therefore willow rhizosphere microbial community dynamics and mechanisms of heavy metal uptake and accumulation in the plants were identified. Additionally, several strains of bacteria were obtained which could be employed to improve phytoextraction efficiency of Zn, Cd, Cu, Ni, and Mo in *Salix purpurea* grown in circum-neutral histosols. Several of the strains tested were capable of mobilizing heavy metals, as well as possibly conferring other important functions in the rhizosphere such as pesticide degradation and disease resistance.

Overall the bacteria cultured from the Elba soilswould likely provide a number of plant-growth promoting services in addition to nutrient or heavy metal

mobilization and should be explored further for their possible use in enhancing the efficiency of *Salix* spp. metal phytoextraction and phytoremediation strategies.

Microbial siderophores are involved in complex interactions amongst microbial populations including quorum sensing, biofilm formation, and pathogenecity. These same siderophores are also capable of modulating microbial interactions with the environment and "higher" organisms through nutrient mobilization and pathogen suppression. Each specific siderophore has unique binding affinities and a unique kinetics profile, and we are just beginning to realize how microorganisms may use each of them under very specific circumstances to a specific purpose.

For logistical reasons, many studies on siderophore production focus on one organism and typically only one siderophore produced by that organism. However, an old African proverb advises, "A single bracelet does not jingle," and presumably this concept can be applied in the study of the microorganisms and mechanisms that underlie rhizosphere processes. Thus, it is only through continued study on multifaceted microbial consortia and their interactions with the evolving life forms and ecosystems around them that we will truly understand the role that siderophores play heavy metal bioremediation, and even, in our world.

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