PHYTOSTABILIZATION POTENTIAL OF THE KLONDYKE MINE TAILINGS SITE AND ITS ASSOCIATED MICROBIAL COMMUNITY

by

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DEDICATION

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BACTERIAL COMMUNITY CHARACTERIZATION OF A HISTORIC SEMIARID LEAD-ZINC MINE TAILINGS SITE

ABSTRACT

Phytostabilization is an emerging technology for the remediation of mine tailings sites. In arid and semiarid environments, mine tailings disposal sites are a major source of environmental pollution as they are subject to eolian dispersion and water erosion. Mine tailings are acidic to neutral, high in metal content, and nutrient poor. Furthermore, these sites remain unvegetated even after decades of no additional mining activity. In arid and semiarid regions, climatic variables such as high winds, salinity, and drought exacerbate the problem. The Klondyke mine tailings site is a model site for studying plant establishment in mine tailings within semiarid regions. It was a lead and zinc ore- processing operation from 1948 to 1958 and is similar in physicochemical characteristics to other acidic pyritic mine tailings.

In a greenhouse study, a native drought tolerant halophyte, *Atriplex lentiformis* (Torr.) S. Wats., was evaluated for its potential as a phytostabilization candidate in compost-amended tailings from the Klondyke site. Germination, plant growth, and metal uptake of *A. lentiformis* were examined, and the microbial community was monitored by enumeration of autotrophic iron- and sulfur-oxidizing bacteria as well as heterotrophic bacteria. Results demonstrated that with 10 to 15% compost addition, growth of *A. lentiformis* was not affected and shoot metal concentrations were generally not a concern for foraging animals. Furthermore, the heterotrophic bacterial community is severely stressed but recovers with compost addition and successful plant growth. Therefore, *A.*

lentiformis is a good candidate for phytostabilization of mine tailings with compost amendments.

Poor revegetation of mine tailings has been attributed to the microbial community involved in acidifying tailings; however, no thorough microbial studies have been conducted. The second study characterizes the bacterial community of the Klondyke site and compares it to an offsite control sample. Results demonstrate that the heterotrophic community is indicative of soil health as it has a positive relationship with pH, phylotype richness, and diversity. Also, the mine tailings contain an unexplored diversity of acidophiles that are important in maintaining acidity and thus, metal bioavailability. Therefore, the bacterial community in mine tailings should be monitored in phytostabilization studies to evaluate restoration.

CHAPTER 1

INTRODUCTION

Explanation of Dissertation Format

This dissertation comprises two chapters and two appendices. Chapter one includes an introduction to the research problem and a review of the current literature. The literature review is being prepared for submission to Reviews in Environmental Science and Bio/Technology and addresses the implementation of a phytostabilization strategy in arid and semiarid environments as well as factors that affect successful remediation. Chapter two provides a summary of the present study that is discussed in detail in Appendices A and B.

Two manuscripts are presented in Appendices A and B. The manuscript in Appendix A is published in Journal of Environmental Quality, and Appendix B is being prepared for submission to Environmental Microbiology. My advisor Dr. Raina M. Maier provided advice and guidance in the manuscripts presented in Appendices A and B, but the ideas, design, execution, and data analysis are my own and represent my original work. For Appendix A, Dr. Edward P. Glenn provided assistance with the design and statistical analyses.

Explanation of Problem

The hardrock mining industry has left a toxic footprint during its exploration and beneficiation of minerals and metals. In 2000, the EPA's Toxic Release Inventory (OIG-

USEPA 2004) reported the hardrock mining industry as the largest producer of toxic waste that year, releasing 1.5 million tonnes, or 47 percent of the total released by U.S. industry. In comparison, estimates from 1991 were approximately 1500-fold greater with a yearly production of 2.2 billion tonnes of waste, indicating an improvement in extraction technology. However, the current waste production exacerbates the existing environmental problems related to the mining industry's historical contamination. It is estimated that it will cost the EPA approximately $50 million to cleanup only a small portion of the Superfund sites related to mine waste (OIG-USEPA 2004). Therefore, a cost-effective remediation approach must be implemented to reduce this environmental problem.

Mine tailings are the primary component of mine waste after ore-processing for metal extraction and are the source of pollution. The tailings are composed of mostly silt or sand sized particles, lack nutrients supportive of biological growth, and contain almost no organic matter (Ye et al. 2002). The rate of metal contamination from mine tailings is approximately 10 to 600 million-kg y^{-1} worldwide. Also, most tailings disposal sites are devoid of any vegetation and have a stressed heterotrophic microbial community. In arid and semiarid environments, natural revegetation of the site is further impeded by the climatic conditions and increased salinity in the tailings. Thus, mine tailings in arid and semiarid regions are highly susceptible to eolian (wind related) dispersion and water erosion (Munshower 1994).

There are approximately 500,000 mine tailings disposal sites in the U.S. and an estimate of three-quarters of a million worldwide (Munshower 1994). In the United

States, the majority of abandoned mine tailings sites are semiarid regions in the western United States (USEPA 2004). Other major tailings sites are located in arid and arid regions, including South Africa, Australia, Mexico, Chile, Spain, and India (Munshower 1994).

Tailings disposal sites have become a source of pollution for nearby communities. In Mexico, communities near tailings sites demonstrated metal exposure with elevated levels of lead found in the blood of both children and adults, and high concentrations of arsenic detected in the urine of children (Gonzalez & Gonzalez-Chavez 2006). High amounts of particulate matter from tailings measured in fractions of <10 μm (PM_{10}) and <2.5 μm ($PM_{2.5}$) in aerodynamic diameter are a serious problem in communities and towns surrounding mine tailings sites and is even more severe in arid environments (Schwegler 2006). The most common contaminants in abandoned mine tailings sites are lead, arsenic, zinc, and cadmium and can cause various health effects. Lead exposure can cause anemia, damage the brain and kidneys, affect child development, and cause reproductive problems. Arsenic can irritate the throat and lungs, induce circulatory and peripheral nervous disorders, and increase lung cancer. Zinc causes a temporary "metal fume fever". Lastly, cadmium is highly carcinogenic when inhaled (OIG-USEPA 2004).

The Klondyke mine tailings site is located along the Aravaipa Creek in the Aravaipa Valley, Graham County, Arizona. It was primarily a lead and zinc ore-processing site from 1948 to 1958. The Arizona Department of Environmental Quality (ADEQ) first became interested in the site when erosion and runoff of the tailings were observed. Concentrations of lead and arsenic in the tailings exceeded the Arizona Non-

Residential Soil Remediation Levels (SRLs) of 10 and 1,200 mg kg-1, respectively. Elevated levels of Cd and Pb were also found in fish sampled from the Aravaipa Creek. Therefore, ADEQ placed the Klondyke site on the Arizona Water Quality Assurance Revolving Fund (WQRF) Registry in 1998 (ADEQ 2001). Since the abandonment of the mining operation in Klondyke, the site has remained unvegetated (Fig. 1).

Phytostabilization, a type of phytoremediation, has become appealing for the restoration of mine tailings sites. Phytostabilization involves the use of plants to revegetate mine tailings and reduce further contamination. The plant root system and plant canopy both reduce eolian dispersion and water erosion. Additionally, the roots and rhizosphere provide an environment for interactions with metals, including adsorption, precipitation, and accumulation. This "biological capping" provided by phytostabilization reduces metal mobility, and hence, prevents dispersion of the contamination (Cunningham & Ow 1996).

Poor plant growth in pyritic mine tailings has been attributed to the microbial community involved in the acidification of tailings; however, the microbial community of mine tailings sites is still rather unexplored (Southam & Beveridge 1992; Schippers et al. 2000; Schroeder et al. 2005; Mendez et al. 2007). Very few studies have characterized the microbial community in mine tailings at the genus and species level and even fewer have examined the microbial community in phytostabilization studies. Studies have indicated that the heterotrophic microbial community increases with plant growth and a substantial presence of acidophilic iron- and sulfur-oxidizers is related to plant death (Schippers et al. 2000; Mummey et al. 2002; Moynahan et al. 2002). Data from a 20-year

mine tailings revegetation study revealed that the microbial community did not recover to its previous state as compared to an undisturbed site and that plant diversity actually decreased (Mummey et al. 2002). However, more long-term studies are required in order to understand the plant-microbe interaction during phytostabilization.

This dissertation focuses on determining the potential phytostabilization capabilities of a native drought tolerant halophyte, *Atriplex lentiformis*, and characterizing the microbial community of the Klondyke site that will affect phytostabilization. Results from this research will help future phytostabilization trials in arid and semiarid mine tailings sites. Also, knowledge of the microbial community will provide beneficial information for both evaluating and implementing revegetation of mine tailings.

Literature Review: Phytostabilization of Mine Tailings in Semiarid/Arid Environments – A Review of an Emerging Remediation Technology

Introduction

Mine tailings disposal sites from either inactive or abandoned mine sites are prevalent throughout semiarid and arid regions throughout the world, including western North America, South America, Spain, India, South Africa, and Australia (Munshower 1994; Tordoff et al. 2000). The global impact of mine tailings disposal sites is enormous; the United States estimates greater than 500,000 sites, Chile 800 sites, Australia 600 sites, and Mexico is affected by 27.1 million hectares of mining activity (Munshower 1994; USEPA 2004; Gonzalez & Gonzalez-Chavez 2006). Unreclaimed mining sites generally remain unvegetated for tens to hundreds of years, and exposed tailings can spread over tens of hectares via eolian dispersion and water erosion (Munshower 1994; Warhurst 2000). In arid and semiarid environments, tailings are a major source of dust that contaminate nearby communities and sensitive environmental areas (Schwegler 2006; Gonzalez & Gonzalez-Chavez 2006). For example, windblown tailings have contaminated nearby agricultural areas and increased metal concentrations in forage material, agricultural crops, and groundwater, resulting in increased human exposure (Castro-Larragoitia et al. 1997; Gonzalez & Gonzalez-Chavez 2006).

Mine tailings, or mill tailings, are the materials remaining after extraction and beneficiation of ores. What prevents the natural revegetation of mine tailings? It is generally a combination of factors beginning with metal toxicity. Tailings are

characterized by elevated concentrations of metals such as As, Cd, Cu, Mn, Pb, and Zn (1 g kg^{-1} to 50 g kg^{-1}). Further, tailings contain no organic matter or macronutrients, and usually exhibit acidic pH although some tailings may be alkaline (Johnson & Bradshaw 1977; Krzaklewski & Pietrzykowski 2002). For these reasons, tailings remain without normal soil structure, and support a severely stressed microbial community (Southam & Beveridge 1992; Mendez et al. 2007). In arid and semiarid regions, plant establishment on mine tailings is further impeded by a number of physicochemical factors including extreme temperatures especially at the tailings surface, low precipitation, and high winds. These factors help contribute to the development of extremely high salt concentrations at 0.1 to 22 dS m^{-1} due to high evaporation and low water infiltration (Munshower 1994).

Emerging Issues

Disposal of mine wastes historically involved either returning the materials to the mining site, disposing into the ocean, a stream, or lake, or placing them into a receiving pond. Today, surface containment of tailings within embankments remains popular. In 1995, it was estimated that on an annual basis over 700 million-kg of metals in mine tailings were disposed on land (Warhurst 2000). Alternatively, tailings may be returned to the mine (in-pit storage or backfilling) or mixed with coarse mine waste (co-disposal). In arid regions, dry-stacking facilities are most common wherein tailings are dried, spread out, and compacted. However, they remain unstable and subject to eolian dispersion and water erosion with the potential to contaminate nearby communities (Engels & Dixon-Hardy 2004). While some countries mandate mining companies to remediate or contain

tailings piles, others have no such requirements and still allow dumping of mine tailings into bodies of water, thus escalating the existing problem of thousands of abandoned mine tailings sites worldwide (Munshower 1994; Coates 2005).

The popularity of aboveground impoundments for mine tailings storage is increasingly problematic in arid and semi-arid regions. Prevention of wind erosion by surface-wetting is not practical in arid environments, especially after closure of mining operations. Therefore, tailings are a significant source of air pollution in the form of particulate matter measured in fractions of <10 μm (PM_{10}) and <2.5 μm ($PM_{2.5}$) in aerodynamic diameter (Schwegler 2006). Short-term exposure to particulates ($PM_{2.5/10}$) can lead to premature death in people with heart or lung disease, respiratory conditions, and decreased lung function, while long-term exposure to fine particles can accelerate lung cancer and cause chronic respiratory disease in children (USEPA 2006). Measurements of $PM_{2.5/10}$ are monitored in recently established mining operations in some countries but are not monitored for abandoned mine tailings disposal sites despite a link to respiratory health problems and the proximity of disposal sites to human populations (Schwegler 2006).

Conventional remediation

Conventional technologies for remediation of mine tailings have focused on physical and chemical stabilization. Physical stabilization entails covering mine waste with an innocuous material, generally waste rock from mining operations, topsoil from an adjacent site, or a clay capping, to reduce wind and water erosion. These solutions are

often temporary in nature due to the impermanence of the capping process (Johnson & Bradshaw 1977). For example, clay caps in arid environments crack from the wetting-drying cycles and poor consolidation of the tailings due to its high salinity (Swanson et al. 1997; Newson & Fahey 2003). Chemical stabilization aims to prevent wind and water erosion using a chemical agent such as a lignin sulfonate or a resinous adhesive to form a crust over the tailings, also a temporary stabilization technique since these crusts can eventually fail (Tordoff et al. 2000). Recently, reprocessing historic tailings materials using more advanced technologies to reduce metal concentrations and toxicity has been considered and is economical in some cases (Warhurst 2000). However, the tailings material is still left after reprocessing and must be stabilized in some way. In general, traditional remediation techniques range from approximately US \$30-300 m^{-3} (Cunningham et al. 1995). An emerging remediation technology, phytostabilization, can reduce this cost to an estimated US \$0.40-11.00 m^{-3}, and stabilize tailings (Ford & Walker 2003).

Phytostabilization for remediation

Phytostabilization creates a vegetative cap for the long-term stabilization and containment of the tailings. The plant canopy serves to reduce eolian dispersion while plant roots prevent water erosion, immobilize metals by adsorption or accumulation, and provide a rhizosphere wherein metals precipitate and are stabilized. Unlike phytoextraction, or hyperaccumulation of metals into shoot/root tissues, phytostabilization primarily focuses on sequestration of the metals within the rhizosphere

but not in plant tissues (Fig. 1). As a result, metals become less bioavailable and livestock, wildlife, and human exposure is reduced (Munshower 1994; Cunningham et al. 1995; Wong 2003).

Although phytostabilization of mine tailings sites in semiarid/arid regions has been experimented with by mining companies, documentation of this remediation technology only occasionally appears in published literature and so general understanding of this technology is limited. In this review we address the current knowledge of phytostabilization in semiarid/arid environments as well as potential problems that determine the long-term success of this technology.

Phytostabilization of mine tailings in arid and semiarid environments

Phytostabilization of mine tailings in arid and semiarid environments involves the use of drought-, salt- and metal-tolerant plants for immobilization of heavy metals in the tailings substrate. In theory, metal bioavailability (and hence toxicity) will decrease as plants facilitate the precipitation of metals to less soluble forms, enhance reduction of metals, complex metals with organic products, sorb metals onto root surfaces, and accumulate metals into root tissues (Cunningham et al. 1995; Wong 2003). Furthermore, the presence of plants in mine tailings enhances the heterotrophic microbial community which may in turn, promote plant growth and participate in metal stabilization (Mummey et al. 2002; Glick 2003; Wong 2003; Mendez et al. 2007). The ultimate objective for successful phytostabilization is the long-term succession of the plant community in mine

tailings to promote soil development processes, microbial diversity, and finally, to restore soil ecosystem functions to a state of self-sustainability.

Candidate requirements for phytostabilization

Phytostabilization of mine tailings in arid environments requires plants that are drought, metal, and salt tolerant and do not hyperaccumulate metals of concern into shoot tissues. Therefore, candidates for phytostabilization should ideally be native to the area in which the mine tailings are found since they have evolved survival mechanisms appropriate to the harsh climate of arid and semiarid environments. A secondary but also important consideration is that the use of native plants avoids introduction of nonnative and potentially invasive species that may result in decreasing regional plant diversity. To date, many field trials have not taken advantage of native plant diversity, resulting in poor plant colonization and soil conditions.

Arid soils are often saline since evaporation rates exceed rainfall and natural salts originate from saline rainfall, unweathered minerals, and fossil salts. In mine tailings operations within arid environments, hypersaline groundwater is used in the beneficiation process and recycled throughout ore-processing. As tailings dry, salt crusts form on the surface (Munshower 1994; Newson & Fahey 2003). Therefore, halophytes (salt-tolerant plants) are especially valuable in phytostabilization. Members of the Chenopodiaceae family, specifically *Atriplex* spp., are highly salt tolerant and serve as pioneer species on mine tailings in semiarid western Australia and are used in revegetation of mine tailings in the western U.S. (Glenn et al. 2001; Jefferson 2004). Other halophytic shrubs

recommended in the western U.S. are creosote bush (*Larrea tridentate* DC., Zygophyllaceae) and desert broom (*Baccharis sarothroides* Gray, Asteraceae). Also, leguminous trees that serve as a nitrogen supply such as *Acacia* spp. and *Prosopis* spp. have been reported as successfully colonizing mine tailings in the western U.S. (Day et al. 1980).

Plants used for phytostabilization must be metallophytes, metal tolerant plants, but ones that do not accumulate or limit metal accumulation to root tissues. Although metallophytes have developed mechanisms to impede translocation of metals in the aboveground plant mass, there may still be an excessively high concentration of metals in the shoot material. There are several ways to measure and express metal accumulation in plants. These include: bioconcentration factor (BF) = (total element concentration in shoot tissue/total element concentration in mine tailings); accumulation factor (AF), translocation factor (TF), or shoot:root (S:R) ratio = (total element concentration in shoot tissue/total element concentration in the root tissue). In general these values should not exceed a ratio of 1, which would indicate that the plant is useful for phytoextraction but should not be used in phytostabilization (Brooks 1998). Surveys of native plants colonizing mine tailings have provided promising information, especially plant families of colonizers with relatively low metal accumulation in aboveground tissues (Table 1).

There are also several guidelines that can be used to help evaluate metal toxicity issues that may arise during phytostabilization. The first is plant leaf tissue toxicity limits, which can help assess the long-term potential for plant establishment (Table 2). The second is, domestic animal toxicity limits which can be compared to aboveground

metal accumulation since foragers, including cattle and other wildlife, may consume these plants (Table 2) (Wood et al. 1995). Unfortunately, metal accumulation in field trials has not been thoroughly documented (Johansson et al. 2005). Thus, identification of suitable phytostabilization plant candidates and understanding their metal accumulation patterns is an area where additional research is critically needed for practitioners in the field.

Implementation of Phytostabilization

Seeds vs. transplants

In general direct seeding produces a more patchy vegetation cover than does the use of transplants. However, while the use of transplants produces better results, this approach is more labor intensive and this is one of the factors that must be taken into consideration for each site. Further, seeding can be successful as shown by a platinum mine tailings study in South Africa and a tin mine tailings site in Zimbabwe (Piha et al. 1995; Van Rensburg & Morgenthal 2004).

Amendments

Since the addition of topsoil amended with organic matter and nutrients is not economical for extensive mine tailings sites, organic amendments are generally used as a substitute. Organic amendments immediately help to decrease metal bioavailability, to provide a slow-release fertilizer, and to serve as a microbial inoculum. In addition, organic matter improves soil structure, reduces erosion, and increases infiltration. The

organic matter may be composed of wood chips, straw, biosolids, composted municipal waste, or manure (Munshower 1994). The carbon to nitrogen (C:N) ratio of the organic amendment should range from 12:1 and 20:1 to prevent high rates of organic matter decomposition and nitrogen consumption by the microbial community. For instance, uncomposted organic amendments such as woodchips contain a high C:N ratio; therefore, nitrogen may become immobilized and impede long-term plant establishment (Van Rensburg & Morgenthal 2004). The addition of commercial compost to mine tailings has been shown to enhance plant growth in greenhouse trials. In addition to pH levels increasing with the addition of compost, there is a decrease in the number of iron- and sulfur-oxidizers that are attributed to acid production and vegetation death in pyritic tailings (Southam & Beveridge 1992; Schippers et al. 2000; Schroeder et al. 2005; Mendez et al. 2007). Furthermore, compost can increase the water-holding capacity and cation exchange capacity of mine tailings (Munshower 1994; Mendez et al. 2007). Similar to compost, biosolids amendment can ameliorate the harsh conditions of mine tailings. For example, biosolids have successfully increased plant growth in a gold mine tailings field trial in New Zealand (Mains et al. 2006b). Anaerobically digested biosolids are preferred to aerobically treated biosolids because of higher nitrogen content and a greater enhancement of plant growth as tested in a copper mine tailings site (McNearny 1998). However, biosolids may contain phytotoxic levels of metals depending upon the source of the material (Munshower 1994). Furthermore, there are differing opinions on the acceleration of metal leaching by biosolids addition in arid environments (Pond et al. 2005).

Addition of inorganic fertilizers should be limited since native vegetation used for phytostabilization of mine tailings in arid environments tend to be adapted to low nutrients and tend to respond differently to fertilizer inputs (Piha et al. 1995). Furthermore, if organic amendments are added, there is likely a sufficient or near sufficient concentration of nutrients already present (Munshower 1994; Van Rensburg & Morgenthal 2004). One exception to this rule is the use of phosphorous fertilizers which may be necessary to alleviate phosphorous deficiency due to the formation of insoluble metal-phosphates, it is important to consider phosphorous fertilizers to alleviate phosphate deficiency. However, the addition of phosphorous amendments can increase arsenic uptake into plants as well as leaching in mine tailings because phosphate behaves chemically similar to arsenate (Mains et al. 2006a). In extremely acidic mine tailings, lime may be required to neutralize acidification; however, without organic matter addition the site may require continuous inputs of lime to maintain a pH >5 (Munshower 1994).

Irrigation

Although drought tolerant plants must be used in phytostabilization, initial irrigation is usually required to aid plant establishment. If seeds are directly sown into mine tailings, irrigation is especially crucial. Drip irrigation for at least 3 to 6 months or until plants become established has proven to be successful in revegetation of mine tailings (Tordoff et al. 2000; Williams & Currey 2002). However, irrigation should be

limited for both cost and dependence of the plant community on the availability of water (Munshower 1994).

Evaluation of successful revegetation

The majority of phytostabilization studies in arid environments have focused on plant growth variables such as plant biomass and percent cover; however, other methods of evaluating successful revegetation must be taken into consideration for long-term rehabilitation of mine tailings. As previously mentioned, the aboveground plant biomass may be a source of metal exposure for foraging animals (Wood et al. 1995; Castro-Larragoitia et al. 1997). Therefore, domestic animal toxicity limits must be observed to prevent further contamination of the ecosystem. In addition, microbial communities have been largely ignored in field studies within arid environments (Mummey et al. 2002). Yet, the heterotrophic microbial community can be linked to plant establishment and plant-microbe interaction is important for promoting nutrient cycling, soil aggregation, and plant nutrient uptake (Bearden & Petersen 2000; Moynahan et al. 2002; Mendez et al. 2007). One critical aspect largely missing from the published literature is the long-term success of phytostabilization. Most studies are terminated after one to two years. Long-term information is needed to help evaluate the efficacy of phytostabilization in permanently reducing metal toxicity, in promoting plant succession, and in promoting the formation of soil structure and properties in tailings materials.

Discussion

Phytostabilization studies in arid environments are limited and have not yet addressed several important issues. For instance, plant metal accumulation has not been documented in the majority of field studies (Johansson et al. 2005). This is especially important in determining the long-term fate of plant establishment as well as the metal contaminants. In addition, metallophytes growing in mine tailings require established ranges of metal tolerance since trends in metal accumulation are species-specific. Therefore, a small pilot study of candidate species for the specific tailings site may be important in reducing costs and evaluating the selected combination of amendments and plant species.

Furthermore, the long-term fate of metals in revegetated tailings has not been thoroughly explored. Although organic amendments are favored for their immediate ability to decrease metal bioavailability, weathering and decomposition of organic residues may ultimately enhance metal mobility (Tordoff et al. 2000; Pierzynski et al. 2002; Pond et al. 2005). For instance, Pond et al. (2005) examined both circumneutral and acidic copper mine tailings from Arizona in a simulated weathering study. The addition of biosolids decreased the concentrations of copper, nitrate, and sulfate in leachates in the acidic tailings sample but slightly increased copper and arsenic leachate in the circumneutral sample compared to unamended tailings. In short term studies, metal availability based on plant metal accumulation may be deceiving. A study of a 20-year rehabilitated uranium site in Australia indicated an increase in metal mobility in the soil surface and an increase in plant metal accumulation (Lottermoser et al. 2005).

Therefore, long-term studies on the chemical state of tailings and plants are essential for determining phytostabilization success.

In summary, phytostabilization of mine tailings in arid and semiarid regions is promising. Studies have indicated that plant establishment on tailings piles is possible and helps to reduce erosion and enhance soil formation properties. Although it may not be possible to create an ecosystem equivalent to the surrounding uncontaminated area, phytostabilization success is dependent on the establishment of a self-sustaining biological cap. Therefore, long-term studies are necessary. Future research and evaluation of phytostabilization in arid and semiarid regions will require a thorough consideration of plant physiology, nutrient dynamics, soil chemistry, and microbiological components of the soil system.

Table 1. Plant families of potential phytostabilization candidates.

Plant family[a]	Metal contaminants	Location	Comments and references
Anacardiaceae	Cu	Skouriotissa mine, Cyprus	Field study using chicken fertilizer and 1:1 soil and mine waste (Johansson et al. 2005)
	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)
Asphodelaceae	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)
Asteraceae	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)
	As	Chihuahua, Mexico	Plant survey (Flores-Tavizon et al. 2003)
	Ag, As, Cd, Cu, Pb, Zn	San Bartolome, Ecuador	Plant survey (Bech et al. 2002)
Chenopodiaceae	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)
	As, Cu, Mn, Pb, Zn	Klondyke, Arizona, U.S.A.	Greenhouse study using compost (Mendez et al. 2007)
	As, Hg, Mn, Pb	Boston Mill Site, Arizona, U.S.A.	Field study (Rosario et al. 2007)
Euphorbiaceae	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)
Fabaceae	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)
Plumbaginaceae	Cu, Pb, Zn	Cartega-La Union Mining District, Spain	Plant survey (Conesa et al. 2006)
Poaceae	Cu, Pb, Zn	Cartega-La Union Mining District, Spain	Plant survey (Conesa et al. 2006)
Polygonaceae	Cd, Cu, Mn, Pb, Zn	Zacatecas, Mexico	Plant survey (Gonzalez & Gonzalez-Chavez 2006)

[a] Plant families listed contain native species documented in the respective paper with plant metal accumulation in aboveground biomass that does not exceed domestic animal toxicity limits (USDA-NRCS 2005).

Table 2. Metal toxicity limits.

Toxicity Index	**As**	**Cd**	**Cu**	**Mn**	**Pb**	**Zn**
				(mg kg^{-1})		
Domestic animal toxicity limits[a]	30	10	40	2,000	100	500
Plant leaf tissue toxicity limits[b]	5 - 20	5 – 30	ND[d]	400 -1,000	30 – 100	100 – 400
Soil plant toxicity levels (SPL)[c]	15	3	200	3,000	100 - 500	400

[a] Based on maximum tolerable levels for cattle (USDA-NRCS 2005).
[b] Based on mean values of toxic levels of metals accumulated in agricultural crops (Kataba-Pendias & Pendias 2001).
[c] Based on total metal concentrations that are generally toxic to plant growth (Munshower 1994; Mulvey & Elliott 2000; Kataba-Pendias & Pendias 2001).
[d] Toxicity values for Cu have not been determined (ND).

Figure 1. Klondyke mine tailings site in Klondyke, Graham County, Arizona. (Photo by Monica O. Mendez, 2005).

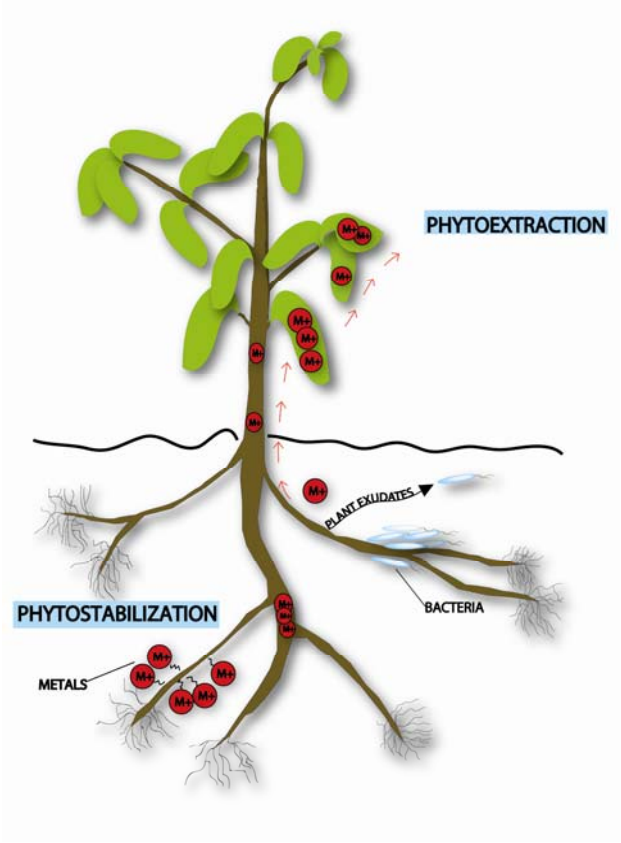

Figure 2. Phytoremediation of metals includes phytostabilization and phytoextraction. (Illustration by Monica O. Mendez, 2003).

CHAPTER 2

PRESENT STUDY

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the most important findings in these papers.

Summary

Objective 1. The objective of the first study presented in this dissertation (Appendix A) was to determine the level of compost addition required for *Atriplex lentiformis* in an acidic lead-zinc mine tailings site, metal accumulation in shoot tissues, and the effect of plant establishment on the microbial community. Physicochemical characteristics of the mine tailings, controls, and all treatments were characterized. Required compost addition was determined by evaluating seed germination and plant growth in compost-amended tailings (75, 85, 90, 95, and 100% tailings) and comparing these variables to results from plants grown in an offsite and compost control. Two mine tailings samples were evaluated: an extremely acidic (K4, pH 3) and moderately acidic (K6, pH 6) mine tailings samples. Additionally, the microbial community was examined by monitoring numbers of bacteria in the initial bulk treatments and post-harvest in both bulk treatment and rhizosphere samples by MPN analysis of the autotrophic iron- and sulfur-oxidizers along with heterotrophic plate counts.

Physicochemical characteristics of the Klondyke mine tailings are very different in comparison to both the offsite sample and compost control. Both tailings contained a low C:N ratio and cation exchange capacity (CEC) as well as minimal inorganic nutrient content comparison to the offsite and compost control. However, these factors increased with increasing amounts of compost addition. Furthermore, metal concentrations of As, Cu, Pb, and Zn in both tailings were higher than background levels, but Mn concentrations were extremely high in only the K6 tailings. Compost addition did not have a significant effect on the plant availability of metals except for Mn.

Plant data indicated that seed germination is not a valuable tool for determining plant establishment in stressed environments, while total dry mass provides a more useful measurement. Seed germination was only significantly inhibited in the extremely acidic (pH 2.7) 100% K4 treatment ($p < 0.05$), while growth of *A. lentiformis* was inhibited in the 95 and 100% tailings treatments despite pH differences. Furthermore, plants did not survive the 100% K4 treatment. *Atriplex lentiformis* did not preferentially accumulate metals of concern in the shoot tissues. In addition, metal concentrations in shoot tissues were generally within the range of domestic animal toxicity limits except for Zn.

Microbial data indicated that the mine tailings have a severely stressed heterotrophic population. The population estimate of iron- and sulfur-oxidizers was initially higher than the heterotrophic counts, but the heterotrophic bacterial numbers increased post harvest and were similar in the higher compost-amended treatments (75, 85, and 90% mine tailings). Also, heterotrophic counts in the rhizosphere were similar in all treatments with surviving plants and were not affected by compost addition.

This study demonstrates that *Atriplex lentiformis* is a good candidate for phytostabilization as it required only 10 to 15% compost addition and did not accumulate metals of concern. Also, the increase in heterotrophic counts along with both organic matter addition may be important in phytostabilization success but requires further investigation.

Objective 2. The objective of the second manuscript presented in this dissertation (Appendix B) was to characterize the microbial community of an acidic lead-zinc mine tailings site and compare it to an offsite sample. These data are important in understanding how the microbial community prevents plant establishment in acidic mine tailings that are targeted for phytostabilization. Currently, there has never been a thorough investigation of the microbial populations inhabiting acidic mine tailings,

Two samples from the Klondyke mine tailings were examined and compared to the offsite sample (OS, pH 8): an extremely acidic (K4, pH 3) and moderately acidic (K6, pH 6) sample. Bacterial community comparisons were conducted by enumeration of autotrophic iron- and sulfur-oxidizing bacteria as well as neutrophilic heterotrophic bacteria. In addition, the cultured and uncultured bacteria were identified by 16S rRNA gene analysis. Phylogenetic analysis of the bacteria was also employed for comparison of the communities.

Heterotrophic numbers were low in the extremely acidic K4 tailings (26 CFU g^{-1}), while heterotrophic numbers in the moderately acidic K6 tailings were much higher (1.5 x 10^5 CFU g^{-1}). However, numbers of autotrophic iron- and sulfur- oxidizers were

similar between the two samples and were not detected in the neutral offsite sample. Observed phylotype richness in the cultured heterotrophic communities also increased with pH. Similarly, with an increase in pH, the observed and estimated phylotype richness as well as diversity of the uncultured libraries increased.

Phylogenetic analysis of the uncultured library also demonstrated that diversity of the community was affected by pH. The number of phylogenetic groups increased from 4 groups in K4 to 7 and 11 groups represented in K6 and the offsite sample, respectively. In addition, the proportion of phylotypes closely related to obligate heterotrophic bacteria was highest in the offsite sample (88%) and relatively low (25%) in the K6 mine tailings, while no phylotypes were related to obligate heterotrophs in the K4 mine tailings.

Furthermore, some of the cultured autotrophic phylotypes and uncultured phylotypes of the K4 and K6 communities were similar and included a diverse group of acidophiles. Shared phylotypes between the mine tailings are closely related to acidophiles previously identified in acid mine drainage and mine tailings (*Acidithiobacillus ferrooxidans*, *Acidimicrobium*, *Leptospirillum ferrooxidans*, and *Sulfobacillus*). Additionally, a few phylotypes are closely related to bacteria not found in the uncultured bacterial community of mine tailings but reported in acid mine drainage communities. These bacteria were related to *Thiomonas*, *Acidimicrobium*, and two unidentified gamma-proteobacteria.

Therefore, the bacterial community of acidic mine tailings can be linked to abiotic factors and present a more diverse community of acidophiles than formerly concluded. This study provides a baseline for the monitoring of acidic mine tailings during a

phytostabilization study, as well as an examination of the bacteria that may be of concern for successful revegetation of mine tailings.

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APPENDIX A

PHYTOSTABILIZATION POTENTIAL OF QUAILBUSH FOR MINE TAILINGS: GROWTH, METAL ACCUMULATION AND MICROBIAL COMMUNITY CHANGES

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Abstract

Abandoned mine tailings sites in semi-arid regions remain unvegetated for extended periods of time and are subject to eolian dispersion and water erosion. This study examines the potential phytostabilization of a lead-zinc mine tailings site using a native, drought tolerant halophyte, quailbush [*Atriplex lentiformis* (Torr.) S. Wats.]. In a greenhouse study germination, growth, and metal uptake was evaluated in two compost-amended mine tailings samples, K4 (pH 3) and K6 (pH 6) at 75, 85, 90, 95, and 100% mine tailings, and two controls, off-site and compost. Microbial community changes were monitored by performing MPN analysis of iron- and sulfur-oxidizing bacteria as well as heterotrophic plate counts. Results demonstrate that germination is not a good indicator for phytostabilization since it was only inhibited in the unamended K4 treatment. Plant growth was significantly reduced in 95 and 100% mine tailings, while growth in 75, 85, and 90% treatments was similar to the off-site control. Quailbush accumulated elevated levels of the nutrient metals Na, K, Mn, and Zn in the shoot tissues; however, metal accumulation was generally below the domestic animal toxicity limit. Initially, autotrophic population estimates were four to six logs higher than heterotrophic counts, indicating extremely stressed conditions. However, post-harvest, heterotrophic bacterial counts increased to normal levels (~ 10^6 CFU g^{-1} dry tailings) and dominated the rhizosphere. Therefore, with compost amendment, quailbush has good potential as a native species candidate for phytostabilization of mine tailings in semi-arid environments.

Introduction

Mine tailings sites are extensive throughout semi-arid regions of the world including South Africa, Australia, North America, and Mexico (Munshower, 1993). Historically, waste products from ore processing were returned to the mine, placed into streams or lakes adjacent to the site, or discharged into a receiving pond. These practices have resulted in an emerging and extensive problem for the following reasons. Metals such as Cu, Fe, Zn, Ni, Pb, Cd, and the metalloid As are present in tailings material at concentrations that range from as low as 1 to 50 g kg^{-1} in older sites (Bradshaw et al., 1978; Walder and Chavez, 1995; Boulet and Larocque, 1998). Mine tailings are usually characterized by the absence of organic matter, nitrogen, and phosphorus and by their neutral to low pH and high acid-producing potential (Wong et al., 1998; Krzaklewski and Pietrzykowski, 2002; Ye et al., 2002). Additionally, tailings piles are generally devoid of vegetation and have no soil structure (Munshower, 1993; Krzaklewski and Pietrzykowski, 2002). In semi-arid areas these problems are exacerbated by high salt content resulting from conditions wherein a high proportion of rainfall undergoes evaporation rather than infiltration into the tailings (Munshower, 1993). Finally, tailings support severely stressed microbial communities. Heterotrophic populations are low in number, while acidophilic autotrophic bacteria such as *Acidothiobacillus* spp. and *Leptospirillum ferrooxidans* thrive (Southam and Beveridge, 1992). In general, the physical, chemical, and biological characteristics of mine tailings interact to almost completely suppress seed germination and plant growth (Yang et al., 1997). As a result,

tailings are spread throughout the environment via eolian dispersion, water erosion, and leaching which can result in the formation of acid mine drainage.

The establishment of a permanent vegetative cap is recognized as a potentially cost-effective and ecologically sound approach to containment of mine tailings and for initiation of soil formation processes (Munshower, 1993; Brooks, 1998). In particular, there is interest in phytostabilization, a process wherein plants are established and function primarily to accumulate metals into root tissue or aid in their precipitation in the root zone (Cunningham et al., 1995). Use of native plants is a focus of this technology because they often demonstrate tolerance for local environmental conditions and provide a foundation for natural ecological succession. One of the largest cost factors associated with revegetation is the requirement for large amounts of organic amendments, e.g. compost or biosolids. These amendments mitigate the toxicity of the tailings and plants fail to grow in their absence (Sabey et al., 1990; Ye et al., 2001; Brown et al., 2003).

In this study we evaluated quailbush for its ability to establish in extremely and moderately acidic lead-zinc mine tailings typically found in semi-arid areas. Quailbush is a perennial halophytic subshrub that is native to Arizona, California, Nevada, and Utah (USDA-NRCS 2005) and has been examined for use in the reclamation of salt-affected lands (Malik et al., 1991; Blank et al., 1998; Malcolm et al., 2003). Quailbush is considered drought tolerant and has previously been observed encroaching into historical mine tailings sites (USDA-SCS 1977; Booth et al., 1999; Arunachalam et al., 2004; Jefferson, 2004). In addition, *Atriplex* spp. have demonstrated accumulation of metals primarily in the roots, which is favorable for phytostabilization strategies (Williams et al.,

1994; Jordan et al., 2002). The objectives of this study were to determine (i) the minimum level of compost required for establishment of quailbush in lead-zinc tailings by evaluating seed germination and seedling growth; (ii) metal accumulation in shoot tissue during growth of quailbush; and (iii) the impact of plant establishment on the microbial community as measured by enumeration of autotrophic and heterotrophic bacterial community before and after planting.

Materials and Methods

Mine Tailings Site

The Klondyke mill site is located on the eastern bank of Aravaipa Creek in the transition zone between the riparian corridor and the semi-arid uplands in the Aravaipa Valley, Graham County, Arizona. The riparian corridor is classified as a broadleaf riparian forest community with mesquite in the uplands and cotton wood, ash, sycamore, and alder in the riparian corridor. From 1948 to 1958, the Klondyke site was primarily a Pb and Zn ore processing operation that disposed of approximately 100,000 metric tons of flotation tailings that have remained devoid of vegetation (Wilson, 1959). In 1993, erosion and runoff from the tailings piles was observed and the Arizona Department of Environmental Quality (ADEQ) found that levels of arsenic and lead in the tailings exceeded the Arizona Non-Residential Soil Remediation Levels (SRLs) of 10 mg kg^{-1} and 1200 mg kg^{-1}, respectively (ADEQ, 2001b; 2002). In addition, elevated levels of Cd and Pb were found in fish sampled from Aravaipa Creek downstream from the site (King

and Martinez, 1998). As a result, the site was placed on the Arizona Water Quality Assurance Revolving Fund (WQARF) Registry in 1998.

Sampling

Two samples (80 L) were collected from the Klondyke upper tailings pile: K4 from 28 cm to 53 cm below the surface (32°51'0" N and 110°20'34" W) and K6 from 21 cm to 42 cm (35°51'1" N and 110°20'33" W). An off-site control sample (OS) was taken from 17 cm to 28 cm below the surface of a vegetated area adjacent to the tailings pile at 32°51'3" N and 110°20'32" W. The compost (EKO Compost, Richland Turf Food, Inc., Plateville, CO) used in this study was a mixture of poultry manure, forest products, and recycled wood products. All materials were stored at 4°C and thoroughly mixed prior to use. The off-site control sample and compost were sieved with a 5 x 5 mm mesh screen.

Mine Tailings and Compost Analysis

For pH analysis, triplicate samples were air dried for 2 d and sieved through a 2-mm mesh screen. A 10-g aliquot was placed into a 50-mL centrifuge tube and 20 mL deionized water was added to achieve a 2:1 ratio of deionized water to soil (v:w). Each solution was shaken for 1 h, centrifuged for 5 min at 15,000 x g, and the pH of the supernatant was determined. Plant-available metals were analyzed using a DTPA (diethylenetriaminepentaacetic acid) extracting solution as described by Lindsay and Norvell (1978). The DTPA extraction was chosen based on its effective chelation of a mixture of plant micronutrients such as Fe, Zn, Cu, and Mn. In triplicate, an air-dried 10-

g aliquot of each sample was mixed with 20 mL of DTPA extracting solution at pH 7.30. The solution was shaken in a 125-mL flask at 150 rpms for 2 h, vacuum filtered through a Whatman no. 42 filter paper, and finally filtered through a 0.45-μm hydrophilic polyethersulfone membrane (Supor®-450, Pall Life Sciences, East Hills, NY). Samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for As, Cd, Cu, Fe, Mn, Pb, and Zn by The University of Arizona Superfund Basic Research Program's Hazard Identification Core (SBRP-HIC) using EPA method 6020 (USEPA 2004). For the remaining analyses, single composite samples of K4, K6, OS, and compost were oven dried at 105°C and sieved through a 2-mm mesh screen. These samples were then analyzed for texture by the hydrometer method; electrical conductivity (1:1 H_2O extraction); cation exchange capacity (CEC) by the sodium acetate method (Chapman 1965); plant-available PO_4-P by the ammonium bicarbonate method (Olsen et al. 1954); total organic carbon (TOC), total carbon, and total nitrogen by high temperature combustion; and total elements (As, Cd, Cu, Fe, K, Mn, Na, Pb, Zn). These analyses were conducted by the Water Quality Center (WQC) Laboratory (University of Arizona, Tucson, AZ) except the total elemental analysis for which samples were prepared by microwave acid digestion (EPA 3051; USEPA 2004) by the WQC Laboratory and then analyzed by ICP-MS by the SBRP-HIC. For the compost sample the C:N ratio and total nitrogen values were provided by Richland Turf Food, Inc., and used to calculate the TOC.

Germination and Plant Growth Study

Two experiments were performed to determine the minimum amount of compost required for growth of quailbush. Compost was selected to serve as a slow-release fertilizer and an organic amendment for both reduction of metal bioavailability and enhancement of heterotrophic microbial growth. Quailbush was selected because *Atriplex* spp. have performed well in disturbed and contaminated sites (Williams et al., 1994; Booth et al., 1999; Jordan et al., 2002; Arunachalam et al., 2004; Jefferson, 2004), it is native to the area as well as salt tolerant (Blank et al. 1998; USDA-NRCS 2005), and it performed similarly or better compared to other native species in a preliminary screening study.

In the first experiment, seed germination and plant growth were examined in four K4 and K6 tailings:compost mixtures (25, 50, 75, and 100% tailings by mass). Results of this study indicated equally good germination and growth of quailbush at all three compost levels tested. Therefore, a second experiment was performed to further titrate the level of compost required. In this experiment seed germination and plant growth were determined in five K4 and K6 tailings:compost mixtures (75, 85, 90, 95, and 100% tailings), as well as compost (100% CC) and the off-site sample (100% OS) alone.

For each experiment, quailbush seeds (Mistletoe-Carter Wholesale Seeds, Goleta, CA, USA) were sown in 12 x 8.5 x 3 cm plastic pots (with six 1.6-mm drainage holes) containing the various tailings or control treatments. Each treatment contained five replicates with 20 seeds per replicate (100 seeds per treatment). Seeds were irrigated with approximately 84 mL of tap water d^{-1} in a fiberglass greenhouse with temperatures

ranging from 24°C to 38°C. On Day 23, germination was quantified, and seedlings of similar size were transferred into 3.8 L pots (15.2 cm top diameter x 17.8 cm height x 12.7 cm bottom diameter) lined with fiberglass screen and containing the same tailings or control treatment. Fiberglass screen material prevented tailings from leaking and allowed water to drain. All pots were prepared and wetted 3 d prior to seedling transfer. Following transplantation, pots were irrigated with 360 mL of tap water d^{-1}. Each treatment was comprised of five replicates with one plant per pot. During the study, seedling height, number of leaves, and basal diameter of each plant were measured every 7 d. Plants were harvested 68 d after transplantation (Day 91 of the experiment) for determination of shoot and root dry mass and metal content.

Plant Dry Mass and Metal Analysis

At the end of the experiment (Day 91), plants were harvested for shoot and root dry mass measurements. The shoots were separated from the roots and placed in a pre-weighed paper bag. Root tissues were washed with tap water followed by a thorough rinse with distilled water to remove soil and particulate matter and then blotted with a paper towel and wrapped in a pre-weighed piece of aluminum foil. All samples were dried in a forced air oven at 65°C and weighed after three days to obtain the shoot and root dry mass.

Quailbush shoot tissue was analyzed for total metal (Na, K, Mn, Fe, Cu, Zn, As, Cd, and Pb) concentrations. Three plants from each treatment were selected for metal analysis. Plant material was dried at 65°C, ground with a Wiley Mill, and passed through

a 40-mesh (0.419 mm) screen. Shoot tissue was prepared by microwave acid digestion by the WQC Lab and analyzed by ICP-MS by the SBRP-HIC.

Enumeration of Heterotrophic Bacteria

Initial (before seed germination) and final (post-harvest) heterotrophic bacterial counts were measured for all treatments. Ten grams of each treatment were placed in a 250-mL jar containing 95 mL of Zwittergent extractant (8.5 g NaCl and 200 µl of 1% Zwittergent solution per liter), shaken vigorously for 2 min, serially diluted in triplicate, and then plated on R2A agar (Becton, Dickinson and Company, Sparks, MD) amended with 200 mg L^{-1} of cycloheximide to inhibit fungal growth. Plates were incubated for five days at 23^{o}C and then enumerated. Counts are reported as colony forming units (CFU) per gram dry weight of each sample.

For planted treatments, heterotrophic bacterial counts were also performed on rhizosphere samples at the end of the experiment. Three plants from each treatment were removed from the pots and all loose soil or tailings material was shaken off the roots. Roots along with adhering soil were immediately stored at 4^{o}C until processed. For each plant, 0.1 g of fresh root material was consolidated from 3 separate 2-cm root tip sections, 0.5 cm of the root-shoot transition region, and 1.5 cm of the main root axis. The roots were placed in 9.9 mL of 1X PBS, sonicated twice for 30 s, and serially diluted in 1X PBS (Ausubel et al., 1995). All treatments were plated in triplicate on R2A agar amended with 200 mg L^{-1} of cycloheximide, incubated for five days at 23^{o}C, and enumerated.

Enumeration of Autotrophic Bacteria

Initial and final counts of autotrophic bacteria, specifically iron- and sulfur-oxidizing bacteria, were assessed using a modification of the most probable number technique (Woomer 1994; Cochran 1950). Ten-gram samples of each treatment were placed into 250-mL jars containing 95 mL of Zwittergent extractant and shaken vigorously for 2 min. Each slurry was serially diluted from 10^{-2} to 10^{-8} in 4.5 mL with five replicates for each dilution. For the rhizosphere samples, the initial 10^{-1} dilution for each plant within a treatment was consolidated and serially diluted from 10^{-3} to 10^{-6} in 4.5 mL with five replicates for each dilution. All samples were inoculated into both iron- and sulfur-oxidizer enrichment media. Iron-oxidizers were grown in modified 9K minimal salts medium (MSM) containing per Liter: 3.0 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $Ca(NO_3)_2 \cdot 4H_2O$, and adjusted to a pH of 2.3 with 10 N H_2SO_4. The autoclaved 9K MSM was amended with filter sterilized $FeSO_4 \cdot 7H_2O$ at a final concentration of 33.3 g L^{-1} (Silverman and Lundgren, 1959; Southam and Beveridge, 1992). Sulfur-oxidizers were grown in modified Starkey's medium (pH 4.5) consisting of a basal nutrient medium (BNM) and a thiosulfate solution (Starkey, 1925; Knickerbocker et al., 2000). The BNM (900 mL) contained 0.3 g of $(NH_4)_2SO_4$, 3.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.33 g of $CaCl_2 \cdot 2H_2O$, and 180 µl of a 1% solution of $FeSO_4 \cdot 7H_2O$ adjusted to pH 2.3 with H_2SO_4. A 100-mL solution of sodium thiosulfate with 200 mM Na_2SO_3 (20 mM Na_2SO_3 L^{-1}) was autoclaved separately and added to the BNM.

After 47 d of incubation on a shaker at 180 rpm, positive and negative results for growth were documented. Positive results were based on a color change from yellow to orange for the iron-oxidizers and a change in turbidity as well as decrease in pH for the sulfur-oxidizers. Population estimates were calculated as described by Briones and Reichardt (1999) and reported as most probable number (MPN) g^{-1}.

Statistics

Statistical analyses were generated using SAS Version 9.0 of the SAS System for Windows (SAS Institute, 2002). All data were tested for normality prior to analysis. For cases of non-homogeneity of variances, data were log-transformed prior to analyses. Due to plant mortality within some of the treatments, the procedure for unequal sample size was used. The effect of compost addition on mean pH was examined within each mine tailings sample by employing a one-way ANOVA. For plant shoot metal concentrations, values were averaged over all treatments within a sample source and analyzed by a one-way ANOVA. Significant factor effects for both sample source and mine tailings concentration were determined using a two-way ANOVA followed by a one-way ANOVA to compare means for plant growth and microbial counts. For all analyses, significant differences between means at the $p < 0.05$ level were determined by employing the Tukey's studentized range test (Tukey's Honestly Significant Difference [Tukey's HSD]).

Results

Mine Tailings Analysis

The K4 and K6 tailings samples had sandy loam and silt loam textures, respectively, with either an extremely acidic (K4, pH 2.7) or a moderately acidic pH (K6, pH 5.7) (Table 1). Both tailings samples exhibited a low CEC, and had minimal inorganic nutrient content, little organic matter, and a low C:N ratio compared to the OS and CC samples. Compost addition generally improved the soil properties of the tailings by increasing the CEC, TOC, total N, and the C:N ratio (Table 1). EC increased slightly as well but not out of an acceptable range for quailbush. Additionally, pH significantly increased in the K4 mine tailings with each increasing level of compost ($F_{4, 10} = 1149$; $p < 0.0001$), while the pH of the K6 mine tailings significantly increased at the highest compost levels (K6-75 and K6-85; $F_{4, 10} = 9.74$; $p = 0.0018$).

Total As, Cu, Pb, and Zn concentrations were elevated in both tailings samples in comparison to background levels normally found in soil while Mn was higher than background only in the K6 sample (Table 2). Lead and zinc were also elevated in the off-site control sample likely due to eolian dispersion from the adjacent tailings piles. Plant-available metals were extremely low for As, Cd, Fe, and Pb in comparison to the total metal concentrations (0.01 to 2% of total metals) while Cu (6% of total metals) and Zn (13 to 18% of total metals) were slightly higher (data not shown). These values are similar to those reported for other lead-zinc mine tailings sites (Zhu et al., 1999; Ye et al., 2002). Manganese exhibited high availability in K4 (70% of total Mn) but only moderate availability in to K6 (5%) even though total Mn in K6 was 25-fold higher. In general,

plant-available metals increased in the order (mg kg^{-1}): Cd < As < Pb < Cu < Fe < Zn < Mn in the K4 tailings, and As < Cd = Fe = Pb < Cu << Mn << Zn in the K6 tailings. Compost addition had little impact on the plant availability of any metal except Mn (data not shown) which decreased from 70 to 40% of total Mn as compost levels increased in the K4 tailings and from 5.4 to 4.6% as compost was added to K6 tailings.

Germination

Germination of quailbush seeds (data not shown) was evaluated to help determine the stage at which mine tailings inhibit plant growth. Results demonstrated minimal difference in germination among all treatments with the exception of the unamended K4 sample where percent seed germination was significantly inhibited (16%, $F_{11, 48} = 3.37$, $p = 0.0017$) compared to amended treatments (K4-75, K4-85, K4-90, K6-85, and K6-95; 60 to 72%) or compost alone (57%).

Plant Growth and Dry Mass

Following germination, five seedlings of similar size from each treatment were transplanted into larger pots containing the same treatment and evaluated for plant growth. Results for mean height, mean number of leaves, and mean basal diameter (data not shown) were similar, collectively demonstrating that the mine tailings alone treatment significantly reduced the growth of quailbush ($F_{4, 40} > 16$, $p < 0.0001$). In fact, all plants in the K4-100 treatment died two weeks after being transplanted, while plants were severely stunted in the K6-100 treatment. In contrast to seed germination, significant

differences in growth of quailbush could be attributed to both sample source (K4, K6, OS, or CC) ($F_{3, 40} > 12$, $p < 0.0001$) and mine tailings concentration ($F_{4, 40} > 34$, $p < 0.0001$). However, there was no significant interaction between these factors ($F_{4, 40} < 2$, $p > 0.5$).

At the end of the experiment, plants were harvested and dried to determine the effect of mine tailings concentrations on the total dry mass of quailbush (Fig. 1). Both tailings materials significantly inhibited mean total dry mass of quailbush in the 95% and 100% tailings treatment. Plants in the K4-100 treatment all died within two weeks, while those surviving the K6-100 treatment produced extremely low plant mass. For the 95% treatments, the total dry mass in K4 and K6 was 6 and 13% of the OS dry mass, respectively. Growth of quailbush was similar to the off-site control at 75, 85, and 90% mine tailings concentration. Furthermore, growth was enhanced in some of the 75 and 85% mine tailings treatments compared to the off-site control. Although not significant, there was a 14 to 27% increase in total dry mass when quailbush was grown in K4-75, K4-85, and K6-75 compared to the OS sample.

Plant Metal Analysis

Plant metal accumulation in quailbush was examined to assess the metal tolerance and phytostabilization potential of this species. The monovalent cations K^+ and Na^+ were both taken up extensively into shoot tissues as is normal for this halophyte (Table 3). Lead, despite its high concentrations in the tailings was accumulated at very low levels. Patterns for accumulation in shoot tissues were examined in K4 and K6 treatments and

generally followed the order (excluding K and Na): Zn ≥ Fe ≥ Mn > Pb > Cu > As > Cd on a mass basis (Table 3). Thus, accumulation in shoots was selective with nutrient metals, particularly Mn and Zn, taken up preferentially over the three non-essential metals As, Cd, and Pb as well as Cu. Additionally, we observed a trend suggesting that as compost increased, shoot metal accumulation decreased (data not shown).

Microbial Counts

In order to assess the level of stress in the mine tailings treatments, the bacterial community was enumerated and compared. Microbial counts were conducted for autotrophic iron- and sulfur-oxidizing bacteria as well as heterotrophic bacteria in bulk samples prior to planting (initial) and in post-harvest (final) bulk and rhizosphere samples.

For both autotrophic iron- and sulfur-oxidizers, initial bulk counts in K4 and K6 tailings were between 10^5 and 10^6 MPN g^{-1} dry material. These counts were not impacted by the addition of compost to the tailings (Fig. 2). Comparing the K4 and K6 treatments, initial counts for iron-oxidizers were generally one log higher in the K6 treatments, whereas sulfur-oxidizers were ~ 0.5 log greater in the K4 treatments. No iron- or sulfur-oxidizers were present in the off-site or compost samples at the detection limit of 10^2 MPN g^{-1} dry material. Post-harvest autotrophic counts showed a 1 to 5 log reduction in iron-oxidizers and a 0.5 to 2 log reduction for sulfur-oxidizers across all samples. For post-harvest counts, compost addition further decreased autotrophic population estimates, particularly for iron-oxidizers which were not detected in either the

K4-75 or K6-75 treatments. Iron- and sulfur-oxidizers were not detected in any rhizosphere samples (data not shown).

Heterotrophic bacterial counts in initial bulk samples were significantly different ($F_{11, 24} = 4059$, $p < 0.0001$) among treatments (Fig. 3A). The unamended K4 and K6 tailings had low initial heterotrophic counts, 10 and 75 CFU g^{-1}, respectively, that were significantly different from each other and all other treatments ($p < 0.05$). The highest heterotrophic count was observed in the compost control treatment (1.4×10^8 CFU g^{-1}) while the off-site control had 8.0×10^4 CFU g^{-1}. The addition of compost significantly increased heterotrophic counts in all compost-tailings treatments to a level higher, in most cases, than the off-site control. For the initial bulk treatments, significant differences can be attributed to sample source ($F_{3, 24} = 4936$, $p < 0.0001$) and tailings concentration $F_{5, 24} = 6576$, $p < 0.0001$), but there was no significant interaction between these factors ($F_{3, 24} = 0$, $p = 1.000$).

For post-harvest bulk samples, heterotrophic counts were comparable to off-site control levels in all compost treatments (Fig. 3B). Specifically, final bulk heterotrophic counts in the 75 to 95% K4 and K6 tailings treatments averaged 2.6×10^6 CFU g^{-1} in comparison to 1.1×10^6 CFU g^{-1} for the OS treatment. For tailings alone, heterotrophic numbers remained significantly lower than all compost treatments ($F_{11, 24} = 67.03$, $p < 0.0001$) with 131 CFU g^{-1} in the K4 treatment (no plants survived) and 6.3×10^4 CFU g^{-1} in the K6 treatment (plants were severely stunted). Sample source ($F_{3, 24} = 25.16$, $p < 0.0001$) as well as tailings concentration ($F_{5, 24} = 113.5$, $p < 0.0001$) had a significant

effect on final bulk-planted heterotrophic counts with a significant sample source x tailings concentration interaction ($F_{4, 24} = 31.45$, $p < 0.0001$).

Finally, as expected, heterotrophic rhizosphere counts (Fig. 3C) were higher than both initial and final bulk heterotrophic counts ranging from 2.4×10^9 to 2.0×10^{10} CFU g^{-1} with no significant differences among treatments with surviving plants ($F_{11, 23} = 0.64$, $p = 0.7607$).

Discussion

The Klondyke tailings are similar in physicochemical properties to other lead-zinc mine tailings that have been studied for their impedance of plant growth (Wong et al., 1998; Krzaklewski and Pietrzykowski, 2002; Ye et al., 2002). The unamended K4 and K6 mine tailings samples did not support plant growth which was expected since the Klondyke tailings site has remained unvegetated for 48 years. There are many possible reasons why plant growth is suppressed. The K4 sample is extremely acidic and below the optimal plant growth range of pH 5.0 to 7.5 (Marschner, 1995). Although the K6 tailings sample is moderately acidic, it shares the following properties with the K4 sample that contributes to the unsuitability of the Klondyke site for plant growth: low CEC, low organic carbon content, almost undetectable phosphate and nitrogen content, high metal content, moisture stress, low heterotrophic counts, and high iron- and sulfur oxidizer counts (Stevenson and Cole, 1999). Thus, it was not surprising that even under greenhouse conditions with sufficient water, plants did not survive in unamended K4 tailings and only severely stunted plants survived in the unamended K6 tailings.

In terms of metals, both tailings samples contain total As and Pb that exceed the limits of for remedial action in Arizona non-residential areas (10 and 1200 mg kg^{-1} respectively), and thus are considered to be hazardous waste (ADEQ, 2002). In regard to phytotoxicity, several metals at the Klondyke site exceed reported soil plant toxicity levels (SPL). This is true for Pb (SPL = 100 to 500 mg kg^{-1}) in the K4, K6, and OS samples, for As and Cu (SPL = 15 mg kg^{-1} and 200 mg kg^{-1}, respectively) in the K4 and K6 samples, for Zn (SPL = 400 mg kg^{-1}) in the K6 and OS samples, and for Cd and Mn (SPL = 3 and 3000 mg kg^{-1} respectively) in the K6 sample (Munshower, 1993; Mulvey and Elliott, 2000; Kataba-Pendias and Pendias, 2001). The reported values are not specific for quailbush but provide a general reference for plant health.

Establishment of Quailbush in Mine Tailings

This study suggests that germination is not a good indicator for quailbush establishment in mine tailings since germination results were similar for all treatments except the extremely acidic unamended K4 sample. These results are similar to the few studies that have examined seed germination in lead-zinc mine tailings. While none of the studies used *Atriplex* spp., they showed that germination in mine tailings is related to a threshold pH of 3.0 (Yang et al., 1997; Ye et al., 2000; Ye et al., 2001; Ye et al., 2002). Specifically, in the absence of an amendment, germination of Bermuda grass (*Cynodon dactylon* [L.] Pers.) and tall wheatgrass (*Agropyron elongatum* [Host] Beauv.) was not significantly inhibited if the mine tailings had a pH > 3 (Ye et al., 2000). Taken together,

these results suggest that seed germination is dependent on the pH of the mine tailings sample and is impacted only at very low pH.

In contrast, growth data indicate that establishment of quailbush in the Klondyke mine tailings requires an organic amendment such as the compost used in this study. Although establishment occurred with 5% compost, quailbush required at least 10% compost to produce growth statistically similar to that in the off-site control. Other studies have similarly demonstrated stunted plant growth of grasses and shrubs in lead-zinc tailings alone compared to amended tailings material and minimal survival past the seedling stage (Yang et al., 1997; Ye et al., 2000; Ye et al., 2001; Shu et al., 2002). For example, the height and biomass of four-wing saltbush (*Atriplex canescens* [Pursh] Nutt.) grown in an acidic copper mine spoil sample alone was stunted compared to sludge-amended spoil (Sabey et al., 1990). Similarly, Hennessy (1985) found that four-wing saltbush grown in an amended mine spoil sample at 50% v/v produced a normal height compared to plants grown in topsoil alone, and its biomass even exceeded that in the topsoil.

While this study did not identify mechanisms of establishment, compost addition increased the organic matter and nutrient content as well as CEC, pH, and heterotrophic bacteria in the tailings. In general, compost addition to mine tailings is known to increase water-holding capacity, CEC, and help to improve the structure of mine tailings by forming stable aggregates (Ye et al., 1999; Stevenson and Cole, 1999; Schippers et al., 2000; Krzaklewski and Pietrzykowski, 2002). Furthermore, added compost can sorb and stabilize metals thereby decreasing their bioavailability (Stevenson and Cole, 1999),

although we observed little change in metal bioavailability as measured by DTPA extraction in this study.

Quailbush as a Candidate for Phytostabilization

Phytoremediation of metal-contaminated soils can focus on extraction (hyperaccumulation) of metals into plant tissues, phytoextraction, or the stabilization of metals in the plant rhizosphere and roots, phytostabilization. In phytostabilization shoot accumulation of metals is undesirable as these plants may eventually serve as forage material. Thus, information on plant tissue metal accumulation in mine tailings environments is important since *Atriplex* spp. are sometimes the preferred grazing food of livestock or wildlife living in the area of remediated mine tailings sites (Wood et al., 1995). Only a few studies have investigated the accumulation of metals in the shoot tissues of *Atriplex* grown on mine tailings (Sabey et al., 1990; Jordan et al., 2002). Furthermore, only a single study has observed metal accumulation trends in *Atriplex* spp. while grown in mine tailings with organic amendments (Sabey et al., 1990). From the plant metal analysis results of quailbush grown in the Klondyke mine tailings, this species can be considered metal tolerant as well as a good candidate for phytostabilization strategies.

Of the nine metals measured, quailbush accumulated high levels of K and Na, into shoot tissues (Table 3). This was expected since *Atriplex* spp. are commonly found in saline soils (Osmond, 1980; Malik et al., 1991; Blank et al., 1998) and is not of concern for foraging animals. Quailbush also shoot accumulated some metals to levels of concern

for plant growth reaching reported plant leaf tissue toxicity limits for Mn (400 to 1,000 mg kg^{-1}), Pb (30 to 100 mg kg^{-1}), and Zn (100 to 400 mg kg^{-1}) (Table 3). However, this does not seem to have impacted growth in most cases suggesting that quailbush is metal tolerant. Shoot tissue metal concentrations are also of concern with respect to domestic animal toxicity limits. A recent National Research Council report (2005) indicates that these limits are 400 to 2,000 mg kg^{-1} for Mn, 30 mg kg^{-1} for Pb, and 500 mg kg^{-1} for Zn. In examining the data, it appears that quailbush metal accumulation exceeded these limits in some cases, particularly for Zn. However, it is unlikely that the mine tailings site would provide the only forage for wildlife in the area. Thus, it may not be critical (or possible) to use plants that will never exceed the domestic animal toxicity limits in shoot materials.

The Microbial Community as an Indicator of Plant Establishment

This appears to be the first study to have measured both autotrophic and heterotrophic microbial numbers in bulk soil and rhizosphere samples during the revegetation of a tailings site. Autotrophic iron- and sulfur-oxidizers were enumerated because of their ability to create an acidic environment in the tailings and impede revegetation (Schippers et al., 2000). In this study, the initial presence of iron- and sulfur-oxidizers served as an indicator of an acidic, disturbed environment. This was confirmed by the measured acid-generating potential at the site which was extremely high with an acid neutralization potential to acid generating potential ratio of 0.01 (ADEQ, 2001a). Enumeration of the heterotrophic community, which is dependent on available

organic matter and is sensitive to environmental stressors, served both as an indicator of disturbance (low initial numbers) as well as an indicator of improvement of the mine tailings for plant growth (high post-harvest numbers). Although we recognize that culturable techniques are limited in assessing the total microbial community, they can serve as a comparison between the treatments for inferring soil health.

Other studies have shown similar results either measuring heterotrophic numbers during revegetation or characterizing both heterotrophs and autotrophs in bulk tailings. For example, Mummey et al. (2001) and Moynahan et al. (2002) linked increased heterotrophic numbers and biomass to normal plant growth in the revegetation of mine tailings. Southam and Beveridge (1992, 1993) and Schippers et al. (2000) have shown that unamended bulk tailings contained high numbers (up to10^6 MPN g^{-1} dry tailings) of iron and sulfur-oxidizing bacteria while heterotrophic bacteria ranged from as low as 10^1 to 10^5 CFU g^{-1}.

The results of this study demonstrate that the composition of the microbial community in a disturbed environment like mine tailings is an important indicator of the extent of disturbance and the potential success of a remediation strategy such as phytostabilization. For highly disturbed sites, one impact of compost addition is the immediate infusion of a substantial heterotrophic microbial community that is requisite for plant growth and long-term ecosystem health. Heterotrophic bacteria, as well as fungi, are required for a number of critical functions: organic matter cycling, formation of soil aggregates, and enhanced nutrient uptake in metal-contaminated environments (Bearden and Petersen, 2000; Moynahan et al., 2002). Also, bacterial involvement in

redox reactions can decrease metal availability as has been demonstrated with Pb (Blake et al., 1993). Garcia-Meza et al. (2006) demonstrated a reduction in exchangeable Cu, Mn, Pb, and Zn with direct inoculation of tailings with bacteria, as well as an increase in organic matter. In addition, increased plant biomass, enhanced nutrient uptake, and reduced metal accumulation has been documented in plants grown in inoculated mine tailings (Carrillo-Castaneda et al., 2003; Petrisor et al., 2004).

Conclusion

Quailbush is a good candidate for phytostabilization of mine tailings in semi-arid regions of the US Southwest and northern Mexico. Organic matter amendment up to 15% by mass may be required depending on the extent of pH, metal, and microbial community stress that exists in a given site. Attributes of quailbush include its status as a halophyte, its metal tolerance, and the fact that it does not hyperaccumulate toxic metals such as As, Cd, or Pb. This study demonstrates that microbial community composition can be used to indicate the potential for and success of a mine tailings revegetation. Indicators to look for include an increase in heterotrophic counts to "normal" levels of approximately 10^6 CFU g^{-1} and a decrease in iron- and sulfur-oxidizers to undetectable levels.

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Table 1. Physicochemical characteristics of Klondyke mine tailings samples and compost.

TRTMT†	Soil texture			pH‡	EC	CEC	PO_4-P	TOC§	Total N	C:N
	Sand	Silt	Clay							
		%			dS m^{-1}	$cmol_c$ kg^{-1}		g kg^{-1}		
					K4					
K4-75	69	21	11	6.3a	7.4	11	0.10	19	2.2	9
K4-85	65	23	12	4.4b	6.5	13	0.11	15	1.8	9
K4-90	64	25	12	4.2c	5.2	10	0.08	9.5	1.1	9
K4-95	62	26	13	3.4d	5.1	8	0.05	5.3	0.6	9
K4-100	58	31	11	2.7e	5.3	6	< 0.01¶	0.4	< 0.2	< 2
					K6					
K6-75	60	25	15	6.5a	8.6	14	0.09	20	2.1	10
K6-85	59	27	14	6.3ab	8.9	12	0.09	11	1.3	9
K6-90	64	21	15	5.8abc	5.6	8	0.08	9.2	1.0	9
K6-95	66	22	12	5.7bc	4.7	7	0.03	4.4	0.5	9
K6-100	39	51	10	5.7c	3.5	5	< 0.01	0.3	< 0.2	< 2
					Controls					
OS	38	45	17	7.7	1.2	25	0.03	18	1.8	10
CC	ND#	ND#	ND#	8.3	7.5	54	< 0.01	270	11	25

† TRTMT, Treatment. Treatment designations are as follows: K4 (pH 2.7); K6 (pH 5.7); OS (offsite control); CC (compost control); number following K4 and K6 are percent mine tailings.

‡ Within the pH column and mine tailings sample, different letters for each mean (n = 3) represent a significant difference at $p < 0.05$ (Tukey's HSD).

§ TOC, Total organic carbon.

¶ Values preceded by "<" indicate detection limits.

ND, Not determined.

Table 2. Total metal concentrations in the Klondyke mine tailings and compost samples.

Samples†	As	Cd	Cu	Fe	Mn	Pb	Zn
				mg kg^{-1}			
K4	62	< 0.1‡	671	38 100	185	5300	366
K6	72	4	792	29 300	4840	5010	3760
OS	< 0.1	< 0.1	140	21 800	1010	781	844
CC	< 0.1	< 0.1	35	5 650	411	18	209
Background§	1 - 50	0.01 – 0.7	2 - 200		20 – 3000	2 - 200	10 – 300

† Sample designations are as follows: K4 (pH 2.7); K6 (pH 5.7); OS (offsite control); CC (compost control).
‡ Values preceded by "<" indicate detection limits.
§ Background ranges from Swaine (1955).

Table 3. Total metal concentrations of quailbush shoot tissues when grown in mine tailings:compost mixtures.

TRTMT†	As‡	Cd	Cu	Fe	K	Mn	Na	Pb	Zn
					mg kg^{-1}				
K4	1.5 ± 0.4a	0.33 ± 0.37a	28 ± 9a	330 ± 171a	55 700 ± 18 000a	395 ± 439a	16 800 ± 8 890a	44 ± 31a	550 ± 287a
K6	0.5 ± 0.14b	0.49 ± 0.45a	23 ± 8a	251 ± 192ab	52 000 ± 18 400a	171 ± 149a	20 200 ± 10 000a	28 ± 31ab	637 ± 305a
OS	0.1 ± 0.0b	0.03 ± 0.04a	17 ± 0a	103 ± 22b	57 600 ± 9 190a	94 ± 7a	10 100 ± 953a	7 ± 1b	126 ± 20b

† TRTMT, Treatment. Treatment designations are as follows: K4 (pH 2.7); K6 (pH 5.7); OS (offsite control).
‡ Within columns, different letters for each mean ± SD (n = 3) represent a significant difference at $p < 0.05$ between means for all treatments within each sample source.

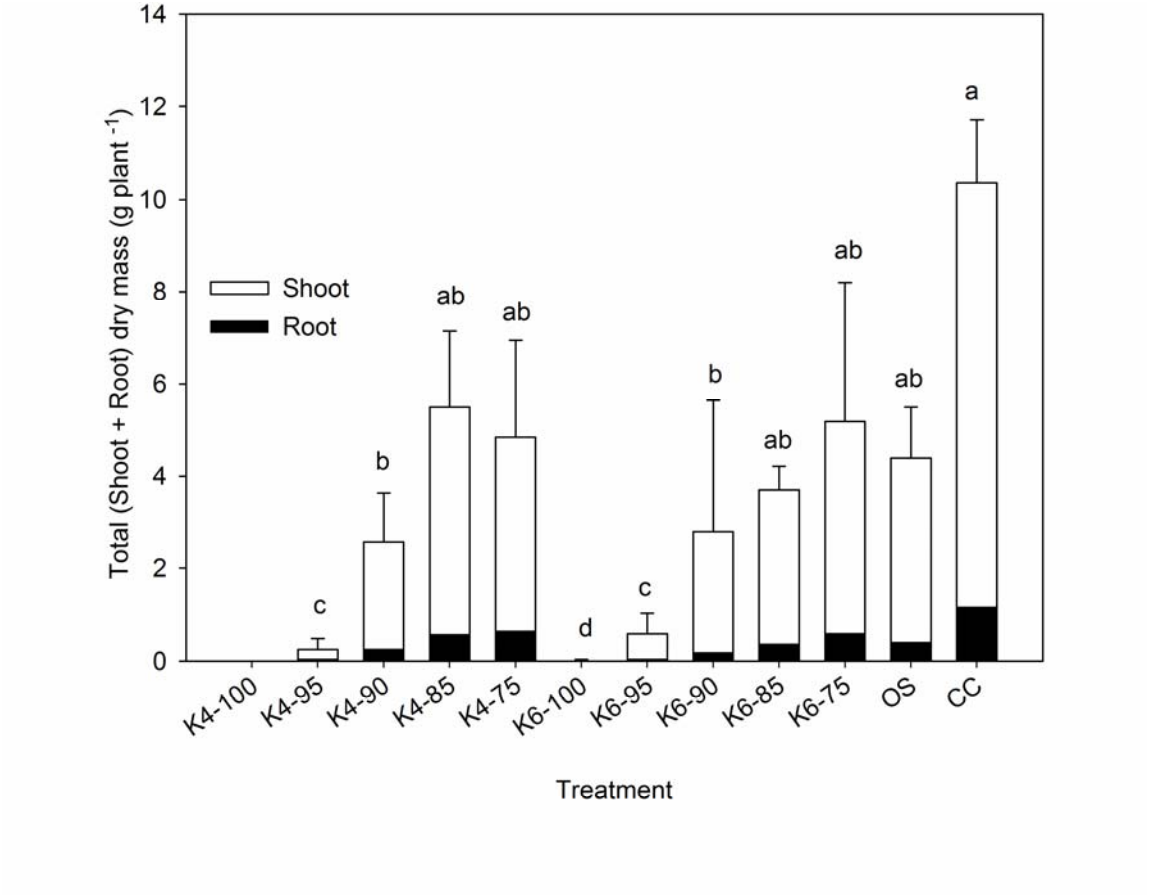
Appendix A Figure Legends

Figure 1. Final mean total (shoot + root) dry mass of quailbush grown in mine tailings:compost mixtures (mean +1 SD). Treatment designations are as follows: K4 (pH 2.7); K6 (pH 5.7); OS (offsite control); CC (compost control); number following K4 and K6 are percent mine tailings. A one-way ANOVA determined there were significant differences between treatments ($p < 0.0001$). All plants in the K4-100 treatment died; therefore, this data was not included in the analysis. Means with different letters are significantly different at $p < 0.05$ (Tukey's HSD test).

Figure 2. Initial and final population estimates (most probable number [MPN] g^{-1}) of autotrophic iron- and sulfur-oxidizers in bulk mine tailings:compost mixtures. Treatment designations are as follows: K4 (pH 2.7); K6 (pH 5.7); OS (offsite control); CC (compost control); number following K4 and K6 are percent mine tailings. Several treatments were undetectable (UD) at the 10^{-2} dilution level. Bars represent the upper and lower limit at a 95% confidence limit ($p = 0.05$).

Figure 3. Initial bulk (A), final bulk-planted (B), and rhizosphere (C) heterotrophic bacterial counts (colony forming units [CFU] g^{-1}) in mine tailings:compost mixtures (mean +1 SD). Treatment designations are as follows: K4 (pH 2.7); K6 (pH 5.7); OS (offsite control); CC (compost control); number following K4 and K6 are percent mine tailings. A one way ANOVA determined there were significant differences between treatments for initial mean bulk and final mean bulk-planted heterotrophic counts ($p < 0.0001$), but there were no significant differences for mean rhizosphere heterotrophic counts ($p = 0.7607$). All plants in the K4-100 treatment died; therefore, rhizosphere counts were not determined (ND) for this treatment. Means with different letters are significantly different at $p < 0.05$ (Tukey's HSD test).

Figure 1


Total (Shoot + Root) dry mass (g plant -1)
14
12
10
8
6
4
2
0
Shoot
Root
c
b
ab
ab
d
c
b
ab
ab
ab
a
K4-100
K4-95
K4-90
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K6-90
K6-85
K6-75
OS
CC
Treatment

Figure 2

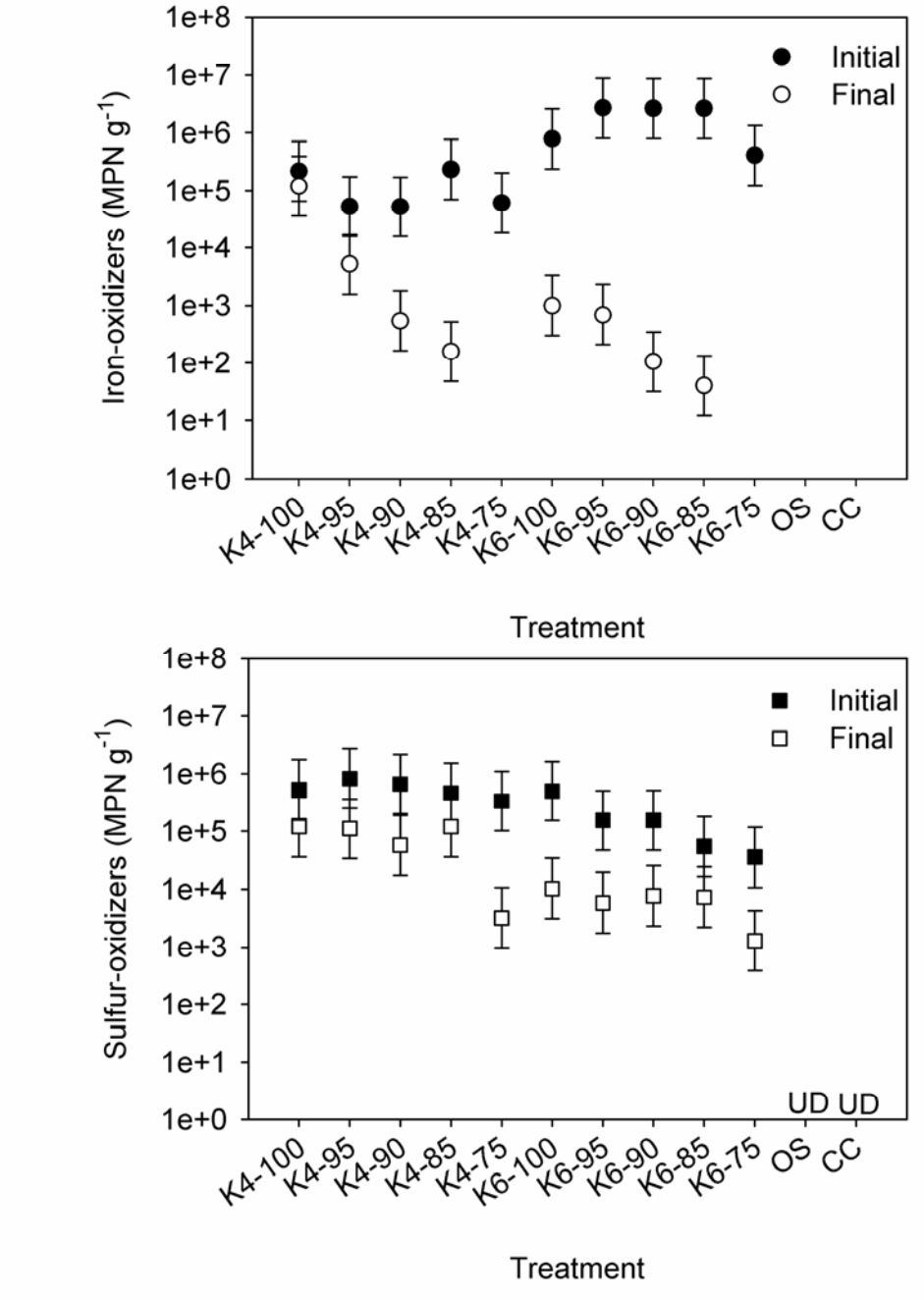
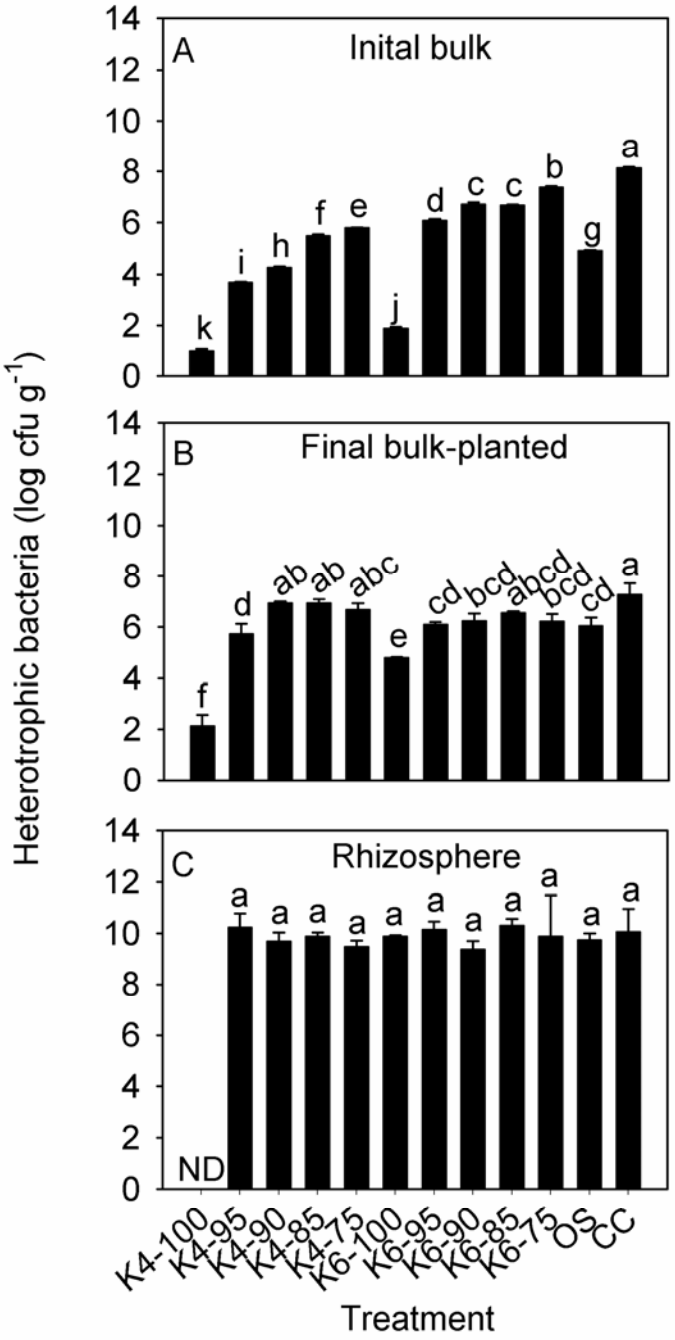

Iron-oxidizers (MPN g-1)
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
Initial
Final
K4-100
K4-95
K4-90
K4-85
K4-75
K6-100
K6-95
K6-90
K6-85
K6-75
OS
CC
Treatment
Sulfur-oxidizers (MPN g-1)
UD UD
Treatment

Figure 3


A
Inital bulk
B
Final bulk-planted
C
Rhizosphere
ND
Heterotrophic bacteria (log cfu g-1)
Treatment
K4-100
K4-95
K4-90
K4-85
K4-75
K6-100
K6-95
K6-90
K6-85
K6-75
OS
CC

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APPENDIX B

BACTERIAL COMMUNITY CHARACTERIZATION OF A HISTORIC SEMI-ARID LEAD-ZINC MINE TAILINGS SITE

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Abstract

The microbiology of mine tailings sites has not been thoroughly investigated despite the contribution of acidophiles to plant death in revegetation of mine tailings (phytostabilization). This study characterizes both the cultured and uncultured bacterial community of an extremely acidic (K4, pH 3) and a moderately acidic (K6, pH 6) lead-zinc mine tailings sample as well as an offsite control sample (OS, pH 8). Bacterial community comparisons were based on numbers of heterotrophic and iron-/sulfur-oxidizing bacteria, identities using the 16S rRNA gene, and phylogenetic analysis. Results demonstrated that heterotrophic bacterial counts, phylotype richness, and uncultured bacterial community diversity increased with pH. Autotrophic iron- and sulfur-oxidizers were present at relatively high numbers in the mine tailings and were not detected in the offsite control sample. In addition, the bacterial community from both tailings was comprised of acidophiles previously observed in acid mine drainage environments. A few of the acidophiles from the uncultured bacterial community are closely-related to genera not previously observed in mine tailings (*Thiomonas*, *Acidimicrobium*, and two gamma-proteobacteria). This study demonstrates that mine tailings are a stressful environment largely affected by pH and a source of previously undetected acidophilic organisms that may impede phytostabilization.

Introduction

Approximately 550,000 abandoned mine sites in the United States have generated 45 billion tons of mine waste, including waste rock and tailings, many of which are in

arid and semiarid regions (Lyon et al., 1993; General Accounting Office., 1996; USEPA, 2004). Characteristically, mine tailings have no soil structure or organic matter, contain high concentrations of metals (As, Cu, Fe, Mn, Ni, Pb, and Cd) ranging from 1 to 50 g kg^{-1}, and are devoid of vegetation (Munshower, 1994; Walder and Chavez, 1995; Krzaklewski and Pietrzykowski, 2002; USEPA, 2004). Recent interest in the reclamation of abandoned mine tailings in arid and semiarid regions focuses on revegetation, or phytostabilization of these sites. Phytostabilization is a low-cost extensive strategy to reduce eolian dispersion and water erosion of the tailings which are often within environmentally sensitive areas and have human populations encroaching (Munshower, 1994; Brooks et al., 1998; Mummey et al., 2002; Moynahan et al., 2002; USEPA, 2004). However, plant establishment in tailings is limited directly by abiotic factors (pH, metal bioavailability, poor soil structure) and indirectly by the microbial community that is intertwined with these physicochemical characteristics (Wong et al., 1998; Tordoff et al., 2000; Schippers et al., 2000; Krzaklewski and Pietrzykowski, 2002). For instance, the presence of high numbers of autotrophic iron- and sulfur-oxidizing bacteria is associated with plant death in acidic mine tailings, while increases in heterotrophic bacteria have been shown to correlate with plant establishment (Schippers et al., 2000; Moynahan et al., 2002; Krzaklewski and Pietrzykowski, 2002). Therefore, increased knowledge of the microbial ecology of mine tailings will provide a baseline for understanding potential complications during revegetation of arid and semiarid environments, as well as a measure of successful remediation

Few microbial diversity studies have examined mine tailings in general and almost no information exists specifically on the microbiology of tailings in arid or semiarid regions. Existing studies have focused on the bacterial community of acid mine drainage (AMD) and subsurface waters of mine tailings piles with few investigations of the overall microbial community associated with the origin of the acid mine drainage (Leduc et al., 2002; Baker and Banfield, 2003; Hery et al., 2005; Bruneel et al., 2005; De La Iglesia et al., 2006). Diversity studies on tailings reveals that acidophilic autotrophs, such as *Acidithiobacillus* spp., *Thiomonas* spp., and *Leptospirillum* spp., and a few acidophilic heterotrophic organisms are linked repeatedly to iron- and sulfur-oxidation. These microorganisms are often present in significantly higher numbers than heterotrophs (Southam and Beveridge, 1992; Schippers et al., 1995; Fortin et al., 1995; Baker and Banfield, 2003; Bruneel et al., 2005). Therefore, the heterotrophic community has largely been ignored except for studies using microbial counts or metabolic diversity as an index of contamination levels or remediation success (Wielinga et al., 1999; Moynahan et al., 2002; Moynahan et al., 2002; Londry and Sherriff, 2005; Mendez et al., 2007). This is problematic for phytostabilization of mine tailings since heterotrophic bacteria are important in nutrient cycling and availability as well as for development of soil physical characteristics (Paul and Clark, 1989).

Taken together, this body of research suggests that the current lack of knowledge concerning the microbial community in arid and semiarid mine tailings sites impedes the implementation of efficient reclamation of these sites using phytostabilization. Therefore, the aim of this study was to begin characterizing this microbial community.

Our focus was on the bacterial community in two tailings samples, one extremely acidic and one moderately acidic, from a semiarid mine tailings site (ADEQ, 2001). We compare the community structure of the tailings samples to an off-site control soil, and relate our findings to physicochemical characteristics of the site. Comparisons of the microbial community are based on enumeration of the culturable heterotrophic and autotrophic iron-/sulfur- oxidizers, identification of cultured and uncultured bacteria by amplification of the 16S rRNA gene from cell lysates and community DNA, and analysis of the phylogenetic composition of the mine tailings community.

Experimental Procedures

Site description and sampling. Samples were collected from the Klondyke mill site in the Aravaipa Valley, Graham County, Arizona where lead and zinc ores were processed from 1948 to 1958 (Wilson, 1959). Approximately 100,000 metric tons of flotation tailings were deposited into two separate piles along the Aravaipa Creek and remain completely unvegetated. Similar to other sites, these (Tordoff et al., 2000; Schippers et al., 2000; Krzaklewski and Pietrzykowski, 2002) lead-zinc mine tailings are acid-generating as they are pyritic and high in sulfur content which prevents natural revegetation and complicates phytostabilization (Wong et al., 1998; Schippers et al., 2000; ADEQ, 2001). In 1998, the Klondyke mill site was placed on the Arizona Water Quality Assurance Revolving Fund (WQARF) Registry due to the levels of Pb and As that exceeded the Arizona Non-Residential Soil Remediation Levels (SRLs) of 10 mg kg^{-1} and 2000 mg kg^{-1},

respectively, and the elevated levels of Cd and Pb in fish sampled downstream from the site (King and Martinez, 1998; ADEQ, 2001).

As previously described (Mendez et al., 2007), two samples (K4 and K6) were collected from the upper tailings pile: K4 from 28 cm to 53 cm below the surface and K6 from 21 cm to 42 cm. Additionally, an off-site control sample (OS) was taken from 17 cm to 28 cm below a vegetated area adjacent to the tailings pile. All material was stored at 4^{o}C and thoroughly mixed prior to use. Samples were analyzed previously for pH, DTPA-extractable metal content, total metal content, particle size distribution, cation exchange capacity, plant-available PO_4-P, total organic carbon, total carbon, and total nitrogen (Mendez et al., 2007).

Enumeration and isolation of heterotrophic bacteria. Heterotrophic bacteria from 10 g of each sample (K4, K6, and OS) were enumerated by dilution plating as described by Mendez et al. (2007) on R2A amended with 200 mg L^{-1} of cycloheximide to inhibit fungal growth and incubated for 5 days at 23^{o}C. R2A provided a selective medium for the enumeration of neutrophilic heterotrophic bacteria. After incubation, bacteria were enumerated and reported as colony forming units (CFU) per gram dry weight of each sample. Counts were log-transformed for normalization prior to the analysis of variance using SAS System for Windows (SAS Institute, 2002). Significant differences between means were determined by employing the Tukey's studentized range test (Tukey's Honestly Significant Difference [Tukey's HSD]).

Bacterial isolates were picked continuously over a two week time period to select for bacteria with different growth rates. Unique colonies were selected based on size, shape, color, and concavity and grouped into morphologically distinct groups. Single isolates were selected from each group and replicate colonies from ten percent of the groups were selected to confirm accuracy of morphological grouping. Isolates were streaked repeatedly on R2A plates to obtain pure cultures. Purity was determined by gram-staining (Murray et al., 1994). Pure isolates were labeled with the corresponding source (K4-, K6-, and OS-) followed by the isolate number.

For 16S rRNA gene amplification, cell lysates were prepared as follows. Isolated colonies were inoculated into 5 ml of R2A broth and incubated on a gyratory shaker (180 rpm) for 3 to 14 days at 23^{o}C. A 1 ml aliquot of bacterial culture was then centrifuged at 14,000 x *g* for 10 min, decanted, and resuspended in 1 ml of sterile distilled water. Cells were lysed by three cycles of freeze-thaw followed by a 15 min boil and stored at -20^{o}C.

Enumeration and DNA extraction of autotrophic bacteria. Iron- and sulfur-oxidizing autotrophic bacteria were enumerated using a modification of the most probable number (MPN) technique (Cochran, 1950; Woomer, 1994). Ten g samples were serially diluted from 10^{-2} to 10^{-6} in 4.5 ml with 5 replicates for each dilution of an iron- or sulfur-oxidizer enrichment medium (pH 2.3 and pH 4.5, respectively) as previously described (Mendez et al., 2007). After 47 days of incubation on a shaker at 180 rpm, positive and negative results for growth were documented. Positive results were based on a color change from yellow to orange for the iron-oxidizers and a change in turbidity as well as decrease in pH

for the sulfur-oxidizers. Population estimates were calculated and reported as most probable number (MPN) g^{-1} (Briones and Reichardt, 1999).

It was not possible to obtain pure cultures of autotrophs for identification; therefore, the 16S rRNA gene of mixed autotrophic cultures was isolated by PCR amplification of the extracted DNA followed by cloning. DNA extractions were performed on a composite of 5 replicate tubes from the 10^{-2} dilution of the MPN analysis. The composite culture was aliquoted into several microcentrifuge tubes and iron precipitates were allowed to settle prior to removal of iron by a series of EDTA washes. For each tube, 500 µl of 0.05 M EDTA was added and vortexed for 5 minutes followed by centrifugation at 14,000 x g for 5 minutes. From each tube, the supernatant was discarded and the remaining pellet was subjected to subsequent washes or resuspended in 500 µl of sterile distilled water for consolidation. This procedure was repeated twice for iron-oxidizers and once for sulfur-oxidizers. Consolidated cell suspensions were extracted using a modification of the CTAB-phenol-chloroform procedure (Ausubel et al., 1995). The modification was a single freeze-thaw cycle after incubation at 37^{o}C. Extracted DNA was resuspended in 100 µl of sterile distilled water.

Soil DNA extraction of uncultured bacteria. Total community DNA was extracted from 0.5 g samples from each mine tailings sample (K4 and K6) and the off-site sample (OS) using the FastDNA® SPIN Kit for Soil **(**Qbiogene Inc., Carlsbad, CA) as specified by the manufacturer.

16S rRNA gene amplification. The 16S rRNA genes were amplified from heterotrophic bacterial cell lysates, mixed autotrophic bacterial DNA, and community DNA extracted form tailings and soil. The universal bacterial primers 27f and 1492r were used to amplify a 1500 bp product from the 16S rDNA (Lane, 1991). Each 50 µl PCR reaction contained 1X buffer (10mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$ - pH 8.3), 0.5 µM each primer, 400 ng/µl bovine serum albumin, 0.2 mM each dNTP, 5% DMSO, 1 U of Taq DNA polymerase, and 5.0 µL DNA template (cell lysate or extracted DNA). The amplification protocol used was 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.15 min and a final extension at 72°C for 10 min followed by 4°C. The entire 1,500 bp product was visualized on a 1.0% agarose gel (GenePure LE, ISC Bioexpress, Kaysville, UT).

16S rRNA gene clone libraries and ARDRA. Clone libraries were created from the 1.5 kb 16S rRNA gene products amplified from DNA extracts using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, San Diego, CA) according to manufacturer's directions. For each autotrophic bacteria clone library, 50 clones were selected, and for each soil DNA clone library, 166 clones were selected. Insert size was verified (approximately 1.5 kb) by amplification using plasmid-specific primers T3 and T7, and the previously described conditions. Autotrophic bacterial clones were designated as Fe-K4-C (K4 iron-oxidizers), S-K4-C (K4 sulfur-oxidizers), Fe-K6-C (K6 iron-oxidizers), or S-K6-C (K6 sulfur-oxidizers) followed by the clone number. Soil DNA clones were labeled with the corresponding source (K4-C, K6-C, and OS-C) followed by the clone number.

PCR products amplified from each clone were then used for amplified ribosomal DNA restriction analysis (ARDRA). Autotrophic clones were subjected to restriction enzyme digest using both BstUI and RsaI, while soil DNA clones were digested with only BstUI. Corresponding clones (7.5 µl and 10 µl of 16S rRNA PCR product, respectively) were digested separately with 5 U BstUI and 5 U RsaI (New England Biolabs, Mississauga, Ontario, Canada), in 25-µl reactions. Digested PCR products were separated by gel electrophoresis on a 3% agarose gel in 1X TBE buffer (Sambrook et al., 1989). Clones were grouped based on ARDRA patterns and each unique pattern was defined as an operational taxonomic unit (OTU). Duplicate clones representing each OTU were selected for sequencing to determine successful grouping. Plasmid DNA was isolated and purified for sequencing with the QIAPrep Spin Miniprep Kit (Qiagen Inc., Valencia, CA).

Sequencing. Amplified 16S rRNA gene products from heterotrophic isolates and purified plasmid DNA from clones were submitted to the University of Arizona Research Labs Genomic Analysis and Technology Core (UARL-GATC) for quantification and sequencing with the ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Primers used were 27f, 518f, 1070r, and 1492r for 16S rRNA gene products, and T3, 518f, 1070r, and T7 primers for the cloned 16S rRNA gene insert (Lane, 1991). Similarity searches were performed by BLAST analysis (Altschul et al., 1997).

Data analysis. All expected phylotypes were evaluated for chimeric sequences using the NAST sequence alignment and chimera check tools from Greengenes (DeSantis et al., 2006a; DeSantis et al., 2006b). Sequences with putative chimeras were checked manually using Pintail as described by Ashelford et al. (2005) and confirmed chimeras were removed. Unique phylotypes were identified based on OTU's with < 99% 16S rDNA sequence similarity as determined by pairwise comparison in BestFit of the GCG Package Version 10.0 (Wisconsin Package, 1999). The number of distinct phylotypes was used for calculation of richness (*S*). Rarefaction curves were created for evaluating the observed species richness among all OTU's identified (data not shown). In addition, the Shannon diversity index (H') was calculated as follows: $H' = -\Sigma(p_i)(\ln p_i)$ where p_i is the proportion of clones or isolates in the *i*th OTU. Evenness (*E*) was also calculated from the Shannon diversity index as $E = H'/\ln S$ (Magurran, 1988). Coverage (*C*) was used as a measurement of captured diversity and calculated using $C = 1 - n/N$, where n the number of singletons (OTU's captured only once) (Good, 1953). To estimate species richness, the nonparametric Chao1 estimate was calculated with log-linear transformed confidence intervals at 95% as described by Hughes et al. (2001). Analyses were performed with EstimateS version 8.0 (Colwell et al., 2004). For the purpose of inputting data into the program, each clone or isolate was treated as a separate sample with 100 randomizations.

Phylogenetic analysis. Nearly complete 16S rRNA gene sequences from both cultured and uncultured bacteria were grouped by sample source and used to construct two

separate trees (K4/K6 and OS). Putative phylogenetic groupings were determined by BLAST analysis (Altschul et al., 1997) and results from the RDP Sequence Match and Classifier (Cole et al., 2005) programs. Nearly complete sequences of reference strains (GenBank) from the *Bacteria* phylum were included in the analysis. Three sequences each from the *Planctomycetes* and *Deinococcus-Thermus* phyla were used as outgroups for the K4/K6 tree and OS tree, respectively, for rooting purposes. Sequences were aligned using Clustal X (Thompson et al., 1997) and the alignments were adjusted manually using MacClade v. 4.08 (Maddison and Maddison, 2001). The most parsimonious trees were constructed from aligned sequences by heuristic search using TBR (tree bisection reconnection) branch swapping on starting trees generated by random sequence addition as implemented by using PAUP 4.0 Beta 10 (Swafford, 2006). Confidence in tree topologies was determined by bootstrap analysis with 100 replicates using maximum parsimony criteria.

Community phylotypes of the cultured iron-/sulfur- oxidizers and uncultured bacteria shared between the samples were analyzed. All unique phylotypes in the study were represented by a single 16S rRNA sequence. Sequences were aligned using Clustal X (Thompson et al., 1997) and imported into DNADIST in PHYLIP version 3.6 (Felsenstein, 2004) to generate distance matrices using the Juke-Cantor correction for multiple substitutions. Operational taxonomic units (OTUs) were assigned by DOTUR (Schloss and Handelsman, 2005). A distance of 0.03 ($OTU_{0.03}$) was examined to determine shared species between the samples. The Sørenson (S_{class}) similarity index was also calculated as an estimate of the ratio of OTUs shared between the two communities

as follows: $S_{class} = (2S_{12})/(S_1 + S_2)$ where S_1 and S_2 are the numbers of OTUs observed or in A and B, respectively, and S_{12} is the number of shared OTUs between A and B.

Results

Bacterial counts. In comparison to the off-site control soil, the Klondyke tailings exhibited a severely stressed culturable neutrophilic heterotrophic community with numbers decreasing as the sample pH decreased with an elevated acid-generating potential (Table 1). Thus, although heterotrophic counts were significantly lower in both tailings samples compared to the OS sample ($F_{2, 6} = 555.53$, $p < 0.0001$), the K4 (pH 2.7) tailings had remarkably low counts, 26 CFU g^{-1}, while the K6 (pH 5.7) tailings count, 1.5 x 10^5 CFU g^{-1}, was only 10-fold lower than the OS (pH 7.7) sample, 2.5 x 10^6 CFU g^{-1}. Also indicating a stressful environment, the numbers of cultured autotrophic iron- and sulfur-oxidizers were elevated to between 4.6 x 10^3 and 1.5 x 10^4 CFU g^{-1} in both tailings samples, whereas these autotrophs were not detectable in the OS sample.

Diversity of cultured and uncultured bacteria. Unique phylotypes in each bacterial library were identified based on < 99% similarity between 16S rDNA sequences. Rarefaction curves (data not shown) as well as percent library coverage suggested that heterotrophic cultured isolates (data not shown) were not sampled well; however, cultured autotrophs and uncultured bacteria were sampled adequately based on percent coverage (Table 2). Therefore, only cultured autotrophs and uncultured bacteria were analyzed further for diversity characterization.

Similar to trends in heterotrophic numbers, observed phylotype and estimated Chao1 richness of cultured autotrophic and uncultured libraries increased with an increase in pH. In terms of Chao1 richness, the K4 (12) uncultured bacteria sample was significantly lower than both the K6 (25) and OS (41) samples ($p < 0.05$). Although there was a large difference between the phylotype and Chao1 estimates of richness for the K6 and OS samples, this difference was not significant (Table 2).

Diversity, as demonstrated by the Shannon diversity index and confirmed by the Shannon evenness factor, followed the same pattern with the Shannon diversity index increasing as pH increased. Furthermore, there is evidence of phylotype dominance in the K4 uncultured library (low Shannon Evenness factor) in comparison to the OS uncultured library for which the Shannon Evenness factor approached 1 indicating little phylotype dominance. For the cultured autotrophic iron-and sulfur oxidizers, there was no diversity in the K4 cultures (Fe-K4 and S-K4, respectively) while K6 cultures (Fe-K6 and S-K4, respectively) demonstrated some diversity in phylotypes. This is not surprising as the media was targeted to a specific group of bacteria.

Phylogenetic analysis of bacterial libraries.

The cultured heterotrophic bacteria from the K4, K6, and OS libraries represented at least three phylogenetic groups *(Actinobacteria*, *Firmicutes*, and *alpha-Proteobacteria*). Each library was dominated by *Actinobacteria* (Table S1). The K4 library with 12 phylotypes ($S = 12$) was represented by the three aforementioned phyla.

The K6 library (S = 16) additionally had members of *Bacteroidetes*, and the OS library (S = 35) was additionally represented by *beta-Proteobacteria* and *gamma-Proteobacteria*.

Most phylotypes of the cultured iron- and sulfur- oxidizers were also found as clones from the uncultured library. These bacteria were related to the genera *Acidiphilium* (Fe-K6-C47 and K6-C83), *Acidithiobacillus* (Fe-K6-C12 and K6-C79; S-K6-C16 and K6-C19), *Leptospirillum* (Fe-K4-C09 and K4-C86), *Thiomonas* (S-K6-C18 and K6-C101), and two separate unidentified iron-oxidizing *gamma-Proteobacteria* (Fe-K6-C35 and K6-C11; S-K6-C04 and K6-C13) (Tables 3 and S2; Fig. 2). Additionally, strains (Fe-K6-C47 and K6-C83) related to *Acidiphilium*, an iron-reducer.

The uncultured bacterial libraries demonstrated a strong trend with pH (Fig. 1). Specifically, the number of phylogenetic groups increased as a function of pH from 4 groups in the K4 sample to 7 groups in the K6 sample to 11 groups in the OS sample. Further, this analysis indicates that there are distinct differences in the nutrient status of the phylotypes detected in the uncultured libraries (Table S2). The extremely acidic K4 sample contained the highest percentage of autotrophic phylotypes (38%) and the remaining phylotypes (62%) were all facultative heterotrophs (heterotrophs capable of autotrophic growth). In the K6 sample, an equal number of autotrophic and obligately heterotrophic (heterotrophs unable to grow as autotrophs) phylotypes (25%) were detected while the remaining cloned sequences (50%) were related to facultative heterotrophs. Finally, the OS uncultured library was dominated by obligate heterotrophic 16S rRNA sequences (88%) with only 10% and 2% of sequences related to autotrophs and facultative heterotrophs, respectively.

When phylotypes in the cultured autotrophic and uncultured bacterial libraries were compared, the extremely acidic K4 and moderately acidic K6 mine tailings communities (Fig. 2) were related with a similarity index of 0.28 (S_{class}) at an $OTU_{0.03}$ definition but neither were similar to the OS sample ($S_{class} = 0$, Fig. 3). Approximately 57% and 18% of the unique phylotypes in K4 and K6, respectively, were shared and these were related to acidophilic iron- and sulfur-oxidizers (Fig. 2).

The largest number of phylotypes shared by K4 and K6 represented the *Gamma-Proteobacteria* (K4-C03, K6-C12, K6-C79, S-K4-C38, Fe-K6-C12, and Fe-K6-C27) and were related to autotrophic iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans*-related strains (Tables 3 and S2). Also in common were K4-C160 and K6-C16 which shared 100% sequence similarity and were related to the *Actinobacteria* (Table S2). These two clones also shared 98% sequence identity to the uncultured bacterium clone TakashiB-B11 and are 84% likely belonging to the *Acidimicrobium* genus, a genera of iron-oxidizing facultative heterotrophs (RDP Classifier; Cole et al., 2005). A third group of related sequences is K4-C26 and K6-C156 (which have 96% sequence identity to *Sulfobacillus* sp. 4G) and K4-C93 (which has 95% sequence identity to uncultured bacterium clone E8c017). These three clones are 100% likely to belong to the genus *Sulfobacillus* which are iron- and sulfur-oxidizing facultative heterotrophs in the *Firmicutes* (RDP Classifier; Cole et al, 2005). Lastly, the clones K4-C86, Fe-K4-C09, and K6-C22 are 100% likely to belong to the autotrophic iron-oxidizing *Leptospirillum* genus (RDP Classifier; Cole et al. 2005).

Discussion

The presence of heterotrophic bacteria as an indicator of soil health. This study is the first to document the cultured and uncultured bacterial community of a historically-contaminated acidic mine tailings site in a semi-arid region and suggests there is a strong relationship between pH and the microbial community. Results demonstrate a decline in heterotrophic bacterial numbers and diversity as samples become more acidic and the acid-generating potential increases. Specifically, neutrophilic aerobic heterotrophs, as enumerated on R2A medium, were detected at lower numbers in the Klondyke mine tailings compared to the neutral OS sample. However, R2A heterotrophic counts in the K4 and K6 samples were comparable to other acidic mine tailings samples (10^1 to 10^5 CFU g^{-1}) (Alexander, 1961; Southam and Beveridge, 1992; Southam and Beveridge, 1993; Wielinga et al., 1999; Schippers et al., 2000). Furthermore, as heterotrophic numbers decreased, counts of autotrophic iron- and sulfur-oxidizers increased as observed in other tailings disposal sites (Southam and Beveridge, 1992; Southam and Beveridge, 1993; Schippers et al., 2000; Enders et al., 2006).

With a decrease in pH, the diversity of the uncultured bacterial community decreased with a shift in the commonly observed uncultured bacteria in soils to those observed in acid mine drainage or mine tailings. Most uncultured bacterial libraries in soils are dominated by the *Proteobacteria*, in particular the *Alpha-Proteobacteria*, *Acidobacteria*, and *Actinobacteria* (Janssen, 2006) Similarly, the OS sample was dominated by *Alpha-Proteobacteria* and contained phylogenetic groups generally represented in soils. However, the K4 and K6 mine tailings samples were dominated by

Firmicutes and *Gamma-Proteobacteria* which is similar to AMD environments as well as other acidic mine tailings sites (Hugenholtz et al., 1998; Bruneel et al., 2005; Janssen, 2006; De La Iglesia et al., 2006).

Additionally, the ratio of total heterotrophic phylotypes to autotrophic phylotypes (Het:$Auto_{phyl}$) of the uncultured community in K4 and K6 is indicative of the harsh environment of the mine tailings and the dominance of the autotrophic bacteria capable of surviving in this environment. The Het:$Auto_{phyl}$ ratio in K4 and K6 was 1.7 and 3.0, respectively. This ratio increased with pH and organic carbon in the OS library with a Het:$Auto_{phyl}$ ratio of 9.5. Therefore, the number of heterotrophic phylotypes has a positive relationship with pH and organic carbon, as well as heterotrophic numbers and bacterial diversity.

Diversity of acidophiles in mine tailings. Individual acid mine drainage sites demonstrate very low diversity, but this study suggests that the source, mine tailings, has an unexpectedly diverse group of acidophilic bacteria. This further suggests that physicochemical and biological factors must select for the limited communities that have been observed in AMD. Understanding these selection factors would enhance understanding of AMD formation.

Bacteria previously detected in both mine tailings and AMD sites were found in the K4 and K6 mine tailings samples. These bacteria were related to *Acidiphilium*, *A. ferrooxidans, A. thiooxidans*, *Acidobacterium* spp., *Alicyclobacillus* spp., *Sulfobacillus* spp., and *Leptospirillum ferriphilum* (Southam and Beveridge, 1992; Southam and

Beveridge, 1993; Goebel and Stackebrandt, 1994; Schippers et al., 2000; Bryan et al., 2006; Mendez et al., 2007; Diaby et al., 2007). However, this is the first detection of bacteria related to *Acidimicrobium*, *Thiomonas*, and two unidentified *gamma-Proteobacteria* in the uncultured bacterial community of mine tailings. *Acidimicrobium*, an iron-oxidizing facultative heterotroph, has been cloned from biofilms of AMD sites at Iron Mountain, CA and can enhance pyrite oxidation when co-cultured with sulfur-oxidizers (Bond et al., 2000; Johnson et al., 2003). Strains of *Thiomonas* spp., have been shown to oxidize As(III) to As(V), a less toxic form with high adsorption capabilities to Fe- and Al-oxides (Bruneel et al., 2003; Battaglia-Brunet et al., 2006). The iron-oxidizing acidophile m-1 and Gamma proteobacterium WJ2 strains have not been detected in mine tailings and may belong to novel genera (Harrison, 1982; Hallberg and Johnson, 2003). Therefore, the mine tailings community possesses a larger diversity of acidophiles than previously determined. Furthermore, bacteria found in AMD sites are assumed to originate from a distant location because of their close relationship to bacteria widely distributed. However, our study demonstrates that the microorganisms found in AMD sites are present in mine tailings and may thrive in AMD by selective pressures rather than an unknown dispersal mechanism facilitating colonization (Baker and Banfield, 2003).

Importance of understanding microbial community in relation to successful restoration. Although the mine tailings were sampled from a semiarid region of the United States, the application of this study extends to other acidic pyritic mine tailings throughout the

world. The Klondyke physicochemical properties are similar to other lead-zinc mine tailings sites that have been targeted for phytostabilization (Wong et al., 1998; Ye et al., 2002; Krzaklewski and Pietrzykowski, 2002). Failure of revegetation attempts or poor plant growth in greenhouse studies have been attributed to acidic conditions that are linked to the presence of cultured iron- and sulfur-oxidizers that are assumed to be *A. ferrooxidans* and *A. thiooxidans* (Wong et al., 1998; Schippers et al., 2000; Ye et al., 2002; Krzaklewski and Pietrzykowski, 2002; Mendez et al., 2007). However, this study indicates that additional acidophilic iron- and sulfur-oxidizers should be monitored for phytostabilization studies even in semiarid environments where acid mine drainage is not a concern but isolated areas of metal leaching within the tailings piles is problematic (Enders et al., 2006).

Phylotypes shared between the K4 and K6 communities indicate that the K4 community is a subset of the K6 community; therefore, microbial communities should be monitored during phytostabilization. These shared phylotypes were related to iron- and sulfur-oxidizers (*Acidithiobacillus ferrooxidans*, *Acidimicrobium*, *Leptospirillum ferriphilum*, and *Sulfobacillus*) and are important in the perpetuating the acidity and metal availability in mine tailings (Southam and Beveridge, 1992; Southam and Beveridge, 1993; Goebel and Stackebrandt, 1994; Bryan et al., 2006). Both *Acidimicrobium* and *Leptospirillum ferriphilum* are iron-oxidizers, while *A. ferrooxidans* and *Sulfobacillus* are both iron- and sulfur-oxidizers. Interestingly, *Acidimicrobium ferrooxidans* and *Sulfobacillus* are facultative heterotrophs that accelerate oxidative dissolution of Fe, in effect decreasing pH. Furthermore, these bacteria consume dissolved organic carbon that

is inhibitory to autotrophs (Bond et al., 2000; Okibe and Johnson, 2004; Bryan et al., 2006). Therefore, interactions between the shared bacteria may be maintaining the acidity in the tailings and their disappearance could be crucial in increasing pH.

The addition of high volumes of organic matter is costly; therefore, the Klondyke study provides a basis for the heterotrophic bacteria that may be tracked for monitoring and understanding revegetation success. Mine tailings are difficult to revegetate without the addition of organic matter or topsoil and an increase in heterotrophic counts has been associated with plant establishment (Gutierrez and Hoffmann, 1991; Ye et al., 2000; Moynahan et al., 2002; Brown et al., 2003; Mendez et al., 2007). However, the heterotrophic bacteria present in tailings that may be contributing to plant establishment are unknown. Although the heterotrophic bacteria cultured in this study are those generally observed in culture collections and were not observed in the uncultured libraries, the presence of a high proportion of the cultured spore-forming gram-positive *Firmicutes* and *Actinobacteria* in the mine tailings samples indicates that these bacteria may be dormant. Furthermore, they are common in nutrient-limiting soils and are drought tolerant (Alexander, 1961; Hugenholtz et al., 1998). Also, heterotrophic bacteria may be linked to unfavorable biogeochemical cycles. Enhancement of heterotrophs from the *Alpha-Proteobacteria* subphylum (*Caulobacter*-, *Sphingomonas*-, and *Rhizobium*-related) in lime-amended mine tailings have been linked to As(V) reduction, enhancing As mobilization (Macur et al., 2001).

Currently, the permanent success of phytostabilization of mine tailings is unknown. Furthermore, most studies only monitor vegetative growth, ignoring the

microbial community and the potential effects that it may have on future ecosystem function. This study has provided a foundation for the reclamation efforts of acidic lead-zinc mine tailings in semiarid regions. Knowledge of the microbial community structure as well as monitoring changes in the microbial diversity is important in evaluating reclamation efforts in terms of amendment addition, plant establishment, and metal immobilization. Restoration ecology is dependent on establishing a reference in order to evaluate restoration goals and success; therefore, this study is an important starting point for future biological stabilization of mine tailings disposal sites.

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Table 1. Physicochemical characteristics and bacterial counts in mine tailings samples.

Sample	pH[a]	TOC (g kg^{-1})	ANP (kg $CaCO_3$ $Tonne^{-1}$)[b]	AGP	ANP/ AGP	Heterotrophs (log CFU g^{-1})[c]	Iron-oxidizers (log MPN g^{-1})[d]	Sulfur-oxidizers (log MPN g^{-1})
K4	2.7	0.4	< 0.3	34.1	0.01	1.41 ± 0.32c	3.66 (3.14, 4.18)	4.19 (3.67, 4.70)
K6	5.7	0.3	< 0.3	13.1	0.02	5.18 ± 0.03b	4.19 (3.67, 4.71)	4.10 (3.58, 4.62)
OS	7.7	18	ND[e]	ND	ND	6.41 ± 0.09a	UD[f]	UD

a. The pH and TOC (Total Organic Carbon) values were determined by Mendez et al. (2007).
b. Values for acid-neutralization potential (ANP) and acid-generating potential (AGP) were determined by ADEQ (2001). An ANP/AGP < 1 indicates that the material is potentially acid-generating.
c. Different letters for each mean ± SD (n=3) represent a significant difference at $p < 0.05$ between each sample source.
d. Population estimates are reported as log most probable number (MPN) per gram of mine tailings with the upper and lower limits at a 95% confidence limit ($p = 0.05$).
e. Acid-neutralization potential and acid-generating potential were not determined (ND).
f. Autotrophic bacteria were undetectable (UD) below the 10^{-2} dilution level.

Table 2. Summary of diversity analyses of uncultured bacterial libraries and cultured autotrophic bacterial libraries.

Source[a]	Phylotype Richness	Chao1 Estimate[b]	Shannon Diversity Index (H')	Shannon Evenness (E)	Coverage (%)	Total # clones
K4	8	12 (12,19)	1.17	0.56	99	155
K6	24	25 (21,49)	2.32	0.73	96	161
OS	42	41 (36,61)	3.49	0.93	87	123
Fe-K4	1	1 (1,1)	0	N/A	100	82
Fe-K6	4	4 (3,7)	0.6	0.43	96	49
S-K4	1	1 (1,1)	0	N/A	100	48
S-K6	3	3 (3,3)	0.36	0.33	98	43

a. Sources of data are from the following libraries: uncultured bacteria from K4, K6, and OS samples; cultured autotrophic iron-oxidizing bacteria (Fe-K4 and Fe-K6) and sulfur-oxidizing bacteria (S-K4 and S-K6) from K4 and K6 mine tailings samples, respectively.
b. Chao1 estimates are followed by log-linear transformed confidence intervals at 95%.

Table 3. Identities of 16S rRNA gene sequences from cultured autotrophic bacteria.

OTU[a]	Putative group	Closest BLAST match (GenBank accession no.)	% Identity	Source
Fe-K4-C09	*Nitrospira*	Uncultured bacterium clone SX3-20 (DQ469238)	99	Acid mine drainage (China)
Fe-K6-C47	*α-Proteobacteria*	*Acidiphilium* sp. (D30769)	99	Acid mine drainage (Japan)
S-K6-C18	*β-Proteobacteria*	*Thiomonas* sp. RCASK1 (AJ879998)	99	Acid mine drainage (France)
S-K4-C38	*γ-Proteobacteria*	*Acidithiobacillus ferrooxidans* YTW (DQ062116)	99	Acid mine drainage (China)
Fe-K6-C12	*γ-Proteobacteria*	Uncultured bacterium clone G28 (DQ480479)	99	Acid mine drainage (China)
Fe-K6-C27	*γ-Proteobacteria*	*Acidithiobacillus ferrooxidans* YTW (DQ062116)	99	Acid mine drainage (China)
Fe-K6-C35	*γ-Proteobacteria*	Iron-oxidizing acidophile m-1 (AF387301)	99	coal mine refuse (USA)
S-K6-C04	*γ-Proteobacteria*	Gamma proteobacterium WJ2 (AY096032)	99	Wetland ecosystem constructed to remediate mine drainage (UK)
S-K6-C16	*γ-Proteobacteria*	Uncultured bacterium clone MS140BH1062003 (DQ354750)	97	Mine subsurface water (South Africa)

a. OTU's are designated as Fe-K4-C (K4 iron-oxidizers), S-K4-C (K4 sulfur-oxidizers), Fe-K6-C (K6 iron-oxidizers), and S-K6-C (K6 sulfur-oxidizers).

Table S1. Identities of 16S rRNA gene sequences from cultured heterotrophic bacteria.

OTU	Putative group	Closest BLAST match (GenBank accession no.)	% Identity
K4-06	*Actinobacteria*	*Kocuria* sp. L5, MI-46a (DQ192212, DQ180950)	99
K4-07A	*Actinobacteria*	*Microbacterium* sp. VKM Ac-2016 (AB042081)	97
K4-07B	*Actinobacteria*	*Rhodococcus* sp. UFZ-B520 (AF235011)	100
K4-07D	*Actinobacteria*	*Brevibacterium antarcticum* DVS 5a2 (AJ577724)	99
K4-07E	*Actinobacteria*	*Microbacterium* sp. EP10 (AM398219)	98
K4-10C	*Actinobacteria*	Uncultured bacterium clone 655937 (DQ404723)	99
K6-01	*Actinobacteria*	*Microbacterium oxydans* strain B5 (DQ350825)	99
K6-02	*Actinobacteria*	*Curtobacterium* sp. 1594 (AY688357)	99
K6-05	*Actinobacteria*	*Streptomyces humidus* subsp. *antitumoris* NBRC 13976, *S. humidus subsp. antitumoris* NBRC 13825, *Streptomyces* sp. PC22 (AB184556, AB184510, DQ385869)	99
K6-06	*Actinobacteria*	*Streptomyces zaomyceticus* strain 14125, *Streptomyces* sp. SM3 (EF063456, DQ887330)	99
K6-08	*Actinobacteria*	*Amycolatopsis decaplanensis* (AJ508237)	99
K6-15	*Actinobacteria*	*Amycolatopsis nogabecetica* (AJ508238)	99
K6-25C	*Actinobacteria*	*Arthrobacter* sp. MHS027, Uncultured bacterium clone AKAU359, *A. aurescens*, *A. nitroguajacolicus* (DQ993331, DQ125595, DQ016989, AJ512504)	99
OS-01	*Actinobacteria*	*Arthrobacter* sp. Ens13 (DQ339622)	99
OS-02	*Actinobacteria*	*Arthrobacter nitroguajacolicus* strain CCM4924T (AJ512504)	99
OS-06	*Actinobacteria*	*Clavibacter michiganensis* (DQ507208)	100
OS-07	*Actinobacteria*	Uncultured soil bacterium clone 623-1 (AF423282)	99
OS-08	*Actinobacteria*	*Arthrobacter* sp. 108 (AY238501)	99
OS-09B	*Actinobacteria*	*Arthrobacter* sp. 16.22 (DQ157987)	100
OS-10A	*Actinobacteria*	Uncultured soil bacterium clone 425-1 (AF423261)	100
OS-10C	*Actinobacteria*	*Arthrobacter* sp. AG1, 94, 83b (AY651317, AJ879125, AJ879123)	98
OS-11	*Actinobacteria*	*Rhodococcus corynebacterioides* (X80615)	99
OS-12A	*Actinobacteria*	Actinobacterium TB3-4-I, *Frigoribacterium* sp. 301 , 227 (AY599739, AF157479, AF157478)	99
OS-13	*Actinobacteria*	Uncultured soil bacterium clone UB9 (DQ248232)	99
OS-14	*Actinobacteria*	*Streptomyces rubrogriseus* NBRC 15455, DSM 41477 (AB184681, AF503501)	99
OS-16A	*Actinobacteria*	*Promicromonospora* sp. 82449 (DQ008600)	99
OS-18	*Actinobacteria*	*Streptomyces venezuelae* NBRC 12595, *S. venezuelae* NBRC 13096, *S. flaveus* NBRC 12375, *S. venezuelae* (AB184836, AB184308, AB184087, AB045890)	100
OS-20	*Actinobacteria*	*Rhodococcus fascians*, *R. fascians* DSM20669 (Y11196, X79186)	100
OS-22C	*Actinobacteria*	Uncultured bacterium clone AKIW474 (DQ129372)	99
OS-23	*Actinobacteria*	*Nocardiodes albus* (AF005003, AF004999, AF004989)	99
OS-29	*Actinobacteria*	Uncultured bacterium clone SC-35, *Microbacterium oxydans* NJ6, *M. oxydans*, *M. oxydans* AC44, *M. oxydans* CV711a, *Microbacterium* sp. B-1132, *M. oxydans* S15-M2, *M. oxydans* SW366-KB-3 (AB255091, DQ403811, DQ105974, AJ717357, AJ717358, DQ347555, AM234158, AM234157)	99
OS-30B	*Actinobacteria*	*Microbacterium* sp. ORS 1417 (AJ968703)	97
OS-31	*Actinobacteria*	*Arthrobacter* sp. J3.41, J3.36 (DQ157999, DQ157996)	99
K6-27	*Bacteroidetes*	Uncultured CFB group bacterium (AJ583191)	98
OS-21A	*Bacteroidetes*	Uncultured Bacteroidetes bacterium clone (AY922040)	94
OS-26C	*Bacteroidetes*	Uncultured bacterium clone AYRV1-015 (DQ990929)	96
K4-01	*Firmicutes*	*Paenibacillus cineris* strain LMG 18439T (AJ575658)	99
K4-03	*Firmicutes*	*Paenibacillus* sp. W-61 (AB110989)	99
K4-04	*Firmicutes*	*Bacillus megaterium* (DQ105968)	99
K4-05	*Firmicutes*	*Bacillus* sp. MHS014, MHS023, MHS038, I101-7, 101-4, PNP12, PDD-	99

		3b-6, *B. simplex* strain WN579, *B. simplex* WN570, *Bacillus* sp. Bt176, 9B-1, Uncultured soil bacterium clone 1296-1, *B. simplex*, Uncultured bacterium clone AKIW1128, *Bacillus* sp. LMG 21002, *B. simplex* LMG 17636, LMG 17633, LMG 21002, *B. macroides* (DQ993310, DQ993303, DDQ993299, DQ192053, DQ192051, DDQ887519, DQ512741, DQ275178, DQ275175, AY965249, AY689061, AF423217, DDQ105977, DDQ129440, AJ316308, AJ628747, AJ628746, AJ628745, AF157696)	
K4-10A2	*Firmicutes*	*Bacillus pumilus* strain Tbl (AB195283)	100
K6-03	*Firmicutes*	*Bacillus cereus* clone AND1313R (DQ289993)	100
K6-09	*Firmicutes*	*Brevibacillus formosus* LMG16101 (AF378234)	99
K6-17B	*Firmicutes*	*Staphylococcus pasteuri* ATCC51129T (AB009944)	99
K6-18	*Firmicutes*	Uncultured bacterium clone AKIW724 (DQ129275)	99
K6-19	*Firmicutes*	*Paenibacillus lautus* (AB073188)	99
OS-03	*Firmicutes*	Uncultured bacterium clone AKAU380 (DQ223134)	99
OS-04	*Firmicutes*	*Bacillus cereus* strain Y1 (AY651924)	100
OS-05	*Firmicutes*	Uncultured bacterium clone AKIW956 (DQ129506)	99
OS-13B	*Firmicutes*	Bacterium Ellin505 (AY960768)	99
OS-16B	*Firmicutes*	*Bacillus licheniformis* (AB039328)	
OS-33	*Firmicutes*	Bacterium K2-24 (AY345429)	98
K4-11	*α-Proteobacteria*	*Sphingomonas* sp. JQ1-11 (DQ132883)	96
K6-11	*α-Proteobacteria*	*Methylobacterium* sp. F48 (MTBF48A13)	99
K6-22	*α-Proteobacteria*	Uncultured bacterium clone RB379 (AB240360)	99
K6-28	*α-Proteobacteria*	*Nordella oligomobilis* (AF370880)	99
OS-09	*α-Proteobacteria*	*Caulobacter leidyi* (AF331660)	99
OS-12B	*α-Proteobacteria*	*Caulobacter* sp. strain FWC38 (AJ227774)	98
OS-15B	*α-Proteobacteria*	*Caulobacter* sp. BBCT22 (DQ337549)	99
OS-30A	*α-Proteobacteria*	*Methylobacterium* sp. GW2, *M. extorquens*, *Methylobacterium* sp. P1, iRIII1, *M. exotorquens* NCIMB 9399, *M. extorquens* IAM 12631, *M. dichloromethanicum* DSM 6343, *M. extorquens*, Uncultured alpha proteobacterium clone MTAA10 (DQ400509, AF531770. AF148859, AY358000, AB175633, AB175632, AB175631, AF293375, AJ964950)	100
OS-15A	*β-Proteobacteria*	Uncultured soil bacterium clone TD2 (DQ248271)	99
OS-17B	*γ-Proteobacteria*	Uncultured bacterium clone HSM-SS-019, Uncultured bacterium clone HSM-SS-001, *Pseudomonas* sp. AEBL3, *Pseudomonas* sp. TB2-8-I, Uncultured bacterium clone Phe56, Uncultured bacterium clone Phe10, Uncultured bacterium clone Glu3 (AB238782, AB238764, AY247063, AY599703, AF534214, AF534205, AF534197)	99
OS-28	*γ-Proteobacteria*	*Dyella ginsengisoli* strain LA-4 (EF191354)	98

Table S2. Identities of 16S rRNA gene sequences from uncultured bacteria.

OTU	Putative group	Closest BLAST match (GenBank accession no.)	% Identity	Nutrient Status[a]
K6-C55	*Acidobacteria*	Uncultured bacterium clone TakashiAB-B21 (AB254782)	98	Facultative heterotroph
K6-C86	*Acidobacteria*	Uncultured forest soil bacterium clone DUNssu362 (AY913535)	98	Facultative heterotroph
OS-C11	*Acidobacteria*	Uncultured soil bacterium clone M25_Pitesti, M01_Pitesti (DQ378245, DQ378223)	95	Obligate heterotroph
OS-C54	*Acidobacteria*	Uncultured soil bacterium clone 529 (AY493931)	99	Obligate heterotroph
OS-C85	*Acidobacteria*	Uncultured bacterium clone PH10-21 (DQ444058)	93	Obligate heterotroph
OS-C103	*Acidobacteria*	Uncultured soil bacterium clone 1526 (AY493923)	96	Obligate heterotroph
OS-C128	*Acidobacteria*	Uncultured bacterium clone E4 (AM085462)	92	Obligate heterotroph
OS-C161	*Acidobacteria*	Uncultured soil bacterium clone 572 (AY493933)	97	Obligate heterotroph
K4-C41	*Actinobacteria*	Uncultured bacterium clone ASL4 (AF543498)	99	Facultative heterotroph
K4-C160	*Actinobacteria*	Uncultured bacterium clone TakashiB-B11 (AB254793)	98	Facultative heterotroph
K6-C10	*Actinobacteria*	Uncultured bacterium clone AKAU4087 (DQ125855)	99	Obligate heterotroph
K6-C16	*Actinobacteria*	Uncultured bacterium clone TakashiB-B11 (AB254793)	98	Facultative heterotroph
OS-C07	*Actinobacteria*	*Couchioplanes cauruleus* (X93202)	99	Obligate heterotroph
OS-C43	*Actinobacteria*	Uncultured bacterium clone ORCA-17F18 (DQ823199)	96	Obligate heterotroph
OS-C82	*Actinobacteria*	*Actinomadura viridis* strain DS (AJ420141)	97	Obligate heterotroph
OS-C102	*Actinobacteria*	Uncultured bacterium clone ORCA-17F18 (DQ823199)	95	Facultative heterotroph
OS-C108	*Actinobacteria*	Uncultured bacterium clone AKAU3763 (DQ125684)	95	Obligate heterotroph
OS-C131	*Bacteroidetes*	Uncultured bacterium clone RB374, clone RB375 (AB241539, AB240357)	96	Obligate heterotroph
OS-C154	*Bacteroidetes*	Uncultured bacterium clone DSBR-B020 (AY302119)	95	Obligate heterotroph
K4-C07	*Firmicutes*	Uncultured bacterium clone SX3-1 (DQ469233)	98	Facultative heterotroph
K4-C26	*Firmicutes*	*Sulfobacillus* sp. 4G (AY371272)	96	Facultative heterotroph
K4-C93	*Firmicutes*	Uncultured bacterium clone E8c017 (DQ455569)	95	Facultative heterotroph
K6-C04	*Firmicutes*	Uncultured bacterium clone D3-28 (DQ464143)	94	Facultative heterotroph
K6-C05	*Firmicutes*	Uncultured bacterium clone YNPFFP9 (AF391988)	93	Obligate heterotroph
K6-C25	*Firmicutes*	Uncultured bacterium clone RCP2-66 (AF523921)	95	Autotroph
K6-C31	*Firmicutes*	Gram-positive iron-oxidizing acidophile SLC66 (AY040739)	93	Obligate heterotroph
K6-C81	*Firmicutes*	*Sulfobacillus* sp. 4G (AY371272)	95	Facultative heterotroph
K6-C107	*Firmicutes*	Uncultured bacterium clone D3-28 (DQ464143)	97	Facultative heterotroph
K6-C109	*Firmicutes*	Uncultured bacterium clone D3-5	97	Facultative heterotroph

		(DQ464142)		
K6-C143	*Firmicutes*	*Sulfobacillus yellowstonensis* strain YTF-1 (AY007665)	94	Facultative heterotroph
K6-C156	*Firmicutes*	*Sulfobacillus* sp. 4G (AY371272)	96	Facultative heterotroph
OS-C17	*Gemmatimonadetes*	Bacterium Ellin5220 (AY234571)	91	Obligate heterotroph
OS-C21	*Gemmatimonadetes*	Uncultured Gemmatimonadetes bacterium clone AKYH1194 (AY921682)	94	Obligate heterotroph
OS-C25	*Gemmatimonadetes*	Uncultured bacterium clone AKAU417 (DQ125912)	91	Obligate heterotroph
OS-C31	*Gemmatimonadetes*	Uncultured Gemmatimonadetes bacterium clone AKYG1614 (AY921912)	93	Obligate heterotroph
OS-C40	*Gemmatimonadetes*	Uncultured low G+C Gram-positive bacterium (AY177765)	96	Obligate heterotroph
OS-C77	*Gemmatimonadetes*	Uncultured soil bacterium clone C114 (AF507703)	95	Obligate heterotroph
OS-C105	*Gemmatimonadetes*	Uncultured soil bacterium clone UC8 (DQ297986)	98	Obligate heterotroph
OS-C150	*Gemmatimonadetes*	Uncultured bacterium clone KCM-B-38 (AJ581612)	94	Obligate heterotroph
K4-C86	*Nitrospira*	Uncultured bacterium clone SX3-20 (DQ469238)	99	Autotroph
K6-C22	*Nitrospira*	Uncultured bacterium clone SX3-20 (DQ469238)	99	Autotroph
OS-C76	*Nitrospira*	Unidentified bacterium clone 1013-28-CG51 (AY532586)	93	Autotroph
OS-C18	*Planctomycetes*	Uncultured soil bacterium clone S165 (AF507705)	93	Obligate heterotroph
K6-C56	*α-Proteobacteria*	Uncultured bacterium RB312 (AB2440333)	98	Obligate heterotroph
K6-C83	*α-Proteobacteria*	*Acidiphilium* sp. (D30769)	99	Obligate heterotroph
K6-C124	*α-Proteobacteria*	Uncultured bacterium clone RB386 (AB240363)	99	Obligate heterotroph
OS-C02	*α-Proteobacteria*	Uncultured soil bacterium clone 565-2 (AF423277)	98	Obligate heterotroph
OS-C20	*α-Proteobacteria*	Uncultured bacterium clone 17RH (AJ863377)	94	Obligate heterotroph
OS-C38	*α-Proteobacteria*	Uncultured bacterium clone AKIW985 (DQ129262)	95	Obligate heterotroph
OS-C44	*α-Proteobacteria*	Sphingomonadaceae bacterium Gsoil 359 (AB245346)	96	Obligate heterotroph
OS-C79	*α-Proteobacteria*	Uncultured bacterium clone AKAU4071, AKAU359 (DQ125843, DQ125596)	98	Obligate heterotroph
OS-C81	*α-Proteobacteria*	Uncultured forest soil bacterium clone DUNssu049 (AY913269)	91	Obligate heterotroph
OS-C92	*α-Proteobacteria*	Uncultured marine bacterium clone SJC1.32 (DQ071107)	98	Obligate heterotroph
OS-C99	*α-Proteobacteria*	Uncultured bacterium clone JSC9-G2 (DQ532251)	99	Obligate heterotroph
OS-C159	*α-Proteobacteria*	Uncultured alpha proteobacterium clone AKYH1192 (AY921758)	98	Obligate heterotroph
K6-C101	*β-Proteobacteria*	*Thiomonas* sp. RCASK1 (AJ879998)	99	Facultative heterotroph
OS-C01	*β-Proteobacteria*	Uncultured bacterium clone 29 (AY250094)	98	Obligate heterotroph
OS-C12	*β-Proteobacteria*	Uncultured bacterium clone L013.1 (AF358001)	96	Autotroph
OS-C14	*β-Proteobacteria*	Uncultured bacterium clone JH-GY05	97	Autotroph

		(DQ351927)		
OS-C19	*β-Proteobacteria*	*Janthinobacterium* sp. IC161 (AB196254)	99	Obligate heterotroph
OS-C27	*β-Proteobacteria*	Uncultured bacterium clone L013.1 (AF358001)	97	Obligate heterotroph
OS-C129	*β-Proteobacteria*	Uncutlured bacterium clone JH-GY05 (DQ351927)	98	Obligate heterotroph
K4-C03	*γ-Proteobacteria*	Uncultured bacterium clone ff5 (DQ303263)	99	Autotroph
K4-C116	*γ-Proteobacteria*	Uncultured bacterium clone ff5 (DQ303263)	98	Autotroph
K6-C11	*γ-Proteobacteria*	Iron-oxidizing acidophile m-1 (AF387301)	99	Autotroph
K6-C12	*γ-Proteobacteria*	Uncultured bacterium clone ff5 (DQ303263)	99	Autotroph
K6-C13	*γ-Proteobacteria*	Gamma proteobacterium WJ2 (AY096032)	98	Facultative heterotroph
K6-C19	*γ-Proteobacteria*	Uncultured bacterium clone MS140BH1062003319 (DQ354750)	98	Facultative heterotroph
K6-C62	*γ-Proteobacteria*	Uncultured bacterium clone TakashiAB-B3 (AB254777)	95	autotroph
K6-C79	*γ-Proteobacteria*	Uncultured bacterium clone G28 (DQ480479)	99	Autotroph
OS-C47	*γ-Proteobacteria*	Uncultured bacterium clone ELB19-045 (DQ015790)	94	Autotroph
OS-C03	*δ-Proteobacteria*	Uncultured soil bacterium clone UH1 (DQ297965)	98	Obligate heterotroph
OS-C118	*δ-Proteobacteria*	Uncultured bacterium clone MIZ29 (AB179520)	97	Obligate heterotroph
OS-C04	*Verrrucomicrobia*	Uncultured soil bacterium clone UF7 (DQ297970)	95	Obligate heterotroph

a. Based on closest relative at the genus level (RDP Classifier; Cole et al., 2005) or published data of the closest BLAST match.

Appendix B Figure Legends

Figure 1. Distribution of phylotypes in uncultured libraries from mine tailings samples. Diagrams show the relative abundance of 16S rRNA phylotypes of clones from the K4 (A), K6 (B), and OS (C) samples with a total of 8, 24, and 42 unique phylotypes, respectively.

Figure 2. Most parsimonious tree generated from 16S rRNA gene sequences from reference bacterial strains (GenBank) and unique phylotypes of both cultured and uncultured bacteria in K4 (indicated by "•") and K6 (indicated by "••") mine tailings. The rooted tree based on nearly full-length sequences was generated using the maximum parsimony analysis by heuristic search (tree bisection reconnection branch swapping) as implemented in PAUP 4.0 Beta (Maddison and Maddison 2001). Three members of the *Planctomycetes* (*Planctomyces brasililensis*, *P. maris*, and *P. llimnophilus*) were used as the outgroup. Bootstrap values (100 replicates) are given for nodes with ≥ 50% support. Accession numbers for references strains are shown in parentheses and type strains ('T') are indicated where possible; accession numbers for 16S rRNA gene sequences from this study are included in Tables S1 and S2.

Figure 3. Most parsimonious tree generated from 16S rRNA gene sequences from reference bacterial strains (GenBank) and unique phylotypes of both cultured and cultured bacteria in the OS sample. The rooted tree based on nearly full-length sequences was generated using the maximum parsimony analysis by heuristic search (tree bisection reconnection branch swapping) as implemented in PAUP 4.0 Beta (Maddison and Maddison 2001). Three members of the *Deinococcus-Thermus* (*Deinococcus radidurans*, *Thermus aquaticus*, *Meiothermus rubber*) were used as the outgroup. Bootstrap values (100 replicates) are given for nodes with ≥ 50% support. Accession numbers for references strains are shown in parentheses and type strains ('T') are indicated where possible; accession numbers for 16S rRNA gene sequences from this study are included in Tables S1 and S2.

Figure 1

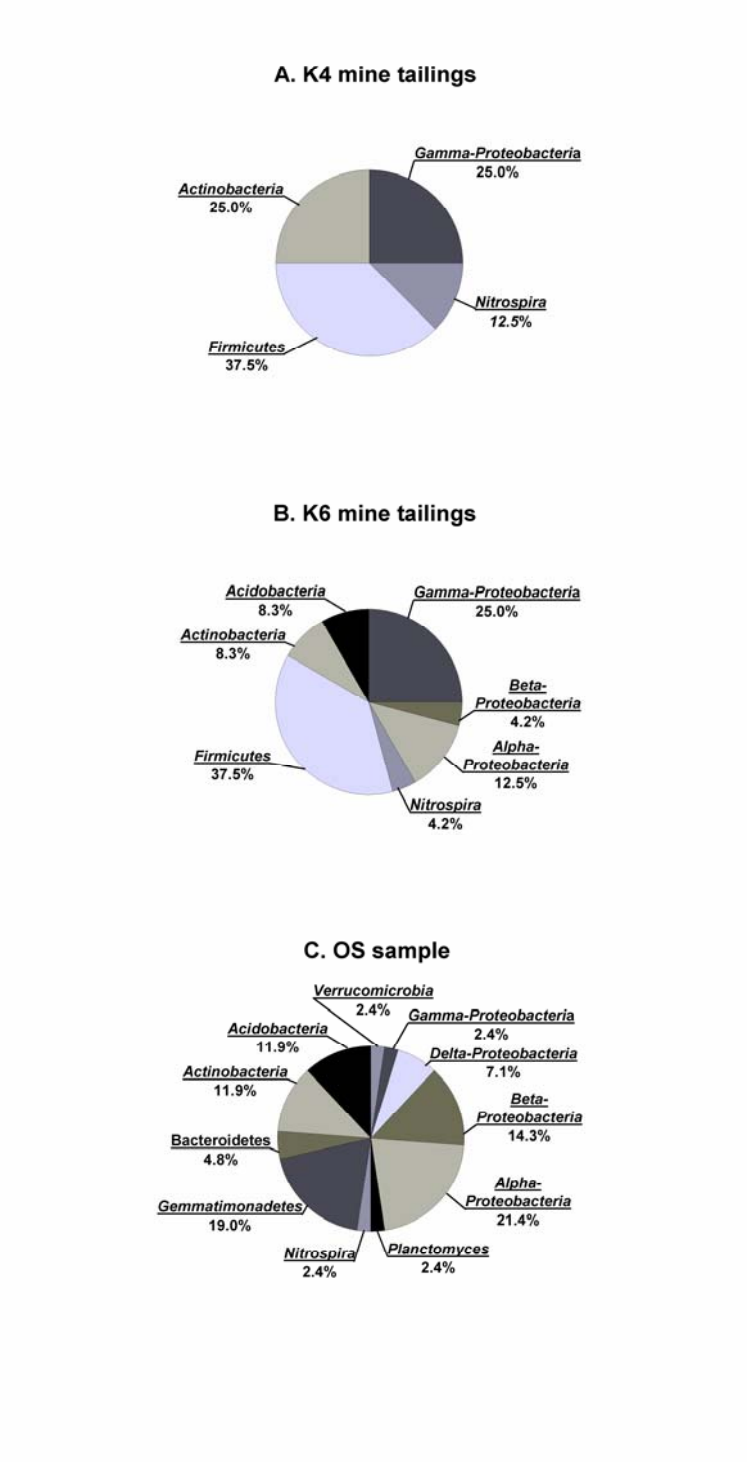

A. K4 mine tailings
Gamma-Proteobacteria
25.0%
Actinobacteria
25.0%
Nitrospira
12.5%
Firmicutes
37.5%
B. K6 mine tailings
Acidobacteria
8.3%
Gamma-Proteobacteria
25.0%
Actinobacteria
8.3%
Beta-
Proteobacteria
4.2%
Alpha-
Proteobacteria
12.5%
Firmicutes
37.5%
Nitrospira
4.2%
C. OS sample
Verrucomicrobia
2.4%
Gamma-Proteobacteria
2.4%
Acidobacteria
11.9%
Delta-Proteobacteria
7.1%
Actinobacteria
11.9%
Beta-
Proteobacteria
14.3%
Bacteroidetes
4.8%
Alpha-
Proteobacteria
21.4%
Gemmatimonadetes
19.0%
Nitrospira
2.4%
Planctomyces
2.4%

Figure 2

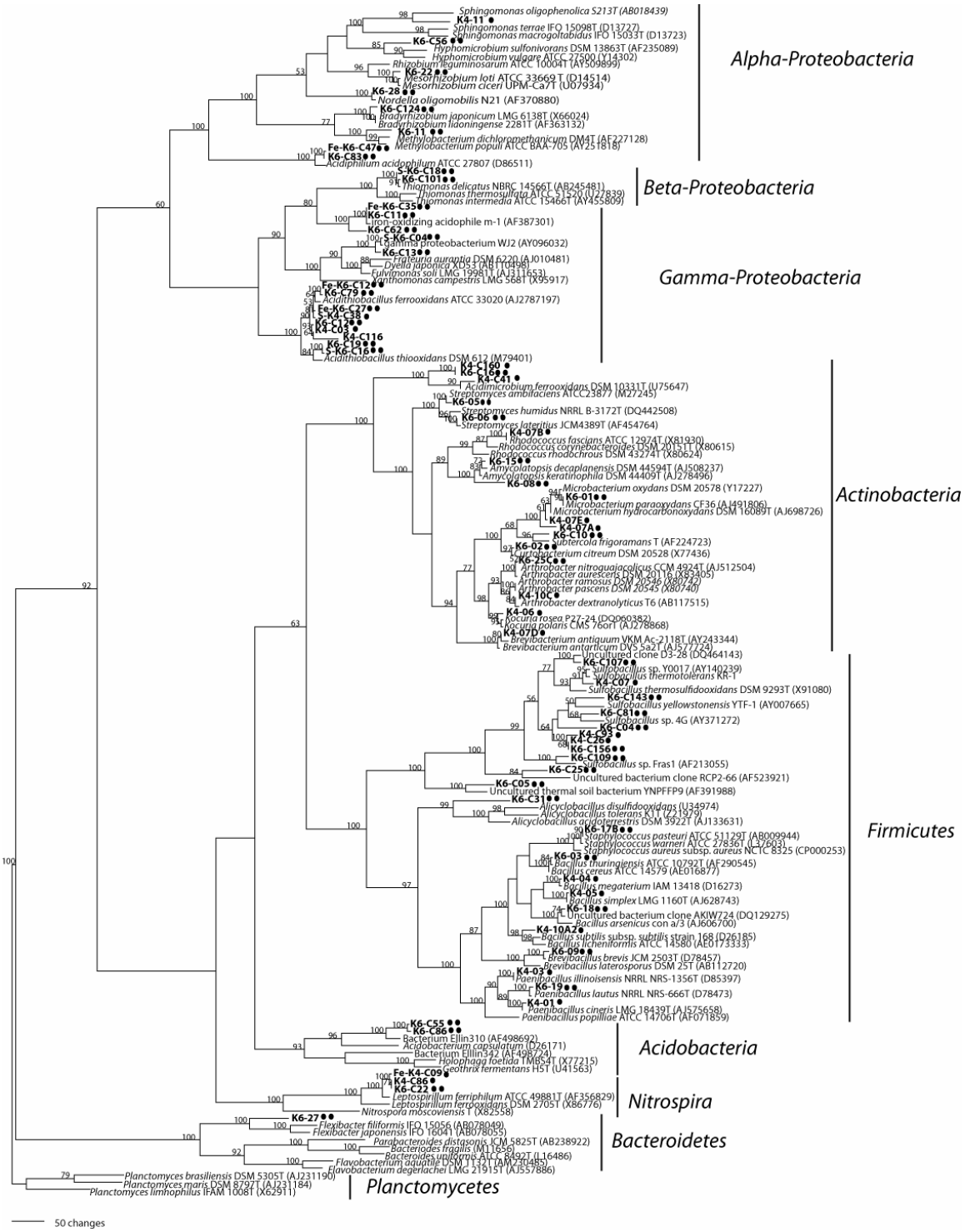
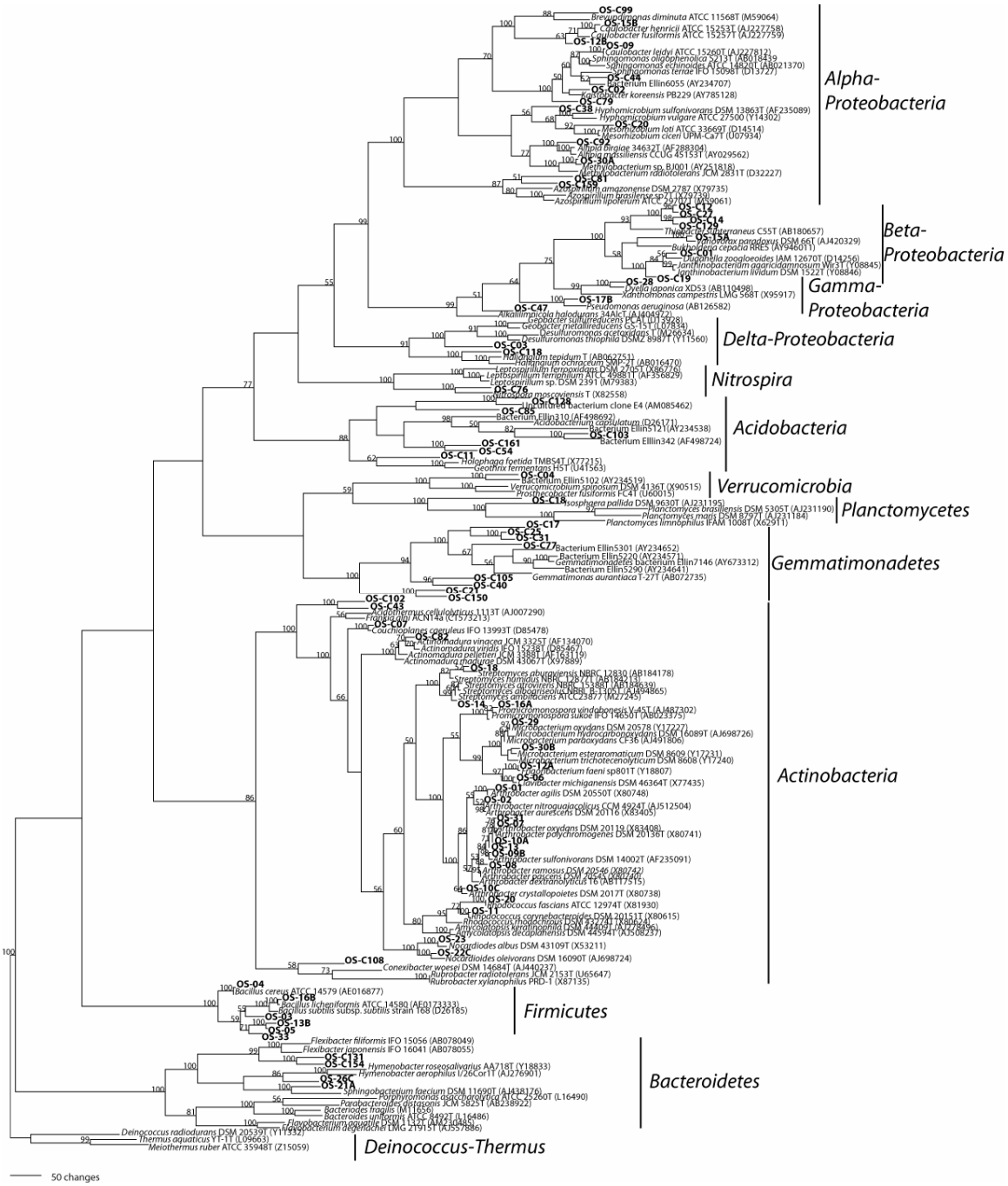

Sphingomonas oligophenolica S213T (AB018439)
K4-11
Sphingomonas terrae IFO 15098T (D13727)
Sphingomonas macrogoltabidus IFO 15033T (D13723)
K6-C56
Hyphomicrobium sulfonivorans DSM 13863T (AF235089)
Hyphomicrobium vulgare ATCC 27500 (Y14302)
Rhizobium leguminosarum ATCC 10004T (AY509899)
K6-22
Mesorhizobium loti ATCC 33669 T (D14514)
Mesorhizobium ciceri UPM-Ca7T (U07934)
K6-28
Nordella oligomobilis N21 (AF370880)
K6-C124
Bradyrhizobium japonicum LMG 6138T (X66024)
Bradyrhizobium liaoningense 2281T (AF363132)
K6-11
Methylobacterium dichloromethanicum DM4T (AF227128)
Fe-K6-C47
Methylobacterium populi ATCC BAA-705 (AY251818)
K6-C83
Acidiphilium acidophilum ATCC 27807 (D86511)
Alpha-Proteobacteria
S-K6-C18
K6-C101
Thiomonas delicatus NBRC 14566T (AB245481)
Thiomonas thermosulfata ATCC 51523 (U27839)
Thiomonas intermedia ATCC 15461 (AY455809)
Beta-Proteobacteria
Fe-K6-C35
K6-C11
Iron-oxidizing acidophile m-1 (AF387301)
K6-C62
S-K6-C04
Gamma proteobacterium WJ2 (AY096032)
K6-C13
Thiateuria aurantia DSM 622D (AJ010481)
Dyella japonica XD53 (AB110498)
Fulvimonas soli LMG 19981T (AJ311653)
Xanthomonas campestris LMG 568 (X95917)
Fe-K6-C12
K6-C79
Acidithiobacillus ferrooxidans ATCC 33020 (AJ2787197)
Fe-K6-C27
S-K4-C38
K6-C15
K4-C03
K4-C116
K6-C19
S-K6-C16
Acidithiobacillus thiooxidans DSM 612 (M79401)
Gamma-Proteobacteria
K4-C190
K6-C169
K4-C41
Acidimicrobium ferrooxidans DSM 10331T (U75647)
Streptomyces ambofaciens ATCC23877 (M27245)
K6-05
Streptomyces humidus NRRL B-3172T (DQ442508)
K6-06
Streptomyces lateritius JCM4389T (AF454764)
K4-07B
Rhodococcus fascians ATCC 12974T (X81930)
Rhodococcus corynebacterioides DSM 20151T (X80615)
Rhodococcus rhodochrous DSM 43274T (X80624)
K6-15
Amycolatopsis decaplanensis DSM 44594T (AJ508237)
Amycolatopsis keratiniphila DSM 44409T (AJ278496)
K6-08
Microbacterium oxydans DSM 20578 (Y17227)
K6-01
Microbacterium paraoxydans CF36 (AJ491806)
Microbacterium hydrocarbonoxydans DSM 16089T (AJ698726)
K4-07E
K4-07A
K6-C10
Subtercola frigoramans T (AF224723)
K6-02
Curtobacterium citreum DSM 20528 (X77436)
K6-25C
Arthrobacter nitroguajacolicus CCM 4924T (AJ512504)
Arthrobacter aurescens DSM 20116 (X83405)
Arthrobacter ramosus DSM 20546 (X80742)
Arthrobacter pascens DSM 20545 (X80740)
K4-10C
Arthrobacter dextranolyticus T6 (AB117515)
K4-06
Kocuria rosea P27-24 (DQ060382)
Kocuria polaris CMS 76or1 (AJ278868)
K4-07D
Brevibacterium antiquum VKM Ac-2118T (AY243344)
Brevibacterium antarticum DVS 5a2T (AJ577724)
Uncultured clone D3-28 (DQ464143)
Actinobacteria
K6-C107
Sulfobacillus sp. Y0017 (AY140239)
Sulfobacillus thermotolerans KR-1
K4-C07
Sulfobacillus thermosulfidooxidans DSM 9293T (X91080)
K6-C143
Sulfobacillus yellowstonensis YTF-1 (AY007665)
K6-C81
Sulfobacillus sp. 4G (AY371272)
K6-C04
K4-C93
K4-C26
K6-C156
K6-C109
Sulfobacillus sp. Fras1 (AF213055)
K6-C25
Uncultured bacterium clone RCP2-66 (AF523921)
K6-C05
Uncultured thermal soil bacterium YNPFFP9 (AF391988)
K6-C31
Alicyclobacillus disulfidooxidans (U34974)
Alicyclobacillus tolerans K1T (Z21979)
Alicyclobacillus acidoterrestris DSM 3922T (AJ133631)
K6-17B
Staphylococcus pasteuri ATCC 51129T (AB009944)
Staphylococcus warneri ATCC 27836T (L37603)
Staphylococcus aureus subsp. aureus NCTC 8325 (CP000253)
K6-03
Bacillus thuringiensis ATCC 10792T (AF290545)
Bacillus cereus ATCC 14579 (AE016877)
K4-04
Bacillus megaterium IAM 13418 (D16273)
K4-05
Bacillus simplex LMG 1160T (AJ628743)
K6-18
Uncultured bacterium clone AKIW724 (DQ129275)
Bacillus arsenicus con a/3 (AJ606700)
K4-10A2
Bacillus subtilis subsp. subtilis strain 168 (D26185)
Bacillus licheniformis ATCC 14580 (AE017333)
K6-09
Brevibacillus brevis JCM 2503T (D78457)
Brevibacillus laterosporus DSM 25T (AB112720)
K4-03
Paenibacillus illinoisensis NRRL NRS-1356T (D85397)
K6-19
Paenibacillus lautus NRRL NRS-666T (D78473)
K4-01
Paenibacillus cineris LMG 18439T (AJ575658)
Paenibacillus popilliae ATCC 14706T (AF071859)
Firmicutes
K6-C55
K6-C86
Bacterium Ellin310 (AF498692)
Acidobacterium capsulatum (D26171)
Bacterium Ellin342 (AF498724)
Holophaga foetida TMBS4T (X77215)
Geothrix fermentans H5T (U41563)
Acidobacteria
Fe-K4-C09
K4-C86
K6-C22
Leptospirillum ferriphilum ATCC 49881T (AF356829)
Leptospirillum ferrooxidans DSM 2705T (X86776)
Nitrospira moscoviensis T (X82558)
Nitrospira
K6-27
Flexibacter filiformis IFO 15056 (AB078049)
Flexibacter japonensis IFO 16041 (AB078055)
Parabacteroides distasonis ICM 5825T (AB238922)
Bacteroides fragilis (M11656)
Bacteroides uniformis ATCC 8492T (L16486)
Flavobacterium aquatile DSM 1132T (AM230485)
Flavobacterium degerlachei LMG 21915T (AJ557886)
Bacteroidetes
Planctomyces brasiliensis DSM 5305T (AJ231190)
Planctomyces maris DSM 8797T (AJ231184)
Planctomyces limnophilus IFAM 1008T (X62911)
Planctomycetes
50 changes

Figure 3


Alpha-Proteobacteria
Beta-Proteobacteria
Gamma-Proteobacteria
Delta-Proteobacteria
Nitrospira
Acidobacteria
Verrucomicrobia
Planctomycetes
Gemmatimonadetes
Actinobacteria
Firmicutes
Bacteroidetes
Deinococcus-Thermus
50 changes

Appendix B References

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