INTERACTIVE EFFECTS BETWEEN LIME, ORGANIC MATTER, AND BACTERIA IN THE ESTABLISHMENT OF *LEYMUS CINEREUS* IN MINE TAILINGS

by

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ABSTRACT

The landscape legacy of historical metal-mining activity can persist for decades. The most frequent strategies used for the remediation of contaminated soils include: the use of synthetic membranes to isolate contaminants (>$0.5 million/acre), direct revegetation (less expensive but difficult to sustain), or lime amendments ($5000/acre). Looking for more cost-effective bioremediation approaches, we performed a set of greenhouse studies to determine what combinations of soil amendments would lead to the best vegetative response, and potentially associated reductions in soil arsenic (As) levels, in "slickens" collected from the Lampert Ranch along the upper Clark Fork near Warm Springs, MT. In our first greenhouse experiment, we planted *Leymus cinereus* (basin wildrye) and compared (after 12 weeks) plant growth and foliar metal concentrations across treatments. Amendments included single or factorial additions of 5% lime, organic matter (+OM), and an arsenic-oxidizing (+oxbact) strain of *Agrobacterium tumefaciens (Agtu)*. Surprisingly, the OM+oxbact treatment revealed among the best plant growth and arsenic uptake response. We then performed a second greenhouse experiment with two levels of OM (1.5% and 5%) and an additional treatment: a mutant (reducing strain) of *Agtu.* Basin wildrye grown in soils amended with 5% OM generally did better than those grown in soils amended with 1.5% OM. At the same time, foliar As uptake (biomass multiplied by As concentration) was unexpectedly high (0.020 mg pot^{-1}) for plants grown in soils amended with 5% OM + oxbact, 3 times greater than foliar arsenic uptake in plants grown in soils amended with 5% OM+ lime and 4 times greater than foliar uptake by plants grown in soils amended with 5% OM and the reducing strain of *Agtu*. These results suggest the combination of OM and *Agtu* oxbact strain could provide a potentially cost- effective approach to remediating As-contaminated soils. Finally, our study results imply that soil restoration approaches could be improved through a greater consideration of microbial communities supported by these re-establishing vegetation communities, which could lead to more sustainable ecosystem successional trajectories.

CHAPTER ONE

INTRODUCTION

Statement of the Problem

There are thousands of acres of land that have been disturbed by mining activities around the world. These lands are associated with heavy metals and metalloids (among them arsenic), which represent a threat to human health and the environment. The most frequent strategies used for the remediation of mining soils include: the use of layers to isolate the toxicants within a confined area, physical removal of the contaminant, and chemical or physical transformation of the contaminants to render them less toxic (Mackova et al. 2006) . Unfortunately, the implementation of these strategies can be very costly (2.5 million USD per hectare) (Berti & Cunningham 2000) and do not offer a permanent solution (Johnson & Bradshaw 1977). Alternatives to these approaches include the use of microorganisms and plants, with two new terms becoming more widely used: bioremediation, from around 50 years ago (Mackova et al. 2006), and phytoremediation, from 1991 (Licht et al. 1995; Visoottiviseth et al. 2002).

Bioremediation and phytoremediation are relatively inexpensive and promising new technologies that refer to the use of microorganisms and green plants to assimilate or detoxify metals, metalloids and organic chemicals (Visoottiviseth et al. 2002). The use of plants is one of the most employed strategies in mining reclamation; however, their successful establishment in mining soils can be limited by low pH, high metal concentrations, and lack of nutrients (Bradshaw 1997).

General approaches to cope with harsh mine soil conditions typically include the use of lime (as $CaCO_3$ equivalents) and organic matter amendments that are believed necessary to address the problems associated with mine soils. Lime or organic matter amendment effectiveness in mine soil remediation have been extensively studied (Kabata-Pendias & Pendias 1992; Kelly 1997; Gadepalle et al. 2007; Kabata-Pendias & Pendias 2001; Bolan et al. 2003; Cao et al. 2003; Walker et al. 2004). These amendments can reduce the availability of most of the metals; however, in the particular case of arsenic they can unfortunately increase its availability and toxicity.

Arsenic is present in soil in two states, as arsenite As(III) or arsenate As(V). As(III) is more toxic and mobile than As(V) (Mandal & Suzuki 2002). The use of lime, and in some cases organic matter, can reduce As(V) in soil to As(III), increasing the toxicity to the plant (Raven et al. 1998), or if As(III) is oxidized to As(V), arsenic assimilation by a plant can be improved, because As(V) has geochemical behavior similar to phosphate $(PO_4)^{-3}$ (Meharg & Macnair,1992; Esteban et al. 2003; Moreno 2010).

Because many soil reduction and oxidation reactions are catalyzed by microorganisms, including the reduction of As(V) and oxidation of As(III) , the soil microbial community is likely to play a crucial role in the establishment of plants in toxic mine soils. These microbial communities can change redox conditions in the soil, influence plant growth, increase or decrease metal toxicity, and influence metal uptake by plants. Therefore, we examined the interactions between bacteria and common mine soil amendments on the germination and growth of basin wildrye (*Leymus cinereus*) through two principal greenhouse studies.

Research Objectives and Hypothesis

The purpose of this research project was to evaluate interactions between lime, organic matter, and an As(III)-oxidizing bacterium *Agrobacterium tumefaciens* in the germination, growth, and arsenic assimilation of the plant *Leymus cinereus* (basin wildrye) in tailings.

The primary objectives for this project were:

1. To quantify germination of *Leymus cinereus* seeds sown in tailings under differing amendment regimes.
2. To quantify plant growth and establishment success of *Leymus cinereus* in mine soils under differing amendment regimes.
3. To quantify arsenic uptake in *Leymus cinereus* roots and shoots under differing amendment regimes.
4. To test whether amending mine soils with As-oxidizing or As-reducing bacteria altered the speciation of bulk arsenic in tailings soils under differing amendment regimes.

Hypothesis: Addition of the bacterium *Agrobacterium tumefaciens* in combination with elevated levels of organic matter and lime amendments would result in greater germination rates, greater biomass growth, and greater foliar and root concentrations of arsenic by basin wildrye in our Montana tailings.

Thesis Structure

This thesis has five main chapters. Chapter One (the present chapter) is an introduction to this study that contains the social and scientific reasons that support

the development of this project. Chapter Two is a review of prior studies that provides the conceptual foundation to our soil arsenic remediation approach, and which provides the scientific basis for our experiments. Chapter Three presents the results of the first pilot greenhouse study, which allowed us to select promising treatment combinations for subsequent, refined experimentation. Chapter Four presents the results from a second greenhouse experiment where we evaluated more specifically the role of bacteria in soil arsenic redox transformations for a subset of treatment combinations, quantifying effects on biomass and arsenic uptake concentrations. Finally, Chapter Five contains the overall conclusions for this thesis.

CHAPTER TWO

LITERATURE REVIEW

Arsenic in the Environment

Arsenic (As) is an element widely distributed in the environment; it is ranked 20th in crustal (terrestrial) abundance, 14th in seawater, and 12th in the human body (Woolson 1975; Mandal & Suzuki 2002). In the Earth's crust, the total arsenic is estimated to be 4×10^{16} kg based on typical concentrations in rock material (Matschullat 2000). In regions with abundant volcanic rocks or sulfide ores, however, arsenic concentrations can be much greater. For example, arsenic concentrations reached 20,000 mg kg^{-1} in gold-mine-associated rocks (Smith et al. 1998). In nature, arsenic is present in more than 200 different mineral forms including As(V) (60%), sulfides and sulfosalts (20%), as well as other forms such as arsenides, As(III), oxides and silicates (20%) (Mandal & Suzuki 2002). Of these, arsenopyrite (AsFeS) is the most abundant (Smedley & Kinniburgh 2002).

Though arsenic is widespread at naturally low concentrations, human activities are responsible for over 80% of all the arsenic introduced to the environment (Nriagu et al. 2007). Sixty percent of these anthropogenic arsenic emissions can be accounted for from only two sources: copper-smelting and coal combustion. Other sources include: the application of herbicides, fungicides, pesticides, glass-production, wood preservation, and waste incineration. Globally, arsenic production is between 75,000 and 100,000 Mg per year (Adriano 2001).

Arsenic in Soils

The range for background total arsenic concentrations in uncontaminated topsoils worldwide is 1–100 mg kg^{-1} (McLaren et al. 2006). However, mean arsenic concentrations determined for individual countries or soil types are usually less than 10 mg kg^{-1} (Kabata-Pendias & Pendias 2001). The variation in arsenic concentrations between soils is related to differences in soil parent material and degree of weathering during soil development. For example, sedimentary rocks contain much higher concentrations of arsenic than igneous rocks (Bhumbla & Keefer 1994). In addition to the arsenic derived from soil parent materials, some arsenic has accumulated in soils as a result of atmospheric deposition. Although a substantial proportion of the current emissions of arsenic to the atmosphere is of anthropogenic origin, natural sources such as volcanic activity and low-temperature volatilization are also important (O'Neill 1990).

Arsenic in soil occurs mainly as inorganic species, typically as arsenite As(III) or as arsenate As(V) (Atker & Naidu 2006). Under reducing conditions (relatively low soil oxygen levels), the predominant inorganic compound in soils is As(III) which is more toxic, more soluble, and more mobile than As(V) (Mandal & Suzuki 2002). For example, in a study conducted by Yoon et al. (2015), the half maximal effective concentration (EC_{50}) for germination in pea was 200 mg kg^{-1} for As(III) but 413 mg kg^{-1} for As(V). The As (III) is highly toxic because it inactivates protein function by reacting with sulfhydryl groups (e.g. cysteine residues). At the whole plant level, this results in degradation of membranes, disruption of root function, and rapid cell death and necrosis (Wauchope 1983).

Toxicity of As(V) stems from it replacing phosphate in many biochemical reactions that produce unstable reaction products (Dixon 1996). As specific examples in plants, As(V) uncouples oxidative phosphorylation in the mitochondria, inhibits the foliar absorption of other chemical elements, and has a profound effect on many critical enzyme reactions. As(V) can be found under oxidizing conditions and can be absorbed onto clays, iron and manganese oxides/hydroxides and organic matter. Compounds of As(V) with Fe and Al ($FeAsO_4$,$AlAsO_4$) are the dominant phases in acidic soils and are less soluble than calcium-As(V) componds ($Ca_3[AsO_4]_2$), which is the main chemical form in alkaline and calcareous soils (Fordyce et al. 1995).

Mobility and Solubility of Arsenic in Soil

The retention of elements by soils involves biochemical processes of considerable complexity, which are strongly interrelated with the chemical, physical and biological properties of the soil. These processes control the solubility, bioavailability, mobility and toxicity of the elements (Sparks 2005; Adriano 2001). The most important of these processes are: plant absorption, retention on the surfaces of organic matter and soil colloids, transport through the soil profile via leaching or transport associated with colloids, precipitation as a new phase in the soil, dissemination through the micro- and macro-pores present in the soil, solubilization in the soil solution, and interactions with microorganisms through redox reactions and methylation.

Many elements in the soil can be found in various forms. This may occur in soluble form, interchangeably (associated with the anion or cation exchange capacity of soil colloids such as clays or organic matter), retained in oxides, hydroxides, and

oxyhydroxides (mainly Fe, Mn and Al), associated with soil organic matter, bounded with carbonates; all these secondary forms are derived from the chemical weathering of trace-element-containing primary minerals such as arsenopyrite (residual fraction) (Moreno-Jimenez 2010).

Sequential extraction methodologies show that arsenic is often associated with oxides and hydroxides in soils (McLaren et al. 2006). Exchange surfaces of silicates and organic matter are usually negatively charged, so they are more likely to retain cations. Low pH conditions (with a predominance of positive charges) can retain arsenic present in molecules such as ($H_2AsO_4^-$), $HAsO_4^{2-}$, H_3AsO_3, or AsO_3^{-3}. Once in the soil solution, elements can have different speciation forms and ionic activity (Sauvé 2001), and it is in solution that environmental risk of arsenic is often greatest.

Bioavailability of a contaminant in the soil is nearly impossible to measure because of the complexity of soil physical, chemical, and biological processes over very short time and space scales (Moreno-Jimenez 2010). In the case of plants, the term phytoavailablity has been used, referring to the “the amount of arsenic that a plant could take from the soil", but this concept has not often been operationally defined and lacks predictive power (Fitz & Wenzel 2006). However, traditionally phytoavailability has been estimated by correlating the concentration of an element in soil and in plant tissue growing in that soil (Feng et al. 2005; Vázquez et al. 2008).

Available and unavailable fractions of the soil contaminants are usually in equilibrium, but, any change in environmental factors (pH, Eh, climate, hydrology, biology, organic material) or the alteration of minerals (dissolution-precipitation, oxidation-reduction, complexation-dissociation, adsorption-desorption) can alter the availability of the element (Mench et al. 2009). Methods evaluating the availability of

an element in the soil suggest that it is not the total quantity of the element in the soil which is available to the plant because primary mineral forms in sand-sized grains, for example, and fractions of secondary forms strongly retained by soil colloids reduce the fraction of an element that is soluble and can be absorbed by a root. Therefore, the liquid phase of the element and the balance between the solid and liquid phases are the most important concepts (Sauvé 2001).

Factors Affecting the Availability of Arsenic in Soil

Effect of pH and pE

Soil colloids have negative and positive charges simultaneously. One part of that charge is fixed, and the other part is pH-dependent. The pH-dependent charge appears in the presence of oxyhydroxides of metals such as Fe, Al and Mn in clay minerals and organic matter. The functional groups on the edges of these materials can be protonated (positively charged) or deprotonated (negatively charged) as a function of pH. When soil pH increases, deprotonation can occur and is at about pH 4 when this occurs in the organic matter. The point of zero charge (PZC) of each soil component is a characteristic value of the soil pH. Below the PZC, the soil component is positively charged (protonation) and above the PZC, the soil will be negatively charged (deprotonation). When soil components are positively charged, these surfaces can retain anions such as As(V) or As(III) (Moreno-Jimenez 2010).

As(V) is the predominant form of arsenic in soils where pH + pE (electron activity) > 10, while As(III) is the dominant form found in soils where pH + pE < 6 (Sadiq 1997). Under aerobic conditions, sulfides are easily oxidized, and as a consequence arsenic is released into the environment (Adriano 2001); when soil pH is

between 3 and 13, the major species found are $H_2AsO_4^-$ and $HAsO_4^{2-}$ (Smedley & Kinniburgh 2002). In reducing environments, arsenic is found as As(III), the predominant species of which are H_3AsO_3 and AsO_3^{-3}.

Generally, an increase in pH causes a release of anions to the exchange positions, triggering a release of As(V) and As(III) from the exchange complex (Smith et al. 1999; Fitz & Wenzel 2002). However there are experiments that show that in the presence of high pH and sulfates and carbonates, arsenic coprecipitation may occur in the form of sulfates and oxyhydroxides (García et al. 2009) or even precipitation as calcium As(V) which is less insoluble than calcium phosphate. In well-aerated alkaline soils, the solubility of arsenic is limited by its precipitation as Ca- or Fe-As(V) (Xie & Naidu 2006). In soils that contain high pH, carbonates can play an important role in the retention of As(V). When the pH drops below 2.5, As(V) becomes completely protonated (Zhang & Selim 2008) making it harder to be retained by soil particles. Figure 2.1 summarizes the charges of some colloids as a function of pH. Shaded areas indicate ranges in which colloids can retain As oxyanions. At acidic pH, the oxides are quite effective. In basic pH, arsenic retention may occur over the carbonates or oxides of aluminum and magnesium (Sadiq 1997).

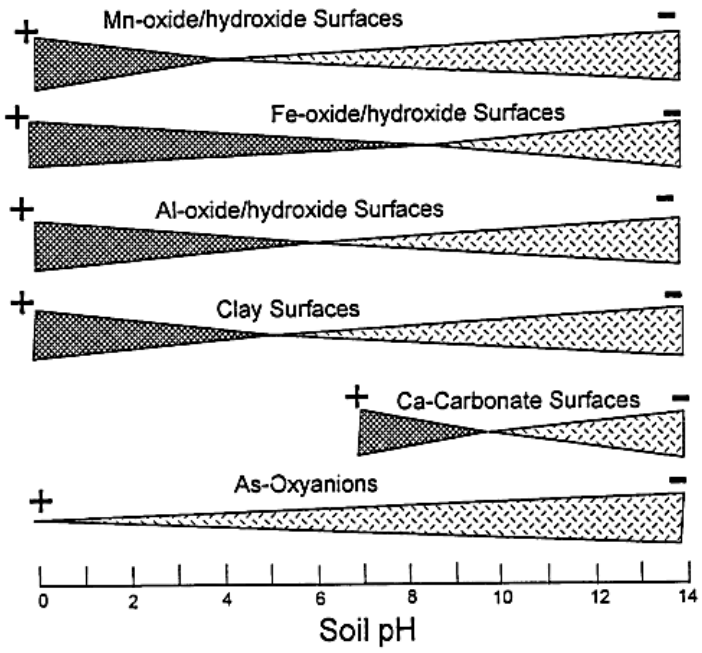

Figure 0-1 General distribution of arsenic with respect to different soil colloids and pH (Sadiq 1997).

The arsenic chemical forms in oxic and anoxic soil solution are generally negatively charged; therefore, the use of some amendments affecting pH can have different results in arsenic adsorption. The results of arsenic adsorption vary largely in studies. Jones et al. (1997) found that the solubility of arsenic in tailings increased with pH following liming. This can be explained because arsenic in soil is present as negative molecule, so an increase of pH will increase the negative charge in the soil decreasing the anion exchange capacity. For example, in a study conducted by Taboada et al. (2008) calcium As(V) compounds such as ($CaHAsO \cdot H_2O$), ($CaHAsO_4 \cdot 2H_2O$) and (CaHAsO) were soluble in water with a range of leachable arsenic from 900 to 4400 mg L^{-1}. As(III) levels increased from 2 to 75 times following the liming of three mine waste sites in Anaconda, Montana (Reclamation

Research Unit 1997). Maeda and Teshigori (Yan-Chu 1994) found that soluble arsenic increases with decreasing redox potential and increasing pH. Goldberg (2002) determined that arsenic adsorption by oxides and clays decreased with pH above 5 for clays, pH 7 for Fe oxides, and pH 9 for aluminum oxides. In contrast with these studies, Kelly (1997) found that soluble arsenic concentrations in pyritic mine wastes decreased after amendment with lime or other dolomitic products. Krueger (1997) observed that liming reduced soluble arsenic concentrations in mine tailings from three different sites in Montana.

Effect of Clay Minerals

There are many factors involved in the soil adsorption and desorption processes that affect arsenic mobility. Among these factors is the structure of primary and secondary minerals with which arsenic comes into contact (Moreno-Jiménez et al. 2010). Clays have been used largely as adsorptive media for heavy metals (Celis et al. 2000). The availability of arsenic is generally higher in sandy textured soils than in clay (Adriano 2001).

Arsenic retention in clays is less effective than in the oxides (Gräfe & Sparks 2006). The anionic character of arsenic suggests that the retention mechanism of this metalloid in the clays could be similar to the retention mechanism of phosphorus (P) in soils mediated by calcium (Fixen & Grove 1990). Studies such as Frost & Griffin (1977) show that montmorillonite can adsorb more As(V) and As(III) than kaolinite, because of the higher surface charge of the montmorillonite. Lin & Puls (2000) found that halloysite and chlorite have a greater capacity to adsorb As(V) than the rest of the

clay minerals, and also that the kaolinite, illite and illite-montmorillonite have a moderate adsorption of As(V).

Interactions with Organic Matter

Addition of organic matter to contaminated soils has been used for many years to improve soil fertility, enhance revegetation and decrease availability of toxic metals (Abbott et al. 2001). The addition of organic amendments to soils can reduce the availability of metals by changing these from bioavailable forms to less-available forms in the fractions associated with organic matter, metal oxides, or carbonates (Walker et al. 2004). The effects of organic matter on metal mobility, however, depend on composition of the organic matter. In general, compounds with larger molecular weight will more effectively retain trace elements, whereas more soluble and lighter compounds might dissolve elements by chelating them or can compete with As(V) and As(III) for soil retention positions (Bernal et al. 2009; Moreno-Jimenez 2010).

Because soil organic matter is complex and often poorly characterized, its effects on arsenic are still poorly understood. Most research has shown a reduction of the uptake of metals and arsenic by plants after the addition of composted materials to soils (Gadepalle et al. 2007, Sabeha K Ouki, et al. 2007). For example, Cao & Ma (2004), showed that biosolid compost application reduced plant uptake of arsenic in carrots by 79–88% and in lettuce by 86–96% compared to untreated soil. Arsenic was adsorbed by the organic matter from the compost, and the fractionation analysis performed showed a reduction of arsenic in soluble water in soil by 45%. Similarly, Cao et al. (2003) reported that dried municipal solid waste and biosolid compost

amendments spiked with arsenic at a ratio of 50 g/kg soil, reduced water soluble arsenic and the uptake of arsenic by Chinese brake fern (*Pteris vittata L.*). The authors hypothesized that arsenic adsorption onto the organic matter was responsible for the observed reductions. A possible theory that explains how organic matter reduces availability of arsenic and metals refers to the relationship between humic acids and the arsenic. Humic acids constitute a major part of the organic matter and they can reduce metal solubility by the formation of stable metal chelates (Ross 1994), or some humic acids may form humic-clay complexes that appear to have the capacity to retain arsenic (Saada et al. 2003).

Contradictorily, some authors maintain that organic matter reduces the degree of adsorption of both forms of arsenic (As(III) and As(V)) and influences their mobility (Redman et al. 2002). This is due to the blocking of adsorption sites of the soil by the soluble fraction of organic matter (fulvic and humic acids) (Mayorga-Moreno 2010). Cao et al. (2003) reported that when biosolid compost was added to either acidic or neutral soil the adsorption of arsenic increased resulting in a reduction of water-soluble arsenic. Likewise, dried municipal solid waste and biosolid compost were mixed with chromated-copper-As(V) (CCA) soil and arsenic-spiked contaminated soil at a ratio of 50 g/kg soil. In this research, both municipal solid waste compost and biosolid compost added to CCA contaminated soil with a neutral pH induced an anaerobic environment in the soil, which favored the conversion of As(V) to more mobile As(III) . In this study, the concentration of As(III) in solution was increased from 10 to 20% or 24% for municipal solid waste or biosolid compost, respectively. Mench et al. (2003) and Clemente et al. (2008) also report similar arsenic behavior after organic matter application.

Arsenic-Microbe Interactions

Microbial biotransformations of arsenic play an important role in the biogeochemical cycling of arsenic, influencing arsenic mobility, distribution and bioavailability. The mechanisms of bacterial resistance to arsenic are associated with genetic determinants, which give them the ability to perform transformations, mainly oxidation and/or reduction (Gadd 2000).

The microbial processes involved in the removal of metal or metalloids in soils and sediments are grouped into three categories: bioaccumulation, oxidation/reduction and methylation/demethylation (Bolan et al. 2006). In terms of this thesis only the processes of oxidation/reduction will be addressed.

Microbial Oxidation of As(III) to As(V)

Abiotic arsenic oxidation can occur naturally at very slow rates; however, this process can be performed faster through the use of microorganisms such as bacteria (Bolan et al. 2006). Oxidation of arsenic can be done by heterotrophic or autotrophic bacteria. Bacterial oxidation of As (III) to As (V) was first described in 1918, but it was not until 1992 that the first As(III) oxidase enzyme from *Alcaligenes faecalis* was isolated (Anderson et al. 1992; Pacheco-González et al. 2013).

As(III) oxidase enzyme Aio (formerly Aox, Aro or Aso) is one of the most important enzymes in arsenic oxidation. As(III) oxidase is involved in the detoxification of arsenic in heterotrophic bacteria (Muller et al. 2003) and in energy generation for chemolithotrophic bacteria (Santini & Vanden Hoven 2004). As(III)-oxidizing bacteria are phylogenetically diverse (Battaglia-Brunet et al. 2006) but all perform the oxidation of As(III) by the Aio enzyme.

Autotrophic As(III) oxidation does not require an organic source of carbon for growth, because the microorganisms are able to fix inorganic carbon from CO_2 or HCO_3^-. In this case these microorganisms gain energy using As(III) as an electron donor (Osborne & Santini 2012). The research of Dastidar & Wang (2009) suggests that autotrophic As(III) oxidation process is apparently preferred over the heterotrophic because of its lower nutritional requirements and lower potential production of harmful organic metabolites.

The oxidation of As(III) to As(V) is a complex process where the pathways are still being researched. A hypothetical pathway is presented in Figure 2.2. In this figure, As(III) enters into the periplasm of the cell and it is detected directly by the protein AioX and indirectly by the AioS sensor kinase. This AioS enzyme, combined with a phosphoryl group, activates the AioR transcriptional response regulator. AioR enzyme with the assistance of a factor (RpoN) will induce Aio operon expression, which encodes the Aio enzyme which is responsible for the oxidation of As(III) to As(V) (Wojnowska & Djordjevic 2012).

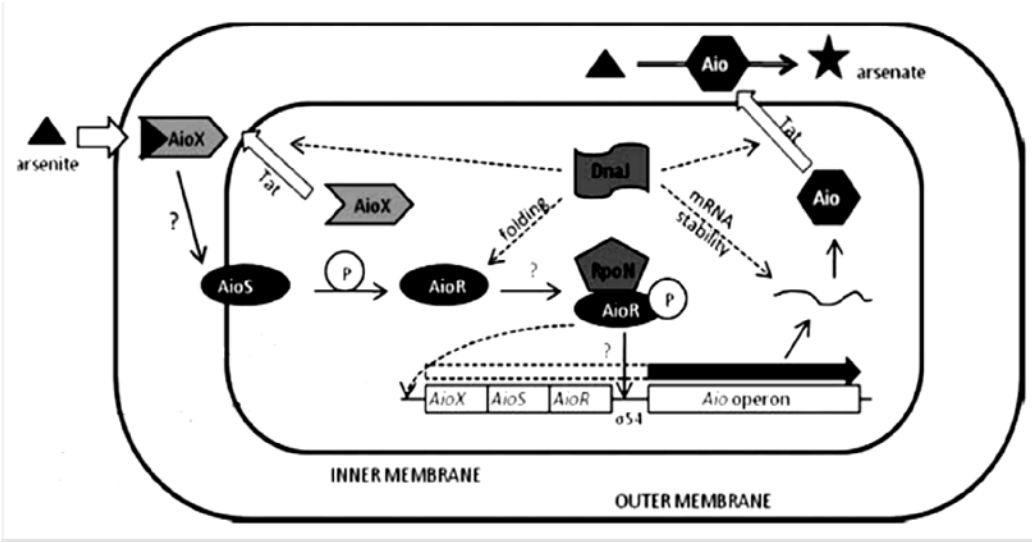

Figure 0-2 Conceptual diagram of As(III) oxidase expression (Santini & Ward 2012). P: phosphoryl group, Tat-twin-arginine translocation export pathway, question marks indicate unknown mechanisms, dashed arrows mean putative pathways.

Microbial Reduction of As(V) to As(III)

Two different arsenic reduction activities have been found in bacteria: 1) dissimilatory reduction (respiration) and 2) a detoxification mechanism. Dissimilatory reduction for anaerobic respiration involves As(V) as the terminal acceptor of electrons and the second one has the purpose of detoxifying arsenic by converting As(V) to As(III). Detoxification mechanisms are often considered as the main mechanism responsible for the rapid reduction of As(V) observed in anaerobic environments (Macur et al. 2001). Detoxification-based microbial As(V) reduction is controlled by the ArsC As(V) reductase, with the resulting As(III) removed from the cell via the efflux pump, ArsB (Tamaki & Frankenberger 1992; Cervantes et al. 1994), which is an inner-membrane protein.

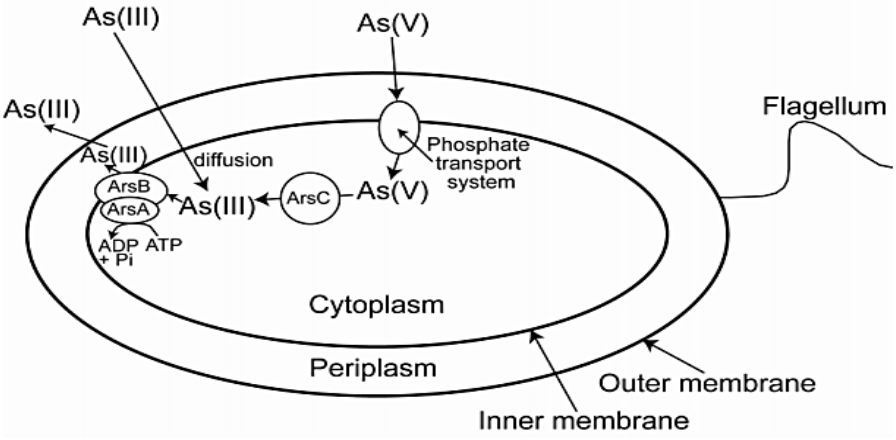

Figure 0-3 Conceptual diagram of arsenic resistance mechanism (Hudson-Edwards & Santini 2013)

Agrobacterium tumefaciens

Agrobacterium tumefaciens is a gram-negative, rod-shaped bacterium that belongs to the *Agrobacterium* genus, *Rhizobiaceae* family, *Rhizobiales* order, *Alphaproteobacteria* class, and the *Proteobacteria* phylum (Smith & Townsend 1907). It is found in soils and is most known because of its ability to induce crown-gall tumors on dicotyledonous plants and its use in biotechnology to transfer genes into plants (Duckely & Hohn 2003).

There are a number of studies that show the ability of this bacterium to live in areas with very high toxic levels of contaminants such as arsenic. Macur et al. (2001) showed the ability of this bacterium to oxidize or reduce arsenic in unsaturated soils contaminated with arsenic. Lehr et al. (2007) studied antimony and arsenic oxidation performed by this bacterium. Santini et al. (2000) performed some studies with *Agrobacterium/Rhizobium* and reported this organism to be the most rapidly growing chemolithoautotrophic As(III) oxidizer known. Hao et al. (2012) isolated *A.*

tumefaciens from the nodules of *Robinia pseudoacacia* growing in zinc-lead mine tailings and found that this bacteria showed high metal resistance and enhanced the growth of *Robinia* plants, suggesting that this bacteria can be used as a plant-growth-promoting bacterium (PGPB). Kashyap et al. (2006) and Lehr et al. (2007) showed the ability of *A. tumefaciens* to rapidly oxidize As(III) to As(V).

Arsenic-Plant Interactions

Plants need macronutrients and micronutrients from the soil (which include metals such as potassium, copper, or zinc) to develop properly. Some plants can take up or tolerate more micronutrients than others and in this case, these plants can be called metal hyperaccumulators or metal-tolerant plants (Lasat 2002).

Depending on the concentration of the metal or metalloid, plants can be classified as excluders, indicators, accumulators, or hyperaccumulators. The excluders avoid the accumulation of the contaminant into the aerial part of the plant. Indicators maintain the same relative proportion of the contaminant in the foliage as in the soil. Accumulators show a high concentration of a trace element in the aerial part, higher than that of the soil in which they live (Baker 1981). A special case are plants that can hyperaccumulate contaminants, and these are called hyperaccumulators, which are plants capable of growing in soils with very high concentrations of metals, absorbing these metals through their roots, and concentrating extremely high levels of metals in their tissues compared to other plants (Padmavathiamma & Li 2007). Hyper-accumulators generally refer to plants able to accumulate >0.1% of dry weight of elements such as Ni, Cu or Pb. For Zn, the limit is >1%, and for Cd, >0.01% of dry

weight (Clemens 2001) . The most common features characterizing hyperaccumulators are:

- The concentrations of elements in aboveground tissues are high, in the case of arsenic, > 0.1% or >1 g kg^{-1}.
- The concentration in aboveground tissues is 10-500 times higher than aboveground tissues of the same plant in unpolluted environments.
- The ratio of the concentrations of the element in the shoots to that in the roots is greater than 1 (Branquinho et al. 2007).

Plants that hyperaccumulate arsenic include: *Pteris vittata* (22,000 mg kg^{-1}); *Agrostis tenuis* and *Agrostis stolonifera* (10,000 mg kg^{-1}); *Pityrogramma calomelanos*, *Mimosa pudica*, and *Melastoma malabratrhicum* (8350 mg kg^{-1}); *Jasione montana* (6,640 mg kg^{-1}); and *Calluna vulgaris* (4,130 mg kg^{-1}) (Wang & Mulligan 2006).

Phytoremediation

The use of plants and associated microorganisms to eliminate, remove, transfer, stabilize and/or degrade contaminants in soil, sediment and water environment is called phytoremediation. Although it usually refers to the decontamination and redevelopment of soils contaminated by pollutants, it also includes the use of plants in soil conservation (Evangelou et al. 2015). This technique is now considered a potential low-cost and an environmentally adequate alternative to soil replacement, excavation and washing techniques (Clemens 2001).

Depending on the contaminants, the site conditions, the level of clean-up required, and the types of plants, phytoremediation technology can be divided into

phytoextraction, rhizofiltration, phytostimulation, phytostabilization, phytotransformation, and phytovolatilization.

Phytoextraction

Also called phytoaccumulation, phytoextraction refers to the uptake and translocation of metal contaminants in the soil by plant roots to aboveground biomass of the plants (Padmavathiamma & Li 2007). This technique is used primarily for the remediation of metals and other toxic inorganic compounds such as: Se, As, Hg, radionuclides, etc. Plants used in phytoextraction must have the following features: the ability to tolerate and accumulate high concentrations of metals in harvestable parts, a high rate of growth, and a large volume of biomass.

Phytostabilization

Phytostabilization is a technique where plants are used to immobilize pollutants in the soil through its absorption and immobilization in the roots or by precipitation in the area of the rhizosphere. During phytostabilization, accumulation of metals in plant shoot tissues is undesirable (Mendez & Maier 2008). This process should reduce the contaminant mobility and prevent migration of the contaminant in groundwater, air, and soil.

Rhizofiltration. Rhizofiltration involves the use of plants to remediate and extract contaminants from groundwater, surface water and wastewater. Roots are the most common organs used to absorb, precipitate, and concentrate heavy metals from contaminated liquid effluents. Rhizofiltration plants are known for their abundant

branching in the roots and ability to remove contaminants over prolonged periods. Aquatic plants are often used in this technique (Moosavi & Seghatoleslami 2013).

Phytotransformation

Phytotransformation is a technique that includes phytostimulation and phytovolatilization. Phytostimulation implies the use of plants and microorganisms to uptake, metabolize, and degrade the contaminants in the soil. Thus, plants provide a habitat for microbial communities and increase the size and activity of microbial populations. Plants with the largest and most dense root systems are used for this process because they are more likely to live in association with the microorganisms (Garbisu & Alkorta 2001). Phytovolatilization converts metals into a gaseous state (where possible), which is evaporated or volatilized into the atmosphere (Singh & Jain 2003).

Leymus cinereus (Scribn. & Merr.) Á. Löve

Leymus cinereus, commonly call basin wildrye, is a plant well-adapted to disturbed soils affected by mining and fire. This species is a native, perennial, cool-season bunch grass of the high plains and intermountain region of the western US. Features of basin wildrye used in mining reclamation include: winter hardiness; adaptability to acid, alkaline, and saline soil conditions; drought tolerance; high biomass production (this grass can grow into clumps that are 3 feet [1 m] in diameter and 3 to 6 feet [1-2 m] tall); well-developed, coarse fibrous root systems; and broad adaptability to different soil textures (NRCS 2011).

Basin wildrye has been seeded in many revegetation and phytostabilization projects in the Upper Clark Fork River basin. It can grow in soils that contain high levels of arsenic (1334 mg kg^{-1}; Martin 2009) . This study also showed that basin wildrye was one of the species that responded best to contaminated soils in terms of percent emergence, shoot height, and biomass production. Other researchers such as Knudson et al. (2003), reported this plant growing naturally in soils with elevated arsenic content and limited nutrient availability. Marty (2000) reported that basin wildrye was one of the top-performing species growing in Montana soils affected by mining activities. Other studies where basin wildrye was researched as a species used in restoration include Paschke & Redente (2002) and Knudson et al. (2003).

Absorption of Arsenic by Plants

Given the speciation of arsenic in the soil, predominantly in inorganic forms such as As(III) and As(V), any reductions of arsenic in the soil will occur primarily by absorption through the root, similar to nutrients or other trace elements. Arsenic absorption in the roots will depend on the biogeochemical modification of the conditions in the direct area of the root and the relationship with microorganisms. Although there is relatively little information on the capacity of plants to mobilize or immobilize arsenic, the phosphate-As(V) analogy is a conceptual approach that allows us to draw certain parallels in the dynamics of phosphorus (P) and arsenic in the rhizosphere (Fitz & Wenzel 2002). Arsenic can enter into the root system through two main channels which are the aquaporins or phosphate transporters (Moreno-Jimenez 2010).

The uncharged molecules like the arsenous acid $As(OH)_3$ can enter the root through aquaporin transporters, and researchers such as Isayenkov & Maathuis (2008) and Ma et al. (2008) have shown the involvement of aquaporins in As(III) absorption. On the other hand, the phosphate transporters can allow the entrance of As(V) into the plant through the same interaction that occurs between P at the root uptake level. Studies such as Meharg & Macnair (1992) and Esteban et al. (2003) have shown the role of these phosphate transporters in the absorption of As(V) by the plant.

Accumulation and Transport

Once arsenic has been absorbed by the plant, the compounds of this element (particularly As(V)) flow through the plant in a few hours symplastically (active transport of cytoplasm to cytoplasm) or apoplastically (extracellular transport Wauchope 1983). As(III) transport from the roots is limited by its high toxicity to root membranes. As(V) is most rapidly absorbed and translocated because of its lower toxicity to the roots; the uptake speed is 3 to 4 times greater for As(V) than for As(III) (Carbonell-Barrachina et al. 1995). A more recent study (Verbruggen et al. 2009) suggests that no matter the form of arsenic absorbed, once it is in the cell, it is converted into As(III). As(III) can be incorporated into the vacuole or into the cytoplasm, and it can leave the cell as As(III) or may be transported to the aerial parts through the xylem. In the case that it remains as As(V), it will be transported through the xylem. Arsenic transport as As(V) or As (III) to the shoots is generally not very effective; therefore, it remains in the vacuole of the cells of the roots. This accumulation in the vacuole is a way of reducing toxicity of metals in the cytosol (Hall 2002). An exception to this storage occurs in plants able to accumulate arsenic

in aboveground parts (Zhao et al. 2003). The reducing process in roots may constitute a physiological mechanism by which plants limit the flow of arsenic into aboveground tissues, thus protecting them from the effects of this metalloid (Moreno-Jiménez et al. 2010).

A schematic pathway of uptake of arsenic into the cell and subsequent translocations is shown in figure 2.4.

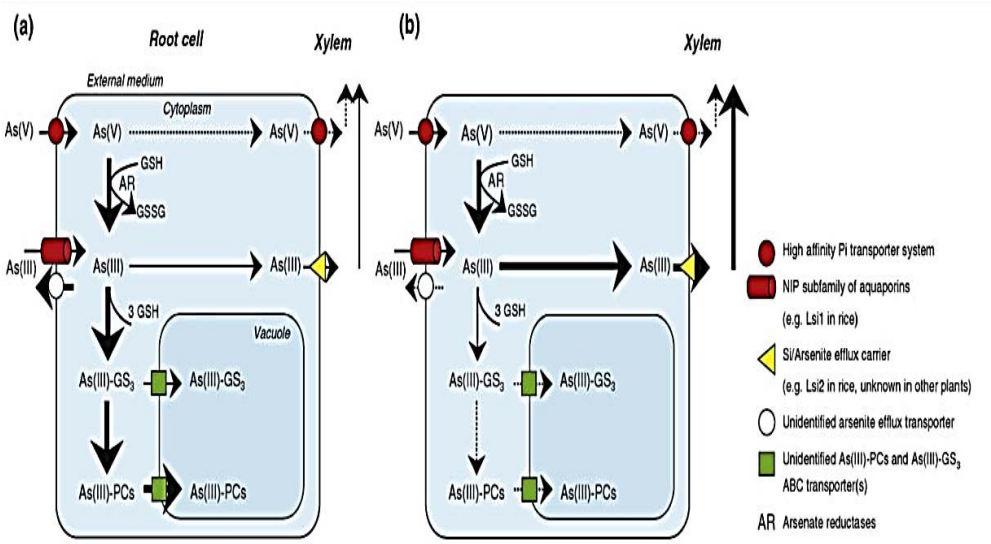

Figure 0-4. Mechanisms to cope with As excess in roots (Verbruggen et al., 2009)

Arsenic Toxicity in Plants

As mentioned above, As(III) is more toxic than As(V). As(III) reacts with disulfide groups on proteins, inhibiting enzyme reactions in the case of enzymes, which can lead to cell death. On the other hand, As(V) is potentially toxic because phosphate can be substituted by As(V) in phosphorylation reactions, including ATP synthesis which can result in an inadequate supply of energy at the cellular level (Scott-Fordsmand & Pedersen 1995; Horswell & Speir 2006). Phytotoxicity

symptoms caused by arsenic include: leaf wilting, violet coloration, root discoloration, cell plasmolysis, and growth reduction that may result in death (Carbonell-Barrachina et al. 1995).

Kabata-Pendias & Pendias (1992) and Neuman & Ford (2006) have noted that normal or sufficient levels of arsenic in mature leaf tissue range from 1 to 1.7 mg kg^{-1}, and excessive or toxic levels for plants range from 5 to 20 mg kg^{-1} . Similarly, Carbonell-Barrachina et al. (1995) reported that foliar arsenic concentrations higher than 5 mg kg^{-1} are toxic to sensitive species whereas 50 mg kg^{-1} in less sensitive plants can result in a growth reduction of more than 50%.

CHAPTER THREE

FIRST EXPERIMENT: INTERACTIVE EFFECTS OF A. TUMEFACIENS AND ORGANIC MATTER IN THE ESTABLISHMENT \OF LEYMUS CINEREUS IN MINE TAILINGS IN MONTANA

Introduction

Arsenic (As) is one of the top ten chemicals that the World Health Organization (WHO) considers to be of concern to public health (Davis et al. 2014). Although arsenic is found naturally in the environment at levels between 1–100 mg kg^{-1} (McLaren et al. 2006), human activities, particularly mining, can result in soil arsenic concentrations up to 20,000 mg kg^{-1} (Smith et al. 1998). Arsenic is generally found in two states in soils: as arsenite As(III) and arsenate As(V). As(III) is more toxic and mobile than As(V) (Tamaki & Frankenberger 1992). Since 1991, the use of phytoremediation technology has been recognized as a novel and less expensive technique for mining reclamation (Licht et al. 1995; Visoottiviseth et al. 2002) and specific plants have been proposed as a viable approach to reducing soil metals and arsenic concentrations. However, in mine tailings, amendment recommendations to promote plant establishment and growth are still lacking. The use of lime (as $CaCO_3$ equivalents) and organic matter are believed necessary in mine tailings because of associated problems such as soil acidity, poor physical structure, low nutrient availability, and toxic levels of arsenic and co-occurring heavy metals such as lead, zinc, and cadmium (Bradshaw 1997). Amendments such as lime, however, may abiotically reduce As(V) to As(III), increasing the toxicity of As to the plant (Raven

et al. 1998) or they may improve plant assimilation if As(III) is oxidized to As(V), which has geochemical behavior similar to that of phosphate $(PO_4)^{-3}$ (Meharg & Macnair 1992; Esteban et al. 2003; Moreno-Jimenez 2010).

The use of lime to increase soil pH and "immobilize" heavy metals has been extensively studied (Andersson & Siman 1991; Kelly 1997; Fang & Wong 1999; Ye et al. 2000; Abbott et al. 2001; Orndorff et al. 2002; Bolan et al. 2003; Garau et al. 2007). Reduction of metal availability by lime occurs because higher pH induces an increases in negative charge, and triggers a greater competition for Ca^{2+} (Bolan et al. 2003). However, an increase in pH can also mobilize soil arsenic and change arsenic speciation (Moreno-Jimenez 2010). As (III) is the predominant adsorbed form of arsenic in soils with high pH, while the As(V) is the dominant adsorbed form in soils with low values of pH (Sadiq 1997). Smith et al. (1999) and Fitz & Wenzel (2002) showed that while an increase in pH of the soil can cause a release of As(V), mainly As(III) is released. Chemical immobilization of arsenic in soil is due to bi- and tri-dendate bonding with Fe-oxy-hydroxides.

The use of organic matter amendments generally improves soil structural properties such as aggregation, reduces leaching, and can increase nutrient availability (Chiu et al. 2006; Walker et al. 2004). However, the influence of organic matter amendments on soil arsenic levels is actively debated; in some studies, addition of organic matter has reduced the mobility of As(III) and As(V) (Gadepalle et al. 2007) but in others there has been a release of arsenic after application of compost or a high correlation between soluble carbohydrates and soluble arsenic in soils (Mench et al. 2003; Clemente et al. 2008). One of the main components of organic matter is the humic and fulvic acids. Thus humic acids can reduce metal solubility by the formation

of stable metal chelates (Ross 1994) or may form humic-clay complexes that have the capacity to retain arsenic (Saada et al. 2003). On the other hand, humic and fulvic acids can influence arsenic mobility because the soil adsorption sites can be blocked by these acids (Mayorga-Moreno 2010).

Bacterial biotransformation plays an important role in the biogeochemical cycling of arsenic, intervening in its mobility, distribution, speciation, and bioavailability. Thus, inoculum of bacteria can influence positively plant growth, metal transformations and metal immobilization (Wu et al. 2006). This makes these microorganisms advantageous in phytoremediation through their direct influences on metal speciation, stimulation of plant growth and protection to the plant of metal toxicity.

Despite the important role of microorganisms in plant interactions with the soil environment in general, phytoremediation practices with microbial inoculation and/or indigenous communities introduced to the remediated sites have been largely ignored (Wu et al. 2006). Therefore, the purpose of this study project was to evaluate interactions between amendments such as lime, organic matter, and the oxidizing strain of *A. tumefaciens* in the germination, growth and arsenic assimilation of the plant *Leymus cinereus* in contaminated tailing soils. To achieve these goals, a greenhouse study was performed to evaluate the variables described previously as single (+ lime; + OM; or + oxidizing bacteria), double (+ lime + OM; + Lime + oxidizing bacteria; or + OM + oxidizing bacteria), or triple amendments (+ lime + OM + oxidizing bacteria).

Materials and Methods

Greenhouse Experiment

We performed a greenhouse study using a completely randomized design with four replications for each treatment. Five single treatments were evaluated: lime (±L), organic matter (±OM), oxidizing strain of *Agrobacterium tumefaciens,* a positive control (uncontaminated soil obtained from the greenhouse or potting mix) and a negative control (unamended tailings). Three combinations of two treatments included lime + OM, lime + oxidizing bacteria, and OM +oxidizing bacteria. Finally a combination of three treatments included lime + organic matter + oxidizing bacteria.

Soil Sample-Collection-Preparation and pH Measurement

Tailings to 0.20-m depth were collected from along the Clark Fork River on the Lampert Ranch Anaconda, Montana in 2013 ($46^0$01'N; $112^0$0' E). This area was placed on the National Priority List in 1983 due to metal contamination following ~100 years of copper smelting at Anaconda, approximately 20 kilometers southwest of the soil collection area (Neuman et al. 1993). Soil was transported to the Plant Growth Center (PGC) at Montana State University (MSU), homogenized, air dried, and sieved (<2 mm). Soil pH was measured in a 1:2 solution of soil: deionized water.

Determination of Lime, Organic Matter and Nitrogen Levels

The total lime requirement was determined by the Acid Base Account (ABA) test, by modified Sobek method (Sobek et al. 1978) and the Shoemaker, McLean and Pratt (SMP buffer) method (Shoemaker et al. 1961; Neuman et al. 2005). Analyses

were performed by Analytical Energy Labs (Billings, Montana) and results were applied to Equation 1.

Tons $CaCO_3$ /1000 tons soil = (% HNO_3 extractable S + % Residual S) * 31.25 + (% HCl extractable S) * 23.44 + SMP Lime Requirement **(1)**

Lime as $Ca(OH)_2$ was applied based on a calcium carbonate equivalence of 100:136 to otherwise unamended tailing soils. Each pot for +lime treatments contained ~1900 g of soil including 50 g of $Ca(OH)_2$. Full amendment calculations are included in appendix A. As the initial lime application caused an increase in pH, limed tailings were allowed to equilibrate before additional treatments were applied or seeding occurred.

Organic matter (OM; "EKO-compost-original" [Missoula, MT]) containing 1.4% nitrogen, 1.9% phosphorus (as P_2O_5) ;0.65% potassium (as K_2O), and 0.48% magnesium was incorporated into the soil at a rate of 5% weight (95 g per pot), to simulate ideal OM conditions for Montana soils, which typically range between 2 and 4% OM (McCauley et al. 2005). Nitrogen was added as KNO_3 following the recommendations of the Natural Resources Conservation Service (NRCS 2011) of 70 pounds per acre. A potassium nitrate solution was prepared based on the nitrogen fraction (14/101) at a final concentration of 1.65 g KNO_3 per liter and 0.6 L of this nutrient solution was added to each pot.

Bacteria Culture

Cultures of oxidizing strains of *A. tumefaciens* were obtained from the McDermott laboratory at MSU. The oxidizing strain of *A. tumefaciens* wild type is able to oxidize As(III) into As(V) at fast rates from 0.7 to 1.7 umol d^{-1}(0.05 to 0.12

mg d^{-1}) (Macur et al. 2004). Every culture was added into the soil before seeding through the same nitrate solution described previously.

Plant Seeds

Basin wildrye has been reported as a good candidate for revegetation in areas impacted by mining activities in the Upper Clark Fork River basin. This species can grow in soils with high total arsenic concentrations (1334 mg kg^{-1}) (Martin 2009), which makes it a good candidate for phytoremediation. Seeds of basin wildrye were provided by USDA NRCS Plant Materials Center, Bridger, Montana, and sown at a rate of eight seeds per pot.

Greenhouse Conditions

Plants were grown in the Plant Growth Center (PGC) at MSU. Temperatures in the greenhouse were maintained at about 22ºC during the day and about 20ºC during the night. Additional lighting to extend day length to a 16-hour photoperiod was provided by 420-watt high-pressure sodium lamps. Pots were rotated once per week to avoid edge/position effects. Plants grew for 12 weeks from March to May 2014.

Metal and Arsenic Analysis

To evaluate the total metal and arsenic in unamended tailings, triplicate samples were sent to a commercial geochemistry laboratory (ALS; Reno, NV) where they were analyzed following multi-acid digest by ICP-MS.

For foliar analyses, at harvest, plants were washed with tap water and rinsed with distilled water. After washing, plants were separated into roots and shoots, oven-

dried at 60°C for six days, and then weighed. After drying, plant material (0.5 g or less from roots or shoots) was digested following standard methods (Lozano-Rodriguez et al. 1995; Moreno-Jimenez 2010). Briefly, root or shoot materials were digested in an autoclave with 3 mL HNO_3 (67%), 2 mL H_2O_2 (30%) and 10 mL deionized water at 125^0C and 1.25 kPa for 30 min, filtered with Whatman paper (number 38), diluted to 25 mL with DI water, and analyzed by ICP-MS at the Center for Biofilm Engineering at MSU.

Statistical Analysis

Plant biomass, germination, and heavy metal concentration data were analyzed using Info stats software 2013 (Balzarini et al. 2008). In cases where data were not distributed normally, natural logarithmic transformations were necessary (e.g., biomass and metal uptake) although in the case of arsenic accumulation in shoots, we used square root transformation for the same reason. All these transformations were applied using rounded lambda values from a Box-Cox function. Treatments differences were tested by employing one-way analysis of variance (ANOVA); statistically significant results are reported for P value <0.05. Means were compared using the least significant difference test (LSD) ($P<0.05$).

Results and Discussion

Tailing Characterization

Arsenic concentrations ranged from 1080 to 3600 mg kg^{-1} (35-115 fold higher than typical background levels of the area (Neuman et al. 2005).Total metal concentrations of Cu, Pb, and Zn were also elevated in comparison with background

soils (Table 3-1). Tailing soils also contained 0.23% P_2O_5, 12.7% loss-on-ignition (waters of hydration and organic matter), pH 3.8, and the texture was silty clay.

Table 3-1 Average of three samples (means±SE) of arsenic and metal concentrations in tailing soils used in this study and in soils around the area.

Metal (loid)	Tailing soil,this study (mg kg^{-1}) Mean ± SE	Back ground soils (mg kg^{-1}) (Neuman et al.,2005)	CV (%)	Min	Max
As	2367±728	31.2	53	1080	3600
Cd	6±0	NR	0	6	6
Cu	5673±1894	45.7	58	3720	9460
Pb	1115±174	29.6	27	804	1405
Zn	1913±281	110	25	1390	2350

Tailings soils also presented low pH (the average of 10 samples measured with a 1:2 solution of soil: deionized water was 3.86) allowing for increasing of metal solubility, which can lead to phytotoxic conditions for plants (Munshower 1994). With these soil characteristics, the establishment of natural revegetation in these areas is quite limited; therefore, revegetation must be assisted using techniques that promote plant growth. Studies to promote plant growth in Anaconda have been conducted since at least 1946 (Ford & Neuman 2005), however, there is always interest in less expensive alternatives for increased establishment of vegetation in these types of heavily contaminated soils.

Greenhouse Study: Germination

After 15 days, no germination of basin wildrye seeds was observed in unamended tailings soils. Seeds in tailings amended only with oxidizing *A. tumefaciens* bacteria reached an average of 19% germination, not statistically different from unamended tailing soils. Treatments that included both OM and

bacteria or just OM had better germination responses: 72% and 69%, respectively. Somewhat lower, though not statistically significant, responses were observed in the positive control (greenhouse potting soil), as well as soils amended with lime + OM + bacteria, lime +bacteria, lime + OM, or just lime (Figure 3-1).

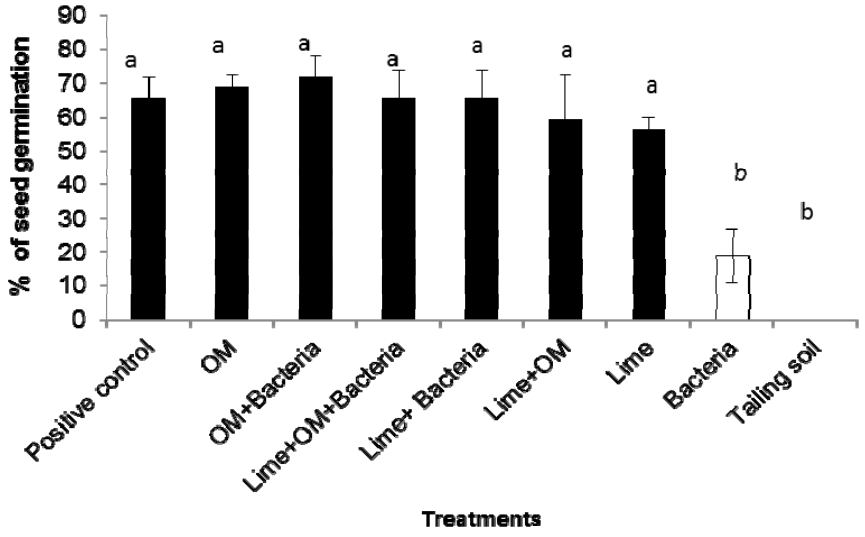

Figure 0-1. Germination of basin wildrye in a greenhouse experiment (means ± SE). Means with same letter are not statistically different ($p > 0.05$). Eight seeds were planted per pot.

Germination in many treatments can be explained by the increased soil pH (See appendix regression analysis between pH and germination $R^2=0.79$, $P<0.0001$), which may have increased nutrient availability from organic matter and/or decreased metal bioavailability (Andersson & Siman 1991; Hart et al. 1996; Basta & Sloan 1999; Brown et al. 2009). No or poor germination in the unamended tailing soil or the tailing soil amended only with bacteria could be the result of the high concentrations of metals present in the soil, which we estimated as 2367 mg As kg^{-1} (as well as 5673

mg Cu kg^{-1}, 1115 mg Pb kg^{-1}, and 1913 mg Zn kg^{-1}), the low pH (3.86), and/or the lack of nutrients. Our results highlight how initial natural plant colonization in these types of metal-contaminated soils can be impaired due to potential soil toxicity issues, corroborating earlier work (Martin 2009).

Greenhouse Study-Plant Biomass Production

Foliar and root biomass varied widely depending on treatment. As we expected, the uncontaminated soil (positive control) had the highest biomass. No detectable biomass was produced in unamended tailings. Pots amended with just bacteria had very poor biomass as well as few surviving plants (6), therefore, these results are excluded from figure 3-2.

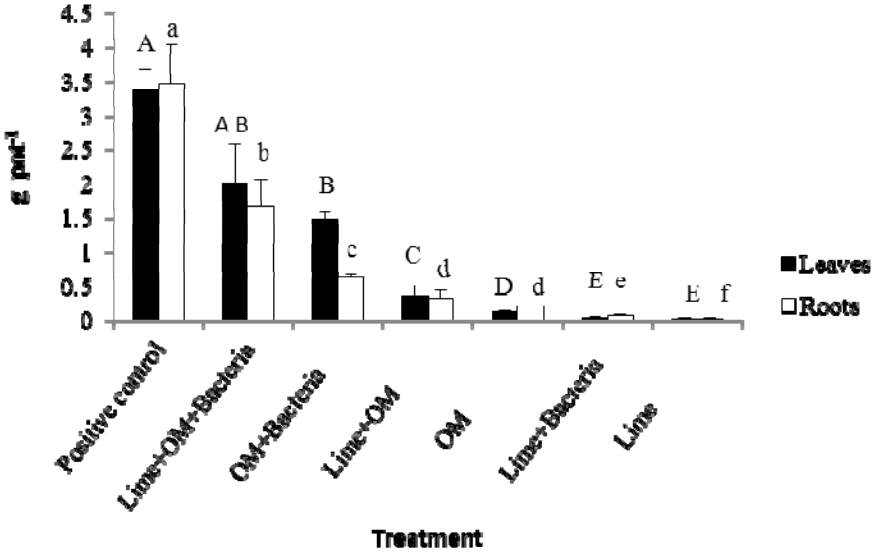

Figure 0-2. Shoot and root biomass (g pot^{-1}) in different treatments 12 weeks after seeding (mean +SE). No plants survived in unamended tailings. Means with the same letter are not statistically different (P >0.05)

The greatest biomass production in shoots was observed in the positive control, combination of lime + OM +bacteria, and OM + bacteria treatments (3.4, 2.0, and 1.5 g pot $^{-1}$, respectively), all of which were not statistically different. The same pattern was observed in root biomass; however, root biomass in the positive control soil (3.38 g pot $^{-1}$) was higher and statistically different from lime + OM +bacteria and from OM + bacteria treatments (1.67 and 0.64 g pot $^{-1}$, respectively).

Addition of OM as well as lime or the use of just OM yielded intermediate levels of aboveground biomass (0.37 and 0.12 g pot^{-1}, respectively) and belowground biomass (0.32 and 0.20 g pot^{-1}, respectively). These results suggest that OM additions could have lowered solubility and mobility of the arsenic (Cao & Ma 2004; Cala et al. 2005; Clemente et al. 2008; Walker et al. 2004)

Low leaf and root biomass was registered in soils amended just with lime (0.02 g pot $^{-1}$) or with lime + bacteria (0.03 leaf and 0.07 root g pot^{-1}). Although soil pH shifted across the different treatments, there was a weak but positive correlation between pH and leaf biomass production ($r=0.48$, $p=0.0079$). These results imply the addition of just lime is not as effective on plant biomass in these soils. Thus, it is possible that lime may have increased As(III) and/or As mobility (Fitz & Wenzel 2002), rendering the soil slightly more toxic for the basin wildrye plants in terms of As even though it is likely that lime reduced the toxicity of other metals present.

Generally, plant benefit appears most pronounced when oxidizing strains of *A. tumefaciens* are present. The role of bacteria in phytoremediation has not been researched extensively (Santini & Ward 2012; Wu et al. 2006). To the best of our knowledge, this is one of the few reports quantifying plant growth outcomes on tailings as a function of amendments including bacterial strain inoculation.

Across a number of our treatments, bacteria amendment could have catalyzed the conversion of As(III) into As(V). This conversion could have helped the plant tolerate more arsenic in the form of As(V). Carbonell-Barrachina et al. (1995) observed that 10 times more As(V) is required to have similar phytotoxic effects as As(III). In addition, plants could confuse As(V) with phosphate. Several reports show the high affinity between phosphate/ As(V) in plant-uptake (Meharg & Macnair 1992; Esteban et al. 2003; Zhao et al. 2003; Zhao et al. 2009).

Thus, treatments combining organic matter and bacteria could have had a synergistically advantageous effect on biomass production, with the bacteria acting as arsenic-tolerant plant growth-promoting bacteria (PGPB). Studies have shown that arsenic-tolerant bacteria possessing PGP properties were able to enhance above and belowground biomass. For example, bacterium inoculation with *Rhizobacteria* promoted growth above normal biomass in *Brassica juncea* in mine tailings in China (Wu et al. 2006). In South Korea, different As-tolerant bacteria possessing PGP traits enhanced root growth and tolerance index of maize in the presence of As (Charlotte et al. 2014).

Arsenic Accumulation in Shoots and Roots

Unfortunately, not all treatments attained the necessary minimum dry weight required (0.5 g) for accurate plant arsenic analysis. Therefore, some arsenic uptake data for specific treatments are not available (figures 3-3; 3-4).

Maximum arsenic concentrations in basin wildrye shoots occurred in soils amended with lime + bacteria (average ± 1 SE: 40±6.6 mg kg^{-1}), which was not statistically different from shoot concentrations observed in soils amended with just

OM (29±1.4 mg kg^{-1}). Intermediate arsenic concentrations in basin wildrye shoots were recorded for soils amended with lime (19±3.4 mg kg^{-1}) or OM +bacteria (20± 5.9 mg kg^{-1}). The lowest arsenic concentration in shoots was observed either in the lime + OM treatment (7±1.4 mg kg^{-1}) or the lime + OM + bacteria treatment (13±0.6 mg kg^{-1}), aside from the uncontaminated soil positive control treatment (1±0.1 mg kg^{-1}).

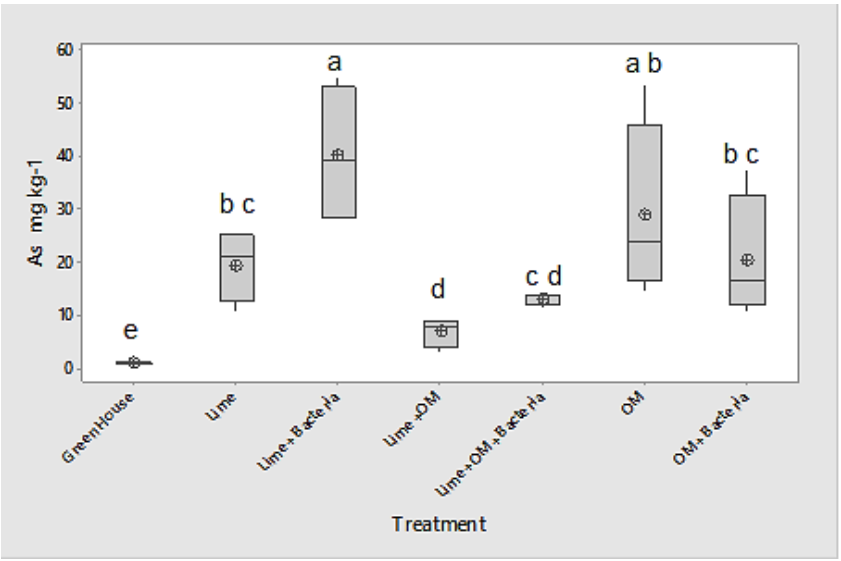

Figure 0-3 Total arsenic concentrations (mg kg^{-1}) in basin wildrye shoots after 12 weeks. Circles in the middle of each boxplot indicates mean value per treatment. Means with the same letter are not statistically different (P>0.05).

The highest arsenic concentrations in roots occurred in soils amended with OM + bacteria (55±3.4 mg kg^{-1}), OM (54± 13.5 mg kg^{-1}), lime + OM + bacteria (35± 13 mg kg^{-1}), or lime + OM (32±9.6 mg kg^{-1}). Statistically, however, there were no differences between these treatments. The lowest arsenic concentrations in roots were reported in soils amended with lime + bacteria and just lime (12.8 and 7.4 mg kg^{-1}, respectively) aside from the uncontaminated control (6±2.7 mg kg^{-1}).

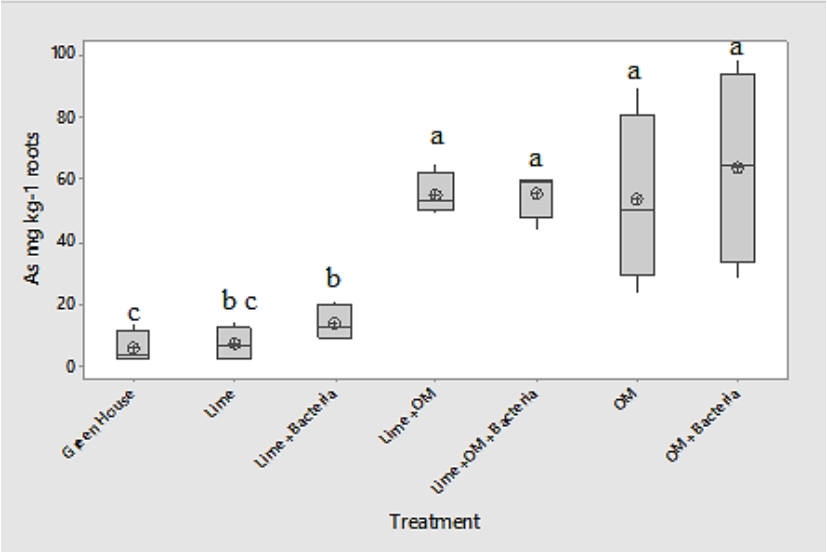

Figure 0-4 Total arsenic concentrations roots (mg kg^{-1}) in basin wildrye roots after 12 weeks. Circles in the middle of each boxplot indicate mean values per treatment. Means with the same letters are not statistically different (P>0.05).

Generally, arsenic concentrations in roots were greater than those in shoots, which could reflect a protective physiological response by the plants to reduce the toxicity of arsenic (Carbonell-Barrachina et al. 1995). An unexpected pattern was observed for soils amended with lime or lime + bacteria. These treatments yielded generally low arsenic concentrations in roots, and higher arsenic concentrations in shoots, which suggests arsenic may have been translocated to the shoot, potentially inhibiting plant growth (these treatments yielded very low shoot biomass).

All treatments that involved any combination with OM resulted in the higher accumulation of arsenic in roots which suggests that basin wildrye may have an ability to store a certain amount of arsenic in its root system when it is exposed to high levels of organic matter and bacteria. It is important to mention that after cleaning the roots, small particles may have been stuck to the roots which could have inflated our measures of arsenic absorbed by roots. Basin wildrye is considered a

good candidate for revegetation in disturbed mine sites (NRCS 2011), but there are few reports on arsenic concentrations in basin wildrye growing directly in tailing soils. The maximum value of arsenic accumulation in basin wildrye shoots previously reported was 16.5 mg kg^{-1}(Neuman 2009), about 37% less than the maximum shoot concentration we observed. Other experiments have been conducted in soils with either lower arsenic concentrations (15 mg kg^{-1} vs this study's average of ~1100 mg kg^{-1}) (Knudson et al. 2003) or with a greater focus on arsenic concentrations in soils than in plant biomass (Tafi 2006). Therefore comparisons are very restricted. However, it is important to highlight that all the plants that grew in our treatments did not exceed the maximum tolerable dietary intake threshold of arsenic for livestock (50 mg kg^{-1}) recommended by United States, Australian and New Zealand guidelines (NRC 1980; Neuman et al. 2005; Mains et al. 2006).

Arsenic Uptake by Shoots

Arsenic shoot uptake can be expressed as a function of arsenic concentration in plant shoots (mg As [kg dry biomass]$^{-1}$) multiplied by biomass production (kg dry biomass pot^{-1}; Fig. 3-5). Treatments that showed the highest arsenic uptake were soils amended with OM + bacteria or lime + OM + bacteria (0.03 mg pot^{-1}). Intermediate values of arsenic uptake were observed for soils amended with OM (0.0036 mg pot^{-1}), lime + OM (0.0027 mg pot^{-1}), and our uncontaminated positive control (0.0041 mg pot^{-1}). Treatments that showed the lowest arsenic uptake were lime + OM (0.0012 mg pot^{-1}) and just lime (<0.001 mg pot^{-1}).

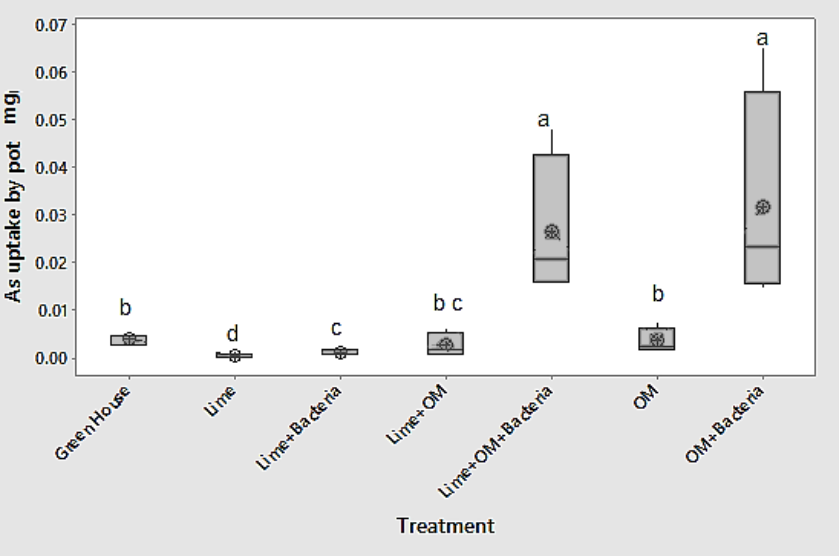

Figure 0-5 Arsenic uptake by basin wildrye shoots after 12 weeks. Circles in the middle of each boxplot indicate mean value per treatment. Means with the same letter are not statistically different (P>0.05).

One of the main observations in our study was that the greatest arsenic uptake value was mainly influenced by biomass production even if arsenic concentration values were not the highest (OM + bacteria). Thus, our results suggest that one revegetation approach for these types of heavily contaminated tailing soils should include the combination of these two amendments. On the other hand, soil treatments that included only lime or lime + bacteria had greater arsenic concentrations in shoots but because of their overall low biomass production, these treatments had low uptake of arsenic compared to OM + bacteria or lime + OM + bacteria. This makes the lime or lime + bacteria treatments less desirable for cost-effective revegetation purposes. Our results clearly show that the use of OM + bacteria has enhanced basin wildrye growth. Other studies have shown the same results with just bacterial inoculation for different metals. For example, Li et al. (2007) showed that in soils contaminated with Cd (8 mg L^{-1}), inoculation with the bacterium *Burkholderia cepacia* enhanced shoot

and root biomass (54% and 46%, respectively) in the *Sedum alfredii* plant. Using the same bacteria and plant, shoot biomass was enhanced by 110% when it was exposed to Zn (8 mg L^{-1}), which resulted also in a Cd and Zn uptake increase of 243% and 96.3%, respectively. Wang et al. (2011) reported that the use of *Agrobacterium radiobacter* lowered soil arsenic concentrations by 54% versus 43% for uninoculated soils, where soils were contaminated with 300 mg As kg^{-1}.

Finally, although this experiment suggested the potential for phytoremediation success from our bacterial inoculation in combination with one level of organic matter, several questions remain. The mechanisms responsible for improved plant growth remain unclear. It is known that some bacteria interact with plants to improve their growth by producing phytohormones (Fallik et al. 1994) capable of inhibiting toxic effects from heavy metals (Gupta et al. 2002). However, we also point out that bacteria could have influenced soil arsenic speciation, changing As(III) into As(V) (Macur et al. 2004) and probably providing some arsenic resistance to the plant. More research on arsenic speciation and plant-growth promotion is warranted. In spite of this, our findings demonstrate that the combination of organic matter and bacteria plays an important role in growth, accumulation and arsenic resistance in plants that can grow directly in tailings soils; moreover, our results can lead us to new techniques that can help to improve phytoremediation outcomes.

Future Work

Our study showed that the combination of high levels of organic matter with bacteria can potentially increase plant biomass and arsenic uptake. However, in the field, organic matter is usually one of the more expensive amendments; therefore, an

evaluation lowering the amount of organic matter could give us better understanding about efficiency. In addition, in this research we hypothesize that bacteria potentially convert As(III) to As(V); therefore, a future study will consider the use of two different strains of *Agrobacterium tumefaciens,* one able to oxidize As(III) to As(V) and the other unable to complete the same arsenic transformation.

CHAPTER FOUR

SECOND EXPERIMENT: EFFECTS OF BACTERIA ON ENHANCED BIOMASS AND METAL UPTAKE OF ARSENIC IN LEYMUS CINEREUS PLANT

Introduction

Thousands of acres of land have been disturbed by mining activities around the world. Soils on these lands are associated with high concentrations of heavy metals and metalloids such as arsenic, which represent a threat to human health and the environment. In order to clean up metal-contaminated soils, conventional technologies such as capping or physical removal of the soil are generally too costly, harmful to soil characteristics, and do not offer a permanent solution (Johnson & Bradshaw 1977; Berti & Cunningham 2000).

The use of plants to stabilize or extract the contaminant in the soil or water is called phytoremediation. It is an emergent technique that offers a potential cost-effective solution for remediation (Licht et al. 1995; Visoottiviseth et al. 2002). Thus, since the discovery of the first arsenic-hyperaccumulator plant, *Pteris vittata* (Ma et al. 2001), great interest has been generated in phytoextraction, especially in arsenic-contaminated soils (Yang et al. 2012). Unfortunately, most plants are not suitable for field phytoextraction. Even those plants that are metal-resistant or metal-accumulating cannot develop well in tailing soils because the lack of nutrients, proper soil characteristics, and the high concentrations of various metals can inhibit their growth and lead to revegetation failure.

Plants used in phytoremediation should be able to grow fast, tolerate high level of metals, and produce high biomass (Shen & Liu 1998; Ma et al. 2009). Regarding these considerations, the use of microorganisms such as bacteria have attracted attention for their biotechnological potential to change redox conditions, improve metal removal from soils, promote plant growth and metal tolerance, or improve transfers of accumulated metals from soil to plants (Li Guo 1996; Macur et al. 2004).

Although there is some evidence of microbially enhanced phytoremediation in small-scale laboratory studies (Knudson et al. 2003; Li et al. 2007; Ma et al. 2009; Wang et al. 2011; Yang et al. 2012), best practices for bacterial enhancement of phytoremediation remain poorly understood or are largely ignored (Santini & Ward 2012; Wu et al. 2006). The present study was therefore designed to investigate (i) the effects of As(III) and As(V) in germination of basin wildrye, (ii) the influence of As(III) oxidation as catalyzed by the bacterium *A. tumefaciens* on arsenic speciation in tailing soils; (iii) the effect of this microbe on biomass production in *Leymus cinereus*; and (iv) the effects of these strains of bacteria on the accumulation and translocation of arsenic in *Leymus cinereus* growing in tailing soils.

Materials and Methods

First, we evaluated germination of *Leymus cinereus* seeds under different concentrations of As(III) and As(V) in a laboratory assay to determine which of the two arsenic species might be more harmful to seeds. Subsequently, we performed a greenhouse study to manipulate growth conditions for basin wildrye over 12 weeks. Our five categories of treatments were (i) lime + OM, (ii) lime + OM + a mutant

As(V)- reducing bacterial strain incapable of oxidizing As(III) to As(V) (*A. tumefaciens* strain), (iii) OM + the same reducing bacterial strain, (iv) lime + OM + an As(III)-oxidizing strain of the same bacterium (wildtype), and (v) OM + the same oxidizing bacterial strain. Each treatment had two levels of OM (1.5 or 5%). We chose the higher OM level to simulate ideal OM conditions for Montana soils (McCauley et al. 2005); the lower level was designed to determine the linearity of potential interactive effects between OM and bacteria. Each treatment had a total of ten replications (ten pots).

Laboratory Assay

We assessed the effects of different concentrations of As(III) and As(V) on the germination of *Leymus cinereus* in laboratory conditions. Water agar plates amended with 0.0, 0.05, 0.5 or 5.0 mM arsenic trioxide (arsenic as As[III[) or sodium arsenate (arsenic as As[V]) were sown with 30 seeds of basin wildrye. Seeds were covered with a lid, and incubated in the dark at room temperature. Results were expressed as total number of seeds germinated or percent. Seeds were considered germinated when both the plumule and radicle extended more than 2 mm from their junction. Each treatment was replicated eight times.

Bacterial Culture

Pure cultures of an oxidizing strain of *A. tumefaciens* wildtype, as well as a reducing strain of *A. tumefaciens* mutant type aioA::Tn-B22 were obtained from Tim McDermott (Montana State University). The oxidizing strain of *A. tumefaciens* wildtype is able to oxidize As(III) to As(V) rapidly (Lehr et al. 2006); critically, the mutant type cannot oxidize As(III) to As(V), but will reduce As(V) (T. McDermott,

unpublished data). Every culture was added into the soil through a nitrate solution (33.3 g KNO_3 into 20 L of deionized water) at a rate of 0.6 L pot^{-1} (each pot contained ~2 kg soil).

Soil Collection and Preparation

Contaminated soils were collected from along the Clark Fork River near to the Warm Springs area downstream of the city of Anaconda and the town of Opportunity, Montana. This area was placed on the National Priority List in 1983 due to metal contamination following ~100 years of copper smelting (Neuman et al. 1993).

Soil was transported to the Plant Growth Center (PGC) at Montana State University (MSU), homogenized, air dried, and sieved (<2 mm).

Soils were limed at rates of 50 g $Ca(OH)_2$ per 2000-g pot, as suggested by an Acid Base Account (ABA) test modified by Sobek method (Sobek et al. 1978) and the Shoemaker, McLean and Pratt (SMP) buffer method (Shoemaker et al. 1961; Neuman et al. 2005). The ABA test was performed by Analytical Energy Labs (Billings, Montana). Because the soil pH initially rose quite rapidly, limed soils were allowed to equilibrate ~4 months. Organic matter (OM) was then added at a rate of 1.5% (28 g pot^{-1}) or 5% (95 g pot^{-1}) (Eko-compost-original, Missoula, MT). Nitrogen fertilizer (in addition to the bacterial inoculum) was added as 1 g of KNO_3 to each pot following the recommendations of the Natural Resources Conservation Service (NRCS 2011). Characterization of tailings and OM were described in Chapter Three.

Plant

Basin wildrye has been reported as a good candidate for revegetation efforts in areas impacted by mining activities in the Upper Clark Fork River. This plant

grows under soils with relatively high arsenic concentrations (1334 mg kg^{-1}) of total arsenic (Martin 2009), which makes it a good candidate for phytoremediation. Seeds of basin wildrye were provided by USDA NRCS Bridger Plant Materials Center, Bozeman, Montana, and sown at a rate of ten seeds per pot approximately eight days after treatments were prepared.

Arsenic Speciation

Arsenic speciation was performed following hydride generation which is one of the most frequent used methods for arsenic determination (Goessler & Kuehnelt 2002). It is based on the production of volatile arsines through sodium borohydride/acid mixtures. For total arsenic, 5 g of soil from each pot in every treatment category was mixed with 50 ml of deionized water at room temperature one week after treatments were prepared. The samples were shaken for two hours and filtered (Whatman paper number 38) by decantation, diluted with 5% nitric acid, and then analyzed by ICP-MS. For arsenic speciation, the protocols described by Lehr et al. (2006) were followed. As(III) was estimated as the difference between total As and As(V). Five samples per treatment were evaluated for arsenic speciation.

Plant Harvest and Arsenic Analysis

Samples were harvested after 12 weeks. Plants were washed with tap water and rinsed with distilled water. After washing, plants were separated into roots and shoots, oven dried at 60°C for six days, and then weighed. Plant material (0.5 g roots or shoots) digestion followed standard methods (Lozano-Rodriguez et al. 1995; Moreno-Jimenez 2010). Briefly, root or shoot materials were digested in an autoclave with 3 mL HNO_3 (67%), 2 mL H_2O_2 (30%) and 10 mL deionized water at 125^0C and

1.25 kPa for 30 min, filtered with Whatman paper (number 38), diluted to 25 mL with DI water, and analyzed by ICP-MS (Energy Laboratories, Billings, Montana).

Statistical Analyses

Two-way analysis of variance (ANOVA) was used to test for treatment differences in biomass, arsenic accumulation and arsenic uptake ($p<0.05$). We used treatment and level of organic matter amendment (1.5 or 5%) as main effects (Software Minitab 17 Statistical 2010). Where residuals were not distributed normally, data were transformed using the rounded lambda value from a Box-Cox function (Software Minitab 17 Statistical 2010). Means were compared using the least significant difference test (LSD) and determined to be different using an α value of 0.05.

Results and Discussion

Germination Assay

The first and last germination of basin wildrye seeds occurred on the fourth and eleventh day, respectively. The mean germination percentage compared to the control decreased significantly ($p<0.01$) with increasing concentrations of As(III) or As(V), though the effect was more dramatic for As(III) (Figure 4.1). At the lowest arsenic concentration (0.05 mM), for example, there were no significant differences in seed germination (both >80%), but at 0.5 mM As(III), germination fell to 30% while 80% of seeds exposed to the same concentration of As(V) germinated. Similarly, none of the 30 seeds germinated when exposed to 5 mM As(III), though almost 60% germinated when exposed to the same elevated concentration of As(V).

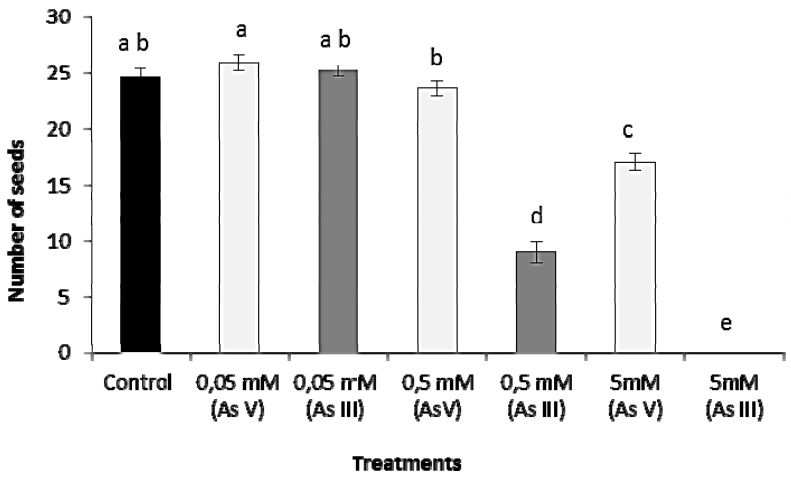

Figure 0-1. Average germination (means ± 1 SE) of basin wildrye seeds (n=30) in the presence of varying arsenic concentrations (0 [dark bar], 0.05, 0.5, and 5 mM), and present as either As(V) in lighter bars or As(III) in gray bars). Means with the same letter are not statistically different ($p > 0.05$).

These results suggest As(III) at concentrations higher than ~0.05 mM are toxic to seeds, and that these toxic thresholds are lower for As(III) than As(V). These results extend the findings of Abedin & Meharg (2002) and Bhattacharya et al. (2012), who observed that germination of rice and chickpea seeds was significantly lower for seeds exposed to As(III) than those exposed to As(V), though these studies used relatively low arsenic concentrations (maximum concentration: 8 mg L^{-1} or 0.11 mM). Our results showed substantial germination of basin wildrye seeds even at concentrations of As(V) of 5 mM. Other research has concluded that these types of germination differences are likely due to the much higher toxicity of As(III) than As(V) to plants (Tamaki & Frankenberger 1992; Petrick et al. 2000).

Arsenic Speciation

The two-way analysis of variance determined that there were significant differences in As(V) between treatments ($p<0.001$). However, there were no

significant differences between treatments and percentage of OM (1.5 or 5%; p=0.067). Therefore, we combined the extraction results for soils amended with 1.5 and 5% OM. Tailings extracted with room-temperature DI water yielded an average As concentration of 137 mg kg^{-1} with 49% as As(V) and 51% as As(III). After mixing with different amendments, the results of arsenic speciation in treatments that had bacteria changed drastically (Figure 4-2). The largest difference was observed in treatment 2 (lime + OM +reducing bacteria) where As(III), the reduced form, increased to 86%; total water-extractable As for this treatment was 2275 mg kg^{-1}, a number greater than the total As and therefore a value that failed to meet our quality control requirements. Similarly, As(III) increased 14% in treatments 3 and 4. The opposite effect was observed in treatment 5 where As(III) decreased by 9%.

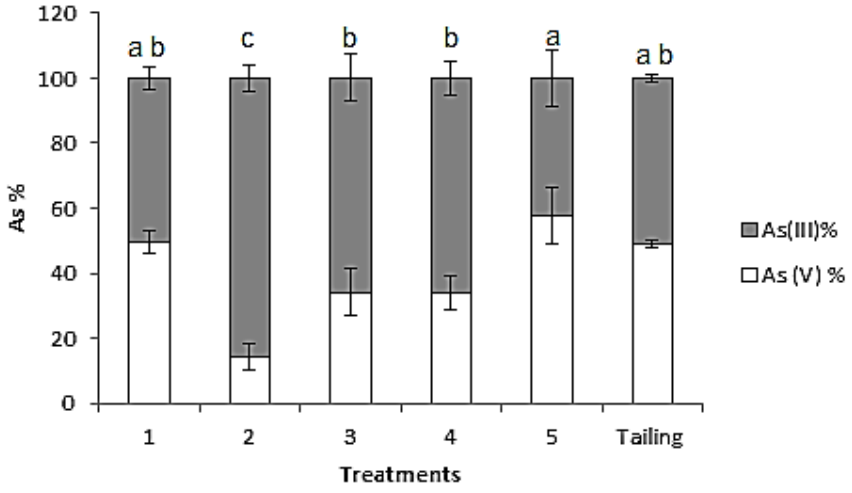

Figure 0-2 Arsenic speciation after mixing treatments in tailing soil (mean ± SE). White bars represent As(V) while grey gars represent As(III). Treatment 1= lime + OM, treatment 2= lime + OM + reducing bacteria, treatment 3= OM + reducing bacteria, treatment 4=lime + OM + oxidizing bacteria, treatment 5= OM+oxidizing bacteria. For each amended set of soils, the average water-extractable total As concentrations were 109, 2275, 579, 192, and 78 mg kg^{-1}.

These results tentatively suggest changes in arsenic speciation for treatments 2, 3 and 4. The changes in arsenic speciation in treatments 2 and 3 can be explained by the action of reducing bacteria which reduces As(V) to As(III) rapidly (T. McDermott, unpublished data). However, it is important to recognize that arsenic speciation is a very delicate technique and that changes in speciation can happen during the storage and preparation of samples in days (Gong 2002). Treatment 4, which was supposed to yield increased As(V), had unexpected results because As(V) actually decreased instead. In this case, lime may have promoted greater reduction of As, so changes in arsenic oxidation were slower than in treatment 5. One example of this has been previously reported (Reclamation Research Unit 1997), where As(III) levels increased from 2 to 75 times following the liming of three mine waste sites in Anaconda, Montana. Although this is a possible explanation for the general patterns we observed, we did not see the same effect in treatment 1 which combined lime + OM, though our relatively short sampling window could have meant that even this treatment might have shown the same trend given more time.

Germination in the Greenhouse

Plant germination in all treatments occurred during the first 20 days. There were no interactions between treatments and percentage of OM ($p=0.141$) in germination effects (two way ANOVA). Treatment 5 (OM + oxidizing bacteria) showed the highest germination (55%), although this was not statistically different from germination for treatments 2 (lime + OM + reducing bacteria) or 4 (lime + OM + oxidizing bacteria). Germination in soils amended with just lime + OM (treatment 1) were not significantly different from treatments 2 or 4. The lowest germination for

basin wildrye (27%) was observed in soils amended with OM + reducing bacteria (treatment 3).

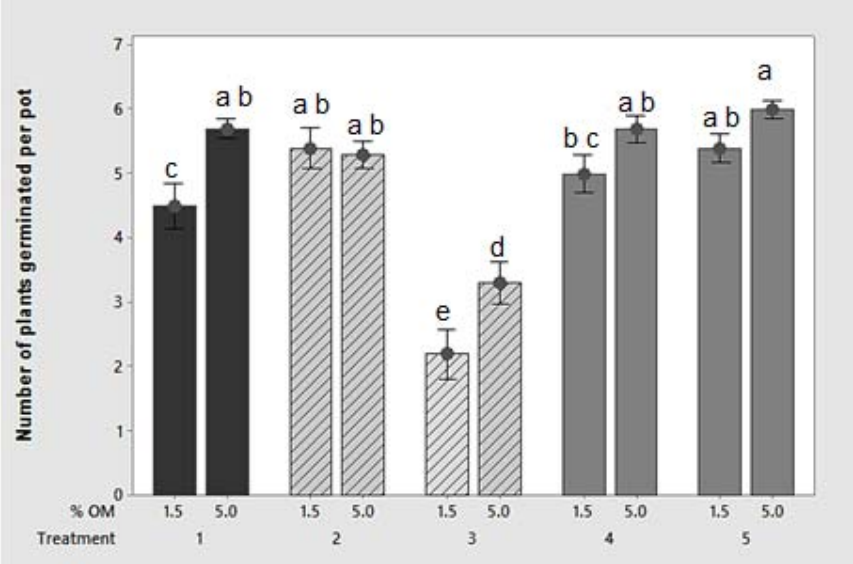

Figure 0-3 Germination of basin wildrye in a greenhouse experiment (means ± SE). 1= lime + OM, 2= lime + OM + reducing bacteria, 3= OM + reducing bacteria, 4= lime + OM + oxidizing bacteria, 5= OM + oxidizing bacteria. Ten seeds per pot were sown. Means with the same letter are not statistically different (P >0.05).

The relatively high germination in treatments 5, 4, 2 and 1 could have been associated with soil pH increase (pH=7), or the nutrients provided by OM. Relationships between pH and metal availability were discussed in the previous chapter. For this experiment, OM consisted of wood products and sewage sludge with a nutrient content of 1.4% nitrogen, 1.9% phosphate (as P_2O_5), 0.65% potassium (as K_2O), and 0.48% magnesium. The properties of the OM influence the pH buffering capacities in soils.

The lowest germination occurred in treatment 3 with 1.5% OM. This could have been caused by the increase of As(III) in soils caused by the reducing bacteria and/or the increase of As(III) in solution due to the lower pH (5). However, in the

same treatment with 5% OM there was a pH of 7 and it still had relatively low germination. It is unclear why the combination of lime + OM + reducing bacteria leads to relatively high germination rates. Similar inhibition of seed germination by As(III) has been reported by Abedin & Meharg (2002) and Bhattacharya et al. (2012). In addition, it is well known that lime decreases bioavailability of other metals (Kabata-Pendias & Pendias 2001) and therefore, the lack of lime in this treatment possibly allowed arsenic and the other metals such as Cu, Pb and Zn to inhibit the germination of seeds.

Plant Biomass

Plant biomass differed between treatments as well with levels of OM amendments (Two Way ANOVA, $p<0.001$). Plants were larger grown with 5% OM than 1.5% OM. The greatest aboveground biomass was observed for soils amended with ± lime + 5% OM + oxidizing bacteria (treatments 4 and 5; 1.64 and 1.54 g pot^{-1}, respectively; Figure 4-4). Treatments 4 and 5 were not different statistically. The lowest biomass responses were observed in treatment 3 with 5% OM (0.22 g pot^{-1}) or 1.5% OM (0.05 g pot^{-1}).

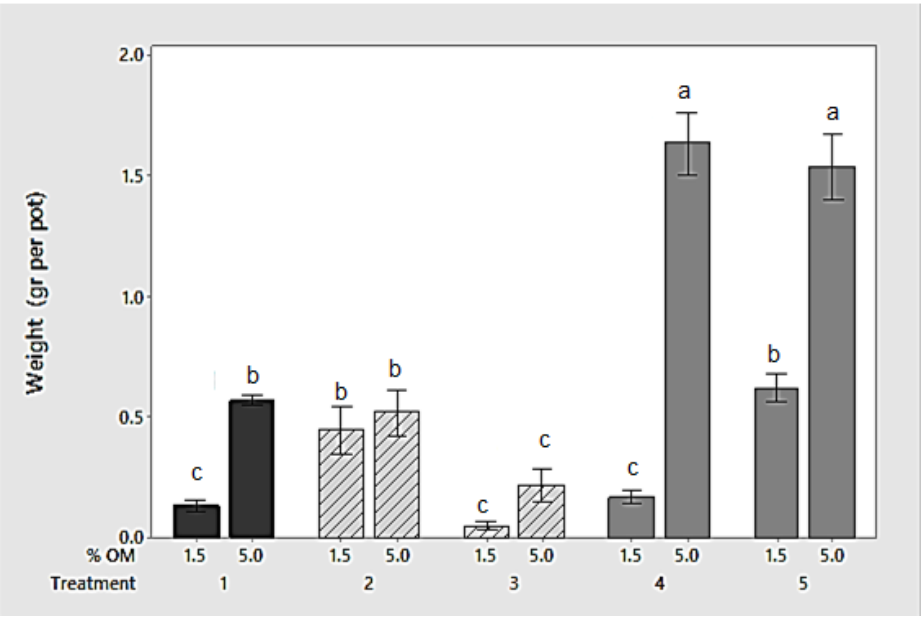

Figure 0-4 Shoot biomass in basin wildrye (mean ± 1 SE). 1= lime + OM, 2= lime + OM + reducing bacteria, 3= OM + reducing bacteria, 4= lime + OM + oxidizing bacteria, 5= OM + oxidizing bacteria. Means with the same letter are not statistically different (P > 0.05).

Root biomass had similar behavior to that of foliar biomass. Thus, the greatest root biomass was found in treatments 4 and 5 with 5% OM (1.47 and 1.42 g pot^{-1}, respectively); these treatments were not significantly different. The lowest belowground biomass production was observed with plants amended with 1.5% OM and reducing bacteria (0.04 g pot^{-1} [treatment 3] ;Figure 4-5). Root biomass was only slightly greater for two other treatments: soils amended with just lime and 1.5% OM (0.12 g pot^{-1}) and soils amended with 5% OM and reducing bacteria (0.16 g pot^{-1}).

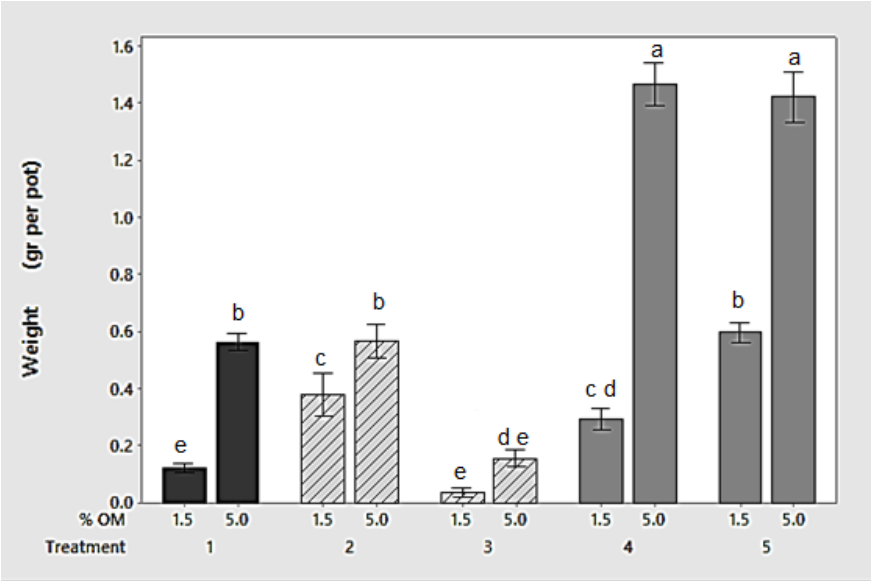

Figure 0-5. Root biomass in basin wildrye (mean ± 1 SE). 1= lime + OM, 2= lime + OM + reducing bacteria, 3= OM + reducing bacteria, 4= Lime + OM + oxidizing bacteria, 5= OM + oxidizing bacteria. Means with the same letter are not statistically different (P>0.05).

Generally, our results showed that high levels of OM are necessary to obtain better biomass production. Likewise, inoculation of oxidizing bacteria greatly increased foliar and root biomass showing that the presence of lime is not required to achieve this result when this bacteria is combined with 5% OM. Wu et al. (2006) states that inoculation with rhizobacteria generally produces larger aboveground biomass and alters metal availability in the soil. Several studies have shown the use of different bacteria able to enhance plant biomass (Lucy et al. 2004; Wu et al. 2006; Li et al. 2007; Wang et al. 2011; Yang et al. 2012; Charlotte et al. 2014). However, little information is available regarding the mechanisms by which microbes enhance phytoremediation and biomass. Only a few of these studies report the role of bacteria as conferring arsenic resistance. In our study, the addition of *A. tumefaciens* could have accelerated redox reactions in the soil, which helped the uptake of the less toxic

arsenic form (As[V]) by the plant (Figure 4.2). As a result, there may have been less growth inhibition in the plants as observed in treatment 5. A similar response was observed in treatment 3 where reducing bacteria could have changed redox conditions making this environment more toxic for the plant and therefore inhibiting its growth. This explanation is supported by the fact that soil redox conditions in treatment 1 (with no bacteria) did not change as drastically as the other treatments (Figure 4-2). Another possible explanation is thatPO_4^{-3} could interfere or inhibit the uptake of As(V). Since PO_4^{-3} in solution is known to decrease plant affinity for arsenic and reduce plant arsenic influx rate (Meharg & Macnair 1990), there is potentially less arsenic in plant tissues which allowed for better growth. In either case, the role of this bacterium in soil and the plant should be further investigated as it could be applied to other plants for phytoremediation roles.

Arsenic Accumulation in Shoots and Roots

Arsenic concentration in basin wildrye shoots varied between levels of OM (Two Way ANOVA p=0.002) but there was no difference between treatments (p=0.98) or the interaction treatment*OM (p=0.52). Plants grown in soils amended with ± lime + 1.5% OM + reducing bacteria (treatments 2 and 3) had the highest arsenic concentrations in shoots (46 and 54 mg kg^{-1}, respectively); the lowest shoot As concentrations were unexpectedly associated with plants grown in soils amended with 5% OM, with concentrations ranging from 9.3 mg kg^{-1} (treatment 4) to 23.2 mg kg^{-1} (treatment 2).

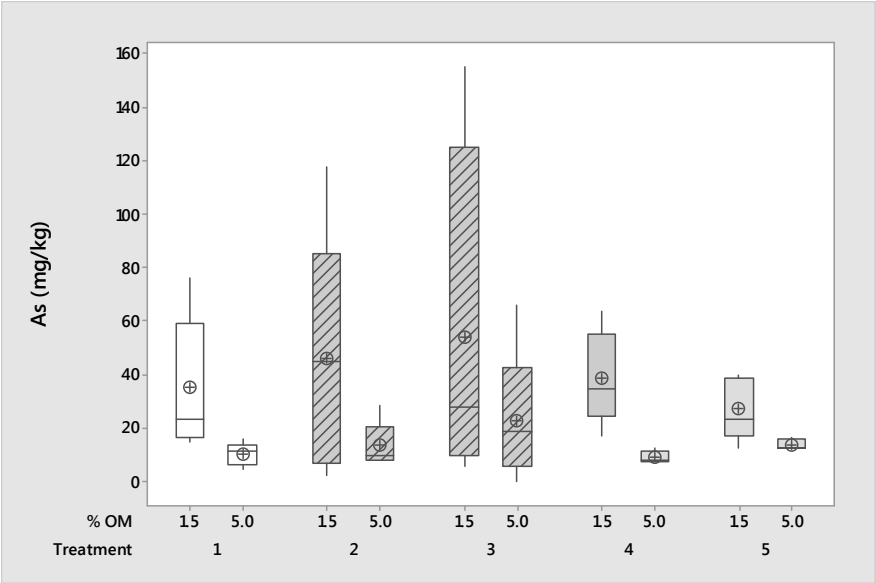

Figure 0-6 Box plot of arsenic concentrations in aboveground biomass. Circles indicate mean values. White bars corresponds to treatment 1 without bacteria, crosshatching indicates treatments with reducing bacteria , and grey shading indicates treatments with oxidizing bacteria (1= lime + OM, 2= lime + OM + reducing bacteria, 3= OM + reducing bacteria, 4= lime + OM + oxidizing bacteria, 5= OM + oxidizing bacteria).

Arsenic concentrations in basin wildrye roots, in general, were about 3- to 20-fold greater than foliar concentrations (Fig. 4-6). The greatest root concentrations were observed for treatment 5 (+5% OM + oxidizing bacteria: 571 mg kg^{-1}) or treatment 4 (+ lime + 5% OM + oxidizing bacteria: 427 mg kg^{-1}). The smallest root concentrations were observed for soils amended with lime, 1.5% OM, and oxidizing bacteria (treatment 4; 46 mg kg^{-1}). For four of the treatments, basin wildrye amended with 5% OM accumulated more arsenic in roots than plants amended with only 1.5% OM. The exception to this trend was treatment 2, where plants were amended with lime, OM, and reducing bacteria, and where average arsenic concentrations dropped from 337 to 275 mg kg^{-1} as OM increased from 1.5 to 5%.

The two-way analysis of variance in root arsenic showed significant differences between treatments, OM, and their interaction (P<0.001).

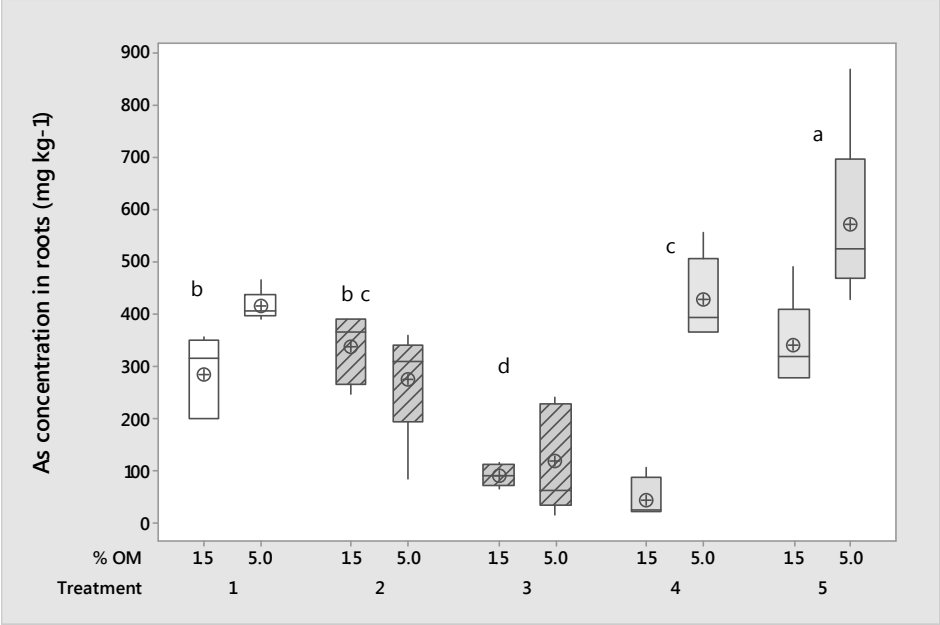

Figure 0-7 Box plots of arsenic concentrations in basin wildrye roots. Circles indicate mean values. White bars corresponds to treatments without bacteria, crosshatching indicates treatments with reducing bacteria, and grey bars indicate treatments with oxidizing bacteria (treatment 1= lime + OM, 2= lime + OM + reducing bacteria, 3= OM + reducing bacteria, 4= lime + OM + oxidizing bacteria, 5= OM + oxidizing bacteria). Means with the same letter are not statistically different (P>0.05).

The highest arsenic accumulation in roots was observed in treatment 5 which also showed higher levels of As(V) than As(III) (Figure 4.2). Meharg & Macnair (1992) and Meharg & Hartley-Whitaker (2002) stated that plants can assimilate high levels of As(V) because of their high affinity with (PO_4^{3-}) for the same uptake carriers in the roots. It is possible that the roots took up more As(V) in this treatment and assimilated more arsenic in comparison with the other treatments that showed higher levels of As(III). Treatments 2 and 3 showed higher levels of As(III), which can also enter the roots cells through the aquaporin system (Isayenkov & Maathuis 2008).

However, since it is more toxic than As(V), plant roots might not have tolerated this species of arsenic. As we mentioned before, there are many studies that have shown an increase in metal accumulation with the use of bacteria (Li et al. 2007; Ma et al. 2009; Wang et al. 2011; Xu et al. 2014), but the description of specific mechanisms by which microbes enhance arsenic accumulation is still not clear.

On the other hand, since arsenic in shoots did not differ between treatments, it is possible that arsenic in the roots may not have been translocated to the shoots for two reasons. First, it is also possible that some arsenic in the roots may have been excreted by the plant roots back into the soil. Second, it is possible that some arsenic in the roots may have been stored in the vacuoles. These scenarios mean it is difficult to quantity the fate of any arsenic absorbed by roots. However, it is clear that the treatments with oxidizing bacteria performed better than the treatments with either reducing bacteria or without bacteria in enhancing plant shoot growth and arsenic uptake by shoots.

Shoot Arsenic Uptake

Shoot arsenic uptake (shoot biomass multiplied by shoot As concentration) differed between treatments as well as between OM levels (Two Way ANOVA $p=0.001$), but the interaction between the two was not significant ($p=0.073$). The highest arsenic uptake was reported for basin wildrye grown in soils amended with the higher levels of OM. For example, treatment 5 (5% OM + oxidizing bacteria) yielded the highest arsenic uptake by shoots (0.020 mg pot^{-1}), with the two next highest results being treatment 5 with 1.5% OM as well as treatment 4 (lime + 5% OM + oxidizing bacteria; 0.014 mg pot^{-1}). Finally, treatment 3 (OM + reducing

bacteria) resulted in the lowest arsenic uptake with 0.005 mg pot^{-1} (5% OM) or 0.003 mg pot^{-1} (1.5% OM, Figure 4-8).

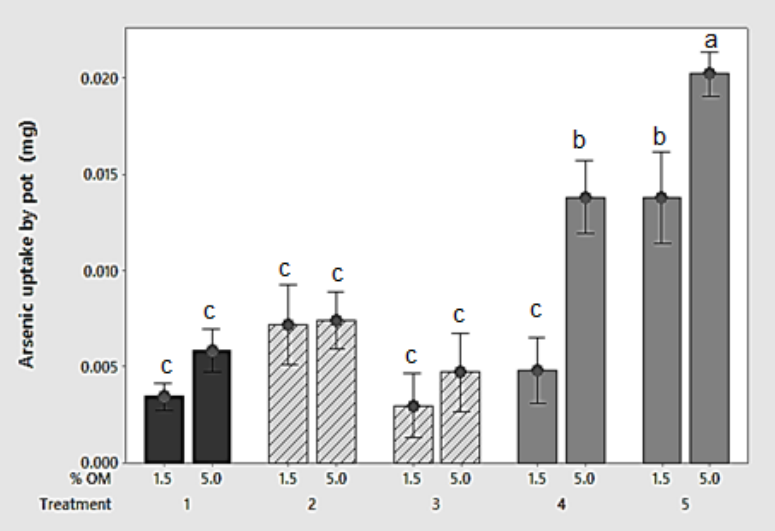

Figure 0-8 Arsenic uptake by shoots in different treatments and different levels of OM (=treatment 1= lime + OM, 2= lime + OM + reducing bacteria, 3= OM + reducing bacteria, 4= lime + OM + oxidizing bacteria, 5= OM + oxidizing bacteria). Means with the same letter are not statistically different (P >0.05).

As we previously reported, the higher arsenic uptake in the treatments was mainly influenced by plant biomass. As a general conclusion, the application of *A. tumefaciens* oxidizing strain in two cases significantly increased arsenic uptake in basin wildrye (Figure 4-8). There are very few studies reporting arsenic concentrations in basin wildrye grown on tailings, thus the comparisons are very limited. In this study, we reported the highest arsenic uptake by pot with a mean of 0.02 mg As pot^{-1} in 12 weeks of basin wildrye growth. In the pilot study, we reported 0.03 mg pot^{-1}. Knudson et al. (2003) reported an average arsenic uptake by basin wildrye after 16 weeks with 4 plants pot^{-1} of 0.04 mg pot^{-1} when plants were exposed

to 50 mg kg^{-1} arsenic and up to 15 mg kg^{-1} phosphorus (our unamended tailings soils averaged 2367 mg kg^{-1} total arsenic and ~1000 mg kg^{-1} phosphorus [0.23% P_2O_5]). The same researchers found that more arsenic was accumulated as more phosphorus was applied to pots. However, these are contradictory with other studies (Meharg & Macnair 1992; Carbonell-Barrachina et al. 1998), which found less arsenic accumulated where more phosphorus was added. In either case, the use of *A. tumefaciens* oxidizing strain in combination with organic matter seems to be a promising, inexpensive, and innovative technique that can be used in areas affected by arsenic.

CHAPTER FIVE

CONCLUSIONS

This project was motivated by the original question: "Which is more critical to the establishment of *Leymus cinereus* in metal-contaminated soils: lime or organic matter?" We used severely contaminated soil collected along the Clark Fork River, Montana, which contained 17- to 126-fold greater concentrations of As, Cu, Pb, and Zn than uncontaminated soils in the area; total arsenic concentrations averaged 2400 mg kg^{-1}, with a maximum value of 3600 mg kg^{-1}.

Our greenhouse study (Experiment 1; Figure 3-2) showed that tailings amended with just 5% organic matter yielded about 6-fold more aboveground biomass (0.12 g pot^{-1}) than tailings amended with just lime (0.02 g pot^{-1}). These relatively low biomass results were much smaller than biomass produced through combinations of these amendments, which suggests it might be important to consider amendment cocktails, consisting of combinations of amendments, instead of just single amendments such as OM or lime.

In our first experiment we introduced the use of the bacterium *Agrobacterium tumefaciens*, a bacterium normally capable of oxidizing As(III) to As(V). When growing in the presence of this bacterium in combination with 5% OM and lime, basin wildrye yielded 5-fold greater biomass than when growing with a combination of lime + OM. Moreover, the double-amendment combination of 5% OM + bacteria yielded 12-fold greater biomass than the 5% OM single amendment alone. Importantly, these two combinations (lime + OM + oxidizing bacteria; OM + oxidizing bacteria) were not statistically different, suggesting the use of organic

matter and oxidizing bacteria can be used as revegetation amendments to improve plant biomass.

For the first experiment, only one level of OM amendment was tested (5%), therefore, we wanted to know whether the same results could be obtained with a lower level of OM amendment (1.5%). Thus, we designed a second greenhouse experiment with two levels of OM (1.5% and 5%). In this second experiment we observed that high levels of organic matter yielded higher plant biomass; however, the combination of lime + 5% OM was not statistically different from the treatment consisting of 1.5% OM + oxidizing bacteria which could represent a cost- saving approach.

The second experiment also highlighted the value of contrasting the roles of wildtype and especially mutant strains of *A. tumefaciens* (developed at Montana State University) as a component of these amendment combinations. The mutant strains are incapable of oxidizing As(III) to As(V), which may represent an important rhizosphere detoxification mechanism. As dramatic as the experimental results were for the differences between soils amended with 1.5% or 5% OM in combination with oxidizing (wildtype) strains of the bacterium, the differences were even greater between nearly identical treatments where only the bacterial strain differed. These results suggest that some bacterial additions may do more harm than traditional amendments such as OM and lime.

The second experiment clearly highlighted the importance of oxidizing (wildtype) vs. non-oxidizing (mutant) strains of bacteria. If oxidizing bacteria enhance basin wildrye biomass yields, an open question was how basin wildrye seed exposure might be affected by different arsenic species. After all, if As(V), one product of

oxidizing bacteria, is less toxic than As(III), how might exposure to these different arsenic species affect basin wildrye seed germination? The germination assay experiment provided dramatic evidence that As(III) is far more toxic than As(V) for a given concentration applied to the agar. These results highlight the importance of evaluating the arsenic species present in the soil before implementing a reclamation plan.

Shifting from a phytostabilization to a phytoextraction perspective, we asked which combination of amendments yielded the greatest As uptake into shoots and roots. Our first and second experiment showed us that arsenic accumulation was always greater in roots than shoots which implies a potential defense mechanism of basin wildrye toward arsenic. In experiment one, the highest arsenic concentration was observed in lime + bacteria and the OM only treatments; however, since these treatments produced low biomass, the final arsenic uptake levels in these treatments were low. On the other hand, treatments that had OM + bacteria or lime + OM + bacteria had higher biomass production which resulted in the highest arsenic uptake in our experiment.

In the second experiment we observed again that the highest arsenic uptake occurred in treatments that produced the highest biomass; therefore, plants growing in treatments with higher organic matter with the oxidizing strain of *A. tumefaciens* removed more arsenic from the soil. We observed that treatments that had oxidizing bacteria accumulated more arsenic in roots probably because this bacterium helped to convert As(III) to As(V); therefore, the plant may have been able to hold more arsenic in a less toxic form. Contrasting treatments with reducing bacteria appeared to uptake less arsenic because plants may not have been able to tolerate high levels of As(III).

Arsenic concentrations in aboveground biomass were very low, making it difficult to categorize basin wildrye in terms of phytoextraction potential. However, they should be considered as possible phytostabilization technology. In addition, none of our treatments exceeded the limit of animal consumption (horses: 50 mg kg^{-1}); therefore, the risk of contamination by consumption of this plant is very low (NRC 1980; Neuman et al. 2005; Mains et al. 2006).

Finally, our arsenic speciation results suggest that the two strains of *A. tumefaciens* were able to modify the arsenic speciation of tailings shortly after inoculation.

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APPENDICES

APPENDIX A

AMENDMENT CALCULATIONS

1. Lime Calculations

Soil Mass (M):

Volume: 0.20 m x 10000m^2 = 2000m^3 Density of soil =1.3 Mg m^3

$$M_{soil} = \frac{1.3\ Mg}{m^3} x\ 2000 m^3 = 2600 Mg = \text{Soil mass (Weight)}$$

Acid Base Account Test:

Average of extractable HNO_3 =0.37
Average of extractable HCL =0.49
Average residual =0.09
Lime SMP =11.4

$$\text{Tons of CaCO3} = \frac{(\%\ HNO3\ S + \%\ Residua) x\ 31.25 + (\%\ HCL\ extractable\ S) x\ 23.44 + SMP}{}$$

$$\text{Tons of CaCO3} = (0.37 + 0.09)\ x\ 31.25 + (0.49) x\ 23.44 + 11.4 = \mathbf{37.26}$$

$$\frac{(37.26\ \text{tons CaCO3})}{2600\ \text{tons of soil}} = \frac{1000}{x} = 96.87\ \mathbf{tons\ CaCO3}$$

$$\frac{(\text{CaCO3})}{\text{Ca(OH)2}} = \frac{100}{136} = \mathbf{0.73}$$

$$96.87\ x\ 0.73 = \mathbf{70.71\ tons\ of\ Ca(OH)2\ per\ ton\ of\ soil}$$

$$\frac{2600000\ kg\ soil}{1.9\ kg\ pot} = \frac{1070710\ kg\ CA(OH)2}{x} = \mathbf{0.05\ kg\ per\ pot}$$

2. Nitrogen Calculations

Nitrogen requirement = 78.4 kg ha^{-1}

Area per pot =195 cm^2

$$\frac{10000\ m2}{0.0195\ m2} = \frac{78\ kg}{x} = 0.15\ gr\ of\ N\ per\ pot$$

Tailing soils had 0.0178 gr of Nitrogen therefore 0,132 gr of Nitrogen per pot was required to be aggregated.

$$\frac{N}{KNO3} = \frac{14}{101} = 0.13$$

Since the source of nitrogen is KNO_3 1 gr per pot should be aggregated.

APPENDIX B

DATA AND OUTPUTS FIRST EXPERIMENT

1. General data Aboveground First Experiment

Treatment	Number of Plants Germinated	Surviving Plants	Soil pH	Weight (gr pot^{-1})	As (mg kg^{-1})	As Uptake by Pot (mg)
Green House	5	5	7.86	3.2700	1.3860	0.0045
Green House	6	6	7.95	3.8300	1.2427	0.0048
Green House	4	4	7.80	2.5700	0.9094	0.0023
Green House	6	6	7.82	3.8300	1.2237	0.0047
Tailing	0	0	3.90	0.0000	0.0000	0.0000
Tailing	0	0	3.86	0.0000	0.0000	0.0000
Tailing	0	0	3.76	0.0000	0.0000	0.0000
Tailing	0	0	3.95	0.0000	0.0000	0.0000
Lime	5	4	7.24	0.0100	24.0900	0.0002
Lime	4	4	7.28	0.0300	25.4600	0.0008
Lime	4	4	7.22	0.0200	10.7400	0.0002
Lime	5	3	7.27	0.0100	17.9200	0.0002
OM	6	6	7.10	0.1450	53.1200	0.0077
OM	5	5	6.45	0.1000	24.4100	0.0024
OM	5	5	6.36	0.1200	14.4700	0.0017
OM	6	5	6.10	0.1100	23.7800	0.0026
Lime+OM	5	2	7.75	0.2600	3.2100	0.0008
Lime+OM	7	4	7.97	0.4100	7.1330	0.0029
Lime+OM	2	1	7.94	0.1000	9.0200	0.0009
Lime+OM	5	6	7.93	0.6900	9.0200	0.0062
Bacteria	2	1	4.5	0.0000	0.0000	0.0000
Bacteria	1	0	4.26	0.0000	0.0000	0.0000
Bacteria	3	1	4.35	0.0000	0.0000	0.0000
Bacteria	0	0	4.35	0.0000	0.0000	0.0000
Lime+Bacteria	5	7	6.40	0.0400	29.8800	0.0012
Lime+Bacteria	7	5	6.88	0.0200	28.0800	0.0006
Lime+Bacteria	5	5	6.93	0.0400	48.3900	0.0019
Lime+Bacteria	4	4	7.13	0.0200	54.4200	0.0011
OM+Bacteria	5	6	7.82	1.7400	37.5350	0.0653
OM+Bacteria	6	4	7.00	1.1800	16.1400	0.0190
OM+Bacteria	5	5	6.96	1.3600	10.7400	0.0146
OM+Bacteria	7	6	6.99	1.5800	17.3400	0.0274
Lime+OM+Bacteria	7	3	7.94	1.8200	14.0900	0.0256
Lime+OM+Bacteria	5	3	7.91	1.3700	11.7000	0.0160
Lime+OM+Bacteria	4	2	7.96	1.1500	14.0570	0.0162
Lime+OM+Bacteria	5	6	7.95	3.6400	13.2530	0.0482

2. General data Roots First Experiment

Treatment	Number of plants germinated	surviving plants	Soil pH	Weight (gr per pot)	As mg kg-1	Arsenic uptake by pot (mg)
Green House	5	5	7.86	3.66	4.8	0.0177
Green House	6	6	7.95	4.88	14.2	0.0695
Green House	4	4	7.8	3.23	2.3	0.0073
Green House	6	6	7.82	2.03	2.7	0.0056
Tailing	0	0	3.9	0	0	0
Tailing	0	0	3.86	0	0	0
Tailing	0	0	3.76	0	0	0
Tailing	0	0	3.95	0	0	0
Lime	5	4	7.24	0.02	5.346	0.0001
Lime	4	4	7.28	0.02	14.32	0.0003
Lime	4	4	7.22	0.02	2.444	0
Lime	5	3	7.27	0.01	7.59	0.0001
OM	6	6	7.1	0.21	23.77	0.005
OM	5	5	6.45	0.2	54.07	0.0108
OM	5	5	6.36	0.19	47.25	0.009
OM	6	5	6.1	0.2	89.36	0.0179
Lime+OM	5	2	7.75	0.19	64.97	0.0123
Lime+OM	7	4	7.97	0.39	49.54	0.0193
Lime+OM	2	1	7.94	0.1	51.85	0.0052
Lime+OM	5	6	7.93	0.6	54.5	0.0327
Bacteria	2	1	4.5	0	0	0
Bacteria	1	0	4.26	0	0	0
Bacteria	3	1	4.35	0	0	0
Bacteria	0	0	4.35	0	0	0
Lime+Bacteria	5	7	6.4	0.09	9.05	0.0008
Lime+Bacteria	7	5	6.88	0.07	16.28	0.0011
Lime+Bacteria	5	5	6.93	0.06	9.917	0.0006
Lime+Bacteria	4	4	7.13	0.05	21.47	0.0011
OM+Bacteria	5	6	7.82	0.62	79.645	0.0494
OM+Bacteria	6	4	7	0.59	98.765	0.0583
OM+Bacteria	5	5	6.96	0.58	28.185	0.0163
OM+Bacteria	7	6	6.99	0.76	48.95	0.0372
Lime+OM+Bacteria	7	3	7.94	1.41	59.11	0.0833
Lime+OM+Bacteria	5	3	7.91	1.42	43.95	0.0624
Lime+OM+Bacteria	4	2	7.96	1.04	59.73	0.0621
Lime+OM+Bacteria	5	6	7.95	2.79	59.92	0.1672

3. Anova Analisys Germination

Analysis of Variance

Variable	N	R²	R² Aj	CV
Germination	36	0.79	0.73	27.05

Table of analysis of variance (SC tipo III)

F.V.	SS	DF	CM	F	p-value
Model	20451.39	8	2556.42	12.71	<0.0001
Treatment	20451.39	8	2556.42	12.71	<0.0001
Error	5429.69	27	201.1		
Total	25881.08	35			

Test:LSD Fisher Alfa=0.05 DMS=1.57357

Error: 1.2589 gl: 28

Treatment	Means	N		
OM+Bacteria	71.88	4	A	
OM	68.75	4	A	
Lime+OM+Bacteria	65.63	4	A	
Lime+Bacteria	65.63	4	A	
Green house soil	65.63	4	A	
Lime+OM	59.38	4	A	
Lime	56.25	4		B
Bacteria	18.75	4		B

3. Anova Analisys in Shoots Biomass

Analysis of variance

Variable	N	R²	R² Aj	CV
LN_Weights per pot (g)	28	0.96	0.95	35.97

Table of analysis of variance (SC tipo III)

F.V.	SS	DI	CM	F	p-value
Model.	106.49	6	17.75	84.29	<0.0001
Treatment	106.49	6	17.75	84.29	<0.0001
Error	4.42	21	0.21		
Total	110.91	27			

Test:LSD Fisher Alfa=0.05 DMS=0.67477

Error: 0.2106 gl: 21

Treatment	Means	n				
Positive control	1.2	4	A			
Lime+OM+Bacteria	0.59	4	A	B		
OM+Bacteria	0.37	4		B		
Lime+OM	-1.23	4			C	
OM	-2.14	4				D
Lime+Bacteria	-3.57	4				E
Lime	-4.16	4				E

4. Anova Analisys Root Biomass

Analysis of variance

Variable	N	R²	R² Aj	CV
LN_Weights per pot (g)	28	0.96	0.95	32.75

Table of analysis of variance (SC tipo III)

F.V.	SS	DF	CM	F	p-value
Modelo.	79.11	6	13.18	81.58	<0.0001
Treatment	79.11	6	13.18	81.58	<0.0001
Error	3.39	21	0.16		
Total	82.5	27			

Test:LSD Fisher Alfa=0.05

Error: 0.1616 gl: 21

Treatment	Means	n				
Positive control	1.19	4	A			
Lime+OM+Bacteria	0.44	4		B		
OM+Bacteria	-0.46	4			C	
Lime+OM	-1.35	4			D	
OM	-1.61	4			D	
Lime+Bacteria	-2.72	4				E
Lime	-4.09	4				F

Means with the same letter are statistically no significant (P >0,05)

5. Anova Analisys Arsenic Concentration in Shoots

Analysis of variance

Variable	N	R^2	R^2 Aj	CV
RAIZ_As in shoots (ppm	28	0.8	0.75	22.98

Table of analysis of variance (SC tipo III)

F.V.	SS	DF	CM	F	p-value
Modelo.	69.89	6	11.65	14.18	<0.0001
Treatment	69.89	6	11.65	14.18	<0.0001
Error	17.25	21	0.82		
Total	87.14	27			

Test:LSD Fisher Alfa=0.05 DMS=1.33281

Error: 0.8215 gl: 21

Treatment	Means	n	E.E.				
Lime+Bacteria	6.27	4	0.45	A			
OM	5.23	4	0.45	A			
OM+Bacteria	4.4	4	0.45		B	C	
Lime	4.37	4	0.45		B	C	
Lime+OM+Bacteria	3.64	4	0.45			C	D
Lime+OM	2.62	4	0.45				D
Green House	1.09	4	0.45				E

Means with the same letter are statistically no significant (P >0,05)

6 Anova Analisys Arsenic Concentration in Roots

Analysis of variance

Variable	N	R^2	R^2 Aj	CV
LN_As (Mg/kg)	28	0.83	0.78	17.27

Table of analysis of variance (SC tipo III)

F.V.	SS	DF	CM	F	p-value
Modelo.	30.36	6	5.06	17.39	<0.0001
Treatment	30.36	6	5.06	17.39	<0.0001
Error	6.11	21	0.29		
Total	36.46	27			

Test:LSD Fisher Alfa=0.05 DMS=0.79317

Error: 0.2909 gl: 21

Treatment	Means	n	E.E.
OM+Bacteria	4.05	4	A
Lime+OM+Bacteria	4.01	4	A
Lime+OM	4.01	4	A
OM	3.88	4	A
Lime+Bacteria	2.59	4	B
Lime	1.81	4	B C
Green House	1.51	4	C

Means with the same letter are statistically no significant (P >0,05)

7 Anova Analisys Arsenic Uptake in Shoots

Analysis of variance

Variable	N	R^2	R^2 Aj	CV
LN_Arsenic uptake by sh	28	0.88	0.85	11.22

Table of analysis of variance (SC tipo III)

F.V.	SS	DF	CM	F	p-value
Modelo.	62.87	6	10.48	25.61	<0.0001
Treatment	62.87	6	10.48	25.61	<0.0001
Error	8.59	21	0.41		
Total	71.46	27			

Test:LSD Fisher Alfa=0.05

Error: 0.4092 gl: 21

Treatment	Medias	n			
OM+Bacteria	-3.63	4	A		
Lime+OM+Bacteria	-3.74	4	A		
Green House	-5.54	4		B	
OM	-5.8	4		B	
Lime+OM	-6.25	4		B	
Lime+Bacteria	-6.82	4			C
Lime	-8.15	4			D

APPENDIX C

DATA AND OUTPUTS SECOND EXPERIMENT

1. General data Shoots Second Experiment

DESCRIPTION	Treatment	% OM	Plants germinated	Soil pH	Weight (gr per pot)	As (Mg/kg)
Lime+OM	1	5	6	7.85	0.585	16.04
Lime+OM	1	5	6	8.29	0.525	8.23
Lime+OM	1	5	6	8.36	0.515	12.06
Lime+OM	1	5	6	8.34	0.555	11.82
Lime+OM	1	5	6	8.32	0.6	4.67
Lime+OM	1	5	6	8.27	0.435	
Lime+OM	1	5	6	7.98	0.6	
Lime+OM	1	5	5	8.23	0.555	
Lime+OM	1	5	5	8.3	0.605	
Lime+OM	1	5	5	8.34	0.745	
Lime+OM	1	1.5	6	5.37	0.076	76.25
Lime+OM	1	1.5	3	5.6	0.092	42.5
Lime+OM	1	1.5	4	5.66	0.092	18.75
Lime+OM	1	1.5	5	5.65	0.256	15
Lime+OM	1	1.5	5	5.49	0.084	23.75
Lime+OM	1	1.5	6	5.62	0.072	
Lime+OM	1	1.5	3	5.55	0.172	
Lime+OM	1	1.5	4	5.65	0.24	
Lime+OM	1	1.5	4	5.48	0.13	
Lime+OM	1	1.5	5	5.6	0.092	
Lime+OM+Mutant	2	5	5	6.88	0.425	28.44
Lime+OM+Mutant	2	5	5	7.65	0.64	13.27
Lime+OM+Mutant	2	5	5	7.95	0.785	9.69
Lime+OM+Mutant	2	5	6	8.16	0.645	8.41
Lime+OM+Mutant	2	5	6	7.77	0.42	8.13
Lime+OM+Mutant	2	5	5	7.8	0.245	
Lime+OM+Mutant	2	5	4	7.68	0.07	
Lime+OM+Mutant	2	5	6	7.9	0.865	
Lime+OM+Mutant	2	5	5	8.02	0.98	
Lime+OM+Mutant	2	5	6	7.45	0.12	
Lime+OM+Mutant	2	1.5	6	8.07	0.785	12.05
Lime+OM+Mutant	2	1.5	6	8.01	0.095	53.75
Lime+OM+Mutant	2	1.5	4	7.91	0.1	117.5
Lime+OM+Mutant	2	1.5	6	7.96	0.07	2.5
Lime+OM+Mutant	2	1.5	7	8.02	0.21	45
Lime+OM+Mutant	2	1.5	5	7.99	0.89	
Lime+OM+Mutant	2	1.5	6	8	0.73	
Lime+OM+Mutant	2	1.5	5	7.95	0.64	
Lime+OM+Mutant	2	1.5	5	8.05	0.46	
Lime+OM+Mutant	2	1.5	4	7.9	0.49	
OM+Mutant	3	5	3	7.25	0.165	65.83
OM+Mutant	3	5	5	7.1	0.22	18.75
OM+Mutant	3	5	3	7.71	0.39	19.69
OM+Mutant	3	5	3	7.36	0.095	0.42
OM+Mutant	3	5	3	7.75	0.08	11.25
OM+Mutant	3	5	2	7.38	0.805	
OM+Mutant	3	5	3	7.15	0.125	
OM+Mutant	3	5	4	7.27	0.15	
OM+Mutant	3	5	5	7.56	0.135	
OM+Mutant	3	5	2	7.52	0.015	

1. General data Shoots Second Experiment-Contiued

DESCRIPTION	Treatment	% OM	Plants germinated	Soil pH	Weight (gr per pot)	As (Mg/kg)
OM+Mutant	3	1.5	2	5.21	0.165	5.83
OM+Mutant	3	1.5	3	4.8	0.06	155
OM+Mutant	3	1.5	2	5.12	0.09	35
OM+Mutant	3	1.5	4	4.98	0.075	21.25
OM+Mutant	3	1.5	4	4.97	0	0
OM+Mutant	3	1.5	2	4.95	0.095	
OM+Mutant	3	1.5	2	5.1	0.011	
OM+Mutant	3	1.5	1	5.18	0.0055	
OM+Mutant	3	1.5	2	4.98	0	
OM+Mutant	3	1.5	0	4.85	0	
Lime+OM+Wild	4	5	5	7.24	0.835	10.74
Lime+OM+Wild	4	5	6	7.12	1.55	7.58
Lime+OM+Wild	4	5	5	7.34	1.765	7.86
Lime+OM+Wild	4	5	5	7.66	1.64	12.5
Lime+OM+Wild	4	5	7	7.28	1.745	8.07
Lime+OM+Wild	4	5	6	7.16	2.165	
Lime+OM+Wild	4	5	6	7.37	1.3375	
Lime+OM+Wild	4	5	5	7.68	2.125	
Lime+OM+Wild	4	5	6	7.44	1.96	
Lime+OM+Wild	4	5	6	7.4	1.26	
Lime+OM+Wild	4	1.5	5	8.06	0.225	47
Lime+OM+Wild	4	1.5	6	7.22	0.125	31.67
Lime+OM+Wild	4	1.5	3	8.08	0.03	35
Lime+OM+Wild	4	1.5	5	6.77	0.125	17.5
Lime+OM+Wild	4	1.5	6	8.01	0.1	63.75
Lime+OM+Wild	4	1.5	6	7.25	0.205	
Lime+OM+Wild	4	1.5	5	7.82	0.155	
Lime+OM+Wild	4	1.5	4	6.9	0.205	
Lime+OM+Wild	4	1.5	5	7.55	0.155	
Lime+OM+Wild	4	1.5	5	7.65	0.33	
OM+Wild	5	5	5	7.72	1.45	12.76
OM+Wild	5	5	6	7.87	1.605	12.11
OM+Wild	5	5	6	7.79	1.155	15.22
OM+Wild	5	5	6	7.84	1.845	12.84
OM+Wild	5	5	6	7.75	1.32	16.62
OM+Wild	5	5	6	7.83	2.14	
OM+Wild	5	5	6	7.62	1.09	
OM+Wild	5	5	7	7.91	1.08	
OM+Wild	5	5	6	7.7	1.45	
OM+Wild	5	5	6	7.87	2.26	
OM+Wild	5	1.5	5	8.2	0.52	12.5
OM+Wild	5	1.5	6	7.21	0.49	22.25
OM+Wild	5	1.5	4	7.63	0.45	37.92
OM+Wild	5	1.5	5	8.01	0.615	23.75
OM+Wild	5	1.5	6	7.25	0.5	40
OM+Wild	5	1.5	6	7.52	0.8	
OM+Wild	5	1.5	5	8.12	0.89	
OM+Wild	5	1.5	6	7.95	0.855	
OM+Wild	5	1.5	5	7.75	0.78	
OM+Wild	5	1.5	6	7.8	0.335	

2. General Data Roots Second Experiment

DESCRIPTION	Treatment	% OM	Soi pH	Weight (gr per pot)	As (Mg/kg)	Arsenic uptake by pot (mg)
Lime+OM	1	5	7.85	0.47	407.5	0.1915
Lime+OM	1	5	8.29	0.67	392.3077	0.2628
Lime+OM	1	5	8.36	0.57	465.9091	0.2656
Lime+OM	1	5	8.34	0.52	412.5	0.2145
Lime+OM	1	5	8.32	0.7	402.2727	0.4425
Lime+OM	1	5	8.27	0.4		
Lime+OM	1	5	7.98	0.54		
Lime+OM	1	5	8.23	0.5		
Lime+OM	1	5	8.3	0.59		
Lime+OM	1	5	8.34	0.68		
Lime+OM	1	1.5	5.37	0.08	198.75	0.0175
Lime+OM	1	1.5	5.6	0.096	203.75	0.0196
Lime+OM	1	1.5	5.66	0.104	341.6667	0.0355
Lime+OM	1	1.5	5.65	0.244	317.0833	0.0774
Lime+OM	1	1.5	5.49	0.092	357	0.0685
Lime+OM	1	1.5	5.62	0.098		
Lime+OM	1	1.5	5.55	0.108		
Lime+OM	1	1.5	5.65	0.18		
Lime+OM	1	1.5	5.48	0.12		
Lime+OM	1	1.5	5.6	0.092		
Lime+OM+Mutant	2	5	6.88	0.33	86.42857	0.0285
Lime+OM+Mutant	2	5	7.65	0.645	309.6154	0.1997
Lime+OM+Mutant	2	5	7.95	0.69	358.3333	0.2150
Lime+OM+Mutant	2	5	8.16	0.54	322.5	0.1742
Lime+OM+Mutant	2	5	7.77	0.585	300	0.1755
Lime+OM+Mutant	2	5	7.8	0.23		
Lime+OM+Mutant	2	5	7.68	0.42		
Lime+OM+Mutant	2	5	7.9	0.758		
Lime+OM+Mutant	2	5	8.02	0.75		
Lime+OM+Mutant	2	5	7.45	0.715		
Lime+OM+Mutant	2	1.5	8.07	0.6	391.129	0.2347
Lime+OM+Mutant	2	1.5	8.01	0.105	285.5	0.0699
Lime+OM+Mutant	2	1.5	7.91	0.1	367.5	0.0368
Lime+OM+Mutant	2	1.5	7.96	0.08	247	0.0556
Lime+OM+Mutant	2	1.5	8.02	0.23	393	0.0904
Lime+OM+Mutant	2	1.5	7.99	0.42		
Lime+OM+Mutant	2	1.5	8	0.725		
Lime+OM+Mutant	2	1.5	7.95	0.45		
Lime+OM+Mutant	2	1.5	8.05	0.645		
Lime+OM+Mutant	2	1.5	7.9	0.45		
OM+Mutant	3	5	7.25	0.158	242.5	0.0158
OM+Mutant	3	5	7.1	0.21	17.5	0.0023
OM+Mutant	3	5	7.71	0.35	216.875	0.0466
OM+Mutant	3	5	7.36	0.0987	55	0.0017
OM+Mutant	3	5	7.75	0.093	62.5	0.0034
OM+Mutant	3	5	7.38	0.23		
OM+Mutant	3	5	7.15	0.13		
OM+Mutant	3	5	7.27	0.15		
OM+Mutant	3	5	7.56	0.142		
OM+Mutant	3	5	7.52	0.016		

2. General Data Roots Second Experiment-Continued

DESCRIPTION	Treatment	% OM	Soi pH	Weight (gr per pot)	As (Mg/kg)	Arsenic uptake by pot (mg)
OM+Mutant	3	1.5	5.21	0.12	95	0.0000
OM+Mutant	3	1.5	4.8	0	90	0.0000
OM+Mutant	3	1.5	5.12	0.105	117.5	0.0123
OM+Mutant	3	1.5	4.98	0.055	65	0.0036
OM+Mutant	3	1.5	4.97	0	no sample	no sample
OM+Mutant	3	1.5	4.95	0.087		
OM+Mutant	3	1.5	5.1	0.012		
OM+Mutant	3	1.5	5.18	0.004		
OM+Mutant	3	1.5	4.98	0		
OM+Mutant	3	1.5	4.85	0		
Lime+OM+Wild	4	5	7.24	1.55	394.6429	0.2763
Lime+OM+Wild	4	5	7.12	1.295	367.3077	0.4757
Lime+OM+Wild	4	5	7.34	1.415	362.5	0.5129
Lime+OM+Wild	4	5	7.66	1.53	455.5556	0.2027
Lime+OM+Wild	4	5	7.28	1.69	557	0.6907
Lime+OM+Wild	4	5	7.16	1.53		
Lime+OM+Wild	4	5	7.37	1.245		
Lime+OM+Wild	4	5	7.68	1.54		
Lime+OM+Wild	4	5	7.44	1.85		
Lime+OM+Wild	4	5	7.4	1.005		
Lime+OM+Wild	4	1.5	8.06	0.47	107	0.0503
Lime+OM+Wild	4	1.5	7.22	0.34	27.5	0.0094
Lime+OM+Wild	4	1.5	8.08	0.052	ND	N.D
Lime+OM+Wild	4	1.5	6.77	0.4	23.125	0.0093
Lime+OM+Wild	4	1.5	8.01	0.27	24.5	0.0066
Lime+OM+Wild	4	1.5	7.25	0.315		
Lime+OM+Wild	4	1.5	7.82	0.295		
Lime+OM+Wild	4	1.5	6.9	0.325		
Lime+OM+Wild	4	1.5	7.55	0.125		
Lime+OM+Wild	4	1.5	7.65	0.36		
OM+Wild	5	5	7.72	1	867.5	0.8675
OM+Wild	5	5	7.87	1.5	523.8095	0.5500
OM+Wild	5	5	7.79	1.102	508.3333	0.4575
OM+Wild	5	5	7.84	1.742	526.087	0.6050
OM+Wild	5	5	7.75	1.21	428.5714	0.4071
OM+Wild	5	5	7.83	1.82		
OM+Wild	5	5	7.62	1.58		
OM+Wild	5	5	7.91	1.53		
OM+Wild	5	5	7.7	1.2		
OM+Wild	5	5	7.87	1.53		
OM+Wild	5	1.5	8.2	0.6	328.3333	0.1970
OM+Wild	5	1.5	7.21	0.65	318.9286	0.2073
OM+Wild	5	1.5	7.63	0.45	281.6667	0.1268
OM+Wild	5	1.5	8.01	0.58	491.6667	0.2213
OM+Wild	5	1.5	7.25	0.5	278.3333	0.1392
OM+Wild	5	1.5	7.52	0.7		
OM+Wild	5	1.5	8.12	0.6		
OM+Wild	5	1.5	7.95	0.75		
OM+Wild	5	1.5	7.75	0.698		
OM+Wild	5	1.5	7.8	0.45		

3. Germination Assay

Analysis of Variance

Variable	N	R^2	R^2 Aj	CV
Germination	48	0.91	0.9	9.79

Table of analysis of variance (SC tipo III)

F.V.	SS	DF	CM	F	p-value
Model	1811.17	5	362.23	86.08	<0,0001
Treatment	1811.17	5	362.23	86.08	<0,0001
Error	176.75	42	4.21		
Total	1987.92	47			

Test:LSD Fisher Alfa=0,05 DMS=2,0699

Error: 4,2083 gl: 42

Treatment	Means	N				
0,05 mM As V	26	8	A			
0,05 mM As(III)	25.25	8	A	B		
Control	24.75	8	A	B		
0,5 mM As(V)	23.88	8		B		
5 mM As V	16.88	8			C	
0,5 mM As(III)	9	8			D	
5 mM As III	0	8				E

4. Germination Greenhouse

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	4	113.900	28.4750	38.08	0.000
% OM	1	12.250	12.2500	16.38	0.000
Treatment*% OM	4	5.300	1.3250	1.77	0.141
Error	90	67.300	0.7478		
Total	99	198.750			

Treatment* % OM

% OM	N	Mean	Grouping
5	5.0	10	6.0 A
1	5.0	10	5.7 A B
4	5.0	10	5.7 A B
2	1.5	10	5.4 A B
5	1.5	10	5.4 A B
2	5.0	10	5.3 A B
4	1.5	10	5.0 B C
1	1.5	10	4.5 C
3	5.0	10	3.3 D
3	1.5	10	2.2 E

5. Anova Analisys in Shoots Biomass

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	4	12.308	3.07695	46.99	0.000
% OM	1	9.434	9.43411	144.07	0.000
Treatment*% OM	4	6.762	1.69055	25.82	0.000
Error	90	5.894	0.06548		
Total	99	34.398			

Grouping Information Using Fisher LSD Method and 95% Confidence

Treatment* OM

% OM	N	Mean	Grouping
4 5.0	10	1.63925	A
5 5.0	10	1.53950	A
5 1.5	10	0.62350	B
1 5.0	10	0.57200	B
2 5.0	10	0.51950	B
2 1.5	10	0.44700	B
3 5.0	10	0.21800	C
4 1.5	10	0.16550	C
1 1.5	10	0.13060	C
3 1.5	10	0.05015	C

Means that do not share a letter are significantly different.

6. Anova Analisys in Root Biomass

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	4	11.455	2.86366	104.54	0.000
% OM	1	7.515	7.51456	274.33	0.000
Treatment*% OM	4	3.943	0.98566	35.98	0.000
Error	90	2.465	0.02739		
Total	99	25.377			

Grouping Information Using Fisher LSD Method and 95% Confidence

Treatment* %OM

% OM	N	Mean	Grouping
4 5.0	10	1.46500	A
5 5.0	10	1.42140	A
5 1.5	10	0.59780	B
2 5.0	10	0.56630	B
1 5.0	10	0.56400	B
2 1.5	10	0.38050	C
4 1.5	10	0.29520	C D
3 5.0	10	0.15777	D E
1 1.5	10	0.12140	E

7. Anova Analisys Arsenic Concentration in Shoots

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	4	1.554	0.3884	0.10	0.983
% OM	1	45.296	45.2962	11.29	0.002
Treatment*% OM	4	13.220	3.3051	0.82	0.518
Error	40	160.416	4.0104		
Total	49	220.487			

8. Anova Analisys Arsenic Concentration in Roots

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	4	638389	159597	19.34	0.000
% OM	1	240685	240685	29.16	0.000
Treatment*% OM	4	280707	70177	8.50	0.000
Error	38	313617	8253		
Total	47	1444368			

Grouping Information Using Fisher LSD Method and 95% Confidence

Treatment* % OM

% OM	N	Mean	Grouping
5 5.0	5	570.860	A
4 5.0	5	427.401	B
1 5.0	5	416.098	B
5 1.5	5	339.786	B C
2 1.5	5	336.826	B C
1 1.5	5	283.650	C
2 5.0	5	275.375	C
3 5.0	5	118.875	D
3 1.5	4	91.875	D
4 1.5	4	45.531	D

Means that do not share a letter are significantly different.

9. Anova Analisys Arsenic Uptake in Shoots

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	4	0.001110	0.000278	19.53	0.000
% OM	1	0.000195	0.000195	13.73	0.001
Treatment*% OM	4	0.000132	0.000033	2.33	0.073
Error	40	0.000568	0.000014		
Total	49	0.002006			

Treatment* %OM

% OM	N	Mean	Grouping
5 5.0	5	0.0202265	A
4 5.0	5	0.0138333	B
5 1.5	5	0.0138142	B
2 5.0	5	0.0074039	C
2 1.5	5	0.0071881	C
1 5.0	5	0.0058553	C
4 1.5	5	0.0048292	C
3 5.0	5	0.0047210	C
1 1.5	5	0.0034530	C
3 1.5	5	0.0030012	C

Means that do not share a letter are significantly different.