Phytoremediation of Metal Contamination using Salix (Willows)

Gordon J. Kersten

University of Denver
Phytoremediation of Metal Contamination using *Salix* (willows)

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Gordon J. Kersten

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Advisor: Martin F. Quigley
ABSTRACT

Abandoned hardrock mines and the resulting Acid Mine Drainage (AMD) are a source of vast, environmental degradation that are toxic threats to plants, animals, and humans. Cadmium (Cd) and lead (Pb) are metal contaminants often found in AMD. In my mine outwash water samples, cadmium and lead concentrations were 19 and 160 times greater than concentrations in control waterways, and 300 and 40 times greater than EPA Aquatic Life Use water quality standards, respectively. I tested the phytoremediation characteristics of three montane willows native to the Rocky Mountains: Salix drummondiana, S. monticola, and S. planifolia. I tested the willows’ accumulation and tolerance characteristics of cadmium and lead contamination. I found that S. drummondiana accumulated more cadmium in stems than both S. monticola and S. planifolia, and that all three willow species accumulated similar concentrations of lead. I found similar trends for leaf accumulation. I also found that S. monticola had a greater growth and tolerance to the lower lead concentrations than high lead concentrations in addition to containing higher field stem concentrations of lead than S. planifolia. Salix planifolia contained nearly 2.5 times greater concentrations of cadmium in field stems than S. drummondiana. Based on my results, S. drummondiana could aid in aboveground accumulation of cadmium polluted watersheds, and S. monticola could aid in aboveground accumulation and tolerance of lead pollution.
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CHAPTER I: LITERATURE REVIEW ON CHARACTERISTICS OF SALIX SPP. NATIVE TO NORTH AMERICA FOR PHYTOREMEDIATION OF METAL CONTAMINATION

Abandoned Hardrock Mine Pollution

Geography of abandoned hardrock mines in the U.S.

Abandoned mines in the North America are a physical as well as an environmental danger. Abandoned mines are environmentally destructive; mine tailings contain sulfides and discharge acid mine drainage (AMD), which is a severe water pollution problem (Hoffert 1947; Tuttle et al. 1969). AMD contains mobile inorganic contaminants such as heavy metals that precipitate and degrade ecological systems, starting with periphyton and benthic invertebrate communities (McKnight and Feder 1984). Heavy metals from the tailings dissipate through AMD in outwash, groundwater, and wind (Hoffert 1947; Roberts and Johnson 1978; McKnight and Feder 1984). The heavy metal contaminants flow through the ecosystem infiltrating soil, watersheds, flora, and the ecological food chain (Roberts and Johnson 1978; Pulford et al. 2002; Govind and Madhuri 2014).

Abandoned mines in the USA are heavily concentrated in the Mountain West and Southwest (Figure 1). I focused on these regions, which overlap with two floristic regions: the Rocky Mountain and Madrean Regions, adapted from Takhtajan (1986) and Thorne (1993). These regions are further broken down into provinces and sub provinces. Estimates of abandoned hardrock mines vary widely. The US Government
Accountability Office’s (GAO) report in 2008 estimated 161,000 abandoned hardrock mines in 12 western states and Alaska with 33,000 sites that have degraded the environment (Nazzaro 2008). Abandoned hardrock mines are a known and costly remediation focus of the USA with combined expenditures of $2.6 billion between 1998 and 2007 for abandoned hardrock mine reclamation, funded among the BLM, US Forest Service, USEPA, and the Office of Surface Mining. This review focuses on abandoned hardrock mines, which are separate from abandoned coal and uranium mines. Coal mines are concentrated in the eastern states such as West Virginia, Pennsylvania, and Kentucky. Uranium mines and their wastes have unique remediation methods.

Hardrock Mining Pollution Effects

Abandoned mines are sources of physical and environmental dangers. Open shafts of abandoned mines are extremely dangerous, given instability of structures and depth of openings. Environmentally, abandoned mines’ most damaging characteristic is AMD, which has chemical, physical, biological, and ecological ramifications (Gray 1997). AMD and outwash from mines pollute watersheds and ecosystems for decades; this is exacerbated when they are abandoned with no further accountability for environmental consequences (Jung and Thornton 1996; Wahsha et al. 2012). Mine tailings, or the basins of disposal areas, of these abandoned mines are also numerous in arid and semiarid regions in the world, making recovery of vegetation even more unfavorable (Tordoff et al. 2000). AMD reduces species and habitat diversity, as well as modifying the food chain (Gray 1997).
Heavy metal pollutants deposit or leach from mines into adjacent topsoil and watersheds through AMD. Coal mines often have alkaline limestone in the vicinity which neutralizes the acidity; however, hardrock mines lack the limestone’s neutralizing presence and have more acidic waste (Fields 2003). Higher concentrations of metals are dissolved in water and more mobile throughout the ecosystem as the pH becomes more acidic. Hardrock mine tailings left behind after mining are distinguished by increased concentrations of arsenic, cadmium, copper, manganese, lead and zinc (Boulet and Larocque 1998; Walder and Chavez 1995).

**Metal Pollution Effects**

Metals pose a large amount of environmental degradation and toxicity to life since they cannot be chemically degraded (Salt *et al.* 1995). Dry and loose tailings have caused acute and chronic respiratory diseases in populations near mine disposal sites (Mendez 2008). Metals have negative effects on humans, as well as plants and animals (Fernandes and Henriques 1991; Das *et al.* 1997; Valko *et al.* 2005; Nagajyoti *et al.* 2010). Biologically essential metals, such as zinc and copper, can be toxic in high concentrations. Research has shown the negative effects of zinc (Niyogi *et al.* 2001). Zinc causes neurological deficiencies in newts (Taban *et al.* 1982), correlation of hepatic degeneration (Mitranescu *et al.* 2011), and decreased growth and reproduction efficiency of plants (Leano *et al.* 2010). Copper is highly toxic to aquatic plants and inhibits photosynthesis, membrane integrity, and other biochemical processes (Fernandes and Henriques 1991). Copper in excess creates reactive oxygen species (ROS) damaging DNA, which causes cancer in humans (Theophanides and Anastassopoulou 2002).
Cadmium and lead are two examples of metal pollutants that are not biologically essential. These metals produce ROS that cause DNA damage and alter homeostasis (Stohs and Bagchi 1995). Cadmium is a carcinogen and causes kidney defects and skeletal damage (Jarup 2003), neurological damage (Méndez-Armenta and Rios 2007), and reproductive system damage (Thompson and Bannigan 2008). Lead causes damages to blood, intestinal and renal tissues, and neurological systems (Stohs and Bagchi 1995; Jarup 2003). Cadmium and lead in plants causes toxic results, such as stunting and chlorosis (Das et al. 1997; Pandey et al. 2012).

Abandoned hardrock mines continue to pollute and contaminate ecosystems. They are more than 161,000 abandoned hardrock mines in the western United States with already a remediation expense report of over $2.6 billion. Their resulting AMD and metal contaminants are toxic to biota at all levels including plants, animals, and humans. Metal concentrations in the ecosystem need to be reduced to pre-mining levels with cheaper and sustainable methods for the sake of diversity and health of the environment.

**Remediation of Abandoned Hardrock Mine Lands**

*Conventional Techniques*

Since the 1940s, remediation methods have progressed from no tested method of treating AMD, to microbial sulfate reduction (Tuttle et al. 1969), to chemical treatments, and many isolation and containment practices. Physical and chemical methods are costly, and often completely remove biological activity from the soil and water at the site (Baker et al. 1994). The most common approach for taking care of mine waste material is piling the waste tailings and containing them with embankments or impoundments for isolation
(Mendez and Maier 2008; Fall et al. 2009). Other containment methods include hydraulic isolation curtains or using concrete to entrap the pollutants (Cunningham and Berti 1993). Another costly but common method is the removal and burial of contaminated soil (Kumar et al. 1995). Another physical method of mine tailing treatment is cemented paste tailings (CPT). Using CPT, 60% of tailings are stored appropriately underground, minimizing environmental damage due to runoff (Fall et al. 2008). Compacted bentonite-paste tailings (BPT) have also been investigated for liner or cover for mine waste tailings (Fall et al. 2009). Although isolation and containment do prevent some pollutants’ escape, the risk and the amount of environmental pollutants are not decreased (Cunningham and Berti 1993). Chemical passive treatments, such as alkaline drain treatments using limestone, are also used to raise pH and precipitate and then filter out heavy metals. Other decontamination methods are soil washing and vapor extraction that simply reduce quantity of pollution, but also reduce productive biological activity (Cunningham and Berti 1993).

Over the decades, working on making these sites safe using traditional techniques, such as embankments, has been very expensive. Necessary remediation efforts for heavy metal and organic contaminations in the U.S. are costly, with estimates of $7 - $42 billion (Salt et al. 1995) and $32 – $72 billion (Fields 2003). Conventional techniques such as soil washing and removal of contaminants are three and six times more costly, respectively, than phytoextraction methods, and 13 and 27 times more expensive, respectively, than phytostabilization methods (Cunningham and Berti 2000).
Phytoremediation (Phytoextraction, Rhizofiltration, Phytostabilization)

Phytoremediation is an alternative and cheaper approach for cleaning up contamination from abandoned mine sites. As opposed to isolation and containment techniques such as vaults and caps, and decontamination techniques, such as soil washing, phytoremediation is a very cost-effective method (Cunningham and Berti 1993). Phytoremediation utilizes plants to clean up soils and water contaminated with acidic mine drainage and metals. Rather than further impairing biological activity or simply covering up the problem, phytoremediation utilizes nature for a more ecosystem-friendly transition in the restoration process. Three common phytoremediation types are phytoextraction, rhizofiltration, and phytostabilization (Salt et al. 1995)

Phytoextraction, using the plant’s natural solar-driven pump, is much cheaper than landfill excavation (Vangronsveld et al. 2009). Phytoextraction, first coined as a concept by Chaney (1983), is a biotic accumulation of metal contaminants into plant tissues after the uptake of metals by roots growing in the contaminated soil. Phytoextraction requires above-ground accumulation and concentration of contaminants for harvesting and removal of contaminants (Kumar et al. 1995; Salt et al. 1998). Natural accumulation of metals in aboveground biomass, such as the presence of nickel in leaves, is thought to prevent microbial infection and herbivory (Martens and Boyd 1994). The effectiveness of phytoextraction varies with contamination level, number and ratio of metal contaminants, depth of contamination, translocation efficiency of the plant itself, total biomass, and amount of extractable metal in plant biomass (Ernst 2005). Once metals are in plant biomass, appropriate measures are taken for the recovery, and re-use,
through leaching or smelting; alternatively, the metals may be stabilized in the soil matrix or disposed in a secure landfill (Cunningham and Berti 1993).

Rhizofiltration is the accumulation, precipitation, or adsorption of aqueous metal pollution (Salt et al. 1998; Raskin et al. 1994). Roots of plants are used to remediate contaminated flowing water, wetlands, and drainages. Aquatic plants such as cattails and submergent algae are examples of rhizofiltration agents used in ground or wastewater treatment of metals such as lead, cadmium, copper, nickel, and zinc (Dietz and Schnoor 2001).

Phytostabilization is the sequestration or trapping of metals in the rhizosphere, either in the plant tissues or the soil matrix (Cunningham et al. 1995). Rather than accumulating metals into the upper biomass, phytostabilization immobilizes the contaminants from dispersion, thus protecting other plants, herbivores, and aquatic biota (Cunningham et al. 1995; Mendez and Maier 2008; Sarma 2011). Phytostabilization is ideal for metals such as chromium and lead that are not easily translocated to aboveground biomass (Chaney et al. 1997).

I focus here on all three of these aspects of phytoremediation, through published accumulation and tolerance experiments. Accumulation experiments include uptake by roots and sequestration or storage of metals within plant tissues. Uptake of metals into plants involves mobilizing and gathering metals into the roots via rhizospheric secretions. These secretions induce uptake of metals via metal-chelating molecules and protons that acidify the soil, mobilizing more metals, or via plasma-membrane-bound metal reductases (Salt et al. 1995). It has been suggested that plants differ in expression of genes that determine the sequestration of heavy metals in vacuoles or cell walls (Rascio
and Navari-Izzo 2011). However, not all metals taken up by plants are accumulated into harvestable parts, or stored internally. Adsorption of metals to roots is equally important for phytostabilization and rhizofiltration.

The terms tolerance and resistance are similar, and often are used interchangeably. It should be noted that resistance is a broader term including both metal exclusion and internal tolerance of metal contaminants (Baker 1987; Zhu et al. 2011). Baker (1987) described exclusion as the restriction of the uptake of metals and tolerance as surviving internal stress of metal contaminants. High tolerance is often associated with larger biomass growth, or at least maintenance of normal growth. Tolerance indices include measurements of root length, root number, and height of new shoots of plants grown in metal treatments, compared to those same measurements of plants grown in control treatments (Punshon and Dickinson 1999). However, Evlard and colleagues (2014) suggested that higher biomass growth is not necessarily a good tolerance strategy by plants, but that reduction in growth rate indicates plants are adjusting to tolerate metals and maintain homeostasis.

Phytoremediation is not only used for heavy metal contaminants. Phytoremediation has demonstrated usefullness in accumulation and tolerance of cyanide (Larsen et al. 2005), uptake of ethanol and benzene (Corseuil and Moreno 2001), uptake of uranium (Dushenkov et al. 1997), stabilization of BTEX (Barac et al. 2009), metabolism of TNT (Schoenmuth and Pestemer 2004), and tolerance and accumulation of common veterinary antibiotics found in fertilizer (Michelini et al. 2012). Schwitzguebel and colleagues (2002), as well as Vangronsveld and colleagues (2009), provide reviews of phytoremediation uses.
Many plant families are utilized for metal phytoremediation purposes. About 500 plant species are known to accumulate toxic inorganic elements, representing about 100 land plant families (Kramer 2010). Brassicaceae is a significant taxon of metal hyperaccumulators (Baker and Brooks 1989). A phytoremediation review also found Salicaceae, including Populus and Salix genera, to be a highly investigated plant family (Tangahu et al. 2011). Salix are widely distributed across North America and the geographic range of abandoned hardrock mines in the United States. It is best practice to use native vegetation since it requires less management and is already acclimated to the area’s climate and seasons (Sarma 2011).

Willows’ characteristics for effective Phytoremediation

In this review, I focused on phytoextraction and tolerance research and results utilizing native willows of North America. Phytoextraction and tolerance are the focus due to the metal contaminants in AMD from abandoned mines. Willows are great phytoremediation agents not only because they accumulate and tolerate metals, but also because they form the dominant vegetation in the upper watersheds at higher elevations, where most of the abandoned mine sites occur across North America.

There are many traits that make willows ideal for phytoextraction. A superior plant for phytoextraction of metal pollutants requires a high translocation rate from roots to shoots (Greger and Landberg 1999). Willows are useful for phytoextraction of metal polluted soils due to their rapid growth, relatively high biomass, (Pulford and Watson 2003; Punshon and Dickinson 1997), and metal translocation ability (Wahsha et al. 2003).
They are also easily propagated due to their rapid development of a deep root system (Vangronsveld et al. 2009). *Salix spp.* (willows) also have broad genetic variability and high transpiration rates (Dietz and Schnoor 2001), grow very quickly, and can tolerate high concentrations of heavy metals (Pulford and Watson 2003; Punshon and Dickinson 1997). Accumulated metals in aboveground biomass are ideal for harvesting and permanent removal of metals. Willows also stabilize ecosystems by tolerating metal contamination with little accumulation, which is still beneficial for the ecosystem and food chain dynamics (Pulford and Watson 2003). While remediating contaminated brownfields or contaminated abandoned mine lands, willows have the added cash benefit of producing woody biomass that can be converted to fuel (Dickinson 2006; Lord et al. 2008; French et al. 2006).

**Finding and Gaps in Literature Research**

To thoroughly review phytoremediation research involving willows native to North America, I searched various combinations of the following terms: willows, *Salix*, phytoremediation, phytoextraction, phytostabilization, rhizofiltration, metals, uptake, accumulation, tolerance, resistance. I coupled these combinations with scientific names of native willow species to North America as well. I used database search “Summons” provided by the University of Denver that searches many databases such as Web of Science and JSTOR, as well as Google Scholar. I limited my search to peer reviewed, published articles that focused on the phytoremediation capabilities of willows native to North America for heavy metal contamination.
With an estimated 450 species worldwide, species in the genus *Salix* are notoriously difficult to distinguish (Percy et al. 2014). Disagreement about the number of species also lends to the difficulty of specific categorization, with worldwide estimates ranging from 350 to 500 (Skvortsov 1999; Rechinger 1992). Lauron-Moreau and colleagues’ (2015) recent phylogenetic analysis of *Salix* spp. is a source used for North American willow species. In this phylogenetic analysis, 122 native and introduced willow species in America were analyzed, using three DNA markers to obtain a biogeographic framework, and yielding two subgenera for American *Salix* spp., *Salix* and *Vetrix*. These agree with Dorn’s (1976; 1977) classification. In my literature search for *Salix* related phytoremediation research, the majority of willow species of focus were native to Europe and Asia. Phytoremediation is best practiced using native flora to provide low maintenance diversity that is already known in the ecosystems of the region. Here I address only those species of *Salix* spp. native to North America, and more specifically of the western and southwestern states where abandoned hardrock mines are most numerous.

I found that 19 of the 99 *Salix* spp. native to North America have been investigated for phytoremediation purposes (Table 1), leaving up to 80 species that could also be useful for phytoremediation. I found that 10 of the 24 papers investigated *S. nigra* (Table 2), which is interesting because it is not in the geographic range of abandoned hardrock mines. Abandoned hardrock mines in the US are mainly in the Rocky Mountain and Madrean floristic regions (Figure 1; Argus 2007). Of the 19 *Salix* spp. native to North America investigated, 14 species were only in one or two studies. Also, there are
74 unique *Salix* spp. and varieties in the abandoned hardrock mine regions of the Mountain West. I found that only 15 of the 74 unique *Salix* spp. and varieties in the abandoned mine regions have been investigated for phytoremediation of metal contamination. Phytoremediation investigations should focus on recommendations of the viability of species for further study or not. This would confirm or rule out the use of species for phytoremediation to save time and money on future investigations or projects. I suggest investigating the phytoremediation abilities of the other 59 *Salix* spp. in the geographic range of abandoned hardrock mines in the United States.

*Metals of Phytoremediation Research*

Overall, I found the investigations focused on appropriate metal contaminants. I found that Cd, Cu, Zn, and Pb were investigated the most often, with 13, 12, 14, and 13 papers respectively, out of 24 total research articles (*Table 2*). The most common metal contaminant in soil is Pb (USEPA 1993). In EPA’s watershed assessments for 11 western US states, Fe, Pb, Cu, Zn, and Cd impaired the most miles of streams (*Table 3*). Iron is a metal contaminant of interest, but is proportionally less investigated as to its level of contamination. However, state specific issues are important to distinguish from one another. Montana’s most damaging metal pollutant is lead, with 74 impairments affecting over 3200 miles of streams. California’s main metal contaminant is Al, with 9 impairments affecting over 3100 miles of streams. In general, resource extraction (i.e. Acid Mine Drainage (AMD), abandoned mine lands (inactive), surface mining, Petroleum/Natural Gas Activities) impairs over 14,000 miles of streams in 8 western states (3 states’ data were unavailable).
Accumulation vs. Tolerance

Accumulation and tolerance were both highly investigated aspects of willow phytoremediation. I found that most research focuses on accumulation of metals, more than 1.5 times more than studies of tolerance (Table 2). Without knowing metal accumulation, metal tolerance can still be useful for phytostabilization purposes. However, without known degrees of tolerance, metal accumulation and extraction cannot be successful because the plant would die and not be able to continue to remediate metal contamination.

Soil vs. Hydroponic Experiments

In willow phytoremediation investigations, I found twice as many focused on contaminated soil than on water or hydroponics. Soil investigations included experiments that performed field sampling or the laboratory use of contaminated tailings or topsoil. A benefit of a soil medium experiment is that metal bioavailability in soil is an issue that only experiments in soil matrices can investigate.

However, hydroponic experiments provide a faster and cheaper screening process, as well as measuring potential for watershed remediation. Hydroponic experiments used for willow screening for metal tolerance correlate with field performance and provided valuable information on accumulation and tolerance (Watson et al. 2003; Huang and Cunningham 1996; Dos Santos et al. 2007; Zhivotovsky et al. 2011). Willows are phreatophytic (deep, water-seeking roots), have demonstrated tolerance and accumulation of metals, and provide large biomasses for higher metal extraction potential. Another
advantage of the use of willows is their ability to thrive in highly wet conditions such as in riparian zones, which increases the bioavailability of metals (McBride 2007).

Length of Experiments

I found that rapid experiments of tolerance or accumulation (four weeks or less) consisted of less than 20% of the 24 research articles. Rapid screening experiments are valuable, cheap, and fast. While longer experiments are obviously valuable to see long-term tolerance effects and metal accumulation, we can utilize shorter and cheaper investigations. Comparisons of shorter duration greenhouse experiments with long-term field studies are extremely valuable on showing any correlations between the two types of experiments. The comparison allows us to gauge the value and reliability of cheaper, faster, and controlled greenhouse studies with field studies. The results from a long-term field studies can be corroborated and extrapolated from short-term hydroponic experiments (McBride 2007; Watson et al. 2003). Comparisons between short- and long-term studies must continue, but I also recommend the use of the cheaper and informative short-term experiments.

Willow Phytoremediation Research Improvements

Microorganisms/Fungi Collaborations

Bioremediation techniques have been used since the 1980s. Microorganisms were used initially for remediation of metals via biosorption, or by reducing metals to lower redox states (Lovley and Coates 1997), using a fungus (Aspergillus niger) to produce acids in a sucrose based substrate to leach out the more bioavailable copper (Mulligan and Galvez-Cloutier 2003), and immobilization of metals from aqueous solutions (Gadd
Plants support diverse communities of microorganisms in the rhizosphere. Phytodegradation is the use of plants and their associated microbes to ameliorate organic contaminants (Salt et al. 1998).

Research into improvements of willow phytoremediation capabilities has been underway for the past two decades, including their associated mycorrhal fungi and bacteria. Understanding the benefits of microorganism and fungal relationships with willows is important. For example, the inoculation of a fungus, *Trichoderma harzianum Rifai 1295-22* or “T22”, increased willows’ biomass by 39% and height by 16% in metal contaminated soil compared to control soil (Adams et al. 2007). Kuffner and colleagues (2010) investigated bacterial associations with *S. caprea* accumulation of Cd and Zn, and found that bacteria, such as *Actinobacteria*, are involved in metal accumulation. It was also found that colonization of dark septate endophytes (DSE), a fungus, increased around roots of *S. caprea* with increasing Pb contamination in soils (Regvar et al. 2010). DSE inoculation also contributes to lower leaf Cd and Zn concentrations while increasing transpiration rates (Likar and Regvar 2013), which is helpful for phytostabilization purposes or the protection of herbivores. Inoculation of microorganisms such as *Streptomyces* sp., *Agromyces* sp., and *C. finlandica* increased the accumulation of Cd and Zn to shoots, most likely by increasing bioavailability of Cd, Zn, and K in polluted soil (De Maria et al. 2011). Mycorrhal treatments, such as *Rhizophagus irregularis*, increased Cu accumulation and shoot biomass (Cloutier-Hurteau et al. 2014). Very recently, willow associated bacteria were isolated and certain strains, such as *Rahnella* sp., increased accumulation by increasing willow twig biomass (Janssen et al. 2015).
Microorganism and fungal relationships with *Salix* spp. native to abandoned mine land regions in the USA are sources of enhancement for phytoremediation using willows.

**Organic Additions**

Another relationship that needs to be investigated further is the optimization of organic additions for metal tolerance or accumulation in willows. Bourret and colleagues (2005) demonstrated that increasing depth of saturation increased Mn bioavailability and accumulation in willows. Also, the additions of EDTA increased accumulation of metal contaminants in above-ground tissues of willows (Zhivotovsky *et al.* 2011; Milan *et al.* 2012). Purdy and Smart (2008) found that phosphate additions in hydroponic experiments decreased the toxicity of arsenic and increased accumulation in aboveground willow tissues. Other additions for soil fertility management, such as nitrogen application for increased biomass, are important in total phytoextraction ventures (Li *et al.* 2003). Exploring organic additions in future investigations should also be a focus for willow phytoremediation research.

**Biofuel and Remediation**

Willows are not only an untapped source for remediation, but also an energy producer with their fast growing and very hardy biomass. With oil and natural gas stores eventually running out or becoming too expensive, diversifying energy sources is a beneficial venture. According to the USEPA, the US electricity generation in 2013 was dominated by coal, natural gas, and nuclear production of 1600, 1100, and 800 Megawatt hours respectively. Hydro, wind, solar, biomass, and other sources together produce only 500 Megawatt hours. Biomass is a carbon neutral alternative: the amount of CO$_2$ emitted
when willow is burned is the same as CO$_2$ captured by the plant during growth (Kuzovkina and Quigley 2005). Willows are hardy, biomass producing plants that require minimal energy input from a management perspective. The simultaneous benefits of using willows as remediation and biofuel sources could be an economical and environmental productive venture.

**Biomining / Phytomining**

An intriguing economic venture for willows in phytoremediation is biomining or phytomining. Low metal content in large areas is not economically viable to extract with conventional method, necessitating cheaper alternatives, such as phytomining (Sheoran *et al.* 2009). Sheoran and colleagues (2009) and Brooks and colleagues (1998) both provide an overview of phytomining by discussing hyperaccumulating plants, their biomass production, and the metal concentrations needed to make the venture worthwhile. *Salix* spp. are not the focus of either of these reviews, even though they can concentrate heavy metals in aboveground biomass while providing large amounts of biomass to fuel the smelting process. Phytomining capabilities of willows need further investigation.

As already discussed, coordination of microorganisms with woody hyperaccumulators has been well researched, but it can also be considered for biomining. Microorganisms often make metals in the rhizosphere more bioavailable, such as by acidifying the soil near roots. The enhanced Cd and Zn phytoextraction in *Salix* spp. via the inoculation of rhizobacteria and fungus is one of many examples of improved metal accumulation using microorganisms (De Maria *et al.* 2011). Bacteria in the rhizosphere acidify the contaminated soil or waste increasing the bioavailability of the metals, which
are then more easily removed or taken up via bacterial sulfate reduction (White et al. 1997; Gadd 2004). The economic benefit of biomining can benefit from the use native vegetation, such as willows, and their rhizospheric microorganisms.

**Willow Genotype Database for North America**

Willows have extremely high variability within species. Therefore, it is important to investigate specific genotypes and genets of *Salix* spp. in addition to *Salix* spp. as a whole. Genets, or genetically unique plants of a species, vary widely in characteristics. For example, two specific genets of *S. purpurea* in an accumulation experiment contained the highest concentrations of metals relative to genets of five other *Salix* spp. tested, while another genet of *S. purpurea* had the lowest concentration of metals in the same experiment (Mleczek et al. 2009). This one example is representative of many willow phytoremediation experiments where high variability and presumed hybridization among *Salix* spp. is prevalent (Dorn 1977; Percy et al. 2014; Karrenberg et al. 2002). A willow database focusing on genotype specific remediation characteristics, such as phytoextraction of Pb or tolerance to Cd, would be valuable for the optimization of phytoremediation. An example of genotype specific research was finding common gene regulation mechanisms to Cr contamination among four *Salix* spp. (Quaggiotti et al. 2007). With a well organized database for specific phytoremediation characteristics, willow genets can be specifically hybridized for phytoremediation of specific metals, time frames, biofuel production, or even biomining purposes.

With the capabilities of GIS mapping, soil and water impairments can be cross referenced with appropriate willows for remediation. EPA documents already detail...
metal impairments for specific watersheds. Along contaminated watersheds, one could propagate metal specific willows for phytoremediation in small, controlled areas. The same practice can be applied to soil on US Forest Service, BLM, or private lands. Specific elemental contaminations in soil are well known. One can create an interactive GIS map of willow species and their associated metal phytoremediation capabilities with soil and water contaminations.

*Dangers to Herbivores in Phytoremediation*

Metal hyperaccumulating willows in the wild are potential dangers to herbivores. But plants accumulating metals as an herbivore deterrent is a leading reason for the accumulation known as the Elemental Defense Hypothesis, elemental allelopathy, and simply as a plant chemical defense (Martens and Boyd 1994; Boyd and Martens 1998). Other natural deterrents exist for willows such as salicin (Markham 1971) and phenol glycoside compounds (Tahvanainen et al. 1985). Herbivore risks can also be minimized further with proper land management is used, such as exclosures or predator urine. Furthermore, it is possible to breed willow clones that have some of these additional herbivore repellent characteristics (Greger and Landberg 1999). Genetically manipulating willows with anti-herbivorous characteristics, such as salicylate-rich leaves or higher phenol glycosides in stems, with willows that have metal accumulation properties is a worthwhile phytoremediation venture. The database would provide the opportunity to choose willows that are endemic with phytoremediation characteristics and have naturally herbivore deterrent characteristics.
Conclusion

With only 19 of 99 Salix spp. native to North America having been investigated for phytoremediation, more studies of investigations into other native willows species are needed. More specifically, willows native to the Rocky Mountain and Madrean floristic regions are the most important to investigate due to their geographic location relative to the nearly 160,000 abandoned hardrock mines. Future phytoremediation investigations of Salix spp. native to North America should focus equally, if not more, on tolerance (resistance to damage) relative to accumulation capabilities of willows. Short term greenhouse experiments, especially hydroponic screening experiments, should be utilized and be continued to be compared to long term field experiments.

Here I investigated the literature on phytoremediation characteristics of metal contamination of willows native to North America. More specifically, I found that there are numerous willow species left to be investigated, especially in the areas of abandoned hardrock mines in the United States. I also found that of the 19 willow species investigated in 24 articles, many are only investigated once or twice. It would be valuable to have more experiments involving many variables including soil, water, metal combinations, pH levels, and duration. These data would aid in the more widespread use and utilization of native plants for cheaper and more sustainable phytoremediation throughout North America.
CHAPTER II: PHYTOREMEDIATION OF CADMIUM AND LEAD-POLLUTED WATERSHEDS

Introduction

Anthropogenic disturbances, including hard rock mining and the resulting outwash, are significant sources of metal pollution in the global environment over the last century (Jung 2001; Wahsha et al. 2012). There are over 500,000 abandoned hardrock mine sites in the U.S., of which 38,500 are on National Forest System lands, polluting watersheds and ecosystems for decades or more (Carr 2005). Acid Mine Drainage (AMD) from the abandoned mines contains toxic concentrations of metals that cause significant environmental damage because the metals cannot be chemically degraded (Salt et al. 1995).

Metal contaminants not only accelerate environmental degradation, but also are detrimental to humans as well as other biota. Dust from dry and loose tailings causes and exacerbates respiratory diseases in human populations near abandoned mine disposal sites (Mendez and Maier 2008). Cadmium and lead are common, biologically non-essential metal pollutants in mine tailings and outwash. Cadmium causes kidney defects and reproductive toxicity (Thompson and Bannigan 2008). Cadmium also causes skeletal damages and is designated a group 2a carcinogen by the International Agency for Research on Cancer (Jarup 2003), and impairs signaling through the blood-brain barrier (Mendez-Armenta 2007). Lead exposure causes renal damage and neurotoxicity (Jarup 2003) as well as high blood pressure (Suruchi and Khanna 2011). Once in the food chain,
lead concentrations in small mammals are the greatest in bone and kidney tissues (Roberts et al. 1978).

Cadmium and lead damage plants as well. The presence of lead causes decrease of growth and reproductive efficiency of plants such as mangroves (Leano and Pang 2010), causes stunting and chlorosis (Pandey et al. 2012), and concentrates mostly in roots and leaves (Feleafel and Mirdad 2012). Cadmium also causes chlorosis and interferes with accumulation and transport of biologically essential elements (Das et al. 1997). In addition, cadmium contributes to cytogenetic damage by inhibiting cell proliferation (Rosas et al. 1984).

The most common approach to mine tailings and waste is piling and containing the waste tailings without chemical or metal removal treatments (Mendez and Maier 2008). Isolating tailings with embankments does prevent the escape and spread of pollutants but does not decrease or remove them (Cunningham and Berti 1993). Common in-situ treatment methods of AMD and such metals as cadmium and lead are merely expensive containments of the problem. There are a wide range of estimates for the cost of hardrock mine remediation in the USA. The Mineral Policy Center estimated the remediation of metal contaminated waste from abandoned hardrock mines will cost the U.S. in total between $32 and $72 billion (Lyon et al. 1993). The US Government Accountability Office found that the US EPA spent $2.2 billion on abandoned hardrock mine land remediation between 1997 and 2008 (Nazzaro 2008). BLM estimates that abandoned hardrock environmental remediation costs are over $400 million for the 22,104 abandoned mine lands in the US (BLM 2013).
Phytoremediation is a cheap alternative to complement conventional methods. Phytoremediation is a broad term for using plants for cleanup of environmental metal pollution through phytoextraction, rhizofiltration, or phytostabilization (Salt et al. 1995). Phytoextraction is the ability of plants to accumulate metal contaminants in their aboveground biomass, for harvesting and contaminant removal (Salt et al. 1998). For example, phytoextraction for one contaminated acre is 16%-20% of the cost of traditional techniques such as removal and appropriately storing contaminated soil (Salt et al. 1995).

Best practices of phytoremediation, such as phytoextraction, use regionally appropriate plants. Many plant families have been found to be good candidates for phytoremediation, including Brassicaceae (mustards), Poaceae (grasses), and Salicaceae (poplars and willows) (Salt et al. 1995). Here I focus on the phytoextraction ability of native montane willows (Salix spp.) of the Central Rockies in Colorado, near abandoned mine lands at elevations above 2,400 meters. Not only are willows the dominant riparian vegetation at these elevations, but willows are also easy to propagate and establish in the field with very deep and extensive root systems (Vangronsveld et al. 2009). Willows are known phytoremediation agents; they accumulate and concentrate metals, grow rapidly with a relatively high biomass (Pulford and Watson 2003; Punshon and Dickinson 1997), and translocate metals from roots to aboveground biomass (Wahsha et al. 2012).

Abandoned mine land remediation is important due to cadmium and lead presence in mine outwash, especially in Colorado where mining activities have been active since the mid-1800s. One third of EPA’s Region 8 (6 states: MT, WY, ND, SD, CO, and UT) superfund sites are in Colorado as of 2014. 2,100 km of streams in Colorado are significantly polluted with AMD and many metals such as cadmium and lead. 89% of
Total Maximum Daily Load (TMDL) of Colorado water impairments are due to many of the 23,000 abandoned mine features according to the Colorado Department of Public Health and Environment (CDPHE 2012). According to the most recent USEPA watershed assessment for Colorado (2010), nearly 1,300 and 300 km of streams are impaired by cadmium and lead, respectively. As of 2011, 938 million gallons of water per year are treated near abandoned mining sites in the Colorado, which is effective but expensive (CDPHE 2012).

Here I investigated the phytoremediation abilities of three common willows: _S. drummondiana, S. monticola_, and _S. planifolia_. These willows are found along EPA impaired and BLM high priority watersheds near abandoned mine lands above elevations of 2,400 meters in Colorado. Mine is the first study to directly compare these three dominant willow species’ phytoremediation characteristics for cadmium and lead contamination. I tested the phytoremediation characteristics of these willows via hydroponic experiments, which are useful for screening willows’ tolerance and accumulation characteristics (Watson _et al._ 2003; Huang and Cunningham 1996; Dos Santos _et al._ 2007; Zhivotovsky _et al._ 2011).

I tested the accumulation and tolerance of cadmium and lead for suitability in phytoremediation of cadmium and lead contaminated watersheds of three Colorado native willow species. With increased information on the three willow species’ phytoremediation characteristics of cadmium and lead, more efficient and diverse remediation practices can be used through propagation of willows along the 1,600 km of cadmium and lead polluted streams across Colorado.

Here, I propose two hypotheses:
1) Three common native willow species differ in cadmium and lead concentrations in stem and leaf metal content after exposure to environmental concentrations of cadmium and lead.

2) Three common native willow species differ in tolerance to environmental concentrations of cadmium and lead.

Within these two hypotheses, I investigated four statistical questions:

1) Do three common native willow species differ in stem and leaf accumulation concentrations after growing in cadmium and lead treatments?

2) Do metal concentration levels of cadmium and lead affect stem accumulation concentrations for each willow species differently?

3) Do three common native willow species differ in their relative tolerance (growth) to cadmium and lead treatments?

4) Do the three common native willow species differ in total and field stem concentrations, represented by cuttings in control treatment, of cadmium and lead?

Methods

Collections

I sampled 160 individual willow genets (unique individual willow shrubs, not connected by roots) in November 2013, and from April to June, 2014, at elevations above 2,400 meters throughout five counties in Colorado: San Juan, Ouray, Lake, Clear Creek, and Summit. These areas were near abandoned mine lands and designated by the BLM as high priority watersheds and by the USEPA as impaired watersheds (Figure 2). Of the
160 individual willow collections, I identified 8 unique willow species. I collected for diversity and found the three most common willows to be *Salix monticola*, *S. planifolia*, and *S. drummondiana*.

Once I identified the three most common willow species, I collected 12 cuttings (20-40 cm in length) from 69 genets in August and October of 2014 for the greenhouse experiment. I collected cuttings from 32 genets of *S. monticola*, 19 genets of *S. drummondiana*, and 18 genets of *S. planifolia*. Individual genets (shrub masses) of same species were at least 20 meters apart, to ensure genetic diversity. Genets were tagged in the field with sequential numbers on aluminum tags. Furthermore, previous studies of willow phytoremediation focused on plants from a limited geographic or genetic range. I selected plants from 10 sites from both eastern and western slopes of the Rocky Mountains with at least 18 unique genets of each species.

*Hydroponic Greenhouse Experiment*

I conducted a four week accumulation and tolerance greenhouse experiment with the three willow species of interest: *S. drummondiana*, *S. planifolia*, and *S. monticola*. I brought cuttings to the greenhouse at the University of Denver immediately after field collections, and placed in bunches of 12 cuttings in each deepot cone (6.4 cm by 36 cm) in every other slot in 20 slot support trays (Stuewe & Sons, Corvallis, OR). I used cupcake papers to prevent cuttings from slipping out of the cones. I submerged each cone in each slot tray in 17 cm of deionized (DI) water supplemented with 132 mL FloraGro (2-1-6 NPK ratio) nutrient solution per 100 L of water. University of Denver’s Olin Hall
The greenhouse has natural, south facing light. I completely replaced the water every other week and supplemented with FloraGro fertilizer once a week.

I grew cuttings for 6 weeks for root development. I discarded cuttings that did not show signs of root or leaf development. For living cuttings, I assigned a root score (0 – 4) at the beginning and end of experiment (Figure S2). For each genet, I chose five living cuttings giving them a letter (a, b, c, d, and e) and then assigned them randomly to each treatment. I successfully rooted 405 cuttings of 828 collected from the 69 willow genets.

I conducted the experiment with five hydroponic treatments: control (DI water; FloraGro), low cadmium (11 ppb or ug/L; 0.10 uM), high cadmium (300 ppb; 2.56 uM), low lead (15 ppb; 0.07 uM), and high lead (145 ppb; 0.70 uM). Hydroponic experiments that use unrealistically high metal concentrations can lead to ‘forced’ metal accumulation and their results have no biological relevance (Van der Ent et al. 2013). Here, I used relatively lower (~ 1 uM) concentrations of metals, representing more environmentally and biologically relevant levels (Table 1). I added metals via a stock solution of 7.6 mM of cadmium chloride (CdCl₂) and 2.2 mM of lead chloride (PbCl₂) weekly to obtain the treatment concentrations. I placed one cutting from three unique genets of each of the three species in a 26.5 L Sterilite storage bin (“block”), for a total of 9 cuttings in each block. I established a randomized block factorial design, randomizing the location of each cutting. Out of the 69 willow genets, there were eight genets that had 10 or more successfully growing cuttings that were included as replicates for genets in treatments and were nested for the genet. I filled remaining blocks with these replicate cuttings. I replicated each treatment block nine times for a total of 45 blocks and a total of 405 cuttings.
I conducted the experiment for four weeks from December 2014 to January of 2015, with diurnal temperatures ranging from 16°C to 23°C. Growing lights were on for 12 hours per day, from 6 am to 6 pm. I randomly organized blocks on the greenhouse benches, and moved all blocks each week to randomize lighting conditions in the greenhouse and minor irregularities of depth profiles in the blocks.

I counted leaves weekly, survival at week 3 and 4, and the biomass of each cutting before and after the experiment for the percent change. Survival was the presence of healthy roots or leaves (Figure S3). I only counted fully developed, healthy leaves. Leaves that were shriveling or showing chlorosis were considered dead and were not counted. At the beginning and end of the experiment, I recorded biomasses of the entire cutting to the nearest 0.1 gram.

Preparation of Stem and Leaf Samples for ICP-MS Analysis

At the conclusion of the greenhouse experiment, I dried the cuttings in paper bags in an oven at 70°C for 72 hours. I finely ground the leaves and stems of the cuttings separately using a bead beater (BioSpec Mini-Beadbeater-16) courtesy of Denver Botanic Gardens. In the atmospheric particulate matter lab at the University of Denver, I prepared the samples for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) by digesting samples with acids. For each leaf and stem sample, I massed out samples between 5 and 40 mg and then added 750 uL nitric acid (HNO₃), 250 uL hydrochloric acid (HCl), 100 uL hydrofluoric acid (HF), and added 100 uL hydrogen peroxide (H₂O₂) dropwise, due to reaction volatility. I then combined 1 mL H₂O₂ with 10 mL of ultrapure H₂O (18.0 M ohm-cm) in each digestion chamber (total of 10 for each round) before heating to 210°C.
in the acid digestion for 1 hour and 25 minutes. After acid digestion, I diluted samples to 15 mL by adding the digested sample to 13.8 mL with ultrapure H$_2$O. I measured the validity of the prepared samples via ICP-MS with soil and spinach SRMs as well as reference metal standards for cadmium and lead at low, medium, and high concentrations: 10, 50, and 100 parts per billion (ppb or ug/L) respectively. I found excellent recovery of my references.

**Analysis**

I analyzed my data using a mixed model with JMP (Version 11.0). I analyzed the biomass percentage change and metal accumulation in stems and leaves using the mixed model. Fixed effects comprised metal type, metal level, and willow species with a full factorial of all three interactions and the willow genet was a random effect. I used the Tukey HSD for post hoc analysis of significant differences. For weekly leaf counts, I used a MANOVA with repeated measures of each week using the Roy’s Max Root test statistic. I calculated values for metal concentration accumulation in stems and leaves, as well as biomass percent change relative to the cuttings in control treatments. For example, biomass percent change was calculated as differences between treatment cuttings’ biomass percent change and their respective control treatment cutting’s biomass percent change (cutting from same genet in different treatment). A negative value means the cutting of a genet grown in a metal treatment had less biomass growth than a cutting from the same genet in the control treatment. This removes the beginning concentrations’ effect on results if, for example, the willow genet was originally growing in a highly cadmium contaminated area. I performed the same calculations relative to control for
metal accumulation concentrations in stems and leaves. The concentration of each metal in a stem or leaf in a control treatment was subtracted from the concentration in another cutting from of the same genet in a metal treatment. It is important to note that I excluded four outlier data points from my results for the impossible accumulation and biomass changes they represented.

**Results**

I found three significant differences testing the accumulation and tolerance of cadmium and lead for the three willow species (Table 5). I found a significant interaction with willow species and metal type for stem accumulation concentration. I found a significant difference among species for leaf accumulation concentration. I also found a significant difference for biomass change for the interaction between Willow Species, Metal Type, and Metal Level.

1) Do three common native willow species differ in stem and leaf accumulation concentrations after growing in cadmium and lead treatments?

I found that *S. drummondiana* accumulated more cadmium in stems than did both *S. monticola* and *S. planifolia* (Willow Species * Metal Type: F=6.43, df=2, p=0.002; Table 5; Figure 3a). I found no differences in lead accumulation in stems between the treatments (Figure 3a).

Similar to stem accumulation results, I found that *S. drummondiana* accumulated more cadmium in leaves than did *S. planifolia* (Willow Species: F=4.07, df=2, p=0.03; Table 5; Figure 3b). *Salix drummondiana* was the only species to have a greater accumulation of cadmium in leaves than control in the pooled metal treatments. I found
no differences in lead accumulation concentration in leaves with all three species accumulating small amounts.

2) Do metal concentration levels of cadmium and lead affect stem accumulation concentrations for each willow species differently?
I found that neither the environmental cadmium nor lead concentration levels in the water affected the accumulation concentration of these metals in stems for any of the species (Figure 4a; Figure 4b; Table 5).

3) Do three common native willow species differ in their relative tolerance (growth) to cadmium and lead treatments?
I found that leaf counts as a measure of tolerance to treatments decreased but differed between species over time. (Manova Repeated Measures: Roy’s Max Root= Time*Willow Species*Treatment: F=3.52, df=8,386, p=0.0006; Figure 5).

I also found a significant interaction for biomass percentage growth (Willow Species * Metal Type * Metal Level: F=4.31, df=2, p=0.01; Table 5; Figure 6). *monticola demonstrated greater tolerance with a 2% increase in biomass over the four week greenhouse experiment for cuttings in the Lead Low treatment, compared to the over 5% biomass loss of cuttings in the Lead High treatment. I found that the three species reacted similarly to the pooled metal treatments of cadmium and lead with an overall decrease in biomass growth relative to controls (Figure S4).

4) Do the three common native willow species differ in total and field stem concentrations, represented by cuttings in control treatment, of cadmium and lead?
I found differences in total stem accumulation concentration for stems in metal treatments (Willow Species*Metal Type: F=29.22, df=2, p<0.0001; Figure 7a). Total stem accumulation is the metal accumulated during the experiment in addition to the metal concentration already present in stems before the experiment. I found that all three species accumulated more and contained higher concentrations of cadmium in stems than each did for lead. I also found differences in field stem concentrations, represented by cuttings in control treatments, of cadmium and lead (Willow Species*Metal Type: F=5.93, df=2, p=0.004; Figure 7b). *S. planifolia* contained the highest concentration of cadmium in stems, twice as much as cuttings from *S. drummondiana*. *Salix monticola* contained the highest lead stem concentration, nearly two times that of cuttings of *S. drummondiana* and three times that of cuttings of *S. planifolia*.

**Discussion**

Based on my field sampling and experimental results, *S. drummondiana* and *S. planifolia* should be equally well investigated for phytoremediation in addition to *S. exigua*, *S. monticola*, and *S. geyeriana* in Colorado, especially for cadmium and lead contamination. Using diverse and native plant material is essential for optimal phytoremediation, and these species provide diverse options for phytoremediation through their metal tolerance and aboveground biomass metal accumulation for permanent removal of cadmium and lead. Overall, all three species contained higher concentrations of cadmium than lead, ranging from 2.5 to 19 times higher concentrations of cadmium than lead in stems based on existing field concentrations. my four week accumulation and plant tolerance experiment demonstrated *S. drummondiana* to be a
better accumulator of cadmium than both *S. monticola* and *S. planifolia* shown by stem and leaf concentrations relative to control treatments. *S. planifolia* is also highly recommended for cadmium remediation based on the fact it contained 2.5 times higher stem field concentrations of cadmium than *S. drummondiana*. All three willow species accumulated low lead concentrations in leaves during the experiment. *Salix monticola* is the best candidate of the three species for lead remediation. *Salix monticola* contained nearly 6 times higher stem field concentrations of lead than *S. planifolia*. I also found that *S. monticola* demonstrated greater tolerance in the lower lead concentration by increasing 2% biomass compared to the higher lead treatment where it lost 5% biomass.

Willows are capable of metal tolerance by increasing biomass and growth over time. However, in this experiment, most cuttings from all three species lost biomass and leaves over the period of the experiment in all treatments. One possible source of biomass loss across the board could be the cupcake papers that were wrapped around roots of the cuttings in the first five days of the experiment. The papers may have caused cavitations in the cuttings disrupting growth. The papers were removed immediately once the wrapping at the base of cutting around roots was noticed. Biomass loss is not typical of willows in metal accumulation and tolerance experiments. For example, *S. drummondiana* increased in biomass over a two month experiment on amended tailings (Meiman *et al.* 2012) and *S. monticola* had an 87% survival rate after 4 years of growth on amended mine tailings consisting of both cadmium and lead, at concentrations higher than my experiment. However, it is also interesting to note that increase in biomass may not be considered appropriate metal tolerance (Evlard *et al.* 2014). I also found that *S. monticola* and *S. planifolia* demonstrated a decrease of metal concentrations over the
course of the experiment relative to their control cuttings (Figure 3; Figure 4). This could have been due to plants translocating the metals to leaves to be senesced or to dying stem or roots parts.

I tested the broad differences in metal accumulation and tolerance between these native willow species with high genetic and geographic diversity. I addressed these three common montane willow species at a significant scale by using 18 to 32 unique genets of each species and by collecting from ten sites throughout five counties in Colorado. This provides overarching trends of each species, rather than results of specific genets or ramets from one location representing the entire species.

Other longer-duration experiments demonstrated higher accumulation abilities of these willow species for Pb and Cd. For example, Bourret and colleagues (2009) demonstrated S. monticola leaves of staked cuttings concentrated nearly 13 times higher concentrations of lead after two years of growth on amended mine tailing deposits than my findings of total lead content in leaves. This supports that the lead accumulation continues with duration of metal exposure. Meiman and colleagues (2012) reported S. drummondiana concentrated twice the total Cd concentration in its aboveground biomass in 2 months than I found in stems of S. drummondiana. As for S. monticola, Bourret and colleagues (2009) found nearly 9 times higher concentration of Cd in leaves pre-rooted cuttings after two years, and Boyter and colleagues (2008) found 11.5 times and 10 times higher concentrations of Cd after grown in amended tailings for 4 months in live and senesced leaves, respectively, compared to my total leaf concentrations. In the same experiment, Boyter and colleagues (2008) also found 17 times higher concentrations of
Cd in bark compared to total Cd concentrations in stems grown in cadmium treatments in my experiment.

**Future Directions**

A genet database containing information to native ranges, metal hyperaccumulation abilities and herbivore deterrent characteristics would be an essential next step for optimizing phytoremediation. Further research into manipulating and hybridizing hyperaccumulating willow genets with herbivore-deterrent willow genets would be a worthwhile research endeavor. Collaborative phytoremediation techniques should also be included in the genet database, such as the interaction with microorganisms and organic additions. Also senesced leaves are of interest with nearly 16 and 46 times more lead accumulated and concentrated in senesced leaves than living leaves demonstrated by Boyter and colleagues (2008). Lastly, willows are fast-growing, metal hyperaccumulating woody plants already commonly used in short rotation coppice biomass production making them ideal candidates for the economic benefit of biomining. With all future research endeavors, the investment in researching mitigation restoration for reducing heavy metal contamination to pre-mining levels remains an important environmental priority.
REFERENCES


Landberg, T, and M Greger. 1994. “Can Heavy Metal Tolerant Clones of Salix Be Used as Vegetation Filters on Heavy Metal Contaminated Land?” In Willow Vegetation Filters for Municipal Wastewaters and Sludges: A Biological Purification System, 133–144.


Zinc Water-Pollution.” *Experientia* 38: 683–685.


Takhtajan, Armen. 1986."Floristic regions of the world." *Berkeley, etc.: (Transl. by TJ Crovello.) Univ. Calif. Press* 581

Tangahu, Bieby Voijant, Siti Rozaimah Sheikh Abdullah, Hassan Basri, Mushrifah Idris, Nurina Anuar, and Muhammad Mukhlisin. 2011. “A Review on Heavy Metals (As, Pb, and Hg) Uptake by Plants through Phytoremediation.” *International Journal of Chemical Engineering*


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(a) Stem concentrations of cadmium in two levels of cadmium treatments for three willow species: *S. drummondiana*, *S. monticola*, and *S. planifolia*. The stem concentrations are the differences between cuttings in low and high cadmium treatments with cuttings of the same genet in control (no metal) treatment. Mixed Model: Willow Species (F=4.03, df=2, p=0.02). Post-hoc Tukey HSD, willow species not connected by same letter are significantly different. One error bar equals one standard error from the mean. 

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b Stem concentrations of cadmium and lead cuttings in the control treatment for three willow species: S. drummondiana, S. monticola, and S. planifolia. Metal concentrations in cuttings in control treatment represent field concentrations. Mixed Model using log transformed metal concentration values: Willow Species*Metal Type (F=7.51, df=2, p=0.001), Metal (F=131.12, df=1, p<0.0001), Willow Species*Metal Type (F=5.93, df=2, p=0.004). Post-hoc Tukey HSD, levels not connected by same letter are significantly different. One error bar equals one standard error from the mean.
Table 1. Willow species (willow) native to North America that have been investigated for phytoremediation characteristics. Floristic Provinces and Sub-provinces are adapted: Takhtajan 1986; Thorne 1987. Floristic Provinces: 4a. Vancouverian Province, 4b. Rocky Mountain Province, 9a. Great Basin Province, 9b. Californian Province, 9c.1. Mojavean sub-province, 9c.2. Sonoran sub-province. These floristic provinces represent the area of abandoned hardrock mines in the USA covering 11 western states.

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<td>X</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td>S. interior</td>
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<td>X</td>
<td>X</td>
</tr>
<tr>
<td>S. lucida</td>
<td>3</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>S. monticola</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
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<td>10</td>
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<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>S. phyllicola</td>
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<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>#</td>
<td>Citation</td>
<td>Salix spp.</td>
<td>Metal(s)</td>
<td>Study Focus</td>
<td>Time</td>
</tr>
<tr>
<td>----</td>
<td>----------------------------</td>
<td>------------</td>
<td>----------</td>
<td>--------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>1</td>
<td>Borsje et al. 2008</td>
<td>S. nigra, S. alba, S. matsuana</td>
<td>Cd, Ni, Pb</td>
<td>Accumulation</td>
<td>70 days = Cd and Ni, 95 days= Pb (due to chlorosis and necrosis in Cd and Ni plants)</td>
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<tr>
<td>2</td>
<td>Bourret et al. 2009</td>
<td>S. monticola, S. geyeriana</td>
<td>Cd, Mn, Pb, Zn</td>
<td>Accumulation and tolerance (growth) in mine tailings</td>
<td>4 growing seasons</td>
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<tr>
<td>3</td>
<td>Bourret et al. 2005</td>
<td>S. monticola, S. geyeriana</td>
<td>Mn, Cd, Cu, Pb, Zn</td>
<td>Tolerance (Survival, growth), accumulation compared to water table</td>
<td>20 weeks</td>
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<td>4</td>
<td>Boyer et al. 2008</td>
<td>S. monticola, S. geyeriana</td>
<td>Cd, Mn, Pb, Cd</td>
<td>Tolerance and Accumulation</td>
<td>4 months</td>
</tr>
<tr>
<td>5</td>
<td>Brieger et al. 1992</td>
<td>S. amygdaloideus, S. interior</td>
<td>Se, Cr, Zn, Co, Sb, Cd, As</td>
<td>Accumulation in leaf, stem, and fruit</td>
<td>N/A</td>
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<tr>
<td>6</td>
<td>Eriksson et al. 1990</td>
<td>S. glauca, S. phylicifolia</td>
<td>Cu, Zn, Pb, Cd</td>
<td>Sequestration, accumulation in leaves and buds for herbivores’ diet</td>
<td>N/A</td>
</tr>
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<td>7</td>
<td>Fisher et al. 2000</td>
<td>S. geyeriana</td>
<td>N/A</td>
<td>Growth and tolerance of mine tailing amendments</td>
<td>4 months</td>
</tr>
<tr>
<td>#</td>
<td>Citation</td>
<td>Salix spp.</td>
<td>Metal(s)</td>
<td>Study Focus</td>
<td>Time</td>
</tr>
<tr>
<td>----</td>
<td>----------------------------------</td>
<td>-------------------------------------</td>
<td>----------------</td>
<td>-----------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>8</td>
<td>Jóźwik 2000</td>
<td>S. polaris, S. reticulata</td>
<td>Cu, Mn, Zn, Pb, Cd</td>
<td>Sequestration, accumulation</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>Kuzovkina et al. 2004</td>
<td>S. nigra, S. discolor, S. eriocephala, S. exigua, S. lucida</td>
<td>Cu, Cd</td>
<td>tolerance uptake, translocation, accumulation</td>
<td>2 months</td>
</tr>
<tr>
<td>10</td>
<td>Kuzovkina et al. 2010</td>
<td>S. smithiana, S. eriocephala, S. lucida, S. nigra, S. purpurea, S. sachalinensis, S. miyabeana, S. s. dasyclados</td>
<td>Pb</td>
<td>accumulation and tolerance</td>
<td>90 days</td>
</tr>
<tr>
<td>11</td>
<td>McBride 2007</td>
<td>S. exigua, S. eriocephala, S. viminalis, S. ampyralotes, S. discolor, S. purpurea, S. dasyclados, S. nigra, S. alba</td>
<td>Cu, Mn, Zn, Cd</td>
<td>accumulation</td>
<td>1 month</td>
</tr>
<tr>
<td>12</td>
<td>Meiman et al. 2012</td>
<td>S. geyeriana, S. drummondiana, S. planifolia, S. bebbiana</td>
<td>Gd, Cu, Pb, Zn</td>
<td>Accumulation in shoots and roots and tolerance (growth)</td>
<td>61 days</td>
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<tr>
<td>13</td>
<td>Mielecky et al. 2010</td>
<td>S. purpurea, S. alba, S. fragilis, S. petiolaris, S. nigra, S. japonica</td>
<td>Gd, Co, Cr, Cu, Ni, Pb, Zn</td>
<td>Ranking of 12 Salix genotypes in hydroponic experiment of accumulation</td>
<td>30 days</td>
</tr>
<tr>
<td>14</td>
<td>Mielecky et al. 2009</td>
<td>S. alba, S. petiolaris, S. purpurea, S. fragilis, S. japonica, S. nigra</td>
<td>Gd, Co, Cr, Cu, Ni, Pb, Zn</td>
<td>accumulation based on genotypes</td>
<td>N/A (collected from field, ratios)</td>
</tr>
<tr>
<td>15</td>
<td>Puckett et al. 2012</td>
<td>S. viminalis x miyabeana, S. eriocephala</td>
<td>As</td>
<td>As accumulation and tolerance in sensitive/tolerant Salix clones with or w/o phosphate</td>
<td>7 and 3 weeks</td>
</tr>
</tbody>
</table>
### Table 2 cont’d 24 peer-reviewed, research articles investigating phytoremediation characteristics of *Salix* spp. native to North America.

<table>
<thead>
<tr>
<th>#</th>
<th>Citation</th>
<th><em>Salix</em> spp.</th>
<th>Metal(s)</th>
<th>Study Focus</th>
<th>Time</th>
<th>Media</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Pullford et al. 2002</td>
<td><em>S. viminalis, S. triandra x viminalis, S. burjatica, S. spathuliflora, S. aquatica, S. candida, S. eriocarpa, S. dasyclados, and other hybrids</em></td>
<td>Cd, Zn, Ni, Cu</td>
<td>metal accumulation</td>
<td>1 - 2 years</td>
<td>contaminated sewage sludge soil</td>
<td>11/20 willow clones demonstrated phytoremediation uses (good survival and biomass production with high metal uptake). This group accumulated Cd and Zn in the wood, but low Ni and Cu in bark, with a good survival rate and biomass production. S. candida was best Ni accumulator, S. aurita x cinerea x viminalis was best accumulator of Zn and Cd, and S. burjatica was best accumulator of Cu.</td>
</tr>
<tr>
<td>17</td>
<td>Punshon et al. 2003</td>
<td><em>S. nigra</em></td>
<td>Ni, Cr, Cu, Zn, Pb, U</td>
<td>geochemical signatures in annual rings due to past contaminations</td>
<td>N/A</td>
<td>tree cores in South Carolina</td>
<td>Metal contents in willows at contaminated sites were orders of magnitude higher than references site’s. Ni was enriched in cores in 1991 corresponding with spillway breaches in 1991 of Ni. Dendro-chrono analysis is limited due to other physiological processes that influence trees’ growth.</td>
</tr>
<tr>
<td>18</td>
<td>Punshon et al. 2005</td>
<td><em>S. nigra</em></td>
<td>Mn, Ni, Fe</td>
<td>Root accumulation with x-ray</td>
<td>N/A</td>
<td></td>
<td>U, Ni, Mn, and Fe concentrations were analyzed in roots of <em>S. nigra</em> on radiological settling pond. Uranium was found to be located outside of epidermis in roots, and Nickel inside the cortex. Ni-rich substance contained in xylem vessel in root. Mn concentrated evenly in parenchyma tissue of stelar cortex. Fe was in the outer cortical region.</td>
</tr>
<tr>
<td>19</td>
<td>Purdy and Smart 2008</td>
<td><em>S. purpurea, S. viminalis x miyabeanana, S. sachalinensis x miyabeanana, S. eriocarpa</em></td>
<td>As</td>
<td>Tolerance and Accumulation</td>
<td>3 weeks</td>
<td>hydroponic: 100, 250-phosphate, 250+phosphate uM Arsenate</td>
<td>Phosphate presence increased aboveground accumulation of As, alleviated toxic effects on growth/biomass.</td>
</tr>
<tr>
<td>20</td>
<td>Shanahan et al. 2007</td>
<td><em>S. geyeriana, S. monticola</em></td>
<td>Mn, Zn</td>
<td>metal toxicity thresholds, tolerance</td>
<td>50 days</td>
<td>6 hydroponic levels of Mn and Zn: 50-16,000 mg/L Mn and Zn 100-1000 mg/L</td>
<td>Geyer and mountain willow have good resistance to Mn and good for phytostabilization of Mn contamination. Willows showed moderate resistance to Zn contamination. Overall, Geyer willow showed greater resistance to both Mn and Zn than did Mountain willow.</td>
</tr>
<tr>
<td>21</td>
<td>Watson et al. 2003</td>
<td><em>S. viminalis, S. triandra, S. burjatica, S. dasyclados, S. candida, and S. spathuliflora</em></td>
<td>Ni, Cu, Zn</td>
<td>comparison of greenhouse hydroponic experiment to field metal tolerance and Ni and Cu wood accumulation</td>
<td>6 weeks</td>
<td>hydroponic with Hoagland’s solution compared with field study</td>
<td>S. burjatica, S. dasyclados, S. candida and S. spathuliflora were more resistant to elevated metal concentrations than S. triandra and S. viminalis. The more resistant clones produced more biomass in greenhouse and field experiments and accumulated higher metal concentrations in wood.</td>
</tr>
<tr>
<td>22</td>
<td>Zalesny and Bauer 2007</td>
<td><em>S. purpurea, S. eriocarpa, s. sachalinensis</em></td>
<td>Zn, Mn, Fe, Cu, Al</td>
<td>uptake and sequestration, accumulation</td>
<td>18 weeks</td>
<td>soil-filled containers</td>
<td>Salix accumulated more Zn, Fe, and Al than Populus. Populus accumulated more Mn in leaves and stems, while Salix accumulated more in roots. Genotypes for species varied.</td>
</tr>
</tbody>
</table>
Table 2 cont’d 24 peer-reviewed, research articles investigating phytoremediation characteristics of *Salix* spp. native to North America.

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Zhivotovsky et al. 2011</td>
<td><em>S. purpurea, S. eriophala, S. nigra, S. dasyphylos, S. miyabean</em></td>
<td>Pb</td>
<td>accumulation, tolerance, and translocation comparison between pot and field</td>
<td>6 months (greenhouse pot experiments) and 4.5 months in field</td>
</tr>
<tr>
<td>24</td>
<td>Zhivotovsky et al. 2011</td>
<td><em>S. lucida, S. serissima, S. nigra, S. sachalinensis, S. miyabean</em></td>
<td>Pb</td>
<td>toxicity threshold tolerance and accumulation</td>
<td>2 weeks</td>
</tr>
</tbody>
</table>
Table 3: Impaired Rivers and Streams in miles by western states

(a) miles of impaired streams by metals other than mercury (includes the non-metal selenium and metals overlap in impaired miles), (b) number of impairments by metals other than mercury for all water bodies, and (c) miles impaired by resource extraction: Acid Mine Drainage (AMD), inactive abandoned mine lands, and subsurface hardrock mining (Oregon, Utah, and Washington had 0 miles). Source: USEPA Watershed Assessment, Tracking & Environmental Results. Data reported by states to EPA under Section 305(b) and 303 (d) of the Clean Water Act. Blank boxes represent no data report and can be considered values of 0.

<table>
<thead>
<tr>
<th>a</th>
<th>Impaired Rivers and Streams from Metals other than Mercury (miles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>State (Data Year)</td>
<td>TOTAL</td>
</tr>
<tr>
<td>Arizona (2010)</td>
<td>540</td>
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<tr>
<td>California (2010)</td>
<td>5,640</td>
</tr>
<tr>
<td>Colorado (2010)</td>
<td>9,642</td>
</tr>
<tr>
<td>Idaho (2012)</td>
<td>545</td>
</tr>
<tr>
<td>Montana (2014)</td>
<td>5,496</td>
</tr>
<tr>
<td>Nevada (2012)</td>
<td>1,149</td>
</tr>
<tr>
<td>New Mexico (2014)</td>
<td>471</td>
</tr>
<tr>
<td>Oregon (2006)</td>
<td>2,453</td>
</tr>
<tr>
<td>Utah (2010)</td>
<td>448</td>
</tr>
<tr>
<td>Washington (2008)</td>
<td>23</td>
</tr>
<tr>
<td>Wyoming (2012)</td>
<td>440</td>
</tr>
</tbody>
</table>

(b) Number of Water Impairments from Metals other than Mercury

| State (Data Year) | TOTAL | Al | As | Be | Cd | Cr | Cu | Fe | Pb | Mn | Mo | Ni | Ag | Tl | Zn |
| Arizona (2010) | 24 | 2 | 1 | 1 | 16 | 2 | 2 |
| California (2010) | 209 | 9 | 9 | 13 | 1 | 62 | 2 | 23 | 1 | 9 | 1 | 1 | 26 |
| Colorado (2010) | 111 | 1 | 7 | 28 | 24 | 20 | 7 | 4 | 20 |
| Idaho (2012) | 95 | 5 | 26 | 5 | 30 | 29 |
| Montana (2014) | 331 | 15 | 49 | 38 | 9 | 55 | 52 | 74 | 3 | 6 | 2 | 28 |
| Nevada (2012) | 84 | 9 | 4 | 4 | 47 | 7 | 6 | 7 |
| New Mexico (2014) | 60 | 51 | 1 | 1 | 5 | 1 |
| Oregon (2006) | 155 | 20 | 12 | 3 | 2 | 13 | 55 | 8 | 30 | 1 | 5 | 6 |
| Utah (2010) | 5 | 4 | 1 |
| Washington (2008) | 68 | 5 | 6 | 3 | 21 | 18 | 3 | 12 |
| Wyoming (2012) | 6 | 2 | 4 |

(c) Rivers / Streams Impacted by Resource Extraction (miles)

| State (Data Year) | Total Resource Extraction | AMD | Abandoned Mine Lands (inactive) | Subsurface (Hardrock) Mining |
| Arizona (2010) | 591 | 555 | 501 |
| California (2010) | 10,354 | 18 | 13 | 501 |
| Colorado (2010) | 564 | 564 | 7,458 |
| Idaho (2012) | 327 | 31 | 267 |
| Montana (2014) | 2,945 | 697 | 2079 |
| Nevada (2012) | 336 | 4 | 195 |
| New Mexico (2014) | 80 | 8 | 23 |
| Wyoming (2012) | 17 | 7 | 10 |
**Table 4** Total cadmium and lead concentrations, in parts per billion (ppb = ug/L) with ICP-MS uncertainties for water samples (Outwash Embankments, Outwash and Drainage, and Control Samples from background water) from 5 counties in Colorado: Clear Creek, Summit, Lake, San Juan, and Ouray County. EPA fresh water aquatic life Continuous Concentration Criterion (CCC) standard and drinking water maximum contaminant level goal (MCLG) are for reference.

<table>
<thead>
<tr>
<th>Water Sample Type</th>
<th>#</th>
<th>Cadmium (ppb)</th>
<th>Uncertainty +/-</th>
<th>Lead (ppb)</th>
<th>Uncertainty +/-</th>
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</thead>
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<td>Embankment</td>
<td>2</td>
<td>13,970</td>
<td>230</td>
<td>6,290</td>
<td>230</td>
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<tr>
<td>Outwash and Drainage</td>
<td>15</td>
<td>76</td>
<td>8</td>
<td>106</td>
<td>5</td>
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<td>Control</td>
<td>18</td>
<td>4.01</td>
<td>0.20</td>
<td>0.63</td>
<td>0.11</td>
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<td>EPA Aquatic Life CCC</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
<td>2.50</td>
<td>-</td>
</tr>
<tr>
<td>EPA Drinking Water MCLG</td>
<td>-</td>
<td>5.00</td>
<td>-</td>
<td>0.00</td>
<td>-</td>
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</table>
Table 5 Mixed Model analysis results of fixed main and interaction effect sources for a) stem accumulation, b) leaf accumulation, and c) biomass percent change. Random effect was willow genet.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>DFDen</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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</thead>
<tbody>
<tr>
<td>a Willow Species</td>
<td>2</td>
<td>66.4</td>
<td>2.63</td>
<td>0.08</td>
</tr>
<tr>
<td>Metal Type</td>
<td>1</td>
<td>197.0</td>
<td>0.59</td>
<td>0.44</td>
</tr>
<tr>
<td>Willow Species*Metal Type</td>
<td>2</td>
<td>197.0</td>
<td>6.43</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Metal Level</td>
<td>1</td>
<td>197.0</td>
<td>0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>Willow Species*Metal Level</td>
<td>2</td>
<td>197.0</td>
<td>1.55</td>
<td>0.22</td>
</tr>
<tr>
<td>Metal Type*Metal Level</td>
<td>1</td>
<td>197.0</td>
<td>0.34</td>
<td>0.56</td>
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<tr>
<td>Willow Species<em>Metal Type</em>Metal Level</td>
<td>2</td>
<td>197.0</td>
<td>0.33</td>
<td>0.72</td>
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<tr>
<td>b Willow Species</td>
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<td>21.5</td>
<td>4.07</td>
<td><strong>0.03</strong></td>
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<td>Metal Type</td>
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<td>58.2</td>
<td>0.32</td>
<td>0.58</td>
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<tr>
<td>Willow Species*Metal Type</td>
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<td>58.1</td>
<td>0.75</td>
<td>0.48</td>
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<td>Metal Level</td>
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<td>54.8</td>
<td>1.21</td>
<td>0.28</td>
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<td>Willow Species*Metal Level</td>
<td>2</td>
<td>54.8</td>
<td>0.86</td>
<td>0.43</td>
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<td>Metal Type*Metal Level</td>
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<td>54.2</td>
<td>0.32</td>
<td>0.57</td>
</tr>
<tr>
<td>Willow Species<em>Metal Type</em>Metal Level</td>
<td>2</td>
<td>54.1</td>
<td>0.23</td>
<td>0.80</td>
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<tr>
<td>c Willow Species</td>
<td>2</td>
<td>66.2</td>
<td>0.49</td>
<td>0.61</td>
</tr>
<tr>
<td>Metal Type</td>
<td>1</td>
<td>196.5</td>
<td>4.97</td>
<td>0.16</td>
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<tr>
<td>Willow Species*Metal Type</td>
<td>2</td>
<td>196.5</td>
<td>0.30</td>
<td>0.74</td>
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<tr>
<td>Metal Level</td>
<td>1</td>
<td>196.5</td>
<td>0.10</td>
<td>0.75</td>
</tr>
<tr>
<td>Willow Species*Metal Level</td>
<td>2</td>
<td>196.5</td>
<td>2.35</td>
<td>0.10</td>
</tr>
<tr>
<td>Metal Type*Metal Level</td>
<td>1</td>
<td>196.5</td>
<td>3.80</td>
<td>0.05</td>
</tr>
<tr>
<td>Willow Species<em>Metal Type</em>Metal Level</td>
<td>2</td>
<td>196.5</td>
<td>4.31</td>
<td><strong>0.01</strong></td>
</tr>
</tbody>
</table>
Figure S1 a Mine tailing with extremely contaminated evaporation pond located east of Leadville, CO. b Willows, including S. planifolia and S. monticola, growing along metal contaminated stream near the Red Mountain Mining District between Ouray and Silverton, CO.
Figure S2 Stems with no root growth with white fungus (a), with a few, single roots (b), with initial adventitious root growth (c), with multiple dense, adventitious sets of roots (d), and with very dense, adventitious roots that filled up the deepot cells, typically representative of cuttings grown an extra two months (e).
Figure S3  

**a** Healthy, fully developed leaves.  
**b** Leaves with chlorosis.  
**c** Leaves with spider mite infestation.
Figure S4 The pooled differences of percent changes of biomass for cuttings in treatment levels for both metals, cadmium and lead, with their corresponding cutting in control treatment (no metals) for three willow species: *S. drummondi*ana, *S. monticola*, and *S. planifolia*. One error bar equals one standard error from the mean.