Salix discolor: Prospects for phytoremediation of lead and polycyclic aromatic hydrocarbons

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Abstract

Phytoremediation is the application of green plants and their associated microbial communities for the removal, stabilization or detoxification of contaminants in the environment. *Salix discolor*, commonly known as the pussy willow, is a common Canadian shrub that was evaluated for phytoremediation potential of lead and polycyclic aromatic hydrocarbons (PAHs) using a short-term hydroponic species. *Salix discolor* was chosen because of the documented ability of willows to tolerate both lead and PAH contamination and their ability to sequester lead. The willows were grown in Hoagland's nutrient solution for four weeks for four weeks, and was subsequently spiked with lead and/or PAHs for an additional 4 weeks. The tissues were then dried and the lead and PAHs were extracted for analysis via atomic absorption spectrometry and gas chromatography-mass spectrometry, respectively. Issues with the gas chromatography-mass spectrometry instrument prevented analysis of the PAHs. There was no accumulation of lead within the leaf/shoot tissue of the willows, but there was between 2940-3450 ppm of lead accumulated within the root tissue. Furthermore, analysis of the growth medium at the conclusion of the experiment showed a decrease in lead concentration from 5 ppm to 0-0.2 ppm. Presence of either lead and/or PAHs did not significantly decrease the willows shoot growth [$p=0.06$] or decrease the transpiration rate of the willows [$p=0.979$]. *Salix discolor* appears to possess potential for the phytoremediation of lead and PAHs and should be evaluated in a more rigorous study using a soil medium.

Keywords: Phytoremediation, *Salix*, *discolor*, lead, PAH, polycyclic aromatic hydrocarbons, willows

Introduction

Phytoremediation is the application of green plants and their associated microbial communities for the removal, stabilization or detoxification of contaminants in the environment (USEPA 2015). Common targets of phytoremediation include heavy metals, hydrocarbons and persistent organic pollutants. There are five major phytoremediation processes, each of which are useful for certain classes of contaminants: phytoextraction (accumulation of contaminants within plant tissues) and phytostabilization (immobilization of contaminants via complexation/chelation) are most useful for the remediation of metals. Phytovolatilization (volatilization via transpiration) and phytotransformation (degradation via plant metabolic processes) are most effective for the remediation of organic compounds. Rhizofiltration, which is the degradation, accumulation or stabilization of contaminants by the microorganisms within the biosphere, is potentially useful for organic or inorganic pollutants (Ghosh and Singh 2005). Therefore, it is necessary to match the process to the intended target in order for remediation to be effective.

Similarly, it is necessary to select a suitable plant for the process. Phytoremediation relies almost exclusively upon the plant having direct root contact with the contaminant, while high productivity is required to promote accumulation or transpiration – limiting the selection of potential candidates. An ideal candidate would accumulate biomass rapidly, have high transpiration rate, possess extensive root systems and would be easily to propagate. Members

of the genus *Salix*, commonly known as willow, possess all of these qualities (Kuzovkina and Quigley 2005). In addition to phytoremediation, these qualities make willows useful in a variety of ecological engineering projects including effluent filtration, erosion stabilization and carbon sequestration (Wani et al. 2011). The rapid biomass accumulation of willow species also adds significant value by providing biomass that may be converted to energy or through the recovery of contaminants from within their tissues (Jiang et al. 2015). This sort of integrated approach would lessen the burden of waste management of contaminated, thereby increasing the economic and ecological feasibility of phytoremediation.

Another important consideration is the ability of a plant to grow in the contaminated zone. The use of a native species would solve this issue from an ecological standpoint. Willows are extensively distributed throughout the Northern Hemisphere, allowing them to be used virtually anywhere. The chosen test species for the present experiment is widely distributed throughout Canada and the United States (Gucker 2007). The use of such a widely distributed species will ultimately allow for wide deployment of a promising technology and will lessen the need to research as many potential phytoremediation candidates – an expensive and time-consuming proposition.

Lead is an ubiquitous pollutant that is naturally present in the soil but is found in much higher concentrations in urban and industrial areas (Smith et al. 2013). Because lead imitates calcium within the body, it is able to be deposited in bones and teeth and can interact with a wide

variety of proteins – one explanation for its high toxicity to mammals. In humans, even small blood concentrations (14 μg dL^{-1}) may cause serious developmental issues during pregnancy, while adults may suffer toxic effects starting at 40 μg dL^{-1} (CDC 2010). Smith et al. (2013) reported mean lead soil concentrations of 25.8 mg kg^{-1}, 22.2 mg kg^{-1} and 16.6 mg kg^{-1} in the surface layer (0-5 cm depth), A horizon and C horizon, respectively in the United States. There is, however, high variability in concentration, particularly in the surface soil, which had a standard deviation of 185 mg kg^{-1}. This implies that most of this deposition is either relatively recent or that lead is quite immobile after deposition. While a significant amount of lead is likely bound in several soil pools, much of the contamination is recent, owing to the many commercial and industrial uses of lead, including as an anti-knock agent in obsolete gasoline blends and lead-based paints (Wuana and Okieimen 2011). Lead concentrations in urban and industrial are normally between 50-200 mg kg^{-1}, while the EPA publishes a human health risk starting at 400 mg kg^{-1} in play areas and 1200 mg kg^{-1} in all other areas. Lead is only sparingly soluble in water and the EPA has set a maximum concentration of 15 μg L^{-1} for potable water (CDC 2010). Remediation of lead contamination is needed to lessen the likelihood of lead exposure directly from the environment, as well as ingestion from contaminated food.

Polycyclic aromatic hydrocarbons (PAHs), similarly to lead, are a group of ubiquitous environmental contaminants that may be formed naturally but are more prevalent in urban and industrial areas. PAHs occur naturally as a component of crude oil and are formed through the incomplete combustion of several fuels – most notably gasoline – and are deposited atmospherically (CDC 2010; Bari et al. 2014). Generally, PAHs are considered to be carcinogenic

with chronic exposure but present low acute toxicity to humans due to robust metabolic degradation (CDC 2010). Because PAHs occur as complex mixtures of several species, benzo(a)pyrene, a known carcinogen, is used as a reference compound for toxicity. Most PAHs present lower toxicity to humans but the EPA assumes there is equal risk (Nisbet and LaGoy 1992). The EPA sets limits on the amount of benzo(a)pyrene that may be present in drinking water at 0.0001 mg L^{-1}, while maximum soil concentration are unregulated. It is noted that urban soil concentrations are usually less than 2 mg kg^{-1} (CDC 2010). Because PAHs can be volatilized from soil and water (Cabrerizo et al. 2011), remediation will decrease atmospheric exposure – the most common route of exposure (CDC 2010).

Research using willow for phytoremediation has been quite extensive, exploring the remediation of several heavy metals and organic compounds (Marmiroli et al. 2011). Zhivotovsky et al. (2011) showed that several willow species can tolerate extremely high lead soil concentrations, growing in concentrations 21,360 mg kg^{-1}. Under these conditions, various species were able to accumulate between 200-1000 mg kg^{-1} of dry tissue. The effect of willows on PAH concentrations is not well understood, however. Spriggs et al. (2005) demonstrated that willows have a significant effect on PAH degradation, while research conducted by Vervaeke et al. (2003) showed that willows significantly decrease PAH degradation. Furthermore, despite widespread lead and PAH co-contamination, there is little literature addressing the issue.

There is a significant knowledge gap that the present study was designed to address. There are four fundamental research questions that will be answered. Is *S. discolor* able to tolerate lead and/or PAH contamination? If so, how does lead and/or PAH contamination affect the growth of *S. discolor*? Is *S. discolor* able to remove lead and/or PAH contamination from the environment? And, if so, in which tissues do lead and/or PAHs accumulate? It is predicted that the presence of lead and PAHs will not significantly decrease the shoot growth of *S. discolor* and that lead will be sequestered mainly within roots tissues, while the PAHs will be degraded. This is expected because willows have been shown to grow effectively in even extremely high concentrations of lead and accumulate within root tissue (Zhivotovsky et al. 2011), while PAHs, when present in low to moderate concentration, do not significantly decrease growth, and may actually stimulate growth (Thygesen and Trapp 2002). The goal of this research is to apply this knowledge in order to create a viable, cost-effective and ecologically sound method to remediate these two serious environmental contaminants.

Methods

Willow procurement and propagation

Fresh softwood *S. discolor* branches were taken from dormant trees on an acreage local to Edmonton, Alberta. Sixty 20 cm long cuttings were prepared from the branches. The fresh weight and diameter, measured at the midpoint, of each cutting was recorded. The cuttings were placed overnight in a distilled water bath and then transferred to a bath containing ½-

strength Hoagland's nutrient solution, which was selected because it is an appropriate medium for hydroponic cultivation (Trejo-Tellez and Gomez-Merino 2012). The solution was changed at 2 and 3 weeks, and the willows were placed into individual beakers containing Hoagland's solution at the beginning of week 4. The initial change interval was longer because willows produce indolebutyric acid – a natural rooting hormone – in order to encourage root formation in the dormant cuttings. Shoot growth was measured once weekly. Additionally, the temperature and relative humidity were recorded in duplicate once every hour. One sensor was placed at the front left side of the chamber, while a second sensor was placed in the rear right side of the chamber in order to monitor environmental homogeneity. The 25 cuttings displaying the most growth were moved to the hydroponic phytoremediation experiment after 30 days.

Hydroponic Experiment

The willows growth solution was spiked with lead (as lead nitrate) and/or PAHs in approximately 500 mL of ½-strength Hoagland's solution at the following concentrations: 0 ppm Pb/0 ppb PAH (control), 5 ppm Pb/0 ppb PAH, 0 ppm Pb/180 ppb PAH and 5 ppm Pb/180 ppb PAH. A fifth treatment of 4.2 mL/L of 70% ethanol was prepared to isolate any effects of the ethanol used to solubilize the PAHs. Although environmental concentrations would be unlikely to reach these levels for either contaminant, these concentrations were used to establish the ability of *S. discolor* to grow in highly contaminated media. The position of the willows within the chamber was rotated at random once weekly and the transpiration rate was measured once weekly for 3 weeks using a mass potometer. All other environmental conditions and

measurements were kept as before applying the spiked growth solutions. A hydroponic experiment was selected in order to simplify the experiment, control variables (i.e. binding of contaminants within different soil pools) and to shorten the length of the experiment (Marmiroli et al. 2011).

Tissue Analysis

The tissues were split into root, shoot/leaf fractions to determine contaminant sequestration and a sample of the remaining spiked solutions to determine total contaminant decrease. The tissues were dried to constant weight at a temperature of 60 ^{o}C for 72 hours (NECi 2012). The lead was extracted using wet digestion method ISO 11466.3, using 10 mL of aqua regia and a 0.05 g tissue sample. This method was demonstrated to effectively extract Pb^{2+} for analysis (Pena-Icart al. 2011). Any remaining plant tissue was removed via filtration and the resulting solution was analyzed using atomic absorption spectrometry (AAS). The samples were prepared using a variation of EPA Method 3350C developed by Song et al. (2002). The PAHS were extracted by placing 15 mL of dichloromethane into a sealed container along with a 0.05 g tissue sample, which was subsequently placed into a heated ultrasonic bath at a temperature of 40 C^{o} for 2 hours. The extract was then filtered to remove any plant tissue and then analyzed using gas chromatography-mass spectrometry (GC-MS).

Statistical Analysis

Analysis of the data was performed using Microsoft Excel 2016 and IBS SPSS version 24 (2015). Methods used include regression analysis, independent t-tests assuming equal variance, one-way ANOVA, two-way ANOVA and one-way ANCOVA. The specific test used for analysis is identified immediately preceding the respective analysis and all tests were performed with a significance level of $\alpha=0.05$.

Results

Environmental Conditions

The significance between the mean temperature and relative humidity recordings of sensor 1 and sensor 2 was tested using independent t-tests assuming equal variance. There was a significant difference between the mean temperature reported by sensor 1 [M=21.78 ^{o}C, SD=2.61 ^{o}C] and sensor 2 [M=22.84 ^{o}C, SD=2.96 ^{o}C]; t(1887)=-8.27, p=2.60x10^{-16}. Similarly, there was a significant difference between the mean relative humidity reported by sensor 1 [M=22.51%, SD=6.30%] and sensor 2 [M=29.77%, SD=9.31%]; [t(1887)=-19.86, p=3.66x10^{-80}]. The minimum temperature recorded was 7.2 ^{o}C, while the maximum was 41.2 ^{o}C and the minimum relative humidity recorded was 7.8%, while the maximum was 56.6%. All of the minimum and maximum recordings were recorded by sensor 2. The standard deviation of sensor 2 was approximately 2.5-3-fold greater than that of sensor 1, leading to large

discrepancies in the recorded temperature and relatively humidity of the environmental chambers. This is clearly visible in figures 1 and 2, which show the change in temperature and relative humidity over the course of the experiment, respectively. Overall, the shape of the data was very similar, but there is a clear offset between the measurements recored by sensor 1 and sensor 2 of approximately 2 ^{o}C for the temperature and 5-10% for the relative humidity.

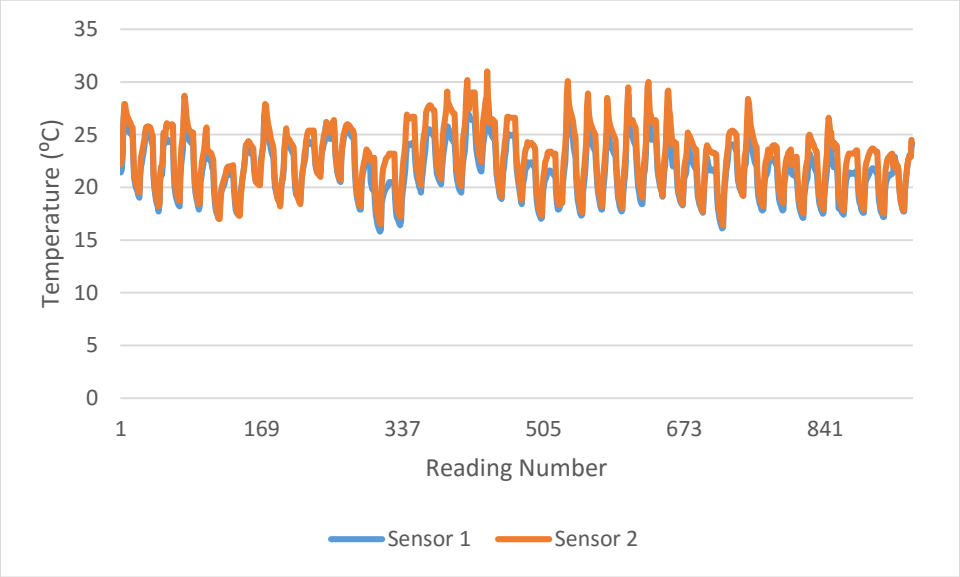

Figure 1: Temperature (^{o}C) change of the environmenal chamber over 8 weeks. Sensor 1 - [M=21.78 ^{o}C, SD=2.613 ^{o}C, min=15.8, max=28.8 ^{o}C], Sensor 2 - [M=22.84 ^{o}C, SD=2.962 ^{o}C, min=7.2 ^{o}C, max=41.2 ^{o}C]. The temperature was recorded once hourly and the willows were randomly rotated within the chamber once weekly.

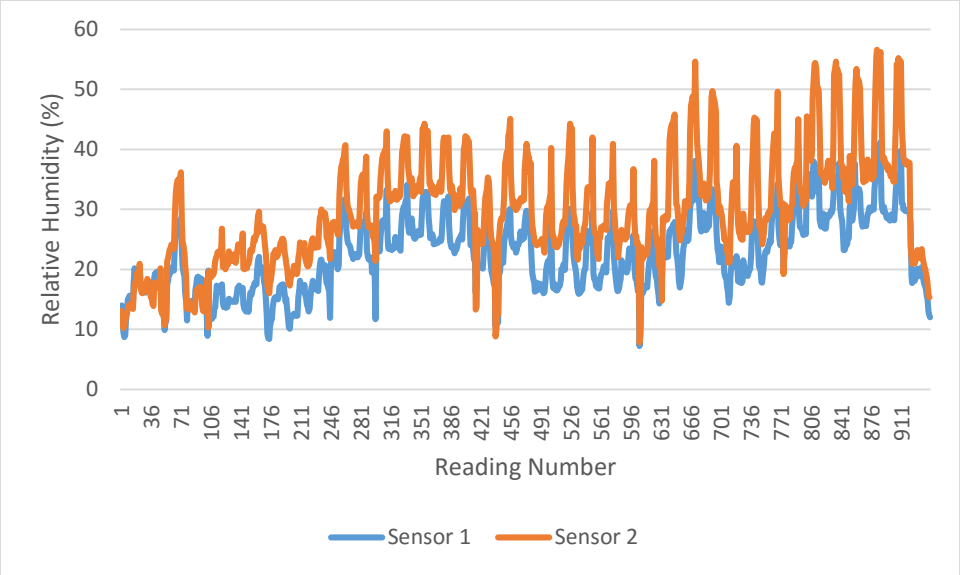

Figure 2: Relative humidity (%) change of the environmental chamber over 8 weeks. Sensor 1 - [M=22.51%, SD=6.302%, min=16.4%, max=31%] and sensor 2 [M=29.77%, SD=9.307%, min=7.8%, max=56.6%]. The relative humidity was recorded once hourly and the willows were randomly rotated within the chamber once weekly.

Willow Viability

Cuttings were classified as "viable" if they displayed some shoot growth during the first four weeks of the experiment (i.e. before the growth solutions were spiked) and "non-viable" if they did not display any growth. The difference between the mean fresh weight and diameter were tested for significance using independent t-tests assuming equal variance. Of the 60 cuttings used, only 28 were viable. There was a significant difference in the mean fresh weight of the viable [M=11.320 g, SD=4.320 g] and non-viable [M=9.129 g, SD=3.016 g) cuttings; [$t(58)=2.301$, $p=0.0250$]. There was also a significant difference in the mean diameter of the viable [M=9.0 mm, SD=1.64 mm] and the non-viable [M=7.8 mm, SD=1.5 mm] cuttings; [$t(58)=2.81$, $p=6.80\text{x}10^{-3}$]. Figure 3 shows a regression analysis of the fresh weight and diameter of the 60 cuttings. There was a relatively high correlation between the two variables [$r^2=0.829$],

indicating that there is a relationship between the weight and diameter of the cuttings. Figure 4 shows the same data after the cuttings have been separated into viable and non-viable groups. Figure 4 clearly demonstrates that larger cuttings tended to be viable, while smaller cuttings tended to be non-viable. It is worth noting that the values of the diameter are given as whole numbers because of the limited resolution of the instrument that was used to measure them. It is unlikely, however, that the relationship would have changed significantly, but a higher resolution would "smooth" the appearance of the figures.

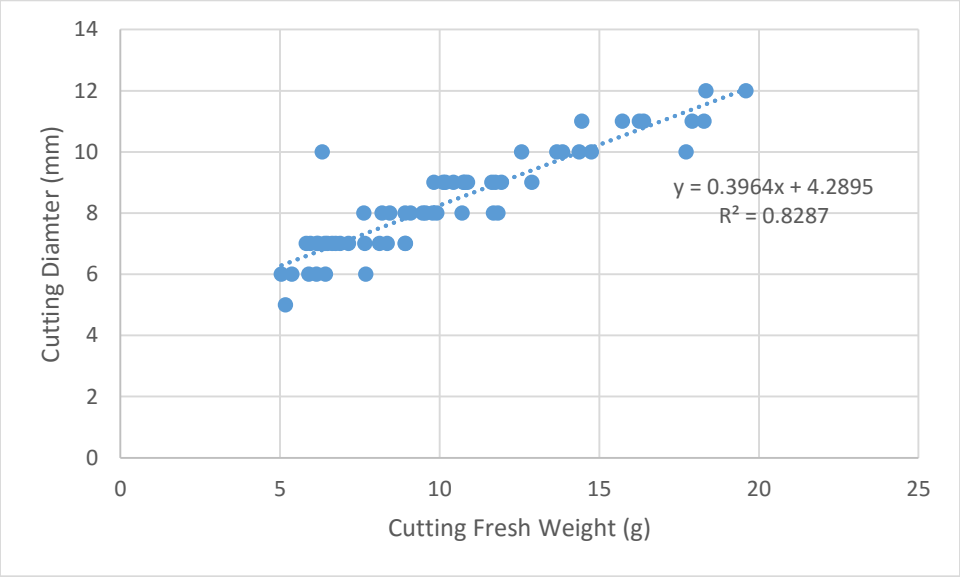

Figure 3: Regression analysis of the fresh weight and diameter of the whole willow cohort. There is good correlation between the fresh weight and the diameter of the cuttings.

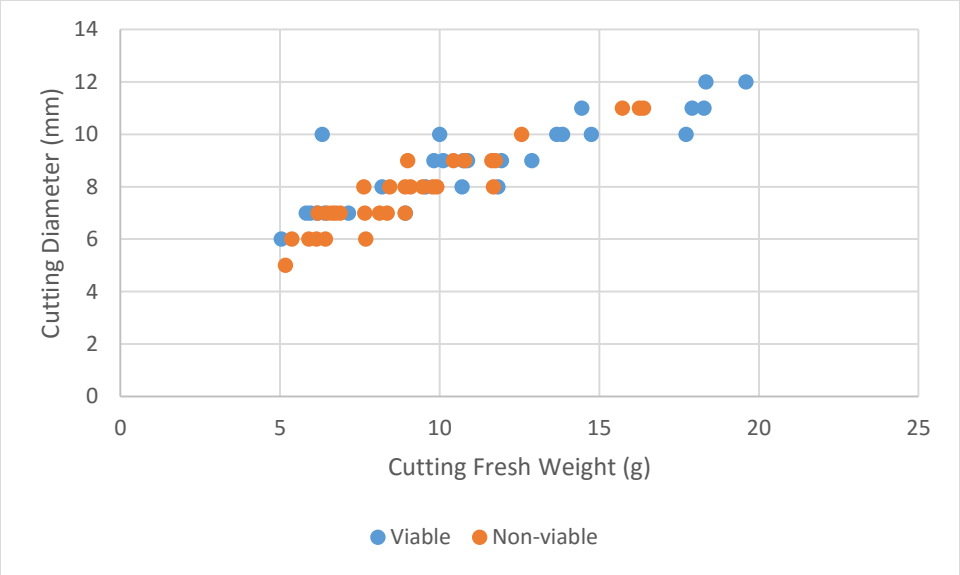

Figure 4: Scatterplot of diameter vs. fresh weight of the willows when split into viable and non-viable groups.

Phytoremediation Experiment

An ANCOVA test was used to test for any significant difference between the mean shoot growth of the five treatment groups, while controlling for the pre-treatment shoot growth. There was no significant difference between the mean shoot growth of any of the treatments [$F(2,4)=2.790$, $p=0.056$]. Figure 5 shows the mean shoot growth and standard deviation for each of the five treatments. There is no clear trend in the data, but the variability of the data is extremely high – in several cases the standard deviation exceeds the mean. This is likely an effect of the relatively small sample size, which was five cuttings per treatment.

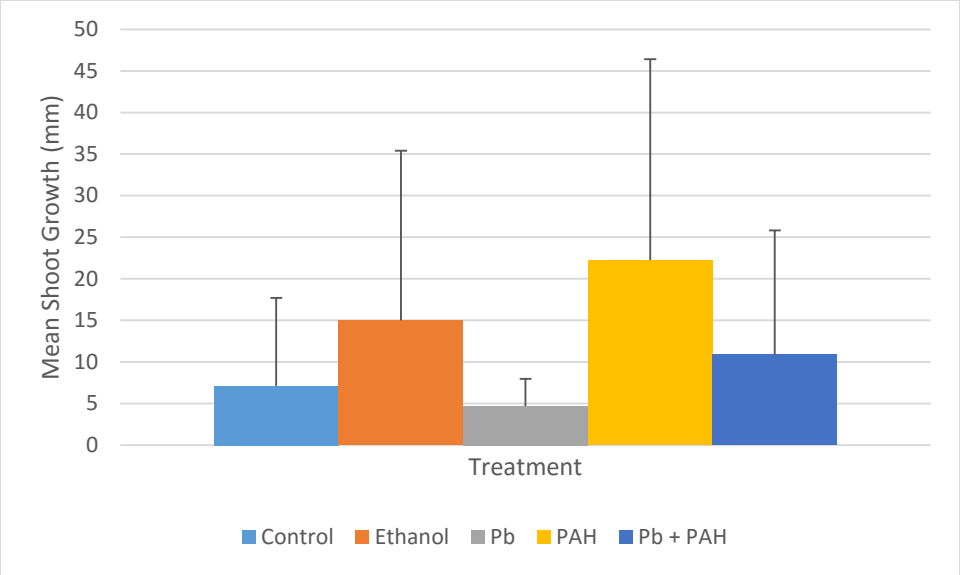

Figure 5: Mean shoot growth and standard deviation of the willows in the five spiked solutions. No clear trend is discernable, but the shoot growth is highly variable between individual cuttings – possibly a result of the small sample size (five replicates per treatment).

Transpiration Rate

The transpiration rate of the willows was tested using a two-way ANOVA with replication. The analysis indicated that there was not a significant difference between transpiration week-over-week [$F(4,60)=1.38$, $p=0.252$], that there was a significant difference in transpiration rate between the treatments [$F(2,60)=3.29$, $p=0.044$], but that the interaction between the variables did not differ significantly [$F(8,60)=0.248$, $p=0.979$]. Figure 6 shows the transpiration rates of each sample for each week measured. There appears to be a trend that as time progressed the transpiration rate increased (i.e. transpiration rate increased with shoot growth), but the interaction was not significant, likely owing to the large variation. Regression analysis shows that there is significant correlation between the amount of shoot growth and the transpiration

rates of the willows [R^2 for weeks 1, 2 and 3 =0.8117, 0.8736 and 0.9136, respectively], which may be found in figure 6.

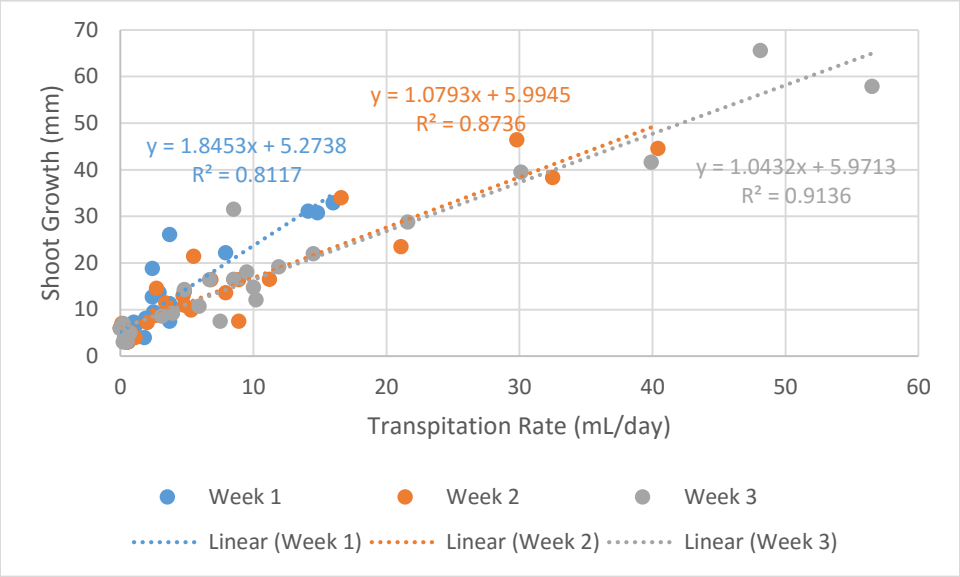

Figure 6: Regression analysis of willow shoot growth vs. transpiration rate. Overall, there is good correlation between the transpiration rate and the shoot growth of the willows.

Tissue Analysis

Unfortunately, tissue analysis of the PAHs could not be carried out because of issues with the instrumentation. While attempting to create a standard curve, the instrument was not able to detect any PAH, only the solvent, which was dichloromethane. Analysis of the PAHs will continue to be analyzed once new standards are received.

The lead content of the leaves/shoots, roots and the growth solutions were analyzed using AAS. Each sample was analyzed twice, if possible. There was no sequestration of lead within the leaf tissue of the willows. Calculating the Pb concentration using the regression equations created

using the standard curves yielded results that were all slightly negative (-0.2 to -0.8 ppm), which were assumed to be 0 ppm (i.e. negative lead values are not possible). Several samples did not have enough leaf tissue to be analyzed, but there were at least two samples for each treatment that were able to be analyzed. Analysis of the root tissues showed that two samples (both from the Pb + PAH treatment) contained 2940 and 3450 ppm (dry weight) of Pb. Only five root samples could be tested because most of the willows did not grow root system of sufficient size to sample. Analysis of the growth solutions taken at the conclusion of the phytoremediation experiment showed that there was a decrease from 5 ppm Pb to 0 to 0.2 ppm Pb. Interestingly, there was no residual Pb found in the Pb + PAH treatment, but in the Pb treatment there was a small amount of residual Pb [M=0.11 ppm, SD=0.084 ppm]. This information is displayed in figure 7.

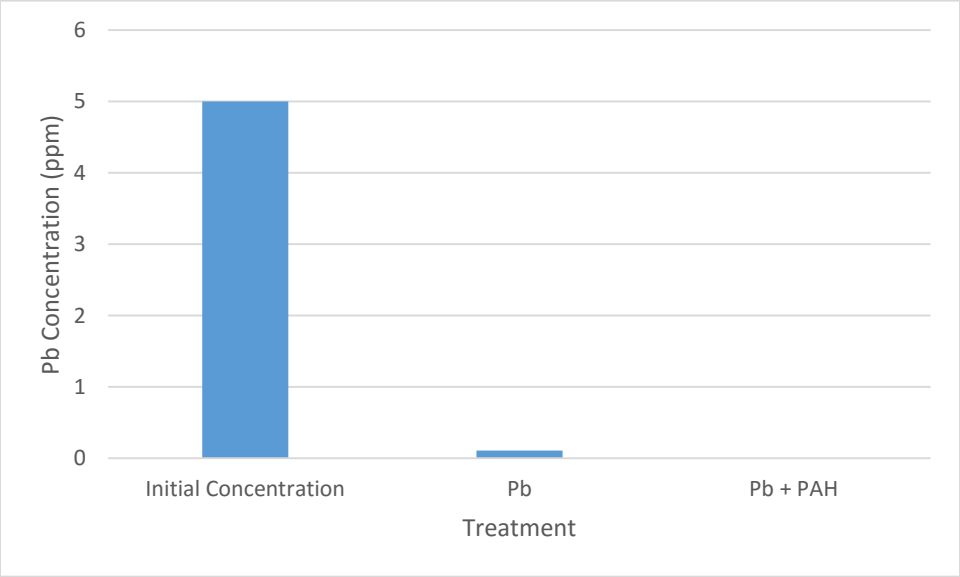

Figure 7: Decrease in lead (Pb) concentration of the two lead containing treatments. None of the Pb + PAH treatment replicates contained any residual Pb, while the Pb treatment replicates contained 0.02-0.20 ppm residual Pb [M=0.11 ppm, SD=0.084 ppm].

Algal Invasion

There were two species that invaded the growth solutions. The species observed were the algae *Protococcus* sp. and the cyanobacteria *Gloeocapsa* sp. A picture of the two species may be found in figure 8.

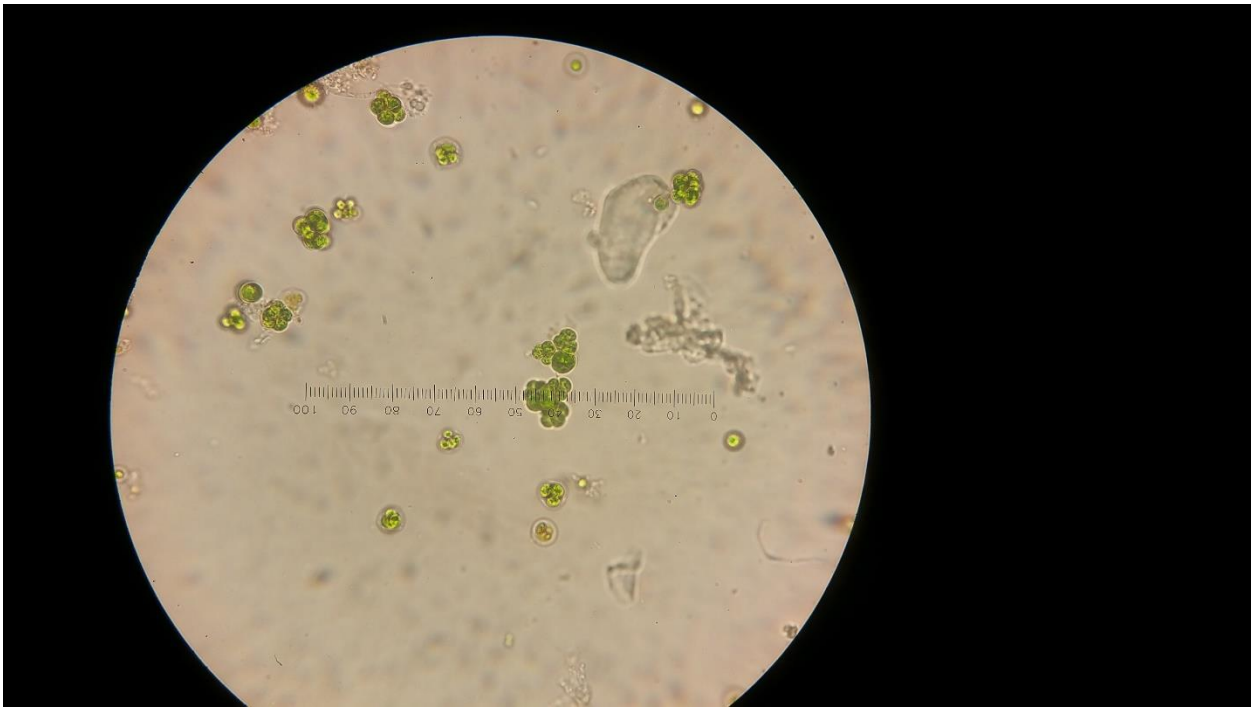

Figure 8: *Protococcus* sp. (algae) and *Gloeocapsa* sp. (cyanobacteria) at 100x magnification. The large cluster crossing the micrometer is an example of *Protococcus* – individual cells are between 7.5-12.5 µm. The cluster immediately below the 70 marking on the micrometer is an example of *Gloeocapsa* – individual cells are between 5-7.5 µm.

Discussion

Effects of Environment on Growth

As noted in the results, there was a significant difference between the mean temperature and relative humidity recorded by the 2 sensors, but as seen in figures 1 and 2, the shape of the data is similar with a small offset between the sensors. However, because the willows were randomly rotated within the environmental chamber weekly, any potential effects on growth would have been minimized. A more important consideration is how the temperature affects the growth of the willows. During July, the hottest month of the year, Edmonton experiences a daily mean high temperature of 22.8 ^{o}C, a mean temperature of 16.2 ^{o}C and a daily mean low temperature of 9.5 ^{o}C (Statistics Canada 2015). The temperatures experienced by the willows during the experiment (approximately 22-23 ^{o}C) was similar to what occurs in the Edmonton region. An experiment performed by Labrecque and Teodorescu (2003) showed that *S. discolor* is highly productive in short-rotation coppices. The temperature conditions that the willows experienced in that experiment were approximately 2 ^{o}C on average. Therefore, it is expected that the willows in the present experiment did not experience significant heat stress and that in terms of temperature, they likely grew at or near their full-potential. Equally important is the effect of relative humidity on growth. The Edmonton region experiences mean relative humidity in the range of 45-60% (Government of Canada 2015) – much higher than the mean relative humidity experience by the willows during the course of this experiment (22.51-29.77%). Generally speaking, chronic low humidity conditions result in water stress to the plant. Low humidity results in a higher vapour pressure deficit, which can lead to increased transpiration, which may be viewed as positive in terms of phytoremediation (Rawson et al. 1977). Because contaminants are absorbed via solution, the greater the amount of solution transpired, the greater the amount of contaminant removed – at least in theory. However,

since low humidity also causes water stress, plant growth is reduced and may be quite severe in some cases, resulting decreases in dry mass yield of 20-30% (Ford and Thorne 1974). Of course, since metals must be deposited within plant tissues to be remediated, maintaining optimum growth is an important consideration. It is very likely that, in terms of relative humidity, the willows were not subjected to ideal conditions, which may have reduced their growth.

Willow Viability

The viability of any plant to be used in any phytoremediation experiment is one of utmost importance. If a plant is not able to stablish itself, then it is entirely useless, thus it important to know under what conditions a plant will be able to grow. It is common knowledge that a great number of tree species may be propagated vegetatively from cuttings and *S. discolor* is no exception. In this experiment, it was found that *S. discolor* may be propagated quite easily in this manner, but the size of the cutting appears to be a major factor in predicting cutting viability. Either the fresh weight or the diameter appear to be good predictors of this, but must be taken relative to the length of the cutting. At a length of 20 cm, viable cuttings had a mean fresh weight of 11.320 g and a mean diameter of 9.0 mm, while non-viable cuttings had a mean fresh weight of 9.129 g and a mean diameter of 7.8 mm. Additionally, there was a significance difference in the means of the fresh weight [$p=0.0250$] and the diameter [$p=6.80 \times 10^{-3}$] of the viable and non-viable group. There is a relatively simple explanation for this. In order for a cutting to establish itself, it must grow new roots in order to access nutrients in the soil and leaves in order to capture carbon so that it may from carbohydrates for both structure and

energy, but the amount of carbohydrate that is available for new growth and energy in a cutting is quite limited (Hoag 2007). Thus, a larger cutting has a higher probability to be viable because it has access to a larger pool of available carbohydrates. The time that the cuttings were harvested may have played an important role in determining which cuttings were viable and non-viable. The cuttings were gathered well into dormancy (mid-December), meaning that there was likely minimal stored carbohydrate within the cuttings. Since the majority of carbohydrate reserves are stored within the root tissue overwinter, with a small amount available in above-ground tissues for the purposes of respiration. Cuttings gathered during bud break would likely have a much higher probability of being viable across all sizes due to greater local availability of carbohydrates (Loescher et al. 1990). Additionally, Camp et al. (2012) found that longer cuttings lead to increased early growth of willows. Therefore, in order to promote both increases in viability and increases in early growth – an important consideration when used for intense short-rotation coppices – larger cuttings should be used for any project where establishing willows from cuttings is necessary.

Willow Growth

The most difficult part of this experiment to reconcile is how well the willows grew. There was an extremely high amount of variability, with the standard deviation of a group being nearly equal to, or even exceeding the mean of that group (see figure 5). Although the total shoot growth of any group was not significantly different from that of any other group, the extreme variation decreases the reliability of the data. It has been noted in several studies that willows

tend to be quite variable as a group (Wang and MacFarlane 2012; Berthod et al. 2015). One simple explanation for this is simply genetic variability (Aradottir et al. 2007). Another possible explanation is the degree of development of the root systems of the cuttings. Cuttings that displayed little to no shoot growth had extremely poorly developed root systems – some of which were non-existent – while the willows with the largest shoots had extremely well developed roots. Intuitively, plants with larger roots (i.e. more surface area over which to acquire nutrients) will be able to grow larger. A third possible explanation is that the size of the cutting played a part in total growth. The largest cuttings, in general, grew much larger shoots than smaller cuttings, but the high variability between the cuttings resulted in no significant differences between the different treatments ($p=0.056$). Similarly, there was no significant differences in the transpiration rates of the willows over time ($p=0.979$). Repetition of the experiment using a larger sample size and more consistently sized cuttings would help to reduce variability and would result in easier to interpret results. The results do, however, conform with the original prediction that lead and PAHs would have little to no effect on shoot growth at the respective concentrations, either singly or in combination. This is unsurprising given that previous studies have shown that willows can tolerate high concentrations of lead in the soil (Zhivotovsky et al. 2011), despite lead being extremely toxic to most plants (Pourrut et al. 2011). The toxicity of PAHs to plants is likely limited at low to moderate concentrations because of their extreme hydrophobicity, which would lead to low bioavailability (Kusmierz et al. 2016).

Lead Removal and Sequestration

The amount of Pb removed from each of the replicates was surprisingly homogenous, despite the fact that there was large variation in the growth of the cuttings indicates that the near complete removal of Pb is likely not attributable solely to the willows. As mentioned in the results, there was algae and cyanobacteria present in the solution of each replicate, which may have affected removal of Pb from the growth solutions. Several species of algae have been shown to be capable of removing Pb from the environment (Chekroun and Baghour 2013), as have bacteria (Valls and Lorenzo 2002). It was expected that *S. discolor* would be capable of sequestering Pb within the root tissue, which is confirmed by this experiment. This result requires further confirmation, however, as only 2 samples (out of 10 exposed to Pb) were able to be analyzed, but the results are encouraging, regardless. Sequestration within root tissue is also preferable to sequestration within leaf tissue from a management standpoint. The roots are much less mobile than the leaves in the environment (i.e. leaves drop, are spread via wind and are eaten), though they present a challenge in terms of removal, but *S. discolor* may be useful for the long-term stabilization and eventual removal of Pb.

Improvements to Experimental Design

There are several ways in which this experiment could be improved in the future. The most obvious improvement is increasing the sample size. Unfortunately, only so many cuttings were available and their viability was questionable since this project was started in the winter. Another major improvement would be gathering cuttings that are more homogenous in size.

Together, these two factors would lessen the variability of the data, leading to more defensible conclusions. Because of the limited amount of cuttings available for the experiment, compromises had to be made in both of these areas. Another method to reduce variability may be to block the samples and then to randomize which treatment the blocks are applied to. Because of the possibility for large genetic variability, it would be desirable to block cuttings sourced from the same tree. The ability to exclude other species in their entirety would also exclude another confounding factor. In this experiment, some rouge algae in the hydroponic solution was a source of frustration because it was not possible to separate it from the roots of the willows, thus it was not possible to separate the effects of the algae on lead/PAH uptake from that of the willows. However, given the scope and intent of this project, these nuisance factors do not completely invalidate the conclusions.

Conclusion

This project did provide a wealth of information regarding the use of *S. discolor* for the phytoremediation of Pb. Based on the tissue that lead is sequestered within, the proposed phytoremediation mechanism of *S. discolor* on Pb is phytostabilization. Within the scope of this experiment, the majority of the research objectives have been achieved. It does appear that *S. discolor* is able to tolerate a significant amount of Pb and/or PAH contamination and that this contamination does not significantly decrease the growth of the plant. Furthermore, it appears that Pb accumulates within the root tissue of *S. discolor*. Unfortunately, sequestration of PAHs could not be evaluated at the present time, but will be followed up with in the near future. It is

recommended that *S. discolor* be evaluated further, preferably for a longer period of time and in a soil medium, to determine if this species is capable of phytoremediation of Pb and/or PAH from soil.

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Literature Cited

Aradottir AL, Svavardottir K, Bau A. 2007. Clonal variability of native willows (*Salix pylicifolia* and *Salix lanata*) in Iceland and implications for use in restoration. Icel. Agric. Sci. 20:61-72. [Internet] [cited 09 April 2016]. Available from: http://www.landbunadur.is/landbunadur/wgsamvef.nsf/8bbba2777ac88c4000256a89000a2ddb/b7ffc48bede95e1a002573d300412e02/$FILE/Clonal%20variability.pdf

Bari MA, Kindzierski WB, Cho S. 2014. A wintertime investigation of atmospheric deposition of metals and polycyclic hydrocarbons in the Athabasca Oil Sands Region, Canada. Science of the Total Environment. 485-486: 180-92. [Internet] [cited 06 March 2016]. Available from: http://www.sciencedirect.com.ezproxy.aec.talonline.ca/science/article/pii/S0048969714004288

Berthod N, Brereton NJB, Pitre FE, Labrecque M. 2015. Five willow varieties cultivated across diverse field environments reveal stem density variation associated with high tension wood abundance. Frontiers in Plant Science. 6(948): 11 pp. [Internet] [cited 09 April 2016]. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4628129/#B27

Cabrererizo A, Dachs J, Moeckel C, Ojeda MJ, Caballero G, Barcelo D, Jones KC. 2011. Ubiquitous net volatilization of polycyclic aromatic hydrocarbons from soil and parameters influencing their soil-air partitioning. Environmental Science Technology. 45(11): 4740-47. [Internet] [cited 06 March 2016] Available from: http://pubs.acs.org/doi/abs/10.1021/es104131f

Camp JC, Rosseau R, Gardiner ES. 2012. Longer black willow cuttings result in better initial height and diameter growth in biomass plantation. Proceedings of the 16th Biennial Southern Silvicultural Research Conference: 43-46. [Internet] [cited 09 April 2016]. Available from: http://www.srs.fs.usda.gov/pubs/41373

[CDC] Center for Disease Control. 2010. Lead toxicity. Agency for Toxic Substances and Disease Registry. 71 pp. [Internet] [cited 06 March 2016] Available from: http://www.atsdr.cdc.gov/csem/lead/docs/lead.pdf

[CDC] Center for Disease Control. 2009. Toxicity of polycyclic aromatic hydrocarbons (PAHs). Agency for Toxic Substances and Disease Registry. 68 pp. [Internet] [cited 06 March 2016]. Available from: http://www.atsdr.cdc.gov/csem/pah/docs/pah.pdf

Chekroun KB, Baghour M. 2013. The role of algae in phytoremediation of heavy metals: A review. J. Mater. Environ. Sci. 4(6): 873-80. [Internet] [cited 13 April 2016]. Available from: http://www.jmaterenvironsci.com/Document/vol4/vol4_N6/118-JMES-449-2013-BenChekroun.pdf

[EPA] United States Environmental Protection Agency. 2015. Learn about lead.

Ford MA, Thorne GN. 1974. Effects of atmospheric humidity on plant growth. Annals of Botany. 38(2): 441-52. [Internet] [cited 09 April 2016]. Available from: http://aob.oxfordjournals.org/content/38/2/441.short

Ghosh M, Singh SP. 2005. A review on phytoremediation of heavy metals1 and utilization of its by-products. Asian Journal on Energy and Environment. 6(04) 214-31. [Internet] [cited 06 March 2016]. Available from: http://www.ecology.kee.hu/pdf/0301_001018.pdf

Government of Canada. 2015. North America – Relative Humidity. Government of Canada. Ottawa, On. 1 webpage (46 maps). [Internet] [cited 09 April 2016]. Available from: https://weather.gc.ca/astro/meteo_animation_e.html?id=hr&utc=12

Gucker, Corey L. 2007. Salix discolor. In: Fire Effects Information System. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer). [Internet] [cited 06 March 2016]. Available from: http://www.fs.fed.us/database/feis/plants/shrub/saldis/all.html#DISTRIBUTION AND OCCURRENCE

Hoag JC. 2007. How to plant willows and cottonwoods for riparian restoration. United States Department of Agriculture. Boise, Idaho. 22 pp. [Internet] [cited 09 April 2016]. Available from: http://www.nrcs.usda.gov/Internet/FSE_PLANTMATERIALS/publications/idpmctn7064.pdf

Kusmierz m, Oleszczuk P, Kraska E, Andruszczak S. 2016. Persistence of polycyclic aromatic hydrocarbons (PAHs) in biochar-amended soil. Chemoshpere. 146: 272-79. [Internet] [cited 09 April 2016]. Available from: http://www.sciencedirect.com/science/article/pii/S0045653515304690

Kuzovkina YA, Quigley MF. 2005. Willows beyond wetlands: use of *Salix* L. species for environmental projects. Water and Soil Pollution. 162(1): 183-204. [Internet] [cited 06 March 2016]. Available from: http://link.springer.com/article/10.1007%2Fs11270-005-6272-5

IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.

Jiang Y, Lei M, Duan L, Longhurst P. 2015. Integrating phytoremediation with biomass valorisation and critical element recovery: A UK contaminated land perspective. Biomass and Bioenergy. 83: 328-39. [Internet] [cited 06 March 2016]. Available from: http://www.sciencedirect.com/science/article/pii/S0961953415301240

Labrecque M, Teodorescy TI. 2003. High biomass yield achieved by *Salix* clones in SRIC following two 3-year coppice rotations on abandoned farmland in Southern Quebec, Canada. Biomass and Bioenergy. 25(2): 135-46. [Internet] [cited 09 April 2016]. Available from: http://www.sciencedirect.com.ezproxy.aec.talonline.ca/science/article/pii/S0961953402001927

Loescher WH, McCamant T, Keller JD. 1990. Carbohydrate reserves, translocation and storage in woody plant roots. HortScience. 25(3): 274-81. [Internet] [cited 09 April 2016]. Available from: http://hortsci.ashspublications.org/content/25/3/274.full.pdf+html

Marmiroli M, Pietrini F, Maestri E, Zacchini M, Marmiroli N, Massacci A. 2011. Growth, physiological and molecular traits in Salicaceae trees investigated for phytoremediation of heavy metals and organics. Tree Physiology. 31(12): 1319-34. [Internet] [cited 06 March 2016]. Available from: http://treephys.oxfordjournals.org/content/31/12/1319

Microsoft Corp. Released 2016. Microsoft Excel 2016. Seattle, WA. Microsost Corp.

[NECi] The Nitrate Elimination Co., Inc. 2012. Suggested protocols for drying samples to constant weight. 1 pp. [Internet] [cited 06 March 2016]. Available from: http://nitrate.com/sites/default/files/attached_files/Suggested%20Protocols%20for%20Drying%20Samples%20to%20Constant%20Weightpdf_0.pdf

Nisbet ICT, LaGoy PK. 1992. Toxic equivalency factor (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Regulatory Toxicology and Pharmacology. 16(3): 290-300. [Internet] [cited 06 March 2016]. Available from: http://www.sciencedirect.com/science/article/pii/027323009290009X

Pourrut B, Shahid M, Dumat C, Winterton P, Pinelli E. 2011. Lead uptake, toxicity, and detoxification in plants. Rev. Environ. Contam. Toxicol. 213: 113-36 [Internet] [cited 09 April 2016]. Available from: http://link.springer.com/chapter/10.1007%2F978-1-4419-9860-6_4

Pena-Icart M, Villanueva Tagle ME, Alonso-Hernandez C, Rodriguez Hernandez J, Behar M, Pomares Alfonso MS. 2011. Comparative study of digestion methods EPA 3050B (HNO_3-H_2O_2-HCl) and ISO 11466.3 (aqua regia) for Cu, Ni and Pb contamination assessment in marine sediments. Marine Environmental Research. 72(1-2): 60-6. [Internet] [cited 06 March 2016]. Available from: http://www.sciencedirect.com/science/article/pii/S0141113611000559

Rawson HM, Begg JE, Woodward RG. 1977. The effect of atmospheric humidity on photosynthesis, transpiration and water use efficiency of leaves of several plant species. Planta. 134(1): 5-10. [Internet] [cited 09 April 2016]. Available from: http://link.springer.com/article/10.1007/BF00390086

Smith DB, Cannon WF, Woodruff LG, Solano F, Kilburn JE, Fey DL. 2013. Geochemical and mineralogical data for soils of the conterminous United States: U.S. Geological Survey Data Series 801. 19 pp. [Internet] [cited 06 March 2016]. Available from: http://pubs.usgs.gov/ds/801/

Song YF, Jing X, Fleischmann S, Wilke BM. 2002. Comparative study of extraction methods for the determination of PAHs from contaminated soils and sediments. Chemosphere. 48: 993-1001. [Internet] [cited 06 March 2016]. Available from: http://www.researchgate.net/profile/Yufang_Song/publication/11166361_Comparative

_study_of_extraction_methods_for_the_determination_of_PAHs_from_contaminated_soils_and_sediments/links/0deec530e88f1acd62000000.pdf

Spriggs T, Banks MK, Schwab P. 2005. Phytoremediation of polycyclic aromatic hydrocarbons in manufactured gas plant-impacted soil. Journal of Environmental Quality. 34(5): 1755-62. [Internet] [cited 06 March 2016]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16151227

Statistics Canada. 2015. Canadian Climate Normals 1981-2010 Station Data: Edmonton International Airport. Government of Canada. Ottawa, On. 1 webpage. [Internet] [cited 09 April 2016]. Available from: http://climate.weather.gc.ca/climate_normals/results_1981_2010_e.html?stnID=1865&lang=e&StationName=edmonton&SearchType=Contains&stnNameSubmit=go&dCode=1

Thygesen RS, Trapp S. 2002. Phytotoxicity of polycyclic aromatic hydrocarbons to willow trees. Journal of Soils and Sediments. 2(2): 77-82. [Internet] [cited 13 April 2016]. Available from: http://link.springer.com/article/10.1007%2FBF02987875

Trejo-Tellez LI, Gomez-Merino FC. 2012. Nutrient solution for hydroponic systems. InTech. Rijeka, Croatia. 23 pp. [Internet] [cited 13 April 2016]. Available from: http://cdn.intechopen.com/pdfs-wm/33765.pdf

[USEPA] United States Environmental Protection Agency. 2015. Remediation technology descriptions for cleaning up contaminated sites. 1 webpage. [Internet] [cited 13 April 2016]. Available from: https://www.epa.gov/remedytech/remediation-technology-descriptions-cleaning-contaminated-sites

Valls M, Lorenzo VD. 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiology Reviews. 26: 327-38. [Internet] [cited 13 April 2016].Available from: http://femsre.oxfordjournals.org/content/26/4/327.abstract

Vervaeke P, Luyssaert S, Mertens J, Meers E, Tack FM, Lust N. 2003. Phytoremediation prospects of willow stands on contaminated sediment: a field trial. Environmental Pollution 126(2): 275-82. [Internet] [cited 06 March 2016]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12927498

Wang Z, MacFarlane DW. 2012. Evaluating the biomass production of coppiced willow and poplar clones in Michigan, USA, over multiple rotations and different growing conditions. Biomass and Bioenergy. 46: 380-88. [Internet] [cited 09 April 2016]. Available from: http://www.sciencedirect.com.ezproxy.aec.talonline.ca/science/article/pii/S0961953412003170

Wani BA, Khan A, Bodha RH. 2011. *Salix*: A viable option for phytoremediation. African Journal of Environmental Science and Technology. 5(8): 567-71. [Internet] [cited 06 March 2011]. Available from: http://www.ajol.info/index.php/ajest/article/view/72048/0

Wuana RA, Okieimen FE. 2011. Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. ISRN Ecology. 2011: 20 pp. [Internet] [cited 06 March 2016]. Available from: http://www.hindawi.com/journals/isrn/2011/402647/cta/

Zhivotovsky OP, Kuzovkina YA, Schullthess CP, Morris T, Pettinelli D. 2011. Lead uptake and translocation by willows in pot and field experiments. International Journal Phytoremediation. 13(8): 731-49. [Internet] [cited 06 March 2016]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21972515