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Chemically Induced Phytoextraction of Lead (Pb) Contaminated Soil by Switchgrass (Panicum virgatum L.), assisted with BAP and NTA applications

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Chemically Induced Phytoextraction of Lead (Pb) Contaminated Soil by Switchgrass (*Panicum virgatum* L.), assisted with BAP and NTA applications

by
Genna Hart

A Thesis Presented in Partial Fulfillment of Requirements for the Master of Science in Integrative Biology for the Department of Ecology, Evolution and Organismal Biology

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Thesis Chair: Dr. Sigurdur Greipsson
Associate Professor of Biology
Kennesaw State University

Committee Chair: Dr. Marina Koether
Professor of Chemistry
Kennesaw State University

Committee Chair: Dr. Thomas McElroy
Associate Professor of Biology
Kennesaw State University

Abstract

The accumulation of lead (Pb) deposits in soil is a growing global concern. Phytoextraction of Pb-contaminated soil can be enhanced by soil chelation to increase the bioavailability of Pb. In the initial study nitrilotriacetic acid (NTA) 5mM, the alkyl polyglucoside (APG) Triton X-100 (2%), and nano-silica were applied to switchgrass (*Panicum virgatum* L.) growing in 5L pots of Pb-contaminated soil. The second experiment used 10mM NTA, the alkylpolyglycoside Triton X-100 (2%), foliar application of the plant growth regulator 6-benzylaminopurine (BAP) twice per week, Triple-12® nutrients, and Infuse™ a soil fungicide which were applied to switchgrass Plants were grown in a controlled greenhouse environment in 5L pots with Pb-contaminated soil (5802.5 mg kg^{-1}) collected from a former Superfund site in Cedartown, GA. Following harvest, plant material was dried, acid digested and chemically analyzed for lead (Pb), aluminum (Al) and iron (Fe) using an ICP-OES. Switchgrass treated with 5mM NTA showed an increased average Pb concentration of 858 mg kg^{-1}, but this was further improved with applications of 10mM NTA, with an average concentration of 3196.5 mg kg^{-1} in plants foliage. Combined applications of NTA and APG and foliar applications of BAP and nutrients resulted in the highest average Pb concentration of 5,355 mg kg^{-1}. The results suggested that soil applications of 10 mM NTA and Triton X-100, Infuse™ and foliar applications of BAP could be a beneficial technique for phytoremediation of Pb contaminated soils.

Acknowledgements

I would like to thank my supervisor and PI Dr. Sigurdur Greipsson for his knowledge, guidance, graduate program advice, and patience during my thesis work. Thank you for building the foundations for this project and encouraging me to learn additional skills along the way. I would also like to thank my committee chairs Dr. Marina Koether, for the use of the ICP-OES, her expansive knowledge and encouragement throughout this process and Dr. Thomas McElroy for their support. I would also like to thank Dr. McElroy for his assistance with my thesis proposal, his helpful advice about graduate school and for teaching foundational skills related to graduate coursework. Thank you, Dr. Melanie Griffin, Dr. Troy Mutchler, Dr. Dovid Kozlovsky, Dr. Susan Smith, Dr. Paula Jackson, Desiree Day, Dr. Joel McNeal, Dr. Mario Bretfeld, Dr. Daniel Ferreira and many others for your support and guidance.

Thank you, Will Beeson and my lab mate Kylie Stover, for your assistance in the lab. Many thanks to my undergraduate research assistants, Nailah Carter, and Nikki Shahi for support in lab and the greenhouse during this time. To my fellow graduate students, thank you for your encouragement and friendship, this cohort has made my time here at KSU even more enriching.

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List of abbreviations

Calculations:

ANOVA	Analysis of Variance
Fisher's LSD	Fisher's Least Significant Difference

Chemical names:

APGs	Alkylpolyglucosides
BAP	6-Benzylaminopurine
EDTA	Ethylenediaminetetraacetic acid
GA_3	Gibberellic acid
PGRs	Plant growth regulators
SA	Salicylic acid
SNP	Nitric oxide donor
Tergitol™ 15-S-9	Polyethylene glycol ether

Elements:

Al	Aluminum
Pb	Lead
Fe	Iron
K	Potassium
N	Nitrogen
P	Phosphorus

General:

AMF	Arbuscular Mycorrhizal Fungi
EPA	Environmental Protection Agency
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectrometry

Introduction

Sources of lead (Pb) soil contamination

Accumulation of lead (Pb) deposits in soil is a growing concern due to the health implications and associated environmental damage [Anyanwu et al. 2018; Eiró et al. 2021]. Common causes of Pb contamination include run-off by factory operations, impurities in fertilizers used in farming, leaded gasoline emissions, smelting and mining operations [Zhuang et al. 2006]. Proximity to industries and former mining sites can also put communities at risk of Pb exposure [Mufalo et al. 2021]. Also, previous locations for smelting and recycling maintain high levels of Pb in nearby soil long afterwards [De Araújo and Do Nascimento 2010; Greipsson et al 2013; González-Chávez et al 2019]. Prior application of pesticides, including herbicides, previously aided in plant growth overall were identified as sources of heavy metal contamination [Alengebawy et al. 2021; Varshney et al. 2012]. Several studies have demonstrated that Pb contamination can come from a variety of sources and can affect the surrounding environment [Al-Sabbak et al. 2012; Chen et al. 2012; Fasinu and Orisakwe 2013; Wu et al. 2016].

Lead contamination and exposure

Lead deposits in soil is a rising global concern in environmental analyses along with the dangerous future implications of soil contamination [Alsafran et al. 2021; Fasinu and Orisakwe 2013; He et al. 2015; Lwin et al. 2018]. Growing concentrations of heavy metal deposits in soils and their impact on the human body is a concern [Jan et al. 2015; Joosse 2022]. One drawback of commercial agriculture is the use of pesticides or herbicides which increases the number of heavy metals being deposited into soil [Alengebawy et al. 2021]. Also, sewage run off into residential areas in countries that are developing, and improper chemical dumping, can both have lasting effects on the soil [Brtnický et al. 2019]. As deposits continue to accumulate in the soil, concerns have been raised regarding translocation of Pb into consumables and water sources [Alsafran et al. 2019; Chen et al. 2012; Fasinu and Orisakwe 2013; Zhuang et al. 2006]. Urban populations are most at risk for Pb exposure and the most effective and least invasive way to reduce this risk is to remove Pb from soils through phytoremediation [Filippelli and Laidlaw 2010]. The reduction of heavy metals in soil can benefit future crops and humans living in urban locations close to these contaminants [Filippelli et al. 2005; Zhuang et al. 2006].

Risks of Pb in soil for humans

Recurring accumulating deposits of Pb in the soil or water systems has the potential to negatively impact its environment [He et al. 2015; Nazir et al. 2020]. Accumulation of Pb in soils has raised concerns about potential translocation of heavy metals from plant roots into the leaves [Zhuang et al. 2006]. Although federal organizations like the Environmental Protection Agency (EPA) have made guidelines to limit further Pb contamination and inform the public of the risks associated with Pb exposure, the current task at hand is to reduce the number and concentration of metals present in the soil [ACCLPP 2012; Filippelli and Laidlaw 2010]. In fact, there is no safe level of Pb exposure for humans, therefore any exposure to deposits of Pb in the environment has the potential to cause damaging human interactions later in life [Eiró et al. 2021, McFarland et al. 2022]. The ability to cross the blood-bone barrier is a major concern as Pb can remain present in the body for an unspecified length of time [Filippelli et al. 2005]. Urban populations have an increased risk of Pb exposure [Brtnický et al. 2019; Filippelli and Laidlaw 2010]. Long-term exposure to low-level Pb is associated with neurological damages which includes cognitive dysfunction in children [ACCLPP 2012; Canfield et al. 2006; Martinez-Finley et al. 2012; Paulson et al. 2019], along with Alzheimer's [Bakulski et al. 2012; Fathabadi et al.

2018] and Parkinson's diseases later in life [Chen et al. 2017]. Negative cognitive outcomes have been associated with children who had blood Pb levels of 10 mg/l or less [ACCLPP 2012; McFarland et al. 2022; Skerfving et al. 2015].

Switchgrass as a second-generation biofuel crop

Switchgrass (*Panicum virgatum* L.) can grow in diverse environments and is a resilient plant once it is established [Fike at al. 2017]. The phytoremediation literature focuses mainly on grasses and similar monocots as they have a short growth process, are cost effective and are typically readily available in the region of interest [Chen et al. 2012; Johnson et al. 2015]. Switchgrass has several ideal characteristics coveted for phytoremediation: it has a high yield biomass and has shown resistance to heavy metal tolerance in previous studies [Chen et al. 2012; Gilly 2020; Greipsson 2011]. Switchgrass is utilized in the energy industry as a second-generation biofuel grass because it consistently yields acceptable harvestable biomass, has large ethanol conversion potential, and can grow in diverse ecosystems [Boyer et al. 2013; Hernández-Allica et al. 2008; Min et al. 2017]. Similar grasses easily show the effects of phytotoxicity or chlorosis rapidly and present a yellow or purple color as a positive indicator of heavy metal toxicity [Foy et al. 1978]. The outcome of the phytoextraction process has the potential to remove significant amounts of heavy metal contaminants from soil and restore the impacted soil for future uses [Greipsson et al. 2022; Huang et al. 1997).

Chelation and the phytoextraction process

The removal of Pb-contaminants from soil can be accomplished through phytoextraction, an emerging cost-effective heavy metal remediation technique [Garbisu and Alkorta 2001;

Greipsson 2011]. Phytoremediation strategies include phytoextraction, using plants to remove heavy metals from contaminated soils [Greipsson 2011; Yan et al. 2020]. Phytoextraction usually requires acidification or chelation of soil which further facilitates the absorption and isolation of Pb in plant material [Foy et al. 1978]. Soil chelation may be improved based on the activation efficiency of the chelating agent used, but that is widely dependent on specific heavy metal interactions within soil [Liu et al. 2020]. Nitrilotriacetic acid (NTA), a derivative of EDTA (ethylenediaminetetraacetic acid) was the chelator used in this study. Successful chelation of Pb in soil has been shown to increase the bioavailability of Pb [Aderholt et al. 2017; Beavers 2016]. Increased motility of Pb in soil is often associated with higher concentrations of Pb in plants [Johnson et al. 2015]. One method of mobilization involves lowering the pH of the contaminated soil which allows Pb ions to mobilize and translocate ions into plant tissues, where it is stored in the leaves [Johnson et al. 2015]. Several other methods of Pb mobilization exist such as, oxidation or heating the soil of interest, but these processes can be expensive and may degrade the soil even further [Filgueiras et al. 2002].

Role of Arbuscular Mycorrhizal Fungi (AMF) in phytoremediation

Symbiosis between the arbuscular mycorrhizal fungi (AMF) in the soil environment and the plant may be beneficial for long-term sustained growth and stress resistance [Cabral et al. 2015; Cruz-Paredes et al. 2021; Yang et al. 2016]. One benefit of AMF is the ability to aid in adaptive tolerance when plants undergo stress or exposure to a contaminated environment. [Azcón-Aguilar et al. 2002; Hovsepyan and Greipsson 2004]. A symbiotic site for chemical exchange and nutrient exchange is created between plants and AMF [van der Heijden et al. 2015]. The addition of plant hormones and uptake enhancers can be further regulated depending on the specific AMF present [Greipsson 2011; Phieler et al. 2014]. Studies have suggested that the suppression of AMF can encourage the plant's heavy metal uptake from the soil and aid the plant in defense against metal toxicity [Hovsepyan et al. 2004; Perry et al 2012]. Other studies identified AMF tubules as a direct deterrent against Pb translocation from the roots into other foliage that could be isolated during harvest [Hildebrandt et al. 2007; Xu et al. 2014]. Soil fungicide may be used to arrest AMF activities to encourage phytoextraction of Pb into tissues of interest [Gilly 2020; Hart et al. 2022]. Further consideration of AMF use may be beneficial to future phytoremediation processes as alteration of the pH range may make plants more susceptible to heavy metal toxicity when AMF is suppressed [Coninx et al. 2017].

AMF suppression

Infuse™ and its properties of pathogenic fungal resistance continue to be considered for future studies [Hovsepyan and Greipsson 2004]. Infuse™(benomyl) is a soil fungicide used to suppress AMF colonization within soil in phytoremediation research [Gilly 2020; Hart et al. 2022; Johnson et al. 2015]. Previous studies indicated that applications of another soil-fungicide benomyl prior to applying chemical chelates improved the uptake of Pb [Perry et al. 2012]. The application of Infuse™ reduced AMF colonization of root cells and treated plants showed an increase in Pb in switchgrass [Aderholt et al. 2017]. Additional studies specific to AMF suppression and its benefit to uptake of Pb and other heavy metals is suggested.

Utilizing the active growth regulator BAP

Plant growth regulators are used to modify the plant's hormonal balance and stress endurance in reference to external mycorrhizal environments [Beavers et al. 2021; Evangelou et al. 2007].

Several studies have included the addition of growth regulators to further enhance the plant's ability to accumulate Pb [Aderholt et al. 2017; Gilly 2020; Perry et al. 2012]. A common application of exposing plants to growth regulators is foliar application or direct soil exposure [Benedetto et al. 2011; Matteo et al. 2015]. The growth regulator utilized in this experiment was Promalin®, which contained 6-benzylaminopurine (BAP). The BAP is a cytokine stimulator that has been shown to encourage plant bud growth and further stimulate multiple root growth via cell division [Bouré et al. 2019]. Combined applications of growth regulators can benefit the phytoremediation process and aid the plant's ability to uptake heavy metals [Aftab and Hakeem 2021].

Chemical chelates used in phytoremediation

The addition of chemical chelates have been used to benefit the phytoremediation process and improve Pb availability. Varying concentrations of chelates were used depending on the soil, type of plants grown and focus of the phytoremediation study [Aderholt et al. 2017; Hovsepyan and Greipsson 2004]. Chelates are chemicals that can increase heavy metal availability during phytoremediation [Aderholt et al. 2017; Beavers 2016; Perry et al. 2012]. EDTA has been largely studied and identified as a successful chelator in several phytoremediation studies but has raised environmental concerns related to its limited biodegradability [Aderholt et al. 2017; Elliott and Brown 1986; Johnson et al. 2015; Gilly 2020; Greipsson 2011]. To limit potential environmental impacts, biodegradable chemicals other than EDTA including NTA and natural chelates like citric acid have been tested in phytoremediation studies as well [Chen et al. 2012; Shinta et al.2021]. Citric acid has been identified as a natural chelate alternative to EDTA due to its ability to be broken down by microorganisms within soil [Aderholt et al. 2017; Beavers 2016;

De Araújo et al. 2010; Freitas et al. 2013]. A benefit to this limited amount of time spent in soil is a reduction in risk for groundwater contamination [Oviedo and Rodríguez 2003]. Future research regarding alternative chemical chelates and their associated applications may benefit the phytoremediation process [Aghelan et al. 2021; Chen et al. 2012].

Foliar nutrient application

The foliar nutrient solution impacts plant growth by acting as a growth regulator to plants that undergo stress from heavy metal uptake [Sharma et al. 2020; Zottini et al. 2007]. Additional research focusing on salicylic acid and other alternative growth regulators in this field could benefit remediated plant’s ability to uptake heavy metals during the phytoremediation processes [Beavers et al. 2021; Greipsson et al. 2021].

Focus of the study

The focus of the experiments presented in this thesis was to determine the efficacy of Pb uptake through the phytoextraction process using switchgrass. The first experiment examined methods of Pb, Al and Fe uptake in switchgrass by evaluating the effect of chemical chelates (NTA) with the addition of permeating agent, Triton-X (100) and nano-silica. The second experiment determined the efficacy of Pb, Al and Fe uptake in switchgrass through the combined addition of chelating agents, phytohormones, surfactants, and a complete nutrient solution which included: nitrilotriacetic acid (NTA), Triple-12® (foliar nutrients), alkyl polyglycosides (APG ±Triton X-100) and the foliar application of Promalin® (includes BAP).

Objective 1 To examine methods of Pb uptake in switchgrass (Panicum virgatum L.) by evaluating the effect of chemical chelates 5mM Nitrilotriacetic acid (NTA) with the addition of permeating agent, Triton X-100 or Nano silica.

Objective 2: To determine the efficacy of lead uptake in switchgrass (Panicum virgatum L.) through the addition of chelating agents, phytohormones, surfactants, and uptake agents including: 10mM Nitrilotriacetic acid (NTA), Triple-12®, Triton X-100 (APG), Promalin® (includes BAP (6-benzyladenine)) and Infuse™ (fungicide).

Materials and Methods

One hundred switchgrass seeds were allowed to germinate in a sandbox environment in the greenhouse for one month before the seedlings were transplanted into twelve, 5L pots containing Pb contaminated soil from a former Superfund Site in Cedartown, GA. Plants were grown in a controlled environment in Kennesaw State University's Science greenhouse. Plants were supplemented with white, fluorescent light for approximately 16 hours per day. Temperature (average 25 °C) and humidity was monitored and maintained at a near-constant level.

Soil

Soil for this experiment was collected from a previous contaminated Superfund site in Cedartown, GA. Following the Agency for Toxic Substances and Disease registry (ASTR) reports in 2006, the soil remains Pb-contaminated with some high levels up to 260,000 mg kg^{-1} present [ATSDR 2006]. Soil samples were randomly selected from the site and were homogenously mixed prior to planting to ensure uniform heavy metal concentrations across experimental treatments.

Experiment 1

In the first experiment, twelve switchgrass plants were grown with three plants in each treatment group. The goal of the first experiment was to determine the most effective alkyl polyglucoside (APG) with respect to Pb uptake.

Switchgrass seeds were germinated (as described above) and transplanted into 5L pots containing the Pb-contaminated soil from Cedartown, GA. In this study, chemically enhanced phytoextraction using the chelator nitrilotriacetic acid 5mM (NTA) with and without nano-silica or the alkyl polyglucoside (APG) Triton X-100 (TX 100) was tested [Hart et al. 2022]. At 56 dap the plants were subjected to four different experimental treatments: (1.) control (2.) nitrilotriacetic acid (NTA), (3.) NTA + nano-silica; and (4.) NTA + APG. The NTA (5 mmol kg^{-1} soil) solution was made by mixing powdered NTA with an aqueous 10 N NaOH solution. The Triton X-100 (2%) was added to the NTA + APG solution in 100mL / 1L increments.

Experiment 2

In the second experiment sixteen switchgrass plants were grown in the greenhouse. Switchgrass seeds were germinated (as described above) and transplanted into 5L pots containing the Pb-contaminated soil. Plants were grown under controlled conditions in the Kennesaw State University Research Greenhouse. Four replicated plants served as the control and did not receive any phytohormone or APG treatments throughout the duration of the experiment. Four replicated pots were in each of the following treatments groups: (1.) BAP and NTA + Triton X-100 (2%), with foliar applications of Promalin® (1.67 mL/L), (2.) NTA 10mM and Triton X-100, (3.) Triple-12® (10mL/L) and NTA 10mM + Triton X-100, with foliar applications of Triple-12®, and (4.) control. The Triple-12® nutrient solution made by Growth Products Ltd. was used by mixing 5mL Triple-12® into 1L H_20. The Triple-1®2 foliar nutrient solution contained slow-release Nitrogen (N), and including 12% Nitrogen, 2% Phosphorous and 12% soluble Potash. The NTA (10 mmol kg−1 soil) solution was made by mixing powdered NTA with an aqueous 10 M NaOH solution. The Triton X-100 (2%) was added to the NTA + APG solution in 100mL / 1L increments. The soil-fungicide Promalin® (propiconazole) solution, created by Valent BioSciences was made by mixing 1.67mL of aqueous Promalin® into 1L H_20. A complete nutrient solution containing: 332 mg L^{-1} Nitrogen (N), 111 mg L^{-1} Phosphorous (P) and 222 mg L^{-1} K (Potassium) was supplied to all plants twice a week for 8 weeks until 64 DAP. All plants were watered with 200mL of water three times per week until 80 days after planting (dap). After 80 dap, plants with foliar applications were sprayed three times per week. The soil fungicide Infuse™ was applied after the 152 dap. The soil-fungicide was given to all plants for two weeks for suppression of symbiotic arbuscular mycorrhizal fungi. After 166 dap the 10mM NTA + Triton X-100 (2%) solution was applied to treatment groups 2, 3, and 4 three times per week until plants were harvested at 179 dap.

Harvesting

Plants in all treatments and control groups were grown until fully mature. Plants were harvested following appearance of chlorosis and/or heavy metal toxicity and the presence of purple leaf tips. The longest leaf, most affected leaves (visible chlorosis) and remaining leaves were collected from mature plants in all treatments. Harvested plant leaves were placed in paper bags, labeled, and dried in an oven at 65°C for 48 hours prior to acid digestion.

Acid digestion and chemical analysis

Dried plant samples were weighed, placed in 100mL disposable acid digestion tubes (Environmental Express®, Inc) following a modified EPA method 3050 B and digested in a combined solution of 38% HCL (10.0mL) and 70% HNO_3 (10.0mL) [U.S. EPA 1996]. Plant samples were capped and placed in the solution for 24 hours until materials were mostly digested, and leaves appeared colorless. Digestion tubes were then placed in the Hotblock Express System™ and refluxed for 55 minutes at 90-95°C [U.S. EPA 1996]. Digestion tubes were allowed to cool to room temperature. Cooled digestion tubes had deionized water added to them up to the 100mL line and were vacuum filtered through 0.45-micron filters. Following filtration each sample was pipetted into 50 mL centrifuge tubes stored in a refrigerator until chemical analysis was performed by Dr. Marina Koether using inductively coupled plasma mass spectrometry (ICP-OES: Perkin Elmer Avio 200). Trace metals Al, Fe and Pb concentrations were identified in each sample collected and tested in triplicate against each standard. Standards for the metals of interest were generated by Dr. Marina Koether from Kennesaw State University's Department of Chemistry and Biochemistry.

Statistical Analysis

Concentrations of Al, Pb and Fe were calculated and statistically analyzed through Analysis of Variance (ANOVA), average and standard deviation calculations, and Fisher's least significant difference (LSD) for post-hoc analyses. Excel's data analysis package along with the R studio's packages ggplot2, ggpubr, tidyverse, broom, AICcmodavg were used to perform the statistical tests of interest [Robinson et al. 2021; Rstudio Team 2020]. Lead data was transformed, and log transformation was employed via Excel. In terms of exploratory data analysis methods, normality tests were not applicable. The main statistical method used for analysis was one-way ANOVA through Excel. Data reorganization, determination of ANOVA type and data variance were verified in Rstudio with a combination of the following packages: ggplot2, ggpubr, tidyverse, broom and AICmodavg [Rstudio Team 2020; Wickham 2020]. The ANOVA tables were calculated to identify interactions between variables. Fisher's (LSD) was used to determine the significance of p-values. Statistically significant p-values were accepted at $p<0.05$ and 5% (LSD) confidence levels, $\alpha=0.05$.

Results

Experiment 1: Effect of chemical chelates on switchgrass Pb, Al, Fe foliar concentrations

Application of 5mM NTA + Triton X-100 resulted in statistically significant p-values of average Pb concentrations in the foliage when compared to the average Pb concentration from the control plants (Figure 1a). Plants in treatments receiving 5mM NTA showed an increase in average Pb, Al and Fe concentrations compared to plants in the control treatment (Figure 1a – 1c). Plants treated with NTA ± Triton X-100 had higher average Pb concentrations following statistical analysis compared to all other treatment groups (Figure 1a). Switchgrass treated with NTA + Triton X-100 had statistically significant p-values when compared to other treatments (Figure

4b). Overall, applications of 5mM NTA and Triton X-100 had a positive effect on Pb uptake. During the continued applications of NTA, the tips of plant leaves turned yellow and purple as the effect of metal induced chlorosis and toxicity were observed. None of the plants from any treatments had statistically significant p-values associated with Fe uptake (Figure 1c).

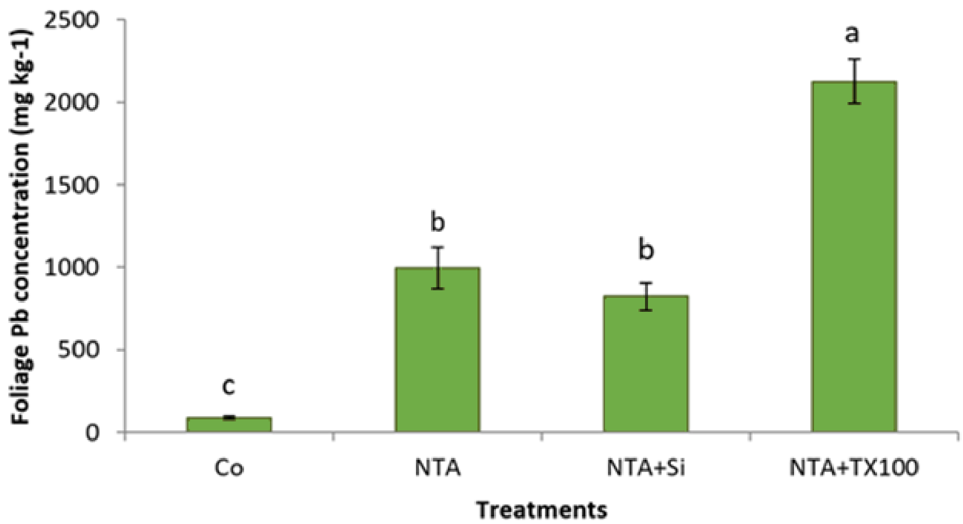

Figure 1a: Average Pb concentration (mg/L) ±SD of *Panicum virgatum* as identified by ICP-OES analyses. Treatment group identifiers are as follows: Control = no treatment, NTA = 5mM nitrilotriacetic acid, NTA+Si = 5mM nitrilotriacetic acid + nano silica, NTA+TX100= 5mM nitrilotriacetic acid + Triton X-100. Means for columns with same letters are not significantly different ($\alpha = 0.05$).

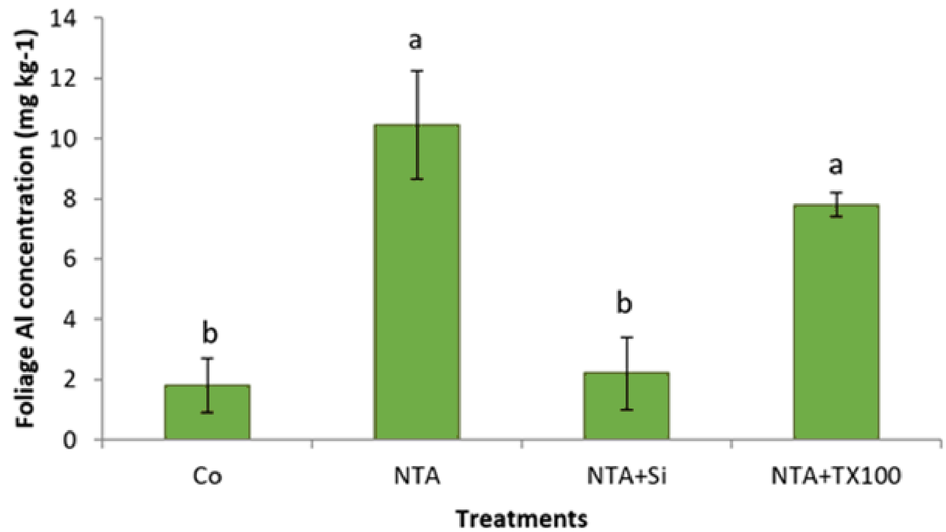

Figure 1b: Average Al concentration (mg/L) ±SD of *Panicum virgatum* as identified by ICP-OES analyses Treatment group identifiers are as follows: Control = no treatment, NTA = 5mM nitrilotriacetic acid, NTA+Si = 5mM nitrilotriacetic acid + nano silica, NTA+TX100= 5mM nitrilotriacetic acid + Triton X-100. Means for columns with same letters are not significantly different (α = 0.05).

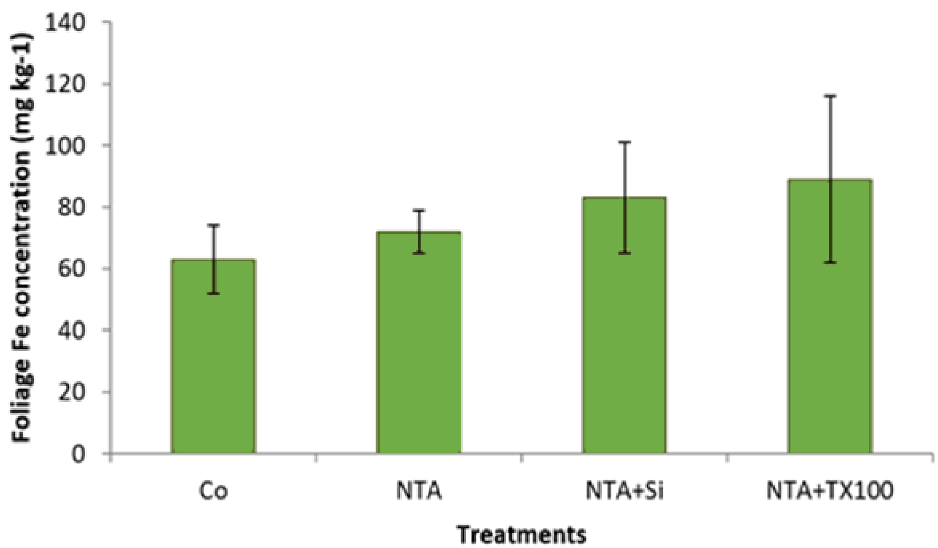

Figure 1c: Average Fe concentration (mg/L) ±SD of *Panicum virgatum* as identified by ICP-OES analyses. Treatment group identifiers are as follows: Control = no treatment, NTA = 5mM nitrilotriacetic acid, NTA+Si = 5mM nitrilotriacetic acid + nano silica, NTA+TX100= 5mM nitrilotriacetic acid + Triton X-100. Means for columns with same letters are not significantly different (α = 0.05).

Figure 2: Untreated *Panicum virgatum* L. plants located on a wire benchtop in KSU's Research Greenhouse.

Figure 3: Isolated *Panicum virgatum* L. leaves displaying signs of chlorosis (yellowing).

Figure 4: Isolated *Panicum virgatum* L. leaves displaying early signs of necrosis.

Experiment 2: Effect of APG and chemical chelates on switchgrass heavy metal concentration and shoot length

Two measurements were collected from each plant during harvest. Shoots were organized into two categories: longest leaf and leaves most affected by chlorosis. All plants treated with additional chemicals had longer average shoot lengths compared to the control plants (Figure 5). Plants with NTA + Triton X-100 had the longest average shoot length across all of the compared treatments (Figure 5).

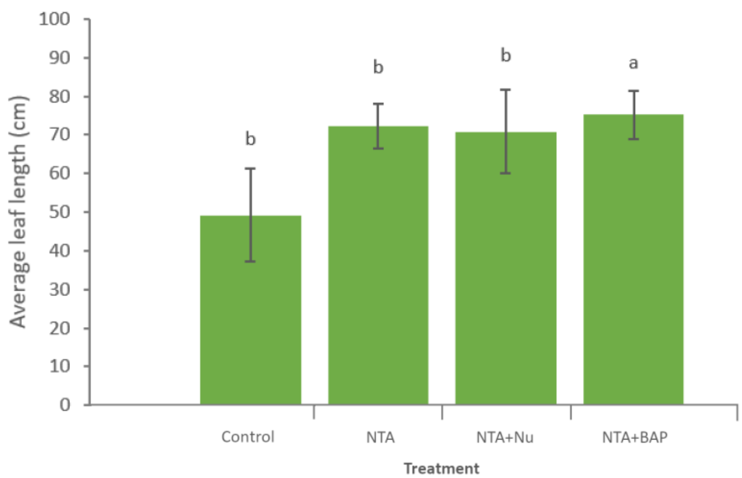

Figure 5: Average shoot length (cm) (±SD) of *Panicum virgatum* as measured by longest leaf length after 8 weeks of growth following applications of benzyl aminopurine (BAP), Triple-12® and 10mM nitrilotriacetic acid (NTA). Treatment group identifiers are as follows: NTA+Nu = Triple-12® + NTA 10mM + Triton -100, NTA = NTA 10mM + Triton X-100, NTA+BAP = Promalin® + NTA 10mM + Triton X-100. Means for columns with same letters are not significantly different ($\alpha = 0.05$).

Treatment groups with 10mM NTA and BAP had the highest average Pb concentration across all treatments (Figures 6a-6c). Plants treated with 10mM NTA had a greater increase in average Pb concentrations than plants treated with 5mM NTA alone (Figure 6a, Figure 6a). One plant in the

10mM NTA and BAP treatment showed the highest Pb concentration of 10,820 mg kg^{-1} Pb (Figure 6a). Experimental treatments of Triple-12®, BAP and NTA + Triton X-100 showed statistically significant p-values associated with Pb concentrations (Figure 6a). Lead analysis for plants treated with NTA + Triton X-100, BAP, and Triple-12® respectively, showed a p-value, $p<0.01$. Plants treated with NTA + Triton X-100, BAP and Triple-12® had a statistically significant p-value for Al, ($p<0.01$), when compared to the control plants. In reference to Fe, all treatments had a significant p-value, $p<0.05$. Combined applications of NTA, APG (Triton X-100) and BAP showed a significant increase in Pb, Al and Fe compared to the control (Figures 6a-6c). During the continued applications of NTA, the tips of plant leaves turned yellow and purple as the metal induced effect of chlorosis and metal toxicity were observed. Chlorosis was first observed following NTA applications after 170 dap.

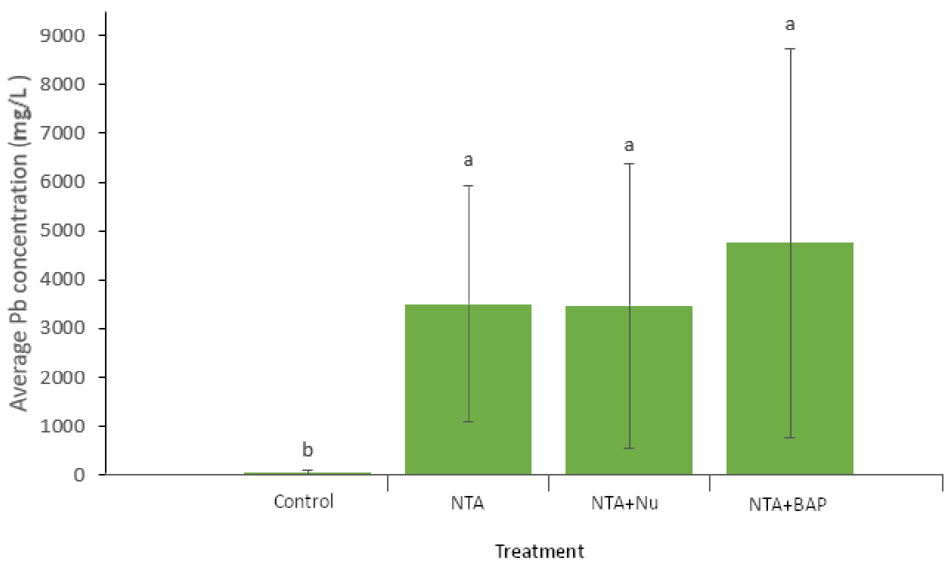

Figure 6a: Average Pb concentration (mg/L) ±SD of *Panicum virgatum* as identified by ICP-OES analyses. Treatment group identifiers are as follows: NTA = NTA 10mM + Triton X-100, NTA+Nu = Triple-12® + NTA 10mM + Triton -100, NTA+BAP = Promalin® + NTA 10mM + Triton X-100. Means for columns with same letters are not significantly different ($\alpha = 0.05$).

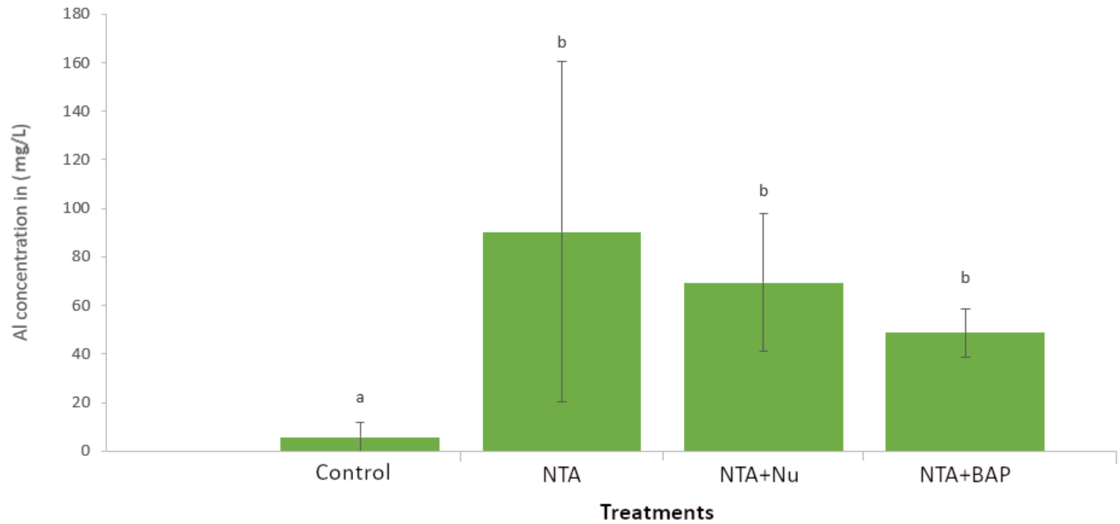

Figure 6b: Average Al concentration (mg/L) ±SD of *Panicum virgatum* as identified by ICP-OES analyses. Treatment group identifiers are as follows: NTA = NTA 10mM + Triton X-100, NTA+Nu = Triple-12® + NTA 10mM + Triton -100, NTA+BAP = Promalin ®+ NTA 10mM + Triton X-100. Means for columns with same letters are not significantly different (α = 0.05).

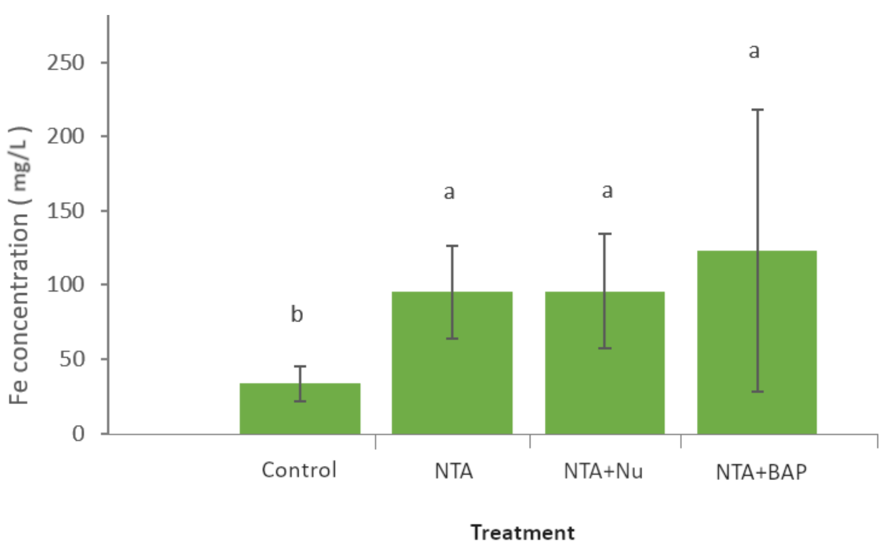

Figure 6c: Average Fe concentration (mg/L) ±SD of *Panicum virgatum* as identified by ICP-OES analyses. Treatment group identifiers are as follows: NTA = NTA 10mM + Triton X-100, NTA+Nu = Triple-12® + NTA 10mM + Triton -100, NTA+BAP = Promalin® + NTA 10mM + Triton X-100. Means for columns with same letters are not significantly different (α = 0.05).

Discussion

Effects of chemical chelates on switchgrass growth

The addition of chemical chelates has been shown to enhance Pb uptake in switchgrass (*Panicum virgatum* L.) [Beavers et al. 2021; Evangelou et al. 2007; Hart et al. 2022]. The combined application of NTA +APG (Triton X-100), BAP® and Infuse™ may benefit future phytoextraction experiments. This was further supported by the largest Pb accumulation in one plant from 10mM NTA + APG and BAP treatment group (Figure 3a). Lead bioavailability increased with the application of NTA, and the use of this chelate could be beneficial as it degrades in remediated soil [Gilly 2020; Hart et al. 2022; Hu et al. 2017]. Further exploration of NTA and APG application can be investigated to determine their phytoremediation efficacy [Gilly 2020; Hart et al. 2022].

Chemical chelates and exploration of additional APGs

Additional APGs can be explored in future studies as their applications may result in increased Pb uptake in switchgrass. Nitrilotriacetic acid (NTA) has been utilized to increase heavy metal bioavailability [Gilly 2020; Hart et al. 2022; Hu et al. 2017] The NTA utilization has shown to be effective in increasing the bioavailability of Pb when applied 10mM doses [Hart et al. 2022]. The NTA is more biodegradable in soil compared to its predecessor EDTA [De Araújo, J., and Do Nascimento, C 2010; Elliot and Brown 1989]. The environmental benefit, cost of NTA, and chelating effect are strong motivators to utilize this chemical [Hu et al. 2017]. Although EDTA has previously been utilized as an effective phytoextraction agent, its environmental persistence has encouraged researchers to utilize other chelates [Oviedo et al. 2003; Shinta et al. 2021]. Concerns with EDTA included Pb contamination of groundwater, resistance to breaking down in soil, and EDTA remaining present in soil for a long time after chelation [Aderholt et al. 2017; Evangelou et al. 2007; Gilly 2020; Shinta et al. 2021]. The EDTA application was also associated with decreased plant growth and biomass [Meers et al. 2010]. Despite the current environmental concerns, EDTA has been widely utilized in previous studies as a successful chelating agent that increases the availability of Pb [Aderholt et al. 2017; Greipsson 2011; Johnson et al. 2015].

Several countries in the EU and Canada have limited EDTA use due to its harmful environmental impact and potential to impact aquatic organisms [EU Chemical Bureau 2004; Health Canada 2018]. Limitations of EDTA use including the impact of exposure and risks of extended environmental contamination was addressed in the EU's regulatory agencies [European Chemical Bureau 2004]. Continued assessment of this chemical and defined limits of its use to prevent environmental damage is an area for future research.

Further studies specific to the phytoextraction efficiency of combined applications of surfactants and chelating agents would be beneficial. Triton X-100 was the most effective alkyl polyglycoside (APG) identified in this study [Hart et al. 2022]. Dual applications of a surfactant and NTA improved the absorption, accumulation, and translocation of Pb when applied to triangular club-rush (*Scirpus triqueter* L.) [Hu et al. 2017]. The increased concentration from 5 mM NTA to 10mM NTA along with the APG Triton X-100 was associated with the largest Pb accumulation in switchgrass [Hart et al. 2022]. While applications of APGs and NTA could be successful in phytoremediation research, alternative ratios and timing of applications of these combined chemicals need to be examined further [Liu et al. 2020]. Future research might include the results of APG applications when paired with the optimal concentration of NTA for different plant remediators and different soil types.

Effects of growth promoters and regulators

Research on phytohormones, their effects on plant growth and Pb translocation has been outlined in several studies [Aderholt et al. 2017; Di Matteo et al. 2015]. Growth regulators like BAP have increased plant growth and assisted with heavy metal accumulation in plants in phytoremediation studies [Hart et al. 2022]. Previous studies have shown that exogenous applications of growth regulators were associated with an increase in Pb remediation [Gilly 2020; Greipsson et al. 2022]. When compared to other switchgrass related phytohormone treatments, BAP promoted the largest concentration of Pb in the foliage [Gilly 2020]. Although phytohormone use can benefit the phytoremediation processes, excessive use of these chemicals can be detrimental to plant health [Varshney et al. 2012].

Dual applications of SNP and GA_3

Previous study has shown that phytoextraction processes were improved with combined foliar applications of SNP (nitric oxide donor) and GA_3 (gibberellic acid) [Beavers et al. 2021]. Previous literature has addressed nitric oxide in further attempts to understand its role in cell signaling and the stress response [Khan et al. 2021; Namdjoyan et al. 2013; Neill et al. 2002]. The overall benefit of dual applications of SNP and GA_3 in the phytoremediation process is an area for further examination.

Salicylic acid and plant stress

Salicylic acid (SA) has been utilized in studies as a plant hormone that assists with plants abiotic stress responses and has been shown to promote plant growth [Vicente et al. 2011; Zottini 2007]. Plants treated with SA displayed increased plant growth despite persistent heavy meal stress exposure [Horvath et al. 2007]. Zottini's study (2007) showed that some plants treated with SA were associated with reduced nitrogen reductase activity inhibitors. A decrease in nitrogen reductase inhibitors may result in increased switchgrass growth. Salicylic acid use was addressed in recent literature based on its ability to counteract heavy metal stress experienced by plants [Sharma et al. 2020]. Plants treated with low doses of SA tolerated more stress related to heavy metal uptake [Sharma et al. 2020]. Future studies utilizing SA applications and associated stress tolerance of plants undergoing phytoremediation is recommended.

A recent review article discussed salicylic acid (SA) use and its relationship with plant stress [Sharma et al. 2020]. The SA was used to help plants tolerate oxidative stress and endure the ongoing effects of heavy metal uptake [Zottini et al. 2007]. Exogenous applications of SA were beneficial for plants under stressful conditions [Sharma et al. 2020]. A recent study suggested that combined applications of SA alongside growth promotor DA-6 (Diethyl aminoethyl hexanoate) was associated with an increase in Pb uptake [Greipsson et al. 2022].

Switchgrass as a biofuel and remediator

Switchgrass has previously been identified as a source of biofuel in the energy sector and successful remediator in phytoremediation literature [Balsamo et al. 2015; Min et al. 2017]. This is largely due to the plants amount of harvestable biomass, ethanol conversion potential and its ability to be grown in diverse climates as a remediator [Boyer et al. 2013; Hernández-Allica et al.

2008; Min et al. 2017]. Knowledge of plant biomass allowed researchers to determine a rate of remediation which would provide an environmental solution for Pb contaminated sites that are not currently remediated [Balsamo et al. 2015; Beavers 2016]. Continued research regarding switchgrass and its associated remediation biomass could solidify its use as an effective source of biofuel.

Previous studies on phytoextraction efficiency

Previous experiments have tested the efficacy of phytohormones, varying chelating agents and Pb phytoremediation by switchgrass [Aderholt et al. 2017; Greipsson et al. 2022]. While Aderholt's (2017) use of EDTA did improve the bioavailability of Pb, applications with additional APGs showed greater Pb concentrations in the foliage of plants. The application of EDTA with the addition of phytohormone BAP and citric acid suggested more Pb uptake efficacy compared to EDTA alone [Aderholt et al. 2017;15, Johnson et al. 2015]. Phytohormone use and varying chelating agents paved the way for future experiments focused on switchgrass phytoremediation [Gilly 2020; Johnson et al. 2015]. Symbiotic use of AMF, several chelating agents including phytohormones, fertilizer treatments, and mycorrhizal treatments created a more tolerant environment for plant growth throughout the duration of the experiments [Gilly 2020; Hart et a. 2022]. Exogenous applications of plant growth regulators have been associated with an increase in phytoextraction efficiency [Beavers et al. 2021]. The switch to NTA from its predecessor EDTA was still associated with an increase in Pb bioavailability and a more effective soil degradation time following the experiment [Gilly 2020; Hart et al. 2022]. Alterations of the pH via sodium hydroxide suggests an effective manner to increase Pb uptake in phytoextraction processes [Foy et al. 1978]. The combined applications of phytohormones, chelating agents, and AMF suppression treatments yielded higher levels of Pb in chemical analyses and leaves room for future studies regarding their effect on phytoremediation processes [Beavers et al. 2021; Hart et al. 2022] A more effective remediation process could help reclaim contaminated soils in a shorter time period and would limit heavy metal exposure to individuals in sensitive populations [Fasinu and Orisakwe 2013; Filippelli and Laidlaw 2010; Nissim and Labreccque 2021].

Future research directions

Further study is required to identify the effects of increased concentrations of additional chemical chelates for switchgrass used in phytoextraction. Future experiments can specifically explore increased NTA applications to determine if the additional concentrations continue to be positively associated with Pb uptake. Successful removal of Pb from contaminated soils may benefit more sensitive human populations who are at risk of Pb exposure [Filippelli et al. 2005; Zhuang et al. 2006]. Switchgrass and similar monocots may be ideal models for phytoextraction efforts due to their short growth cycle, large biomass yield and resilience to phytotoxicity in the presence of Pb [Balsamo et al. 2015; Chen et al. 2012; Gilly 2020; Greipsson 2011]. Research encompassing combined applications of NTA, Triton X-100, foliar BAP, and Infuse™ yielding high Pb concentrations, and additional treatment combinations might improve upon past remediation efforts. Successful efforts of heavy metal uptake and Pb removal through phytoextraction could be applied through large scale practices to prevent further Pb accumulation and to remove current deposits from soil.

As demand for work in brownfield sites grows, so does its association with phytoremediation as these sites have high potentials for reclamation [Nissim and Labreccque 2021]. Brownfield sites utilize previously developed areas to continue development, or expansion [EPA 2020]. These redevelopments are further complicated if the soil at the site in question is contaminated with heavy metals [EPA 2018]. Specific government grant funding has been distributed towards remediation of eligible contaminated sites and there is a push to reclaim this soil [EPA 2018]. Often contaminated farm sites lack access to additional viable soil and remediation is a clear path to continued vegetation growth or utilization of sites [Yan et al. 2020]. There is a growing need for additional advances in effective phytoremediation practices to sustain the environment in affected cities [Anyanwu et al. 2018; Lee et al. 2021; Zhuang et al. 2006]. Continued phytoremediation research on the reclamation of brownfield sites and contaminated farmland may benefit the environment and associated redevelopment projects.

Several areas of continued research are recommended to strengthen the phytoremediation process and limit associated environmental concerns. The impact of additional APGs other than Triton X-100 could be researched in terms of phytoremediation including Tergitol™ 15-S-9 (Polyethylene glycol ether) [Dalton, 2019]. Varying concentrations of PGRs like benzyl aminopurine and stress tolerance from salicylic acid applications is another area for continued scientific research [Aderholt et al. 2017; Gilly 2020; Greipsson et al. 2022]. Utilization of paired SNP and gibberellic acid applications in previous studies had a positive association with plant growth and tolerance to chemical chelate applications and should continue to be researched [Beavers et al 2021]. Continued research focusing on the benefits of APGs, PGRs and chemical chelate applications within phytoremediation could broaden areas of interest with current knowledge gaps.

Conclusion

In conclusion, various approaches to phytoremediation may be beneficial to remediate contaminated soils. Different combinations of PGRs, APGs and chemical chelates may increase levels of heavy metal uptake. This study suggests that soil applications of 10 mM NTA and Triton X-100, Infuse™ and foliar applications of BAP could be a beneficial technique for phytoremediation of Pb contaminated soils. Lead uptake was amplified by the combined application of chemical chelates, growth regulators, and a soil fungicide. With the potential for Pb increase via phytoextraction, specific AMF and plant-soil interactions can be studied further. Phytoremediation studies involving switchgrass and arrested AMF activities can be studied further to determine the implications of suppression on phytoremediation. Increased Pb availability and the environmental impact of NTA, citric acid and EDTA use can be further assessed. Future research of additional chelating agents and chemical chelates may also be beneficial in the phytoremediation process of contaminated soils.

Statement of Integration

This study integrated the following fields, biology, chemistry, sociology, and environmental sciences. Biology was used to assess plant growth regulators, surfactants, phytohormones and chelating agents to encourage Pb uptake in switchgrass plants. Concentrations of Pb, Al and Fe were isolated by chemical reflux reactions and later identified through chemical analysis and implementation of the ICP-OES. The chemistry of heavy metal interactions within soil and the effect of chemical chelates were used to create the combined applications that experimental plants were treated with. Sociology was used in the study to identify how phytoremediation may benefit the environment and sensitive urban populations living near Pb-contaminated soils. Environmental science was implemented by identifying a need for phytoremediation and a reduction in heavy metal contamination in a specific soil sample.

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