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Assessing Moringa oleifera Plants as Exposure Pathways for Heavy Metals in Highly Contaminated Areas

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Assessing *Moringa oleifera* Plants as Exposure Pathways for Heavy Metals in Highly Contaminated Areas

by

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We hereby approve the thesis of

Assessing *Moringa oleifera* Plants as Exposure Pathways for Heavy Metals in Highly Contaminated Areas

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PREFACE

This thesis has been written in journal format and conforms to the style appropriate to my discipline. This manuscript will be submitted for publication in the International Journal of Phytoremediation, a peer reviewed interdisciplinary scientific journal, and therefore reflects the required formatting for this publication. This thesis does not contain a list of tables or a list of figures since these are not included in the submission directions for contributors to this journal. Figures and tables follow the text of the manuscript as required by the International Journal of Phytoremediation and this thesis committee.

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Abstract

This study examined the uptake of heavy metals by *Moringa oleifera* trees in both field and laboratory settings. The main goal of this study was to determine whether using Moringa, grown in heavily contaminated areas, as food or herbal supplements increases the risk of heavy metal exposure. The field component of this study was carried out in the Southern part of Kabwe, Zambia where a lead-zinc mine has left a legacy of severe environmental pollution in the neighboring areas. Moringa trees have several medicinal properties which is one of the reasons they were first introduced to the area. The trees were planted in this part of Kabwe to aid in remediation and so that residents could use them to counter the harmful effects of lead exposure. However, since the trees can potentially hyperaccumulate heavy metals in their shoots, residents consuming them for medicinal purposes could be unknowingly exposing themselves to toxic metals. The secondary goal of this study was to investigate the potential of Moringa to perform phytoremediation. Plant and soil samples were collected from 60 locations in the Mine Neighborhood, which is heavily polluted. Heavy metal concentrations in the plant samples were quantified using X-Ray Fluorescence (XRF) while soil samples were analyzed using Atomic Absorption Spectroscopy (AAS). In the greenhouse experiments, Moringa plants were grown using two experimental designs. The first group included plants grown for 15.5 weeks while the second had plants that were grown for 7 weeks. Plants were spiked with a 10, 000 ppm lead-nitrate solution. Mycorrhizae were added to some treatments to determine its impact on metal uptake. The plants and soil samples were quantified using XRF. The results from both the lab and field showed that Moringa grown in contaminated soils can only be consumed in quantities of less than a gram per day without risk and can be used for phytostabilization.

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Chapter 1

Introduction

Heavy metal mining globally

The negative effects of mining on human and environmental health are experienced in numerous countries around the globe (Stewart 2019). People can experience a multitude of health issues related to heavy metal exposure in places that are heavily contaminated. Bello et al. (2016) examined blood lead levels (BLLs) of people who lived near a lead-zinc mine in Adudu, Nigeria. They cross referenced those BLLs with soil, dust, drinking water and crop lead concentrations. Their results showed that over 10% of children and adults tested had BLLs above the CDC reference limit of 5 μg/dL (Bello et al. 2016). The study showed that there was a positive correlation between the BLLs in adults and the amount of lead (Pb) in the drinking water. However, none of the tested BLLs showed any correlation with the lead concentrations seen in soil and dust samples. The results also showed that children who lived within 12 kilometers of the mine had the highest BLLs. These findings raise concerns not only in Adudu but in other areas where children live close to a mine, since proximity to a mine increases the risk of exposure and potential health issues.

The Gaza Strip in Palestine faces a similar issue since the area is a hub for mining and manufacturing operations. Safi et al. (2019) tested the BLLs of children from all five parts of the Gaza Strip. The children were separated into two groups: those living in close proximity to a major Pb processing zone and those who lived between 100 to 500 meters away from a lead hotspot. Of the 1,705 children sampled, 326 of them lived near a lead hotspot. The BLL reference limit used for this study was 10 μg/dL and approximately 26% of the sampled children had BLLs over this value. Most of the individuals who exceeded this limit lived within 100 m of a lead hotspot or had family members who worked in a lead-based industry. The lack of corrective action by the government is a major problem in the Gaza Strip and it leaves the residents at high risk. The dangers these activities pose to people, especially children, living in close vicinity to a heavy metal operating site are well known yet mining and related activities continue.

Mathee et al. (2018) investigated health risks associated with mining in Johannesburg, South Africa. Gold mining and ore processing pose a major threat to the health of people living near or working at the mine. However, many individuals do not have the option to move or find other means of employment so they must continue to reside and work in heavy metal contaminated hotspots. One of the four neighborhoods studied (which is located near an industrial cotton plant) had very high levels of Pb in soil, over 2000 mg/kg. The second highest Pb concentrations of about 900 mg/kg were found in an area within 200 meters of the mine tailing operations. Both sites exceeded the South African lead reference limit of 230 mg/kg as well as the Canadian lead limit of 120 mg/kg used for this study. These two areas also had the highest levels of arsenic (60.5 mg/kg and 65.3 mg/kg respectively) which also exceeded both the South African limit of 48.0 mg/kg and the Canadian limit of 18 mg/kg. A positive correlation between arsenic (As) concentration in soils and blood arsenic levels in the residents was observed. High levels of Pb and As in humans can cause serious illness and in certain cases death. Proper measures to reduce exposure would be the best course of action for Johannesburg and similar areas.

Research in Zamfara, Nigeria was conducted by Chukwu and Oji (2018) regarding 163 gold mining related deaths that occurred between March and June of 2010. Mining in this area ceased after 85 years, however, the land was not remediated prior to people using it for housing and agriculture. Evaluation of heavy metal exposure risk via contaminated agricultural soils was the main focus for their study. The metals of interest were lead, zinc, arsenic, nickel, copper, chromium, and cadmium. Of these metals, zinc and lead were the most concentrated metals in the soil. A pollution-load index developed for this study showed that the soil was only polluted by lead, cadmium, and zinc while the concentrations of the other metals did not pose a need for concern. The occurrence of these three metals was linked to the mining that occurred previously, therefore, the metals' presence in the soil was correlated with the negative health of people living on the old mine. The need for a viable way to lower metal concentrations in the soil so they do not contribute to metal exposure risk especially when individuals live so close to a mining site is seen once again.

Two years prior, Tirima et al. (2016) conducted a study of their own regarding the mining related deaths that occurred in Zamfara between March and June of 2010. This study looked at additional neighborhoods in order to determine the true extent of the mining related health issues in Zamfara. Upon investigating six more villages, nearly 500 additional deaths were linked to heavy metal pollution from the mines. A quarter of the deceased were comprised of children below the age of five. The government conducted a remediation project which included providing medical aid to affected individuals. The remediation process was tedious and costly due to the economic state and location of this area. Contaminated soils and waste were removed and subsequently replaced via a four-phase process. As a result of the government's efforts, lower heavy metal concentrations (below 400 mg/kg) were seen in three-fourths of the treated areas. The remaining issue is that these expensive remediation efforts could easily be undone. For that to not occur, residents need to be aware of the risks in order to decrease the likelihood that the area will face recontamination by tasks such as illegal small-scale mining practices. Not all mining areas are fortunate enough to have the money available to invest in large scale, commercial remediation projects which is why our study aims to determine if the use of plants to remediate mining communities would be effective since it is a more financially practical option for many areas.

The environmental effects of mining can persist long after mining operations have ceased. The country of Kosovo in Europe offers insight into the impact Pb and zinc (Zn) mining can have on the health of individuals even after those operations have stopped. Ćorac et al. (2017) found that some people illegally sell and process waste from the closed mine to make a living. However, these practices put everyone in the nearby vicinity at risk, not just the people selling the mining waste. This study measured BLLs of 42 children who permanently resided in a Roma camp in which many adults processed mine waste for a living and found levels that averaged around 19.1 µg/dL. These results were compared to BLLs of 36 children from outside of the camp which at 4.87 µg/dL averaged just below the CDC reference limit. While the Roma camp was closer to the industrial area than the other site, both were located in areas of low industrial contamination according to a pollution diffusion model the researchers conducted. Ćorac et al. (2017) believes the most significant difference in exposure was due to the processing of lead waste in the camp communities, however, they were not granted permission by the residents of the Roma camp to measure environmental lead concentration so this conclusion is not fully supported. Despite this setback, the study still provides evidence that even minimal exposure to heavy metals can cause health problems. The results from Kosovo were concerning to researchers and they believe environmental remediation of the site is necessary but have no recommendations for how to do so.

In the Derebaci lead mining gallery in Turkey, Bakirdere et al. (2016) investigated lead, cadmium, copper, nickel, and manganese concentrations in soils and plants in areas surrounding the now closed mining gallery. Part of this study included evaluating water's ability to move contaminants. In the Derebaci gallery, many of the heavy metals were leached from the mine via surface currents flowing through the area. Results showed that plants located 15 meters away from the gallery had the highest concentrations of heavy metals. The high accumulation there is most likely due to leaching caused by surface currents flowing towards the Karakaya Dam Lake, located just north of the sampling site. Natural processes like wind and water are instrumental in the movement of toxic materials often increasing exposure. These natural processes make heavy metals harder to contain and in turn the metals affect a greater area than they would have if not for these mechanisms. If metals could be removed from the soil or stabilized, the chance of them getting redistributed lessens which in turn reduces the chance of potential exposure.

Mining and its health risks in Kabwe, Zambia

In many of the other mining areas, both mentioned above and otherwise, proximity to the mine increases the risk of exposure. Since it is often not feasible to relocate the individuals to a new location, this study aims to determine if phytoremediation using Moringa trees, or another plant, would help lower the overall environmental metal concentration so that it would be safer for these individuals to continue residing there. Phytoremediation and various means of stabilizing and immobilizing those metals could also aid in reducing the effect of natural elements, such as wind and water, on heavy metal contamination. By identifying a remediation method that is both cost effective and efficient it could be useful in many mining towns. Since we do not have the resources necessary to do studies in multiple locations, we picked Kabwe as our model site. Many of the major characteristics shared amongst mining communities such as being rural, economically disadvantaged, and having a number of health problems related to heavy metal exposure are present in Kabwe making it an ideal area to conduct this research.

Decades of mining occurred at the Broken Hill Mine in Kabwe, Zambia starting in 1904 and ending in 1994. Mining operations caused the surrounding neighborhoods to become extremely polluted and contaminated. A highly mobile and heavily contaminated soil layer covers the ground of the surrounding neighborhoods making the metals hard to avoid, especially since more polluted dust is continuously being blown in from the mine. Additionally, water flowing through tailing ponds and slag deposits continually remobilizes previously settled contaminants. Little progress has been made on remediating these soils, so the economy and the health of living organisms in the area continue to suffer as a result (Carrington 2017; Kribek et al. 2019; Nyambe et al. 2018; Yabe et al. 2015).

Despite getting help from various research groups such as the World Bank on projects aimed to aid in the clean-up of the environment near the former mine and to address the health issues of the individuals living in the area, there still remains a lot to be accomplished. When the World Bank was working in the area, one of their main goals was to address the lead poisoning problem in children, who are disproportionally affected by Pb exposure. This was a pilot project that ended with the World Bank establishing some baseline data on BLLs in Kabwe and making recommendations to use chelation therapy to lower the impact of heavy metals on children (World Bank 2011). Chelation therapy is a process that involves taking a medicine that binds to heavy metals in the blood and thus removes the metals from the body (Yabe et al. 2015). The World Bank also proposed the idea of using natural containment methods such as planting trees and grass to immobilize metals (World Bank 2011). Children living in the area near the former Broken Hill Mine had BLLs averaging nearly ten times the limit of 5 μg/dL set by the CDC

(Caravanos et al. 2014). This is beyond the level for which chelation therapy was deemed necessary (45 μg/dL). Plus, many individuals are above the 60 μg/dL mark which is where some painful symptoms become prevalent in younger individuals (Yabe et al. 2015). Another study looked at 246 children in three neighborhoods and found that all the children had BLLs over 5 μg/dL and 25% of them had BLLs exceeding 65 μg/dL. At least half of the tested children needed immediate medical treatment with four children having BLLs over 300 μg/dL (Bose-O'Reily et al. 2018).

Once an individual has lead poisoning, its effects stay with them forever, so exposure prevention is the best way to protect human life. Lead can cause damage to the brain, kidneys, muscles, and almost every other body system (Cronan 2019). During its developmental stages, the body tends to absorb lead in greater quantities than in an already developed body (Yabe et al. 2015). As a result, **c**hildren are more likely to get lead poisoning because their bodies are still developing (Cafasso 2018; Wani et al. 2015). The risk of children encountering lead is increased in part due to the tendency of children to play in the contaminated dirt and then put their hands in their mouths. Intellectual problems including learning disabilities, lower IQs and memory problems are common amongst children experiencing the effects of lead poisoning. Individuals suffering from deficiencies of minerals necessary for body function such as calcium and iron are more likely to experience lead poisoning symptoms (Cafasso 2018).

One study in Kabwe by Yabe et al. (2015) focused on children under the age of seven living near the mine to help the most at risk individuals. A total of 246 blood samples were taken and 100% of the results came back above the CDC level of concern (5 μg/dL). Some of the BLLs recorded were even above the reference limit for brain damage (encephalopathy) and death (65 μg/dL).

The chance of getting lead poisoning in the neighborhood around the old mine remains high due to the living conditions. Many of the roads remain unpaved and as a result the contaminated dust from the roads easily ends up back in the air when disturbed. Residents typically do not have the means to take any major protective measures and people must take the few jobs available whether the working conditions are ideal or not (Carvanos et al. 2014).

The health of the environment is also a major concern when dealing with the negative effects of mining. A study performed in three highly impacted neighborhoods in Kabwe found immense amounts of pollution in the soil. The Kasanda neighborhood saw median soil lead concentrations as high as 3008 mg/kg which is well above the international safety level set at 400 mg/kg (Bose- O'Reilly et al. 2018). Toxic metal concentrations in the ecosystem have resulted in a decrease in biodiversity since many of the organisms that previously inhabited those areas cannot survive in the present conditions. Many aquatic organisms cannot live in the water since it has been acidified by the constant runoff coming from the mines and smelters. It has been shown that the most realistic and cost-effective remediation technique for the area is phytoremediation (Nyambe et al. 2018).

In order to counteract the amount of lead that has been introduced into the area, researchers suggest removing the topsoil where many heavy metals are concentrated and planting trees to decrease the mobility of those metals. Another suggestion is that the areas where children encounter lead should be covered so that the dust is contained and not continually being redistributed into the air. At this point, reducing exposure is likely the best step individuals in Kabwe can take to protect their health (Kriberk et al. 2019).

Heavy metals in general

There are health and environmental risks associated with exposure to certain heavy metals. Metals are naturally present on the Earth, but many are trapped. However, anthropogenic activities such as mining have freed more metals from their constraints and introduced them into the open environment. Approximately 35 metals pose a health risk when individuals are exposed to them in any capacity. Of these toxic metals, 23 are classified as heavy metals (Jaishankar et al. 2014). Some heavy metals are necessary in low quantities for normal biological processes to occur. However, an accumulation of high concentrations of these metals will cause numerous health issues. Some heavy metals will replace necessary cations and can cause a deficiency of a needed metal as well as stop normal biological processes in the body from happening. The toxicity of a metal to an individual is dependent on several factors including the pathway of exposure, duration of exposure, and the concentration. Each heavy metal poses its own set of health, environmental, and biological risks and some are significantly more dangerous than others (Jaishankar et al. 2014).

Arsenic is a metalloid which has properties of both a metal and nonmetal although it is usually included in the heavy metal category. It has been shown to be toxic and carcinogenic, and it can cause errors in mitosis as well as other cell functions. Various industries contribute to the increasing concentration of arsenic in the environment (Jaishankar et al. 2014). Lead is a commonly encountered metal that is also extremely toxic. Soil lead concentrations have increased due to smelting, mining, additives in gas, fertilizers, pesticides, burning of fossil fuels, and battery waste. As a result, lead has been at the root of many environmental and health issues globally (Jaishankar et al. 2014). Lead can accumulate in plants and disrupt their ability to germinate or grow (Zulfiqar et al. 2019). It serves no function in any biological process and causes significant problems when concentrated. When lead is present in living cells, it causes an imbalance of highly reactive atoms (free radicals) and the antioxidants needed to counteract them causing oxidative stress. Lead is also able to substitute itself for necessary cations in the body which in turn interferes with multiple biological processes such as enzyme regulation, neurotransmitter release, and protein maturation since lead is unable to do the job of the original element. This interference subsequently causes many issues in multiple systems of the body (Jaishankar et al. 2014).

Uranium is another heavy metal that poses a health threat to humans and the environment. Due to the metal being both radiotoxic and chemotoxic, it poses a significant danger to human and ecological health (Rump et al. 2019). The bone surface, bone marrow, and colon are commonly affected by uranium due to its ability to efficiently penetrate and accumulate in those areas of the body. Many kidney related diseases are also tied to uranium deposition since the kidneys can absorb significant quantities of the metal (Rump et al. 2019).

A fourth heavy metal of interest to this study is selenium. This metal offers certain benefits in human health in small quantities (Natasha et al. 2018). Selenium is essential to metabolic processes in the body and works as an antioxidant protector. It also has both positive and negative effects on various plants. Studies have shown that much of the selenium in the edible portion of plants is directly correlated with the presence of the metal in soils, leading to serious health problems in non-remediated agricultural soils (Natasha et al. 2018). Several natural and anthropogenic processes like volcanic eruptions, coal burning, and element extraction introduce selenium into the environment. The duality of selenium being both toxic and biologically necessary leaves a small margin between it being beneficial and detrimental. These four heavy metals and others not mentioned can cause severe harm to humans (especially younger children), animals, plants, and the environment as a whole (Natasha et al. 2018; Yabe et al. 2018).

Phytoremediation

Environmental metal concentrations are increased by anthropogenic activities such as mining. These activities cause excess amounts of metals to be present in the soil which can cause health problems. These excess metals need to be removed; however, an issue arises since conventional remediation methods are costly. In-situ bioremediation has recently become a major point of interest when it comes to rehabilitating contaminated soils. Phytoremediation is a type of bioremediation that uses plants to improve the conditions of the environment. Phytoremediation is generally cheaper and more sustainable than other remediation methods like surface capping, soil washing, soil flushing, and solidification (Soliman and Sugiyama 2016). Surface capping generally can only cover a small area not making it useful in larger sites. Both soil washing and soil flushing require having the necessary chemicals to treat the metals in the soil and any form of solidification is used as a last resort due to cost. While phytoremediation still needs to be studied more, plants do offer a more feasible method of clean up for contaminated areas especially those that are economically disadvantaged (Liu et a. 2018).

There are five major types of phytoremediation: phytoextraction, phytostabilization, phytodegradation, phytovolatilization, and hydraulic control. Phytoextraction occurs when the contaminants in the soil are absorbed and stored in the plant during nutrient and water uptake. Phytostabilization is a method in which contaminants in the soil are immobilized by the plant roots. It helps lower the spread of metals and does not introduce them into the food chain (Soliman and Sugiyama 2016). It also stops the metals from entering the bloodstream and becoming bioavailable which is when metals begin to effect human health (Yan et al. 2019).

Phytovolatilization is when the contaminants are taken up by the plants and then released into the atmosphere in a less toxic vapor form. The gaseous materials typically do not resettle in the environment which lowers the overall concentration of toxins. Phytodegradation either uses enzymes in the plant or the metabolic processes of the plant to break down the contaminants so the metals that remain are less toxic. The last method of phytoremediation is hydraulic control in which trees are used to manipulate the flow of groundwater and control where contaminants go (Soliman and Sugiyama 2016).

The types of plants that are capable of phytoremediation vary as do the metals they can uptake (Muthusaravanan et al. 2018). Every plant cannot be used for every method of phytoremediation and a variety of limitations exist as well. Some plants can only remediate the top few inches of soil due to root length. Other plants that accumulate metals risk introducing them into the food chain due to subsequent herbivore consumption. Plus, the process of phytoremediation can take multiple growing seasons to work (Muthusarvanan et al. 2018).

Moringa oleifera and phytoremediation

Moringa is a tree grown in the tropics and subtropics. It is widely known for its medicinal value, nutritional content, industrial uses, ability to grow in hot, dry areas or during times of drought and low demand for soil nutrients. It can also uptake metals alongside nutrients and water due to its hyperaccumulation properties. Moringa is a unique plant in the tropics since it is in full bloom at the end of the dry season when many other plants are dormant (Anwar et al. 2007).

Due to its hyperaccumulation properties, *Moringa oleifera* (Moringa) has the potential to be used in phytoremediation of contaminated soils (Soliman and Sugiyama 2016). Hyperaccumulators can concentrate metals from the soils into different parts of the plant structure which will reduce the concentration of those metals in the surrounding soil. Moringa uptakes metals such as lead and arsenic from the soil and it can both store and tolerate toxins so that the plant itself is not adversely affected. Moringa accumulates those metals in its above ground parts such as its stems and leaves (Soliman and Sugiyama 2016).

The nutritional composition of Moringa is highly dependent on the environment where the tree is grown, the genetic makeup of the tree, and how it is cultivated. The nutritional content of the soil has a significant impact on what nutritional, therapeutic, and medicinal benefits the tree provides. There is a large overlap of where Moringa trees are naturally found (parts of Asia and Sub-Saharan Africa) and where people who would need access to low-cost medical treatments live. However, research shows that many of the people living in countries where Moringa is naturally found are unaware of the benefits that the tree could provide (Moyo et al. 2011).

Moringa oleifera and water treatment

Moringa can also be used to improve water quality. Treatment of the water in rural areas is typically necessary because access to clean freshwater is often limited. As a result, the contaminated water is used for irrigation, which increases the chance that fruits and vegetables will take up heavy metals and that those metals will then be transferred to people upon consumption. However, many times in these places water is not treated due to the cost of treatment (Petersen et al. 2016). Seed powder from Moringa trees is a commonly used natural coagulant due to its low cost (Hellsing et al. 2014). There is a strong cationic protein present in Moringa that gives it the ability to act as a natural coagulant (Keogh et al. 2017). A lot of turbid matter that needs to be removed from water is negatively charged allowing it to readily attract to the cations in the seed powder (Foidl et al. 2001). In addition, the proteins in the seeds bind permanently to the silica interfaces present in sediment (Nouhi et al. 2018).

Moringa seed powder can soften hard water and rivals some synthetically made coagulants such as alum in its ability to clear turbid water (Muyibi and Evison 1995). Individuals in Sudan have been using Moringa seed powder as a coagulant due to it not having the adverse effects of its chemical counterparts. Moringa has also been shown to be very effective at reducing turbidity, with some studies showing a decrease in turbid conditions as great as 99%. The powder causes the turbid material to settle out of the water column, making it easier to reduce the turbidity of water (Anwar et al. 2007; Keogh et al. 2017). The removal of turbid matter increases as the amount of seed powder added increases (Muyibi and Evison 1995). However, the ratio of Moringa to water has to be kept carefully balanced because if there is too much powder added it will remain in the water in excess, which in addition to being wasteful also causes an increase in oxygen demand which can result in oxygen depletion and hypoxia (Kwaambwa et al. 2015). The use of Moringa to treat water is desirable because it has very little effect on the natural pH, alkalinity, and conductivity of water. It has been used to prohibit the growth of microorganisms in water as well. In fact, studies show that Moringa powder impedes the growth of *Escherichia coli*, a pathogenic bacterium**.** Moringa seed powder is capable of binding to both Gram-positive and Gram-negative bacteria making the bacteria settle to the bottom of the water. This ability makes Moringa seed powder an effective, natural antimicrobial. Two studies conducted by Eilert et al. (1981) and Fahey (2005) both attribute Moringa's antimicrobial trait to the benzyl isothiocyanate present in the plant.

Moringa powder has also been used in combination with other methods to treat water such as Solar Water Disinfection (SODIS), a method that works most efficiently in low turbidity water. In SODIS, Moringa is used as pretreatment. The ideal Nephelometric Turbidity Units (NTUs) range for SODIS to be employed is less than 30 NTUs, however in many cases water can reach well over 200 NTUs making SODIS virtually ineffective. Moringa has been shown to lower the turbidity enough to make SODIS effective. SODIS without Moringa treatment allows for the regrowth of bacteria if left overnight, however, if the water is treated with Moringa powder the chance of bacteria regrowth is greatly reduced (Keogh et al. 2017).

Petersen et al. (2016) tested the ability of Moringa to remove the protozoan parasite *Cryptosporidium parvum* oocysts from water. Results showed that the addition of extracts taken from Moringa seeds reduced the presence of the oocysts in wastewater by 38% after 15 minutes in comparison to a 0.02% reduction in untreated water after the same amount of time. After 90 minutes, the treated wastewater showed a 94.7% reduction in turbid material. There was also a general increase in overall quality of the water as well as an increase in settling time for oocysts. Oocysts settled in water treated with Moringa seed extract in 3.6 hours as compared to the 74.6 hours it took for oocysts to settle in untreated water. Treated stream water also had a significant reduction of *C. parvum* oocysts. When samples were spiked with 100, 1,000, and 10,000 oocysts, there was an average reduction of oocyst accumulation by 80% due to the use of Moringa seeds. While the oocysts are not completely removed from the water, the reduction has a significant enough impact on water quality that treating the water is still beneficial because it makes it easier to disinfect via other methods.

Moringa oleifera and health

Moringa is rich in a variety of minerals, proteins, and the simple sugar, rhamnose. The tree has been used for antihypertensive, diuretic, antispasmodic, antitumor, antiulcer, hepatoprotective, antibacterial, and antifungal remedies. The tree also provides a supply of Vitamin A and C, potassium, calcium, iron, and other nutrients (Fahey 2005; Foidl et al. 2001).

However, the medicinal traits of the tree are not just specific to one part of the tree, Moringa is one of the few plants in which nearly all parts of the tree have a use. The roots can be used for infertility treatments, counteracting inflammation, or working against the formation of kidney stones as an antilithic. The roots also have a variety of antibacterial and antimicrobial properties. Leaves have been used as a purgative and to treat ear and eye infections. The leaves' ability to act as an antioxidant is also a widely exploited property of the tree. The liquid extract from the leaves can be used to prevent the growth of microorganisms. The stem bark has been used as a rubefacient to increase blood circulation or as a treatment for ulcers and tumors. Bark extract is commonly used as an antibacterial treatment against *Staphylococcus aureus*. The gum of the tree has been mixed with sesame oil to alleviate headaches or on its own to treat teeth cavities. Flowers have medicinal value as a treatment for muscle disorders and tumors, and they are commonly used as a stimulant. The flowers are also used for antibacterial and antifungal treatments. Extracts from seeds can be used to prevent the production of cancer-causing enzymes in the liver (Foidl et al. 2001).

Since the 1920s in Nicaragua, Moringa has been used as a live natural fence around properties. The presence of the tree around the perimeter of the property helps lower the amount of dust from the road that enters someone's home, yard, and garden. Another trait of the tree that has been utilized in Nicaragua is combining Moringa leaf extract with 80% ethanol to promote the growth of other plants (Foidl et al. 2001; Makkar and Becker 1996). Evidence from Uganda in the 1980s shows the tree is capable of treating many of the symptoms of HIV/AIDS (Kasolo et al. 2010). As a result of these properties and many more, Moringa is a popular source of food, nutrients, and medical treatments for over 300 diseases for people living in places where the tree is commonly found, earning it the title of one of the world's most useful trees (Anwar et al. 2007; Gopalalkrishnan et al. 2016; Keogh et al. 2017; Moyo et al. 2011).

Due to its nutritional value, Moringa has the potential to reduce food insecurity in rural areas. While many parts of the tree are full of nutrients, the leaves are thought to be the most beneficial as they are full of a variety of vitamins, minerals, and amino acids. For this reason, the leaves have been used as a treatment for malnutrition in infants since it increases the amount of nutrients present in breast milk (Gopalakrishnan et al. 2016). Dried Moringa leaves show an approximate crude protein concentration of 30.3% (Moyo et al. 2011). This level of proteins has the potential to meet the dietary requirements of many animals, making Moringa a sufficient feed additive (Makkar and Becker 1996). Many parts of the tree including the stems, seeds, and oil extracts are rich in proteins as well. The protein in Moringa is easily digestible due to the presence of lipid and sulfur rich amino acids (Ferreira et al. 2008). According to Moyo et al. (2011) dried Moringa leaves have higher mineral concentrations than their fresh counterparts. Dried leaves are highly enriched in calcium which impedes the onset of osteoporosis and they possess copper which increases the body's defense system. Without copper, the body risks losing the ability to produce lymphocytes and antibodies. The iron found in the leaves increases the production of hemoglobin and myoglobin which is essential for oxygen transport and cell division and growth. Iron is also essential to ensuring that the central nervous system (CNS) functions normally. Moringa has high percentages of zinc which are essential in the diet of animals, including humans, since it is necessary for cell division and growth and the production of DNA, RNA, insulin, and a variety of enzymes. Moringa leaves have a low enough concentration of phenols that there are no harmful effects when animals ingest them. However, the concentration of phenols is high enough that they provide a variety of benefits such as preventing platelet aggregation and acting as an antitumor, anti-inflammatory, antioxidant and antimicrobial (Moyo et al. 2011).

The general nutritional composition in Moringa can potentially reduce the possibility of disease in animals and increase their ability to fight off parasites making livestock stronger and more resistant to diseases. Due to Moringa having the amino acids, methionine and cysteine, it can remove toxins from the body. Those characteristics of the tree have also been known to help increase the body's defense against radiation. Plus, the greater concentration of polyunsaturated fatty acids (PUFA) versus saturated fatty acids in the tree gives Moringa a greater ability to ward off diseases since higher amounts of PUFA increase the performance of the body's immune system (Moyo et al. 2011).

Pollution in Kabwe, Zambia

Vast amounts of heavy metals contaminate the area around the former Broken Hill Mine in Kabwe, Zambia. The mine was in operation for approximately nine decades. As a result of its operation, lead, selenium, arsenic, and other toxic metals contaminate the soils of the nearby schools and neighborhoods. Pollution from the mine is responsible for daily heavy metal exposure in individuals living in the Mine Neighborhood and attending the David Ramushu Combined School. Heavy metal poisoning is a major health concern for the people living there. Research performed by Bose O'Reilly et al. (2018), Carrington (2017), Carvanos et al. (2014), Nyambe et al. (2018), and Yabe et al. (2018) shows that the blood lead levels (BLLs) in children near the former mine are much higher than the recommended safe level of 5 μg/dL set by the Centers for Disease Control and Prevention (CDC).

A study conducted by the World Bank (2011) tried to help combat the negative effects of lead poisoning in the area by planting the medicinal tree *Moringa oleifera* (Moringa) for residents to use as a treatment method. Unfortunately, a risk assessment was not carried out to assess the potential for the plants to be an additional exposure pathway for heavy metals since Moringa can hyperaccumulate metals (Amadi and Tanee 2014). As a result, the people living in Kabwe could be facing unknown risks by consuming the plant. The current study explores potential ways to counteract the harmful effects of heavy metal pollution. Heavy metal poisoning is a serious issue in communities with nearby mines, whether they are still operational or not. While treatments are available, many places where heavy metal poisoning is a prevalent issue do not have the resources necessary to remediate the area. This study will be beneficial because it intends to address the problem of potential exposure and analyze the potential of using phytoremediation to rehabilitate these areas.

Previous research has been conducted in the area by Dr. Sam Mutiti, as part of his Fulbright fellowship (2016-2017). He found that some Moringa trees and vegetables grown in this area contained considerable amounts of lead and other heavy metals (Ngoma 2018). The present study examined Moringa trees grown in contaminated soils to better understand how heavy metals are accumulating within the trees and what underlying exposure risks exist.

Moringa has the potential to reduce the heavy metal concentrations in the soil of mining neighborhoods worldwide. However due to the hyperaccumulation abilities of the trees a potential exposure risk is present during the consumption of Moringa. This study’s aim was to determine whether the medicinal use of Moringa grown in contaminated soils is helpful or harmful to the health of people living near mines due to the risk of further exposure to heavy metals.

Goals, objectives, and hypotheses

The overarching goal of this study is to understand the role of Moringa in human exposure to heavy metals and assess its remediation potential when grown in heavily contaminated soils. This study will be conducted in the Mine Neighborhood near Broken Hill Mine in Kabwe, Zambia since it possesses many of the common characteristics of a typical mining town.

Objective 1: Examine the risk of Moringa trees as an additional exposure pathway by quantifying metal uptake and distribution by Moringa and determine the safe daily consumption limit.

- Hypothesis: Moringa trees planted in contaminated soils will have significantly higher concentrations of heavy metals than those grown in uncontaminated soils, therefore making them unsafe for consumption.

Objective 2: Assess the phytoremediation potential of the Moringa trees to see if it will be a cost-effective and reliable remediation method for individuals living in areas polluted by mining.

- Hypothesis: Due to its hyperaccumulation capabilities, Moringa will uptake lead and other heavy metals in its leaves and other structures making the contaminants less mobile.

Objective 3: Examine if the addition of soil amendments will increase the uptake potential of heavy metals by Moringa plants therefore enhancing its phytoremediation abilities.

- Hypothesis: Various soil characteristics and amendments such as fertilizer and mycorrhizae will increase the ability of Moringa plants to take up heavy metals from contaminated soils.

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Chapter 2

Evaluating the Phytoremediation and Exposure Risk of *Moringa oleifera* Grown in Kabwe, Zambia

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Abstract

*Moringa oleifera (*Moringa*)* is a medicinal plant that has hyperaccumulating properties. Due to its medicinal benefits, Moringa was planted in the highly contaminated Mine Neighborhood near the former Broken Hill Mine in Kabwe, Zambia. However, an analysis of potential exposure risks to heavy metals via the use of Moringa grown in the area as nutritional and medicinal supplements was not performed prior to the tree's introduction in the area. This study evaluated this risk and determined whether the ability of medicinal plants like Moringa to hyperaccumulate heavy metals makes them unsafe for consumption if grown in polluted areas. The study also explored the phytoremediation potential of Moringa trees under field conditions. Plant and soil samples were collected from 60 households and the David Ramushu school (which serves children pre-K to 12th grade) in the Mine Neighborhood. The field samples were then analyzed for heavy metal concentrations. The results showed that it was unsafe to consume more than 1 g/day of Moringa plant parts if the trees are grown in contaminated areas. It was also determined that Moringa needed to be used in combination with other known phytoremediation plants to efficiently remediate the contaminated soils of the Mine Neighborhood.

Keywords: phytostabilization, phytoextraction, blood lead levels, heavy metal poisoning, mining

Introduction

There are currently twenty-three known toxic heavy metals on Earth (Jaishankar et al. 2014). Their concentrations in surface soils have increased due to anthropogenic activities releasing them from their confines into the open environment. Their toxicity to humans varies depending on the metal, exposure pathway, length of exposure, and concentration of the contaminant. Some of the heavy metals, such as selenium, are biologically beneficial and necessary in small quantities, however, in large amounts they can be toxic and dangerous. In small amounts, selenium is required by living things for the performance of metabolic processes in the body but negatively affects the health of the kidneys, liver, and muscles in high quantities (Natasha et al. 2018). On the other hand, heavy metals such as lead have no known function in living organisms and can cause serious harm, even in small amounts. Lead, together with arsenic (a metalloid) and uranium, can cause deficiencies in essential metals and nutrients, as well as disrupting biological processes in the body (Jaishankar et al. 2014). For example, arsenic will disrupt mitosis, while uranium can cause kidney failure and/or weakening of the bones. Lead can replace essential metals and subsequently causing normal bodily functions to halt and can cause weak bones when it substitutes for calcium in the bones (Jaishankar et al. 2014; Natasha et al. 2018; Rump et al. 2019).

High concentrations of heavy metals in the soils, which are increased due to anthropogenic processes, must subsequently be removed from the open environment. However, traditional remediation techniques for contaminated areas are costly. In recent years, phytoremediation has emerged as a cost-effective method for rehabilitating contaminated areas in lieu of their more traditional counterparts such as surface capping, soil removal, and solidification (Liu et al. 2018). Phytoremediation is a form of bioremediation that uses plants to improve the conditions of the environment. Plants can be used to absorb toxic contaminants from soils into their structures (phytoextraction) or they can use their roots to immobilize the metals and keep them in place (phytostabilization). Plants can also take up the contaminants and release them back into the environment in a less harmful form via transpiration (phytovolatilization), break down the contaminants using their natural enzymes and various metabolic processes (phytodegradation), or manipulate the flow of contaminated groundwater to keep the pollution in one area (hydraulic control) (Muthusaravanan et al. 2018). Various plants can perform different types of phytoremediation, however, not all plants are capable of remediation (Muthusaravanan et al. 2018; Soliman and Sugiyama 2016). Plants that can take up significant amounts of metals, typically over 1000 ppm, are known as hyperaccumulators. These plants can perform phytoremediation. Over 400 identified hyperaccumulators already exist with more being evaluated and added annually as the search for efficient remediation techniques continues (Soliman and Sugiyama 2016).

One plant of interest is Moringa, which has been shown to have numerous medicinal properties while also being a heavy metal hyperaccumulator (Amadi and Tanee 2014; Anwar et al. 2007). This plant has great potential for use in contaminated areas due to its ability to either improve negative health effects caused by heavy metals or reduce the concentrations of the metals in the environment. One question asked in this study is whether these properties can be utilized at the same time by planting the trees in contaminated areas where both human and environmental health need treatment or if their use are mutually exclusive. The main goal of this study was to answer the above question by assessing the phytoremediation potential of Moringa in the field and evaluate the potential of different plant parts to increase heavy metal exposure if they are grown and consumed in heavily contaminated areas.

Materials and Methods

Site description

This study was conducted in Kabwe, Zambia which is the home of the former Broken Hill Mine (latitude -14.45528, longitude 28.43360). The study focused on one area called the Mine neighborhood shown in Figure 1 below. The mine operated for nearly nine decades extracting and processing lead and zinc ores. The mine was shut down in 1994 for economic reasons by the Zambian government who had been operating it under the Zambia Consolidated Copper Mines (BMR 2016; Mumba 2014). Despite being closed, the mine continues to act as the pollutant source of lead, zinc, cadmium, arsenic and other metals. These heavy metals have been and continue to g mobilized from the mine site as dust particles before traveling into the surrounding neighborhood. This has resulted in a layer of heavy metals blanketing the soil and covering other surfaces in the neighborhood. The presence of these toxic metals continuously puts the health of the residents at risk (Carrington 2017; Kribek et al. 2019; Nyambe et al. 2018). Of the four metals/metalloids (lead, arsenic, uranium, and selenium) explored in this study, lead is the one that has received the most attention and has been extensively studied in the area. Numerous studies have investigated the impact of lead on the people of Kabwe by testing lead levels in their blood stream (Bose-O’Reilly et al. 2018; Yabe et al. 2015; Yabe et. 2020)

Most children living in these neighborhoods (around the old mine) have blood lead levels (BLLs) averaging over ten times the 5 μg/dL safety limit set by the United States’ Centers for Disease Control and Prevention (CDC) (Caravanos et al. 2014; Yabe et al. 2015). To help combat the negative health effects of lead pollution in Kabwe, the World Bank conducted a pilot study in the area. One of the products of the project was planting *Moringa oleifera* (Moringa) trees in the residential areas. The trees are used to stabilize the soils, provide shade during the hot season, and counter the negative health effects of lead exposure when consumed for the plant's medicinal properties (Amadi and Tanee 2014; World Bank 2011). Unfortunately, there is a lack of information in literature about the risk associated with simultaneously using the Moringa tree for phytoremediation and as a medicinal supplement. This area, therefore, provides a perfect site for conducting this project and answering the project's main research question.

Sample collection

Moringa plant samples (leaves, barks, stems, pods/seeds, and tree cores) were collected in the Mine Neighborhood (Figure 1) in June and December of 2019 (dry and wet season respectively) from sixty locations around peoples' houses and the David Ramushu Combined School which is located on the northwest edge of the neighborhood. Plant samples were collected from every other house that had Moringa trees or from the next available plant if the spatial gap between Moringa trees was too big. The plant parts were collected based on availability, because not all the trees had all the parts of interest present.

Leaves, stems, and pods were collected based on what could be reached from ground level as shown in Figure 2A. Leaves and stems were collected from trees by snapping them off the branches at the budding point and placing them in a labeled clean sample bag. Pods were collected in a similar manner and placed in separate bags to minimize cross contamination.

Bark samples were collected using a knife to remove the external layer until the fleshy green interior was exposed (Figure 2B). The samples were collected at convenient heights for the person sampling but at least a meter above ground. The bark was peeled off the trees directly into clean sample bags without touching them. All the bags were properly labeled with the location and Global Positioning System (GPS) coordinates as well as other general information about the sites. All this information was also recorded in a field notebook.

Plant cores were collected from the trunks using a Jim-Gem© 12" Increment Borer. Only trees that had a diameter of at least 20 cm were selected for core sampling. Each core was placed in a straw to keep them intact during transportation to the lab (Figure 2C) and then placed in a labeled bag.

Composite soil samples were collected from the top 5 cm at each sampling location using a clean shovel and Ziploc® bags (Figure 2D). The initial plan was to collect soil samples from around the roots of the trees but since some trees were located in places that were not conducive for soil disturbance (soil collecting would have ruined the landscaping) the soil at those houses was collected from a couple meters away from the trees (the closest convenient spot).

Sample analysis

All samples were transported to the Soil Physics lab at the University of Zambia (UNZA) for processing. The tree cores were air dried for ~48 hours before being analyzed. The leaf, bark, stem, and pod samples were carefully, but thoroughly, washed in the lab to remove dust residue (Figure 3) before being placed in a Binder© oven to dry for ~ 48 hours at 70 °C. After drying, the samples were crushed to a powder and placed in labeled bags before metal analysis. Heavy metal quantification on the tree parts was conducted using an Olympus© X- ray Fluorescence (XRF) machine. The machine was calibrated daily before any samples were run to ensure that the instrument was performing as expected. For extra quality control, the samples were analyzed six times and the average was calculated.

Soil samples were analyzed for total lead concentrations at UNZA's Soil Science Analytical lab. The soils were digested using the Aqua Regia method and the metals were quantified using Atomic Absorption Spectroscopy (AAS) as described by Hamvumba et al. (2014).

Statistical analysis

Analysis of variance (ANOVA) tests and descriptive statistics were utilized to gain insight into the data and understand relationships between sample locations and plant parts. An ANOVA was used to test whether there was a significant difference in the uptake of metals across plant parts. ANOVAs were also employed to determine which of the heavy metals examined (lead, arsenic, selenium, and uranium) was taken up in significantly higher quantities relative to the other metals. For all analyses, the confidence level was set at 95% (alpha value of 0.05).

Safe daily intake

Recommended safe daily intake levels for Moringa consumption (using the average concentrations of lead in the field samples) were determined based on the Interim Reference Level (IRL) established by the United States' Food and Drug Administration (FDA) for daily consumption of a lead contaminated food source. Lead was the focus metal for intake level calculations because it was the most concentrated metal at the field site. The reference level is set at 3 μg/day for children and 12.5 μg/day for adults. The allowable amounts of Moringa (dry mass in grams) that can be consumed without raising an individual's BLL over the CDC safety limit (5 μg/dL) based on the above IRLs are provided in Table 1.

Results

Uptake and distribution within the plant

The highest average uptake of lead was seen in the bark at an average concentration of 309.5 ppm, while the lowest average was in the cores at 8.04 ppm (Figure 4). The average concentration of lead in the various plant parts were all significantly different from each other. The p-values were less than 0.001 for all the comparisons except between leaves and stems which still showed a weak significant difference at a p-value of 0.049. Arsenic had the second highest average concentration of the four metals of interest. Its highest concentration was in the bark (50.66 ppm) and lowest in the cores (3.62 ppm). Comparisons of arsenic uptake among the different parts showed significant differences in uptake (p-values less than 0.02). The only exceptions were the comparisons between seeds and cores (p-value of 0.29) and between leaves and stems (p-value of 0.3). Uranium followed similar uptake patterns to lead and arsenic with the highest concentration in the bark (19.5 ppm) and the lowest in the cores (1.1 ppm). At the set 95% confidence level, uranium uptake in the different parts were all significantly different from each other (p-values less than 0.02). Selenium ranged in concentration from a low of 2.03 ppm in the cores to a high of 8.8 ppm in the leaves. The only parts of the plant that were significantly different from each other were the bark and cores (p-value of 0.003). The rest of the concentrations were not significantly different from each other with p-values all greater than 0.1.

Soil concentrations

Soil lead concentrations ranged from a low of 296 ppm to a high of 5017 ppm. The average concentration of lead in soil was 2158 ppm. The highest concentrations were closest to the mine and decreased with distance from the old mine site. There is some spatial variation in the soil concentrations within the neighborhood (Figure 5).

Daily intake estimates

Estimates of the safe daily intake quantities of Moringa (based on field samples) ranged from contaminated soils 0.001 g/day to 0.5 g/day (Table 1).

Discussion

Uptake and distribution within the plant

This study evaluated the uptake of lead, arsenic, selenium, and uranium in various parts of the Moringa tree to gain some insight on where it stores the heavy metals and also whether it acts as an additional pathway for heavy metal exposure to people consuming it when grown in contaminated areas. For three of the four metals (lead, arsenic, and uranium) the highest concentrations were found in the bark whereas the lowest amounts were in the cores. Several plants are known to take up heavy metals without their health deteriorating, but they must be in a homeostatic balance for this to occur (Alscher and Erturk 2002; Dat et al. 2000; Foyer et al. 1994). To achieve this balance, the metals must be distributed throughout the plants. If the metals are not processed properly within the plant, they can potentially replace essential metals and cause stress in the plants (Malayeri et al. 2008).

The first route of transport for metals and nutrients in plants is through the transpiration stream located in the xylem (Khan et al. 2014; Page et al. 2012). The xylem consists of vascular tissues that move water and nutrients into plants (Page et al. 2012). The metals and nutrients get moved to photosynthetic and chlorophyll-rich parts of the plants like the leaves (Page and Feller 2005, 2015; Page et al. 2006). A secondary transport pathway is found in the phloem. It can relocate many non-essential metals into other parts of the plants (Richau et al. 2009; Yang et al. 2006). While transportation via the xylem is virtually the same for all heavy metals, transport via the phloem varies. There are three main methods of phloem redistribution. The first is efficient mobility via the phloem into new plant growth. Nickel travels in the phloem this way. The second method also has high phloem mobility but at a slightly slower rate. These metals which include zinc and cadmium are deposited into the meristems that include growing root and shoot tips. The third method involves little to no mobility in the phloem. This causes metals such as iron and manganese to be stored in the leaves where they were first transported via the xylem (Herren and Feller 1994, 1996; Page and Feller 2015; Richau et al. 2009; Riesen and Feller 2005; Van Bel 2003; Van Bel and Gamalei 1992; Yang et al. 2006; Zeller and Feller 1998).

The high accumulation of arsenic, lead, and uranium in the bark in comparison to the other three plant parts could suggest that they are redistributed through the phloem via the same pathway as nickel. They seem to rapidly move into expanding plant growth such as the new layers of bark or young leaves and stems. As the bark continues to grow, it offers a place to store these metals while having little to no interference on plant growth. The low accumulation of these same metals in the cores is most likely due to the xylem pushing out these non-essential metals so they do not accumulate there, metals are just passing through this part. Due to the core samples being comprised mostly of xylem cells, the low concentration of uranium, arsenic, and lead in the cores is expected. Another possible reason for the higher concentrations of metals in the barks is due to Moringa being a heavy metal hyperaccumulator. Hyperaccumulators tend to compartmentalize metal ions in the parts of a plant where the metals would not have access to cellular sites that are necessary for maintaining plant life (Amadi and Tanee 2014; Richau et al. 2009; Tangahu et al. 2011; Yang et al. 2006). In either case, since the nutrient stream (xylem) and the leaves are vital to the wellbeing and functioning of the plant it makes sense that non-essential heavy metals would be moved away from the xylem.

New leaves and stems do not contribute to photosynthesis as significantly as their mature counterparts so there would be minimal interruption to plant function by distributing the metals here. It is possible that as they grow bigger the plants stop distributing the metals to these parts and the increase in biomass dilutes the earlier concentrations of lead, arsenic, and uranium. The relatively high presence of lead and arsenic in the pods could also be due to the redistribution of metals via the phloem into new plant growth. By going into the newly formed fruiting bodies, the metals do less harm to the plant as a whole (Herren and Feller 1994).

The difference in the concentration of metals in the bark versus the leaves could also be due to Moringa being a deciduous tree (Chukwuebuka 2015) and atmospheric deposition in porous bark (Moreira et al. 2018). The leaves are shed off on a seasonal basis while the bark is not. The longer lifespan of bark in comparison to the leaves could be the reasoning behind the higher accumulation of metals there. The bark is showing years of accumulation of metals while the leaves are just showing a single growing season's worth of metal concentrations (Ugulu et al. 2016).

Secondary to bark, the concentrations for uranium were highest in the pods/seeds followed by stems then leaves. Its accumulation could also be explained with the same concept of the phloem redistributing metals into new plant growth. However, little is known about what causes certain metals to be redistributed to certain parts of the plants and metals do not behave the same way, so discrepancies are expected (Van Bel 2003).

Another possible reason Moringa trees accumulate heavy metals and push them outwards into the bark and leaves could be to deter herbivores or disease-causing microorganisms from consuming it (Behmer et al. 2005; Boyd and Martens 1998; Fones et al. 2010; Galeas et al. 2007; Hanson et al. 2004; Horger et al. 2013; Jiang et al. 2005; Pollard and Baker 1996; Poschenrieder et al. 2006; Rascio and Navari- Izzo 2011; Rathinasabapathi et al. 2007). Insect deterrence is a reasonable explanation for why Moringa in this study accumulated metals in certain parts of its structure. Based on previous research conducted with other plants, it is more likely that deterrence would be an explanation for why metals accumulated in the leaves. However, there is no evidence against this hypothesis being used to explain the heavy metal presence in the barks as well. Hanson et al. (2004) saw the presence of selenium acting as a deterrence against phloem-feeding aphids. Additionally, many studies have been carried out on plants using arsenic, cadmium, nickel, zinc, and selenium as active deterrents against insect consumption while none studied the use of lead. There is a need for research on the use of lead as an active deterrent to insects and other pests by hyperaccumulator plants as well as to understand the transport and distribution of heavy metals in Moringa trees. This was beyond the scope of this current study.

Results for the current study show that lead was the most absorbed and translocated metal across all the plant parts. This could be explained by the fact that Broken Hill Mine, which is the source of the pollutants, was a lead-zinc mine. The other metals are generally found in relatively lower concentrations in the soils around the neighborhood. Selenium was the least translocated heavy metal of the four metals of interest in this study. This is not unusual as McLean and Bledsoe (1992) reported in their study. Selenium is a very common metal detected in contaminated areas but not necessarily in large amounts (McLean and Bledsoe 1992).

Phytoremediation potential

Moreno et al. (2008), Prasad and De Oliviera Frietas (2003), Rodriguez et al. (2005), and Van Ginneken et al. (2007) describe phytoremediation as the use of plants to solve environmental degradation problems by removing hazardous materials from the environment. At all of the sites sampled around the Mine Neighborhood, in this study, lead concentration in soils (with the lowest detected value being 296 ppm) exceeded the standard safe limits set by South Africa (230 ppm) and Canada (120 ppm) (Mathee et al. 2018) but not all exceeded the international and USEPA safe limit of 400 kg/kg (Figure 5). Two sites in the current study (296 and 349 ppm) had values below the international safety limit of 400 ppm but most values were above this safety level with the next lowest reading being 430 ppm and the maximum being 5850 ppm (Bose-O’Reily et al. 2018).

Despite every household in the neighborhood receiving a Moringa tree to plant in their yards, the plant was not present at every house visited. It appears not every household was able to successfully grow the tree. Even though Moringa is supposed to be a hardy tree (resistant and able to grow in areas with harsh climates and low soil nutrients), its low presence in the Mine Neighborhood suggests the tree would not be able to perform phytoremediation alone in this type of area (Foidl et al. 2001). Successful phytoremediation requires full coverage of the contaminated area to reduce mobilization, and the ideal plants would either be native plants or ones that can survive in extreme conditions (Gajic et al. 2018). Of the known phytoremediation methods, Gajic et al. (2018) suggest using phytostabilization to treat areas experiencing mine waste. They also suggest that phytoextraction, to a lesser degree, would be useful in these areas as well.

A combination of phytoremediation plants such as self- propagating sunflowers, stress tolerant members of the Brassicaccae family, and deep-rooted tree and grass species for stabilization (Forte and Mutiti 2017; Mourato et al. 2015; Santibanez et al. 2008; Schiemer 1940; Schnoor 1997) would be ideal additions to supplement the Moringa trees in contaminated areas. By utilizing both phytostabilization and phytoextraction, the metals would not only be made less mobile but also be removed from the soil. This would ideally reduce overall exposure to heavy metals in these areas.

Exposure through consumption of Moringa

While bioremediation using hyperaccumulators is important in polluted soils, it could potentially harm human health if the plants are both concentrating metals and being consumed. Since hyperaccumulators move a lot of the heavy metals into the shoots, it makes it dangerous to consume them as they would act as an additional exposure pathway for the people. Heavy metals do not tend to break down and as a result they get passed through the food chain. Based on the lead concentrations detected in the different parts of Moringa trees throughout the Mine Neighborhood, safe daily intake limits were calculated for people living in similarly contaminated areas. To consume the contaminated Moringa without risking raising one's BLL over the CDC safety limit of 5 µg/dL, one can only consume <1 g/day of Moringa. No plant part could be eaten in quantities larger than 1 g/day without potentially increasing the BLLs above the critical value if 5 µg/dL. Due to the small amount of the tree that could be consumed daily and still be deemed safe, it is not recommended that the people use Moringa medicinally if it were grown in contaminated soils. Consumption of Moringa for its health benefits would be negated by the negative health effects that come with the consumption of lead (Chibuike and Obiora 2014; Flannery et al. 2020; Huang et al. 1997; Kramer 2010; Mathee et al. 2018; Pehlivan 2009; Rahman et al. 2013). This consumption risk is further increased since other heavy metals such as arsenic and selenium are present in those plant parts as well.

Conclusion

This study evaluated the role Moringa plants play in phytoremediation and exposure to heavy metals in heavily polluted areas. The study also provides some knowledge about how Moringa distributes lead, arsenic, uranium, and selenium within the plant. Additionally, the study provided estimates of daily safe intake values for consuming Moringa that is grown in contaminated areas. The chosen study site was Kabwe, Zambia in an area that is heavily polluted with heavy metals such as lead, arsenic, selenium and uranium amongst others. This site was chosen because it also had numerous Moringa trees that were planted to aid in remediation and provide nutritional supplements to combat the negative health effects of lead exposure in people.

The study concluded that despite Moringa trees accumulating significant amounts of heavy metals in their shoots, Moringa is a poor to moderate phytoremediation plant for areas with high heavy metal contamination. The plant did not grow in every yard that planted the tree. Additionally, several Moringa plants did not have enough biomass to effectively remove heavy metals from the soil. However, Moringa trees could potentially be used for phytoremediation in combination with other plants that have larger biomass and are more tolerant to harsh conditions.

The high accumulation of lead in the different parts of the Moringa plant makes it unsuitable for use as a phytoremediator and a nutritional supplement at the same time. The calculated safe daily intake estimates show that consuming more than half a gram of the plant could increase an individual’s BLL to values above the established safe level of 5 µg/dL.

Results from this field study also showed that Moringa stored most of the lead, arsenic, and uranium in the bark and selenium in the leaves. The cores concentrated the least amount of all the metals studied. The relatively high accumulation of metals in the bark could be explained by the redistribution of metals to parts of the plant that are not essential to photosynthesis via the phloem. It could also potentially be due to plants using metals as a method of deterrence against herbivore consumption.

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Table 1. The amount of Moringa tree (in grams) that can be consumed by children and adults, respectively, without exceeding the IRL for daily allowable limit suggested by the US FDA.

Plant Part	Average Concentration (ppm)	Daily allowable intake for children to not exceed 3 ug (g)	Daily allowable intake for adults to not exceed 12.5 ug (g)
Bark	309.5	9.69×10^{-4}	4.03×10^{-3}
Pods/Seeds	27.0	1.11×10^{-2}	4.62×10^{-2}
Leaves	150.8	1.98×10^{-3}	8.28×10^{-3}
Cores	8.0	3.74×10^{-2}	$1.56 \times 10^{\cdot 1}$
Stems	106.8	2.80×10^{-3}	1.17×10^{-2}

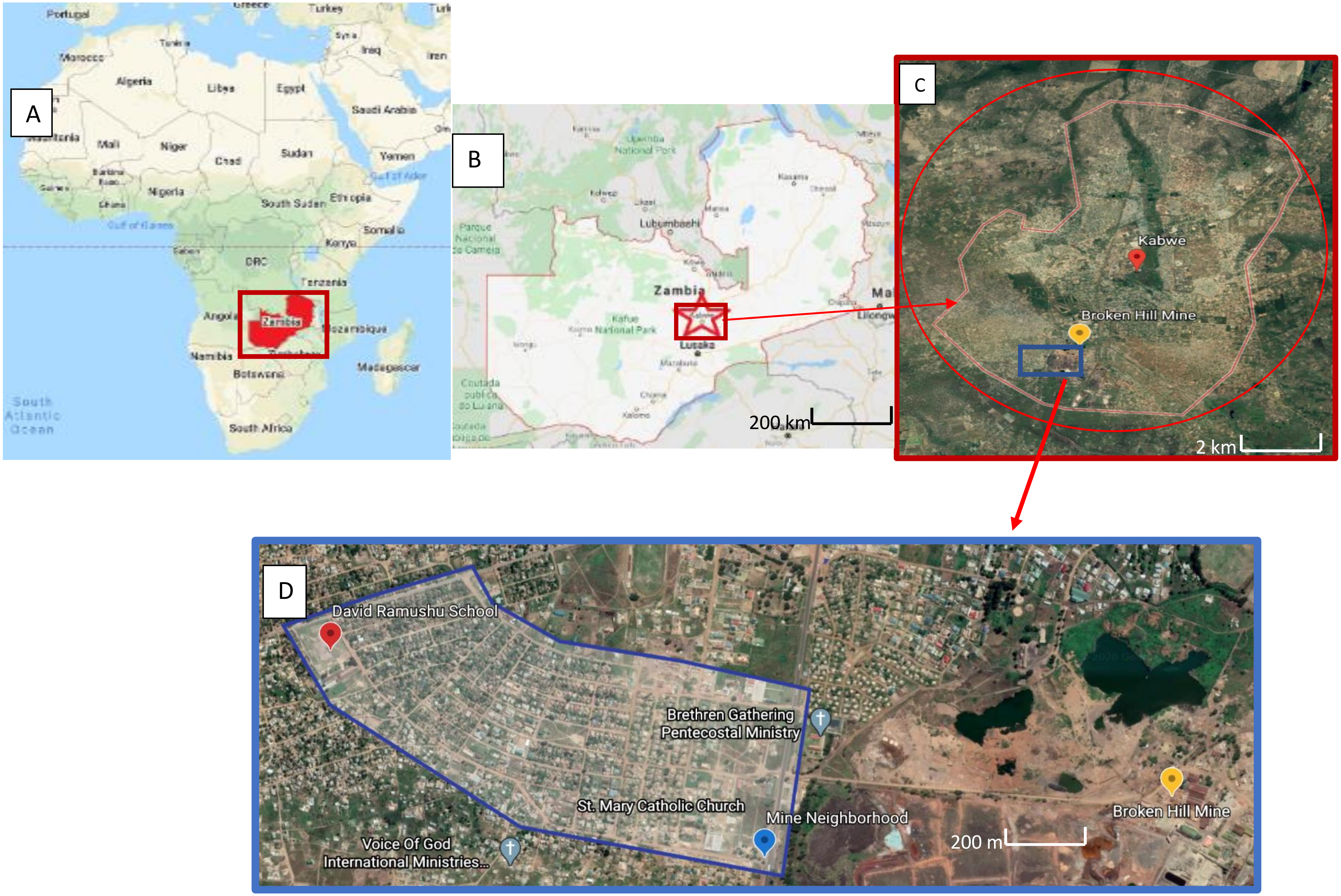
A
Portugal
Turkey
Morocco
Tunisia
Algeria
Libya
Egypt
Syria
Iraq
Iran
Saudi Arabia
Mali
Niger
Chad
Sudan
Yemen
Nigeria
South Sudan
Ethiopia
Somalia
Kenya
DRC
Tanzania
Angola
Zambia
Mozambique
Namibia
Botswana
Madagascar
South Africa
South Atlantic Ocean
B
Lubumbashi
Zambia
Kafue National Park
Lusaka
200 km
C
Kabwe
Broken Hill Mine
2 km
D
David Ramushu School
Brethren Gathering Pentecostal Ministry
St. Mary Catholic Church
Mine Neighborhood
Voice Of God International Ministries...
Broken Hill Mine
200 m

Figure 1

Figure 2

Figure 3

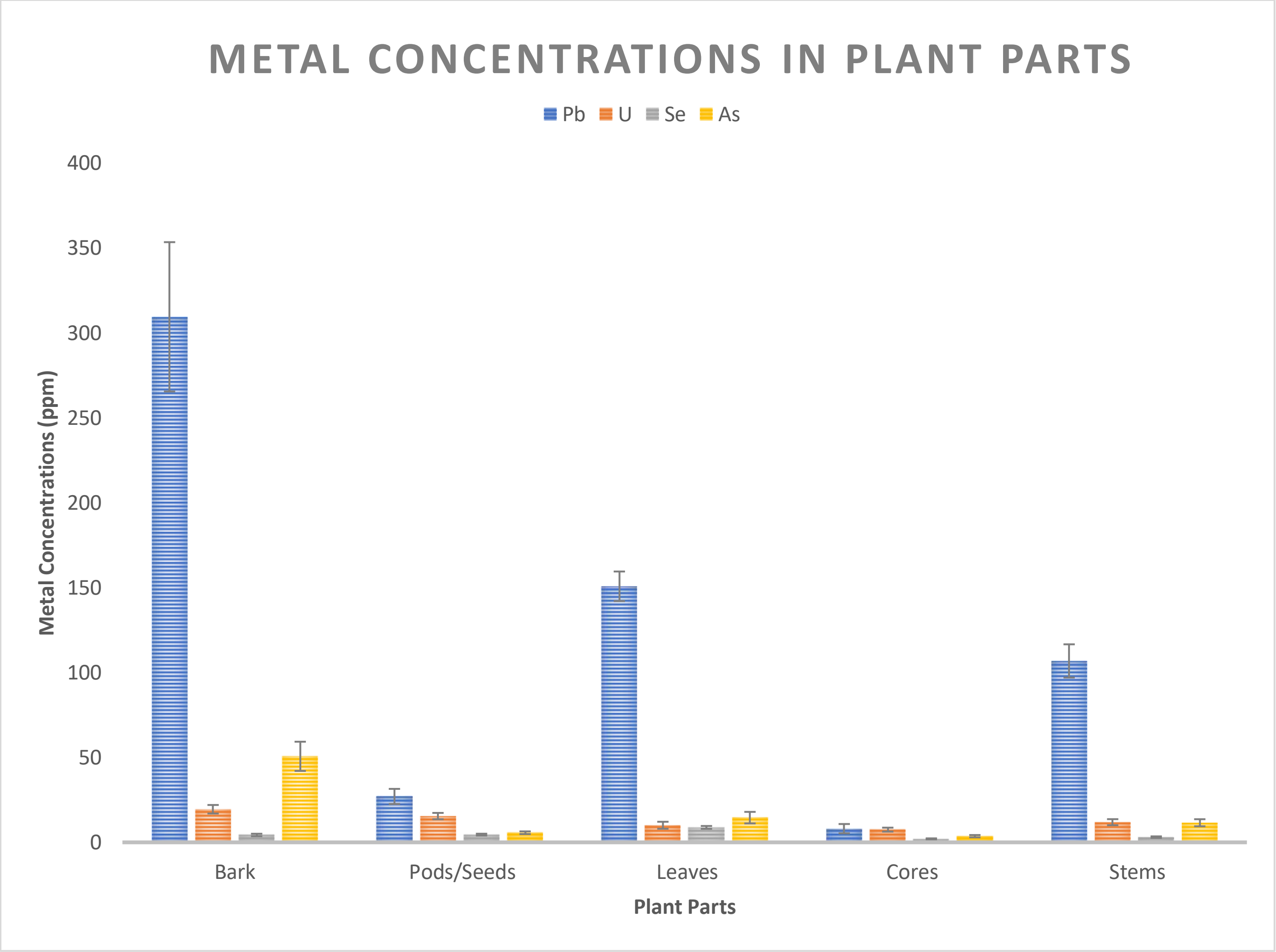
METAL CONCENTRATIONS IN PLANT PARTS
Pb
U
Se
As
Metal Concentrations (ppm)
400
350
300
250
200
150
100
50
0
Bark
Pods/Seeds
Leaves
Cores
Stems
Plant Parts

Figure 4

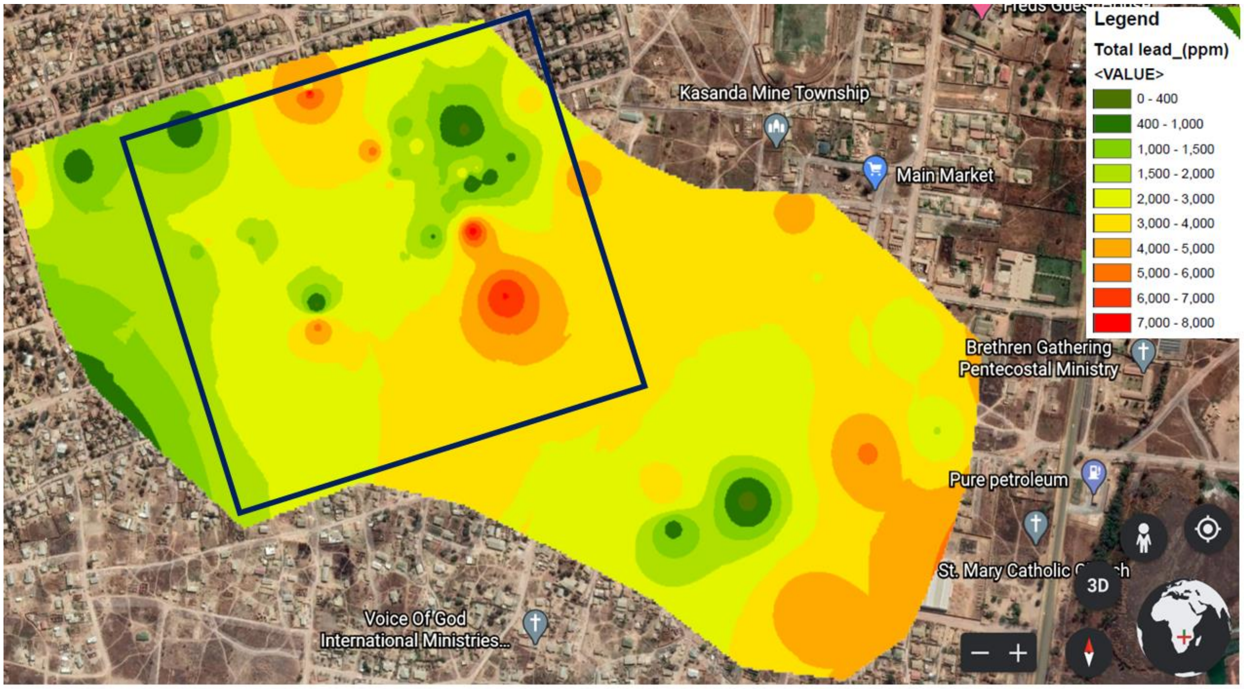
Legend
Total lead_(ppm)
<VALUE>
0 - 400
400 - 1,000
1,000 - 1,500
1,500 - 2,000
2,000 - 3,000
3,000 - 4,000
4,000 - 5,000
5,000 - 6,000
6,000 - 7,000
7,000 - 8,000
Kasanda Mine Township
Main Market
Brethren Gathering Pentecostal Ministry
Pure petroleum
St. Mary Catholic Church
Voice Of God International Ministries...
3D

Figure 5

Figure captions

Figure 1. A) A map of Africa highlighting Zambia in red, B) The town of Kabwe in Zambia is marked with a red star, C) The entire town of Kabwe, with the location of Broken Hill Mine labeled by a yellow marker, D) The study sites for sample collection, the David Ramushu Combined School and the Mine Neighborhood, as well as Broken Hill Mine are marked.

Figure 2A. The collection of leaves from the Moringa tree. B). Bark sample collection using a knife to expose the green inner layer is shown. C). A core sample placed in a straw for protection from breakage. D). The collection of soil from the homes of houses with Moringa.

Figure 3. Members of the research team washing the dust residue from the samples.

Figure 4. Concentrations of Pb, U, Se, and As in the bark, pods/seeds, leaves, cores, and stems of the Moringa trees studied.

Figure 5: Spatial distribution of lead in soils around the Mine Neighborhood in Kabwe, Zambia. The highest concentrations are in areas closest to the old Broken Hill Mine and some isolated pockets within the neighborhood.

Chapter 3

Evaluating the Difference in Metal Uptake of *Moringa oleifera* Plants Treated with Various Soil Amendments

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Abstract

Moringa oleifera (Moringa) is a tree that has the ability to hyperaccumulate large amounts of heavy metals. As a result, Moringa can be used for phytoremediation. Studies have shown that amending the soils can enhance a plant's ability to perform phytoremediation. The primary goal of this study was to investigate the effects of mycorrhizae on the uptake of lead by Moringa plants and their phytoremediation potential when grown under lab conditions. The second goal was to assess the potential risk of consuming Moringa grown in lead contaminated soils. In this study, two sets of Moringa plants were treated with 10, 000 ppm solutions of lead nitrate as well as mycorrhizae to understand the uptake potential of the trees. The presence of lead in the plants and soil was then quantified using XRF spectrometry. Using ANOVA tests as well as bioconcentration, translocation, and enrichment factors it was determined that Moringa trees are weakly suitable, but not very ideal, for phytoremediation in highly contaminated areas. This study also determined that the consumption of Moringa grown in contaminated soils is likely to pose a serious threat to the health of children and adults despite the presence or absence of additional amendments.

Keywords: phytoremediation, lead, metal accumulation, mycorrhizae, exposure risk

Introduction

The effect that mining has on the health of the environment and its inhabitants is a global issue (Stewart 2019) that continues to plague humanity. Many countries depend on mining as a major source of national gross domestic product and personal incomes. Unfortunately, in many developing countries the problems that accompany mining are not always considered before, during, or after mining operations begin. Even in industrialized countries where environmental consciousness and regulation exist these issues occur. As a result, water, soil, crops, air, and wildlife in the surrounding environments are contaminated. Many people living in areas close to the mines are exposed to elevated levels of pollutants and their health suffers as a result. Blood lead levels (BLLs) over the safety limit (5 μg/dL) set by the United States' Centers for Disease Control and Prevention (CDC) are commonly seen in individuals living near such areas (Bello et al. 2016; Safi et al. 2019; Tirima et al. 2016; Yabe et al. 2015). Without rehabilitative action, the health of these people will continue to deteriorate, sometimes to the point of death, even when exposure is acute. Moreover, the environment becomes more difficult to remediate the longer these conditions persist, and the contamination goes unchecked (Chukwu and Oji 2018).

To make matters worse some of these mines are found in less affluent neighborhoods resulting in these populations being disproportionately affected compared to their affluent counterparts. Whatever the reason, the people living near these mines are economically disadvantaged and do not have the option to relocate, making their health dependent on the remediation of their neighborhoods (Mathee et al. 2018). The rehabilitation of heavy metal contaminated areas has occurred for many decades and as a result many remediation technologies exist. In one of the heavily contaminated areas of Kabwe, Zambia, a project was initiated to lessen the effect of lead pollution on the neighborhoods surrounding a closed mine.

The mine is located in the southern end of the town and was called Broken Hill Mine. This was a very productive lead and zinc mine. However, this resulted in extensive lead contamination in the area that permeates the neighborhoods, and subsequently impacts the residents' livelihood. As part of a pilot project carried out by the World Bank, *Moringa oleifera* (Moringa) trees were given to the residents of the Mine Neighborhood to plant in their yards because of the trees' hyperaccumulating and medicinal abilities (World Bank 2011).

Moringa trees are well known for their medicinal and nutritional value. They have the capability to treat nearly 300 diseases and the plant offers many benefits due to its antibacterial, antifungal, diuretic, and antihypertensive properties (Anwar et al. 2007; Fahey 2005; Foidl et al. 2001). Most parts of the tree can be consumed as they offer either a nutritional or health benefit. In addition to its medicinal attributes, Moringa is also capable of hyperaccumulating heavy metals. This gives it the potential to perform phytoextraction, a type of phytoremediation, in contaminated areas. Phytoremediation is a form of bioremediation in which plants are used to remediate contaminated areas. Phytoextraction involves the plants absorbing heavy metals while performing normal plant processes and then storing the metals in various parts of the plant (Amadi and Tanee 2014; Soliman and Sugiyama 2016).

One of the most prevalent heavy metals in the world is lead, which is usually accompanied by other metals. Lead is one of 23 known toxic heavy metals. While some heavy metals are necessary, in low quantities, for biological processes to occur, lead is not required for any known biological function. In fact, lead is known to replace important cations and cause deficiencies in the body subsequently interrupting some of these biological functions (Jaishankar et al. 2014). Lead can damage nearly every system of the body and cause physical, mental, and intellectual disabilities. Additionally, the effects of lead poisoning can be fatal. Lead more readily targets developing body tissues, therefore placing children are at higher risk for lead poisoning than fully developed adults (Cafasso 2018; Cronan 2019; Yabe et al. 2015).

This study investigated the ability of Moringa to perform phytoremediation in soils with elevated levels of lead. Due to Moringa's ability to hyperaccumulate heavy metals, it could potentially remove enough lead from the soil rendering it safe to use for agricultural purposes. The efficiency of Moringa to transport lead from the soils into and throughout the plants was also assessed together with the effect of mycorrhizae on Moringa's ability to take up lead and remove it from contaminated soil. Secondarily this study also investigated whether further exposure risk to heavy metals exists when consuming parts of the tree for its medicinal value, when Moringa is grown in contaminated soils.

Materials and Methods

Seed germination and Moringa growth

Rorchio™ Moringa *oleifera* seeds were purchased from Amazon©. The hard, exterior shell of at least 50 Moringa seeds were cracked prior to submerging them in water for 24 hours. The seeds were then planted in small plastic cups for germination using Sta-Green © Moisture Max Potting Mix plus Fertilizer soil. Two weeks after germination the seedlings were transplanted into larger pots (Figure 1A,1B,1C). Daily checks were conducted on the plants and they were watered, as necessary (typically every two days).

Experimental design 1

This experiment was carried out in a temperature-controlled greenhouse (21.1°C and 55% relative humidity) at Georgia College and State University (GCSU) in Milledgeville, GA. The plants were germinated during the end of the fall season (10/25/2019) before being transplanted into pots with equal amounts of soils. The plants were subjected to three different treatments in the winter season (01/15/2020). The first treatment involved the application of 100 mL of a 10,000 ppm lead nitrate solution to the plants (referred to as Lead Only treatment). This treatment had a total of ten plants. The second treatment included the application of 100 mL of a 10,000 ppm lead nitrate solution and a solution of Root Naturally© Granular Endo Root Mycorrhizae to the plant soils (referred to as the Lead & Mycorrhizae treatment). One gram of powder mycorrhizae was also mixed into the soil of each of the ten plants in this treatment. The third treatment was a control treatment with five plants and no amendments added (referred to as Control, Figure 2A,2B).

The lead nitrate solution was made by dissolving 3 grams of lab-grade powdered lead nitrate with 3 liters of distilled water while the mycorrhizae solution was made by mixing 42.5 grams of mycorrhizae into 3.8 liters of water. The plants were grown for another four weeks and were watered every other day before being harvested at the end of winter 02/11/2020. During this period of the experiment the temperature in the greenhouse stayed relatively close to the set temperature of 21^{o}C.

Plant harvest and sample preparation

Plant growth was tracked by measuring their heights before treatment application and prior to harvest. The height differences for each treatment were then calculated by subtracting the two readings to compare differences in growth between treatments for the same time frame. During harvest, the leaves (any part of the plant starting from the bud outward, Figure 3A) were removed from the plant first and then the stems were cut as close to the soil line as possible (Figure 3B). Finally, the roots were pulled from the soil (Figure 3C) and thoroughly washed and cleaned using a clean toothbrush to remove all of the soil particles. Samples of the soils in the pots were collected and bagged for drying. All the samples for the experiment were then dried at

70°C in the Binder© oven and then placed into sealable bags for crushing and storage prior to analysis.

Experimental design 2

Another set of seeds were prepared for germination using the same methods as those in the 'Seed Germination and Moringa Growth' section above. After germination (which began in winter on 01/08/2020), the seedlings were placed in small pots and allowed to grow for three weeks. After the three-week growth period ended, one set of plants was removed from their pots and their roots were dipped in Root Naturally© Granular Endo Root Mycorrhizae (Figure 4A) before being replanted in mycorrhizae enriched soil. This treatment is referred to as 'Soil Pb Mycorrhizae' (Figure 4B). Another set of plants referenced as 'Liquid Pb Mycorrhizae' (Figure 4C) were watered with a liquid mycorrhizae solution. The solution was made by dissolving 14.3 grams of Wildroot© Pure Premium Mycorrhizae Fungi Organic Concentrate in 3.8 L of water. A third set of plants labeled as 'Lead Control' (Figure 4D) consisted of plants that were not treated with anything other than 50 mL of the lead nitrate solution that was also applied to the other two treatments.

There were 12 plants in the Soil Pb Mycorrhizae treatment group and 10 plants in both the Liquid Pb Mycorrhizae and Lead Control treatments. The plants were contaminated with 50 mL of the 10,000 ppm lead nitrate solution on 02/05/2020. The plants were allowed to grow and watered as necessary for two more weeks before being harvested on 2/20/2020. This experiment was carried out for a shorter time frame than initially planned because there were significant fluctuations in the internal greenhouse temperature (21°C to 30°C) due to the cooling system malfunctioning. This caused observable plant stress and resulted in early harvesting. The shoots (both the leaves and stems of the plants, Figure 5A) were harvested from each plant. The roots were extracted from the soil (Figure 5B) but were frozen instead of being dried like the other set of plants due to the small amount of biomass present. These roots will be analyzed at a later time. After root removal, soil samples were collected and bagged for drying. The shoots and soil were dried in the Binder© oven, crushed as necessary, and bagged for analysis.

Sample analysis

The metal concentrations of the samples were quantified using the Delta X-Ray Fluorescence (XRF) instrument by Olympus © (Boston, MA). The XRF instrument was calibrated daily to ensure that it produced accurate results. The data from the XRF analysis was processed using basic statistics and Analysis of Variance (ANOVA) tests in Microsoft Excel. The ANOVAs were used to compare lead uptake amongst the treatments and various plant parts using a 95% confidence level (alpha value of 0.05).

Three indices were calculated to determine the potential of Moringa to perform phytoremediation including the bioaccumulation factor (BCF), translocation factor (TF), and enrichment factors (EF). The formulas (Table 1) were adapted from the version used by Brankovic et al. (2019).

Safe daily intake values

The average amount of Moringa parts that would be safe to consume when the plant is grown in soils with lead concentrations between 3000 ppm and 6500 ppm is less than a gram daily. The safe intake limit values were calculated based on the amount of lead taken up in the different plants parts and the Interim Reference Level (IRL) for safe consumption of food set by the Food and Drug Administration (FDA) of 3.0 μg/day for children and 12.5 μg/day for adults (Flannery et al. 2020). The estimated safe quantities were computed by dividing the reference values by the concentrations (ppm) in the plant parts.

Results

Experimental design 1

No significant difference existed between lead concentrations in the soils of the Lead Only (3050.38 ppm) and Lead & Mycorrhizae treatments (3453.30 ppm), Figure 6. An ANOVA test showed a p-value of 0.41. However, the control group which was not treated with any of the 10,000 ppm lead nitrate solution did show a significant difference (p-values < 0.0001) from the soils of the other two treatments with an average concentration of 6.77 ppm.

The average concentrations of lead uptake in the leaves, stems, and roots of Moringa trees grown in contaminated soil are shown in Figure 7. The leaves of all three treatments were not significantly different from each other (p-values > 0.05). However, the greatest average uptake was seen in the leaves of the Lead Only treatment at a value of 29.83 ppm. The uptake of lead by the stems also showed that there was not a significant difference between any of the treatments (p-values ranged from 0.51 to 0.66). However, the stems of the Lead & Mycorrhizae group saw the greatest uptake of lead averaging 69.35 ppm. For both the leaves and the stems, the Control treatment had the least amount of lead uptake. The roots of the Lead Only and the Lead & Mycorrhizae treatments were not significantly different (p-value of 0.99) from each other with averages of 80.05 ppm and 80.65 ppm, respectively. The roots of the Control treatment showed no lead uptake.

For the Lead Only treatment there were statistically significant differences (p-values < 0.05) between ‘soil v leaves’, ‘soil v stems’, and ‘soil v roots’ while the other three comparisons (‘stems v roots’, ‘stems v leaves’, and ‘roots v leaves’) were not significantly different from one another. Within the Lead & Mycorrhizae treatments, there were two sets of comparisons out of the six that did not show a significant difference from one another (‘stem v roots’ and ‘stem v leaves'). Due to the lack of lead uptake in the roots of the Control treatment, the only parts compared were the stems, soil, and leaves. The 'stem v soil' and 'soil v leaves' did not show a significant difference. Comparing the three treatments to each other showed no significant difference in the stem, leaf, and root uptake. However, the soil of the Control treatment versus both the Lead Only and Lead & Mycorrhizae treatments did show a significant difference because lead was added to the latter two treatments.

Table 2 shows the BFC, TF, and EF results of the plants in Experimental Design 1. Except for one enrichment factor for the Control treatment, all of the factors were less than 1. No TFs could be calculated for the control plants as there was no lead detected in the roots. The TFs and EFs for the stems were greater than the TFs and EFs for the leaves in both the Lead Only and Lead & Mycorrhizae treatments. The BCF for the Control treatment was zero while the other two treatments were 0.026 (Lead Only) and 0.023 (Lead & Mycorrhizae).

Figure 8 shows the height difference of the plants before and after contamination in the Lead Only, Lead & Mycorrhizae, and Control treatments. There was an increase seen in all three treatments that averaged approximately 20 centimeters. While there was no significant difference seen in height increase, the Lead & Mycorrhizae treatment averaged slightly more growth than the other two treatments.

Experimental design 2

Results from the soil analysis showed that the treatments were not significantly different from each other based on the 95% confidence level used. The Liquid Pb Mycorrhizae treatment had the highest average at 6327.62 ppm followed by the Soil Pb Mycorrhizae treatment (5282.40 ppm) and then the Lead Control treatment (5579.07 ppm), Figure 9.

The Soil Pb Mycorrhizae treatment averaged a lower concentration of lead in the shoots at 6.84 ppm compared to Lead Control and Liquid Pb Mycorrhizae treatments which showed mean accumulations of 13.57 ppm and 11.96 ppm respectively (Figure 10). All three treatments were not significantly different from each other. The shoots took up a very small fraction of the total lead in the soil (< 0.3%).

The enrichment factors for the plants in Experimental Design 2 were also all less than one. The Lead Control had the greatest enrichment factor followed by Liquid Pb Mycorrhizae and then Soil Pb Mycorrhizae (0.0024 > 0.0019 > 0.0013).

Daily safe intake values

The safe daily quantities that a person can consume without exceeding the limit set by the FDA ranged from 0.004 g to 0.1 g. Table 3 and Table 4 show the amount of lead in grams that could be consumed daily without causing the blood lead level of an individual to exceed 5 μg/dL.

Discussion

The results from Experimental Design 1 showed that the roots had the greatest concentration of lead of the studied plant parts. This is likely due to the roots being the first point of contact the plant has with the contaminated soil. Additional energy would be required to move any metals from the roots into the shoots. Another potential explanation is that lead, due to its insolubility, is commonly retained in root cells so that it will not reach the xylem for further transportation into the plant shoots (Bravin et al. 2008; ITRC 2009; Kosegarten and Koyro 2001; Richau et al. 2009; Yang et al. 2006). It is also not unusual amongst heavy metals, in general, for only a minor amount to make it into the shoots (Page and Feller 2005; Page et al. 2006). Heavy metals in the soil frequently become immobilized in the roots, increasing accumulation and subsequently detection rates (Gong and Tian 2019). High accumulation rates in the roots is not a rare trend as many plant species preferentially store lead in their roots when compared to other parts of the plant (Tangahu et al. 2011). Root metal accumulations have been known to increase with the use of mycorrhizae according to Li et al. (2009), however, this was not observed in this study. Root uptake by the Lead Only (80.05 ppm) and Lead & Mycorrhizae (80.65 ppm) treatments did not show a significant difference in uptake averaging a difference of only 0.60 ppm. Another possible explanation is that metals are sometimes found in high concentrations on the roots when excess metal solution precipitates onto them, however, this is likely not the case for this study because samples were thoroughly washed so excess lead precipitants should not be present (Prasad and De Olivieira Freitas 2003; US EPA 2000).

The Lead Only treatment averaged the highest accumulation for lead in leaves. However, there was no significant difference in uptake between any of the three treatments. This means that the addition of mycorrhizae to the soil did not have any effect on the uptake of lead in the leaves. For stem lead uptake, there was also no significant difference observed, but the Lead & Mycorrhizae treatment had the highest uptake of lead in the stems. The addition of mycorrhizae could potentially have caused this increase in lead uptake and over an increased period of time a significant difference between the treatments might have been observed. It is possible that the mycorrhizae and Moringa would have been able to establish a stronger relationship and produce different results over a longer time frame. A higher concentration of lead in the stems versus in the leaves was observed as well. According to Forte and Mutiti (2017) when studying *Helianthus annuus*, lead is not easily moved within plants and is more likely to be stored in stems rather than the leaves. The current study supports these findings since stem lead concentrations were higher than leaf lead concentrations. Tangahu et al. (2011) observed similar trends in their study in which five different species of *Brassica* plants had higher concentrations of lead in the stems than the leaves. This could be due to plants trying to exclude excess harmful metals from the leaves to keep them clear to perform photosynthesis. It is well known that the xylem transports lead to the leaves and then the phloem redistributes it to other parts of the plant. However, in the case of the Moringa plants used for this study the only other places to store the lead is in the stems or roots which could potentially explain the results (Khan et al. 2014; Page and Feller 2005, 2015; Page et al. 2006; Riesen and Feller 2005; Yang et al. 2006).

Moringa is known to hyperaccumulate metals and hyperaccumulators tend to move metals from the soil into the shoots without retaining them in the roots (Amadi and Tanee 2014; Kramer 2010; Memon and Schroder 2009). However, the Lead Only and Lead & Mycorrhizae treatments had the highest concentration of lead in the roots versus any other plant parts. Liu et al. (2000) observed that many of the hyperaccumulators within the Genus *Brassica* also concentrate lead in their roots. Vamerali et al. (2009) noted the preferential retention of heavy metals in the roots of various members of the Genus *Populus*. Shtangeeva et al. (2004) found that lead, in comparison to other heavy metals, does not move efficiently from the roots of *Triticum vulgare* into its shoots. While hyperaccumulators should have more metals in their shoots, these studies show that lead tends to concentrate in the roots in a lot of plant species including some other hyperaccumulators. Potentially, lead behaves differently from other heavy metals causing this pattern of root accumulation seen in all these studies including the current one.

Many hyperaccumulators can perform phytoextraction which typically becomes more efficient with the addition of microorganisms like mycorrhizae (Chibuike and Obiora 2014; Van Ginneken et al. 2007). When studying *Brassica juncea* and *Pteris vittata*, Salido et al. (2003) determined that this addition can be crucial for mobilizing lead since it is typically very stationary due to its insolubility. While sometimes the use of mycorrhizae increases uptake into the shoots like Liphadzi et al. (2003) observed in *Helianthus annuus*, *Brassica rapa, and Brassica juncea* this was not the case in Experimental Design 1. The lead accumulation in the leaves does not support this claim since all three treatments were not significantly different from each other despite one being treated with mycorrhizae. However, similarly to these results, a study by Rydlova and Vosatka (2003) found that the addition of mycorrhizae did not change lead uptake in *Agrostis capillaris* between mycorrhizae and non-mycorrhizae lab treatments that spanned a 20-week time frame.

Soil from the Lead Only and the Lead & Mycorrhizae treatments were not significantly different from each other in terms of lead accumulation (p-value of 0.51). This means any differences in lead uptake by the leaves, stems, and roots should not have been impacted by soil lead concentrations. In other studies, it has been concluded that the addition of mycorrhizae should reduce the soil lead concentration, mostly by making lead more bioavailable than normal so it can be concentrated in plants (Bano and Ashfaq 2013; Gong and Tian 2019; Meier et al. 2012). The soil lead concentration results from this study do not support those conclusions. Additionally, the soil from the Lead & Mycorrhizae showed a slightly higher average lead concentration than the Lead Only soil by a few hundred parts per million meaning mycorrhizae did not reduce the lead concentration of the soil.

Hussain et al. (2013) and Kabir et al. (2010) found that lead can reduce plant height. Results from the current study did not exactly experience this effect. All three treatments in Experimental Design 1 saw a continued increase in height after contamination, and there was no significant difference between any of the three treatments. However, the Lead Only treatment had the lowest average increase in height while the Lead & Mycorrhizae had the highest. There is a possibility the mycorrhizae allowed the latter treatment to have a higher average growth, but more experimentation would need to be conducted to confirm this assertion. The lack of difference among the treatments could also be an artifact of the growth time, the current study was not conducted for a long enough period of time for the Moringa to grow into trees. If the effects of mycorrhizae on tree growth is slow then these effects would be missed in a time-limited study such as this one.

The BCFs, TFs and EFs can be used to determine whether plants would be able to perform phytoremediation or stabilize the metals. The BCF looks at how much of the metal in the soil gets moved into the roots of the plant. The TF shows how much of the roots' metal concentrations can be moved into the stems or leaves. The EF looks at various parts of the shoots and the quantity of metals from the soil that were moved those parts (Brankovic et al. 2019; Nirola et al. 2015; Sagiroglu et al. 2006; Sinha et al. 2007; Wu et al. 2011). TFs with values > 1 mean that the metals move easily throughout the plant, which suggests it is a potential hyperaccumulator and may be good at performing phytoremediation (Kastratovic et al. 2014; Majid et al. 2014; Sagiroglu et al. 2006; Wu and Sun 1998). If the EF is > 1, also shows that there is a high likelihood the plant can effectively perform phytoremediation (Adesodun et al. 2010). A BCF > 1 also means the plants have a high phytoremediation potential. However, if the value of the BCF is < 1 the plant can absorb metals but not accumulate them (Alaboudi et al. 2018; Fitz and Wenzel 2002; Mendez and Maier 2008; Satpathy et al. 2014; Yoon et al 2006). The results from the current study showed that all of the values calculated were < 1 except for the EF for the leaves of the Control treatment. It can be concluded that *Moringa oleifera* that is grown for just a few weeks does not have the potential to perform phytoextraction. Moreover, these results also show higher concentrations of lead in the roots than in any other plant parts.

Based on Yoon et al. (2006), the young *Moringa oleifera* from our study would not be useful for phytostabilization. However, Gajic et al. (2018) suggest that plants with either a BCF < 1 and TF > 1 or plants with a BCF and a TF both < 1 can phytostabilize and the young Moringa from our study falls into the latter category. Based on the results from this study (Table 2), we would suggest pairing Moringa with other plants that can either phytostabilize or phytoextract until more research is conducted to see the effect of growth time on *Moringa oleifera's* ability to phytoremediate metals (Schnoor 1997).

The concentrations of lead in the soil from Experimental Design 2 were not significantly different from each other. This was the expected result as all three treatments were contaminated with 50 mL of a 10,000 ppm lead nitrate solution. This means that the shoot uptakes should not have been affected by any variation in soil lead concentration since no statistical difference was observed.

Lead concentrations in the shoots of the three treatments were not significantly different from each other based on ANOVA test results. The application of mycorrhizae did not affect the uptake of lead. This greenhouse experiment was conducted over a shorter time period (66 days overall), than Experimental Design 1 and shows mycorrhizae had less effect on uptake. It appears that mycorrhizae added to plants with a shorter growth time had less impact on lead uptake by *Moringa oleifera* plants. This could potentially be a result of the mycorrhizae not being able to effectively colonize the roots in a short time and/or the general immobility tendencies of lead. More time is likely needed for the plant to create an effective symbiotic relationship with the fungi and for it to mobilize lead. Further research is needed to better explain the results from this study.

Due to the minute amount of the plants that could be consumed safely, it is not recommended that individuals consume Moringa grown in highly contaminated soils. The likelihood that an individual could consume enough Moringa to reap the medicinal benefits of the plant but not risk raising their BLL over the CDC safety limit is very low. The practice of consuming Moringa grown in highly contaminated areas such as the neighborhood near the Broken Hill Mine in Kabwe should not continue due to the risk of lead exposure. This practice becomes more dangerous when one thinks of the other toxic metals present in the plants (Flannery et al. 2020).

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Table 1. Formulas for calculating the various phytoremediation factors.

Phytoremediation Factor	Formula	Formula Key
Bioconcentration (BCF)	$BCF = M_{root} / M_{soil}$	M_{root} – metal concentration in root
		M_{stem} – metal concentration in stem
Translocation (TF)	$TF_{stem} = M_{stem} / M_{root}$	M_{leaf}- metal concentration in leaf
	$TF_{leaf} = M_{leaf} / M_{root}$	M_{soil} – metal concentration in soil
Enrichment (EF)	$EF_{stem} = M_{stem} / M_{soil}$	M_{shoot} – average metal concentration in leaf & stem
	$EF_{leaf} = M_{leaf} / M_{soil}$	
	$EF_{shoot} = M_{shoot} / M_{soil}$	

Table 2. The results for Experimental Design 1 from the calculated phytoremediation factors.

Phytoremediation Factor	Lead Only Treatment	Lead & Mycorrhizae Treatment	Control Treatment
Translocation Factor Stems	0.41	0.86	-----
Translocation Factor Leaves	0.37	0.15	-----
Enrichment Factor Stems	0.011	0.020	0.59
Enrichment Factor Leaves	0.0098	0.0034	1.48
Bioconcentration Factor	0.026	0.023	0.0

Table 3. Daily allowable intake of lead in grams for children and adults based on the plant parts from Experimental Design 1. The amounts were calculated using the FDA's Interim Reference Level for daily lead consumption.

Treatment	Plant Part	Average Concentration (ppm)	Daily allowable intake for children to not exceed 3ug (g)	Daily allowable intake for adults to not exceed 12.5ug (g)
Lead Only	Leaves	29.8	1.00×10^{-2}	4.19×10^{-2}
	Stems	32.6	9.20×10^{-3}	3.83×10^{-2}
	Roots	80.1	3.74×10^{-3}	1.56×10^{-2}
Lead & Mycorrhizae	Leaves	11.8	2.54×10^{-2}	1.05×10^{-1}
	Stems	69.4	4.32×10^{-3}	1.80×10^{-2}
	Roots	80.7	$3.71 \times 10^{.3}$	1.54×10^{-2}
Control	Leaves	10.0	2.99×10^{-2}	1.24×10^{-1}
	Stems	4.0	7.49×10^{-2}	3.12×10^{-1}
	Roots	0.0	N/A	N/A

Table 4. Daily allowable intake of lead in grams for children and adults based on the shoots of Experimental Design 2. The values were calculated using the FDA's Interim Reference Level for daily consumption of lead.

Treatment	Average Concentration (ppm)	Daily allowable intake for children to not exceed 3 ug (g)	Daily allowable intake for adults to not exceed 12.5 ug (g)
Liquid Pb Mycorrhizae	12.0	2.49×10^{-2}	1.04×10^{-1}
Soil Pb Mycorrhizae	6.8	4.41×10^{-2}	1.83×10^{-1}
Lead Only	13.6	2.20×10^{-2}	9.19×10^{-2}

Figure 1

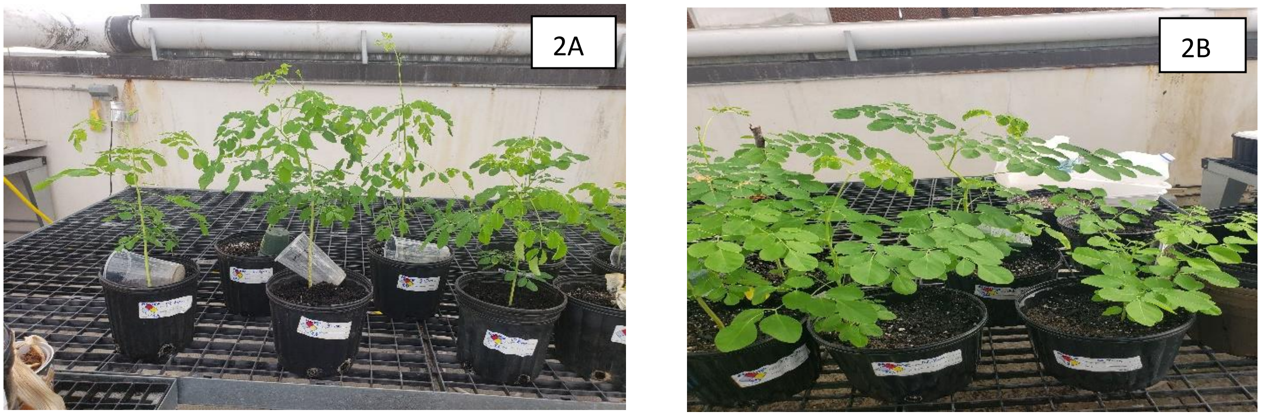

Figure 2

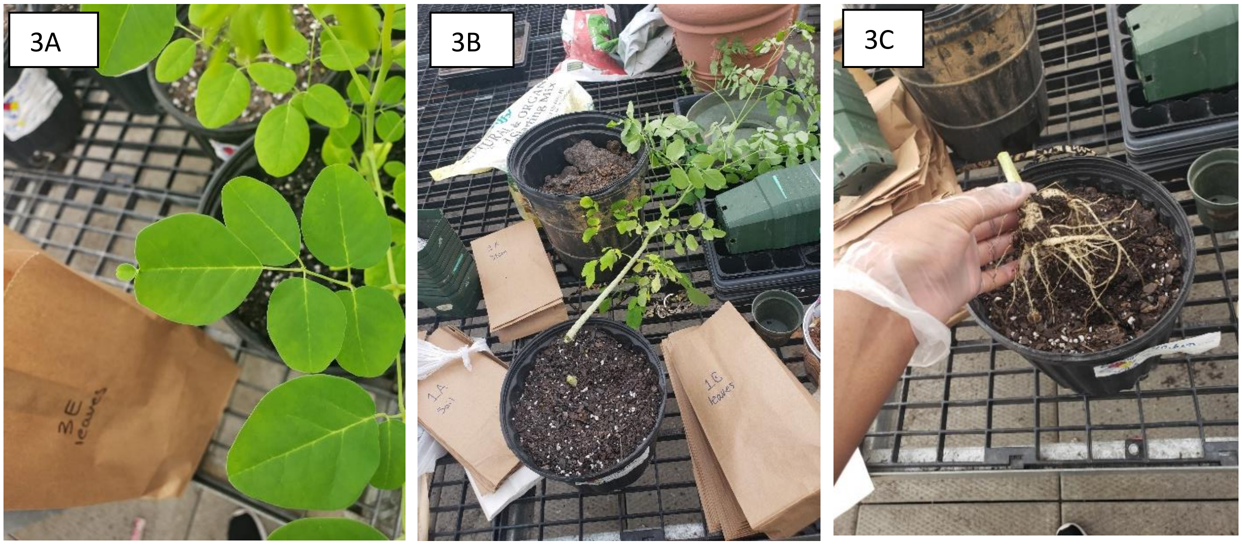

Figure 3

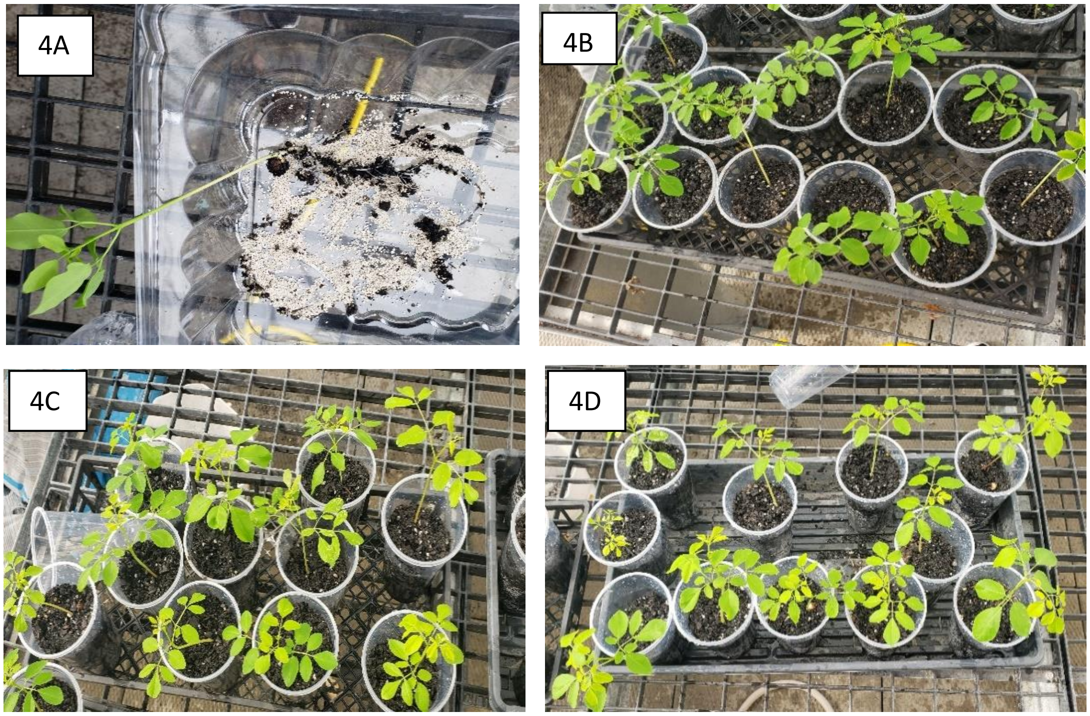

Figure 4

Figure 5

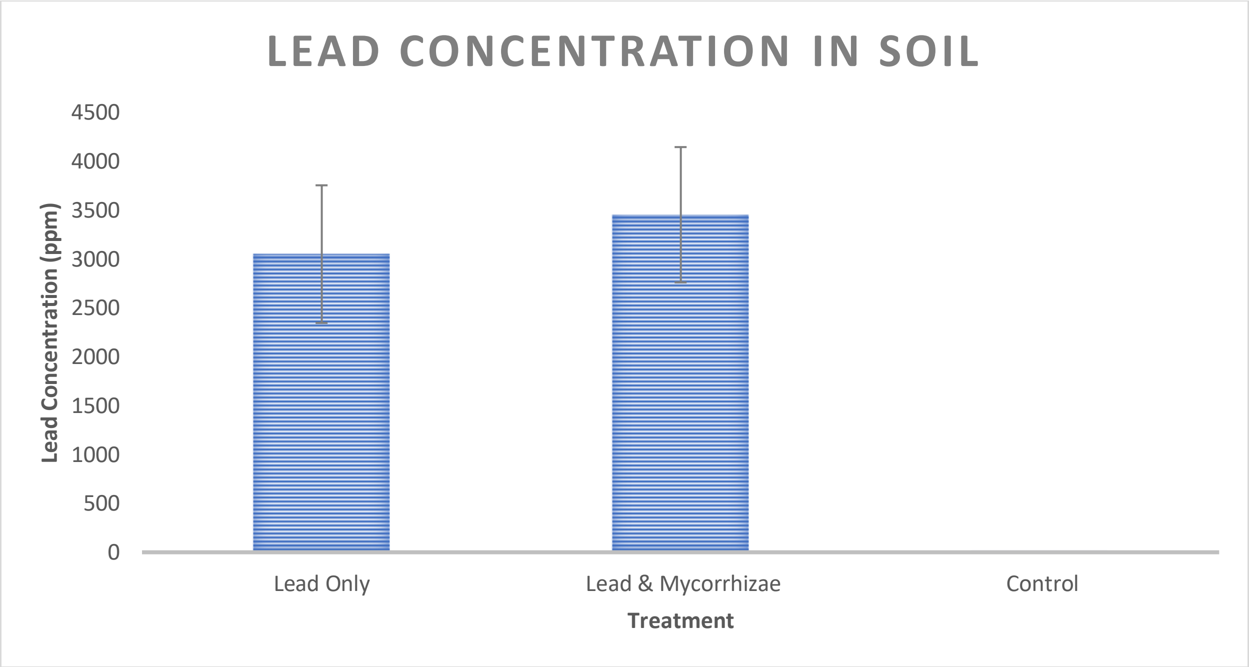
LEAD CONCENTRATION IN SOIL
Lead Concentration (ppm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Lead Only
Lead & Mycorrhizae
Control
Treatment

Figure 6

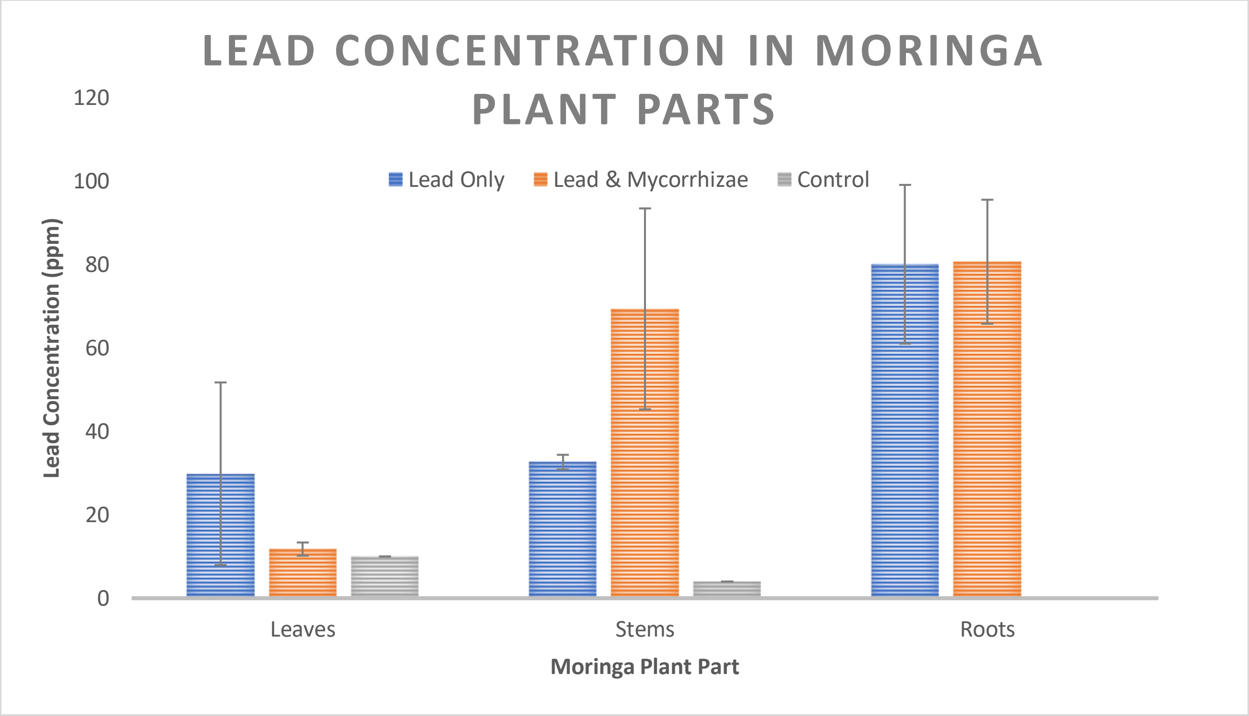
LEAD CONCENTRATION IN MORINGA PLANT PARTS
Lead Only
Lead & Mycorrhizae
Control
120
100
80
60
40
20
0
Lead Concentration (ppm)
Leaves
Stems
Roots
Moringa Plant Part

Figure 7

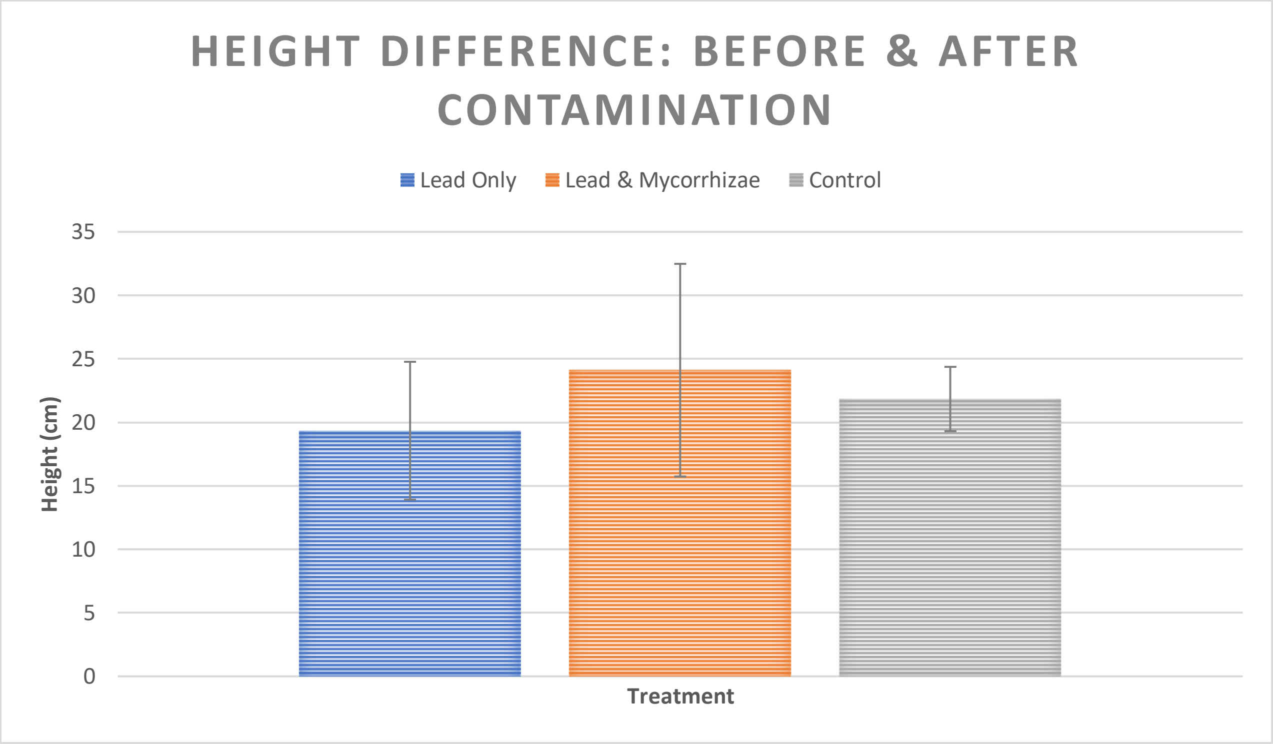
HEIGHT DIFFERENCE: BEFORE & AFTER CONTAMINATION
Lead Only
Lead & Mycorrhizae
Control
35
30
25
20
15
10
5
0
Height (cm)
Treatment

Figure 8

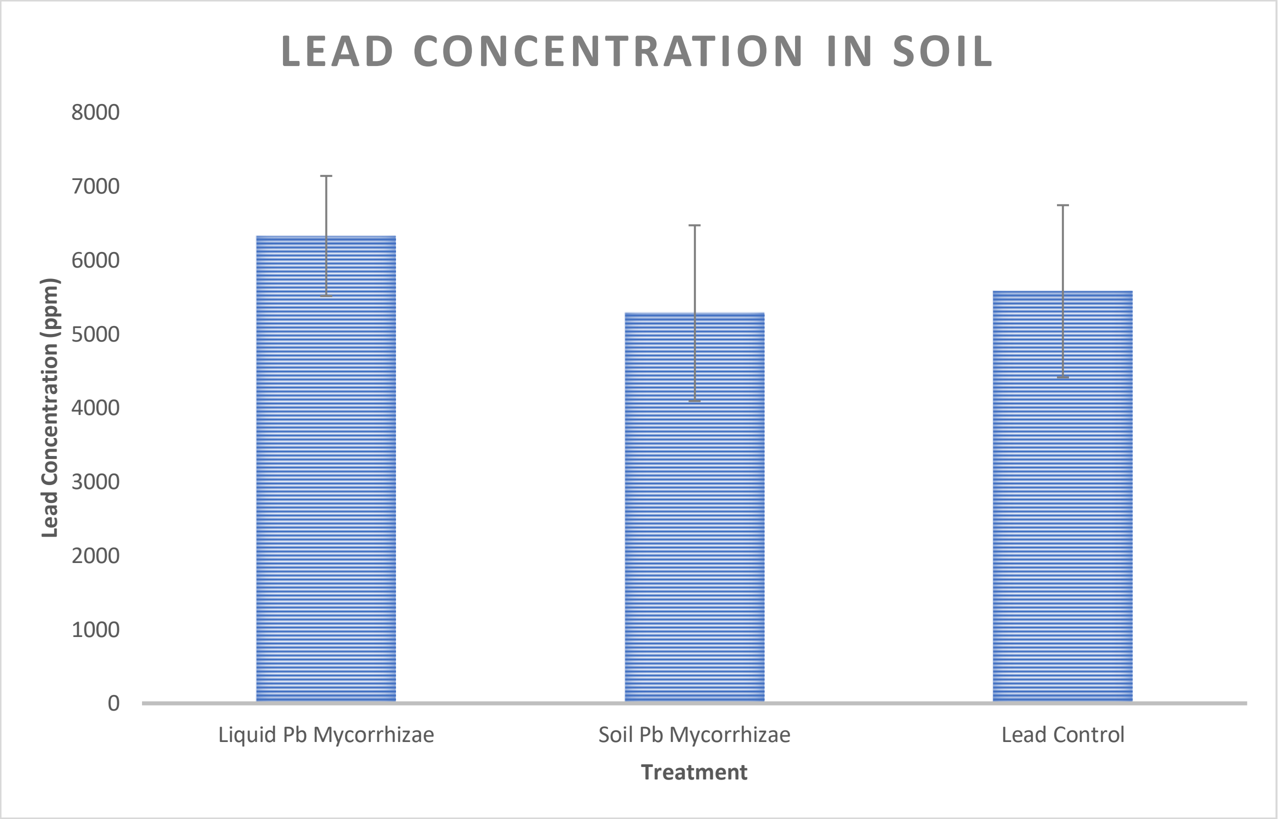
LEAD CONCENTRATION IN SOIL
Lead Concentration (ppm)
8000
7000
6000
5000
4000
3000
2000
1000
0
Liquid Pb Mycorrhizae
Soil Pb Mycorrhizae
Lead Control
Treatment

Figure 9

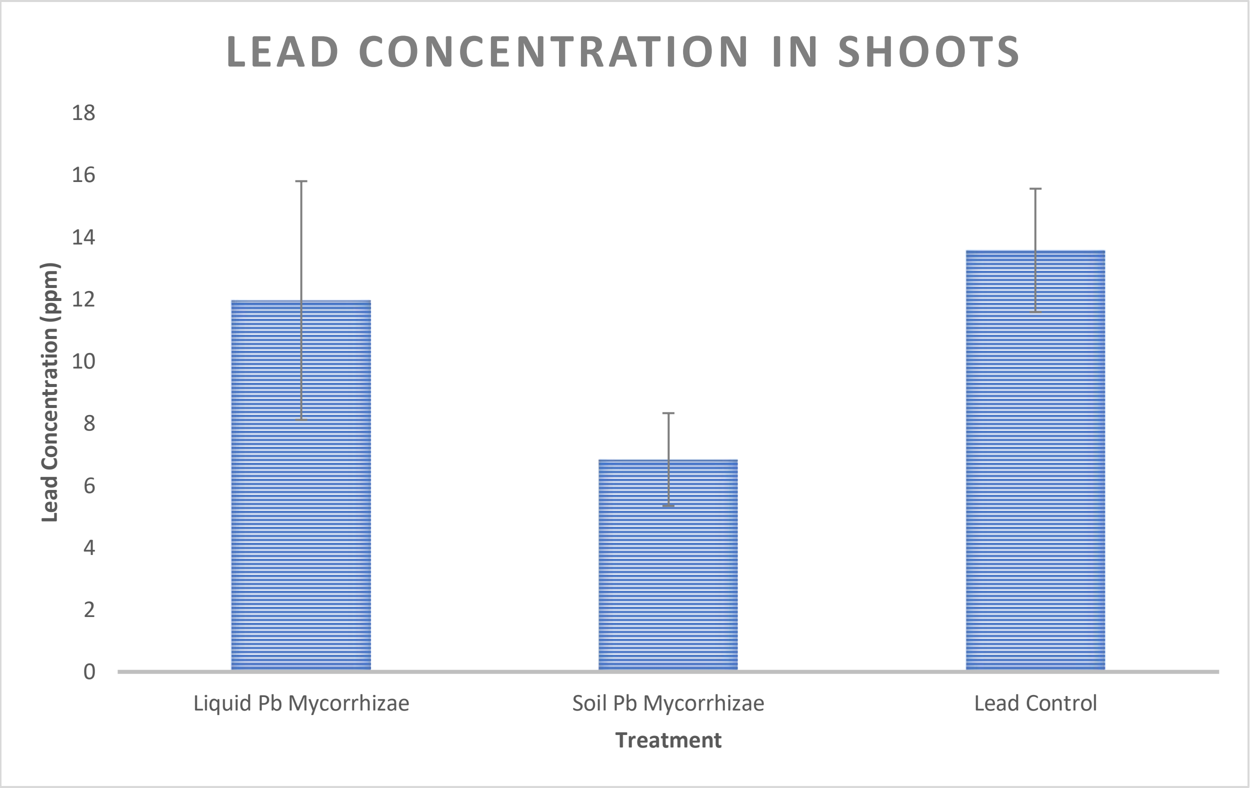
LEAD CONCENTRATION IN SHOOTS
Lead Concentration (ppm)
0
2
4
6
8
10
12
14
16
18
Liquid Pb Mycorrhizae
Soil Pb Mycorrhizae
Lead Control
Treatment

Figure 10

Figure Citations

Figure 1A) The early stage of Moringa plant germination. Figure 1B) The Moringa plants once they were transplanted into larger pots. Figure 1C) The transplant of one of the Moringa plants.

Figure 2A, 2B) The Moringa plants in the control treatment of Design 1. They were not contaminated with lead solution.

Figure 3A) The leaves from the Moringa plants. 3B) The stems of the tree were cut at the soil line. 3C) The roots extracted from the soil.

Figure 4A) The roots of the Soil Pb Mycorrhizae being dipped in mycorrhizae. 4B) The plants from the Soil Pb Mycorrhizae treatment. 4C) The Liquid Pb Mycorrhizae treatment plants. 4D) The plants in the Lead Control.

Figure 5A) An example of the shoots (the combined leaves and stems) of the plants of Experimental Design 2. 5B) The roots of some of the smaller Moringa plants.

Figure 6) Lead Concentration in the soil from Experimental Design 1.

Figure 7) A graph depicting the uptake of lead by Moringa plant parts from Experimental Design 1.

Figure 8) The height difference of the Lead Only, Lead & Mycorrhizae, and Control treatments from Experimental Design 1.

Figure 9) Soil lead concentrations in the Liquid Pb Mycorrhizae, Soil Pb Mycorrhizae, and Lead Control treatment groups.

Figure 10) Lead Concentration in Shoots of Moringa trees in Experimental Design 2.

Chapter 4

Conclusion

This study reveals new information on how Moringa could potentially work in remediating contaminated soils since most of the previous research has been on the use of dried, powdered Moringa seeds in aqueous environments. The study also provides some knowledge about how Moringa distributes lead, arsenic, uranium, and selenium once taken up within the plant. Plus, it offers some insight about how Moringa responds to different applications of mycorrhizae.

Results from the field research showed that Moringa stored most of the lead, arsenic, and uranium in the bark and selenium in the leaves. The cores concentrated the least amount of all the metals studied. The relatively high accumulation of metals in the bark could be explained by the redistribution of metals to parts of the plant that are not essential for photosynthesis via the phloem. It could also potentially be due to plants using metals as a method of deterrence against herbivore consumption.

In the lab experiment of this study, it was observed that mycorrhizae application did not significantly affect the uptake of lead nitrate solution in the roots. The addition of mycorrhizae to the roots and soil only showed a minor difference in uptake. Despite a slightly higher uptake rate of lead in the absence of mycorrhizae, there was no significant difference in the uptake of lead in the leaves and plant growth (height) of any of the three treatments. Results from this study show that the method of mycorrhizae application (liquid mycorrhizae poured onto the soils or solid mycorrhizae application directly onto the roots and in the soil) did not produce a significant difference in lead uptake even though the use of a liquid mycorrhizae solution resulted in higher lead concentrations being detected in that treatment. The results also showed that this was not due to a difference in soil lead concentration since there was no significant difference in accumulation.

Through the calculation of the enrichment, bioconcentration, and translocation factors, the lab study determined Moringa would be better suited for phytostabilization versus phytoextraction. The field study showed that unless enough trees could be successfully planted Moringa would not be an ideal means of phytoremediation in a heavy metal contaminated area. Moringa could likely be used to stabilize some of the soils in contaminated areas but there are other options that would be more beneficial for remediating mining areas.

This study concluded that the consumption of Moringa trees grown in contaminated soils should not be continued as it increases potential exposure to heavy metals. Across both the lab and field studies, the varying lead concentrations showed that no more than 1 gram of Moringa could be consumed a day without risking raising one's blood lead level over the CDC recommended safety limit (5 ug/dL). The benefit from the medicinal values of the tree would be cancelled out by the damage done by putting high concentrations of lead and other toxic metals in their bodies because of the high amounts that need to be consumed for medicinal purposes. Based on results from this it can be concluded that Moringa trees should not be planted for simultaneous use as a nutritional supplement and as a phytoremediator.