INVESTIGATING BENEFICIAL MICROBES FROM MARSH ECOSYSTEMS FOR SUSTAINABLE AGRICULTURAL AND ENVIRONMENTAL APPLICATIONS

by

PETRINA TANESHA MCKENZIE-REYNOLDS

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This thesis is approved by the following members of the Final Oral Committee:

Dr. Gulnihal Ozbay, Committee Chairperson, Department of Agriculture and Natural Resources, Delaware State University
Dr. Venugopal Kalavacharla, Committee Member, Department of Agriculture and Natural Resources, Delaware State University
Dr. Richard Barczewski, Committee Member, Department of Agriculture and Natural Resources, Delaware State University
Dr. Cyril Broderick, Committee Member, Department of Agriculture and Natural Resources, Delaware State University
Dr. Kalpalatha Melmaiee, Committee Member, Department of Agriculture and Natural Resources, Delaware State University
Dr. Lathadevi Karuna Chintapenta, External Committee Member, Department of Agriculture and Natural Resources, Delaware State University

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Petrina Tanesha McKenzie-Reynolds

Faculty Advisor: Dr. Gulnihal Ozbay

ABSTRACT

Microorganisms are often thought of as pests and harmful organisms, but there are numerous important microbes that are beneficial to both human and environmental health. Some microbes develop symbiotic associations with plants helping in their growth and survival while many others develop resistance to metals toxicity and remove them from our soils and water. Effective and efficient bioaccumulation of soluble and particulate forms of metals by microorganisms can be implemented to reduce heavy metal environmental pollution. Reports on global climate change also predicts in loss of 50% of arable land worldwide due to increased salinity, indicating the need for sustainable agriculture. These threats have inspired the need for use of these environmental friendly microbes in amending these issues. For these reasons, this study was focused on studying microorganisms from tidal marsh zones with special attention on heavy metal tolerant bacteria and mycorrhizae from the Blackbird Creek Marsh, located in Townsend, Delaware. We assume that the stressors in marsh might enhance the development of mycorrhizae with special abilities to withstand these conditions. These marsh ecosystems are exposed to various abiotic/biotic stresses such as tidal inundations, temperature, salinity and excess nutrients and naturally they harbor these microorganisms that can remove excess heavy metals accumulated in these soils. For the isolation of lead and cadmium tolerant bacteria, soil samples were inoculated in Luria Broth (LB) and enriched with various concentrations of lead nitrate and cadmium chloride. Heavy metal tolerant bacterial colonies were enumerated and genomic DNA was isolated using phenol: chloroform method. DNA was amplified using universal bacterial primers (27F/ 1492R) and the PCR amplicon was identified by Sanger sequencing.

Growth of bacteria tolerating high levels of lead (concentrations up to 2500 mg/kg) and cadmium (up to 500 mg/kg) was observed.
Most of the bacteria identified are 97% similar to the cadmium tolerant aerobic bacteria, *Bacillus cereus*. Analysis also identified *Enterobacter Sp.* to be 98% similar to most of the lead tolerant bacteria. A pilot plant study was conducted through greenhouse experiments, and marsh soils were used to grow *S. alterniflora.* The soils were autoclaved to kill the spores. Salinity treatments were conducted, and plant measurements were recorded both for the treatment and control samples. Positive VAM effect was observed especially in the root masses of the harvested Spartina plants. Root samples from *S. alterniflora* were collected and stained with acid fuschin. They were also observed under light microscope which confirmed the presence of mycorrhizal spores. Nested PCR confirmed the Mycorrhizae present in the samples belonging to the *Glomus, Aculospora, Archaeospora, Gigaspora and LETC* groups.

Keywords: Mycorrhizae, symbiosis, heavy metals, bacteria, marsh wetlands, sustainable, *Spartina alterniflora, Phragmites australis,* bioremediation

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CHAPTER 1

INTRODUCTION

Wetlands perform a variety of ecological functions. These ecosystems are habitats to many important micro- and macro-organisms that are extremely beneficial to our environment (Washington State Department of Ecology 2001). An immense number of microbes and plants are a part of the wetland ecosystem. These organisms share a complex and dynamic relationship that helps to sustain the wetland (Koretsky et al. 2005). However, there have been major concerns as it relates to the health of these ecosystems. Globally, many of these landscape features are altered due to natural and anthropogenic factors. Anthropogenic factors include: improper land use for agricultural production, industrialization, and residential development (Houlahan et al. 2006) while natural factors include storm surges, hurricanes, flooding among others. Decrease in arable land is caused by many factors, but mainly from environmental pollution, including heavy metal pollutants. These factors do not only affect our wetlands, but are also major problems for terrestrial ecosystems and their inhabitants. Proper management is therefore required for the preservation of these resources.

Many of these pollution issues may be associated with human induced activities in and around these areas. The buildup of these pollutants can cause negative impacts on both human and environmental health. Chemical build up and the growing impacts of climate change are considered major factors that are affecting these ecosystems. Many of the arable lands and plant species are affected by these phenomena. Abiotic (pollutants in the air, soil and water and climate change) and biotic (pathogens and pests) factors have contributed to the rapid degradation of the agricultural industry (Higa and Parr, 1994, Yang et al. 2009).

It is for these reasons that attention is being given to the development of sustainable agriculture and environmental protection (Reganold et al. 1990, Vance, 2001, Noble and Ruaysoongerm 2010). As an approach to remedy these problems, the initiative was taken to develop sustainable methods/practices to enhance plant growth, suppress plant diseases, restore soil structure and hydrological functions and at the same time reduce environmental pollution caused by heavy metals build up (Adesemoye et al. 2009).

Microorganisms' uniqueness and their effective biosynthetic capabilities in adequate environmental and cultural conditions have made them likely candidates for addressing these challenging environmental and agricultural problems. Many of these beneficial organisms can be found in wetlands and they play significant roles in maintaining these areas in a sustainable way. Microbes found in these ecosystems share diverse types of relationships and have tremendous potential to improve the environment. The importance of understanding the roles that these wetland microbes play in both agricultural and environmental sustainability is extremely important.

These microbial communities include **symbiotic fungi and chemotrophic bacteria** which were investigated in this research. The relationships created between these microbes and their hosts are considered beneficial to both organisms.

Plant growth, development and survival are often influenced by negative external variables termed as stress factors. These are classified as abiotic or biotic stress factors. Salinity, drought, flood, intense sunlight, pH and extreme temperatures are all categorized as abiotic stressors that are naturally occurring and may cause serious damages to plants in the affected location (Higa and Parr, 1994, Yang et al. 2009).

1.1 Plant response to abiotic stress: Salinity

Overtime, abiotic stress such as high soil salinity and heavy metal toxicity have become common adverse environmental conditions that affect and limit crop productivity worldwide (Moses et al. 2013). Koca et al. (2007) reported salinity as one of the major abiotic stresses that adversely affect arable lands worldwide, resulting in the loss of crop productivity and yield of most economically important crops (El-Din et al. 2016).

Plants subject to increase salinity stress resulting from high saline soils decrease the plants' ability to absorb water. The decreased potential of a plant to absorb water into the root zone also decreases the water potential of the soil (Sabir et al. 2009). Water availability deficiency due to high saline conditions increases the potential of the plant cells to be dehydrated and results in osmotic stress (Hasegawa et al. 2000). Abiotic stressor such as salinization of soil is a fundamental problem and is a major cause of crop loss (Bor et al. 2010, Quilambo 2003). These losses are expected to increase worldwide due to the anticipated rise in global warming (Giri et al. 2003, Al-Karaki 2006). Seven percent of the earth's lands consist of saline soils (Ruiz-Lozano et al. 2012) and an increase in salinization of arable land will result in land loss of 50% (Wang and Chen 2009).

To date research has focused on developing methods that enable plants to be tolerant to abiotic stresses such as drought, salinity, heat, cold, flooding, and nutrient limitation (Bhatnagar-Mathur et al. 2008; Collins et al. 2008, Witcombe et al. 2008). The effects of salinity on agricultural yields are of major concern (Tester and Davenport 2003) as it reduces the growth and development of plants and considerable loss in crop production (Giri et al. 2003).

Crops can be affected at any level by high salinity and are more susceptible at their germination stage.

For seeds with coats that are permeable to salts, germination ability might be decreased due to the presence of salinity (Tobe et al. 2004). Considering these consequences, mitigation approaches for the negative impacts of salinity stress is a crucial factor, especially in this increasing need for sustainable agriculture (Kumar et al. 2014). Biological methods such as the use of symbiotic fungi have been explored and are considered as an excellent choice for both agricultural and environmental sustainability (Mayak et al. 2004). Studies have reported that there is a significant association between fungal symbionts and the population of plants in natural ecosystems (Singh et al. 2011, Zhang et al. 2011). These organisms colonize and reside partially (facultative) or entirely (obligated) in the internal tissue of their host plant (Bhoopander et al. 2009). Different host plants and the fungal endophytes may express their lifestyle through a range of associations including symbiotic, mutualistic, commensalism and parasitism depending on the host plant and environmental factors (Glick et al. 2004). These relationships can enhance growth, increase reproductive success and aid in biotic and abiotic stress tolerance to the host plant (Bothe 2012, Sijam and Tahat 2012). It is therefore a beneficial and promising alternative to exploit the use of fungal symbionts to alleviate the problems associated with crop production caused by both abiotic and biotic stress.

The development of biotechnological applications using microbial symbionts in improving plant stress tolerance and sustainable food crops is now becoming a major interest (Abdel-Fattah et al. 2013, Jeong and Muneer 2015).

Prevention of crop losses due to pests and diseases is another benefit from these symbiotic relationships as well as speeding up decomposition of organic matter and toxic compounds, improving nutrient cycling and soil structure (SP-IPM 2005).

Plant host and environment influence the role these fungi play in each organism's survival. Collectively, these fungi promote tolerance for plants that are subjected to habitat imposed abiotic and biotic stresses that are serious threats to agricultural and environmental degradation (Ducic et al. 2007). Using environmentally friendly methods such as the application of Vesicular Arbuscular Mycorrhizae (VAM) fungi to plants that are moderately tolerant to salt, can help to achieve growth and development in these adverse conditions (Mayak et al. 2004).

The application of the symbiotic VAM fungi in saline soils has the potential to improve plant growth and tolerance against adverse saline conditions (Al-Karaki 2006, Daei et al. 2009; Kumar et al. 2010; Abdel-Fattah and Asrar 2012, Asrar et al. 2014). Symbiotic relationships with VAM fungi are formed with most plants species (Ardekani et al. 2009, Al-Karaki, 2001). Vesicular Arbuscular Mycorrhizae (VAM) are the most abundant members of the fungi that develops and produces the best known symbiotic associations with plant roots and contributes to plant growth (Powell and Bagyuraj 1984). These microbes are highly dispersed in our marsh ecosystems, many of which are not thoroughly researched. Conflicting environmental factors and harsh conditions with stressful levels of salinity contributes to an elevated level of disturbed productivity in plant species.

Research has shown that 7% salinity prevents most salt marsh species from becoming established. *Spartina* is one of the few plants capable of tolerating and growing in the higher saline environment (Zedler and Varty 2008, Krishnamoorthy et al. 2015) and for such reason it was chosen as the primary experimental plant species in this research.

An alteration in microbial communities ultimately leads to significant break down in ecosystem's structure and function.

Some wetland/marsh ecosystems are more affected than others and may require more attention. Other areas are less disturbed but still require significant attention. Blackbird Creek is one such ecosystem, located in north-central Delaware. It drains approximately 80 square kilometers (km^2) into Delaware Bay and over 50% as agricultural land use, about 48% forested, and 1% developed (Klemas 2011). The intertidal creek ecosystem is poorly understood, especially as climate continues to change at unprecedented rates. It is also a natural habitat for the native *Spartina alterniflora* and the invasive *Phragmites australis* (DNREC 2016). Therefore, Blackbird Creek was selected as the ideal system to develop a greater understanding of how the system acts as a nursery and to determine what are the mechanisms associated with VAM that supports these plant species subjected to elevated levels of salinity.

1.2 Spartina alterniflora

Spartina alterniflora, the smooth cordgrass, saltmarsh cordgrass, oyster- grass or salt-water cord grass is a perennial deciduous grass which is found in intertidal wetlands, especially estuarine salt marshes.

It is native to the Blackbird Creek and it is the single most important marsh plant species in the estuary of Chesapeake Bay (USDA NRCS Plant Materials Program 2016). *Spartina alterniflora* is one of the few plants capable of growing in a wide range of salinities and tolerant to drought. It is also known as environmental engineers and is intolerant of shade. According to USDA Forest Service Fire Effects Information System, *Spartina* can grow in levels of salinities ranging from 34-40 parts per thousand (ppt) (USDA NRCS Plant Materials Program 2016).

On sites where seedlings are transplanted, stem and rhizome growth respond well to a well-balanced distribution of commercial fertilizer. Nursery beds prior to transplanting are adequate for good growth and development.

Spartina alterniflora are affected by the flower beetles that may limit seed production. Decline and death of the stems are often caused by the sugar cane borer. A rust-like fungus is commonly found on the *Spartina* grass but is considered to be non-threatening to the plant (citation). New transplanted plants can be affected because problems associated with nutria *(Myocaster coypus)* and will need predator protection. *Spartina* populations are also affected by marsh vegetation that are considered invasive in the specific location (USDA NRCS Plant Materials Program 2016).

Spartina is widely used for erosion control along shorelines, canal banks, levees, and other areas of soil water interface. It is used effectively in marsh restoration to stabilize soils. *Spartina* plants are also capable of absorbing wave energies and uptake nutrients from sediments (USDA NRCS Plant Materials Program 2016). These grasses provide food and cover to various marsh birds and mammals.

It is also a very important source of fodder used by live-stock producers (USDA NRCS Plant Materials Program 2016). Moreover, bedding, thatch, packing for pottery, metal and icehouse installation, musical instruments, baskets, arrow shafts and cigarette casings are also by products produced from the *Spartina* marsh grass (USDA NRCS Plant Materials Program 2016).

1.3 Heavy metal toxicity and beneficial bacteria

Another environmental concern is the toxic pollution caused by heavy metals. A build-up of these pollutants can have long term negative effects and in some cases, have fatal outcomes (citation). Heavy metals occur naturally in soils; however, geological and anthropogenic activities can increase the concentrations of the elements excessively. This can cause harmful effects on both plants and animals. Some of these sources include mining, burning of fossil fuels, use of agricultural fertilizers and pesticides.

Production of batteries, use of lead bullets, paints and sewage sludge can be other contributors to pollution in many ecosystems (Low et al. 2000). The pollution of the environment with these toxic heavy metals is spreading at an alarming rate globally (Cheng 2003). A build up in the soils and water is a significant environmental problem (Cheng 2003). Research data have reported that the physiological-biochemical processes in plants growing on heavy metal contaminated soils have been negatively affected, which results in the plant growth reduction (Chatterjee and Chatterjee 2000, Keles et al. 2000, Foca et al. 2005). A decline in plant growth eventually reduces crop yields and results in food insecurity.

Heavy metals can modify the biological properties of soils as well as the number and diversity of soil microorganisms' activities (Cheng et al. 2003).

Plants do require certain metal elements for their growth and survival but excessive amounts can become toxic to the plant. Plants ability to accumulate essential metals equally enables them to absorb non-essential metals. When these heavy metal concentrations within the plant exceed the optimal levels, plants are affected directly or indirectly.

For instance, a reduction in beneficial soil microorganism's population due to high metal concentration often leads to a decrease in organic matter decomposition (Cheng et al. 2003).

This results in a decline of soil nutrients available to the plant. Enzyme activities necessary for plant metabolism is also affected and can result in plant death (Schaller and Diez, 1991). Research performed by Ahmad et al. (2012) showed the reduction in shoot and root growth in wheat plants occurred when cadmium levels in the soil was as low as 5 mg/L. In addition, presence of these heavy metals also reduces the environmental health such as soil's structure, hydraulic functions, nutrient exchange and human health.
These toxic effects on human health and environment are permanent and sometimes fatal (Rehman et al. 2008).

Marsh microbes and marsh grasses play an important role in removing pollutants from the soils (Kadlec et al. 1996). These wetland communities are very important and require the best efforts to keep them protected against degradation. However, with increase in human population and industrialization, elevated levels of pollution and contamination are recorded in many parts of the country.

There are several heavy metals present in the environment including wetland/marsh ecosystems and understanding their quantitative presence and effect on the system and its components is extremely important (Burke et al. 2002, Byers et al. 1998). Although some marshes may be less polluted than others, there is still a major concern associated with protecting them and developing different management methods to deal with heavy metal contaminations (Kadlec 1996). This also is expected to foster a reduction in adverse impacts on human lives and the environment.

Removal of heavy metals can be achieved with the use of microorganisms, plants or a combination of both organisms (Komori et al. 1989).

Studies have reported that *Phragmites* is being used as a phytoremediation agent against various heavy metals (Ghassemzaddeha et al. 2008). Several microorganisms especially bacteria (*Bacillus subtilis, Pseudomonas putida and Enterobacter cloacae*) have been used in the reduction of Cr (V1) to the less toxic Cr (111) with successful results (Garbisu et al. 2003, Batta et al. 2013).

Further studies have also reported that *Bacillus cereus and Bacillus thuringiensis* have increase extraction of Cd and Zn from Cd-rich soils and soils polluted with effluent from industry (Arasuc et al. 2010, Benavides et al. 2005). For these reasons, this project was also designed to isolate and identify heavy metal tolerant bacteria, specifically lead and cadmium tolerant bacteria from *Spartina* and *Phragmites* soils of Blackbird Creek. They are two of the four most hazardous heavy metals found in our environment (WHO 2016).

1.3.1 Lead (Pb) in the Environment

Lead (Pb) is a naturally occurring metal found in the earth's crust and is ranked number 2 on "The Agency for Toxic Substances and Disease Registry's (ATSDR) list. The extensive use of this metal has resulted in major environmental contamination (WHO 2016). Lead is one of the most toxic heavy metals that has profound damaging effects on human and environmental health. It is a major pollutant found in soil, air, and water (Low et al. 2000).

Lead has no biological function; it is a cumulative toxicant and is a major ecological hazard. Young children are particularly susceptible to lead poisoning and it can cause adverse effects such as brain development. Adults can also experience risks of high blood pressure and kidney disease. Pregnant women are at risk for miscarriage, still births, premature births and low birth weight along with minor cases of malformations (WHO 2016).

Lead is absorbed into the body mainly by inhalation and ingestion. Some traditional cosmetics and medicines use can also result in lead exposure. Sources of lead contamination in the environment include mining, paints, leaded gasoline and aviation fuel.

Lead can also be found in ammunitions, ceramic, jewelry, toys, pesticides and solder (WHO 2016). Lead can also cause poisoning in terrestrial and marine animals. Target organs affected by lead are the bones, brain, kidneys and also the blood. Plant growth is also affected in areas where soils are highly contaminated (WHO, 2016).

High metal concentration may result in the inhibition of cytoplasmic enzymes and damage to the cell structures due to oxidative stress in plants, Plants also experience low growth rates, reduced nutrient uptake, chlorosis and yield depression. The presence of lead gradually delays seed germination due the prolong incubation of the seeds.

The toxic effect cause seed neutralization by some mechanisms such as leaching and chelation (Neklyudov 2008). These activities sometimes result in plant death. Heavy metals when released into aquatic systems bonds with particulate matters. This eventually settles and become incorporated into sentiments. These sediments often reduce water quality and are accumulated by organisms (example, benthic organisms) and passes up the food chain. Some functions of the organism's cell structures and organs are often disrupted and may also result in death (Neklyudov 2008).

1.3.2 Cadmium (Cd) in the Environment

Cadmium is poisonous for plants, animals and humans (Gupta and Gupta 1998). Cadmium is ranked number 7 on ATSDR's top 20 list (WHO 2016). The metal is usually released into the environment through agricultural chemicals.

These chemicals include: pesticides, insecticides, fungicides and fertilizers. Cigarettes, sludge, mining, smelting activities, incineration of PVC plastics and batteries along with the burning of fossil fuels also releases cadmium into the environment. Reservoirs containing shellfish also contain cadmium (Tang et al. 2013).

Cadmium can have both acute and chronic effects on human through inhalation or oral exposure. A buildup of cadmium in the kidney can cause disease. Target organs for cadmium also include the lung, brain, liver, placenta, and bones. This metal is also classified as a group B1 probable human carcinogen by the United States Environmental Protection Agency (EPA 2016). Limited evidence exists for a reduction in sperm number and viability, decreased birth weight, decreased reproduction and testicular damage associated with cadmium (EPA 2016).

High toxicity of cadmium in plants results in stunting and chlorosis (Shahid et al. 2017). Cadmium also interferes with the uptake and transportation of several elements such Calcium (Ca), Magnesium (Mg), Potassium (K) and Phosphorus (P) (Benavides et al. 2005). The alteration in mineral uptake by plants affected by cadmium toxicity is mainly due to the reduction of population in soil microbes. Cadmium toxicity has been shown to affect stomatal opening, transpiration, photosynthesis and water and nutrient uptake (Benavides et al. 2005).

Physical and chemical methods have been implemented with the intention of removing these pollutants. However, there are some disadvantages from using these conventional methods. Cost-limitation and generation of hazardous by-products when polluted materials concentrations below 100 mg/L are the main effects associated with these methods (Gavrilescu 2004, Wang and Chen 2009). Using biological methods to combat these challenges is an effective approach since they are easily operated and do not produce secondary pollution (Chen et al. 2009).

The utilization of organisms, primarily microbes as cleaning agents for contaminated soil, water and air is an area of environmental biotechnology that has the potential to effectively and efficiently reduce contaminants using more environmentally friendly and economical methods. There are several factors that contribute to the increasing degradation of the environment both naturally and anthropogenic.

However, the use of biotechnology can be an effective solution for these problems (Figure 1.1). The use of these microorganisms in addition to being a long-term prospective to pollution amelioration measures is an innovative technology. This is also a low-cost approach for the remediation of heavy metal contaminants from the environment (Garbisu and Alkorta 2003). Report by Baylock et al. (1997) showed that 50% to 65% saving was achieved by using microbes for the treatment of Pb polluted soils compared to the use of a conventional method for the same purpose.

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Beneficial microbes and the environment: A relationship summary

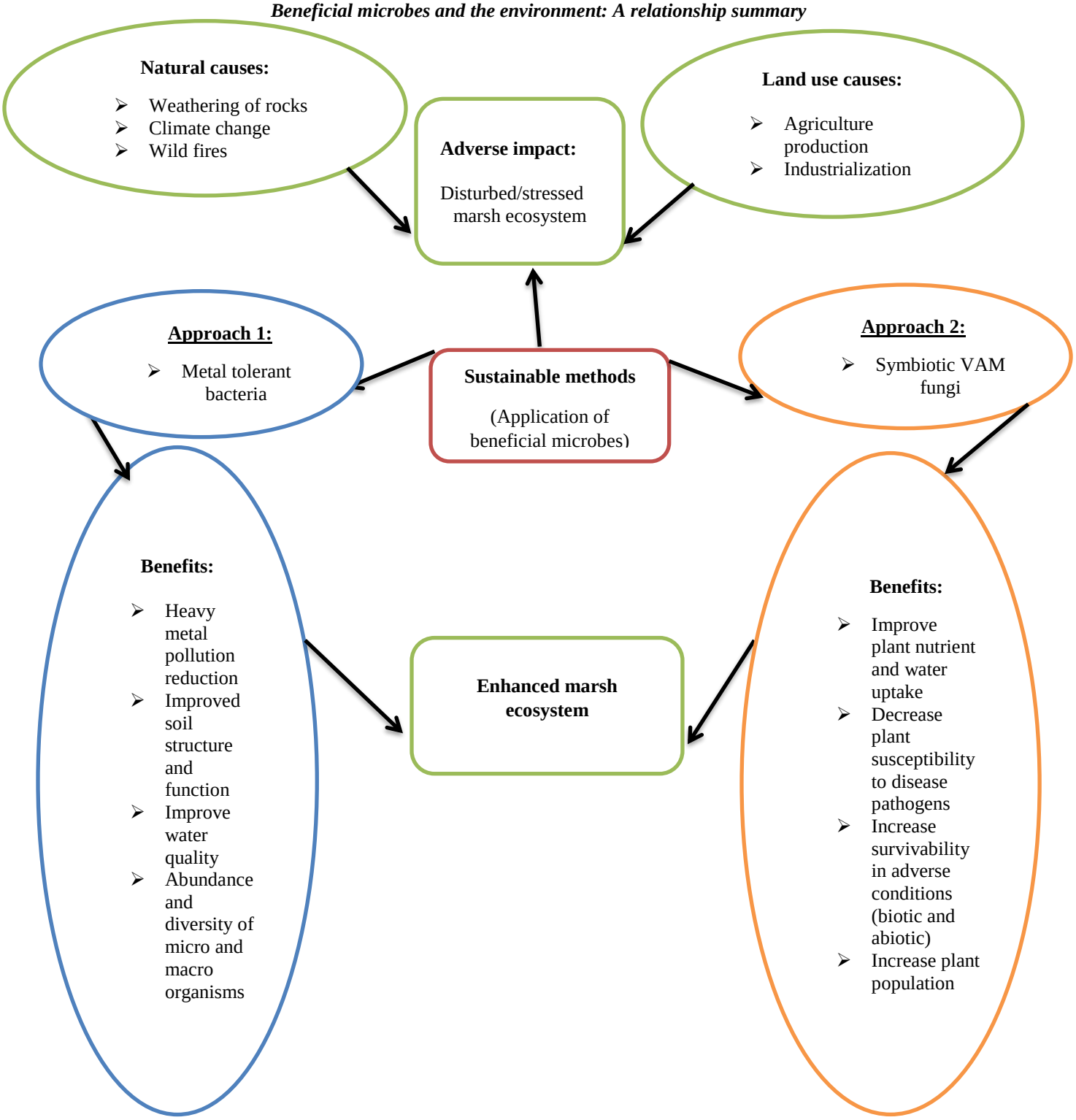


Figure 1.1. Summary of the relationship between environment, its components and the effects of the beneficial microbes' present.

1.4 Problem statement

Wetlands provide important ecosystem functions and values, such as wildlife habitat, water filtration and flood protection. Wetland plant communities play a fundamental role in maintaining these functions but are thought to be increasingly threatened by human modifications of the landscape for industrialization, residential communities and agricultural production. The increased use of poisonous chemicals and the growing impacts of climate change have contributed tremendously to the degradation of these ecosystems. In response to those changes, this project focused on identifying heavy metal tolerant bacteria from marsh soils, with a future goal of investigating their uses for bioremediation. Also, this study has explored VAM fungi in marsh soils with an aim of using them for sustainable agricultural methods and helping plants easily adopt to impacts of climate change through greenhouse studies. Various sampling methods have provided a representation of the abundance and effectiveness of the microbes that have been proposed while greenhouse studies have been conducted to further investigate the effects of VAM on plants exposed to elevated levels of salinity stress. This may provide a basis on how these microbes can be utilized as an environmentally friendly approach to better conserve and manage our ecosystems.

1.5 Research questions

1. What kinds of microbes are present in these tidal marshes for environmental sustainability?
    - Heavy metal tolerant bacteria (HMTB).
2. What kinds of microbes are present in these tidal marshes for agricultural sustainability?
    - Symbiotic (VAM) fungi.

1.6 Research objectives

The research goals are to further increase the knowledge on different beneficial microbes present in the Blackbird Creek and their relationships with the plants and soils communities.

Research objectives include:

1. Determining the abundance of lead and cadmium tolerant bacteria in *Spartina alterniflora* and *Phragmites australis* soils in Blackbird Creek.
2. Isolating and characterizing cadmium and lead tolerant bacteria from marsh soils.
3. Identification of VAM fungi presence in marsh soils and marsh plant roots in Blackbird Creek.
4. Conduct greenhouse experiments to study if VAM will help plants tolerate slat stress.

1.7 Research Hypothesis

H_o1: *Spartina* and *Phragmites* soils will have the same abundance of lead and cadmium tolerant bacteria.

H_a1: *Spartina* and *Phragmites* soils will not have the same abundance lead and cadmium tolerant bacteria.

H_o2: Marsh VAM fungi may have a positive impact on plant growth characteristics under salt stress.

H_a2: Marsh VAM fungi may not have any impact on plant growth characteristics under salt stress.

CHAPTER 2

REVIEW OF LITERATURE

2.0 Experiment#1: Isolation, Identification and Characterization of Lead and Cadmium Tolerant Bacteria from Marsh Soils

2.1 What are heavy metals?

Heavy metals are generally metallic elements that have a relatively high density compared to water (Fergusson 1990). Some metalloids are also considered heavy metals based on the interrelation of heaviness and toxicity. Arsenic for example is able to induce toxicity at low levels of exposure. Based on the inter-relatedness of heaviness and toxicity, arsenic is a metalloid that is also considered a heavy metal (Duffus 2002). The elevation of these elements has been associated with environmental contamination. This have contributed to increasing concerns for both ecological and global public health. Reports have also shown that a dramatic rise in human exposure was a result of their increase in industrial, domestic and technological applications (Brad 2002). Studies by He et al. (2005) have reported that the most substantial sources of heavy metals in the environment include industrial, agricultural, pharmaceutical, domestic effluents and atmospheric sources. Prominent point sources of environmental pollution caused by heavy metal involve areas such as mining, foundries and smelters (Fergusson 1990, Duffus 2002, Brad 2002).

Heavy metals are naturally occurring elements found throughout the earth's crust (Shallari 1998).

However, environmental contamination can also occur because of metal corrosion, atmospheric deposition, soil erosion of metal ions and leaching of heavy metals, sediments re-suspension and metal evaporation from water resources to soil and ground water (Nriag 1989).

Heavy metal pollution can also result from natural phenomena such as weathering and volcanic eruptions. Coal burning, petroleum combustion, nuclear power stations, high tension lines, plastics, textiles, microelectronics, wood preservations and paper processing plants are industrial sources for heavy metal pollution (Arruti et al. 2010). Because of their presence in trace concentrations in various environmental matrices, heavy metals are also considered as trace elements (Kabata- Pendia 2001). The bioavailability of these elements can be influenced by biological factors (Hamelink et al. 1994). The biological factors include species characteristics, trophic interactions, and biochemical/physiological adaptation, also play an important role.

Several studies have also demonstrated that reactive oxygen species (ROS) production and oxidative stress significantly influences the toxicity and carcinogenicity of metals such as arsenic (Yedjou et al. 2008), cadmium (Tchounwou et al. 2001), chromium (Patlolla et al. 2009), lead (Yedjou et al. 2008, Tchounwou et al. 2004), and mercury (Tchounwou et al. 2008).

They are also ranked among the priority metals that are of great public heath significance. These elements are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. Studies from the United States Environmental Protection Agency (U.S. EPA 2016), and the International Agency for Research on Cancer (IARC) have led to the classification of these five elements as probable human carcinogens.

These reports are based on epidemiological and experimental studies demonstrating associations between exposure and cancer incidents in human and animals.

Various health problems such as headache, irritability, abdominal pain, anxiety, nervous system issues, cancer of the kidney and bladder are also associated with heavy metal toxicity (Chatterjee and Chatterjee 2000).

This occur when the vital nutritional minerals from their original place and hindering their biological function thus interrupting the vital organs and glands inclusive if the brain, kidneys, bone, heart, liver etc. Environmental issues are of major concern since these elements accumulate in the plants and are transferred to the human body through consumption. Research have shown that higher concentrations of heavy metals have also been an environmental problem in aquatic ecosystems globally (Chatterjee and Chatterjee 2000). Accumulation of these heavy metals in seafood or in plants when they reach ground water and is naturally a major toxic source for human. However, it has been discovered that some soil microbes have been very efficient in manipulating these elements and reducing the build-up in the soils (Chatterjee and Chatterjee 2000). According to Oancea et al. (2005) the microbial world is varied and as a result some microorganisms have altered to the noxious concentrations of heavy metals and become "metal resistant". Approaches to overcome the toxic effects of heavy metals and metalloids have progressed through the use of these microbes. These microbial activities are amplified in the rhizosphere and simplifies the removal of toxic heavy metals. They also enhance the accumulation, degradation and biomethylation of trace elements.

Current studies have proven the extensive variability of microorganisms (bacteria, fungi, yeast, algae) that can be used in bioremediation processes. Some of these have already been employed as bio absorbents of heavy metals (McBride and Martínez 2000, Friedlová 2010).

The use of microbes in bioremediation assays versus the conventional treatment methods comprise low cost, chemical and biological sludge minimization and no requirement for nutrient addition (Nannipieri et al. 1997 and Baath 1989).

2.1.1 What are heavy metal tolerant bacteria?

Resistance to heavy metals in water, soil and industrial waste have been demonstrated by many organisms. These microbes exhibit genes that are located on chromosomes, plasmids or transposons that encode species resistant to a variety of metal ions. Even though some metals may be beneficial to plants, most metals are nonessential and have no essential nutrient value and may be toxic to microorganisms (Hughes et al. 1989, Poole et al. 1989, Silver et al. 1994).

These toxic metals interact with the organism's cellular components through covalent and ionic bonding. At high levels, these metals damage the cell membranes, alter enzyme specificity, disrupt cellular functions and damage DNA structure. However, microorganisms have adopted to the presence of these metals by developing resistance mechanisms (Poole et al. 1989).

Studies have discovered six types of metal resistance mechanisms. These include: exclusion by permeability barrier, intra- and extra-cellular sequestration, active transport efflux pumps, enzymatic detoxification, and reduction in the sensitivity of cellular targets to metal ions (Silver, 1992, Rouch et al. 1995).

Environmental conditions due to pressures from metal-contamination have led to the development of resistance systems to almost all toxic metals (Rouch et al. 1995). These systems are mostly plasmid-mediated and very specific and have been found in most eubacterial groups studied (Silver et al.1984). In fact, a significant number of researches have been conducted since the early 1970s and have identified several microorganisms as being resistant to certain metals. Aerobic microorganisms were dominant in these reports. Examples of these resistant microbes include: *Staphylococcaus* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus Sp*.

Resistance has also been reported for mercury [Hg(II)] and organomercurials in obligate anaerobes such as the Bacteroides and *Clostridium* species (Nakahara et al. 1977, Marques et al. 1979, Harnett and Gyles, 1984, Schwarz and Hobel, 1989, Belliveau et al. 1991, Wang and Shen, 1995).

In most situations, the soil may be polluted with more than one heavy metal and the antagonistic and synergistic relationships influences plant metal toxicity and hence the abundance of the metal tolerant bacteria present. According to Nicholls and Mal (2003) Pb and Cu at both high and low concentrations resulted in leaves and stem death of the *Lythrum salicaria*. Reports showed that there was no synergistic interaction between both metals. In another study by Ghani (2010) the effect of six heavy metals (Cd, Cr, Co, Mn, and Pb) on the growth of maize was examined. The result showed that the presence of these metals in soil reduced the growth and protein content of maize. It is important to understand that the type of heavy metal involved determines the effect of toxicity on the growth and development of the host plant.

Metals such as Pb and Cd which do not have any beneficial role in the plant's development have shown adverse effects at even very low levels Their presence also influences the presence of microbes' resistant to these metals (Taiz and Zeiger 2002).

2.1.2 Lead tolerant bacteria

Even though Pb has been known to have high levels of toxicity, many micro-organisms have developed various mechanisms that enables them to survive in these conditions.

Based on previous experiments micro-organisms, resistant to Pb have been isolated from soils polluted by elevated levels of the metal.

Some of the species identified from these isolates are as follows: Gram-positive bacteria *Bacillus cereus, Arthrobacter sp*. and *Corynebacterium sp.;* the Gram-negative bacteria *Pseudomonas marginalis, Pseudomonas vesicularis* and *Enterobacter sp.* and *Saccharomyces cerevisiae* and *Penicillium sp.* Psf-2 (Chen and Wang 2007). Some lead-resistant bacteria have been found to play a significant role in development of plants that have been exposed to lead. For example, the endophyte *Bacillus sp*. MN3-4 increases Pb(II) accumulation in *Alnus firma,* and *Pseudomonas fluorescens* G10 and *Mycobacterium sp*. G16 promote plant growth and reduce Pb toxicity in *Brassica napus*. Studies have also shown activities of the lead-resistant bacteria *Streptomyces sp.* and *Ps. Vesicularis*. These bacteria were capable of producing a red and red–brown pigment, identified as the lead tetroxide, minimum (red lead, Pb3O4) (Chen and Wang 2007).

2.1.3 Cadmium tolerant bacteria

The removal of cadmium from the environment has been achieved using a variety of microbes. Several studies have isolated these bacterial strains. The strains of cadmium tolerant bacteria isolated include: *Pseudomonas putida, Escherichia coli, Pseudomonas aeruginosa, Pseudomonas syringae, Pseudomonas fluorescens, Comamonas testosterone, Staphylococcus aureus, Alcaligenes eutrophus, Gluconobacter oxydans* and *Bacillus subtilis.*

Staphylococcus lugdunensis, Alcaligenes xylosoxidans, Ralstonia metallidurans, Lactobacillus plantarum, Serratia liquefaciens, Klebsiella planticola, Paenibacillus sp. and *Bacillus thuringiensis* are also among the strains identified. Bioaccumulation of cadmium by some gram-negative bacteria has also been established.

These bacteria include: *Escherichia coli* (Cohen et al. 1991), *Pseudomonas putida*, *Pseudomonas syringae* and *Pseudomonas aeruginosa* (Higham et al. 1984).

Another Gram-negative rod bacterial strain that have been identified to be heavy metal biosorbent the *Alcaligenes eutrophus (Ralstonia metallidurans)* strain CH34 (Diels et al. 1990).

2.1.4 Metal tolerant bacteria and bioremediation prospects

Soils, water and sediments polluted by heavy metals can be remediated using biological encoded changes in the oxidation state of these metals. This microbial-mediated process clears or immobilizes the contaminants including toxins such as: hydrocarbons, agrochemicals and other organic toxicants. Inorganic toxic compounds (heavy metals) are more challenging in the break down process for microbes.

The inability to simplify these metals into harmless compounds have resulted in these organisms need to be used according to their specialization for the heavy metal type. Therefore, bioremediation assays for heavy metals depends on the active metabolizing capabilities of microorganisms.

Microorganisms differ in their requirements for heavy metal as essential micronutrients for growth and development. For example, all bacteria require ferric ion (Fe^{3+}) while for anaerobic bacteria essentially require ferrous ion (Fe^{2+}). (Ahemad M 2014). Several studies have been completed to explore the use of these microbes and their ability to remove or detoxify toxic products from the environment (Garbisu and Alkorta 2003).

Evidently, microorganisms have the potential for remediation of soil pollutants and increasing the production of agricultural crops at very low input cost.

Understanding the mechanisms of absorption and mobilization of the heavy metal and trace elements in the soil is very important in the selection of the Rhizospheric microbes. This is essential in the restoration of soil health.

2.1.5 Mechanisms of Bioremediation

Heavy metal-contaminated areas are widely populated by microorganisms that can easily convert / mineralize the organic contaminants into carbon dioxide and water as end products. These organic compounds may also be converted into metabolic intermediates which are used primarily as substrates for cell growth. Degradative enzymes for target pollutants and resistant to relevant heavy metals are two capable defense mechanisms of these microorganisms. There are different mechanisms of bioremediation such as: biosorption, metal-microbe interactions, bioaccumulation, biomineralization, biotransformation and bioleaching (Comte et al. 2008). Microorganisms use the chemical removed from the soil for their growth and development. This is done through reducing or oxidizing these metals. The process of binding, oxidizing, immobilizing, transformation and volatizing are different methods by which these microbes restore the environment. Understanding the mechanisms that control growth and activity of microorganisms in contaminated locations can be very successful and beneficial in these areas. Their metabolic capabilities and response to environmental changes are very important attributes that determines the success of the bioremediation process. Even though a considerable number of contaminants are organic solvents that interferes with membranes, cells can develop various defense mechanisms (Comte et al. 2008).

2.1.6 Bioremediation by Adsorption

Biosorption of heavy metals can occur effectively by microbes without significant energy need at their binding site in their cellular structure.

These bacterial cells walls contain various reactive compounds but the polymeric substances are very important and have significant effects on acid-base properties and metal adsorption (Guiné et al. 2006).

Studies have reported that the metal binding behavior of extracellular polymeric substances (EPS) have a great ability to degrade complex heavy metals via various mechanisms, including proton exchange and micro-precipitation of metals. The characterization and quantification of proton and adsorption of metals on bacterial cells and EPS free cells have been revealed by recent studies. This was done to determine the relative importance of EPS molecules in metal removal (Fang et al. 2011).

2.1.7 Bioremediation by Physio-Bio-Chemical Mechanism

The process of biosorption resembles a similar relationship of bio-sorbent towards sorbate (metal ions), this is until equilibrium is established between the two components. One microorganism, *Saccharomyces cerevisiae* acts as a biosorbent in the removal of Cd (II) through the ion exchange mechanism (Talos et al. 2009). Studies have also revealed the potential of *Cunninghamella elegans* as a sorbent against heavy metals released by textile wastewater (Tigini et al. 2010). The cell metabolic cycle gain energy from heavy metal degradation. Bioaccumulation involves both active and passive modes of toxic metal bioremediation. (Brierley 1990). Fungi have also reveal exceptional capabilities emerged as a biocatalyst that can access heavy metals and transform them into less toxic compounds (Pinedo-Rivilla et al. 2009).

Fungi including *Klebsiella oxytoca*, *Allescheriella* sp., *Stachybotrys* sp., *Phlebia* sp. *Pleurotus pulmonarius* and *Botryosphaeria rhodina* are also known for their metal binding potential. Other fungal species like *Aspergillus parasitica* and *Cephalosporium aphidicola* can also be used in the biosorption process in the biodegradation of Pb (II) contaminated soils (Akar et al. 2007).

Bacteria such as the *Synechococcus* sp. (cynobacterial strains) has been reported to have the expression of the smtA gene and the capability of producing metal-binding protein (Huckle et al.1993). Some species, for example the *Ralstonia eutropha* has been genetically modified to express mouse metallothionein on the cell surface. This decreases the toxic effect of Cd (II) in the contaminated sites (Valle et al. 2000). The range of cadmium accumulation is regulated by the expression of different proteins and peptides by the *Escherichia coli* bacteria species (Mejare et al. 2001).

2.1.8 Bioremediation a promising tool in environmental and agricultural sustainability

Bioaccumulation is the fundamental process in bioremediation. Metal removal requires the metabolic energy of the microbes involved in the process. This includes the transportation of the metals across a cell membrane and its transformation (Lors et al. 2004). Metal tolerant bacteria usually have a high surface area to volume ratio, hence their capability for a large contact area to aid in the interaction with the metals.

The absorption of metals by these microorganisms is a complex procedure and depends largely on the chemistry of the metal ions, surface properties of the organism, cell physiology and physio-chemical parameters (pH, temperature, metal concentration).

Sequestration is also dependent on the diversity of microorganisms (Lozano et al. 2013, Malik, 2004). Microorganisms employ various methods in controlling intracellular metal levels.

These methods comprise various influx and efflux mechanisms and metal complexation by cellular components (Mohsenzadeh 2012). Heterotrophic aerobes and anaerobes from bacteria and heterotrophic sulfur reducers from *Archaea Sp*. are the main organisms (Naik et al. 2012).

Metal adsorption by cell wall components is one of the more significant interaction mechanisms (Nakajima et al. 2004).

Globally, environmental pollution caused by heavy metals has become an overwhelming phenomenon. The persistence nature of heavy metals poses a significantly high degree of environmental threat to the life of both plants and animals (Deeb et al. 2009). Restoration of soils polluted by metal toxicity normally entails numerous elements essential for sustainability.

Cost effectiveness, suitability and sustainability, compatibility to environmental change, biological systems and food contamination and anthropogenic impacts are some of the elements that are of major concern. Bioremediation is considered a more effective technique compared to conventional physiochemical methods. This requires microbes to aid in the clean-up of contaminated environments. Bioremediation is considered to be safe, cost effective and environmentally friendly. This process utilizes numerous agents which include bacteria, yeast, fungi, algae and higher plants when treating contaminated areas (Deeb et al. 2009).

Currently, there are various microorganisms that are being studied for bioremediation. Many of these microbes are already been used as bio sorbents of heavy metals (Deeb et al. 2009).

Some of the most dominant metal bio sorbents in the bacteria class consist of *Bacillus Sp.* (Hameed 2006), *Pseudomonas Sp.* (Huston et al. 2002) and *Streptomyces Sp.* (Infante et al. 2014, Jain et al. 2012).

2.2 Experiment #2: Application of Marsh Mycorrhizae to promote plant growth and aid in plant development under salt stress

2.2.1 Beneficial organisms: symbiotic relationships and their impact in plant growth

Naturally, plants abundantly form beneficial associations with soil-borne microbes that are important to their survival and therefore are important to plant biodiversity and ecosystem functioning. Major examples of symbiotic microbes include mycorrhizal fungi that aid in the uptake of water, nutrients and helps in pathogen infection defense. Rhizobium bacteria is another important microbe that fix atmospheric nitrogen for the plant (Pirozynski and Malloch 1975).

There are other types of beneficial soil-borne microbes, such as plant-growth-promoting rhizobacteria and fungi that have biological control capabilities which can stimulate plant growth by directly suppressing deleterious soil-borne pathogens or by developing aboveground plant parts for enhanced defense against foliar pathogens or insect herbivores. There are even some algae that can facilitate symbiotic relationships. The establishment of beneficial associations requires mutual recognition and the essential coordination of plant and microbial responses (Pirozynski and Malloch 1975).

Bacteria and fungi together have created environmental conditions that are favorable for the evolution of plants. Soil microbes are abundant in most terrestrial environments.
Bacterial and fungal species work together to support and enhance plant development in the rhizosphere (the soil root zone) by transporting nutrients and preventing disease.

These microbes continually increase soil nutrient availability to plants by transforming unavailable nutrients into bioavailable forms. Microbes releases critical nutrients when they die also making them a source of biofertilizers. Apart from nutrient cycling, microbes produce hormones and other chemicals that promote plant growth (Pirozynski and Malloch 1975).

Soil microbes also act as agents for preventing pathogen infection by influencing plant systemic disease resistance and coating the root surfaces and creating a shield for the plant preventing it from getting infected by pathogens (Pirozynski and Malloch 1975).

Plants have evolved with microbes since their existence and mycorrhizal fungi are likely to have co-evolved with these plants and is believed to have enabled early land plant colonization (Pirozynski and Malloch 1975). The colonization of plants is predominantly by endophytic and mycorrhizal fungi, biofilms formed by bacteria on root and leaf surfaces.

Endophytic bacteria living inside plant tissues, nitrogen-fixing bacteria living inside root or stem nodules, and pathogenic organisms forms infectious structures on leaves and in roots (Laila and Heil 2011). In many cases, plants have gained specific advantages from this intimate relationship with microbial agents, for example: transportation of fixed nitrogen and other nutrients in addition to pathogen protection. Research have shown that microorganisms affect plant fitness through direct or indirect contact with plant functional traits such as nutrient provision, changes to photosynthesis, alteration of plant development and stress tolerance (Friesen et al. 2011, van der Heijden et al. 2008). Prominent plant-microbe interactions are the symbioses of plants with nitrogen fixing bacteria and with mycorrhizal fungi, which enhances the plant's ability to grow and develop on soils that are affected by abiotic and/or biotic factors. For example, nodulation in legumes that are in contact with rhizobia enables plants to obtain nitrogen under low nitrogen availability.

It has also been argued that this association enables legumes to have a ‘high nitrogen lifestyle’ Phosphorus uptake is also an advantage for most terrestrial plants with mycorrhizae association (Mckey 1994).

Symbiotic relationships are also identified in other organisms such as algae. They too have significant real-life application that is important to the survival and sustainability of corals and coral reefs. These relationships are very important to all the parties involved (host and symbiont) (Mckey 1994).

2.2.2 Beneficial soil fungus

Seven distinct groups of fungus have been classified by scientists who identified more than 70,000 unique fungal species. The hyphae or filaments (hair like) structures of the fungi grows through the soil and along the root surfaces or mycelium.

A protective sheath is developed allowing the fungus to protect their DNA environmental damage. Soil- fungi is known as decomposers but the beneficial soil fungi can either be decomposers or mutualists. These mutualists are further classified into three specific functional categories known as saprophytic, ectomycorrhizal and arbuscular mycorrhizal fungi (Houlahan, et al. 2006).

2.2.3 Saprophytic fungi

Saprophytic fungi are found in all terrestrial ecosystems and are the primary decomposers. They are capable of degrading extremely resistance substances. These fungi also help in improving soil quality by decomposing complex carbon compounds and increasing soil organic matter. This will help the soil to retain nutrients and moisture (Houlahan, et al. 2006).

2.2.4 Ectomycorrhizae (EcM)

A symbiotic relationship is form with the ectomycorrhizal fungi and some plant species by forming a dense hyphal sheath/mantle which surrounds the root surface.

The Ectomycorrhizae, fungi do not normally infect their plant host's cortical cell walls, a hyphal net between the plant's epidermal and cortical root cells is formed. This is commonly known as a Hartig net.

These microbes benefit plants by transporting nitrogen from the surrounding soil to improve plant nitrogen uptake. In exchange, plants supply the fungi with carbon that can be easily broken down by the root exudates. EcM fungi have low host specificity, thus many different fungal species form symbiotic relationships with various plant species (Houlahan, et al. 2006).

2.2.5 Arbuscular Mycorrhizae (AM) fungi

It is believed that approximately 80% of the plant species on earth are associated with AM fungi. Glomus is considered the largest genus of arbuscular mycorrhizal (AM) fungi. Currently, scientists have identified approximately 85 different AM fungal species. The symbiotic interactions between plants and AM fungi originated many decades ago.

These associations evolved from the interactions with both free-living saprophytic fungi that over time became endosymbiotic with plants and from parasitic fungal interactions that developed into mutually beneficial relationships.

There are several characteristics that differentiate Arbuscular mycorrhizae from Endomycorrhizae. Arbuscular fungi generally infect their host plant by penetrating the root cortical layer.

AM fungi transport soluble phosphorus to the plant to aid in development as oppose to transporting nitrogen to the host. They also act as transport paths that extend the plant roots and allow them to efficiently take up nutrients and water from the surrounding environment.

Many studies have also shown that the symbiotic relationship created with arbuscular mycorrhizae and their host have increased drought tolerance for these plants.

Environmental conditions such as moisture, cation exchange capacity and soil pH can strongly influence AM fungal colonization with plants. Studies have shown that AM fungal hyphal growth significantly declines in soils that are highly fertilized. Tillage is also believed to optimize the establishment of arbuscular mycorrhizae colonization of plants (Houlahan, et al. 2006).

2.2.6 What are Vesicular Arbuscular Mycorrhizal (VAM) Fungi?

Vesicular Arbuscular Mycorrhizal Fungi (Mycorrhizae: plural or Endomycorrhizae) is a group of fungus that penetrates the cortical cells of the roots of vascular plants (Brundrett 2002). Characterization of mycorrhizae is based on their structures, arbuscules and vesicles formed by fungi from the Glomeromycota phylum. Phosphorus, sulfur, nitrogen, other micronutrients and water are captured by plants from the soil with significant help from these fungi (Brundrett 2002). These microbes help the plant by creating a symbiotic relationship with the root of the vascular host. A bi-directional movement of nutrients is created when the cortical tissue of the roots is colonized. This normally takes place in during the active growth period of the plant.

The fungus benefit from the photosynthetic carbon created and in return, inorganic soil nutrients become available and is transported to the plant through its root.

A critical linkage is created between root and soil. This relationship is significant to the plant's development and the association between fungi and roots angiosperms represent a two-way beneficial mechanism (Brundrett 2002).

A mycelial network of vesicles and arbuscules is formed when colonization of the root occurs. The aseptate or septate mycelial branches out with minimal damage to the plant tissues.

These arbuscules have an absorptive function and are formed intracellularly. The vesicles are actual swellings of the hyphae having a storage function that are formed both inter and intracellularly (Brundrett 2002).

The six genera of these beneficial fungi include: *Glomus Sp., Gigaspora Sp., Acaulospora Sp., Entrophospora Sp., Scutellospora Sp.* and *Sclerocystis Sp.* belonging to the *Endogonaceae* group.

Mycorrhizal associations formed by these fungi are generally identified by the spores and sporocarps characteristics. These are generally found in the soil surrounding the host roots and rarely inside the roots. Identification of VAM fungi directly from the roots is challenging and timely.

The host range for VAM fungi is numerous and includes almost all the families of the angiosperm species. Some aquatic plant roots are also known to be colonized by VAM fungi (Prakash et al.1991).

The physiology and ecology of VAM fungi are been extensively researched for a greater understanding of their roles and function in the ecosystem. This has a greater influence their applicability in ecosystem restoration and sustainable agriculture (Brundrett 2002).

2.2.7 Classification of Mycorrhizae

Mycorrhizae have been classified on three different bases. This classification system is according to tropic level, morphological and anatomical features and a general category.

2.2.8 Classifications

A. Tropic level by Frank (1988) classified mycorrhizae into:

1. Ectotropic Mycorrhiza
2. Endotropic Mycorrhiza

B. Morphological and anatomical feature mycorrhiza divided into three types:

1. Ectomycorrhiza
2. Endomycorrhiza
3. Ectendomycorrhiza

C. General classification:

2.2.9 Vesicular- Arbuscular Mycorrhizae (VAM)—Endomycorrhizae

The term VAM was originally used in reference to symbiotic associations formed by all fungi in the Glomus group. However, AM has become a preferred acronym when referring to these species since some of the major suborder cannot effectively form vesicles in plant roots. Based on the spore formation of the members in the Glomus group, they are further divided into families and genera. These spores are distinctive and range in diameter from 10 μm for Glomus tenue to more than 1,000 μm for some *Scutellospora sp*. Spores color can vary from hyaline (clear) to black. surface texture of spores may also be smooth or ornamented.

Glomus generally form spores at the end of the hyphae. Spores of the *Acaulospora Sp*. laterally from the neck of a swollen hyphal terminus, while *Entrophospora* forms spores within the neck of the hyphal terminus.

The *Gigasporineae* are divided into two genera because of the presence of the inner membranous walls and a germination shield (structure from which the germ tube can grow) for either the *Scutellospora* or the absence of these structures for *Gigaspora* (Hayat 2010).

The AM symbiosis does not generally specify in host selection and may colonize a wide range of taxonomic species of both herbaceous and woody plants. However, AM fungi colonization patterns, plant response and effectiveness differ in the level at which they produce in root systems.

This affect the efficiency of nutrient and water uptake contributing to plant growth and development. Thus, the importance of distinguishing between specificity, natural ability to colonize, effectiveness of colonization and plant response to colonization (Hayat 2010).

2.2.10 Ectomycorrhizae (EM) fungi

Ectomycorrhizae (EM) is identified by the presence of hyphae between root cortical cells and producing a netlike structure called the Hartig net. Hartig is considered the father of forest biology. A sheath, or mantle is often observed in many EM, these tissues may completely cover the absorbing root (usually the fine feeder roots/root hairs). The mantle can vary in thickness, color, and texture depending on the type of plant-fungus association. The mantle usually increases the surface area of absorbing roots and generally affects the morphology of the root hairs. Root bifurcation (division) and clustering is often a result.

The mantle and hyphal strands shares a common boarder that extends into the soil. Rhizomorphs are often formed from the aggregation the hyphal strands that may be visible to the unaided eye. The internal portion of rhizomorphs can also develop into tube-like structures that are specialized for long-distance transport of nutrients and water. Ectomycorrhizae are generally found on woody plants ranging from shrubs to forest trees. Many of the host plants belong to the families *Pinaceae, Fagaceae, Betulaceae* and *Myrtaceae*. Ectomycorrhizae are known to form in over 4,000 fungal species, belonging primarily to Basidiomycota and Ascomycota kingdom (Hayat 2010).

2.3 Ericoid

Fungal hyphae grow over the root surface but not forming a true mantle. Plants such as *Calluna* (heather), *Rhododendron* (azaleas and rhododendrons) and *Vaccinium* (blueberries) are usually associated with ericoid. These plants have very fine root systems and generally grow in acid, peaty soils. *Ascomycetes* of the genus *Hymenoscyphus* are the fungi involved (Hayat 2010).

2.3.1 Arbutoid

This group have characteristics of both EM and *Endomycorrhizae*. There is potential for Intracellular penetration, a mantle is formed, and a Hartig net is present. Some species of the *Pyrolaceae, Arbutus* (e.g., Pacific madrone), *Arctostaphylos* (e.g., bearberry) are plants associated with this type of relationship. *Basidiomycetes* is the fungi involved and may be the same fungi that colonize EM tree hosts in the same region (Hayat 2010).

2.3.2 Monotropoid

This type of fungi colonizes achlorophyllous (lacking chlorophyll) monotropic plants (e.g., Indian pipe). These associations produce the Hartig net and mantle.

The same fungi also form EM associations with trees and create a connection/path through which carbon and other nutrients can flow from the autotrophic host plant to the heterotrophic and parasitic plant (Hayat, 2010).

2.3.3 Orchidaceous Mycorrhizae

Mycorrhizal fungi are very beneficial in the life cycle of plants of the *Orchidaceae* family. Orchids generally have very small seeds and a small nutrient reserve.

The plant depends on the mycorrhizal fungus for carbon and vitamins that aid the development of the embryo. Dependency of carbon from these fungi by the achlorophyllous species is a life-long relationship. The fungus grows into the plant cell and covers the cell membrane which results in the formation off hyphal coils within the cell (Hayat 2010).

The life span of these coils is very short, they quickly lose turgor and physical qualities. The nutrient contents are then absorbed by the developing orchid.

The fungi in the symbiotic relationship are *Basidiomycetes* similar to those involved in decaying wood (e.g., *Coriolus, Fomes, Marasmius*) and pathogenesis (e.g., *Armillaria* and *Rhizoctonia*). Mycorrhizae are very important in nutrient uptake and translocation in mature orchids (Hayat 2010).

2.3.4 Beneficial soil bacteria

Soil bacteria are very important in biogeochemical cycles and have been used extensively for crop production for decades. Plant–bacterial associations in the rhizosphere helps to promote plant health and soil fertility. Free-living soil bacteria are very beneficial to plant growth and are usually referred to as plant growth promoting rhizobacteria (PGPR).

These microbes can also be referred to as plant health promoting rhizobacteria (PHPR) or nodule promoting rhizobacteria (NPR).

Cyanobacteria of the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Sinorhizobium* and *Mesorhizobium* are all symbiotic bacteria that interact with plants, aids in their growth potential and significantly contribute to sustainable plant production. They synthesize specific compounds for the plants, facilitate the uptake of certain nutrients from the soil and lessens or prevent the plants susceptibility to diseases (Hayat 2010).

They also help to retain soil organic nitrogen and other nutrients in the plant–soil system this reduces the need for the use of nitrogen and phosphorus fertilizers. (Hayat 2010).

2.3.5 Symbiotic algae relationships

Since algae can produce their own food by photosynthesis, why do they need association with other organisms? These interactions enable both organisms to benefit through a symbiotic association known as commensalism. The blue-green algae are the most primitive and are basically bacteria that have photosynthetic capabilities. Their cells have no nucleus and are their scientifically termed as *Cyanobacteria*.

Most blue-green algae are free-living but some have developed symbiotic relationships with animals as sponges and *Echiuroid* worms (Paracer and Ahmadjian 2000).

These algae gain protection from predators by living in the tissues of these organisms, in return they fix nitrogen and promote photosynthesis. This association provides an extra food source for their hosts. They also provide a secondary function by providing color for their hosts, this helps to reduce the effects of coral bleaching. Some algae enhance the process of photosynthesis which allows them to feed themselves and their hosts in the process. There are several other symbiotic relationships created by algae and some sea animals (Paracer and Ahmadjian 2000). Studies have shown that many organisms have established symbiotic associations.

These interactions especially in plant-microbe relationships enable the ability for both organisms to efficiently acquire the water and nutrients needed. In many cases plant survival and development is dependent on these relationships. The association of VAM fungi with plant communities is one that has been investigated for decades and developed increasing significance in the agriculture sector. This is especially the case since sustainable agricultural practices are important in environmental and human health (Paracer and Ahmadjian 2000).

2.4 How are VAM Fungi useful in sustainable agriculture?

2.4.1 Sustainable nutrient supply

Microbes such as nitrogen fixing bacteria or phosphorus solubilizing bacteria are believed to synergistically interact with AM fungi.

This association may improve phosphorus nutrition, enhance nitrogen uptake, improve disease resistance in their host plant and thereby benefit plant growth and development.

This mycorrhizal symbiosis is very important in sustainable agricultural systems especially where nutrient levels are low.

Naturally, the interaction with VAM mycelium and other bacteria or fungi plays a significant role in nutrient mobilization from crop residues (Paracer and Ahmadjian 2000). Studies by Hodge et al. (2009) showed that decomposition of plant litter in soil which resulted in increased nitrogen capture from the *Lolium perenne* leaves litter was enhanced by the presence of the VAM symbiont *Glomus hoi*.

It is also believed that bacteria associated with VAM can also assist in the mobilization of nutrients from soil. According to Minerdi et al. (1996) there may be potential for improved nitrogen supply to mycorrhizal plants through fixation of atmospheric nitrogen.

2.4.2 Biocontrol

Considering the need for a reduced use of pesticides due their threats to human health and non-targeted organisms, other alternatives need to be considered. Microbial inoculants can be considered as potential alternatives for controlling these pests and diseases. There has been ongoing research on the use of VAM as a protective agent against pathogens. Studies have suggested that these basic mechanisms of VAM has improve plant nutrition and competition for photosynthesis.

The presence of VAM in the suppression of root pathogens, stimulation of saprotrophs and the enhancement of plant growth promoting microorganisms have been important biocontrol mechanisms (Paracer and Ahmadjian 2000).

2.4.3 Support system and soil structure enhancement

VAM also acts as a support system for seedling establishment for many plants. The microbes can also influence plant invasion success. Plant diversity and community structure are also influenced by the presence of VAM (Heijden 2008). VAM fungi also influence plant communities by improving soil structure and soil aggregation. Soil aggregation and soil structure stabilization occur when there is binding of soil particles into bigger and compact aggregates. Ecosystem functioning is largely dependent on soil aggregate formation. This improves the penetration of the soil by water and air. It also protects the soil from erosion, making these areas suitable for crop production. The nature of the soil's microflora is a very important determinant in crop growth and development, especially those in close to host plant roots (Heijden 2008). Since the evolution of most life forms on earth and their environment are sustained by microorganisms, most biological activities are influenced by the state of these units of life. Therefore, in order to enhance food production, these soil microorganisms and their relationships with their plant hosts are very important. They are essential in enabling the plant's ability to compete particularly under environmental stress (e.g salinity, drought, nutrient deficiencies and disease/pathogen attacks. Studies have shown that low agricultural production efficiency is mostly because of a poor coordination of energy conversion (National Academy of Sciences, 1989).

These microorganisms effectively help to maintain a suitable environment of plant growth, and improve the quality both soil and crop. A wide range of positive results are possible depending on the VAM predominance and activities at any one time.

However, it is strongly believed that it is possible to attain maximum economic crop yields of very high quality, at higher net returns, without the application of chemical fertilizers and pesticides.

Understanding the most effective soil and crop management practices is always important in promoting a more sustainable agricultural approach. This also enhances the growth, numbers and activities of beneficial soil microorganisms. This will also improve the growth, yield and quality of crops. In essence, the foundation of sustainable agriculture is dependent on the quality of the soil (National Academy of Sciences 1989).

2.4.4 VAM as a biofertilizer

"An area that appears to hold the greatest promise for technological advances in crop production, crop protection, and natural resource conservation is that of beneficial and effective microorganisms applied as soil, plant and environmental inoculants " (Dr. Teruo Higa 1994).

Agricultural management practices and strategies are mainly dependent on the use of in-organic chemical based fertilizers. These chemicals are often a serious threat to human and environmental health, leading to the exploitation of beneficial microbes being used for biofertilizer as an alternative approach (Adesemoye et al. 2009). This has become very a significant development in the agriculture sector, considering their potential role in food safety and sustainable crop production (Adesemoye et al. 2009).

Studies have shown that end-mycorrhizal fungi can improve nutrient uptake, plant growth and tolerance to abiotic and biotic stress (Singh et al. 2011).

Even though new methods for massive production have developed in recent years, the production of VAM inoculum on a large scale remains a challenge.

The challenge is due to the obligate symbiotic behavior of the VAM that need to have a host plant for growth and completion of their life cycles (van der Heijden et al. 2015).

Hence, the propagation step must include cultivation with the host plant. Therefore, the production and management of the high amount of inoculum necessary for large-scale application can be considered a demanding process.

However, VAM fungi inoculation can be easily carried out for plant production systems that involve a transplant stage, this is because smaller amounts of inoculum are needed. Open field inoculation treatment may seem technically impractical and economically challenging but once the VAM biodiversity is restored and established, the VAM community will persist. Maximum results can be achieved with the implementation of a VAM-friendly management such as fall cover cropping and conservation tillage (van der Heijden et al. 2015).

Once these management practices are maintained and there are no detrimental effects either before or after cultivation, there can be tremendous contributions. Studies shows that the biodiversity of mycorrhizal hyphal network will remain unaltered and effective in the future.

For these reasons, VAM inoculation only represents an initial cost since the persistence of the fungi once it is favored by the soil could be distributed over the years.

Van der Heijden et al. 2015, demonstrated that VAM inoculation can be economically profitable, in comparison to conventional fertilization.

This provides substantial savings for growers especially for sustainable productions. Global environmental and economic crisis has forced growers to use alternative management strategies to sustain their agricultural systems. The potential for the use of VAM in this situation has drawn the attention of the commercial sector, and several companies nowadays produce and sell VAM-based inoculums.

Some manufacturers have used a single formulation approach, using only a few VAM species as components. The few species that are often used can easily be propagated and are normally preferred. They are normally found in association with a large variety of host plants in different habitat types and can be readily available for the crops (Van der Heijden et al. 2015).

CHAPTER 3

MATERIALS AND METHODS

3.0 Site Selection

Blackbird Creek is located in Townsend, Delaware and is one of the least disturbed marsh ecosystems in the state. It is approximately forty kilometers (km) north of the Delaware State University main campus. Blackbird Creek is one of the twenty-eight National Estuarine Research Reserves (NERR), and also is one of two reserves established by National Oceanic and Atmospheric Association (NOAA) in Delaware. Blackbird Creek is like many other ecosystems affected by human-induced changes which can ultimately alter its structure and functions. Some human activities of concern in this area are the building of residential and industrial infrastructures and agriculture practices near the marsh that can cause heavy metal contamination from the use of agriculture chemicals (WHO 2016). This has driven the need to examine heavy metal tolerant microorganisms from soil samples from this area that can tolerate heavy metals and possibly be used for bioremediation. The presence and abundance of VAM fungi in this area is also investigated to help determine its benefits for plant development and agricultural production.

3.1 Experiment#1: Isolation, Identification and Characterization of Lead and Cadmium Tolerant Bacteria from Marsh Soils

3.1.1 Sample collection

Soil samples (100 g) were collected by composite sampling method from the areas dominated by *Phragmites* and *Spartina* in Blackbird Creek during spring 2016. These samples were collected at a depth of 0-15 cm and 10 cm away from the plant. In composite sampling method, soil samples were collected from the center of the selected area and its surroundings.

A total of ten samples were collected from each sample site (*Spartina* and *Phragmites* dominated) were combined and treated as single sample for each site.

This was done to improve spatial coverage and include all the possible characteristics without increasing sample number/size. All soil samples were placed in clean 3.7 L Ziploc bags (S.C. Johnson and Son, Inc., Wisconsin, USA) and stored at 80˚C. Water samples (20 L) were collected and stored at room temperature. Marsh water samples were used to prepare culture media (agar plates and liquid media) for isolating bacteria.

3.1.2 Screening of Metal Tolerant Bacteria

For isolation of both lead and cadmium tolerant microbes, 100ml Luria-Broth (LB) was enriched separately with 1,000 mg/kg of lead (0.3997 g) in the form of lead nitrate and cadmium (0.407 g) in the form cadmium chloride. They were prepared using LB nutrient broth (6 g of LB powder dissolved in 250 ml of marsh water), the medium was autoclaved (Tuttnatuer Autoclave-Steam Sterilizer 3870 M, Hauppauge, New York, USA) at 15 lb pressure and 121˚C for 15 mins.

This medium containing heavy metal was inoculated with 10 g of soil separately as shown in Table 3.1.

These tubes were then incubated at 37˚C under agitation at 200 revolutions per minute (rpm) for 48 hours (New Brunswick Scientific Edison New Jersey, USA I 24 Incubator Shaker Series).

Table 3.1. Liquid nutrient media for enrichment culture.

Heavy metal (HM)	HM vol. (g)	H_20 Vol. (ml)	Soil vol. (g)	LB broth (g)
Cadmium (Cd)	0.407	250	10	6
Lead (Pb)	0.3997	250	10	6

3.1.3 Spread plating

Luria-Bertani (LB) agar plates were prepared using 4 g of agar per 100 ml of marsh water containing cadmium up to 500 mg/kg and lead concentrations up to 1,000 mg/kg.

Stock solutions of the heavy metals were prepared using 0.814 g of $CdCl_2$ in 500 ml of water and 1.9985g of $PbNO_3$ in 500 ml of water separately. Working solutions (diluted solution) of 100, 200,300, 400 and 500 mg/kg of $CdCl_2$ solution was prepared for cadmium (Table 3.2), while 2,100, 2,200, 2,300, 2,400 and 2,500 mg/kg of $PbNO_3$ was made from lead as shown in (Table 3.3). Initial dilutions up to 500, 1,000 and 2,000 mg/kg of lead showed overgrowth of colonies that were uncountable. The enriched medium was then autoclaved for 15 min at 121 ˚C and 15 lb pressure. After the medium was autoclaved 20 ml of medium containing heavy metal and LB agar was poured into Petri dishes. After solidification of agar, 100 µl of the cultured suspension from enriched cultures (step described in 3.1.3) was spread platted on the LB agar plates and incubated at 37˚C (Fisher Scientific, Isotemp Incubator, 6841 (Hampton, New Hampshire, USA). The platting was performed in triplicates for each dilution.

Table 3.2. Cadmium concentrations for solid nutrient growth medium.

Final Cd conc. (mg/kg)	Diluted conc. (mg/kg)	H_2O vol. (ml)	Stock vol. (ml)	Agar vol. (g)
500	100	80	20	20
	200	60	40	
	300	40	60	
	400	20	80	
	500	0	100	

Table 3.3. Lead concentrations for solid nutrient growth medium.

Final Pb conc. (mg/kg)	Diluted conc. (mg/kg)	H_2O vol. (ml)	Stock vol. (ml)	Agar vol. (g)
2,500	2,100	16	84	20
	2,200	12	88	
	2,300	8	92	
	2,400	4	96	
	2,500	0	100	

3.1.4 Enumeration of bacteria colonies

The well isolated colonies were calculated as colony forming units (CFU) using the formula CFU/10 grams of soil = Number of colonies×0.1 (amount plated in ul) ×10.

Ten isolated colonies were picked from the plates for each metal concentration and were further inoculated into tubes of LB nutrient broth with heavy metals prepared as above for further identification studies. The LB broth tubes were then incubated at 37°C in an incubator shaker overnight.

3.1.5 Genomic DNA (Deoxyribonucleic acid) extraction and molecular analysis

Genomic DNA was isolated using the phenol-chloroform method (He 2011).

Nucleic concentrations were examined using the Nano drop 200 Spectrophotometer, (Thermo Scientific, Waltham, Massachusetts, USA 200). DNA integrity and quality were further confirmed by performing gel electrophoresis using 1% (w/v) agarose gel. 1% Agarose gel was prepared with 1x Tris acetate-EDTA (TAE) buffer (30 ml), ethidium bromide (0.5 µg/ml) and Agarose powder (0.3 g). The aim of this procedure was to obtain DNA which has a 260/280 value close to 1.8 and 260/230 value greater than 2.0.

This confirms that the DNA is free from protein and other contaminants and is of good quality (Thermo Scientific, Waltham, Massachusetts, T009-Techical Bulletin).

3.1.6 Polymerase Chain Reaction (PCR) analysis

Primers for PCR were designed targeting the 16S rDNA gene sequence for bacteria as this sequence is present in all bacterial domains. 100 ng of genomic DNA was used as a template and universal bacterial primers,**27F 5' AGAGTTTGATCCTGGCTCAG 3'** and **1492R 5' GGTTACCTTGTTACGACTT 3'** (Byers et al. 1998) were used in the PCR reaction. The PCR mixture (25µl) contained 1µl of template, 5 µl of Taq DNA polymerase buffer (5x), 1µl of dNTPs (20mM) 0.5µl polymerase DNA primers (each), 0.25 µl Taq polymerase DNA enzyme and 16.75µl autoclaved water. The PCR was performed in a S1000 Thermal Cycler, (BIO-RAD, Hercules, California USA).

The PCR program for this reaction is 94˚C for 5 m, followed by 30 cycles of 94˚C for 1m, annealing temperature of 44.9˚C for 1m and 72˚C for 1m followed by a final extension performed at 72˚C for 10 m. The PCR products were examined on a 1% (w/v) agarose gel electrophoresis in 1x TAE buffer with ethidium bromide.

This was examined using the GENE Box program, SYNGENE. The amplified products (1500 bp) were purified to remove the PCR reagents using a Qiagen PCR clean up kit (QIAGEN, Germantown, Maryland, USA) to perform further sequencing analyses.

3.1.7 Sequencing analysis

The purified PCR products were sequenced by Sanger sequencing at the University of Delaware Institute of Biotechnology Sequencing Laboratory, Newark, Delaware USA.

The FASTA sequences from the chromatogram files were read using free software Finch TV 1.4 (Geospiza Utah, USA). The sequences were then searched against previously published bacteria 16SrDNA sequences in the NCBI databases using Advanced BLAST (Altschul et al. 1997). BLAST takes a query sequence and searches a given database of sequences for significant matches, generating local alignments that vary in length, providing descriptive parameters as well as statistical evaluation of any matches. The lower the E-value, or the closer it is to zero, the better the match is and % Maximum identity is the percentage of residues that match up in the alignment. For the analysis, we used sequences that have an E- value of <0.05. The scoring sequences of the most similar sequences were aligned with the sequences of other representative bacteria in the 16S rDNA bacterial regions by the Mega 4.0.2 software version and a neighbor joining phylogenetic tree was constructed.

3.1.8 Statistical analysis

Data analysis was performed using the Microsoft Office Excel and the computing Statistical Package for Social Sciences (SPSS) (Chicago, Illinois, USA).

General linear model was used to determine any significant differences in Cd and Pb tolerant bacterial populations in marsh soils dominated by *Spartina* and *Phragmites* grasses at the level of $p<0.05$. Pairwise analysis was used to evaluate the comparison among bacterial growth for each metal concentration.

3.2 Experiment#2: Application of Marsh Mycorrhizae to promote plant growth and aid plant development under salt stress

3.2.1 Sample collection

Soil and plant root samples were collected from the Blackbird Creek and transported to the Aquatic Sciences Laboratory at Delaware State University, Dover, Delaware for storage and experimental use. A series of testing techniques were carried out to identify the presence of Vesicular Arbuscular Mycorrhizae (VAM). These techniques include acid fuchsin root staining (Gerdemann et al. 1963), light microscopy (Nikon Eclipse E200-40x, 100x), Scanning Electron Microscopy (SEM) (Hitachi S-2600N) for identifying spores and wet sieving of soils to isolate the spores (Gerdemann et al. 1963).

Molecular methods were also performed to confirm the presence of VAM fungi. Genomic DNA was isolated from roots and soils using the DNeasy Plant Mini Kit (Qiagen Sample and Assay Technologies, (QIAGEN, Germantown, Maryland, USA) and C-TAB method (Doyle and Doyle 1987, Cullings 1992) for root samples and the Power Soil Extraction Kit (MO Bio, California, USA) for soil samples. Nested PCR method was performed for amplification and identification of VAM fungi.

3.2.2 Pre-greenhouse laboratory studies

3.2.3 Acid fuchsin staining of roots (Gerdemann et al. 1963)

Roots were rinsed with tap water to remove debris; they were heated in KOH at 90˚C for 1 hour. Roots were then removed, rinsed and soaked in 10% HCl for 5 min. After removing the roots from HCl, they were heated in 0.01% lactic-acid fuchsin for 60 min and observed under a light microscope at 40X magnification for deep pink spore structures within the root.

3.2.4 Wet sieve method

VAM spores were isolated from 100 g of soil by wet sieving and decanting methods (Gerdemann and Nicolson 1963). The soil samples were mixed with 1 L of tap water and the suspension was passed through a series of sieves (250 µm and 38 µm) for collecting spores. Spores were then isolated in 50% and 15% sucrose solution using density gradient centrifugation. The samples were then centrifuged at 4,000 rpm for 5 min (Walker et al. 1982). The spores were observed using light microscopy. This procedure was performed with an intention to inoculate the plants in the greenhouse set up with VAM spores.

3.2.5 Molecular methods

3.2.6 DNA extraction and PCR (Nested)

Roots were ground in liquid nitrogen using a mortar and pestle and approximately 1 g of ground sample was transferred to a 1.5 ml tube. DNA was extracted from roots using the DNeasy Plant Kit (QIAGEN, Germantown, Maryland, USA) according to the manufacturer's instructions. PCR was performed by a nested procedure. The first-round amplification was performed using the universal eukaryote primers NS5 and ITS4.

Primer sequences are **NS5: 5' AAC TTA AAG GAA TTG ACG GAA G 3'** and **ITS4: 5' TCC TCC GCT TAT TGA TAT GC 3'** (White et al. 1990). The cycling parameters were in 3 min at 94°C, followed by 28 cycles of 45 sec at 94°C, 50 sec at 94°C, 50 sec at 51°C and 0 sec at 72°C. The program was concluded by a final extension phase of 10 min at 72°C. The PCR product (100 bp) was then used as templates in the second round. In order to check the success of amplification, PCR products were run on agarose gels (1%) in Tris-acetate buffer (TAE). Five separate PCRs were performed using the primer pairs (Table 3.4 and Figure 3.2).

Table 3.4. Five primer sets used to identify VAM specific groups.

Primer Set	Forward sequence	Reverse sequence	Annealing temperature (Tm)	Product size (bp)	Reference
GLOM1310/ITS4	*5' AGC TAG GCT TAA CAT TGT TA 3'*	*5'TCC TCC GCT TAT TGA TAT GC 3'*	*54.9*	*100*	(Redecker 2000)
LETC1670/ITS4	*5' GAT CGG CGA TCG GTG AGT 3'*	*ITS4: 5'TCC TCC GCT TAT TGA TAT GC 3'*	*54.9*	*100*	(Redecker 2000)
ACAU1660/ITS4	*5' TGA GAC TCT CGG ATC GGG 3'*	*ITS4: 5'TCC TCC GCT TAT TGA TAT GC 3'*	*54.9*	*100*	(Redecker 2000)
ARCH1311/ITS4	*5' TGC TAA ATA GCT AGG CTG Y 3'*	*ITS4: 5'TCC TCC GCT TAT TGA TAT GC 3'*	*62*	*100*	(Redecker 2000)
GIGA5.8R /NS5	*5' ACT GAC CCT CAA GCA KGT G 3'*	*5' AAC TTA AAG GAA TTG ACG GAA G 3'*	*49.8*	*100*	(Redecker 2000)

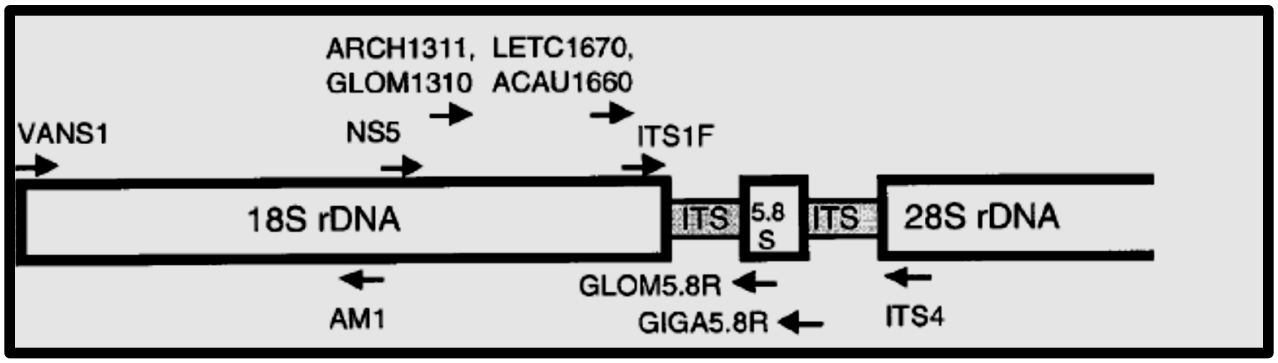


Figure 3.2. Schematic representation of ribosomal rDNA genes with annealing sites of primers (Redcker 2000).

The PCR parameters for the second round differed from the first one only in annealing temperatures. To check the success of amplification, PCR products were ran on agarose gels (1%) in Tris-acetate buffer (TAE)

3.3 Greenhouse Cultivation of Spartina alterniflora

Greenhouse experiments were conducted using soils and VAM roots collected from the *Spartina* dominated sites in Blackbird Creek salt marsh, located in Townsend, Delaware. Soils were collected at a depth of 0-10 cm and 0-5 cm away from the plant. Plant VAM roots were collected to depths of 30 cm. Experiment was conducted in the greenhouse facility of College of Agriculture and Related Sciences, Delaware State University main campus in Dover, Delaware in the spring of 2016.

Soils (2 bags each 37 L approximately) were collected from the Blackbird Creek marsh, and a day after collecting the soil (1 bag) it was autoclaved for use in the treatment experiment (Figure 3) to remove microbes (specially to kill VAM spores and other beneficial microbes) present in the soil. One bag of marsh roots with root hairs were also collected and placed in a cooler. The roots were brought to the laboratory, washed with tap water, patted dry and chopped into small pieces.

The soils were then potted according to the randomized complete block experimental design with autoclaved soils and un-autoclaved soils. Also, some pots were filled with soils and roots in 2:1 ratio and labelled as “VAM” and pots without roots were used as control.

This was performed because our earlier experiment identified the presence of VAM fungi in the roots of *Spartina* and the aim of this experiment was to check if the spores in the roots would infect the plants through plant-trap culture method and help them tolerate salinity stress.

3.3.1 Experimental Design: Randomized Complete Block Design (RCBD)

A Randomized Complete Block Design was set up with a total of 4 treatments and 2 controls. Three blocks were used in this design, namely: A, B and C as shown in (Figure 3.3).

Each block received the same level of irrigation for both saline and non-saline treatments. Each treatment had a total of 9 sub-samples randomly arranged in each replicate blocks. The pots were color coded **Yellow=UAs+VAM, Blue=UAs+VAM+Salt, Grey=UAs Only-Control, Green=As+VAM, Pink=As+VAM+Salt and Red=As Only-Control.**

The soils were filled into 6cm pots. Three replicas of each treatment were placed in each block, resulting in 18 pots per block. This resulted in a total of 54 pots for planting of seedling.

The experimental bench had 7.3 m x 1.5m (11.24 m^2) area. All 3 blocks were oriented parallel to the cooling fan air flow. The length of the blocks was approximately 270cm with spacing of 30cm between each pot. Each of the 3 blocks were separated by approximately 45cm.

As VAM fungi are obligate endophytes and they can survive only in a living host, plant trap culture method was used to inoculate the pots with these fungi.

The plant trap culture method (Walker et al. 1982) was used to inoculate fungal spores and mycelia into soils (2:1 ratio) cultivated with *Spartina alterniflora*. This was done by washing and chopping the VAM roots and used for application in each pot for the different treatments.

The treatments consisted of **Autoclaved soil (As) +VAM, Autoclaved soil (As)+VAM+Salt, Un-autoclaved soil (UAs)+VAM, Un-autoclaved soil (UAs)+VAM+Salt, Autoclaved soil (As) only and Un-autoclaved soil (UAs) only.** A layer of soil was placed in the bottom of each pot. Chopped VAM roots were then placed in the pots creating another layer above the soil. A second layer of soil was placed on the top. The very top layer was mixed for even distribution of both roots and soil.

BLOCK A

UAs + VAM	As + VAM	As + VAM
As + VAM + S	UAs (control)	As (control)
UAs + VAM	UAs + VAM	As (control)
UAs + VAM + S	As (control)	UAs (control)
UAs + VAM + S	As + VAM	UAs + VAM + S
As + VAM + S	UAs (control)	As + VAM + S

BLOCK B

As + VAM	As (control)	As + VAM
As (control)	UAs + VAM	UAs + VAM + S
UAs + VAM + S	UAs + VAM	AUs (control)
UAs + VAM	As (control)	As + VAM + S
UAs + VAM + S	As + VAM	As + VAM + S
As + VAM + S	UAs (control)	UAs (control)

BLOCK C

As + VAM	As + VAM + S	UAs (control)
As + VAM +S	UAs + VAM	As + VAM + S
UAs (control)	As (control)	As (control)
UAs + VAM	UAs + VAM + S	UAs+ VAM + S
As + VAM	UAs + VAM	UAs (control)
As + VAM	UAs + VAM + S	As (control)

COLOR CODE

TREATMENT	COLOR
As (CONTROL)	
UAs +VAM + S	
As +VAM	
UAs + VAM	
As +VAM + S	
UAs (CONTROL)	

Figure 3.3. Randomized Complete Block Design used in this study.

Randomized Complete Block Design of the experiment was set-up to investigate the role of VAM fungi in the marsh grass, *Spartina alterniflora* affected by abiotic stress (salinity). Treatments were designed to identify the physical responses of the *S. alterniflora.* Plants in each block are arranged randomly to ensure balanced distribution in air flow, temperature and sunlight.

3.3.2 Germination

Spartina alterniflora seeds were obtained from Pineland Nursery in Columbus, New Jersey. These seeds were stored in the refrigerator to inhibit germination and aid viability in my study. *Spartina alterniflora* seedlings that were pre-germinated in the lab and were placed in the pots. The seeds were germinated using the culture dish method (Biber et al. 2008), where seeds were washed on a 38-micron meter sieve (Fisher Scientific Company, U.S.A Standard Test Sieve, ASTM E-11) and then placed in a glass culture dish filled with tap water. Water levels were checked each day to ensure that seeds do not dry out and germination began in five days. This method was chosen and compared to the paper towel method because there was a higher percentage of germination with relatively shorter timeline.

3.3.3 Seedlings transplantation

These seedlings were then transplanted to pots for the greenhouse and watered daily to saturation level. Overall plant appearance and growth were checked daily. Plant numbers in each experimental block were thinned to two plants per pot to reduce competition for nutrients, water, and space.

3.3.4 Irrigation

Water regimes were implemented after full plant emergence. Pots were watered with 200 ml of tap water using a graduated cylinder. The soil: water volumetric percentage content was measured and recorded for each pot in order to monitor the water saturation level of all treatments using Time-Domain Reflectometry (TDR 100).

3.3.5 Stress imposition: salinity stress

Salinity stress was imposed after 12 weeks of plants' maturity using a 5% (W/V) solution of commercial synthetic sea salt equivalent to salt concentration of the sea water ((approximately34 parts per thousand-(ppt) (Baisakh et al. 2007). These applications were made with 200 ml per pot for 24 hours, with each treatment replicated 9 times in the greenhouse trial.

3.3.6 Harvesting

Spartina alterniflora plants were harvested after 24 hours of salt treatment (September 1, 2016). One plant was carefully removed from each treatment in all the blocks (A, B and C). The roots were washed to remove excess soil. The physical plant characteristics such as number of leaves, root length, root mass, shoot length and plant heights were measured. Roots were also collected into plastic storage bags and kept on ice and then transferred to refrigerator for storage. These roots were stored for spore's identification, microscopic and molecular studies.

3.4 Post greenhouse study

3.4.1 Physical and molecular study of plant from greenhouse study

Plant's physical characteristics were measured and compared in between the treatments for the effects of VAM fungi on the plant's growth and development. Molecular methods were used to determine the effect of salt shock treatment on the plants. These results were compared to plants that were not exposed to salt shock treatment. Comparative assessment for these characteristics was also performed based to the types of soils (autoclaved or un-autoclaved) that these plants were grown in. Some soils were autoclaved to remove VAM and other beneficial organisms that were present.

This was done to test the effect of VAM on the plants' physical development as a lone agent. Results were compared to the physical characteristics of those plants that were grown in soils not autoclaved. The un-autoclaved soils would have maintained their natural characteristics.
This comparison enabled me to tell if there were any benefits of these VAM fungi and if these benefits had any significant differences on the plants in autoclaved soils**.**

3.4.2 Post-greenhouse laboratory studies

Confirmations of presence of VAM in the harvested roots were performed using the acid fuchsin staining of roots. The spores were then identified by light microscopy. Molecular analysis was further performed by conducting DNA isolation using the C-TAB method and confirmed using the Nanodrop and gel electrophoresis. Amplification of DNA was also performed using nested PCR.

3.4.3 DNA extraction using C-TAB **(Doyle and Doyle 1987, Cullings 1992) and PCR (Nested)**

Plant root tissue was gently washed and placed on ice. Approximately 1 g of roots tissue was grounded in liquid nitrogen using a chilled mortar and pestle. The ground tissue was transferred into 15 ml plastic centrifuge tube and capped. Five ml of preheated cetyl-trimethylammonium bromide (C-TAB) isolation buffer was added into those centrifuge tubes and the samples were incubated for 30 min. After incubation, 5 ml of chloroform: isoamyl alcohol (24:1) (this *prevents DNA from shearing)* was added and solution was centrifuged for 15 min at 3,500 rpm. The aqueous (top) layer was then removed with a pipette and transferred to a clean tube.

Cold isopropyl alcohol in the amount of 2.5 ml was added to precipitate the DNA. The samples were then centrifuged at approximately 3,500 rpm for 15 min and then the supernatant was removed.

Five ml of DNA wash solution was added and samples was allowed to sit for 20 min in the cooler (*pellets were dislodged in-order to remove traces of chloroform*). Samples were centrifuged for 15 min at 3,000 rpm.

DNA wash step was repeated one more time; supernatant was removed and allowed to be air dried. Pellets were re-suspended in 100µL of TE with RNase A.

The concentrations were checked using the Nano-drop and then quantified with 1% agarose gel. Figure 3.4 below shows the summary of the methods applied in my study. Nested PCR was performed similar to the pre-greenhouse study. This PCR was also performed using the five primer sets designed to identify VAM fungi.

3.4.4 Statistical analysis

Data analysis was performed using the Microsoft Office Excel and the computing package called SPSS. A fixed-effect one-way analysis of variance (ANOVA) was used to determine any significant differences in VAM treatment effects on the chosen physical characteristics of the *Spartina alterniflora*. Statistical significance of $p<0.05$was used in this study. Comparison of the various means was performed using the Tukey's Test. The Shapiro Wilk's Test was used to confirm normality of the data.

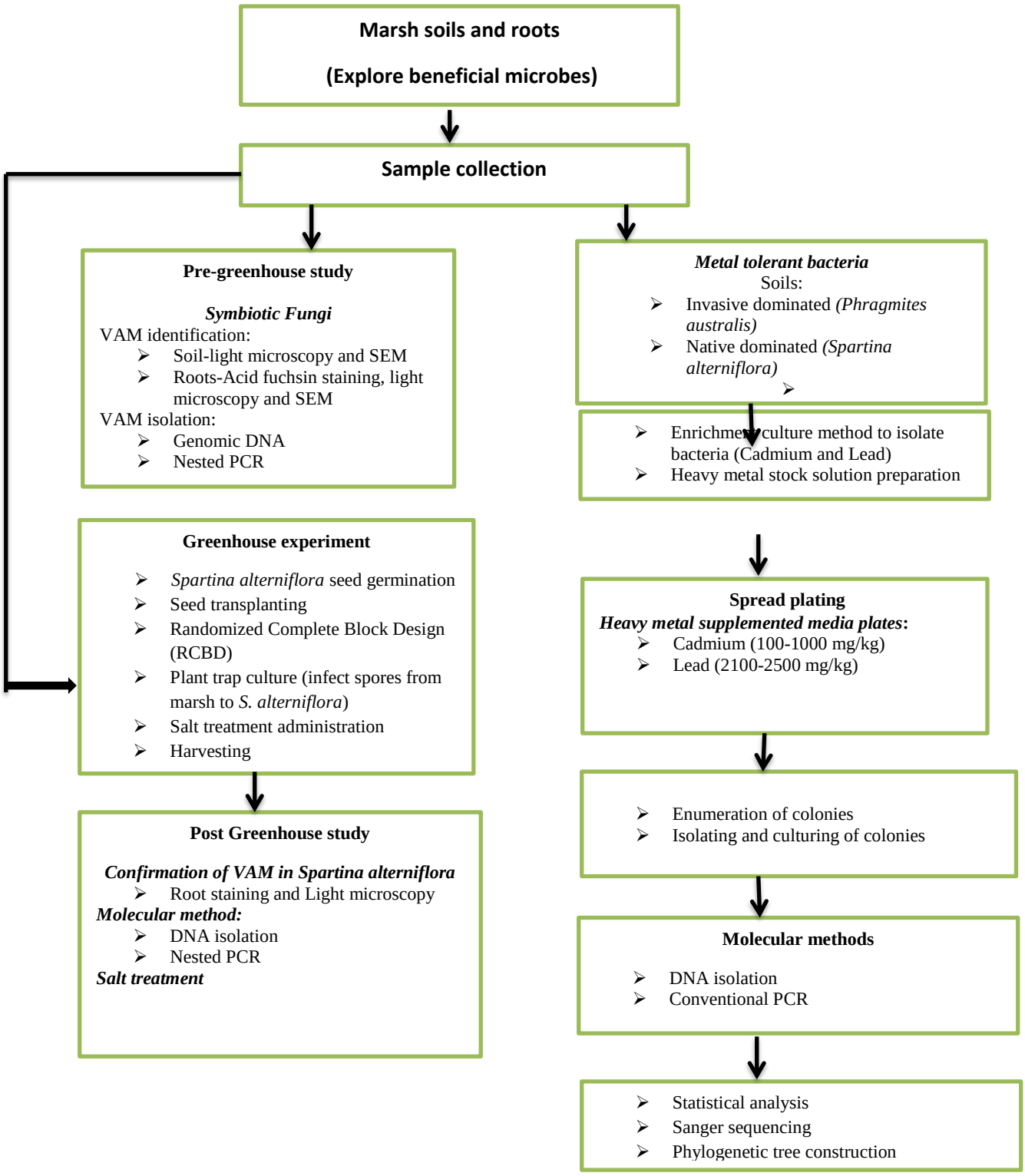


Figure 3.4. Summary of the materials and methodology used.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.0 Experiment#1: Isolation, Identification and Characterization of Lead and Cadmium Tolerant Bacteria from Marsh Soils

4.1 Isolation of heavy metal resistant bacteria

Heavy metal resistant bacteria were isolated and identified from marsh soils. Over 500 colonies were screened from initial LB agar plates supplemented with each heavy metal concentration. One hundred isolates were selected in the secondary screening from the marsh soils and inoculated into LB medium for growth.

4.1.1 Growth studies of marsh soil bacteria

Bacteria from marsh soils were able to grow up to 25000 mg/kg of lead and 500 mg/kg of cadmium concentration levels. An observational comparative analysis in the cadmium concentrations showed a higher level of tolerant bacteria in the *Spartina alterniflora* soils than in the soils of the *Phragmites australis.* This was also identical in observations made for the abundance of lead tolerant bacteria found in both soils. For both cadmium and lead concentrations, each level showed a decrease in the numbers of colonies as the concentration of the heavy metal increases. Thus, the higher the concentration of cadmium or lead the lower the ability of some bacteria to tolerate the level of the heavy metal being exposed to.

Colonies were counted as Colony Forming Units (CFU) CFU/ 10 grams of soil = Number of Colonies X 0.1X 10 as shown in (Tables 4.5-4.8 and Figure 4.5 and 4.6).

Table 4.5. Colony forming units for cadmium tolerant bacteria in *Spartina* soils.

CFU/10gms of soil	Cadmium concentrations (mg/kg)				
	100	200	300	400	500
Spartina alterniflora					
Replica #1	200	150	90	75	23
Replica #2	144	100	96	30	28
Replica #3	250	78	38	33	30
Average CFU	198	109	75	45	28

Table 4.6. Colony forming units for cadmium tolerant bacteria in *Phragmites* soils.

CFU/10gms of Soil	Cadmium concentrations (mg/kg)				
	100	200	300	400	500
Phragmites australis					
Replica #1	77	99	68	25	20
Replica #2	135	70	35	25	10
Replica #3	145	40	35	25	14
Average CFU	119	70	43	28	15

Table 4.7. Colony forming units for lead tolerant bacteria in *Spartina* soils.

CFU/10gms of soil	Lead concentrations (mg/kg)				
	2100	2200	2300	2400	2500
Spartina alterniflora					
Replica #1	275	265	200	150	48
Replica #2	270	265	190	100	55
Replica #3	270	261	186	85	65
Average CFU	271.6	263.6	192	111.6	56

Table 4.8. Colony forming units for lead tolerant bacteria in *Phragmites* soils.

CFU/10gms of Soil	Lead concentrations (mg/kg)				
	2100	2200	2300	2400	2500
Phragmites australis					
Replica #1	200	165	150	100	38
Replica #2	196	163	113	86	30
Replica #3	190	163	108	53	70
Average CFU	195.3	163.6	123.6	79.6	46

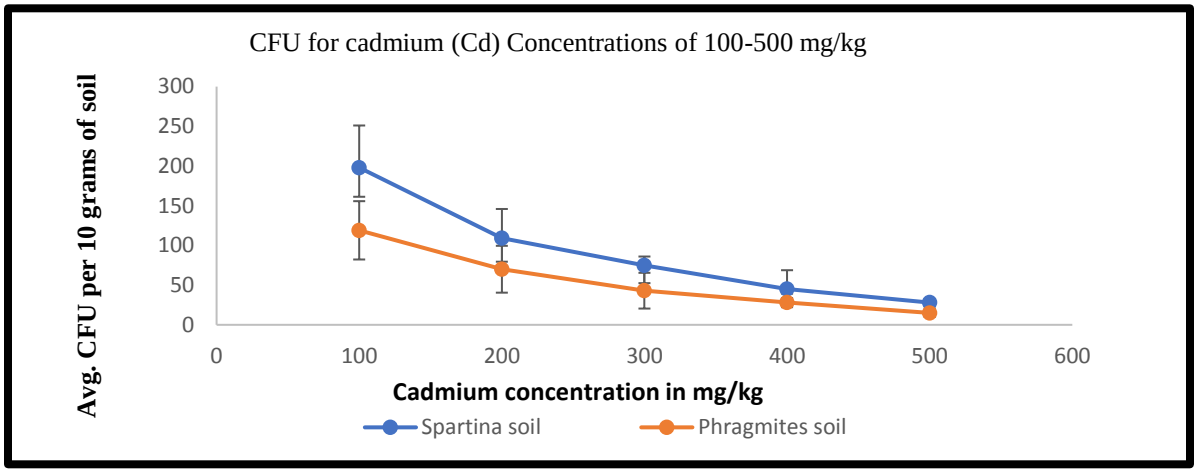


Figure 4.5. Comparison of cadmium tolerant bacteria from *Spartina alterniflora* and *Phragmites australis* soils.

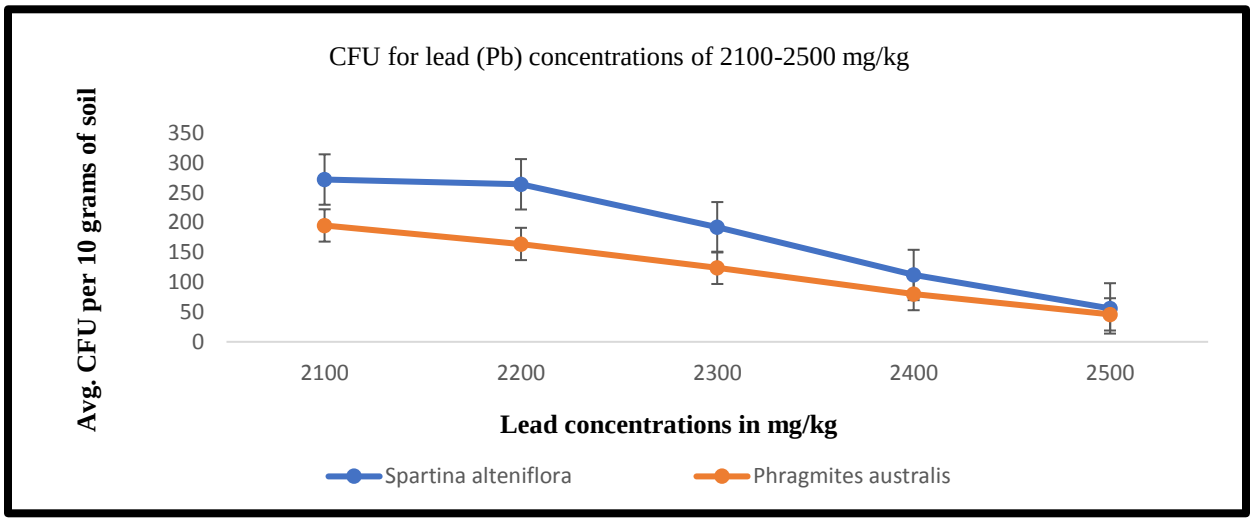


Figure 4.6. Comparison of lead tolerant bacteria from *Spartina alterniflora* and *Phragmites australis* soils.

Plates inoculated initially with lead concentrations of 100 to 1000 mg/kg grew a lot of colonies and were uncountable. Therefore, LB plates were made up 1600 mg/kg to assess tolerance of bacteria in both *Spartina* and *Phragmites* soils. Uncountable numbers of colonies were still observed on LB plates with concentration of 1600 mg/kg. Concentration levels for lead were again increased to 2000 mg/kg for continued observation of lead tolerant bacteria.

The results continued to produce uncountable colonies and hence the need to increase the levels of concentration. Conclusive results showed the ability for these bacteria to grow up to 2500

mg/kg, which demonstrated well isolated colonies within the countable range. Lead tolerant bacteria also showed a greater presence in *Spartina* soils at each concentration level when compared to *Phragmites* soils.

4.1.2 Molecular analysis results

4.1.3 DNA results

Nano drop results for soil genomic DNA at 260/280 ranged from 1.6 to 2.5 and 260/230 values were 2.0 to 2.3. This indicates that the isolated DNA was pure of protein and other contaminants displaying good quality DNA (Figure 4.7).

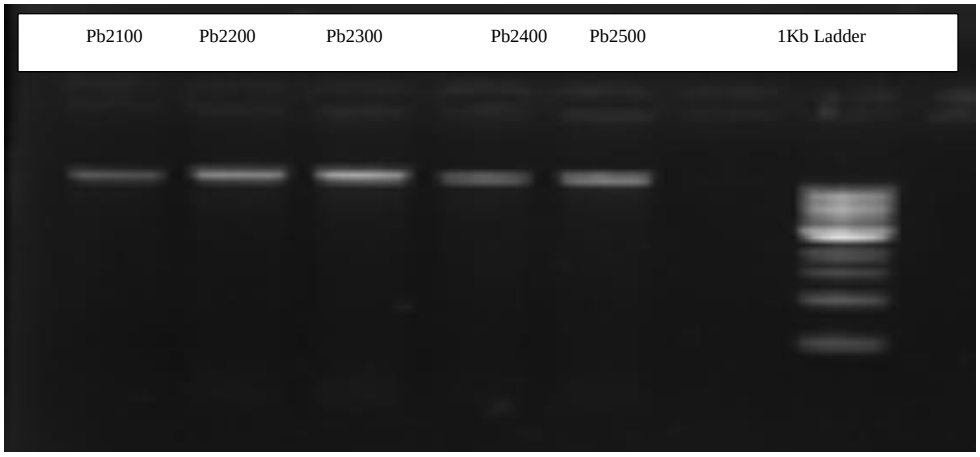


Figure 4.7. Genomic DNA of heavy metal tolerant bacteria on 1% Agarose gel.

4.1.4 PCR results

PCR results with the 16S rDNA universal primers (27F and 1492R) showed the presence of heavy metal tolerant bacteria in soil samples *(Spartina* and *Phragmites*) with a product size of approximately 1.5 kb (Figures 4.8a, 4.8b and 4.8c).

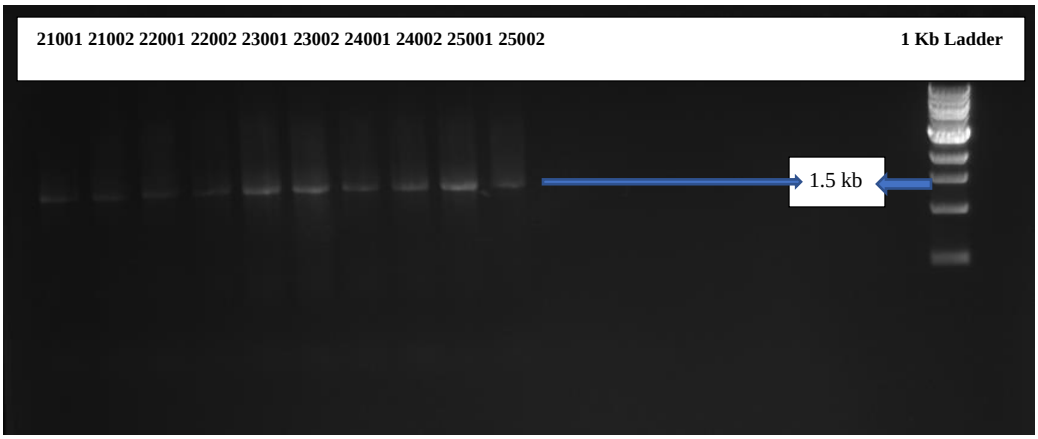


Figure 4.8a. Amplification of genomic DNA isolated from Pb tolerant bacteria at different (2100-2500 mg/kg) concentrations in *Spartina* soils.

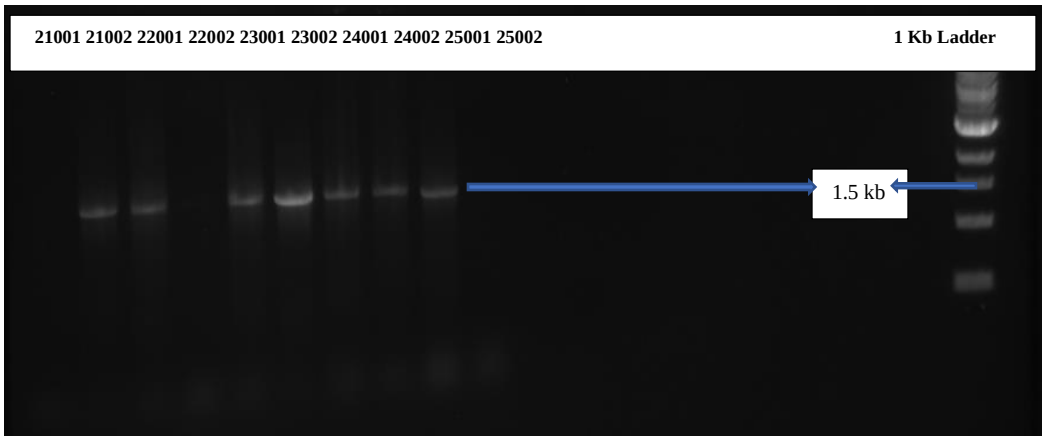


Figure 4.8b. Amplification of genomic DNA isolated from Pb tolerant bacteria at different (2100-2500 mg/kg) concentrations in *Phragmites* soils.

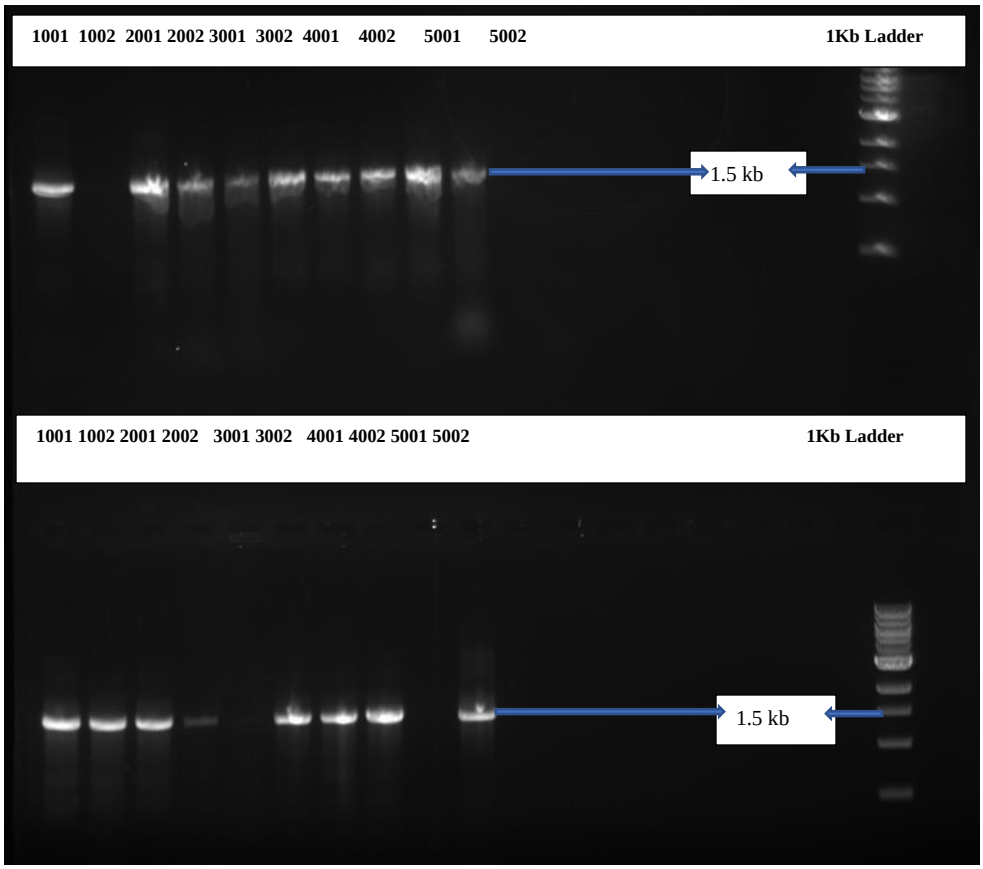


Figure 4.8c. Amplification of genomic DNA isolated from Cd tolerant bacteria at different (100-500 mg/kg) concentrations in *Spartina* (top) and *Phragmites* (bottom) soils

4.1.5 Sequencing results

Basic Local Administration Tool (BLAST) used for analysis from NCBI with 16s gene database as a reference showed different strains of cadmium and lead tolerant bacteria that were isolated (Tables 4.9 and 4.10 respectively). Most of the bacteria identified are 97% similar to cadmium tolerant *Bacillus cereus* and 98% similar to lead tolerant *Enterobacter Sp*. (Figures 4.9 and 4.10 respectively).

Sequences for the cadmium tolerant bacteria isolated from the marsh soil samples were assessed on September 7th, 2017 and the accession ID is given in the table below.

Table 4.9. Identification of different cadmium tolerant bacterial strains isolated from marsh soils samples.

Sample ID	Sample ID	Accession ID
cd_ph_100-1.27F	*Bacillus thuringiensis* strain B16 16S ribosomal RNA gene, partial sequence	gi|1159676871|KX977387.1
cd_ph_100-2.27F	*Bacillus cereus* strain Y1 16S ribosomal RNA gene, partial sequence	gi|442558065|KC247316.1
cd_ph_100-5.27F	*Bacillus* sp. P1(2013) 16S ribosomal RNA gene, partial sequence	gi|472456042|KC701469.1
cd_ph_100-6.27F	*Photobacterium ganghwense* strain SX1 16S ribosomal RNA gene, partial sequence	gi|384563699|JQ394838.1
cd_ph_100-6_#1.27F	*Bacillus thuringiensis* strain 9 16S ribosomal RNA gene, partial sequence	gi|494594994|KC870057.1
cd_ph_200.27F_	*Bacillus cereus* strain BM2 16S ribosomal RNA gene, partial sequence	gi|1159842943|KY773607.1
cd_ph_200-1_#2.27F	*Pantoea* sp. F10-PCAi-T3P21 16S ribosomal RNA gene, partial sequence	gi|358365190|JN853250.1
cd_ph_300-1.27F	*Citrobacter youngae* strain ZF2 16S ribosomal RNA gene, partial sequence	gi|1200762100|KX639818.1
cd_ph_300-2_#1.27F	*Citrobacter freundii* partial 16S rRNA gene, strain TRS1-B4	gi|300393901|FN997639.1
cd_ph_300-2_#2.27	*Citrobacter freundii* strain JX3-1-1 16S ribosomal RNA gene, partial sequence	gi|1236049007|MF716709.1
cd_ph_300-3.27F	*Bacillus cereus* strain H3 16S ribosomal RNA gene, partial sequence	gi|451964224|KC441784.1
cd_ph_400_#1.27F	*Pantoea* sp. YY9 16S ribosomal RNA gene, partial sequence	gi|1028916021|KU298560.1
cd_ph_400_#2.27F	*Citrobacter freundii* strain S3-12 16S ribosomal RNA gene, partial sequence	gi|451935970|KC210829.1
cd_ph_400-1.27F	*Citrobacter freundii* strain C12 16S ribosomal RNA gene, partial sequence	gi|675296543|KM222618.1
cd_ph_400-2.27F	Unknown sequence	
cd_ph_400-3.27F	*Citrobacter* sp. NCCP-837 gene for 16S ribosomal RNA, partial sequence	gi|645910646|AB938205.1
cd_ph_500.27F	*Enterobacter* sp. strain IMBL10 16S ribosomal RNA gene, partial sequence	gi|1210467549|KY937912.1
cd_ph_500-1.27F	*Pantoea agglomerans* strain T224 16S ribosomal RNA gene, partial sequence	gi|479284716|KC764985.1
cd_ph_500-1.27F	*Bacillus* sp. DYJL8 16S ribosomal RNA gene, partial sequence	gi|313292094|HQ317151.1
cd_ph_500-1_#1.27F	*Enterobacter cloacae* strain AceB-2 16S ribosomal RNA gene, partial sequence	gi|239505185|FJ605378.1
cd_ph_500-1_#2.27F	*Enterobacter* sp. strain FA1-153 16S ribosomal RNA gene, partial sequence	gi|1151331579|KY476169.1
cd_ph_500-2.27F	*Bacillus cereus* strain 0083 16S ribosomal RNA gene, partial sequence	gi|745739503|KP236219.1
cd_ph_500-3.27F	*Bacillus* sp. NOB4 16S ribosomal RNA gene, partial sequence	gi|158530235|EU232724.1
cd_ph_500-5.27F	*Bacillus cereus* strain BM2 16S ribosomal RNA gene, partial sequence	gi|1159842943|KY773607.1

cd_ph_500-6.27F	*Bacillus anthracis* strain WTA-24 16S ribosomal RNA gene, partial sequence	gi|612340525|KJ210673.1
cd_sp_100.27F	*Bacterium fjat-scb-2* 16S ribosomal RNA gene, partial sequence	gi|318069040|HQ873708.
cd_sp_100-2.27F	*Bacillus* sp. strain TC2-30 16S ribosomal RNA gene, partial sequence	gi|1151332107|KY673671.1
cd_sp_100-3.27F	Uncultured bacterium clone S16-31 16S ribosomal RNA gene, partial sequence	gi|1150392820|KY344433.1
cd_sp_100-4.27F	*Enterobacter cloacae* subsp. dissolvens strain 478 16S ribosomal RNA gene, partial sequence	gi|303270787|HM162426.1
cd_sp_200.27F	*Bacillus* sp. PVL04 16S ribosomal RNA gene, partial sequence	gi|557816825|KF648904.1
cd_sp_200-1.27F	Uncultured bacterium clone YCB2011 16S ribosomal RNA gene, partial sequence	gi|452772928|KC463798.1
cd_sp_200-4.27F	*Bacillus cereus* strain GX S-2 16S ribosomal RNA gene, partial sequence	gi|1042745053|KU879246.1
cd_sp_200-5.27F	*Bacillus* sp. WR-15 16S ribosomal RNA gene, partial sequence	gi|1023530408|KU159254.1
cd_sp_300_#1.27F	Uncultured bacterium clone U7 16S ribosomal RNA gene, partial sequence	gi|222092519|FJ538298.1
cd_sp_300_#2.27	*Bacillus cereus* strain x2 16S ribosomal RNA gene, partial sequence	gi|671761216|KJ812209.1
cd_sp_300-1.27F	*Bacillus* sp. BG1-2 16S ribosomal RNA gene, partial sequence	gi|928470778|KP992119.1
cd_sp_300-1_#1.27F	*Bacillus cereus* strain YN01 16S ribosomal RNA gene, partial sequence	gi|675152628|KJ948669.1
cd_sp_300-2.27F	*Escherichia coli* strain BEBJ3 16S ribosomal RNA gene, partial sequence	gi|147886637|EF560787.1
cd_sp_300-3.27F	*Bacillus* sp. strain BC1-19 16S ribosomal RNA gene, partial sequence	gi|1151331967|KY625535.1
cd_sp_300-4.27F	*Shigella dysenteriae* strain TYN 130605 16S ribosomal RNA gene, partial sequence	gi|1043111670|KX162657.1
cd_sp_300-6.27F	Bacterium JP60 16S ribosomal RNA gene, partial sequence	gi|472834871|KC602291.1
cd_sp_400.27F	Uncultured bacterium clone S10-34 16S ribosomal RNA gene, partial sequence	gi|1140239992|KY363837.1
cd_sp_400-1.27F	Uncultured Bacillus sp. clone N38 16S ribosomal RNA gene, partial sequence	gi|383280638|JQ622566.1
cd_sp_500.27F	*Bacillus cereus* strain BM2 16S ribosomal RNA gene, partial sequence	gi|1159842943|KY773607.1
cd_sp_500-2.27F	*Bacillus cereus* strain DZ4 16S ribosomal RNA gene, partial sequence	gi|320119951|HQ143564.1
cd_sp_500-3.27F	*Bacillus cereus* strain B105 16S ribosomal RNA gene, partial sequence	gi|924658862|KP966475.1
cd_sp_500-6.27F	*Bacillus cereus* strain RJ06 16S ribosomal RNA gene, partial sequence	gi|992324593|KT718054.1

cd: Cadmium
ph: Phragmites
sp: Spartina

Sequences for the lead tolerant bacteria isolated from the marsh soil samples were assessed on July 20th, 2017 and the accession ID is given in the table below.

Table 4.10. Identification of different lead tolerant bacterial strains isolated from marsh soils samples.

Sample ID	Description	Accession ID
pbsp2-1.27F	*Escherichia coli* strain TYN 130606 16S ribosomal RNA gene, partial sequence	gi|1043111671|KX162658.1
pbsp2-2.27F	*Shigella* sp. CH-43 16S ribosomal RNA gene, partial sequence	gi|928241861|KR148992.1
pbsp2-3.27F	Unknown sequence	
pbsp1-1.27F	*Escherichia coli* strain TYN 130606 16S ribosomal RNA gene, partial sequence	KX162658.1
pbsp1-2.27F_010	Bacterium OC8(2011) 16S ribosomal RNA gene, partial sequence	gi|320148365|HQ179025.1
pbsp1-3.27F	*Escherichia coli* strain TYN 130606 16S ribosomal RNA gene, partial sequence	gi|1043111671|KX162658.1
pbsp3-1.27F	*Citrobacte*r sp. 17.6 KSS partial 16S rRNA gene, strain 17.6 KSS	gi|377550055|HE575919.1
pbsp3-2.27F	*Escherichia coli* strain RCB249 16S ribosomal RNA gene, partial sequence	gi|927337723|KT260461.1
pbsp3-3.27F	Unknown sequence	
pbsp3-4.27F	Unknown sequence	
pbph1-1.27F	*Enterobacter* sp. CAB 1099 16S ribosomal RNA gene, partial sequence	gi|607837692|KJ194592.1
pbph1-2.27F	*Enterobacter cloacae* strain RCB980 16S ribosomal RNA gene, partial sequence	gi|927338454|KT261192.1
pbph1-3.27F	*Enterobacter aerogene*s strain KNUC5001 16S ribosomal RNA gene, partial sequence	gi|383793416|JQ682628.1
pbph2-1.27F	Unknown sequence	
pbph2-2.27F	*Citrobacte*r sp. 519C4 16S ribosomal RNA gene, partial sequence	gi|1002096754|KT764985.1
pbph3-1.27F	*Enterococcus faecalis* strain 18g 16S ribosomal RNA gene, partial sequence	gi|725611150|KM392091.1
pbph3-2.27F	*Klebsiella pneumoniae* strain QLR-2 16S ribosomal RNA gene, partial sequence	gi|690377123|KM096434.1
pbph1-4.27F	Unknown sequence	
pbph3-3.27F	*Citrobacter* sp. 519C4 16S ribosomal RNA gene, partial sequence	gi|1002096754|KT764985.1
pbph3-4.27F	*Enterobacter cloacae* strain AA4, complete genome	gi|1158824263|CP018785.1

cd: Cadmium
ph: Phragmites
sp: Spartina

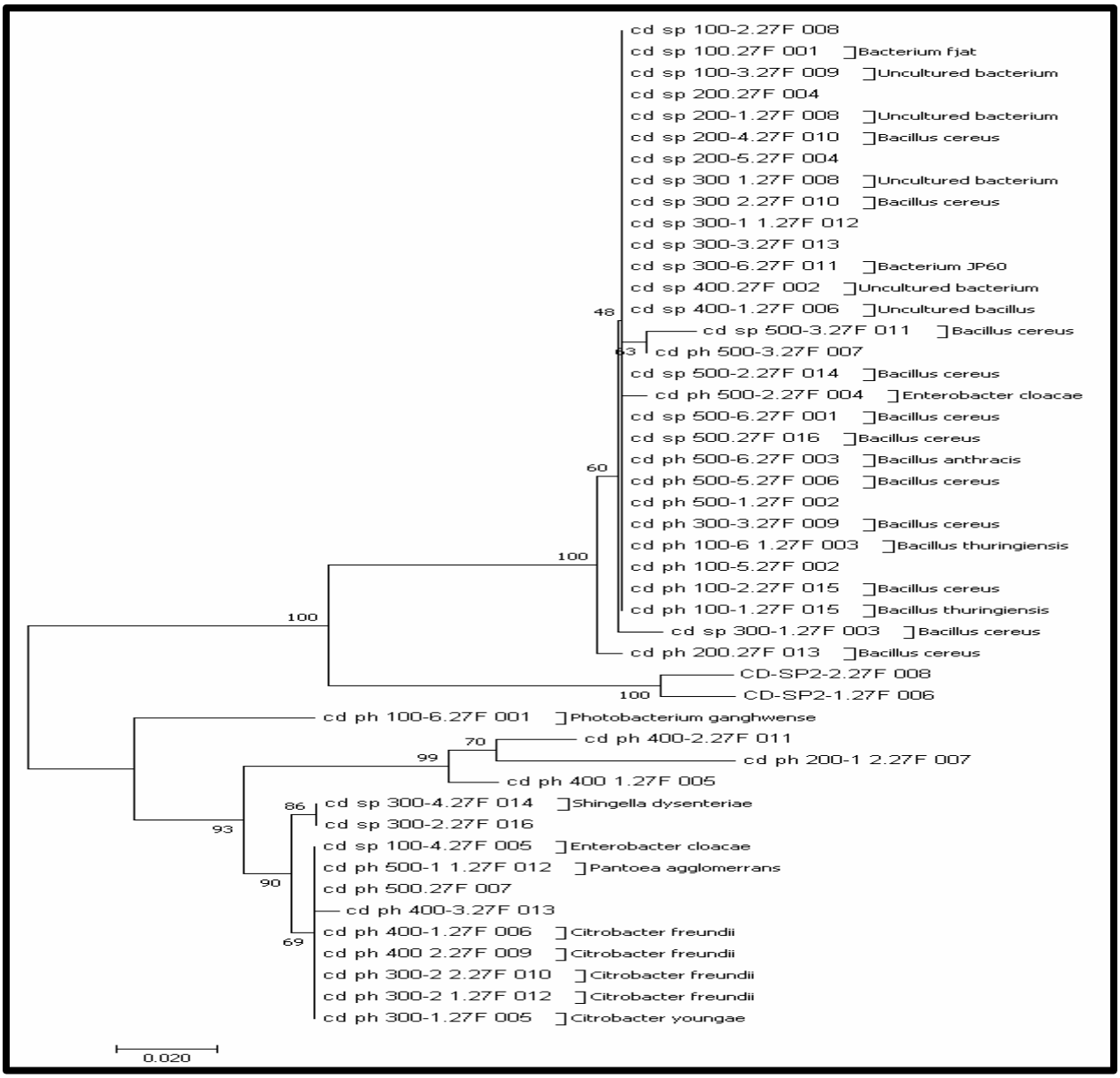


Figure 4.9. Neighbor joining tree for cadmium tolerant bacteria using MEGA 4.
Cadmium (cd), *Phragmites* (ph), *Spartina* (sp)

Cadmium tolerant bacteria identified were mostly similar (97%) to the *Bacillus cereus* bacteria.

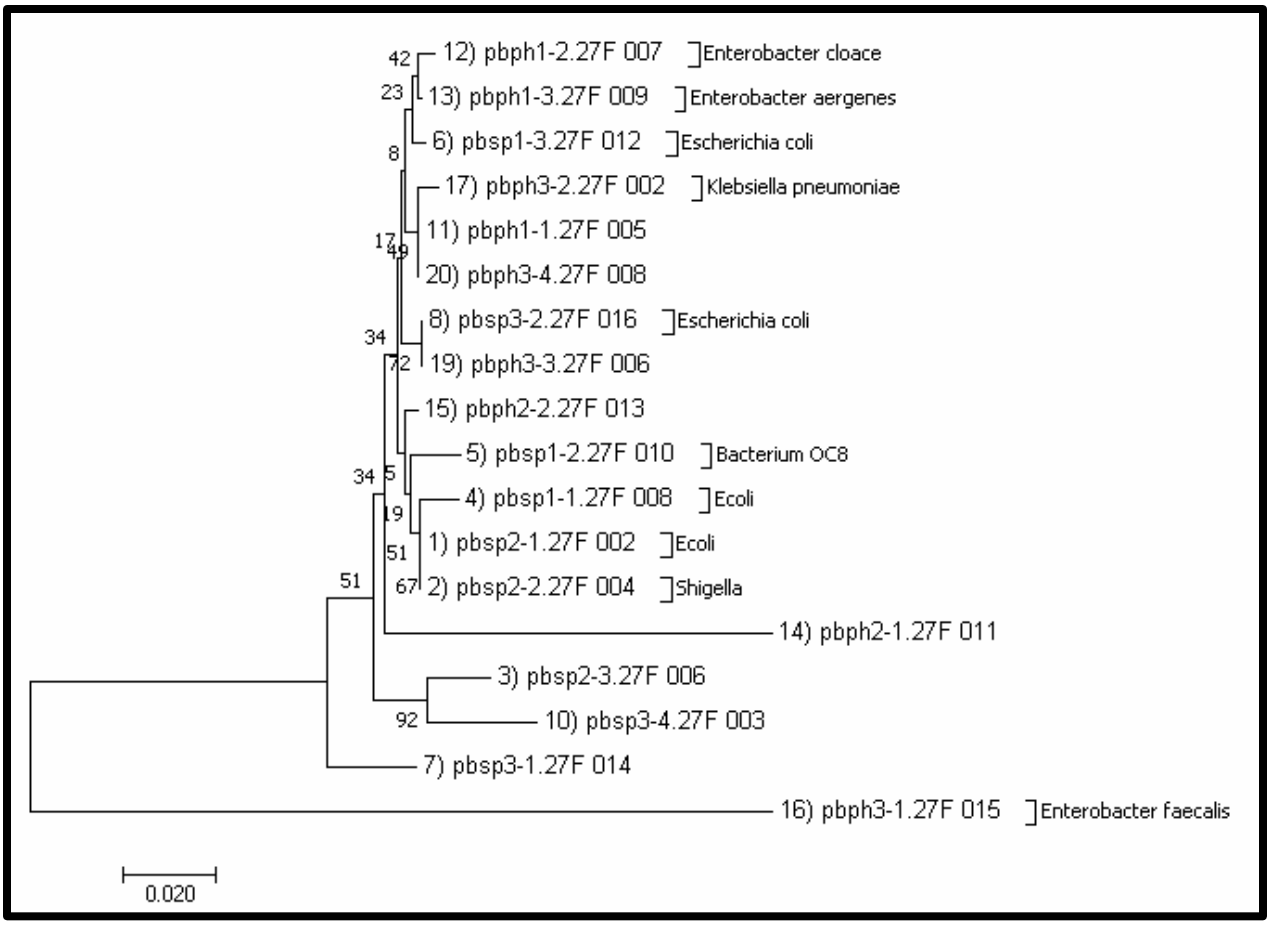


Figure 4.10. Neighbor joining tree for lead tolerant bacteria using MEGA 4.0.
Lead (pb), *Phragmites* (ph), *Spartina* (sp)

Lead tolerant bacteria identified were mostly similar (98%) to the *Enterobacter* Sp.

4.1.6 Statistical analysis results

4.1.7 Cadmium tolerant bacteria analysis

Bacterial growth (colony forming units (CFU)) data was exported into the SPSS program and generated a result of ($p \leq 0.01$), as the mean difference is significant at a level of ($p < 0.05$). This indicates that there is a significant difference in the amount of cadmium tolerant bacteria between sites (Table 4.1.1). Significant difference was observed between the numbers of cadmium tolerant bacteria growing in both soil types. Pairwise comparison estimated the marginal means based on the original scale of the dependent variable, bacterial abundance. Significant difference ($P \leq 0.01$) was observed among the numbers of bacterial populations grown in *Spartina* soils at 100 mg/kg compared to the bacterial numbers observed at 200-500 mg/kg in *Phragmites* soils.

Bacterial growth in *Spartina* soils with cadmium concentration of 200 mg/kg was also significantly different from the bacterial growth in *Phragmites* soil with concentration of 100, 300, 400 and 500 mg/kg.

There was no difference observed for the number of bacteria growing in *Spartina* soils with cadmium concentrations of 300 mg/kg to that of *Phragmites* soil with 400 mg/kg cadmium concentration ($P = 0.12$). The abundance of bacteria present in this region (300-400 mg/kg) may have been a result of the tolerance level being similar, thus the abundance levels in both soil types (*Spartina* and *Phragmites*) could be the same at cadmium concentration of 300-400 mg/kg. Bacteria grown at cadmium concentration of 400 mg/kg in *Spartina* soils showed differences compared to cadmium concentration of 500 mg/kg in *Phragmites* soils ($P = 0.27$) as shown in (Table 4.1.2).

Table 4.1.1. General linear model for test effects of Cd tolerant bacterial abundance observed within the two soil types.

Source	Wald Chi-Square	df	Sig.
(Intercept)	124.948	1	0.01
Soil type	18.332	2	0.01
Cad	120.226	4	0.01
Dependent Variable: Bacterial abundance			
Model: (Intercept), Species, Cad, Species * Cad			

Table 4.1.2. Pairwise comparison for bacterial abundances at varying concentrations of cadmium per soil type.

Cad/Sp mg/kg	Cad/Ph mg/kg	Mean Difference (Sp-Ph)	Std. Error	df	Sig.	95% Wald Confidence Interval for Difference	
						Lower	Upper
100	200	69.0000a	13.68806	1	0.000	42.1719	95.8281
	300	99.8333a	13.68806	1	0.000	73.0052	126.6614
	400	121.7222a	14.42848	1	0.000	93.4429	150.0015
	500	137.3333a	13.68806	1	0.000	110.5052	164.1614
200	100	-69.0000a	13.68806	1	0.000	-95.8281	-42.1719
	300	30.8333a	13.68806	1	0.024	4.0052	57.6614
	400	52.7222a	14.42848	1	0.000	24.4429	81.0015
	500	68.3333a	13.68806	1	0.000	41.5052	95.1614
300	100	-99.8333a	13.68806	1	0.000	-126.6614	-73.0052
	200	-30.8333a	13.68806	1	0.024	-57.6614	-4.0052
	400	21.8889	14.42848	1	0.129	-6.3904	50.1682
	500	37.5000a	13.68806	1	0.006	10.6719	64.3281
400	100	-121.7222a	14.42848	1	0.000	-150.0015	-93.4429
	200	-52.7222a	14.42848	1	0.000	-81.0015	-24.4429
	300	-21.8889	14.42848	1	0.129	-50.1682	6.3904
	500	15.6111	14.42848	1	0.279	-12.6682	43.8904
500	100	-137.3333a	13.68806	1	0.000	-164.1614	-110.5052
	200	-68.3333a	13.68806	1	0.000	-95.1614	-41.5052
	300	-37.5000a	13.68806	1	0.006	-64.3281	-10.6719
	400	-15.6111	14.42848	1	0.279	-43.8904	12.6682

4.1.7 Lead tolerant bacteria analysis

A generated result of ($p \leq 0.01$), when ($p < 0.05$) indicates that there is a significant difference in the amount of lead tolerant bacteria between sites (Table 4.1.3). Pairwise comparison estimated the marginal means based on the original scale of the dependent variable "bacterial abundance". Significant difference ($P \leq 0.01$) was observed among bacteria grown in *Spartina* soils at 2100 mg/kg compared to bacteria grown at 2300-2500 mg/kg in *Phragmites* soils. Bacterial numbers in *Spartina* soils with cadmium concentration of 2200 mg/kg was also significantly different from the number of bacteria observed in *Phragmites* soil with a concentration of 2300, 2400 and 2500 mg/kg. Comparative analysis showed that there was no significant difference between bacterial populations observed in *Spartina* soils with lead concentration of 2100 mg/kg and *Phragmites* soils with 2200 mg/kg of lead. This too could be as result of bacteria in these regions (2100 mg/kg and 2200 mg/kg) having similar tolerant mechanisms. This may suggest that in both soil types, the bacteria isolated from 2100 mg/kg were also withstanding 2200 mg/kg concentrations of lead with similar tolerance capabilities (Table 4.1.4).

Table 4.1.3. General linear model for test effects of Pb tolerant bacterial abundance within the two soil types.

Source	df	Mean square	F	Sig.
Corrected model	5	33538.200	59.458	0.01
Intercept	1	678003.333	1201.986	0.01
Soil type	1	24653.333	43.706	0.01
Pb	4	35759.417	63.395	0.01

Table 4.1.4. Pairwise comparisons for bacterial abundance of varying concentrations of lead per soil type.

Pb/Sp mg/kg	Pb/Ph mg/kg	Mean difference (Sp-Ph)	Std. error	Sig.[b]	95% Confidence interval for difference[b]	
					Lower bound	Upper bound
2100	2200	19.833	13.712	0.161	-8.467	48.134
	2300	75.667*	13.712	0.000	47.366	103.967
	2400	137.833*	13.712	0.000	109.533	166.134
	2500	182.500*	13.712	0.000	154.200	210.800
2200	2100	-19.833	13.712	0.161	-48.134	8.467
	2300	55.833*	13.712	0.000	27.533	84.134
	2400	118.000*	13.712	0.000	89.700	146.300
	2500	162.667*	13.712	0.000	134.366	190.967
2300	2100	-75.667*	13.712	0.000	-103.967	-47.366
	2200	-55.833*	13.712	0.000	-84.134	-27.533
	2400	62.167*	13.712	0.000	33.866	90.467
	2500	106.833*	13.712	0.000	78.533	135.134
2400	2100	-137.833*	13.712	0.000	-166.134	-109.533
	2200	-118.000*	13.712	0.000	-146.300	-89.700
	2300	-62.167*	13.712	0.000	-90.467	-33.866
	2500	44.667*	13.712	0.003	16.366	72.967
2500	2100	-182.500*	13.712	0.000	-210.800	-154.200
	2200	-162.667*	13.712	0.000	-190.967	-134.366
	2300	-106.833*	13.712	0.000	-135.134	-78.533
	2400	-44.667*	13.712	0.003	-72.967	-16.366

The presence of heavy metal tolerant bacteria (lead and cadmium) were found to be abundant in these marsh soils. The presence of these bacteria in the BBC marsh soils have been found to be tolerable to cadmium levels of up to 500 mg/kg and lead levels up to 2500 mg/kg (Tables 5-8). Most of the bacteria identified based on sequencing analysis are 97% similar to the cadmium tolerant aerobic bacteria, *Bacillus cereus*. The anaerobic *Clostridium* bacteria were also among the cadmium tolerant species identified. Analysis also identified *Enterobacter Sp.* to be 98% similar to most of the lead tolerant bacteria (Figures 4.9 and 4.10 respectively). Results also showed that Soils from areas dominated by the *Spartina alterniflora* had a greater presence of bacteria tolerant to high concentrations of cadmium and lead when compared to the soils from the areas dominated by *Phragmites australis* (Figures 4.5 and 4.6 respectively). Pairwise comparison proved that there were significant differences in the abundance of different heavy metal tolerant bacteria present in soils dominated by *Spartina* compared to those present in soils dominated by the *Phragmites* (Tables 4.1.2 and 4.1.4). Significance level was set at $p<0.05$ and a value of ($p\leq0.01$) was generated, resulting in significant differences in the abundance of bacteria present at each site (Tables 4.1.1 and 4.1.3). Results may also suggest that the bacteria abundance in soils with cadmium concentrations of 300-400 mg/kg may be similar. There were similarities in the abundances of bacteria that can tolerate lead concentrations of 2100-2200 mg/kg in both soil types (*Spartina* and *Phragmites*).

Less presence of the cadmium and lead tolerant bacteria found in *Phragmites* dominated soils may be attributed to the ability of this grass species to effectively remove pollutants from the soil through phytoremediation. Ghassemzaddeha et al. (2008) have also reported the use *Phragmites* as a phytoremediation agent against various heavy metals.

Studies carried out by Komori et al. (2005) concluded that removal of heavy metals can be achieved with the use of microorganisms, plants or a combination of both organisms.

Generated results concluded that there were higher abundances of lead tolerant bacteria in these soils than that of cadmium tolerant bacteria. Higher lead tolerance may be attributed to the contribution from previous human induced activities that caused contamination (EPA, 2016). This is similar to a report from Ahmed et al. (2005) which stated that bacteria, when exposed to high levels of heavy metals in their environment have adapted to this stress by developing various mechanisms for resistance. Based on these results the hypothesis "*Spartina* and *Phragmites* soils will have the same abundance of lead and cadmium tolerant bacteria" has been rejected. It is evident that heavy metal tolerant bacteria are present in the marsh soils. These bacteria are present in in varying numbers according to sites and type of heavy metal. However, based on their tolerance levels they may be effectively applied in bio-removal/bioremediation assays. This will undoubtedly contribute to significant environmental sustainability measures.

4.2 Experiment#2: Application of Marsh Mycorrhizae to promote plant growth and aid plant development under salt stress

4.2.1 Pre-Greenhouse Study

4.2.2 Acid Fuchsin root staining method results

VAM fungal spores were identified in the roots of the *Spartina alterniflora* using the Acid Fuchsin root staining method (Figures 4.1.1a and 4.1.1b).

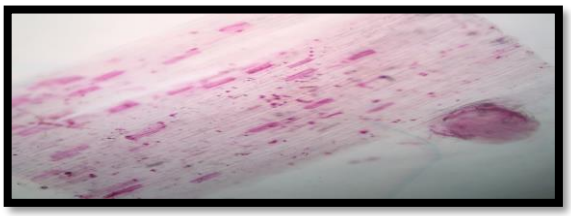


Figure. 4.1.1a

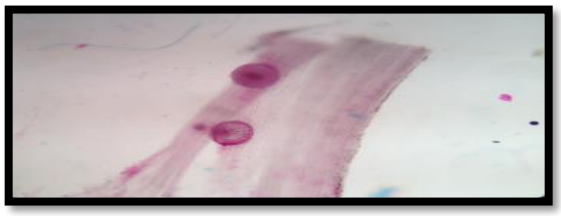


Figure. 4.1.1b

Figures 4.1.1a and 4.1.1b. Purple colored areas indicate VAM spores.

4.2.3 Wet Sieve method result

VAM spores were isolated and identified from the marsh soils using the Wet Sieve method. Further confirmation of these spores was made using the light microscope under magnification of 40x and oil immersion 100x (Figures.4.1.2a, 4.1.2b and 4.1.2c).

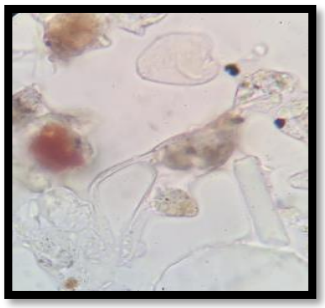


Figure. 4.1.2a

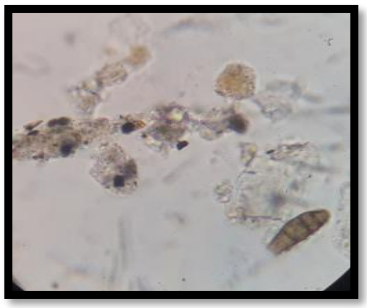


Figure. 4.1.2b

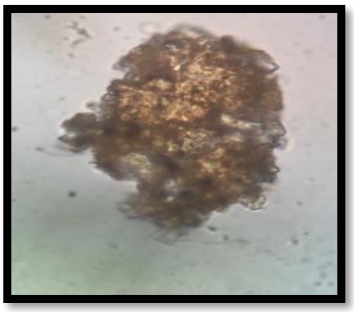


Figure. 4.1.2c

Figures 12a, 12b and 12c. Observation of fungal spores including VAM under 40 and oil immersion (100x)..

4.2.4 Molecular results

4.2.5 DNA results

Genomic DNA was extracted from the root samples. The tight bands without streaks displayed that there is good quality of the DNA without any degradation (Figures 4.1.3).

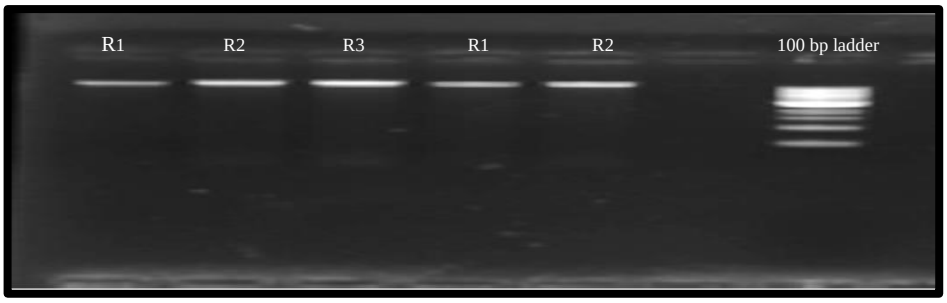
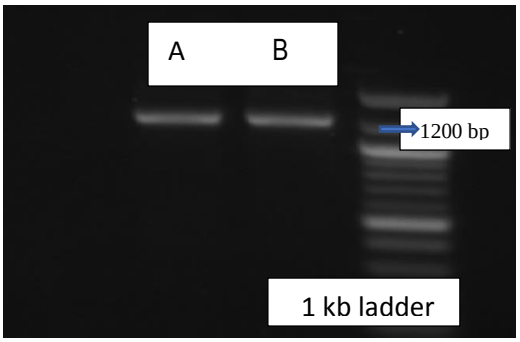


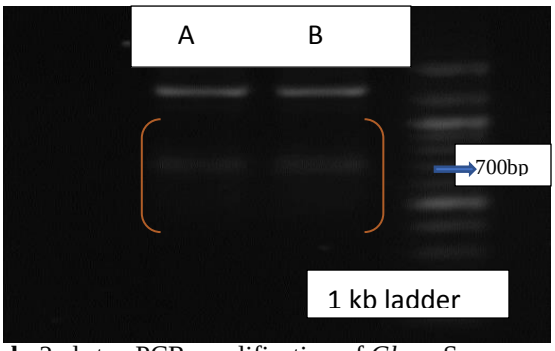
Figure 4.1.3. Genomic DNA of roots on 1% Agarose gel.

4.2.6 PCR results

PCR results showed the presence of fungi in the roots samples with a product size of 1200 bp. A positive band with VAM specific primers identified the presence of the VAM species from Glomus group with a product size of 700 bp (Figures 4.1.4a and 4.1.4b respectively).



a. 1st Step of PCR amplification of VAM



b. 2nd step PCR amplification of *Glom.* Sp.

Figures 4.1.4a and 4.1.4b. Identification of VAM fungi in marsh grass roots by nested PCR on a 1% agarose gel.

4.3 Greenhouse Experiment

4.3.1 Germination results

Evidence of germination was observed after five days using the culture dish method. At approximately eight days into the germination process, over 80% of germinated seeds was recorded.

4.3.2 Seedlings transplantation and growth

All pots were successfully potted using three seedlings each and resulting in a total of 162 healthy seedlings. There was approximately 80% in the growth rate and survival of the seedlings transplanted.

4.3.3 Harvesting and physical measurements

Physical measurements resulted in RL-root length, SL- shoot length and number of leaves showing approximate normality for all treatments. The number of leaves for each selected plant were in the same quantity range among all treatments. Shoot length was also similar in all selected plants and root lengths among all treatments also ranged in the same proximity. However, plants lengths did show moderate differences in measurement among treatments, while variability was observed in root masses resulting in significant differences among treatments. Table 4.1.5 and Figures 4.1.5 through 4.1.9 shows direct comparison for each of the different treatments that were measured in accordance to the specific characteristic for these plants. Plant characteristics such as root length, shoot length and number of leaves may be similar due to various factors such as seed type, which may affect the plant length and even leaf production of the plant.

The growth environment, such as greenhouse potted versus the natural environment for *Spartina alterniflora* could have an effect on all these characteristics. Containerization and Pot size may also have an effect on the length of the roots as they are restricted with limited area for growth and expansion.

Tables 4.1.5. Average measurements for *Spartina alterniflora* physical characteristics of randomly selected plants from blocks A, B and C. **As: Autoclaved soil, UAs: Unautoclaved soil, S: Salt; PL: Plant length, RL: Root Length, RM: Root Mass, SL: Shoot Length.**

BLOCK A					
TREATMENT	**P L (cm)**	**# OF LEAVES**	**R L (cm)**	**R M (g)**	**S L (cm)**
As+VAM+S	67	16	45	4	43
As+VAM	72	18	39	5	37
UAs+VAM+S	79	11	24	3	55
UAs+VAM	75	14	30	5	45
As (Control)	77	12	27	4	51
UAs (Control)	71	14	25	4	46
BLOCK B					
TREATMENT	**P L (cm)**	**# OF LEAVES**	**R L (cm)**	**R M (gm)**	**S L (cm)**
As+VAM+S	74	8	18	3	43
As+VAM	62	11	18	3	44
UAs+VAM+S	87	12	38	2	49
UAs+VAM	82	15	31	25	51
As (Control)	87	19	33	6	53
UAs (Control)	80	11	26	6	54
BLOCK C					
TREATMENT	**P L (cm)**	**# OF LEAVES**	**R L (cm)**	**R M (gm)**	**S L (cm)**
As+VAM+S	80	13	24	5	56
As+VAM	65	17	25	4	40
UAs+VAM+S	86	20	31	4	55
UAs+VAM	66	21	36	7	48
As (Control)	89	19	33	9	56
UAs (Control)	56	17	32	7	51

4.3.4 Statistical analysis

4.3.5 Plant length

At the end of the growth period, the trends in mean plant lengths of the *Spartina alterniflora* showed relatively small differences for the treatment effects (p=0.09) (Table 16). The differences among means were observed for treatments effects of **As+VAM, As (control)** and **UAs+VAM+S** (66.2±5, 84.5±4.5 and 82±4 cm respectively, mean±1 standard error). The Tukey's test for comparison confirmed that there were trends of moderate differences among the treatments effects. However, these differences were not significant (Figure 4.1.5).

4.3.6 Number of leaves

The trend in the differences among means for leaf abundance per plant for each treatment showed no major differences (p=0.5) (Table 4.1.7). Confirmation was given by the Tukey's test for comparison. (Figure 4.1.6).

4.3.7 Root lengths

Generated results for the differences among means of the root lengths for the *Spartina alterniflora* showed no major differences in treatment effects(p=0.6) (Table 4.1.8). The Tukey's test for comparison confirmed that the differences in treatment means were not significant (Figure 4.1.7).

4.3.8 Root masses

The differences among means for treatments effects on root masses were significantly different (p=02) (Table 4.1.9). The mean root masses for the *Spartina alterniflora* were mostly affected by treatments, **As+VAM, As (control) and UAs+VAM+S** (3.9± .7, 7.6± 1.5 and 2.9± .2 grams respectively, mean±1 standard error). The Tukey's test for comparison confirmed that were significant differences among treatment effects on the root masses (Figure 4.1.8).

4.3.9 Shoot lengths

The *Spartina alterniflora* mean shoot lengths after plants were affected with by the different treatments showed no significant differences (p=0.2) (Table 4.20). The Tukey's test for comparison confirmed there were no differences among treatments effect (Figure 4.1.9).

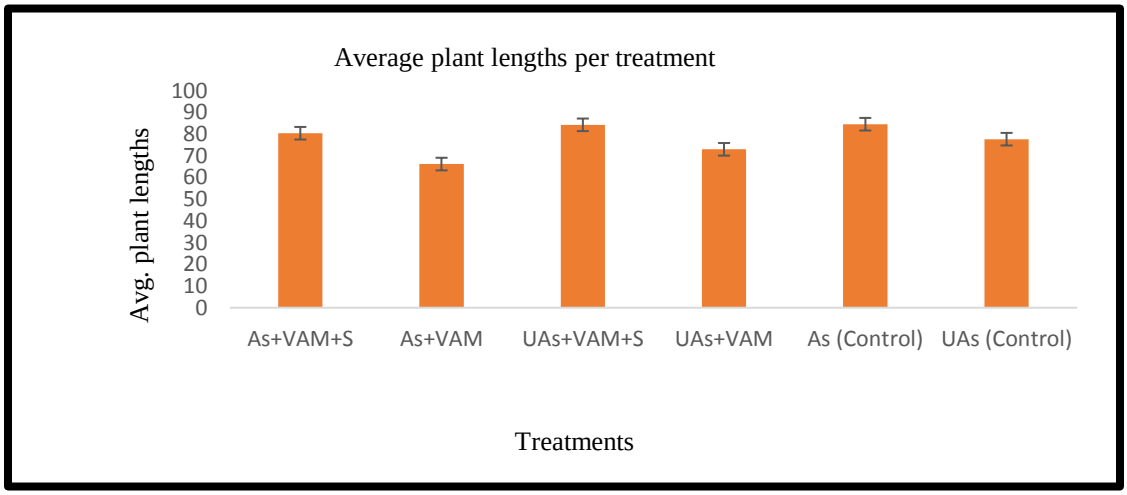


Figure 4.1.5. Average plant lengths of *Spartina alterniflora* responses to treatment effects manipulated by un-autoclaved (UAs) and autoclaved (As) soils with and without VAM roots. (average ±1 standard error).

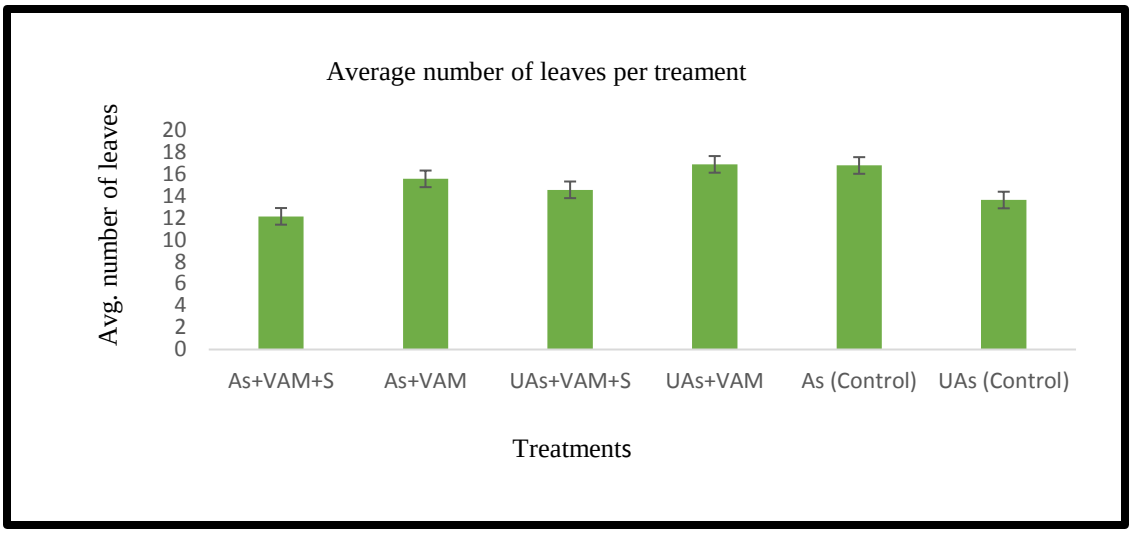


Figure 4.1.6. Average number of leaves of *Spartina alterniflora* responses to treatment effects manipulated by un-autoclaved (UAs) and autoclaved (As) soils with and without VAM roots. (average ±1 standard error).

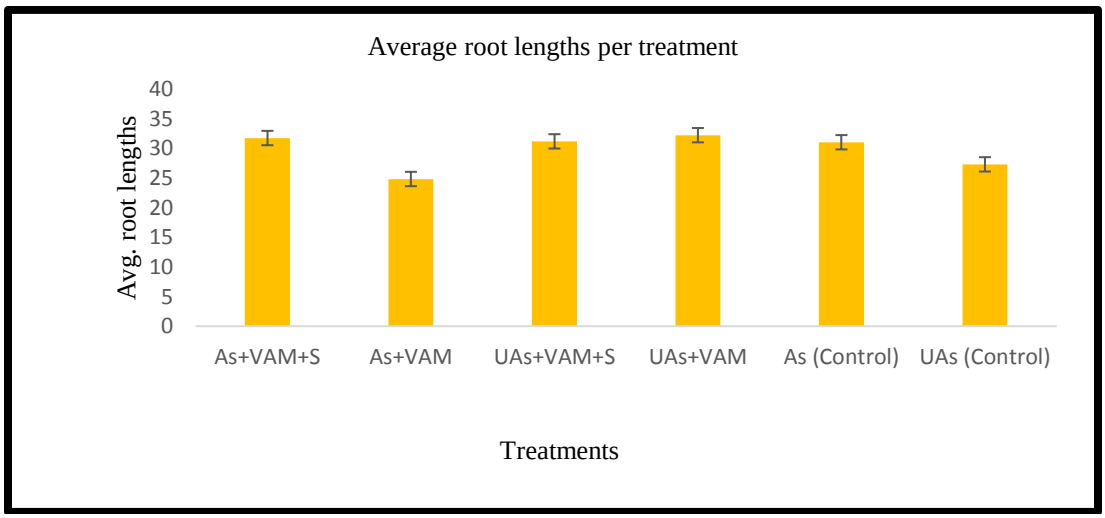


Figure 4.1.7. Average root lengths of *Spartina alterniflora* responses to treatment effects manipulated by un-autoclaved (UAs) and autoclaved (As) soils with and without VAM roots. (average ±1 standard error).

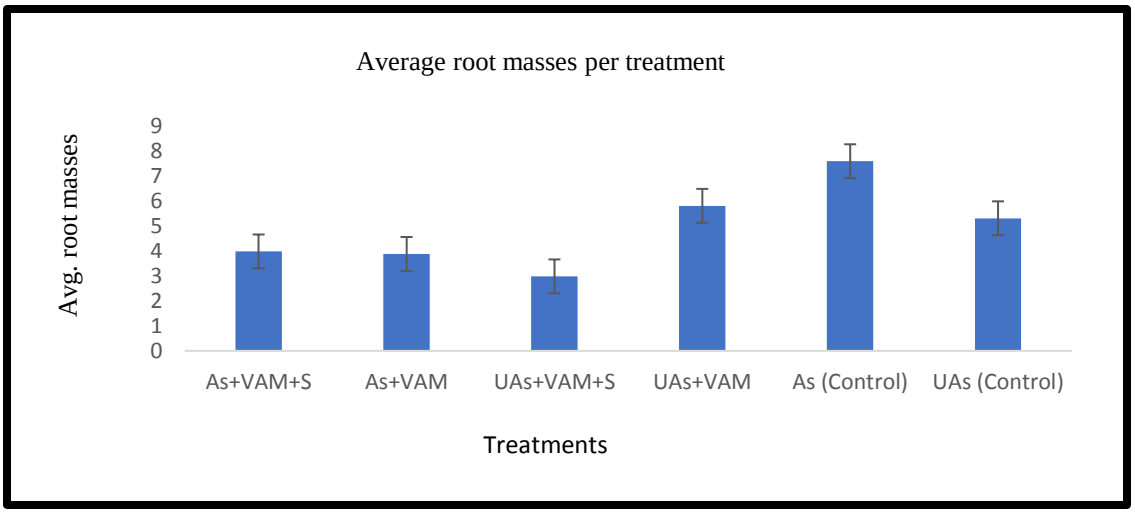


Figure 4.1.8. Average root masses of *Spartina alterniflora* responses to treatment effects manipulated by un-autoclaved (UAs) and autoclaved (As) soils with and without VAM roots. (average ±1 standard error).

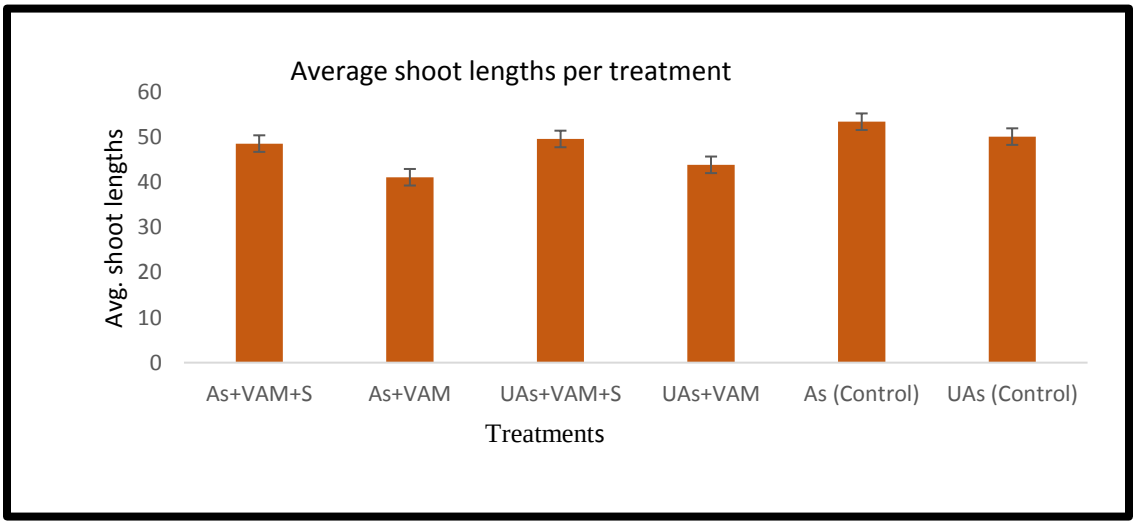


Figure 4.1.9. Average shoot lengths of *Spartina alterniflora* responses to treatment effects manipulated by un-autoclaved (UAs) and autoclaved (As) soils with and without VAM roots. (average ±1 standard error).

Table 4.1.6. One-way ANOVA for treatments effects on *Spartina alterniflora* plant lengths.

Source	df	Mean Square	F	Sig.
Plant characteristics (PL)* treatments	5	446.026	2.044	.091

Table 4.1.7. One-way ANOVA for treatments effects for *Spartina alterniflora* number of leaves.

Source	df	Mean Square	F	Sig.
Plant characteristic (# of L)* treatments	5	26.675	.883	0.501

Table 4.1.8. One-way ANOVA for treatments effects on *Spartina alterniflora* root lengths.

Source	df	Mean Square	F	Sig.
Plant characteristic (RL)* treatments	5	75.099	.761	0.583

Table 4.1.9. One-way ANOVA for treatments effects on *Spartina alterniflora* root masses.

Source	df	Mean Square	F	Sig.
Plant characteristic (RM)* treatments	5	23.541	2.998	0.021

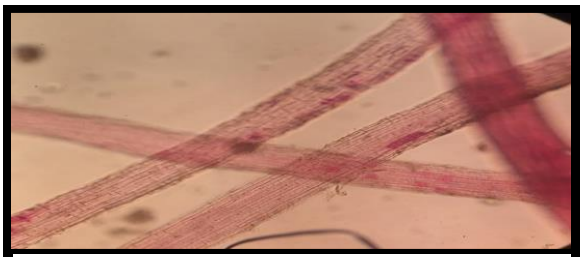
Table 4.20. One-way ANOVA for treatments effects on *Spartina alterniflora* shoot lengths.

Source	df	Mean Square	F	Sig.
Plant characteristic (SL)* treatments	5	177.192	1.521	0.203

4.4 Post-Greenhouse Laboratory results

4.4.1 Root staining

VAM fungi were identified in the *Spartina alterniflora* roots using the acid fuchsin method. These spore and sporangium were further confirmed using the light microscopy with magnification of 40x and 100x (Figures 4.20a and 4.20b).



a. Identification of VAM spores with 40x magnification

b. Identification of VAM spores with 100x magnification

Figures 4.20a and 4.20b. Purple colored areas indicate VAM spores.

4.4.2 Molecular results

4.4.3 DNA results

Genomic DNA was extracted from the root samples. The tight bands of genomic DNA without streaks displayed that there is good quality of the DNA without any degradation (Figure 4.2.1).

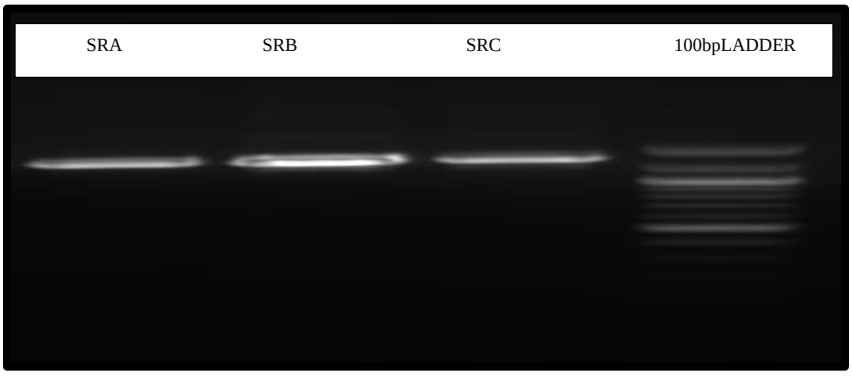


Figure 4.2.1. Genomic DNA of roots from group A, B and C on 1% Agarose gel.

4.4.4 PCR results

PCR results showed the presence of fungi in the roots samples with product sizes ranging from 600 bp to 1200 bp. A positive band with VAM specific primers **GLOM, GIGA, ACAU, ARCH** and **LETC** identified the presence of the VAM species from each group (Figures. 4.2.2a, 4.2.2b, 4.2.2c, 4.2.2d and 4.2.2e).

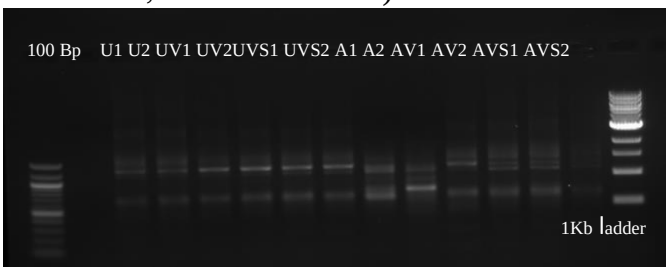


Figure 4.2.2a. VAM specific primer (GlOM1310) identified species from the *Glomus* group.

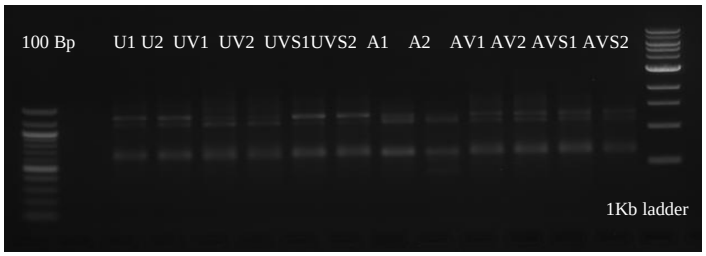


Figure 4.2.2b. VAM specific primer (*GIGA5.8R)* identified species from the *Gigaspora* group.

Figure 4.2.2c. VAM specific primer (ACAU1660) identified species from the *Aculospora* group.

Figure 4.2.2d. VAM specific primer (ARCH1311**)** identified species from the *Archaeospora* group.

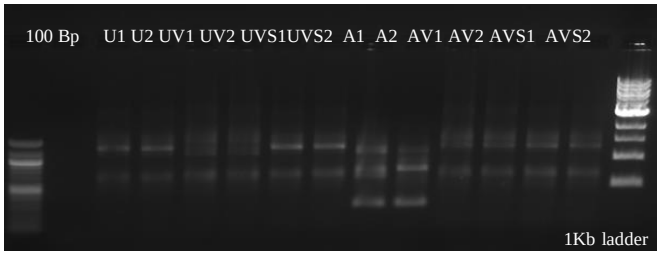


Figure 4.2.2e. VAM specific primer (LETC1670) identified species from the *LETC-Glomus etunicatum group.*

Figures 4.2.2a, b, c, d and e. Identification of VAM fungi in marsh grass roots by nested PCR on 1% Agarose gel.

The current research has identified the presence of VAM fungi belonging to the Glomus, Aculospora, Archaeospora, and Gigaspora groups in marsh roots from the Blackbird Creek as shown in the (Figure. 4.2.3) below.

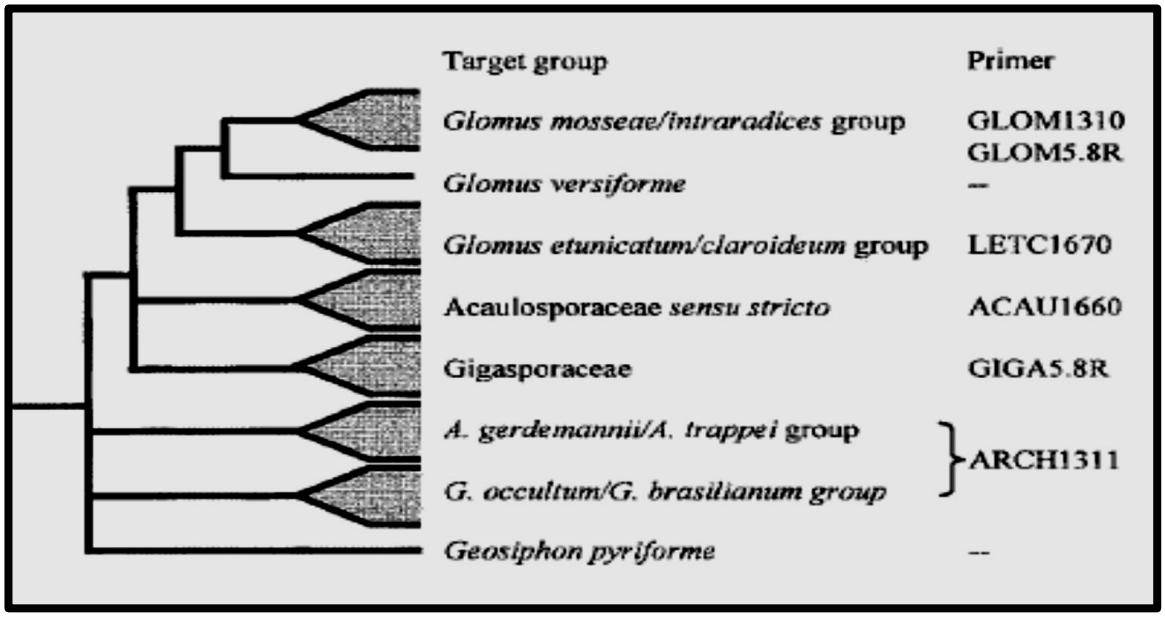


Figure 4.2.3. VAM phylogenetic groups targeted by the five-primer set used in this study (Redcker 2000).

These microbes have been known to provide substantial benefits to the growth and development of almost all plant species. These benefits are especially expressed in plants affected by biotic and abiotic factors (Powell and Bagyuraj 1984). Studies have been carried out to prove the benefits of these microbes. The current project is considered a novel approach in investigating the benefits of identifying and applying the VAM Fungi from marsh plant roots of the Blackbird Creek.

The marsh grass *Spartina alterniflora* was subjected by six treatments to analyze their effects on five different physical characteristics of the plant after a three months' growth period and twenty-four hours' salinity imposition under greenhouse conditions.

Spartina alterniflora plants were subjected to six distinct treatment conditions (Figure 3.3) that influenced their responses, respectively. The treatments were distributed in a randomized format that ends in defining the PL-plant length, # of L-number of leaves, RL-root length, RM- root mass and SL-shoot length.

Some differences but not necessarily significant were observed in the effect of the treatments on the abundance of the plant leaves. One way fixed-effect ANOVA concluded a p-value of (**0.5)**. similarly, Shoot and root lengths showed no significant differences among treatment effect, as the ANOVA analysis p-values resulted in (**0.2)** and (**0.6)** respectively. It is assumed that the similarities in plant characteristics may have been due to a combination of factors such as seed type and/or growth environment.

Some seeds are extremely viable and may be able to germinate/produce plants that are very robust and are able to develop adequately despite its environmental conditions.

Spartina alterniflora are capable of withstanding extreme conditions such as high salinity and will be able to survive milder or less invasive conditions (Reganold et al. 1990, Noble et al. 2010). Regardless of the treatments, these plants were exposed to a milder environment therefore, they may have been able to develop normally and even at a better rate with the addition of the VAM fungi. This is evident when the characteristics are measured against As only (control). Plants seem to exhibit similar response when compared to the other five treatments.

The comparison was made since the As soils were soils which should be missing the elements of VAM and other microbes. The remaining soils were either un-autoclaved or possessed one element or both. The presence of VAM in the soils of the As treatment and the plants development associated with this treatment may be due to factors such as:

Some species of VAM and other beneficial microbes having the potential to survive temperatures higher than 121°C (autoclave conditions).

When the response of plants affected by As is compared to other treatments, the similarity may be due to a reduced VAM diversity and or integrity in the soils of the those treatments. It is also assumed that when VAM spores from marsh environment were added to autoclaved and un-autoclaved soils, the efficiency in the survival of VAM spores might have decreased due to the disturbance in soil structure or change in the natural environment of the VAM fungi. This might have led to no significant differences in the growth characteristics of plants in autoclaved, autoclaved + VAM, un-autoclaved and un-autoclaved + VAM treatments. The greenhouse exhibits a milder environment compared to the natural environment of the *Spartina alterniflora* and again may have allowed for adequate growth and plant development. This environment also takes in the use of containers which could cause uniform growth patterns among plants. Container cultivation may have caused some limitations in the growth expansion of the plant roots, affecting both length and probable mass (Reganold et al. 1990, Noble et al. 2010). This is a result of the roots having limited and equal space, hence the similarity in lengths among all treatments.

Root masses did however produce a significant difference among treatments exhibiting a p-value of (**0.02)** when significant level is set at **(p<0.05)** analyzed by a one-way fixed ANOVA.

This would have been expected since VAM associations occur directly within the plant roots and are expected to help in increasing root size, hence it's mass. When this happens, the plant develops a broader root surface area that is extremely beneficial for the plant.

This aids the ability for adequate nutrient and water uptake for the plant in especially adverse environmental situations (Laila and Heil 2011). Similar results have been identified in studies by

previous researchers. According to experiments carried out by Beruti et al. (2015), inoculation was performed in both greenhouse and open-field conditions. Most of the experiment were performed in greenhouse while the remaining were in open-field conditions. Fungal colonization in inoculated plants compared to non-inoculated controls were significantly more frequent in the greenhouse than in the open field. Naturally, the non-inoculated control portion of a field often contains VAM fungal propagules, while control pots in greenhouses are filled with sterilized soils that are VAM free or highly reduced in VAM diversity. Interestingly, observation showed that the root biomass benefits were more from inoculation in field conditions compared to that of greenhouse conditions. Assessment showed that containerized roots stop growing due to the constraints of the pot boundaries at a specific point during cultivation period (Beruti et al. 2015). Additionally, inoculated plants are often less prone to invest in root growth in pots. This may be as a result of containerized inoculated plants being more likely to rely massively on fungal-mediated uptake and can reach a maximum level of exploration of the substrate sooner than non-inoculated plants, without increasing the root biomass. This may have also contributed to the physical results of the plants cultivated in the **As** control treatment being similar to that of the other treatments.

However, the effectiveness of VAM inoculation on shoot biomass, yield, and plant nutrition does not seem to be affected by the experimental conditions, and has been shown to be equally successful in greenhouse and open-field conditions. Researches have also shown that VAM is responsible for plant root development (Laila and Heil 2011).

Molecular results also confirmed the presence of VAM fungi in the samples of Spartina roots. Nested PCR amplification further confirmed the presence of VAM fungi from five different groups which include *Glomus, Acaulospora, Archaeospora, Gigaspora* and *LETC- Glomus*

etunicatum. The *Gigaspora* group showed very minor differences among the treatments. In the Un-autoclaved + VAM treatments some species within this group are present but absent in other treatments. This is specifically in the autoclaved +VAM treatments. Species in the *Glomus* group are normally abundantly present in natural soils and also in commercial VAM. However, in this research it was observed that the patterns in the bands were varying and irregular among the treatments. This made the results for this primer set inconclusive. The *LETC* group also from the *Glomus* but have different species only showed significant differences in the patterns observed in autoclaved Only Soils (Control). In the *Acaulospora* group of species, very faint and less number of bands were identified in roots from the autoclaved soils when compared to the un-autoclaved and un-autoclaved + VAM. For the *Archaeospora* species, roots from the autoclaved soils have less bands than those roots from the un-autoclaved treatments. The treatments with autoclaved and un-autoclaved + VAM + Salt, there were evidents of prominent bands.

Which means the percentage distribution of VAAM species is changing depending on the soil type and condition. Differences in the patterns of *Aculospora* and *Archaeospora* species were observed with respect to the different treatments. These group showed dominance in the species present, which led to the assumption that these species may be specific to marsh soils.

The identification of the presence of these targeted groups and thee species present also proved a successful greenhouse experiment.

These results influenced the acceptance of the hypothesis “Marsh VAM fungi may be more efficient in helping plant growth characteristics and aid in development under abiotic (salinity) stress”. Since VAM helps the plant root to develop a broader surface area (positively effecting root mass), this will allow better anchorage for the plant against physical environmental factors. These factors include wind and flooding events. With larger and healthier plant roots, soil

structure is also maintained. These roots will be able to better hold soil particles together. This helps to reduce soil erosion, aid in nutrient retention and also helps in soil hydrological function. Improved soil structure helps the soil to efficiently retain moisture and nutrient which is significant for plant growth and development (National Academy of Sciences, 1989, Parr et al. 1994). These data will be significant in performing future research on testing their effectiveness/benefits on plant development and growth when under abiotic stress.

CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Having the essential background knowledge of the sources, chemistry, and potential risks of toxic heavy metals in contaminated soils is extremely necessary for the selection of appropriate and effective remedial options. Remediation of contaminated soils is necessary to reduce the associated risks, make land resources available for agricultural production, enhance food security and reduce land tenure problems. Several methods of bio-application including bioremediation are frequently listed among the most suitable technologies for cleaning heavy metal contaminated soils. This type of applicability and commercialization is necessary since environmental degradation caused by urbanization, industrialization and agriculture are frequently reported and are of major concerns. The data gathered for this research can facilitate future research in other similar marsh ecosystems and help to restore areas that are affected by various environmental factors. This will help in designing the appropriate remediation assay for that location based on the heavy metals that are present.

VAM fungi are significant biotic soil components and when missing or impoverished as a result of anthropogenic input, can ultimately result in a less efficient ecosystem functioning.

Re-establishing the natural level of VAM fungi richness is a representation of a promising alternative to conventional fertilization practices. This has significant potential for sustainable agriculture. The recommended approach in adaptation and to achieve this goal is the direct re-introduction of VAM inoculum into the target soils.

Effective exploitation of these fungi in applicative assays requires the knowledge of how VAM fungi adapt and react to the target ecosystem and soil management.

The events that lead to the establishment of a functional symbiosis, including the mechanisms involved in nutrient transfer are also important in establishment and success of the program.

Research has estimated that global population will exceed nine billion by 2050 (Rodriguez and Sanders, 2015). It is also estimated that global agriculture will have to face the task of almost doubling food production but also of reducing the dependence of producers on agrochemicals in order to safeguard human and environmental health. This is due to the forecast which predicts that necessary yield increase exceeds the current global capacity to produce food (Rodriguez and Sanders, 2015). This intensifies the need to implement or revitalize eco-friendly technologies, such as VAM-based fertilization. Future work of this study may investigate the benefits of VAM in plant development under extended period of salinity stress. This is intended for both *Spartina* and other terrestrial crops. The transfer of salt tolerant genes in the tested terrestrial crops will also be a potential project through application of salinity stress on these plants.

This will help with development of salt resistance crops as the concerns of climate change rises. This will eventually create a future for sustainable agriculture and food security.

In conclusion, there are beneficial microbes present in marsh ecosystems in the Blackbird Creek. Their abundance and tested capabilities have shown the potential to be utilized in both environmental and agricultural sustainability. This approach of microbial use can be applied through bio-remediation using heavy metal tolerant bacteria and the use of VAM fungi as bio fertilizers.

It is recommended that these types of technologies are introduced to small farmers through education and training. An incentive program can also be implemented for farmers and natural resources managers at all levels with the intention to adapt and use these technologies long term. Education on the cost-effectiveness and simplicity of using these biotechnological approaches in amending agricultural and environmental issues should be disseminated to individuals at all levels starting with programs such as 4-H clubs in schools. These approaches should be a priority for developing countries to ensure future generation adapt and use those practices to sustain themselves.

Globally, both human and environmental health is affected by abused land use practices and climate driven changes. Habitat management, crop growth protection and health are continuously challenged by biotic and abiotic factors. This has increased the use of agricultural chemicals that has led to environmental pollution. The use and build-up of these toxic chemicals mainly in the form of heavy metals in the environment is spreading at an alarming rate (Cheng 2003). This has become a major problem in many ecosystems, especially the wetlands. This has prompted the need to develop solutions to promote conservation and sustainability. Therefore, this study focused on utilizing beneficial/environmentally friendly microbes in addressing these issues. My research provided baseline information to address those challenges and help in promoting sustainable agricultural practices.

The use of these microorganisms has been explored and implemented by agencies such as the United States Environmental Protection Agency (USEPA) and United States Geological Survey (USGS).

This approach provides alternative or addition to different conventional methods for farm management and environmental restoration. The data in my study may expand our current knowledge in relation to the use of beneficial organisms.

Investigating the presence and roles of these microbes in the Blackbird Creek ecosystem and how best they can be effectively applied to ecosystem management gives a clearer understanding of marsh microbes and their applicability.

The opportunity to continue further investigation for different use of these microbes is also a potential outcome of this research. Overall benefits include reduced environmental risks associated with agriculture, improve crop productivity, reduced heavy metal pollution, improved soil quality, sustainable agriculture, protection of local natural resources and ultimately human health.

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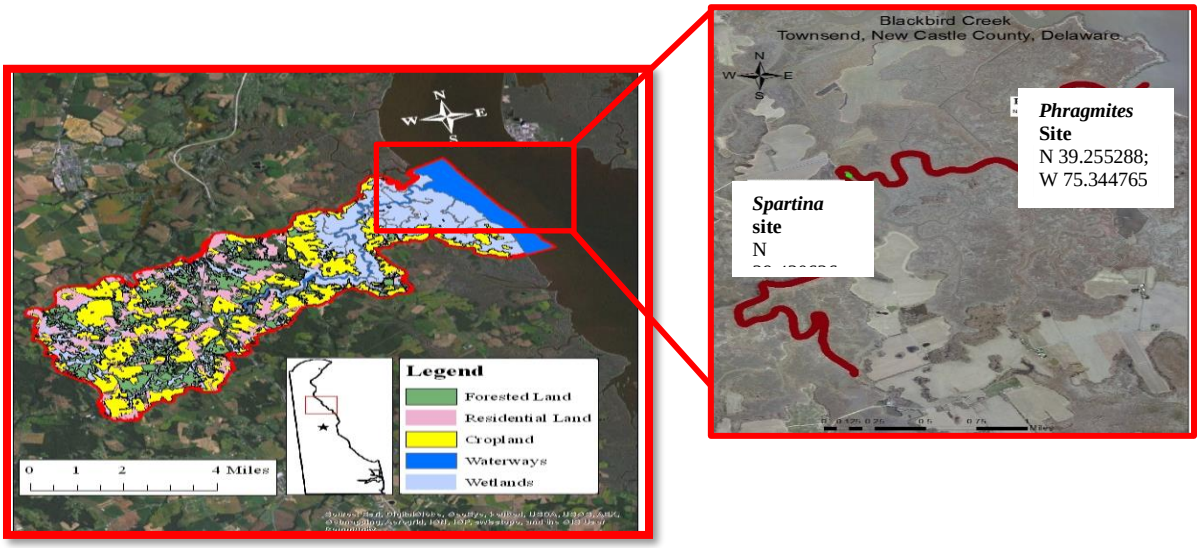
APPENDIX 1

Sample Site Location and Experiment Equipment

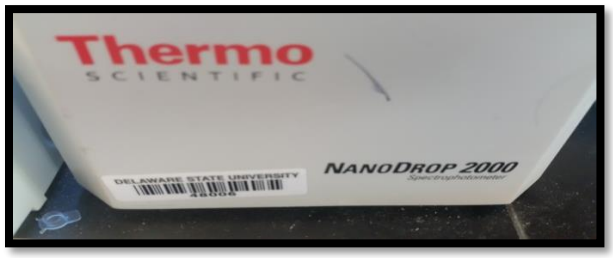


Blackbird Creek (BBC) with specific study sites (http://delawarewatersheds.org/the-delaware-bay-estuary-basin/blackbird-creek).

Aerial view of the Blackbird Creek (BBC), Townsend, Delaware (Photo credit DNREC).

DNA S1000 Thermal Cycler, BIO-RAD:
This performs the amplification of the DNA samples by a polymerase chain reaction (PCR) process.

Sorvall RC 6+ Centrifuge, Thermo Fisher Scientific:
Used in the separation of liquids and purification of cells.

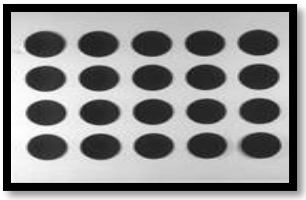


NanoDrop 2000, Thermo Scientific:
Full spectrophotometers and Flurospectrometer used to quantify and assess purity of DNA, RNA, Proteins etc.

Light Microscope:
Uses focused light and lenses to magnify specimen, usually a cell. When used in combination with staining methods, diverse types of cells can be identified (example: gram positive and gram-negative bacteria cells).

Scanning Electron Microscope, Oxford Instruments:
This is used to reveal information about the sample including external morphology (texture), chemical composition, crystalline structure and orientation of materials.

SEM Stubs



SEM Carbon Discs

These are placed in the SEM machine with the samples to reveal the structure of the cells present.

Time-Domain Reflectometry 100 (TDR) meter:
This gives the ratio of the volume of water in a given volume of soil to the total soil volume and at saturation; the percentage VWC will be equivalent to the soil pore space.

APPENDIX 2

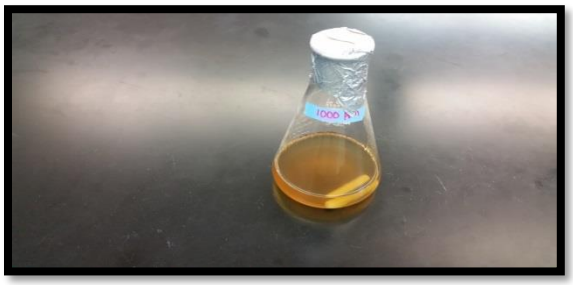
Isolation, Identification and Characterization of Lead and Cadmium Tolerant Bacteria from Marsh Soils

Sample collection at BBC from soils dominated by *Spartina alterniflora.*

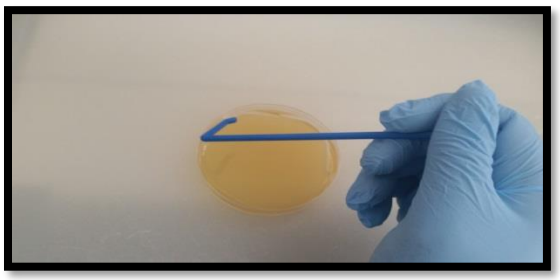
Sample collection at BBC from soils dominated by *Phragmites australis.*

Spartina and Phragmites dominated soil media inoculated with lead and cadmium concentrations for bacterial growth.

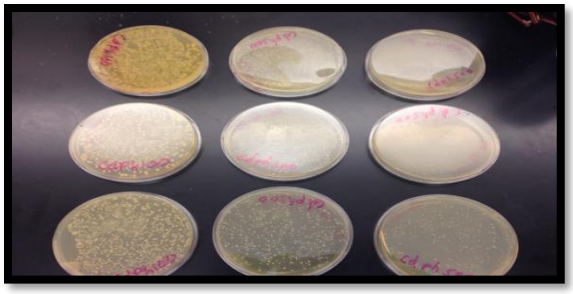
LB broth stock solution inoculated with lead and cadmium concentrations.



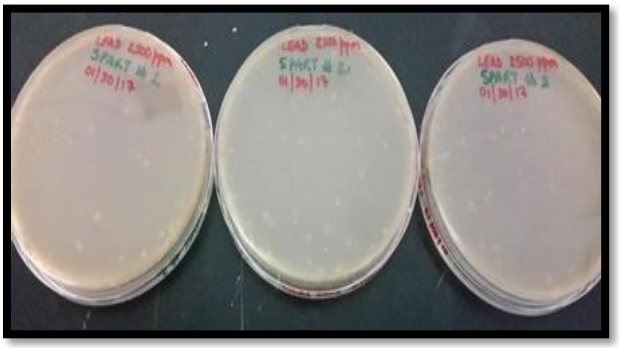
LB agar media prepared at 1000 mg/kg for use in making agar plates for bacterial culture.



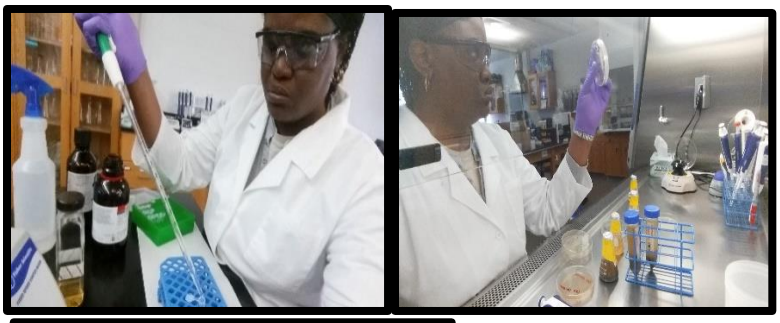
Spread Plating:
Prepared soil media form both *Spartina* and *Phragmites* dominated soil is spread plated for bacterial culturing.



Cadmium Tolerant Bacteria growth up to 500 mg/kg.



Lead Tolerant Bacteria growth up to 2500 mg/kg.



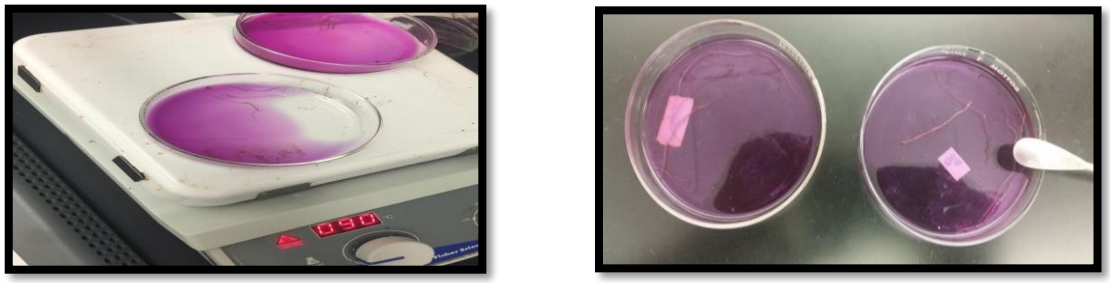
Molecular methods of bacteria isolation:

Isolation and confirmation of the metal tolerant bacteria using Phenol/Chloroform DNA extraction, Nano-drop and PCR.

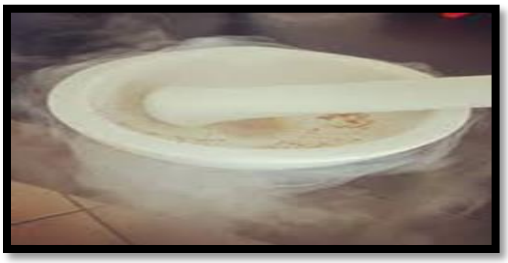
APPENDIX 3

Application of Marsh mycorrhizae to promote plant growth and aid in plant development under salt stress

Wet Sieve Method:
Used in the separation of particles for sample analysis.



Acid Fuchsin Staining of *Spartina alterniflora* Roots.



Mortar and pestle:
Used for the grinding of root samples in preparation for genomic DNA extraction.

Spartina alterniflora seed germination in preparation for greenhouse expirement.

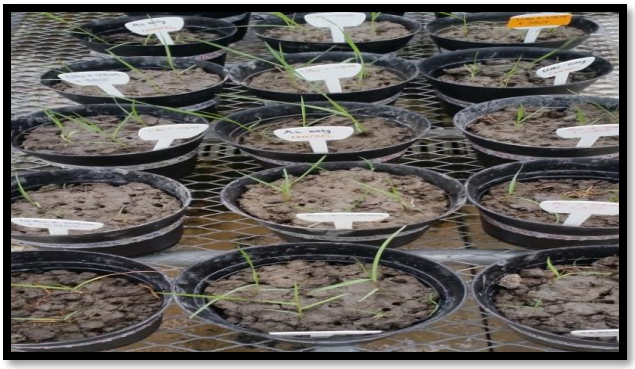
Rinsing of Spartina roots for use in greenhouse experiment.

Preparation of roots for innoculation of soil for greenhouse experiment.

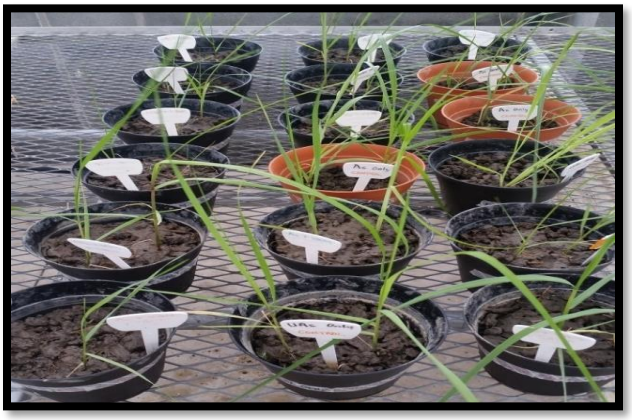
Prepared VAM roots for greenhouse experiment.

Prepared soil media inoculated with VAM roots for greenhouse experiment (plant trap method).

Prepared pots for seed transplant to be used in greenhouse experiment.



Initial stages of seedlings growth under greenhouse conditions.



Mature *Spartina alterniflora* seedlings grown under greenhouse conditions.

Mature *Spartina alterniflora* seedlings grown under greenhouse conditions.

Spartina alterniflora grass without salt treatment, harvested for the identification of the effects of VAM on its physical characteristics.

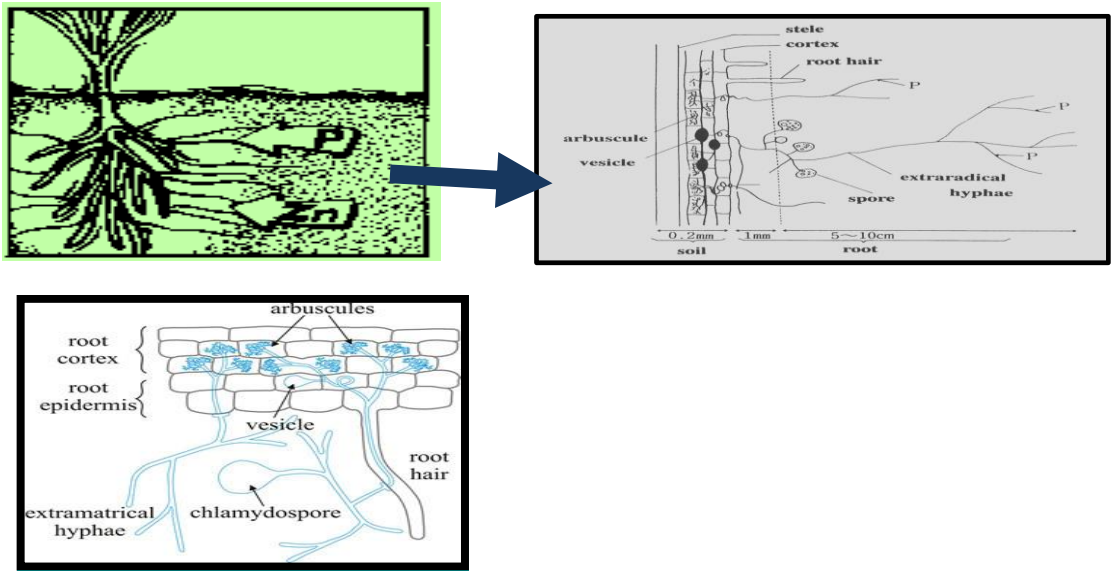
Spartina alterniflora root samples separated for length and mass measurements. These roots were not exposed to salt treatment/shock.

Spartina alterniflora leaf samples separated for length and quantity measurements. These samples were not exposed to salt stress.

Spartina alterniflora grass with salt treatment, harvested for the identification of the effects of VAM on its physical characteristics.

Spartina alterniflora root samples separated for length and mass measurements. These roots were exposed to salt treatment/shock.

Spartina alterniflora leaf samples separated for length and quantity measurements. These samples were not exposed to salt stress.



Schematic picture of arbuscular mycorrhizal fungi colonizing roots and their hyphal extension into soil.

GLOSSARY

TERMS	DEFINITIONS
A	
Abiotic Factors	These are non-living chemical and physical factors within the environment that affect both living organisms and the functioning of ecosystems.
Achlorophyllous	These are plants that are unable to engage in photosynthesis because they do not have ant chlorophyll.
Aerobic bacteria	These organisms survive only in the presence of oxygen.
An-aerobic bacteria	An anaerobic organism or anaerobe can survive without the presence of oxygen. Some anaerobes may even die in the presence of oxygen. Obligate anaerobes are harmed by the presence of oxygen, aerotolerant organisms cannot use oxygen for growth but tolerate its presence and facultative anaerobes can grow without oxygen but use oxygen if it is present.
Angiosperm	These are seed-bearing plants in which the ovules are enclosed in an ovary. These ovaries develop into the fruits after fertilization. Angiosperms include herbaceous plants, shrubs, grasses, and most trees.
Arbuscular mycorrhizal fungus	An arbuscular mycorrhizal fungus is a type of mycorrhiza in which the fungus penetrates the cortical cells of the roots of a vascular plant and establish a symbiotic relationships. Arbuscular mycorrhizas are characterized by the formation of unique structures, arbuscules and vesicles which helps the plants to capture nutrient and water.
Autoclave	An effective and reliable means of sterilizing laboratory materials. The process uses saturated steam under pressure.
B	
Bacteria	These are a group of single celled microscopic living organisms. Bacteria are found everywhere in nature. They can be both dangerous (cause infections) and beneficial (fermentation and decomposition) to both human and the environment.
Biotic	The living things in the ecosystems are referred to as biotic components. This community are made up of the animals, plants and microorganisms. Waste products from dead and living organisms are also biotic components.
Bifurcation	This is the point at which the main body is split into two parts.
Bioremediation	This is the use of biological organisms to remove or neutralize contaminants in soils and water. Bioremediation agents can be bacteria or fungi that can effectively remove salts and metals from the environment.
C	
Carcinogens	Any substance or radiation that promotes the formation of cancer in living cells.

Chemotrophs	Chemotrophs are organisms that obtain energy through chemical process called chemosynthesis. This process involves the transfer of electrons across the plasma cells. of some microorganisms which oxidize and reduce these inorganic compounds into carbon dioxide and water for growth and development.
Composite sampling	This a method used to collect multiple temporally or spatially discrete samples. These are combined to make a homogenized single sample.
D	
Deleterious	This means being harmful to a person, thing, place etc. Any cause that can make a situation worse.
E	
Ectomycorrhizae	Ectomycorrhizas is a group of fungi that forms a symbiotic relationship with the roots of various plant species. The fungal spores colonize the outer root surfaces of its host (plant).
Endomycorrhizae	Endomycorrhizae is the mycorrhizal fungi that penetrates the root cell walls of the plant root instead of existing on the epidermal layer of the root.
F	
Filamentous	Having thin thread-like or fibrous structural built.
Fungi	Fungi are heterotrophs, they are a diverse group of eukaryotic single-celled or multinucleate organisms that live by decomposing and absorbing the organic material in which they grow.
G	
Gel-electrophoresis	Laboratories commonly use this technique to separate charged molecules like DNA, RNA and proteins according to their size.
H	
Heavy metal	Any metallic chemical element that has a relatively high density. They are highly toxic or poisonous even at low concentrations.
Hyphae	These are the thread-like long, branching filaments. They make up the structure of a fungus, oomycete, or actinobacterium. Hyphae helps release enzymes to absorb nutrients from food sources.
I	
Inoculum	The most active material that is used for an inoculation.

M	
Marsh	Marsh wetlands area/ecosystems dominated by herbaceous rather than woody plant species. Marshes can often be found at the edges of lakes and streams.
Mycorrhizae	Mycorrhizae are symbiotic relationships that form between fungi and plants. The root system of a host plant is colonized fungi which helps to provide increased water and nutrient absorption capabilities for the plant. The plant in return provides the fungus with carbohydrates formed from photosynthesis.
Microbes	A microorganism or microbe is a microscopic organism, which may exist in its single-celled form, or in a colony of cells. These organisms are found everywhere in nature (soil, water, plants, animals and human body).
Monotropic	Any material that exists in multiple forms, only one of which is stable at all temperatures and pressures.
Morphology	The study of the form and structure of organisms and their specific structural features. This include the outward appearance (shape, structure, color, pattern, size), which is the external morphology (or eidonomy), as well as the form and structure of the internal/internal morphology parts like bones and organs, (or anatomy).
Mycelia	This is the vegetative part of a fungus or fungus-like bacterial colony, that is made up of a mass of branching, thread-like hyphae. The mass of hyphae is sometimes called Shiro.
P	
Photosynthesis	A process by which plants, algae, and certain microorganisms convert light energy from the sun into the chemical energy of food. During photosynthesis, energy from sunlight is harnessed and used to convert carbon dioxide and water into organic compounds, such as sugar molecules-and oxygen.
Phycomycetous	Of or relating to any of a primitive group of fungi, formerly included in the class *Phycomycetes*, but now classified in different phyla: includes certain mildews and molds.
Primers (DNA)	A primer is a short nucleic acid sequence that provides a starting point for DNA synthesis. The primer therefore serves to prime and lay a foundation for DNA synthesis. These DNA primers are commonly used to perform the polymerase chain reaction to copy pieces of DNA or for DNA sequencing.
R	
Rhizomorphs	These are threadlike or cordlike structure in fungi that is made up of parallel hyphae that leads certain fungi across various substrates like a root through soil. Rhizomorphs act as an absorption and translation organ of nutrients.

Rhizosphere	This is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms. The rhizosphere contains many bacteria, fungi and other microorganisms.
S	
Sanger sequencing	A method of DNA sequencing based on the selective incorporation of chain-terminating deoxynucleotides by DNA polymerase during in vitro DNA replication.
Septate	This is the inability of cell division by spores or hyphae due to the lack of hyphae.
Symbiosis	An interaction between two different organisms living in close physical association, typically to the advantage of both.
Sustainability	Sustainability can defined as a socio-ecological process in which the property of biological systems to remain diverse and productive indefinitely.
T	
Turgor	The normal distention or rigidity of plant cells, resulting from the pressure exerted by the cell contents on the cell walls.
U	
Un-autoclave	Remains in the original state without being processed by heat and pressure.
V	
Vesicular	A membrane-bound sac in eukaryotic cells that stores or transports the products of metabolism in the cell and is sometimes the site for the breaking down of metabolic wastes.
W	
Wetlands	A wetland is a land area that is saturated with water, either permanently or seasonally, such that it takes on the characteristics of a distinct ecosystem. Wetlands links land and water, and are some of the most productive ecosystems in the world. Some common names for the diverse types of wetlands are swamp, marsh and bog.