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Assessment of Thelypteris palustris, Asparagus sprengeri, and Lolium perenne for their potential use in the phytoremediation of arsenic-contaminated soils

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ASSESSMENT OF *THELYPTERIS PALUSTRIS, ASPARAGUS SPRENGERI*, AND *LOLIUM PERENNE* FOR THEIR POTENTIAL USE IN THE PHYTOREMEDIATION OF ARSENIC-CONTAMINATED SOILS

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ABSTRACT

The goal of this study is to evaluate the potential of three plants, *Thelypteris palustris* (marsh fern), *Asparagus sprengeri* (asparagus fern), and *Lolium perenne* (perennial ryegrass) for use in phytoremediation of arsenic-contaminated soils. Specifically, the objectives of this study are to (1) confirm if arsenic accumulation occurs in the selected plants, (2) to examine morphological effects of arsenic in the selected plants, and (3) to evaluate the oxidation state of arsenic upon accumulation in the selected plants. The analytical method combination of ICP-MS, SEM, and XANES was used to accomplish the objectives of this study.

The results indicate marsh fern, asparagus fern, and perennial ryegrass all uptake arsenic. Bioaccumulation factors of all plants except ryegrass are > 1, indicating that they are accumulators of arsenic. The bioaccumulation factors of marsh fern were found to be in the range of the bioaccumulation factors (>10) of the known hyperaccumulator, *Pteris vittata*. After arsenic exposure, plants exhibited necrosis or vascular system degradation and collapse. All plants contained a mixture of the arsenic oxidation states of As (V), As (III), and/or As (0). Ryegrass was the only plant to contain As (III) chemically associated with sulfur. As (V) and/or As (0) were the dominant oxidation states in above-ground biomass of asparagus fern and ryegrass. As (V) was the dominant oxidation state in the roots of marsh fern.

In conclusion, marsh fern, asparagus fern, and ryegrass have the ability to survive arsenic exposure and accumulate arsenic into above-ground parts. Marsh fern is a good candidate for phytoextraction of areas contaminated with low levels of arsenic. Although, asparagus fern and ryegrass are not good candidates for phytoextraction, their potential in phytostabilization should be further investigated.

CHAPTER 1
INTRODUCTION

Environmental contamination by metals such as lead, chromium, arsenic, zinc, cadmium, copper and mercury can adversely affect the environment and human health (Kos et al., 2002). Arsenic is known to increase the risk of the development of cancer; in addition, arsenic can cause abnormalities in the human reproductive and neurological systems (O'Connor, 2002). Contamination of drinking water by arsenic affects millions of people (Association for the Environmental Health of Soils, 1998). The largest mass arsenic poisoning in history is occurring because groundwater used as drinking water has become contaminated with naturally occurring inorganic arsenic (Smith et al., 2000). After Hurricane Katrina resulted in extensive flooding of New Orleans, Louisiana, floodwater samples contained arsenic possibly released from destroyed industrial buildings and critical city infrastructure services, such as water and sewer services (Presley, 2006). The average concentration of arsenic contained in floodwater was 30 ug/L and this concentration was consistently higher than the suggested maximum arsenic drinking water limit of 10 ug/L (Pardue et al., 2005).

The remediation of toxic metals in the environment is an urgent and challenging environmental issue (Koch et al., 2000). Metals are not degradable, so the cleanup of metals generally requires the immobilization of the metal, reduction of the toxicity of the metal, or removal of the metal from the contaminated areas (Kos et al., 2002). For most metal-polluted soils, the most common types of remediation strategies are soil excavation, removal, and landfilling (Vangronsveld et al., 1998). In the near future, these traditional techniques of remediation of metal contaminated areas will eventually

decrease in economic feasibility and in public acceptance because of the development of less expensive and new environmentally friendly remediation techniques (Lombi et al., 2001) . Phytoremediation uses plants to remove or neutralize contaminants in contaminated environments in soil and water (Van Der Lelie et al., 2001). Phytoremediation causes minimal environmental disturbance, allows soils to be left in a useable condition after the removal of the contamination (Schwitzguebel, 2001) and has a low cost of implantation (Lombi et al., 2001). Phytoextraction, one type of phytoremediation (Zynda, 2001), is the transfer of the metal into plant parts that can be easily harvested and removed from the cleanup site (Pierzynski et al., 2001). The harvested plant parts, rich in accumulated metals, can be safely processed by drying, ashing or composting. Furthermore, some of the metals in the ash may be retrievable for future uses (Garbisu et al., 2001). The efficient movement of metal from root to shoots is an important factor in considering a plant for phytoextraction processes (Cai, 2003; Ernst, 1996). Certainly, there is the need for the identification of plants that have the ability to accumulate metals in high levels from contaminated areas (Lasat, 2002).

The goal of this study was to evaluate the potential of three plants, *Thelypteris palustris* (marsh fern), *Asparagus sprengeri* (asparagus fern), and *Lolium perenne* (perennial ryegrass) for use in phytoremediation of arsenic-contaminated soils. Specifically, the objectives were (1) to confirm if arsenic accumulation occurs in the selected plants, (2) to examine morphological effects of arsenic in the selected plants, and (3) to evaluate the oxidation state of arsenic upon accumulation in the selected plants. The plants were chosen because of the ease of their cultivation, their ability to survive in various environmental habitats and because of the limited information in literature

concerning their mechanisms of arsenic accumulation or tolerance. The marsh fern was of particular interest because of the recently-documented ability of the ladder brake fern (*Pteris vittata*) to hyperaccumulate arsenic (Ma et al., 2001; Tu et al., 2002).

Greenhouse studies were used to gain further insight into the behavior of arsenic in the selected plants and to screen the potential of each plant as a hyperaccumulator of arsenic. Plants were exposed to various arsenic treatment levels, then plant samples were examined by inductively coupled plasma-mass spectrometry (ICP-MS) (Goessler et al., 2002) to determine arsenic concentrations, scanning electron microscopy (Blaszczak et al.) to observe the morphological changes that occur plants after exposure to arsenic (Langer et al., 2004; Tryton, 1971), and x-ray near edge structure (XANES) to determine arsenic speciation (Webb et al., 2003). The determination of arsenic speciation by XANES is important because it provides helpful information for the better understanding of mechanisms of arsenic accumulation, translocation, and detoxification inside of the selected plants (Zhang et al., 2002).

The hypotheses of this research are:

1. *Thelypteris palustris, Asparagus sprengeri*, and *Lolium perenne* will accumulate arsenic into above-ground plant parts.
2. The morphology and growth patterns of all three plants will be altered after arsenic exposure.
3. As (V) will be the dominant arsenic oxidation state in exposed plant roots, while As (III) will be the dominant arsenic oxidation state in above-ground plant parts.

CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

2.1 Arsenic in the environment

Arsenic is a toxic trace element widely distributed in soils and aquifers from geologic and anthropogenic sources (Zhang et al., 2006). Soils worldwide have an average natural arsenic content level of 5 mg/kg (Materia et al., 2001), with soil parent materials, such as sedimentary rocks and arsenic-abundant minerals (Francesconi, 2002), serving as the main source of arsenic in soils (Chen et al., 2002). Determining background or existing arsenic concentrations in soils is an important factor in determining if a soil is polluted (Chen et al., 2002).

Arsenic has been used in agriculture to control weeds, insects, and rodents (Abdullah 1997). For example, calcium arsenate has been used as an insecticide in ant killers and animal dips (National Toxicity Program, 1998). Throughout the United States, calcium arsenate was applied as a general insecticide on an array of vegetable crops. In the southern United States from 1920 to 1950, calcium arsenate was very extensively and frequently used for the control of boll weevils in cotton (Murphy, 1998). Soils in Louisiana have an established background arsenic level of 7 mg/kg, but soils used for cotton production in Louisiana possess an average arsenic concentration of 23 mg/kg because of the application of arsenic-containing agricultural chemicals (Cox et al., 1996). Leaching of arsenic from chromate copper arsenate (CCA)- treated wood is another major source of soil contamination (Debran et al., 2006). Mulches with mixtures of CCA-treated wood at a level of 0.1% have a greater arsenic concentration level than Florida's residential clean soil guideline level of 0.8 mg/kg (Townsend et al., 2003).

Groundwater is contaminated by arsenic most commonly by dissolution of minerals or ores (Pinsker, 2001) or from man-made sources such as mineral extraction or agricultural practices (Nordstrom, 2002). Arsenic solubility in groundwater can be influenced by the concentrations of phosphate, bicarbonate, silicate and organic matter contained in groundwater (Nordstrom, 2002).

Arsenic predominately occurs in natural systems as the four oxidation states -3, 0, +3, and +5. Arsenic is most commonly found in water systems as inorganic arsenite (+3) and inorganic arsenate (+5) (Le, 2002). Arsenate (As (V)) and arsenite (As (III)) are the oxidation states of arsenic mostly found in soils (Zhang et al., 2006). In oxidized soils, arsenate is expected to be the dominant arsenic oxidation state while arsenite is expected to be the dominant oxidation state in anaerobic conditions (Sadiq, 1997). In water-saturated soils experiencing anaerobic conditions, 15-40% of arsenic is found to be below the +5 oxidation state (Materia et al., 2001). Arsenate exhibits a strong interaction with solid matrix (Zhang et al., 2006). As (V) can become chemically absorbed by iron and aluminum oxides, non-crystalline aluminosilicates, and, to a smaller extent, layer silicate clays (McBride, 1994). Solid phases of arsenic (III) (+3) and arsenic (V) (+5) that can display a significant importance in natural systems, such as soils, are iron, manganese, and calcium arsenates, as well as, arsenic (III) sulfides (Inskeep et al., 2002). For example, formation of sulfides under anaerobic conditions may cause the co-precipitation of arsenic in its lower oxidation states (McBride, 1994). Additionally, the chemical absorption of arsenic to the surface of Fe oxide/hydroxides is believed to be the common mechanism in the formation of arsenic solid phases (Sadiq, 1997).

The bioavailability of a metal determines its toxicity in the environment (Isphording, 1999). Arsenite (III) is considered to have greater bioavailability than arsenate (V) because when arsenite is present at a neutral pH, it becomes the uncharged oxidation state, (As(0)) with the ability to easily permeate biological membranes (Webb et al., 2003).

2.2 Human health effects of arsenic contamination

Contamination of drinking water by arsenic presents a severe health risk to millions of people worldwide (Huang et al., 2004). Of the 125 million people who live in Bangladesh, approximately 33 million to 77 million are at risk from drinking contaminated water (Smith et al., 2000). Waters from wells in Bangladesh and China contain inorganic arsenic concentrations up to several thousand ug/L (Le, 2002). Human epidemiological studies conducted in China and Argentina established a direct relationship between exposure through human ingestion of water containing elevated arsenic concentrations and the predominance of skin, bladder and lung cancers (Le, 2002). The World Health Organization has established a maximum water arsenic contamination limit of 10 ug/L. However, it is not an uncommon occurrence to have natural groundwater concentrations that exceed the drinking water standard of 10 ppb (Nordstrom, 2002). The Canadian maximum water arsenic contaminant limit of 25 ppb is currently under review by Health Canada (Le, 2002).

Samples taken from floodwaters after hurricane Katrina in New Orleans, Louisiana, displayed an average arsenic concentration level of 30 ug/L and these concentrations were consistently greater than the maximum arsenic water contamination level of 10 ug/L (Tillet, 2006). Concentrations of arsenic, chromium, and nickel were

found to be significantly higher at the bottom of floodwater columns in Mid City New Orleans (Pardue et al., 2005). Toxicant and pollutant levels in New Orleans, Louisiana, after Hurricane Katrina in some soil/sediment samples were found to exceed the established United States Department of Environmental Protection Region VI human health screening criteria for arsenic, iron and lead (Presley et al., 2006).

2.3 Phytoremediation of metal-contaminated soils

Phytoremediation, the use of plants to remove or neutralize contaminants in contaminated environments in soil and water, (Van Der Lelie et al., 2001) has emerged as a promising technique for remediation of metal-contaminated soil. Phytoremediation is applicable to a wide range of contaminants, reduces risk of exposure to the community by providing plant groundcover (Belz, 1997), and allows the soil to be left in usable condition after contaminants are removed (Schwitzguebel, 2001). Phytoremediation is more economical when compared to alternative remediation methods. (Environmental Protection Agency, 2001; Dunphy, 2000; Wantanabe, 1997).

The two phytoremediation processes most suited to address metal contamination are phytoextraction and phytostabilization (Pierzynski et al., 2001). Phytoextraction is the mechanism by which plants uptake a contaminant and store the contaminant in plant parts and is the most applicable mechanism to the remediation of heavy metals (Belz, 1997). The ultimate goal of phytoextraction is the transfer of metals from soils to the plant portions that have the capability to be easily harvested and removed from the contaminated site (Pierzynski et al., 2001). Phytostabilization is the use of plants to stabilize contaminated soils to minimize leaching and wind or water erosion (Pierzynski et al., 2001). It has been successfully used for remediation of many metal-contaminated

sites (Pierzynski et al., 2001). For example, *Festica rubra*, a known metal excluder plant, has been used in phytostabilization of erosion-susceptible metal contaminated soils (Lasat, 2000). *Agrostis cappillaris* L. applied in combination with a soil amendment effectively stabilized a metal contaminated sandy soil for over 10 years (Munch et. al, 2003).

Selection criteria for plants used in remediation of metal-contaminated soil include metal tolerance for both phytoextraction and phytostabilization, metal accumulation for phytoextraction, and high production of above-ground biomass (for rapid remediation) for both methods, but particularly for phytoextraction (Clemens et al., 2002; Rumens et al., 2002). The bioaccumulation factor, the ratio of metal concentration in the shoot to the metal concentration in the exposure media, is important when considering a plant for phytoextraction (Zhao et al., 2003). Plants that maintain metal concentrations in shoots that remain at a constant or low level after exposure to a wide range of metal concentrations species, i.e., plants that have bioaccumulation factors < 1, may be suitable for phytostabilization because low above-ground concentrations reduce food-chain contamination and reduce potential for groundwater contamination at polluted sites (Lehmann et al., 2004). A bioaccumulation factor > 1 indicates that a plant is effective in the transportation of metal from the soil to the shoots of the plant and may be an indication of the usefulness of a plant for phytoextraction (Lehmann et al., 2004). A plant with the ability to accumulate concentrations of metals into its above-ground parts that are higher than the concentration of the metal to which it was exposed is termed a "hyperaccumulator" (Emhart et al., 2002). Hyperaccumulating plants are mostly favored for use in phytoextraction processes (Dertilis-Tartar et al., 2006).

2.4 Hyperaccumulators

Hyperaccumulators have the ability to tolerate and actively uptake non-essential metals at high concentration into their aerial parts (Robinson et al., 2003.). There are several definitions of hyperaccumulators (CIA, 2003;Reeves et al., 2000; Robinson et al., 2003.) some of which do not take into account the metal concentration in the medium, but only the concentration in the plant tissue (CIA, 2003). For this study, Megharg's definition of a hyperaccumulator as a plant with the ability to translocate and accumulate concentrations of metals in above-ground parts that are higher than the concentration of metals in the exposure media (Meharg, 2003) will be used.

Metal hyperaccumulation is a costly energy-consuming plant process (Lasat, 2000). It has been hypothesized that hyperaccumulation occurs as a defensive strategy in plants (Boyd, 1998). Decomposition of leaf litter from hyperaccumulators on the soil surfaces reintegrates the metal into the soil causing the hyperaccumulator to obtain competitive advantage over the non-hyperaccumulator through growth inhibition (Boyd, 1998). Hyperaccumulation of nickel in the annual *Streptanthus polygaloides* (milkwort) was shown to have increased defenses against pathogen attack. The bacterial pathogen, *Xanthomonus campestris pv. campestris*, and the necrotrophic fungi, *Alternaria brassicicola* were unable to grow in the milkwort containing high nickel concentrations (Boyd, 1998).

Hyperaccumulators documented in the literature are mainly crop plants (Ghosh et al., 2000). Many wild hyperaccumulating plants are slow growing, have low biomass production, and specific growth requirements. These natural factors have resulted in more focus being placed on specific agricultural groups, such as *Zea mays L*. (corn),

Brassica spp. (mustards), and *Nicotiana spp.* (tobacco) that could be grown efficiently once agricultural practices were established for them (Klassen et al., 2000).

Pteris vittata (brake fern) (Ma et al., 2001) and few of its relatives (Meharg et al., 2002) (Srivastava et al., 2006) are the only known hyperaccumulators of arsenic. *Pteris vittata*, the most documented arsenic hyperaccumulator, has shown great potential for phytoextraction processes (Ponyton et al., 2004; Tu et al., 2002). When *Pteris vittata* was grown in 1500 mg/kg of arsenic, arsenic in its fronds increased from 29.4 ug/g to 15,861 ug/g after two weeks of exposure. Basal frond pinnae of *Pteris vittata* have been reported to contain arsenic concentrations of 6000-9000 ug/g (Lombi, 2002). *Pteris vittata* exposed to 6 mg/kg of arsenic accumulated a 126 fold enrichment concentration of 775 ug/g of arsenic (Christen, 2001). *Pteris vittata* found growing on a site contaminated with chromate copper arsenate contained arsenic concentrations up a level of 5000 mg/kg (Visoottiviseth et al., 2002). Ponyton et al (2004) reported that after 24 hours of exposure to a solution of 200 ug/L of arsenic-labeled arsenate, *Pteris vittata* completely depleted the arsenic from the solution. In a concentration dependant study of arsenic removal, *Pteris vittata* displayed the ability to reduce arsenic water concentrations below 10 ug/L during a 6 hour period and to reduce arsenic water concentrations to 0.5 ug/L in a 24 hour period. It was concluded that *Pteris vittata* was able to remove arsenic from contaminated water over a varied range of arsenic contamination levels (Huang et al., 2004). Arsenic concentrations increase in *Pteris vittata* as water-soluble concentrations of arsenic is increased (Tu et al., 2002).

Pteris cretica (cretan brake fern) *, Pteris longifolia* (long-leaved brake fern), and *Pteris umbrosa* (Japanese lady fern) have been identified as additional arsenic

hyperaccumulators in the *Pteris* genus; fronds contained dry weight arsenic concentrations levels of 6200-7600 ug/g of arsenic after exposure to 500 mg/kg of arsenic (Zhao, 2002). Arsenic concentrations in the fronds and roots of these species increased linearly as additional arsenic treatments were placed into the substrate (Zhao, 2002). When *Pteris cretica* was exposed to an initial concentration of 200 ug/L arsenic, the arsenic concentration in solution was reduced by 98.6 % to 2.8 ug/L within a 24 hour exposure period (Huang et al., 2004). *Pteris cretica* very quickly transports arsenic into its shoots; for example, 50% of the 60% of accumulated arsenic was located in the shoots of *Pteris cretica* within 6 hours of arsenic exposure (Ponyton et al., 2004).

Pteris liaurite L.*, Pteris quadriautita* Retz*,* and *Pteris ryukyuensis* Tagawa were recently identified as arsenic hyperaccumulators (Srivastava et al., 2006). After being grown in soil containing an arsenic concentration of 100 mg/kg, the newly reported arsenic hyperaccumulating ferns and *Pteris cretica* all contained an average frond dry weight arsenic concentration range of 1770 to 3650 ug/g and an average root dry weight arsenic concentration range of 182 to 507 ug/g. Arsenic (III) composed a greater percentage of arsenic speciation in fronds than arsenate (Srivastava et al., 2006).

Pityrogramma calonmelanos (silver fern) is the only non-*Pteris* genus of fern to be identified as an arsenic hyperaccumulator (Meharg, 2003). When the silver fern was found growing in the an industrial district in Thailand, the fronds of the silver fern had a maximum of 8350 ug/g of arsenic in its dry mass, while the roots contained a lower arsenic concentration range (Francesconi et al., 2002). Because the silver fern was capable of accumulating a high concentration of arsenic into its fronds, it had the estimated potential of removing 2% of the total load of soil arsenic per year (Francesconi

et al., 2002). Although arsenic concentrations in spores of silver fern slightly contribute to total arsenic concentrations in the plant, spores of the silver fern exhibit concentration levels up to 3500 ug/g of arsenic (Visoottiviseth et al., 2002).

2.5 Plant responses to metal contamination

The two basic strategies used by higher plants for metal tolerance in their environments are exclusion and accumulation (Zhao, 2002). Exclusion allows the plant to maintain metal concentrations in shoots at a constant or low level (Baker, 1981). Exclusion occurs when the entrance of metals into the root of the plant is restricted and/or the translocation of metals from the roots to the shoots of the plant is restricted (Zhao, 2002). For example, resistance to the uptake of arsenic is generally accomplished by the suppression of the high affinity of the phosphate/arsenate uptake system in order to reduce the influx of arsenic to a level that plant detoxification can occur (Meharg, 1994).

Accumulation is the process of the metals being concentrated into the plant parts (Zhao, 2002). Accumulator plant species do not prevent the entry of metals into the roots (Lasat, 2000) and may concentrate metals in above-ground plant parts from low or high soil metal concentrations (Baker, 1981). Most metal uptake, except for mercury, occurs in the aqueous state (Lasat, 2002) and translocation of metals from root to shoot mostly occurs in the xylem with help from the transpiration stream (Cai, 2003).

Accumulator plant species have developed specific mechanisms, such as detoxification of high levels of metals contained in their cells (Lasat, 2000). In response to accumulation high amounts of metals, such as arsenic, plants may produce phytochelatins (Rabb et al., 2005). Exposure to arsenic has been shown to stimulate larger production and accumulation of phytochelatins in different plant species (Zhao et

al., 2003). It is critical to understand that it is not the total quantity of a metal that determines toxicity, but rather its bioavailability (Isphording, 1999). In plants that do not accumulate extremely high concentrations of arsenic, the detoxification mechanism of reduction of As (V) to As (III) is followed by complexation of arsenic to phytochelatins (McGrath et al., 2003). Immobilization of metals in the cell walls is another mechanism used by plants to reduce the toxicity of exposure to excess metals (Kabata-Pendias, 2001). Therefore, accumulators may reduce metals to other chemical forms in order to protect against the reduction of metabolic activities (Francesconi et al., 2002).

Plant uptake of metals, except for mercury, occurs when metal is dissolved in water (Lasat, 2002). Water enters the cell walls of the root epidermal cells and the intracellular space of the epidermis and root hairs and then moves along the cell walls (apoplast) of the cortex until it meets the casparian strip (Stern, 1994). The casparian strip, composed of suberin bands in the inner walls of endodermal cells, obstructs movement of water along the endodermal cells because the cell membrane is fused to the suberin bands (Delvin et al., 1983). Therefore, water and dissolved substances are forced to pass through the cell membrane instead of the cell walls, thus regulating the materials absorbed and transported into other plant parts (Delvin et al., 1983). Root absorption of metals occurs by diffusion of ions from the external solution and by metabolic movement of ions against chemical gradients (Kabata-Pendias, 2001). Metals are considered "taken" into the body of a plant when the metal ion passes the cellular membranes of the root cells (Tuner, 1994). The transport of metals across cellular membranes is the beginning step of metal absorption into plant tissues (Lasat, 2000). Electrical charges prevent metal ions from flowing freely across the cellular membrane into the internal

fluid of the cell. Therefore, metal ions can cross the cellular membrane with assistance from transport membrane proteins (Lasat, 2002), such as metallothions (Vijver et al., 2004).

The movement or translocation of metals from roots to shoots mostly occurs in the xylem with assistance of the transpiration stream (Cai, 2003). Two internal plant mechanisms controlling translocation are root pressure and leaf transpiration (Lasat, 2000). It is theorized that metals are chelated in the xylem sap (McBride, 1994). Without chelation of metals in xylem sap, movement of the metal from the roots to the shoot would become severely retarded because of the high cation exchange capacity of xylem cell walls (Cai, 2003) and fixation of trace metals inside of the xylem walls would possibly occur (Hughes, 1981). After translocation of a metal to the leaves, leaf cells absorb metals from the metal concentrated xylem sap (Lasat, 2000).

Arsenate (As (V)) and arsenite (As (III)) are inorganic forms of arsenic that are phytoavailable in soil solutions (Meharg et al., 2002) because both inorganic forms are water soluble (Wang et al., 2002). In a typical soil, most arsenic is provided to plants as As (V) (Webb et al., 2003). The reduction of As (V) to As (III) can be an essential part in the detoxification of arsenic upon entrance into plant systems (Zhang et al., 2002). An initial reduction of arsenic is believed to occur directly after uptake of arsenic into plants and before transportation of arsenic to the shoots of the plant (Webb et al., 2003), although arsenic has the tendency to primarily accumulate in roots and has a low rate of transport toward other plant parts (Baghour et al., 2001). On the other hand, it has been proven that reduction of As (V) to As (III) can occur during translocation of arsenic from the root to the shoot (Zhang et al., 2002) and can provide a means to transport As (III)

without interference to cell phosphate status (Duan et al., 2005). Furthermore, the presence of As (V) in the roots of exposed plants (Francesconi et al., 2002) and the presence of As (III) in the shoots of exposed plants (Webb et al., 2003) supports the idea that reduction of arsenic does occur at some time after arsenic has entered the plant (Zhang et al., 2002). This reduction is essential to the detoxification process of arsenic in the plant (Zhang et al., 2002).

In a hydroponic investigation, pH and phosphorus were found to have interactive effects on *Pteris vittata*'s ability to accumulate arsenic. Phosphorus inhibited arsenic uptake regardless of the arsenic exposure concentration levels (Tu et al., 2003). Therefore, arsenate is taken into the plant by the phosphate transport system because arsenate and phosphate are chemical analogs (Wang et al., 2002). *Pteris liaurite* L., *Pteris quadriautita* Retz, and *Pteris ryukyuensis* Tagawa increased their uptake of phosphorus into fronds during arsenic exposure (Srivastava et al., 2006). In response to a possible phosphorus deficiency caused by arsenic accumulation, these ferns may have increased their capacity for phosphate uptake by producing additional phosphate transport molecules (Wang et al., 2002).

Arsenate competes with phosphorus for uptake carriers in the root cellular membranes (Meharg et al., 1991). For example, active transport inside the plant depends on the adenosine triphosphate (ADP) complex to provide the energy to move an ion from an area of low concentration to higher concentrations. Arsenate blocks the phosphorylation of the ADP, thus resulting in loss of energy by the plant (Dixon et al., 1958). While As (V) can replace phosphorus, it does not have the ability to duplicate the function of phosphorus in plants; therefore, plants can exhibit phosphorus deficiency

symptoms (Cox, 1995). Furthermore, the reduction of As (V) to As (III) may provide a means of transporting arsenite without interfering with the phosphate status of cells (Duan et al., 2005) because the toxicity of arsenic does depend on the species of arsenic available to and inside of plants (Tamaki et al., 1992). Seventy-five percent of arsenic in *Pteris vittata* fronds was in the As (III) oxidation state and the remaining arsenic concentration was As (V) (Lombi, 2002). Another experiment indicated, *Pteris vittata* with concentrations of ~10,000 ug/g had an arsenic sulfide (As_2S_2) concentration of 6 $\pm$ 2%, as well as, 94% $\pm$ 2% aqueous As (III) in fronds. Therefore, it is possible arsenic coordination with sulfur is attributable to drying of plant resulted in the arsenic changing from oxidation state (+3) to (+5) (Webb et al., 2003). In *Pteris vittata* containing accumulations of 1000 ug/g arsenic, young, old, half dried and fully dried fronds mainly contained arsenic (III); however, regardless of the age or condition of the fronds, no detectable amounts of arsenic coordination with sulfur were found (Webb et al., 2003).

2.6 Effects of arsenic on plant health

Arsenic's effect upon plant growth has been demonstrated in rice, tomatoes and smooth cordgrass (Clemens et al., 2002). Arsenic toxicity in rice plants causes symptoms of straighthead disease, such as lack of grain development resulting in the mature heads of the rice to remain in an upright position (Han, 2005). Tomatoes exposed to 50 and 100 mg/kg of arsenic exhibited a decrease in vegetative and root system growth (Miteva, 2002). Cordgrass root, shoot, and total dry matter production was significantly increased after arsenic exposure (Carbonell-Barrachina, 1998).

In *Pteris vittata,* arsenic concentrations below 334 uM in hydroponic solution showed a benefit to plant growth and a benefit to phosphorus uptake (Tu et al., 2003). A

range of arsenate additions to soil up 100 mg/kg were shown to stimulate an increase in fern biomass by 64 to 107% (Tu et al., 2002). The fern exhibited a relatively high biomass at low pH/low arsenic levels or high pH/high arsenic levels (Tu et al., 2003). The addition of 50 mg/kg of arsenic to the soil resulted in the highest fern plant biomass production (3.9 g / plant), highest ratio of shoot to soil arsenic concentrations (bioaccumulation factor = 63), and highest ratio of shoot to root arsenic concentrations (Translocation factor = 25) (Tu et al., 2002).

Arsenic incorporation into the structure of cellular components is one of the primary causes of inorganic arsenic toxicity (Wang et al., 2002). As (V) decouples phosphorylation in the mitochondria and in seed germination (Kabata-Pendias, 2001). Once reduction of arsenate occurs, As (III) reacts with sulfydryl to produce thiol complexes, degrades cell membranes and eventually causes death of cells (Peterson et al., 1981). Similar to phosphorus deficiency symptoms, arsenic toxicity symptoms include central pith cells becoming disintegrated, large and succulent, thin-walled or developing large intercellular spaces. In addition, phloem and xylem elements become thin-walled and vascular tissues display minimum development (Delvin et al., 1983).

Visual indications of arsenic toxicity are wilting of leaves, necrosis around the margin of leaves, discoloring of roots and plasmolysis of roots. All of these symptoms indicate that movement of water into the plant is reduced (Carbonell-Barrachina et al., 1998) and metabolic activity is affected (Cox, 1995). Overall, plants grown under the stress of elevated concentrations of metals will have an alteration in morphology (Kabata-Pendias, 2001) and the accumulation of high concentrations of metals may result in

irreversible damage to the plant and eventually may causes the death of the plant (Barocsi et al., 2003).

2.7 Characteristics of plants used in this study

Thelypteris palustris, the common marsh fern, is native to the eastern United States (United States Department of Agriculture Natural Resources Conservation Service, 2006) and it is commonly found in bogs or swampy stream banks (Tryton, 1971). Worldwide, the marsh fern inhabits a wide geographic range. In addition to being found in North America, the marsh fern can be found in Africa from the Kenya to Cameroon (Tryton, 1971). Although arsenic hyperaccumulating ferns have been reported (Ma et al., 2001) (Srivastava et al., 2006), no reports have been made on the ability of *Thelypteris palustris* to accumulate arsenic into its above ground parts or its ability to tolerate arsenic. Therefore, this study of the marsh fern will be the first to document its ability to tolerate and accumulate arsenic.

Asparagus sprengeri (asparagus fern) thrives in coastal areas consisting of rocky and woodland terrain (Ellison, 1995). The asparagus fern is not a true fern, but a member of the lily family and member of the same genus as edible asparagus (Reid, 2004). The root system of asparagus fern is thick and tuberous (Muttart Conservatory, 2005); therefore, the roots function in the underground accumulation of food (Stern, 1994). The asparagus fern has been documented to accumulate 1150 ug/g of arsenic with the assistance of chelates (Bagga et al., 2001), but no research on arsenic uptake without chelates or the uptake mechanisms in asparagus fern has been published.

Lolium perenne (perennial ryegrass) is a cool season grass species widely distributed across the United States and is used to establish turf in areas such as

residential areas, parks, athletic fields, and golf courses (Martiniello et al., 2006), Ryegrass is best suited for mild climates because it does not withstand severe winters or hot-dry weather very well. Ryegrass grows best in dark, wet, and well drained surface soils (United States Department of Agriculture Natural Resources Conservation Service, 2004). The fibrous root system of a single mature ryegrass can produce as many as 15 million individual roots and branch roots (Stern, 1994). Ryegrass was found to be able to tolerate 1,230 mg/kg of arsenic in soil and exhibited a 20% reduction in growth after arsenic exposure in comparison to control plants (Bagga et al., 2001). Studies indicate that ryegrass can accumulate arsenic to high levels when mobilization is assisted by chelates; during six weeks of experimental arsenic exposure, ryegrass was found to accumulate an arsenic concentration of 1,200 mg/kg (Bagga et al., 2001). Another grass, *Agrostis tenuis,* has been documented to accumulate arsenic, but is not considered a hyperaccumulator (Visoottiviseth et al., 2002; Cai, 2003). The uptake mechanism of arsenic into grasses has not been documented.

2.8 Review of analytical methods used in this study

A combination of the following complementary analytical methods was used in this study: (1) Scanning electron microscopy (SEM) was used to observe the morphological changes that occur in plants after exposure to arsenic; (2) inductively coupled plasma mass spectroscopy (ICP-MS) was used to determine arsenic concentrations and (3) X-ray absorption near-edge spectroscopy (XANES) was used to determine the oxidation states of arsenic contained in samples.

Scanning electron microscopy (SEM) provides topographical and elemental information at a magnification range of 10x – 100,000x with a virtually unlimited field of

depth (PhotoMetrics Inc., 2005). Samples are bombarded with an electron beam to produce a variety of signals. This study employed secondary electron detection, which can produce images of the topography of samples just a few nanometers across (Goldstein et al., 1992) (Figure 1). SEM was chosen as an analytical method for this study because it is a valuable technique for the visualization of structural alteration (Blaszczak et al., 2005). SEM images have been used to examine various morphological effects, such as changes in stomata openings of *Phyllanthus amarus* after cadmium exposure (Rai et al., 2005), crystalline structures of copper in leaves of the creosote bush (Aldrich et al., 2003), and margins of the pinnate of *Pteris vittata* to examine arsenic toxicity symptoms (Lombi, 2002). SEM has also been used to investigate areas of necrosis on potato tubers following bacterial infection (Blaszczak et al., 2005).

Inductively coupled plasma-mass spectrometry (ICP-MS) is a powerful analytical tool for the analysis of trace metals. ICP-MS combines the power of inductively coupled plasma with a mass analyzer (Goessler et al., 2002). The argon gas plasma atomizes and ionizes elements in samples before they enter the mass analyzer. After elements enter the mass analyzer, elements are identified by their individual mass to charge ratios (University of Missouri Research Reactor Center, 2003). The concentration of elements in the sample is proportional to the intensity of the elemental peaks detected by the mass analyzer (University of Missouri Research Reactor Center, 2003). ICP-MS is a sensitive, accurate and precise tool for analysis of multiple elements at once (Story et al., 1992). ICP-MS has detection limits in the ppb to ppm concentration range (University of

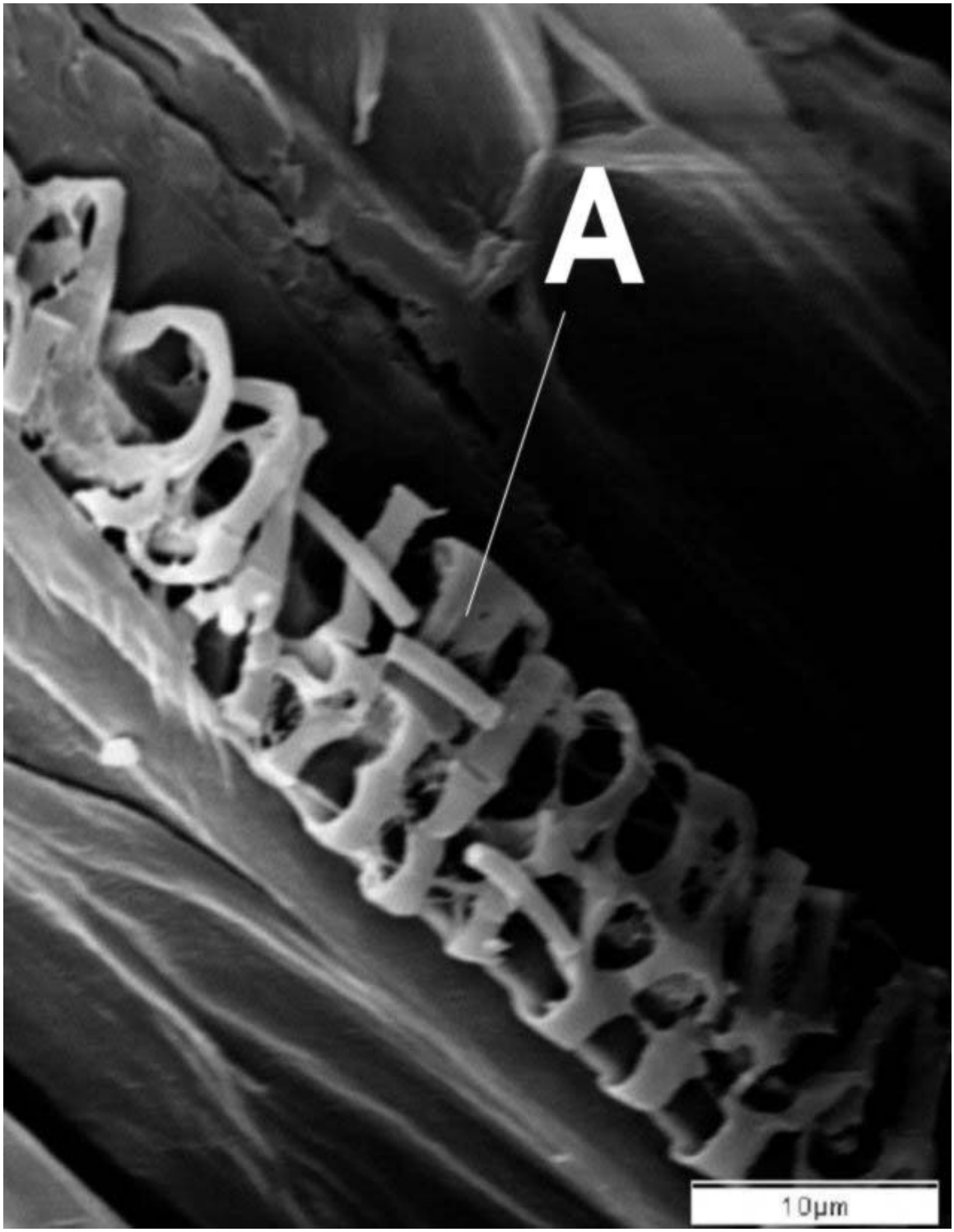

Figure 1: Example of a secondary electron image produced by SEM. This is an image of the tracheary elements (A) in the xylem of the asparagus fern.

of Missouri Research Reactor Center, 2003) and can detect arsenic at very low concentration levels (Pongratz, 1998). ICP-MS has been used in studies investigating the total concentration of arsenic in the xylem of cucumbers (Mihuez et al., 2005), and arsenic uptake in sunflowers (Rabb et al., 2005).

X-ray absorption near edge structure (XANES) can be used to characterize oxidation states, atom types, arrangements of elements contained in samples and probable chemical environments surrounding the element of interest in samples (Smith et al., 2005). X-ray absorption near edge structure refers to the absorption edge region of the sample at an electron volt (eV) range estimated to be 50ev below and above the absorption edge (McNear et al., 2005). Oscillations occur when X-rays directed at a sample have sufficient energy to free or excite a bound electron within a sample (Kirkman, 1999). The small oscillations that appear on the edge step of the spectrum graph are a visual indication of the XANES information in the sample (Kirkman, 1999) (Figure 2). XANES is an attractive alternative to the use of traditional non-physiological extraction techniques to determine metal speciation that may cause an alteration of metal species contained in the sample (Lombi, 2002) and chemical complexes in samples may not to be preserved (Zhao et al., 2003). Moreover, traditional methods are time consuming and indirect (Smith et al., 2005), while XANES does not require extensive sample preparation (McNear et al., 2005) and can be used on both liquid and solid samples (Smith et al., 2005). Unlike strong acid or methanol/water extraction methods, XANES can analyze specific elements and determine specific oxidation states (Smith et al., 2005). XANES can be used to evaluate the movement of metal and metalloid ions in plant cells (Pickering et al., 2000) , to evaluate the speciation of metals contained in plant

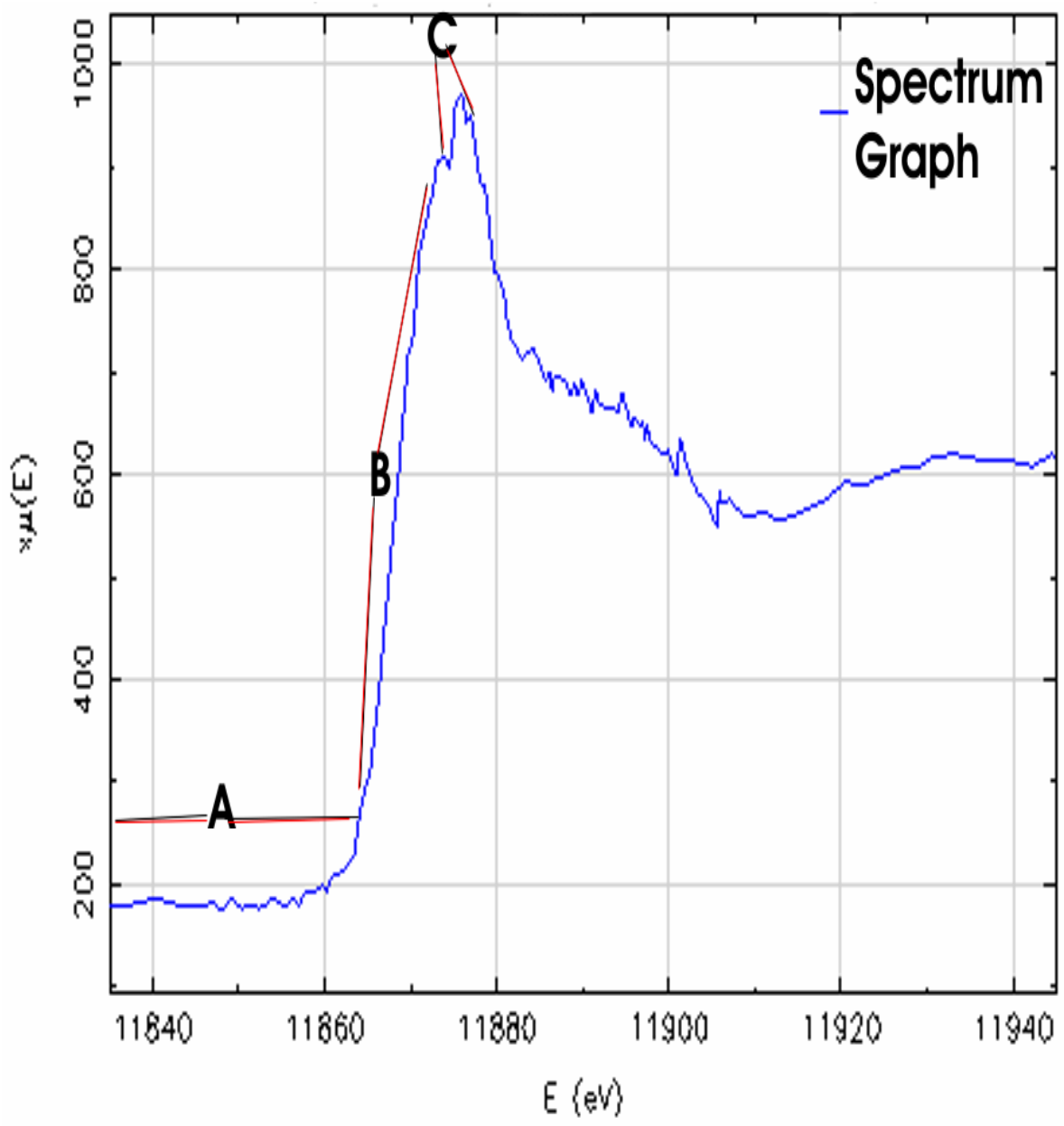

Figure 2: Sections of XANES spectrum graph. A= pre-edge region (before absorption of energy from the beam). B=absorption edge (interaction with electron beam and energy is released) and C= XANES oscillations.

parts (Pickering et al., 2006) to a detection level of 20g/kg (Cornelis, 2005), and to evaluate the potential of plant species for phytoremediation (Tu et al., 2002). For example, XANES was used to investigate the mechanisms of cadmium uptake in mesquite plants (Aldrich et al., 2003), to investigate the speciation of arsenic contained in plant parts of the known arsenic accumulator, *Pteris vittata* (Webb et al., 2003), and to investigate the speciation of arsenic contained in the xylem sap of cucumbers after exposure to arsenic (Mihuez et al., 2005).

CHAPTER 3
MATERIALS AND METHODS

Greenhouse studies were conducted using hydroponic and soil cultivation to examine the effect of arsenic exposure on marsh fern, asparagus fern, and perennial ryegrass. All plants, except for the marsh fern in the large scale hydroponic study, were placed into the hydroponic experiments while still potted in their growth media. Arsenic treatment levels for each study were accomplished by the addition of potassium arsenate to the hydroponic solution. The arsenic treatment levels used in the experiments were based on previous hydroponic studies of wetland plants (Carbonell et al., 1998) and hyperaccumulating plants (Ma et al., 2001). ICP-MS, SEM and XANES analysis were performed. These analytical methods were adapted from previous studies of plants (Tryton, 1971; Rabb et al., 2005; Webb et al., 2003).

3.1 Acquisition of plant materials

Two hundred marsh fern *(Thelypteris palustris*) root cuttings were purchased through Southern Tier Consulting and Nursery of West Clarksville, New York, and 24 mature marsh ferns were purchased through Crownsville Nursery of Strasburg, Virginia. Both the marsh fern root cuttings and the mature marsh ferns were placed into ½ gallon plastic pots and cultivated in greenhouse space. Growth media for the root cuttings was mixed at the rate of 5 oz of Osmocote ® extended time release fertilizer per cubic foot of Scotts® Metrol Mix 300 potting soil. Mature marsh ferns were placed into a growth media which consisted of a mixture of 5 oz of Osmocote® per cubic foot medium grade vermiculite. Vermiculite was used because it is not chemically reactive. Landscaping

fabric obtained from Lowe's ® Home Improvement Store in Baton Rouge, Louisiana, was used to line the inside of these pots to reduce the loss of growth media through their underside openings.

Twenty-four asparagus ferns *(Asparagus sprengeri)* were purchased from Clegg's Nursery of Baton Rouge, Louisiana. The roots of the plants were pressure-washed to remove the potting soil growth media used by the supplier. The asparagus ferns were then potted into ½ gallon plastic pots containing growth media mixed at a rate of 5 oz of Osmocote ® per cubic foot of medium grade vermiculite. The undersides of the plastic pots were covered with cheese cloth to reduce the loss of vermiculite through the underside openings.

Pennington ® Fairway Classic perennial ryegrass seeds were purchased from Lowe's ® Home Improvement Store in Baton Rouge, Louisiana. Twenty-four ½ gallon plastic pots containing a growth medium mixture of 5 oz of Osmocote® per cubic foot of medium grade vermiculate were seeded with 8 oz of ryegrass. The ½ gallon pots were lined with landscape fabric to prevent the loss of growth media.

3.2 *Thelypteris palustris* experiments

One preliminary study and two hydroponic studies were performed to evaluate the ability of *Thelypteris palustris* (marsh fern) to accumulate arsenic. Potassium arsenate (KH_2AsO_4) was dissolved in the watering solution for the preliminary studies and the nutrient solution for the hydroponic studies. Arsenic treatment levels for all *Thelypteris palustris* studies were 0 (control), 250, and 500 ug/L.

In order to test how quickly arsenic is taken into the marsh fern, a preliminary non-replicated experiment was conducted with stock marsh ferns that were not previously

exposed to arsenic. Three plants propagated from root cuttings were exposed to one of the three treatment levels (0,250, and 500 ug/L) by manual overhead of watering cans containing arsenic solution. One frond sample from each plant was harvested at 0, 24, and 48 hours, respectively.

A large scale hydroponic experiment was performed using 36 marsh ferns placed into a thirty-six unit gravitational nutrient fed hydroponic system. The hydroponic system was set up in a Randomized Complete Block Design (RCB) consisting of 36 marsh ferns, four blocks, three cells per block, three replications per cell and three arsenic treatment levels randomized by cell in each of the blocks (Figure 3). Approximately 125 liters of water was used for each hydroponic arsenic treatment level 0, 250, and 500 ug/L, respectively. Foxfarm ® GrowBig Hydroponic Concentrate (3-2-6) hydroponic solution was added at 15 ml per 3.8 L of water. The growth media of the ferns was removed and the ferns were permitted to float in the solution. At four weeks, fiddleheads were counted and plants were harvested. The limitation of this study was that the fronds of the ferns died before the termination of the experiment as the result of an insect infestation. Therefore, only the roots of the marsh ferns from this study were analysed. Oven-dried samples were taken from this study for the determination of the speciation of arsenic following arsenic exposure.

A small-scale hydroponic study was initiated to investigate the accumulation of arsenic in the fronds of the marsh fern. Twelve marsh ferns were placed into plastic dishpans filled with four liters of arsenic solution (Figure 4). Aquarium air pumps were inserted into the arsenic solution in each dishpan to aerate the solution and to decrease algae growth. Approximately one week after exposure, the arsenic solution was changed

and the plastic dishpans were cleaned to remove any algae formation. After two weeks of arsenic exposure, plants were harvested and separated into above-ground and below-ground biomass. Fresh frond samples were taken from this study to determine the speciation of arsenic after accumulation.

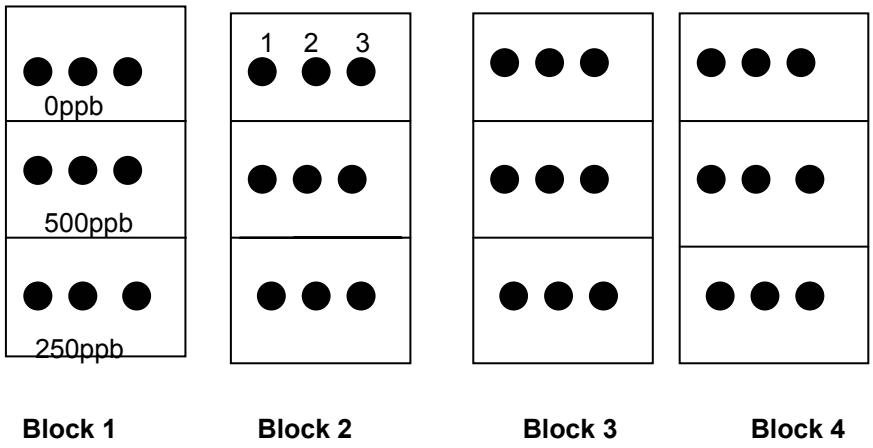

Figure 3: Large scale hydroponic setup for the marsh fern *(Thelypteris palustris)*. 0, 250, and 500 ug/L were the treatment levels and 1, 2, and 3 represent the number of plants per treatment level in each of the four blocking sections, respectively.

The reproductive system components (sporangia, spore mother cells, and roots) of a healthy stock marsh ferns that had not been exposed to arsenic were evaluated to establish baseline reproductive system measurements for the marsh fern. A second hydroponic setup consisting of one fern per arsenic treatment levels of 250 and 500 ug/L was initiated to evaluate morphological changes in reproductive system components after arsenic exposure. However, only roots from the 500 ug/L treatment level were evaluated because roots exposed to 250 ug/L were destroyed during the freeze-drying process. Frond samples were taken from ferns in the small-scale hydroponic study to examine morphological changes in the vascular system after arsenic exposure.

Figure 4: Small-scale hydroponic experiment for the marsh fern *(Thelypteris palustris)*. Twelve plastic pans containing one marsh fern each (n=12) and 4 liters of hydroponic solution were arranged in a randomized complete block design consisting of four replications and three treatment levels (0,250, and 500 ug/L).

3.3 *Asparagus sprengeri* experiments

One small scale hydroponic study was performed using *Asparagus sprengeri* (asparagus fern) to evaluate the accumulation of arsenic into fronds. Arsenic treatment levels of 0, 250, and 500 ug/L were produced by the addition of potassium arsenate to the hydroponic solution. The hydroponic system setup consisted of 12 plastic dishpans containing two liters of hydroponic solution and 12 repotted asparagus ferns arranged into a randomized complete block design (Figure 5)**.** Air pumps were inserted into the two liters of hydroponic solution of each dishpan to reduce algae growth. At one week of arsenic exposure, the hydroponic solution evaporated and fresh hydroponic solution was placed into the dishpans; in addition, the plastic dishpans were cleaned to remove algae.

After two weeks of arsenic exposure, ferns were harvested and separated into fronds and stems. Stem, leaf, and roots samples were taken from this study for evaluation of the morphological effect of arsenic upon the vascular system of the asparagus fern.

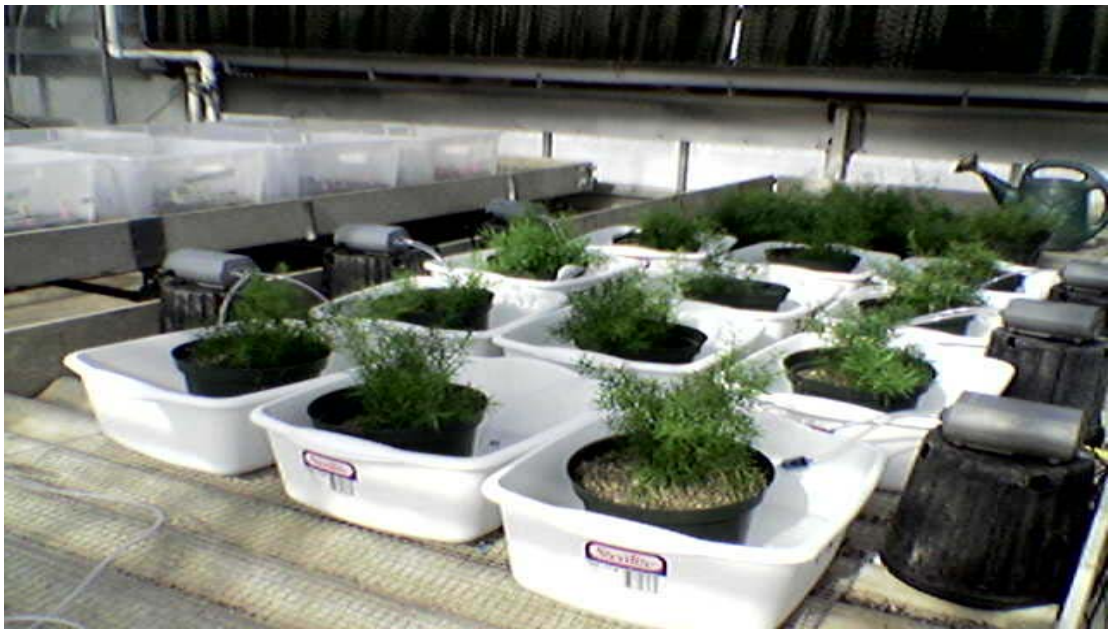

Figure 5: Small-scale hydroponic setup for the asparagus fern *(Asparagus sprengeri)*. Twelve plastic pans containing two liters of hydroponic solution and one plant (n=12) each were arranged into a randomized complete block design with three arsenic treatment levels (0, 250, and 500 ug/L) and four replications of each treatment level.

3.4 *Lolium perenne* experiments

A small-scale hydroponic study was conducted with twelve ½ gallon pots seeded with 8 oz of Lolium *perenne* (ryegrass). The pots were placed into 12 plastic dishpans that contained two liters of hydroponic solution that were arranged into a randomized complete block (Figure 6). Ryegrass was exposed to the arsenic treatment levels (0, 1800, and 2500 mg/L) for five days. After five days of exposure, the blades of the ryegrass were harvested. Samples to evaluate the morphological effect of arsenic upon the vascular system of ryegrass were collected from this study.

A hydroponic setup consisting of a single ½ gallon pot seeded with ryegrass was placed into a plastic dishpan containing two liters of hydroponic solution containing 4500 mg/L of arsenic. An aquarium pump was inserted into the hydroponic solution to decrease the formation of algae. Samples used for the determination of arsenic speciation were collected after 8, 9, and 10 days of exposure.

Figure 6: Small scale hydroponic study experimental set up for ryegrass *(Lolium perenne)*. Twelve plastic pans contained two liters of hydroponic solution and one ½ gallon plastic pot of seeded ryegrass. The plastic pans were arranged into a randomized complete design with three arsenic treatment levels (0, 1800, and 2500 mg/L) and four replications of each treatment level

3.5 Analytical methods

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was used to determine the total amount of arsenic accumulated in plant tissues. In preparation for ICP-MS analysis, all plant samples, except for samples from the marsh fern large scale study, were placed in a subzero freezer for four days. Samples from the marsh fern large scale study were oven-dried for 24 hours at 65^{o}C and then ground into a homogenous mixture. After samples were placed into a subzero freezer or oven-dried and grounded, they were

digested in an Anton Par Instruments ® Multiwave 3000 in accordance with EPA Method SWA 846-3052 (United. States Environmental Protection Agency, 1986). After sample digestion, total arsenic concentration of the samples was determined using a Perkin Elmer ® Elan 9000 ICP-MS.

Scanning electron microscopy (SEM) was used to investigate the effects of arsenic exposure on the vascular system of exposed plants. Freeze-dried samples were mounted on a 25x25 mm SEM stub and gold sputter-coated more than once to reduce the possibility of charging upon exposure to the electron beam. Secondary electron images of plant vascular system cross-sections and morphological features were produced using a JOEL ® 840A SEM. Analysis of secondary electron images was accomplished with IMAGEJ software (Rasband, 2003).

Fresh samples and freeze-dried samples of all three plants were analyzed by x-ray absorption near edge structure for the determination of the species of arsenic contained in exposed plant tissues. First, samples were placed between two layers of kapton tape. Next, XANES spectra for each sample were produced by using a synchrotron double-crystal monochromator beamline to determine the oxidation states of arsenic in the samples. Arsenic (0) and sodium arsenate were used as calibration standards for the beamline. Athena ® extended x-ray absorption fine structure software (Ravel, 2001-2006) was used for the analysis of XANES scans. If spectra sample data were collected less than 200 eV about the absorption edge of the sample, the data were normalized using the Cromer-Liberman (CL) normalization function instead of the autobk normalization function. Autobk attempts to remove components in the sample spectra attributed to background interruptions to normalize data; while CL uses information of the absorbing

atom to normalize sample spectra data. In addition, CL is effective when the XANES spectra do not have a large range of data. Athena ® is capable of fitting a linear combination of standard spectra (Table 1) to unknown sample spectra. For this study, the linear combination fit all combinations option was used to determine the fractional contribution of the standard spectra to the fit of the sample. The fractional contributions are equivalent to the fractional amounts of each element and compound in the sample (Pickering et al., 2006). The combination with the lowest R-value represents the best linear combination fit to the sample spectrum (Ravel, 2001).

Table 1: Arsenic Standards for Athena ® linear combination fit

Arsenic Standards
As (0)
As (III)
As (III) sulfate
As (V)
As (V) oxide
As2S2 (arsenic sulfide
Na2HAs04 (sodium arsenate)
75% As (V)/25% As (III)
94% As (III) oxide/6% As2S2

3.6 Bioaccumulation and translocation factors

The bioaccumulation factor of a plant for a given metal is the ratio of the compound in the above-ground plant parts in relation to the amount of metal in the growth medium. The translocation factor is the ratio of its concentration in the above-ground parts in comparison to its concentration in the roots (Wei et al., 2006). Translocation factors are used to determine the effectiveness of a plant in the translocation of metals from the roots to the shoots (Tu et al., 2002). Both the

bioaccumulation factor and the translocation factor should be considered when investigating whether a plant is a hyperaccumulator of a metal (Cruse et al., 1972).

Bioaccumulation Factors and Translocation Factors were calculated as follows:

A. Bioaccumulation Factor = Concentration of metal in shoots/concentration of metal in exposure media

B. Translocation Factor = Concentration of metal in shoots/concentration of metal in roots

Mean arsenic accumulation concentrations determined by ICP-MS analysis were used to calculate mean bioaccumulation factors for all three plants studied. Translocation factors were calculated for the asparagus fern only; above- and below-ground plant materials from the same experiment were not available for the other two plants. Bioaccumulation factors >1 indicate the plant is an "accumulator", <1 indicate the plant is an "excluder" (Baker, 1981), and greater than >10 indicate a plant has the potential to be a "hyperaccumulator" (Ma et. al, 2001). Translocation factors > 1 indicate the plant is effective in the translocation of a metal from its root to the shoot (Ma et al. 2001).

3.7 Plant health assessment

Plant health indexes were established to document plant response to arsenic exposure. A significant decline in plant pigmentation with increasing metal levels can be an indication of arsenic toxicity and moreover, a lack of adaptability to high arsenic levels (Miteva, 2002). Photographs of the marsh ferns and perennial ryegrass from their respective small scale studies were taken in order to visually characterize plant health (Figure 7, 8). A ranking of 1= healthy, 2=slightly necrotic, and 3= severely necrotic.

Figure 7: Marsh fern (*Thelypteris palustris)* plant health index. 1= healthy, 2 = slightly necrotic and 3 = severely necrotic.

Figure 8: Ryegrass (*Lolium perenne)* plant health index. 1 = healthy, 2 = slightly necrotic, and 3 = severely necrotic.

3.8 Statistical analysis

Microsoft ® Excel software ANOVA one-way tool with replication option was used for data analysis of the large scale marsh fern hydroponic study. All other hydroponic study data analyses were performed using the ANOVA one way tool without replication option. For hydroponic studies, the statistical significance of means was determined by Least Significance Difference Test (LSD) at a 5% alpha. The significance of vascular system cross-sections for all exposed plants and the significance of asparagus root accumulation vs. frond accumulation were analyzed by the Microsoft ® Excel software option of two sample Z-test with a 5% alpha level.

CHAPTER 4
THELYPTERIS PALUSTRIS
RESULTS AND DISCUSSION

4.1 Arsenic accumulation

After 48 hours of arsenic exposure, marsh fern exposed to 250 and 500 ug/L in the preliminary study did not have a significantly greater uptake of arsenic than control plants (Table 2). Fiddlehead production was also not significant in marsh fern after 48 hours of arsenic exposure. Marsh fern exposed to 500 ppb exhibited the highest bioaccumulation factor (4.94).

Table 2: Arsenic concentration in fronds, bioaccumulation factors, and fiddlehead production after 48 hours of exposure in the preliminary uptake study

Arsenic Treatment (ug/L)	Arsenic in Frond Mean ± SD (ng/g)	Range of Arsenic in fronds (ug/g)	Mean BF	Fiddlehead Production Mean ± SD
0	50 ± 0.04 a	10.2-97.9	0.00	1.67 ± 0.58a
250	640 ± 0.47 a	111.0-1010.0	2.56	4.33± 0.58 a
500	2470 ± 2.87 a	517.0-5770.0	4.94	4.33± 0.58 a

Means followed by the same letter are not significantly different at 5% alpha. BF= bioaccumulation factor.

The marsh fern exposed to 500 ug/L in the preliminary study displayed the highest arsenic accumulation range (1120 to 5770 ng/g) and the highest bioaccumulation factor (4.94). Marsh ferns exposed to 250 and 500 ug/g b arsenic treatment levels were found to have the largest average fiddlehead production over 48 hours of exposure (4.33).

Marsh fern roots exposed to 500 ug/L in the large scale hydroponic study were found to have significantly different arsenic accumulation concentrations from the control plants (0 ug/L) and fern roots exposed to 250 ug/L. Marsh fern roots that were exposed to 250 ug/L displayed the highest average fiddlehead production (7.25) (Table 3).

In the small scale hydroponic study, no significant difference was found in the ability of the common marsh fern to accumulate arsenic into its fronds (Table 4). Exposed ferns displayed bioaccumulation factors > 1. Ferns exposed to 500 ug/L had a bioaccumulation factor of 96.68. Arsenic exposure had adverse effects upon the qualitative plant health of the marsh fern. Control ferns and ferns exposed to 250 ug/L had an average plant health ranking of 1.25 (healthy) and ferns exposed to 500 ug/L exhibited an average plant health index ranking of 2.25 (slightly necrotic) (Table 4).

Table 3: Fiddlehead production and arsenic concentrations in the roots of marsh fern after four weeks of exposure in the large-scale hydroponic study.

Arsenic Treatment (ug/L)	Fiddleheads Produced	Range of Fiddleheads Produced	Arsenic in Roots Mean +/- SD (ng/g)	Range of Arsenic in Roots (ng/g)
0	2.83 +/- 4.00 a	0-13	250 +/- 2100 a	0-3300
250	7.25 +/- 4.17 a	2-16	29500 +/- 21400 a	2800-76500
500	2.25 +/- 2.89 b	0-10	54700 +/- 3930 b	2800-2255750

Means that are followed by the same letter are not significantly different at 5% alpha.

4.2 Plant response to arsenic exposure

Images of the reproductive system structures of a healthy marsh fern that were not exposed to arsenic were used to establish baseline reproductive measurements. Mean reproductive structure measurements for the healthy unexposed marsh fern were 0.23 um (sporangia), 41.6 um (spores), and 218.9 um (root vascular system cross-sections). Sporangia baseline mean measurements displayed the least amount of variations in baseline measurements (Table 5). Marsh fern roots exposed to 500 ug/g were found to have significantly different cross-sectional vascular system measurements from the roots of the healthy unexposed marsh fern. The size of the spores of the healthy unexposed marsh fern did not significantly differ from the size of the spores found on marsh ferns

exposed to 250 or 500 ug/g (Table 5). However, the outer layer of spores in exposed ferns exhibited an altered morphology (Figure 9). Vascular cross-sectional diameters of the marsh fern stems exposed to 250 and 500 ug/g were not significantly different from the control (0 ug/g) (Table 5). Examination of the vascular system cross-sections of the stems of the marsh fern fronds with SEM showed that the stem ferns exposed to 500 ug/g exhibited vascular collapse and degradation (Figure 10).

Table 4: Accumulation of arsenic in the fronds, bioaccumulation factors and plant health index rankings of marsh fern after two weeks of exposure in the small-scale hydroponic study.

Arsenic Treatment (ug/L)	Arsenic in Fronds Mean +/ - SD (ng/g)	Range of Arsenic in Fronds (ng/g)	Mean BF	Mean PH Ranking
0	`8330 +/- 13100`a	5360-50230	0	1.00
250	176000 +/- 4000`a	106090-185800	70.72	2.25
500	483000 +/- 47800a	40210-112500	96.68	2.25

Means that are followed by the same letter are not significantly different at 5% alpha. BF= bioaccumulation factor and PH = plant health. 1= healthy, 2=slightly necrotic, and 3= severely necrotic.

4.3 Arsenic species

Arsenate (+5) was found to be the dominant arsenic species contained in the oven-dried roots of the marsh fern that were exposed to 250 and 500 ppb in the large-scale hydroponic study. One marsh fern root sample that was analyzed did not contain arsenate in detectable amounts (Figure 11).

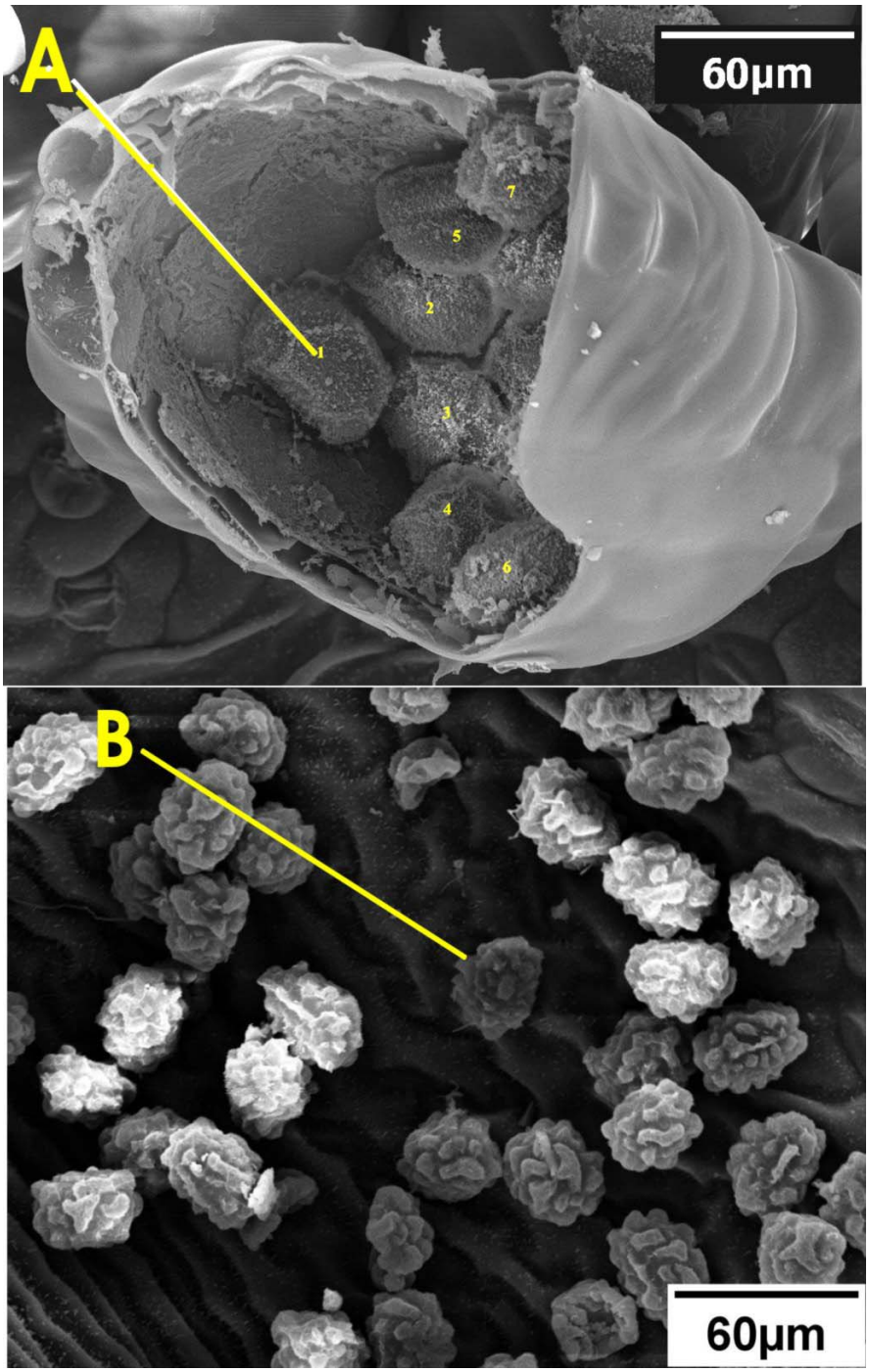

Figure 9: SEM micrographs showing changes in the outer layer of spores after arsenic exposure. A= healthy spores and B = spores exposed to 500 ug/L arsenic. The yellow line from the letter is showing the location of a single spore.

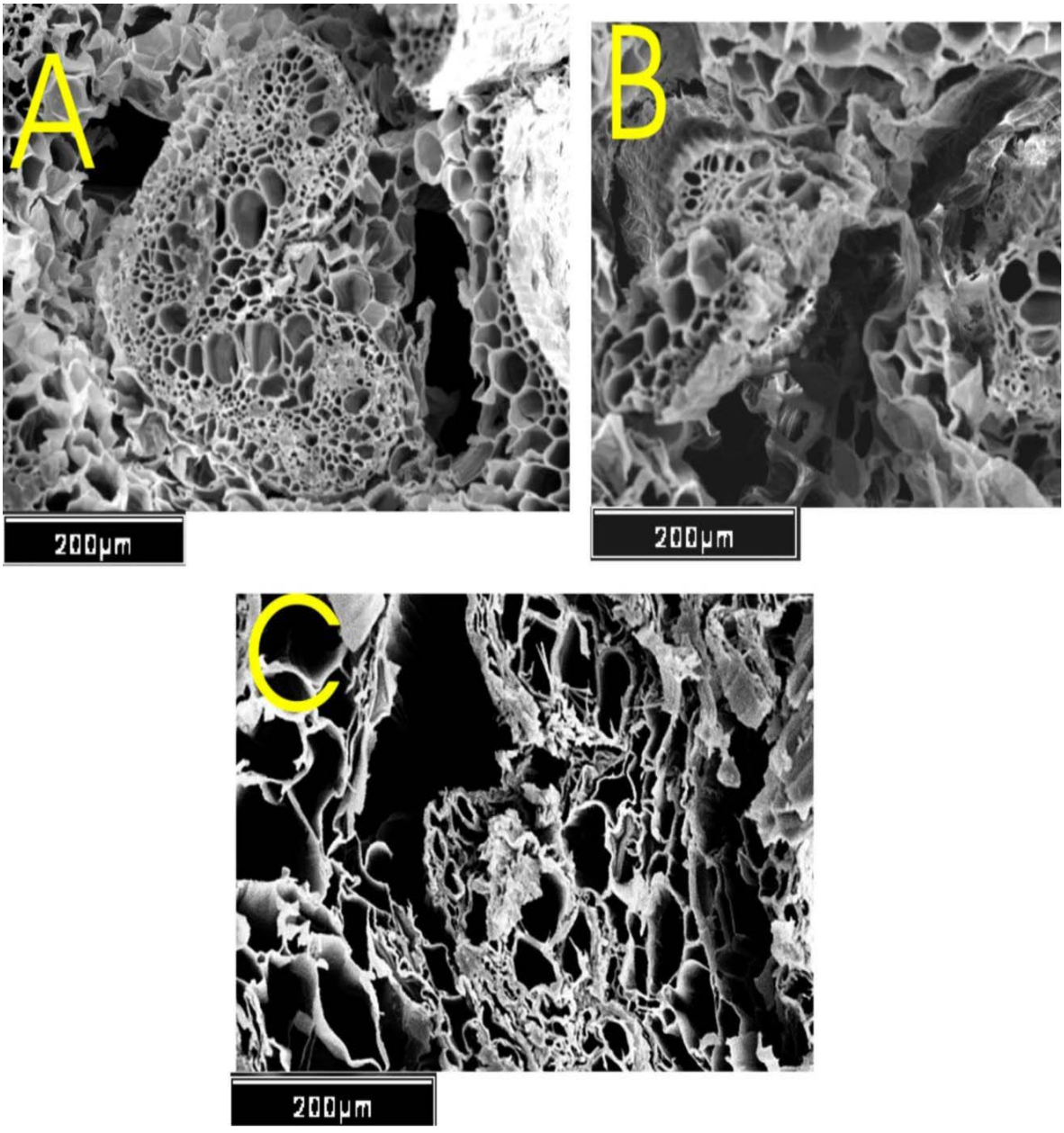

Figure 10: SEM micrographs of the cross-sections of the vascular system in the stem of the marsh fern. A= Fern exposed to 0 ppb arsenic (control) B= Fern exposed to 250 ug/L arsenic, C= Fern exposed to 500 ppb arsenic.

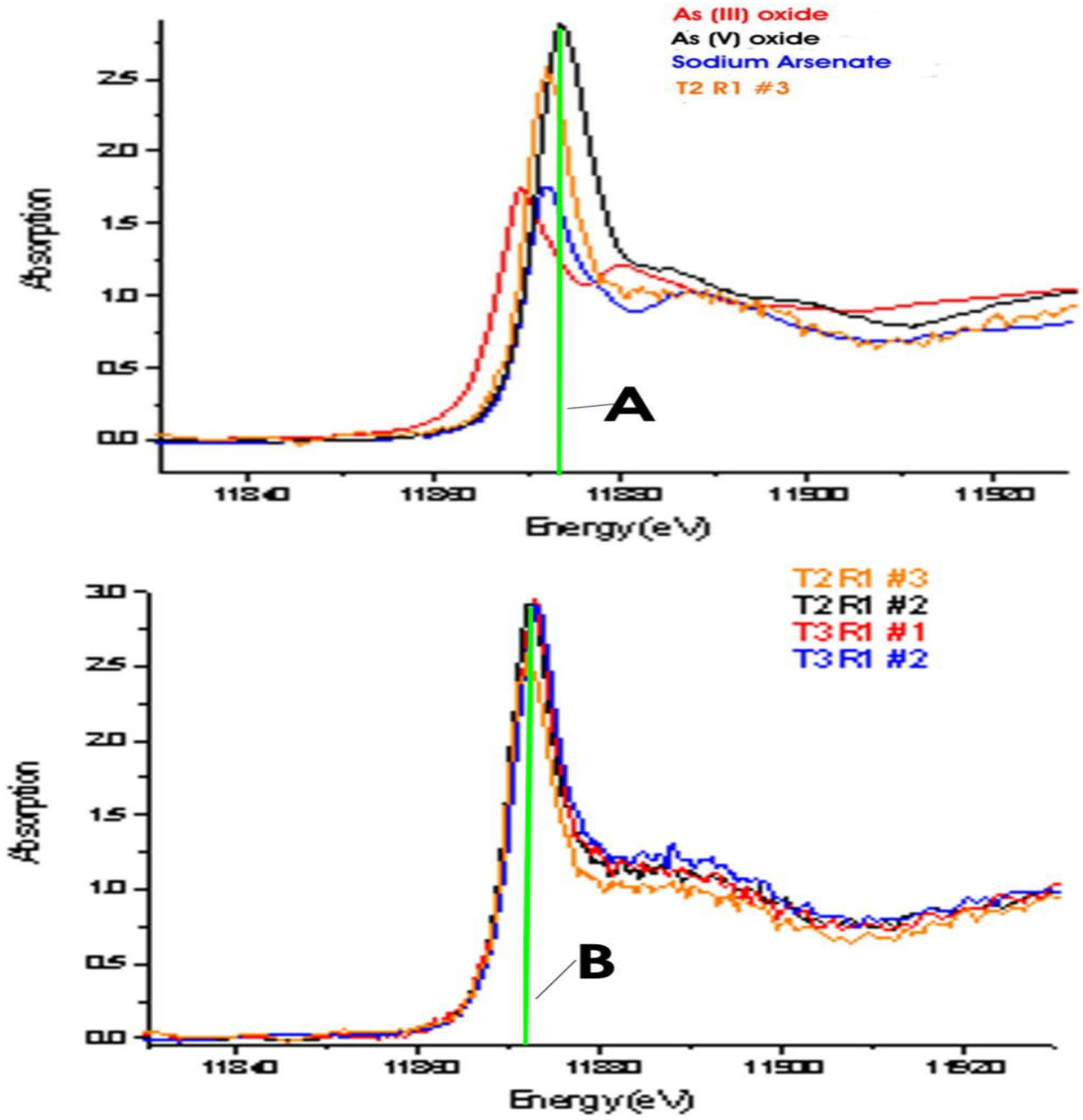

Figure 11: Qualitative analysis of the oxidation states in oven-dried marsh fern roots by comparing the XANES spectrum (B) absorption to the arsenic standard XANES spectrum (A) absorption of the following: As (III) Oxide (red line), As (V) oxide (black line), and sodium arsenate (blue line). The majority of samples had similar absorbance to As (V) oxide; therefore As (V) is the dominant oxidation state of arsenic contained in these samples.

The concentration of arsenic contained in the fronds taken from the small scale hydroponic study was below the detection limits of the XANES analytical method so the speciation of arsenic contained in these frond samples was not determined.

Table 5: Marsh fern plant measurements as determined by SEM

Arsenic Treatment (ug/g)	Sporangia (um)	Spores (um)	Root Vascular Cross-Sectional Diameter (um)	Stem Vascular Cross-Sectional Diameter (um)
0	0.23 ± 0.001	41.62 ± 5.22 a	218.9 ± 5.22 a	23.54 ± 14.43 a
250	*	32.72 ± 13.95 a	*	22.55 ± 18.51 a
500	*	30.46 ± 3.76 a	30.46 ± 3.76 b	41.76 ± 19.88 a

Means followed by the same letter are denoted as not significantly different at 5% alpha.
*Statistical analysis of sporangia at higher levels were not analyzed because the release of spores in exposed plants occurred before SEM analysis and the vascular system cross sections of roots exposed to 250 ug/g were not analyzed because they were destroyed during the free-drying process.

4.4 Discussion

The results of this study indicate that the marsh fern have the capability to accumulate arsenic in the roots and fronds at higher levels than in the growth medium. Bioaccumulation factors for marsh fern fronds (70.72-96.68) lie within the range of documented bioaccumulation factors of the Chinese brake fern (10-120) after exposure to soil arsenic levels less than 400 mg/kg. In addition, bioaccumulation factors of the marsh fern exceed the combined bioaccumulation factors range of three recently reported arsenic accumulators, *Pteris quadriarita, Pteris cretica*, and *Pteris ryukyuenisis* (17.7-36.5) (Ma et al., 2001; Chen et al., 2002;Tu et al., 2002). High bioaccumulation factors can be an indication of strong phytoremediation potential (Wei et al., 2006).

Arsenic treatment levels had a significant effect upon arsenic accumulation in the roots, but did not have a significant effect on the accumulation of arsenic in the fronds of marsh fern. These findings differ from the results of studies using other ferns. For example, arsenic concentrations in the ladder brake fern fronds increased as more water-soluble arsenic became available to the plant (Tu et al., 2002) and concentrations of arsenic in both fronds and roots of *P.vittata*, *P.cretica*, *P.longfoila* and *P.umbrosa* increased linearly with increasing additions of substrate arsenic concentrations (Zhao, 2002). Therefore, the significant accumulation of arsenic into the roots of the marsh fern and the non-significant arsenic accumulation in fronds are maybe the result of small amounts of arsenic becoming stored in vacuoles of the root cells and later being released from the vacuoles of back into the back into the plant (Ponyton et al., 2004).

Arsenic exposure does cause necrosis on fronds of the marsh fern. The replacement of phosphorus by arsenic in the plant causes symptoms of phosphorus deficiency (Cox, 1995). Therefore, arsenic toxicity and phosphorus have similar symptoms, such as necrosis (Delvin et al., 1983). Arsenic toxicity causes necrosis on leaf tips and margins and limits the movement of water into the plant (Carbonell-Barrachina et al., 1998). Arsenic toxicity symptoms are visual indicators of arsenic having an effect upon the metabolism of the plant (Cox, 1995). Arsenic cannot duplicate the functions of phosphorus as an essential plant nutrient within the plant (Carbonell-Barrachina et al., 1998). Arsenic affects plant metabolism by substituting for phosphorus in the ATP (adenosine triphosphate) active transport molecule. The new arsenic substituted molecule causes energy loss by the plant (Dixon et al., 1958) by uncoupling oxidative phosphorylation (Woolson, 1972). The appearance of necrosis on fronds of the marsh

fern is a visual indication that arsenic was limiting the movement of water into the plant and oxidative phosporylation was being interrupted.

Marsh fern root vascular system cross-sectional diameters, marsh fern stem vascular system tissue, and the topography of the outer layer of spores was altered after arsenic exposure. Biological and structural properties of plant root cells can be altered when concentrations of elements exceed threshold values for physiological constraints (Kabata-Pendias, 2001). Inorganic toxicity of metals in plants may be primarily caused by incorporation of arsenic into cellular components (Wang et al., 2002). Phosphorus deficiency may cause pith cells to become disintegrated and the development of vascular tissue to become minimized (Delvin et al., 1983). Additions of cobalt have been reported to cause root nodules of exposed plants to increase in size, even when exposed plants have a high nitrate supply available to them (Hallsworth et al., 1960). Arsenic exposure possibly has the same ability to increase root vascular system cross-sectional diameters of marsh fern even when a high supply of phosphorus maybe available to the plant. Cadmium has been reported to have the ability to reduce growth in plant stems by altering the production of ribosomal RNA (Rai et al., 2005). Therefore, the degradation of marsh fern vascular tissue after higher levels of arsenic exposure may be the visual exhibition of ribosomal RNA synthesis destruction, phosphorus deficiency, or toxicity from arsenic becoming integrated into cellular components.

The alteration of the outer layer of marsh fern spores may be an indication of structural adaptation to changes in ecological conditions (Tryton, 1971). Spores of *P. calomelanos* can contain a maximum arsenic concentration of at least 3500 ppb (Visoottiviseth et al., 2002). Arsenic accumulation is excluded from spores of *Pteris*

vittata (Pickering et al., 2006). Therefore, the alteration in outer layers may be a visual indication of marsh fern spores having the ability to accumulate arsenic.

CHAPTER 5
ASPARAGUS SPRENGERI
RESULTS AND DISCUSSION

5.1 Arsenic accumulation

Accumulation of arsenic in asparagus fern root was significantly different from the accumulation of arsenic in asparagus fern fronds. However, the accumulation of arsenic into the fronds of asparagus ferns exposed to 250 and 500 ug/L was significantly different from the accumulation of arsenic in control plants (0 ppb) (Table 6). Ferns exposed to 250 ppb had an average arsenic accumulation of 520 ppb, while ferns exposed to 500 ppb had an average arsenic accumulation of 550 ppb. Arsenic accumulation ranges were 110 to 820 ppb and 480 to 730 ppb for asparagus fern exposed to 250 and 500 ppb of arsenic respectively. The average bioaccumulation factor for ferns exposed to 250 ppb was 2.08 and ferns exposed to 500 ug/L had an average bioaccumulation factor of 1.1.

Table 6: Arsenic concentrations in fronds and roots, bioaccumulation factors (BF), and translocation factor (TF) of the asparagus fern.

Arsenic Treatment (ug/L)	Arsenic in Fronds Mean +/- SD (ng/g)	Range of Arsenic in Fronds (ng/g)	Mean BF	Arsenic in Roots Mean +/- SD (ng/g)	Range of Arsenic in Roots (ng/g)	Mean TF
0	80 +/- 0 a	60-110 a	0.00	52.52 +/- 0.00 a	10-70	0.69
250	520 +/- .36 b *	110-820 b	2.08	10047.5 +/- 8.87 a *	4270 – 23130	0.01
500	550 +/- .14 b *	480-730 b	1.10	9032.6+/- 6.55 a *	4770 to 18810	0.07

Means in the same column followed by the same letter are not significantly different at 5% alpha and Means in the second and third rows followed by * are significantly different at 5% alpha

Treatment levels did not have a significant effect on the accumulation of arsenic in the roots of the asparagus fern (Table 6). Roots of the asparagus fern exposed to 500 and 250 ug/g had average arsenic accumulation concentrations of 9,030 ng/g and 10,050 ng/g, respectively. In addition, roots of asparagus fern displayed an arsenic accumulation concentration range of 4,270 to 23,130 ng/g during exposure to 250 ug/L and an arsenic accumulation range of 4,770 to 18,810 ng/g during exposure to 500 ug/L. Asparagus fern roots exposed to 250 ug/L had an average translocation factor of 0.01 while asparagus fern roots exposed to 500 ug/L had an average translocation factor of 0.07.

5.2 Plant response to arsenic exposure

Arsenic exposure levels did not have a significant effect on the size of the vascular system cross-sectional measurements in the leaves, stems, or roots of the asparagus fern (Table 7). Asparagus fern roots exposed to 0, 250 and 500 ug/L displayed mean vascular system cross-sectional measurements of 28.8 um, 63.03 um, and 50.42 um, respectively. Fronds of the control asparagus ferns had a mean vascular system cross-sectional measurement of 20.77 um. Fronds of asparagus ferns exposed to 250 ug/L exhibited a mean vascular system cross-sectional measurement of 29.13 um, while leaves exposed to 500 ug/L exhibited a mean vascular system cross-sectional measurement of 30.53 um (Table 7). Stems of asparagus fern displayed mean cross sectional measurements of 35.05 um for ferns exposed to 0 ug/L, 39.21 um for ferns exposed to 250 ug/L and 39.72 um for ferns exposed to 500 ug/L. A visual characterization of these sections revealed a degradation of the vascular system cross-sections in the stems (Figure 12) and roots of asparagus fern exposed to 250 and 500 ug/L (Figure 13) and an alteration in the appearance of stomata (Figure 14).

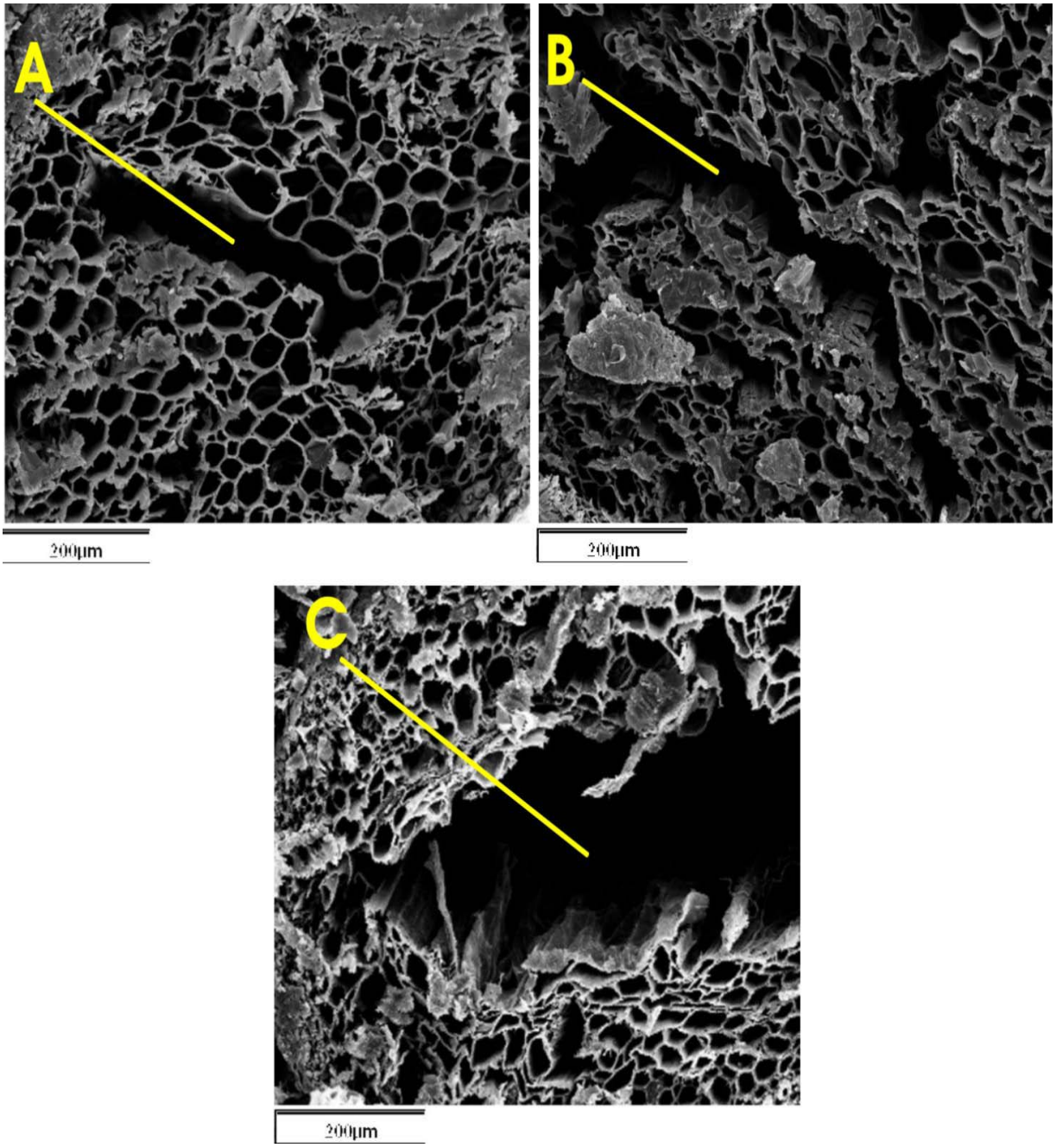

Figure 12: SEM micrographs of the degradation and collapse of asparagus fern stem vascular system cross-sections after arsenic exposure. A= control asparagus fern roots, B= asparagus fern roots exposed to 250 ppb, C= asparagus fern roots exposed to 500 ug/L. The yellow lines from the letters are showing areas of vascular degradation.

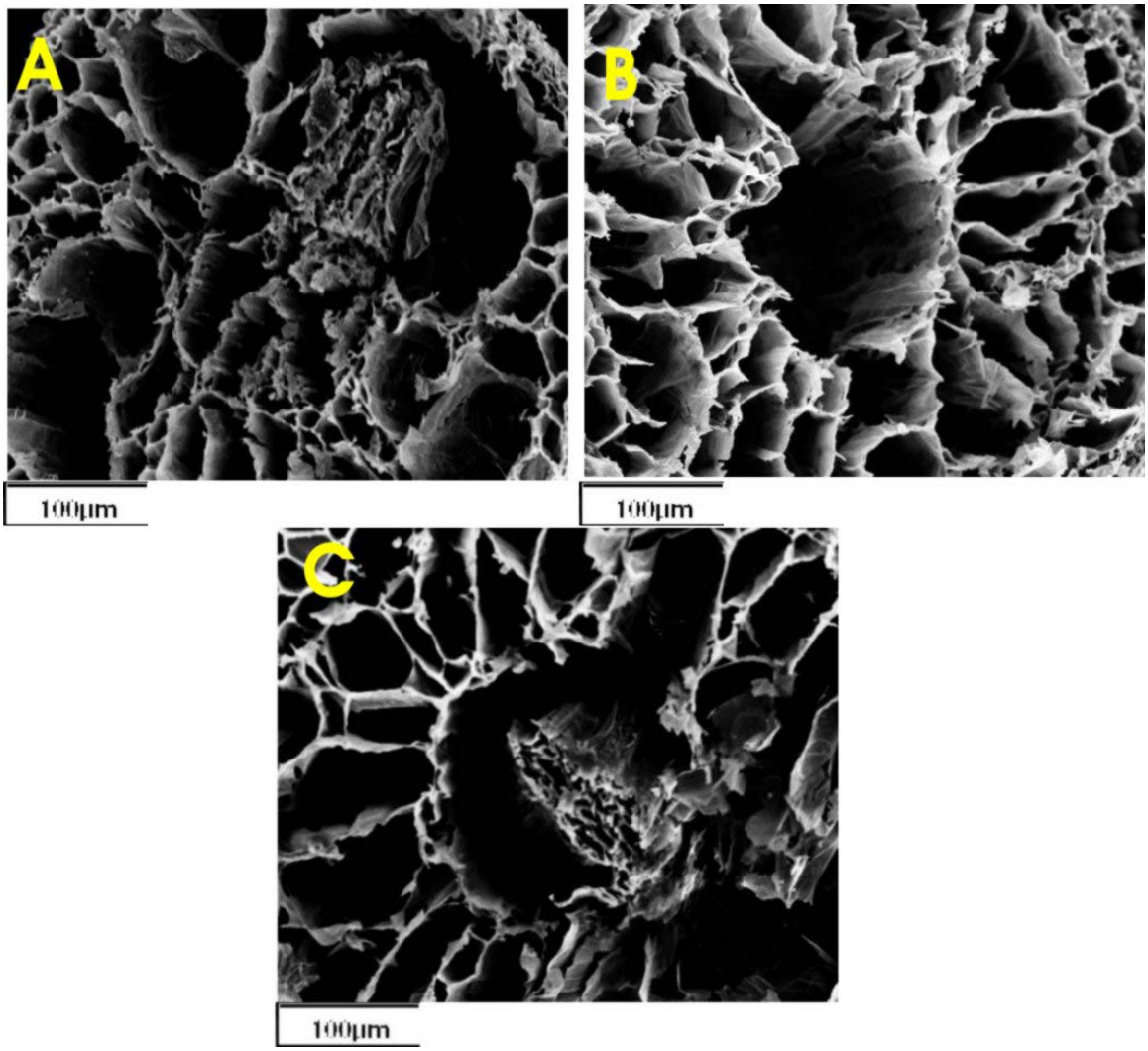

Figure 13: SEM micrographs of the degradation of asparagus fern root vascular cross-sections after arsenic exposure. A= control asparagus fern roots, B= asparagus fern roots exposed to 250 ppb, C= asparagus fern roots exposed to 500 ug/L.

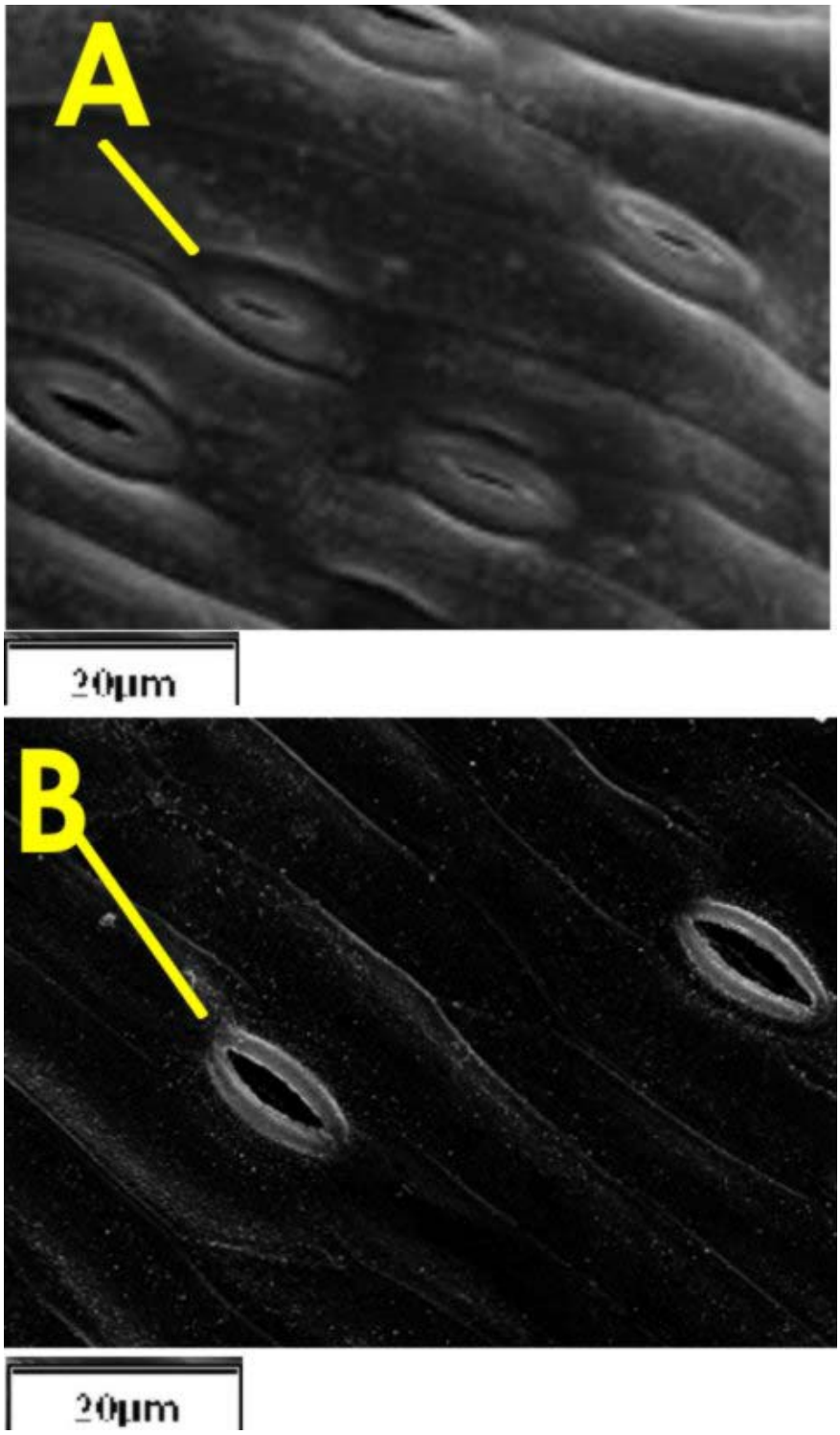

Figure 14: SEM micrographs of morphological changes in stomata openings of asparagus fern after arsenic exposure. , A = 250 ug/L, and B =500 ug/L.

Table 7: Asparagus fern root, leaf, and stem measurements.

Arsenic Treatment Levels (ug/L)	Root Vascular System CS ± SD (um)	Leaf Vascular System CS ± SD (um)	Stem Vascular System CS± SD (um)
0	28.8 ± 20.17 a	20.77 ± 12.38 a	35.05 ± 9.78 a
250	63.03 ± 47.90 a	29.13 ± 12.57 a	39.21 ± 14.8 a
500	50.42 ± 29.70 a	30.53 ± 9.41 a	39.72 ± 14.61 a

Means in the same column with the same letter are not significantly different at the 5% alpha level. CS= cross-sections

5.3 Arsenic species

Fresh samples exposed to 4500 mg/L and freeze dried samples of asparagus fern samples exposed to 250 ug/L contained combinations of various arsenic oxidation states when their respective linear combinations were compared to arsenic standards spectra. Fresh young asparagus fern leaves were found to contain arsenic species of 50% As (0) and 50% As (III) oxide (Figure 15). However, fresh mature asparagus fern fronds exhibited arsenic species of 79% As (0) and 21% As (III) oxide (Figure 16). In addition, fresh asparagus fern fronds had arsenic species of 85% As (V) oxide, 8% As (0) and 7% As (III) oxide (Figure 17), while freeze-dried fronds had 69% As (0) and 31% of 75% As (V) oxide/25% As (III) oxide (Figure 18).

5.4 Discussion

In this study, the ranges of arsenic accumulation in asparagus fern did not achieve the reported arsenic accumulation level of 1130 mg/L when chelating agents were used to increase availability of arsenic to the plants (Bagga et al., 2001). Bioaccumulation factors of the asparagus fern are > 1, thus indicating asparagus fern is an accumulator of arsenic. However, these bioaccumulation factors are far below the typical values of the arsenic hyperaccumulator *Pteris vittata* (>10) (Wang et al., 2002). Concentration of arsenic in

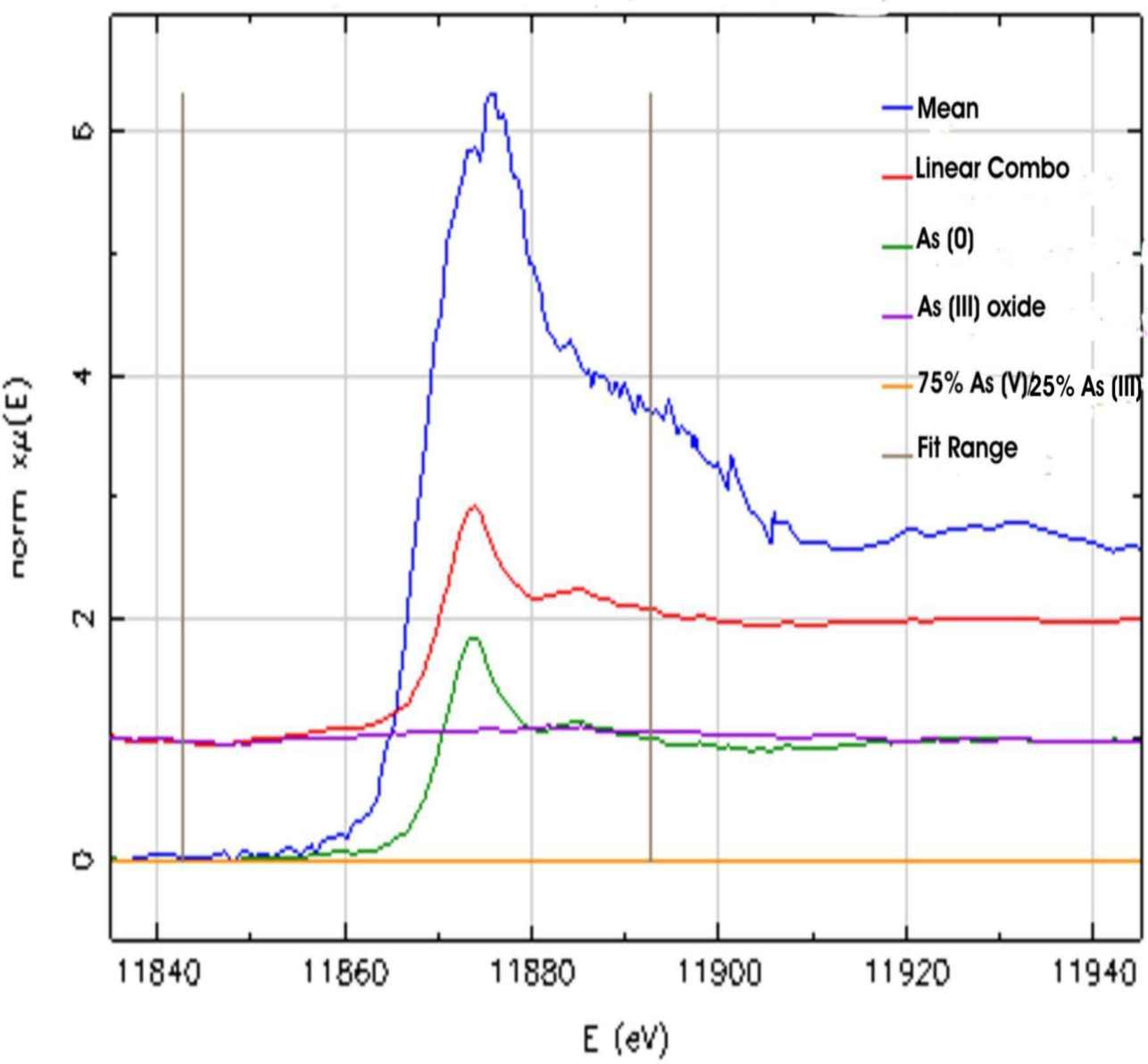

Figure 15: Arsenic oxidation states in young fresh asparagus fern fronds exposed to 4500 mg/L of arsenic. A quantitative analysis of XANES spectrum by fitting the sum of the standard spectra was performed**.** The figure shows the sample data as the blue line (mean) and the best fit of arsenic standard spectra as the red line (linear combination). The components of the best fit of arsenic standard spectra fit are shown as follows: 50% As (0) [green line] and 50% As (III) oxide [purple line]. 75%As (V)/25% As (III) [yellow line] did not make a contribution to the arsenic standard spectra fit.

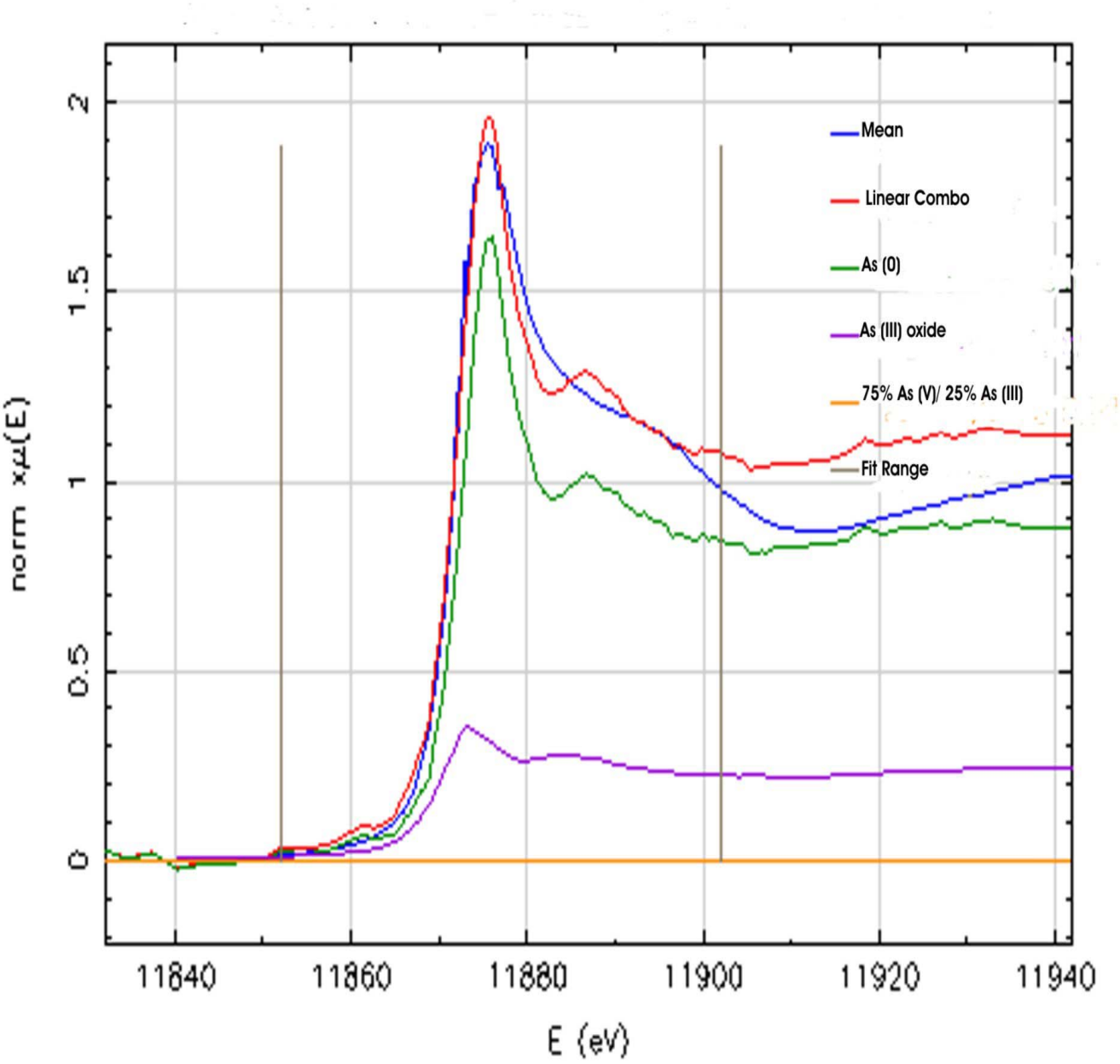

Figure 16: Arsenic oxidation states in mature fresh asparagus fern fronds exposed to 4500 mg/L of arsenic. A quantitative analysis of XANES spectrum by fitting the sum of the standard spectra was performed. The figure shows the sample data as the blue line (mean) and the best fit of arsenic standard spectra as the red line (linear combination). Components of the fit are shown as follows: 79% As (0) [green line] and 21% As (III) Oxide [purple line]. 75%As (V)/25% As (III) [yellow line] did not make a contribution to the arsenic standard spectra fit.

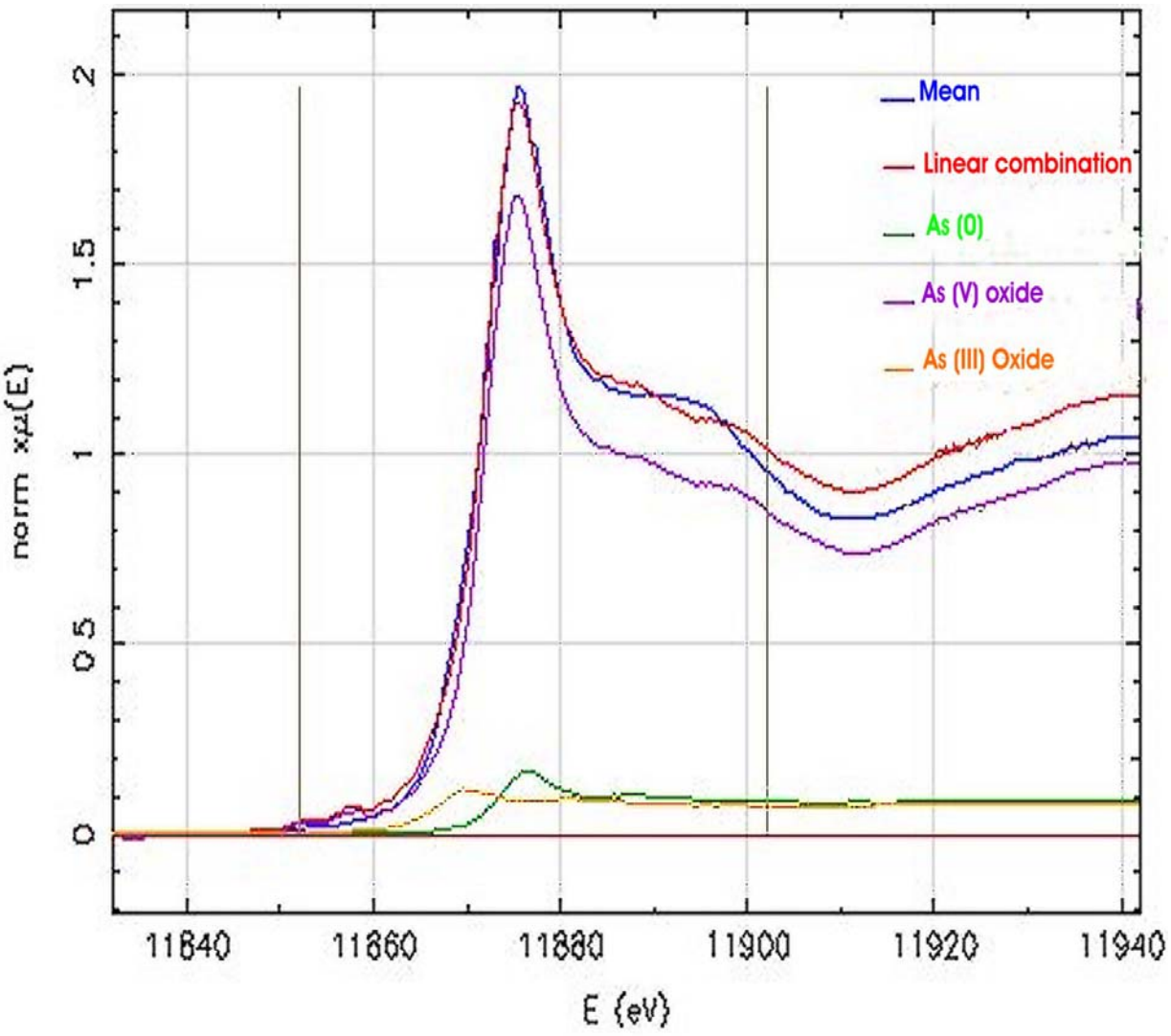

Figure 17: Arsenic oxidation states in fresh asparagus fern fronds exposed to 4500 mg/L of arsenic. A quantitative analysis of XANES spectrum by fitting the sum of the standard spectra was performed. The figure shows the sample data as the blue line (mean) and the best fit of arsenic standard spectra as the red line (linear combination). Components of the fit are shown as follows: 85% As (V) oxide [purple line], 8% As (0) [green line], and 7% As (III) Oxide [yellow line].

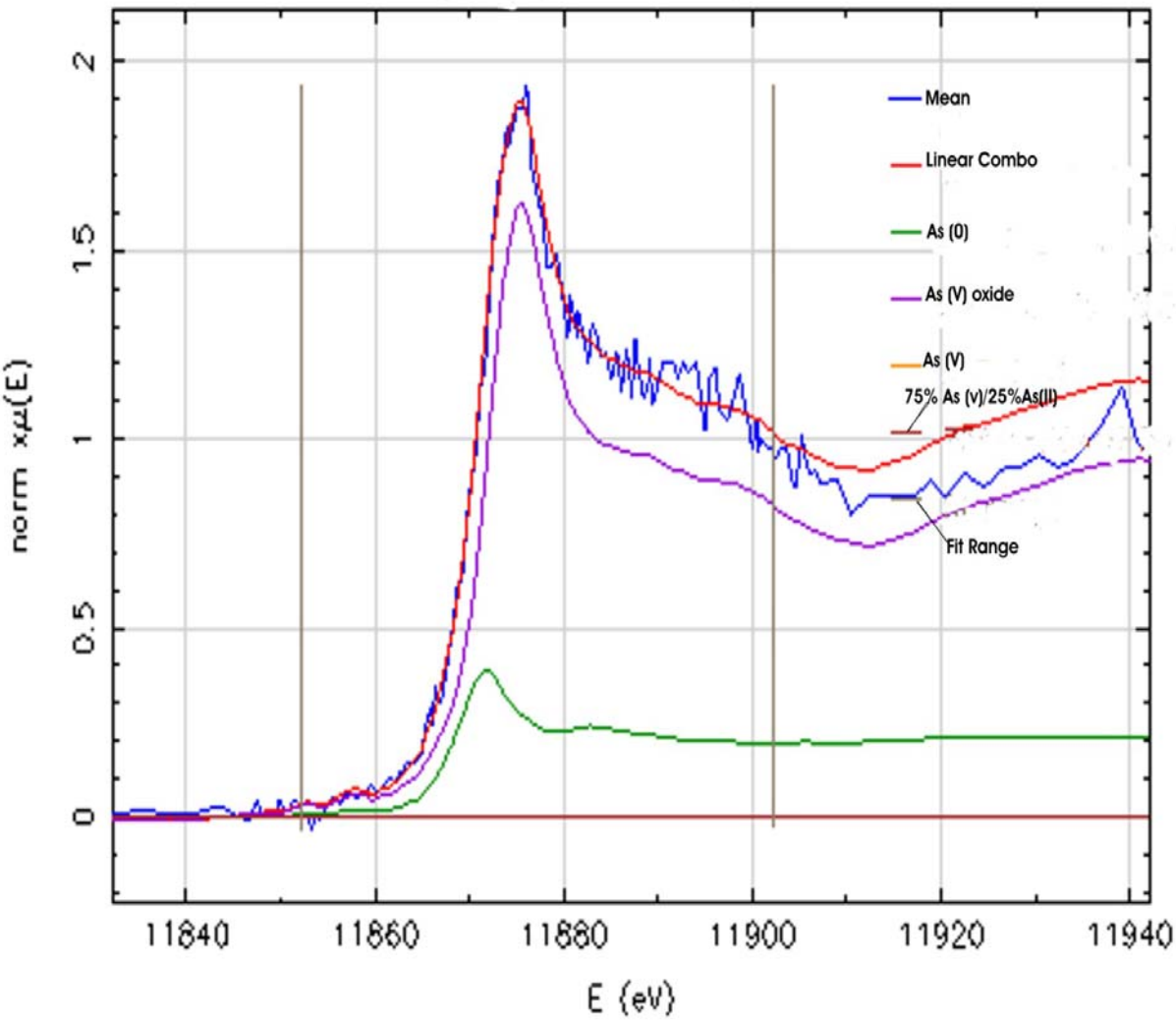

Figure 18: Arsenic oxidation states in freeze-dried asparagus fern fronds exposed to 500 ug/L of arsenic. A quantitative analysis of XANES spectrum by fitting the sum of the standard spectra was performed. The figure shows the sample data as the blue line (mean) and the best fit of arsenic standard spectra as the red line (linear combination). Components of the fit are shown as follows: 50% As (V) oxide [purple line] and 50% As (0) [green line], 75%As (V)/25% As (III) [brown line] did not make a contribution to the arsenic standard spectra fit.

in fronds or roots of the asparagus fern. Asparagus fern translocation factors were < 1, therefore, it is not efficient in the movement of arsenic from roots to shoots (Cai, 2003). Inefficient root to shoot translocation may be the result of increased sequestration of metals into the vacuoles of root cells or enhanced loading of arsenic into xylem (McGrath et al., 2003).

Cross-sectional dimensions of vascular tissue sections of leaves, stems, and roots of asparagus fern did not differ significantly among the treatments. However, SEM examination indicates that some of the vascular tissue sections in the roots of asparagus fern exhibited collapse and vascular tissue in stems of asparagus fern exhibited degradation. Phosphorus deficiency, which has similar symptoms of arsenic toxicity, can cause the disintegration of pith cells or cause pith cells to become succulent with thin-walls and abnormally large intercellular spaces. Phosphorus deficiency can reduce vascular tissue development (Delvin et al., 1983).

Tuberous roots of asparagus fern exposed to 500 ppb visually exhibited blockage in majority of the vascular cross-sections. It is possible that suberization of these vascular sections may have occurred from exposure to high levels of arsenic. Suberization limits the rate of respiration and water loss in tubers, such as potatoes. Water loss in badly suberizied areas may occur at very high rates (Dioup, 1998). Therefore, the blockage in the tuberous roots of the asparagus fern may be the result of clotted depositions of arsenic. Vascular bundles in stems of *Brassica juncea* exhibited blockage after exposure to chromium (Han et al., 2004).

The asparagus fern exhibited reshaped stomata openings after arsenic exposure. An increase in stomata openings in *Phyllanthus amarus* after exposure to cadmium has

been reported (Rai et al., 2005) Cadmium influence on stomata opening seems to be related to a reduction of turgor pressure in the assisting cells of the stomata. The rapid and preferential absorption of metal into assisting cells and the alteration in the permeability of cell membranes result in decreased turgor pressure and are possibly the internal plant factors affecting the reshaping of asparagus fern stomata following arsenic exposure (Rai et al., 2005). Transpiration is controlled by plant biophysical factors, such as stomata size, density and degree of opening (Langer, 2004) Thus, the reshaped stomata openings after arsenic exposure may be caused by changes in cell membranes of the plant contributing to a decrease in cell turgor pressure and/or an alteration in plant transpiration.

Freeze-dried and fresh asparagus fern above-ground biomass contained a combination of arsenic oxidation states. Fresh young asparagus fern fronds contained 50% As (0) (uncharged arsenic oxidation state) and As (III) oxide, respectively. As (0) composed most of the arsenic oxidation states in fresh mature asparagus fern fronds with the remainder of arsenic existing as As (III) oxide. This contradicts the data from previous studies showing of arsenate to be the dominant oxidation state in true ferns, with As (III) being the most common form of arsenic in young fronds and As(V) the dominant species in older fronds (Francesconi et al., 2002). The appearance of As (0) and As (III) oxide is consistent with the suggestion that plants protect themselves from interruption of oxidative phosphorylation by reducing and storing arsenic as arsenite (Mattusch et al., 2000).

Fresh asparagus fern stems mostly contained As (V) with a smaller fraction of arsenic as As (III) oxide. This combination of arsenic species in fresh asparagus fern

fronds indicates that the reduction of arsenic is most likely taking place inside of the leaves of asparagus fern frond, unlike in the true fern species, *Pteris vittata,* where the initial reduction of arsenic occurs immediately after uptake into the plant and before transportation of arsenic into shoots and roots of the plant (Webb et al., 2003) .

Arsenic in freeze-dried fronds mainly contained the uncharged oxidation state of arsenic, As (0), with the remaining percentage of arsenic being a combination of As (V) oxide/As (III) oxide. Dried plants will normally show a change in oxidation state from As (III) to As (V) (Webb et al., 2003). However, in asparagus fern fronds, most of the arsenic remained as the uncharged As (0) oxidation state. Therefore, the presence of the uncharged species, As (0), in asparagus fern is an indication that As (V) is being reduced to and accumulated as an uncharged species of arsenic. This reduction of As (V) to As (0) provides arsenic the ability to easily permeate biological membranes inside of the asparagus fern (Webb et al., 2003). Drying of asparagus fern fronds does not cause the total oxidation of As (0) to As (V). Although the asparagus fern fronds had been freeze-dried, As (0) remained the dominant oxidation state in the fronds of the asparagus fern. This finding is not consistent with reports that indicate that regardless of the form of arsenic applied to a plant, arsenic will eventually become oxidized and metabolized to arsenate (Peterson et al., 1981).

CHAPTER 6
LOLIUM PERENNE RESULTS AND DISCUSSION

6.1 Arsenic accumulation

Ryegrass exposed to 2500 mg/L arsenic was found to have significantly different accumulation into the blades than ryegrass exposed to the 0 mg/L (control) and 1800 mg/L (Table 8). Arsenic accumulation ranges in the blades of the ryegrass were 1.64 to 5.30 ug/g for the control ryegrass, 84.79 to 532.61 ug/g for ryegrass exposed to 1800 mg/L, and 402.08 to 1154.76 ug/g for ryegrass exposed to 2500 mg/L. Average bioaccumulation factors for the blades of ryegrass exposed to 1800 mg/L and 2500 mg/L were 0.12 and 0.32, respectively

Table 8: Arsenic concentrations, bioaccumulation factors and plant health rankings for ryegrass.

As Treatment (mg/L)	Arsenic in Blades Mean +/- SD (ug/g)	Arsenic Accumulation Ranges (ug/g)	Mean BF	Mean PH Ranking
0	2.80 a	1.64 - 5.30	0	1.00
1800	209.97 b	84.79 - 532.61	0.12	1.75
2500	801.19 b	402.08 -1154.76	0.32	1.75

Means followed by the same letter are not significantly different at 5% alpha. BF= bioaccumulation factor and PH = plant health 1= healthy, 2=slightly necrotic, and 3= severely necrotic.

6.2 Plant response to arsenic exposure

Arsenic treatment levels did not have a significant effect on the size of the vascular system cross-sectional diameters (Table 9). The control ryegrass displayed a mean vascular system cross-section measurement of 70.76 um, whereas ryegrass exposed

In addition, ryegrass exposed to 2500 mg/L exhibited a mean vascular system measurement of 16.93 um. Vascular system cross-sections of ryegrass exposed to 1800 ppm arsenic are thin (Figure 19). Blades of ryegrass had variable responses after arsenic exposure (Figure 20). Plant health index means indicate that necrosis occurred in ryegrass exposed to arsenic (Table 8).

Table 9: Vascular cross-sectional measurements for blades of ryegrass.

Arsenic Treatment (mg/L)	Mean Vascular System Cross-sectional Diameter (um) ± SD
0	70.76 ± 45.67 a
1800	96.40 ± 83.23 a
2500	16.93 ± 12.18 a

Means followed by the same letter in the same column are not significantly different at 5% alpha

6.3 Arsenic species

Freeze-dried samples of plants exposed to 2500 ppm and fresh samples of plants exposed to 4500 ppm both contained a combination of arsenic species. However, fresh samples of ryegrass exposed to 4500 ppm were found to contain 90% As (V) oxide and 10% arsenic sulfide (As_2S_2) (Figure 21). Freeze dried samples of ryegrass contained 71% As (V) oxide and 29% As (III) sulfate (Figure 22).

6.4 Discussion

Ryegrass is an arsenic "hypertolerant" species rather than being a true arsenic hyperaccumulating species (Cai, 2003). Although the highest treatment level of arsenic had a significant effect upon the bioaccumulation factors of ryegrass, the bioaccumulations factors are < 1. Bioaccumulation factors < 1 indicate a plant is not effective in removing metals from its exposure media (Tu et al., 2002).

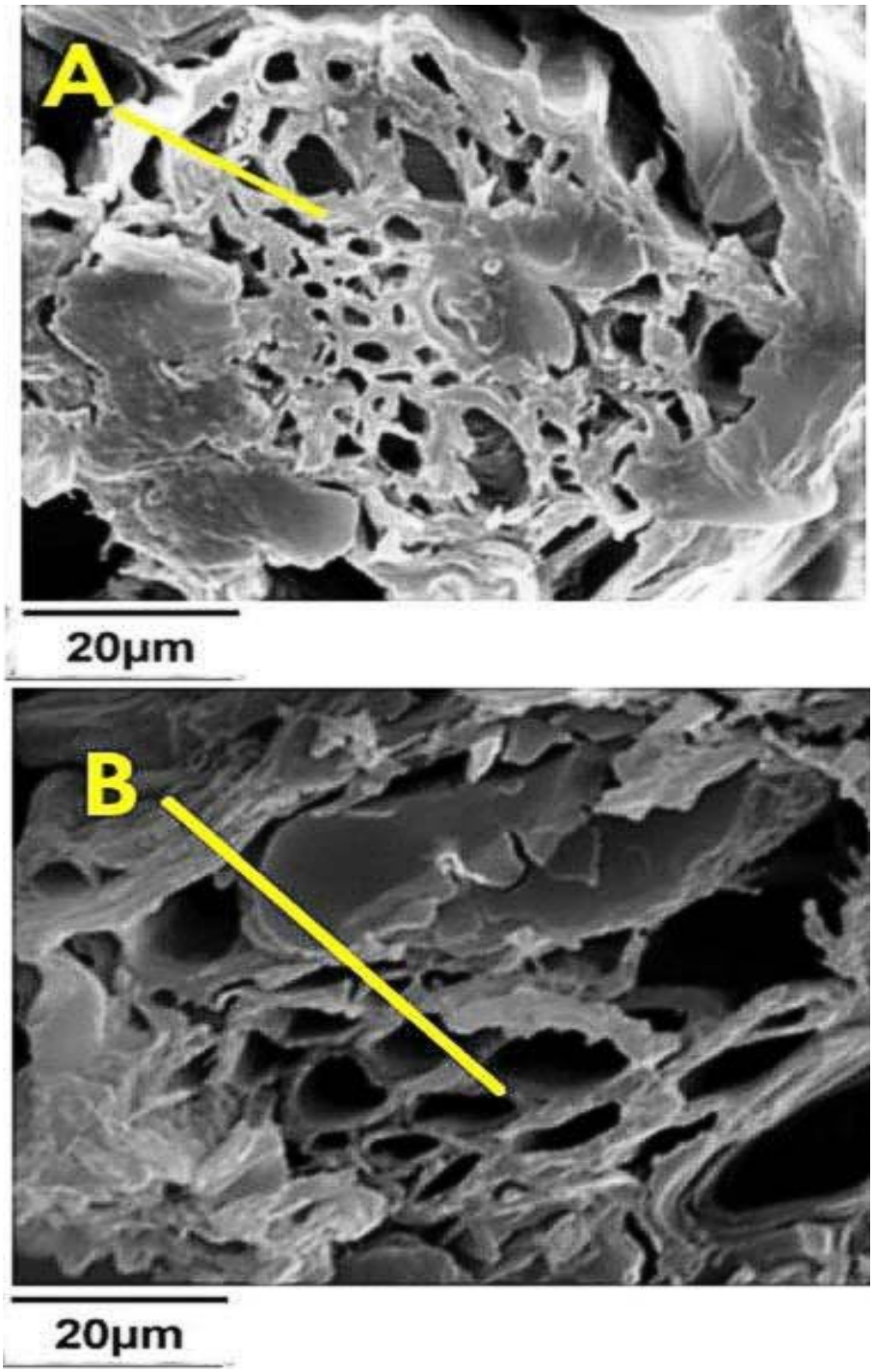

Figure 19: SEM micrograph showing thinning of ryegrass vascular system cross sections. A= control and B= ryegrass exposed to 1800 mg/L. Yellow letters from letters are pointing to areas of vascular thinning.

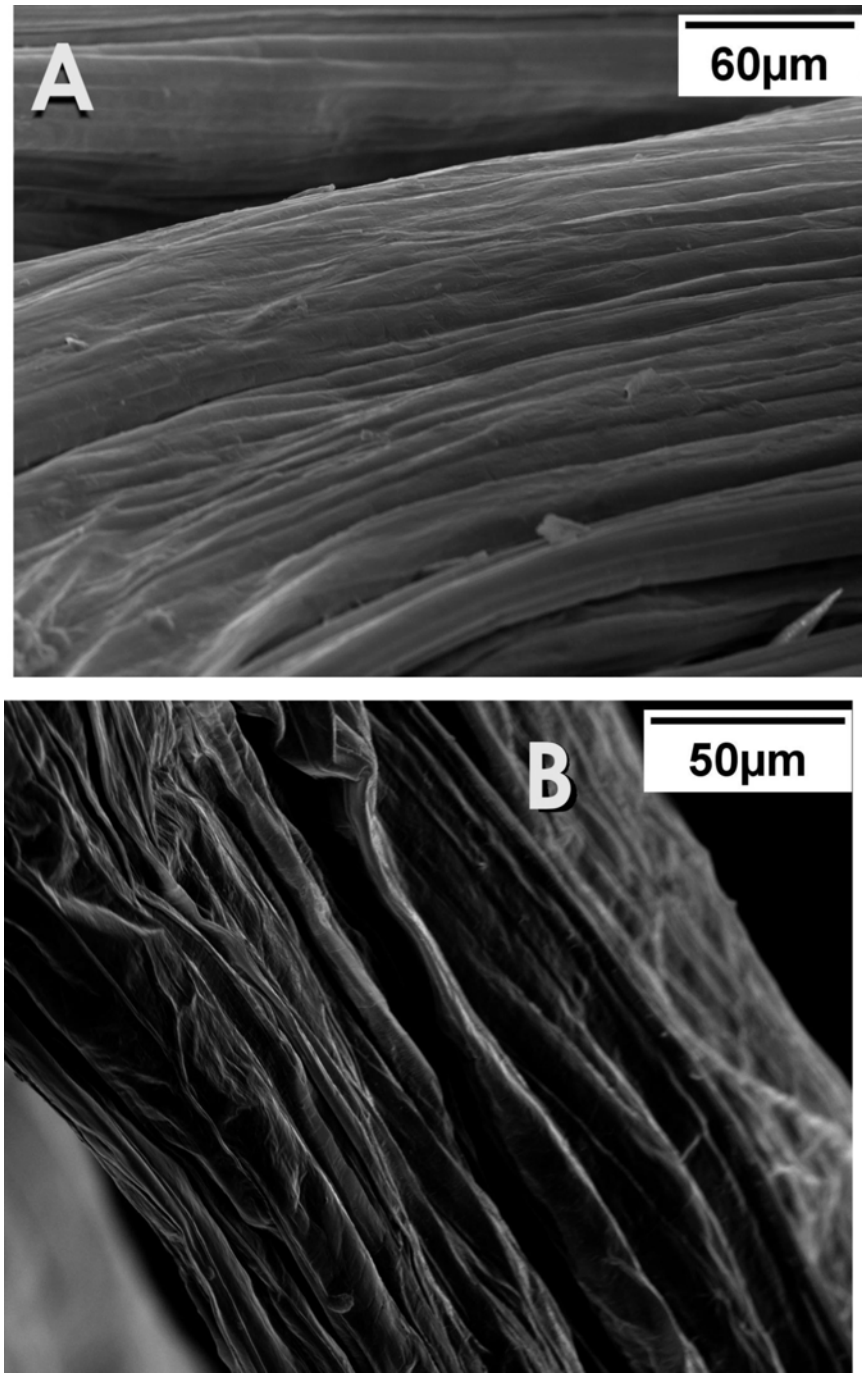

Figure 20: SEM micrographs showing the variable response in wilting of ryegrass blades after exposure to 2500 mg/L. A= healthy blade and B = wilted blade

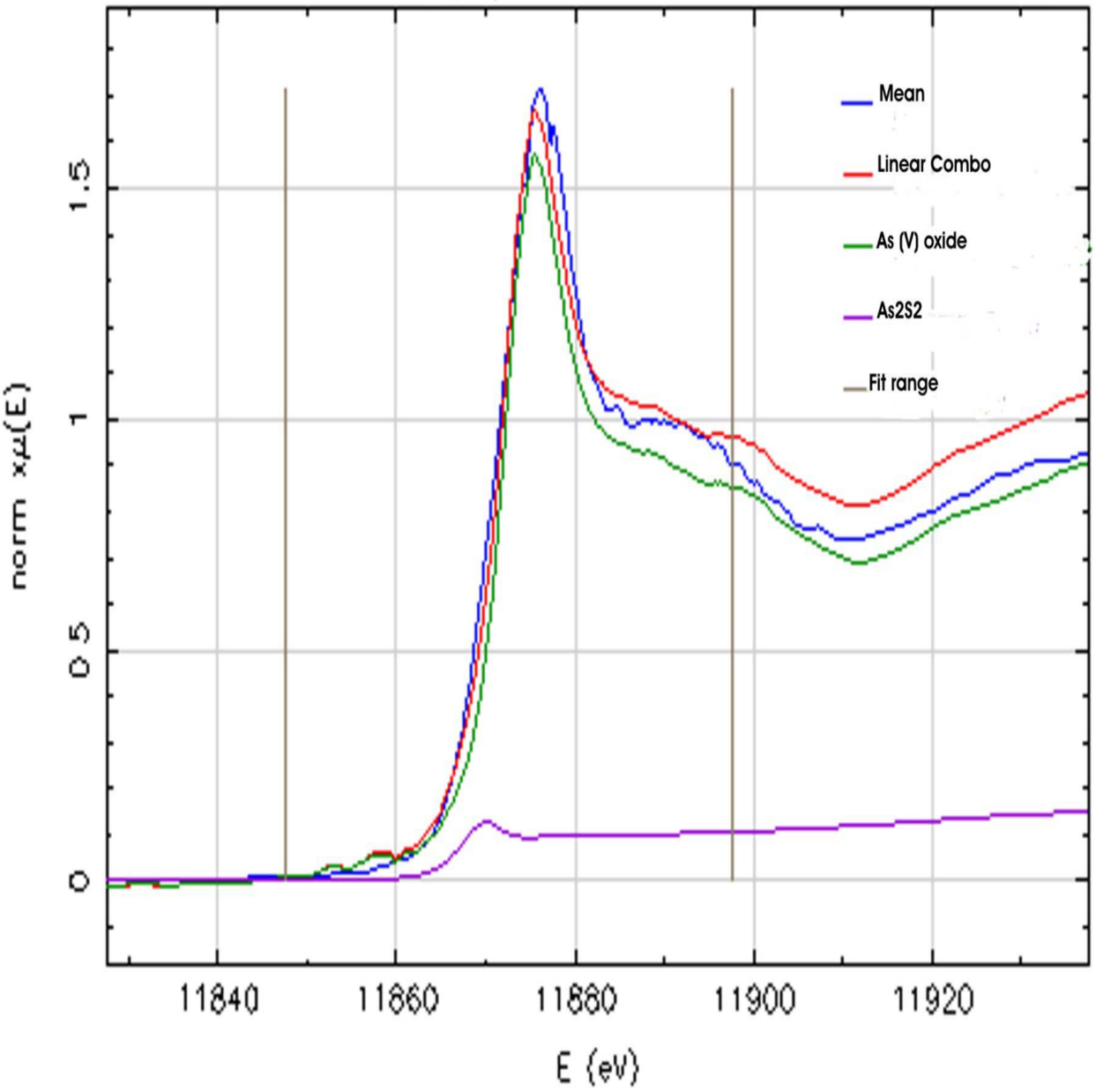

Figure 21: Arsenic oxidation states in fresh ryegrass exposed to 4500 mg/L of arsenic. A quantitative analysis of XANES spectrum by fitting the sum of the standard spectra was performed. The figure shows the sample data as the blue line (mean) and the best fit of arsenic standard spectra as the red line (linear combination). Components of the fit are shown as follows: 90% As (V) oxide [green line] and 10% of As_2S_2 [purple line]. Fresh ryegrass was exposed to 4500 mg/L of arsenic.

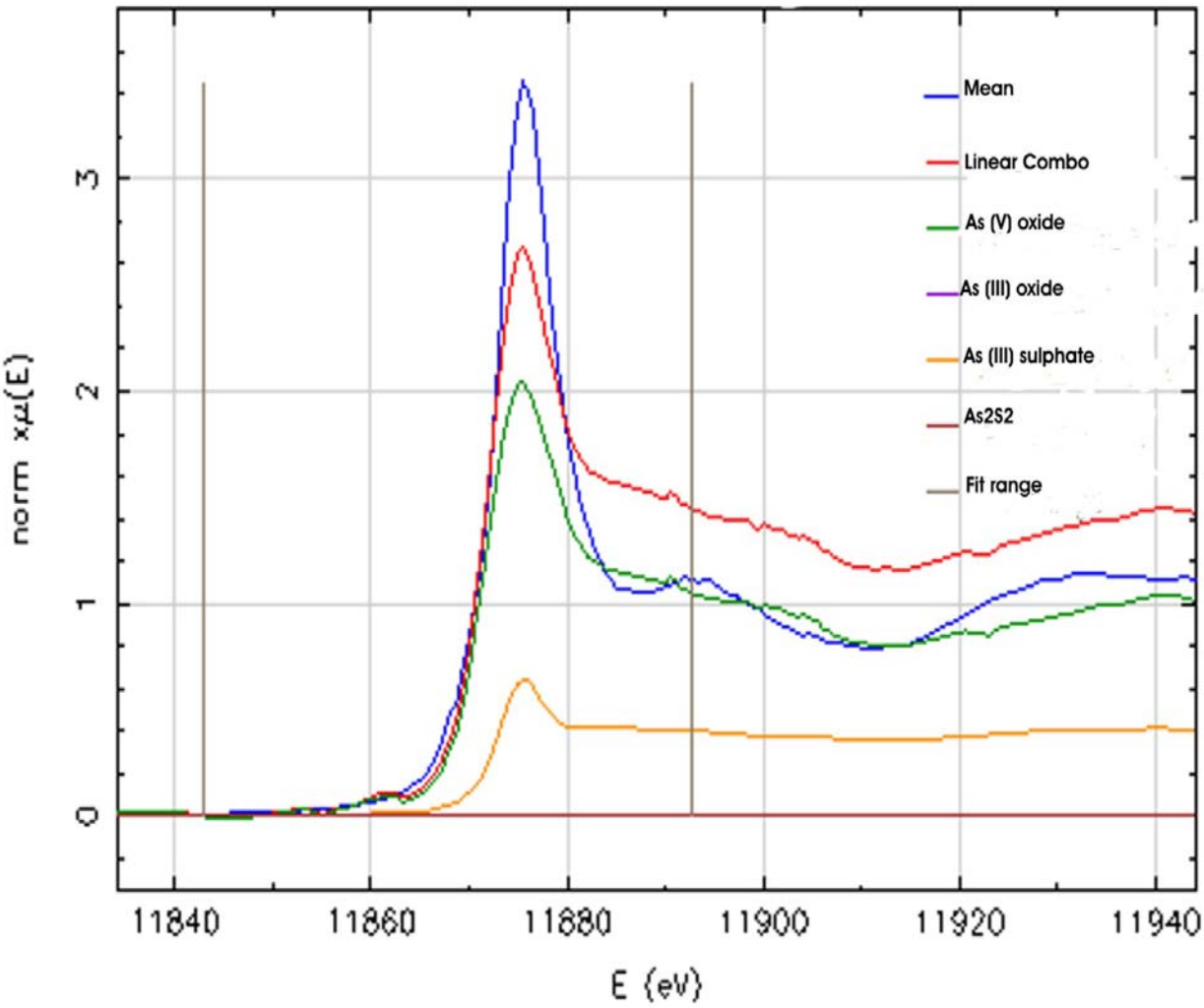

Figure 22: Arsenic oxidation states in freeze-dried ryegrass exposed to 2500 mg/L of arsenic. A quantitative analysis of XANES spectrum by fitting the sum of the standard spectra was performed. The figure shows the sample data as the blue line (mean) and the best fit of arsenic standard spectra as the red line (linear combination). Components of the fit are shown as follows: 71% As (V) oxide [green line] and 29% of As (III) sulfate [yellow line]. Freeze-dried ryegrass was exposed to 2500 mg/L of arsenic.

An external indication of arsenic altering the plant health of ryegrass is the appearance of slight necrosis in exposed plants. Necrotic blades of ryegrass will exhibit wilting after arsenic exposure. High levels of arsenic have been found to be located in areas of necrosis on *Pteris vittata* (Lombi, 2002). Arsenic cannot duplicate the phosphorus requirement in the plant. Therefore, the plant displays phosphorus deficiency symptoms (Cox, 1995), such as necrosis (Delvin et al., 1983). The level of necrosis in ryegrass was equal for all arsenic treatment levels. Hypertolerance is achieved by plants detoxifying internally accumulated metals and probably compartmentalizing and complexing accumulated metals (McGrath et al., 2003). This equal level of necrosis in ryegrass may be the result of immobilization of arsenic in cell walls. Immobilization of metals is a key reaction of plants to reduce the toxicity of exposure to excess trace metals (Kabata-Pendias, 2001).

Thinning of ryegrass vascular system cross section walls resulted from arsenic exposure. Plants growing under the stress of elevated trace metal concentrations will display an altered morphology (Kabata-Pendias, 2001). Arsenic complexation with structural components of cells is one of the primary causes of inorganic toxicity in plants (Wang et al., 2002). Phosphorus deficiency symptoms, which can occur when arsenic replaces phosphorus (Cox, 1995), cause thinning of phloem and xylem walls and minimizes the development of vascular tissues (Delvin et al., 1983).

Combinations of arsenic species were contained in ryegrass. Ryegrass mostly contained As (V). As (V) should be the dominant species of arsenic in ryegrass (Tlustos et al., 2002). As (V) is more thermodynamically stable than arsenite and decreases the loss of enzyme activities in cells (Francesconi et al., 2002). Therefore, the action of As

(V) on plant functions is more subtle than arsenite; although As (V) has the ability to uncouple phosphorylation in plant cells (Peterson et al., 1981).

A small fraction of arsenic was found to be in arsenic-sulfur chemical compounds. As (III) has an affinity for sulfur-hydrogen groups (Webb et al., 2003). As_2S_2 and As (III) sulfate were a small percentage of the species of arsenic accumulated in freeze dried and fresh ferns, respectively. The presence of these arsenic-sulfur compounds in the blades of the ryegrass agrees with findings that arsenic can be coordinated with sulfur atoms in above-ground parts of plants after exposure to high arsenic concentrations (Webb et al., 2003).

CHAPTER 7
CONCLUSIONS

The overall goal of this research was to determine the potential of *Thelypteris palustris* (marsh fern), *Asparagus sprengeri* (asparagus fern), and *Lolium perenne* (perennial ryegrass) for the phytoremediation of arsenic contaminated soils. The research presented here evaluated the potential of these plant species on the basis of their ability to accumulate arsenic into above-ground parts, alteration in plant morphology after arsenic exposure, and the species of arsenic accumulated in plant parts.

The first hypothesis of this research was that *Thelypteris palustris, Asparagus sprengeri*, and *Lolium perenne* will accumulate arsenic into above-ground plant parts. This was demonstrated to be correct. Marsh fern, asparagus fern, and ryegrass can accumulate arsenic into above-ground plant parts. Asparagus fern and ryegrass had the ability to withstand exposure to 4500 mg/L of arsenic. Marsh fern was able to accumulate more arsenic into fronds than the arsenic treatment levels in hydroponic solution and its bioaccumulation factors were in the range of known arsenic hyperaccumulators, such *Pteris vittata*. Therefore, the marsh fern should be considered a hyperaccumulator of arsenic. Although asparagus fern does have the ability to accumulate arsenic in above-ground parts, bioaccumulation factors and translocation factors indicate the asparagus fern is not effective in the translocation of arsenic from a hydroponic solution. Ryegrass can accumulate high levels of arsenic into above-ground parts from a hydroponic solution; however, the concentration of arsenic accumulated in above-ground parts was not greater than the concentration of arsenic in the hydroponic solution. Therefore, ryegrass is tolerant of arsenic, but does not hyperaccumulate arsenic.

The second hypothesis for this study was that the morphology and growth patterns of all three plants would be altered after arsenic exposure. This study supports this hypothesis that arsenic exposure affected growth patterns and caused alterations in morphology. Marsh fern, asparagus fern, and ryegrass all displayed changes in morphology following arsenic exposure. Necrosis, a visual sign of arsenic toxicity, was exhibited in marsh fern and ryegrass after exposure to arsenic. Vascular system cross-sectional diameters of different plant parts were altered after exposure to arsenic. Marsh fern root vascular system cross-sections significantly increased in diameter with exposure to 500 ug/g of arsenic. Asparagus fern roots displayed degradation as arsenic concentrations in the hydroponic solution increased. The open space in asparagus fern stems increased as arsenic concentrations increased. Tuberous roots of asparagus fern exposed to 500 ug/g had blockages in of most vascular system cross-sections. Ryegrass vascular system cross-sections exhibited thinning of section walls after arsenic exposure. Alteration in the morphology of marsh fern, asparagus fern, and ryegrass did occur after arsenic exposure. Arsenic exposure caused stimulation of stem vascular tissue production, and changes in spore outer layers in the marsh fern. Exposure to arsenic resulted in alteration of the morphological appearance of stomata in asparagus fern. Blades of ryegrass exposed to arsenic began to wilt.

The final hypothesis was that As (V) would be the dominant arsenic species in exposed roots; while As (III) would be the dominant arsenic species in above-ground plant parts. Arsenate (As (V)) was the dominant species of arsenic found in the roots of the marsh fern. However, arsenite (As (III)) was not the dominant species found in the above-ground parts of ryegrass or asparagus fern. The combination of arsenic species

found in the fronds of asparagus fern was influenced by the age of the fronds. Young fronds contained 50% As (0) and 50% As (III); while mature fronds contained As (0) as the dominant arsenic species. As (V) is the dominant oxidation state of arsenic contained in ryegrass and As (III) in ryegrass is associated with sulfur. Therefore, the total reduction of As (V) to As (III) does not occur during translocation of arsenic to above-ground parts or after accumulation of arsenic into above-ground parts.

In conclusion, marsh fern, asparagus fern, and ryegrass have the ability to survive arsenic exposure and accumulate arsenic into above-ground parts. Marsh fern has the potential to be used in phytoextraction of areas contaminated with low levels of arsenic. Asparagus fern and ryegrass do not exhibit a potential for phytoextraction, but their potential in phytostabilization should be further investigated.

The analytical method combination of ICP-MS, SEM, and XANES was used in this study. ICP-MS data determined arsenic accumulated in exposed plant to the ug/g concentration range and provided information needed to calculate how effective a plant is in the accumulation (BF) and the movement (TF) of arsenic to above-ground plant parts. Therefore, ICP-MS is an excellent analytical method to determine the ability of a plant to accumulate arsenic and to determine how effective a plant is in the translocation of arsenic before field applications.

SEM was used to evaluate the extent of morphological changes in arsenic exposed plants. In this study, SEM micrographs of exposed plants exhibited degradation and collapse of vascular system cross-sections, alteration in stomata opening, and alteration in spore outer layers. These morphological changes in plants after exposure are an indication of plant tolerance or sensitivity, modification of metabolic processes, and

variation in plant growth patterns. Thus, SEM would be a valuable tool to obtain insight into the sustainability of exposed plants in field applications.

XANES was used to determine the oxidation states and chemical forms of arsenic in arsenic exposed plant samples. Results of XANES analysis for this study indicated that exposed plants contained diverse combinations of arsenic oxidations states and chemical forms. These combinations presented information on reduction-oxidation processes during arsenic translocation to above-ground plant parts, the oxidation state and chemical form of accumulated arsenic in plant parts, and possible arsenic detoxification mechanisms in plant parts. Because of the information presented in XANES data, researchers will be given a better idea of how to deal with plant biomass after removal from contaminated sites.

In conclusion, the analytical method combination of ICP-MS, SEM, and XANES were very effective in the assessment of the potential of arsenic exposed plants for phytoremediation. This analytical method combination could be used to identify other species of plants that are applicable for phytoremediation of contaminated soils, sediments, and water systems, such as those systems with elevated contamination caused by natural arsenic sources, natural disasters like Hurricane Katrina, and industrial waste from mining and chemical production.

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VITA

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