

A STUDY OF THE PHYTOREMEDIATION PROCESS BY TWO ARSENIC  
HYPERACCUMULATORS GROWN IN A HYDROPONIC ENVIRONMENT

By

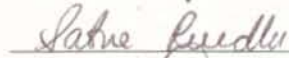
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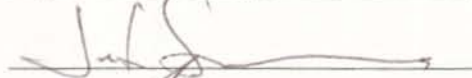
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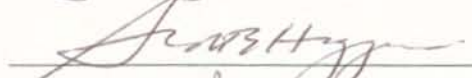
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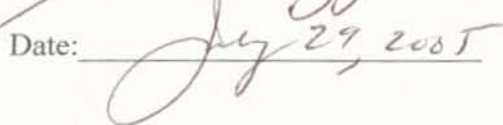
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Summer 2005  
Western Carolina University  
Cullowhee, North Carolina

A STUDY OF THE PHYTOREMEDIATION PROCESS BY TWO ARSENIC  
HYPERACCUMULATORS GROWN IN A HYDROponic ENVIRONMENT

A thesis presented to the faculty of the Graduate School of Western Carolina University  
in partial fulfillment of the requirements for the degree of Master of Science.

By

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July 2005

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Dedication and Acknowledgements

This thesis is dedicated in the memory of Earl Ingle, my grandfather who passed away before the completion of this project. He was truly one of the wisest and kindest individuals that I have yet to meet.

In addition, I would like to acknowledge several individuals. First, I would like to acknowledge my thesis committee, Dr. David J. Butcher (Director), Dr. Sabine S. Rundle, and Dr. Jack S. Summers. Without their guidance and support, the completion of this project and thesis in a timely process could not have been achieved.

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## Abstract

### A STUDY OF THE PHYTOREMEDIATION PROCESS BY TWO ARSENIC HYPERACCUMULATORS GROWN IN A HYDROPONIC ENVIRONMENT

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Western Carolina University (Summer 2005)

Director: Dr. David J. Butcher

Arsenic contamination has become a global problem for both developed and developing nations. However, traditional remediation is a very expensive process. Therefore, alternate methods are being developed. One type of alternate method is called phytoremediation. This type of remediation uses vascular plants to cleanup contaminated environments. This project consisted of an investigation of the phytoremediation process by two arsenic hyperaccumulating plants (*P. vittata* and *P. cretica cv Mayii*) grown in a controlled propagation system. The primary method of the investigation was the measurements of arsenic and nutrient (macro- and micro-) uptake by the plants exposed to different forms of arsenic. The results of the arsenic analysis showed that *Pteris vittata* extracted both forms of arsenic. In addition, the arsenic analysis for *Pteris cretica cv Mayii* showed that the root tissue contained the lowest concentration of arsenic, compared to the stem and leaf tissue. The macronutrient analysis for *Pteris vittata* and *Pteris cretica cv Mayii* determined calcium to be the most common nutrient. Of the four macronutrients analyzed, sulfur was the least common nutrient detected in *Pteris vittata* and *Pteris cretica cv Mayii* tissue. The results of micronutrient analysis for *Pteris vittata*

determined iron to be the most common nutrient. The most common micronutrient detected in the root tissue for *Pteris cretica* cv. *Mayii* was also determined to be iron. However, the most common micronutrient in the stem and leaf tissue was determined to be sodium. Based on these finding, a more detail analysis of the role of macro- and micronutrient on arsenic uptake needs to be conducted.

## Chapter 1: Introduction

In the 21<sup>st</sup> century the planet will face many global issues. One of most important issues affecting the entire human species is an increase in environmental contamination. Since the industrial revolution the human species has drastically increased the amount of environmental contamination. All different environments (air, land, and aquatic) are being affected by this contamination. An example of this contamination can be seen in the land and water contamination by arsenic.

Arsenic contamination of the environment can be seen throughout the entire world. In the United States, the drinking water sources of more than 18,000 community have been found to contain arsenic [1]. In undeveloped nations, the arsenic contamination of local drinking water is even higher than in developed nations (note: some of arsenic contamination is due to natural sources). In addition, most commercially purchased food has been found to contain some arsenic contamination [1]. Besides the environmental exposure to arsenic, many industrial workers are occupationally exposed to this toxic substance (approximately 900,000 workers may be exposed annually) [2].

Currently, remediation of arsenic contaminated environments is very expensive. Many traditional remediation projects expense millions of dollars to remove the toxic material from contaminated site [3]. Though developed nations of the world can afford the expensive cost of traditional remediation projects, the undeveloped nations can

not possible afford such high cost. However, a new type of remediation has been developed that could drastically reduce the expense of these remediation projects (allowing even poor undeveloped nations to remediate their environments). This new type of remediation is called phytoremediation.

Phytoremediation is the use of green or vascular plants to cleanup sites of environmental pollution [3]. Phytoremediation is an inexpensive but useful method of environmental remediation. By use of phytoremediation, many contaminated sites, which had been previously too expensive to be remediated, can now be cleaned. In this project, the phytoremediation by two arsenic hyperaccumulators was investigated.



## Chapter 2: Background

### 2.1 Phytoremediation

#### 2.1.1 Advantages

Phytoremediation provides several advantages over traditional approaches to environmental cleanup. One of the leading advantages of phytoremediation is cost efficiency [3]. This form of remediation is very inexpensive when compared to traditional forms of remediation. The major costs of phytoremediation are soil preparation (not a factor in hydroponic or aquatic-based phytoremediation), weed and pest control, and the harvesting and disposal of the plants' biomass (containing the contaminants) [3]. A consensus estimate of the cost for phytoremediation treatment of the soil is about \$25-\$100 per ton [3]. However, the costs for the traditional forms of soil remediation (e.g. thermal desorption and chemical treatment) ranges from \$100-\$1500 per ton. The cost estimation for phytoremediation treatment of the water ranges from \$0.60-\$6.00 per 1000 gallons of contaminated water [3]. The cost of water remediation by the more traditional approaches will generally be higher than the phytoremediation method, ranging from \$0.60-\$3400 per 1000 gallons of contaminated water. Another advantage of phytoremediation for environmental remediation is that it offers site-performance enhancements [3]. Phytoremediation can be a performance enhancement in that it offers a permanent treatment solution (by removing contamination from the media). There are some traditional remediation methods that may not offer a permanent treatment

solution for contaminated environments, such as soil excavation (top layer of the contaminated soil is removed from pollution site). In addition, phytoremediation is a performance enhancement because it will not permanently disrupt these environments [3]. Traditional methods of remediation, such as chemical treatment, may permanently alter the local soil and landscape ecology of the cleanup site causing ecological stress.

Another advantage of phytoremediation is that this method has more public acceptance [3]. Some traditional forms of remediation require lots of equipment, and create substantial dust and noise (which disrupts public harmony). However, phytoremediation does not require sophisticated equipment and will not generate dust and noise. As result of this fact, the public is more accepting of phytoremediation, compared to traditional remediation. In addition, phytoremediation has more public acceptance because plants are very aesthetically pleasing [3].

### 2.1.2 Disadvantages

There are several disadvantages to phytoremediation. One of the main disadvantages for phytoremediation is the amount of time required to treat a contaminated site. Phytoremediation is a very lengthy process, requiring as much as 18-60 months for site remediation [3]. The time-consuming nature of phytoremediation is partially due to the plants' life cycle.

Another disadvantage of phytoremediation is that this method will not allow 100% removal of contaminants [3]. The plants involved in this remediation approach will not completely remove all contaminants. In addition, only certain plant species can

be used in this remediation method (many plants will not survive in a contaminated environment). As a result of this fact, this method depends on specific plant species to be used in this remediation process [3].

### 2.1.3 Types of Phytoremediation

The Phytoremediation process can be divided into three different categories: phytoextraction, rhizofiltration, and phytostabilization. In phytoextraction, high-biomass, metal-accumulating plants are used to transport and concentrate metals from the soil into the above-ground shoots. Once the metals have been concentrated in the above-ground shoots, this plant material can be collected [3].

In order for phytoextraction to effectively remediate a site with metal contamination, it requires the following factors: (1) metal solubility and availability for uptake, and (2) the ability of the plant to accumulate metal in harvestable plant tissue (leaf and stem tissue) [3]. Metal solubility is dependent on the pH of the environment (aquatic or soil-based). The pH of the environment will either enhance or inhibit metal availability for plant extraction. For soil-based environments, a pH below 5.5 will allow for more extraction of metals by a plant species [3]. An example of this effect can be seen in lead uptake by a plant. As the pH decreases, the lead in the soil becomes more soluble and more available for uptake by the plant. However, a decrease in pH may inhibit the plant's normal growth. Because the pH does affect phytoextraction, it can be adjusted to get the maximum extraction without the more harmful effects on the plant (the pH effect on a plant will vary with species).

Another required factor for effective remediation by phytoextraction is the ability of the plant to accumulate metal. Plants that tolerate high concentration of toxic metal (such as zinc, arsenic, and lead) are known as hyperaccumulators [5]. Hyperaccumulators can absorb and concentrate trace elements, heavy metals, and radionuclides to 100 times the normal level (possible even higher). It is believed that some hyperaccumulators absorb and concentrate such substances to produce toxic foliage, which helps them to evade predators such as caterpillars, fungi, and bacteria [5].

Currently, there are more than 400 plant species that have been identified as being able to hyperaccumulate heavy metals [5]. Most of the plant species are nickel hyperaccumulators. However, many of these plants are poorly suited for phytoextraction remediation of metal contaminated sites [3]. The reason for this fact is due to the various plant species' biomass. In previous studies, plants with a low biomass have been shown to be poorly suited for phytoextraction remediation of metal contaminated soil [5]. As result of these findings, only plants with large biomass should be used in phytoextraction remediation. In addition, the concentration and storage of toxic metal may vary between different hyperaccumulating plant species. Some plant species will concentrate and store most of the toxic metal in their root system. However, some plant species will concentrate and store most of the toxic metal in their stem and leaves. The practical use of

phytoextraction is restricted to plants which store the toxic metals in their stems and leaves (in order to allow easy harvesting of contaminated plant material).

The phytoextraction process uses the roots to absorb and translocate the toxic metals from the soil to the aboveground harvestable plant tissues (such as stems and leaves). Toxic metals will primarily enter a plant system by the root absorption of the cation from the soil solution [3]. This penetration of toxic metals is due to negative membrane potentials and the low intracellular metal activity causing an influx of these metals into the root cells. Once the toxic metals are in the root system, the metals will be concentrated. After the concentration of the toxic metals occurs, the metals will be distributed throughout the plant system.

A number of plant physiological processes are involved in the long-distance transport of the toxic metal from the roots to the aboveground plant tissues (biochemical process will be discussed in another section) [3]. The first step in this process involves unloading of the toxic metals into the root xylem cell. The standard model for metal unloading into the xylem cells involves the absorption of the metals from the root symplasm into the mature xylem vessels. Once the metals are in the xylem vessels, they are transported to the aboveground plant tissues by the transpiration stream.

The rate for the metal translocation from the root to stem varies among the different hyperaccumulating plant species. For many hyperaccumulating plant species, the rate of metal translocation from the root to stem is much lower than the

rate of uptake of metals (the roots will have a higher concentration of the metals than the stem and leaves). A possible reason for this lower rate of translocation is that most of the metals are divalent cations [3]. Because of this fact, these cations have to be complexed to organic molecules within the cell in order to travel throughout the plant system.

However, for some hyperaccumulating plant species, the rate for metal translocation is high. An example of this type of hyperaccumulating plant is *Pteris vittata* (Chinese ladder brake fern), an arsenic hyperaccumulator. *Pteris vittata* has been found to have a higher concentration of arsenic in its leaves and stem, compared to its roots [6]. In this fern, the leaves contained 96% of the total arsenic accumulated by the fern (making it a good plant for phytoextraction) [7]. If both the high metal solubility and hyperaccumulating plants are used at a metal contaminated site, then effective phytoextraction can occur.

Another type of phytoremediation is called rhizofiltration. Rhizofiltration is a type of remediation where the plant's roots (grown in aerated water) precipitate and concentrate toxic metals from contaminated aquatic environments [3]. This type of phytoremediation is very useful in cleaning up polluted surface water and groundwater contaminated with heavy metals and radionuclides.

Rhizofiltration is very similar to the phytoextraction process. Both types of remediation use hyperaccumulating plants and have similar requirements for efficient removal of contamination. In addition, both are believed to use a small biomolecule,

called phytochelatin, in their contaminate detoxification [3]. However, the main difference between these two types of phytoremediation is the site location (soil or aquatic site).

The storage process of rhizofiltration can occur as a result of several mechanisms. One process is by having extracellular and cell wall precipitation [3]. Another process is by having intracellular uptake and storage in the vacuole (in plants, the vacuole will be the waste product storage site). The uptake mechanism for rhizofiltration may involve the membrane transport systems, such as aqueous pores, ion efflux pumps, or ion selective channels [3].

A rhizofiltration plant should exhibit characteristics that provide the maximum toxic removal from a contaminated water site [3]. The plants should be able to accumulate significant amounts of the contaminant of concern and to tolerate high levels of a toxic metal. In order for these plant to effectively filter the contaminated water, they must have a high root:shoot ratio for metal uptake.

Another type of phytoremediation is called phytostabilization. In phytostabilization, plants are used to stabilize and prevent the spreading of contaminants in the soil (thus rendering the contaminants harmless) [3]. However, this method is expensive and harmful to the environment. Phytostabilization will not remove the harmful contaminants but will reduce the possible health hazards for the human species. The plant species used in phytostabilization will be different from phytoextraction plant species [3]. For phytostabilization, the plants should be poor

transducers of metal contaminants (preventing accumulation of toxic metals in aboveground plant tissue). In addition, these plants must be fast growers with a dense root system and have a high transpiration rate in order to remove moisture from the ground.

#### 2.1.4 Site Requirements for Phytoremediation

In site selection for phytoremediation, there are two limiting factors based on current techniques. One factor is root contact of the plant (primary limitation) [3]. Phytoremediation requires the plant's root zone to be in contact with the contaminants. Because of this limitation, the plant must either have extended roots or the contaminated media must be moved into the plant's root zone. To compensate for this limitation, standard agricultural equipment and practices can be used to bring the contaminated media within the root zone.

Another limiting factor for site selection is the concentration of contaminants in the water or soil. A very high concentration of contaminants may inhibit plant growth and possibly kill the plants. As result of this toxicity, phytoremediation will be limited to sites where there are low concentration levels of contaminants (can not be used at acute risk levels).

As a result of these limitations, sites for phytoremediation should contain medium-level contamination within the plant's root zone [3]. Some of the possible sites that can be repaired by phytoremediation are superfund sites (15% of sites



contaminated with heavy metals, Department of Defense sites (15% contain heavy metal contamination), and Department of Energy sites (53% contain heavy metal or radionuclide contamination) [3].

## 2.2 Arsenic

### 2.2.1 Chemical Properties and Environmental Sources

Arsenic is a metalloid and the 20<sup>th</sup> most abundant element on the planet [4].

Arsenic can occur naturally in the environment as either a trivalent or pentavalent form [1]. It is commonly present in the earth's crust. High concentrations of arsenic are released into the environment as a result of volcanic activity [2]. However, these natural sources of arsenic rarely occur as a pure substance. Instead, arsenic will be found in both inorganic (e.g. sodium arsenite, calcium arsenate, and lead arsenate) and organic forms (e.g. arsenobetaine) [2].

Besides natural sources, anthropogenic sources of arsenic can be found in the environment [1]. These sources of arsenic are generally produced from manufacturing industries (it has been estimated that 60,000 tons of arsenic is produced annually) [2]. These industries include manufacturers of glass, pesticides, wood preservatives, and semiconductors [2]. In addition, these anthropogenic sources will come from primary copper, zinc, and lead smelters [1]. However, artificial sources can also be the result of mining and the application of arsenic-based pesticides (e.g. Barber's Orchard).

### 2.2.2 Toxicity in Humans

Arsenic toxicity for humans will vary depending on the chemical form and exposure length to the arsenic source [2]. The primary toxic form of arsenic is the trivalent compound [1]. This chemical form of arsenic will affect the enzymatic activities, including mitochondrial enzymes. For example, a number of sulfhydryl-containing enzyme systems can be altered due to trivalent arsenic exposure [1]. However, pentavalent arsenic compounds have been shown to have little effect on the enzymatic process. This toxicity of the chemical form can be further enhanced by the biotransformation of arsenic in the human body [1].

The exposure length will also affect arsenic toxicity. An acute exposure will produce a variety of symptoms. Symptoms for an acute exposure include the following: fever, vomiting, anorexia, convulsions, paralysis, and possible death [2]. However, chronic exposure will also cause a number of toxic effects on the human body. This exposure may cause neurotoxicity of both the peripheral and central nervous systems (symptoms includes sensory changes, paresthesias, and demyelination of axon nerve fibers) [1]. Liver and peripheral vascular disease could also develop from this chronic exposure [1].

In addition, chronic exposure to arsenic has been linked to causing cancer (both trivalent and pentavalent arsenic has been shown in laboratory to be capable of producing chromosome breaks) [1]. Depending on the type of chronic exposure (oral, dermal, or inhalation), the possible type of cancer will vary. If the skin is chronically exposed to arsenic, then cancer tumors will develop on the skin. An inhalation-type of chronic

exposure will cause various types of upper respiratory tract cancers. Also certain liver cancers have been linked to chronic exposure of arsenic [1].

### 2.3 Phytochelatins: A Biomolecule Involved in the Phytoremediation Process

Before the translocation of the metal compounds can occur, hyperaccumulating plants must detoxify these compounds to prevent phytotoxicity of plant cells (an important step in the phytoextraction process). One of the common methods of detoxification is by chelation of the metals by a ligand. In phytoextracting plants, the most common ligands are believed to be a type of compound called metallothioneins [8].

All metallothionein compounds share three similar characteristics [3]. One characteristic is that all metallothioneins have a low molecular weight (<10kD). The second characteristic is that these compounds contain a large fraction of cysteine residues (with their -SH group). The third characteristic of metallothionein compounds is forming of metal-thiolate clusters when exposed to metal ions.

A formal classification system has been developed for these metallothionein compounds [3]. The first category is called Class I Metallothioneins. This category consists of all metallothionein compounds with a highly conserved arrangement of cysteine residues and is common to mammals. The second category is called Class II Metallothioneins and consists of all metallothionein proteins not included in Class I. The final category is called Class III Metallothioneins. This category consists of

cysteine-rich metal-binding peptides [3]. However, the metallothioneins in this category are not produced by the transcription-translation process. In this category, a group of compounds called phytochelatins are located [3].

The first detection of phytochelatins was seen in studies involving exposing plants to cadmium [8]. Since these studies, phytochelatins have been found throughout the plant kingdom. Currently, the phytochelatins are believed to be the main metallothionein compounds involved in the metal and metalloid detoxification in plants [8].

### 2.3.1 Structure of the Phytochelatins

The basic structure of phytochelatins has been shown to consist of three amino acids (Glutamic acid, Cysteine, and Glycine) [8]. Of these three amino acids, the Cysteine and Glutamic acid are linked together in the phytochelatins by a  $\gamma$ -carboxylamide bond. The general structure is similar to a compound called glutathione (GSH) [8].

The phytochelatins make up a family of structures with increasing repetitions of the  $\gamma$ -Glu-Cys dipeptide (repetition units). This dipeptide chain has been found in various plants with a range of 2 to 11 repetition units (2 to 5 units is the most common types found) [8]. The terminal end of these structures was determined to be the Glycine. In addition, the phytochelatins have been shown to structurally vary between different plant species (for example, the terminal Glycine is replaced by

Serine in some plant species) [8]. However, all phytochelatins appear to bind metals and metalloids by the same mechanism (no matter the structural variation of the phytochelatins between species).

### 2.3.2 Biosynthesis of Phytochelatins

Phytochelatins are believed to be synthesized from an organic molecule called glutathione (GSH) [8]. In various studies involving different hyperaccumulating plants, the increase in phytochelatins corresponds to the decrease in GSH levels in plant cells [3]. In studies involving cadmium hyperaccumulating plants, inhibition of GSH synthesis resulted in prevention of phytochelatin production [8]. This prevention of phytochelatin production was reversed by the addition of GSH to the growth medium [8].

The initial step of phytochelatin synthesis occurs by the transpeptidation of the  $\gamma$ -Glu-Cys moiety from one GSH molecule onto another GSH molecule, which forms the first phytochelatin ( $PC_2$ ). In later stages of this synthesis process, this  $\gamma$ -Glu-Cys moiety will be transpeptidated onto a phytochelatin molecule (resulting in different types of phytochelatins being produced) [8]. The transpeptidation occurring in this synthesis is due to an enzyme called PC synthase. However, in order for this synthase to be activated, the enzyme must be exposed to metals or metalloid compounds.

The initiation of the biosynthesis of phytochelatins has been connected to the exposure of plant cells (primarily root cells) to metal or metalloid contamination

(causing activation of PC synthase) [3]. In plants exposed to only trace amounts of heavy metals, low concentration of phytochelatin have been detected [3]. However, a large exposure of plant tissue to heavy metals has been shown to cause a high level of phytochelatin to be produced. Within a few hours, an entire plant system will have a high concentration of phytochelatin in its cells [3].

### 2.3.3 Function of Phytochelatin

Phytochelatin can be detected in plant tissues exposed to only trace levels of essential metals [3]. In addition, *in vitro* studies involving these compounds in the cell cultures have found a strong correlation between the concentration of depleted metal ions in the culture medium and phytochelatin levels. These findings have been used as evidence of phytochelatin's role in the homeostasis of essential metal ion metabolism [3].

The clearest evidence of phytochelatin's role in heavy metal detoxification can be found in the characterization of the phytochelatin-deficient mutant, *cad1-3* of *Arabidopsis* [3]. *Cad1-3* mutants were compared to wild type plants (normal, nonmutated plants) and semi-quantitative data of metal detoxification by phytochelatin were collected (both wild types and mutants were exposed to different types of metal and metalloid ions). When both mutant and wild type plants were exposed to cadmium ions, a 40-fold difference in detoxification ability was detected, with wild types being better at detoxification. When the plants were exposed to  $\text{AsO}_4$

anion, there was a 20-fold difference in detoxification between the mutant and wild type [3]. This finding indicates that the phytochelatins have a significant role in the detoxification of both these types of ions. However, when the plants (wild type and mutant) were exposed to zinc and nickel ions, no difference in the detoxification ability between wild type and mutant plants was found. It appears that the detoxification of metals by phytochelatins is a very selective process (e.g. will detoxify only certain types of metals) [3]. Based on these findings, the detoxification step of phytoextraction is very complex in nature [3].

The detoxification of metals or metalloids with phytochelatins involves the formation of complexes [3]. These complexes form by using the –SH group of the phytochelatins to bind to the metal or metalloid ion. A complete phytochelatin-metal complex will consist of at least two phytochelatins. However, there are two types of complexes. The type of complexes are called high molecular weight (HMW) and low molecular weight (LMW). The transition between the two types of complexes may depend on the time period and level of exposure to a metal or metalloid contamination (at least for plants) [3]. In *Arabidopsis*, exposure to a high concentration of cadmium resulted in a higher production of HMW complexes [3]. In addition, the type of complex formation may also be depended on the amount of total phytochelatins synthesized (HMW complexes favor a large concentration of phytochelatins) [3].

#### 2.3.4 Location of Phytochelatin Complexes

Once phytochelatins have complexed with a metal, the complexes will be transported throughout the plant system [3]. Within individual plant cells, these phytochelatin-metal complexes have been shown to be sequestered inside the cell's vacuole (a vacuole is a large membrane-bound structure inside an individual plant cell, which stores waste products of the cell) [8]. The sequestering of these complexes in vacuoles have been shown in tobacco plants [3]. In tobacco plants exposed to a heavy cadmium contamination, the largest concentration of cadmium and phytochelatins has been found in the plant's vacuole. Currently, the method of transporting these complexes into the vacuole is unknown. However, it is believed that membrane-bound carrier proteins are responsible for this transportation of the complexes [3].

#### 2.4 Hyperaccumulators of Arsenic

The first known arsenic hyperaccumulating plant was identified to be *Pteris vittata* (Chinese Brake Fern) [9]. Recently, a second arsenic hyperaccumulator called *Pteris cretica cv Mayii*, has been identified [6]. Normally, arsenic is very toxic to plants [10]. Arsenic affects nonaccumulating plants in several ways including interfering with phosphate metabolism and reacting with plant proteins [11]. These effects will ultimately result in prevention of cellular function and death [11]. However, both *P. vittata* and *P. cretica cv. Mayii* detoxify arsenic.

In a study by Huang et al. (2004), the phytoremediation of arsenic-containing water by *P. vittata* and *P. cretica cv. Mayii* was investigated [6]. In this study, both



arsenic hyperaccumulators were grown in water containing  $^{73}\text{As}$ -labeled arsenic (arsenic concentration varied for water samples between 20 and 500ppb). In addition, a nonhyperaccumulating fern (*Nephrolepis exaltata*) was exposed to the arsenic water samples. In this study, they determined the efficiency of arsenic removal from the water by continuously monitoring the depletion of the  $^{73}\text{As}$ -labeled arsenic in the water. At the end of the study, Huang et al. found that both arsenic hyperaccumulators (*P. vittata* and *P. cretica cv. Mayii*) efficiently and rapidly removed the arsenic from the water samples (arsenic concentration in water samples was below the current legal limit for arsenic concentration in water). In addition, they detected no difference in arsenic uptake levels between the different arsenic hyperaccumulating species.

A study by Lombi et al. investigated the localization and speciation of arsenic in *P. vittata* [7]. In this study, they found 93% of the total arsenic accumulated to be concentrated in the fronds, with most of the arsenic stored in the pinnae parts of the frond. In addition, Lombi et al. found very little arsenic in the developing spores (only about 2%). Using energy dispersive x-ray microanalyses, they determined elements with a high concentration in the fronds. The results of this analysis showed a positive correlation between potassium and arsenic concentration in the frond cells. This analysis also showed that the arsenic may be stored in the cell's vacuole. Using x-ray absorption near edge spectroscopy analysis, Lombi et al. showed that 75% of the arsenic in the fronds exists in the  $\text{As}^{3+}$  form [7].

In a recent study by Gumaelius et al. (2004), an investigation of possible arsenic accumulation by *P. vittata*'s gametophytes was conducted [10]. Ferns have a type of life

cycle called alternation of generation which consists of two independent stages, gametophytes (haploid) and sporophytes (diploid). In this study, spores were harvested and grown in a medium containing different concentrations of arsenate. Using ICP-MS, Gumaelius et al. measured the arsenic uptake levels in the developing gametophytes. In this study, they found that gametophytes are also able to uptake arsenic like the sporophyte stage of *P. vittata*. They found that gametophytes accumulate arsenic up to 2.5% of their dry weight when grown in a medium contained between 5 to 10mM arsenate.

A study by Wang et al. investigated the mechanism of arsenic uptake by this fern [11]. They found that the fern will take up arsenate by a phosphate transport system. This finding was supported by varying the concentrations of phosphate in their nutrient solution. When they increased the phosphate concentration, the arsenic levels in the roots and shoots decreased. However, when the phosphate concentrations were low, the roots and shoots increase arsenic uptake. Based on their findings, however, the uptake of arsenite is conducted by some other unidentified transport system (variation in phosphate concentration did not affect the uptake of arsenite). In addition, they found that phosphate concentration may not affect the arsenic transportation from the root to the fronds [11].

The possible role of phytochelatin in arsenic tolerance in *P. vittata* was investigated by Zhao et al. [12]. In this study, they exposed *P. vittata* to arsenate ( $\text{Na}_2\text{HAsO}_4$ ) for a specific amount of time (no more than 7 days). In this study, they detected only one species of phytochelatin ( $\text{PC}_2$ ). The arsenic concentration correlated

significantly with concentration of phytochelatins in the root and shoot (the shoot contained the higher concentration of phytochelatins). When Zhao et al. exposed the ferns to BSO, an inhibitor of GSH synthetase, the concentrations of phytochelatins decreased in response to the arsenate. However, the degree of phytotoxicity appeared to remain the same (with or without BSO exposure). According to Zhao et al., this observation supported the idea of phytochelatins playing a limited role in hypertolerance of arsenic in *P. vittata* [12].

### Chapter 3: Goals of Project

The major objective of this project was the investigation of the phytoremediation by two arsenic hyperaccumulators (*P. vittata* and *P. cretica cv Mayii*). The project had three goals. The first goal was the construction of a system for the controlled propagation of arsenic hyperaccumulators. A controlled propagation system is required in order to insure that hyperaccumulators are provided maximum exposure to arsenic. In some previous studies, the arsenic hyperaccumulators were propagated in a soil-based system. The problem with this system is that a soil matrix provides only limited exposure of the hyperaccumulators to the arsenic. However, this problem is prevented in a controlled propagation system. In addition, a controlled propagation system will allow for the controlled exposure of macronutrients and micronutrients. The controlled exposure of the hyperaccumulators is required due to previous studies showing possible links between arsenic uptake by the hyperaccumulators and nutrient concentrations [1].

The second goal of the project was the growth and measurement of arsenic uptake by hyperaccumulators. In addition, the macro- and micro- nutrient levels of the hyperaccumulators were measured. The nutrient levels were measured in order to establish any possible link between certain nutrients and arsenic uptake of the plant.

The third goal of the project was conducting a series of arsenic-based experiments using Ion Chromatography. This goal was designed to determine if arsenic speciation could be detected by ion chromatography. If possible, the arsenic speciation by ion

pchromatography would prove support evidence for specific species of arsenic concentrated by the hyperaccumulators.

## Chapter 4: Methodology

### 4.1 Propagation of Arsenic Hyperaccumulators

#### 4.1.1 Construction of Arsenic Hyperaccumulator Propagation System

The growth medium for the hyperaccumulators was a hydroponic system. This hydroponic system was constructed and used in the greenhouse at Western Carolina University (Figure 1 and 2). The system was designed and constructed for the maximum growth of 60 plants. The hydroponic system consists of six plastic growth trays, two wooden growth racks (for holding the growth trays), and six solution reservoirs. In addition, this system used six hydroponic-type pumps and timer-set growth lights.



Photo by: Ralph Patterson

Figure 1: Site of Western Carolina University Hydroponic System



Photo by: Patrick Baldwin

Figure 2: Overview of Western Carolina University's Hydroponic System

#### 4.1.2 Arsenic Hyperaccumulators

Two different types of arsenic hyperaccumulators were grown in the hydroponic system, *Pteris vittata* (Chinese Brake Fern) and *Pteris cretica cv Mayii* (Moonlight Fern) (Figure 3 and 4). These arsenic hyperaccumulators were purchased from the Edenspace Corporation. *P. vittata* was grown for two cycles in the hydroponic system (ninety cellpak type of this species were grown). However, *P. cretica cv. Mayii* was grown for only one cycle in the system.

In the hydroponic system, both species of hyperaccumulators were exposed to three types of arsenic solutions. The solutions contained either zero arsenic, arsenite, or arsenate (10 liters of each solution were prepared). The arsenite solutions were prepared by adding 500 $\mu$ M of NaAsO<sub>2</sub> to the hydroponic nutrient solution. The arsenate solutions were prepared by the addition of 500 $\mu$ M of Na<sub>2</sub>HAsO<sub>4</sub>•7H<sub>2</sub>O to the nutrient solution. The zero arsenic solutions contain only hydroponic nutrient solution.



Photo by: Ralph Patterson

Figure 3: Photo of *Pteris vittata* (Chinese Brake Fern)



Photo by: Patrick Baldwin

Figure 4: Photo of *Pteris cretica cv Mayii* (Moonlight Fern)



#### 4.1.3 Operation of Arsenic Hyperaccumulator Hydroponic System

The operation of the hydroponic system consisted of growing thirty plants per four week cycle. Cycle 1 was conducted during the months of June and July. Cycle 2 was conducted during the months of October and November. Prior to the insertion of plants into the system, each plant was carefully rinsed with ultrapure water to remove any possible contamination. Each growth tray was lined with all-weather black plastic sheets. In these sheets, the plants were placed 60 cm apart from each other (Figure 5). In order to prevent the plants from being accidentally moved from their location, industrial-strength plastic clothes pins were placed on both sides of each plant.

Each solution reservoir had its own hydroponic pump and tube. The tubing was run under the black plastic sheet during a growth cycle (to minimize possible algae growth). In addition, the reservoirs were covered with plastic. The growth racks were tilted slightly to allow the hydroponic nutrient solution to naturally flow back into the reservoirs (Figure 6). The flow rate was maintained at 4 L/min in order to keep good aeration of the roots (controlled by the tilting of the racks). The hydroponic nutrient solution used in this experiment was prepared from three different solutions. The hydroponic solutions were as follow: Floragro, Floramicro, and Florabloom (General Hydroponics). The solutions were prepared by adding Floramicro (26.4mL), Floragro (39.6mL), Florabloom (13.2mL) to ultrapure water (9.020L).



Photo by: Patrick Baldwin

Figure 5: A Side-view of the Western Carolina University Hydroponic System with Hyperaccumulators (*Pteris cretica cv Mayii*)



Photo by: Patrick Baldwin

Figure 6: Hydroponic Solution Returning to the Reservoir

Before the plants were inserted into the system for a cycle, the growth trays, pumps, and reservoirs were cleaned with 10% nitric acid (these washes were then analyzed to ensure that no arsenic contamination occurred). The plastic sheets and pump tubing were replaced after each cycle.

Once the plants were inserted into the system, they were cultivated for four weeks under growth lights (the plants were exposed to 15 hours of light). During the course of a cycle, the volumes of hydroponic solutions were maintained at 15 liters (replacement liquid used to maintain this volume was ultrapure water). On a weekly basis, a 30 mL sample of hydroponic solution was collected from each reservoir. These samples were then analyzed for pH and electrical conductivity levels.

At the end of the 4-week growth cycle, the plants were removed from the system. The plastic sheets and tubing were removed from the system. Both the sheets and the tubing were then analyzed for the presence of arsenic.

## 4.2 Arsenic Uptake Analysis

### 4.2.1 Sample Preparation of Plant Tissue Samples for Inductively Coupled Plasma-Optical Emission Spectrum

Following the removal of the hyperaccumulators from the hydroponic system, each fern was weighed (wet weight). Once the initial weights of the ferns were collected, they were oven-dried for 24 hours at 40°C. After the 24 hour period, each fern was removed from the oven and reweighed (dry weight). The *P. cretica cv* Mayii samples were then divided into three different portions: fronds, stems, and roots. However, the *P.*

*vittata* samples were not divided into different portions due to their smaller size (not enough plant tissue of each type for ICP analysis). Next, the samples were pulverized for homogenization using a Spex 8000 Mixer/Mill.

Once the samples were pulverized, each sample (0.200g) was weighed in triplicate into a test tube. Concentrated nitric acid (5mL) was added to each test tube. In addition, 5mL of concentrated nitric acid was added to an empty test tube (a reagent blank). The samples were heated on a lab constructed hotplate for 2 hours at 90°C.

After the samples had heated for 2 hours, the samples were allowed to cool to room temperature (~40 minutes) and 0.5mL of 30% H<sub>2</sub>O<sub>2</sub> was added to each sample and the reagent blank. Next, the samples were reheated on a hotplate for 1 hour at 90°C.

Once the final heating phase was finished, the samples were allowed to cool to room temperature. Next, each sample was gravity filtered in a volumetric flask. After filtration, the samples were diluted to volume using ultrapure water.

#### 4.2.2 Inductively Coupled Plasma-Optical Emission Spectrum Analysis of Plant Tissue Samples

Arsenic concentration of the plant tissue samples was determined by an Inductively Coupled Plasma-Optical Emission Spectrum (ICP-OES). The model used for this analysis was Perkin Elmer Optima 4100 DV (Figure 7). The operation conditions for the ICP-OES are given in Table 1.

In addition to the arsenic concentration, both macronutrient and micronutrient concentration were measured by ICP-OES. The macronutrients were calcium, magnesium, phosphorus, and sulfur. The micronutrients were sodium, iron, molybdenum,

manganese, copper, and nickel. The emission lines analyzed by the ICP-OES for both macronutrient and micronutrient can be seen in Table 2 and 3.

In order to accurately measure the arsenic concentration in the plant tissue samples, aqueous standards were prepared for calibration of the ICP-OES (stock standard was 1000 ppm As/Pb). The linear calibration range was from 0.025 ppm to 40.0 ppm. Before the calibration occurred, a calibration blank (a reagent blank) was analyzed to remove any background interference from the analysis. Once the ICP-OES analysis was complete, the original concentration and statistical analysis was conducted. The average concentration, standard derivation and Student's t-test was conducted using Microsoft Excel.



Photo By: Patrick Baldwin

Figure 7: Perkin Elmer Optima 4100 DV (ICP-OES)

Table 1: The Operation Conditions of Perkin Elmer Optima 4100 DV

Power: 1.3 kW
Argon flow rates: Nebulizer gas: 0.8 L/minute Auxiliary gas: 0.2 L/ minute Plasma gas: 15 L/minute
Emission lines for arsenic: 188.979, 193.696, 197.197, and 228.812nm

Table 2: The Emission Lines for the Macronutrient Analyzed By Perkin Elmer Optima 4100 DV

Element	Emission Line
Calcium	315
Magnesium	279
Phosphorus	213
Sulfur	181

Table 3: The Emission Lines for the Micronutrient Analyzed By Perkin Elmer Optima 4100 DV

Element	Emission Line
Sodium	330nm
Iron	239nm
Molybdenum	203nm
Manganese	259nm
Copper	224nm
Nickel	232nm

#### 4.3 Arsenic-based Experiments using Ion Chromatography

A series of arsenic-based ion chromatography experiments were conducted using DX-120 Ion Chromatograph (Dionex Corporation) (Figure 8). These experiments consisted of separation and detection of different anionic species of arsenic (arsenite and arsenate). To conduct these arsenic experiments, two different 1000 ppm arsenic stock solutions (arsenite and arsenate) were prepared. The arsenite solution was prepared by dissolving 173.43 mg of  $\text{NaAsO}_2$  in 1 L of ultrapure water. The arsenate solution was prepared by dissolving 416.58mg of  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  in 1 L of ultrapure water.

The first type of arsenic-based ion chromatography experiments consisted of detection of the peak retention time for each species of arsenic using 10 ppm standard solution of arsenite and arsenate. The second type of arsenic-based ion chromatography

experiments consisted of separation and detection of different species of arsenic in a mixed solution. To conduct this type of experiment, a series of 10 ppm and 20 ppm mixed solutions were prepared from arsenic stock solutions. These samples were transferred to 0.5 mL vials and then analyzed by the Ion Chromatograph.



Photo by: Patrick Baldwin

Figure 8: Photo of HPLC and IC



## Chapter 5: Results and Discussion

### 5.1 Arsenic Uptake by Hyperaccumulating Plants

#### 5.1.1 Arsenic Uptake by *Pteris vittata*

An analysis of the arsenic concentration was measured by ICP-OES. The arsenic concentrations for two *Pteris vittata* growth cycles are shown in figure 9. The average arsenite concentration for the first growth cycle of *Pteris vittata* was  $1400 \pm 520 \mu\text{g/g}$ . The average arsenate concentration for the first growth cycle of *Pteris vittata* was  $1170 \pm 260 \mu\text{g/g}$ . For the second growth cycle, the average arsenite concentration of *Pteris vittata* was  $1530 \pm 450 \mu\text{g/g}$ . The arsenate concentration for this cycle was  $1040 \pm 320 \mu\text{g/g}$ .

A Student's t-test analysis was conducted to determine if *Pteris vittata* extracted more of one form of arsenic. The Student's t-test results for analysis of growth cycle 1 arsenic uptake determined that there was no significant difference in uptake between arsenite and arsenate (T value was 1.27 and T Critical value was 2.16). However, the results for Student's t-test for growth cycle 2 determined that there is a significant difference between the uptake by different arsenic forms (T value was 2.78 and T Critical value was 2.12). The discrepancy in the uptake between different arsenic forms could be due to the effect of certain macro- or micronutrient uptake (Note: please see page 40).

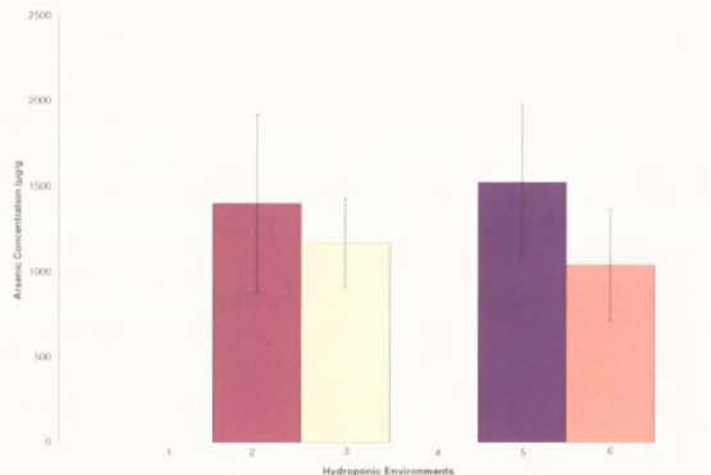


Figure 9: Comparison of Arsenic Concentration in Entire Tissue Samples from *Pteris vittata* Grown in Three Different Hydroponic Environments (n= 10, Concentration of Arsenite and Arsenate in solution= 500µM, 1= Cycle 1 Control Solution, 2= Cycle 1 Arsenite Solution, 3= Cycle 1 Arsenate Solution, 4= Cycle 2 Control Solution, 5= Cycle 2 Arsenite Solution, and 6= Cycle 2 Arsenate Solution)

### 5.1.2 Arsenic Uptake by *Pteris cretica cv Mayii*

The analysis of the arsenic concentration in various tissues of *Pteris cretica cv Mayii* can be seen in Figure 10. The average arsenite concentration for the root tissue of *Pteris cretica cv Mayii* was  $27.0 \pm 42.0 \mu\text{g/g}$ . The stem tissue of *Pteris cretica cv Mayii* contained an average arsenite concentration of  $1300 \pm 370 \mu\text{g/g}$ . The leaf tissue contained an average arsenite concentration of  $1090 \pm 330 \mu\text{g/g}$ . A Student's t-test analysis was conducted to determine if leaf tissue contained significantly more arsenic than stem tissue. The results of the Student's t-test determined that there was no significant difference in arsenic concentration between the leaf and stem tissue (T value was 0.977 and T Critical value was 2.30). These findings correspond with previous

studies conducted on *Pteris vittata*. A previous study by Lombi et al. showed 93% of arsenic uptake by *Pteris vittata* to be concentrated in the above ground biomass (stem and leaf material) [6]. In the Chinese Brake Fern, Lombi et al. found only a little arsenic present in the root tissue [6]. Based on the findings of this project, it appears that *Pteris cretica cv Mayii* has a similar pattern of arsenic distribution and concentration as *Pteris vittata*.

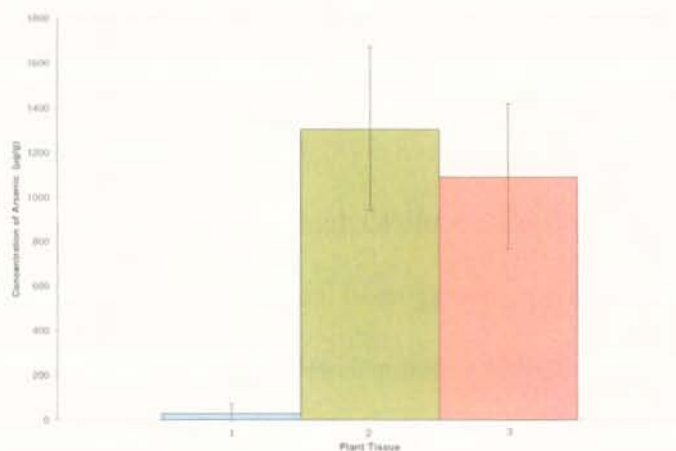


Figure 10: Comparison of Arsenic Concentration in Various Tissue Samples from *Pteris cretica cv Mayii* Grown in an Arsenite Hydroponic Environment (n= 5, Concentration of Arsenite in solution= 500µM, 1= Root Tissue, 2= Stem Tissue, and 3= Leaf Tissue)

## 5.2 Nutrient Uptake by Arsenic Hyperaccumulators

### 5.2.1 Macronutrient Concentration Levels in *Pteris vittata*

A macronutrient analysis using ICP-OES was conducted on the tissue samples from the arsenic hyperaccumulators. A plant macronutrient is an element with a

concentration greater than  $30 \mu\text{mol g}^{-1}$  per dry weight [13]. The four macronutrients analyzed were calcium, magnesium, phosphorus, and sulfur. A summary of macronutrient data is available in Appendix 1.1.

The most common macronutrient detected for arsenic-free, arsenite, and arsenate-exposed *Pteris vittata* was calcium (Growth Cycle 1 and 2) (Figure 11). The average concentrations of calcium for the arsenic-free plants were  $7450 \pm 1080 \mu\text{g/g}$  (Cycle 1) and  $9200 \pm 2200 \mu\text{g/g}$  (Cycle 2). The calcium concentrations in the plants, grown in arsenite, were  $10,500 \pm 2700 \mu\text{g/g}$  (Cycle 1) and  $9700 \pm 1700 \mu\text{g/g}$  (Cycle 2). The average concentrations of calcium in the arsenate-grown plants were  $9500 \pm 1450 \mu\text{g/g}$  (Cycle 1) and  $9300 \pm 2400 \mu\text{g/g}$  (Cycle 2).

The most probably reason for the high calcium concentration levels in *Pteris vittata* is due to the element's role in normal plant growth and development (Figure 11). Calcium is required for normal cell wall development. Also, the plants use calcium as a cofactor in certain enzymes and as a secondary messenger in metabolic regulation [13].

The least common of the four macronutrients analyzed for the arsenic-free, arsenite-grown, and arsenate-grown *Pteris vittata* was sulfur (Figure 12). The average concentrations of sulfur in the arsenic-free plants were  $2740 \pm 400 \mu\text{g/g}$  (Cycle 1) and  $3410 \pm 800 \mu\text{g/g}$  (Cycle 2). The sulfur concentrations in the arsenite-grown plants contained  $3440 \pm 700 \mu\text{g/g}$  (Cycle 1) and  $3600 \pm 540 \mu\text{g/g}$  (Cycle 2). The average concentrations of sulfur in the arsenate-grown plants were  $3160 \pm 510 \mu\text{g/g}$  (Cycle 1) and  $3250 \pm 800 \mu\text{g/g}$  (Cycle 2). The most probable reason for the sulfur concentrations is that it was used basically as a component of cysteine, methionine, and proteins [13].

A Student's t-test analysis was conducted to determine if *Pteris vittata* contained more calcium when grown in the arsenic-free solution or arsenic-containing solutions (Figure 11 shows average of calcium for all environments). The Student's t-test results showed that there was a significant difference in calcium concentration between arsenic-free and arsenite-grown plants for Cycle 1 (T value was 3.33 and T Critical value was 2.17). However, the results of the Student's t-test showed no significant difference for Cycle 2 (T value was 0.583 and T Critical value 2.11). In addition, the Student's t-test showed that there was a significant difference in calcium concentration between the arsenic-free and arsenate-grown plants for Cycle 1 (T value was 3.65 and T Critical value was 2.11). The results of arsenic-free and arsenate-grown plants showed no significant difference in calcium concentration (T value was 0.125 and T Critical value 2.10). Also, Student's t-test showed that there was no significant difference in calcium concentration between the arsenite and arsenate-grown plants (T value was 0.995 and T Critical value was 2.14 for Cycle 1; T value was 0.415 and T Critical value 2.12 was for Cycle 2). A similar comparison was made for magnesium (data not shown). There was no significance difference between any of the replicates.

A Student's t-test analysis was conducted to determine if *Pteris vittata* extracted more phosphorus from the arsenic-free solution or arsenic-containing solutions (Figure 13 shows average of phosphorus for all environments). The Student's t-test results showed that there was a significant difference in phosphorous uptake between arsenic-free and arsenite-grown plants (T value was 2.34 and T Critical value was 2.12 for Cycle 1; T value was 3.75 and T Critical value 2.10 was for Cycle 2). Also, Student's t-test

showed that there was no significant difference in phosphorous uptake between the arsenic-free and arsenate plants (T value was 1.57 and T Critical value was 2.10 for Cycle 1; T value was 0.602 and T Critical value 2.11 was for Cycle 2).

Next, a Student's t-test analysis was conducted to determine if arsenite-grown plants contained significantly more phosphorus than arsenate-grown plants. The results of this analysis for Cycle 1 showed that there was no significant difference in phosphorus concentrations between arsenic exposures (T value was 0.987 and T Critical value was 2.11). However, the results of the Student's t-test for Cycle 2 showed that there was a significant difference in phosphorus concentrations between arsenite and arsenate-grown plants (T value was 4.70 and T Critical value was 2.11).

The significant difference in phosphorus concentration between arsenite and arsenate-grown plants for cycle 2 could possibly have been a factor in arsenic uptake by these plants. In cycle 2, a significant difference was detected in the amount of arsenite uptake compared to arsenate. For arsenite-grown plants, a higher concentration of phosphorous was detected than the arsenate-grown plants. In a previous study by Wang et al., the concentration of phosphorus was shown to directly affect the amount of arsenate uptake [11]. In their study, they found that increasing the amount of phosphate in the nutrient solution decrease the concentration of arsenic in the plant tissue. However, they found that the phosphorus concentration does not affect arsenite uptake by *Pteris vittata*. Based on their findings, the results of this project can be better understood. The significant difference in arsenic uptake between the arsenite and arsenate exposed plants (cycle 2) is partially due to concentration of phosphorus. Since phosphorus concentration

does not affect arsenite uptake, the arsenite-grown plants had no form of interference in their arsenic uptake (thereby having higher concentration of both arsenic and phosphorus compared to the arsenate-grown plants). However, the arsenate-grown plants may be a competitive interference from the phosphorus concentration in the hydroponic solution. As a result of this fact, the amount of arsenate uptake was reduced due to having to compete with the phosphorus for uptake by the roots (reducing both the amount of phosphorus and arsenate uptake by the plant).

Another Student's t-test analysis was conducted to determine if *Pteris vittata* extracted more sulfur from the arsenic-free solution or arsenic-containing solutions (Figure 12 shows average of sulfur for all environments). The Student's t-test results showed that there was a significant difference in sulfur uptake between arsenic-free and arsenite-grown plants (T value was 2.76 and T Critical value). However, the results of the Student's t-test showed no significant difference in sulfur uptake between arsenic-free and arsenite-grown plants of Cycle 2 (T value was 0.600 and T Critical value 2.12). Also, Student's t-test showed that there was no significant difference in sulfur uptake between the arsenic-free and arsenate-grown plants (T value was 2.05 and T Critical value was 2.11 for Cycle 1; T value was 0.464 and T Critical value 2.10 was for Cycle 2). In addition, Student's t-test showed that there was no significant difference in sulfur uptake between the arsenite and arsenate-grown plants (T value was 1.03 and T Critical value was 2.11 for Cycle 1; T value was 1.14 and T Critical value 2.12 was for Cycle 2).

Next, sulfur to arsenic ratio was calculated for the arsenic-exposed *P. vittata*. The results of this ratio are shown in Table 2. These findings showed the highest ratio

belonged to the arsenate-grown plants. In these plants, cysteine-containing phytochelatins are believed to be involved in the transportation and detoxification of arsenic [8]. Based on the findings of this study, no increase sulfur uptake in the arsenic-grown plants was detected. Therefore, the phytochelatins may be broken down and recycled by plant's cells for later synthesis of new phytochelatins (resulting in no increase in sulfur uptake for arsenic-grown plants).

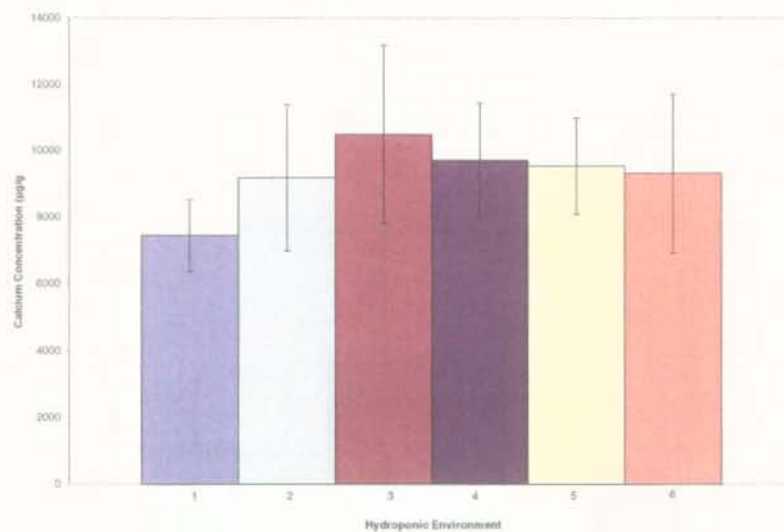


Figure 11: Comparison of Calcium Concentration in Entire Tissue Samples from *Pteris vittata* Grown in Three Different Hydroponic Environments (n= 10, Concentration of Arsenite and Arsenate in solution= 500µM, 1= Cycle 1 Control Solution, 2= Cycle 2 Control Solution, 3= Cycle 1 Arsenite Solution, 4= Cycle 2 Arsenite Solution, 5= Cycle 1 Arsenate Solution, and 6= Cycle 2 Arsenate Solution)



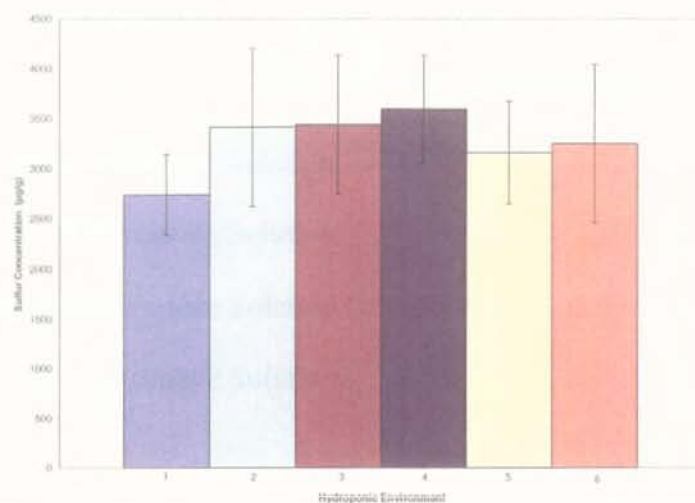


Figure 12: Comparison of Sulfur Concentration in Entire Tissue from *Pteris vittata* Grown in Three Different Hydroponic Environments (n= 10, Concentration of Arsenite and Arsenate in solution= 500 $\mu$ M, 1= Cycle 1 Control Solution, 2= Cycle 2 Control Solution, 3= Cycle 1 Arsenite Solution, 4= Cycle 2 Arsenite Solution, 5= Cycle 1 Arsenate Solution, and 6= Cycle 2 Arsenate Solution)

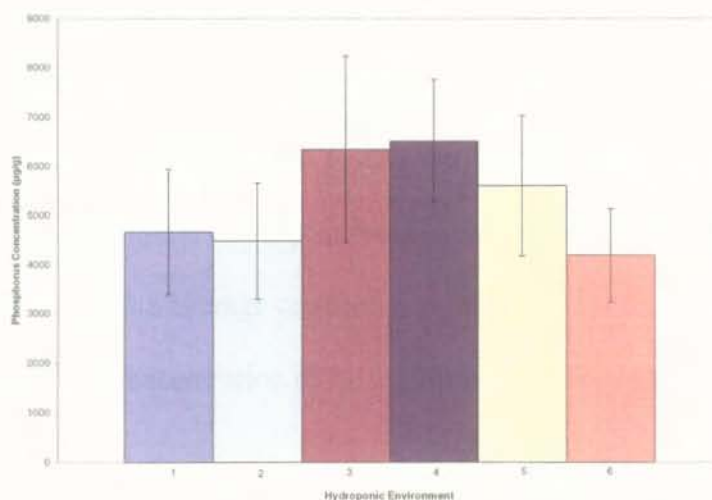


Figure 13: Comparison of Phosphorus Concentration in Entire Tissue Samples from *Pteris vittata* Grown in Three Different Hydroponic Environments (n= 10, Concentration of Arsenite and Arsenate in solution= 500 $\mu$ M, 1= Cycle 1 Control Solution, 2= Cycle 2 Control Solution, 3= Cycle 1 Arsenite Solution, 4= Cycle 2 Arsenite Solution, 5= Cycle 1 Arsenate Solution, and 6= Cycle 2 Arsenate Solution)

Table 4: The Sulfur to Arsenic Ratios for Arsenic-exposed *Pteris vittata*

Hydroponic Environment	Sulfur/Arsenic Ratio
Cycle 1 Arsenite Solution	2.45
Cycle 2 Arsenite Solution	2.70
Cycle 1 Arsenate Solution	2.40
Cycle 2 Arsenate Solution	3.12

#### 5.2.2 Macronutrient Concentration Levels in *Pteris cretica cv Mayii*

A macronutrient analysis was also conducted by ICP-OES on the tissue samples from *Pteris cretica cv Mayii*. A summary of macronutrient data is available in Appendix 1.2. The most common macronutrient detected in the root tissue was calcium (Figure 14). The average concentrations of calcium for the arsenic-free plants were  $29,500 \pm 5,020$   $\mu\text{g/g}$ . The calcium concentrations in the arsenite-grown plants contained  $22,300 \pm 1,150$   $\mu\text{g/g}$ . The least common macronutrient in root tissue for both types of arsenic exposure was sulfur (Figure 16). The average concentration of sulfur for the arsenic-free roots was  $3,320 \pm 550$   $\mu\text{g/g}$ . The concentration of sulfur in the arsenite-grown roots was  $1,970 \pm 350$   $\mu\text{g/g}$ .

The most common macronutrient detected in the stem tissue was phosphorus (Figure 15). The average concentrations of phosphorus for the arsenic-free plants were  $5740 \pm 440$   $\mu\text{g/g}$ . The phosphorus concentrations in the arsenite-grown plants contained

4,760 ± 600 µg/g. The least common macronutrient in stem tissue for both types of arsenic exposure was sulfur (Figure 16). The average concentration of sulfur for the arsenic-free stem was 1,280 ± 140 µg/g. The concentration of sulfur in the arsenite-grown stem was 1,780 ± 280 µg/g.

The most common macronutrient detected in the leaf tissue was phosphorus (Figure 15). The average concentrations of phosphorus for the arsenic-free plants were 12,200 ± 1,500 µg/g. The phosphorus concentrations in the arsenite-grown plants contained 9,800 ± 1040 µg/g. The least common macronutrient in stem tissue for both types of arsenic exposure was sulfur (Figure 16). The average concentration of sulfur for the arsenic-free stem was 2,940 ± 230 µg/g. The concentration of sulfur in the arsenite-grown stem was 2,700 ± 181 µg/g.

A Student's t-test analysis was conducted to determine if arsenic-free *Pteris cretica Mayii* contained more calcium than the arsenite plants (Figure 14 shows average of calcium for both environments). The Student's t-test results for the root tissue showed that there was a significant difference in calcium concentration between arsenic-free and arsenite-grown plants (T value was 3.13 and T Critical value was 2.78). Also, Student's t-test for stem tissue showed that there is a significant difference in calcium concentration between the arsenic-free and arsenite-grown plants (T value was 2.55 and T Critical value was 2.36). Finally, the Student's t-test results for leaf tissue showed a significant difference in calcium concentration between arsenic-free and arsenite-grown plants (T value was 3.23 and T Critical value was 2.57). Based on these findings, it appears that

for certain tissue the arsenic-free *Pteris cretica cv Mayii* will concentrate more calcium than the *Mayii* ferns exposed to arsenite.

A Student's t-test analysis was conducted to determine if arsenic-free *Pteris cretica Mayii* contained more phosphorus than the arsenite plants (Figure 15 shows average of phosphorus for both environments). The Student's t-test results for root tissue showed that there was a significant difference in phosphorous concentration between arsenic-free and arsenite-grown plants (T value was 8.11 and T Critical value was 2.30). The Student's t-test results for the stem tissue showed that there was a significant difference in phosphorous concentration between the arsenic-free and arsenite-grown plants (T value was 2.93 and T Critical value was 2.36). Finally, the Student's t-test for leaf tissue showed a significant difference in phosphorous concentration between arsenic-free and arsenate-grown plants (T value was 2.91 and T Critical value was 2.36). Based on these findings, it appears that the arsenic-free *Pteris cretica cv Mayii* extracted and concentrated more phosphorus than the *Mayii* ferns exposed to arsenite.

Another Student's t-test analysis was conducted to determine if the arsenic-free *Pteris cretica cv Mayii* significantly concentrated more sulfur than the arsenite-grown *Mayii* (Figure 16 shows average of sulfur for both environments). The Student's t-test results for the root tissue showed that there was a significant difference in sulfur concentration between arsenic-free and arsenite-grown plants (T value was 4.61 and T Critical value was 2.36). Also, Student's t-test for stem tissue showed that there a significant difference in sulfur concentration between the arsenic-free and arsenite-grown plants (T value was 3.49 and T Critical value was 2.45). Finally, the Student's t-test

results for leaf tissue showed no significant difference in sulfur concentration between arsenic-free and arsenite-grown plants (T value was 1.69 and T Critical value was 2.31). Based on these findings, it appears that for certain tissue the arsenic-free *Pteris cretica cv Mayii* will concentrate more sulfur than the *Mayii* ferns exposed to arsenite.

A similar comparison was made for magnesium for the various tissues (data not shown). There was no significance difference between any replicates.

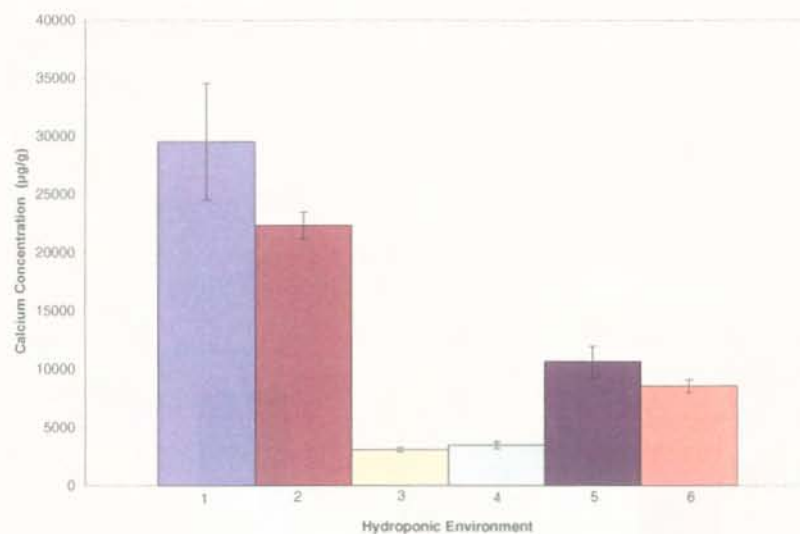


Figure 14: Comparison of Calcium Concentration in Various Tissue from *Pteris cretica cv Mayii* Grown in Two Different Hydroponic Environments (n= 5, Concentration of Arsenite in solution= 500µM, 1= Root Control Solution, 2= Root Arsenite Solution, 3= Stem Control Solution, 4= Stem Arsenite Solution, 5= Leaf Control Solution, and 6= Leaf Arsenite Solution)

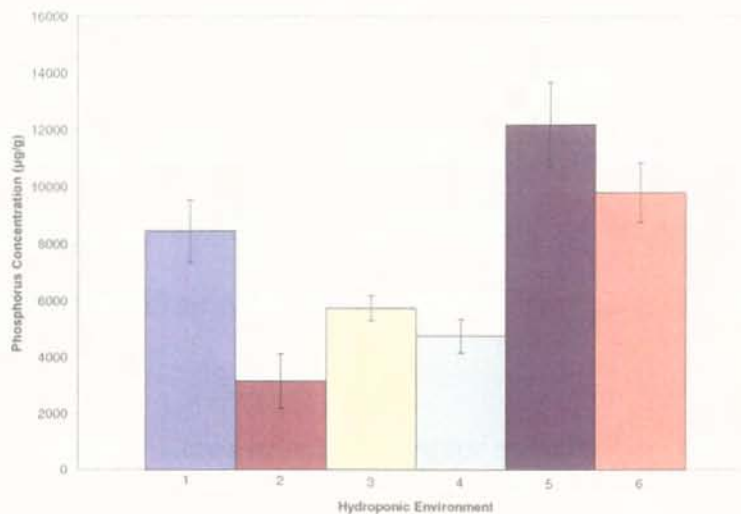


Figure 15: Comparison of Phosphorus Concentration in Various Tissue from *Pteris cretica cv Mayii* Grown in Two Different Hydroponic Environments (n= 5, Concentration of Arsenite in solution= 500µM, 1= Root Control Solution, 2= Root Arsenite Solution, 3= Stem Control Solution, 4= Stem Arsenite Solution, 5= Leaf Control Solution, and 6= Leaf Arsenite Solution)

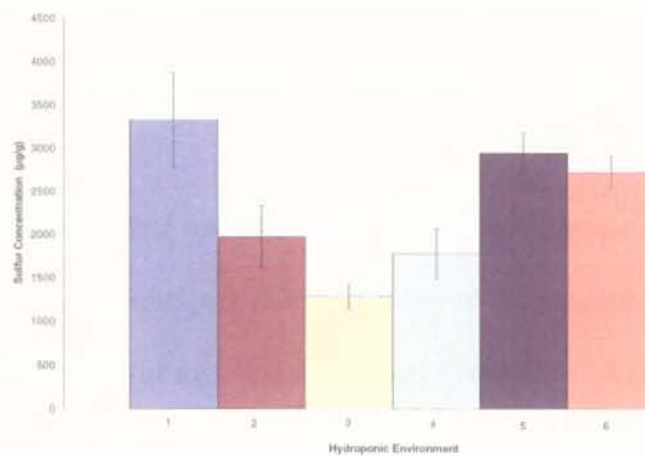


Figure 16: Comparison of Sulfur Concentration in Various Tissue from *Pteris cretica cv Mayii* Grown in Two Different Hydroponic Environments (n= 5, Concentration of Arsenite in solution= 500µM, 1= Root Control Solution, 2= Root Arsenite Solution, 3= Stem Control Solution, 4= Stem Arsenite Solution, 5= Leaf Control Solution, and 6= Leaf Arsenite Solution)

### 5.2.3 Micronutrient Concentration Levels in *Pteris vittata*

A micronutrient analysis using ICP-OES was conducted on the tissue samples from the arsenic hyperaccumulators. A micronutrient is an element with a concentration less than  $3\mu\text{mol g}^{-1}$  per dry weight [13]. The five micronutrients were iron, molybdenum, manganese, copper, and nickel. A summary of micronutrient data is available in Appendix 1.3.

The most common micronutrient detected for arsenic-free, arsenite, and arsenate exposed *Pteris vittata* was iron (Growth Cycle 1 and 2) (Figure 17). The average concentrations of iron for the arsenic-free plants were  $1,530 \pm 810 \mu\text{g/g}$  (Cycle 1) and  $3,170 \pm 1,250 \mu\text{g/g}$  (Cycle 2). The iron concentrations in the arsenite-grown plants were  $2,040 \pm 700 \mu\text{g/g}$  (Cycle 1) and  $7,170 \pm 1,720 \mu\text{g/g}$  (Cycle 2). The average concentrations of iron in the arsenate plants were  $2,990 \pm 850 \mu\text{g/g}$  (Cycle 1) and  $3,420 \pm 1,970 \mu\text{g/g}$  (Cycle 2). The most probable reason for iron being the most common micronutrient detected is due to its role in plant growth and development. Iron is a constituent of specific proteins involved in photosynthesis, respiration, and  $\text{N}_2$  fixation [13].

Besides iron, the other micronutrients were detected in a concentration of  $250 \mu\text{g/g}$  or less. In fact, no nickel was detected in any of the plant tissues of *Pteris vittata*. The reason for the absence of nickel is due in part to the fact that hydroponic solutions contained no nickel. Also, the detection of nickel in the plant tissue may require an analysis by an ICP-MS, which can measure elements in the nanogram per gram range. In fact, nickel is employed by the plants as a constituent of urease [13].

A Student's t-test analysis was conducted to determine if *Pteris vittata* contained more iron when growth in the arsenic-free solution or arsenic-containing solutions (figure 17 shows average of iron for all environments). The Student's t-test results showed that there was no significant difference in iron uptake between arsenic-free and arsenite-grown plants for Cycle 1 (T value was 1.52 and T Critical value was 2.10). For Cycle 2, there was a significant difference in iron uptake between arsenic-free and arsenite-grown plants (T value was 5.95 and T Critical value 2.12). Also, Student's t-test showed that there was a significant difference in iron uptake between the arsenic-free and arsenate-grown plants for Cycle 1 (T value was 3.95 and T Critical value was 2.10). However, no significant difference was detected for iron uptake between arsenic-free and arsenate-grown plants for Cycle 2 (T value was 0.345 and T Critical value 2.13 was for Cycle 2).

Similar comparisons were made for molybdenum, manganese, and copper (data not shown). There was no significant difference between any replicates.

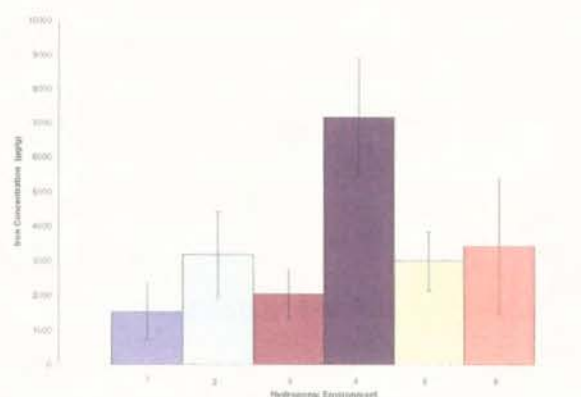


Figure 17: Comparison of Iron Concentration in Entire Tissue Samples from *Pteris vittata* Grown in Three Different Hydroponic Environments (n= 10, Concentration of Arsenite and Arsenate in solution= 500µM, 1= Cycle 1 Control Solution, 2= Cycle 2 Control Solution, 3= Cycle 1 Arsenite Solution, 4= Cycle 2 Arsenite Solution, 5= Cycle 1 Arsenate Solution, and 6= Cycle 2 Arsenate Solution)



#### 5.2.4 Micronutrient Concentration Levels in *Pteris cretica cv Mayii*

An ICP-OES micronutrient analysis was also conducted on the tissue samples from *Pteris cretica cv Mayii*. The six micronutrients were sodium, iron, molybdenum, manganese, copper, and nickel. A summary of micronutrient data is available in Appendix 1.4. The most common micronutrient detected in the root tissue was iron (Figure 18). The average concentrations of iron for the arsenic-free plants were  $2510 \pm 450 \mu\text{g/g}$ . The iron concentrations in the arsenite-grown plants contained  $1970 \pm 390 \mu\text{g/g}$ . Beside iron, the other macronutrients were detected in only in a trace concentration. In fact, no nickel was detected in any of the plant tissues of *Pteris cretica cv Mayii*.

The most common micronutrient detected in the stem and leaf tissue was sodium (Figure 19). The average concentrations of sodium for the arsenic-free plants (stem tissue) were  $730 \pm 83.0 \mu\text{g/g}$ . The sodium concentrations in the arsenite-grown plants' stem tissue contained  $900 \pm 164 \mu\text{g/g}$ . The average concentrations of sodium for the arsenic-free plants' leaf tissue were  $1710 \pm 250 \mu\text{g/g}$ . The sodium concentrations in the leaf tissue of arsenite-grown plants contained  $1700 \pm 301 \mu\text{g/g}$ . The most probably reason for sodium being the most common micronutrient is its role in respiration [13].

A Student's t-test analysis was conducted to determine if arsenic-free *Pteris cretica Mayii* contained more sodium than the arsenite-grown plants (Figure 19 shows average of sodium for both environments). The Student's t-test results for root tissue showed that there was a significant difference in sodium concentration between arsenic-free and arsenite plants (T value was 2.71 and T Critical value was 2.31). The Student's t-test results for the stem tissue showed that there was no significant difference in sodium

concentration between the arsenic-free and arsenite-grown plants (T value was -2.09 and T Critical value was 2.45). Finally, the Student's t-test results for leaf tissue showed no significant difference in sodium concentration between arsenic-free and arsenite-grown plants (T value was 0.0540 and T Critical value was 2.31).

A Student's t-test analysis was conducted to determine if arsenic-free *Pteris cretica Mayii* contained more iron than the arsenite-grown plants (Figure 18 shows average of iron for both environments). The Student's t-test results for root tissue showed that there was no significant difference in iron concentration between arsenic-free and arsenite plants (T value was 2.00 and T Critical value was 2.31). The Student's t-test results for the stem tissue showed that there was no significant difference in iron concentration between the arsenic-free and arsenite-grown plants (T value was 1.49 and T Critical value was 2.36). Finally, the Student's t-test results for leaf tissue showed no significant difference in iron concentration between arsenic-free and arsenate-grown plants (T value was 1.31 and T Critical value was 2.57).

Similar comparisons were made for molybdenum, manganese, and copper (data not shown). There was no significant difference between any replicates.

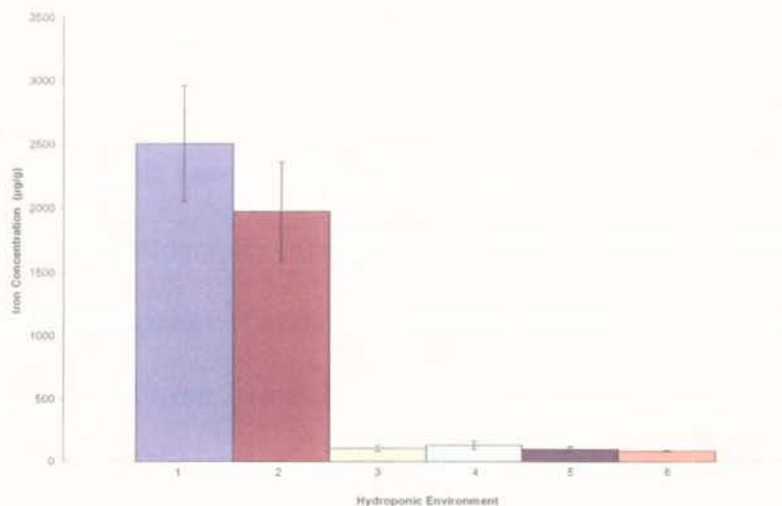


Figure 18: Comparison of Iron Concentration in Various Tissue from *Pteris cretica cv Mayii* Grown in Two Different Hydroponic Environments (n= 5, Concentration of Arsenite in solution= 500 $\mu$ M, 1= Root Control Solution, 2= Root Arsenite Solution, 3= Stem Control Solution, 4= Stem Arsenite Solution, 5= Leaf Control Solution, and 6= Leaf Arsenite Solution)

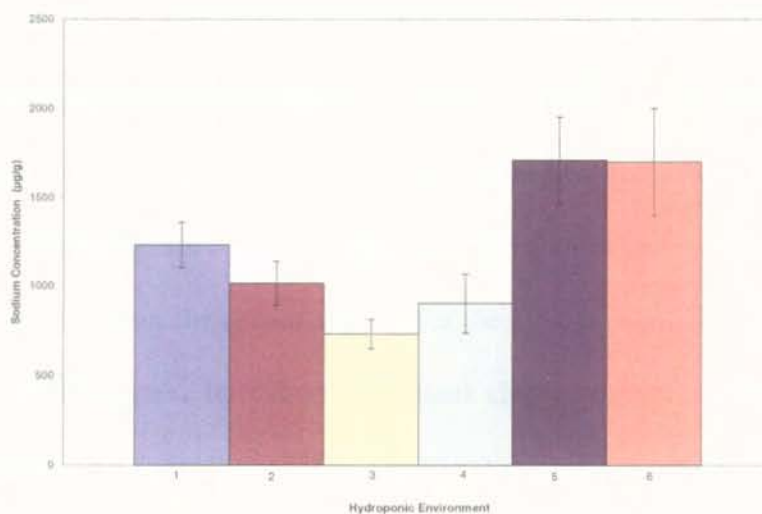


Figure 19: Comparison of Sodium Concentration in Various Tissue from *Pteris cretica cv Mayii* Grown in Two Different Hydroponic Environments (n= 5, Concentration of Arsenite in solution= 500 $\mu$ M, 1= Root Control Solution, 2= Root Arsenite Solution, 3= Stem Control Solution, 4= Stem Arsenite Solution, 5= Leaf Control Solution, and 6= Leaf Arsenite Solution)

### 5.3 Arsenic-based Experiments Using Ion Chromatography

In order to establish a method for identification of arsenic species, a series of arsenic-based experiments were conducted using ion chromatography. The first type of arsenic-based ion chromatography experiments consisted of detection of the peak retention time for each species of arsenic. The chromatograms for each arsenic species detected several peaks (Figure 20 and 21). When the arsenate chromatogram was compared to the blank solution chromatogram, the peak near 8 minutes was selected as a possible candidate for being the arsenate anion (Figure 20). The rest of the peaks on the arsenate chromatogram were eliminated because they appeared on the blank chromatogram (Chromatogram not shown).

When the arsenite chromatogram was analyzed, a peak near 5 minutes was selected as a possible candidate for being the arsenite anion. However, a slight peak near 5 minutes was also seen on the blank chromatogram. To determine whether this peak on the arsenite chromatogram was the anion, a second experiment was conducted using 100ppm arsenite solution (Figure 22). If the peak around 5 minutes was the arsenite anion, then the chromatogram for the 100ppm solution should have a large peak at the 5 minute position. However, the chromatogram for the 100ppm sample failed to show a large peak around 5 minutes. In addition, the blank chromatogram for this experiment had a peak at around 5 minutes (similar in height to the peak seen in the blank chromatogram for 10ppm arsenite experiment). Based on these findings, ion chromatography can not detect arsenite.

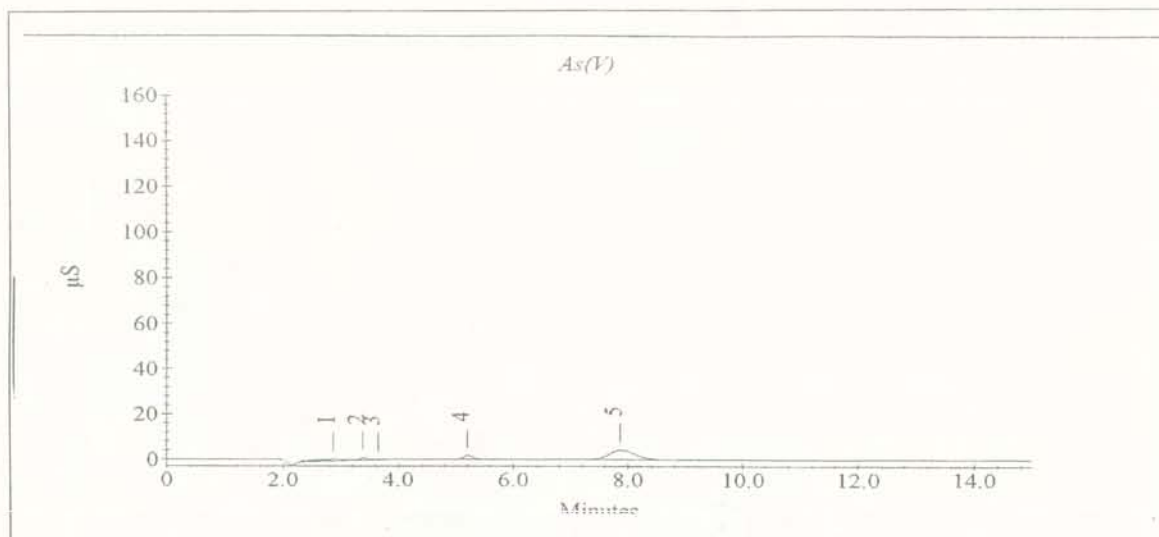


Figure 20: Ion Chromatogram for a 10ppm Sample of Arsenate

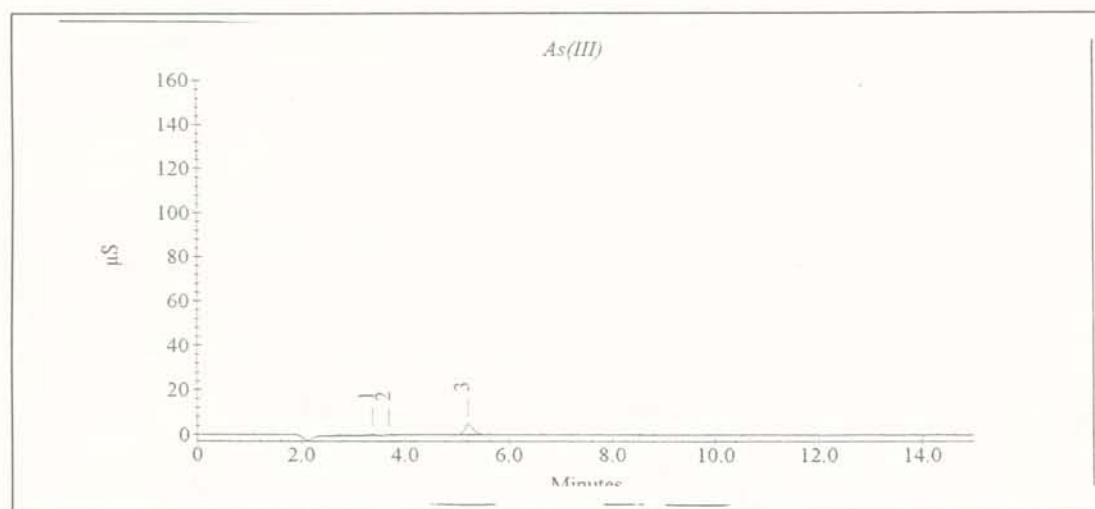


Figure 21: Ion Chromatogram for a 10ppm Sample of Arsenite

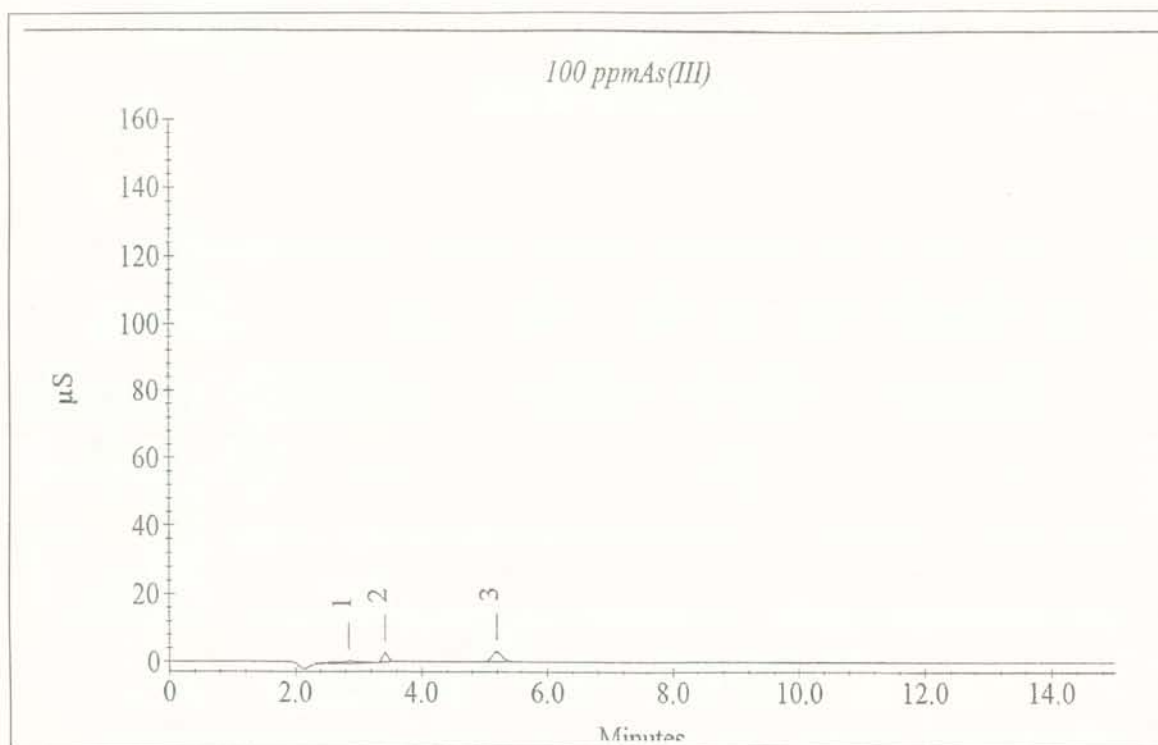


Figure 22: Ion Chromatogram For a 100ppm Sample of Arsenite

## Chapter 6: Conclusion

Arsenic contamination of the environment has become a global problem, affecting both the developed and developing nations. In particular, sources of drinking water have been shown to contain high levels of arsenic [14]. One method of environmental remediation is called phytoremediation [3]. Phytoremediation is the use of vascular plants to remediate sites of environmental contamination. This project consisted of an investigation of the phytoremediation process by two arsenic hyperaccumulating plants (*P. vittata* and *P. cretica cv Mayii*).

In this investigation, 75 hyperaccumulators were grown and analyzed (60 *Pteris vittata* and 15 *Pteris cretica cv Mayii*). The results of the arsenic analysis showed that *Pteris vittata* extracted both forms of arsenic. For the first growth cycle, there was no significant difference in the amount of arsenic extracted between the arsenite and arsenate exposed plants. However, for the second growth cycle, there was a significant difference in the amount of arsenic extracted (more arsenic was extracted by arsenite-grown plants). This difference between growth cycle could possibly be due to a macronutrient concentration in the hydroponic solution. The results of the arsenic analysis for *Pteris cretica cv Mayii* showed that the root tissue contained the lowest concentration of arsenic, compared to the stem and leaf tissues. In addition, no significant difference in arsenic concentration was found for the stem and leaf tissues.

The results of the macronutrient analysis for *Pteris vittata* and *Pteris cretica cv Mayii* determined calcium to be the most common nutrient. Of the four macronutrients analyzed, sulfur was the least common nutrient detected in *Pteris vittata* and *Pteris cretica cv Mayii* tissue. For *Pteris vittata* cycle 1, no significant difference in phosphorus concentration was found between arsenite and arsenate-grown plants. However, phosphorus concentration for cycle 2 arsenite plants was determined to be significantly different from the arsenate plants. This significant difference in phosphorus concentration could possibly be a factor in the higher arsenic concentration found in the arsenite-grown plants.

The results of micronutrient analysis for *Pteris vittata* determined iron to be the most common micronutrient. The most common micronutrient detected in the root tissue for *Pteris cretica cv. Mayii* was also determined to be iron. However, the most common micronutrient in the stem and leaf tissue was determined to be sodium. Of the five micronutrients, no nickel was detected in the plant tissue (for both hyperaccumulating species). The other micronutrients (molybdenum, manganese, and copper) were found in only trace amounts.

Based on the results of this investigation, two areas of new research should be conducted on these arsenic hyperaccumulators. The first area of new research should be a more detailed analysis of macro- and micronutrient role in these hyperaccumulators. In previous studies, phosphate uptake has been found to directly affect arsenate uptake [11]. Since phosphate (a macronutrient) has been shown to affect arsenate uptake, then other macronutrient may also affect arsenite uptake. Also an ICP-MS analysis of micronutrient



may provide a better understanding of various factors allowing for arsenic uptake by the hyperaccumulators.

Another area of new research should be an arsenic speciation analysis of various plant tissues of the hyperaccumulators. Previous studies have shown that most of the arsenic stored in the leaf tissue is in arsenite form. Because of this fact, a tissue analysis for arsenic speciation will provide information on location at which most of the arsenate is converted to arsenite [7].

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## Literature Cited

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Appendix

## Appendix

### Appendix 1.1 Macronutrients in *Pteris vittata*

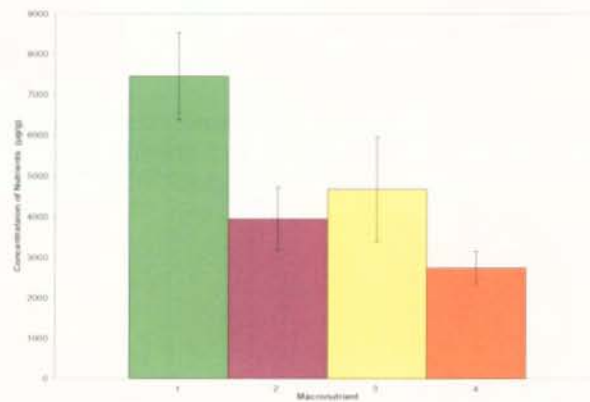


Figure 23: Comparison of Macronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenic-free Environment (Growth Cycle 1) [n=10, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur]

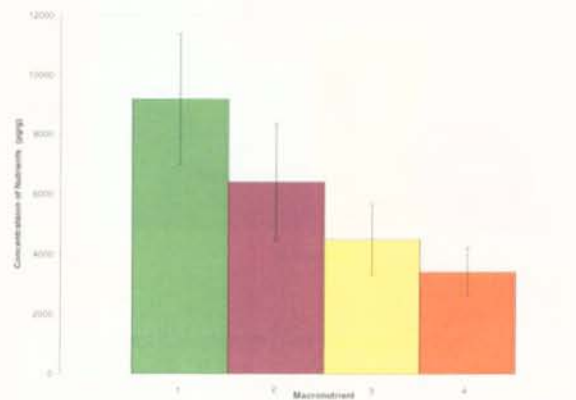


Figure 24: Comparison of Macronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenic-free Environment (Growth Cycle 2) [n=10, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur]

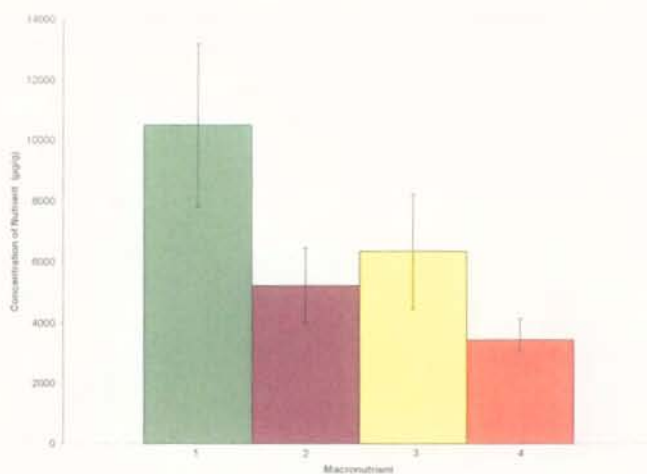


Figure 25: Comparison of Macronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenite Environment (Growth Cycle 1) [n=10, concentration of Arsenite in solution = 500µM, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur]

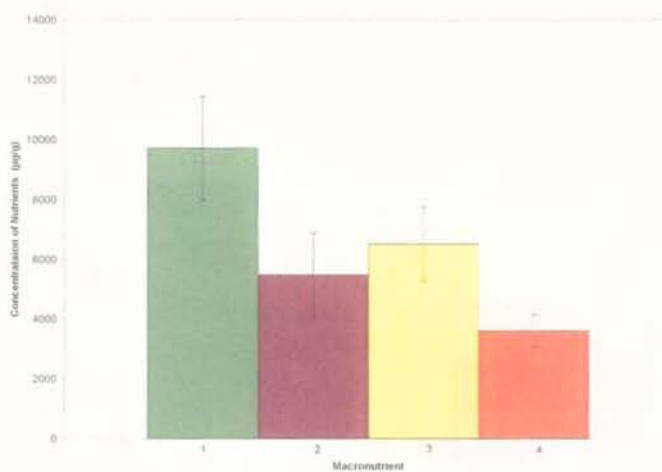


Figure 26: Comparison of Macronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenite Environment (Growth Cycle 2) [n=10, concentration of Arsenite in solution = 500µM, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur]

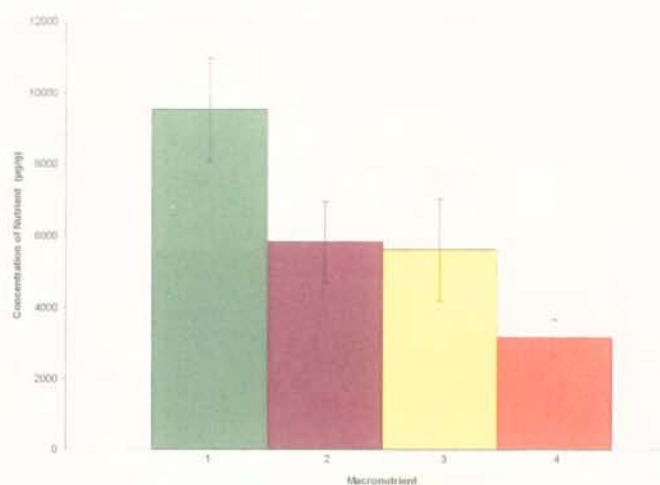


Figure 27: Comparison of Macronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenate Environment (Growth Cycle 1) [n=10, concentration of Arsenate in solution =  $500\mu\text{M}$ , 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur]

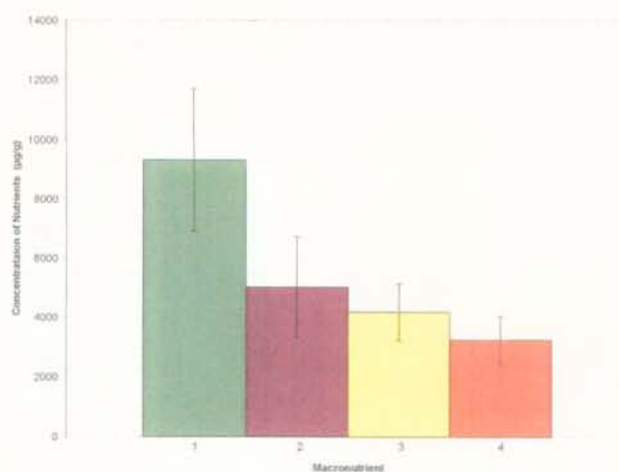


Figure 28: Comparison of Macronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenate Environment (Growth Cycle 2) [n=10, concentration of Arsenate in solution =  $500\mu\text{M}$ , 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur]



## Appendix 1.2 Macronutrients in *Pteris cretica* cv *Mayii*

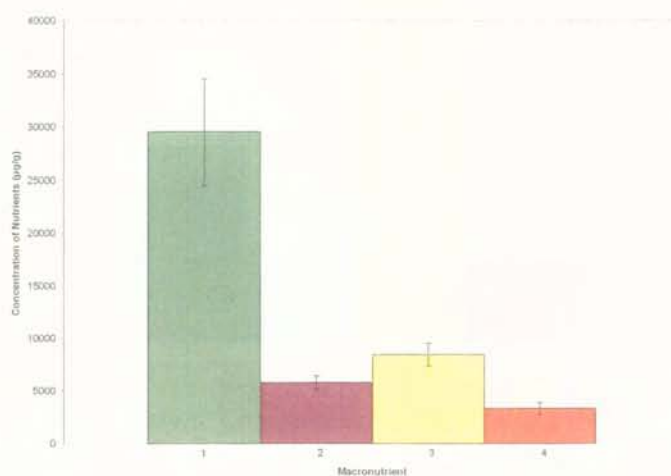


Figure 29: Comparison of Macronutrient Concentration Levels in Root Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenic-free Environment (n= 5, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur)

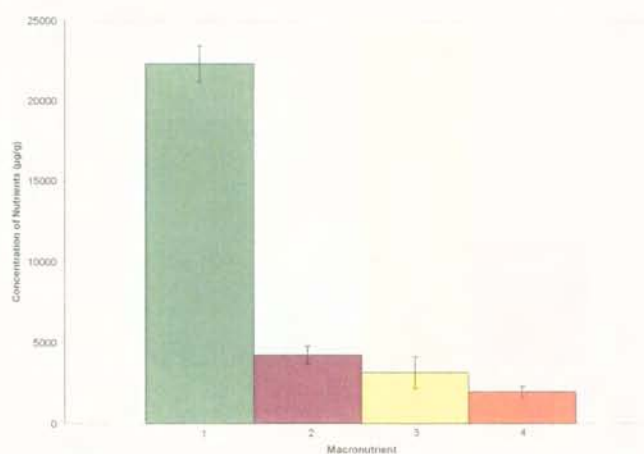


Figure 30: Comparison of Macronutrient Concentration Levels in Root Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenite Hydroponic Environment (n= 5, Concentration of Arsenite in solution= 500µM, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur)

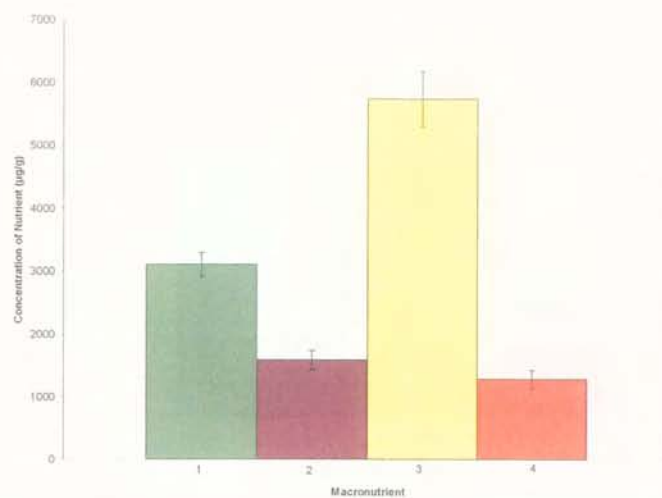


Figure 31: Comparison of Macronutrient Concentration Levels in Stem Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenic-free Hydroponic Environment (n= 5, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur)

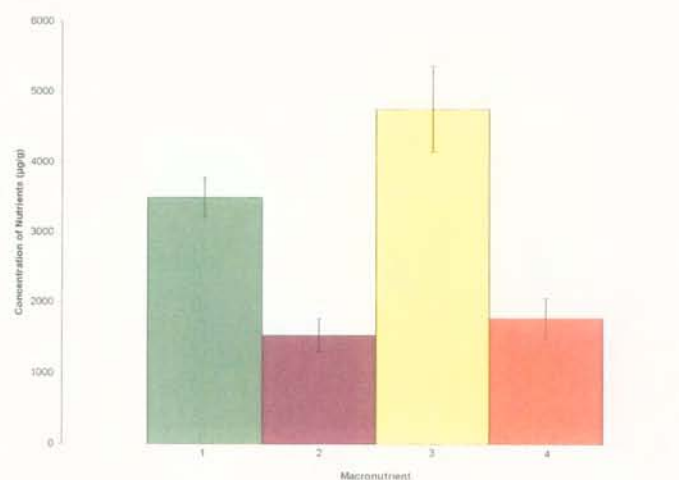


Figure 32: Comparison of Macronutrient Concentration Levels in Stem Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenite Hydroponic Environment (n= 5, Concentration of Arsenite in solution= 500µM, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur)

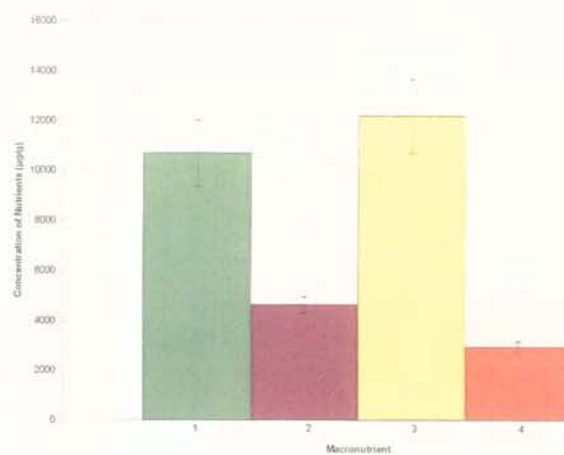


Figure 33: Comparison of Macronutrient Concentration Levels in Leaf Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenic-free Hydroponic Environment (n= 5, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur)

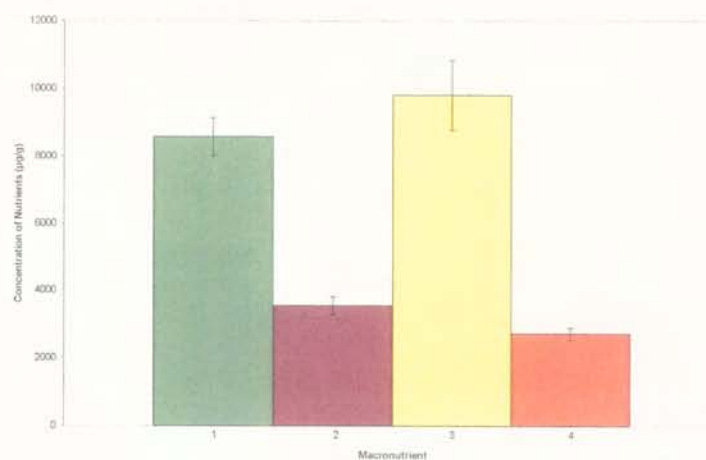


Figure 34: Comparison of Macronutrient Concentration Levels in Leaf Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenite Hydroponic Environment (n= 5, Concentration of Arsenite in solution= 500 $\mu$ M, 1= Calcium, 2= Magnesium, 3= Phosphorus, and 4= Sulfur)

### Appendix 1.3 Micronutrients in *Pteris vittata*

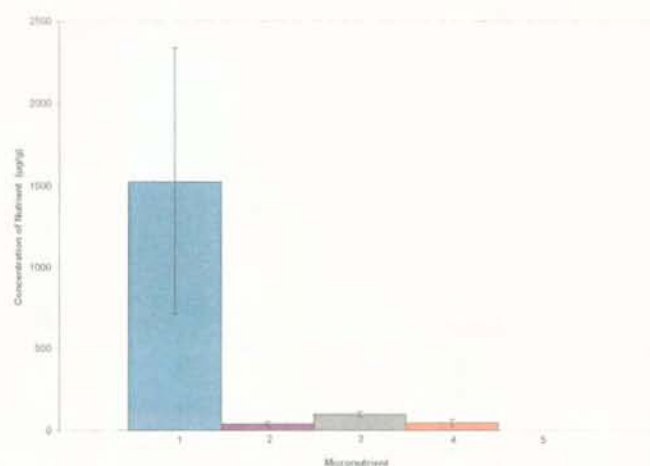


Figure 35: Comparison of Micronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenic-free Environment (Growth Cycle 1) [n=10, 1= Iron, 2=Molybdenum, 3= Manganese, 4= Copper, and 5= Nickel]

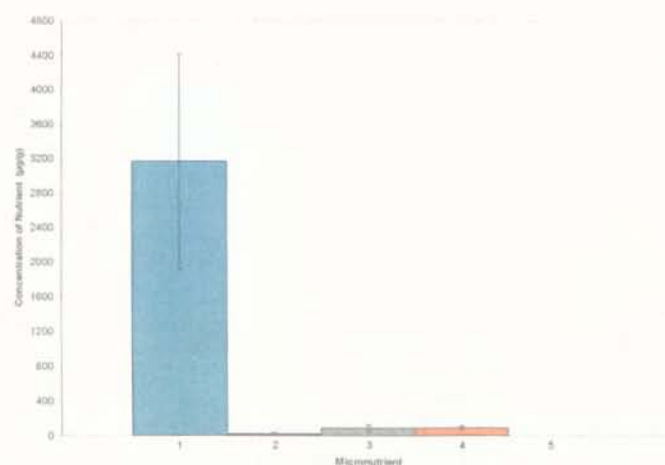


Figure 36: Comparison of Micronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenic-free Environment (Growth Cycle 2) [n=10, 1= Iron, 2=Molybdenum, 3= Manganese, 4= Copper, and 5= Nickel]

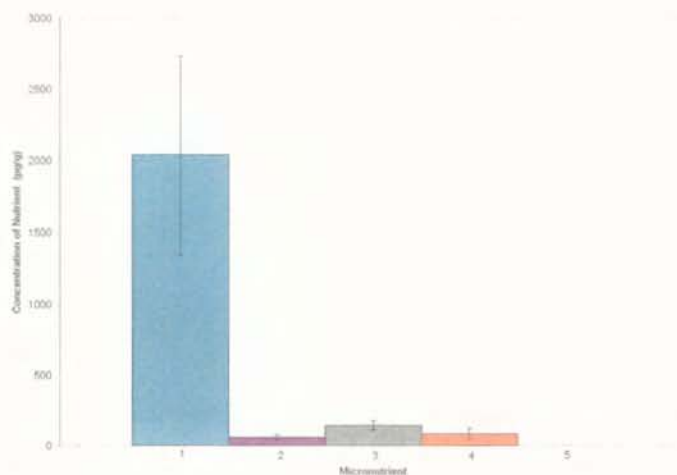


Figure 37: Comparison of Micronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenite Environment (Growth Cycle 1) [n=10, concentration of Arsenite in solution = 500 $\mu$ M, 1= Iron, 2=Molybdenum, 3= Manganese, 4= Copper, and 5= Nickel]

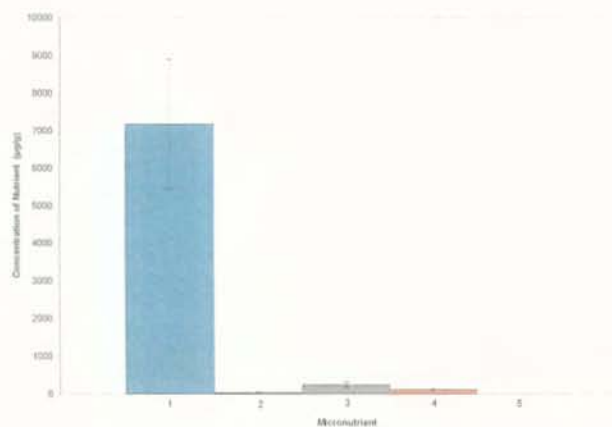


Figure 38: Comparison of Micronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenite Environment (Growth Cycle 2) [n=10, concentration of Arsenite in solution = 500 $\mu$ M, 1= Iron, 2=Molybdenum, 3= Manganese, 4= Copper, and 5= Nickel]

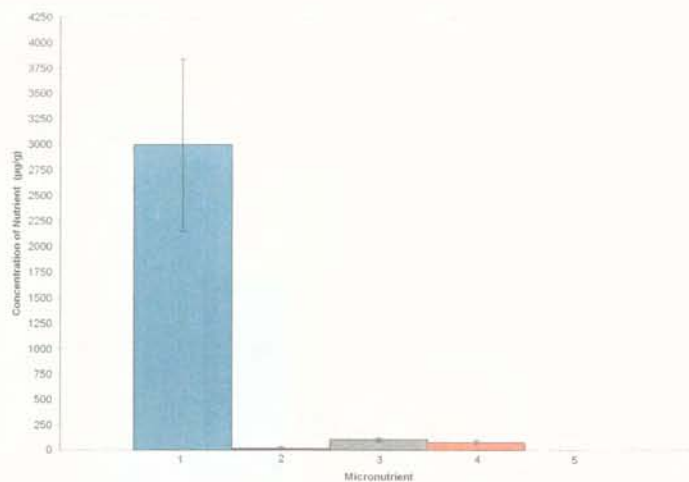


Figure 39: Comparison of Micronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenate Environment (Growth Cycle 1) [n=10, concentration of Arsenate in solution = 500µM, 1= Iron, 2=Molybdenum, 3= Manganese, 4= Copper, and 5= Nickel]

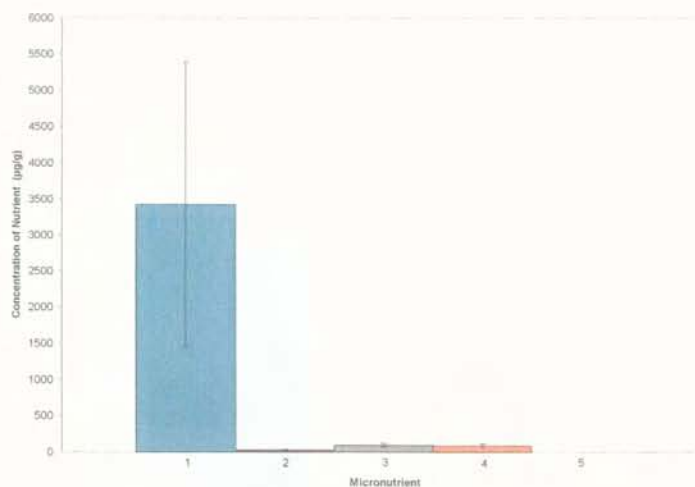


Figure 40: Comparison of Micronutrient Concentration Levels in Tissue Samples from *Pteris vittata* Grown in an Arsenate Environment (Growth Cycle 2) [n=10, concentration of Arsenate in solution = 500µM, 1= Iron, 2=Molybdenum, 3= Manganese, 4= Copper, and 5= Nickel]

#### Appendix 1.4 Micronutrients in *Pteris cretica* cv *Mayii*

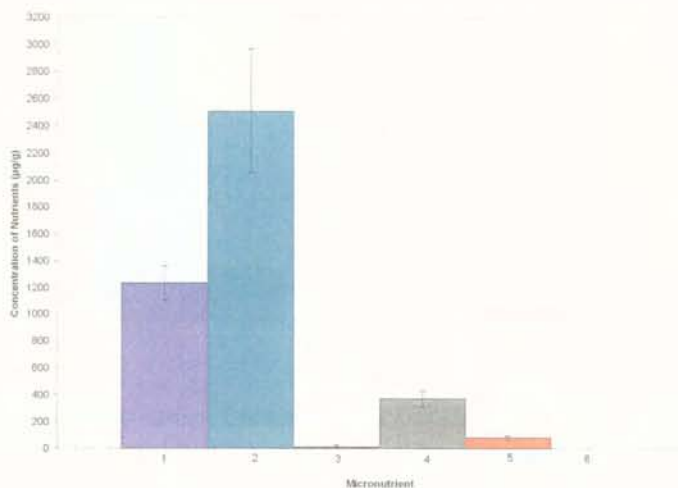


Figure 41: Comparison of Micronutrient Concentration Levels in Root Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenic-free Environment (n= 5, 1= Sodium, 2= Iron, 3=Molybdenum, 4= Manganese, 5= Copper, and 6= Nickel)

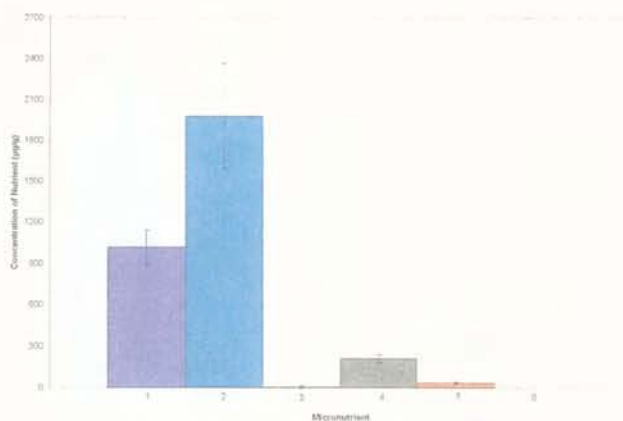


Figure 42: Comparison of Micronutrient Concentration Levels in Root Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenite Environment (n= 5, concentration of Arsenite in solution = 500 $\mu$ M, 1= Sodium, 2= Iron, 3=Molybdenum, 4= Manganese, 5= Copper, and 6= Nickel)

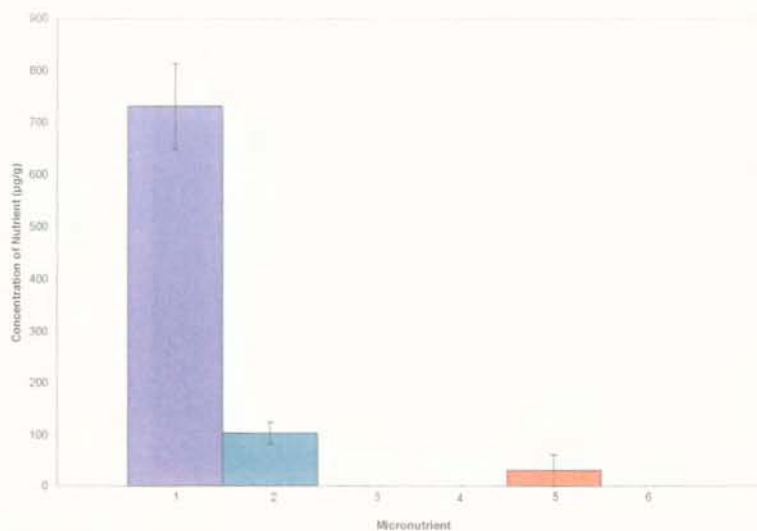


Figure 43: Comparison of Micronutrient Concentration Levels in Stem Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenic-free Environment (n= 5, 1= Sodium, 2= Iron, 3=Molybdenum, 4= Manganese, 5= Copper, and 6= Nickel)

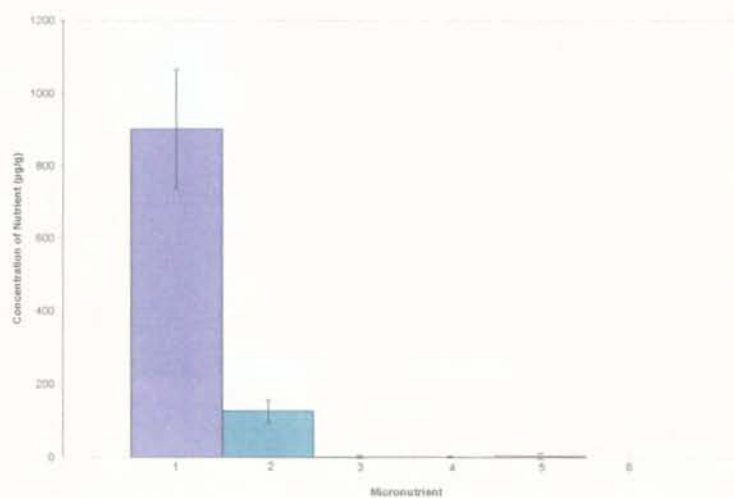


Figure 44: Comparison of Micronutrient Concentration Levels in Stem Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenite Environment (n= 5, concentration of Arsenite in solution = 500µM, 1= Sodium, 2= Iron, 3=Molybdenum, 4= Manganese, 5= Copper, and 6= Nickel)



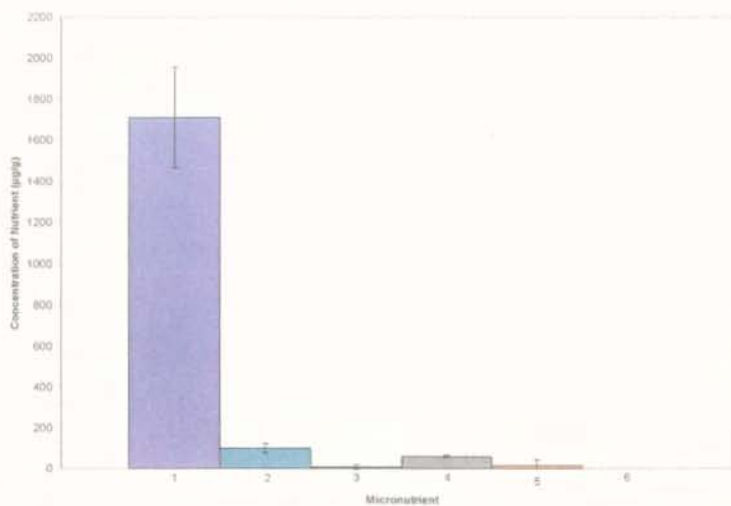


Figure 45: Comparison of Micronutrient Concentration Levels in Leaf Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenic-free Environment (n= 5, 1= Sodium, 2= Iron, 3=Molybdenum, 4= Manganese, 5= Copper, and 6= Nickel)

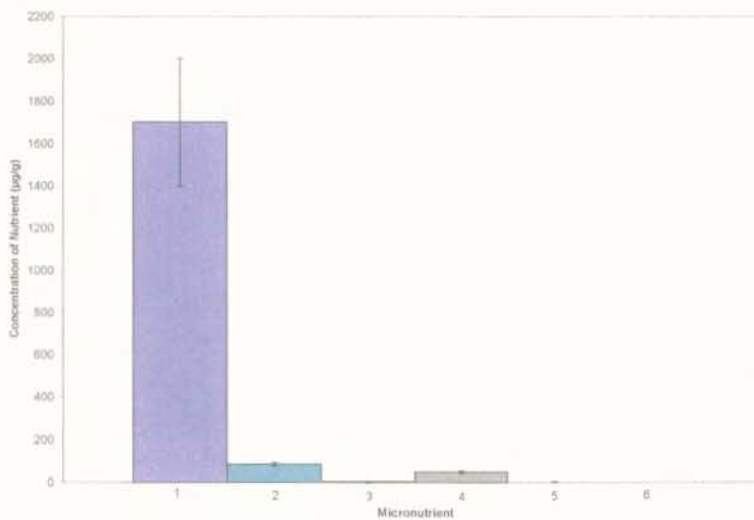


Figure 46: Comparison of Micronutrient Concentration Levels in Leaf Tissue Samples from *Pteris cretica* cv *Mayii* Grown in an Arsenite Environment (n= 5, concentration of Arsenite in solution = 500µM, 1= Sodium, 2= Iron, 3=Molybdenum, 4= Manganese, 5= Copper, and 6= Nickel)